BICISTRONIC VECTORS FOR BREAST AND PROSTATE CANCER RESEARCH

BICISTRONIC VECTORS FOR ANIMAL MODELS OF BREAST AND PROSTATE CANCER

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Abstract:

The improving of our understanding of cancer development still depends on cancer research at the molecular level. In his project, bigenic vectors for animal models of breast and prostate cancer are created.

Bigenic constructs are useful because they create animals expressing two genes of interest at a time, with one injection step and no need for crossings. In order to produce these vectors, previous animal models have been analyzed, and the elements that worked successfully in previous models were gathered in a new arrangement for the creation of an improved model. In order to create a bigenic vector, the viral internal ribosomal entry site was utilized, as a means of obtaining two protein products from one transcript.

One vector, the MMTV-Neu1842-IRES-Cre was successful in generating a line of transgenic mice. Female founders of this line already express the expected phenotype, tumors of the mammary tissue. Once this line is established, it can be crossed with the Rosa26 line, to determine the pattern of Cre expression.

Other vectors were created for models of prostate cancer, using the probasin promoter and the MT oncogene. While transgenic mice were attempted, there were no phenotype differences between wild type and transgenic mice.

All created vectors were tested for expression of the two genes carried in tissue culture experiments. All the experiments were successful, indicating a working oncogene (by means of a focus assay) and Cre activity (by excision assay).

The new breast cancer animal model carrying the MMTV-Neu1842-IRES-Cre construct is promising and can be used in combination with existing models to answer some of the remaining questions regarding breast cancer signaling pathways.

Acknowledgments:

I would like to dedicate this work to my daughter Izabela. She has been very patient and understanding, during the time I had to spend working on this project, time that I should have been spending with her. I also like to thank my husband for his moral help during the thesis write-up sessions.

I would like to thank my supervisor and committee members for their time and comments. I appreciate the opportunity I had to work on this project. I have learned a lot.

I have been very fortunate to meet and work with Rob Perry. I would like to thank him for being a wonderful teacher, and showing me all the little secrets of the cloning business. He has been a wonderful advisor and helped me improve my writing skills considerably. His comments were always to the point and his constructive criticisms helped me improve my working techniques and my thinking pattern.

I would also like to thank my laboratory colleagues, who have helped locate various things in the chaotic arrangement of the three labs we had. I would like to mention especially Adele and Niki. Adele has guided me through tissue culture techniques and has been a great advisor. Niki was a good friend and offered moral support when I needed it most.

Overall I am just happy and thankful to finish this project. I hope my work will participate in any minute way to the world of cancer research. This thought justifies the long nights, weekends and emotional stress that this project has put me through. This being said, I will take a break from laboratory research at least for now. Again, thank you everybody that helped me and guided me through this work.

Table of Contents

List of Illustrations

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 1: Introduction

Cancer is still one of the most prevalent human diseases. A danger to anybody, regardless of lifestyle, cancer is still a main focus of researchers, at the molecular level, in clinical studies, in new diagnosis and treatment procedures. Prostate and breast cancers are second only to lung cancer in their abundance in men and respectively women. These two types of malignancies were studied intensively and they still deserve attention from researchers. The biochemical mechanisms underlying their development are still not completely clear. Both have several animal models but more are needed to mimic different stages and types of the human diseases.

Prostate cancer represents more than 26% of the diagnosed cancers in Ontario men and is the leading form of diagnosed cancer in Canadian men (National Cancer Institute, 2001). One in 8 men will develop the disease in their lifetime (Canadian Cancer Society, 2003). Breast cancer represents 29.8% of all newly diagnosed cancers in females in Canada in 2001. One in 9 women will be diagnosed with the disease in their lifetime (Canadian Cancer Society, 2003).

The survival rate for patients with any type of cancer still depends on an early and correct diagnosis. Diagnosis tools are improving and this is observed in the increasing survival rate trend for both prostate and breast cancer. The most common breast cancer detection methods in Canada are mammograms (Health Canada, 2003). They are x-rays of the breast tissue that can show abnormalities in the breast tissue. A mammogram result can also identify the presence cysts and calcifications. To confirm malignancy a second diagnostic tool is used, such as an ultrasound or a biopsy. A mammogram has a 20% chance of missing a cancerous nodule (Cancer Care Ontario, 2003). The molecular background of tumor progression is needed for less invasive diagnostic methods. Canbreal developed a new blood test to diagnose breast cancer by measuring the levels of two estrogen dependent markers, and this new test is currently in clinical trials in Mexico (Canbreal, 2003). More similar initiatives are needed.

The primary diagnostic tool for prostate cancer screening in Canada is still an annual digital rectal exam for men over 50, while the American Cancer Society recommends an annual prostate serum antigen (PSA) test. Elevated PSA levels indicate a malfunction of the prostate gland, while not necessarily indicating malignancy. PSA is a member of the kallikrein family, and other members of this family are biomarkers in many other cancers, including ovarian, but their role in the development of the tumor is not yet understood (Yousef *et al* 2002). Studies on other members of the kallikrein family, found another potential diagnostic tool for prostate cancer, kallikrein 11. Serum levels of kallikrein 11 in combination with PSA levels, can distinguish between benign prostatic hyperplasia and malignancy, thus reducing the number of unnecessary biopsies (Nakamura *et al* 2003). This variety of new and improving diagnosis techniques promises to increase the rate of early detection, and thus improve survival rates.

This ultimate goal of improving survival rate and quality of life relies on research for better diagnostic and treatment tools as well as potential cures. In order to obtain these tools, the mechanisms underlying the development of cancer have to be understood. To study the evolution of cancer and the cellular signaling pathways affected by cancer, good study models are needed.

1.1 Animal Models

Research is continuously aiming to complete the mapping of the intricate molecular pathways involved in cancer development. Understanding interactions at molecular level can eventually be translated into more efficient treatments of human disease. The interaction of various gene products at molecular level is studied using bacterial systems, yeast models, insect studies or animal models. A good model is essential in understanding a signaling pathway and in determining its downstream effects. When studying a human disease, such as breast or prostate cancer, the ideal animal model has to be very similar to its human counterpart at the molecular level.

While some prostate gland diseases are observed in canines and rodents, prostate cancer is unique to humans. Therefore, in order to obtain a greater understanding of the etiology of prostate cancer, the generation of sufficient animal models is required. Breast cancer is naturally occurring in dogs, cats and rodents. The mouse is a useful tool in the lab and its natural capability of developing breast malignancies is further beneficial.

A good animal model has tissue specific oncogene expression, which does not interfere with the organ s normal growth and development. The oncogene expression can be controlled and can eventually be delayed until adulthood; tumor development phases are similar to the stages of the human disease. Cancer development also depends on other factors, such as angiogenic factors. In order for a tumor to grow it needs to create new blood vessels with the help of angiogenic factors. If these factors can be limited in the proximity of the tumor, the tumor may not progress (review by Folkman 2002). The ideal model is a reflection of the human disease and could be used for preliminary drug testing besides the signaling pathway studies.

The choice of the oncogene is the most important in a cancer animal model. Oncogenes are either mutated versions of a normal gene, extra copies of a normal gene, or viral genes. The presence of an oncogene in a cell can cause deregulation of cellular functions and transformation. An oncogene can trigger one or more signaling pathways. The downstream components of a signaling pathway depend sometime on the tissue type, as genes can be differentially expressed. In order to understand the signal transduction capacity of a single oncogene, it is essential to know the downstream members of the signaling web.

Signal transduction pathways have been heavily studied. Some of the most common oncogenes used in animal models are p53, fos, myc, ras, neu, polyoma

middle T (MT), and SV40 large T. The choice of oncogene varies depending on the type of cancer researched.

Another important choice in building an animal model is the promoter used to express the oncogene. One can choose the promoter of an ubiquitous protein, or a strong viral promoter, to express the oncogene in all tissues, to assess the potential of an oncogene. On the other hand, if one type of cancer is specifically researched, a tissue specific promoter is used. Important characteristics of a promoter are its dependence on hormones or other inducers, its spatial and temporal expression pattern, and its potency to promote transcription of the downstream gene. Temporal expression patterns are as important as spatial expression patterns, because studies may need oncogene expression after or during embryonic development.

1.2 Purpose Statement

The purpose of this project is to design animal models for breast and prostate cancer relying on previous studies, trying to combine various elements into a better model. The constructed transgenes contain previously used promoters and oncogenes, in new combinations. One of the goals of this project is to study the effect of MT-driven tumorgenesis of the prostate, by creating a mouse model of prostate cancer, involving the tissue specific promoter probasin, the MT oncogene, the encephalomyocarditis virus (EMCV) IRES (internal ribosomal entry site) and Cre recombinase, in one bicistronic vector. This vector was also constructed with the firefly luciferase following the IRES site, instead of Cre recombinase. The second part of the project focuses on creating a line of transgenic mice carrying MT or a mutated version of Neu (Neu1842, Siegel *et al*, 1994) under the murine mammary tumor virus (MMTV) promoter, followed by the EMCV IRES and Cre recombinase.

1.3 Breast Cancer Animal Models

The first mouse models of breast cancer were created using the MMTV virus. This retrovirus was shown to integrate at a specific locus in the mouse genome, denoted int-1. Mice infected with the virus developed mammary tumors (Nusse *et al* 1982). The tumors were caused by cellular proteins activated by the genome integrated provirus (Peters *et al* 1984). Mammary tumors were also induced with various chemicals, but were shown to be the result of mutated versions of cellular proteins, such as H-ras mutations induced by nitroso-methylurea treatment (Sukumar *et al* 1983).

These and other studies proved the efficiency of the MMTV LTR (long terminal repeat) promoter, isolated from the MMTV virus. It was shown to localize to the breast tissue, with expression observed in the salivary glands and

male reproductive tract (Ponta *et al* 1985). This promoter was used in the majority of breast cancer animal models that followed, such as MMTV-myc (Stewart *et al* 1984) and MMTV-neu (Bouchard *et al* 1989).

Newer models use the endogenous ErbB2 promoter (Andrecheck *et al*, 2003), but ErbB2 is normally expressed in other tissues besides the breast, such as the prostate, thus this promoter does not offer expression localized to the breast. Other promoters have been used, such as the prolactin promoter, but its strict dependence on steroids makes it undesirable for transgenic studies (Crenshaw *et al* 1989). Currently there is one protein known to express solely in the breast, the whey acidic protein. Its promoter has been used in transgenic mouse studies, in conjunction with the SV40 large T antigen, but the disadvantage of this model is that the promoter is active only in mammary epithelial cells during late pregnancy and lactation (Tzeng *et al* 1993).

The viral MMTV LTR promoter is not limited by pregnancy and lactation, thus remains the best choice for mouse breast cancer model. MMTV-MT transgenic mice (Guy *et al* 1992) were one of the best-studied breast cancer animal models.

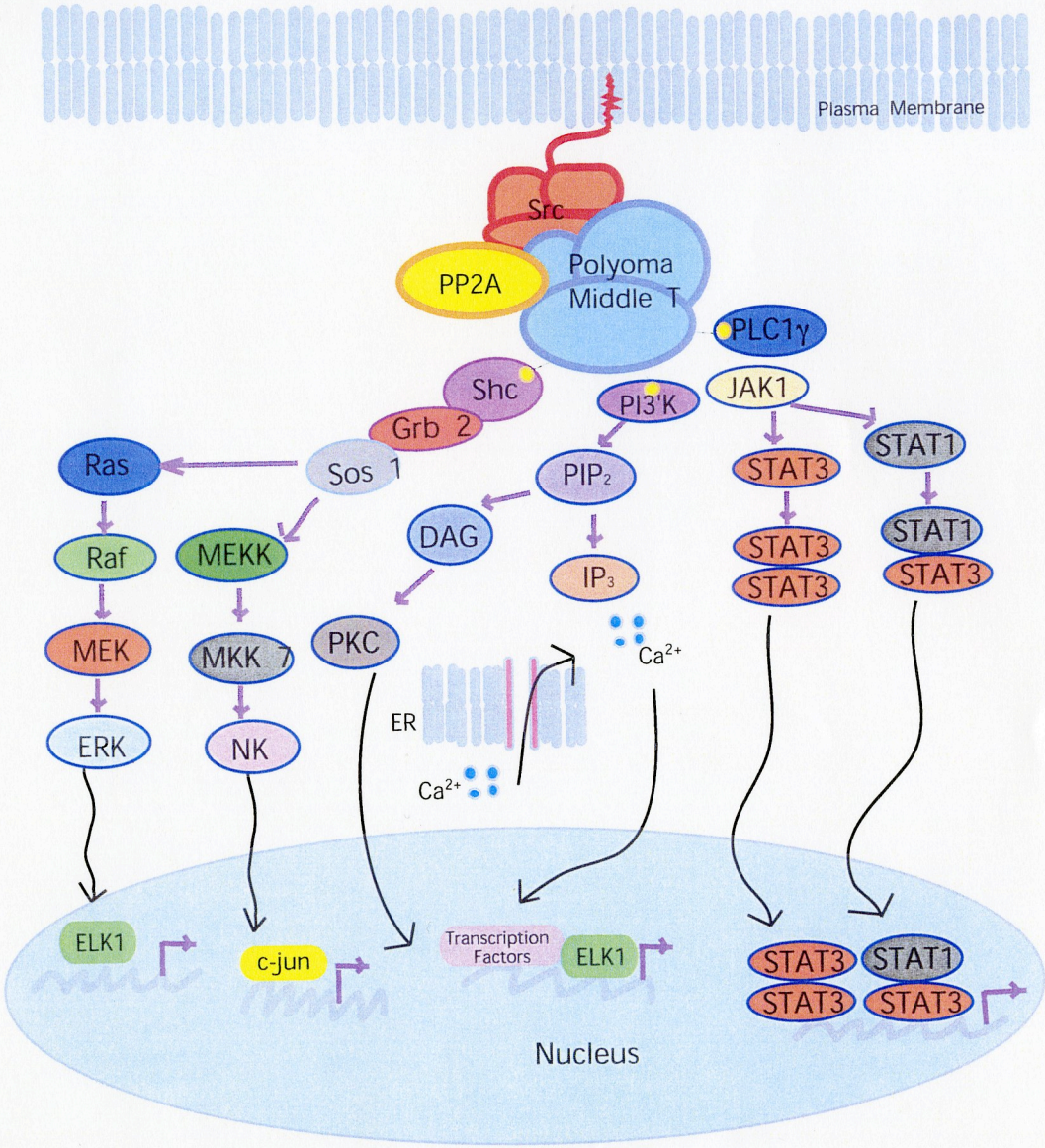
1.3.1 Polyomavirus Middle T antigen

MT is one of the viral proteins produced by polyoma virus during its lytic cycle (Norbury *et al* 1987). Together with the large T antigen and small T antigen, they act on the infected cell to increase the levels of mRNA of cellular genes that allow for DNA replication, in order to provide a suitable cellular environment for replication of the viral genome. When infection does not kill the cell, the T-antigens are still produced, resulting in cellular transformation and tumorgenicity (Benjamin *et al* 1979). The T antigens have been shown to increase the levels of proto-oncogenes, such as c-myc and c-fos (Zullo *et al* 1987), which can lead to transformation (Calabretta *et al* 1985, Terrier *et al* 1988).

MT does not appear to express any enzymatic activity of its own, but functions by interfering with and disturbing the normal activities of essential growth and regulatory factors in the cell. Through a series of interactions, MT assembles a large multi-protein complex at the cell membrane (Schaffhausen *et al* 1982). The complex consists of MT, the core dimer of protein phosphatase 2A (Pallas *et al* 1990), and a *src*-family tyrosine kinase, p60c-src, p62c-yes or p59c-fyn (Courtneidge *et al* 1984, Kypta *et al* 1988, Kornbulth *et al* 1990). Subsequently, the kinases phosphorylate themselves and MT, creating phosphotyrosine sites for interaction with the SH2 domain of phosphoinositol-3 kinase (PI3 K) (Auger *et al* 1992), the phosphotyrosine binding domain of ShcA (Dilworth *et al* 1994, Blaikie *et al* 1997), and PLCγ-1 (Gorga *et al* 1990).

The interaction with ShcA and PI3 K causes phosphorylation of the tyrosines on the ShcA molecule, creating a docking space for the Grb2 adapter. Once Grb-2 binds to the complex, it brings in the nucleotide exchange factor

Figure 1: MT signaling pathways.
MT anchors itself to the cellular membrane by connecting to various proteins. It forms a multi-protein complex that recruits Shc, PLCγ1 and PI3 K. These molecules signal downstream using pathways similar to the EGFR (see Figure 2). The MAPK, STAT and PLCγ1 cascades are activated in the absence of an external stimulus, causing deregulations in cell growth and cell cycle.


Plasma Membrane
Src
PP2A
Polyoma
Middle T
PLC1γ
Shc
Grb 2
PI3'K
JAK1
Ras
Sos 1
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STAT1
STAT3
Raf
MEKK
DAG
STAT1
STAT3
IP3
STAT3
MEK
MKK 7
PKC
Ca2+
ER
ERK
NK
Ca2+
ELK1
c-jun
Transcription
Factors
ELK1
STAT3
STAT1
STAT3
STAT3
Nucleus

mSos, and due to the relocation of this factor to the membrane, the MAPK pathway can be activated, through Ras (Rozakis-Adcock *et al* 1992, Dilworth *et al* 1994). The MAPK pathway is responsible for upregulating the transcription of many genes important in the growth and development of the cell. Deregulation and aberrant activation of the MAPK pathway will lead to uncontrolled production of molecules involved in cell cycle and growth regulation, leading to a transformed phenotype. By this mode of action middle T mimics the behavior of activated growth factor receptor tails (Glover *et al* 1999).

Activated growth factor receptor tails as well as MT, interact with PLC1γ (Meisenhelder *et al* 1989, Gorga *et al* 1990). PLC-1γ is capable of activating Ras and thus signal via the MAPK pathway (Smith *et al* 1990). MT indirectly activates Akt, via the PI3 K pathway, but not via Shc. Akt is involved in insulin stimulation response and apoptosis. Its uncontrolled activation leads to deregulation of these processes (Kitamura *et al* 1998, Kulic *et al* 1997). MT also interacts with the 14-3-3 family of molecules (Summers *et al* 1998). The 14-3-3 family of proteins consists of conserved regulatory molecules expressed in all eukaryotes, which control cell cycle progression, transcriptional alterations, and apoptosis (Fu *et al* 2000).

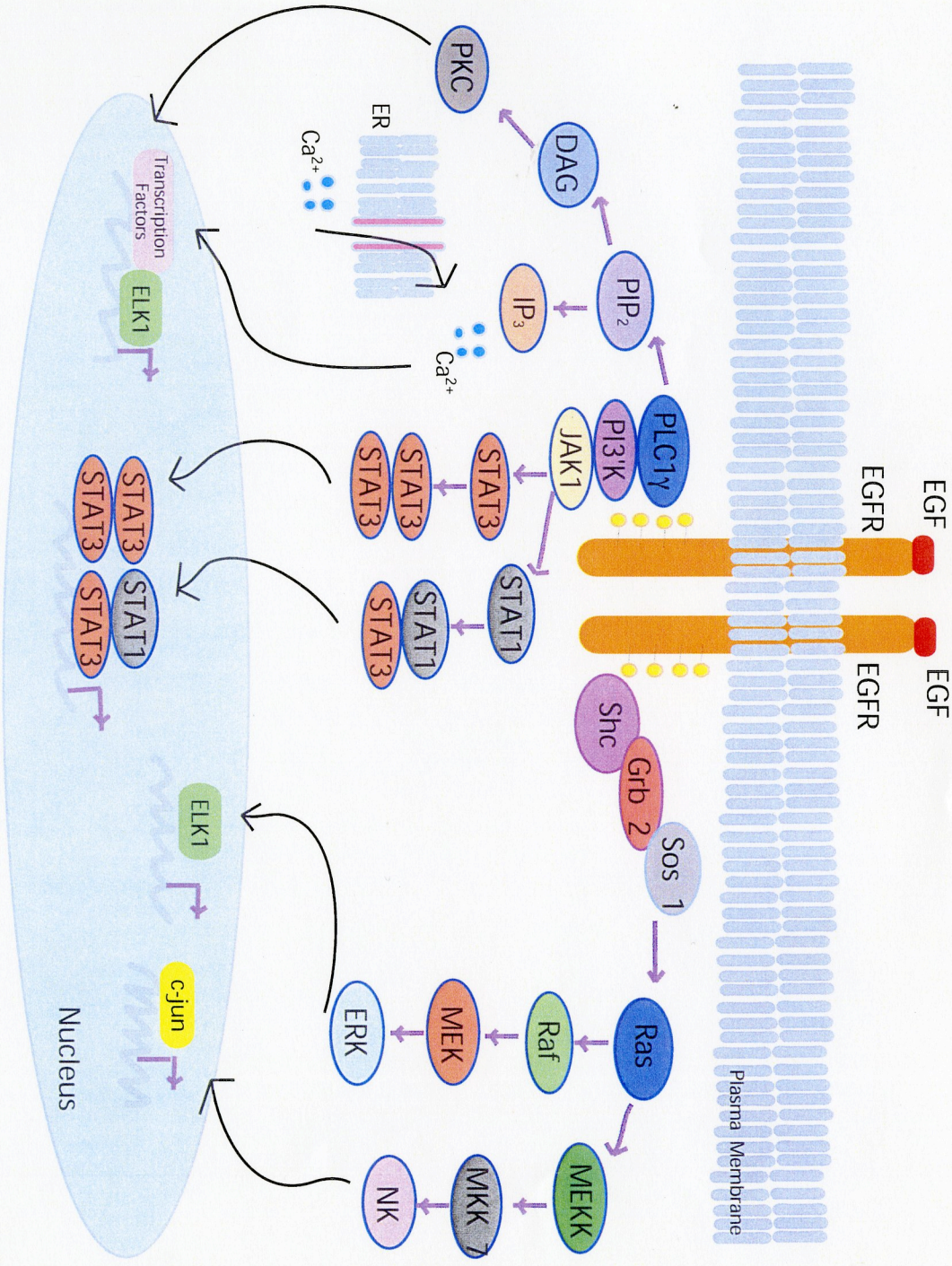
The newer mouse breast cancer models are used to study genes overexpressed, mutated or deleted in human tumor samples. These animals helped resolve unanswered questions about breast cancer development, and they are still needed for further research. As many as 150 oncogenes have been associated with the spontaneous non-inherited forms of breast cancer, out of which 40 have been shown to be important at the crucial step towards metastasis. The Her2 oncogene was first identified in breast cancer samples (Varley *et al* 1987) and now studies show that Her2 is overexpressed/activated in 25% of breast cancers (Canadian Cancer Society, 2003). Drugs such as herceptin were designed for treatment of her-2 overexpressing cases of breast cancer (Baselga *et al* 1998), achieving the end goal of developing a potential treatment for the disease.

Her2 is the homologue of Neu in rats and ErbB2 in mice (Schechter *et al* 1989, Slamon *et al* 1987). This gene has been studied intensively and mice models are widely available (Muller *et al* 1988, Guy *et al* 1992, Andrecheck *et al* 2003). Transgenic mice expressing wild type and mutated versions of erbB-2 have helped in mapping a net of interaction pathways between various proteins involved in normal mammary gland development and tumor progression.

1.3.2 Erb B2 (Neu, Her2) Signaling Pathways

ErbB2 is a receptor tyrosine kinase (RTK) and a member of the epidermal growth factor receptor (EGFR) family. Upon ligand binding, ErbB2 can form homo- and heterodimers with other EGFR family members, such as ErbB1, ErbB3 and ErbB4 (Cochet *et al* 1988, Goldman *et al* 1990, Alimandi *et al* 1995,

Figure 2: Signaling pathways activated by the epidermal growth factor receptor upon binding to its ligand.
The MAPK, STAT and PLCγ1 pathways are activated upon receptor homo- or heterodimer formation, as a result of ligand binding. In a particular cell type, one or more of these pathways can be activated at once, depending on the expression patterns of the signaling molecules. The end result is the alteration of transcription due to direct activation/inhibition, or to variations in the levels of transcription factors present in the nucleus. This process is strictly regulated and has an active role in cell cycle and cell growth.


EGF
EGF
EGFR
EGFR
Plasma Membrane
PLC1γ
PI3'K
JAK1
PIP2
DAG
IP3
PKC
ER
Ca2+
Ca2+
Shc
Grb 2
Sos 1
Ras
Raf
MEK
ERK
MEKK
MKK 7
NK
STAT3
STAT3
STAT3
STAT3
STAT1
STAT3
STAT3
STAT3
STAT1
Transcription Factors
ELK1
ELK1
c-jun
Nucleus

Cohen *et al* 1996). Activated ErbB2 can signal downstream using either the MAPK pathway or the PI3 K pathway (Gallego *et al* 1992, Ram *et al* 1996). Both these pathway are responsible for regulating key events in cell growth, and a signaling error in these pathways can lead to deregulation and ultimately to a transformed phenotype. Both these pathways are influenced by a variety of factors and can enhance proliferation as well as protect cells from apoptosis.

ErbB2 interacts with PLC1γ via its SH2 domain, to activate PKC (protein kinase C) (Meisenhelder *et al* 1989). PLC1γ splits phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3). The IP_3 interacts with Ca^{++} channels on the membrane of the endoplasmic reticulum. Ca^{++} flows into the cytosol where it interacts with calmodulin (Berridge *et al* 1986). The Ca^{++}-calmodulin complex activates target proteins, such as PI3 K (Yamaguchi *et al* 1987). Furthermore, arachidonic acid is hydrolyzed from DAG. The free arachidonic acid participates in the biosynthesis of prostaglandins (Schiess *et al* 1992), which can modulate cellular responses to hormones (Bergstrom *et al* 1963). DAG also activates the PKC pathway. PKC binds DAG, which causes a slight conformational change, causing increased affinity for Ca^{++}. When bound to DAG and Ca^{++}, the active PKC transfers a phosphate from ATP to specific serine or threonine of target proteins (Nishizuka *et al* 1983, Hunter *et al* 1984, Cochet *et al* 1984).

ErbB2 can be either mutated or overexpressed to induce transformation. The first activating ErbB2 mutation has been identified in mouse tumor samples (Bargmann *et al* 1986) as a single point mutation in the transmembrane region of ErbB2. This mutation, denoted NeuNT caused dimerization of the receptors in the absence of ligand, thus rendering the downstream signaling pathway active. A series of deletions proximal to the transmembrane domain of Neu were discovered as being the cause for oncogenic potential of other mutated versions of Neu. Out of the 4 mutations discovered by this experiment, Neu1842 was the most potent. Neu1842 has a 36 bp in frame deletion close to the transmembrane domain of the protein. However, this new mutant has less oncogenic potential than the previously described NeuNT (Siegel *et al* 1994).

The oncogenes used to explore breast cancer are good candidates for studying other cancer types, if they are expressed under the right promoter. Both MT and ErbB2 are good candidates for a prostate cancer animal model. MT would provide a fast transformation of the tissue, while ErbB2 has been shown to express in the prostate tissue, thus its deregulation may participate to tissue transformation in the prostate, as it does in the breast tissue.

1.4 Prostate Cancer Animal Models

One of the earliest mouse models of prostate cancer was designed using the polyomavirus middle T oncogene, under the control of the C3 promoter, of rat

prostate steroid-binding protein. This transgenic line was not valid, as the males could not reproduce and the females developed mammary tumors, due to leaky expression under the C3 promoter (Cleutjens *et al* 1997).

The next model had tumors are induced by the presence of the SV40 Large T antigen of polyomavirus (Foster *et al* 1997, Kasper *et al* 1998) under the probasin promoter. This is the well-recognized TRAMP (Transgenic Adenocarcinoma of Mouse Prostate) model, currently the best prostate cancer model. The advantage over the previous model was a better promoter choice.

The prostate gland develops postnatal until up to 5 weeks of age. The probasin gene is expressed at puberty, when the animal becomes sexually mature. A probasin-CAT transgenic mouse demonstrates that the probasin promoter becomes active between 2 and 7 weeks of age, expressed strictly in the prostate, and is only active in the presence of steroid hormones. The probasin promoter has two androgen-binding sites, thus expression of the downstream gene is strictly regulated. The 426 basepairs of the rat probasin (PB) gene promoter and 28 basepairs of 5'-untranslated region is sufficient to target a reporter construct to the prostate The probasin promoter has been shown to be prostate specific, and is active in the epithelial cells of the rodent prostate (Greenberg 1994). The tumors developed by these mice were shown to be strictly androgen dependent, due to the two steroid binding sites on the promoter (Asamoto *et al* 2001). This proves that the existing models are not sufficient to imitate the human condition, as human prostate cancer is either androgen dependent or independent.

Other promoter choices were the human PSA promoter, MMTV LTR, and fetal globin promoter. None of these promoters is strictly specific to the prostate and androgen independent, thus none can overcome the weakness of the current best model. Various oncogenes used include the SV40 large T antigen, with or without the small T antigen, rasT24, human bcl-2 and keratinocyte growth factor (Green *et al* 1998). More studies of the oncogenes responsible for the human disease are needed in order to create a model more comparable to the human disease.

Rodent models have proven useful in providing accurate models in signaling pathways, especially in breast cancer. Until recently, transgenic mice have been created with only one modified gene at a time. The procedure involved injecting the foreign gene in the fertilized eggs, then genotype the progeny for the foreign fragment. The effect of one modified gene in the animal system is useful and interesting to study, but more complex research is needed to acquire information on gene-gene interaction. For these studies, a mouse carrying two independent constructs is required. This can be achieved by crossing two different transgenic lines in order to obtain litter of the desired genotype. This process is long and the success rate is low as the desired genotype often represents a very low percentage of the litter and several matings have to occur until enough animals are obtained. This process is expensive and time consuming, both issues

being important factors in research. In order to speed this process, new technology and new research methods have to be developed.

To continue the odyssey and study the complete pathways of interactions between oncogenes and other proteins, one must be able to look at phenotypes of animals where the function/expression of two or more genes is altered. Transgenic mice carrying two constructs simultaneously reduce greatly the time needed to study the impact and relationship of two genes. For this purpose, bicistronic constructs, similar to those carried by several viruses, can be made and used to produce transgenic mice. Bigenic mice carry and express two separate genes at the same time, under the control of one promoter. The construct contains a promoter of choice, one gene of interest, IRES and the second gene of interest. Some of the first bigenic mice were constructed using EMCV IRES followed by Cre and GFP (Wen *et al* 2003).

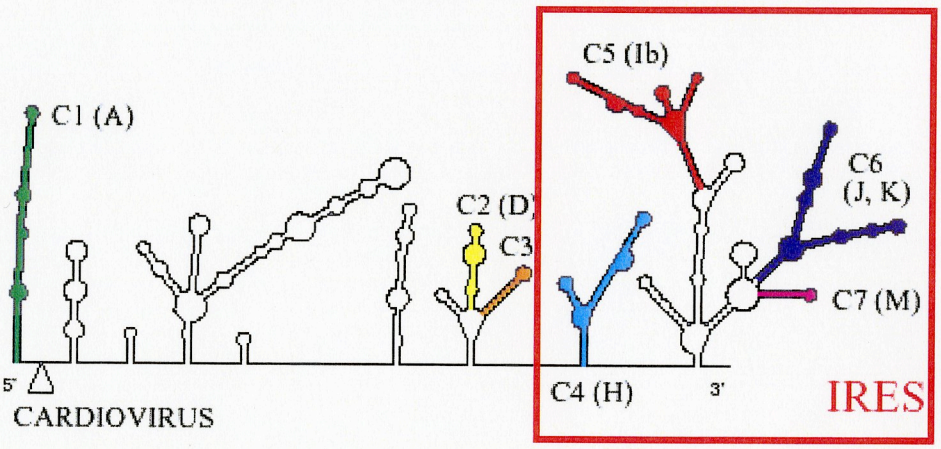
1.4.1 Encephalomyocarditis virus IRES

The EMCV IRES sequence is used in this project to allow the translation of Cre simultaneously with the upstream oncogene, once the promoter is induced. The EMCV IRES chosen proved effective previously in other bicistronic vectors (Ghattas *et al* 1991). The way the ribosome is recruited to the IRES is 5 cap independent. EMCV is a picornavirus. Picornaviruses have RNA genomes and replicate by inhibiting the host cell s cap-dependent translation. They use the internal ribosomal entry site as a cap-independent option. The IRES has an intricate secondary structure and looks somewhat like a clover leaf (Figure 3). The ribosome is recruited to this structure, binds it and begins scanning for initiation codons.

There is a polypyrimidine tract followed by an AUG codon about twenty nucleotides downstream. This motif was suggested to represent the eukaryotic analog of the prokaryotic Shine/Dalgarno sequence. Ribosomes bind directly to this 12^{th} AUG codon (the initiation codon for viral protein synthesis). They do not utilize the 11^{th} AUG codon located only 8 nucleotides upstream. Mutations in the flanking sequences of the initiator AUG codon which render it suboptimal for initiation lead to recognition of downstream AUG codons most likely because ribosomes begin to scan the mRNA in the 5' to 3' direction. Also, the 11 AUG may be hindered due to the tertiary structure of the IRES/ribosome binding (Witwer *et al* 2001).

The expression of the second cistron in a bicistronic vector containing an EMCV IRES is about 1/3 of the first cistron (Novagen). In the vectors designed for this project, the ones carrying the MT oncogene will experience a further reduced expression of the downstream gene. MT contains an alternative poly (A) site at the 3 end of the gene, with an approximate 50% transcript termination efficiency (Norbury *et al* 1987). This factor will reduce the amount of the second

Figure 3: Secondary RNA structure of IRES sequences.
Both aphtovisuses and cardioviruses have IRES structure they utilize for cap independent translation. The clover-leaf like structure is similar for the two virus families but the cardiovirus IRES is most common in bicistronic studies. A-M are conserved patterns, various secondary structure elements important in ribosomal recruitment. The figure was published in Nucleic Acids Research,29(24), p.5079-89 (Witwer *et al* 2001).


C5 (1b)
C1 (A)
C6
(J, K)
C2 (D)
C3
C7 (M)
5'
CARDIOVIRUS
C4 (H)
3'
IRES

transcript to about a sixth of the amount of the first transcript. The second transcript has to produce a highly processive protein in order to be efficient.

1.4.2 Cre recombinase

Cre recombinase was the choice for our second transcript, following MT and IRES. This protein is highly processive and only several copies per cell are sufficient for efficient excision. Cre recombinase is a useful tool in transgenic mice studies, as it allows for tissue specific gene ablation. This is important, as some genes are necessary for proper development in organs other than that of interest. With the help of the Cre-loxP system, the gene of interest can be removed only from the tissue where Cre is expressed, at the time the tissue specific promoter of Cre becomes active.

Cre recombinase is a protein encoded by the genome of bacteriophage P1 that recognizes specific loxP sequences which it excises (Abremski *et al* 1984). Floxed genes are flanked by loxP sites in order to be excised when Cre recombinase is expressed. Cre recombinase in a transgenic mouse is best to be expressed locally, in the organ of interest, to be active in a restricted area and remove the gene of interest.

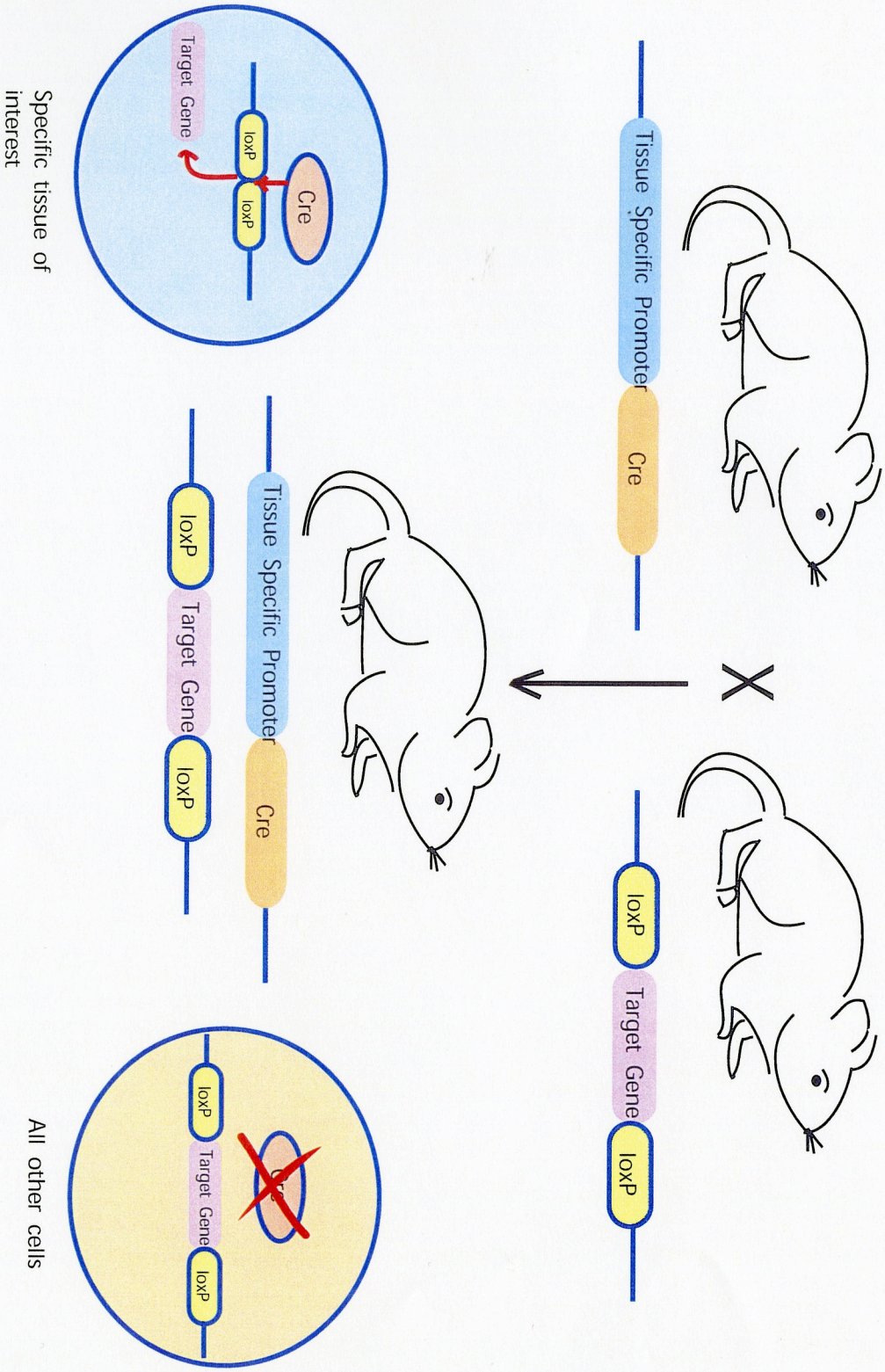
This lab has used the Cre-loxP system in order to study Neu effects in breast cancer. Thus a line of mice containing floxed Neu is already established (Andrecheck *et al* 2002). The Cre system has been used with various other floxed lines, for example: the tumor inhibitor p53 (Marino *et al* 2000), angiogenic factor VEGF (Gerber *et al* 1999), downstream effector β-catenin (Harada *et al* 1999), STAT3 (Takeda *et al* 1998) and oncogenes BRCA1 (Xu *et al* 1999), BRCA2 (Ludwig *et al* 2001), c-fos (Zhang *et al* 2002).

Figure 4 depicts the use of the Cre-loxP system to generate tissue specific gene ablation. In bicistronic models, the effect of tissue specific ablation of one gene of interest can be observed during oncogenesis, in one mouse crossing step.

The excising capability of Cre can be tested by mating the bigenic mice produced by Cre containing constructs, to the Rosa26 strain of mice. Also known as a reporter strain, or gene-trap system, the Rosa26 line was constructed to monitor Cre mediated excisions. In this line, retroviral sequences and a b-gal-neomycin resistance fusion gene (bgeo) are integrated in the locus ROSA26. Transcription of the bgeo gene is initiated from exon-1 of the ROSA26 allele and is ubiquitous during embryonic development and adult tissues. The gene-trap strain has a floxed stop fragment that abolishes the b-gal expression in the absence of Cre. (Mao *et al* 1999). This system can be used to detect spatial and temporal expression of Cre in the transgenic line.

Crossings between these bigenic mice and existing lines of floxed genes of interest, will excise locally a gene of interest, allowing observation of mammary gland/prostate development in the absence of a gene of interest. Mice carrying two genes under the control of one promoter will aid in monitoring tumor

Figure 4: The Cre-loxP system.
A gene flanked by loxP sites can be excised by Cre recombinase. Using tissue specific promoters, Cre can be expressed only in localized tissues, thus being able to function and remove the target gene only in the desired area. The advantage of a bicistronic vector is that it will carry an oncogene in the same transgene as Cre. Through the recombination process, one can observe tissue transformation in the absence of a gene of interest.


Tissue Specific Promoter
Cre
loxP
Target Gene
loxP
X
Tissue Specific Promoter
Cre
loxP
Target Gene
loxP
Target Gene
loxP
loxP
Cre
Specific tissue of interest
loxP
Target Gene
loxP
All other cells

development and will be a useful tool in following experiments testing gene-gene interaction *in vivo* for various cancer types.

1.4.3 Luciferase

Monitoring *in vivo* development of various organs can now be achieved with the help of modern technology. The Xenogen IVIS apparatus (www.xenogen.com) can be used to visualize luciferase presence and activity in a living organism. The firefly luciferase gene is a common reporter gene. In the presence of its luciferin substrate is emits light, which can be measured in order to assess the activity of the enzyme. Luciferase expression in mammalian tissue does not influence normal development. This reporter has been used in several lines of transgenic mice, initially in studies of promoter activity (DiLella *et al* 1988) and recently in gene regulation studies (Zhang *et al* 2003).

Visualizing an organ *in vivo* allows researchers to follow disease progression and normal organ development, using a lower number of animals. This technology also offers the advantage of using the same animal for measuring parameters at different age points, thus minimizing errors.

The models of breast cancer proposed in this study make use of the MMTV promoter and either the MT oncogene, or the Neu1842 mutant. For prostate cancer, the MT oncogene was chosen, and the probasin promoter, to limit the oncogene expression to the target tissue. The addition of the IRES and Cre after the oncogenes, make these vectors useful tools in studying gene-gene interactions in breast cancer induced by either MT or mutated Neu. The transgenic lines of mice proposed will be used in combination with new or existing lines of transgenic mice, to study gene interactions in a more complex manner, by modifying two or more genes at a time.

Chapter 2: Materials and Methods

2.1 DNA Constructs

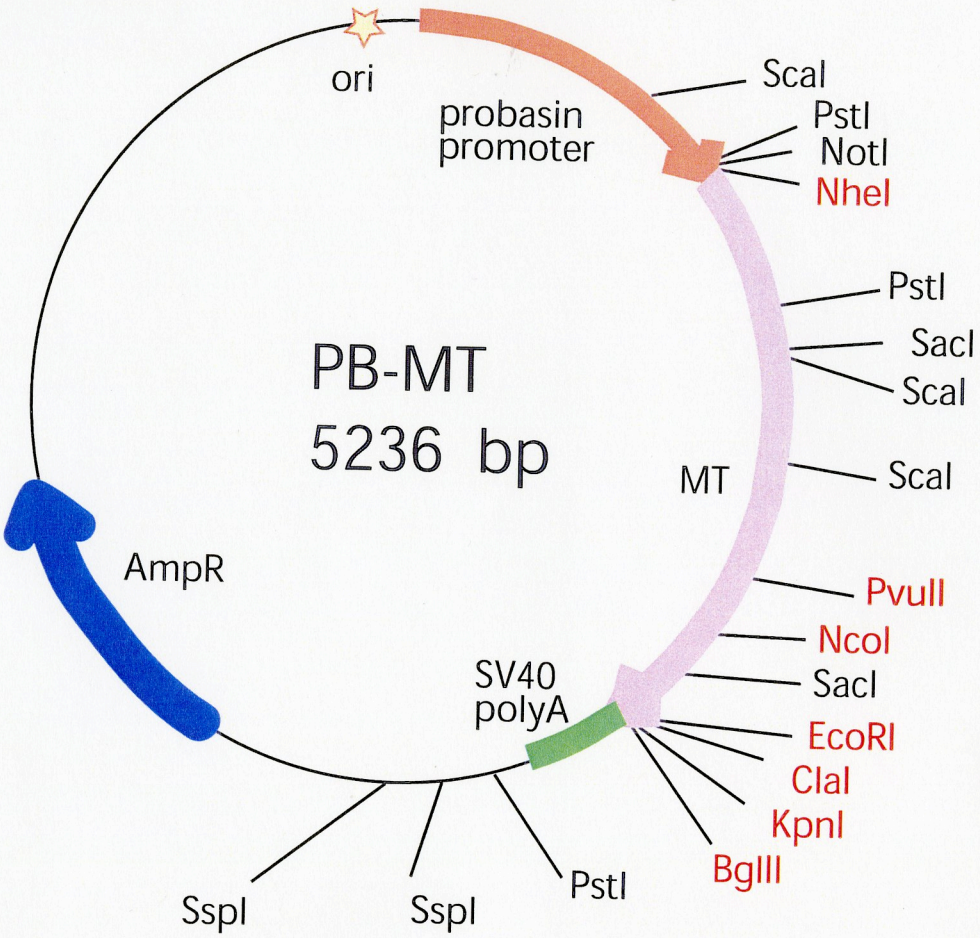
Three bicistronic vectors were constructed and used to make transgenes and transgenic mice. The intermediate vectors and the final vectors of interest are presented below. All agarose gels used for purification and size analysis were 1%. All restriction digests were performed in 20µl volumes. All ligations were performed using T4 DNA Ligase (Invitrogen). Ligation mixture was used to transform DH5α cells. The cells were made competent by the following protocol:

1. Grow 50µl of frozen cell stock overnight in 100 ml of LB at 37°C
2. Grow 1ml of overnight stock in 100 ml LB for 3 hrs at 37°C
3. Spin cells down at 4,000rpm for 15 minutes at 4°C in 50 ml Falcon tubes
4. Discard supernatant and resuspend cells in 25 ml of cold ddH2O, spin down for 10 minutes at 4000 rpm, 4°C and repeat
5. Resuspend cells in 25 ml 10% cold glycerol, and spin down 5 minutes at 4000 rpm at 4°C
6. Discard supernatant and resuspend cells in glycerol leftover on tube walls
7. Aliquot 50µl in 1.5 ml microfuge tubes and use in electroporation
8. Electroporate using 2.5µl ligation mix

2.1.1 PB-MT

The probasin-Cre vector (supplied by Eran Andrecheck) was digested with PstI (Gibco) and HaeII (NEB) in 1x Y+/TANGO buffer (Fermentas) and a 736bp fragment was isolated. The pJ4ΩMT vector (supplied by John Hutchnson; MT oncogene was inserted in the pJ4Ω vector (Rodríguez 1992) in the EcoRI/HindIII sites of the MCS) was digested with HindIII (Gibco) and HaeII (NEB) in buffer 2 (NEB) with BSA, and a 4.5 kb band was isolated. The two fragments were ligated with a phosphorylated linker, obtained by annealing two 5' phosphorylated oligonucleotides: 5'GCGGCCGCTAGC3' and 5'ACGTCGCCGGCGATCGTCGA3'. The ligation reaction was set at a 1:2 ratio probasin:MT, with 1ng and 2ng of linker present. Both combinations gave positive colonies. The final resulting vector was screened with SacI (800bp drop) and an EcoRI/NotI double digest (1400bp drop). The construct was also sequenced using primer 5'AAGCTACTCTGCACCTTGTCAGT3' to ensure the proper junction between the probasin promoter and the MT oncogene.

Figure 5: PB-MT
The 5236bp vector contains the probasin promoter, MT oncogene followed by a SV40 polyA tail. The vector also has an ampicillin resistance gene, AmpR for selection and the bacterial origin of replication, ori, in the pJ4Ω vector backbone. The most common restriction enzyme sites are shown on the map. The red highlighted sites are unique.


ori
probasin
promoter
ScaI
PstI
NotI
NheI
PstI
SacI
ScaI
MT
ScaI
PvuII
NcoI
SacI
EcoRI
ClaI
KpnI
BglII
PstI
SspI
SspI
AmpR
SV40
polyA
PB-MT
5236 bp

2.1.2 PB-MT-IRES-Luciferase

The vector was created in two sub-cloning steps. In the first step, the NcoI/SalI (Gibco, in buffer 2x Y+/TANGO from Fermentas) fragments of pGL3-promoter vector (Promega, 1924bp) and pCITE vector (Promega, 3664bp) were ligated. Previous to ligation, the pCITE vector was dephosphorylated by calf intestinal alkaline phosphatase (CIAP) (Gibco), following the manufacturer's protocol, to avoid background colonies due to vector self ligation. The obtained vector was designated CITE-Luciferase. It was screened with ScaI (2586bp and 3002bp), HindIII (2303bp and 3285bp) and BglI (1422bp, 3815bp and 351bp).

The PB-MT vector, previously described, was digested with HaeII (NEB), Klenow (GIBCO) blunted then digested with EcoRI (Gibco). All these reactions were done in EcoRI buffer (Fermentas). A 2087bp piece was obtained and gel isolated in low melt agarose (Invitrogen). The CITE-Luciferase vector was cut with PvuII (Roche) and EcoRI (Gibco) in EcoRI buffer (Fermentas). A 5546bp piece was obtained and isolated in low melt agarose (Invitrogen). The two pieces were ligated in an overnight reaction, using a 1:1, 2:1 and 1:2 ratios of vector to insert. All produced viable colonies. The resulting vector was screened with NcoI (880bp and 6753bp) and NheI (3506bp and 4127bp). The vector was also sequenced with the following primers:

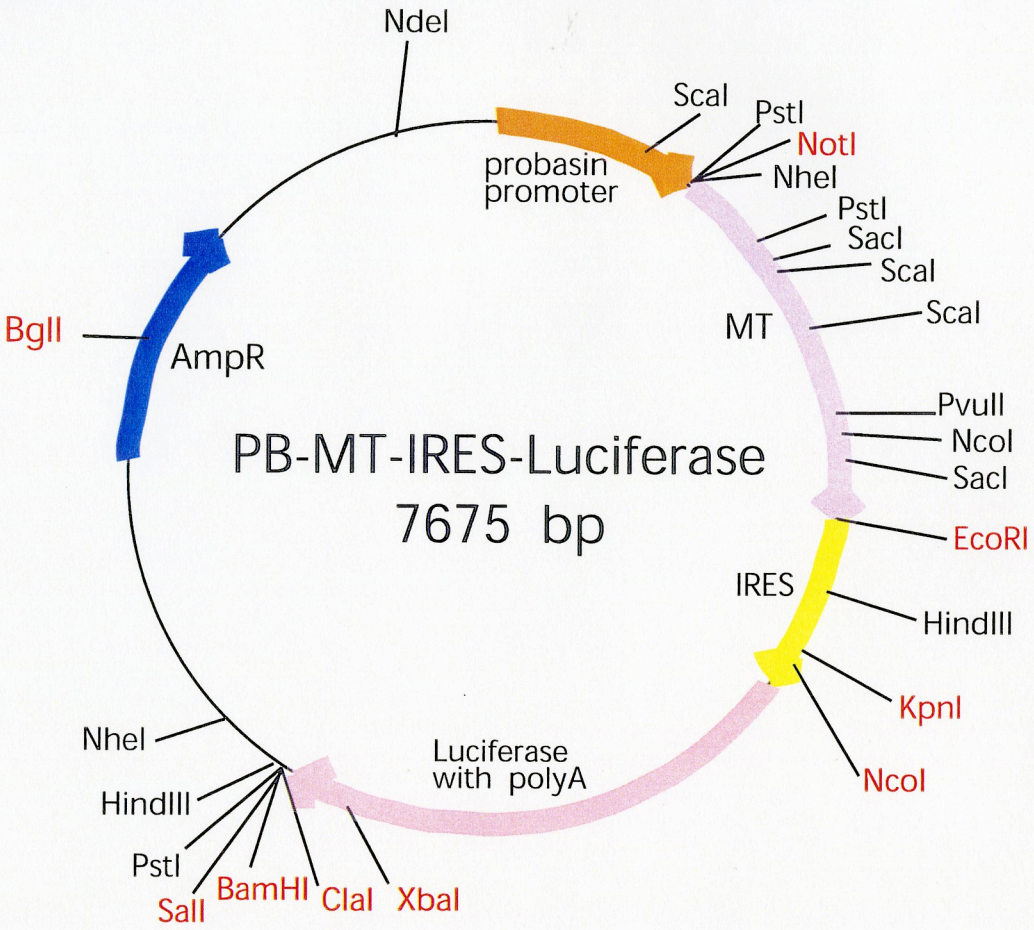
- 5'AAGCTACTCTGCACCTTGTCAGT3' to ensure the proper junction between the probasin promoter and the MT oncogene
- 5'GCAGCCTTACTGGGTATTTGTC3' to ensure a correct MT-CITE junction
- 5'TGAAGGATGCCCAGAAGGTA3' to ensure a correct CITE-Luciferase junction
- 5'CATTGAACCTGACTTGTGGATT3' to ensure the proper junction between the probasin promoter and the pCITE backbone.

The transgene fragment was obtained by digesting the vector with NdeI and BamHI and isolating the 4604bp fragment.

2.1.3 PB-MT-IRES-Cre

The 5' end of the Cre gene from the MMTV-Cre vector (provided by Eran Andrecheck, containing p206 backbone, with the Cre recombinase cloned in the EcoRI/HindIII sites of the MCS), was amplified by PCR using the primers: 5'CTGCAGAATTCCATGGTCGACTCGACCATGCCCAAGAAGAAGAAGA AGGTGTCC3' and 5'CGCTAGAGCCTGTTTTGCACGTTCACCGGC3'. These primers are modifying the 5' end of the gene, by introducing a NcoI site before the start codon and eliminating the HindIII and XbaI sites present. The PCR reaction resulted in a 400bp fragment, which was gel purified and digested with

Figure 6: PB-MT-IRES-Luciferase
The 7675bp vector contains the probasin promoter, MT oncogene followed by EMCV IRES, and Luciferase with a polyA tail. The vector also has an ampicillin resistance gene, AmpR for selection and the bacterial origin of replication, ori, in the pGL3 vector backbone. The most common restriction enzyme sites are shown on the map. The red highlighted sites are unique.


NdeI
ScaI
PstI
NotI
NheI
probasin
promoter
PstI
SacI
ScaI
MT
ScaI
PvuII
NcoI
SacI
EcoRI
IRES
HindIII
KpnI
NcoI
Luciferase
with polyA
NheI
HindIII
PstI
SalI
BamHI
ClaI
XbaI
BglI
AmpR
PB-MT-IRES-Luciferase
7675 bp

NcoI/BamHI (Gibco, in buffer 6). The resulting fragment was gel purified using Qiagen columns.

The NcoI/EcoRI (Gibco, in buffer 5) fragment (593bp) of the pCITE vector (Promega, 4830bp), the EcoRI/BamHI (Gibco, in buffer 3) fragment (2943bp) of the pBluescript KSII+ vector (Stratagene, 2961bp) and the digested PCR fragment were ligated in a three way ligation. The ligation was set up in a ratio of 1:1:3 pCite:pBluescript:Cre PCR product. The result was tested with BglI (expected sizes ~ 800bp, 1600bp and 1800bp) and KpnI (expected 500bp drop). This vector was then cut with XhoI/EcoRI (Gibco, in buffer H, Roche) to isolate a fragment containing the IRES site followed by the 5'end of Cre. Because of the close proximity of the two sites, XhoI was added first to the digest, left on for 3 hours at 37 C, then EcoRI was added and left overnight at 37°C.

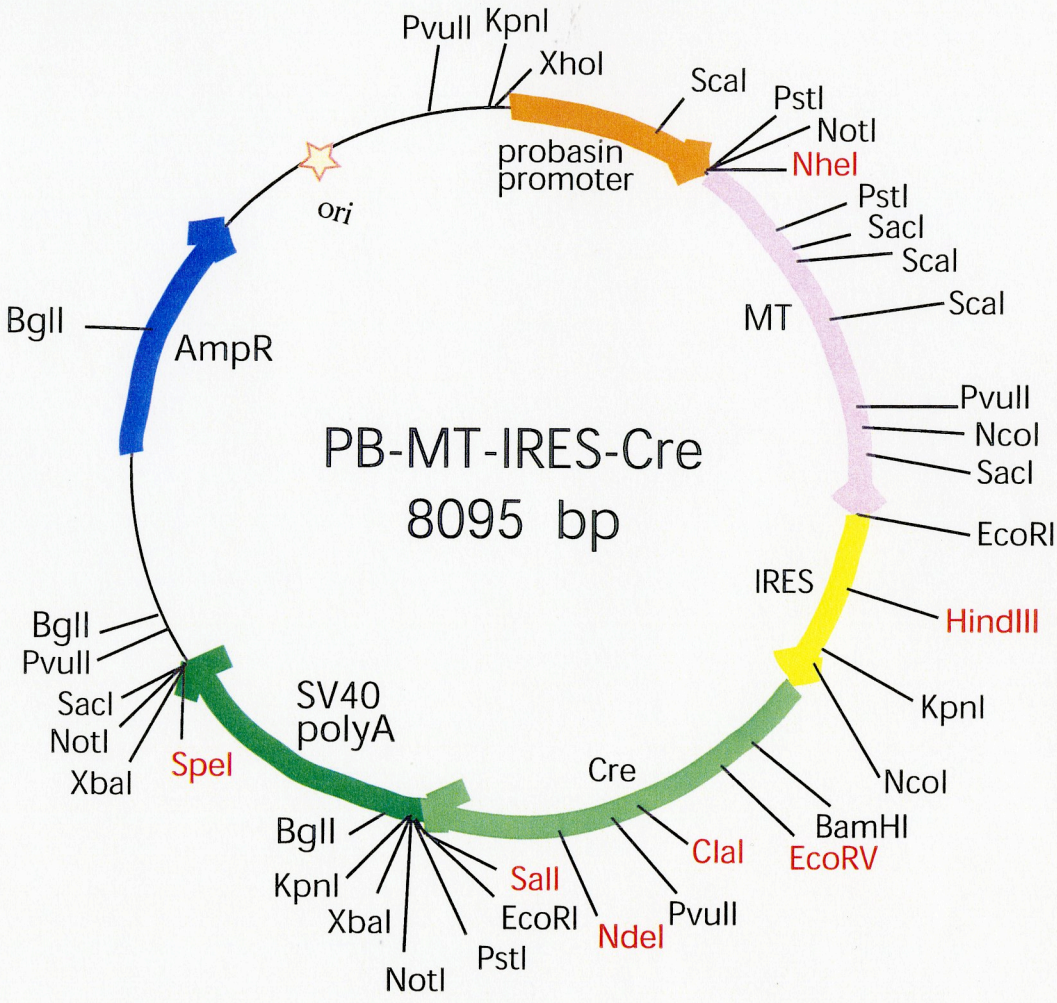
The PB-MT vector was digested with HaeII (NEB), blunted with Klenow (Gibco), heat inactivated at 72°C for 15 minutes, then digested with EcoRI (Gibco), all in EcoRI buffer (Fermentas). The resulting 2087bp fragment was ligated in the EcoRV/EcoRI (Gibco, in buffer 3) sites of the pcDNA3 vector (Invitrogen, 5446bp). EcoRV was added to the DNA first, left on for 3hrs at 37°C, then EcoRI was added and left overnight. The resulting plasmid was verified by PstI digest (expected sizes ~5000bp and 2500bp). This vector was cut with XhoI/EcoRI (Gibco, buffer H, Roche) and the IRES-5'Cre fragment was inserted. The resulting vector carries PB-MT-Ires-5'Cre and was checked by KpnI digest (2300bp and 3700bp expected) and NcoI digest (800bp drop expected). This fragment was digested with BamHI (Gibco)/SpeI(Roche) in buffer 4 (Gibco) to open the backbone and introduce the BamHI/SalI (Gibco, buffer 10) fragment of MMTV-Cre (1210bp) and the SalI (Gibco)/SpeI(Roche) fragment (digested in buffer 4, Gibco) of MMTV-Cre(~1000bp).

The ligated product was checked with BamHI (expected bands at 2200bp, 6000bp) and NotI (expecting approximate sizes: 750bp, 4000bp, 3400bp). The resulting positive clones 25 and 33 were sequenced using the following primers:

- 5'GCAGCCTTACTGGGTATTTGTC3' to ensure a correct MT-CITE junction
- 5'TGAAGGATGCCCAGAAGGTA3' to ensure a correct CITE-Cre junction
- 5'CGTTTGTTCGGATCGGTCG3' to ensure a proper Cre-backbone junction
- 5'CGCTAGAGCCTGTTTTGCACGTTCACCGGC3' to ensure a correct Cre junction, between the 5' fragment and the 3' end.

The transgene was obtained by digesting this vector with XhoI and SpeI and isolating the 5300bp band.

Figure 7: PB-MT-IRES-Cre
The 8095bp vector contains the probasin promoter, MT oncogene followed by EMCV IRES, and Cre followed by a SV40 polyA tail. The vector also has an ampicillin resistance gene, AmpR for selection and the bacterial origin of replication, ori, in the pBluescript KSII+ vector backbone. The most common restriction enzyme sites are shown on the map. The red highlighted sites are unique.


PB-MT-IRES-Cre
8095 bp
PvuII
KpnI
XhoI
ScaI
PstI
NotI
NheI
probasin
promoter
ori
PstI
SacI
ScaI
MT
ScaI
PvuII
NcoI
SacI
EcoRI
IRES
HindIII
KpnI
NcoI
BamHI
EcoRV
ClaI
PvuII
NdeI
EcoRI
SalI
PstI
NotI
XbaI
KpnI
BglII
SV40
polyA
SpeI
XbaI
NotI
SacI
PvuII
BglII
AmpR
BglII

2.1.4 MMTV-Neu1842-IRES-Cre

The MMTVNeu8142 vector (provided by Niki Sharan, Siegel *et al* 1994) was cut with EcoRI (Fermentas, Y+/Tango buffer) and dephosphorylated using CIAP (Gibco). PB-MT-IRES-Cre was digested with EcoRI (Fermentas, Y+/Tango buffer) and ligated with the MMTVNeu8142 vector backbone. The correct orientation was monitored by NcoI digest (~1600bp drop) and HindIII digest (~4000bp and 8000bp).

The plasmid was sequenced with:

- 5'CCCACTGCAGAGAACCCTGAGTACC3' to ensure a correct Neu8142-CITE junction
- 5'CGTTTGTTCGGATCGGTCG3' to ensure a proper Cre-backbone junction
- 5'GGAGTTTCAATACCGGAGATCA3' to check the 3' end of Cre

The transgene was obtained by digesting with SpeI to linearize construct.

2.1.5 MMTV-MT-IRES-Cre

The MMTV-Cre vector was cut with HindIII/EcoRI (Gibco, buffer EcoRI, Fermentas), dephosphorylated, and the 7kb backbone was gel isolated by Qiagen columns. The pJ4Ω-MT vector was cut with EcoRI (Gibco, buffer EcoRI, Fermentas), dephosphorylated with CIAP (Gibco), then cut with HindIII (Roche, buffer H). The 1.6 kb band was gel isolated by Qiagen column. The dephosphorylation steps were done to ensure that the EcoRI sticky end produced by these two cuts will not self-ligate. PB-MT-IRES-Cre was cut with EcoRI (Gibco, buffer EcoRI, Fermentas), to release a 2.2kb fragment containing the IRES site and Cre.

The three pieces were ligated in a 3:1:1 ratio, of 7kb:2.2kb:1.6kb fragments. The ligation was left overnight at 16°C. The resulting fragment was checked for orientation using NcoI digest, which would result in a 800bp drop for the correct orientation and a 1.6kb drop for reverse orientation. The construct was sequenced with the following primers:

- 5'GCAGCCTTACTGGGTATTTGTC3' to ensure a correct MT-CITE junction
- 5'CGTTTGTTCGGATCGGTCG3' to ensure a proper Cre-backbone junction

A transgene could have been obtained by linearization with SpeI.

Figure 8: MMTV-Neu1842-IRES-Cre
The 13kb vector contains the MMTV promoter, Neu1842 oncogene followed by EMCV IRES, and Cre followed by a SV40 polyA tail. The vector also has an ampicillin resistance gene, AmpR for selection and the bacterial origin of replication, ori, in the p206 vector backbone. The most common restriction enzyme sites are shown on the map. The red highlighted sites are unique.

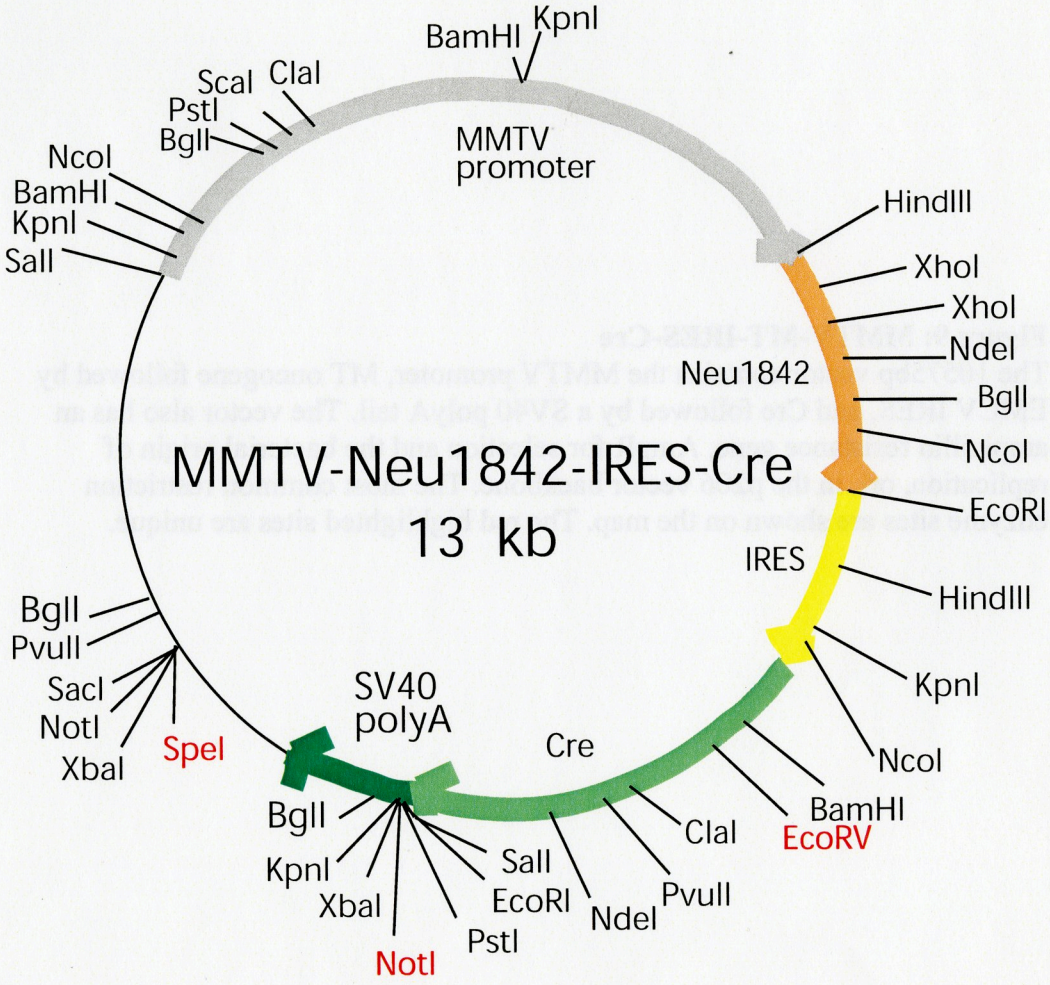
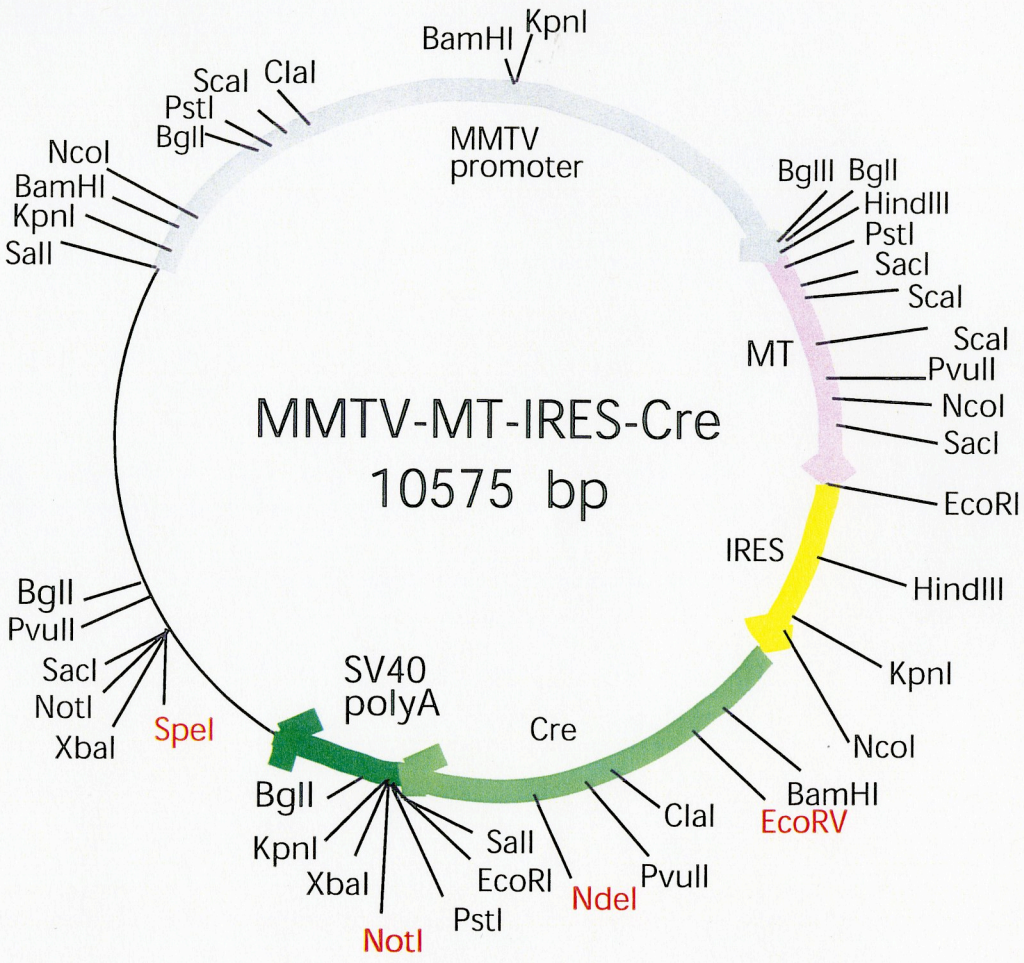

MMTV-Neu1842-IRES-Cre
13 kb
BamHI
KpnI
MMTV
promoter
ScaI
ClaI
PstI
BglI
NcoI
BamHI
KpnI
SalI
HindIII
XhoI
XhoI
NdeI
Neu1842
BglI
NcoI
EcoRI
IRES
HindIII
KpnI
NcoI
BamHI
EcoRV
ClaI
PvuII
NdeI
SalI
EcoRI
PstI
NotI
XbaI
KpnI
BglI
Cre
SV40
polyA
SpeI
XbaI
NotI
SacI
PvuII
BglI

Figure 9: MMTV-MT-IRES-Cre
The 10575bp vector contains the MMTV promoter, MT oncogene followed by EMCV IRES, and Cre followed by a SV40 polyA tail. The vector also has an ampicillin resistance gene, AmpR for selection and the bacterial origin of replication, ori, in the p206 vector backbone. The most common restriction enzyme sites are shown on the map. The red highlighted sites are unique.


MMTV-MT-IRES-Cre
10575 bp
MMTV promoter
MT
IRES
Cre
SV40 polyA
BamHI
KpnI
ScaI
ClaI
PstI
BglII
NcoI
BamHI
KpnI
SalI
BglII
HindIII
PstI
SacI
ScaI
ScaI
PvuII
NcoI
SacI
EcoRI
HindIII
KpnI
NcoI
BamHI
EcoRV
ClaI
PvuII
NdeI
SalI
EcoRI
PstI
NotI
XbaI
KpnI
BglII
SpeI
XbaI
NotI
SacI
PvuII
BglII

2.2 Tissue Culture

Rat-1 fibroblasts were grown in 10%FBS DMEM media (Gibco). Dihydrotestosterone (SIGMA) was added to a final concentration of $1x10^{-8}$ M (Zhang 2000) to induce the probasin promoter. Dexamethasone (SIGMA) was added to a final concentration of $1x10^{-6}$ M (Muthuswamy 1994) to induce the MMTV promoter. The cells were transfected using 6 equivalents of ExGen500 (Fermentas) and manufacturer's instructions. The co-transfection of experimental vectors and selection vectors was done with a 10:1 ratio of experimental vector to selection vector. The selection vector contained PGK-driven puromycin resistance (provided by Niki Sharan). For selection and stable expression, cell lines incubation was performed in the presence of puromycin (SIGMA), at a final concentration of 3μg/ml as per manufacturer's instructions.

For cotransfection of MMTV-Cre with PGK-polyA-lacZ, equal amounts of DNA were used (5μg of each per 10 cm dish) and 6 equivalents of ExGen500. The PGK-polyA-lacZ construct carries a neomycin resistance gene. Selection of positive clones was done with Geneticin (Sigma) at a final concentration of 0.3μg/μl, as per manufacturer's instructions.

Focus assays were performed in the presence of 2% FBS DMEM for two weeks. Controls were puromycin treated experimental plates and pcDNA3 control transfections. Stable cell lines of MMTV-Neu1842-IRES-Cre were created by growing cells in 10% FBS DMEM and 3μg/ml puromycin, then collecting the surviving colonies onto 24-well plates, in the presence of 10μl of 1xTrypsin EDTA (Gibco) and 1ml 10% FBS DMEM. Stable cell lines were grown in the presence of puromycin.

Stable cell lines for the PB-MT-IRES-Cre and PB-MT-IRES-Luciferase constructs were obtained by growing cells in 10% FBS DMEM and 3μg/ml puromycin. The surviving colonies were pooled together and propagated. They were treated as one batch of stable cell lines.

Cells were fixed with 10% formalin and stained with Wright's Giemsa. Cells were also harvested and lysed; these cells were washed 3 times in ice-cold PBS prior to lysis. 10cm plated were added 600μl lysis buffer, scraped down and spun 10 minutes at 13,000g at 4°C. The supernatant was transferred to a clean tube and kept at -80°C.

2.3 X-Gal Staining

Cells were washed with PBS, fixed for 2 minutes (1x PBS, 2% formaldehyde, 0.4% glutaraldehyde) at room temperature. The plates were then washed several timed with 1x PBS and stained (5mM K_4CN_6Fe, 5mM $K_6CN_{12}Fe_2$, 2mM $MgCl_2$, 1x PBS, 2mM X-Gal) and incubated overnight at 37°C. The plates were then inspected visually, and under the microscope for blue patches.

2.4 Western Blots

Protein lysate and immunoblots were prepared as previously described (Webster et al 1998) with few exceptions: protein was loaded at ~ 70μg total protein per well (measured by BioRad assay) on a 9% PAGE gel. The primary antibodies used were: rat-αMT antibody pAB762, 3E1, pAB815 (provided by John Hutchinson), rabbit α-Cre antibody (Novagen) and rabbit α-Grb2 antibody (Novagen). Secondary HRP antibodies were used for detection on film, using the ECL detection system and the manufacturer's instructions (Amersham).

2.5 Histology

The prostates of one PB-MT-IRES-Luciferase mouse heterozygous by PCR genotyping, and a wild-type mouse were sent for a histological analysis. The tissue was fixed in formalin and stained with hematoxylin and eosin by the McMaster University Pathology Research Services.

2.6 PCR for Mouse Genotyping

DNA extracted from mouse tails (Tortorice, 1997) was amplified by PCR. The following primers were used to identify the foreign fragments:

- 5'ACGTTCCCGGCATTTCTAGT3' forward primer overlapping MT and 5'CTCTTCTTCTTGGGCATGGT3' reverse primer, overlapping Cre recombinase, resulting in a ~700bp fragment.
- 5'CAGCTATAAAAAGCAGGAAGCTACTC3' forward primer overlapping the rat probasin promoter and 5'TGCAGAACCGCTGGTAGTAT3' reverse primer, overlapping MT oncogene, resulting in a ~400bp fragment.

The PCR conditions were optimized by varying the concentration of MgCl2 and a concentration of 1mM was found sufficient. Each reaction contained 0.01μg genomic tail extracted DNA, 1mM each primer, 150mM dNTP mix and 1U Taq polymerase (Invitrogen).

2.7 Transgenic mice

The PB-MT-IRES-Cre and PB-MT-IRES-Luciferase transgenes were sent to UC Davis and used in FVB background mice. Similarly, the MMTV-Neu1842-IRES-Cre transgene was sent to McGill, Montreal and also used in FVB mice.

Chapter 3: Results

In the attempt to create new animal models for breast and prostate cancer, several experiments have been completed. Firstly, plasmid vectors carrying bicistronic transgenes were designed and created. Secondly, these plasmids were tested in *in vitro* conditions to assess their potency and viability. Once they show promising results in *in vitro* studies, the transgenes can be used to create a transgenic mouse model.

After each of the four plasmid constructs were created, they were sequenced to ensure the expected junctions, as detailed in the materials and methods section. Only three of the four plasmids were tested and used in further experiments. To test transformation potential, focus assays were performed. Western blots were attempted to measure the amount of expressed protein. This was unsuccessful due to unsatisfactory antibodies. There were positive results only for the Grb2 band, indicating protein presence. No other protein bands were visible in any of the cases. When blotting against luciferase and Cre the negative results may be due to the low amount of protein, due to lower transcription rates of the secondary gene, as described in the introduction. This problem may be solved by loading more whole cell lysate per well, or performing a TCA precipitation to concentrate the protein in the lysate. In the case of MT, the antibodies were previously used in immunoprecipitation assays alone, thus there was no guarantee they will be efficient in Western blotting. Increasing amount of antibody and incubation time, changing the composition of the incubation buffer are methods that may help with optimization of antibody-antigen binding.

Each of the constructs tested showed transformation potential and activity of the secondary gene product Cre. The results were obtained by analysis of transfected Rat-1 fibroblast cells.

3.1 PB-MT-IRES-Cre

The gene was cloned to start at the 12^{th} AUG in the IRES sequence. The vector was cloned in such a way that the start codon of the Cre gene is in the same reading frame as the ATG start codons present in the IRES sequence, because it is necessary that the gene start codon be the 11^{th} or 12^{th} ATG codon in the IRES for proper expression of the gene. Interestingly, if the start codon is the 12^{th} AUG, the translation efficiency is increased compared to a start codon at the 11^{th} AUG (Qiao *et al* 2002)

This construct was tested both by focus assay and by stable cell line analysis. Focus assays showed that the oncogene is active, as foci resulted in the test plates not containing dihydrotestosterone, and had a significant increase in the plates where dihydrotestosterone was added. The results are tabulated in Table 1 and Figure 10. The result was expected, as the probasin promoter is induced by steroid presence (Greenberg *et al* 1994).

Table 1: Focus assay performed in Rat-1 cells showing the transformation potential of the PB-MT-IRES-Cre construct

The focus assay was performed as described in detail in Chapter 2. For each trial, the standard deviation (SD) and the standard error (SE) were calculated. The average number of foci increases from 3.2 ± 0.2 with a standard deviation of 0.8, to 12.8 ± 0.7 with a standard deviation of 3.8, after addition of dihydrotestosterone (DHT). The difference is significant with a 99% confidence according to the t-test.

Table 1: Focus assay performed in Rat-1 cells showing the transformation potential of the PB-MT-IRES-Cre construct

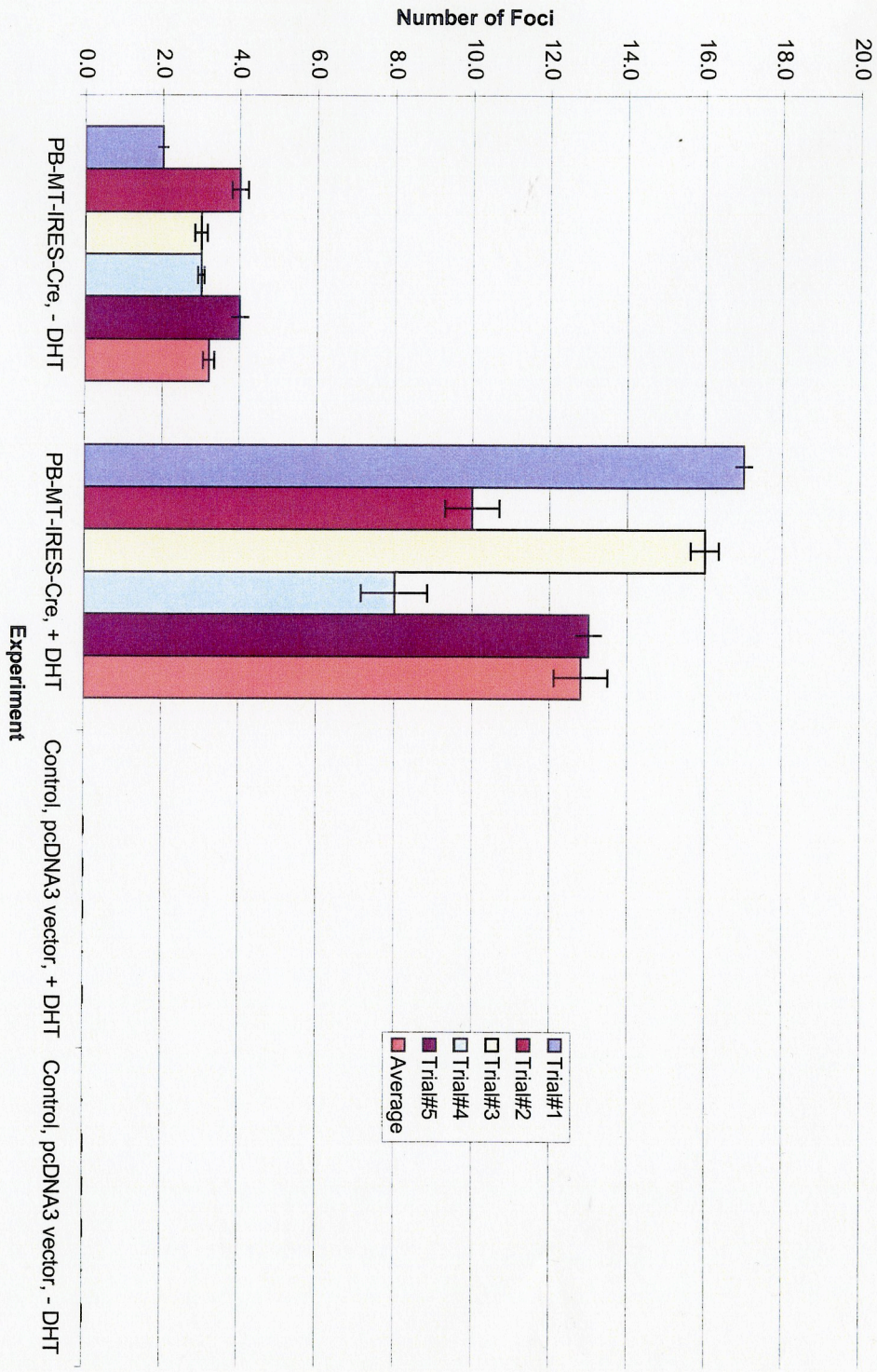
Experiment - Focus assay	Trial#1	SD	SE	Trial#2	SD	SE	Trial#3	SD	SE	Trial#4	SD	SE
PB-MT-IRES-Cre, - DHT	2.0	0.3	0.1	4.0	0.5	0.2	3.0	0.4	0.2	3.0	0.2	0.1
PB-MT-IRES-Cre, + DHT	17.0	0.5	0.2	10.0	1.7	0.7	16.0	0.9	0.4	8.0	2.1	0.9
Control, pcDNA3, + DHT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Control, pcDNA3, - DHT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

	Trial#5	SD	SE	Average	SD	SE
PB-MT-IRES-Cre, - DHT	4.0	0.5	0.2	3.2	0.8	0.2
PB-MT-IRES-Cre, + DHT	13.0	0.8	0.3	12.8	3.8	0.7
Control, pcDNA3, + DHT	0.0	0.0	0.0	0.0	0.0	0.0
Control, pcDNA3, - DHT	0.0	0.0	0.0	0.0	0.0	0.0

Each trial represents the average of six 10cm experimental plates.

Figure 10: Focus assay of PB-MT-IRES-Cre

The focus assay was performed as described in Chapter 2. Each trial represents the average number of foci from six 10cm experimental plates. The numbers are presented in Table 1. The error bars represent the standard error for each trial. The average number of foci increased from 3.2 ± 0.2 with a standard deviation of 0.8, to 12.8 ± 0.7 with a standard deviation of 3.8 after adding the inducer, dihydrotestosterone (DHT). This difference is significant with a 99% confidence according to the t-test.


PB-MT-IRES-Cre Focus Assay
Number of Foci
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
Trial#1
Trial#2
Trial#3
Trial#4
Trial#5
Average
PB-MT-IRES-Cre, - DHT
PB-MT-IRES-Cre, + DHT
Control, pcDNA3 vector, + DHT
Control, pcDNA3 vector, - DHT
Experiment

Focus assays in rat-1 fibroblasts demonstrate the transformation ability of the construct. A focus assay is a transformation assay, useful to measure the capacity of an oncogene to transform cells. Normal cells grow by adhering themselves to the plastic of the plate, while transformed cells lose this anchoring dependence and pile up in top of each other in an irregular fashion. The transfected cells are grown for 2 weeks on a plastic plate in low serum conditions, to minimize the growth rate of untransfected cells, fixed to the plate with formalin and visualized by staining with Giemsa. The number of foci is relative to the transforming capacity of the tested oncogene.

Tissue culture experiments were performed for five days, in cells stably expressing the construct, with or without dihydrotestosterone. After five days, the cells were fixed and stained, and the number of foci counted. The results are displayed in Table 2 and Figure 11. The probasin promoter is functional in the absence of steroid hormone, but the addition of the hormone increases (almost doubles) the amount of foci observed.

To show the activity of Cre, X-gal staining was performed on plates of stably expressing cells, after two days of culture (Figure 18). The cells were transfected with a construct containing a floxed polyA site in front of a lacZ gene (Figure 17). If the Cre molecule is present and active, the floxed polyA sequence is removed and the lacZ gene is restored. In the presence of x-gal, beta-galactosidase reacts to produce a visible blue pigment. Under the microscope, many individual blue colonies were observed, as well as blue foci. The blue foci indicate the co-expression of the oncogene and the Cre recombinase, as expected.

Transgenic mice were attempted using this construct. While several males and females were identified as positive by PCR genotyping, there were no phenotypical differences between wild type and transgenic littermate males. There was no homozygous male or female obtained for the transgene. Disections were performed on mice that were between 5 and 8 months of age.

3.2 PB-MT-IRES-Luciferase

Tissue culture studies were performed in the same manner as for the PB-MT-IRES-Cre construct. The results were also similar, indicating a potent oncogene. A luciferase assay was not performed to determine the activity of luciferase in this construct.

Transgenic mice for this construct were also attempted. There were only genotypically positive males obtained by PCR screening. Several attempts were unsuccessful at obtaining positive females. Dissections of mice from 2 to 5 months of age showed no phenotypical differences in the size and appearance of the prostate gland. One representative result is shown in Figure 12.

The results of the Rat-1 focus assay are presented in Table 3 and Figure 13, while the results from stable expression cell line analysis are shown in Table

Table 2: Stable expression of the PB-MT-IRES-Cre construct in Rat-1 cells
The cells were grown in 10cm dishes for 5 days after seeding at $1x10^6$ cells per dish. The number of foci apparent after Giemsa staining was counted. For each trial, the standard deviation (SD) and the standard error (SE) were calculated. The average number of foci increased from 62.4 ± 0.6 with a standard deviation of 5.4, to 139 ± 1.1 with a standard deviation of 6.6, after addition of dihydrotestosterone (DHT). The difference is significant with a 99% confidence according to the t-test.

Table 2: Rat-1 cells stably expressing the PB-MT-IRES-Cre construct, under puromycin selection

Experiment - Stable cell lines	Trial#1	SD	SE	Trial#2	SD	SE	Trial#3	SD	SE	Average	SD	SE
PB-MT-IRES-Cre, - DHT	65.0	2.3	0.9	72.0	4.2	1.7	70.0	4.3	1.8	69.0	3.6	0.8
PB-MT-IRES-Cre, - DHT	56.0	3.1	1.3	49.0	2.8	1.1	53.0	4.2	1.7	52.0	3.5	0.8
PB-MT-IRES-Cre, - DHT	64.0	3.5	1.4	48.0	3.9	1.6	53.0	4.5	1.8	55.0	8.2	1.9
PB-MT-IRES-Cre, - DHT	71.0	5.2	2.1	58.0	2.6	1.1	66.0	3.4	1.4	65.0	6.6	1.5
PB-MT-IRES-Cre, - DHT	67.0	2.5	1.0	69.0	3.1	1.3	77.0	2.9	1.2	71.0	5.3	1.2
Control, pcDNA3, - DHT	0.0	0.0	0.0	0.2	0.4	0.2	0.0	0.0	0.0	0.0	0.1	0.0
PB-MT-IRES-Cre, + DHT	131.0	6.4	2.6	121.0	5.2	2.1	123.0	7.1	2.9	125.0	5.3	1.2
PB-MT-IRES-Cre, + DHT	162.0	6.1	2.5	149.0	7.3	3.0	148.0	4.9	2.0	153.0	7.8	1.8
Control, pcDNA3, + DHT	7.0	0.5	0.2	5.0	1.0	0.4	3.0	0.2	0.1	7.0	2.0	0.5

Each trial represents the average of six 10cm experimental plates.

Figure 11: Stable expression of the PB-MT-IRES-Cre construct in Rat-1 cells.
The cells were grown in 10cm dishes for 5 days after seeding at $1x10^6$ cells per dish. The number of foci observed after Giemsa staining was counted. The error bars represent the standard error for each trial. Each trial represents the average of 6 experimental plates. The numbers are presented in Table 2. The number of foci increases from an average of 62.4 ± 0.6 with a standard deviation of 5.4 , to 139 ± 1.1 with a standard deviation of 6.6 after addition of dihydrotestosterone (DHT). This difference is significant with a 99% confidence according to the t-test.

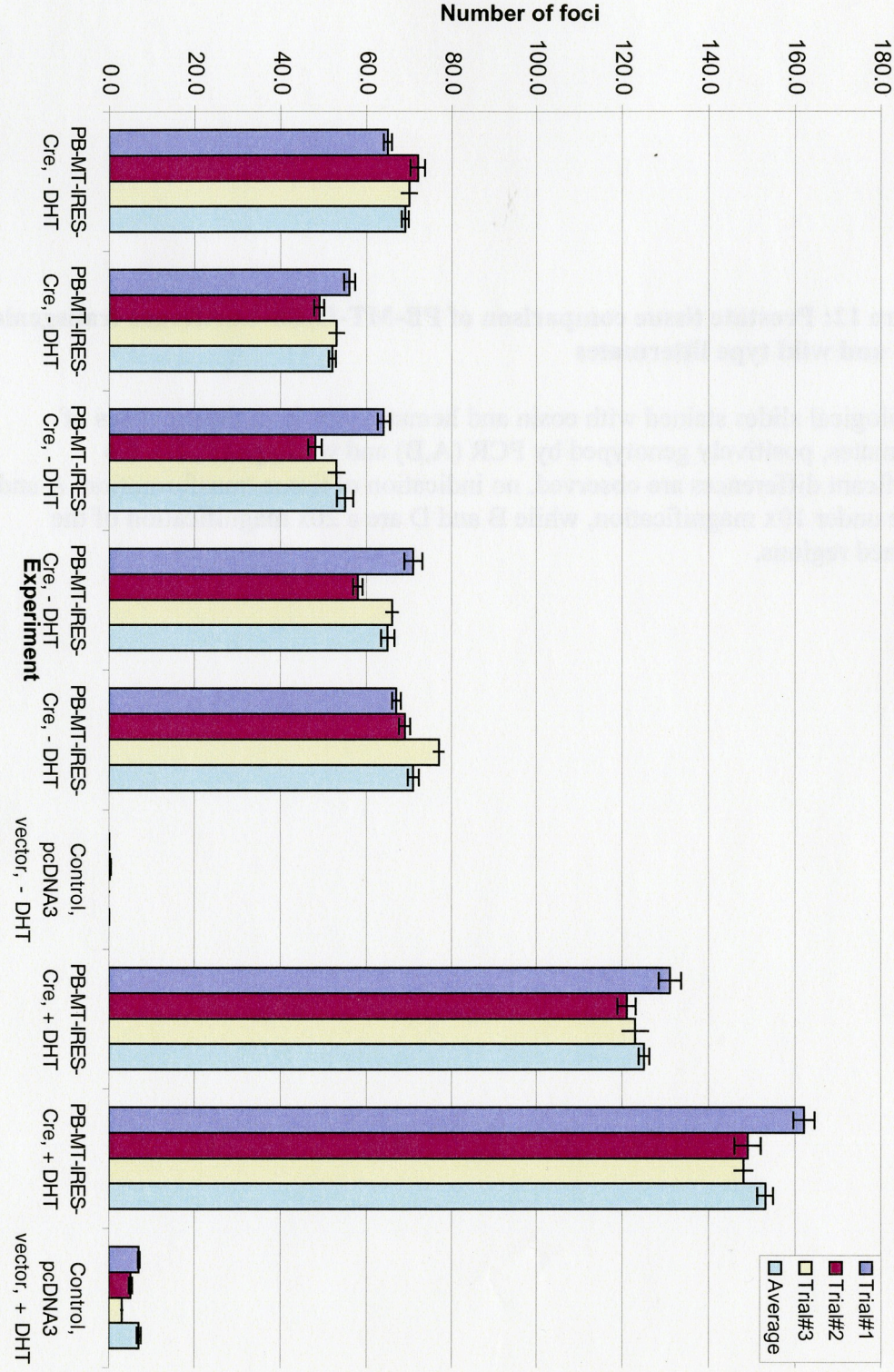

PB-MT-IRES-Cre
Number of foci
0.0
20.0
40.0
60.0
80.0
100.0
120.0
140.0
160.0
180.0
Trial#1
Trial#2
Trial#3
Average
PB-MT-IRES-Cre, - DHT
PB-MT-IRES-Cre, - DHT
PB-MT-IRES-Cre, - DHT
PB-MT-IRES-Cre, - DHT
PB-MT-IRES-Cre, - DHT
Control, pcDNA3 vector, - DHT
PB-MT-IRES-Cre, + DHT
PB-MT-IRES-Cre, + DHT
Control, pcDNA3 vector, + DHT
Experiment

Figure 12: Prostate tissue comparison of PB-MT-IRES-Luciferase transgenic mice and wild type littermates

Histological slides stained with eosin and hematoxylin form the prostates of littermates, positively genotyped by PCR (A,B) and wildtype (C, D). No significant differences are observed, no indication of tissue transformation. A and C are under 10x magnification, while B and D are a 20x magnification of the outlined regions.

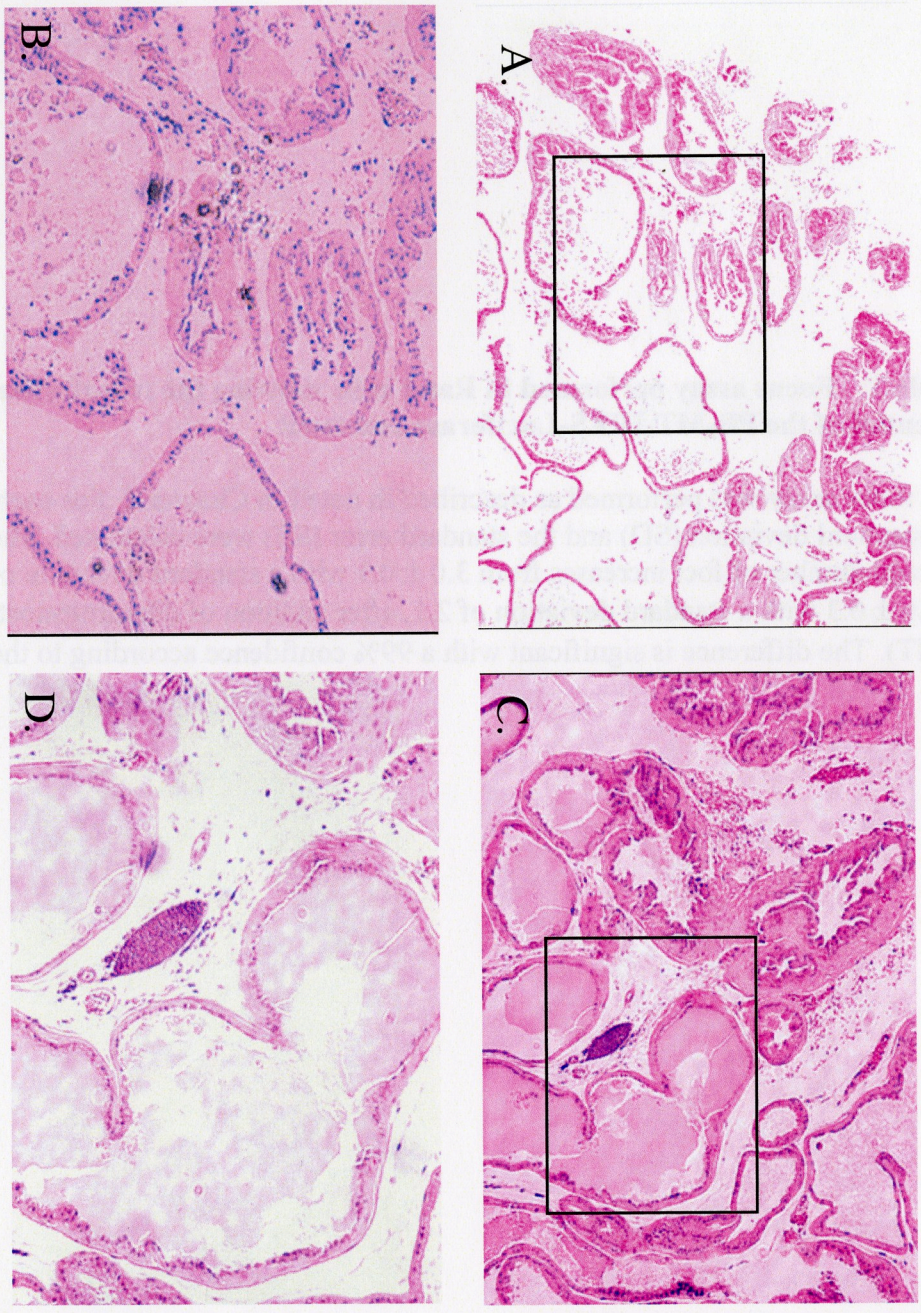

B.
A.
D.
C.

Table 3: Focus assay performed in Rat-1 cells showing the transformation potential of the PB-MT-IRES-Luciferase construct

The focus assay was performed as described in detail in Chapter 2. For each trial, the standard deviation (SD) and the standard error (SE) were calculated. The average number of foci increases from 3.0 ± 0.1 with a standard deviation of 0.8, to 8.9 ± 0.3 with a standard deviation of 2.1, after addition of dihydrotestosterone (DHT). The difference is significant with a 99% confidence according to the t-test.

Table 3: Focus assay performed in Rat-1 cells showing the transformation potential of the PB-MT-IRES-Luciferase construct

Experiment - Focus assay	Trial#1	SD	SE	Trial#2	SD	SE	Trial#3	SD	SE	Trial#4	SD	SE
PB-MT-IRES-Luc, - DHT	3.0	0.6	0.2	2.0	0.4	0.2	4.0	0.7	0.3	2.0	0.3	0.1
PB-MT-IRES-Luc, + DHT	8.0	0.5	0.2	7.0	0.5	0.2	10.0	0.3	0.1	9.0	0.7	0.3
Control, pcDNA3, + DHT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Control, pcDNA3, - DHT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Trial#5	**SD**	**SE**	**Trial#6**	**SD**	**SE**	**Trial#7**	**SD**	**SE**	**Average**	**SD**	**SE**
PB-MT-IRES-Luc, - DHT	3.0	0.2	0.1	3.0	0.4	0.2	4.0	0.2	0.1	3.0	0.8	0.1
PB-MT-IRES-Luc, + DHT	7.0	0.8	0.3	8.0	0.5	0.2	13.0	0.3	0.1	8.9	2.1	0.3
Control, pcDNA3, + DHT	0.0	0.0	0.0	1.0	0.3	0.1	0.0	0.0	0.0	0.1	0.4	0.1
Control, pcDNA3, - DHT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Each trial represents the average of six 10cm experimental plates.

Figure 13: Focus assay of PB-MT-IRES-Luciferase
The focus assay was performed as described in Chapter 2. Each trial represents the average number of foci from six 10cm experimental plates. The numbers are presented in Table 3. The error bars represent the standard error for each trial. The average number of foci increased from 3.0 ± 0.1 to 8.9 ± 0.3 after addition of dihydrotestosterone (DHT). The difference is significant with a 99% confidence according to the t-test.

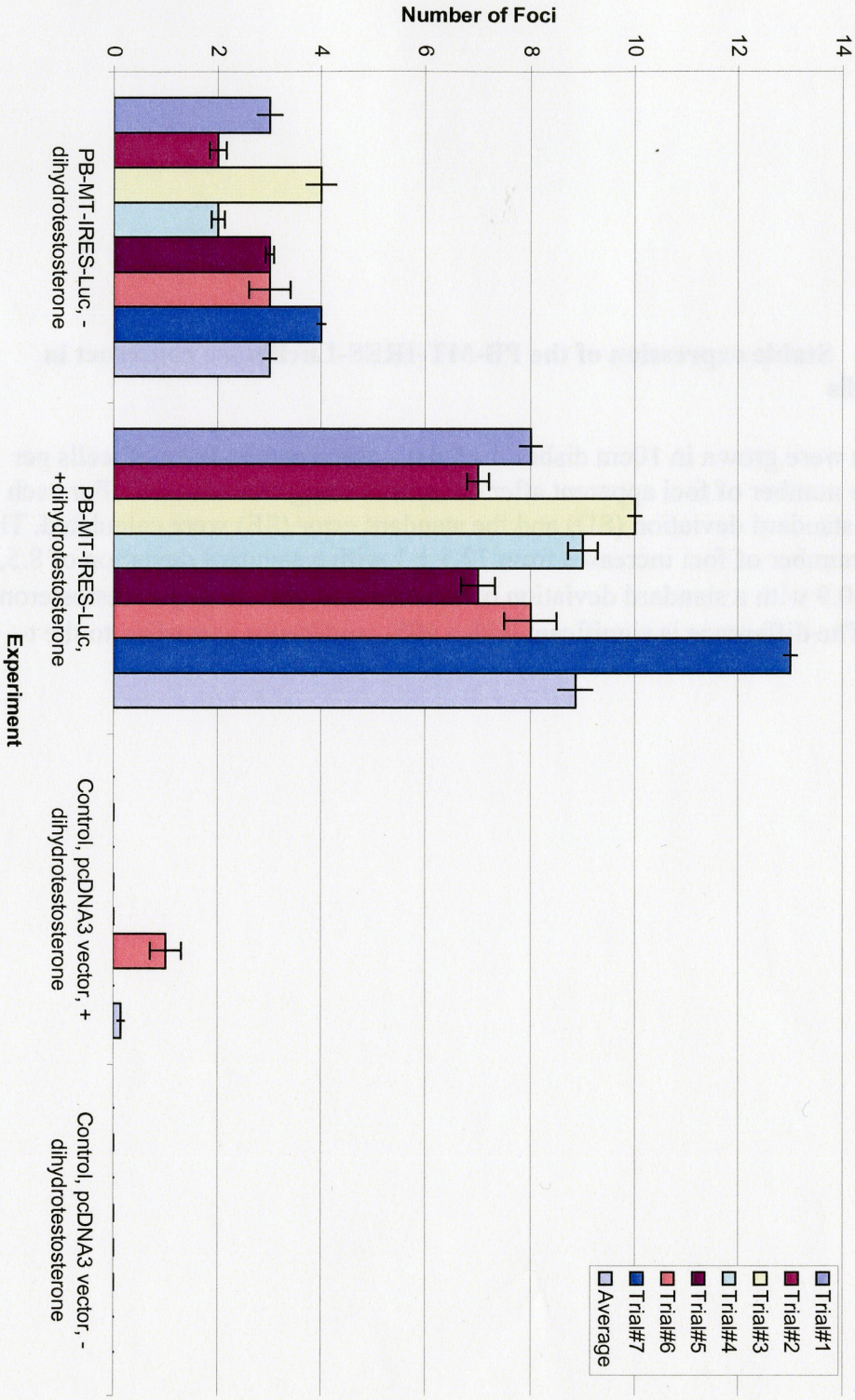

PB-MT-IRES-Luc Focus Assay
Number of Foci
0
2
4
6
8
10
12
14
Trial#1
Trial#2
Trial#3
Trial#4
Trial#5
Trial#6
Trial#7
Average
PB-MT-IRES-Luc, -dihydrotestosterone
PB-MT-IRES-Luc, +dihydrotestosterone
Control, pcDNA3 vector, +dihydrotestosterone
Control, pcDNA3 vector, -dihydrotestosterone
Experiment

Table 4: Stable expression of the PB-MT-IRES-Luciferase construct in Rat-1 cells

The cells were grown in 10cm dishes for 5 days after seeding at $1x10^6$ cells per dish. The number of foci apparent after Giemsa staining was counted. For each trial, the standard deviation (SD) and the standard error (SE) were calculated. The average number of foci increases from 72.5 ± 1 with a standard deviation of 8.5, to 144 ± 0.9 with a standard deviation of 5.5 after addition of dihydrotestosterone (DHT). The difference is significant with a 99% confidence according to the t-test.

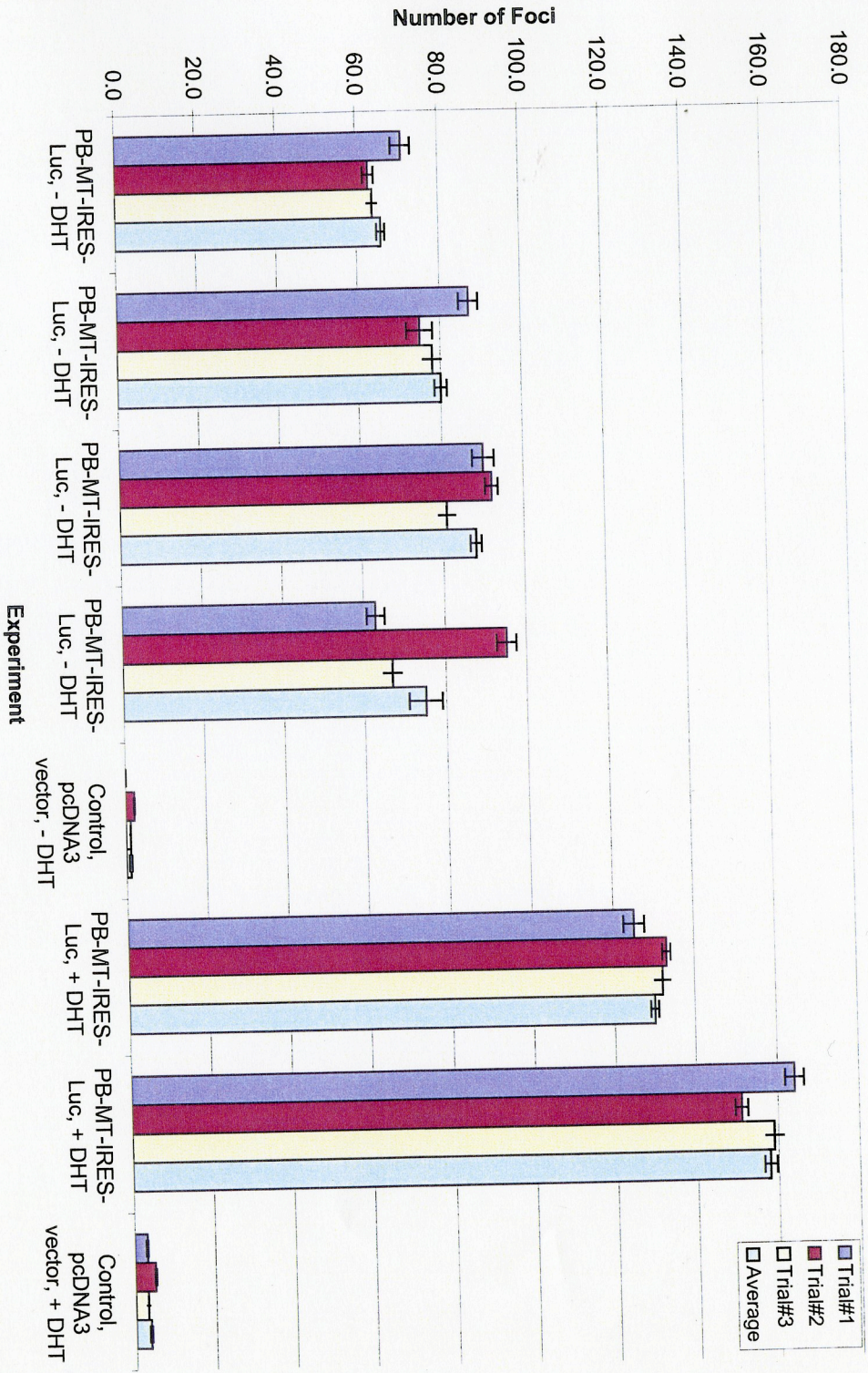
Table 4: Rat-1 cells stably expressing the PB-MT-IRES-Luciferase construct, under puromycin selection

Experiment - Stable cell lines	Trial#1	SD	SE	Trial#2	SD	SE	Trial#3	SD	SE	Average	SD	SE
PB-MT-IRES-Luc, - DHT	71.0	6.0	2.4	63.0	3.4	1.4	64.0	2.9	1.2	66.0	4.4	1.0
PB-MT-IRES-Luc, - DHT	87.0	5.8	2.4	75.0	7.8	3.2	78.0	5.4	2.2	80.0	6.2	1.5
PB-MT-IRES-Luc, - DHT	90.0	6.7	2.7	92.0	3.9	1.6	81.0	4.8	2.0	88.0	5.9	1.4
PB-MT-IRES-Luc, - DHT	63.0	5.6	2.3	95.0	6.2	2.5	67.0	5.2	2.1	75.0	17.4	4.1
Control, pcDNA3, - DHT	0.0	0.0	0.0	2.0	0.4	0.2	1.0	0.2	0.1	1.0	1.0	0.2
PB-MT-IRES-Luc, + DHT	125.0	6.3	2.6	133.0	2.5	1.0	132.0	4.6	1.9	130.0	4.4	1.0
PB-MT-IRES-Luc, + DHT	164.0	5.7	2.3	151.0	3.8	1.6	159.0	5.1	2.1	158.0	6.6	1.5
Control, pcDNA3, + DHT	3.0	0.6	0.2	5.0	0.4	0.2	3.0	0.7	0.3	3.7	1.2	0.3

Each trial represents the average of six 10cm experimental plates.

Figure 14: Rat-1 cells stable expression of the PB-MT-IRES-Luciferase construct.

The cells were grown in 10cm dishes for 5 days after seeding at $1x10^6$ cells per dish. The number of foci apparent after Giemsa staining was counted. Each trial represents the average of 6 experimental plates. The error bars represent the standard error for each trial. The numbers are presented in Table 4. The number of foci increases from an average of 72.5 ± 1 with a standard deviation of 8.5, to 144 ± 0.9 with a standard deviation of 5.5 after addition of dihydrotestosterone. This difference is significant with a 99% confidence according to the t-test. One negative control is showing some foci most likely due to contamination.


PB-MT-IRES-Luc
Number of Foci
0.0
20.0
40.0
60.0
80.0
100.0
120.0
140.0
160.0
180.0
Trial#1
Trial#2
Trial#3
Average
PB-MT-IRES-Luc, - DHT
PB-MT-IRES-Luc, - DHT
PB-MT-IRES-Luc, - DHT
PB-MT-IRES-Luc, - DHT
Control, pcDNA3 vector, - DHT
PB-MT-IRES-Luc, + DHT
PB-MT-IRES-Luc, + DHT
Control, pcDNA3 vector, + DHT
Experiment

4, Figure 14. As expected, in the presence of testosterone the amount of foci was significantly greater than in the absence of testosterone.

3.3 MMTV-Neu1842-IRES-Cre

As with the previous constructs, tissue culture experiments resulted in a viable oncogene and an active Cre recombinase, by similar techniques. Focus assay results are presented in Table 5, Figure 15, while stable expressing cell line analysis is presented in Table 6, Figure 16. The results of the Cre assay are presented in Figure 18.

The potential of the Neu1842 oncogene is evident from the focus assays. As expected, there is little expression of the transgene in the absence of the dexamethasone inducer, because very few foci are present in the absence of the inducer. The MMTV promoter is dependent on glucocorticoid presence for activation. The amount of foci observed in the absence of the inducer is lower than 5 in both the focus assay and the stable cell line analysis. This level is much lower than observed for the probasin promoter.

This transgene was injected in FVB background mice at McGill. Founder mice that express the desired phenotype were already obtained and are currently under observation. Genotyping was done by PCR. Founder females have already developed mammary tumors. This line is currently being propagated.

Table 5: Focus assay performed in Rat-1 cells showing the transformation potential of the MMTV-Neu1842-IRES-Cre construct
The focus assay was performed as described in detail in Chapter 2. For each trial, the standard deviation (SD) and the standard error (SE) were calculated. The average number of foci increases from 2.3 ± 0.1 with a standard deviation of 0.6, to 9.5 ± 0.3 with a standard deviation of 1.3, after addition of dexamethasone. The difference is significant with a 99% confidence according to the t-test.

Table 5: Focus assay performed in Rat-1 cells showing the transformation potential of the MMTV-Neu1842-IRES-Cre construct

Experiment - Focus assay	Trial#1	SD	SE	Trial#2	SD	SE
MMTV-Neu1842-IRES-Cre, -dexamethasone	3.0	0.7	0.3	2.0	0.8	0.3
MMTV-Neu1842-IRES-Cre, +dexamethasone	8.5	2.0	0.8	9.0	1.6	0.7
Control, pcDNA3 vector, +dexamethasone	0.0	0.0	0.0	0.0	0.0	0.0
Control, pcDNA3 vector, -dexamethasone	0.0	0.0	0.0	0.0	0.0	0.0
	Trial#3	**SD**	**SE**	**Average**	**SD**	**SE**
MMTV-Neu1842-IRES-Cre, -dexamethasone	2.0	0.1	0.0	2.3	0.6	0.1
MMTV-Neu1842-IRES-Cre, +dexamethasone	11.0	0.6	0.2	9.5	1.3	0.3
Control, pcDNA3 vector, +dexamethasone	0.0	0.0	0.0	0.0	0.0	0.0
Control, pcDNA3 vector, -dexamethasone	0.0	0.0	0.0	0.0	0.0	0.0

Each trial represents the average of six 10cm experimental plates.

Figure 15: Focus assay of MMTV-Neu1842-IRES-Cre

The focus assay was performed as described in Chapter 2. Each trial represents the average number of foci from six 10cm experimental plates. The numbers are presented in Table 5. The error bars represent the standard error for each trial. The average number of foci increases from 2.3 ± 0.1 with a standard deviation of 0.6, to 9.5 ± 0.3 with a standard deviation of 1.3, after addition of dexamethasone. The difference is significant with a 99% confidence according to the t-test.

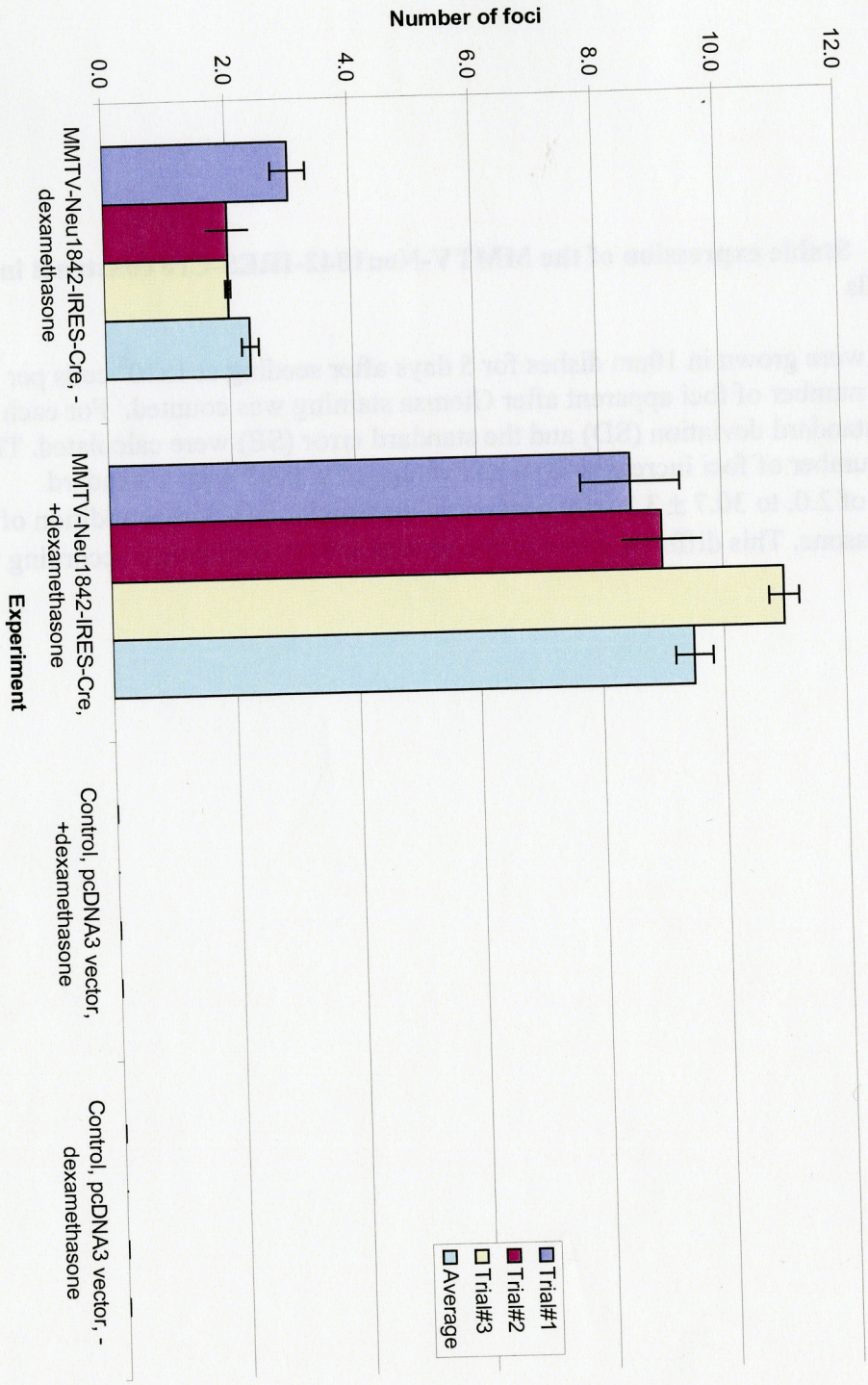

MMTV-Neu1842-IRES-Cre Focus assay
Number of foci
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Trial#1
Trial#2
Trial#3
Average
MMTV-Neu1842-IRES-Cre, -dexamethasone
MMTV-Neu1842-IRES-Cre, +dexamethasone
Control, pcDNA3 vector, +dexamethasone
Control, pcDNA3 vector, -dexamethasone
Experiment

Table 6: Stable expression of the MMTV-Neu1842-IRES-Cre construct in Rat-1 cells

The cells were grown in 10cm dishes for 5 days after seeding at $1x10^6$ cells per dish. The number of foci apparent after Giemsa staining was counted. For each trial, the standard deviation (SD) and the standard error (SE) were calculated. The average number of foci increases from an average of 5 ± 0.5 with a standard deviation of 2.0, to 30.7 ± 3.2 with a standard deviation of 13.5 after addition of dexamethasone. This difference is significant with a 99% confidence according to the t-test.

Table 6: Rat-1 cells stably expressing the MMTV-Neu1842-IRES-Cre construct, under puromycin selection

Experiment - Stable cell lines	Trial#1	SD	SE	Trial#2	SD	SE
MMTV-Neu1842-IRES-Cre, -dexamethasone	5.0	0.8	0.3	7.0	0.5	0.2
MMTV-Neu1842-IRES-Cre, +dexamethasone	17.0	1.9	0.8	44.0	2.5	1.0
Control, pcDNA3 vector, +dexamethasone	0.0	0.0	0.0	0.0	0.0	0.0
Control, pcDNA3 vector, -dexamethasone	0.0	0.0	0.0	0.0	0.0	0.0
	Trial#3	**SD**	**SE**	**Average**	**SD**	**SE**
MMTV-Neu1842-IRES-Cre, -dexamethasone	3.0	0.2	0.1	5.0	2.0	0.5
MMTV-Neu1842-IRES-Cre, +dexamethasone	31.0	1.4	0.6	30.7	13.5	3.2
Control, pcDNA3 vector, +dexamethasone	0.0	0.0	0.0	0.0	0.0	0.0
Control, pcDNA3 vector, -dexamethasone	0.0	0.0	0.0	0.0	0.0	0.0

Each trial represents the average of six 10cm experimental plates.

Figure 16: Stable expression of the MMTV-Neu1842-IRES-Cre construct in Rat-1 cells

The cells were grown in 10cm dishes for 5 days after seeding at $1x10^6$ cells per dish. The number of foci apparent after Giemsa staining was counted. Each trial represents the average of 6 experimental plates. The error bars represent the standard error for each trial. The numbers are presented in Table 6. The number of foci increases from an average of 5 ± 0.5 with a standard deviation of 2.0, to 30.7 ± 3.2 with a standard deviation of 13.5 after addition of dexamethasone. This difference is significant with a 99% confidence according to the t-test.

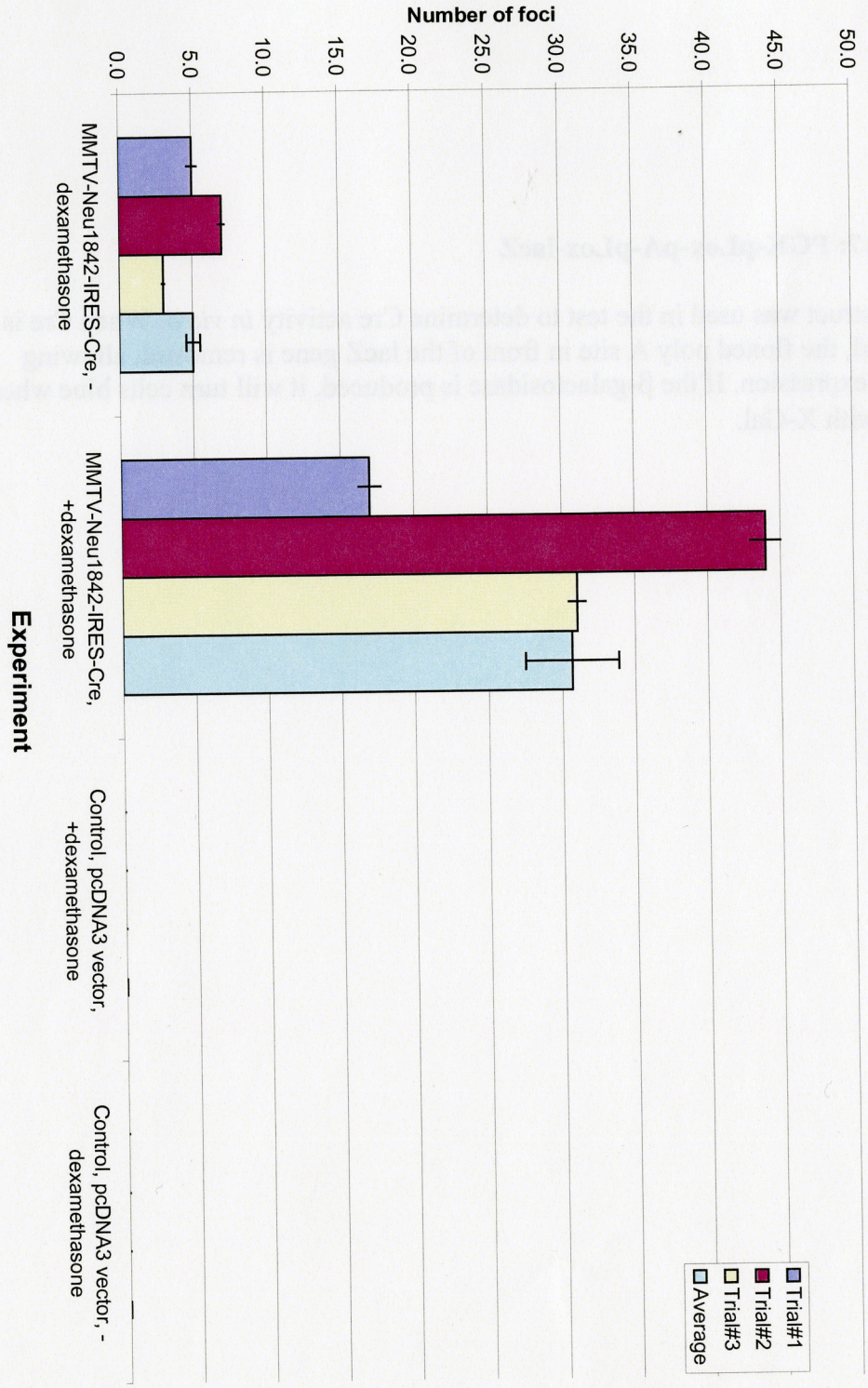
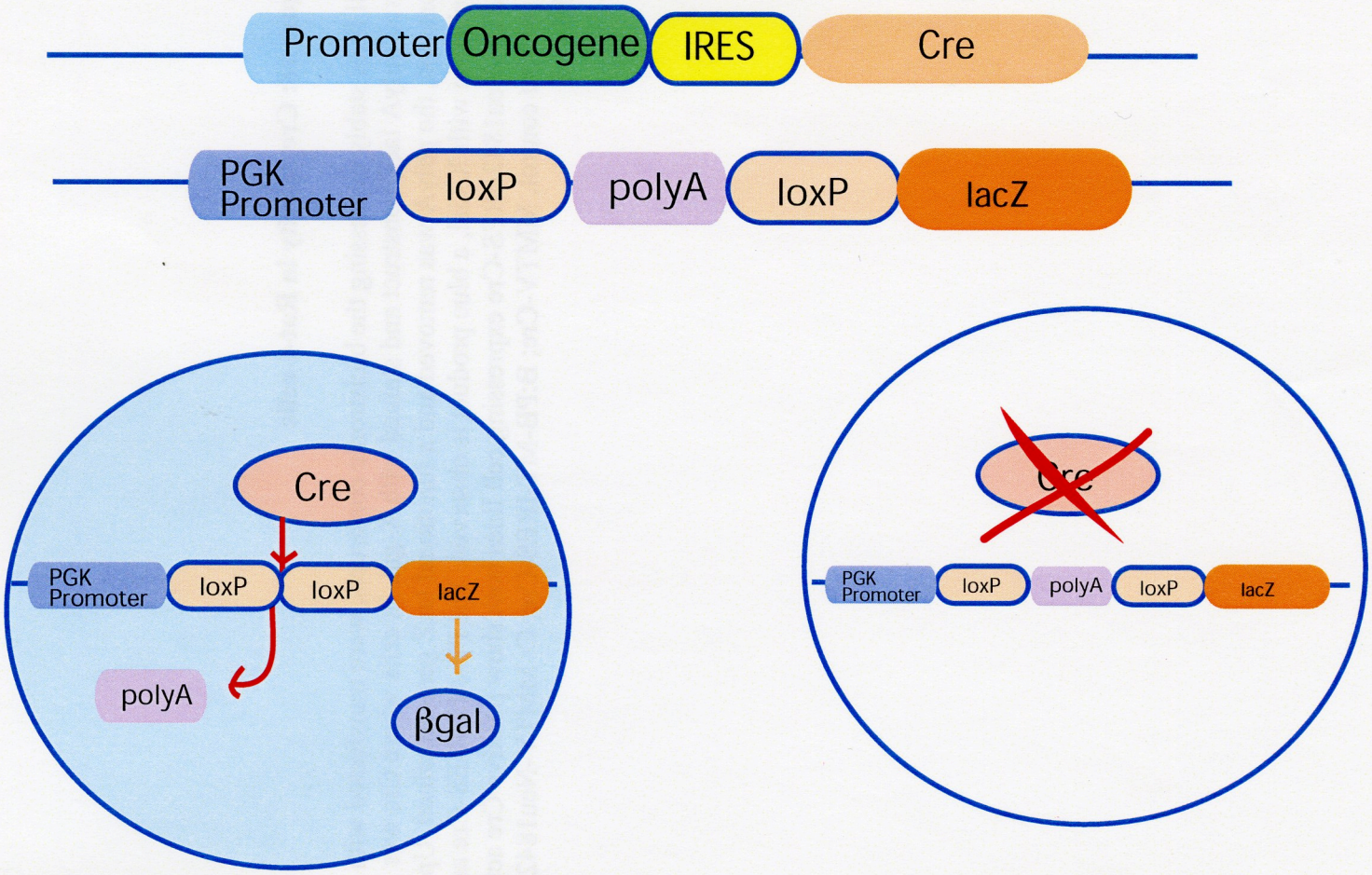

MMTV-Neu1842-IRES-Cre
Number of foci
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
Trial#1
Trial#2
Trial#3
Average
MMTV-Neu1842-IRES-Cre, -dexamethasone
MMTV-Neu1842-IRES-Cre, +dexamethasone
Control, pcDNA3 vector, +dexamethasone
Control, pcDNA3 vector, -dexamethasone
Experiment

Figure 17: PGK-pLox-pA-pLox-lacZ

The construct was used in the test to determine Cre activity *in vitro*. When Cre is expressed, the floxed poly A site in front of the lacZ gene is removed, allowing for lacZ expression. If the β-galactosidase is produced, it will turn cells blue when stained with X-Gal.

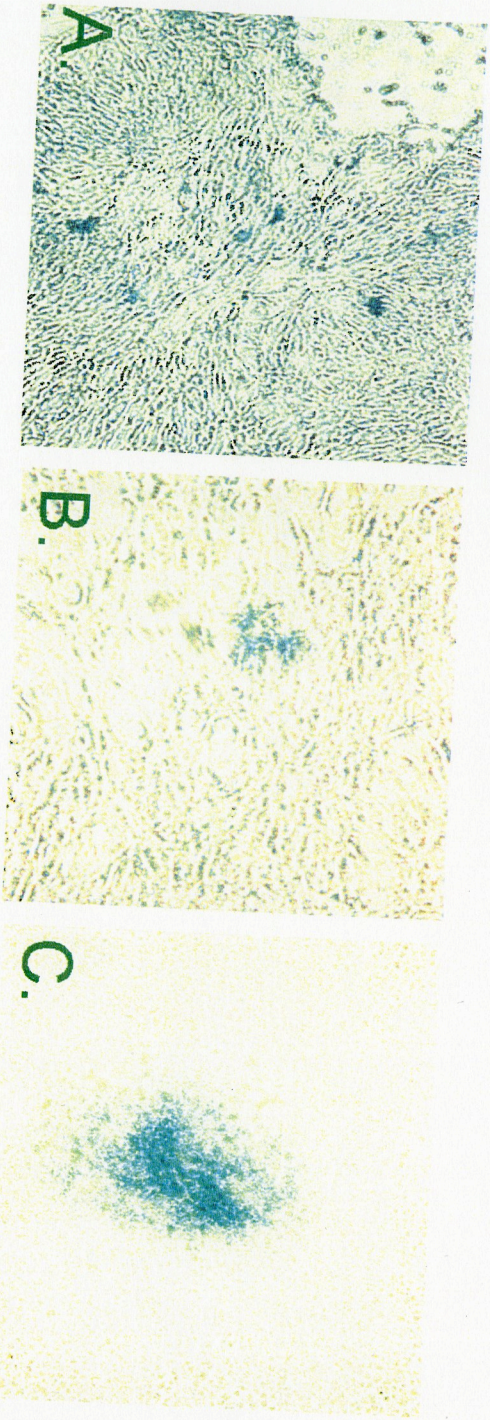


Cells where Cre is expressed and active

All other cells

Figure 18: Cre activity in Rat-1 cells

Cell lines stably expressing the bicistronic constructs, were transfected with a floxed polyA lacZ construct and stained with X-gal. In cells where Cre was expressed, the polyA was removed and a functional lacZ gene remained. Upon incubation with X-Gal, a blue product is observed. The PB-MT-IRES-Cre and MMTV-Neu1842-IRES-Cre expressing cell lines both show positive Cre activity. A: positive control, MMTV-Cre; B:PB-MT-IRES-Cre; C: MMTV-Neu1842-IRES-Cre


A.
B.
C.

Chapter 4: Discussion

This project was designed to continue and improve the study of breast and prostate cancer by creating new animal models. Animal models are still an essential part of research as there are still many unanswered questions about the signaling pathways involved in cancer onset and development. These new models have the advantage of expressing two genes under the same promoter, in a bicistronic construct. They will also utilize the Cre-loxP system for future studies, in combination with existing models. Their design is meant to ease the study of gene-gene interactions, by allowing tissue specific gene ablation with the help of the Cre-loxP system.

In the first stage of the project, four plasmid constructs were designed and built, and sequenced to ensure the presence and right positioning of all components. Out of these four constructs, only three were used in further studies. The MMTV-MT-IRES-Cre construct was designed and built only. For the other three constructs, the transformation potential was assessed by a focus assay in Rat-1 cells, and by examining cell lines with stable expression of each of the constructs. Transgenic animals were also attempted, and the MMTV-Neu1842-IRES-Cre construct is the most promising since founder animals are already expressing the expected phenotype, tumors of the mammary tissue.

The three constructs tested for functionality are the PB-MT-IRES-Cre, PB-MT-IRES-Luciferase and the MMTV-Neu1842-IRES-Cre. They were all tested for transformation efficiency by focus assay. As expected, all gave positive results, showing the potential for tissue transformation (see Figures 10, 13 and 15). In all cases, as expected, the number of foci present increased significantly upon addition of inducer, DHT or dexamethasone. The results for the probasin driven constructs were very similar, as expected, because the transformation potential was tested for the same oncogene, under the same promoter.

The focus assays were all performed in Rat-1 fibroblasts. These cells have been used for a long time in researching the signaling pathways downstream of the EGF receptor (Haley *et al* 1989, Osei-Frimpong *et al* 1994). This study was designed to be qualitative rather than quantitative. For comparison of transformation potential between the constructs, one can subclone the transgene with a potent viral promoter, such as the PGK or CMV promoter. The promoters used in this study were not very efficient in the Rat-1 cell line, as they require outside stimuli, and these cells have few receptors for the steroid or glucocorticoid hormones. A more accurate test would have used a prostate cell line for the probasin driven promoters and a mammary epithelial tissue cell line for the MMTV driven construct. Nontumorigenic rat prostate cell lines that could be used in future studies for quantitative analysis are the newly developed DP-153 (Song *et al* 2003) or the CA-25 line (Gordon *et al* 1995). These cell lines are more suitable because they have androgen receptors that can enhance the response of the construct to the presence of steroids in the media. For the MMTV driven construct, a nontumorigenic breast cell line, such as HBL100 (Rao *et al* 1987) or

the MCF-10A (Ciardiello *et al* 1990) can be used. These cell lines bind both EGF and glucocorticoids, providing cellular context similar to *in vivo* breast tissue. The tests performed in Rat-1 cells demonstrated that the constructs have transformation capacity even in less than ideal conditions.

The transformation ability of the constructs was observed not only in a transient expression setting, but also in stable expression of the constructs. Stable cell lines of the PB-MT-IRES-Cre, PB-MT-IRES-Luciferase and the MMTV-Neu1842-IRES-Cre constructs were grown for 5 days and the foci formed in this period counted. The probasin promoter showed a high amount of gene expression in the absence of DHT. The promoter is known to allow a basal expression level of downstream genes in the absence of steroid inducer (Greenberg *et al* 1994). The MMTV promoter showed very low levels of expression in the absence of dexamethasone. The low number of foci formed in the absence of dexamethasone is expected, as the basal levels of expression of the unstimulated MMTV promoter are very low (Marcellus *et al* 1991).

These cell lines were also used to test the activity of Cre recombinase, the second gene in the built constructs. Cre activity was assessed by X-Gal staining assay. The stable cell lines expressing PB-MT-IRES-Cre and MMTV-Neu1842-IRES-Cre were transfected with a PGKlacZ construct that has a floxed poly A region in front of the lacZ gene (see Figure 17). An active Cre recombinase is able to remove the floxed polyA sequence and allow expression of a functional lacZ gene. In the positive control MMTV-Cre and the test construct were cotransfected in Rat-1 cells and selected with geneticin. This was used as a positive control, as the expression of the Cre recombinase from the MMTV-Cre construct, in dexamethasone treated Rat-1 cells in was previously confirmed by Eran Andrecheck. The results were positive for all three constructs, as shown in Figure 18. Lower amounts of blue patches were observed for the bicistronic constructs, as expected. The test was performed on pooled puromycin selected colonies for both constructs. The selection marker is not on the same plasmid as the bigenic construct, thus some of the surviving cells may have only the puromycin selection vector and not the experimental construct. However, this test showed an important fact, that the Cre recombinase and the upstream oncogene are co-expressed. Blue colored foci on the plates indicate cellular transformation in the presence of Cre recombinase. This confirms the goal of the designed vectors, to have the two genes expressed simultaneously under the same promoter.

For the PB-MT-IRES-Luciferase construct, the simultaneous expression of the two genes was not tested. For this construct, transgenic mice were obtained and histological analyses were performed. Mice were genotyped by PCR. Dissection of six positive male mice between the ages of 2 and 5 months, and 12 wild type littermates, two for each positive mouse, showed no apparent difference in prostate gland appearance or size. Histological analysis shows no difference between genotypically positive and negative tissues after 5 months of age (Figure 12). For this construct, female positive mice could not be obtained after several crosses were set-up. Males of positive and negative genotype results were found

in each litter, but only negative females. One hypothesis to explain this situation is that the insertion point of the transgene in both founder males used is lethal to heterozygous females.

Transgenic animals were also obtained for the PB-MT-IRES-Cre construct. This line had three founder males and two founder females. Heterozygous males had no palpable phenotype or differences in prostate gland appearance and size by dissection in males 3 months of age. Homozygous animals were not obtained.

Founder animals from the third transgenic mouse line were successful and showed the expected phenotype. Female mice, expressing the MMTV-Neu1842-IRES-Cre construct developed breast tumors. These founder animals are currently used to propagate the line.

Efforts were made to produce a viable transgenic line for all three constructs used. FVB mice were used for injections because FVB embryos have pronuclei that are easily manipulated for transgenic microinjection projects. This, coupled with high reproductive performance, makes the FVB ideally suited for transgenic studies (Taketo *et al* 1991).

Analysis of the transgenic mouse prostates could include RNA protection assays and Western blots to determine whether the transgene is transcribed and translated properly. An RNA protection assay would show that the message is transcribed. A Western blot could indicate the presence of protein from the construct. Western blots were attempted, but the optimization trials were not sufficient and the antibodies were not sensitive enough to detect any of the target proteins. Several attempts were unsuccessful due to either too little target protein in the loaded lysate, for Cre and Luciferase detection, or inadequate antibodies against MT.

These methods would still not produce enough information to determine whether the proteins detected are also active. To test the Cre recombinase activity, one can cross a transgenic line of mice expressing Cre to the Rosa26 transgenic line, as described in the introduction.

The progeny resulting from a Rosa26 gene-trap mouse and the PB-MT-IRES-Cre transgenic line will confirm that Cre is expressed only at puberty and not during embryogenesis, and that Cre expression is localized to the prostate. The expected results are uncolored embryos and adult males with a blue prostate tissue.

For the MMTV-Neu1842-IRES-Cre transgenics, the progeny resulting from a cross with the Rosa26 line will show when and where Cre recombinase is expressed. The mice heterozygous for each construct will show blue tissue where the MMTV promoter is active. As mentioned, the MMTV promoter is leaking to the male reproductive tract and to the salivary glands, thus one expects colored mammary tissue as well as some coloration of the salivary glands and the male reproductive tract.

To test the activity of luciferase in the PB-MT-IRES-Luciferase construct, a luciferase assay can be performed after treatment of cell lysates with luciferin.

The intensity of light emitted afterwards can be monitored and measured, as a quantitative assay of luciferase activity (Promega). This construct is special as it offers researchers the chance to monitor in vivo development of an organ. It can also be used to monitor organ changes due to tumorgenesis. The most important feature is the ability to monitor metastatic spread in live animals. All these extraordinary features can be monitored by using the Xenogen IVIS system. Upon luciferin injection, the luciferase activity in the tissue of interest can be observed on a computer screen. This modern technology can provide a wonderful tool for future studies, to research the development of an organ in live animals, without sacrificing them, with the ability to keep track of the organ of the same animal over long periods of time. This is the tool for future research in organ development and cancer studies.

New animal models for cancer studies were designed in this project. While not all constructs provided transgenic animals, the ideas behind the design of these mice are still of interest. Transgenic mice expressing functional MT and Cre proteins under the probasin promoter would provide great insight into modern prostate oncology studies. The PB-MT-IRES-Cre construct was designed such that the oncogene is expressed when natural steroid hormones are released, at puberty, thus offering the opportunity of a normal phenotype during development. With a properly developed prostate gland, the oncogenic process can be studied, as it advances. The advantage of the Cre recombinase is exploited only in transgenic lines crossing studies. Progeny of animals bearing a floxed gene of interest and animals with the PB-MT-IRES-Cre construct would develop normally to puberty and afterwards, the oncologic development can be followed in the absence of the gene in question. This approach is extremely beneficial, since it allows the animal to have a normal embryogenesis and organ development. This approach can be modified either by changing the target floxed gene or by changing the oncogene, in order to observe and compare the development of prostate malignancy under the influence of various oncogenes, and in the absence of different genes of interest.

One of the main questions of prostate cancer, researched and hypothesized currently, would benefit from this model. It has been shown that ErbB2 is important in cancer development in the breast tissue and the prostate tissue. Recently it was shown that ErbB2 is involved in metastasis, enhancing the tumor spread in breast malignancies (Zhau *et al* 1996, Ma *et al* 2003). Using this mouse and the ErbB2$^{Flox/Flox}$ mice (Andrecheck *et al* 2002), we could obtain progeny carrying both the ErbB2$^{Flox/Flox}$ and the PB-MT-IRES-Cre construct. This line of mice would start expressing the MT oncogene and Cre recombinase only at puberty, allowing for normal development. From this point on, oncogenesis will start, in the absence of ErbB2. Analyzing these mice would answer a very important question about the role of ErbB2 and would test the hypothesis whether ErbB2 is crucial for metastasis in prostate as well as in the breast tissue.

As prostate cancer research advances, more genes involved in tumorgenesis will be found. As these novel genes are discovered, mice carrying

floxed versions of them can be made. The mating of these mice with the probasin-MT-IRES-Cre will cause localized knockout of the novel genes, making it possible to study their involvement in cancer progression and development, without disturbing the normal development of the animal. Once tumorgenesis is achieved, the effect of transformation on the organ and the proteins expressed locally can be assessed. This will permit the identification of other factors involved in facilitating or obstructing transformation. This model is not designed to overcome the androgen dependent limitations of the TRAMP model, but rather offer a better alternative to monitoring prostate tumor development and will be an useful tool in following experiments testing gene-gene interaction *in vivo*.

The MMTV-Neu1842-IRES-Cre construct was also designed to take advantage of the Cre-loxP system. This model is promising, since it expresses a mutated form of the ErbB2 gene, rather than using a foreign oncogene such as MT. MT models of breast cancer have been studied intensively and successfully. This model was the only successful line of transgenic animals. This line of transgenic animals is invaluable, as it can be used in crossing with other already existent lines. Crossing this line with $^{Flox/Flox}$ mice versions of a gene of interest would show the role of this gene in ErbB2 (Neu) mediated oncogenesis. This approach can be used to map signaling pathways and test various hypotheses about gene interactions. The cDNA microarray study by Mackay *et al* (2003) can provide a good starting point of genes that are upregulated during ErbB2 induced tumorgenesis of the breast. The effect of the absence of these gene products can be studies *in vivo* using animal models as described. As an ultimate goal, more complex mouse models can be built with the help of this model, leading to one or more, that can best mimic the human condition. This advancement will help scientists better understand the mechanism of oncogenesis.

Both PB-MT-IRES-Cre and MMTV-Neu1842-IRES-Cre transgenic lines can be used to cross with various other lines containing floxed genes. There are several lines of transgenic mice containing floxed genes already established. Several of these floxed genes are known to have important roles in tumorgenesis. Thus, results of transgenic mice resulting from this study mating with floxed p53, β-catenin, stat3, c-fos, BRCA1, BRCA2, and VEGF can provide insightful results.

The removal of tumor suppressors from the cell should accelerate tumor progression. p53 is a known tumor suppressor. It is able to block MT mediated signaling (Doherty *et al* 1999) and it was shown to be degraded or mutated in Neu mediated oncogenesis (Li *et al* 1997). The existing p53 floxed transgenic line (Marino *et al* 2000) can be used to analyze tumor progression in the absence of the p53 gene.

Wnt signaling is involved in breast cancer onset and the wnt pathway has been shown to cooperate with the EGF/EGFR signaling pathway to induce mammary gland tumors (Schroeder *et al* 2002). β-catenin is an effector of the wnt pathway. An animal model with localized removal of β-catinen can be obtained by crossing one of the bigenic Cre constructs with the floxed β-catenin mouse

(Harada *et al* 1999). Studies of the progeny resulting from this cross can show how important is wnt signaling collaboration in tumor formation and progression in either breast or prostate tissue.

The absence of a downstream effector of either MT or Neu oncogenes should negatively affect the development of tumors. Stat3 is a downstream effector for both oncogenes (see Figures 1 and 2). Mice having floxed stat3 genes (Taked *et al* 1998), crossed with bigenic mice bearing the PB-MT-IRES-Cre or MMTV-Neu1842-IRES-Cre genotypes, can help determine how critical stat3 signaling is in tumor formation and development, in either breast or prostate tumorgenesis.

Collaboration with other oncogenes is sometimes critical in rapid tumor development. Transgenic mice carrying floxed versions of known oncogenes are available and can be used in crossing with the PB-MT-IRES-Cre or MMTV-Neu1842-IRES-Cre mice. The progeny of crosses with Floxed c-fos mice (Zhang *et al* 2002) will stress the importance of downstream signaling through Ras and the MAPK pathway (see Figures 1 and 2). Tumor progression is expected to be delayed in tissues where c-fos is not present. BRCA 1 and 2 oncogenes are known to be involved in hereditary breast cancer (Bowcock *et al* 1993, Schutte *et al* 1995). The relationship of these two oncogenes with the ErbB2 receptors and p53 has been studied briefly in human tissue samples, without conclusive results (Kim *et al* 2003). This confirms the fact that there is still much to be resolved in the signaling pathway and protein interactions of Erbb2 and other cellular components.

Tumor development does not depend solely on the malfunction and activation of an oncogene. The extracellular conditions have to be permissive for the tumor to grow. New blood vessels are needed to form to feed the growing tumor. This is achieved with the help of angiogenic factors. Restriction of the amount of angiogenic factors around the tumor should stop progression (Billington *et al* 1991). VEGF is an angiogenic factor that helps in the creation of new blood cells, necessary for tumor development. It has been shown to be present in ductal carcinomas, together with mutated p53 and overexpressed neu (Lee *et al* 2002). Studies with floxed VEGF (Gerber *et al* 1999) can show how this factor affects tumor progression. One would expect tumors to be inhibited or have a slower development in the absence of this factor.

New potential partners for MT and Neu are discovered every day, especially due to the use of microarrays. Microarray data for breast cancer and prostate cancer is already available from recent studies (Mackay *et al* 2003, Glinsky *et al* 2003). Information generated by these new methods can be exploited to better understand the pathways of cancer.

Chapter 5: Summary

The DNA constructs presented here can open a new way to study signal transduction pathways. The MMTV-Neu1842-IRES-Cre line can be used in future studies to answer important questions about other key players in Neu mediated breast tumorgenesis. The prostate-directed vectors have great potential and are worth another try to obtain expressing transgenic mice. These animals will provide an easier way to study the signal transduction pathways in prostate cancer, as this field has not been as scrutinized as in breast cancer research.

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