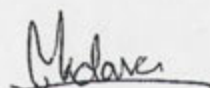


**Identification of Pathways Involved in Breast Cancer
Susceptibility – Contribution of *SuprMam* Loci**

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of The Australian National University

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Abstract

Two genomic regions that modify mammary tumorigenesis, *SuprMam1* and *SuprMam2* have been identified in the BALB/c mouse strain in the *Trp53*^{+/-} mouse model [7]. We studied mammary gland morphology, tumour growth and expression levels of potential candidate genes and pathways in SM09 congenic mice (BALB/c *SuprMam* loci in C57BL/6 background) in comparison to parental strains to identify the genes within the *SuprMam* loci that might be responsible for higher breast cancer susceptibility in BALB/c mice.

While the analysis of mammary gland morphology revealed that there is a significant difference between the number of ductal branches between the two parental strains, there was no significant difference between SM09 and control mice. A similar pattern was observed for total epithelial area, suggesting that BALB/c *SuprMam* alleles alone do not contribute significantly to the morphological differences of the parental strains.

The tumour suppressor DMBT1 (deleted in malignant brain tumors) has been identified as a candidate modifier gene within *SuprMam1* [7]. Using semi-quantitative RT-PCR, *Dmbt1* mRNA was found to be significantly lower in mammary glands of susceptible BALB/c mice compared to C57BL/6, while SM09 mice had similar levels to the control C57BL/6. This indicates that the lower level of *Dmbt1* expression in BALB/c mice must be due to transcription factor differences outside the region, which are not present in SM09 mice.

Assessment of the rate of tumour growth in SM09 and control mice demonstrated no significant difference in the tumour growth rate between the two strains indicating that the BALB/c *SuprMam* alleles do not affect the growth rate of the tumours in SM09 mice. However, this does not exclude the potential of the BALB/c *SuprMam* alleles to increase the susceptibility of normal mammary epithelium to tumorigenesis in the SM09 mice.

Transcriptional profiling of the mammary glands of SM09 and control mice revealed that the majority of the differentially expressed genes are located within the *SuprMam* loci, demonstrating a direct effect of the loci. We studied the vitamin D pathway as a potential candidate pathway as a number of genes (*Cyp2r1*, *PTH*, *Calca*, *Calcb*)

involved in the pathway are located within the *SuprMam* loci. Further, increasing scientific evidence suggests an association of vitamin D and breast cancer risk.

While plasma levels of 25(OH)D, calcium and phosphate were not different between the two strains, there was an approximate 3-fold increase in plasma PTH levels in SM09 female mice compared to controls. *Cyp2r1* contained potential promoter polymorphisms, but was not differentially expressed in the liver, and *Pth*, *Calca* and *Calcb* genes had no distinct polymorphisms. However, *Cyp27b1*, which is located outside the *SuprMam* loci, had a 9-fold reduction of expression in the kidney of the SM09 female mice. Further experiments suggest that reduced *Cyp27b1* expression results in lower levels of 1,25(OH)₂D leading to increased plasma PTH levels. If this is a significant contributor to BALB/c and human breast cancer susceptibility, then there is a practical opportunity for risk reduction. Identification of the genetic factors within the *SuprMam* region causing the differential expression of *Cyp27b1* will require further analysis.

1. Blackburn, A.C., et al., *Genetic mapping in mice identifies DMBT1 as a candidate modifier of mammary tumors and breast cancer risk*. The American Journal of Pathology, 2007. **170**(6): p. 2030.

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Abbreviations

ANU	Australian National University
APF	Australian Phenomics Facility
BSA	Bovine serum albumin
CaSR	Calcium sensing receptor
CT	Cycle threshold
DDT	Dichlorodiphenyltrichloroethane
DEPC	Diethylpyrocarbonate
DMBA	7,12-Dimethylbenz(a)anthracene
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ES	Enrichment score
FDR	False discovery rate
FEW	Family-wise types 1 error
GO	Gene ontology
GWAS	Genome wide association study
H&E	Haemotoxylin and eosin
hCG	human Chorionic Gonadotropin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
JCSMR	John Curtin School of Medical Research
KAl(SO ₄) ₂	Aluminium potassium sulphate
LC-MS	Liquid chromatography–Mass spectrometry
LFS	Li-Fraumeni Syndrome
NBF	Neutral buffered formalin
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCA	Principal components analysis
PMSG	Pregnant mare's serum gonadotropin
PTH	Parathyroid hormone
PVDF	Polyvinylidene difluoride
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SD	Standard deviation
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SuprMam	Suppressor of mammary tumours
TEB	Terminal end bud
TPER	Tissue protein extraction reagent
UNSW	University of New South Wales
UPLC	Ultra performance liquid chromatography

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Chapter 1 - Introduction

1.1 Introduction

Breast cancer is the second most common cancer in the world, the fifth most common cause of cancer death and the leading cause of cancer death in women [4, 9]. Breast cancer is a complex disease with both environmental and genetic factors contributing to an individual's risk of developing the disease. The identification of two important genes *BRCA1*, *BRCA2* [10, 11] and the emergence of new and different approaches during the last two decades has led to a gradual and a much clearer understanding of genetic susceptibility to breast cancer. Epidemiological studies and the identification of unusual familial clustering and phenotypes associated with breast cancer from clinical observations as well as quantification of contribution of, and interactions between genetic factors to breast cancer have provided essential foundations to this understanding.

1.2 Genetic models of cancer susceptibility

Although the extensive genetic studies related to tumorigenesis emerged just a few decades ago, the possible involvement of genetic alterations in tumorigenesis was first suggested by Theodor Boveri in 1914 [12]. In his paper titled "The origin of malignant tumours", he explained that the alterations in the chromosome content of a cell might contribute to tumorigenesis [12]. From there it took many more years to identify specific chromosomal abnormalities in cancer genomes such as the Philadelphia chromosome in chronic myeloid leukaemia patients in 1960 [13]. Even though it was relatively easy to characterize hematological malignancies as specific chromosomal abnormalities, this became a much more difficult task in most solid tumors which exhibit numerous clonal and nonclonal chromosomal abnormalities [14].

In the mid 1970s, Peter Nowell suggested a model which explained the genetic heterogeneity of solid tumours [15]. He explained that the genetic instability acquired in a normal cell can convert it into a neoplastic cell and that the accumulation of these genetic alterations and the selective growth of these subpopulations of cells leads to cancer progression. The work on cancer gene identification continued after this, which was greatly supported by the launch of the Human Genome Project in 1990. The completion of the Human Genome Project in 2003, the availability of a completed map

of the human genome, and technical advances in genomics, facilitated the process of identifying candidate cancer genes.

1.2.1 A polygenic model of breast cancer susceptibility

Breast cancer, like other common cancers, shows familial clustering. It is about twice as common in the first-degree relatives of women with the disease than in the general population, consistent with variation in genetic susceptibility to breast cancer [16]. In the 1990s, two major susceptibility genes for breast cancer, *BRCA1* and *BRCA2* were identified using genetic linkage studies and it was demonstrated that the inherited mutations in these genes significantly increase the risk of breast cancer [10, 11] in carriers. However, the germ line mutations in *BRCA1*, *BRCA2* and a few other rare variants account for only 15-20% of breast cancer that clusters in families and 5% of breast cancer overall [17, 18]. Subsequent genetic linkage studies have failed to identify additional major breast cancer susceptibility genes. The model of a single susceptibility gene such as *BRCA1* or *BRCA2* failed to explain familial breast cancer adequately [19, 20].

These observations have led to the hypothesis that breast cancer susceptibility is largely 'polygenic', that is, susceptibility is due to a large number of genes which have a small but multiplicative effect on risk [1]. A number of comprehensive analyses using both population-based cases of breast cancer and large familial clusters has strongly favored the polygenic model [8]. The polygenic model is also consistent with multiple observations that the excess of familial breast cancer is distributed across many families, each usually consisting of a modest number of cases, rather than just a few families with high incidence of breast cancer [21, 22].

Several extensive studies have also demonstrated that the familial aggregation of breast cancer can be adequately explained by the effects of highly penetrant mutations such as those in *BRCA1*, *BRCA2* along with a polygenic component [21-23]. According to the polygenic model, although the risk alleles are common, a relatively small proportion of carriers develop the disease because the increase in risk is small, or carriers may carry other compensatory alleles that reduce the risk, or carriers must be exposed to certain environmental factors to result in breast cancer [24]. Several environmental risk factors for breast cancer in the general population are identified in epidemiologic studies and

many of these relate to estrogen exposure including timing of menarche and menopause, parity, age of first live birth, breast-feeding and administration of contraceptives and hormone replacement therapy [25]. It is also possible that some of the familial aggregation may be due to a clustering of lifestyle risk factors such as diet or reproductive factors within the family. Therefore, the interaction between these environmental factors and the genetic variants will determine the penetrance of the low risk alleles contributing to a polygenic model of breast cancer susceptibility.

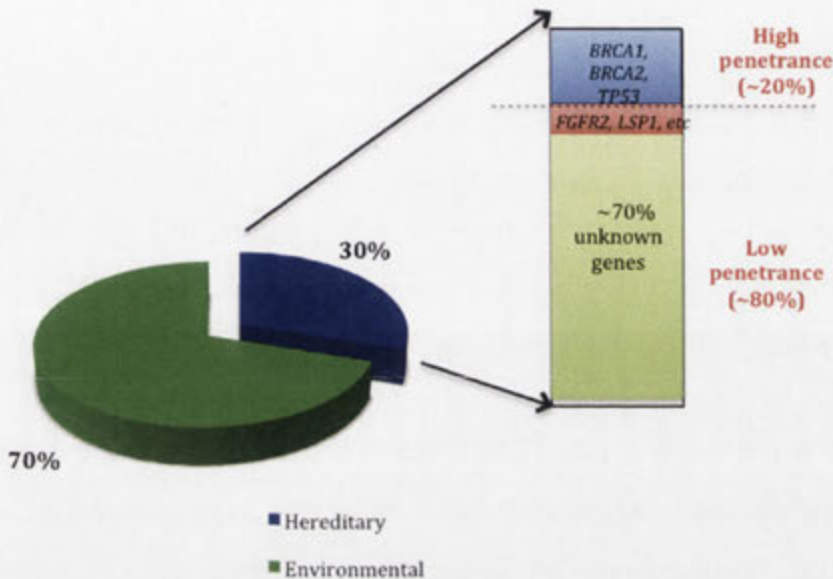


Figure 1.1: The genetic and environmental contribution to breast cancer risk

1.3 High penetrance breast cancer susceptibility genes

Mutations in the three well established high-penetrance breast cancer susceptibility genes, *BRCA1*, *BRCA2* and *TP53*, are known to result in a greater than 10-fold increase in relative risk of breast cancer [8].

1.3.1 *BRCA1* and *BRCA2*

BRCA1 and *BRCA2* were identified through linkage analysis and positional cloning. *BRCA1* and *BRCA2* are involved in DNA double strand breakage repair and therefore play an important role in maintaining genomic integrity. Both *BRCA1* and *BRCA2* are large genes encoding nuclear proteins and are known to have multiple loss of function mutations. However, most of these mutations are rare within the population. Early studies on large cancer families suggest that the risk of breast cancer by the age of 70 for *BRCA1* and *BRCA2* mutation carriers can be as high as 87% and 84% respectively [26, 27]. However, the risks are lower in populations unselected for family history of

breast cancer (65% for *BRCA1* and 45% for *BRCA2*) [28].

BRCA1 and *BRCA2* are also identified as high penetrance ovarian cancer genes (20-40% for *BRCA1* and 10-20% for *BRCA2*) and confer a slightly increased risk of other cancers [17, 28]. However, larger studies are required to confirm whether these findings reflect the real increased risk of these cancers. However, while *BRCA1* is not considered as a male breast cancer susceptibility gene, men with germ line mutations in *BRCA2* have an estimated 6% lifetime risk of breast cancer [17].

1.3.2 *TP53*

TP53 was identified as a high-risk breast cancer gene in 1990, in a study to identify the candidate genes leading to Li-Fraumeni syndrome [29]. In 1969, Li and Fraumeni reviewed medical records and death certificates of childhood sarcoma patients and identified who also had a high rate of breast cancer and other neoplasms. These observations lead to the suggestion of a new familial cancer syndrome of diverse tumours (Li-Fraumeni Syndrome/ LFS). The range of cancers in the syndrome has been determined to include breast carcinomas, brain tumours, soft tissue sarcomas, leukemia, osteosarcomas and adrenocortical carcinoma. These tumours develop at unusually early ages and exhibit frequent multiple primary tumours in LFS patients [30].

Using segregation analysis, Williams and Strong demonstrated in 1985 that the distribution of cancers in LFS families best fit a rare autosomal dominant gene model [31]. This model also suggested that the probability of developing invasive cancers in LFS families is almost 50% by the age of 30 years, while it is only 1% in the general population. In 1990, Malkin *et al* in trying to identify candidate genes for LFS, targeted tumour suppressor genes, the class of genes most strongly associated with familial tumours [29]. They studied the tumour suppressor gene *TP53* because of previous indications that this gene is inactivated in most cancers that are associated with LFS, and found inherited germ line *TP53* mutations in all of the LFS families analyzed.

Further studies have confirmed that the germ line mutations in *TP53* cause LFS [32] and *TP53* is the only gene so far identified in which mutations are definitively associated with LFS. The total life-time cancer risk is found to be significantly higher in women with Li-Fraumeni syndrome, especially in relation to their increased risk for breast cancer [33]. Females from LFS families who carry germ line mutation in *TP53*

have almost a 100% lifetime risk of developing breast cancer, while the risk is around 73% for affected males. This difference may be due to the smaller breast tissue in males as well as differences in reproductive hormones. However, the contribution of *TP53* mutations to the overall risk of familial breast cancer in the general population is very low, as LFS is rare and germ line *TP53* mutations are uncommon in non-Li-Fraumeni breast cancer families [34, 35].

1.3.3 Other high penetrance susceptibility genes

Mutations in several other genes including, *PTEN*, *STK11* and *CDH1* have also been identified as increasing breast cancer risk [36-38]. These mutations are considerably more rare when compared to *BRCA1*, *BRCA2* or *TP53* and the magnitude of the associated risk for each of these genes remains uncertain. However the relative risks are likely to be intermediate and in the range of 2-10 suggesting a low attributable risk of mutations in these genes to familial breast cancer [8].

Four other intermediate-penetrance breast cancer genes, *CHEK2*, *ATM*, *BRIP1* and *PALB2* have been identified through mutational screening [39-42]. Mutations in these genes are rare and together they confer a relative risk of breast cancer of 2-4 [8].

1.4 Low penetrance breast cancer susceptibility genes

More than 70% of genetic predisposition to breast cancer remains unexplained and if any further genetic predisposition factors are identified, they are unlikely to be high-penetrance dominant susceptibility genes as such genes would have been identified by linkage analysis. Population studies have revealed that 67% of families with more than 3 cases of breast cancer were not linked to mutations in *BRCA1*, *BRCA2* or other more common susceptibility alleles [27]. Multiple studies [43, 44] with a similar outcome indicate that there are common elements of risk in the population that are shared between women with breast cancer and their relatives, and low-penetrance susceptibility alleles may be responsible for a large fraction of the risk in these families.

Penetrance	Gene/Locus	Relative risk of breast cancer	Carrier frequency	Method of identification
High	<i>BRCA1</i>	>10	0.1%	Linkage
	<i>BRCA2</i>	>10	0.1%	Linkage
	<i>TP53</i>	>10	Rare	Candidate resequencing
Uncertain	<i>PTEN</i>	2-10	Rare	Linkage
	<i>STK11</i>	2-10	Rare	Linkage
	<i>CDH1</i>	2-10	Rare	Linkage
Intermediate	<i>ATM</i>	2-3	0.4%	Epidemiology; Candidate resequencing
	<i>CHEK2</i>	2-3	0.4%	Candidate resequencing
	<i>BRIP1</i>	2-3	0.1%	Candidate resequencing
	<i>PALB2</i>	2-4	Rare	Candidate resequencing
Low	10q26 16q12 2q35 8q24 5p12	1.08-1.26	24-50%	GWAS
	11p15 5q11	1.07-1.13	28-30%	GWAS
	2q33	1.13	0.87	Candidate association

Table 1: Summary of known breast cancer susceptibility factors (GWAS: Genome Wide Association Studies) (Adapted from [4, 8])

Thus, low-penetrance modifier alleles are proposed to play a major role in breast cancer susceptibility. While the low penetrant alleles have predictable frequencies in the population and may be very common, breast cancer would occur in only a small fraction of individuals carrying these alleles. In this situation, it would be difficult to recognize familial clustering, and therefore, low-penetrance alleles are likely to contribute to a significant fraction of what is presently identified as sporadic breast cancer [23, 45].

In recent years the search for low penetrance variants was conducted mainly through Genome-Wide Association Studies (GWAS). These studies evaluate a large number of common genetic single nucleotide polymorphisms (SNPs) to identify association with disease based on the patterns of linkage disequilibrium in the human genome [46]. In the past years several novel risk alleles for breast cancer were identified by four recent GWAS (table 1.2): Breast Cancer Association Consortium, Cancer Genetic Markers of Susceptibility, DeCode Islanda, Memorial Sloan-Kettering Cancer Center [1-4]. Comparison of the results obtained from these four major GWAS has highlighted a correlation of allele frequency of some SNPs located on the genes: *FGFR2* (a receptor tyrosine kinase), *TNRC9* (a high-mobility group chromatin-associated protein), *MAP3K1* (the signaling protein mitogen-activated protein kinase1), *LSP1* (F-actin bundling cytoskeletal protein) and *H19* (an untranslated messenger RNA involved in regulation of the insulin growth factor gene 2 [8, 47]. The most strongly associated SNP

was in intron 2 of the FGFR2 gene that is amplified and over expressed in 5-10% of breast cancer [47].

These studies provide a powerful tool to identify new susceptibility factors and prognosis of the disease. Common genetic variants may influence prognosis either by influencing the type of tumour that develops, the host response to tumour or the handling or metabolism of breast cancer directed therapies. However, the use of GWAS for the identification of risk alleles also has several limitations. Apart from requiring accumulation of large number of data, these studies do not reveal the mechanism by which the novel allelic variants cause the susceptibility. Furthermore, some differences were observed between different studies, which could be due to population stratification, differences in sample size or genetic heterogeneity in the setting of genotyping platforms used [4]. On the other hand, while GWAS reveal locations of genetic variation associated with disease, due to the haplotype structure of the human genome, many times there are multiple gene transcripts nominated by positive polymorphisms. Recently, a new multi-SNP GWAS analysis method called Pathways of Distinction Analysis was developed. This method uses GWAS data and pathways-gene and gene-SNP associations to identify pathways that could differentiate cases from controls [48]. This method will enrich the power of GWAS in breast cancer risk prediction. While the significant impact of genomic approaches on our understanding of disease biology is well accepted, the clinical relevance of genomic variation for cancer susceptibility testing remains to be established [49].

Gene	Location	SNP	Study
FGFR2	Chr 10q	rs2981582	Easton <i>et al.</i> , 2007
		rs1219648rs2420946rs11200014 rs28981579	Hunter <i>et al.</i> , 2007
		rs2981582	Gold <i>et al.</i> , 2008
TNRC9	Chr 16q	rs12443621 rs8051542	Easton <i>et al.</i> , 2007
		rs8049226	Hunter <i>et al.</i> , 2007
		rs3803662 rs3112625	Gold <i>et al.</i> , 2008
		rs3803662	Stacey <i>et al.</i> , 2007
MAP3K1	Chr 5q	rs889312	Easton <i>et al.</i> , 2007
		rs726501	Hunter <i>et al.</i> , 2007
LSP1	Chr 11q	rs3817198 rs498337	Easton <i>et al.</i> , 2007
		rs7120258	Hunter <i>et al.</i> , 2007
H19	Chr 11p	rs2107425	Easton <i>et al.</i> , 2007
		rs7120258 rs7578974	Hunter <i>et al.</i> , 2007

Table 1.2: Comparative analysis of SNPs identified in the four major GWAS [1-4]

1.5 Factors affecting penetrance

1.5.1 Population evidence

The significance of low penetrance alleles is also evident in breast cancer families with known mutations. Although mutations in *BRCA1* and *BRCA2* seem to cause strikingly similar clinical disease in twins [45] the penetrance of breast cancer has been shown to vary among different populations carrying *BRCA1* and *BRCA2* susceptibility alleles [23]. For example, the estimated penetrance to age 70 years for breast cancer was found to be 59.9% for the Jewish *BRCA1* mutations and 28.3% for the Jewish *BRCA2* mutations, but is lower in non-Jewish population [50].

1.5.2 Allelic variation

The penetrance of any susceptibility gene is usually assessed as an average for a population, but this penetrance might vary between populations, within populations and between individuals. The allelic variations, which might result from different mutations in a single gene, are one of the several potential sources of variation. For example, the effect of a truncating *BRCA1* or *BRCA2* mutation is likely to differ from that of a missense mutation [51].

1.5.3 Modifier genes of penetrance

The impact of mutations in a major gene might depend on the presence of one or more specific alleles of a genetic modifier. These modifying genes may or may not be linked to the major gene. If a modifying gene is linked to the major gene, then the modifier allele might co-segregate with the mutant allele and most carriers in the family will have at least one copy of the modifier allele in common [51]. It has been proposed that the gene for 17 β -hydroxysteroid dehydrogenase-2 (*EDH17B2*) is a linked modifier of ovarian cancer risk in carriers of *BRCA1* mutations [52]. However, if a modifier is unlinked to the major gene, the carriers in a given family are likely to have different alleles at the modifier locus. The *PROGINS* variant (306 bp Alu insertion in intron 7) of the progesterone receptor is found to be associated with increased risk (OR 2.4) of ovarian cancer in *BRCA1* and *BRCA2* mutation carriers [53]. Recently a polymorphism in *MDM2*, which is a key negative regulator of *TP53*, has been shown to be a candidate as a genetic modifier in *TP53*-mutated cancers and in LFS. Several studies have revealed that the *MDM2* polymorphism is associated with a difference of 19 years (28 versus 47 years, $p=0.01$) [54] and 9 years (18.6 versus 27.6 years, $p=0.0087$) [55] in the average age at the cancer diagnosis in *TP53* mutation carriers. These genetic

variants, while acting as modifier genes in the carriers of major gene mutations, can also act as low penetrance susceptibility polymorphisms in non-mutation carriers, which may contribute to tumour development.

1.6 Mouse models for the study of modifier genes of breast cancer risk

Identifying genes of small effect in a human population is difficult because familial aggregation may be explained by shared genetic, environmental or behavioural risk factors or some combination of these, and the heterogeneity in the general population can often obscure the small contributions of individual genes. The use of mouse models and inbred strains overcome the problems of population heterogeneity, as well as environmental and behavioral factors confounding genetic effects. When compared to human populations, the information that can be obtained from tumours in mouse models has allowed the mapping of loci involved in determining the aggressiveness and metastatic behaviour in addition to the frequency and latency of mammary tumours [56].

It is being found that approximately half of LFS families carry germ line mutations in the tumour suppressor gene *TP53*, which confer a 50% risk of developing breast cancer by 50 years of age [57]. Mice with *Trp53* deletions have been made on three different inbred strains, 129Sv, BALB/c and C57BL/6 to model human LFS. All three strains develop sarcomas, lymphomas and some carcinomas, while each strain also develops a unique tumour type. The 129Sv strain develops teratomas [58] while the BALB/c strain develops mammary carcinomas, both of which are rarely seen in the C57BL/6 strain [59]. These differences in tumour development between the mouse strains can be exploited to map low-penetrance modifying genes in specific tumour types, which might be applied in patients with LFS.

The *TP53* pathway appears to be critical in the protection of the breast epithelium against tumorigenesis. This is evident in the high risk of female LFS patients for developing breast cancer. High rates of somatic *TP53* mutations are found in breast cancer even in the context of mutations in *BRCA1* and *BRCA2* genes [60, 61]. *TP53* participates in many pathways that contribute to breast cancer, such as ErbB2/Neu, resulting in decreased latency and increased incidence of mammary tumours in transgenic mouse models [62]. *TP53* is a commonly mutated gene in sporadic breast

cancer. Therefore, genes identified in mouse models that cooperate with p53 deficiency to increase breast cancer risk are likely to be relevant to both familial and sporadic breast cancer in humans. Mouse models of breast cancer usually lack expression of hormone (estrogen and progesterone) receptors, representing the less common (30%) basal subtype of human breast cancer. In the *Trp53*^{+/-} mouse model, 3/26 (12%) of malignant tumours retain expression of hormone receptors representing the luminal subtype of human breast cancer [63]. This suggests that the BALB/c-*Trp53*^{+/-} model of breast cancer represents aspects of both basal and luminal subtypes of breast cancer.

Mammary tumours are prevalent in BALB/c mice hemizygous for the *Trp53* gene, with 55% of females developing spontaneous mammary tumours between 8-14 months of age [59], while C57BL/6-*Trp53*^{+/-} mice are nearly completely resistant to mammary tumours with less than 1% of females developing tumours [64]. This makes BALB/c-*Trp53*^{+/-} mice a suitable model to study mammary tumorigenesis. However, even though the risk of mammary tumours varied among strains, tumours in other tissues have shown a similar prevalence in BALB/c-*Trp53*^{+/-} and C57BL/6-*Trp53*^{+/-} mice [24]. This highlights the fact that the C57BL/6 mice possess alleles that can compensate for deficiencies in a major tumour suppressor pathway such as *Trp53*, in a mammary gland-specific manner. On the other hand, around twenty percent of BALB/c mice with wild type *Trp53* are known to develop spontaneous mammary tumours while the equivalent rate is around 55% in BALB/c-*Trp53*^{+/-} mice [59, 65]. Therefore this suggests that BALB/c mice possess alleles of one or more modifier genes, which increase their susceptibility to mammary tumours. The genetic polymorphisms underlying these differences in mammary tumorigenesis between BALB/c and C57BL/6 mice in a *Trp53*^{+/-} context is yet to be identified.

1.7 *SuprMam1* and *SuprMam2* loci: Novel breast cancer modifier loci in mice

1.7.1 Identification of *SuprMam1* and *SuprMam2* loci

During their attempt to genetically map the recessive BALB/c alleles contributing to increased spontaneous mammary tumours in *Trp53*^{+/-} mice, Blackburn *et al* identified two new breast cancer susceptibility loci [7]. Over 200 female N2-backcross [(C57BL/6 x BALB/c) x BALB/c]-*Trp53*^{+/-} mice were monitored for tumour development over 18 months. A genome scan was conducted on two groups of N2 mice with and without

mammary tumours to identify mammary tumour modifier loci. The results from this study have indicated a strong genomic region on chromosome 7 that had a significant association with occurrence of mammary tumours in *Trp53*^{+/-} mice. This chromosome 7 locus was confirmed with the analysis of additional SNP markers and was designated *SuprMam1* (for *Suppressor of Mammary* tumours). They observed two potential peaks within this linkage region, which raises the possibility that there may be more than one gene within the region affecting mammary tumour susceptibility. A secondary peak of association was also identified on chromosome 2, which was named *SuprMam2*.

In the context of germ line deficiency in *Trp53* and the occurrence of tumours in other tissues, it is not possible to distinguish the effect of *SuprMam* loci on latency versus incidence of mammary tumours. However, the BALB/c alleles of *SuprMam1* have been shown to both increase the frequency and decrease the median latency (hazard ratio, 1.93; 95% confidence interval, 1.26 to 2.95; *P* = 0.002) of mammary tumours without affecting the occurrence of other tumour types [7] suggesting that the effect of *SuprMam* loci is specific for mammary tumours. Homozygosity at *SuprMam2* significantly, yet less strongly, increased the mammary tumour risk (hazard ratio, 1.54, 95% confidence interval 1.00-2.36, *P* = 0.047) (unpublished data). A preliminary analysis of tumours collected from the F1-*Trp53*^{+/-} mice has found that 2/6 mammary tumours showed loss of heterozygosity (LOH) for the *SuprMam1* peak marker, D7Mit68, with the BALB/c allele being retained, while 0/7 other tumour types showed LOH. This is consistent with a tumour suppressor that is specific for mammary tumours being present in the C57BL/6 strain in the *SuprMam1* region.

The *SuprMam1* and *SuprMam2* regions do not overlap with any of the previously identified breast cancer susceptibility loci in humans or mouse. This includes the dominant mammary tumour susceptibility modifier (*Mtsm1*) mapped on chromosome 7 using a BALB/c-*Trp53*^{+/-} mouse model [66]. It is also important to note that these *SuprMam* loci do not carry any genes in known breast cancer pathways. However, there are several GWAS identified genes that are syntenic with *SuprMam* loci in mice. For example, *FGFR2*, the low-risk gene identified in the human GWAS [2], is located within the *SuprMam1* locus. Therefore it is important to identify the polymorphic genes within these loci that are responsible for BALB/c susceptibility to mammary tumours.

In one approach to identify the polymorphic genes within these loci that are responsible for the BALB/c susceptibility to mammary tumours, Blackburn *et al*, 2007 used oligonucleotide microarrays to identify genes within *SuprMam1* that were differentially expressed in mammary glands from C57BL/6-*Trp53*^{+/-} and BALB/c-*Trp53*^{+/-} mice. They found five genes, *Dmbt1*, *Thumpd1*, *Trim12*, *Spon1* and *Hbb-b2* to be significantly differentially expressed between the two strains (intensity >150, P < 0.005, change more than two-fold). Of these, *Hbb-b2* and *Spon1* were higher in BALB/c, whereas *Trim12* (tripartite motif protein 12), *Dmbt1* (deleted in malignant brain tumours 1) and the *Thumpd1* (a predicted protein) were decreased in BALB/c [7]. Out of these genes, *Dmbt1*, which has been previously described as a tumour suppressor gene [67, 68], showed a 3.8-fold difference in expression with lower levels in the susceptible BALB/c strain [7]. Together with this, the reduced expression of DMBT1 protein in normal breast tissue from women with breast cancer compared with cancer-free controls [7] suggests *Dmbt1* as a candidate modifier of mammary tumours within the *SuprMam1* locus.

1.7.2 Congenic mice for *SuprMam* loci

One approach to study the effect of the identified susceptibility loci is by generating congenic model organisms. This approach has been used successfully in rats to identify eight breast cancer susceptibility loci (*Mcs1-8*) by crossing two rat strains with large differences in their susceptibility to the induction of mammary tumours by the chemical carcinogen dimethylbenzanthracene (DMBA) [69]. A similar approach to study the effect of *SuprMam* loci on increased mammary tumour susceptibility and the analysis of potential candidate genes including *Dmbt1* is to develop C57BL/6 mice congenic for the BALB/c alleles of the *SuprMam* loci. Then it would be possible to look for differences in gene expression and examine their mammary gland biology compared to normal C57BL/6 mice. BALB/c susceptibility loci *SuprMam1* and *SuprMam2* were introduced on to the C57BL/6 background by conventional breeding. These mice were then backcrossed for ten generations and the littermates were inter-crossed to produce homozygotes at *SuprMam1* and *SuprMam2* loci, which were confirmed by genotyping. This congenic mouse line was named SM09 and they were inter-crossed for maintenance of the lines.

Two other congenic lines were also developed to investigate the effect of *SuprMam1* locus on mammary tumorigenesis without the effect of *SuprMam2*. These two lines

were named SM09-pro and SM09-dist, which carry BALB/c alleles in the proximal region of the *SuprMam1* locus and the distal region of the *SuprMam1* region on a C57BL/6 background, respectively. These mice can be used to identify the pathways in the mammary gland whose biology and gene expression is altered by the BALB/c *SuprMam* alleles.

1.8 Hypothesis

This thesis examines the hypothesis that genetic variation within *SuprMam1* and *SuprMam2* contributes to breast cancer susceptibility in mice.

1.9 Project aims

1. **Characterize the mammary gland morphology of SM09 congenic mice:** any alteration in the basic mammary gland morphology of SM09 mice will suggest the involvement of BALB/c *SuprMam* alleles in controlling mammary gland morphology. (Chapter 3)
2. **Analyze expression of *Dmbt1* in mammary glands of SM09 mice:** Lower *Dmbt1* expression in SM09 mammary glands (similar to that of the BALB/c parental strain) will suggest that *Dmbt1* is a tumour suppressor responsible for the increased breast cancer susceptibility in SM09 mice. (Chapter 3)
3. **Transcriptional profiling on congenic mammary glands:** The resulting data will be examined for two categories of genes.
 - i. Genes within the loci with significant differences in expression – This may point directly to genes containing polymorphisms that affect expression and are responsible for mammary tumour susceptibility.
 - ii. Genes outside the loci with significant differences in expression – These differences will expose biological pathways and potential relationship of these pathways to genes within the loci.

This will identify pathways for further analysis. (Chapter 4)

- 4. Identify pathways whose gene expression is altered in *SuprMam* congenic mice:** One or two candidate genes will be selected and the related pathways will be studied to assess the significance of these genes and their pathways in increased breast cancer risk in SM09 mice. (Chapter 5)

The outcomes of this work will be,

- Identification of biological pathways that are altered by BALB/c *SuprMam* alleles.
- Explain the biological differences examined through the identification of the polymorphic genes responsible for the high mammary tumour susceptibility in SM09 mice.

Chapter 2 – Materials and Methods

2.1 Animals

All procedures involving animals were carried out in accordance with institutional and national guidelines for the use of animals and were approved by the Australian National University Animal Experimentation Ethics Committee (Animal Ethics Protocol Number A2011/37).

All the marker positions described in this thesis are in accordance with Ensemble Mouse Genome Build 37.

2.1.1 Generation of the congenic mouse strains

The congenic mouse strains were produced by conventional breeding by Dr Anneke Blackburn and Ms Melissa Rooke at JCSMR, starting in 2004. C57BL/6 females and a BALB/c male mouse were crossed to generate F1[B6xBALB] males, one of which was backcrossed to C57BL/6 females to generate 48 male N2-B6x[B6xBALB] offspring. These mice were genotyped using the microsatellite markers D7MIT68 and D2MIT135, corresponding with the linkage peaks for *SuprMam1* and *SuprMam2* respectively. DNA from mice heterozygous at both loci (n=15) was sent to the Australian Genome Resource Facility (AGRF) for genome scanning to determine which males possessed the least amount of BALB/c alleles across the whole genome, and interesting breakpoints within *SuprMam1*. From this, 2 males with the least amount of BALB/c alleles outside the loci of interest were selected and used to establish the subsequent congenic lines – males #1809 and #1802, giving rise to the SM09 and SM02 lines, respectively.

The lines were then backcrossed for 10 generations to C57BL/6 females. At the final backcross, mice from the SM09 line that carried neither *SuprMam1* nor *SuprMam2* BALB/c alleles were selected and intercrossed to make a control line for comparison to the SM02 and SM09 congenic lines.

2.1.2 Finer mapping of the BALB/c regions in the congenic mice

The majority of the work reported in this thesis was performed on the control and SM09 strain. In chapter 5, two strains from the SM02 congenic line were also analysed. The region of BALB/c alleles carried by each strain was originally mapped using the markers listed in tables 2.1 and 2.2 for *SuprMam1* and *SuprMam2* respectively. The

SM09 congenic mice carry BALB/c alleles for both the *SuprMam1* and *SuprMam2* regions, whereas the SM02 lines carry BALB/c alleles only for the *SuprMam1* region.

Chr 7: Markers	Genotyping method	Position (Mb)	BALB/c	C57BL/6
D7Mit231	Microsatellite	73.70		✓
D7Mit318	Microsatellite	81.10		✓
D7Mit62	Microsatellite	91.80		✓
D7Mit96	Microsatellite	107.70		✓
D7Mit220	Microsatellite	111.54		✓
D7Mit281	Microsatellite	119.40	✓	
rs3680026	Amplifluor SNP	120.46	✓	
rs13479487	Amplifluor SNP	127.20	✓	
D7Mit68	Microsatellite	132.48	✓	
D7Mit105	Microsatellite	135.71	✓	
rs13479540	Amplifluor SNP	141.20	✓	
Dock1	RFLP	141.80		✓

Table 2.1: The markers used for the genotyping of BALB/c alleles on the *SuprMam1* locus. The peak linkage markers are indicated in red colour. (RFLP- Restriction Fragment Length Polymorphism)

Chr 2: Markers	Genotyping method	Position	BALB/ c	C57BL/ 6
D2Mit336	Microsatellite	113.70		✓
D2Mit445	Microsatellite	121.12	✓	
D2Mit398	Microsatellite	125.55	✓	
D2Mit135	Microsatellite	129.19	✓	
rs3726475	Amplifluor SNP	138.40	✓	
D2mit48	Microsatellite	155.95		✓
rs13476872	Amplifluor SNP	159.11		✓

Table 2.2: The markers used for the genotyping of BALB/c alleles on the *SuprMam2* locus. The peak linkage marker for *SuprMam2* is indicated in red colour.

Confirmation of these regions and finer mapping was done by exome sequencing of SM09 and control mice, using Agilent Sureselect XT2 mouse exome kit and Illumina HiSeq 2000 sequencing (This was carried out at the Australian Phenomics Facility). The strain specific SNVs (Single Nucleotide Variations) were used to confirm the BALB/c regions on chromosome 2 and chromosome 7 to define the *SuprMam* loci carried in SM09 mice.

This analysis determined that the BALB/c regions carried by the different congenic strains were:

	<i>SuprMam1</i> (chr 7)	<i>SuprMam2</i> (chr 2)
1) SM09	119.4-141.2 Mb	121.2-138.4 Mb
2) SM02-pro	104.5-132.6 Mb	None
3) SM02-dist	125.3-135.5 Mb	None

(See also Figure 5.6, p65)

2.1.3 Estrous stage determination

Stages of estrus were determined by cytological evaluation of vaginal smears. Vaginal smears were obtained by means of an ordinary pipette. A few drops of sterile PBS solution were drawn into the pipette, introduced into the vagina and then retracted into the pipette. The fluid was transferred to a glass slide and mounted under a cover slip. The mice were staged twice daily and were divided into diestrus, pro-estrus, early estrus, estrus and post-estrus stages according to the following cytological assessment [70].

- 1- Diestrus: almost exclusively leukocytes.
- 2- Pro-Estrous: showing both leukocytes and nucleated epithelial cells in approximately equal numbers.
- 3- Early-Estrous: showing clearly defined epithelial cells, some with distinct nuclei.
- 4- Estrous: large, squamous-type epithelial cells without nuclei.
- 5- Post-Estrous: showing approximately equal numbers of leukocytes and epithelial cells, but the latter are large, folded, and with translucent nuclei.

2.2 Morphological analysis of the mammary glands

2.2.1 Wholemounts

To examine morphology, mammary glands were isolated from the sacrificed mice and carmine-alum whole-mount staining was performed. Both 4th inguinal mammary glands were removed and spread on a microscopic slide at room temperature for several minutes to adhere to the glass. The tissues were then transferred to histology cassettes and fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 1 hour. After 15 min in 70% ethanol, the tissues were rinsed in distilled water and stained overnight at 4°C in carmine alum solution (0.2% carmine, 0.5% KAl(SO₄)). The slides were then dehydrated at room temperature by 15 min each of 70%, 95% and

100% ethanol and cleared in xylenes for 1 hr. The tissues were then mounted on slides with permount and cover-slipped.

Four to 8 low power (4X) fields of view on each slide were analyzed for ductal branches by counting the number of branchpoints within each field under a bright field microscope (Olympus 1X71 (1.210). The mean and SD was calculated for 5-8 mice per strain and estrus stage, and statistical significance ($p < 0.05$) was determined by Student's t-test.

2.2.2 Histological analysis

For histological analysis of the total epithelial area, mammary glands were fixed overnight in neutral buffered formalin (NBF), processed, embedded in paraffin for sectioning and stained with hematoxylin and eosin (H&E). The preparation and staining of these slides was performed by Anne Prins at JCSMR, ANU. The H&E stained slides were digitized using an Olympus 1X71 (1.210) with camera attached and measurements were taken with the ImageJ imaging software (Wayne Rasband, National Institute of Health, Bethesda, Maryland, USA). Three microscopic fields were randomly selected from each slide and examined at 20X magnification using Olympus 1X71 (1.210) with camera attached. Epithelial area was determined by manual tracing of lobuloalveolar units and expressed as a percentage of the total area examined. Four animals per strain and estrus stage was used and statistical significance ($p < 0.05$) was determined by Student's t-test.

2.3 In vivo tumour growth

2.3.1 Cell culture and preparation

Two C57BL/6 mammary tumour cell lines (E0771 and AT-3) were used for the experiments described in Chapter 3. The AT-3 cell line was originally established from a primary mammary gland carcinoma of an MTAG mouse [71] and E0771 is a medullary breast adenocarcinoma cell line originally isolated as a spontaneous tumour from C57BL/6 mouse [72]. Cell culture conditions described by Ewans et al., 2005 [73] were used for the culture and the maintenance of both cell lines. The cells were maintained as a monolayer in RPMI medium supplemented with 10 mM HEPES and 5% fetal bovine serum and 100 units/ml penicillin in a 37°C, humidified, CO₂ (5%) incubator.

On the day of the injection, cells were trypsinized (0.05% trypsin + 0.5mM EDTA in phosphate buffered saline (PBS)), counted and washed with fresh media and resuspended in serum free RPMI at concentrations of 2.5×10^5 cells per 15ul for E0771 and 5×10^5 cells per 15 ul for AT-3 cells.

2.3.2 Assessment of tumour growth

Tumour cells were injected on to the mammary fat pad of both fourth mammary glands of 10 female mice each of SM09 and control strains. After tumour cell injections, mice were examined daily for signs of incidence of tumours. When tumours reached a measurable size, they were measured every 1-2 days using a digital caliper in two perpendicular dimensions parallel to the surface of the animal. The size of the tumour is expressed as $\pi/6 \times L \times W^2$, where 'L' and 'W' are the tumour's longest dimension and shortest dimension respectively. The tumour growth rate was calculated by simple descriptive statistics of mean and standard deviation using Microsoft Excel 2011 software from Microsoft.

2.4 Gene expression analysis (semi-quantitative and quantitative PCR)

2.4.1 RNA extraction

Mammary gland, kidney and liver tissues that were snap frozen in liquid nitrogen and stored at -80°C were used for RNA extraction. Approximately 50-100mg of frozen tissue was homogenized with 1ml of TRIzol reagent (Invitrogen, Catalogue # 15596-026) and incubated at room temperature for 5 minutes to permit the complete dissociation of nuclear protein complexes. The homogenate was then centrifuged at $12,000 \times g$ for 10 minutes at 4°C to remove extra cellular material and the supernatant was transferred to a fresh tube. Chloroform (200ul) was added to each tube and mixed vigorously by hand for 15 seconds and incubated at room temperature for 2 minutes. The tubes were then centrifuged at $12,000 \times g$ for 15 minutes at 4°C . The aqueous phase containing RNA was transferred to a fresh 1.5 ml tube. One volume of 70% ethanol was added to each tube and mixed well by pipetting. Samples were transferred to RNeasy columns and RNA was extracted using RNeasy mini kit (Qiagen, Catalogue # 74104). DNA was removed using TURBO DNA-free Kit (Applied Biosystems, Catalogue # AM1907). RNA was eluted in RNase free water and quantity (ng/ul) and

the purity of RNA (abs 260/280) was measured using a Nano drop (spectrophotometers, ThermoScientific). RNA samples were stored at -80 °C.

The quantity and the quality of RNA used for microarray analysis was measured using a Bioanalyzer (Agilent 2100). Eukaryotic total RNA Nano Series chip was used and all samples had a RIN value (RNA Integrity Number) above 8. All the samples also passed three quality control checks that were carried out at Ramaciotti Centre, UNSW before proceeding to hybridization to gene arrays.

2.4.2 Reverse transcription

Invitrogen First-Strand cDNA Synthesis kit (Catalogue # 18080-044) was used for the conversion of RNA into cDNA. RNA (500 ng) was reverse transcribed to first-strand cDNA by adding 1ul of oligo(dT)₁₂₋₁₈ and 1ul of 10mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH) and made up to 13 ul with DEPC-treated water. This reaction was incubated at 65°C for 5 minutes followed by 1 minute on ice. A master mix consisting of 4 ul of First Strand buffer, 1 ul of 0.1 M DTT, 1 ul of RNaseOUT (Recombinant RNase inhibitor, catalogue # 10777-019) and 1 ul of SuperScript III RT (200 units/ul) were added. Amplification was done at 50°C for 60 minutes, followed by heating at 70°C for 15 minutes to inactivate the reaction. cDNA samples were stored at -20°C for further use.

2.4.3 Selection of primers

All primers used were selected from previous publications and were checked on NCBI primer blast to check specificity and avoid non-specific amplification. Primers were obtained on order from GeneWorks Custom Oligo Service (<http://www.geneworks.com.au/>) and dissolved in nuclease free water to 100uM stock solution and diluted to 10uM working concentration for semi-quantitative PCR and 6uM for real time PCR. The list of primers and their sequences are listed in appendix A.

2.4.4 Semi-quantitative PCR reaction

PCR was performed with 300 pmol/ml of each primer in a 20- μ l reaction volume containing 10 $\times$ PCR buffer, 25mM MgCl₂, 1.25 mM dNTPs, and 5 U/ul of Taq polymerase and amplified 30 cycles (90°C for 30 secs, 60°C for 30 secs, 72°C for 1 minute). PCR products were run on 1.5% agarose/TBE gel with Gelred. The resulting bands were analyzed for expression levels and mouse β -actin was used as a loading control.

2.4.5 Real-time PCR (Quantitative PCR)

SYBR green reaction was used for relative quantification of gene expression in 384 well plates (Applied Biosystems, Catalogue # 4329001) with the aid of robotic pipette. Analyses were carried out in triplicate 10 μ l reactions. The master mix was prepared with 5 μ l SYBR green (Applied Biosystems, catalogue # 4367659), 0.5 μ l (300nM) forward primer and 0.5 μ l (300nM) reverse primer. cDNA was diluted in nuclease free water to make a final concentration of 2.5ng/ μ l. Total 10 μ l reaction volume was used per well with 6 μ l of master mix and 4 μ l of cDNA. The plate was centrifuged and run on a 7900HT real time PCR machine (Applied Biosystems). Genes were amplified at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C 15 sec and 60°C for 1 min. A dissociation curve step was also applied in the amplification protocol to check only a single product was amplified. The expression levels were presented as a fold-change calculated using the $\Delta\Delta CT$ method [74]. Results from at least four mice per group were compared and the statistical significance was determined using Student's t-test.

2.5 Transcriptional profiling

2.5.1 Treatment groups and sample preparation

Two groups of control (C57BL/6) and SM09 female mice at 8-12 weeks of age were used for microarray analysis. *Trp53* was activated by irradiation. Maximal induction of *Trp53* after irradiation requires the mammary glands to first be stimulated to proliferate by hormone treatment [75]. Therefore two different treatment groups were analysed as follows:

1. **Basal** - Untreated, diestrus as determined by vaginal ontology (2.1.3)
2. **Treated** - Mice were injected with 5 IU i.p of pregnant mare serum gonadotrophin (PMSG) (Folligon from Intervet. International B.V., Boxmeer-Holland) followed by 5 IU i.p. human Chorionic Gonadotrophin (hCG/ Chorulon) 48 hours later. Six hours later, mice were subjected to 5 Gy whole-body X-ray irradiation from the X-Rad 320 Biological Irradiator calibrated at 110 rads/min (1.1 Gy equivalent) by exposing mice for 4.5 minutes and the tissues were harvested 6h post-irradiation. Mrs. Debbie Howard at JCSMR performed the irradiation of the mice.

The two pairs of fourth mammary glands were removed from each mouse and the lymph node of each mammary gland was removed prior to snap freezing in liquid

nitrogen. RNA extraction and cDNA synthesis from mammary glands and lymph nodes excised from mammary glands were carried out as described in section 2.4. Four biological replicates were analyzed for each genotype/treatment combination.

The genotype/treatment combinations are:

Treatment	Genotype	
	Control	SM09
	Basal	✓
Treated (HCG_xrt)	✓	✓

2.5.2 Microarray hybridization

The samples were hybridized to Affymetrix MoGene-1_1-st-v1 microarrays at the Ramaciotti Centre for Genomics (<http://www.ramaciotti.unsw.edu.au/microarray.html>). All samples were processed and hybridized in a single batch on an array of 24 chips. Sixteen samples of mammary glands and eight samples of lymph nodes (basal group only) were analysed, however only the data from mammary glands were analysed in this thesis.

The quality of each total RNA sample was verified with an Agilent Bioanalyzer 2100 and the Agilent Eukaryotic Total RNA Nano assay kit (Agilent Technologies). For each sample, biotinylated copy RNA was prepared according to the standard Affymetrix single-amplification protocol from 5 µg of total RNA (Expression Analysis Technical Manual; Affymetrix).

2.5.3 Data analysis

Following microarray hybridizations, CEL files were imported into Partek Genomics Suite version 6.3 (Partek, St Louis, Missouri, USA) using the following parameters: Probes to Import: Interrogating Probes and Control Probes; Probe filtering: skip; Pre-background Adjustment: Adjust for GC Content; Background Correction: RMA Background Correction; Normalization: Quantile Normalization; Log Probes using Base: 2; Probeset Summarization: Mean (This analysis was performed by Dr Stephen Ohms at the ACRF Biomolecular Resource Facility at the ANU).

The library files used during data import were:

C:\Microarray Libraries\MoGene-1_1-st-v1.r4.pgf
C:\Microarray Libraries\MoGene-1_1-st-v1.r4.clf
C:\Microarray Libraries\MoGene-1_1-st-v1.r4.bgp
C:\Microarray Libraries\MoGene-1_1-st-v1.r4.qcc
C:\Microarray Libraries\MoGene-1_1-st-v1.r4.mps
C:\Microarray Libraries\MoGene-1_1-st-v1.na32.mm9.probeset.csv
C:\Microarray Libraries\MoGene-1_1-st-v1.na32.mm9.transcript.csv

In addition to an assessment of microarray quality at the Ramaciotti Centre, we assessed the arrays further by a Principal Components Analysis (PCA) plot and the quality-control metrics implemented in Partek GS. Based on these, the quality of all array hybridizations was assessed as acceptable. We concluded the PCA plot showed no evidence of a batch effect, because the arrays were hybridized on the same date.

The experiment was analyzed in Partek GS using a 2-way ANOVA model based on the method of moments [76]. The two ANOVA factors were Treatment (with 2 levels: Basal, HCG_xrt) and Genotype (with 2 levels: control and SM09).

The ANOVA model is:

$$Y_{ijk} = \mu + \text{Treatment}_i + \text{Genotype}_j + \text{Treatment} * \text{Genotype}_{ij} + \varepsilon_{ijk}$$

Where, Y_{ijk} represents the k^{th} observation on the i^{th} Treatment for the j^{th} Genotype.

μ is the common effect for the whole experiment.

ε_{ijk} represents the random error present in the k^{th} observation on the i^{th} Treatment at the j^{th} Genotype. The errors ε_{ijk} are assumed to be normally and independently distributed with mean 0 and standard deviation δ for all measurements.

The following contrasts [77] were performed:

HCG_xrt vs. Basal

SM09 vs. Control

HCG_xrt * SM09 vs. HCG_xrt * Control

Basal * SM09 vs. Basal * Control

Differentially expressed genes were selected using the following criteria; FDR=0.05, fold change = greater than or equal to +1.5 or less than or equal to -1.5.

Two lists of differentially expressed genes (in the basal and treated states) were uploaded to the DAVID v6.7 Functional Annotation web server (Database for Annotation, Visualization and Integrated Discovery (<http://david.abcc.ncifcrf.gov/>)) to identify enriched functional annotation terms. Enriched annotation terms were identified using two different utilities available in DAVID; the functional annotation chart and functional annotation clustering. This process was carried out separately for the genes differentially expressed in the basal and treated states using the default DAVID settings apart from the stringency filter being increased to high.

Kegg pathway (Kyoto Encyclopaedia of Genes and Genomes) was also used as another annotation method to identify the signaling pathways enriched in the differentially expressed genes (<http://www.genome.jp/kegg/pathway>). Kegg pathway is a collection of manually drawn pathway maps representing the molecular interaction and reaction networks for metabolism, genetic and environmental information processing, cellular processes and human diseases.

2.6 Measurement of systemic markers of the vitamin D pathway

2.6.1 Measurement of plasma 25(OH)D levels

Five females and five males each of SM09 and control mice at 3, 10, 12 and 16 months of age were used. About 200ul of blood was collected into vials containing sodium EDTA by retro orbital bleeding by Ms. Jean Cappello or by animal technician staff of the Australian Phenomics Facility (APF). After centrifugation, the supernatant (plasma) was isolated and stored protected from light at -80°C. For the assay in chapter 5.3.2, frozen plasma samples were sent to RMIT Drug Discovery Technologies (RDDT), Melbourne, Australia for the analysis of vitamin D metabolites [25(OH)D₂ and 25(OH)D₃] by LC-MS/MS. The assay described in chapter 5.3.7.1, was carried out at University of Western Australia using ultra performance liquid chromatography (UPLC) separation coupled tandem mass spectrometry (MS/MS) detection [78] by Asst/Prof Michael Clarke.

2.6.2 Measurement of serum calcium and phosphate levels

Calcium and phosphate levels in the serum collected from mice were measured at Canberra National Hospital. The serum albumin was also measured to calculate the corrected calcium concentration using the formula:

$$\text{Corrected [Ca]} = \text{Measured [Ca]} + [(40 - \text{albumin}) \times 0.02]$$

2.6.3 Measurement of plasma parathyroid hormone level

Sodium EDTA plasma was collected from the orbital vein for parathyroid hormone (PTH) level determination. Intact immunoreactive PTH was measured in duplicate using ELISA (Immutopics Inc., San Clemente, California, USA), which uses two affinity-purified polyclonal antibodies raised to peptides common to mouse PTH. The first antibody recognizes epitopes within the midregion/carboxy-terminal portion of the peptide (region 39–84), while the second antibody, conjugated to horseradish peroxidase for detection, recognizes epitopes in the amino-terminal region (region 1–34). Statistical analysis was performed using Student's t test, and P values less than 0.05 were accepted as significant.

2.7 Western blotting

Protein was extracted from tissues using TPER extraction buffer (Thermo Scientific, catalogue # 78510). Protease inhibitors (complete, Mini, EDTA-free, Roche, catalogue # 04693159001) were used in all the samples in a concentration of 1 tablet per 10 ml of the buffer. Total protein was estimated using BCA protein assay kit (Thermoscientific, catalogue # 23225). Equal amounts of protein (50ug) were loaded on SDS-PAGE and separated by electrophoresis. Proteins were transferred to PVDF membrane. Membrane was blocked with 5% BSA and incubated with primary antibody. Non-specific Anti- β -actin loading control (Abcam, catalogue # Ab8227) was used as a loading control. For detection of protein, anti-CYP2R1 antibody (Abcam, catalogue # ab79924) was used at a 1:50 dilution, while anti-rabbit IgG, HRP-linked antibody (Cell Signaling, catalogue # 7074) was used as secondary antibody. Image was taken with FujifilmFLA/LAS using the ECL detection reagent. The relative OD obtained using the test antibody was divided by the relative OD of β -actin to normalize for variations in sample loading and transfer.

2.8 Rodent diets

Apart from the regular breeders chow (Gordon’s Specialty Feeds, Bargo, NSW), two other customized diets were used in some experiments. Those two diets were purchased from Specialty Feeds (Glen Forrest, WA) and are semi-pure diets which are SPF05-34 supplemented with 2200 IU/kg vitamin D₃ and 1% Ca²⁺ and SPF05-33 supplemented with 2% Ca²⁺ and no added vitamin D. The composition of all the diets used, including the regular diet is given in the table below.

Component	SPF05-34 High vitamin D	SPF05-33 Low vitamin D	Regular breeders diet
Vitamin D	2200 IU/Kg	None added	200 IU/Kg
Calcium	1.00%	2.00%	1.01%
Phosphorous	0.70%	1.20%	0.77%
Magnesium	0.20%	0.20%	0.18%

Table 2.3: The dietary composition of different diets used

Chapter 3 – The Effect of *SuprMam* Loci on the Normal Mammary Gland Morphology, Mammary Tumour Growth and *Dmbt1* Expression

3.1 Introduction

This chapter examines the effect of *SuprMam* loci on mammary tumour susceptibility in SM09 mice in three different aspects.

- A. The effect of *SuprMam* loci on normal mammary gland morphology.
- B. The effect of *SuprMam* loci on tumour growth.
- C. The effect of *SuprMam* loci on *Dmbt1* expression.

In order to explore the effect of *SuprMam* loci on the mammary tumour susceptibility of SM09 mice, it is important to redefine the BALB/c breakpoints on chromosome 7 and chromosome 2. The previous work at our lab defined the *SuprMam1* region on SM09 mice using several micro satellite and SNP markers, ranging from 119.4 to 135.7 Mb and *SuprMam2* region on chromosome 2 ranging from 121.1 to 129.2 Mb (Ensemble Mouse Genome Build 37) (unpublished data). We used Amplifluor SNPs HT Genotyping as well as exome sequencing (both of which were carried out at the Australian Phenomics Facility) to identify the BALB/c break points and redefined the *SuprMam1* locus on chromosome 7 (119.4 to 141.2 Mb) and *SuprMam2* locus on chromosome 2 (121.1 to 138.4 Mb) that are present in SM09 mice (figure 3.1).

3.1.1 The effect of *SuprMam* loci on mammary gland morphology

Most of the organs of vertebrates are usually patterned during embryogenesis and then maintain their basic structure throughout adult life. The breast tissue is distinct in that it continually undergoes dramatic changes in size, shape and function throughout the lifetime of reproductively active females in association with puberty, pregnancy and lactation. It is also the origin of the most common malignancy in women. The risk of developing breast cancer has been linked to both endogenous and exogenous factors [5].

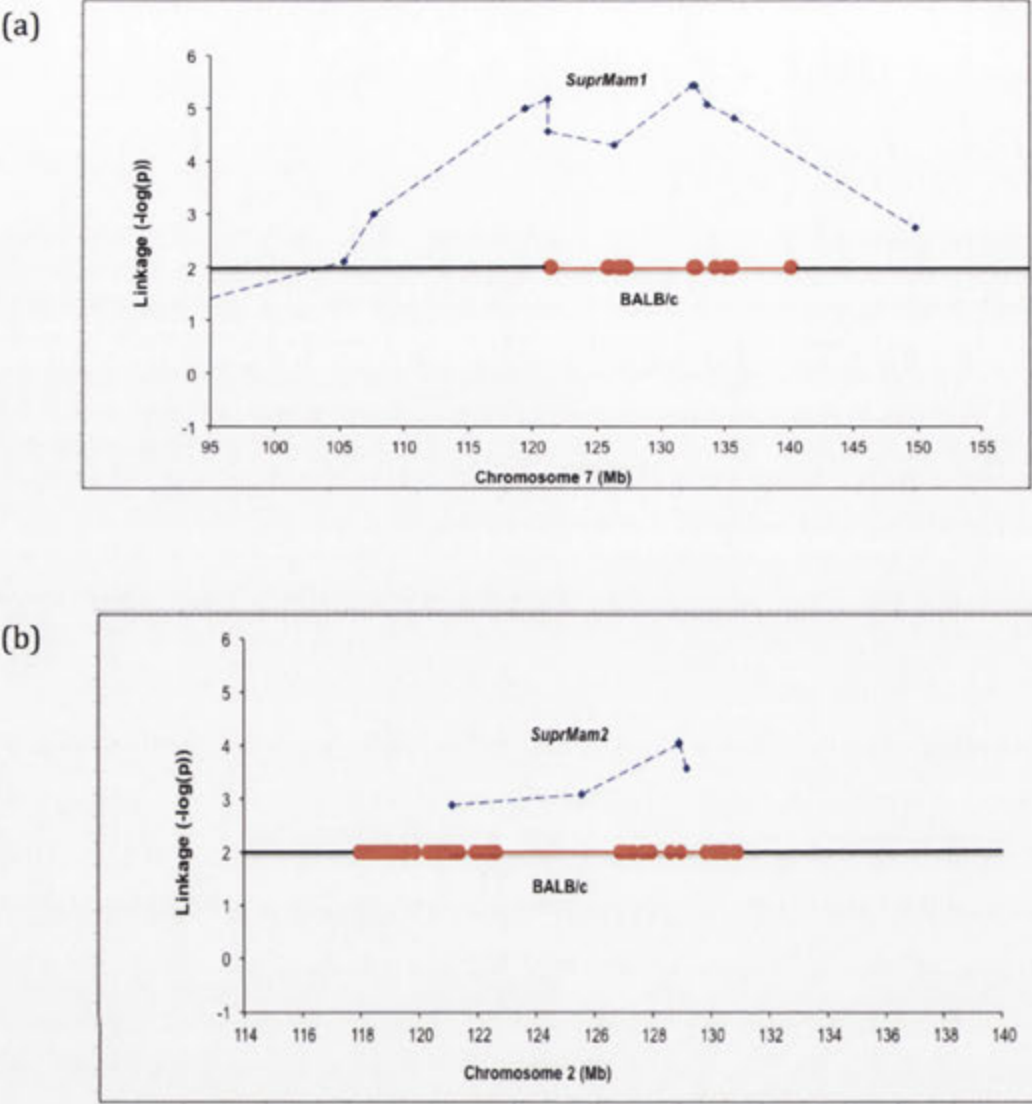


Figure 3.1: Mapping of *SuprMam* loci in SM09 mice. (a) *SuprMam1* region on chromosome 7 ranging from 119.4 to 141.2 Mb. (b) *SuprMam2* region on chromosome 2 ranging from 121.2 to 138.4 Mb. The red dots denote the BALB/c markers, while the blue line indicates the linkage analysis results. Linkage analysis results from Blackburn *et al*, 2007 [7] (All positions are according to the Ensemble Mouse Genome Build 37).

The molecular mechanisms underlying the development of breast cancer are incompletely understood. Increasing evidence points to developmental differences in the breast that may influence the risk of developing cancer. During its normal developmental cycle, the mammary gland exhibits many properties, which are also associated with breast cancer development. Thus, an understanding of normal mammary gland development and morphology, and the biochemical factors that influence them is important for the better understanding of tumour development and progression.

3.1.1.1 Anatomy

The mammary gland of the mouse is the most widely studied as a model for normal mammary gland development, and even though it does not fully represent the mammary glands of all species, particularly those of humans, it has provided many insights into mammary gland biology. In the mouse there are five pairs of mammary fat pads located just below the skin, which extend from the thoracic (three pairs) to the inguinal (two pairs) regions of the animal (figure 3.2). Each fat pad has an exterior nipple to which the primary epithelial duct is connected to allow the release of milk during lactation. A lymph node is located in each of the fat pads and is often used as a landmark when examining histological sections or wholemounts. There is a gradient of differentiation among the glands, with the first thoracic gland being the least differentiated and the fifth inguinal gland the most differentiated [79].

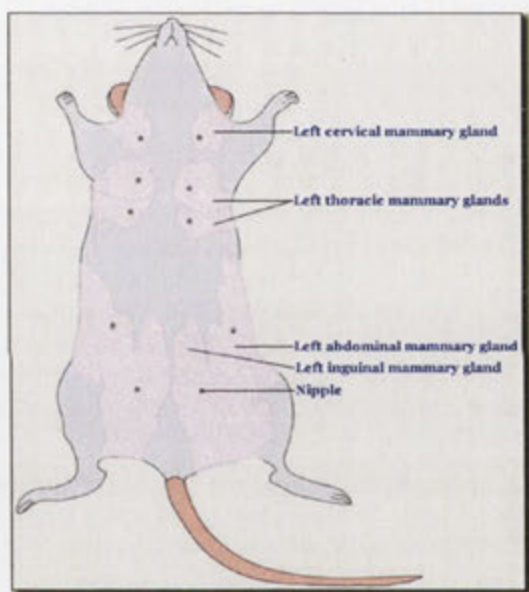


Figure 3.2: The positioning of the five pairs of mammary fat pads in mice. Copied from the Atlas of Laboratory Mouse histology (<http://ctr.genpath.net/static/atlas/mousehistology/>)

3.1.1.2 Normal mammary gland development

In all mammals the mammary gland develops from a localized thickening of the ectoderm or epidermis. The mammary bud forms by the elevation of an epidermal “mammary crest” and a milk-line that forms on both sides of the mid ventral line in the embryo [80].

The mammary gland comprises stromal and epithelial cells that interact with each other through the extra cellular matrix, which is important for the proper patterning and functioning of the normal mammary gland [5]. The interaction between the epithelium and the mesenchyme specifies the mammary bud at mid-gestation (around embryonic day 10) which then invades from the nipple into the mammary fat pad and forms a small, branched ductal network in the proximal corner of the fat pad [81]. After birth, the epithelium grows along with the mouse, but does not begin to fill the fat pad until puberty. The ductal elongation and branching continues after puberty until the mammary fat pad is filled by approximately 9-12 weeks of age, depending on the strain [5]. This expansion occurs with the release of ovarian hormones, which induces the swelling of distal ends of the mammary ducts into bulbous structures called terminal end buds (TEBs). The TEBs are the invading fronts of the ducts that proliferate, extend into the fat pad, and branch by bifurcation until the ducts reach the limits of the fat pad, upon which the TEBs start to regress (figure 3.3) [79].

During pregnancy and lactation, the reproductive hormones stimulate the expansion and terminal differentiation of the mammary epithelium into secretory, milk-producing, lobular alveoli. Therefore, the development of the mammary gland is complete only when pregnancy and lactation occur. When the period of lactation finishes, the mammary gland undergoes the process of involution, where it is remodeled back to that of an adult nulliparous mouse as a result of apoptosis in the secretory epithelium [5]. However, even though the structure may look the same at this stage, breast cancer risk is found to be lower in parous mice than in nulliparous mice, which is also found to be true for women [82]. This difference in the risk is due to the complete development of the mammary gland achieved during pregnancy and lactation, which is manifested by the differentiation of the terminal end buds into secretory units, which have a smaller proliferative compartment due to their longer cell cycle as well as the possession of more efficient DNA excision repair capacity [83]. In addition to morphological differentiation, pregnancy causes permanent changes in the epithelial cells and stroma of the mammary gland [84]. Differential expression of growth regulatory genes such as amphiregulin, insulin-like growth factor-1 and transforming growth factor- β -3 has been reported in parous mammary tissue and may limit carcinogenesis in the mammary epithelium [85].

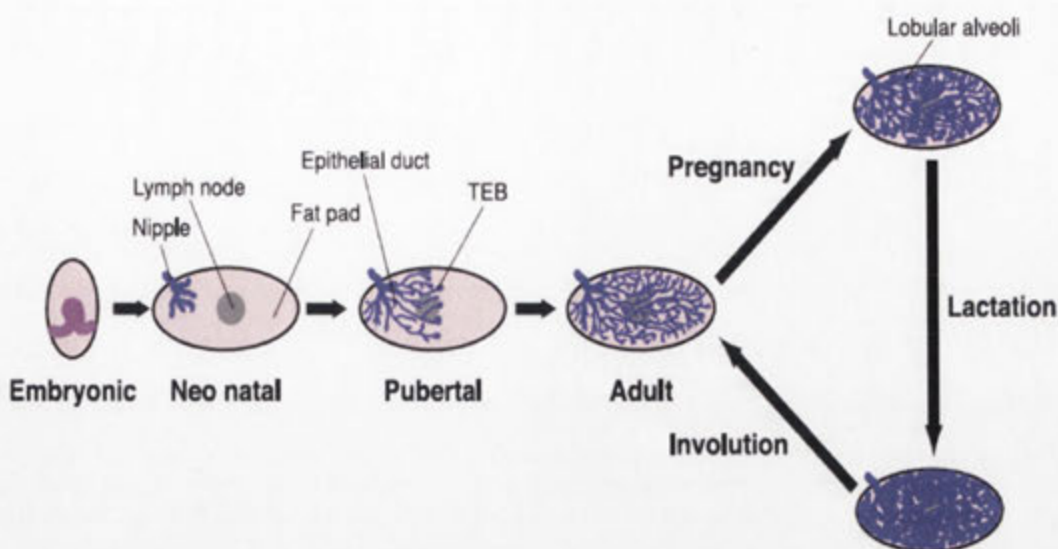


Figure 3.3: Stages of mouse mammary gland development (Copied from Wiseman *et al*, (2002)) [5]. The mouse mammary gland is specified at embryonic day 10 and invades the fat pad to form a ductal network. After puberty, TEBs (Terminal End Buds) form and ducts invade filling the fat pad. During pregnancy the epithelium becomes secretory and expand to fill the mammary gland, which then dies by apoptosis upon involution.

3.1.1.3 Hormonal influences on mammary gland morphology

Normal mammary growth, differentiation and regression are the result of complex interactions between systemic hormones and local cell-cell interactions, which are mediated by different growth factors. The morphologic response of the mammary gland to these complex interactions results in developmental changes that permanently modify both its architecture and biological characteristics [86]. This development includes multiple stages of proliferation and morphogenesis that are largely directed by concurrent alterations in key hormones and growth factors across various reproductive states [80]. The ductal elongation is directed by estrogen, growth hormone, insulin-like growth factor-1 and epidermal growth factor, where as ductal branching and alveolar budding is influenced by additional factors such as progesterone, prolactin and thyroid hormone [80].

The mouse has a 4-5 day estrous cycle that is divided into proestrus, estrus, metestrus and diestrus. The ovarian hormones estrogen and progesterone fluctuate during each estrous cycle and are critical for complete ductal development. The level of estrogen

peaks at estrus and progesterone levels peak at diestrus and the timing of these different stages is extremely strain dependent [87].

Cole *et al* [88] was the first to identify morphological changes within the mammary glands of mice during the estrous cycle. They showed that the maximal TEB formation occurs during proestrus while ductal extension and dilation occurs during estrus and an open network of thin, branched ducts is characteristic of diestrus. Andres and Strange [89] also found that the proliferation of mouse mammary epithelial cells was highest during late proestrus and estrus and minimal during diestrus. In contrast, a recent study by Fata *et al.* in mature (12- to 14-week-old) mice demonstrated increased alveolar development during diestrus without changes in ductal proliferation during the estrous cycle [87]. Studies of Hovey *et al* concur with those of both Cole and Fata *et al* in that maximal TEB development occurs in estrus where as alveolar budding was maximal at diestrus [80]. However, all these studies confirm that the mouse mammary gland morphology does change with the changing hormone levels during different stages of the estrous cycle.

3.1.1.4 Strain differences in mammary gland development

Breast cancer manifests itself in the mammary epithelium. Therefore the properties of the mammary epithelium and its interaction with stromal cells play an important role in tumorigenesis. These interactions are crucial for the proper patterning and functioning of mammary gland and disruption of the interaction between the mammary epithelium and stroma can both induce and promote breast cancer [5].

The normal mammary gland development displays many of the properties associated with tumour development such as invasion, re-initiation of cell proliferation, resistance to apoptosis and angiogenesis. For example, TEBs can be compared to a solid tumour as it is comprised of a rapidly proliferating mass of epithelial cells that invades into the stromal tissue. The mammary gland continues to grow throughout the lifetime of the animal and therefore it retains the ability to initiate proliferation, a property that can also be related to tumour growth. On the other hand, the mammary gland possesses an inherent mechanism to resist apoptotic signals in order to protect the lactating mammary gland from premature involution [5]. One of the other properties in common between normal mammary gland development and tumorigenesis is angiogenic remodeling.

Several features of this process that could possibly contribute to the tumorigenic process differ between C57BL/6 and BALB/c mouse strains. The amount of epithelium present, the extent of ductal branching, and the expression of hormonal receptors have all been reported to be greater in BALB/c than in C57BL/6 mammary glands [90]. Involution of the mammary gland progresses more rapidly in C57BL/6 mice [91] suggesting that apoptotic responses in the BALB/c mammary gland may be attenuated. We examined whether these differences correlate with the BALB/c *SuprMam* alleles.

3.1.2 Determination of the effects of *SuprMam* loci on tumour growth

The two *SuprMam* susceptibility loci were initially identified in BALB/c *Trp53*^{+/-} mouse model of spontaneous breast cancer [7], and therefore it is important to determine the tumorigenic strength of the *SuprMam* loci in the absence of other BALB/c alleles. Ideally this would be done in *Trp53*^{+/-}-SM09 mice, mimicking the original model in which these loci were identified. However, the generation of *Trp53*^{+/-}-SM09 mice and the assessment of the susceptibility to spontaneous mammary tumours were beyond the time frame of this project.

There are many steps in the development of spontaneous mammary tumours to which the *SuprMam* alleles may be contributing. Two major processes that can be considered are the transformation of the epithelial cells, and the growth and survival of the transformed cells to form a detectable tumour. When it comes to the susceptibility studies, the assessment of the transformation would be the ideal measure. However, since that could not be achieved under the circumstances as an alternative approach, we studied the difference in tumour growth of transplanted tumour cells in SM09 and control mice.

3.1.3 Determination of the effect of *SuprMam* loci on *Dmbt1* expression

The gene *DMBT1* (Deleted in malignant brain tumors) was originally isolated based on its deletion in a medulloblastoma cell line. The main sites of *DMBT1* expression are surface epithelial cells and associated glands [92]. In most tissues, low to moderate *DMBT1* levels are expressed under normal conditions. However, a deregulation has been observed in response to various pathophysiological conditions, such as bacterial infection, inflammation, tumour-flanking tissues and carcinogen exposure [92]. *DMBT1*

is now identified as a modifier of susceptibility to mammary tumours in mice and humans and it has been shown to be down regulated in breast cancer [7, 68, 92].

3.1.3.1 DMBT1 as a mammary tumour suppressor in BALB/c mice

As an initial approach to identify the polymorphic genes within these loci that are responsible for the BALB/c susceptibility to mammary tumours, oligonucleotide microarrays were used to identify genes within *SuprMam1* that were differentially expressed in mammary glands from C57BL/6-*Trp53*^{+/-} and BALB/c-*Trp53*^{+/-} mice [7]. Five transcripts were found to be significantly different in expression between the 2 strains. *Dmbt1* emerged as a leading candidate because of its putative role as a tumour suppressor gene in epithelial cancers [93].

Using semi-quantitative rt-PCR, *Dmbt1* expression was found to be more than five-fold higher in mammary glands from C57BL/6-*Trp53*^{+/-} mice than in BALB/c-*Trp53*^{+/-} and this difference in expression is mammary gland specific, as the level of *Dmbt1* expression was similar in small intestines of both the strains [7]. Further using a semi-quantitative immunohistochemical staining score, Blackburn *et al*, (2007) demonstrated that women without breast cancer were more likely to show significant positive staining (~2-fold) in the benign glandular epithelium than women with breast cancer [7]. Therefore lower levels of DMBT1 expression in normal mammary epithelium associate with increased likelihood of breast cancer in patients and mice.

3.1.3.2 DMBT1 as a tumour suppressor in SM09 congenic mice

As the *Dmbt1* gene is located within the *SuprMam1* region (figure 3.4), the SM09 congenic mice carry the *Dmbt1* gene of BALB/c origin (7:138.2 Mb) while the control mice have the *Dmbt1* gene of C57BL/6 origin. Therefore as previously described [7], since *Dmbt1* expression is significantly down regulated in BALB/c mice compared to C57BL/6 mice, the SM09 mice are expected to exhibit a similar down regulation in expression.

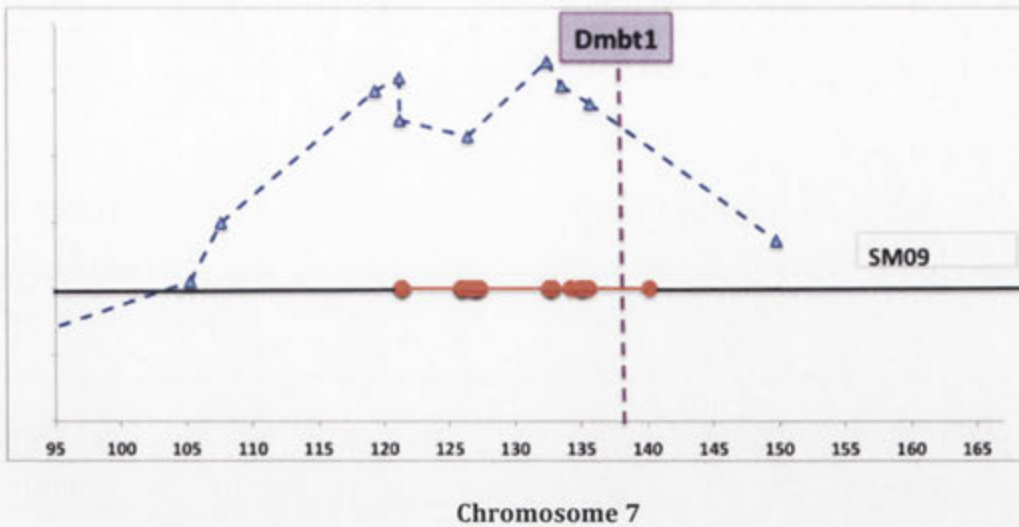


Figure 3.4: Mapping of *Dmbt1* within *SuprMam1* locus on chromosome 7. The red dots denote the BALB/c markers, while the blue line indicates the linkage analysis results. Linkage analysis results from Blackburn *et al*, 2007 [7] (All positions are according to the Ensemble Mouse Genome Build 37).

3.2 Methodology

3.2.1 Analysis of mammary gland morphology of SM09 and control mice

Mature 12 month old virgin female mice of the C57BL/6, BALB/c and SM09 congenic strain (10 mice per group) were staged twice daily for estrous by vaginal smears (2.1.3). The mice that were in estrus or diestrus stages were sacrificed immediately so that all three strains had five mice at each stage. Mammary glands were collected for wholemounts and H&E slides. The wholemounts were used to analyze the ductal branch count whereas H&E slides were used to calculate the percentage epithelial area (details in section 2.2).

3.2.2 Analysis of tumour growth in SM09 and control mice

Two C57BL/6 mammary tumour cell lines, AT3 and E0771 were cultured and prepared as described in chapter 2. Ten female mice of each SM09 and control strains were used for each cell line. The cell preparation was injected to the two 4th mammary fat pads of each mouse and the tumour appearance and growth was assessed as described in chapter 2.

3.2.3 Analysis of *Dmbt1* expression in SM09 and control mice

Total RNA was isolated from snap frozen mammary glands of SM09, BALB/c and C57BL/6 mice and the *Dmbt1* gene was amplified with two different primer pairs using semi-quantitative rt-PCR as described in chapter 2.

3.3 Results

3.3.1 BALB/c *SuprMam* alleles do not alter mammary gland morphology

3.3.1.1 Lateral branching

The number of lateral branching and alveoli present was analyzed and averaged for each mouse. Examination of the wholemounts revealed dramatically different morphologies between the parental C57BL/6 and BALB/c strains (figure 3.5), consistent with the previously published results [90, 91]. In the mammary glands of C57BL/6, there was limited lateral branching at both estrus and diestrus stages, consisting mainly of primary ducts and a very limited number of secondary ducts. In contrast, BALB/c mammary glands exhibited very high lateral branching with increased secondary branching and some tertiary branching with emanating alveolar buds. The difference in lateral branching between BALB/c and C57BL/6 strains was significant at both estrus and diestrus stages (figure 3.6.a). The extent of lateral branching in SM09 mice was unambiguously more similar to that of C57BL/6 mice than BALB/c (figure 3.5). When quantified, the number of lateral branches in SM09 mice was lower than that of C57BL/6 mice, however this difference was not significant (figure 3.6.b).

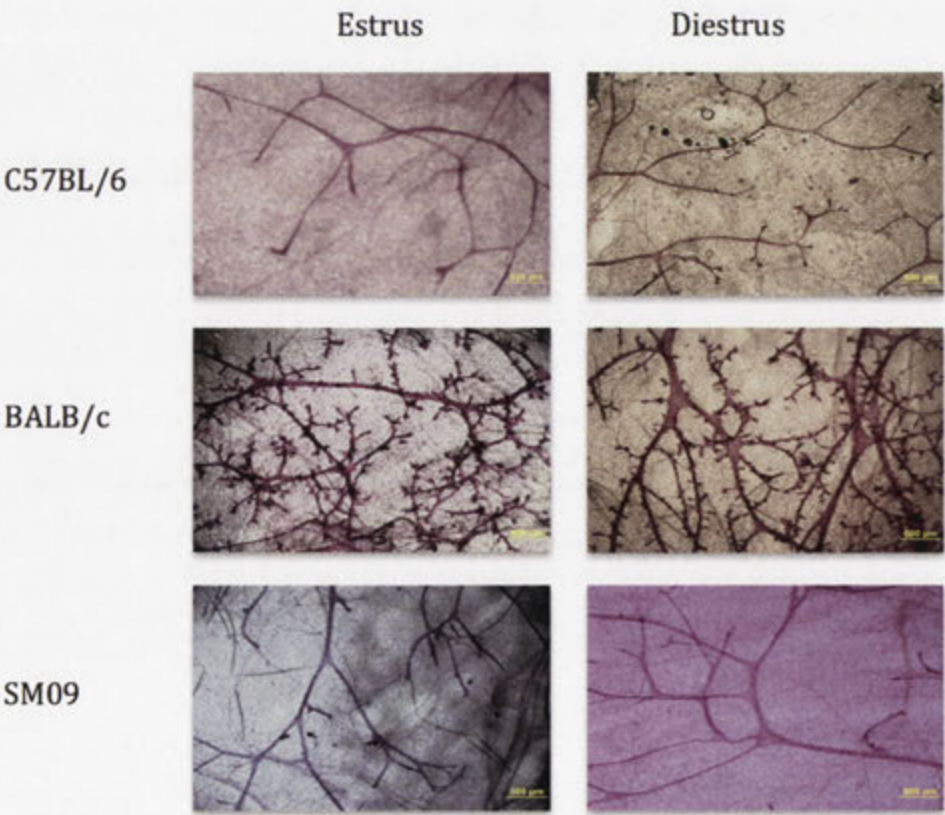


Figure 3.5: The whole mounted mammary glands of C57BL/6, BALB/c and SM09 mice demonstrating the ductal branching at estrus and diestrus stages. (Magnification - 4X under the bright field microscope)

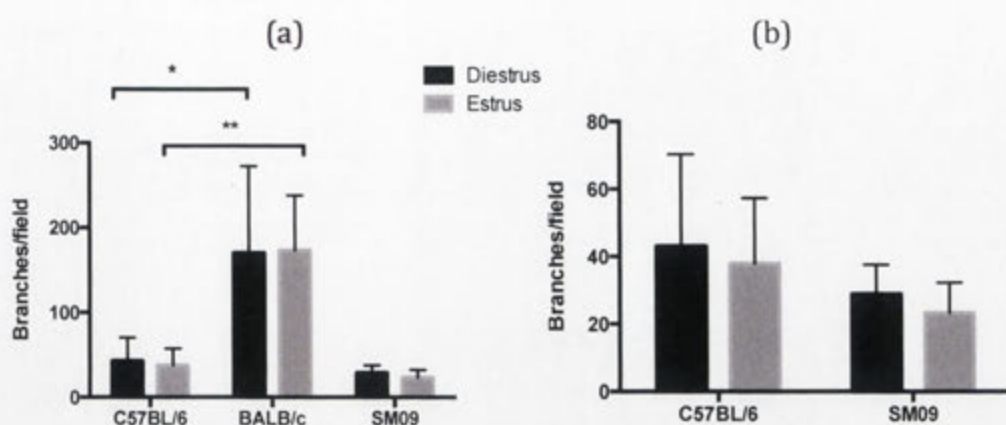


Figure 3.6: Ductal branch counting on whole mounted mammary glands at diestrus and estrus stages. (a) Comparison of C57BL/6, BALB/c and SM09 mammary glands (b) Comparison of C57BL/6 and SM09 mammary glands. Graph represents mean $\pm$ SD of 10 mice per group. * $P < 0.05$, ** $P < 0.01$ by Student's t-test.

3.3.1.2 Percentage epithelial area

The percentage epithelial area in the mammary glands from H&E slides was calculated using the ImageJ software. Confirming the previously published data [90] a clear difference in epithelial area was observed between BALB/c and C57BL/6 mice (figure 3.7). The mammary glands of BALB/c mice exhibited a higher percentage epithelial area compared to C57BL/6 mice at both estrus and diestrus stages. However, this difference was significant only in the mammary glands collected at diestrus stage (figure 3.8.a). The percentage epithelial area in SM09 mice was similar to that of C57BL/6 mice at both estrus and diestrus stages (figure 3.8.b). No significant difference was observed between the estrus and diestrus stages in any of the strains.

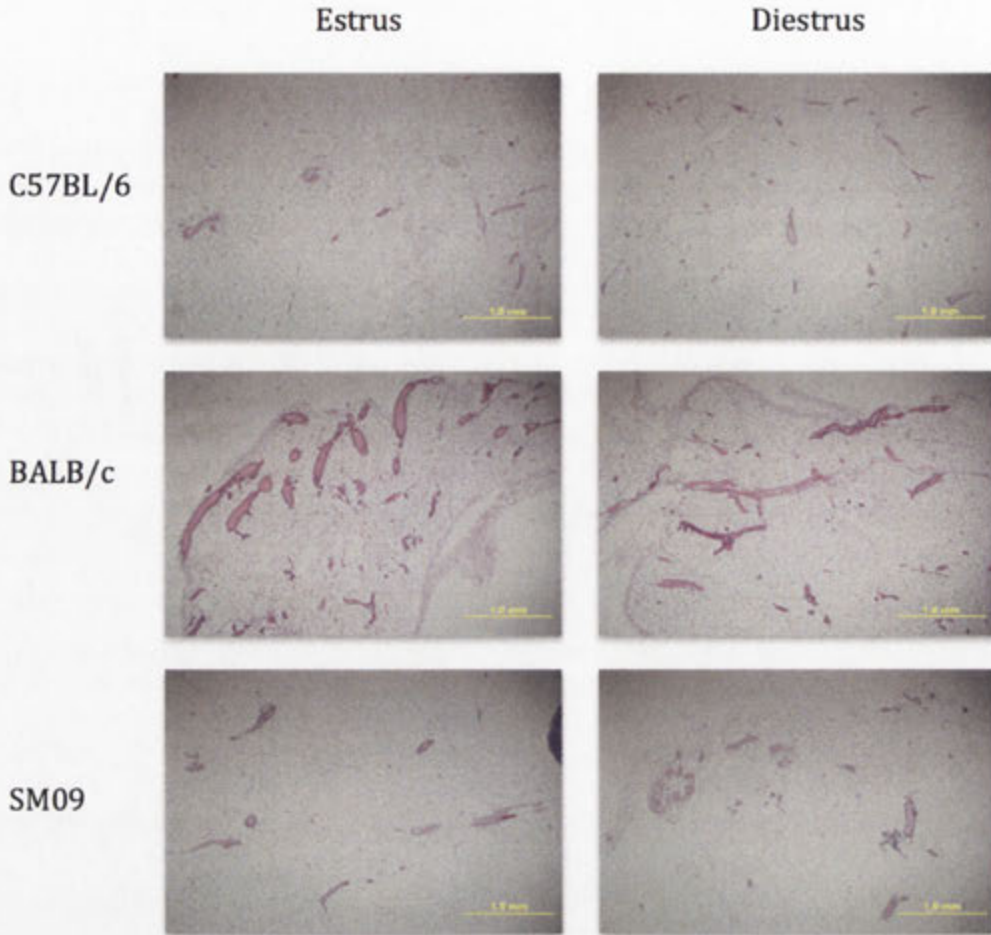


Figure 3.7: H & E slides of mammary glands from C57BL/6, BALB/c and SM09 mice at estrus and diestrus stages exhibiting the total epithelial area. (Magnification – 4X bright field microscope)

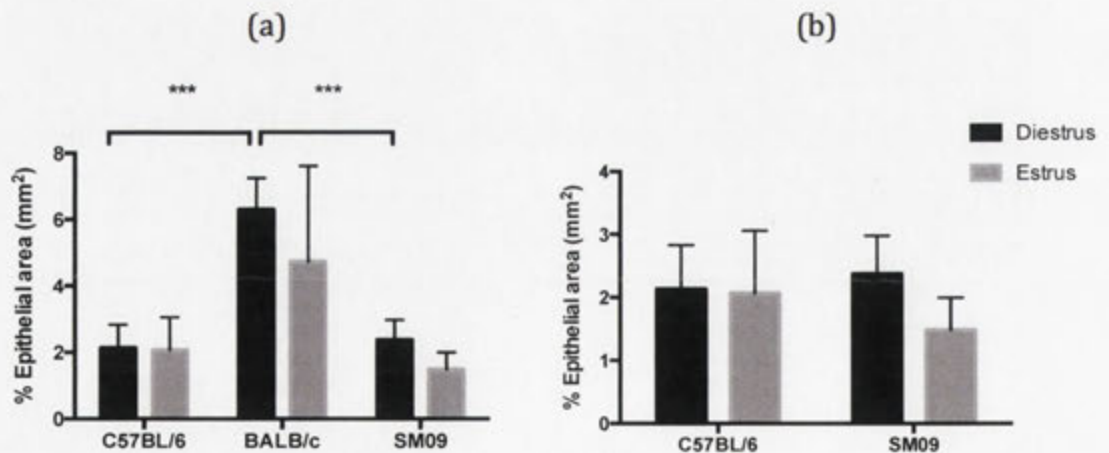


Figure 3.8: The percentage epithelial area in the mammary glands at estrus and diestrus stages. (A) Comparison of C57BL/6, BALB/c and SM09 mammary glands. (B) Comparison of C57BL/6 and SM09 mammary glands. Graph represents mean $\pm$ SD of 10 mice per group. *** $P < 0.001$ by Student's t-test.

3.3.2 BALB/c *SuprMam* alleles do not alter mammary tumour growth rate

The growth of tumours in mice was examined following the injection of cultured E0771 and AT3 cells. The tumour size was measured every 1 to 2 days and the experimental end-point was the tumour size. Mice were considered to have lethal disease and were euthanized when a tumour reached or just exceeded 20 mm in the greatest dimension or when signs of morbidity were observed. Accordingly, mice with E0771 and AT3 tumours were euthanized on day 27 and day 39 post-injections respectively. For E0771 tumours, the sites that did not develop measurable tumours by day 14 were considered as failed injections while it was day 25 for AT3 tumours.

In the mice injected with E0771 cells, the measurable tumours were detected in more than 50% of injection sites by the day 11 post-injections [Cnt – (9/16) 56.3%, SM09 – (12/18) 66.6%]. However, it took 25 days post-injections to detect measurable tumours in at least 50% of sites with the AT3 cell line [Cnt – (12/18) 66.6%, SM09 (10/20) 50%]. The difference in tumour occurrence between the strains was not statistically significant. While the E0771 tumours showed a faster growth rate compared to the AT-3 tumours, there was no significant difference in the tumour growth between SM09 and control mice with either cell line (Figure 3.9).

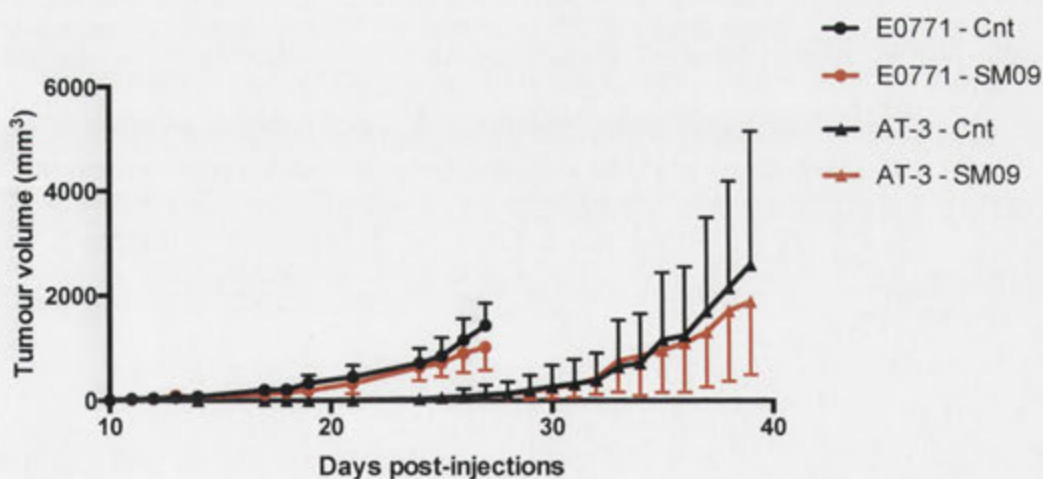


Figure 3.9: The growth of E0771 and AT-3 tumours in SM09 and control mice. Mice were injected with cultured cells [E0771 cells (2.5×10^5 cells/site), AT-3 cells (5×10^5 cells/site)] into both the 4th mammary fat pads of 10 mice per group. The tumour size was measured every 1 to 2 days. The statistical significance was calculated using Student's t-test.

3.3.3 BALB/c *SuprMam* alleles do not alter *Dmbt1* expression.

Confirming previous results, BALB/c mammary glands showed very low or undetectable amounts of *Dmbt1* transcript. This was evident at the end of 40 PCR cycles using the primer pair spanning the CUB and ZP domains of the *Dmbt1* gene. The primers specific for the transmembrane and 3' UTR regions produced similar results. The *Dmbt1* mRNA levels were comparatively high in C57BL/6 and SM09 mice and SM09 mice had similar transcript levels to that of C57BL/6 mice (figure 3.10). The semi-QPCR reaction was repeated for the same set of samples and was also performed for an independent set of mice, all of which produced similar results.

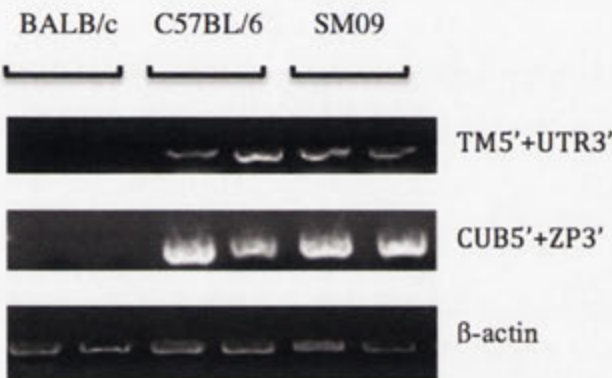


Figure 3.10: Levels of *Dmbt1* expression were compared in the mammary glands of BALB/c, C57BL/6 and SM09 mice using RT-PCR. Figure displays 2 samples representative of 5 mice analysed. An independent set of 5 mice showed similar results. *Dmbt1* transcripts were amplified using two sets of primer pairs (TM5'+UTR3' and CUB5'+ZP3'). All samples were analyzed for β -actin to ensure integrity of RNA.

3.4 Discussion

3.4.1 BALB/c *SuprMam* loci do not alter mammary gland morphology.

The mammary gland relies upon a delicate balance of endocrine signals and a proper interaction between the stromal and epithelial cells to achieve its ultimate morphology during stages from embryogenesis through to sexual maturity [80] [5]. The processes and hormones that are important for ductal growth and morphogenesis change between different developmental stages and may also be involved in mammary gland tumorigenesis. There are also strain and species differences in the profile of these hormones and processes, which may contribute to observed differences in mammary tumour development between strains. Our observations in parental strains concur with

those of Girard *et al* [90] as BALB/c mammary glands exhibited very high level of lateral branching as well as the percentage epithelial area compared to C57BL/6. As demonstrated by Fata *et al*, (2001) the progesterone level peaks at diestrus, which in turn induces maximal ductal branching and lobular-alveolar growth. However, the differences in regard to stages of estrus cycle were not consistent through the different strains.

Even though there is a significant difference in the mammary gland morphology between BALB/c and C57BL/6 mice, such difference was not observed between SM09 congenics and C57BL/6 mice. Therefore it is clear that the BALB/c *SuprMam* alleles do not contribute significantly to the morphological differences observed in the parental strains.

3.4.2 BALB/c *SuprMam* alleles do not affect the tumour growth

The injection of cultured E0771 and AT-3 cells resulted in tumours growing successfully in more than 50% of sites in both strains. The failure of cells to grow in some of the sites of mice was most likely due to technical problems such as cells leaking out of the injection site.

While the AT-3 tumours demonstrated a slower growth rate when compared to the E0771 tumours, there was no significant difference in the tumour growth between the two strains with both cell lines. This indicates that the BALB/c *SuprMam* alleles do not affect the growth rate of the tumours in SM09 mice. However, it does not exclude the potential of the BALB/c *SuprMam* alleles to increase the susceptibility to mammary tumours in the SM09 mice. In an ideal setting, this could be elucidated by generating *Trp53*^{+/-}-SM09 mice, which will allow us to compare the spontaneous tumour occurrence in SM09 mice compared to the control mice.

3.4.3 The *Dmbt1* expression is not regulated by the BALB/c *SuprMam* alleles.

The *Dmbt1* expression in SM09 mammary glands was studied as Blackburn *et al*, (2007), previously identified it as a candidate susceptibility modifier gene located in *SuprMam1*. Along with the reduced *Dmbt1* expression in BALB/c mammary glands, they also demonstrated that the homozygosity for the BALB/c allele at *SuprMam1* results in a 10-week reduction in latency and a twofold increase in the risk of

developing mammary tumours compared with heterozygotes. Therefore, this provides evidence to suggest that a decrease in levels of *Dmbt1* is sufficient to render mammary tissue susceptible to tumour development. However, as the *Dmbt1* expression is not reduced as expected in SM09 mice, who also carry the *Dmbt1* gene from BALB/c strain raises the possibility that this reduction in expression observed in BALB/c mice might not be due to the gene itself. This indicates that the lower level of *Dmbt1* expression in BALB/c mice must be due to a regulatory element located outside the *SuprMam1* region, which is not present or altered in SM09 congenic mice. The fact that the reduction in *Dmbt1* expression is specific to the mammary gland in BALB/c mice, also suggests that it is likely that the enhancers that bind tissue specific transcription factors are disrupted in the BALB/c mice [7]. Blackburn *et al*, (2007) previously sequenced 3 kb upstream of exon 1 and have found no polymorphisms between BALB/c and C57BL/6 strains. Therefore, *Dmbt1* expression might be regulated by a polymorphic regulatory factor residing at a distant site outside the *SuprMam* region.

The association in both mice and humans of lower DMBT1 expression in mammary epithelium with increased likelihood of breast cancer indicates DMBT1 is a relevant modifier of breast cancer risk. However, SM09 mice are not a suitable model to study the effect of *Dmbt1* expression on breast cancer risk, as they do not exhibit the relevant phenotype despite carrying the BALB/c alleles. As the technique applied here is only semi-quantitative, there remains the possibility that intermediate effects on expression of DMBT1 could be present in SM09 mice. This could be assessed by more quantitative techniques such as real time RT-PCR. However, the evidence presented here suggests it is unlikely that DMBT1 is a major contributor to the mammary tumour susceptibility associated with *SuprMam1* and *SuprMam2*.

3.5 Conclusion

SuprMam mice did not differ significantly from controls in mammary gland morphology, tumour growth or *Dmbt1* expression. Therefore it is unlikely that genes involved in these processes are major contributors to the mammary tumour susceptibility of BALB/c-*Trp53*^{+/-} mice. However, it cannot be excluded that more sensitive assays aimed at specific genes could reveal important differences. As the approaches employed in the chapter did not reveal any candidate genes or pathways for further investigation, a global approach was taken as described in the next chapter.

Chapter 4 – Transcriptional Profiling of the Mammary Glands

4.1 Introduction

The previous chapter studied three potential aspects of mammary tumour susceptibility phenotype, which could have been altered in SM09 mice; mammary gland morphology, tumour growth and *Dmbt1* expression. As they failed to explain the biological pathways or genes responsible for increased breast cancer susceptibility, the next step would be the transcriptional profiling, which will provide a much broader insight to the gene expression differences between the two strains.

Gene expression profiling using microarrays is a global approach that allows the investigation of the expression of over 20,000 genes simultaneously. Therefore, microarray-based gene expression profiling can be used to identify the genes whose expression is different between two different strains or in relation to a particular treatment. The application of this approach to mammary glands of SM09 mice will provide not just one candidate susceptibility gene per locus for investigation, but will also identify multiple genes including those located biologically downstream of the *SuprMam* loci and hence the biological pathways contributing to increased breast cancer risk in SM09 congenic mice. While the underlying genetic variation may not be expressed in the mammary gland, the outcome must have biological consequences in the mammary gland that will most likely alter gene expression. Thus the downstream effects should be detectable.

4.1.1 Effect of placental hormones and irradiation on Trp53 status

As explained in chapter 1, the p53 tumour suppressor protein plays an important role and breast cancer susceptibility. Therefore, activation of p53 in normal mammary glands and there by transcriptional activation of target genes will help understand the genetic differences responsible for susceptibility of the mammary glands to tumorigenesis by mimicking a cancer setting. Kuperwasser *et al*, (2000) has shown that high levels of wild-type p53 in the resting nulliparous mammary gland are inactive because of cytoplasmic sequestration and that the mechanism is conserved among humans and mice [75]. One of the simple means to activate p53 would be to irradiate mice to induce DNA damage. However, the transcriptional activation of target genes by

p53 would require p53 to be translocated and get accumulated in the nucleus after DNA damage.

Kuperwasser *et al.*, (2000) demonstrated that the administration of the placental hormones, human Chorionic Gonadotrophin (hCG) and Pregnant Mare Serum Gonadotrophin (PMSG) which induces a superovulatory state, resulted in nuclear localization of p53 after irradiation [75]. Therefore, treatment of placental hormones followed by irradiation would result in nuclear accumulation of p53 protein in mammary epithelium and transcriptional activation of target genes.

On the other hand, since the *SuprMam* loci were identified in BALB/c-*Trp53*^{+/-} model of spontaneous breast cancer [7], activation of p53 in SM09 mice would also help to reveal the gene expression differences and biological pathways regulated by p53.

4.1.2 Differential expression analysis

Microarrays can be used to identify genes whose expression levels change between two or more sample groups. From a statistical point of view, it is testing the null hypothesis that there is no differential expression for a particular gene between the experimental groups. Each hypothesis test (one for each gene) is conducted independently of the tests on each of the other genes on the microarray.

For a single gene, the risk (or probability) of rejecting the null hypothesis when it is in fact true (i.e. a type I error) is often set to a threshold of 0.05. If we are conducting a single hypothesis test, this level of risk is usually regarded as acceptable. However, if we conduct multiple hypothesis tests (one for each gene on the microarray), the problem of multiplicity of hypothesis tests arises. The larger the number of hypotheses, the more likely we are to find extreme differential expression scores, even if all the null hypotheses are true.

Dudoit *et al.*, 2002 [94], was one of the first studies to identify the importance of the multiplicity problem as a key statistical issue in microarray data analysis and various statistical strategies have been developed to overcome the problem of multiplicity. The Dunn-Sidak and the Bonferroni procedures are closely related [95, 96] and can be used to control the “experiment-wise Type I error rate (α_e), which is the probability of making a single Type I error among all the hypotheses tested.

If testing K independent hypotheses, the expected number of Type I errors is given by $K \cdot \alpha$, (e.g. if we test 20,000 genes, we would expect $0.05 \times 20,000 = 1000$ false positives). Thus the significance level of each individual test α' should be adjusted to $\alpha_c = \alpha_c / K$ where α_c is comparison-wise significance level (usually set to 0.05). Alternatively, the p-values may be adjusted to $p_B = p_c \cdot K$, where p_B is the Bonferroni corrected p-value and p_c is the unadjusted p-value.

However, the Dunn-Sidak and Bonferroni corrections are generally considered overly conservative [94]. They assume that all the hypothesis tests are independent, which may not be true in a gene expression profiling experiment. The result is a corrected p-value that may be larger than it should be. They also protect against even a single false positive, which is likely to be too strict if thousands of tests are being conducted.

To put this another way, the multiplicity problem in microarray data does not require protection against even a single type 1 error, so that controlling family-wise type 1 error rate (FEW) might not be useful, because even though this leaves out false positives it hides many true positives as well [97]. Instead it is more appropriate to emphasize the proportion of false positives among the identified differentially expressed genes.

The False Discovery Rate (FDR) is a more lenient procedure to adjust for multiple hypothesis testing. It is a compromise between the uncorrected analysis of the multiple tests and family-wise error rate. The FDR is the proportion of false positives among all positives and this was first introduced by Benjamini and Hochberg [98]. When a study involves a large number of tests, the FDR error measure is a more useful approach to determining a significance cutoff, as the FEW approach is too stringent. A typical FDR approach estimates the cutoff for the significant hypotheses so that the FDR is controlled at a desired level, for example 5% [95]. Various studies have used and discussed the FDR approach for identifying differentially expressed genes in microarray analysis [94, 99-101].

4.2 Methodology

Five female mice of each control (C57BL/6) and SM09 strains at 8-9 weeks of age in basal state and at 10-11 weeks of age in treated state (see section 2.5.1, p21 for details) were used for microarray analysis. Four biological replicates were analyzed for each

genotype/treatment combination. PCA plot was used to assess the quality of the microarray data, while Partek Genomics Suite version 6.3 was used to identify the differentially expressed genes using criteria of FDR=0.05 and fold-change ≥ 1.5 or ≤ -1.5 . DAVID v6.7 Functional Annotation web server and Kegg pathways were used to identify the biological pathways enriched in the differentially expressed gene lists. The sample preparation and data analysis is explained in detail in chapter 2.4 and 2.5 respectively.

4.3 Results

4.3.1 Hormones induce p53 activity in the mammary gland after irradiation

The functional status of p53 can be assessed by evaluating the transcriptional targets and biological responses to ionizing radiation. Induction of *p21/Cdkn1a*, a gene that is transcriptionally activated by p53 in the mammary glands of SM09 and control mice was used as a reporter for transactivation by p53. The microarray results revealed an approximate two-fold increase in the *p21* expression in mammary glands of both SM09 and control mice after the treatment (SM09; 2.2-fold $p=7.24 \times 10^{-7}$, Cnt; 2.1-fold increase $p=1.7 \times 10^{-4}$). The real-time PCR for *p21* on the mammary glands of control and SM09 mice resulted in a 17-fold increase in control mice ($p=0.0012$) and a 23-fold increase in SM09 mice ($p=0.0014$) with the treatment (figure 4.1).

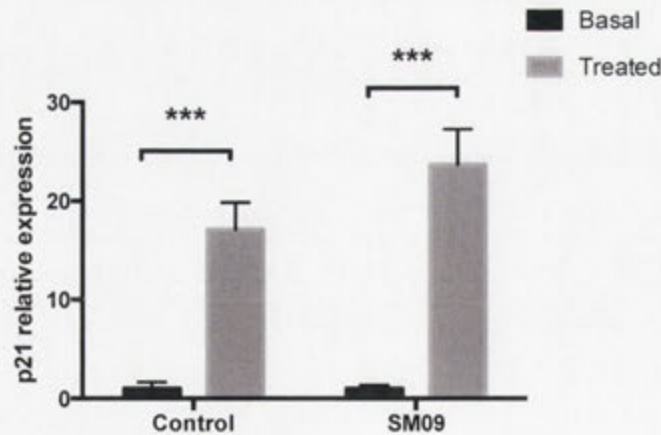


Figure 4.1: Relative expression of p21 in the mammary glands after hormone treatment followed by irradiation as determined by RT-PCR. Both control and SM09 mammary glands have increased p21 expression after treatment confirming transactivation by p53 (n=5). *** $P \leq 0.001$ by Student's t-test.

4.3.2 Principal components analysis (PCA)

Principal components analysis (PCA) is a statistical procedure that can be used in the exploratory analysis of high dimensionality data like microarray data [102]. For instance, the expression values from a set of 10 microarray samples each containing 20,000 genes can be regarded as a dataset with 20,000 data points in a space with 10 dimensions or alternatively as 10 data points in a space with 20,000 dimensions. The latter situation is of most interest in the initial stage of microarray data exploration as it provides a global or high-level view of the entire microarray experiment in a single plot. Because it is impossible to visualize data in 10 dimensions, PCA attempts to find the 3 dimensions that capture the most variability in the data and show the entire dataset in this reduced dimensional space. By doing so, it may be possible to detect the main trends in the data and to determine whether the biological signal in the data is obscured by experimental noise due to technical or other problems with the experiment [102].

The PCA plot shown below shows that the samples from the two treatments cluster separately along the second principal component. Because there were 16 microarrays in the experiment, we are looking at a representation from a 16-dimensional space. When two points are close together in the PCA plot they are similar in 16-dimensional space. Likewise, if two points are very different in 16-dimensional space, they will be far apart in the PCA-mapped visualization of the data. As shown at the top of the plot, approximately 57% of the information content in the entire 16-dimensional space could be visualized with a statistically driven 3-D visualization (which is a typical value for a microarray experiment).

The yellow ellipse denotes the envelope of the control samples while the pink ellipse denotes the envelope for the SM09 samples. Within each ellipse, the basal samples (red) tend to cluster towards the left of the plot along the first principal component (the component explaining the great percentage of variability of all the components) (figure 4.2). Our conclusion from the PCA plot is that the experiment as a whole appears to have successfully separated the effects of genotype and treatment, that the biological replicates are adequately clustered together and that there do not appear to be any major technical problems with the experiment.

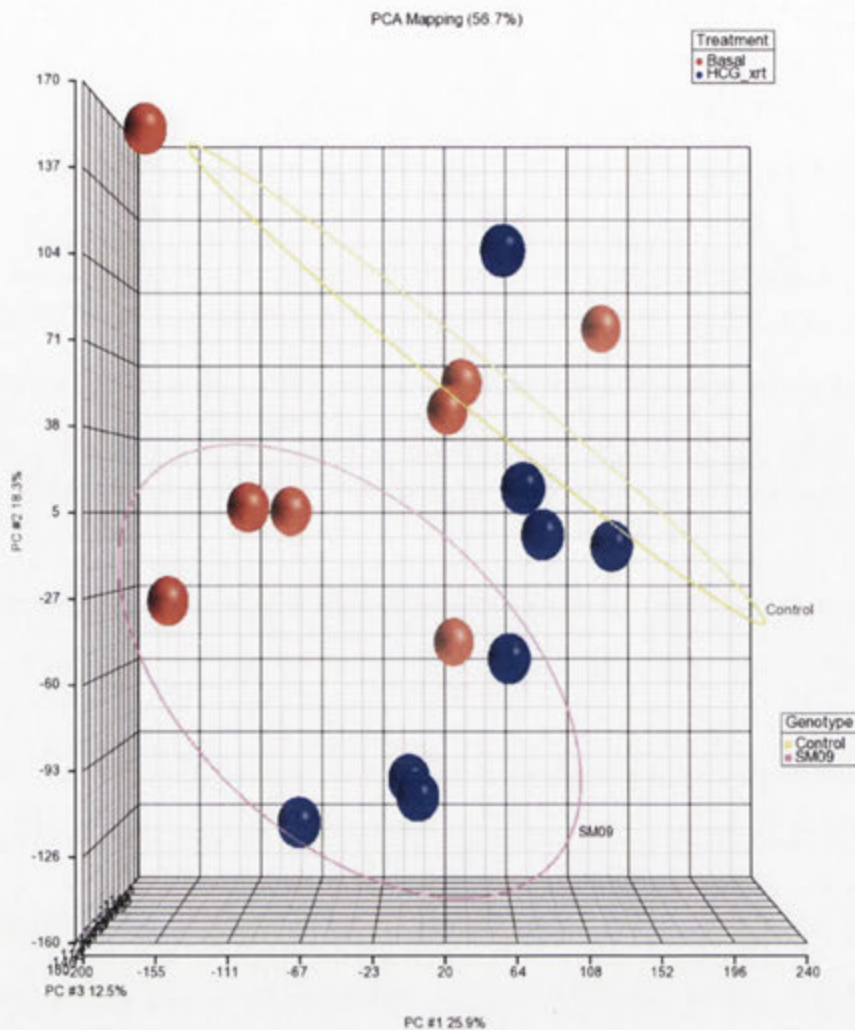


Figure 4.2: PCA plot for the gene expression analysis of mammary glands from control and SM09 mice; red circles indicate the samples at basal state (Basal) while the blue circles indicate the treated samples (HCG_xrt). The ellipses show the segregation of the two genotypes where pink is SM09 and yellow is control.

4.3.2 Identification of differentially expressed genes

Our aim was to generate lists of genes that are differentially expressed between the two strains in basal and treated states. From a statistical point of view, for each gene we are testing the null hypothesis that there is no differential expression across the sample groups. As each sample group contains four replicates, the expression level of a probe is summarized as the mean of the expression levels of the replicates.

4.3.2.1 Differentially expressed genes in the basal state

We first identified differentially expressed genes in the SM09 strain compared to controls at the basal state using the FDR threshold set to 0.05 and fold change of ≤ -1.5 or ≥ 1.5 as described in methods. In the basal state, increased expression was seen in 3

genes with the fold changes ranging from +1.53 to +4.58, while decreased expression was seen in 7 genes with the fold changes ranging from -1.52 to -2.21 (Table 4.1). Out of these 10 genes, which are differentially expressed between the two strains, nine of them (*Gchfr*, *Tgm3*, *Mertk*, *Polr1b*, *1500003O03Rik*, *Cdan1*, *Stard9*, *A430105I19Rik*, *Adam33*) are located within the *SuprMam2* locus. None were located in the *SuprMam1* locus.

Gene Symbol	Fold Change	Chr.	Gene Description	p-value
Gchfr	4.6	2	GTP cyclohydrolase I feedback regulator	6.00E-05
Tgm3	2.2	2	Transglutaminase 3, E	3.54E-05
Fabp2	1.5	3	Fatty acid binding protein 2, intestinal	1.23E-05
Mertk	-1.5	2	c-mer proto-oncogene tyrosine kinase	9.41E-06
Polr1b	-1.7	2	Polymerase (RNA) I polypeptide B	6.35E-05
1500003O03Rik	-1.7	2	RIKEN cDNA 1500003O03 gene	8.38E-05
Cdan1	-1.8	2	Congenital dyserythropoietic anemia, type 1	4.60E-05
Stard9	-1.9	2	START domain containing 9	4.65E-06
A430105I19Rik	-2.0	2	RIKEN cDNA A430105I19 gene	5.88E-05
Adam33	-2.2	2	Disintegrin and metallopeptidase domain 33	4.16E-07

Table 4.1: The differentially expressed genes in basal state of control vs SM09 mammary glands.

The genes that are in *blue* are located within the *SuprMam2* locus. (FDR=0.05, fold change= > +1.5 to < -1.5)

This analysis also revealed 13 other unannotated probe sets to be differentially expressed between control and SM09 mammary glands with the fold changes ranging from -2.5 to +2.1. However, these could not be followed up with pathway analysis due to the unavailability of annotations.

4.3.2.2 Differentially expressed genes in treated (p53 activated) state

In the treated state, twenty-nine genes were differentially expressed between the two mouse strains. Increased expression was observed for 11 genes with the fold changes ranging from +1.50 to +5.93. Eighteen genes showed decreased expression with the fold changes ranging from -1.52 to -3.45 (Table 4.2). Out of the 29 genes that are differentially expressed, 2 genes (*Cyp2r1*, *Itpr1p12*) are located within the *SuprMam1* locus while 11 genes (*Tgm3*, *Gchfr*, *Duoxa1*, *Duox1*, *Gm14085*, *1500003O03Rik*, *Stard9*, *Casc4*, *Nphp1*, *Nop56*, *Polr1b*) are located within the *SuprMam2* locus. The analysis revealed 56 annotated probe sets to be differentially expressed between the two strains during the treated state with the fold changes ranging from -3.0 to +2.3. Five genes, all of which are located within the *SuprMam* loci, (*Tgm3*, *Gchfr*, *1500003O03Rik*, *Stard9*, and *Polr1b*) are differentially expressed in both the basal and

treated states. The differential expression of *Tgm3* was greatly increased with the treatment (+2.20-fold to +5.92-fold), while the other four genes show no significant enhancement of differential expression with treatment.

Gene Symbol	Fold Change	Chr.	Gene Description	p-value
Tgm3	5.9	chr2	Transglutaminase 3, E polypeptide	6.37E-09
Gchfr	4.7	chr2	GTP cyclohydrolase I feedback regulator	5.45E-05
Gm3893	4.4	chr4	Predicted gene 3893	2.19E-05
4933409K07Rik	3.5	chr4	RIKEN cDNA 4933409K07 gene	2.28E-05
Duoxa1	3.3	chr2	Dual oxidase maturation factor 1	1.67E-06
Duox1	2.3	chr2	Dual oxidase 1	1.34E-04
Olfir243	1.7	chr7	Olfactory receptor 243	5.50E-04
Abpe	1.6	chr7	Androgen binding protein epsilon	2.30E-04
Gm14085	1.6	chr2	Predicted gene 14085	4.19E-05
Vmn1r222	1.5	chr13	Vomeranasal 1 receptor 222	2.36E-04
Mageb4	1.5	chrX	Melanoma antigen, family B, 4	9.58E-05
1500003O03Rik	-1.5	chr2	RIKEN cDNA 1500003O03 gene	6.05E-04
Btnl5	-1.5	chr17	Butyrophilin-like 5	1.27E-04
Gm9908	-1.5	chr8	Predicted gene 9908	1.72E-04
Pgf	-1.5	chr12	Placental growth factor	2.62E-04
Stard9	-1.6	chr2	START domain containing 9	1.12E-04
Zfp57	-1.6	chr17	Zinc finger protein 57	6.73E-04
Casc4	-1.6	chr2	Cancer susceptibility candidate 4	4.82E-04
Nphp1	-1.6	chr2	Nephronophthisis 1 (juvenile) homolog (human)	1.78E-07
Zfp296	-1.7	chr7	Zinc finger protein 296	1.18E-04
Gm3696	-1.7	chr14	Predicted gene 3696	4.10E-04
Naa20	-1.7	chr2	N(alpha)-acetyltransferase 20, NatB catalytic subunit	1.15E-05
Hepacam2	-1.8	chr6	HEPACAM family member 2	7.83E-06
Nop56	-1.8	chr2	NOP56 ribonucleoprotein homolog (yeast)	2.20E-04
Gdel	-1.8	chr7	Glycerophosphodiester phosphodiesterase 1	2.44E-04
Homer2	-1.9	chr7	Homer homolog 2 (Drosophila)	6.37E-04
Cyp2r1	-2.0	chr7	Cytochrome P450, family 2, subfamily r, polypeptide 1	3.06E-04
Polr1b	-2.1	chr2	Polymerase (RNA) I polypeptide B	2.00E-06
Itpril2	-3.5	chr7	Inositol 1,4,5-triphosphate receptor interacting protein-like 2	3.33E-05

Table 4.2: The differentially expressed genes in treated state of control vs SM09 mouse mammary glands. The genes that are in *blue* are located within the *SuprMam2* locus, while those that are in *red* are located within the *SuprMam1* locus. (FDR=0.05, fold change= > +1.5 to < -1.5)

4.3.3 Functional Annotation of differentially expressed genes

The majority of genes that were differentially expressed between the two mouse strains have no previously recognized role in mammary tumorigenesis. However, an understanding of their biological functions is a step to elucidating their possible direct or indirect role in mammary tumorigenesis. A commonly used approach is to map a list of differentially expressed genes to biological annotation terms to identify statistically enriched or overrepresented biological annotation terms in the list.

4.3.3.1 Enriched single GO annotation terms and Kegg pathways.

Identification of enriched gene ontology (GO) annotation terms and Kegg pathways was carried out separately for the lists of differentially expressed genes in the basal and treated states. No Kegg pathways were enriched for the differentially expressed genes in both basal and treated states.

Biological process (BP)	P Value
Nucleus organization	0.022
Cellular component organization	0.054
Post-translational protein modification	0.090
Molecular function (MF)	
Nucleoside binding	0.005
Catalytic activity	0.020
Purine nucleoside binding	0.039
Transferase activity	0.051
Purine ribonucleotide binding	0.056
Ribonucleotide binding	0.056
Purine nucleotide binding	0.063
Transferase activity, transferring phosphorus-containing groups	0.084
Nucleotide binding	0.092
Binding	0.093
Hydrolase activity	0.096

Table 4.3: Enriched GO annotation terms for differentially expressed genes in basal state

Molecular function (MF)	P Value
Nucleoside:sodium symporter activity	0.004
Nucleotide and nucleic acid transmembrane transporter activity	0.009
Nucleoside transmembrane transporter activity	0.009
Nucleoside binding	0.023
Solute:sodium symporter activity	0.050
Solute:cation symporter activity	0.078
Cellular component (CC)	
Membrane	0.097

Table 4.4: Enriched GO annotation terms for differentially expressed genes in treated state

Enriched annotation terms for each of the three biological domains of the GO annotation- biological process (BP), molecular function (MF) and cellular component (CC), were identified in the differentially expressed genes. Three BP, eleven MF but no CC annotation terms were enriched in the differentially expressed genes in the basal

state (Table 4.3). In the treated state, six MF annotation terms and one CC annotation term were enriched (Table 4.4).

4.3.3.2 Enriched clusters of functionally related GO annotation terms

This analysis was carried out separately for genes differentially expressed in the basal and treated states. Seven clusters of functionally related GO terms were identified as being enriched in the genes differentially expressed at the basal state, whereas five clusters were enriched in the treated state.

Annotation clusters	ES	GO Terms	Gene Names
Annotation Cluster 1	1.23	Purine nucleoside binding(MF) Ribonucleotide binding(MF) Purine ribonucleotide binding(MF) Purine nucleotide binding(MF) Nucleotide binding(MF)	Stard9 Mertk Cdan1 Tgm3
Annotation Cluster 2	0.85	Post-translational protein modification(BP) Protein modification process(BP) Biopolymer modification(BP) Cellular protein metabolic process(BP)	Mertk Cdan1 Tgm3
Annotation Cluster 3	0.79	Transferase activity(MF) Cellular component organization(BP) Cellular macromolecule metabolic process(BP) Cellular metabolic process(BP)	Mertk Cdan1 Tgm3 Polr1b
Annotation Cluster 4	0.76	ATP binding(MF) Adenyl ribonucleotide binding(MF) Adenyl nucleotide binding(MF)	Stard9 Mertk Cdan1
Annotation Cluster 5	0.56	Binding(MF) Cell part(CC) Cell(CC)	1500003O03Rik Stard9 Gchfr Mertk Cdan1 Fabp2 Adam33 Tgm3 Polr1b
Annotation Cluster 6	0.48	Metal ion binding(MF) Cation binding(MF) Ion binding(MF)	1500003O03RikA dam33 Tgm3 Polr1b
Annotation Cluster 7	0.47	Macromolecule metabolic process(BP) Primary metabolic process(BP) Metabolic process(BP)	Mertk Cdan1 Adam33 Tgm3 Polr1b

Table 4.5: Enriched clusters of functionally related GO annotation terms for differentially expressed genes in basal state. (ES denotes the enrichment score)

Annotation cluster	ES	GO Term	Gene Names
Annotation Cluster 1	0.95	Metal ion binding(MF) Cation binding(MF) Ion binding(MF)	1500003O03Rik Zfp57 Gdel Zfp296 Duox1 Cyp2r1 Tgm3 Polr1b
Annotation Cluster 2	0.21	Purine nucleoside binding(MF) Purine nucleotide binding(MF) Nucleotide binding(MF)	Stard9 Duox1 Tgm3
Annotation Cluster 3	0.09	Transport(BP) Establishment of localization(BP) Localization(BP)	Gm14085 Duoxa1
Annotation Cluster 4	0.05	Intracellular organelle(CC) Organelle(CC) Intracellular part(CC)	Stard9 Gchfr Naa20 Duoxa1 Zfp57 Homer2 Nop56 Cyp2r1 Tgm3 Polr1b, 1500003O03Rik Gdel
Annotation Cluster 5	0.004	Regulation of biological process(BP) Biological regulation(BP) Regulation of cellular process(BP)	Gchfr Pgf Zfp57 Duox1 Olfr243

Table 4.6: Enriched clusters of functionally related GO annotation terms for differentially expressed genes in treated state. (ES denotes the enrichment score).

The functional annotation clustering revealed seven and five annotation clusters in basal and treated states respectively. However, none of these clusters had an Enrichment Score (ES) >1.3 . An ES of 1.3 is equivalent to a p-value on the non-log scale of 0.05 [103]. Therefore more attention should be given to groups with scores >1.3 . However, gene groups with lower scores can be potentially interesting and should be explored as well [103].

4.4 Discussion

The analysis of the microarray data revealed differential expression of a number of genes demonstrating that the *SuprMam* loci alter the gene expression in the mammary

glands of SM09 mice. Most of the differentially expressed genes in the basal state (9 out of 10) and almost 50% in the treated state (13 out of 29) are located within the *SuprMam* loci, which further confirms the direct effect of the susceptibility locus. It is possible that these genes carry polymorphisms that cause this difference in expression, which might play a role in the increased susceptibility of SM09 congenic mice to mammary tumours. Those differentially expressed genes, which are outside the *SuprMam* loci, might be genes that are biologically downstream of the *SuprMam* loci. Therefore it is important to study the pathways related to these genes as they might possibly expose the genes within the *SuprMam* loci containing functional polymorphisms, but that have unaltered transcript levels.

Four out of five genes that are differentially expressed between the two strains during both basal and treated states and are located within the *SuprMam* loci are reported to be cancer related. **Gchfr (GTP cyclohydrolase 1 feedback regulator)** protein is found to be up regulated in a proteome profiling of a mouse model of breast cancer and also identified in proteomic studies of human breast cancer cell lines [104]. Gchfr is known to play a role in regulating phenylalanine metabolism, although the role of *Gchfr* is not well known in breast cancer. Although there is not much evidence demonstrating the relevance of *Gchfr* in breast cancer, around 4-fold up regulation of *Gchfr* in SM09 mice is consistent with the previously reported results [104].

On the other hand Kuo *et al*, (2012) reported **Polr1b (polymerase (RNA) 1 polypeptide B)** to be down-regulated (1.7-fold) in triple negative breast cancer patients, while demonstrating the involvement of *Polr1b* in the tumour necrosis factor (TNF) regulatory network [105]. This makes the downregulation of *Polr1b* in SM09 mice (-1.7-fold in Basal, 2.1-fold in treated) potentially relevant to breast cancer risk. Apart from that, **Stard9 (START domain containing 9)** was shown to associate with centrosomes and to be crucial for mitosis, while *Stard9* depleted cells display mitosis defects leading to apoptosis [106, 107]. Almost 2-fold reduction of *Stard9* expression in SM09 during the treated state indicates the possibility of leading to increased mutations in the presence of *Trp53*^{+/+}, thereby increasing cancer susceptibility in SM09 mice.

Perhaps the most interesting candidate out of the differentially expressed genes is, **Tgm3 (Transglutaminase 3)**, which is located within *SuprMam2*. *Tgm3* is up regulated by 2-fold during the basal state and is further increased up to 5.9-fold with the

treatment. It is the only gene within the list of differentially expressed genes to have such a significant change with treatment. *Tgm3* is a calcium dependent enzyme catalyzing covalent cross-linking between peptides or proteins and is important for effective epithelial barrier formation and the assembly of the cell envelope [108]. *Tgm3* has previously been identified as a candidate tumour suppressor gene in various cancers including breast cancer [109, 110], head and neck cancer [111-113], esophageal squamous cell carcinoma [108, 114] and basal cell carcinoma. However, all these studies suggest an inverse association of *Tgm3* expression and cancer, while it is up regulated in SM09 mice, hence this does not make it a solid candidate for further investigation.

While the differentially expressed genes were not enriched in any Kegg pathways, several clusters of functionally related GO annotation terms were enriched. However, none of them had an ES value greater than 1.3, which is considered significant. This can possibly be due to the fact that the gene expression differences observed here are in relation to the mammary glands. There can be other genes of relevant biological pathways that are differentially expressed in other tissues and thereby having a systemic effect on mammary tumour susceptibility. Therefore, the expression levels of those genes in mammary glands will not provide enough evidence to reveal relevant pathways.

It is important to note that a number of unannotated transcripts were differentially expressed between the two strains. These transcripts that are derived from the previously unannotated portions of the genome are known to possess some biological function instead of merely being transcriptional noise [115]. However, due to the lack of annotations they were not included in any of the functional analyses. As some of the unannotated transcripts could be functionally relevant to tumour susceptibility of SM09 mice, it is important to recover this information. One possible approach in doing that would be to blast the sequences of unannotated probes against the mouse genome. However, most of the unannotated transcripts are known to be small RNAs, which typically lack properties that allow them to be identified from sequence alone. On the other hand, gene prediction based on sequence alone, although reliable, isn't 100% accurate, especially for small proteins without homologs in sequenced genomes. The recent developments in the design of more advanced microarray platforms has addressed this issue by using high resolution tiling to ensure each unannotated transcript

is tiled with many probes to aid in identification (<http://web.stanford.edu/group/caulobacter/CauloHI1/>).

4.5 Conclusion

The transcriptional profiling on mammary glands from SM09 and control mice revealed gene expression differences related to *SuprMam* loci, confirming the possible involvement of *SuprMam* loci in increased breast cancer susceptibility of SM09 mice. This study revealed four potential candidate genes – *Tgm3*, *Gchfr*, *Polr1b*, *Stard9* - which could be playing a role in increased mammary tumour susceptibility in SM09 mice. The investigation of the biology of these genes and their variants in the mammary glands would be an important next step. However, since the transcriptional profiling on the mammary glands did not reveal relevant biological pathways for further analysis, it is important to use alternative approaches to identify the biological pathways responsible for the increased breast cancer susceptibility in SM09 mice.

Chapter 5 – Analysis of Vitamin D Pathway in Relation to Breast Cancer Susceptibility in SM09 Mice

5.1 Introduction

The previous chapter explains the identification of differential gene expression in mammary glands of SM09 and control mice through transcriptional profiling. However, the Kegg Pathway analysis did not result in the enrichment of any biological pathways. Therefore we considered alternative approaches to identify potential biological pathways related to increased breast cancer susceptibility in SM09 mice. The vitamin D pathway was identified as a potential biological pathway because:

- several major genes involved in the vitamin D pathway are located within the *SuprMam1* locus (*Cyp2r1*, *Calca*, *Calcb* and *Pth*);
- increasing scientific evidence suggests there is an association between vitamin D and breast cancer risk in humans [6, 116-119];
- experimental evidence suggests a difference in vitamin D metabolism between the two parental strains, BALB/c and C57BL/6 [120, 121].
- mRNA analysis of activated T-cell gene expression in BALB/c and C57BL/6 found *Cyp2r1* (a major vitamin D hydroxylase gene located within the *SuprMam1* locus) to be differentially expressed between the two strains (unpublished data from Rohan Williams, Molecular Systems Biology Group, JCSMR). Activated T-cells are stimulated to proliferate, and therefore may model the proliferative activity of cancer or pre-cancerous breast epithelial cells.

“Vitamin D” refers to a group of fat-soluble secosteroids. Vitamin D and its metabolites can be categorized into two families of steroids, the cholecalciferols and ergocalciferols, which differ chemically in their side chains. Cholecalciferol (Vitamin D₃) is synthesized in the skin on exposure to sunlight. UV-B light converts 7-dehydrocholesterol to pre-vitamin D₃, which then converts to vitamin D₃ upon thermal isomerization. Ergocalciferol (vitamin D₂) is synthesized by irradiation of ergosterol (pre-vitamin D₂) which is found in a variety of plants and yeasts [122]. Vitamin D can also be acquired through ingestion of foods containing vitamin D. When vitamin D and its metabolites are mentioned without a subscript, it indicates both families of vitamin D, cholecalciferols and ergocalciferols.

Apart from its main physiological role in calcium homeostasis, several studies have demonstrated a wide range of functions of vitamin D, which can potentially protect against cancer. There is strong experimental evidence showing that vitamin D participates in cell growth regulation, apoptosis and proliferation in normal and malignant breast cells [118, 123-127]. While the exact mechanism underlying the protective action of vitamin D against cancer is not clearly understood, experimental evidence showing anticarcinogenic properties of vitamin D have led to a hypothesis that low levels of vitamin D might increase risk of breast cancer.

5.1.1 Vitamin D metabolism

In order to be biologically active and affect mineral metabolism and to have effects on numerous other diverse physiological functions including inhibition of growth of cancer cells, vitamin D must be converted to its active form [122]. The first step of this activation takes place in the liver, where a hydroxyl group is added to form 25-hydroxyvitamin D or calcidiol/25(OH)D by one or more cytochrome P450 vitamin D 25-hydroxylases (CYP2R1, CYP2D11, CYP2D25, CYP27A1).

Out of these vitamin D hydroxylases, CYP2R1 is identified as a key vitamin D 25-hydroxylase in humans and mice, and is located within the mammary tumour susceptibility locus *SuprMam1* on mouse chromosome 7. It is primarily expressed in the liver [122]. According to Cheng et al, low circulating levels of 25(OH)D and classic symptoms of vitamin D deficiency were observed in an individual with a mutation in exon 2 of the *CYP2R1* gene which eliminated the vitamin D 25-hydroxylase activity of CYP2R1 [128]. In mice, *Cyp2r1* knockout mice have serum 25(OH)D levels reduced to one half of the level of wild type mice, further suggesting *Cyp2r1* is an important gene regulating serum 25(OH)D levels [129]. At the population level, Wang *et al*, (2010) demonstrated that common human variants at the *CYP2R1* locus are associated with different circulating 25(OH)D concentrations [130]. Thus, there is strong evidence indicating CYP2R1 is the enzyme underlying the crucial first step in vitamin D metabolism.

25(OH)D is further metabolized to 1,25-dihydroxyvitamin D (1,25(OH)₂D) in the kidney by 1 α -hydroxylase (CYP27B1) which is tightly regulated by parathyroid hormone (PTH), calcium and phosphate concentrations as well as 1,25(OH)₂D itself [122]. Several vitamin D target tissues such as breast, prostate and colon, also express CYP27B1, and this is now known to contribute to local production of 1,25(OH)₂D and

to tissue specific responses to vitamin D [131]. Thus, $1,25(\text{OH})_2\text{D}$ activity can be of an endocrine or auto/paracrine origin. However, the regulation of CYP27B1 in non-renal tissues generally differs from kidney such that its activity in non-renal tissues may be more dependent on the concentration of available $25(\text{OH})\text{D}$ substrate [132]. Therefore, the circulating $25(\text{OH})\text{D}$ levels determine the extent of $1,25(\text{OH})_2\text{D}$ that can be synthesized locally within tissues [131]. However, circulating $1,25(\text{OH})_2\text{D}$ produced by extra-renal tissues, breast tissue for instance, is undetectable in anephric conditions [133]. This suggests that $1,25(\text{OH})_2\text{D}$ produced in non-renal tissues is not released into the blood stream but instead acts locally by binding to vitamin D receptors (VDRs) in the same tissue.

5.1.2 Vitamin D and breast cancer

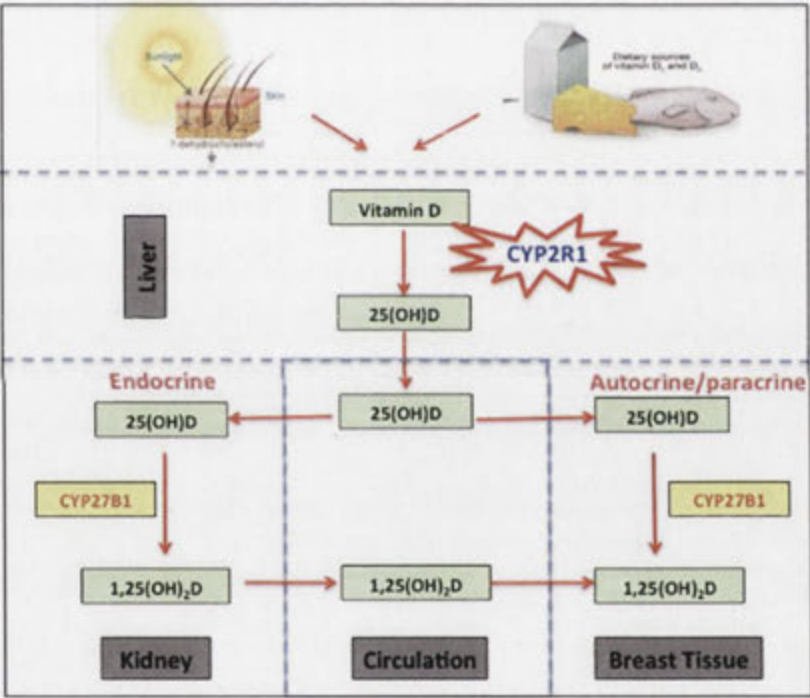


Figure 5.1: Vitamin D metabolism. Vitamin D (from diet and supplements or from synthesis in the skin) is first metabolized to $25(\text{OH})\text{D}$ in the liver and then further metabolized to $1,25(\text{OH})_2\text{D}$ by 1α -hydroxylase (Cyp27b1) in the kidneys (endocrine pathway) and breast tissue (autocrine/paracrine pathway). Adopted from Cui *et al*, (2006) [6].

Both the endocrine pathway and the autocrine/paracrine pathway of vitamin D metabolism have been proposed to be involved in breast cancer [133, 134]. In the endocrine pathway, circulating $1,25(\text{OH})_2\text{D}$ reaches the breast tissue to exert its anti-carcinogenic effect, whereas in the autocrine/paracrine pathway, circulating $25(\text{OH})\text{D}$

reaches the breast tissue and is further catalyzed to $1,25(\text{OH})_2\text{D}$ by local 1α -hydroxylase to regulate cell proliferation, differentiation and apoptosis (figure 5.1).

5.1.4 Regulators of vitamin D pathway and breast cancer risk

There is now considerable evidence from epidemiological, clinical and experimental research that suggests that vitamin D and its regulators, parathyroid hormone (PTH), calcium, phosphate and PTH-related protein, may affect breast cancer risk.

PTH is a major hormone regulating calcium homeostasis through stimulation of renal calcium absorption and bone resorption in response to low serum ionized calcium levels [135]. PTH secretion is regulated by serum ionized calcium concentration through a calcium sensing receptor (CaSR) on the surface of the parathyroid cells [136]. A decrease in serum ionized calcium of as little as 0.1mg/dL (0.025 mmol/L) results in a large increase in serum PTH concentration by deactivating the CaSR. Similarly an equally small increase in serum ionized calcium rapidly lowers the serum PTH concentration by activating the CaSR [135]. PTH also stimulates the conversion of $25(\text{OH})\text{D}$ to $1,25(\text{OH})_2\text{D}$ in the kidney by inducing Cyp27b1 enzymatic activity, thereby stimulating intestinal calcium absorption. Parathyroid cells contain VDRs, and the PTH gene contains a vitamin D response element. Binding of $1,25(\text{OH})_2\text{D}$ to the VDR causes inhibition of PTH gene expression and thereby inhibits PTH synthesis [137]. This feedback loop involving PTH, $1,25(\text{OH})_2\text{D}$ and calcium absorption is the central mechanism for maintaining calcium homeostasis.

Several epidemiological studies have demonstrated an increased risk of breast cancer associated with primary hyperparathyroidism, a condition where serum PTH levels are increased [138-141]. Experimental studies also suggest that PTH has carcinogenic and tumour-promoting effects [142, 143]. A high proportion of cancers including breast cancer, are known to synthesize PTH-related protein (PTHrP), whose N-terminal region is homologous to that of PTH, and activates the PTH receptor with equal affinity [144].

Calcium is an important intracellular messenger that is involved in processes related to proliferation, apoptosis and cell signaling [145]. Increased serum calcium levels have a tumour protective effect, through decreasing proliferation and increasing differentiation in breast cancer cell lines [6].

Given the separate effects of each of these vitamin D-related elements – 25(OH)D, 1,25(OH)₂D, PTH and calcium – but their close interconnection and interaction, it is possible that it is the interaction rather than the effect of any single factor, that is important to breast cancer risk.

5.2 Methodology

5.2.1 Quantitative real-time analysis of vitamin D pathway genes

Real-time PCR was carried out on RNA isolated from snap frozen mammary glands, liver and kidney tissues of SM09, and C57BL/6 mice with at least 4 mice per group as described in Chapter 2. *Gapdh* is used as the internal control in all reactions. The fold change was established by the $\Delta\Delta CT$ method [74] and statistical significance was determined using Student's T-test.

The circulating levels of 25(OH)D, PTH, calcium and phosphate of SM09 and control mice were measured. Details of the methods used and laboratories where each of these assays were carried out is described in Chapter 2.

5.3 Results

5.3.1 Expression profiling of the vitamin D pathway genes

Nine major genes involved in the vitamin D pathway (*Vdr*, *Cyp27a1*, *Gc*, *Calca*, *Cyp2r1*, *Cyp27b1*, *Calcb*, *Pth* and *Cyp24a1*) were analysed from the microarray experiment described in the previous chapter. Out of these, four of the genes (*Cyp2r1*, *Pth*, *Calca* and *Calcb*) are located within the *SuprMam1* locus. While most of the genes appear to be differentially expressed between the two strains in both basal and treated states as demonstrated by the heat map (figure 5.2), the absolute expression/signal intensity levels of many of them were very low (table 5.1). The expression of *Cyp2r1* in the mammary gland, which is clearly differentially expressed as represented by the heat map, was assessed by RT-PCR. Confirming the microarray results, a 2-fold reduction in expression in SM09 mice compared to control mice was observed. However, the CT values were above the accepted threshold value, and the protein in the mammary gland was not detected by western blotting despite it being easily detectable in liver tissue (see figure 5.4).

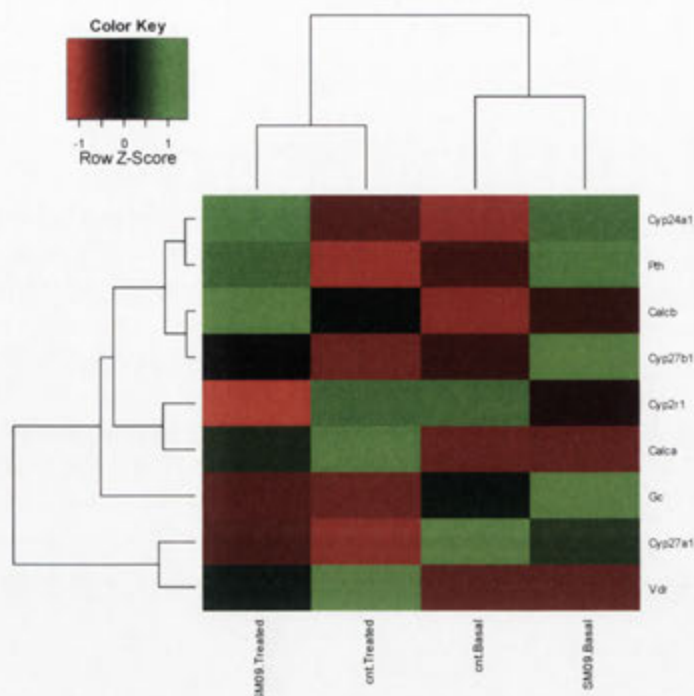


Figure 5.2: Difference in mRNA abundance in mammary glands of SM09 and control mice as determined by microarray analysis. The heat map demonstrates 9 major genes involved in vitamin D pathway at basal (untreated) state and treated (placental hormones + γ irradiation)

Gene symbol	Basal			Treated		
	Fold change	P-value	Intensity range	Fold change	P-value	Intensity range
<i>Cyp24a1</i>	+1.2	0.03	23.1-25.6	+1.2	0.05	23.5-25.8
<i>Pth</i>	+1.2	0.01	21.9-24.7	+1.3	0.004	21.1-24.3
<i>Calcb</i>	+1.1	0.58	19.1-19.5	+1.1	0.22	20.0-21.1
<i>Cyp27b1</i>	+1.1	0.27	19.3-21.4	+1.2	0.01	18.9-19.7
<i>Cyp2r1</i>	-1.5	0.01	30.7-41.2	-2.0	0.0003	27.6-39.6
<i>Calca</i>	+1.1	0.93	28.1-28.4	-1.4	0.25	36.0-42.7
<i>GC</i>	+4.0	0.04	35.7-64.7	+1.1	0.82	21.0-22.8
<i>Cyp27a1</i>	-1.1	0.30	74.8-77.8	+1.1	0.66	69.3-70.5
<i>Vdr</i>	-1.1	0.97	53.9-54.0	-1.3	0.21	58.3-63.5

Table 5.1: The intensity/ absolute expression level, fold change and p values of 9 major genes involved in vitamin D pathway from the microarray expression analysis. Note: The signal intensities ranged from 9.3 to 192 across the entire array

This suggests that the genes with expression intensities close to or below that of *Cyp2r1* (40) in the mammary glands might not be biologically important, as it is not their major site of expression. Only *Cyp27a1* and *Vdr* genes had average expression levels above 40, but both of them had no significant difference in expression between the two strains (table 5.1). However, these results may still indicate that there is a deregulation of the vitamin D pathway in SM09 mice compared to control mice, but it might be a systemic (endocrine) effect that increases the mammary tumour susceptibility rather than a local (autocrine/paracrine) effect of the vitamin D pathway.

5.3.2 No significant difference in the plasma 25(OH)D levels or liver *Cyp2r1* expression

The measurement of 25(OH)D levels is a more direct way of identifying potential differences in vitamin D-related biological functions. *Cyp2r1* is the major vitamin D hydroxylase which produces 25(OH)D. Therefore, if *Cyp2r1* is significantly downregulated in SM09 mice, it may result in reduced plasma 25(OH)D levels. Plasma 25(OH)D levels were measured in male and female SM09 and control mice at 4 different ages. There was no significant difference in the plasma 25(OH)D levels observed between SM09 and control mice at any of the ages considered (figure 5.3).

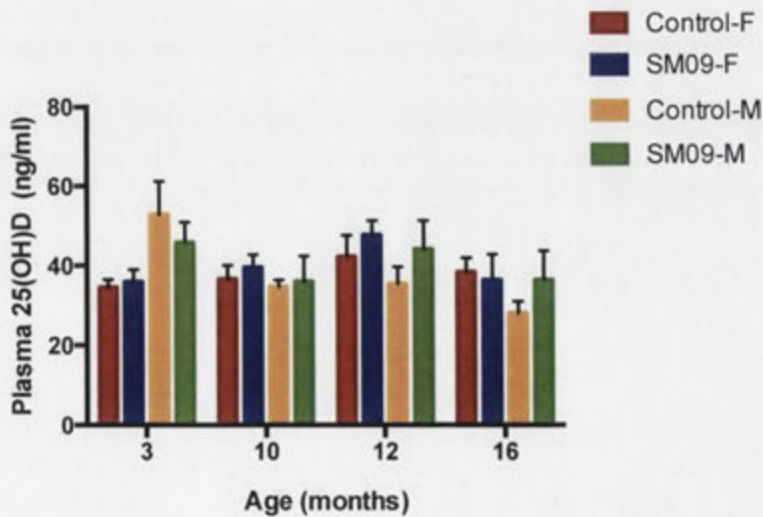


Figure 5.3: Plasma 25(OH)D levels of control and SM09 female(F) and male(M) mice at 3, 10, 12 and 16 months of age. (n=5/group)

While the microarray data showed that *Cyp2r1* was differentially expressed in the mammary gland albeit with expression at a low level, it is also important to investigate its expression in the liver, the major source of systemic 25(OH)D levels. The results demonstrated that there was no difference in *Cyp2r1*, both at the mRNA and protein

levels, between the livers of SM09 and control mice. Lack of any difference in the *Cyp2r1* expression also explains the absence of any difference in plasma 25(OH)D levels between SM09 and control mice.

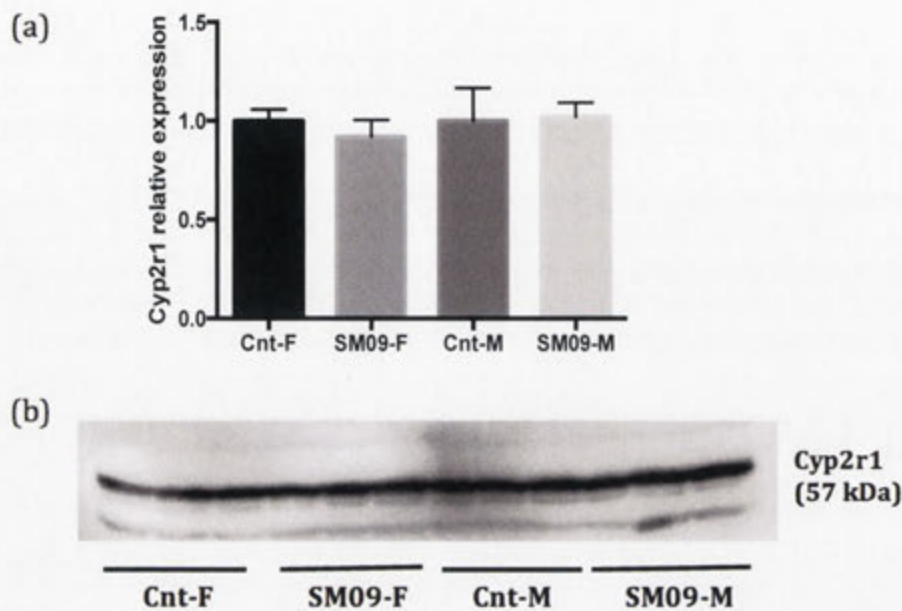


Figure 5.4: *Cyp2r1* expression in the liver of SM09 and control mice. (a) *Cyp2r1* mRNA level by real-time PCR (b) *Cyp2r1* protein level by western blotting (n=3).

5.3.4 Major regulators of vitamin D pathway

Since the vitamin D pathway is known to be tightly regulated, it is possible that any difference might be within one of the regulators or a downstream factor beyond the synthesis of the 25(OH)D. Thus calcium, phosphate and PTH levels were measured in female SM09 and control mice.

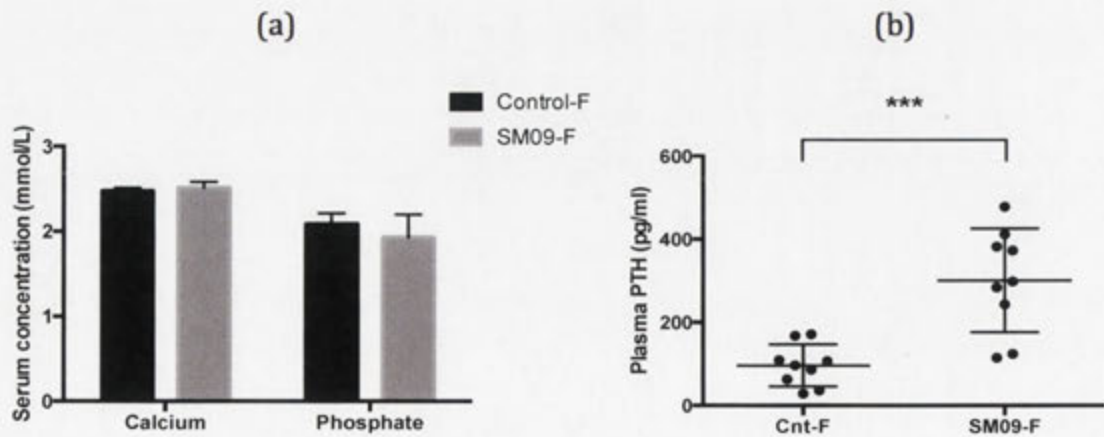


Figure 5.5: Systemic level of major regulators of vitamin D (a) Serum calcium and phosphate levels are not different between SM09 and control mice. (b) Plasma PTH levels in SM09 mice are ~3-fold higher than controls. $P^{***} \leq 0.001$ by Student's t-test.

While the calcium and phosphate levels showed no significant difference between the two strains, SM09 females had more than 3-fold higher levels of PTH compared to controls (301 ± 125 vs 96 ± 50 pg/ml, $p=0.0004$ respectively).

To confirm the association of the elevated PTH levels with the BALB/c alleles of the *SuprMam* loci, the plasma PTH levels were measured in several other mouse strains including BALB/c, F1 and two other congenic lines related to SM09 mice (SM02-pro and SM02-dist) (figure 5.7). SM02-pro and SM02-dist mice carry only the *SuprMam1* locus from BALB/c, while the SM02-pro mice carry a BALB/c region towards the proximal end of the original *SuprMam1* locus (104.5-132.6 Mb), while in SM02-dist mice it is towards the distal end (125.3-135.5 Mb) (Figure 5.6).

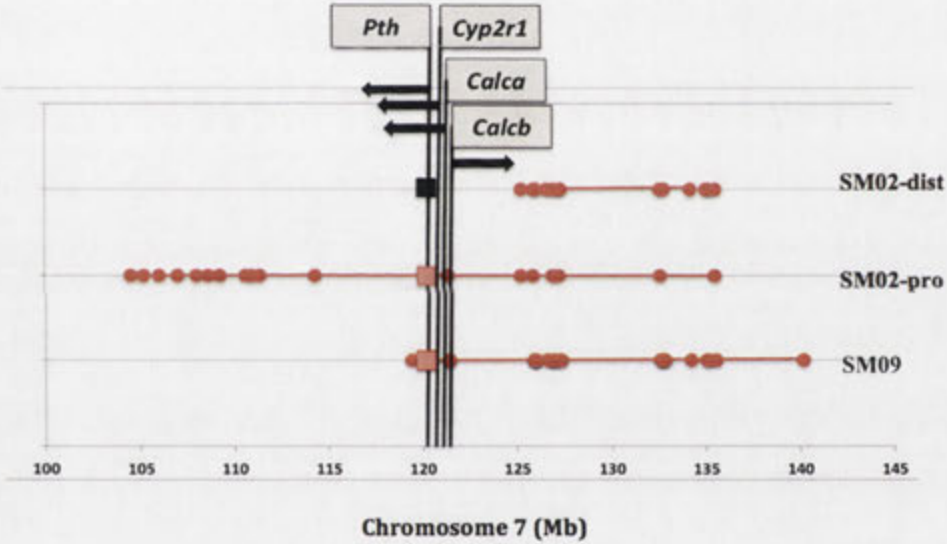


Figure 5.6: Mapping of BALB/c regions on the three congenic mouse strains, SM09, SM09-pro and SM02-dist. SM09 and SM02-pro mice carry BALB/c alleles of all four genes (*Pth*, *Cyp2r1*, *Calca* and *Calcb*) while SM09-dist mice carry alleles from C57BL/6 origin. (The red dots denote the BALB/c regions)

The female mice of SM09, SM02-pro and BALB/c strains have higher plasma PTH levels compared to control mice, while SM02-dist and F1 females have PTH levels similar to that of controls (figure 5.7). All three groups of female mice with increased plasma PTH levels carry all four genes, *Pth*, *Cyp2r1*, *Calca*, and *Calcb* genes from a BALB/c background. In male mice none of the strains had higher plasma PTH levels regardless of their genetic composition. The results confirm that the genetic component

driving the increased PTH levels in SM09 mice is associated with the *SuprMam1* locus on chromosome 7, as SM02-pro mice, which carry only the *SuprMam1* locus but not *SuprMam2*, also demonstrated the same phenotype. This suggests that there is a strong possibility that a genetic difference within *Cyp2r1*, *Pth*, *Calca* or *Calcb* genes, all of which are located within the *SuprMam1* locus, is responsible for the increased plasma PTH levels.

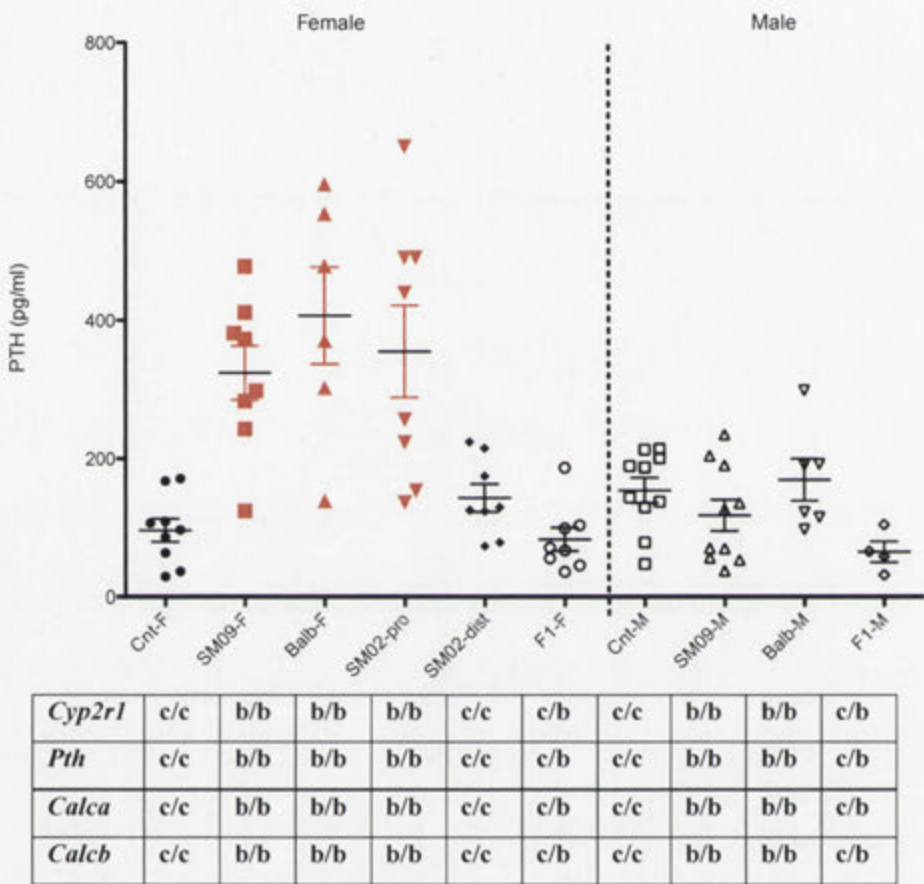


Figure 5.7: Plasma PTH levels (pg/ml) of different mouse strains. The table indicates the origin of *Cyp2r1*, *Pth*, *Calca* and *Calcb* alleles in each strain ('b' denotes BALB/c alleles while 'c' denotes C57BL/6 alleles).

5.3.5 Identification of polymorphisms in *SuprMam1* related to vitamin D pathway genes

Exome sequencing data used for detecting the congenic regions (Chapter 2) were analyzed to identify polymorphisms (Single Nucleotide Variants/SNVs, insertions and deletions) that could change protein function in major vitamin D pathway genes located in *SuprMam1* (*Cyp2r1*, *Pth*, *Calca* and *Calcb*). The results indicated that none of these genes carry polymorphisms within exons. To ensure that there are no unexpected

genetic variants present in our congenic mice, the other major vitamin D pathway genes located outside of *SuprMam1* (as per figure 5.2, section 5.3.1) were also examined. As expected, no differences were found.

To look for genetic influences on mRNA expression levels, promoters of the vitamin D pathway genes within the *SuprMam1* locus (*Cyp2r1*, *Pth*, *Calca* and *Calcb*) were analyzed for differences in transcription factor binding sites using the Genomatix MatInspector software [146]. Since the BALB/c promoter sequences were not available in Ensembl, the DBA strain, which shares haplotype structures with the BALB/c strain in this region (<http://mouse.cs.ucla.edu/>), was used for the analysis, i.e. the analysis examined differences in transcription factor binding sites between C57BL/6 and DBA strains. Analysis of the *Cyp2r1* promoter (600 bp upstream) revealed 14 transcription factor binding sites to be different between the two strains (figure 5.8). These differences in transcription factor sites were associated with seven SNPs reported on Ensembl and listed in table 5.3. (<http://www.ensembl.org/>). However, there were no SNPs present in *Pth*, *Calca* or *Calcb* promoters within 600 bp of the gene start, although some variants were present further upstream.

Although there are differences in transcription factor binding sites, these are all theoretically predicted sites and the *Cyp2r1* expression in the liver was not found to be different between the two strains. Therefore, we did not pursue functional testing of these differences.

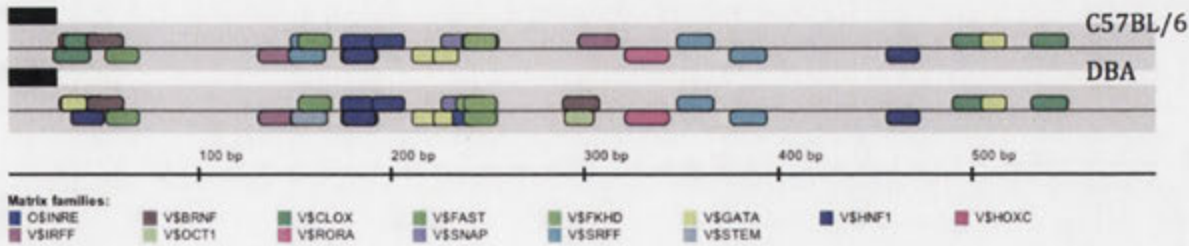


Figure 5.8: Transcription factor binding site differences in the *Cyp2r1* promoter between C57BL/6 and DBA strains. (Results output from Genomatix MatInspector software)

Matrix Family	Detailed Family Information	Binds to	
		C57BL/6	DBA
V\$CLOX	CLOX and CLOX homology (CDP) factors	✓	
V\$HOXC	HOX - PBX complexes	✓	
V\$SRFF	Serum response element binding factor	✓	
V\$SNAP	snRNA-activating protein complex	✓	
V\$IRFF	Interferon regulatory factors	✓	
V\$GATA	GATA binding factors		✓
V\$RORA	v-ERB and RAR-related orphan receptor- α		✓
V\$HNF1	Hepatic Nuclear Factor 1		✓
V\$STEM	Motif of binding sites for stem cell factors		✓
O\$INRE	Core promoter initiator elements		✓
V\$FAST	FAST-1 SMAD interacting proteins		✓
V\$FKHD	Fork head domain factors		✓
V\$OCT1	Octamer binding protein		✓
V\$BRNF	Brn POU domain factors		✓

Table 5.2: Transcription factor binding sites within the *Cyp2r1* promoter (600 bp upstream) that are different between C57BL/6 and DBA strains.

SNP	Alleles (C57BL/6 vs DBA)	Location (bp)
rs50319309	G/A	-192
rs50208777	G/T	-290
rs51368564	T/C	-306
rs49895337	T/G	-358
rs46760458	A/G	-448
rs47896208	A/G	-557
rs46376023	C/T	-562

Table 5.3: Known SNPs within the mouse *Cyp2r1* promoter recorded on Ensembl. The allele variation is C57BL/6 vs DBA, whereas the location is indicated as the number of base pairs upstream from the transcription start site.

5.3.6 Significant down regulation of *Cyp27b1* expression in SM09 mice

Plasma PTH level is directly regulated by active vitamin D ($1,25(\text{OH})_2\text{D}$) via the negative vitamin D response element (VDRE) in the PTH promoter [116]. Therefore assessment of the plasma levels of $1,25(\text{OH})_2\text{D}$ levels has the potential to explain the increased PTH levels in SM09 female mice. However, there are technical difficulties in measuring $1,25(\text{OH})_2\text{D}$ mainly due to its short circulating half-life (4-6 hours) and the

very low circulating levels (1000-fold less than 25(OH)D) [147]. Therefore the expression of *Cyp27b1* in the kidney was measured as an indication of the ability to synthesize active vitamin D. SM09 female mice had an almost 9-fold reduction in *Cyp27b1* expression in the kidney, suggesting a reduced ability to synthesize active 1,25(OH)₂D.

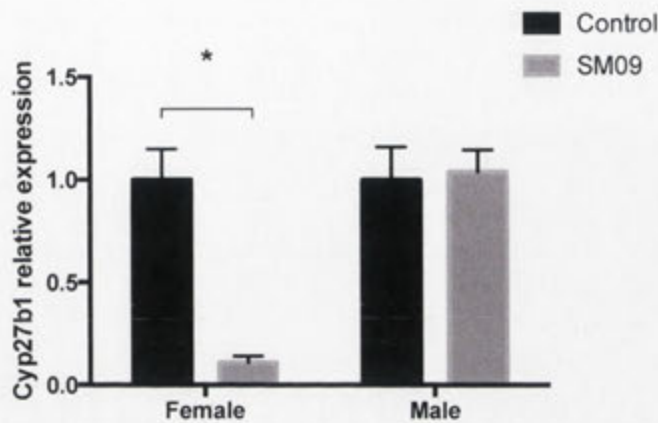


Figure 5.9: *Cyp27b1* mRNA level in the kidneys of SM09 and control mice as assessed by real-time PCR. $P^* \leq 0.05$ by Student's t-test (n=4)

The result that down regulation of *Cyp27b1* expression is seen only in SM09 females, but not males, matches patterns in their plasma PTH levels. This suggests that either lower *Cyp27b1* expression is the cause of higher plasma PTH in SM09 female mice, or that higher plasma PTH is causing downregulation of *Cyp27b1* expression. However, the later seems to be unlikely because, if the primary defect is associated with *Pth* gene leading to synthesize/release excess PTH, it should in turn upregulate the *Cyp27b1* expression. Since *Cyp27b1* expression is significantly downregulated regardless of the higher plasma PTH it supports the argument that the genetic defect, which drives the high PTH phenotype, is associated with the *Cyp27b1* gene.

5.3.7 Manipulation of the PTH levels

The measurement of PTH levels indicated a large amount of variation in plasma PTH levels in SM09 mice (figure 5.7). This suggested that environmental factors might be interacting with the SM09 genotype to determine the PTH levels. Therefore, we examined the effect of diet, hormones and p53 genotype on the PTH phenotype.

5.3.7.1 Effect of dietary vitamin D and calcium on plasma PTH level.

Mice were given special composition diets for two weeks and plasma vitamin D and PTH levels were measured. Both SM09 and control mice that were fed on a low-vitamin D (high calcium) diet had reduced 25(OH)D levels while those fed a high-vitamin D diet did not show an increase in plasma 25(OH)D levels compared to levels on the regular diet (figure 5.10.a).

On the other hand, the plasma PTH levels in SM09 mice that were fed on the low-vitamin D (high calcium) diet returned to normal levels, while in those fed on high-vitamin D diet, PTH levels were significantly reduced by almost 50% compared to SM09 mice on a regular diet, but did not return to normal levels (figure 5.10.b).

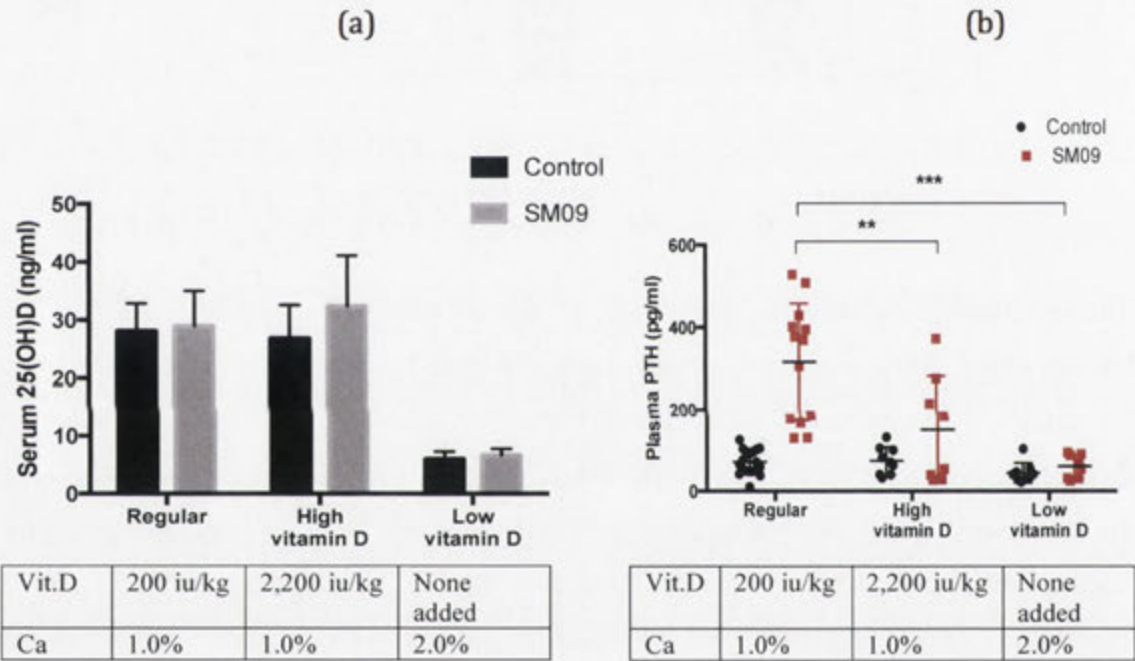


Figure 5.10: Circulating 25(OH)D and PTH levels in female SM09 and control mice fed on regular, high-vitamin D and low-vitamin D (high calcium) diets. (a) 25(OH)D levels in mice fed with three different diets. (b) PTH levels in mice fed on three different diets. (n=10) ***P ≤ 0.001, **P≤ 0.01 by Student's t-test

Thus increasing dietary calcium or vitamin D reduces the plasma PTH levels of SM09 female mice, with the 2-fold increase in dietary calcium being more effective than a 10-fold increase in dietary vitamin D (table 5.3). The high vitamin D diet, while reducing

the plasma PTH levels, was associated with only a small, but not significant, increase in 25(OH)D levels (10-20%).

	25(OH)D (ng/ml)		PTH (pg/ml)	
	Control	SM09	Control	SM09
Regular diet	28.1 ± 4.7	29.0 ± 6.0	71.3 ± 30.1	315.7 ± 141.9
High-vitamin D diet	26.9 ± 5.7	32.4 ± 8.7	74.2 ± 33.8	150.3 ± 130.7
Low-vitamin D diet	6.0 ± 1.3	6.6 ± 8.7	44.8 ± 24.1	55.8 ± 28.8

Table 5.3: Summary of circulating 25(OH)D and PTH levels in SM09 and control mice on three different diets.

5.3.7.2 *Effect of placental hormones on plasma PTH level.*

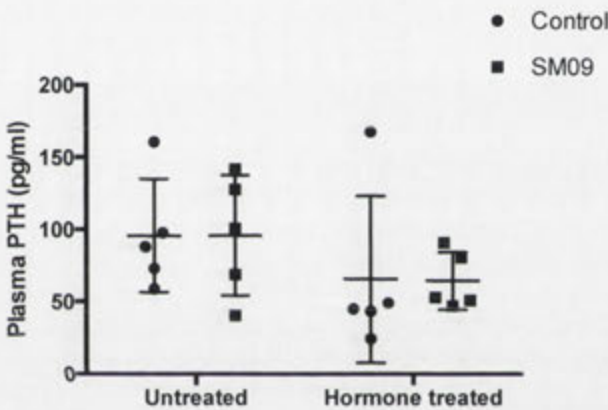


Figure 5.11: Plasma PTH levels in SM09 and control male mice, untreated and treated with placental hormones. (n=5)

The finding that increased PTH levels were seen only in female SM09 mice suggests an interaction of female hormones with the PTH phenotype. Therefore, we injected male mice of SM09 and control strains with placental hormones (PMSG and hCG) to see if the SM09 males, after hormone injections would have a similar phenotype to that of females. In contrast to what was expected, SM09 and control males had lower plasma PTH levels compared to the untreated males, however, this result was not statistically significant. While, this suggests that placental hormones on their own are not governing the increased PTH in SM09 females, it does not exclude the potential involvement of female sex hormones.

5.3.7.C The effect of *Trp53* genotype on plasma PTH level.

As the *SuprMam* loci were identified in a BALB/c-*Trp53*^{+/-} model of spontaneous mammary tumours, it is important to evaluate the potential interaction of p53 deficiency with any phenotype discovered. Thus the association of plasma PTH levels with the *Trp53* genotype was examined. Plasma PTH levels were not significantly different between the three *Trp53* genotypes suggesting that *Trp53* phenotype does not have a major effect on the plasma PTH levels (figure 5.12.a). One cohort of mice from figure 5.12.a was staged for estrous cycle at the time of plasma collection, and the analysis of PTH levels during estrus and diestrus stages were performed. Even though the mice at diestrus stage have slightly higher plasma PTH levels compared to those in estrus stage, this result was not statistically significant (Figure 5.12.b). This trend, however, is consistent with the reduced levels seen after placental hormone treatment in male mice (figure 5.11) as the treatment with placental hormones mimic estrus [75].

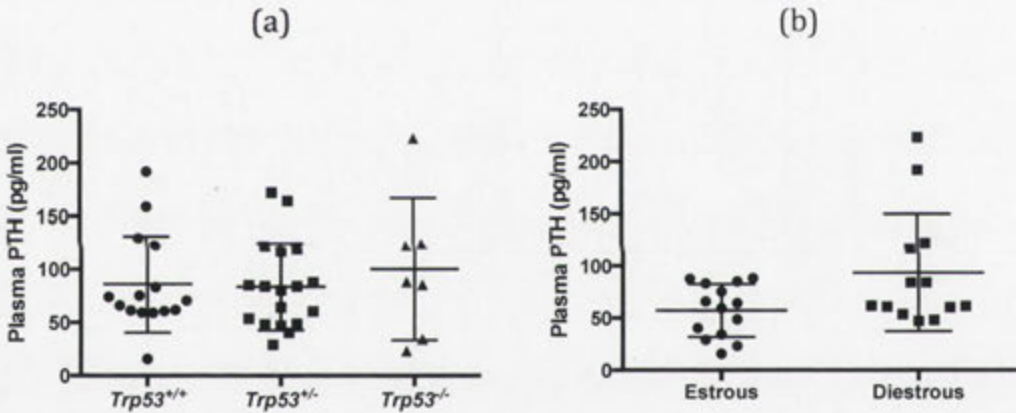


Figure 5.12: Plasma PTH levels in BALB/c female mice of different *Trp53* genotypes (a) Two cohorts of mice grouped according to *Trp53* genotype. (b) One cohort of p53 mice grouped according to estrus and diestrus stages.

However, it is important to note that while these mice are of BALB/c origin, they did not show the high PTH phenotype as expected. We suspect that this may be due to different bedding used for the maintenance of this strain. Some beddings are known to have endocrine modifying effects [148], and as mice ingest some of the bedding, could contribute a different amount of calcium to the diet of the mice. The possibility of the effect of bedding on the PTH phenotype is currently under investigation. Therefore, there is still potential for the PTH levels to be influenced by *Trp53* genotype or estrous stage, but further experiments under better-defined environmental/hormonal conditions are needed to establish this.

5.4 Discussion

Here we have shown that, compared to control mice, female SM09 mice that are at increased risk of developing breast cancer have similar serum 25(OH)D and calcium levels, but higher PTH levels. Gene expression studies showed that kidney *Cyp27b1* expression was significantly downregulated in SM09 mice compared to control mice. It is important to note that both increased plasma PTH levels and reduced *Cyp27b1* expression were seen only in female and not male SM09 mice.

While there is solid evidence of the effect of vitamin D against cancer, [6, 116, 149] the results of studies investigating the association of circulating 25(OH)D and breast cancer have been inconsistent through out. To date, only eight prospective studies of circulating 25(OH)D levels and breast cancer risk have been reported. Out of these only two studies demonstrated an inverse association of circulating 25(OH)D levels and breast cancer risk [117, 150] while six studies showed no significant association between plasma 25(OH)D levels and breast cancer risk [151-156]. These reports along with the results of the current study indicate that the measurement of plasma 25(OH)D levels might not be the ideal measure when it comes to studying the effect of vitamin D on cancer risk. However, it is important to note that most studies including the current study measure the total plasma 25(OH)D which include both the free form and those that are bound to DBP. Therefore, development of an assay to measure only the freely available 25(OH)D which is known to be functional would be more reliable. The measurement of 1,25(OH)₂D would provide a more meaningful measure as it is mostly the actions of 1,25(OH)₂D that is proven to be effective against cancer development. The measurement of 1,25(OH)₂D levels in SM09 female mice compared to control mice will help confirm this finding. However, of all the steroid hormones, the quantification of 1,25(OH)₂D has been the most difficult challenge to analytical biochemists. 1,25(OH)₂D circulates at concentrations too low for direct UV or mass spectrometry quantification and is also highly lipophilic.

On the other hand, experimental studies suggest that PTH has carcinogenic and tumour-promoting effects [144, 157, 158] which may therefore lead to an increased risk of breast cancer. As proposed by McCarthy, PTH can act as a cancer promoter both directly as well as indirectly [144]. A direct role is suggested by recent evidence that many cancers express receptors for PTH/PTH-related protein, which mediate co-mitogenic and pro-invasive signals. Indirectly, PTH increases hepatic production of the

growth factor IGF-1, a likely cancer promoter. This could explain the studies suggesting an association between hyperparathyroidism and increased breast cancer risk [138, 139, 141]. These considerations along with the strong biological interactions between 1,25(OH)₂D and PTH levels raise the possibility that PTH may be the missing link between the association of vitamin D deficiency with increased breast cancer risk. However, to date there have been no reports on pre-diagnostic PTH levels and breast cancer risk in healthy individuals.

Increased PTH levels were seen only in the female SM09 mice and not the males. This suggests that sex hormones may be playing a role in determining PTH levels. Gunnarsson *et al.*, (2009) demonstrated an inverse relationship between testosterone and PTH levels in vitamin D deficient men, and suggested that the serum PTH response to vitamin D deficiency in humans can be modified by a range of factors beyond serum calcium levels [159]. Testosterone is known to affect calcium metabolism at least partly by its conversion to estrogens [160, 161] that cause increased absorption of calcium from the small intestine [162] as well as increasing calcium reabsorption in the kidneys [163]. These processes would lead to calcium preservation and thus may reduce the need for higher PTH levels when testosterone levels are sufficient. On the other hand, Song *et al.*, (2004) has also demonstrated that the PTH response in female mice is more sensitive to changes in serum 1,25(OH)₂D levels than in male mice [164]. In our study, the lower *Cyp27b1* expression was seen only in SM09 female mice, suggesting an absence of vitamin D deficiency in the male mice that would stimulate PTH secretion. However, it is possible that *Cyp27b1* expression itself is regulated by a factor that is modulated by sexual hormones.

Misharin *et al.*, (2009) has investigated the difference in vitamin D metabolism between female mice of the two parental strains used in this study, BALB/c and C57BL/6, and reported that while there is no difference in 25(OH)D levels between the two strains, 1,25(OH)₂D levels are higher in C57BL/6 than BALB/c [121]. Consistent with the strain differences for serum 1,25(OH)₂D, mRNA levels for *Cyp27b1* were 17-fold higher in kidneys from C57BL/6 vs BALB/c mice. These data demonstrate an important difference between BALB/c and C57BL/6 strains in handling vitamin D that has the potential to impact on both systemic and localized responses to this hormone.

Consistent with the results of Misharin *et al*, [121] here SM09 female mice showed an almost 9-fold reduction in expression of *Cyp27b1* compared to controls. This would result in lower levels of 1,25(OH)₂D and hence lower calcium level, both of which have the potential to increase the systemic PTH level. The finding that there was no difference in serum calcium levels between the two strains suggests that increased PTH might be stimulating bone resorption as well as renal calcium reabsorption to maintain the blood calcium levels, regardless of the improper functioning of the *Cyp27b1*.

Feeding mice on a diet with a two-fold increase in calcium but no added vitamin D brought the PTH levels of SM09 mice back to normal. This suggests that the increased calcium in the diet is bypassing the need for proper functioning of *Cyp27b1* and reduces serum PTH levels through the CaSR. However, feeding on a diet with more than a 10-fold higher vitamin D levels and normal calcium resulted in only a 50% reduction in PTH levels. In this case, the excess vitamin D in the diet might be leading to synthesis of more 1,25(OH)₂D to suppress the PTH production and/or 25(OH)D in excess might directly influence the PTH level by binding to the VDR and downregulating PTH gene expression and thereby the synthesis.

The results presented here, indicate an association between the increased plasma PTH level and reduction of kidney *Cyp27b1* expression, but do not provide the causality. PTH is known to upregulate *Cyp27b1* transcription in the kidney, but our results are not consistent with this model. Thus it seems likely that the primary defect is reduced levels of *Cyp27b1* resulting in reduced active 1,25(OH)₂D leading to increased PTH expression and/or secretion. However, the location of the *Cyp27b1* gene on mouse chromosome 10 makes it difficult to directly relate the defect to the *SuprMam1* locus. An unknown factor within the *SuprMam1* locus may be suppressing *Cyp27b1* expression and thereby increasing PTH levels while *Cyp27b1* remains non-responsive to increased PTH levels.

Even though transcriptional regulation of *Cyp27b1* by PTH has been well studied, the exact site of action and any required transcription factors have not been clearly identified. Zierold *et al*, (2007) have demonstrated that nuclear receptor 4A2 (NR4A1) and CCAAT enhancer-binding protein β (C/EBP β) regulate the PTH-mediated transcriptional regulation of *Cyp27b1* [165]. However, none of these genes are located within the *SuprMam1* locus. Therefore it is important to identify other transcription

factors or non-coding RNAs that regulate *Cyp27b1* expression and investigate their relevance to the *SuprMam1* locus.

5.2 Conclusion

The results presented in this chapter suggest that reduced *Cyp27b1* expression in SM09 female mice is resulting in increased plasma PTH levels. Both dietary calcium and vitamin D normalize the plasma PTH, with calcium being more effective than vitamin D. However *Cyp27b1* is located outside the *SuprMam1* locus. Further investigation is required to identify a genetic factor within the *SuprMam1* locus that regulates its expression.

Chapter 6 – General Discussion

6.1 Cancer susceptibility and mouse models.

The recent convergence of two areas of cancer genetics that have developed independently during the last few decades are providing a promising and exciting new phase in our understanding of the genetics of cancer susceptibility. Studies in rodents show multiple genes with relatively small effects control cancer susceptibility [56], and human population studies have also shown that the majority of genetic predisposition to cancer is the result of many low penetrance genes rather than single gene mutations [18]. The convergence of these two independent sources of evidence opens up the possibility of a successful interaction between the study of tumour susceptibility in humans and rodent models.

During the early phases, the identification of single genes that are inherited in a Mendelian manner stimulated interest in the genetics of human cancer. This was followed by the chromosomal mapping and cloning of these important genes to identify the related mutations [166]. The development of mouse models to study oncogenes and tumour suppressor genes confirmed the tumorigenic effects of mutations in these genes [167, 168]. More recent developments in mutating these genes at specific time periods or tissues, enabled the development of mouse models that better represent the development of human cancer [169, 170]. While these mouse models helped to establish the molecular genetic pathways of cancer development, this approach only enabled the investigation of those genes that have been previously identified [171]. The evidence from both epidemiological studies of human cancer and genetic studies of tumorigenesis in experimental animals indicate that it is also important to define a large group of unknown genes that affect the development of human cancer. One approach to achieve this is to first identify and map them in mice, and then to study the role of their homologues in humans.

Inbred strains of mice and rats that differ in susceptibility to tumours provide the parental strains for the crosses that are needed to map tumour susceptibility genes through segregation analysis [172]. For example, in mice heterozygous for deletion of *Trp53*, the incidence of mammary tumours varies among strains, with C57BL/6 being resistant and BALB/c being susceptible [59]. Based on this difference, Blackburn *et al* (2007) identified the *SuprMam* loci which significantly increased the mammary tumour

susceptibility in BALB/c-*Trp53*^{+/-} mice [7]. The generation of a congenic mouse strain carrying the BALB/c *SuprMam* loci on the C57BL/6 background while retaining the *Trp53*^{+/-} genotype would be the ideal approach to identify specific effects of these loci on mammary tumour susceptibility. However, the development of SM09 congenic mouse strain with wild type *Trp53* as described in this thesis was the closest to the ideal setting that could be achieved during the time frame of this project.

Isolation of genes that are responsible for effects of cancer susceptibility loci is an important prerequisite for the understanding of the mechanisms of their action and for their exploitation as targets for therapy or prevention. On this basis this thesis examined different aspects of mammary tumour susceptibility, including mammary gland morphology, tumour growth and expression of previously identified candidate genes (*Dmbt1*). However, these approaches did not reveal any candidate genes or pathways for further analysis. On the other hand, transcriptional profiling of mammary glands, while confirming the effect of *SuprMam* loci on differential expression of genes, revealed four genes worthy of further investigation. These four genes are quite novel and there is little known about the role they may play in cancer development, opening an opportunity for the discovery of new contributors to cancer susceptibility.

Tumour susceptibility genes might affect tumorigenesis in various organs by participating in processes that are organ or tissue specific. This explains why overlapping susceptibility loci with partly opposite effects are involved in tumorigenesis in the intestine and the mammary gland of *APC*^{Min} mice [173]. Therefore, as the initial step, gene expression profiling in mammary glands is a useful step to identify any alterations in gene expression with local effects in the mammary gland. However, some genes that affect tumour susceptibility might function systemically, such as the MTV-linked loci that control the development of MTV-induced mammary tumours [174]. Therefore, when determining candidate genes or pathways involved it is important to consider all functions that may contribute to tumour susceptibility, such as local factors, the interaction between normal or stromal cells and tumour cells, and the role of systemic factors.

6.2 Confirming the tumorigenicity of *SuprMam* loci

While this thesis examined the effect of *SuprMam* loci on the tumour growth of SM09 mice compared to controls, its effect on the spontaneous mammary tumour development was not investigated. As the *SuprMam* loci was identified in BALB/c-*Trp53*^{+/-} mouse model, it is important to confirm that SM09 mice exhibit a similar increase in susceptibility to mammary tumours on a *Trp53*^{+/-} background. This could be achieved by breeding SM09 female mice with C57BL/6-*Trp53*^{+/-} male mice for several generations while confirming that they retain the *SuprMam* alleles from BALB/c background. While it may take sometime to establish SM09-*Trp53*^{+/-} mouse strain and 1-2 years for spontaneous mammary tumour development, it would be the ideal mouse model to confirm the findings and any follow-up of the current study.

6.3 Functional significance of PTH in mammary tumour development

A high proportion of cancers including breast cancer have been shown to synthesize PTH-related protein (PTHrP), which activates the PTH receptor with equal affinity [175, 176]. Several studies have demonstrated that human breast cancers, as well as cells in hyperplastic mammary epithelium, express the PTH receptor [157, 177, 178], suggesting that plasma PTH or PTHrP of autocrine origin have growth promotional activity. Presumably, plasma PTH would have its most significant influence prior to the acquisition of the capacity for substantial autogenous PTHrP production [144].

Recent studies have shown that the PTHrP promoter contains the negative vitamin D response element which is also present in the PTH promoter [179, 180]. This will provide an explanation for the suppression of PTHrP production in cancers by 1,25(OH)₂D [181-183], while suggesting that both PTH and PTHrP might be acting in a similar manner towards cancer progression. Therefore, it is important to evaluate the effect of PTH on cancer cell behaviour. C57BL/6 mammary tumour cell lines, E0771 and AT3, which were used in this study, can be used to assess this *in vivo*. The growth characteristics, including apoptosis and cell proliferation could be assessed in the presence or absence of PTH in the culture media.

The assessment of the effect of systemic PTH level on mammary gland biology, both *in vitro* and *in vivo*, will be important to identify any functional significance of the

findings of this thesis. Since the mammary gland is known to express PTH-receptor, mammary gland organ culture would be one way to identify the effects of PTH on the mammary tumour development, independent of other systemic factors. Peng *et al*, (2009) has reported a similar study to evaluate the effect of 25(OH)D on the development of precancerous lesions in mouse mammary gland organ culture [184]. Mammary glands from control and SM09 mice pretreated with estrogen and progesterone can be cultured separately in media followed by DMBA treatment for the induction of precancerous lesions as described previously [185]. Then the media can be supplemented with commercially available mouse PTH, and the mammary glands can be fixed and processed for histopathological evaluation to determine if PTH in the media increases the formation and development of lesions, and if this effect is different between the mammary glands of two strains. PTH can also be included in the culture for various times as described by Peng *et al*, (2009) to identify the stage specific effects. Including PTH during the early phase of growth will reveal the effect of PTH on the initiation of the lesions, while including PTH in a later stage will determine the growth promotion effect [184].

The increased calcium in the diet normalized the serum PTH levels in SM09 mice, while increased dietary vitamin D brought it down to a lesser extent. This suggests that dietary calcium is more effective than vitamin D in normalizing plasma PTH in SM09 mice, which is probably due to the hypothesized defect in *Cyp27b1* expression. Since mammary glands express the PTHr, it can be hypothesized that PTH acts on the mammary epithelium to increase mammary tumour susceptibility while calcium protects the epithelium by suppressing systemic PTH. A similar hypothesis has been previously proposed in colorectal cancer, suggesting that PTH inhibits differentiation in the colorectal epithelium, while dietary calcium promotes differentiation by suppressing PTH [186]. When investigating this *in vivo*, even though the higher serum PTH in SM09 mice is increasing the mammary tumour susceptibility, we might not be able to identify small differences in the gene expression within mammary glands. One approach to overcome this is to further increase PTH levels either by directly injecting PTH into SM09 control mice or feeding a diet which is deficient in both vitamin D and calcium, and identifying genes differentially expressed in mammary glands, especially markers of differentiation and proliferation. A group of mice of each strain after PTH supplementation can then be fed on a high calcium diet (with normal vitamin D), which will suppress the systemic PTH levels. Expression analysis of differentiation and

proliferation markers within the mammary glands of these mice will help to establish the hypothesis that PTH promotes tumorigenesis by reducing differentiation and increasing proliferation, while dietary calcium protects against tumorigenesis by suppressing PTH.

6.4 Identification of the underlying functional genetic variant(s) in the *SuprMam1* locus

While it is clear that the *SuprMam1* locus alters *Cyp27b1* expression of SM09 mice and increases PTH levels, it is important to identify the specific genetic factors within the *SuprMam1* locus that drive this phenotype. Since both SM09 and control mice have the *Cyp27b1* gene from the C57BL/6 background, it is clear that there are no polymorphisms within the *Cyp27b1* gene sequence or its promoter. Therefore it is likely that the functional polymorphism is within a regulatory gene involved in promoting *Cyp27b1* transcription or RNA stability.

Databases such as Transcription factor prediction database (DBD) (<http://www.transcriptionfactor.org/>) can be used to identify potential transcription factors that would bind to *Cyp27b1* promoter. The identification of the chromosomal location of the predicated transcription factors will determine if they are located within the *SuprMam1* locus. The predicted transcription factors that are not located within the *SuprMam1* locus can also be relevant as they might be regulating *Cyp27b1* expression through other transcription factors within the locus. The exome sequencing data on control and SM09 mice can be used to identify the SNVs within the exons of the predicted transcription factors. While the mouse SNP databases can be used to examine potential functional SNPs within non-coding regions, absence of BALB/c data on most databases is a set back. Therefore, resequencing of the non-coding regions of *SuprMam1* from BALB/c and C57BL/6 DNA will be important to identify any underlying genetic polymorphisms.

6.5 Role of dietary calcium and vitamin D in breast cancer prevention and treatment

If PTH has a cancer promotional activity as suggested by evidence presented in this thesis, the implication is that increased dietary calcium and vitamin D intakes will lower the breast cancer risk of an individual by lowering the plasma PTH levels. However,

according to the mouse experiments conducted during this study, dietary calcium is more effective than vitamin D in normalizing systemic PTH levels, and therefore potentially more important in prevention and treatment of cancer. Even though to date there are no reported studies directly investigating the association between blood levels of PTH with breast cancer risk in humans, there is indirect evidence in humans supporting the hypothesis of the current study. Indirect evidence for a role of PTH in breast cancer risk in humans comes from observations that patients with hyperparathyroidism are more likely to be diagnosed with breast cancer [138]. Therefore, pre-diagnostic serum PTH levels above normal (as in hyperparathyroidism) may increase breast cancer risk, even after controlling for 25(OH)D concentration.

It is also important to note that, while the SM09 mice had almost 3-fold increase in plasma PTH levels, there was no difference in 25(OH)D or calcium levels when compared to control mice. Therefore, it is possible that the 25(OH)D and calcium measurements do not necessarily represent the circulating PTH levels. One of the drawback of the current study was the unavailability of 1,25(OH)₂D measurements, which might possibly reflect the higher plasma PTH levels. Therefore, when it comes to human studies it will be important to measure plasma PTH and 1,25(OH)₂D levels when considering calcium or vitamin D deficiency.

Several studies have suggested an inverse association with dietary calcium and breast cancer risk as reviewed by Cui *et al*, (2006) [6]. While most of these studies did not measure the plasma PTH levels, it is possible that dietary calcium reduces breast cancer risk by regulating PTH levels, as was seen in SM09 mice. The current hypothesis also has a potential to explain the association of obesity with breast cancer risk. Obesity is known to result in an altered calcium metabolism with higher circulating PTH levels [187-191]. This may be due to decreased bioavailability of vitamin D in obese individuals due to sequestration in tissue fat [192]. It has also been speculated that PTH may inhibit catecholamine-induced lipolysis, enhance *de novo* lipogenesis and modulate *CYP27B1* activity in adipose tissue [191, 193]. On the other hand, higher PTH levels were shown to almost double the colon cancer risk among overweight individuals [194]. While these studies suggest a potential interaction between being overweight and higher PTH levels, the exact mechanism explaining that overweight women have an increased risk of breast cancer with higher circulating PTH is not clear, but might be mediated through an interplay between PTH, calcium, vitamin D and their receptors.

The results presented in this thesis suggest that increased calcium and vitamin D intake to be effective in normalizing circulating PTH levels. Human studies have demonstrated that, when hyperparathyroidism is treated with vitamin D, patients can become resistant to vitamin D therapy as a result of significant reduction in the expression of VDRs that negates the actions of vitamin D sterols [195-197]. On the other hand the amount of vitamin D supplementation required to raise systemic vitamin D by a biologically meaningful amount is far above the physiological concentrations, and difficult to achieve [198]. This was reflected in the current study as well, as increasing dietary vitamin D by over 10-fold did not result in a significant increase in the plasma 25(OH)D levels. While calcium appears to be more effective in normalizing systemic PTH level, the potential of using calcium as a therapeutic agent is limited by the risk of causing hypercalcemia. Hypercalcemia also limits the potential of vitamin D therapy as 1,25(OH)₂D facilitates the absorption of calcium from the gastrointestinal tract, thus adding to the calcium load.

Therefore, there is a need of a therapeutic agent that will manage the PTH levels without undesirable effects of increased calcium. The CaSR is identified as a therapeutic target due to its central role in calcium homeostasis and therefore, ligands that mimic the effects of extracellular calcium at the CaSR, which are called calcimimetics are considered to be important [199]. Calcimimetic agents represent a new class of therapeutic agents that increase the sensitivity of the parathyroid gland CaSR to extracellular calcium, thereby inhibiting PTH secretion and rapidly decreasing PTH levels. The typical examples of calcimimetics are cinacalcet HCl, NPS R-568 and NPS R-467 and cinacalcet HCl has been used in various *in vitro* [200] and *in vivo* studies in both rodents and humans [200-202] and has produced successful results in reducing systemic PTH levels without causing hypercalcemia.

6.6 Conclusion

The work presented in this thesis has used congenic mice to identify several novel genes with potential involvement in susceptibility to mammary carcinogenesis. By discovering these genes in a rodent model, they come with a biological system ready for further studies to elucidate their function and contribution to mammary gland biology.

In addition to the four novel genes, the vitamin D/calcium regulation pathway has been identified as potentially playing a role in SM09 mice. The evidence presented in this thesis suggests that increased systemic PTH levels in SM09 female mice due to lower *Cyp27b1* expression in kidney might be leading to increased breast cancer susceptibility in SM09 mice. While further investigation is required to investigate the genetic factor within the *SuprMam1* which drives this phenotype, it is clear that dietary calcium and vitamin D can successfully reverse this phenotype. Therefore, calcimimetic agents, which manage the PTH levels without disturbing the mineral metabolism, would be more effective as a therapeutic agent for PTH-dependent cancers on its own or in combination with other treatments. The *SuprMam* congenic mice would provide an excellent model for understanding the role of PTH in epithelial cancer development and for testing preventive interventions.

Appendix

Appendix A: PCR primers for mRNA expression (F denotes forward primer and R denotes reverse primer).

Name	Gene Symbol	Sequence
Deleted in malignant brain tumours [7]	<i>Dmbt1</i>	<i>CUB5</i>
		<i>ZP3</i>
		<i>TM5</i>
		<i>UTR3</i>
Cyclin-dependant kinase inhibitor 1A [203]	<i>P21/Cdkn1a</i>	
Cytochrome P450, family 2, subfamily r, polypeptide 1 [204]	<i>Cyp2r1</i>	
Cytochrome P450, family 27, subfamily b, polypeptide 1 [205]	<i>Cyp27b1</i>	
Glyceraldehyde-3phosphate dehydrogenase	<i>Gapdh</i>	
Beta actin [7]	<i>Actb</i>	

Appendix B: Promoter sequences used for the identification of transcription binding sites via Genomatix software. Note: *Cyp2r1* promoter (600 bp upstream) contained several SNPs for C57BL/6 Vs. DBA, while *Pth*, *Calca* and *Calcb* had no distinct polymorphisms within this region.

```
>Cyp2r1 - C57BL/6 -7 dna:chromosome
chromosome:NCBIM37:7:121706186:121708486:1-RevCompl 600bp
TGGGGGCTTGGGAGAACAAAGAAGGGTGAAAAGATAGGTGACTAGAACTAATCAGAATCC
TCAAAGGGTCCACCGAGAAATGGCTCATAACAAACAGTGAAATTGCAGACAGAGTGAAGC
AAAATACAATAAGGTTATTTCCCTTTTCTCTTGATATGTGGACTCGCTAATGGAAGTGCT
GGTTATTACCAGACTCGGTTAAAGAATGCAGTTCTAATCTCTTCCCTATCCGTAGACTGA
AAATAAACAGACCAGGCAACGTTAGACACTCTTTAAGGACAGACGTCCAGAGAACTAAGA
TGCACCGAGCCCAGAGAGCGAAGTTCACATGACCAACCTACTGACAACATTCCGGAAAAG
GGCCCTAGGGCTCCGTGCCCCTTATGCGGTCTCACCAGAGCCAAGCCAAACCCAAAGG
TTTACAAACGGACTCTGAGCAAAGCAGATCTGGAGCTGGCTAACCCTAAGGACCCTGAGC
CCACACTGAATGAACCAATGGCCGTAGAGATAAGGCGGGCCGTGAGGCGCCGGGCCAAT
GGGAGTGCGGCAGGGCTGGTGGGTGCGCCCCTGCCTCCTCGCTCCGGACTGGTTTCGTAGC
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>Cyp2r1 - DBA -7 dna:chromosome - RevCompl 600bp
chromosome:NCBIM37:7:121706186:121708486:1
TGGGGGCTTGGGAGAACAAAGAAGGGTGAAAAGATAGATGACCAGAACTAATCAGAATCC
TCAAAGGGTCCACCGAGAAATGGCTCATAACAAACAGTGAAATTGCAGACAGAGTGAAGC
AAAATACAATAAGGTTATTTCCCTTTTCTCTCGATATGTGGACTCGCTAATGGAAGTGCT
GGTTATTACCAGACTCGGTTAAAGAATGCAGTTCTAATCTCTTCCCTATCCGTAGACTGA
ACATAAACAGACCAGGCAACGTTAGACACTCTTTAAGGACAGACGTCCAGAGAGCTAAGA
TGCACCGAGACCAGAGAGCGAAGTTCACATGACCAACCTACTGACAACATTCCGGAAAAG
GGCCCTAGGGCTCCGTGCCCCTTATGCGGTCTCACCAGAGCCAAGTCAAACCCAAAGG
TTTACAAACGGACTCTGAGCAAAGCAGATCTGGAGCTGGCTAACCCTAAGGACCCTGAGC
CCACACTGAATGAACCAATGGCCGTAGAGATAAGGCGGGCCGTGAGGCGCCGGGCCAAT
GGGAGTGCGGCAGGGCTGGTGGGTGCGCCCCTGCCTCCTCGCTCCGGACTGGTTTCGTAGC
```

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>Pth - C57BL/6 - 7 dna:chromosome
chromosome:NCBIM37:7:120532087:120532687: 1-RevCompl 600bp
AATCTAGTTTTTGAAGCTGATGAAAGGTGATATGAGCTGGTTGGAAATGGCTTGTAAGAA
AAAGCATGCTACATGATTTCTTAAGTACGCAAGTGATTTTATAATGTGGCTATGACAAAT
AGCAAATATACTTCAGGCTTTAATTTCAAATAGTAGATAGAATAGCTTTGTTCATATTTGG
AATCAAAACTTTGTAAATGGTAAGGCACAGTGGAAAATGTCTTTAACCCTAGCATTCATG
AAGCAGAGGCAGGTGGATCTCTTGAGTTTTAGCCATCCTGGTCTACATATTGAGTTCCAG
ACCAGCCAGAGTTACATAGTGAGACTTTGTCTCAAAGATTTTTTTTTTAAATATAAACTCA
GTATTATCGATTATGTCAAAAATTTCTAGGTGGACACTTAGTATTTGCTACATCTTCTTT
TGCACCTTTCTTTGTCAGAACCTGCTCATTTTCATCTACTATCTAGTTATCTGAAACTTT
AGAGGAGTGGGCACCAACCCCATGAGGGTATGTGGCTGTTCTGATCCTGTGATTGAGAGCC
AGAGAACCAGGAGTGACATCATCTTAACAATAAAATACTCCTCTTGGTGAGCAAAAAGC
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>Calca - C57BL/6 - 7 dna:chromosome
chromosome:NCBIM37:7:121779871:121780471: 1-RevCompl 600bp
GGCTTTGGGGGTCTTTTGGGGGGTGTCAGATTTTGGAGGATTTTTTTTTTTCATTTTTCAT
TTTACCTGTGGCCACATAACCCCAAATGAGGCAGCTGCCACAAGCCTCGGGTCTAACTGA
GTCCTTGTGTGCAGTACCAACACCTCTGACTATCTGCCTAAACCTGTGTGCCACGCTGCC
AGAAGTAGCCTTGAGATCAAGAATTCATCCCTCAGGTGCACTAACTATTCTAGATTAG
GCACGGACTTAGGTGGGGGTGGGGGTACGATTAGAATCAGACATGCAGCAAGGAGTGCTT
GAGATACTGGACTCTACTCTCCAAGATTCTGAGATTGGAGTCAGGAATATTCAAAGTCAA
GAAGGAAAGAAGAGATGAAATTTATCTTGATGTCAAAGGCCCTCCAAATTCCCGCTAAT
TTTAAGGGTGGCTCTCACTGATCCGTATCATCTCCCCCACTTCCACCAATGACCTCAATT
TAAATTCAAATGGTGTCTCTTGCTAGATGCTCTGAGTTCTGGAAGCACAGAGGTGACGC
AATCTGTCTGGGGCATGGGGCCCTTCCACCTATTGGCTGCCTGGAGCCCCGAGACCCCTC
```

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>Calcb - C57BL/6 - 7 dna:chromosome
chromosome:NCBIM37:7:121861557:121862157:1 - 600bp
CGCAGGATTTGGAAGGTCTGGTACAGGAGGAGAAAGCCAGTTCCCTGTGCAGTCTGTTA
GCCTGCTGCTCCACAATACTCTCTAGTTTCTATCCTTATTAGGTGATGGGAAGCACGCAC
TGCTAGAGTGCCCATTTGGGACAGGTATGACAGAAGTACCCTAATGTATCCAAGGACCCG
CTTCTTCCTGTGACAGTCATCATCGTGGATGTATCTACTGAAGTCCTTTTAGAACCTGGG
AGTGCTACTCAGCCTGCGTGGGAGTCCAGCTACGAGGTTTCAAGTCCCCATTGGAGTGGGC
AGCAAAAGGTTGTAGGCTGGAGTTCAGGTATTAAAGAGGTCGTGATGTCAAACCTCAGGTT
TGCTCACATTCTGGACGAATTCACCCTCTCTGTATCCTTACCCCAACCCCACTCCCACTC
CTACCCGGTTTCTCAGCAATGACCTCAAAGACAGGGAGTGGACTGCTGCCTCCCTCCTGC
AGAAGTGTAAGTAGCTCCAGCTATGACGTTATGGAAGCTCGGTCAGAGCTCTGATTGGTG
GAAGAGCTACTGCGGACCCCCCACACCCTCAAGATCGAGAATAAGAGACCACGGCTCTGG
```

Appendix C: Unannotated transcripts that were differentially expressed in the mammary glands of SM09 and control mice as identified by microarray analysis.

Column ID	Fold change	P value
10343392	2.1	9.09E-05
10342832	1.9	5.10E-05
10340813	1.9	6.90E-06
10430904	1.8	7.30E-06
10344340	1.8	1.48E-05
10340355	1.7	8.47E-05
10343300	1.7	7.34E-05
10435185	-1.9	1.45E-05
10342610	-2.0	7.14E-05
10338539	-2.1	4.33E-05
10341144	-2.2	2.37E-05
10342905	-2.4	7.04E-05

Unannotated transcripts differentially expressed during the basal state

Column ID	Fold change	P value
10343304	2.3	5.51E-05
10343392	2.1	8.05E-05
10341835	2.1	2.16E-04
10339900	2.1	7.30E-04
10339532	2.1	1.49E-04
10343533	2.0	2.51E-04
10340536	2.0	3.92E-05
10342135	1.9	5.62E-04
10341494	1.9	6.29E-04
10338770	1.9	3.05E-04
10339663	1.8	1.55E-04
10339699	1.8	3.93E-04
10338268	1.8	5.61E-04
10343865	1.8	5.63E-04
10341243	1.8	8.09E-05
10342992	1.7	5.20E-04
10342429	1.7	2.29E-04
10343083	1.6	5.51E-04
10603175	1.6	2.17E-06
10434289	1.6	7.74E-04
10338130	1.6	7.85E-04
10343300	1.6	3.16E-04
10342739	1.6	9.33E-05
10339985	1.5	2.93E-04
10340642	1.5	3.87E-04
10341061	1.5	5.47E-04
10416897	-1.5	1.91E-05
10342821	-1.5	3.78E-04
10342720	-1.6	1.10E-04

Unannotated transcripts differentially expressed in the treated state.

Column ID	Fold change	P value
10450197	-1.5	1.27E-04
10435185	-1.5	4.93E-04
10578423	-1.5	1.72E-04
10342619	-1.7	5.15E-04
10417371	-1.7	4.10E-04
10342610	-1.7	7.84E-04
10338320	-1.7	5.78E-04
10338222	-1.7	7.26E-04
10339119	-1.8	7.73E-04
10340484	-1.8	5.92E-04
10378438	-1.8	5.16E-05
10342300	-1.8	1.10E-04
10412488	-1.8	4.27E-04
10340826	-1.9	6.07E-04
10339112	-1.9	3.78E-05
10341144	-2.0	8.18E-05
10340290	-2.0	3.24E-05
10338963	-2.0	2.19E-04
10344406	-2.0	3.15E-04
10340443	-2.1	7.86E-04
10608711	-2.1	1.28E-05
10343494	-2.3	5.96E-04
10340560	-2.7	5.56E-05
10341274	-3.0	2.84E-05

Continuation – Unannotated transcripts differentially expressed in treated state

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