

TRANSGENIC NON-HUMAN MAMMALS EXPRESSING CONSTITUTIVELY
ACTIVATED TYROSINE KINASE RECEPTORS

Field of the Invention

5 The invention is directed toward tumor-susceptible transgenic non-human mammals. The invention further pertains to the use of such mammals in the development of anti-cancer agents and therapies.

BACKGROUND OF THE INVENTION:

10 Recent advances in recombinant DNA and genetic techniques have made it possible to introduce and express a desired sequence or gene in a recipient animal. Through the use of such methods, animals have been engineered to carry non-naturally occurring sequences or genes, that is, sequences or genes that are not normally or naturally present in the unaltered animal. The techniques have also been used to produce
15 animals which exhibit altered expression of naturally present sequences or genes.

 Animals produced through the use of these methods can be either "chimeric", in which only some of the animal's cells contain and express the introduced sequence or gene, or "transgenic", in which all of the cells of the animal contain the introduced sequence or gene. Consequently, in the case of transgenic animals every animal is
20 capable of transmitting the introduced genetic material to its progeny as compared to the chimeric animals in which transmittal to progeny is dependent upon whether the introduced material is present in the germ cells of the animal.

 The high efficiency transformation of cultured mammalian cells has been accomplished by direct microinjection of specific DNA sequences into the cell nucleus
25 (Capecchi, M., Cell 22:479-488 (1980)). More specifically, it has also been demonstrated that DNA could be microinjected into mouse embryos and found in the resultant offspring (Gordon *et al.*, P.N.A.S. U.S.A. 77:7380-7384 (1978)). Thus, the ability to produce certain transgenic mice is described and well known.

 The basic procedure for producing transgenic mice requires the recovery of
30 fertilized eggs from newly mated female mice and then microinjecting into the male

into the mouse. The microinjected eggs are then implanted in the oviducts of one-day pseudopregnant foster mothers and allowed to proceed to term. The newborn mice are then tested for the presence of the microinjected DNA by means known in the art and appropriate to detect the presence of the microinjected DNA. See, for example,
5 Wagner *et al.* P.N.A.S. U.S.A. 78:6376-6380 (1981), U.S. Patent No. 4,873,191, which describes the production of mice capable of expressing rabbit beta-globin in its erythrocytes.

The insulin-like growth factor I receptor (referred to as "IGF-1R" or "IGF1R") is a transmembrane tyrosine kinase which is overexpressed or activated in many
10 human cancers including colon, breast, lung, prostate, glioblastoma and melanoma. IGF1R is thought to be required for the establishment and maintenance of the transformed phenotype and to exert an anti-apoptotic effect in tumorigenesis. Baserga, R., Cancer Research, 55:249-252 (1995). Activation of the receptor is initiated by the binding of either IGF-I or IGF-II to the two α -subunits of the IGF1R, i.e., receptor
15 dimerization, resulting in the autophosphorylation of critical tyrosine residues in the catalytic domain of the β -subunit. Kato *et al.*, J. Biol. Chem. 265:2655-2661 (1993); Gronborg *et al.*, J. Biol. Chem. 258:23435-23440 (1993). Ligand binding also leads to phosphorylation and subsequent activation of downstream substrates thought to be involved in growth regulation and differentiation. Ottensmeyer *et al.*, Biochemistry
20 39: 12103-12112 (2000). *In vitro* models have been developed to study the role of kinase dimerization in the activation of the IGF1R that utilize fusion proteins comprising the soluble kinase domain of IGF1R and the homodimeric glutathione S-transferase (GST), Baer *et al.*, Biochemistry 40:14268-14278 (2001).

To date, the transgenic models established for studying IGF1R signaling have
25 been limited to overexpression of the ligands for IGF1R (Bol *et al.*, Oncogene 14:1725-1734 (1997); DiGiovanni *et al.*, Cancer Research 60:1561-1570 (2000)). These models are further complicated by the existence of a family of IGF binding proteins (IGFBPs), which serve to modulate the bioavailability of free IGF-I and IGF-II, resulting in a long latent period for tumor development.

30 One approach to studying *in vitro* the constitutive stimulation of a receptor in the absence of ligand binding activation has utilized a chimeric oncogenic receptor-type tyrosine kinase comprising the extracellular and transmembrane domains of CD8

with the kinase domain of c-Eyk (Zong *et al.*, EMBO Journal, Vol.15, No. 17, 4516-4525 (1996)). The resulting chimera showed elevated kinase activity and caused cellular transformation. Such chimeric receptors may incorporate the extracellular, transmembrane and cytoplasmic domains from the same or different species. The preparation and use of chimeric receptors comprising cytokines and other kinase domains are known in the art. See, for example, Lawson *et al.*, WO 99/57268; M. Roberts, U.S. Patent No. 5,712,149; and Capon *et al.*, U.S. Patent No. 5,741,899.

It is therefore of interest to develop a transgenic non-human mammalian model for ligand-independent IGF-1 receptor-driven tumorigenesis. It is also desirable that the animal develops tumors that overexpress IGF1R within a fairly short period of time from birth, facilitating the analysis of multigenerational pedigrees. Such a model can be used to study the pathogenesis and treatment of tumors overexpressing or having activated IGF1R. The instant invention represents such a model using a chimeric receptor.

SUMMARY OF THE INVENTION:

The invention is directed to a transgenic non-human mammal whose germ cells and somatic cells express a constitutively activated tyrosine kinase receptor that acts as an oncogene sequence. The constitutive activation of the tyrosine kinase receptor results from a transgene comprising a dimerizable-inducing moiety and the catalytic kinase domain of the tyrosine kinase receptor to be activated. In a preferred embodiment of the invention, the non-human mammal is a mouse, the transgene comprises the extracellular and transmembrane domains of CD8 and the intracellular catalytic kinase domain of IGF1R, and the constitutively activated tyrosine kinase receptor is that of IGF1R. The invention further comprises mammalian embryos carrying the constitutively activated tyrosine kinase receptor capable of developing into viable transgenic animals whose progeny carry the constitutively activated tyrosine kinase receptor after breeding forward by sexual reproduction. The invention further comprises DNA constructs comprising a selected promoter plus the constitutively activated tyrosine kinase receptor cDNA or DNA segments cloned into plasmids for ultimate insertion into the genome of a mammal.

The transgenic non-human mammals of the invention are characterized by specificity for the constitutively activated tyrosine kinase receptor that is expressed by the transgenic non-human mammal. In a preferred embodiment of the invention, the transgenic mice express constitutively active IGF1R which drives tumorigenesis; 5 adenocarcinomas develop in the salivary and mammary glands of the transformed mice as early as eight weeks of age. Tumors from the transgenic mice that express the transgene when grafted into nude mice are sensitive to inhibitors of the specific constitutively activated tyrosine kinase receptor, but not other tyrosine kinase 10 receptors, and are thus capable of being utilized as tools for the identification and optimization of molecules and chemical entities for novel anticancer therapies. Thus, the invention also embodies non-human mammals and methods for the identification of selective modulators of constitutively activated tyrosine kinase receptors and pharmaceutical compositions comprising the selective modulators so identified.

Thus, the invention provides a transgenic non-human mammal whose somatic 15 and germ line cells contain a genome comprised of a transgene encoding a sequence for a constitutively activated tyrosine kinase receptor, wherein the non-human mammal is more susceptible to developing tumors specific to the constitutively activated tyrosine kinase receptor.

The invention also provides a transgenic non-human mammal which expresses 20 a genomic DNA fragment which comprises a coding sequence for a constitutively activated tyrosine kinase receptor, wherein said transgenic non-human mammal can be bred to produce progeny non-human mammals whose genomes comprise said genomic DNA fragment.

The invention further provides a method for obtaining a target mouse whose 25 genome comprises a genomic DNA fragment wherein the DNA fragment comprises a coding sequence for a constitutively activated tyrosine kinase receptor, wherein said mouse can be bred to produce progeny mice whose genomes comprise said genomic DNA fragment, said method comprising the steps of:

- (a) isolating a fertilized egg from a first female mouse;
- 30 (b) transferring a genomic DNA fragment which comprises a coding sequence for a constitutively activated tyrosine kinase receptor into said fertilized egg;

(c) transferring said fertilized egg containing said genomic DNA fragment to the uterus of a pseudopregnant second female mouse; and

(d) maintaining said second female mouse such that:

(i) said second female mouse becomes pregnant with an embryo

5 derived from said fertilized egg containing said genomic DNA fragment;

(ii) said embryo develops into said target mouse; and

(iii) said target mouse is viably born from said second female mouse;

wherein the genome of said target mouse comprises said genomic DNA fragment and

10 wherein said mouse can be bred to produce progeny mice whose genomes comprise said genomic DNA fragment.

The invention also includes a method of screening for selective modulators of a constitutively activated tyrosine kinase receptor for use in treating cancer resulting from a constitutively activated tyrosine kinase receptor which comprises

15 administering an investigational substance to a non-human mammal of the invention and assaying the cancer treating efficacy of said investigational substance in said cancer. Also provided is a pharmaceutical composition for treating cancer resulting from a constitutively activated tyrosine kinase receptor which comprises a substance determined by this method of screening for selective modulators to have cancer
20 treating efficacy in said cancer.

The invention further includes a method for producing a transgenic mouse that develops tumors specific for a constitutively activated tyrosine kinase receptor comprising the steps of: (a) obtaining a tumor fragment from a transgenic non-human animal of the invention that is a mouse; (b) implanting subcutaneously said tumor
25 fragment to a recipient nude mouse; and (c) maintaining said mouse from step (b) under conditions to promote growth of said mouse and said tumor.

Yet another aspect of the invention is a method for producing a transgenic mouse cell line that expresses a constitutively activated tyrosine kinase receptor comprising the steps of: (a) obtaining a tumor fragment from a transgenic non-human
30 animal of the invention that is a mouse;; (b) isolating cells from said tumor sample; and (c) placing the isolated cells under such conditions as to maintain growth and viability of said isolated cells.

BRIEF DESCRIPTION OF THE DRAWINGS:

Figs. 1A and 1B are diagrammatic representations of the dimerized MMTV-CD8:IGF1R (MCI) construct. The cross hatched portion of the diagram represents the CD8 extracellular and transmembrane domains and the open portion of the diagram represents the intracellular, catalytic kinase domain of IGF1R.

Fig. 2 is a diagrammatic representation of the MMTV-CD8:IGF1R (MCI) construct. The CD8:IGF1R fusion gene was cloned into a vector in such a way that it was downstream of a MMTV promoter and was flanked with intron and polyA sequences for efficient message processing

Fig. 3 shows a representative Northern blot analysis of the salivary tissue of a control and five progeny mice found to have passed the transgene (CD8:IGF1R). Each lane contains 20 µg of total RNA isolated from salivary gland tissue resolved on a 1% agarose gel in 17.5% formaldehyde.

Fig. 4 shows a representative Northern blot analysis of control and tumored salivary mammary tissue showing expression of the transgene. Lane a – control mammary tissue; lane b – control salivary tissue; lane c – salivary tissue (line 15); lane d – mammary tissue (line 15); lane e – control salivary tissue; lane f – salivary tissue (line 19); lane g – mammary tissue (line 19); lane h – IGF1R-Sal Cells; lane i – xenografted salivary tissue (line 19); lane j – xenografted lung tissue (line 19); and xenografted mammary tissue (line 19). For each sample polyA mRNA was isolated from 50 µg of total RNA by the methods described in Example 4 below. The isolated mRNA was resolved on a 1% agarose gel in 17.5% formaldehyde.

Figs. 5A (mammary) and 5B (salivary) show the transgenic mouse (line MCI-19) with representative multifocal mammary tumors.

Fig. 6 shows representative normal (right lung) and lung metastases (left lung) of representative salivary gland tumors.

Fig. 7 shows representative Western blot analysis of salivary and mammary tissue from normal and tumored mice. Lane a – normal mammary tissue; lane b – normal salivary tissue; lane c – salivary tissue (line 15); lane d – mammary tissue (line 15); lane e – normal salivary tissue; lane f – salivary tissue (line 19); lane g – mammary tissue (line 19); lane h – IGF1R-Sal cells; lane i – xenografted salivary

tissue (line 19); lane j – xenografted lung tissue (line 19); and lane k – xenografted mammary tissue (line 19).

Fig. 8A shows representative xenografted salivary tumors from transgenic line MCI-19 and Fig. 8B shows representative mammary tumors from transgenic line

5 MCI-15.

Fig. 9 shows representative *in vivo* growth of IGF1R-Sal tumors from transgenic line MCI-19 implanted subcutaneously into nude mice at various points of time following implantation.

Fig. 10 shows representative *in vivo* growth of IGF1R-Mam tumors from
10 transgenic line MCI-15 implanted subcutaneously into nude mice at various points of time following implantation.

Figs. 11A and 11B show representative *in vitro* growth of the IGF1R-Sal cell line. Fig. 11A is a representative photograph of primary cells established on poly-D lysine matrix in monolayer. Fig. 11B is a representative graph of the exponential
15 growth of cells in media.

DETAILED DESCRIPTION OF THE INVENTION:

Cancer in humans develops through a multi-step process, indicating that multiple changes must occur to convert a normal cell into one with a malignant
20 phenotype. One class of involved genes includes cellular oncogenes which, when activated by mutation or when expressed inappropriately, override normal cellular control mechanisms and promote unbridled cell proliferation.

The invention relates to production of transgenic non-human mammals containing within their genomes a chimeric receptor that when expressed results in the
25 constitutive activation of a tyrosine kinase and the development of tumors early in age. The tumors have been found to express high levels of the activated tyrosine kinase, which in turn can be transplanted into nude mice as xenografts. The tumors so generated have been used to establish cell lines for use in biological studies.

The invention also relates to a transgenic non-human mammal whose somatic
30 and germ line cells contain a genome comprised of a transgene encoding a sequence for a constitutively activated tyrosine kinase receptor, wherein the non-human

mammal is more susceptible to developing tumors specific to the constitutively activated tyrosine kinase receptor.

In a preferred embodiment, described in the examples that follow, the construct is the engineered human CD8:IGF1R fusion gene. Those skilled in the art will recognize that other constructs can be generated that will produce similar results, that is, that will lead to the dimerization and constitutive activation of the tyrosine kinase receptor from which the cytoplasmic catalytic kinase domain has been engineered. By way of example, and not intending to be limited thereto, the transgene of the invention may comprise a dimerization-inducible moiety, such as CD8 or glutathione S-transferase (GST), and an intracellular kinase domain selected from the group of receptors with tyrosine kinase activity including, but not limited to, epidermal growth factor receptor (EGFR), HER2, insulin receptor (IR), insulin-like growth factor (IGF1R), platelet-derived growth factor (PDGFR), fibroblast growth factor (FGFR) and hepatocyte growth factor receptor (Met).

The nucleotide sequences (human cDNA) used herein were cloned using standard molecular biology techniques (Maniatus *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982); Ausubel, F.M. *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons, Volume 2 (1991)) based on sequences available in the public domain (GenBank). Nucleotide sequences from other mammals can be employed in the invention herein, provided that membrane associated dimerization causes constitutive activation of the tyrosine kinase receptor, that is, said activity results in signal transduction in the mammalian cells. In the invention, expression of the transgene results in the dimerization of the tyrosine kinase receptor to which the fusion protein binds and in turns results in the constitutive activation of the receptor in the absence of the naturally occurring ligand binding.

The construct may also comprise selected nucleic acid regions associated with the transgene (as by fusion therewith) for mediation of, for example, its introduction into the target genome, its expression loci in the transgenic mammal, on/off external regulation of transgene expression, and other desired features, as generally known in the art.

In one aspect, the invention provides transgenic lines that express activated IGF1R under the control of the mouse mammary tumor virus (MMTV) promoter. Several lines of mice were generated that expressed the transgene in both the salivary and mammary glands; mice harboring the transgene developed adenocarcinomas in these tissues as early as eight weeks of age. In culture, cell lines derived from salivary tumors are sensitive to inhibitors of the IGF1R, but are significantly less sensitive to inhibitors specific for other receptors, such as the EGF receptor. Importantly, when grafted into nude mice, these lines form tumors that grow well and are sensitive to standard cytotoxic agents. The transgenic animals described herein may serve as efficient tools in the identification, development, and optimization of biological and chemical moieties for use as anticancer therapies directed at inhibition of the IGF-1 receptor.

In accordance with the invention, a construct was generated in which the extracellular and transmembrane domains of the human CD8 antigen (Sukhatmet *et al.*, Cell 40(30):591-597 (1985)) were fused to the intracellular, catalytic tyrosine kinase domain of IGF1R (Fig. 1A). This fusion protein alleviates the necessity for IGF ligand to cause dimerization of the IGF1R and subsequent autophosphorylation. Instead, the CD8 portion of the fusion protein dimerizes in a ligand-independent way, resulting in constitutive activation of the tyrosine kinase (Fig. 1B). By constitutive activation it is meant dimerization of the fusion protein, as driven by the CD8 domain, which results in autophosphorylation of the kinase domain, a property needed for intracellular signal transduction.

The engineered human CD8:IGF1R fusion gene was cloned into a vector in such a way that it was downstream of a mouse mammary tumor virus (MMTV) promoter and was flanked with intron and polyA sequences for efficient message processing. The MMTV promoter, when expressed *in vivo* in the animal, drives transcription of downstream sequences in the mammary and salivary glands of mice. One skilled in the art would be able to clone the transgene of the invention into a vector under the control of other promoters that drive tumor development in the skin of the non-human mammal. For example, the human CD8:IGF1R transgene was cloned into a vector under the control of the K14 promoter with similar results.

Fig. 2 provides a diagrammatic representation of the MMTV-CD8:IGF1R transgene construct. The sequence of this transgene construct is provided in SEQ ID NO:1 and a plasmid of this construct was deposited with the American Type Culture Collection ("ATCC"), 10801 University Boulevard, Manassas, Virginia 20110-2209, on January 10, 2003 and has been given ATCC Deposit Designation PTA-4930. The deposit referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-Organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence(s) of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

Microinjection of the MMTV-CD8:IGF1R construct of SEQ ID NO:1 into the pronucleus of fertilized oocytes resulted in the generation of eight founder mice carrying the transgene (CD8:IGF1R) DNA. Of these eight mice, six were bred to produce progeny and five were found to pass the transgene in a Mendelian fashion to offspring. Mice from these five lines were subsequently examined for expression of the transgene in salivary gland tissue. As can be seen in Fig. 3, one transgenic mouse line, identified as MCI-19, had particularly high levels of transgene expression in the salivary glands. Subsequently, another transgenic mouse line, identified as MCI-15, was also shown to have high levels of salivary gland expression (data not shown). Two transgenic mouse lines developed tumors of the salivary and mammary glands (lines MCI-15 and MCI-19) and were retained for further studies. Tumors from both salivary gland and mammary gland tumors were also shown to have high levels of expression of the transgene, as did xenografted tumors and cell lines, as compared to tissues from non-transgenic mice which showed no expression of the transgene (Fig. 4)

Both male and female mice from each transgenic line developed tumors. Female mice began to develop tumors in the salivary gland at about 90 days of age, although enlarged glands are obvious just after weaning. Once tumors fully develop,

they grow rapidly such that mice are often euthanized within a few weeks of the initial observation of the tumor. Mammary tumor development, however, is more latent, often not occurring until the animals are about 150 days of age or older.

Tumors in the mammary tissue are often multifocal, arising simultaneously in multiple regions of the gland with no apparent bias for location (Figs. 5A (mammary) and 5B (salivary)). In male mice, salivary glands develop with the same frequency as in females and within the same time frame. Mammary gland tumors have also been observed in a few male mice, although with reduced incidence compared to females. Interestingly, metastasis of the salivary gland tumors to the lungs has been observed in both male and female mice (Fig. 6). Because of the rapid growth rate of the tumors in the salivary gland, surgical removal of the affected gland is often required to allow the animal to survive long enough to develop substantial metastasis.

In an effort to demonstrate the causal relationship between the oncogene activity of the transgene and tumorigenesis, activity of IGF1R kinase was assessed in a number of samples (Fig. 7). Using antibodies to the CD8 portion of the fusion protein, CD8:IGF1R proteins were immunoprecipitated from lysates of tissue samples, tumors, and cell lines as described in the examples that follow. Resolution of this immunoprecipitate on polyacrylamide gels and subsequent immunoblotting with antiphosphotyrosine antibodies enabled observation of the level of activation of the CD8:IGF1R fusion protein in these samples. As shown in Fig. 7, there is significant phosphorylation of the IGF1R tyrosine kinase in the tumor samples from both transgenic lines, MCI-15 and MCI-19, the xenografted tumors, and the IGF1R-Sal cell line, but not in tissues from the control, i.e., non-transgenic mice.

In order to determine the tumorigenic potential of these lesions observed in the transgenic mice, tumors from both salivary and mammary glands of MCI mice were transferred to nude (*nu/nu*) mice. Fragments from both tumor types grew when implanted subcutaneously on the ventral side of nude mice within a few weeks. Both mammary gland tumors from MCI-15 and salivary gland tumors from MCI-19 grew as xenografts (Figs. 8A and 8B). Fig. 9 shows representative *in vivo* growth of IGF1R-Sal tumors from transgenic line MCI-19 implanted subcutaneously into nude mice at various points of time following implantation. In the early passages of the tumor, the tumors grew slowly and inconsistently, with roughly 20% showing no

growth. With increasing passages, the rate of tumor growth improved and tumor take-rates became 100% by the third sequential transfer of tumors from mouse to mouse (passage 3). Fig. 10 shows representative *in vivo* growth of IGF1R-Mam tumors from transgenic line MCI-15 implanted subcutaneously into nude mice at various points of time following implantation. The growth of the initial passage (p0, from transgenic mouse host to nude mice) was slow with a significant percentage rated as no-take (33%). In subsequent passages both the growth and take-rate improved.

An *in vitro* cell line was established from the IGF1R-Sal tumors grown in nude mice. Tumor cells isolated from tumor xenografts and seeded onto tissue culture dishes coated with various extracellular matrices behaved and grew very differently. Collagens I and IV, fibroectin and laminin all appeared to facilitate and enhance the growth of fibroblasts. Poly-D-lysine coated dishes were found to provide the optimal condition for IGF1R-Sal cells. In this condition, a continuous IGF1R-Sal cell line was established (Figs. 11A and 11B) which grows in exponential fashion. The following cell culture condition were utilized: Complete RPMI 1640 media supplemented with 10% fetal bovine serum and 25 mM HEPES.

The transgenic non-human mammals produced herein, the xenografted tumors, and the cell line established therefrom provide useful tools for reiterative cycles of drug evaluation to develop and optimize properties of useful therapies for cancer.

In a specific embodiment described in the examples that follow, the transgenic mouse so produced is an excellent model for studying specifically the role of IGF1R in tumorigenesis and the identification and development of modulators to affect tumorigenesis. In a particular embodiment of the invention, as exemplified below, a transgenic non-human mammal is engineered to express a constitutively activated tyrosine kinase receptor. In this particular embodiment, expression of the transgene leads to constitutively activated IGF1R without the need for expression of its corresponding ligand and ligand binding. This model is particularly useful in that the tumors produced in these mice are specific to IGF1R. The tumors generated in the mice described herein are exquisitely sensitive to modulators, and more specifically inhibitors, of the engineered kinase domain from which they were generated, while they have limited susceptibility to modulators of other tyrosine kinase receptors. For

example, in culture, cell lines derived from tumor cells from the CD8:IGF1R fusion transgenics are sensitive to inhibitors of IGF1R, but are significantly less sensitive to inhibitors specific to EGFR (data not shown). By use of the invention herein, models can be developed that are specific for a particular tyrosine kinase receptor and thus enable the identification and development of selective tyrosine kinase receptor inhibitors.

The transgenic animals of the invention are accordingly useful inter alia for the development of pharmacotherapies for the treatment of cancer, particularly those characterized by the expression of tyrosine kinase receptors. In a preferred embodiment of the invention, the transgenic animals are useful for the identification of selective modulators of constitutively activated tyrosine kinase receptors. In a particularly preferred embodiment, the transgenic animals of the invention are useful for the development of pharmaceuticals and therapies for cancers in which IGF1R is overexpressed or activated. Such cancers include, but are not limited to, breast, colon, prostate, and lung cancer.

The invention also provides a method of screening for selective modulators of a constitutively activated tyrosine kinase receptor for use in treating cancer resulting from a constitutively activated tyrosine kinase receptor. Methods of screening for modulators using transgenic mice are generally known in the art. See, for example, Omer et al., Cancer Research 60, 2680-2688, May 15, 2000. In one aspect, the method of the invention of screening for selective modulators includes the following steps. Tumors that are derived from transgenic mice are excised and implanted in nude mice. Once tumors reach a size of 100 to 200 mm³, mice are randomized into treatment groups for evaluation of investigational substances or compounds that have demonstrated the desired cell potency and pharmacokinetic properties. Compounds are formulated in a vehicle such as 40% PEG400/0.1% Tween 80, and are administered by oral gavage once or twice daily. Tumor volume in animals treated with vehicle alone or with different doses of compounds is measured twice a week. Inhibition of tumor growth is calculated as %T/C, where T and C are, respectively, tumor volumes in animals treated with compounds and control animals treated with vehicle alone. Compounds that showed %T/C of 50% or less at more than one dose below the maximum tolerated dose are considered efficacious.

The invention is further useful for imparting traits accompanying expression of the constitutively activated tyrosine kinase receptors into a line or breed of non-human mammals by genetically engineering one or more mammals of the line according to the invention to obtain transgenic mammals carrying at least one
5 transgene in each of their genomes which, when expressed, constitutively activates the specific tyrosine kinase receptor from which it was engineered, preferably in the salivary and mammary glands. Transgenic product mammals are then selected for based on the genotyping as described herein.

Having now generally described the invention, the same will be more readily
10 understood through reference to the following examples which are provided by way of illustration and are not intended to be limiting of the invention. The full text of references and published materials cited herein are incorporated by reference as if set forth at length.

15 EXAMPLES:

Example 1 - CD8:IGF1R Transgene Construct.

The MMTV-CD8:IGF1R (MCI) construct shown in Fig. 2 and SEQ ID NO:1 was generated using standard molecular biology techniques, for example, Ausubel, F.M. *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons, Volume 2
20 (1991). A HindIII/BamHI fragment comprising the nucleotide sequence of SEQ ID NO:2 and encoding amino acid residues 1-218 of the human CD8 gene (SEQ ID NO:3) was cloned into the HindIII/BamHI site of pcDNA3.1(+) (InvitrogenTM Corporation, Carlsbad, California). The full length human cDNA for IGF1R was obtained from R. Baserga (Thomas Jefferson University, Philadelphia, PA), Morrione
25 *et al.*, J. Virology, 69:5300-5303 (1995). The cytoplasmic catalytic domain was obtained using standard restriction enzyme techniques and subsequently, as a BamHI fragment of the IGF1R comprising the nucleotide sequence of SEQ ID NO:4 and encoding the amino acid sequence of SEQ ID NO:5, was inserted into at this site of the vector containing the CD8 fragment. Orientation and integrity of the CD8:IGF1R
30 fusion sequence comprising the nucleotide sequence of SEQ ID NO:6 and encoding the amino acid sequence of SEQ ID NO:7 was detected by restriction analysis and confirmed by sequencing. To generate the transgene construct, the CD8:IGF1R

fusion gene was then subcloned into the MMTV vector described by Mueller *et al.*, Cell: 54, 105-115 (1988), resulting in the construct of Fig. 2 and SEQ ID NO:1. Orientation and integrity of the construct was confirmed by sequencing.

5 Example 2 - Generation and Breeding of Transgenic Mice.

Transgenic mice harboring the MMTV-CD8:IGF1R construct (MCI) were generated by microinjection of a PVUII fragment from the above construct into the pronucleus of C57Bl/6XDBA2 F2 (B6D2F2) embryos. Embryos were generated by in-house mating of hybrid stud B6D2 males to virgin females from the same background (Harlan Sprague Dawley, Indianapolis, Indiana) using the techniques described by Hogan *et al.*, Manipulating the Mouse Embryo: a Laboratory Manual, second edition, Brigid Hogan, Rosa Beddington, Frank Constantini, and Elizabeth Lacey, eds, Cold Spring Harbor Laboratory Press (1994). Injected embryos were transferred to pseudopregnant ICR female mice (Harlan Sprague Dawley, Indianapolis, Indiana) and allowed to develop to term. At five to eight days of age, toe and tail samples were taken for DNA analysis of the transgene. Mice harboring the transgene were identified by a polymerase chain reaction (PCR) strategy designed to detect the CD8:IGF1R fusion by using the oligos 5'AGGGTGTGGTGAAAGATGAAC3' (SEQ ID NO:8) for the CD8 portion of the fusion, and 5'CGTCCGAGTAAGTGGTGAAGA3' (SEQ ID NO:9) for the IGF1R portion of the fusion. Founder mice (F0) shown to harbor the transgene were then outbred to the ICR background, and progeny (F1) were again tested for transmission of the transgene in a Mendelian fashion. Mice were observed twice daily for the presence of tumors in either the salivary gland or mammary gland, and the date of first observation of lesions was noted. Animals were euthanized when the tumors grew to about 1000 mg, or when the lesions impaired movement or feeding. All mice were housed in shoebox housing with food and water *ad lib* on a 12/12 light dark cycle, and were humanely handled under the guidelines of the institutional ACUC in an AAALAC accredited facility.

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Example 3 - Gene Expression Analysis.

Detection of CD8:IGF1R transgene expression was performed by Northern blot analysis. To identify transgenic lines that had expression of the transgene, F1 offspring from founders were euthanized and their salivary glands were harvested. In addition, expression was analyzed in tumors from two lines of mice, as well as xenograft and cell line samples. For tissues, the samples were flash frozen in liquid nitrogen and stored until the time of mRNA isolation. Total RNA was isolated using a monophasic solution of phenol and guanidine isothiocyanates, such as the Trizol[®] LS Reagent system as described by the manufacturer (GIBCO[™] Invitrogen Corporation, Carlsbad, California). From these RNA isolates, mRNA was extracted using the Oligotex Direct mRNA kit as recommended by the manufacturer (Operon/QIAGEN, Valencia, California). For cell lines, cells were lysed directly into the Trizol[®] lysis buffer, and stored at -80°C until needed. The mRNA was resolved in 1.0% agarose gel electrophoresis under denaturing conditions with 17.6% formaldehyde and transferred to nylon membrane (Hybond-N, Amersham Biosciences, Uppsala, Sweden) by capillary blotting before hybridization. CD8:IGF1R messages were detected by hybridizing the RNA to a radiolabelled probe (Ready-to-Go kit, New England Nuclear/Perkin Elmer[™] Life Sciences, Boston, Massachusetts) derived from the 3' end of the construct encoding the IGF1R tyrosine kinase domain. Hybridization was done in Rapid-hyb buffer (Amersham Biosciences, Uppsala, Sweden) overnight and nonspecific annealing of the probe was eliminated by multiple washes under stringent conditions (2x20 min in 0.1xSSC, 2%SDS at 65°C). Specific hybridization of the probe to the CD8:IGF1R message was detected by exposing the blot to film (X-OMAT, Kodak Company, Rochester, NY) overnight.

Example 4 - Kinase Activity Analysis.

To ensure that the message expressed from the transgene construct generated a constitutively active IGF1R kinase domain, protein from several sources was analyzed for IGF1R specific activity. Samples analyzed were from the same tissues as described for the Northern Blot analysis. Tissues were homogenized in a Triton, Tris, and glycerol (TTG) lysis buffer (1% Triton X-100, 5% glycerol, 0.15M NaCl, 20mM Tris-HCl, pH 7.6, 1mM EDTA, 1mM sodium vanadate, 40μM ammonium

molybdate) at 4°C. Proteins were quantitated using the micro BCA assay; 3 mg of total tissue lysate or IGF1R-Sal cells were immunoprecipitated with antibodies to CD8 (lanes c, d, f, g, h, i and j of Figs. 4 and 7) or with antibodies to the IGF-1 receptor (lanes a, b and e of Figs. 4 and 7). Proteins were separated on a 10% polyacrylamide gel and subjected to Western blot analysis using an anti-phosphotyrosine antibody (anti-pTyr). The filter was subsequently stripped and re-probed with an antibody to the IGF-1 receptor (anti-IGF1R) to insure equal protein loading.

10 Example 5 - Establishment of Xenografts and Cell Lines.

The establishment of xenografts and cell lines derived from said xenografts were carried out using protocols known to those skilled in the art, such as those described in Berger *et al.*, Immunodeficient Mice in Oncology, Contrib. Oncol. Basel, Fiebig, H.H. and Berger, D.P. (eds), Vol. 42, 321-351, Karger (1992). Each nude mouse was given a subcutaneous implant of a tumor fragment (approximately 20 mg) obtained from a donor transgenic MCI-19 mouse bearing a spontaneously arising tumor. Implantation was accomplished using a 13-gauge trocar inserted subcutaneously in to the ventral side of the mouse, near the mammary gland. Nude (*nu/nu*) mice were obtained from Harlan Sprague Dawley Co. (Indianapolis, Indiana), and maintained in an ammonia-free environment in a defined and pathogen-free colony. Tumor growth was determined by measurement of tumors with a caliper once or twice a week until the tumors reached a predetermined "target" size of 1 gm. Tumor weights (mg) were estimated using the formula: tumor weight = (length x width²) ÷ 2. Xenograft IGF1R-Sal (MCI-19 xenograft tumors) tumors grown in nude mice were excised and minced with scissors, and were dissociated using an enzyme cocktail consisting of 0.025% collagenase (Sigma Chemical Co., St Louis, Missouri), 0.05% pronase (Calbiochem, LaJolla, California) and 0.04% DNase (Sigma) for 1 hour at 37°C. After removal of debris, by passing the cell suspensions through 70 µm nylon screens, the cells were washed in phosphate buffered saline (PBS), counted, and resuspended in complete RPMI™ (GIBCO™ Invitrogen Corporation, Carlsbad, California) 1640 media supplemented with 10% heat inactivated fetal bovine serum (GIBCO™ Invitrogen Corporation, Carlsbad, California). Cells (3 x 10E+5) were

then seeded onto a variety of Biocoat Cellware[®] (Becton Dickinson, Franklin Lakes, New Jersey) coated separately with the following extracellular matrix: collagen I, Laminin, fibronectin, Collagen IV, Poly-D-Lysine.

5 Example 6 - Generation of Transgenic Mice with CD8:Her2 Transgene.

Using the same approach as described in the Examples above, animals were also generated that contained a transgene encoding a fusion of the human CD8 transmembrane and extracellular domains to the intracellular kinase domains of the Her2/erbB2 gene (fusion sequence comprising the nucleotide sequence of SEQ ID NO:10 and encoding an amino acid of SEQ ID NO:11) (Plasmids 57584 and 59296 were obtained from ATCC and joined to make the full length clone of human Her2 (GenBank No. M11730); then used to form the fusion construct with human CD8). In a similar manner to the CD8:IGF1R transgene, the CD8:Her2 fusion protein is expressed under the control of the MMTV promoter. Thus, the construct was identical to that for the CD8:IGF1R transgene, except for the difference in the intracellular kinase domain. The construct was generated using molecular biology techniques known to those of skill in the art where a fragment comprising the nucleotide sequence of SEQ ID NO:12 encoding amino acids 1-218 of the CD8 gene (SEQ ID NO:13) were cloned in frame to a fragment comprising the nucleotide sequence of SEQ ID NO:14 encoding the C-terminal 570 amino acid Her2 kinase domain of SEQ ID NO:15 via an XhoI site and a connector (SEQ ID NO:16). The final construct, where the transcriptional fusion of the recombinant protein was cloned downstream of the MMTV promoter, was handled as described herein for the generation of mice for CD8:IGF1R. The CD8:Her2 mice (Her2 mice) developed mammary gland and salivary gland tumors, similar to the CD8:IGF1R mice and those generated by mutation to the rat neu gene (Mueller *et al.*, Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene, Cell: 54, 105-115 (1988)). As with the CD8:IGF1R mice (MCI-15 And MCI-19), mammary gland and salivary gland tumors were established in xenografts and in cell culture for testing kinase inhibitors for specificity against the constitutively activated Her2 tyrosine kinase receptor.

Example 7 - Generation of Transgenic Mice with CD8:Met Transgene.

Using the same approach as described in the Examples above, animals were also generated that contained a transgene encoding a fusion of the CD8 transmembrane and extracellular domains to the intracellular kinase domains of the cMet gene (fusion sequence comprising the nucleotide sequence of SEQ ID NO:17 encoding an amino acid of SEQ ID NO:18). In a similar manner to the CD8:IGF1R transgene, the CD8:Met fusion protein is expressed under the control of the MMTV promoter. Thus, the construct was identical to that for the CD8:IGF1R transgene, except for the difference in the intracellular kinase domain. The construct was generated using molecular biology techniques known to those of skill in the art where a fragment comprising the nucleotide sequence of SEQ ID NO:19 and encoding amino acids 1-218 of the CD8 gene (SEQ ID NO:20) were cloned in frame to a fragment comprising the nucleotide sequence of SEQ ID NO: 21 encoding the C-terminal 425 amino acid cMet kinase domain of SEQ ID NO:22 via a BamHI site and a connector (SEQ ID NO:23) (the human Met construct was obtained courtesy of G. Van der Woude, P.N.A.S. U.S.A., 84:6379-6383 (1987)). The final construct, where the transcriptional fusion of the recombinant protein was cloned downstream of the MMTV promoter, was handled as described herein for the generation of mice for CD8:IGF1R. The CD8:Met mice (MCM mice, for Mmtv/Cd8/Met) developed mammary gland and salivary gland tumors, similar to the CD8:IGF1R mice described herein. As with the CD8:IGF1R mice (MCI-15 and MCI-19), mammary gland and salivary gland tumors were established in xenografts as well as in cell culture for testing kinase inhibitors for specificity against the constitutively activated cMet tyrosine kinase receptor.

Example 8 - Evaluation of IGF1R Inhibitors.

The proliferative activity of two IGF1R inhibitors in cells was evaluated *in vitro* against a cell line derived from a xenograft tumor generated in a transgenic mouse, IGF1R Sal (CD8-IGF1R), which was engineered to overexpress the catalytic domain of the human IGF1R. The two IGF1R inhibitors evaluated were a benzimidazole compound as described in WO02/079192 and U.S. Application No. 10/263,448, hereby incorporated by reference, and paclitaxel. Inhibition of cellular

proliferation was also determined in a variety of human tumor xenograft cell lines including, Colo205 (colon), Geo (colon), RD1 (rhabdomyosarcoma), H3396 (breast), and MDA PCa2b (prostate). Proliferation was evaluated by incorporation of [^3H]-thymidine into DNA after a 72 hour exposure to reagents. Cells were plated at 1,500
5 cells/well in 96-well microtiter plates and 24 hours later cells were exposed to a range of drug concentrations. After 72 hours incubation at 37°C, cells were pulsed with 4 $\mu\text{Ci/ml}$ [$6\text{-}^3\text{H}$] thymidine (Amersham Pharmacia Biotech, UK) for 3 hours, trypsinized, harvested onto UniFilter-96, GF/B plates (PerkinElmer, Boston, Massachusetts), and scintillation was measured on a TopCount.NXT (Packard,
10 Connecticut). Results are expressed as an IC_{50} , which is the drug concentration required to inhibit cell proliferation by 50% to that of untreated control cells. The IC_{50} values for the compounds evaluated in this assay are shown in Table I.

Table I

Compound	Cytotoxicity Assay IC_{50} (nM) against IGF1R Sal tumor cell line ¹
benzimidazole compound	120
paclitaxel	2.9

15 ¹Inhibition of proliferation was determined after a 72 h exposure by [^3H]-thymidine incorporation.

CLAIMS:

What is claimed is:

1. A transgenic non-human mammal whose somatic and germ line cells contain a genome comprised of a transgene encoding a sequence for a constitutively activated tyrosine kinase receptor, wherein the non-human mammal is more susceptible to developing tumors specific to the constitutively activated tyrosine kinase receptor.
2. The transgenic non-human mammal of claim 1 wherein the transgene comprises a sequence for a constitutively activated tyrosine kinase receptor operably linked to a murine promoter.
3. The transgenic non-human mammal of claim 2 wherein the transgene comprises a sequence encoding for a dimerization-inducible moiety and the intracellular catalytic kinase domain of a tyrosine kinase receptor.
4. The transgenic non-human mammal of claim 3 wherein the dimerization-inducible moiety is a sequence encoding for the extracellular and transmembrane domains of CD8 and the kinase domain is selected from the group consisting of epidermal growth factor receptor (EGFR), HER2, transforming growth factor- α (TGF- α), insulin receptor (IR), insulin-like growth factor (IGFR), platelet-derived growth factor (PDGFR), fibroblast growth factor (FGFR), and hepatocyte growth factor receptor (Met).
5. The transgenic non-human mammal of claim 2 wherein the promoter is MMTV.
6. The transgenic non-human mammal of claim 4 wherein the kinase domain is IGF1R.
7. A transgenic non-human mammal which expresses a genomic DNA fragment which comprises a coding sequence for a constitutively activated tyrosine kinase receptor, wherein said transgenic non-human mammal can be bred to produce progeny non-human mammals whose genomes comprise said genomic DNA fragment.
8. A method for obtaining a target mouse whose genome comprises a genomic DNA fragment wherein the DNA fragment comprises a coding sequence for

a constitutively activated tyrosine kinase receptor, wherein said mouse can be bred to produce progeny mice whose genomes comprise said genomic DNA fragment, said method comprising the steps of:

- (a) isolating a fertilized egg from a first female mouse;
- 5 (b) transferring a genomic DNA fragment which comprises a coding sequence for a constitutively activated tyrosine kinase receptor into said fertilized egg;
- (c) transferring said fertilized egg containing said genomic DNA fragment to the uterus of a pseudopregnant second female mouse; and
- (d) maintaining said second female mouse such that:
 - 10 (i) said second female mouse becomes pregnant with an embryo derived from said fertilized egg containing said genomic DNA fragment;
 - (ii) said embryo develops into said target mouse; and
 - (iii) said target mouse is viably born from said second female mouse;
- 15 wherein the genome of said target mouse comprises said genomic DNA fragment and wherein said mouse can be bred to produce progeny mice whose genomes comprise said genomic DNA fragment.

9. A method of screening for selective modulators of a constitutively activated tyrosine kinase receptor for use in treating cancer resulting from a
20 constitutively activated tyrosine kinase receptor which comprises administering an investigational substance to the non-human mammal according to claim 1 and assaying the cancer treating efficacy of said investigational substance in said cancer.

10. A pharmaceutical composition for treating cancer resulting from a constitutively activated tyrosine kinase receptor which comprises a substance
25 determined by the method of claim 9 to have cancer treating efficacy in said cancer.

11. A method for producing a transgenic mouse that develops tumors specific for a constitutively activated tyrosine kinase receptor comprising the steps of:
(a) obtaining a tumor fragment from the transgenic non-human animal according to claim 1, wherein said transgenic non-human animal is a mouse;
30 (b) implanting subcutaneously said tumor fragment to a recipient nude mouse; and

(c) maintaining said mouse from step (b) under conditions to promote growth of said mouse and said tumor.

12. A method for producing a transgenic mouse cell line that expresses a constitutively activated tyrosine kinase receptor comprising the steps of:

- 5 (a) obtaining a tumor fragment from the transgenic non-human animal according to claim 1, wherein said transgenic non-human animal is a mouse;
- (b) isolating cells from said tumor sample; and
- (c) placing the isolated cells under such conditions as to maintain growth and viability of said isolated cells.

10 13. A transgenic mouse cell line that expresses a constitutively activated tyrosine kinase receptor obtained by the method according to claim 12.