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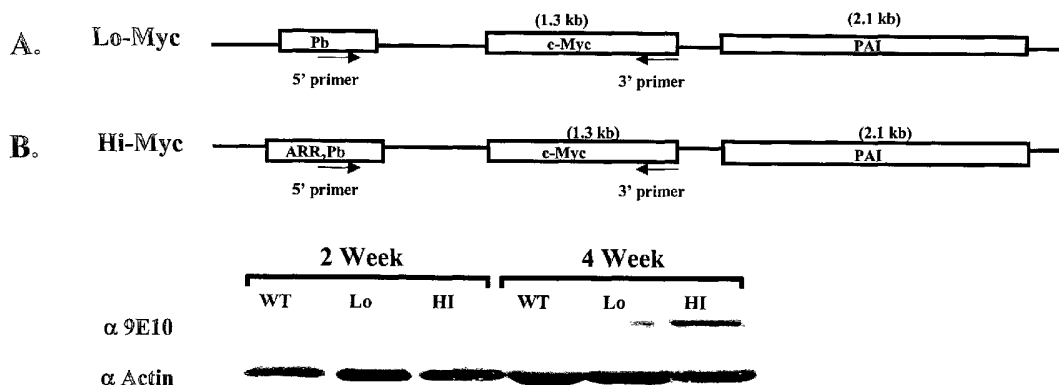
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(54) Title: ANIMAL MODELS OF PROSTATE CANCER AND METHODS FOR THEIR USE



(57) Abstract: The invention disclosed herein is an animal of human prostate cancer consisting of a transgenic mouse having a c-Myc transgene under the control of prostate specific regulatory sequences.

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ANIMAL MODELS OF PROSTATE CANCER AND METHODS FOR THEIR USE**STATEMENT OF GOVERNMENT SUPPORT**

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5 invention.

RELATED APPLICATIONS

This application claims priority under Section 119(e) from U.S. Provisional Application Serial No. 60/390,692 filed June 21, 2002, the contents of which are
10 incorporated herein by reference.

FIELD OF THE INVENTION

The present invention provides animal models of human cancers and methods for their use.

BACKGROUND OF THE INVENTION

15 Cancer is the second leading cause of human death next to coronary disease. Worldwide, millions of people die from cancer every year. In the United States alone, as reported by the American Cancer Society, cancer causes the death of well over a half-million people annually, with over 1.2 million new cases diagnosed per year. While deaths from heart disease have been declining significantly, those resulting from cancer
20 generally are on the rise. In this century, cancer is predicted to become the leading cause of death.

Worldwide, several cancers stand out as the leading killers. In particular, carcinomas of the lung, prostate, breast, colon, pancreas, and ovary represent the primary causes of cancer death. These and virtually all other carcinomas share a common lethal
25 feature. With very few exceptions, metastatic disease from a carcinoma is fatal. Moreover, even for those cancer patients who initially survive their primary cancers, common experience has shown that their lives are dramatically altered.

Adenocarcinoma of the prostate is the most frequently diagnosed cancer in men in the United States, and is the second leading cause of male cancer deaths (Karp et al., Cancer Res. 56:5547-5556 (1996)). The significant susceptibility of this organ to cancer in humans is not understood. While prostatic intraepithelial neoplasia (PIN) is generally
5 believed to be a precursor to adenocarcinoma of the prostate, a comprehensive understanding of this disease progression is lacking. A genetically well-defined and manipulatable model is therefore needed to dissect the molecular events associated with development of adenocarcinoma and its progression from PIN.

Clinical analysis of human prostate tumors has implicated the c-Myc
10 protooncogene in this pathology. Amplification of c-Myc is observed in 29% of all prostatic carcinomas, and overexpression of the protein is also observed in melanomas, primary human colon adenocarcinoma, and multiple myelomas. Amplification of chromosome 8 is also observed in prostate cancer tumors and is of particular interest because the c-Myc protooncogene maps to this region at 8q24. Fluorescence in situ
15 hybridization (FISH), comparative genome hybridization (CGH) and immunocytochemistry have all demonstrated a consistent amplification of c-Myc copy number as well as an increase in c-Myc immunoreactivity in the foci derived from prostatic intraepithelial neoplasia (PIN), localized prostatic carcinomas and metastases. The fact that amplification of c-Myc can be observed in PIN also raises the possibility
20 that c-Myc activation may act as an initiating event for prostate cancer development. Taken together, these data provide evidence that c-Myc plays a key role in the development and onset of prostatic as well as many other cancers.

Mutations in the MXI1 gene also occur in primary prostate cancer tumors (see, e.g. Eagle et al., (1995) Nat Genet 9, 249-55). As this gene can negatively regulate the
25 effects of c-Myc, this data suggests that changes in Mxi activity in prostate cancer cells could disrupt the normal function of c-Myc. Similarly, Mxi knockout mice display prostatic hyperplasia. Taken together, this data provides further evidence that increased c-Myc activation may play a role in prostate cancer progression.

A number of transgenic models of prostate cancer have been reported in the
30 literature (see, e.g. U.S. Patent Nos. 5,907,078, 5,917,124, 6,323,390 and Zhang et al., The

Prostate 43: 278-285 (2000), Buttyan et al., Cancer Metastasis Review 12: 11-19 (1993), Shibata et al., Toxicol Pathol 26: 177-183 (1998) and Greenberg et al., Mol Endocrinol 8: 230-239 (1994) which are incorporated herein by reference). Prostate cancer transgenic mice known in the art include the TRAMP model expressing the viral oncogenes large and small T antigen, probasin driving large T antigen, C3 driving c-Myc, probasin driving 5 androgen receptor, probasin driving IGF-1, probasin driving NAT I and NAT2, probasin driving Ras, C3 driving the SV40 large T antigen, C3 driving the SV40 T early region, C3 driving the polyoma virus middle T gene, C3 driving Bcl-2, MMTV LTR driving the SV40 T antigen, MMTV LTR driving int2/fgf3 and wap, CR2 driving the 10 SV40 T antigen, fetal G- γ globin driving the SV40 large T antigen, ARR₂PB driving the CAT reporter gene, probasin driving Bcl-2, and gp91-phox driving the SV40 early region.

Significantly, the c-myc transgenic mice known in the art, do not exhibit a phenotype which parallels the phenotype that is observed in human cancers of the prostate. Consequently there is a need in the art for c-myc animal models that reproduce 15 the malignant phenotype observed in cancers of the prostate. The invention disclosed herein satisfies this need.

SUMMARY OF THE INVENTION

The invention disclosed herein provides a transgenic mouse that having tissue specific overexpression of the protooncogene, c-Myc in the prostate. These transgenic 20 mice harbor a c-myc transgene under the regulatory control of the naturally occurring prostate specific probasin promoter, or alternatively, a composite probasin promoter comprising two androgen receptor binding sites. These probasin promoters drive high level expression of c-Myc in the prostate at the onset of puberty (approx. 6-7 weeks) when androgen production begins. The transgenic mice having such constructs develop 25 high grade prostatic intraepithelial neoplasia (PIN) as early as 10 weeks of age. Consequently, embodiments of the invention disclosed herein can be used as an in vivo model system for the study of prostate cancer and its progression. In addition, embodiments of the invention disclosed herein can be used in preclinical and clinical

models to test novel diagnostic and therapeutic modalities such as drug therapies relevant to prostate cancer prevention and its progression.

The invention disclosed herein has a number of embodiments. A typical embodiment of the invention is a transgenic mouse whose genome comprises a nucleic acid construct comprising a promoter having the probasin regulatory element shown in SEQ ID NO: 1, wherein the promoter is operably linked to c-myc as shown in SEQ ID NO: 2 such that the c-myc protein encoded therein is expressed in prostate cells of the transgenic mouse at detectable levels. In certain embodiments of the invention, the promoter further comprises the sequence shown in SEQ ID NO: 4 or the sequence shown SEQ ID NO: 5.

Preferred transgenic mice of the invention exhibit a number of phenotypic characteristics. In one embodiment, the transgenic mice develop overt prostatic anaplasia characterized by prostatic intraepithelial neoplasia. In a closely related embodiment, the mice develop a prostatic intraepithelial neoplasia that further progresses to invasive adenocarcinoma. Typically in this embodiment, the adenocarcinomas that develop in these mice exhibit a lack of immunostaining with anti-synaptophysin antibody.

The adenocarcinoma cells that develop in these transgenic mice of the invention exhibit a number of molecular characteristics that observed in clinical specimens of human prostate cancer. In one such embodiment, the adenocarcinoma cells exhibit a decreased expression of the NKX3.1 gene (SEQ ID NO: 6) as compared to cells of the prostatic intraepithelial neoplasia. In yet another embodiment of the invention, the prostate cells of the transgenic mouse exhibit an increased expression of the Pim 1 gene (SEQ ID NO: 7) as compared to the prostate cells from a non-transgenic mouse of the same murine strain.

A related embodiment of the invention is a transgenic mouse susceptible to prostate tumor formation having a genomically-integrated nucleic acid molecule comprising a first segment which is a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and a second segment which is the c-myc gene shown in SEQ ID NO:2, the first and second segments being

operatively linked such that said mouse expresses the c-myc protein specifically in the prostate of the mouse to effect tumor formation therein. In certain embodiments of the invention, the 5' regulatory element further comprises at least two androgen response elements having the sequence shown in SEQ ID NO: 13.

5 In preferred embodiments, these transgenic mice of the invention exhibit a number of phenotypic characteristics. In one embodiment, the transgenic mice develop prostatic intraepithelial neoplasia, wherein the cells that make up lesions of the prostatic intraepithelial neoplasia exhibit large irregular nuclei, a hyperchromatic vesicle pattern, a vesicular chromatin pattern, prominent nucleoli and an amphophilic cytoplasm. In a
10 closely related embodiment, the mice develop a prostatic intraepithelial neoplasia that further progresses to invasive adenocarcinoma characterized by the numerous nests of acini consisting of cytologically atypical cells extending into prostatic stroma and periprostatic adipose tissue. Typically in this embodiment, the cells of these adenocarcinomas do not exhibit morphological features of neuroendocrine carcinomas
15 as shown by a lack of immunostaining with either anti-synaptophysin antibody or anti-chromogranin A antibody.

 The adenocarcinoma cells that develop in these transgenic mice of the invention exhibit a number of molecular characteristics that observed in clinical specimens of human prostate cancer. In one embodiment, the adenocarcinoma cells exhibit a
20 decreased expression of the NKX3.1 gene (SEQ ID NO: 6) as compared to cells of the prostatic intraepithelial neoplasia. In yet another embodiment of the invention, the prostate cells of the transgenic mouse exhibit an increased expression of the Pim 1 gene (SEQ ID NO: 7) as compared to the prostate cells from a non-transgenic mouse of the same murine strain.

25 Yet another embodiment of the invention consists of prostate cells derived from the transgenic mice disclosed herein. A typical example of this embodiment of the invention is an immortal cell line derived from prostatic tissue of a transgenic mouse of the invention and representing a stage of progression of prostate cancer. Preferred
 embodiments include immortalized intraepithelial neoplasia cells and immortalized
30 adenocarcinoma cells.

Yet another embodiment of the invention consists of a method of making a transgenic mouse wherein the transgene comprises c-myc under the regulatory control of the probasin promoter. A typical embodiment of the invention is a method of making a transgenic mouse having a phenotype characterized by the development of prostatic intraepithelial neoplasia, the method comprising introducing into the mouse genome a
5 nucleic acid molecule comprising a first segment which is a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and a second segment which is the c-myc gene shown in SEQ ID NO:2, so that said transgenic mouse is made.

10 Yet another embodiment of the invention consists of a method for screening a compound for antitumor activity, comprising administering the compound to a transgenic mouse wherein the transgene comprises c-myc under the regulatory control of the probasin promoter, and then monitoring the antitumor activity of said compound. A related embodiment of the invention is a method for screening a cancer treatment for
15 antitumor activity, comprising administering the compound to a transgenic mouse wherein the transgene comprises c-myc under the regulatory control of the probasin promoter, and monitoring the antitumor activity of said compound. A preferred embodiment of the invention is a method of testing compounds for an effect on prostate tumors, the method comprising, administering the compound to be tested to a transgenic
20 mouse having a genomically-integrated nucleic acid molecule comprising a first segment which is a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and a second segment which is the c-myc gene shown in SEQ ID NO:2, wherein the transgenic mouse develops prostate tumors and then comparing one or more characteristics of the prostate tumors in the transgenic
25 mouse to which the compound was administered with the same one or more characteristics of the prostate tumors in the transgenic mouse to which the compound has not been administered, wherein a difference in one or more of the one or more characteristics indicates that the compound has an effect on prostate tumors.

Yet another embodiment of the invention consists of a method of identifying
30 one or more markers associated with prostate cancer. An illustrative embodiment

consists of a method comprising comparing the presence, absence, or level of expression of genes in prostatic tissue from a transgenic mouse and prostatic tissue from a second mouse, wherein the genome of the transgenic mouse comprises a nucleic acid construct and the genome of the second mouse does not comprise the nucleic acid construct, wherein the construct comprises a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and the c-myc gene shown in SEQ ID NO:2, wherein and wherein the c-myc gene is expressed in the prostate cells of the transgenic mouse such that the transgenic mouse develops prostate tumors, wherein the difference between the transgenic mouse and the second mouse in the presence, absence, or level of expression of a gene indicates that the expression of the gene is a marker associated with prostate cancer.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the generation and characterization of c-Myc transgenic mice. Figure 1(A) shows the construction of the c-Myc-Lo and c-Myc Hi transgene. cDNA encoding human c-Myc was cloned along with the insulin polyA site downstream of either the rat probasin or the modified small composite probasin promoters. Primers specific to each promoter and human c-Myc were used to confirm germline transmission of the transgene. Figure 1(B) shows the comparative expression of c-Myc in the mouse prostate. Total protein was isolated from 2 and 4 week old wildtype, Myc-Hi and Myc-Lo mice. C-Myc expression was determined by western blot using the human specific α 9E10 c-Myc antibody. Transgene expression is seen as early as 2 weeks in both transgenic mice and expression increases as mice reach puberty by 4 weeks. β -actin was used as a loading control. Figure 1 (C) shows the kinetics of mPIN and cancer progression in Myc-Hi and Myc-Lo transgenic mice. Myc-Hi mice exhibit mPIN as early as 2 weeks compared to 4 weeks in the Myc-Lo transgenic animals. Myc-Hi mice go on to develop invasive prostatic adenocarcinoma by 6 months whereas the Myc-Lo animals continue to exhibit mPIN at the same timepoint as observed with hematoxylin and eosin (H&E).

Figure 2 shows the development of cancer in Myc-Hi and Myc-Lo transgenic animals without neuroendocrine differentiation. Figure 2(A) shows the longer latency for cancer progression in Myc-Lo mice. Prostates from 6 and 12 month transgenic mice reveal invasive adenocarcinoma at 12 months in the Myc-Lo animals. In contrast, invasive tumor is already apparent at 6 months in the Myc-Hi mice (H&E). Figure 2(A) shows the presence of an intact fibromuscular layer, as shown by the positive smooth muscle actin (SMA) immunostaining (arrow, left panel) confirms the in-situ nature of the mPIN lesions. In contrast, SMA staining is absent in the invasive tumors (contractile smooth muscle surrounding blood vessels serve as an internal positive control, arrow-right panel). Figure 2(C) shows that tumors do not undergo neuroendocrine differentiation. Synaptophysin immunostaining identifies the rare neuroendocrine cell normally present in murine prostates (positive staining is also seen in periprostatic ganglion, insert right panel). The invasive adenocarcinoma shows absence of synaptophysin immunostaining (right panel).

Figure 3 shows that c-myc expression gives a proliferative advantage to prostate tissue. Figure 3(A) shows that Ki67 and TUNEL labeling revealed an increase in both proliferation and apoptosis as cells progress from normal to mPIN and finally invasive cancer. Figure 3(B) shows that proliferation outpaces apoptosis in mPIN and invasive cancer. A total of 500 cells were counted from 5 high power fields and the number of Ki67 positive cells (proliferative index) and apoptotic bodies (apoptotic index) were scored and graphed.

Figure 4 shows the evaluation of vascular profile in myc tumors. Figures 4(A-I) shows mice were injected with FITC-conjugated *Lycopersicum esculentum* lectin that binds to the luminal surface of blood vessels. Animals were subsequently perfused with paraformaldehyde, prostates were dissected, sectioned on vibrotome and mounted on histological slides with glycerol containing Topo 3 for visualization of nuclei (in blue). A-C, wild-type animals (A- 2 month, B and C - 1 year). Note that the blood vessels travel in the stroma juxtaposed to the prostate epithelium. There is no increase in vascular density in the prostate of 2 month versus 1 year old wild-type mice; D-F - transgenic animals (2 months). Increased vascular density and tortuosity in vessels is associated with

early transformation stages, G-I - transgenic animals (1 year). Figure 4(J) shows a higher degree of vascular density and disorganization characterizes late stage tumors.

Figure 5 shows the effects of castration on Myc-induced mPIN and prostate cancer. Figure 5(A) shows a schematic of castration experiment on Myc-Hi transgenic mice. Mice were either castrated at 2 months (*) of age with mPIN or castrated at 8 months (*) after tumors had developed. Figure 5(B) shows that castration causes the reversion of mPIN lesions 1 month post-surgery. However, once invasive tumors are established, castration results in only partial regression of the primary lesion, when examined at 1 and 3 months post-surgery. Figure 5(C) shows that androgen independent tumor formation following castration at 8 months. Ki67 immunohistochemistry shows a dramatic decrease in the proliferative rate in 3 month castrates. The decrease was not as evident in 8 month castrates and proliferation began to increase 3 months post surgery indicating the regrowth of androgen independent tumors.

Figure 6 shows that microarray analysis identifies a distinct expression signature of genes in c-myc transgenic animals. Figure 6(A) shows unsupervised clustering classifies samples as either wild-type (wt) or transgenic (mPIN and cancer) with one exception. Figure 6(B) shows genes differentially expressed between wild-type and transgenic mice. The top 60 non-EST genes are included, a full list is available in Supplementary Information. The list includes two genes known to be involved in human prostate cancer, Nkx3.1 and Pim-1 (arrows). Nkx3.1 expression is lost in transgenic samples and Pim-1 is upregulated consistent with published human data (see, e.g. Bowen *et al.* (2000) *Cancer Res* 60, 6111-6115; Dhanasekaran *et al.*, (2001) *Nature* 412, 822-826). Expression values of each gene are normalized to have a mean of zero and standard deviation of one across the samples. Figures 6 (C, D) show that Nkx3.1 protein levels diminish in mPIN lesions and are lost in tumors samples consistent with microarray data. Immunohistochemistry (C) and western blot analysis (D) using Nkx3.1 antibody shows a consistent loss of Nkx3.1 expression as tumors progress.

Figure 7 shows cross-species expression module comparison algorithm. Figure 7(A) shows a schematic of experimental design. Figure 7(B) shows the identification of genes differentially expressed between Myc-like and non-Myc-like human tumors in

multiple datasets. In the graph, each point represents a gene found in both the Prostate A (see, e.g. Dhanasekaran et al., (2001) *Nature* 412, 822-826) and Prostate B (see, e.g. Welsh et al., (2001) *Cancer Res* 61, 5974-5978) human cancer datasets. For each gene, the Student t-test P-values reflecting the degree of differential gene expression between
5 Myc-like and non-Myc-like human tumors (see schematic) were determined and plotted. Genes below the line have a P-value product less than 5×10^{-7} and are listed in Table 2 (red squares and yellow triangle (Pim1) (scatter plot).

DETAILED DESCRIPTION OF THE INVENTION

10 Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are further defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be
15 construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, 2d ed.,
20 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y and Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience Publishers, (1995). As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

25

I. PROBASIN-MYC TRANSGENIC MODEL OF PROSTATE CANCER

The invention disclosed herein provides a transgenic mouse that having probasin promoter mediated tissue specific overexpression of the protooncogene, c-Myc in the
30 prostate. This overexpression confers high grade prostatic intraepithelial neoplasia (PIN)

as early as 10 weeks of age. Consequently, transgenic animals of the invention disclosed herein can be used as an in vivo model system for the study of prostate cancer and its progression. In addition, embodiments of the invention disclosed herein can be used in preclinical and clinical models to test novel diagnostic and therapeutic modalities such as
5 drug and dietary therapies relevant to prostate cancer prevention and its progression. Alternatively, cells from such animals can be isolated, cultured, and contacted with compounds to be screened. Both embodiments significantly accelerate the rate at which new anticancer drugs and treatments can be evaluated.

In an illustration of the present invention as shown in the Examples below a
10 transgenic animal has been created for myc-dependent tumorigenesis. One embodiment of the invention provides a model for tumorigenesis of prostate tumors. The animal is a rodent and in a preferred embodiment, a mouse. As illustrated below we use a probasin promoter or a modified small composite probasin promoter, ARR₂Pb (a core probasin promoter along with two additional androgen response elements), (see, e.g. Zhang et al.,
15 2000; as well as Wu et al., Mech Dev. 2001 Mar;101(1-2):61-9; Andriani et al., J Natl Cancer Inst. 2001 Sep 5;93(17):1314-24; Lowe et al., Gene Ther. 2001 Sep;8(18):1363-71; and Rubinchick et al., Mol Ther. 2001 Nov;4(5):416-26) to drive expression of c-Myc specifically in the prostate. Significantly, this promoter driven c-Myc expression results in mouse prostatic intraepithelial neoplasia (mPIN), which further progresses to become
20 invasive adenocarcinoma. In addition, these tumors are architecturally and morphologically very similar to human prostatic adenocarcinoma

As noted herein, the present invention provides transgenic animals with a modulated phenotype from that of the original/initial transgenic animals. The modulation is an enhancement of the original observed phenotype seen in the initial
25 transgenic animals. By modulation is meant that the characteristic phenotype shown by the transgene is more pronounced, appears earlier or later; and where protein is produced more or less protein is produced than the parent strains or the like. Where earlier or accelerated, it is meant that the observed phenotype is seen at least one month earlier in the life-span than the phenotype in the parental strain or similarly for later appearance.
30 For example, a modulated phenotype for a human disease model (e.g. prostate cancer)

would show a pathology associated with the disease that more accurately reflects the human pathologic state including having more of the characteristics of the disease than the initial transgenic animal parental strains or the like. Alternatively, a modulated phenotype could reflect a faster or slower onset of the pathology of the human disease.

5 Offspring with the modulated phenotype are utilized in animal models as for example testing of treatment modalities in a disease model or for pathogen susceptibility. The transgenic parent can carry an overexpressed sequence, either the nonmutant or a mutant sequence and humanized or not as required. The term transgene is therefore used to refer to all these possibilities. Cells can also be isolated from the offspring which carry a

10 transgene from each transgenic parent and that are used to establish primary cell cultures or cell lines as is known in the art.

A transgenic strain can be heterozygous or homozygous for the transgene and is produced as is known in the art. Any method can be used which provides for stable, inheritable, expressible incorporation of the transgene within the nuclear DNA of an

15 animal. These transgenic animals are constructed using standard methods known in the art and as set forth for example in U.S. Pat. Nos. 5,614,396 5,487,992, 5,464,764, 5,387,742, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,384, 5,175,383, 4,873,191, 4,736,866 as well as PCT patent applications WO 94/23049, WO 93/14200, WO 94/06908, WO 94/28123.

20 More specifically, any techniques known in the art can be used to introduce the transgene expressibly into animals to produce the parental lines of animals. Such techniques include, but are not limited to, pronuclear microinjection (see, e.g. U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines; gene targeting in embryonic stem cells (see, e.g. U.S. Pat. No. 5,614,396); electroporation of embryos; and

25 sperm-mediated gene transfer.

II. CHARACTERIZATION OF PROBASIN-MYC TRANSGENIC RODENT MODELS OF PROSTATE CANCER

The mouse has long been exploited as a model to study the molecular basis of

30 human cancers and test novel therapies. Recent advances in mouse engineering techniques and in genomics tools to query mouse tumor tissue allow a more global

comparison of these models to human cancer. We have addressed these issues in a novel transgenic model of mouse prostate cancer using the human c-Myc gene as the initiating event. Myc has been widely implicated in many human cancers (see, e.g. Nesbit et al., (1999) *Oncogene* 18, 3004-3016) and is sufficient to give cancer phenotypes in various murine tissues when expressed as a transgene (see, e.g. Jensen et al., (2002) *J Biol Chem*; Moroy et al., (1991) *Oncogene* 6, 1941-1948; Nesbit et al., (1999) *Oncogene* 18, 3004-3016; Pelengaris et al., (2002) *Nat Rev Cancer* 2, 764-776; Pelengaris et al., (2002) *Cell* 109, 321-334; Pelengaris et al., (1999) *Mol Cell* 3, 565-577; Zhang et al., (2000) *Prostate* 43, 278-285). We focused our efforts on the role of c-Myc in prostate cancer for two reasons. First, several independent, publicly available gene expression profiling datasets of human prostate tumors (see, e.g. Dhanasekaran et al., (2001) *Nature* 412, 822-826; Magee et al., (2003) *Cancer Cell* 3, 273-283; Singh et al., (2002) *Cancer Cell* 1, 203-209; Welsh et al., (2001) *Cancer Res* 61, 5974-5978) are available for comparison to mouse models. Second, the functional role of Myc in human prostate cancer remains undefined. Numerous studies of human prostate cancer have demonstrated increased c-Myc gene copy number in up to 30% of tumors, even at the preneoplastic stage called prostate intraepithelial neoplasia (PIN) (see, e.g. Jenkins et al., (1997) *Cancer Res* 57, 524-531; Nesbit et al., (1999) *Oncogene* 18, 3004-3016; Qian, J., Jenkins, R. B., and Bostwick, D. G. (1997) *Mod Pathol* 10, 1113-1119; Sato et al., (1999) *J Natl Cancer Inst* 91, 1574-1580). However, Myc is only one of many genes localized to the 8q24 amplicon (see, e.g. Elo et al., (2001) *Ann Med* 33, 130-141).

Efforts to define mechanisms by which Myc induces cancer have found a number of effects such as increased cell proliferation that clearly contribute to tumorigenesis. Among the most perplexing has been the well documented pro-apoptotic activity of Myc, particularly when serum or other survival factors are limiting (see, e.g. Ahmed et al., (1997) *Proc Natl Acad Sci U S A* 94, 3627-3632; Evan et al., (1992) *Cell* 69, 119-128; Pelengaris et al., (2002) *Cell* 109, 321-334; Prendergast, G. C. (1999) *Oncogene* 18, 2967-2987). Recent transgenic models have clarified how the seemingly paradoxical death-promoting activity of c-Myc can lead to cancer. Expression of a hormone-regulated Myc transgene in the skin rapidly induces epidermal hyperplasia and

papillomas, with associated increases in cellular proliferation and minimal evidence of apoptosis (see, e.g. Pelengaris et al., (2002) *Nat Rev Cancer* 2, 764-776; Pelengaris et al., (1999) *Mol Cell* 3, 565-577). However, these tumors rapidly apoptose when cultured ex vivo, presumably due to the lack of critical survival factors present in the mouse dermis.

5 In contrast, transgenic expression of c-Myc in the pancreas caused rapid involution of insulin-producing islet cells, due to increased apoptosis, and subsequent onset of diabetes (see, e.g. Pelengaris et al., (2002) *Nat Rev Cancer* 2, 764-776; Pelengaris et al., (2002) *Cell* 109, 321-334). When complemented with a BclX_L transgene, the mice no longer display the apoptotic phenotype, but develop aggressive islet cell cancers. These experiments
10 demonstrate that the response to Myc expression in different tissues is critically dependent on associated survival signals and suggest a role for secondary cooperating events to rescue cells from Myc-induced apoptosis.

To determine the consequence of increased c-Myc expression in the prostate, we generated transgenic mice expressing human c-Myc from two different strength prostate
15 specific promoters. In all founder lines, c-Myc expression resulted in complete penetrance of mouse prostatic intraepithelial neoplasia (mPIN), which progressed to invasive adenocarcinoma within 6-12 months of age. mPIN lesions were observed as early as 2-4 weeks, providing evidence that c-Myc is sufficient to induce a preneoplastic phenotype in the prostate. These cells demonstrated a high rate of proliferation that
20 overcame the apoptotic effect of c-Myc expression suggesting that prostate tissue contains survival factors that allow the cells to tolerate increased Myc protein. Microarray expression profiling studies defined a Myc expression signature in the mouse prostate that shares features with human prostate cancer and implicates candidate genes in tumor progression. For example, expression of the putative human prostate tumor
25 suppressor gene NXX3.1 (see, e.g. Bowen *et al.* (2000) *Cancer Res* 60, 6111-6115; He et al., (1997) *Genomics* 43, 69-77; Voeller et al., (1997) *Cancer Res* 57, 4455-4459) was detected in Myc-induced mPIN lesions but absent in invasive cancers. Furthermore, analysis of human cancer databases with the murine Myc gene signature uncovered gene clusters whose expression was tightly correlated in human prostate and breast cancer.
30 The murine Myc signature genes most consistently coexpressed in the human cancers

included Pim1 previously shown to cooperate with Myc in mouse tumor models. Collectively, this disclosure provides a novel transgenic mouse model of prostate cancer and demonstrate the utility of comparing mouse and human cancer expression databases.

5 III. CHARACTERISTICS OF PROBASIN-MYC TRANSGENIC MICE

A. Transgenic Expression Of C-Myc In The Prostate Induces MPIN, Then Invasive Adenocarcinoma With Reproducible Kinetics And High Penetrance.

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Two different prostate-specific transgenic expression constructs ((1) probasin-Myc; and (2) ARR₂/probasin-Myc, respectively), were used in the transgenic mice disclosed herein in order to vary the dosage of c-Myc expression specifically in the prostate (Figure 1A). Expression from the probasin promoter begins at a low level in the prostate at 1-2 weeks of age and increases with rising levels of androgen as the mice reach puberty between 4-8 weeks (Figure 1B). The ARR₂/probasin promoter contains two additional androgen response elements which boost the level of androgen-dependent expression (Figure 1B) (see, e.g. Wu, X et al., (2001) *Mech Dev* 101, 61-69; Zhang et al., (2000) *Endocrinology* 141, 4698-4710). Multiple founders were obtained for each construct, prostate-specific c-Myc protein expression was confirmed and independent lines from each construct were expanded for phenotypic analysis. Based on the levels of transgene expression, the probasin-Myc mice and ARR₂/probasin-Myc mice are designated Lo-Myc and Hi-Myc, respectively. Both Lo-Myc and Hi-Myc mice showed histologic evidence of mouse PIN (mPIN) at 4 weeks of age. Of note, mPIN was present in the Hi-Myc mice as early as 2 weeks (Figure 1C, Table 1). Specifically, multifocal proliferative lesions affecting several ductules within the individual lobes were observed in the dorsolateral and ventral lobes and to a lesser extent in the anterior lobe. Cribiform and tufting growth patterns of the secretory epithelial layer were observed in the mPIN lesions. Unlike epithelial hyperplasia, which can also assume these architectural patterns, the cells that make up these lesions exhibited progressive nuclear atypia, demonstrated by the presence of large irregular nuclei, both hyperchromatic and a

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vesicular chromatin pattern, prominent nucleoli and an amphophilic cytoplasm. Finally, the in-situ nature of these lesions was confirmed by the presence of an intact fibromuscular layer, demonstrated by positive smooth muscle actin (SMA) immunohistochemical staining (Figure 2B, left panel).

- 5 The mPIN lesions in both Lo-Myc and Hi-Myc mice subsequently progressed to invasive adenocarcinomas, as seen by the extension of numerous nests of acini consisting of cytologically atypical cells into the prostatic stroma and periprostatic adipose tissue. These acini exhibit crowding, irregular contours and haphazard growth patterns (Figure 1C and 2A). The mPIN/cancer transition was evident by 3-6 months in the Hi-Myc mice and by 10-12 months in the Lo-Myc mice, suggesting that the dosage of Myc may affect the rate of disease progression (Figure 2A). Invasion was confirmed by the absence of SMA staining, which documents penetration through the fibromuscular layer (Figure 2B, right panel). Foci suggestive of lymphovascular invasion were also noted in some cancers and mPIN was identified focally in glands adjacent to the invasive tumor.
- 10 The penetrance of mPIN and cancer was essentially 100 percent in all founder lines with reliable kinetics (Table 1), indicating the potential utility of this model for studying progression from mPIN to cancer and for preclinical therapeutic studies.

- 15 To date, the only known murine models of prostate cancer that progress beyond the mPIN stage are the SV40 large T antigen models of prostate cancer (TRAMP, C3-Tag and LADY) (see, e.g. Garabedian et al., (1998) *Proc Natl Acad Sci U S A* 95, 15382-15387; Greenberg et al., (1995) *Proc Natl Acad Sci U S A* 92, 3439-3443; Kasper et al., (1998) *Lab Invest* 78, i-xv; Masumori et al., (2001) *Cancer Res* 61, 2239-2249; Shibata et al., (1996) *Cancer Res* 56, 4894-4903). Shortcoming of the T antigen models include the high frequency of neuroendocrine differentiation that occurs, as recognized by its typical histologic features and subsequent confirmation by immunohistochemical stains such as synaptophysin or chromogranin A (see, e.g. Masumori et al., (2001) *Cancer Res* 61, 2239-2249). Although human prostate cancers can occasionally possess a completely neuroendocrine phenotype (e.g. small cell carcinoma), the majority do not.

- 25 In contrast to the T antigen transgenic animals, the mPIN and invasive carcinoma lesions detected in the Lo-Myc and Hi-Myc mice do not exhibit the
- 30

morphologic features of neuroendocrine carcinomas and this is further confirmed by the lack of immunostaining with synaptophysin (Figure 2C). Therefore, the Lo- and Hi-Myc mice represent new models for human prostatic adenocarcinoma that offer advantages over current models by providing a phenotype that parallels that observed in human prostate cancer.

B. Myc Expression In The Mouse Prostate Induces Proliferation, Apoptosis And Angiogenesis.

Myc can induce both proliferation and apoptosis (see, e.g. Amati et al., (1998) *Front Biosci* 3, D250-268; Dang, C. V. (1999) *Mol Cell Biol* 19, 1-11; Pelengaris et al., (2002) *Nat Rev Cancer* 2, 764-776; Pelengaris et al., (2002) *Cell* 109, 321-334; Prendergast, G. C. (1999) *Oncogene* 18, 2967-2987). To understand the cellular effects of c-Myc overexpression in the prostate, we tested for proliferation using Ki67 immunohistochemistry and apoptosis using TUNEL assays. Immunohistochemistry showed an increase in Ki67 staining in both mPIN and tumor lesions when compared to wildtype samples as shown in figure 3A. Ki67 staining was quantified by counting a total of 500 cells from 5 high power fields. The proliferative index increased from 20 in wildtype cells to 140 in mPIN lesions and 180 in tumor tissue (Figure 3B). The increased number of Ki67 positive cells indicates that these are rapidly proliferating lesions (Figure 3A, B). TUNEL assays performed on the same tumor showed that c-Myc was also capable of inducing apoptosis in the mouse prostate. Similar to the Ki67 assay, 500 cells were counted and those exhibiting apoptosis were scored. Decreased numbers of positive apoptotic bodies were seen in both mPIN (100 vs. 140) and tumor tissue (100 vs. 175) when compared to Ki67 staining (Figure 3B). These results demonstrate that c-Myc induces both proliferation and apoptosis in the mouse prostate. The rapid development of mPIN suggests that, like epidermal cells, the prostate may contain survival signals that rescue much of the gland from Myc-induced apoptosis.

Myc can also induce angiogenesis in certain tissues, a property that likely contributes to tumor progression and metastasis (see, e.g. Elenbaas et al., (2001) *Genes Dev* 15, 50-65; Hurlin et al., (1995) *Oncogene* 11, 2487-2501; Pelengaris et al., 1999;

Watnick et al., (2003) *Cancer Cell* 3, 219-231). To determine if Myc induces angiogenesis in the mouse prostate, wild-type and transgenic animals at age 2 months and 12 months were evaluated for alterations in the vascular pattern associated with tumor progression (Figure 4). Blood vessels were identified in green after perfusion with FITC-conjugated
5 *Lycopersicum esculentum* lectin as previously described (see, e.g. Rodriguez-Manzaneque et al., (2001) *Proc Natl Acad Sci U S A* 98, 12485-12490). An increase in vascular density was evident at age 2 months in transgenic animals and was associated with mPIN. Vascular density doubled with progression to invasive adenocarcinoma at the 1 year time point. These findings demonstrate that Myc can induce an angiogenesis
10 program in the mouse prostate that is associated with disease progression.

C. Effects Of Hormone Ablation On The Initiation And Maintenance Of Myc-Induced Prostate Lesions.

As hormone ablation therapy is the primary clinical treatment for prostate cancer patients with advanced stage disease, we examined the effect of castration on disease
15 progression in the Hi-Myc transgenic mice. Since the ARR₂Pb promoter is regulated by androgen, interpretation of the effect of hormone ablation is confounded by potential silencing of the transgene. Mice were either castrated at 2 months when they had definite mPIN lesions or at 8 months after tumors had developed, then analyzed either one month or three months post-castration (Figure 5A). Complete regression of mPIN was
20 observed in all three mice within one month of castration (Figure 5B) and was correlated with absence of detectable transgene expression by immunoblot. This result indicates that Myc-induced mPIN is reversible, similar to other Myc-induced neoplastic phenotypes in tetracycline-regulated models (see, e.g. Jain et al., (2002) *Science* 297, 102-104; Karlsson et al., (2003) *Blood* 101, 2797-2803). Conversely, mice with prostate
25 cancer (castrated at 8 months of age) had residual tumor at one and three months post-castration, although there was histologic evidence of partial regression and fibrosis (Figure 5B). Immunoblot studies demonstrated evidence of Myc expression in residual tumor, but precise measures of transgene expression were complicated by sample heterogeneity. The residual tumor foci retained high levels of proliferation post-
30 castration that increased with time, as measured by Ki67 immunohistochemistry (Figure

5C). Larger cohorts of mice will be followed to determine if these mice eventually relapse with progressive, hormone refractory prostate cancer.

D. Expression Signature Of Myc-Driven Murine Prostate Cancer.

5 The high penetrance and reliable kinetics of the PIN/cancer transition in the Myc models disclosed herein provides an experimental opportunity to define the cooperating molecular events involved in Myc-driven prostate cancer progression. In this context, we isolated dorsal, lateral and anterior prostate from Hi-Myc transgenic mice and non-transgenic littermate controls at various timepoints during the mPIN/cancer transition
10 for gene expression profiling experiments. Samples were divided for parallel analysis by mouse Affymetrix arrays for gene expression and comparative genome hybridization arrays (array CGH) for chromosomal gains and losses. Matched tissue was saved for histological and immunohistochemical studies. Remarkably, the expression signatures for wild-type, mPIN and cancer were strong enough to be recognized by unsupervised
15 clustering with only one error (Figure 6A). Initially, we were puzzled by one potential outlier in the mPIN group - a Hi-Myc mouse harvested at age 9 months who was presumed to have cancer based on the kinetics of the model (Figure 6A, see asterisk). However, histologic review of the tissue sections showed that, in fact, this mouse had mPIN without cancer, indicating that the gene expression signature is extremely powerful
20 at recognizing these distinct stages of tumor progression. Parallel array CGH experiments were also conducted using genomic DNA from these samples to look for evidence of chromosomal gains or losses that might accompany these expression changes. To date, we have not observed any genomic changes in mPIN lesions or cancers, but it is important to note that current mouse BAC arrays are limited to 3
25 megabase resolution and we cannot rule out smaller gains or losses.

 Next we generated a supervised gene list that distinguishes wild-type mice from Myc transgenic mice. We ranked genes by the degree of differential expression between wild-type and transgenic mice using the Student's t-test. The 60 most differentially expressed genes, excluding ESTs, are shown in Figure 6B, and the complete list is
30 available in Supplementary Information. The genes on this list can potentially come

from several categories, including genes modulated generally in tumorigenesis, or specifically (see, e.g. Coller et al., (2000) Proc Natl Acad Sci U S A 97, 3260-3265) in prostate tumorigenesis or Myc-driven tumorigenesis. Additionally, the Myc-specific genes can either be Myc transcriptional targets or genes whose up or down regulation complements Myc expression during tumorigenesis. Since the *Myc* transgene is expressed as early as 1-2 weeks of age, the Myc target genes can be either directly or indirectly regulated by Myc transcription. In an attempt to address this issue, we compared our list of Myc-driven tumor associated gene changes to various lists of Myc target genes and found that some genes and gene families are in common, but we were unable to demonstrate any statistically significant overlap. Thus, not unexpectedly, our list does not appear to be dominated by direct Myc transcriptional targets.

Several genes of interest appeared on the list and include L-Myc, normally expressed at high levels in differentiated prostate tissue (see, e.g. Luo et al., (2001) J Urol 166, 1071-1077), Tmprss2, a serine protease overexpressed in a majority of prostate cancer patients (see, e.g. Vaarala et al., (2001) Int J Cancer 94, 705-710), Sparc, an antiadhesive protein that is differentially expressed during human prostate cancer progression (see, e.g. Thomas et al., (2000) Clin Cancer Res 6, 1140-1149), EGF which has been implicated in prostate cancer progression (see, e.g. Kim et al., (2003) Clin Cancer Res 9, 1200-1210), and several Ly6 genes which belong to the same family as prostate stem cell antigen (PSCA), a cell surface antigen overexpressed in human prostate cancer (see, e.g. Jalkut et al., (2002) Curr Opin Urol 12, 401-406) (Figure 6B, asterisks). Among the most interesting genes are Nkx3.1 and Pim-1, for the reasons discussed below (Figure 6B, arrows).

E. Loss Of Nkx3.1 Protein Expression Marks The Transition From mPIN To Invasive Cancer.

The microarray finding of reduced levels of NKX3.1 mRNA in transgenic mice is of particular interest because human NKX3.1 is a putative tumor suppressor gene in human prostate cancer (see, e.g. He et al., (1997) Genomics 43, 69-77). Loss of heterozygosity at the NKX3.1 locus occurs commonly in human prostate tumors due to large deletions at 8p22, but it has proved difficult to directly implicate NKX3.1 as the

relevant gene since mutations do not occur in the remaining allele (see, e.g. Voeller et al., (1997) *Cancer Res* 57, 4455-4459). To distinguish between the possibility that NKX3.1 may be a Myc target gene (since decreased mRNA levels were found in transgenic mice with mPIN and cancer) versus a complementary secondary event, we examined NKX3.1 protein expression in situ using immunohistochemical studies. NKX3.1 protein expression was consistently present in mPIN lesions at variable levels but was undetectable in all the cancers (Figure 6C). Immunoblot studies of prostate lysates from tumor-bearing mice also showed a marked decrease in Nkx3.1 protein expression when compared to lysates from wildtype or mPIN mice (Figure 6D). These results indicate that NKX3.1 loss is distinct from the onset of Myc expression and raises the possibility that Myc gain and NKX3.1 loss may be critical cooperating events in the mPIN/prostate cancer transition.

F. Cross Species Bioinformatic Comparison Of Mouse And Human Datasets Implicates Pim-1 In Cancer Progression.

The large number of genes in the Myc signature emphasizes the need for strategies to prioritize genes for functional evaluation. Accordingly, we searched for gene expression patterns common to both our Myc transgene model and human prostate cancer. A schematic of our 'cross-species expression module comparison approach is shown in Figure 7. We began with our list of genes differentially expressed between wild-type and Myc-transgenic mice. We then identified the human orthologs for these genes using the HomoloGene database (<http://www.ncbi.nlm.nih.gov/HomoloGene/>), and determined which were present in several human cancer gene expression datasets. We ascertained whether the human tumors were more Myc-like or non-Myc-like using a weighted gene voting prediction algorithm (see, e.g. Golub *et al.* (1999) *Science* 286, 531-537), and then made ranked lists of the genes most differentially expressed between the human tumors in these two categories using the Student's t-test. Finally, we identified the genes most consistently differentially expressed between Myc-like and non-Myc-like human tumors based on the overlap between the ranked lists from different human datasets (Table 2a,b). In summary, this method both evaluates whether the coexpressed

mouse Myc signature genes are also coexpressed in human cancers, and identifies the most consistently regulated genes in the Myc expression module.

We first performed this analysis using two publicly available prostate cancer datasets (see, e.g. Dhanasekaran et al., (2001) *Nature* 412, 822-826; Welsh et al., (2001) *Cancer Res* 61, 5974-5978). The most striking result was the presence of Pim1 near the top of our list (Table 2, Fig. 7). Pim1 was on our original list of genes differentially expressed between wild-type and Myc transgenic prostates with a rank of 113 (Figure 5B, Supplementary Information). The reprioritization of Pim1 to a rank of 2 is noteworthy in that Pim1 has previously been shown to cooperate with Myc in lymphomagenesis (see, e.g. van Lohuizen et al., (1989) *Cell* 56, 673-682; van Lohuizen et al., (1991) *Cell* 65, 737-752), and suggests that this approach could be used with other transgenic cancer models to identify complementing oncogenes.

To investigate whether the Myc expression signature identified using our transgenic mice is prostate-specific, we added a breast cancer dataset to our cross-species comparison algorithm. We again found that one of the most consistently regulated genes in the Myc expression module is Pim1 (Table 2). This result is consistent with the fact that Myc expression is implicated in the progression of many tumor types (see, e.g. Nesbit et al., (1999) *Oncogene* 18, 3004-3016).

IV. TYPICAL EMBODIMENTS OF THE INVENTION

The invention disclosed herein has a number of embodiments. A typical embodiment of the invention is a transgenic mouse wherein the transgene comprises c-myc under the regulatory control of 5' regulatory sequence having the tissue specific activity to the probasin promoter. An illustrative embodiment of the invention is a transgenic mouse whose genome comprises a nucleic acid construct comprising a promoter having the probasin regulatory element shown in SEQ ID NO: 1, wherein the promoter is operably linked to c-myc as shown in SEQ ID NO: 2 such that the c-myc protein encoded therein is expressed in prostate cells of the transgenic mouse at detectable levels. The c-myc protein used herein is the well known human protooncogene having for example the NCBI accession no: P01106. The term

“promoter” as used herein refers to a nucleotide sequence on a DNA molecule that facilitates the transcription of a gene to which it is operatively coupled. In certain embodiments of the invention, the promoter further comprises the sequence shown in SEQ ID NO: 4 or the sequence shown SEQ ID NO: 5.

- 5 As is disclosed herein, an essential facet of the invention is the use of probasin based promoter sequences to express c-myc protein in the prostate of transgenic animals. An essential embodiment of the invention is a c-myc transgenic mouse wherein c-myc is under the transcriptional control of a probasin 5' regulatory sequence (e.g. a rat, murine or human probasin 5' regulatory sequence) which is used to express c-myc protein
- 10 specifically in the prostate of the transgenic mouse to effect tumor formation therein. Consequently, c-myc in the transgenic mice of the invention can be under the transcriptional control of a number of probasin promoter based regulatory elements. In one specific embodiment, c-myc is under the regulatory control the minimal probasin promoter based sequence comprising SEQ ID NO: 1. Alternatively, c-myc is under the
- 15 regulatory control the minimal probasin promoter based sequence comprising residues 141-455 of SEQ ID NO: 5. Alternatively, c-myc is under the regulatory control the probasin promoter based sequence comprising SEQ ID NO: 4. Alternatively, c-myc is under the regulatory control the composite probasin promoter based sequence (ARR2PB) comprising SEQ ID NO: 5.
- 20 Preferred embodiments of the transgenic mice of the invention exhibit a number of specific phenotypic characteristics. In one embodiment, the transgenic mice develop prostatic intraepithelial neoplasia by as early as 2-4 weeks of age (see, e.g. Table 2). Typically such transgenic mice develop invasive cancer by as early as 3-6 months of age (see, e.g. Table 2). The finding disclosed herein that mice that express a prostate-specific
- 25 c-myc transgene (e.g. probasin promoter-myc transgene) develop overt prostatic anaplasia (e.g. prostatic intraepithelial neoplasia and adenocarcinoma) is surprising in view of scientific articles that teach that prostate-specific c-myc transgenic animals do not develop overt cancer and that this consistent with observations that the c-myc gene product in and of itself is best known for its properties of cellular immortalization and
- 30 proliferative stimulation rather than direct transformational ability (see, e.g. Zhang et al.,

The prostate 43: 284 (2000); Nesbit et al., Oncogene 1999, 18: 3004-3016 and Schmidt Oncogene 1999, 18: 2988-2896). In addition, the finding disclosed herein that mice that express only a probasin promoter-myc transgene develop exhibit the phenotype disclosed herein is unexpected in view of scientific articles that teach that the transformation of cells (in vitro) by c-myc expression generally requires additional oncogenic events, such as a mutation of the ras protooncogene (see, e.g. Zhang et al., The prostate 43: 284 (2000)).

The adenocarcinoma cells that develop in these transgenic mice of the invention exhibit a number of molecular characteristics that observed in clinical specimens of human prostate cancer. In one such embodiment, the adenocarcinoma cells exhibit a decreased expression of the NKX3.1 gene (SEQ ID NO: 6) as compared to cells of the prostatic intraepithelial neoplasia. In yet another embodiment of the invention, the prostate cells of the transgenic mouse exhibit an increased expression of the Pim 1 gene (SEQ ID NO: 7) as compared to the prostate cells from a non-transgenic mouse of the same murine strain.

A related embodiment of the invention is a transgenic mouse susceptible to prostate tumor formation having a genomically-integrated nucleic acid molecule comprising a first segment which is a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and a second segment which is the c-myc gene shown in SEQ ID NO:2, the first and second segments being operatively linked such that said mouse expresses the c-myc protein specifically in the prostate of the mouse to effect tumor formation therein. In certain embodiments of the invention, the 5' regulatory element further comprises at least two androgen response elements having the transcriptional activity of the sequence shown in SEQ ID NO: 13.

A variety of well known assays in the art allow artisans to easily quantitate the transcriptional activity of a specific sequence (such as the sequences that comprise the probasin promoter and/or androgen response elements disclosed herein) with a minimal amount of experimentation. Such assays can be used to examine the activity of a 5'-regulatory sequence that has been modified so that it is not identical to the specific 5'-regulatory sequences disclosed herein (e.g. has been engineered to contain a site for

restriction endonuclease cleavage) yet will function in the same way to obtain the same result (i.e. to express the c-myc protein specifically in the prostate of a mouse to effect tumor formation therein). Typically such sequences have at least about 80%, preferably at least about 85%, more preferably at least about 90%, most preferably at least about 95%
5 nucleic acid sequence identity with the 5'-regulatory sequences disclosed herein. "Percent (%) nucleic acid sequence identity" with respect to the 5'-regulatory sequences disclosed herein is defined as the percentage of nucleic acid residues in a sequence that are identical with the nucleic acid residues in for example SEQ ID NOS: 1, 4, 5 or 13, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent
10 sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment
15 over the full length of the sequences being compared. The ALIGN software is preferred to determine nucleic acid sequence identity. A preferred assay which measures the transcriptional activity of a prostate specific 5'-regulatory sequence is a quantitative assay for the measurement of chloramphenicol acetyl transferase (CAT) mRNA transcription in a murine PIN and/or adenocarcinoma cell. In such assays one typically uses typically
20 uses a murine cell that is not of the prostate lineage (such as a cell of the lymphoid lineage such as a B-cell) as a comparative control (see e.g. Knuchel et al., J Virol Methods. 1994 Jul;48(2-3):325-38).

The following procedural steps demonstrate the ease at which an artisan can compare the transcriptional activity of a 5'-regulatory sequence of SEQ ID NO: 1 (or
25 SEQ ID NO: 4, 5 or 13) and a sequence having for example at least about an 80%, preferably at least about an 85%, more preferably at least about a 90%, most preferably at least about a 95% identity with this sequence. In a first step one uses well know methods in the art to construct a set of vector constructs comprising; (1) SEQ ID NO: 1 driving a reporter gene; and (2) the test sequence(s) driving this reporter gene. In a
30 subsequent step, one introduces these constructs in to one or more appropriate cell lines

and then measures and compares the amount of mRNA transcripts generated by each these constructs. As used herein a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1 is one which produces an equivalent transcription profile as does SEQ ID NO:1 (e.g. produces an essentially identical amount of mRNA in a given cell type) and which further can be used to
5 expresses the c-myc protein specifically in the prostate of a mouse to effect tumor formation therein.

The transgenic mice of the invention exhibit a number of phenotypic characteristics that are analogous to those observed in human prostate cancer. As in
10 humans, prostates of elderly wild-type mice display regions of increased epithelial cell number, which is referred to as hyperplasia. In addition to age, this increase in cell number can also arise as a consequence of transgene expression. In this context, hyperplasia can either be focal, involving one or several glands or it can be diffuse, involving the majority (> 50%) of the glands. These cells do not have abundant
15 cytoplasm or other atypical changes that are typically found in prostate intraepithelial neoplasia (PIN).

The precursor of prostate cancer in humans is felt to be prostatic intraepithelial neoplasia (PIN), a lesion characterized by cellular proliferation within existing glands accompanied by atypical cytology. PIN in the mouse can be similarly defined and several
20 attempts have been made to classify these lesions. Park et al have subdivided murine PIN (mPIN) into four types (Park et al., Am J Pathol. 2002 Aug;161(2):727-35). PIN I lesions are characterized by having relatively small foci with one to two layers of atypical cells. The stroma surrounding the ducts is intact and the cells are generally more columnar, larger and taller than adjacent normal cells. They also display an abundant
25 amount of cytoplasm with hyperchromatic nuclei. Areas that fall under the classification of PIN II have larger foci that contain two or more layers of atypical cells however, these cells still do not fill the lumen. Cribiform and tufting patterns of these cells may be clearly discernable as well as an abundant pale pink cytoplasm with increasing nuclear pleomorphism and hyperchromasia. The foci found in PIN III lesions contain atypical
30 cells which either do or almost completely fill the lumen of the gland. These atypical

cells are very often poorly oriented with relatively pale cytoplasm along with increasingly severe nuclear pleomorphism and hyperchromasia. The nuclear to cytoplasmic ratio is reversed and mitotic figures are often present. In PIN IV, the foci of atypical cells completely fill the lumen of the ducts, which are no longer smooth but instead look
5 distorted with irregular and bulging shapes.

Recently, a group of pathologists associated with the Mouse Models of Human Cancer Consortium (MMHCC), have redefined the criteria for mouse PIN in an attempt to simplify this classification into a single group. Mouse PIN (mPIN) is now characterized histologically as having multifocal proliferative lesions of epithelial cells
10 affecting several ductules within individual lobes. Cribiform and tufting patterns of growth are also observed but these lesions must also exhibit progressive nuclear atypia. Atypical cells usually characterized by the presence of large irregular nuclei, hyperchromatic or vesicular chromatin patterns, and prominent nucleoli with amphophilic cytoplasm. Finally, in earlier studies of mouse models of prostate cancer
15 dysplasia was often used to describe these lesions, however today it is felt that this term is synonymous with mPIN.

Invasive cancer is characterized by the extension of malignant cells (usually as numerous nests of acini) through the basement membrane extending into the prostatic stroma and periprostatic adipose tissue surrounding the gland. An increased stromal
20 response (desmoplasia), penetration through the basement membrane (confirmed by immunohistochemistry using anti-laminin antibodies), lymphovascular invasion and metastases are also hallmarks of the disease. Prostatic malignancies can arise from either epithelial or neuroendocrine cells. Neuroendocrine differentiation, although very rare (1-5% of human cancers), occurs in several forms including small cell neuroendocrine
25 carcinoma, carcinoid-like tumors and adenocarcinoma with focal neuroendocrine differentiation. Histologically, these tumors have distinguishing architectural features such as rosette formation and "salt and pepper" chromatin, which differs them from adenocarcinomas. Immunohistochemistry can also be used to help classify these types of tumors. Anti-cytokeratin antibodies are used to identify epithelial tumors whereas
30 positive staining with chromogranin A or synaptophysin would indicate neuroendocrine

differentiation. Epithelial tumors exhibit positive staining with anticytokeratin antibodies and negative staining for neuroendocrine markers (chromogranin and synaptophysin A). Neuroendocrine tumors exhibit positive staining for chromogranin A and synaptophysin and either negative or dot like positivity for cytokeratin markers.

5 The transgenic mice of the invention develop tumors characterized as prostatic intraepithelial neoplasia (PIN). As used herein, the criteria for the mouse PIN observed in the transgenic animals of the invention preferably follows the definition of PIN provided by pathologists associated with the Mouse Models of Human Cancer Consortium (MMHCC). Briefly, Mouse PIN (mPIN) is characterized histologically as
10 having multifocal proliferative lesions of epithelial cells affecting several ductules within individual lobes. Cribiform and tufting patterns of growth are also observed but these lesions must also exhibit progressive nuclear atypia. Atypical cells contain large irregular nuclei, both hyperchromatic and vesicular chromatin patterns, and prominent nucleoli with amphophilic cytoplasm. Of note, in earlier studies of mouse models of prostate
15 cancer, dysplasia was often used to describe these lesions. However today this term is synonymous with mPIN.

 In the transgenic animals of the invention, PIN typically progresses to adenocarcinoma. As used herein, the criteria for the adenocarcinoma observed in the transgenic animals of the invention preferably follows the definition of adenocarcinoma
20 provided by pathologists associated with the Mouse Models of Human Cancer Consortium (MMHCC). Invasive adenocarcinoma is defined by MMHCC as the presence of tumor cells or extensions of numerous nests of acini consisting of cytologically atypical cells invading into the prostatic stroma and periprostatic adipose tissue surrounding the gland. An increased stromal response (desmoplasia), penetration
25 through the basement membrane (confirmed by immunohistochemistry using anti-laminin antibodies), lymphovascular invasion and metastasis are also hallmarks of the disease.

 As is known in the art, prostatic malignancies can arise from either epithelial or neuroendocrine cells. The prostatic malignancies observed in the transgenic animals of
30 the invention arise from epithelial cells. In contrast, neuroendocrine differentiation (1-

5% of human cancers), occurs in several forms including small cell neuroendocrine carcinoma, carcinoid-like tumors and malignancies with focal neuroendocrine differentiation. Histologically, these tumors have distinguishing architectural features such as rosette formation and "salt and pepper" chromatin, which differs them from
5 adenocarcinomas. Immunohistochemistry is typically used to facilitate the classification of these different types of tumors. For example, anti-cytokeratin antibodies are used to identify epithelial tumors whereas positive staining with chromogranin A or synaptophysin indicates neuroendocrine differentiation.

In preferred embodiments, these transgenic mice of the invention exhibit a
10 number of phenotypic characteristics. In one embodiment, the transgenic mice develop prostatic intraepithelial neoplasia, wherein the cells that make up lesions of the prostatic intraepithelial neoplasia exhibit large irregular nuclei, a hyperchromatic vesicle pattern, a vesicular chromatin pattern, prominent nucleoli and an amphophilic cytoplasm. In a closely related embodiment, the mice develop a prostatic intraepithelial neoplasia that
15 further progresses to invasive adenocarcinoma characterized by the numerous nests of acini consisting of cytologically atypical cells extending into prostatic stroma and periprostatic adipose tissue. Typically in this embodiment, the cells of these adenocarcinomas do not exhibit morphological features of neuroendocrine carcinomas as shown by a lack of immunostaining with either anti-synaptophysin antibody or anti-chromogranin A antibody.
20

The adenocarcinoma cells that develop in these transgenic mice of the invention exhibit a number of molecular characteristics that observed in clinical specimens of human prostate cancer. In one embodiment, the adenocarcinoma cells exhibit a decreased expression of the NKX3.1 gene (SEQ ID NO: 6) as compared to cells of the
25 prostatic intraepithelial neoplasia. NKX3.1 is a defined Homeobox protein having NCBI accession no: P97436. In yet another embodiment of the invention, the prostate cells of the transgenic mouse exhibit an increased expression of the Pim 1 gene (SEQ ID NO: 7) as compared to the prostate cells from a non-transgenic mouse of the same murine strain. Pim 1 is a defined serine/threonine-protein kinase having NCBI accession no:
30 P06803.

Yet another embodiment of the invention consists of prostate cells derived from the transgenic mice disclosed herein. A typical example of this embodiment of the invention is an immortal cell line derived from prostatic tissue of a transgenic mouse of the invention and representing a stage of progression of prostate cancer. Preferred
5 embodiments include immortalized intraepithelial neoplasia cells and immortalized adenocarcinoma cells.

Yet another embodiment of the invention consists of a method of making a transgenic mouse wherein the transgene comprises c-myc under the regulatory control of the probasin promoter. A typical embodiment of the invention is a method of making a
10 transgenic mouse having a phenotype characterized by the development of prostatic intraepithelial neoplasia, the method comprising introducing into the mouse genome a nucleic acid molecule comprising a first segment which is a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and a second segment which is the c-myc gene shown in SEQ ID NO:2, so that said transgenic
15 mouse is made.

The disclosed transgenic animals can be used as research tool to determine genetic and physiological features of prostate cancer, and for identifying compounds that can affect prostate tumors. In general, the typical method of testing compounds for an effect on prostate tumors involves (a) administering the compound to be tested to a
20 transgenic animal as disclosed herein, and (b) comparing one or more characteristics (e.g. rate of growth) of the prostate tumors in the transgenic animal to which the compound was administered with the same characteristics of the prostate tumors in a control transgenic animal to which the compound has not been administered. Differences in one or more of the one or more characteristics indicates that the compound has an effect
25 on prostate tumors.

Certain specific embodiments of the invention consists of methods for screening compounds for antitumor activity. In an illustrative example, the disclosed transgenic animals can be used to in assays of the effects of a test compound on the inhibition of a physiological processes associated with prostate cancer (e.g. androgen production)
30 and/or on the initiation, progression, or both of prostatic cancer. Typical assays will

examine whether a test inhibitory compound will prevent or slow development of PIN, adenocarcinoma, or metastasis and such assays can compare the disease progression and/or survival of animal treated with such compounds relative to untreated controls.

5 The effects of test compounds on physiological processes associated with prostate cancer (e.g. androgen production) and/or on the initiation, progression, or both of prostatic cancer can be examined in a number of ways. For example test compound can be administered to the c-myc transgenic mice disclosed herein orally, intravenously, intraperitoneally or by another art accepted method of administration beginning at a specified time period such as at 2 weeks of age. The effects of this test compound on
10 tumor initiation and progression can be monitored by performing histopathologic studies of the prostates of untreated c-myc transgenic animals, treated c-myc transgenic animals, and optionally, untreated control c-myc transgenic mice that have been castrated. Animals from each group can be sacrificed at various time points such as 10 weeks, 4 months, 6 months, 1 year etc. Circulating compound and/or pertinent analyte levels (e.g.
15 testosterone, and dihydrotestosterone) levels can be measured at the time of sacrifice. Hematoxylin and eosin-stained sections can be histopathologically scored and factors such as androgen receptor expression can be examined by art accepted methods such as immunohistochemistry.

Disease progression such as the presence and size of tumors in mice exposed to
20 various test compounds can be measured by any one of a wide variety of assays known in the art. For example, Positron Emission Tomography (PET) has been successfully performed on mice (McCarthy et al., J. Chem. Ed. 71:830-836 (1994)). F-androgen receptor ligands have also been developed for PET scanning of humans with prostatic cancer (see, e.g. Bonasera et al., J. Nuclear Med. 37:1009-1015 (1996)). Tumors as small
25 as 4 mm can be resolved in humans with this technique (NB by six months of age, prostatic tumors in the disclosed C-myc transgenic mice are several centimeters in diameter). PET scanning with these ligands as well as fluoro-deoxyglucose (FDG) can allow monitoring of tumor growth and metastatic spread over time in individual C-myc transgenic mice. Thus, PET can be used as one parameter to disease progression.

In general, a typical method of identifying markers associated with prostate tumors involves comparing the presence, absence, or level of expression of genes in prostatic tissue from a transgenic animal as disclosed and prostatic tissue from a matching non-transgenic animal. Differences between the animals in the presence, absence, or level of expression of a gene indicates that the expression of the gene is a marker associated with prostate tumors. The transgenic animals disclosed can also be used to identify molecular markers that can be used to predict whether patients with carcinoma in situ will have indolent or aggressive disease. In addition, the transgenic animals disclosed can be used to identify molecular markers that and may be mediators of progression. Identification of such mediators would be useful since they are potential therapeutic targets.

In yet another comparative analysis using the transgenic mice disclosed herein, prostatic tissue can be recovered from young transgenic animals (e.g. those with PIN) and older transgenic animals (e.g. those with adenocarcinoma), and compared with similar material recovered from age-matched normal littermate controls to catalog genes that are induced or repressed as disease is initiated, and as disease progresses to its final stages. This analysis can also be extended to include an assessment of the effects of various treatment paradigms (including the use of compounds identified as affecting prostate tumors in the transgenic animals) on differential gene expression (DGE). The information derived from the surveys of DGE can ultimately be correlated with disease initiation and progression in the transgenic animals.

In this context, one embodiment of the invention consists of a method for screening a compound for antitumor activity, comprising administering the compound to a transgenic mouse wherein the transgene comprises c-myc under the regulatory control of the probasin promoter, and then monitoring the antitumor activity of said compound. A related embodiment of the invention is a method for screening a cancer treatment for antitumor activity, comprising administering the compound to a transgenic mouse wherein the transgene comprises c-myc under the regulatory control of the probasin promoter, and monitoring the antitumor activity of said compound. A preferred embodiment of the invention is a method of testing compounds for an effect on prostate

tumors, the method comprising, administering the compound to be tested to a transgenic mouse having a genomically-integrated nucleic acid molecule comprising a first segment which is a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and a second segment which is the c-myc gene shown in SEQ ID NO:2, wherein the transgenic mouse develops prostate tumors and then comparing one or more characteristics of the prostate tumors in the transgenic mouse to which the compound was administered with the same one or more characteristics of the prostate tumors in the transgenic mouse to which the compound has not been administered, wherein a difference in one or more of the one or more characteristics indicates that the compound has an effect on prostate tumors.

Yet another embodiment of the invention consists of a method of identifying one or more markers associated with prostate cancer. An illustrative embodiment consists of a method comprising comparing the presence, absence, or level of expression of genes in prostatic tissue from a transgenic mouse and prostatic tissue from a second mouse, wherein the genome of the transgenic mouse comprises a nucleic acid construct and the genome of the second mouse does not comprise the nucleic acid construct, wherein the construct comprises a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and the c-myc gene shown in SEQ ID NO:2, wherein and wherein the c-myc gene is expressed in the prostate cells of the transgenic mouse such that the transgenic mouse develops prostate tumors, wherein the difference between the transgenic mouse and the second mouse in the presence, absence, or level of expression of a gene indicates that the expression of the gene is a marker associated with prostate cancer.

V. ADVANTAGES OF THE INVENTION

The Myc transgenic models of prostate cancer described here offer several advantages over current models such as those in which the SV40 large T antigen serves as the initiating event. First, the histologic features of the mPIN and cancer lesions accurately reflect the predominant adenocarcinoma phenotype observed in human prostate cancer, with no evidence for the neuroendocrine phenotype observed in many

of the T antigen models (see, e.g. Masumori et al., (2001) *Cancer Res* 61, 2239-2249; Perez-Stable et al., (1997) *Cancer Res* 57, 900-906). Second, the fact that mPIN lesions appear with essentially 100 percent penetrance and progress to invasive cancer with reliable kinetics should make this model suitable for preclinical therapeutic studies. In addition, the differences in progression time between the Lo-Myc and Hi-Myc models provides some flexibility in the design of secondary genetic mouse crosses to study the effects of complementing events.

One notable feature of the mPIN lesions observed in our models is their rapid onset relative to the timing of transgene expression. This raises the possibility that Myc is sufficient to induce preneoplastic lesions in the mouse prostate in the absence of any secondary changes, consistent with reports of Myc gene amplification in human PIN lesions (see, e.g. Bubendorf et al., (1999) *Cancer Res* 59, 803-806; Jenkins et al., (1997) *Cancer Res* 57, 524-531; Qian, J., Jenkins, R. B., and Bostwick, D. G. (1997) *Mod Pathol* 10, 1113-1119). The latency for disease onset in other transgenic Myc cancer models varies widely and presumably reflects the availability of cooperating survival signals, as seen in the skin versus pancreatic islet cell models discussed previously (see, e.g. Pelengaris et al., (2002) *Cell* 109, 321-334; Pelengaris et al., (1999) *Mol Cell* 3, 565-577).

There is optimism in the mouse modeling community that genetically engineered mouse models of human cancer will have utility in the preclinical evaluation of new anticancer agents, perhaps serving as better predictors of clinical activity in humans. We provide an example of this utility with the transgenic mice disclosed herein using hormone ablation therapy, a conventional treatment approach for advanced prostate cancer. The results of these studies establish that mPIN lesions are reversible whereas advanced adenocarcinomas are not. Further studies will determine if mice with advanced lesions eventually relapse with full-blown hormone refractory prostate cancer, but the increasing fraction of proliferating cells that we observed in these tumors suggests that this is only a matter of time. These tumors will further serve as models for dissecting mechanisms of resistance to hormone ablation therapy.

An important consideration with this model is the hormone-dependent expression of the transgene. The fact that advanced tumors do not regress and retain

some residual Myc expression suggests that transgene expression may have become hormone independent. There is growing evidence from human studies that hormone refractory prostate cancer is associated with, and perhaps caused by, restoration of androgen receptor signaling despite continued treatment with hormone ablation therapy.

- 5 Postulated mechanisms include androgen receptor gene amplification or mutation, as well as activation of kinase pathways that alter androgen receptor (see, e.g. Craft et al., (1999) *Nat Med* 5, 280-285; Taplin et al., (1995) *N Engl J Med* 332, 1393-1398; Visakorpi, T. (1999) *Ann Chir Gynaecol* 88, 11-16). Further analysis of the transgenic Myc model will likely reveal similar or additional mechanisms that can be evaluated in
10 human samples.

- Another highly desirable characteristic of any mouse cancer model is that it recapitulate the molecular features of the human disease. Mouse-specific genomics tools for expression profiling allowed us to address this question with the disclosed transgenes using a global approach. Among the most interesting initial findings was reduced
15 expression of NKX3.1 in Myc-induced prostate tumors. Our immunohistochemical results clearly demonstrate that Myc expression and NKX3.1 loss are distinct events, separated in time during the mPIN/cancer transition. Of note, our finding appears distinct from the NKX3.1 loss associated with PTEN loss in the accompanying paper (see, e.g. Wu, X et al., (2001) *Mech Dev* 101, 61-69) where reduced NKX3.1 expression
20 is coincident with PTEN loss, implying coordinate regulation in a single pathway. One compelling explanation for our result is that loss of NKX3.1 complements increased Myc expression to promote the mPIN/cancer transition. Two additional findings support this hypothesis. First, comparative genomic hybridization studies of human prostate cancer often report the parallel presence of chromosome 8q24 gain (Myc) and 8p22 loss
25 (NKX3.1) in the same tumor (see, e.g. Tsuchiya et al., (2002) *Genes Chromosomes Cancer* 34, 363-371). Second, mice lacking NKX3.1 loss develop mPIN lesions but not cancer, suggesting that a rate-limiting second hit may be required for full blown tumorigenesis (see, e.g. Bhatia-Gaur et al., (1999) *Genes Dev* 13, 966-977; Kim et al., (2002) *Cancer Res* 62, 2999-3004; Abdulkadir et al., (2002) *Mol Cell Biol* 22, 1495-1503).
30 Alternatively, absence of NKX3.1 expression may be a marker for the cell of origin in the

Myc and PTEN prostate cancer models and play no functional role in the transformation process. These and other hypotheses can now be tested through genetic crosses.

In addition to examining our mouse prostate cancer gene lists for known human prostate cancer orthologues, we asked whether the global Myc signature in the murine prostate can be observed in a subset of human prostate cancers. Despite current
5 limitations due to different microarray expression profiling platforms and the limited number of orthologues represented on mouse and human chips, we were able to verify that genes correlated with Myc status in the mouse can be used to define Myc-like human tumors, and that at least one of the genes most consistently associated with the Myc
10 signature in human tumors, Pim1, is also consistent with our knowledge about the role of Myc in tumorigenesis. Pim-1, a serine/threonine kinase, is known to cooperate with c-Myc in murine lymphoma models (see, e.g. Moroy et al., (1991) *Oncogene* 6, 1941-1948; van Lohuizen et al., (1989) *Cell* 56, 673-682), and increased Pim1 expression was recently observed in a subset of human prostate cancers and shown to correlate with
15 poor clinical outcome (see, e.g. Dhanasekaran et al., (2001) *Nature* 412, 822-826). Although these investigators did not determine the Myc status of these tumors, our mouse model and subsequent analysis of human microarray datasets suggest that these genes are linked in prostate and breast cancer.

While our initial analysis demonstrates the utility of cross species comparisons of
20 global datasets, a number of additional steps will further realize the full potential of this approach. It will be important to standardize expression analysis platforms to provide comprehensive coverage of the mouse and human transcriptome with appropriate cataloguing of orthologues for cross comparison. In addition, human datasets must be linked to independent tissue analysis for specific molecular lesions of interest, such as
25 Myc amplification or PTEN loss for the examples discussed here. Parallel construction of tissue arrays from tumor samples analyzed by expression microarrays will allow such experiments to be performed. Ultimately, we envision using such cross species comparisons to validate the relevance of mouse models for human disease, to help prioritize lengthy gene lists for functional evaluation and to extend gene cohorts that
30 segregate with a specific molecular lesion across multiple tissues.

EXAMPLES

EXAMPLE 1: ILLUSTRATIVE NUCLEIC ACID CONSTRUCTS OF THE INVENTION.

5

Plasmids: The plasmids AR₂Pb-Flag-Myc-PAI and Pb-Flag-Myc-PAI were constructed by ligation of the following gene fragments into the Bluescript (KS+) backbone (Stratagene). For Pb-Flag-Myc-PAI: the poly(A) tail of the insulin receptor gene (PAI) was subcloned into the BamHI/NotI site of the Bluescript KS+ multiple cloning site (MCS). The 5' flanking promoter region (-426/+28) of the rat probasin gene was subcloned into the KpnI/EcoRV restriction sites located in the MCS. The human c-Myc c-DNA was amplified by PCR using a 5' primer containing the Bgl II restriction site and the consensus sequence for the FLAG epitope (5'GGGAGATTCTCATCGCCACCATGGACTACAAGGACGACG

10 ACGACAAGGCCATGCCCCCTCAACGTTAGCTTCACC) (SEQ ID NO: 8). The epitope tag was engineered to aid with immunohistochemistry however, we were unable to detect it via western blot and relied on the human specific anti-9E10 c-Myc antibody (Santa Cruz) to detect transgene expression. The 3' primer contained a BamHI restriction site for cloning purposes (3'GGGGGATCCTTACGCACAAGAGTTCCGTAGCTGTTC) (SEQ ID NO: 9). After PCR amplification, the product was gel purified, digested and filled in using the large fragment Klenow polymerase. Following gel purification, the blunt-ended product was subcloned into the EcoRV site of the Bluescript KS+ Pb-PAI backbone thus generating the PB-Flag-Myc-PAI transgenic construct. The AR₂Pb-Flag-Myc-PAI was generated in the same way except the AR₂Pb promoter sequence was subcloned into the KpnI/EcoRV site instead of PB promoter. The AR₂PB sequence contains the original probasin sequence PB (-426/+28) plus two additional androgen response elements (see, e.g. Zhang et al., (2000) *Endocrinology* 141, 4698-4710). The completed constructs were sequenced and tested for promoter inducibility by androgen in LNCaP cells by transient transfection before microinjection into FVB ova. By transient transfection, the

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ARR₂Pb promoter was able to confer approximately 20x higher levels of expression than the Pb promoter.

EXAMPLE 2: GENERATION OF TRANSGENIC ARR₂PB-MYC-PAI AND PB-MYC-PAI MICE.

ARR₂PB-Myc-PAI and PB-Myc-PAI constructs were linearized with KpnI/NotI, micro-injected into fertilized FVB ova and transplanted into a pseudo-pregnant female (University of Irvine Transgenic Facility). Transgenic founders were screened by PCR using genomic DNA isolated from tail snips. The 5' primer was specific to either the AR₂Pb promoter (5' AR₂Pb-CAATGTCTGTGTACAACCTGCCAACTGGGATGC) (SEQ ID NO: 10), or the Pb promoter (5'Pb-CCGGTCGACCGGAAGCTTCCACAAGTGCATTTA) (SEQ ID NO: 11) and the 3' primer for both reactions was located at the end of the c-Myc cDNA (5'-TTACGCACAAGAGTTCCGTAGCTGTTC) (SEQ ID NO: 12).

A PCR product of 1438 base pairs was generated from the AR₂Pb-Myc-PAI mice and a 1774 base pair product was produced by the Pb-Myc-PAI mice. Seven founder lines were obtained from the AR₂Pb-Myc-PAI construct (designated 1,2,4,7,8,11 and 13) whereas three founders were generated with the Pb-Myc-PAI construct (designated # 6,9,10). Breedings were carried out and germline transmission was obtained by four AR₂Pb-Myc-PAI founders (4, 7, 11 and 13) and two Pb-Myc-PAI mice (6 and 9). These mice were bred and the offspring were aged to determine if prostate cancer developed in the transgene positive male mice. Prostates were isolated "en block" from transgenic and wildtype mice at 2-12 weeks as well as at 6, 9, 12 and 16 months and cut in half along the sagittal plane. Superficial and deep H&E sections were examined on the same tissue in order to document the presence/absence of mPIN, microinvasion and invasive adenocarcinoma (described below).

EXAMPLE 3: MOUSE DISSECTIONS, TISSUE ISOLATION AND CASTRATION.

Urogenital organs were isolated and prostates were micro-dissected in a petri dish containing 10 mls of cold phosphate buffered saline (1x PBS, Gibco-BRL 14190144)

under a dissecting microscope. Adipose tissue surrounding the mouse prostates was cleared using forceps. The mouse prostate is composed of four pairs of lobes (ventral, dorsal, lateral and anterior lobes) which were separated from the urethra using dissecting shears. One half was used to obtain protein and RNA while the other half was fixed in
5 10% phosphate buffered formalin for histology (Fisher SF100-4). The liver, testes, bone from the spine, brain, kidneys and lungs were also isolated for both histological examination as well as protein analysis to check for non-specific expression of c-Myc. For castration experiments, mice were anesthetized using Isoflurane (Abbott Laboratories). The perineal region was cleaned three times with ethanol and a betadine
10 scrub (VWR, AJ159778) and sterile dissecting shears were used to make a 4-5 mm incision in this region. Using two sterile forceps, the testes were located and a ligature was made around the testicular vessels and the tunica albuginea that encases the testes. The testes were amputated with dissecting shears and the scrotum sutured shut with 6-0 Ethilon black monofilament nylon (Ethicon Inc., 1665). A local triple antibiotic was
15 applied over the region of the wound to facilitate healing.

EXAMPLE 4: PATHOLOGICAL AND IMMUNOHISTOCHEMICAL DATA.

Mice were aged to the appropriate time point and then sacrificed for dissection. The prostate tissues that were sent for histology were marked with ink on one side (the
20 cut side), splayed and embedded "en face" to maximize pathologic examination of each lobe. Sections were cut in the same manner on the microtome enabling us to orient the prostatic lobes with the bladder and the seminal vesicles as a reference point. Prostates, testes, lung, liver, bone (spine), kidney and brain were all harvested for western blot analysis and histology. The tissues that were kept for protein analysis were homogenized
25 using a tissue grinder in 2x SDS buffer (100 mM Tris-HCl pH=6.8, 200 mM DTT, 4% SDS, 20% glycerol, 50 mM B-gly-Phosphate, 1 mM NaVo₄, and 40 ug/ml PMSF) and normalized for total protein via Bio-Rad assay. Tissue used for histology was fixed initially in 10% buffered formalin phosphate (Fisher SF100-4) for eight hours followed by gentle washing in running water and finally transferred to 70% ethanol. Serial tissue
30 sections (4 µm thick) were cut from paraffin-embedded blocks and placed on charged

glass slides. H&E and masson trichrome staining were performed using standard procedures. For immunohistochemical analysis using polyclonal antibodies the Vector Laboratories R.T.U. Vectastain Universal Elite ABC Kit (cat PK-7200) was used and for monoclonal antibodies we used the Vector M.O.M. Basic Kit (BMK-2202). Briefly, sections were deparaffinized with xylene and rehydrated through graded alcohol washes followed by antigen retrieval in a pressure cooker for 30 minutes in sodium citrate buffer (10 mM, pH 6.0). Slides were then incubated in 0.3% hydrogen peroxide to quench endogenous horseradish peroxidase for 30 minutes. The slides were then blocked by incubation in normal horse serum (dilution 1:20) in 0.1 M Tris buffered saline pH 6.0 and subsequently incubated for 30 minutes with the following antibodies diluted in Tris-buffered saline: anti-synaptophysin polyclonal antibody (Dako # A0010) diluted (1:5000), anti-alpha smooth muscle actin monoclonal antibody (Dako # M0851) diluted (1:1000), anti-Nkx3.1 polyclonal antibody (kindly provided by Dr. Cory Abate-Shen) diluted (1:6000), anti Ki67 polyclonal antibody (Novacastra Laboratories #NCL-Ki67p) diluted (1:20,000). Negative controls were included in each assay. Slides were then treated with biotin labeled anti-mouse IgG and incubated with preformed avidin biotin peroxidase complex. Metal enhanced diaminobenzidine substrate was added in the presence of horseradish peroxidase and finally, sections were counter stained with hematoxylin, dehydrated and mounted.

20

EXAMPLE 5: TUNEL ASSAYS.

TUNEL assays were performed as described in the In Situ Cell Death Detection Kit, POD from Roche. Prior to the addition of TdT enzyme, sections were deparaffinized with xylene and rehydrated through graded alcohol washes. Antigen retrieval was performed in sodium citrate buffer (10 mM, pH 6.0) by applying microwave irradiation (750 W) for 1 minute. The slides were then incubated for 5 minutes in 3% hydrogen peroxide to quench endogenous horseradish peroxidase. Next, slides were immersed for 30 minutes at room temperature in Tris-HCl, 0.1 M pH=7.5 containing 3% BSA and 20% normal bovine serum. TUNEL reaction mixture containing a 1:20 dilution of TdT enzyme was added to the slides for 2 hours at 37 °C in a humidified

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atmosphere chamber. 50 μ l of Converter-POD was then added to each slide and incubated at 37 °C for 45 minutes in a humidified atmosphere chamber. DAB substrate was applied for 1 minute followed by counterstaining in hematoxylin.

5 **EXAMPLE 6: MICROARRAY MEASUREMENTS.**

Total RNA was isolated from gross prostate tissue following microdissection using the Tri-Reagent RNA Isolation Reagent (Sigma-Aldrich cat# T9424) as described by the manufacturer. Biotin labeled cRNA was generated following Affymetrix protocols. Briefly, first strand cDNA synthesis was carried out by reverse transcribing
10 total RNA using Superscript Reverse Transcriptase (Gibco cat# 18064-014). Second strand synthesis was performed using 10 U/ μ l DNA ligase (Gibco cat# 18052-019) 10 U/ μ l DNA Pol I (Gibco cat# 18005-025) and 2 U/ μ l RNase H (Gibco cat# 18021-014). Double stranded cDNA cleanup was done using the GeneChip Sample Cleanup Module (Affymetrix cat# P/N 900371). Synthesis of biotin-labeled cRNA was carried out using
15 the Enzo Bioarray Kit (Enzo Diagnostics Inc. cat# 42655-20) and following fragmentation, was hybridized to Affymetrix murine chips (U74Av2).

EXAMPLE 7: DATA ANALYSIS.

Hierarchical clustering analysis was performed using the genes with significant
20 variation across all samples (standard deviation (σ) > 2000, coefficient of variation (σ/mean) > 0.05, fraction above background > 0.5) (see, e.g. Eisen et al., (1998) Proc Natl Acad Sci U S A 95, 14863-14868). To identify the most informative set of differentially expressed genes between two sets of samples, we ranked each gene by the probability that the means of its expression values in each set are statistically distinct
25 using the Student's t-test.

EXAMPLE 8: CROSS-SPECIES EXPRESSION MODULE COMPARISON ALGORITHM.

A schematic of our approach is shown in Figure 7. We first generated a ranked
30 list of mouse genes differentially expressed between wild-type and Myc-transgenic mice using the Student's t-test. Each gene on this list was assigned a weighting factor (Golub

et al. (1999) *Science* 286, 531-537). We then identified the human orthologs for these genes using the HomoloGene database (<http://www.ncbi.nlm.nih.gov/HomoloGene/>). We assigned human tumors Myc-like or non-Myc-like status using a weighted gene voting prediction algorithm (see, e.g. Golub *et al.* (1999) *Science* 286, 531-537) using the
5 ranked and weighted mouse gene list. Before applying the algorithm, we normalized the expression of each gene to a mean of zero and a standard deviation of one separately in the mouse dataset and each of the human datasets.

For each gene in the human dataset, multiple prediction pairs of Myc-like and non-Myc-like groups were made, using all possible numbers of orthologous genes
10 corresponding to the ranked and weighted mouse gene list, but always excluding the current gene of interest (if it exists in the mouse dataset) from the prediction calculations. Then for each of the resulting prediction pairs we calculated the Student's t-test P-value for the current gene, and kept the minimal value. Thus, for each gene we retained the minimal P-value possible using the prediction algorithm and the ranked and weighted
15 mouse gene list. This was done to ensure that the number of genes used in the prediction was determined using the exact same optimization procedure individually for each gene. Finally, we identified the genes most consistently differentially expressed between Myc-like and non-Myc-like human tumors by ranking the genes on the overlapping lists by the products of the P-values from each human dataset.

20 Throughout this application, various publications are referenced (within parentheses for example). The disclosures of these publications are hereby incorporated by reference herein in their entireties.

The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single illustrations of individual aspects of the invention,
25 and any that are functionally equivalent are within the scope of the invention. Various modifications to the models and methods of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and teachings, and are similarly intended to fall within the scope of the invention. Such modifications or other embodiments can be practiced without departing from the true
30 scope and spirit of the invention.

TABLES**Table 1 - High Penetrance of mPIN and Cancer in c-Myc Transgenic Mice.**

Mice were aged to the appropriate time point and sacrificed for histological analysis. Age, phenotype and number of mice are listed for both the Myc-Hi and Myc-Lo transgenic models. Generally, in the Myc-Hi model, all mice ≤ 3 months developed mPIN and all animals older than 6 months developed cancer. In the Myc-Lo model, most animals less than 12 months developed mPIN and cancer after 1 year. Only a few exceptions (*) are observed.

Table 1A

Strain	Age	# Mice	Phenotype	Comments
Myc-Hi	≤ 3 months	16	16/16 mPIN	mPIN in 4/4 mice at 2 weeks
	≥ 6 months	20	19/20 invasive cancer	
			1/20 mPIN	
Myc-Lo	≤ 10 months	10	8/10 mPIN	2 mice at 2 weeks were normal
	≥ 10 months	10	10/10 invasive cancer	

Table 1B

	mPIN	mPIN/cancer transition	Invasive cancer
Myc-Hi	2 weeks	3-6 months	> 6 months
Myc-Lo	4 weeks	6-12 months	> 12 months

Table 2A. Myc signature genes consistently regulated in Myc-transgenic and human prostate cancers.

Table 2: <i>myc</i> signature genes consistently regulated in <i>myc</i> transgenic cells relative to prostate cancer.									
Rank	Product (a)	P-values		Datasets					
		Prostate A (n=14)	Prostate B (n=25)	Direction (b)	Unigene ID	Gene Abr	Gene Description		
1	5.2E-08	0.00049	0.00011	up	279009	MGP	matrix Gla protein		
2	8.9E-08	0.0040	2.2E-05	up	81170	PIM1	pim-1 oncogene		
3	2.0E-07	0.0019	0.00011	down	118684	SDF2	stromal cell-derived factor 2		
4	3.2E-07	7.5E-06	0.043	down	79877	MTMR6	myotubularin related protein 6		
5	3.5E-07	0.00079	0.00044	down	52788	FXR2	fragile X mental retardation, autosomal homolog 2		
(a) Genes are ranked by the product of the Student's t-test P-values for each cancer dataset. (b) Up or down regulated in <i>Myc</i> -like tumors. n, number of samples.									

Table 2B. Myc signature genes consistently regulated in Myc-transgenic and human prostate and breast cancers.

Rank	Product (a)	P-values		Datasets					Gene Description
		Prostate A (n=14)	Prostate B (n=25)	Breast (n=117)	Direction (b)	Unigene ID	Gene Abr		
1	9.2E-22	0.00075	0.0044	2.8E-16	down	74637	TEGT	testis enhanced gene transcript (BAX inhibitor 1)	
2	1.7E-18	0.0017	0.00043	2.4E-12	down	174905	KIAA0033	KIAA0033 protein	
3	3.4E-18	0.004	2.2E-05	3.8E-11	up	81170	PIM1	pim-1 oncogene	
4	3.9E-17	0.00039	0.03	3.3E-12	down	215857	RNF14	ring finger protein 14	
5	6.3E-17	0.0023	0.0033	8.3E-12	down	174139	CLCN3	chloride channel 3	

(a) Genes are ranked by the product of the Student's t-test P-values for each cancer dataset. (b) Up or down regulated in Myc-like tumors.
Note that the P-values are substantially lower in general for the Breast dataset because of the much larger number of samples (n).

CLAIMS:

1. A transgenic mouse whose genome comprises a nucleic acid construct comprising a promoter having the nucleotide sequence shown in SEQ ID NO: 1, wherein the promoter is operably linked to c-myc having the nucleotide sequence shown
5 SEQ ID NO: 2 such that the c-myc protein encoded therein is expressed in prostate cells of the transgenic mouse at detectable levels.
2. The transgenic mouse of claim 1, wherein the promoter further comprises the sequence shown in SEQ ID NO: 4 or the sequence shown SEQ ID NO: 5.
10
3. The transgenic mouse of claim 1, wherein the transgenic mouse develops overt prostatic anaplasia, wherein the overt prostatic anaplasia is prostatic intraepithelial neoplasia.
- 15 4. The transgenic mouse of claim 3, wherein the transgenic mouse develops adenocarcinoma.
5. The transgenic mouse of claim 4, wherein cells of the adenocarcinoma exhibit a lack of immunostaining with anti-synaptophysin antibody.
20
6. The transgenic mouse of claim 4, wherein cells of the adenocarcinoma exhibit a decreased expression of the NKX3.1 gene (SEQ ID NO: 6) as compared to cells of the prostatic intraepithelial neoplasia.
- 25 7. The transgenic mouse of claim 4, wherein the prostate cells of the transgenic mouse exhibit an increased expression of the Pim 1 gene (SEQ ID NO: 7) as compared to the prostate cells from a non-transgenic mouse of the same murine strain.
8. Prostate cells isolated from the transgenic mouse of claim 1, wherein the cells
30 express the c-myc protein shown in SEQ ID NO: 3.

9. A transgenic mouse susceptible to prostate tumor formation having a genomically-integrated nucleic acid molecule comprising a first segment which is a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and a second segment comprising the c-myc gene shown in SEQ ID NO:2, the first and second segments being operatively linked such that said mouse expresses the c-myc protein specifically in the prostate of the mouse to effect tumor formation therein.
10. The transgenic mouse of claim 9, wherein the 5' regulatory element further comprises at least two androgen response elements having the transcriptional activity of the sequence shown in SEQ ID NO: 13.
11. The transgenic mouse of claim 9, wherein the transgenic mouse develop prostatic intraepithelial neoplasia, wherein the cells that make up lesions of the prostatic intraepithelial neoplasia exhibit large irregular nuclei, a hyperchromatic vesicle pattern, a vesicular chromatin pattern, prominent nucleoli and an amphophilic cytoplasm.
12. The transgenic mouse of claim 9, wherein the transgenic mouse develop an invasive adenocarcinoma characterized by the numerous nests of acini consisting of cytologically atypical cells extending into prostatic stroma and periprostatic adipose tissue.
13. The transgenic mouse of claim 12, wherein cells of the adenocarcinoma do not exhibit morphological features of neuroendocrine carcinomas as shown by a lack of immunostaining with anti-synaptophysin antibody or anti-chromogranin A antibody.
14. The transgenic mouse of claim 9, wherein cells of the adenocarcinoma exhibit a decreased expression of the NKX3.1 gene (SEQ ID NO: 6) as compared to cells from a non-transgenic mouse of the same murine strain.

15. The transgenic mouse of claim 9, wherein the prostate cells of the transgenic mouse exhibit an increased expression of the Pim 1 gene (SEQ ID NO: 7) as compared to the prostate cells from a non-transgenic mouse of the same murine strain.

5

16. An immortal cell line derived from prostatic tissue of the transgenic mouse of claim 9 and representing a stage of progression of prostate cancer.

17. A method of testing compounds for an effect on prostate tumors, the method comprising,

10 (a) administering the compound to be tested to a transgenic mouse having a genomically-integrated nucleic acid molecule comprising a first segment which is a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and a second segment which is the c-myc gene shown in SEQ ID NO:2, wherein the transgenic mouse develops prostate tumors;

15 (b) comparing one or more characteristics of the prostate tumors in the transgenic mouse to which the compound was administered with the same one or more characteristics of the prostate tumors in the transgenic mouse to which the compound has not been administered, wherein a difference in one or more of the one or more
20 characteristics indicates that the compound has an effect on prostate tumors.

18. A method of identifying markers associated with prostate cancer, the method comprising comparing the presence, absence, or level of expression of genes in prostatic tissue from a transgenic mouse and prostatic tissue from a second mouse, wherein the
25 genome of the transgenic mouse comprises a nucleic acid construct and the genome of the second mouse does not comprise the nucleic acid construct,

wherein the construct comprises a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and the c-myc gene shown in SEQ ID NO:2, wherein and wherein the c-myc gene is expressed in the

prostate cells of the transgenic mouse such that the transgenic mouse develops prostate tumors,

wherein the difference between the transgenic mouse and the second mouse in the presence, absence, or level of expression of a gene indicates that the expression of the gene is a marker associated with prostate cancer.

19. A method of making a transgenic mouse having a phenotype characterized by the development of prostatic intraepithelial neoplasia, the method comprising introducing into the mouse genome a nucleic acid molecule comprising a first segment which is a 5' regulatory element having the transcriptional activity of the probasin promoter shown in SEQ ID NO:1, and a second segment which is the c-myc gene shown in SEQ ID NO:2, so that said transgenic mouse is made.

Figure 1

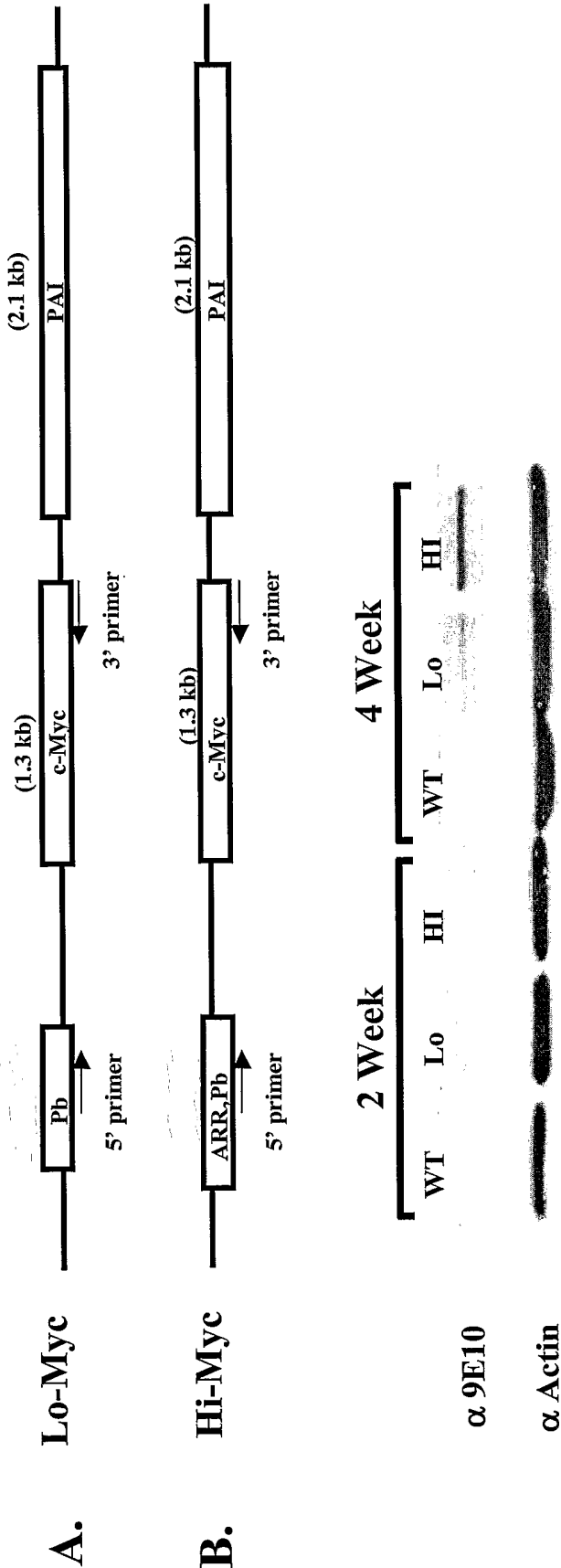


Figure 2

A. Lo-Myc



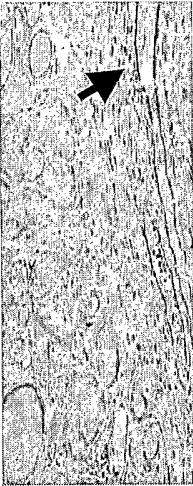
Hi-Myc



6 Months

12 Months

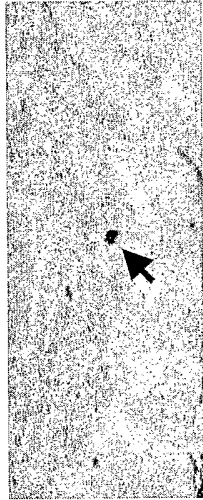
B. SMA
Hi-Myc



mPIN

Cancer

C. Synaptophysin



Benign

Cancer

Figure 3

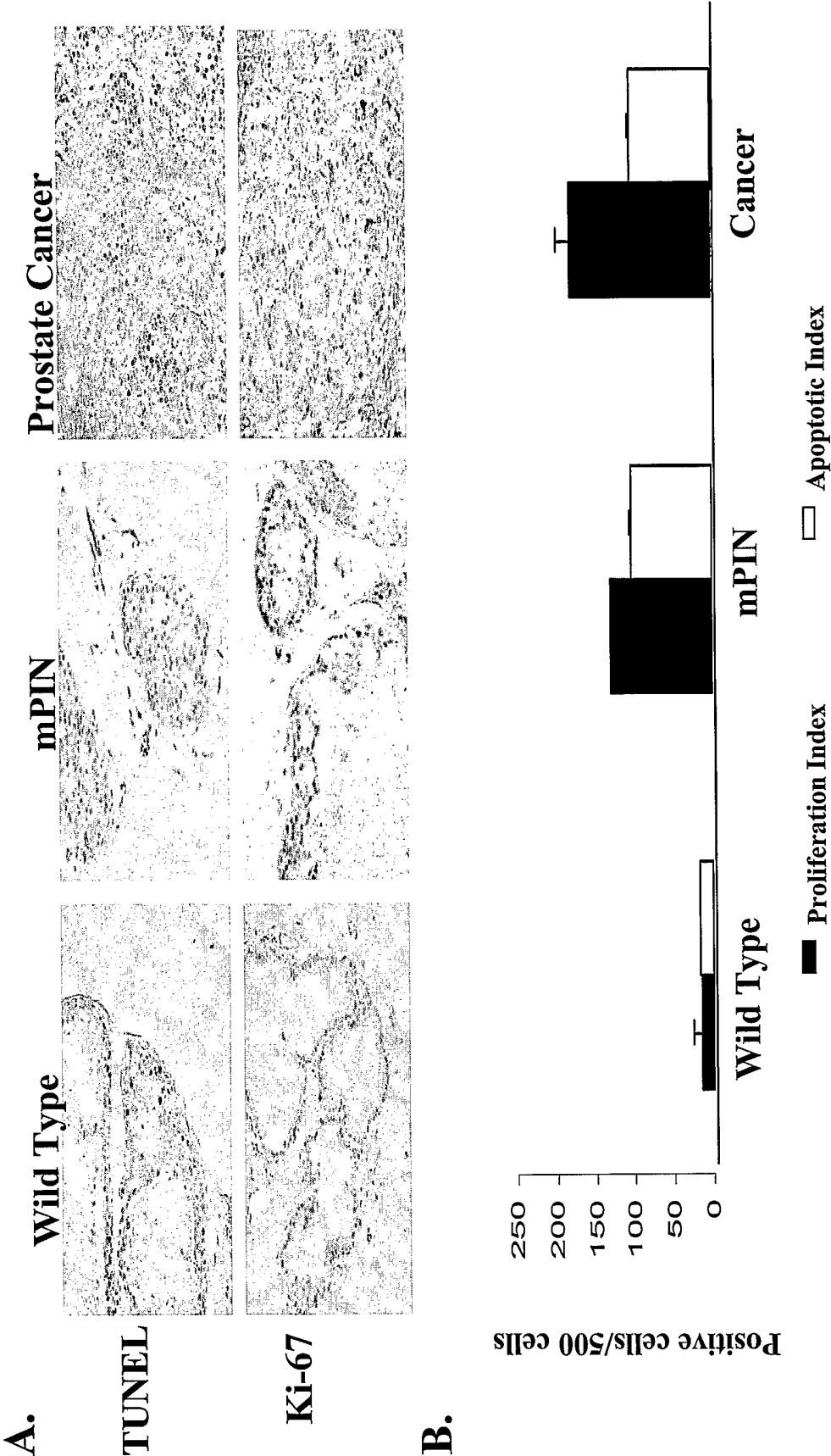
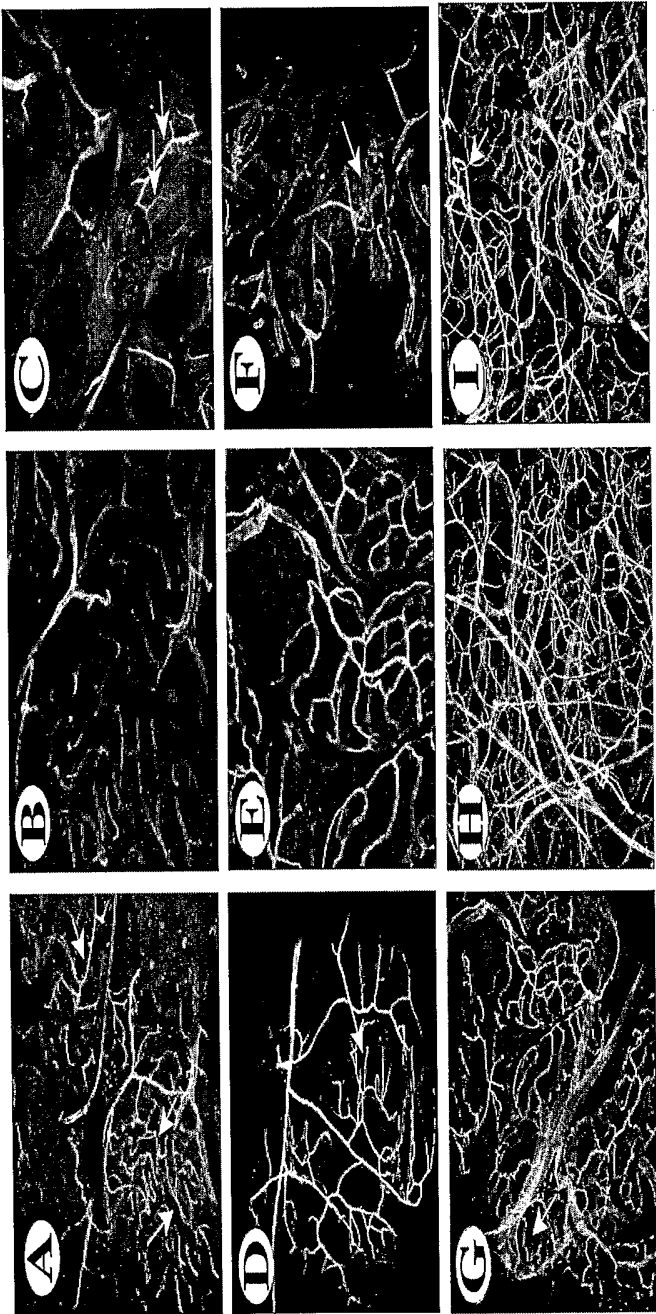


Figure 4



Vascular density (mm²)

	2 months	1 year
Wild-type animals	23 (±3)	21 (±6)
Transgenic animals	34 (±5)	45 (±9)

J.

Figure 5

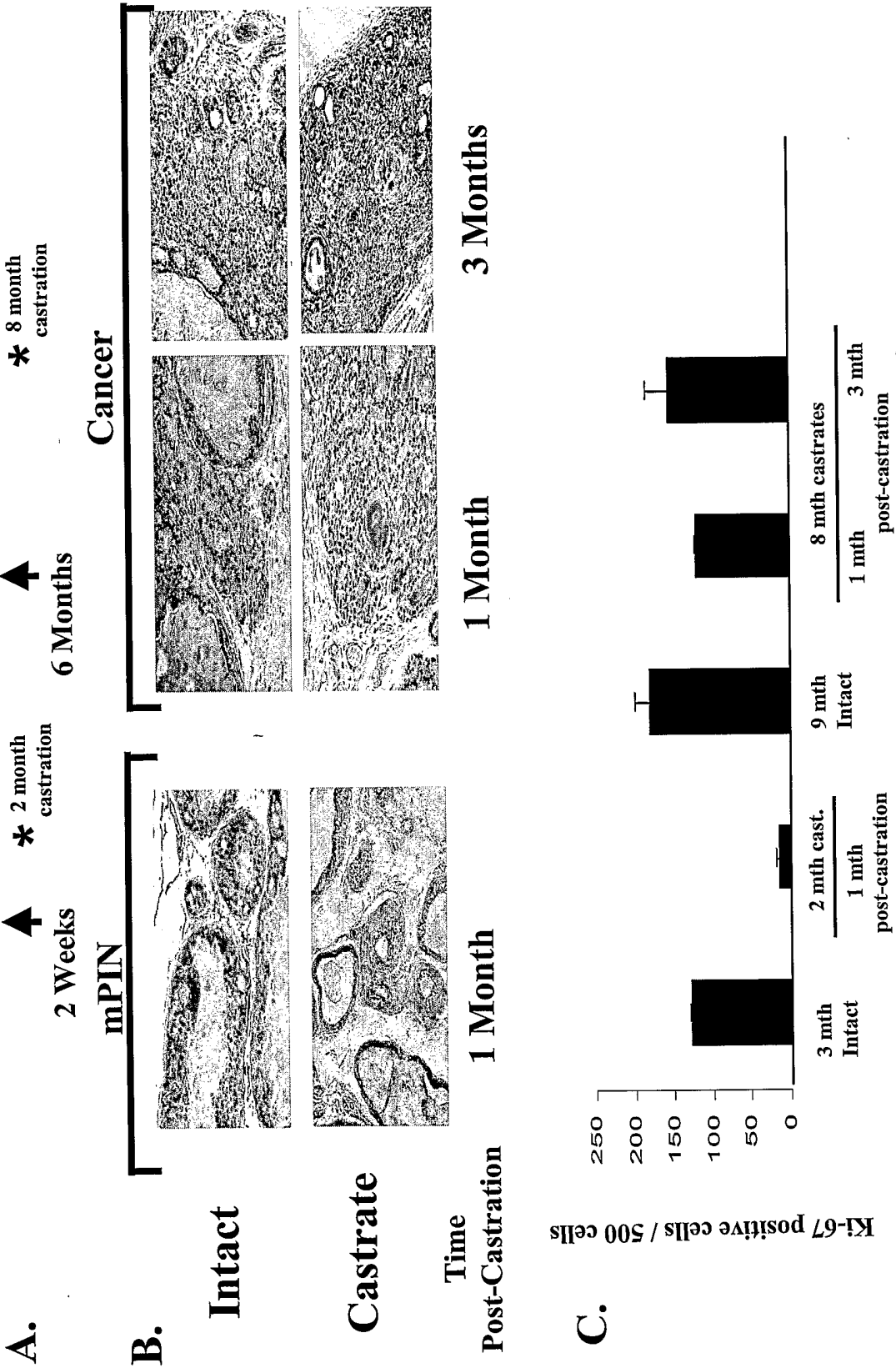
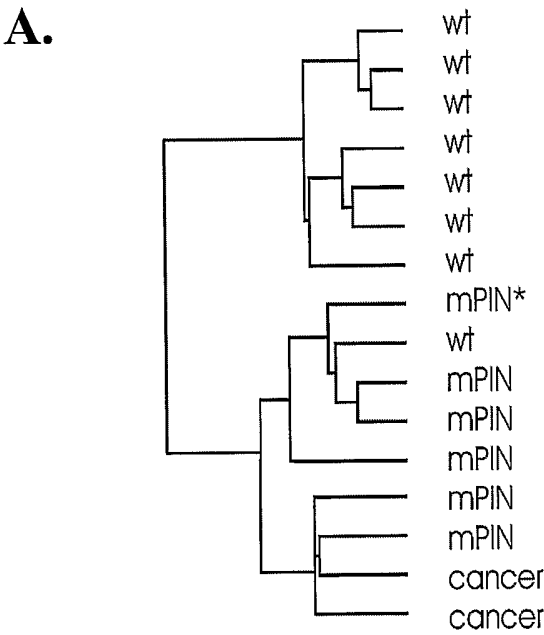
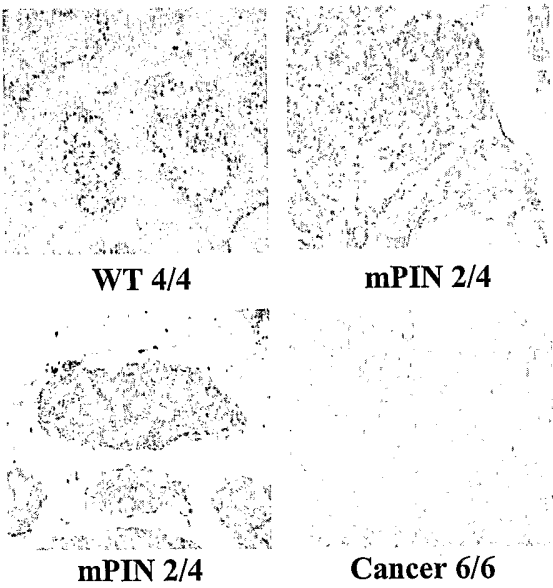


Figure 6



C. **Nkx3.1**



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Figure 6

B.

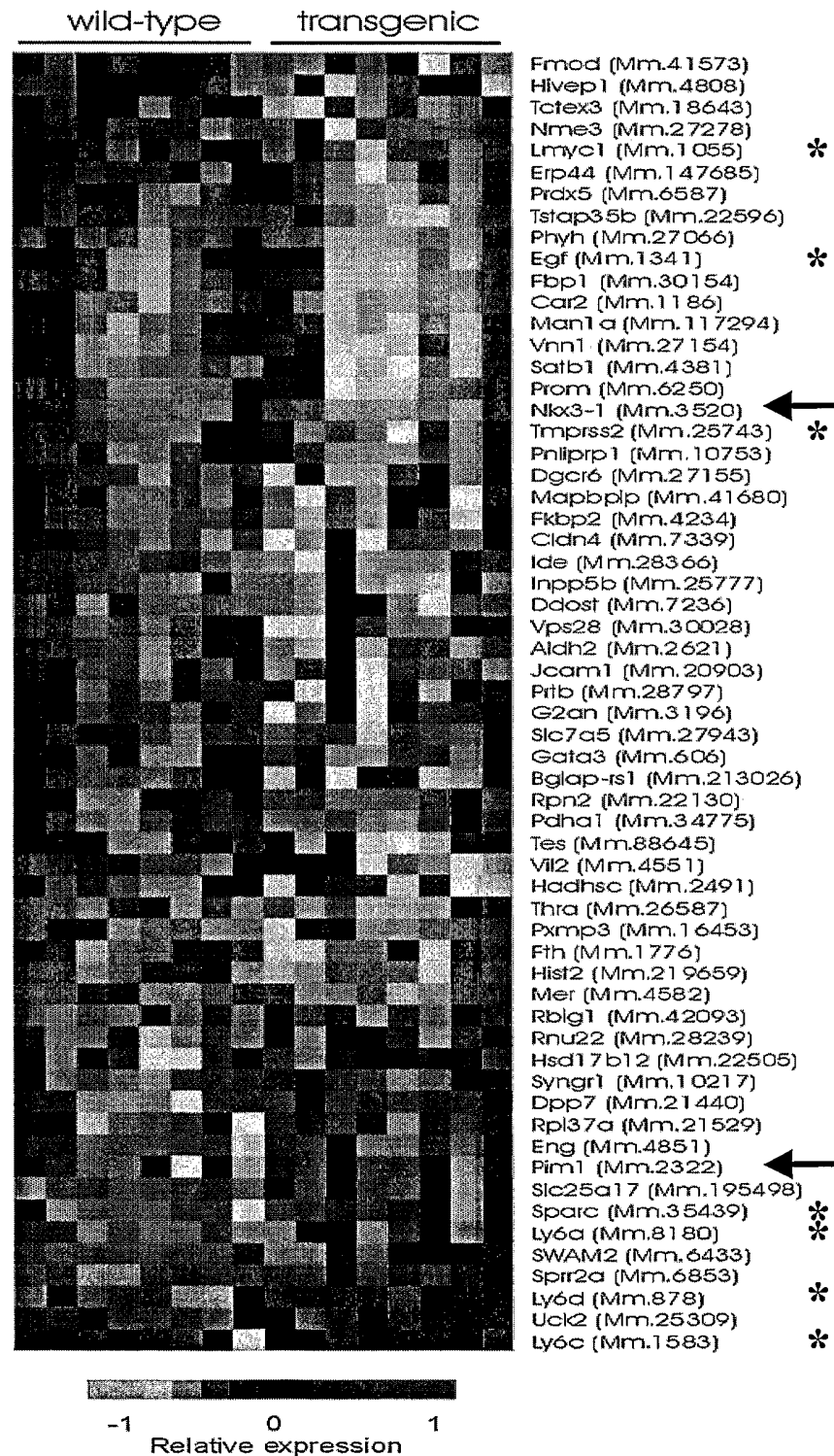
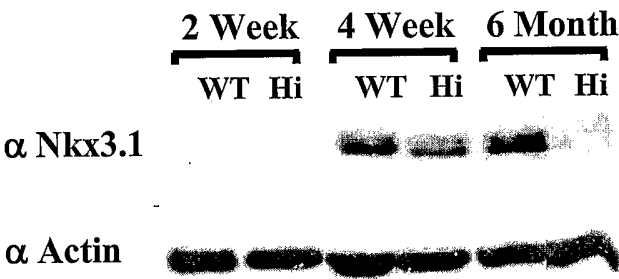


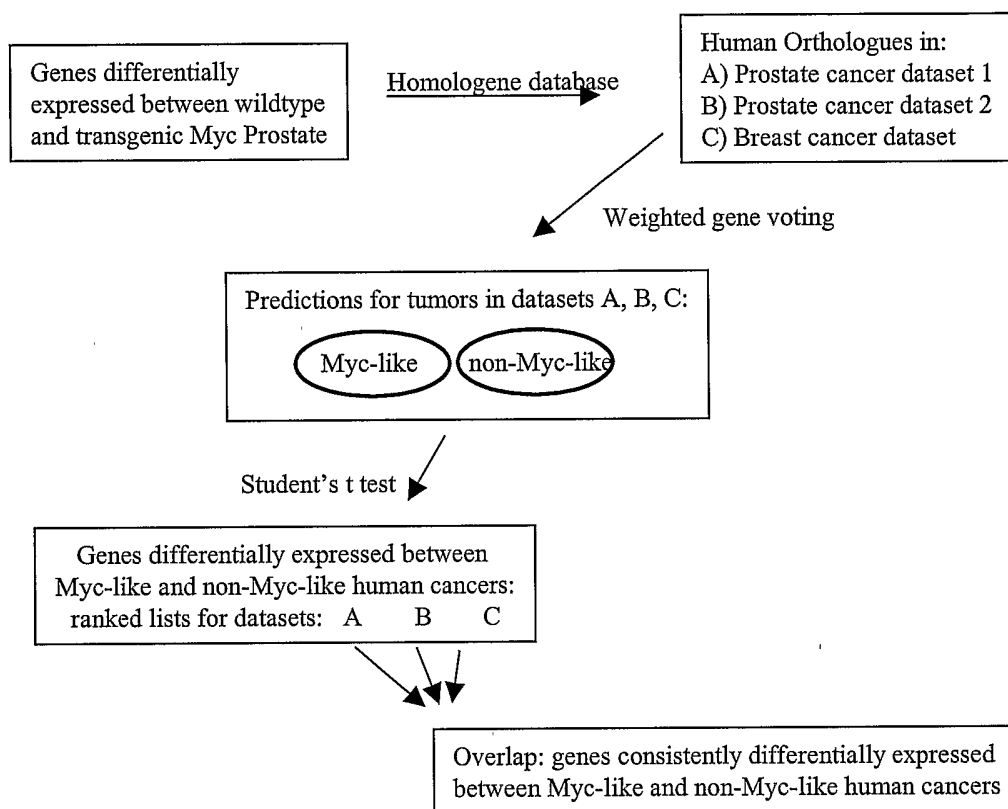
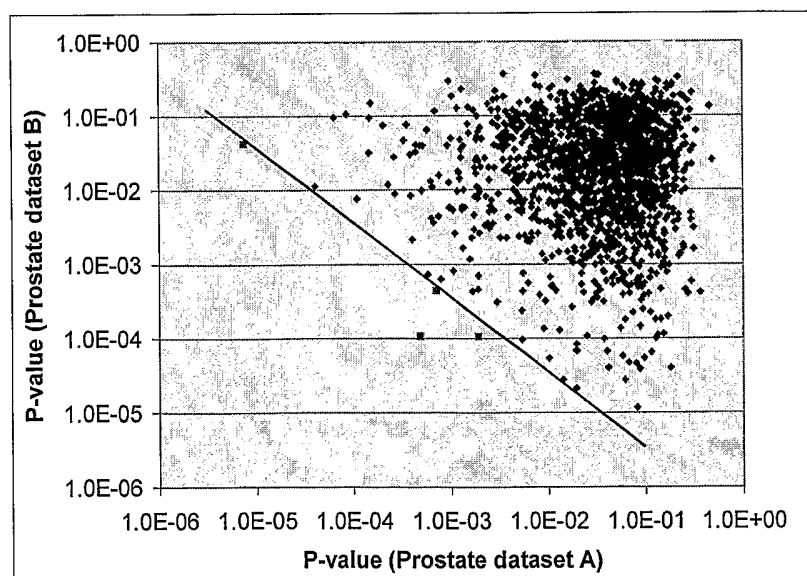
FIGURE 6

D.



9/9

Figure 7

A.**B.**

SEQUENCE LISTING

<110> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA
CHARLES L. SAWYERS
KATHARINE B. ELLWOOD

<120> ANIMAL MODELS OF PROSTATE CANCER AND
METHODS FOR THEIR USE

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<150> 60/390,692

<151> 2002-06-21

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Ser	Leu	Arg	Gly	Asp	Asn	Asp	Gly	Gly	Gly	Gly	Ser	Phe	Ser	Thr	Ala		
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His Ser Gly Asn Pro Gln His Ser Pro Asp Pro Arg Arg Asp Ser Ala
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