CELLULAR MECHANISMS OF RESISTANCE TO MAMMARY TUMORIGENESIS IN THE COPENHAGEN RAT

by

James Eric Korkola

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Medical Biophysics, in the University of Toronto

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CELLULAR MECHANISMS OF RESISTANCE TO MAMMARY TUMORIGENESIS IN THE COPENHAGEN RAT

Doctor of Philosophy, 1999
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Abstract

Studies have identified several genes that play a role in susceptibility to human breast cancer. In contrast, little is known about genes conferring resistance to breast cancer. Resistance is difficult to study in humans, but the Copenhagen (Cop) rat is resistant to mammary tumor induction, and provides an excellent model to study this phenomenon. This thesis focuses on mechanisms that may be responsible for resistance of Cop rats to mammary tumorigenesis.

Since there was evidence that T-cell immunity may be involved in resistance, I bred Cop rats with athymic, nude rats that are T-cell deficient, then interbred the F1 offspring to produce F2 animals, some of which were athymic with partial Cop background. If T-cell immunity is involved in resistance, then athymic F2 rats would be more susceptible to mammary tumorigenesis than their euthymic littermates. No tumors developed in any of the F2 rats, indicating resistance to mammary tumorigenesis is not T-cell related and that the parental nude strain is also resistant.

Next, I examined the cellular structures of mammary glands of Cop and susceptible rats at various times following carcinogen treatment. Preneoplastic lesions known as intraductal proliferations (IDPs) that harbor Ha-ras mutation were evident in both strains at early time points, but unlike susceptible rats, the IDPs in Cop rats failed to progress into
more advanced lesions and instead disappeared. I examined several potential mechanisms that may be responsible for the disappearance of IDPs in Cop rats. Surprisingly, I found that the proliferative index was higher in Cop IDPs than in those of susceptible Wistar-Furth rats at a time when the IDPs in Cop rats were disappearing. There was, however, a concomitant increase in the apoptotic index in the Cop lesions. Finally, I showed that high levels of cyclin D1 expression may be important in progression from IDPs to more advanced lesions, since this gene was highly expressed in all tumors but only in some IDPs. Furthermore, elevated expression of this gene may be of importance in resistance, since Cop IDPs had lower levels of cyclin D1 than IDPs of susceptible rats.
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Finally, thanks to my girlfriend Elisabeth and my parents, who were always supportive throughout the project.
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<td>AAF</td>
<td>3-Acetylamino-fluorene</td>
</tr>
<tr>
<td>AB</td>
<td>alveolar bud</td>
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<tr>
<td>ATM</td>
<td>ataxia-telangiectasia gene</td>
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<td>Buf/7N</td>
<td>Buffalo</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>Cop</td>
<td>Copenhagen</td>
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<td>CTL</td>
<td>cytotoxic lymphocyte</td>
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<td>DES</td>
<td>diethylstilbestrol</td>
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<td>DAH</td>
<td>ductal alveolar hyperplasia</td>
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<tr>
<td>DH</td>
<td>ductal hyperplasia</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma(s) in situ</td>
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<td>DMBA</td>
<td>7, 12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>ENU</td>
<td>N-ethyl-N-nitrosourea</td>
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<tr>
<td>F344</td>
<td>Fischer 344</td>
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<tr>
<td>HAN</td>
<td>hyperplastic alveolar nodule</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<td>IDP</td>
<td>intraductal proliferation</td>
</tr>
<tr>
<td>IDP*</td>
<td>initiated IDP</td>
</tr>
<tr>
<td>IDP**p</td>
<td>initiated plus promoted IDP</td>
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<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>MCA</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>mcs</td>
<td>mammary carcinoma suppressor</td>
</tr>
<tr>
<td>Mcs</td>
<td>mammary carcinoma susceptibility</td>
</tr>
<tr>
<td>MEC</td>
<td>mammary epithelial cell</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumor virus</td>
</tr>
<tr>
<td>MNU</td>
<td>N-methyl-N-nitrosourea</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NSD</td>
<td>inbred Sprague Dawley</td>
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<tr>
<td>O**MeG</td>
<td>O**Methylguanine</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>SD</td>
<td>Sprague Dawley</td>
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<tr>
<td>TD</td>
<td>terminal duct</td>
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<tr>
<td>TEB</td>
<td>terminal end bud</td>
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<td>WF</td>
<td>Wistar Furth</td>
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<td>Wistar Kyoto</td>
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CHAPTER 1

INTRODUCTION
1.1. Human Breast Cancer

1.1.1. Incidence and Risk Factors

Breast cancer is the most common form of cancer diagnosed in North American women. It is estimated that Canadian women have a lifetime risk of 1 in 9 of developing the disease and that in 1998, 19,300 new cases of breast cancer will be diagnosed (1). Despite the improved detection and treatment for the disease, it is estimated that 5,300 deaths will be attributed to breast cancer in 1998, second only to lung cancer related deaths in Canadian women (1).

A number of factors have been associated with an increased risk of developing breast cancer. Diet is thought to be one of the most important factors, and high fat diets correlate positively with increased incidence (2). It is still controversial, however, whether this correlation is actually due to the high fat content (3) or the large number of calories in the high fat diet (4), which may instead ultimately provide the promotional stimulus. Another factor also shown to play an important role is hormones, since early menarche and late menopause, both of which lead to longer exposures to estrogens, are associated with increased risk, while early pregnancy, which exposes the breast to hormones inducing differentiation of the tissue, is protective (5). Furthermore, recent studies indicate that some endogenous estrogen metabolites may themselves be weakly carcinogenic in humans (6), although more studies are still required to confirm this notion. Finally, genetics are
thought to play an important role in the risk of developing breast cancer, since a family history of breast cancer has been shown to be associated with increased risk (7).

1.1.2. The Genetics of Human Breast Cancer

A number of oncogenes and tumor suppressor genes have been shown to be mutated, amplified, or lost in both sporadic and inherited breast cancers. A brief survey of some of the more frequent genetic alterations is presented below.

In studies performed on sporadic breast cancers, one of the most commonly observed mutations is in the p53 tumor suppressor gene (8,9). p53 is thought to be involved in regulating several key functions of the cell, including cell cycle transitions and DNA damage response (10) and apoptosis (11). Up to 40% of breast cancers have demonstrable p53 mutations (8). Mutations in p53 are often dominant-negative mutations resulting in stabilization of the mutant protein (12). Immunohistochemical studies of breast tissue for mutant p53 has shown that overexpression is associated with a poor prognosis (increased likelihood of metastases and decreased survival) (9). As well, there is loss of heterozygosity (LOH) of the short arm of chromosome 17, the location of p53, in 60-65% of breast cancers (8,13). The long arm of chromosome 17 also shows frequent LOH, and several genes implicated in breast cancer lie in this region, including nm23, prohibin, and BRCA-1 (see below) (8). It is unclear which of these genes is the crucial target for this LOH. Indeed, reduced expression of the nm23 gene has been associated with increased likelihood of metastases (14), and prohibin is mutated in some breast tumors (8).
Furthermore, although *BRCA-1* is affected in more than 80% of cases of LOH, it appears that another, unidentified gene may be the target of the LOH in this region (8). Like *nm23*, loss of *e-cadherin* correlates with increased rates of metastasis (15). This gene is involved in cellular adhesion (16), which may explain why loss of this gene leads to increased metastases in breast tumors. Finally, loss of the *Rb* gene is a frequent event in breast cancer (13,14). Like *p53*, this tumor suppressor gene seems to be essential for proper regulation of the cell cycle (17).

Amplification of numerous genes has been reported in sporadic human breast tumors. One of the most commonly amplified genes is *neu* (also known as *ERBB2* or *Her-2*), which has regions of homology to the epidermal growth factor receptor. The *neu* gene is amplified in up to 20% of invasive carcinomas (8), but may be amplified in an even higher percentage of carcinomas *in situ*, which are believed to be precursor lesions for breast cancer (14). In lymph node positive patients, *neu* overexpression is associated with poor prognosis (8). Conflicting reports exist on *neu* overexpression and prognosis in lymph node negative patients, with some reporting no correlation between *neu* expression and survival (8) and others finding poor prognosis associated with *neu* overexpression in these women (18). The *c-myc* oncogene has also been shown to be amplified in 6-30% of breast cancers, and this amplification is associated with lymph node involvement and poor prognosis (8). The amplification of the q13 region of chromosome 11 may result in amplification of the *Int-2* gene in approximately 4-23% of cases, although several other candidate genes that lie within this region may also play a role in development and progression of the disease, as well as an increased risk of metastases (8). The gene
encoding cyclin D1 also lies in this region, and has been shown to amplified in up to 20% of cases (8). Furthermore, there is some evidence that cyclin D1 overexpression may be an important preliminary event in the transition from premalignant to malignant lesions, since approximately 80% of carcinomas in situ overexpress cyclin D1, while less than 20% of premalignant and benign lesions overexpress this gene (19). The cyclin D1 gene seems to be critical in the transition from the G1 to S phase of the cell cycle. Several other genes have been shown to have occasional amplification (2-6%) in breast tumors, such as the IGF-I (insulin-like growth factor I) and EGF (epidermal growth factor) receptors (8). Furthermore, increased activity of telomerase has been associated with more malignant breast tissue compared to normal breast (20). Finally, the Ha-ras proto-oncogene has been shown to be amplified in up to 65% of human breast cancers (21,22), although the results of these studies have not been repeated by other groups.

Studies of breast cancer prone families have identified several genes that play important roles in predisposing women to development of breast cancer. Individuals with Li-Fraumeni syndrome carry germline mutations in the p53 gene, and have an increased risk of developing the disease (23). There is some evidence that individuals who are heterozygous for mutations in the ataxia-telangiectasia gene (ATM) show an increased risk of developing breast cancer (24), although this remains controversial (25). Linkage analysis of breast cancer prone families has led to the identification of two genes, BRCA-1 (26) and BRCA-2 (27), which are associated with increased risk when inherited in a mutated form. BRCA-1 also seems to be associated with an increased risk of developing ovarian cancer (26). Interestingly, although a report shortly after the discovery of BRCA-1
indicated that there may be aberrant subcellular localization of the protein in sporadic breast tumors (28), other groups have been unable to duplicate these results (29). Analyses of BRCA-1 in sporadic breast and ovarian tumors (30,31) and BRCA-2 in sporadic breast tumors (32,33) have shown that these genes are rarely mutated, indicating that mutations are unlikely to be important in the development of sporadic breast cancers. There is, however, some evidence that expression of BRCA-1 is decreased in invasive breast cancer compared to normal mammary tissue (34). Furthermore, the introduction of wild-type BRCA-1 into mammary cell lines derived from sporadic tumors inhibits tumors development and growth when injected into nude mice (35). As mentioned above, BRCA-1 lies on chromosome 17, which often shows deletion of one allele in up to 60% of tumors. The loss of this chromosome and, therefore, loss of BRCA-1 may lead to the lower expression levels that have been observed in breast cancer relative to normal tissue (33). These results indicate that BRCA-1 may be of importance in sporadic tumor formation, presumably not because of mutation of the gene, but instead decreased protein levels (35). BRCA-1 and BRCA-2 have little homology to other known proteins, and their functions are still poorly understood. Furthermore, hundreds of mutations have been identified, and it is unclear which of these are important. Recent evidence suggests that BRCA-1 and 2 may play a role in DNA repair and cell cycle arrest, since they associate with the Rad-51 protein, which is involved in the response to DNA damage (36,37). Finally, although original estimates indicated that inheritance of a mutant form of one of these genes led to a lifetime risk of developing breast cancer of 80-90%, more recent data indicates that the risk may be much lower, at 20-40% (38).
It is likely that both susceptibility and resistance genes are present in the human population. Susceptibility genes are those that do not decrease the likelihood of developing a disease when present in the wild-type form, but greatly increase the risk of developing the disease when inherited in a mutated or polymorphic form. An example of such genes are the BRCA genes, which do not confer resistance to breast cancer in women carrying normal copies of the genes, but greatly increase the risk of breast cancer in women carrying mutated copies. In contrast, carriers of resistance genes would be resistant to development of the disease, whereas women who do not carry such a gene would have no predisposition to developing the disease, but would not be resistant. There are currently no genes that have been identified with this property. It is difficult, if not impossible, to study resistance to breast cancer in the human population because we are unable to determine if an individual did not develop cancer due to genetic resistance or because she was not exposed to the hormonal and environmental factors necessary for initiation and promotion of mammary tumors. Identification of resistance genes in the human population would be important in both screening for and potential treatment of the disease. Fortunately, the induction of mammary tumors in the rat provides an excellent animal model with which to study resistance to breast cancer.

1.2. The Process of Chemical Carcinogenesis

Since the rat mammary tumor model makes use of chemical carcinogens to induce tumors, it is important to review the process of carcinogenesis. Chemical carcinogenesis is
a multistep progress, and, in order to better understand the changes associated with tumor development, it is divided into three defined stages (39). The first stage is initiation, which occurs when cells sustain permanent, heritable changes as a result of the action of the carcinogen upon the target tissue. These changes produce a population of altered cells that each have the potential to become a tumor (39). Initiation is a rapid event that may occur when an activated carcinogen interacts with the DNA of a cell and causes formation of a DNA adduct. If the cell repairs this adduct prior to replication, then no heritable change will occur. If the DNA is replicated prior to repair, however, a base pair mismatch may result. Subsequent divisions will then lead to a permanent change in the DNA (39,40). Since this change is permanent, initiation is thought to be a non-reversible event (39,40). In MNU-induced mammary tumorigenesis in the rat, it is thought that mutation of the c-Ha-ras proto-oncogene is the initiating event (see section 1.3.3.) (41).

The second stage of chemical carcinogenesis is promotion (39). This stage occurs when a subpopulation of initiated cells acquire a selective growth advantage over the surrounding tissue as a result of the action of a promotional agent. These agents may act by inducing a differential effect on the initiated cells. For example, the action of a promotional agent may increase the mitotic rate within initiated cells, or inhibit the growth of the surrounding normal tissue, resulting in the development of focal proliferations of preneoplastic cells (40). Promotional agents can be exogenous xenobiotics, as in skin carcinogenesis (42), or endogenous, as in rat mammary tumorigenesis (43), where hormones are thought to provide the promotional stimulus (see section 1.3.2.). Unlike initiation, promotion occurs over an extended period of time and is reversible (i.e., if the
promotional stimulus is removed, then the preneoplastic cells may lose their growth advantage over the surrounding tissue and thus not develop into tumors). Furthermore, promotional agents are usually unable to induce tumors on their own (38).

The third stage of tumorigenesis is progression, in which some preneoplastic cells develop both morphological and biological changes associated with cancer (39,40). It is a lengthy process, with numerous changes occurring, including phenotypic changes (e.g. loss of organization within a focus of preneoplastic cells compared to the normal surrounding tissue) and genotypic changes (e.g. chromosomal instability leading to amplifications, loss of heterozygousity, and deletions). Cells that sustain such changes display the characteristics of cancer such as loss of cell cycle control and uncontrolled proliferation, resistance to cytotoxic drugs, chemicals, and radiation, and the ability to metastasize (40).

1.3. Rat Mammary Tumorigenesis

1.3.1. An Overview of the Rat Mammary Tumor Model

Mammary tumorigenesis in the rat can be accomplished by administration of one of a variety of chemical carcinogens. The most commonly used mammary carcinogens in the rat model are 7,12-dimethylbenz[a]anthracene (DMBA), first described by Huggins (reviewed in (43)), and N-methyl-N-nitrosourea (MNU), first described by Gullino (44). These two carcinogens differ in their mode of action, but both are capable of inducing
multiple mammary tumors in treated animals. Other less commonly used carcinogens to induce mammary tumors in the rat include ethynitrosourea (ENU) (45), 2-acetylaminofluorene (AAF) (46), 3-methylcholanthrene (MCA) (47), and diethylstilbestrol (DES) (48).

Rats are most commonly treated with the carcinogen at 50-55 days of age (49,50). At this time, the animals are actively cycling through estrous, but the mammary gland has not yet reached full differentiation (51). Carcinogen administration induces multiple mammary tumors in the rats, with a latency of approximately 3-6 months, depending on strain, dose, and carcinogen used (49,50). With both DMBA and MNU, the tumors are usually adenocarcinomas and resemble human breast tumors histologically (49,52). Despite this similarity, rat mammary tumors do differ from human tumors in several respects. Unlike human breast tumors, rat tumors rarely metastasize (53), although there are some reports that metastases can develop (44), particularly if the animals are treated with the carcinogen at a younger age (50).

As reviewed in section 1.1.2., a number of oncogenes and suppressor genes have been implicated in human breast cancer. An analysis of some of the more commonly altered genes in human cancer has shown that they are rarely altered in rat mammary tumors. For example, PCR based studies of p53 mutations in DMBA- or MNU-induced mammary tumors showed mutation rates of 2.5% and 0% respectively, indicating that p53 mutations do not play an important role in rat mammary tumorigenesis (54), unlike human breast cancer development. Similarly, the neu (55) and c-myc (56) genes have not been shown to play a role in any rat mammary tumors, either induced or spontaneous. In
contrast, the Ha-ras oncogene is activated in a high percentage of rat mammary tumors induced by MNU (41) and a lower but not insignificant percentage of tumors induced by DMBA (54). Activation of ras is rarely seen in human breast tumors; overexpression is occasionally seen (21,22), although this remains controversial (57). It is likely, however, that ras is important in human breast cancer, since many receptors that are overexpressed in tumors signal through the ras pathway. Telomerase activity seems to be constitutive in both normal and malignant rat breast tissue, indicating that it is unlikely to be important in progression of rat mammary tumors (58). Alterations that are common to both rat and human mammary tumorigenesis include overexpression of the cyclin D1 gene (19,59), and loss of the nm23 gene (57). The cyclin D1 gene in particular may be of importance. This gene, which is normally involved in the regulation of the G1-S transition of the cell cycle, has been shown to be overexpressed in pre-malignant lesions in humans, and is thought to be of importance in the development of rat mammary tumors as well (19,59). In summary, rat mammary tumors resemble human tumors histologically (see section 1.5.2.) and share several common elements at the molecular level.

As a model for human breast cancer development, the rat has several advantages over the other commonly used model in the mouse (60). Unlike human and rat mammary tumorigenesis, mouse mammary tumorigenesis often has a viral etiology (61), due to the presence of the mouse mammary tumor virus (MMTV). As well, mice tend to be more resistant to both chemical and radiation induction of mammary tumors than rats (60,62). Indeed, chemically-induced mammary tumorigenesis in the mouse often requires pituitary isografting in order to provide the promotional stimulus necessary for growth (63), while
the rat model only requires a single injection of the carcinogen without any further exogenous promotion. This makes the rat system easier to use and manipulate. As well, mouse mammary tumors tend to be hormone independent, while many human and most rat tumors are hormone dependent (60,62). Finally, the histology of rat tumors is similar to human breast cancers, particularly the invasive rat mammary tumors (64).

1.3.2. Hormone Dependence of Rat Mammary tumors

Rat mammary tumors induced by DMBA, MCA, MNU, and ENU have all been shown to be hormone dependent (43). As mentioned in section 1.2., it is thought that the promotional stimulus for the tumors is provided by endogenous hormones within the animal (43). A number of studies have shown that hypophysectomy following carcinogen treatment leads to regression of most tumors (43,65,66). It appears that prolactin is the pituitary hormone that is responsible for formation and progression of both DMBA and MNU induced mammary tumors. This finding is a result of observations that prolactin treatment restores growth of tumors in hypophysectomized animals, and that agents that inhibit prolactin secretion inhibit the growth of tumors in intact carcinogen treated animals (43,65,66). Furthermore, growth of mammary tumors is more rapid when prolactin secretion is enhanced (43). There is evidence that prolactin is mitogenic for DMBA and MNU induced mammary tumor cells in vitro (43). In contrast to prolactin, growth hormone, another pituitary hormone, has been shown to have either a slight stimulatory effect on the growth of DMBA-induced mammary tumors or no effect at all, indicating
that it is less important than prolactin (43). Growth hormone does seem to be somewhat more potent, however, in stimulating growth of MNU-induced mammary tumors than those induced with DMBA (65).

In addition to hypophysectomy having a negative effect on the growth of tumors in DMBA- and MNU-treated rats, ovariecctomy also inhibits growth (43,66,67). Administration of estradiol to ovariectomized animals restores growth of the tumors (43,67). Interestingly, administration of prolactin to these animals can also temporarily restore growth of the tumors (43,66). Like prolactin, administration of estrogens, at least in moderate doses, enhances the growth of tumors (43). Unlike prolactin, however, estrogens are not able to restore the growth of tumors in DMBA treated animals that have been hypophysectomized (43). In contrast, the growth of tumors in MNU-treated animals that have been hypophysectomized can be restored by administration of estrogens (43,68). These results indicate that both DMBA- and MNU-induced mammary tumors are dependent on pituitary and ovarian hormones for their growth (43). There is a difference, however, in the sensitivity of the tumors to the various hormones, with DMBA-induced tumors being strongly prolactin dependent, while MNU-induced tumors require both prolactin and estrogens for their growth (43).

1.3.3. MNU as a mammary carcinogen in the rat

MNU is a direct-acting carcinogen, requiring no metabolic activation. At physiological pH, MNU rapidly breaks down in a series of steps to form a
methyldiazonium ion which is electrophilic, attacking DNA, RNA, and protein, leading to methyl adduct formation (69) (Fig.1-1). MNU is water-soluble and for tumor induction can be administered intravenously (44), intraperitoneally (70), or subcutaneously (71), usually at a dose of 25-50 mg/kg. MNU leads to methylation of several sites in DNA, the most common being N²-methylguanine (72). Approximately 70% of detectable adducts in DNA following exposure to a methylating agent such as MNU are N²-methylguanines, since the N² position of guanine is the most nucleophilic site in DNA (73). The N²-methylguanine adduct is not directly mutagenic, although it can be lost by spontaneous depurination, which may lead to base changes. Approximately 80% of tumors induced by MNU harbor the activated Ha-ras oncogene (41), although there is some variation in this percentage depending on factors such as carcinogen dose (74) and diet (75). The activation of Ha-ras is thought to result from the formation of another DNA adduct, O⁶-methylguanine (O⁶-MeG) in codon 12 of the Ha-ras gene (76). If the O⁶-MeG adduct is not removed by repair enzymes prior to DNA replication, a G-T base pair will be formed by DNA replication. A further round of DNA replication will result in a permanent G to A transition, leading to a change in the codon from a GGA to GAA. As mentioned in section 1.2., this is thought to be the initiating event in MNU-induced mammary tumorigenesis. The G to A transition leads to a glutamic acid residue being incorporated into the protein instead of the normal glycine. This amino acid change results in a constitutively active form of Ha-ras, since the glutamic acid residue alters the site where catalysis of GTP (present in active ras) to GDP (present in inactive ras) normally occurs (77). The notion
Figure 1-1. Breakdown of N-methyl-N-nitrosourea into methylidiazonium and methylation of DNA at the O\(^6\) (a mutagenic adduct) and N\(^7\) (the major adduct) positions of guanine by this product.
that *Ha-ras* is activated by MNU adduct formation is further strengthened by studies that have shown that MNU preferentially methylates the middle guanine in 5′-purine-G-(A/T)-3′ sequences (78), which is similar to the GGA sequence of codon 12 in *Ha-ras*. Furthermore, studies in bacteria have shown that MNU leads to G to A transitions in 100% of cases, and that 82% are in the second guanine of 5′-GG(A/T)-3′ sequences (79). Thus, the second nucleotide of codon 12 of *Ha-ras* appears to be a hotspot for O6-MeG formation and hence point mutation.

Another factor that affects the frequency of mutations in *Ha-ras* caused by MNU is the rate of repair of the lesion. Repair of guanine adducts is carried out by the O6-alkylguanine-DNA alkyltransferase protein (80). This protein is an unusual enzyme, since it is inactivated by the repair process it catalyzes. This involves the irreversible transfer of the alkyl group from guanine to a cysteine residue within the active site of the protein (80). The enzyme repairs adducts with an efficiency in the order methyl>ethyl>butyl>>benzyl (80,81). In the case of codon 12 of *Ha-ras*, however, the enzyme is 18 times slower in its repair of the second guanine than the first, indicating that the sequence itself may play a role in determining repair efficiency (82). Indeed, the lower rate of repair seems to be due to the conformation of the sequence in codon 12 of *Ha-ras*, which makes it less accessible to the repair enzyme, presumably due to steric hinderance (82).

It should be noted that there is some evidence for an alternate mode of action of MNU on *Ha-ras*, in which MNU leads to methylation of the promoter of a *Ha-ras* gene with a pre-existing codon 12 mutation, resulting in up-regulation of the gene product (83).
This notion arises from an observation that mutant *Ha-ras* is detectable by PCR in the glands of untreated animals (83). This idea remains controversial, and has yet to be confirmed by any other group. Furthermore, several lines of evidence argue against this mechanism of activation. For example, it was estimated that 1 in $10^5$ cells in the mammary gland would carry the mutated *Ha-ras* gene (83), which would lead to 160-4000 mutant cells in the mammary glands of the animal (84). The spontaneous tumor rate, however, is only 3% in these rats, which is much lower than would be expected with such a large mutant pool (57). Furthermore, no mutant *Ha-ras* alleles were observed in over 100 spontaneous rat mammary tumors (57).

As well as inducing tumors in 7-8 week old rats, MNU has been shown to be capable of inducing tumors in neonatal animals and animals from 21 days of age onwards (50,85), although its potency decreases after the animal reaches 60 days of age (52). Furthermore, MNU is a much less potent mammary carcinogen when administered to parous animals (51). This is thought to be a result, at least in part, of differentiation of the mammary gland following lactation and involution, thereby decreasing the size of the target cell population (see section 1.5.1) (51).

1.3.4. DMBA as a mammary carcinogen in the rat

In contrast to MNU, DMBA (Fig.1-2A) requires metabolic activation in order to form the ultimate carcinogen. DMBA is altered during several enzymatic steps to form a bay region diol-epoxide (Fig.1-2B), which is thought to be the ultimate carcinogenic form
of the chemical. This epoxide that forms is then thought to interact with DNA leading to formation of adducts, particularly at adenines. DMBA is a fat soluble carcinogen, and is usually administered by intragastric intubation at doses up to 100 mg/kg (43,66), although direct dusting of the carcinogen onto the exposed mammary tissue is also occasionally used (86,87). Unlike tumors induced by administration of MNU, DMBA activates the Ha-ras oncogene at codon 61 by inducing an A to T transversion (88). Recent reports provide conflicting data on DMBA activation of Ha-ras, with some reports finding no activation following DMBA treatment (89), while others report an activation rate of approximately 20% (54), which was the initial estimate of the Ha-ras mutation frequency induced by DMBA (90).

Like MNU, DMBA is effective in inducing tumors in animals younger than 30 days of age (50). Although treatment with DMBA leads to similar numbers of tumors as MNU, the number of preneoplastic lesions observed in the glands of animals is much higher with DMBA (52). This observation will be further discussed in section 1.5.3. DMBA, however, seems to produce tumors that are less aggressive in appearance histologically than those induced by MNU (91). Furthermore, as mentioned in section 1.3.2., DMBA-induced tumors are more dependent on prolactin (43). Like MNU, the potency of DMBA decreases as the animals age, such that administration of the carcinogen to animals older than 60 days of age leads to far fewer tumors than observed with the same dose in younger animals (51). Finally, DMBA is less potent in inducing mammary tumors in parous animals, likely for the same reasons as discussed for MNU in section 1.3.3. (51).
Figure 1-2. (A) DMBA and (B) 3,4-diol-1,2-epoxide-DMBA, the ultimate carcinogenic form of DMBA.
1.3.5. Genetic Influences in Rat Mammary Tumorigenesis

One of the most interesting aspects of the mammary tumor model in the rat is the different susceptibilities of inbred strains to tumor induction (49, 87, 92, 93), as shown in Table 1-1. The majority of strains are susceptible to tumor induction. They develop multiple mammary tumors after a single administration of carcinogen, and the tumors have a relatively short latency. Examples of susceptible strains include Sprague-Dawley (SD), Wistar-Furth (WF), and Buffalo (Buf/N) rats (87, 92, 93). A smaller number of strains, such as AC1 and Fischer (F344) rats, are of intermediate susceptibility, developing fewer than two tumors per animal after a long latency following treatment (87, 92, 93). Finally, there are several strains which are resistant to mammary tumor formation. These animals never or rarely develop mammary tumors. Examples of resistant strains include Wistar-Kyoto (WK) (94) and Copenhagen (Cop) rats (87, 92, 93). Of the two strains, resistance in the Cop rat is better characterized. Interestingly, it is possible the decreased sensitivity of the AC1 rat relative to highly susceptible strains may be partly due to the fact that it is derived from a cross of the resistant Cop rat with the susceptible August strain (95).

There is not a strong correlation between development of spontaneous tumors and susceptibility to chemically-induced tumors (60). For example, the F344 strain is intermediate in its response to chemically-induced tumors while SD and WF are susceptible. The F344 rats, however, develop more spontaneous tumors than either SD or WF rats. There is a correlation between development of spontaneous and induced tumors in Cop rats, since they are resistant to the development of both. Finally, there is a strong
Table 1-1: Susceptibilities of different strains of rat to mammary tumorigenesis induced by MNU or DMBA

<table>
<thead>
<tr>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osborne-Mendel</td>
<td>F344</td>
<td>Cop</td>
</tr>
<tr>
<td>WF</td>
<td>Aug</td>
<td>WK</td>
</tr>
<tr>
<td>NSD</td>
<td>ACI</td>
<td>feral</td>
</tr>
<tr>
<td>Lewis</td>
<td>Wistar</td>
<td></td>
</tr>
<tr>
<td>Buf/N</td>
<td></td>
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</tr>
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</table>
correlation between development of spontaneous mammary tumors and spontaneous pituitary tumors \((r=0.99)\), although the number of strains on which information for both types of tumors is available is very small \((n=4)\) \((60)\). This is likely due to the dependance of rat mammary tumors on pituitary hormones and the overexpression of these hormones in pituitary neoplasms.

1.4. Resistance to Mammary Tumorigenesis

1.4.1. The Copenhagen Rat and Mammary Tumorigenesis

The Cop rat is the most widely studied of the resistant strains of rat. Cop rats are resistant to the induction of mammary tumors by a wide variety of means. Injections of MNU or DMBA \((92,93)\), administration of AAF \((46)\) or DES \((48)\), and direct exposure of the mammary gland to DMBA \((93)\) all fail to induce tumors in Cop rats. This resistance is not restricted solely to the breast. It appears that Cop rats may also be resistant to chemically-induced liver cancer \((46,96)\). Cop rats do, however, develop cancers at other sites such as bladder \((48)\) and leukemias \((93)\), indicating that the resistance is not a general phenomenon, but is instead organ-specific.

Although the Cop rat has been known to be resistant for some time, the mechanism of resistance is still not clear. Several possible explanations for their resistance have been examined and excluded. For example, the failure to induce mammary tumorigenesis is not due to a defect in metabolizing the carcinogen to an active state \((93)\),
since MNU is a direct acting carcinogen but does not induce tumors. Furthermore, Cop rats are resistant to hormone-induced (48) and spontaneous mammary tumors (60). As mentioned in section 1.3.2., endogenous hormones are thought to provide the promotional stimulus necessary for growth of tumors (43). Cop rats and susceptible rats, however, have identical hormone profiles for estrogen, progesterone, and prolactin, indicating that hormonal differences are unlikely to be responsible (87). It is also unlikely that there is a defect in the response of these animals to hormones, since they develop normally and are capable of lactation.

From genetic crosses and tumorigenesis studies, it was postulated some time ago that resistance is due to a single autosomal dominant tumor suppressor gene (93) termed the mammary carcinoma suppressor (mcs) gene (97). Two groups conducted similar transplantation studies in which Cop mammary epithelial cells (MECs) were transferred into the cleared mammary fat pads of F1 (Cop × WF) or (Cop × NSD) animals. Direct dusting of DMBA onto those glands did not lead to tumor formation (87,97). If, however, MECs from the susceptible WF rat or a mixture of WF and Cop MECs were transplanted then dusted, tumors developed (97). The researchers concluded that resistance was not due to any systemic effects such as the immune system or paracrine effects (87,97), although this assumption was not entirely correct, as discussed below in section 1.4.2. Furthermore, the resistance could not be due to any diffusible factors, since the mixture of cells would have conferred resistance to the co-transplanted WF cells. Thus, both groups concluded, resistance must be due to a single, non-diffusible, epithelial cell-specific factor in the Cop MECs (87,97).
Several experiments performed in our lab further clarified the nature of resistance in Cop rats. First, it was shown that resistance is not due to higher levels or greater activity of the O\textsuperscript{6}-alkylguanine alkyltransferase enzyme, since the rates of formation and repair of O\textsuperscript{6}-MeG were equivalent in Cop and susceptible Buf/N rats (98). As well, resistance was not due to a defect in the initiation process, since the Ha-ras gene was shown to be activated in the mammary glands of Cop rats at a similar level to Buf/N rats at 30 days post-MNU treatment (99). By 60 days post-MNU treatment, however, the levels of activated Ha-ras in the Buf/N mammary glands had increased, whereas in the Cop rats, there was no increase (99). This result indicated that the putative suppressor gene likely was acting to prevent the expansion of preneoplastic cells. Finally, it was shown that Cop rats are not completely resistant as was previously thought, since tumors developed when the rats were injected as neonates (100). These tumors had a lower level of Ha-ras activation and some were histologically classified as adenosquamous carcinomas, a tumor type not previously seen in this model, indicating that they may have arisen by a different mechanism (101). Thus, our results indicated that the putative mcs gene is probably developmentally regulated and acts after the initiation phase of carcinogenesis.

Further work into Cop resistance by Gould and co-workers has shown that resistance can be overcome by means of retrovirally-mediated gene transfer. Retroviral vectors carrying either an activated Ha-ras gene or the neu oncogene induced a large number of tumors with short latencies when injected directly into the mammary ductal tree (102). Control vectors carrying the neomycin resistance gene did not induce any tumors in
the animals (103). The authors ruled out overexpression of activated \textit{Ha-ras} alone as the tumor inducing mechanism, since the expression levels of the oncogene in tumors were equivalent to or lower than the levels of the wild type \textit{Ha-ras} gene in the normal mammary tissue (103). Since it is thought that at least two hits are necessary to induce tumors (104), it is likely that tumor formation resulted from the cooperation of the the oncogene carried by the vector with a second oncogene activated by insertional mutagenesis by the retroviral vector. Inactivation of a suppressor gene is a less likely event, since that would require insertional inactivation by the retrovirus plus loss of the second allele.

Another study has shown that resistance in Cop rats can also be partially overcome by direct exposure of the gland to crystalline MNU (105). It was found that 100% of SD rats developed mammary tumors if their glands were directly dusted with MNU. In contrast, only 4 out of 11 Cop rats developed mammary tumors by this method, with two of the tumors being non-palpable microcarcinomas discovered when the experiment was terminated (105). It is likely that tumors developed due to the extremely high local dose of carcinogen. It should be noted that direct exposure of the Cop mammary gland to DMBA leads to much less frequent tumor development (10% (87) or less (93)).

Another study has shown that ACI rats are susceptible to mammary tumorigenesis induced by estrogen (106). ACI rats, as mentioned in section 1.3.5., are a strain derived from a cross of Cop with August rats. Spady \textit{et al} showed that treatment of Cop rats with estradiol resulted in pituitary tumors and hyperprolactinemia (107), but no mammary tumors developed, in contrast to ACI rats. Thus, while the Cop rats responded to the
hormone treatment by developing pituitary tumors, they did not respond like susceptible strains which also developed mammary tumors.

More recent work involving large scale breeding and linkage analysis has indicated that the notion of a single suppressor gene is not correct. Instead, it appears that resistance is due to three distinct genetic loci, now called mammary carcinoma susceptibility genes, termed *Mcs-1*, *Mcs-2*, and *Mcs-3* (108). *Mcs-1* is located on rat chromosome 2, and is thought to modulate tumor number in a semi-dominant manner, since there is an effect of gene dosage (i.e., animals homozygous for the *Mcs-1* locus are more resistant than heterozygous animals, which are in turn more resistant than animals which lack the Cop *Mcs-1* locus completely) (108). This raises the possibility that the Cop *Mcs-1* gene may be a defective sensitivity gene or a semi-dominant tumor suppressor gene. *Mcs-2* and *Mcs-3* are located on chromosomes 7 and 1 respectively, and act in a dominant manner to suppress carcinoma formation (108). These two loci are located in large segments of DNA (36 and 30 cM respectively), so there may be more than one resistance locus within each of these linkage groups (108). *Mcs-1*, -2, and -3 appear to act in an additive manner to produce resistance to mammary tumors in Cop animals (108). There is also a fourth locus in Cop rats termed *Mcs-4*, that, paradoxically, seems to act to increase the number of tumors in animals carrying this allele (108). None of these loci correspond to any known human breast cancer suppressor gene locations, although the *Mcs-4* locus corresponds to a region that has previously been implicated in breast cancer in African-American women (108).
1.4.2. Immunosurveillance as a potential resistance mechanism in Cop rats

As mentioned in section 1.4.1., both groups that conducted transplantation experiments of Cop MECs into F1 animals concluded that resistance could not be due to an immune mechanism (87,97). Both groups neglected to recognize, however, that immune recognition depends not only on the immune system of the host, but also on the cells being transplanted. Thus, an immune mechanism could function if carcinogen-exposed MECs from Cop rats presented an antigen while those from WFs did not. This would result in T-cells recognizing the transplanted Cop MECs and removing them, while WF MECs would not be recognized and thus would have the potential to grow into tumors. Such a differential presentation of antigen could be due to the different major histocompatibility complexes (MHCs) of the Cop rat (RT-1\textsuperscript{a}) versus the WF rat (RT-1\textsuperscript{b}) (109). Antigen presentation on MHC molecules is dependent upon the peptide sequence of the antigen (110). It has been shown that a peptide that is not normally presented on the MHC can be presented following point mutations (111). If a common target was mutated in both strains, it is possible that the peptide sequence could be presented on the Cop MHC molecule but not on the WF MHC. Indeed, such a scenario has been observed in the human population. Some melanoma and breast cancer patients have been shown to overexpress a protein known as $MAGE-1$ (112). A fragment of this protein can be presented by the MHC, but only if the patient has the HLA-A1 haplotype, making them ideal candidates for immunotherapy (113).
Several lines of evidence indicated that such an immune mechanism was tenable. First, as mentioned in section 1.4.1., Cop rats are susceptible to mammary tumor formation if they are injected with MNU as neonates (100). It has long been recognized that the immune system of neonates is unique in its response to antigens (114,115). As a result of neonatal MNU treatment, a peptide that normally would be antigenic in adult animals may instead be recognized as a self antigen. Thus, the T-cells that would recognize the antigen would be eliminated from or made tolerant within the animal, and therefore the initiated cells would be free to progress into tumors.

Other indirect lines of evidence also show that the immune system may be capable of acting to induce resistance in the rat mammary tumor model. For example, administration of thymopentin (thymopoetin) to DMBA-treated SD rats not only reversed the thymic atrophy normally caused by DMBA, but also delayed the onset and growth of mammary tumors (116). This result indicates that the immune system may be capable of acting on preneoplastic cells to reduce tumor incidence even in animals that are not normally resistant to tumorigenesis. As well, adoptive transfer of splenocytes from parous donors to virgin female rats has been shown to decrease the tumor incidence in the recipient rats (117). Thus, since the immune system can be demonstrated to have an effect on tumor incidence and latency in the rat mammary tumor model, it is possible that Cop resistance may be at least partially due to an immune mechanism.
1.5. Mammary Developmental Stage and Tumorigenesis in the Rat

1.5.1. Mammary Gland Structure and Development

There are six pairs of mammary glands in the rat that are distributed along the milk line (52). There is one pair in the cervical region, two pairs in the thoracic region, one pair in the abdominal region, and two pairs in the inguinal region of a mature female rat. The nipples are medial in location, with the glands running subcutaneously in a dorsolateral manner into the mammary fat pads of the animal (53).

In newborn pups, there is little difference in the male and female mammary glands. They are comprised of a main lactiferous duct which branches into several secondary ducts, which in turn branch into a tertiary set of ducts (64). Over the first three weeks of life, the gland continues to grow and branch. Most branches are straight, with the occasional lateral bud sprouting from the branch. To aid in the description of the mammary gland, Russo and Russo divide the gland into three zones (see Fig.1-3), depending on their proximity to the nipple and main lactiferous ducts (64). The first region, or zone A, is that closest to the nipple, and consists mainly of the main lactiferous ducts, with occasional lateral buds (64). The next region, of zone B, is intermediate in its location to the nipple, and contains most of the secondary ducts and has abundant lateral buds (64). The most distal region known as zone C contains large, club-shaped terminal structures known as terminal end buds (TEBs) (64). These structures contain rapidly proliferating epithelial cells that are thought to be the targets for the carcinogen (64,118).
Figure 1-3. Schematic of the rat mammary gland at 50 days of age. Zone A contains the primary lactiferous ducts, Zone B contains numerous lobules and alveolar buds (ABs), and Zone C contains numerous terminal end buds (TEBs). Adapted from (50).
The number of TEBs is maximal at 21 days of age (51). With the onset of the estrous cycle at approximately 35 days of age, the gland progresses further into the fat pad, but the number of TEBs continues to decrease. This decrease may be a result of differentiation into alveolar buds (ABs) as the TEBs cleave and septate, or may be due to shrinkage of the TEBs into smaller and thinner structures known as terminal ducts (TDs), which may also differentiate into ABs (51). By 50 days of age, the number of TEBs has decreased from the high of approximately 24/mm² observed at 21 days of age to approximately 5/mm² (51). These structures decrease in number further such that by 65 days of age there are fewer than 2/mm² (51). Full differentiation of the gland into lobular structures and therefore the complete disappearance of TEBs requires the hormonal influence of pregnancy and lactation (49,51,64). Therefore, TEBs are still present in older virgin animals, although their numbers are reduced even further as the animals age.

1.5.2. Mammary Tumor Morphogenesis

As mentioned in the previous section, the target cells for mammary carcinogens are thought to be located in TEBs. There is a strong correlation between the number of TEBs at time of treatment and subsequent formation of tumors (64). For example, the number of TEBs is maximal at 21 days of age (51). Treatment of rats at this age with DMBA or MNU leads to mammary tumor formation (85,119). The MNU-treated animals develop tumors within 1-2 months (85). The authors have suggested that this rapid tumor development is correlated to TEB number (85). It has also been observed that treatment
of animals older than 60 days leads to a much lower rate of tumor formation. Again, this correlates with the number of TEBs, since they are almost absent from the glands of animals of this age (51,64). Finally, it has also been observed that the six pairs of mammary glands in the rat differ in their degree of differentiation, with the thoracic-cervical glands being less differentiated than the abdominal-inguinal mammary chain (64). This also correlates well with tumor data, since several groups report that tumor development occurs more frequently in the thoracic-cervical area than the abdominal-inguinal region (44,64).

Most studies on development of mammary tumors have used the well characterized model in which rats are treated with carcinogen at 50 days of age. At this age, TEBs are still numerous, although not nearly as abundant as at earlier times (51). The majority of the work on tumor development has been done using DMBA (49,64,120-123), although some characterization of changes has been done with MNU as well (85,120). Following DMBA treatment, several changes are evident in the mammary glands of rats compared to untreated, age-matched controls. First, the carcinogen seems to inhibit differentiation, since the treated animals have more TEBs and fewer differentiated ABs than the controls (51). Second, some TEBs approximately double in size following DMBA treatment (52,124). At this point, they are known as intraductal proliferations, or IDPs. They are first evident in the mammary glands of animals approximately two weeks after treatment and exhibit an elevated mitotic rate, a greater number of cell layers around the central lumen, luminal debris, occasional immune cell infiltration, and moderate stromal reaction (53). Furthermore, changes in the cell types within IDPs are evident
compared to TEBs. Within a TEB, there are three types of epithelial cells, namely dark and intermediate cells, characterized by their electron density and morphological characteristics, and myoepithelial cells, which provide mainly structural support for the duct. The composition of a normal TEB is approximately 80% dark cells, 10% intermediate cells, and 10% myoepithelial cells (125). Following carcinogen treatment, the number of dark cells decreases and the number of intermediate cells increases, such that an IDP has roughly 50% dark and 40% intermediate cells (125). Furthermore, there is hypertrophy of the epithelial cells, and an increase in the intracellular spaces (125). IDPs are prevalent in the glands of DMBA-treated animals, with as many as 200 present per animal by 10 weeks post-treatment (52). An examination of IDPs from MNU-treated SD rats indicates that approximately 65% of the lesions contain mutant Ha-ras alleles, consistent with their classification as preneoplastic lesions (126).

It has been postulated that there are two different subsets of IDPs. The first set, the ‘initiated’ IDP (IDP') shows the characteristics of IDPs (i.e., increased size, high proliferation rate) (49,52). The second set, the ‘initiated plus promoted’ IDP (IDP') shows these characteristics as well, but also has a marked stromal reaction with an increased infiltration of lymphocytes and mast cells (49,52). It is thought that it is the IDP' set that will ultimately develop into tumors. Of particular interest is the notion that mast cells, which are increased approximately threefold in IDP' compared to IDP', may provide a growth promoting stimulus for the IDP' (49,52). It has been shown that mast cells can release factors such as heparin and histamine which may in turn stimulate cell proliferation. Furthermore, mast cells may also release factors that stimulate angiogenesis.
Thus, it is possible that the presence of mast cells may provide a proliferative stimulus for IDPs (49,52), although this notion has yet to be confirmed.

The next observable change is the progression of IDPs into ductal carcinoma *in situ* (DCIS). DCIS are similar to IDPs, likely arising from coalescing IDPs, but in addition to the changes mentioned above, DCIS are larger, have more pronounced immune infiltration, and show the development of multiple secondary lumenal spaces and micropapillae (53). The first DCIS usually appear between the third and seventh weeks (52). Like IDPs from MNU-treated animals, DCIS are *Ha-ras* positive, with approximately 90% harboring mutant *Ha-ras* alleles (126).

The final change seen in the tumorigenic process is the appearance of tumors, which are first palpable around seven weeks post-DMBA treatment (53). The epithelial cells within tumors appear to have the same characteristics as observed in IDPs and DCIS, indicating that they probably originate from these structures (53). The tumors show a prominent infiltration of immune cells. The tumors are classified as adenocarcinomas, often with cribriform, papillary, or comedo patterns (53). Different regions of the same tumor can exhibit each of these patterns. Papillary carcinomas show infiltration of lymphocytes and mast cells, particularly within fibrovascular cores that are present in the tumors. These tumors show many papillae or secondary projections towards the lumen (53). Cribriform carcinomas exhibit similar characteristics as papillary carcinomas with strong immune infiltration, but have distinct secondary lumenal spaces that are variable in shape and size (53). Finally, comedo carcinomas are characterized by intraductal epithelial growth and necrotic debris within lumenal spaces (53).
Following DMBA treatment, altered structures also arise from the alveolar components of the gland. These structures are benign lesions such as hyperplastic alveolar nodules (HANs), adenomas, and cysts (124). HANs and cysts are first evident approximately 5 weeks post-DMBA treatment. Neither of these structures exhibit mitoses or infiltration of lymphocytes (124). Adenomas also appear by weeks 5-6, and tend to resemble the proliferating lobules observed during the early stages of pregnancy. Unlike IDPs, DCIS, and tumors, none of these structures show any modification of the surrounding stroma (124). Finally, fibroadenomas occasionally develop, presumably from existing adenomas. These structures have epithelial ducts surrounded by dense, irregular connective tissue. A full overview of the tumorigenic process in the mammary gland as a result of DMBA treatment is shown in figure 1-4.

1.5.3. Morphogenesis of MNU-Induced Mammary Tumors

Most of the changes that occur in the mammary glands of animals treated with MNU are similar to those seen in DMBA-treated animals. There are, however, some notable differences, outlined below.

The first major difference is the number of preneoplastic IDPs found in the mammary glands of treated animals. While the doses of the two carcinogens typically used in these studies produce roughly equivalent numbers of tumors, there seem to be ten times fewer IDPs in MNU-treated rats. Russo and Russo report finding 20-30 IDPs per gland in animals treated with DMBA (52), while Anderson et al report a peak number of 2.2 per
Figure 1-4. A schematic of mammary lesion formation following treatment with DMBA or MNU (DH and DAH form only with MNU administration; all other lesions are common). Solid lines indicate normal differentiation, dotted lines indicate formation of abnormal lesions.
rat (120) and Sakai and Ogawa report 1-3 IDPs per gland following MNU treatment (126). Furthermore, the size of IDPs seems to be different with the two carcinogens. IDPs are reported to be approximately 200μm in diameter when DMBA is used as the carcinogen (52). With MNU induced IDPs, the size tends to be smaller, approximately 130μm, with 200μm IDPs being virtually absent (120). It is unclear why these two carcinogens produce such disparate results at doses that produce roughly equivalent numbers of tumors.

Another major difference in MNU-induced tumorigenesis is that preneoplastic structures also seem to arise from within the ductal components of the gland. As mentioned in section 1.5.2., only benign lesions seem to develop from the ductal or alveolar components of the glands of DMBA-treated rats. In MNU-treated rats, ductally-derived structures known as ductal alveolar hyperplasia (DAH) and ductal hyperplasia (DH) (120) develop that have the potential to form tumors (see Fig.1-4). The main characteristics of DH and DAH are that they appear larger than the surrounding normal tissue, often exhibiting densely staining bulbous endings, much like IDPs. Unlike IDPs, these structures can arise from any regions of the gland, not just the peripheral regions where the majority of TEBs are found (120). Interestingly, the sum total of IDPs, DAH, and DH in rats 6 weeks post-MNU treatment corresponds well with the expected number of tumors for the animal (120). Finally, while HANs are fairly common in DMBA-treated animals, they are rare in MNU-treated animals, with fewer than 1 per animal on average by 9 weeks post-MNU (120). HANs may be precursors for adenomas, which may explain
why adenomas are fairly common in DMBA-treated animals but rare in MNU-treated animals (120).

1.6. Aims and Organization of the Thesis

As described in section 1.3.5., the Cop rat provides a unique opportunity to study resistance to mammary tumorigenesis. This resistance is not a result of differences in carcinogen metabolism, differences in hormones, or a failure of the carcinogen to initiate the target cells in resistant compared to susceptible rats. Instead, resistance seems to be the result of several dominant resistance genes that act to suppress the growth of preneoplastic cells prior to the time they progress into neoplasia. Thus, the Cop rat may allow us to determine the mechanism by which resistance to mammary tumorigenesis occurs. In this thesis two hypotheses were tested, as follows:

**Hypothesis 1**

The first hypothesis is that resistance occurs through a T cell-mediated immuno-surveillance mechanism. The transplantation experiments outlined in section 1.4.1. designed to answer this question neglected to take into account that immune recognition depends not only upon the host immune system, but also upon the cells being transplanted into the animal. Thus, the conclusion that the immune system could not play a role in resistance was premature. We felt that this mechanism was tenable, and even supported by several observations. As a result, we performed experiments designed to answer whether
or not resistance in Cop rats was dependent upon T-cells. Furthermore, we conducted some experiments to determine if NK cells were responsible for the resistance.

**Hypothesis 2**

The second hypothesis of this thesis is that preneoplastic lesions form in the mammary glands of Cop rats but do not progress into tumors. We had evidence that initiation occurs within Cop animals since mutant *Ha-ras* was present in the glands of treated Cop rats. It was, however, unclear whether these cells exhibit any expansion into focal structures or whether they remain as sporadic cells throughout the gland which would eventually be lost. As well, we felt examination of the stages of preneoplastic growth in Cop versus WF animals might provide us with evidence of the mechanism of resistance. For example, growth inhibition or increased cell death in the preneoplastic lesions in the Cop glands relative to the susceptible WF glands could be of importance. Furthermore, examining genes that may be important in tumor development (e.g. *Ha-ras*, cyclin D1) within preneoplastic structures could provide important clues into progression and resistance.

**Organization of the Thesis**

All of the data presented in the thesis are papers either already published or being prepared for publication, with the exception of chapter 5. Chapter 2 describes the experiment designed to answer the questions posed in specific aim 1. In this chapter, we performed a breeding experiment in which Cop rats were crossed with athymic rats which
lack T-cells. By producing F2 animals and then treating both the normal and athymic animals with MNU, we were able to compare the tumor incidences in the two groups to determine if T-cells played any role in resistance. A version of this work was published in Carcinogenesis 18, 53-57, 1997.

Chapter 3 describes the preparation of mammary gland wholemounts from both treated and untreated Cop and WF rats at various times following MNU treatment, and establishes that resistance occurs during the transition from early preneoplastic lesions (IDPs) to more neoplastic lesions (DCIS). Thus the Mcs genes in the Cop likely function by inhibiting the progression of preneoplastic lesions and/or causing their disappearance. This chapter is a version of a paper that addresses some of the questions raised in specific aim 2, and was published in Carcinogenesis 20, 221-227, 1999.

Chapter 4 continues the work done in chapter 3 by examining the proliferation and apoptotic indices within lesions at several time points following MNU treatment. As well, we look at expression of cyclin D1 which has been shown to be overexpressed in rat mammary tumors and we examine mast cell involvement in the lesions, since mast cells have been proposed to have a potential promotional role in the progression of early lesions in DMBA induced tumors. This chapter is being prepared for submission for publication. Finally, Chapter 5 presents a general discussion and future directions for the project.
References


CHAPTER 2

Resistance to chemically-induced mammary tumors in Copenhagen × nude-derived F2 athymic rats: evidence that T-cell immunity is not involved in Copenhagen resistance

James E. Korkola, Geoffrey A. Wood, and Michael C. Archer

(A version of: Carcinogenesis 18, 53-57, 1997)
2.1. Abstract

Resistance to chemically-induced mammary tumors in the Copenhagen rat is well defined, but the mechanism of resistance has yet to be determined. We have tested whether or not Copenhagen rat resistance is dependent on T-cells, since several lines of evidence supported an involvement of the immune system. We crossed Copenhagen rats with an athymic nude rat to produce F1s, that were interbred to produce F2 animals, some of which were athymic with partial Copenhagen rat background. A comparison of the mammary tumor incidences between the nude athymic F2 animals and their non-nude littermates allowed us to determine what role, if any, T-cells played in resistance. Following treatment with N-methyl-N-nitrosourea, we observed no difference in the tumor incidences between the two groups. Furthermore, the mammary tumor incidences in the F2 nude and non-nude animals was almost zero. These results indicate that T-cells are not involved in Cop resistance, and that nude rats are resistant to N-methyl-N-nitrosourea-induced mammary tumorigenesis.

2.2. Introduction

Chemically-induced mammary tumors in the rat have been particularly useful in furthering our understanding of human breast cancer development (1-3). As discussed in section 1.3.5., susceptibility to chemically-induced mammary tumorigenesis varies considerably among different strains of rat. The Copenhagen (Cop) rat is the best characterized strain that is resistant to tumor development (4). A number of previous
studies that have addressed the mechanism of this resistance (4-6) are outlined in section 1.4.1. Of particular relevance to this chapter are the transplantation experiments performed in an effort to determine if systemic effects could account for resistance using Cop and inbred Sprague Dawley rats (NSD) (5,6). These experiments resulted in the conclusion that resistance must be due to an autosomal dominant tumor suppressor gene that acts mainly within the Cop mammary parenchyma and that neither the immune system nor paracrine effects are likely to account for the resistance (5,6).

The results of these transplantation experiments, however, do not rule out an involvement of the immune system in resistance, as discussed in section 1.4.2. Immune recognition and removal of abnormal cells depends not only on the immune system of the host, but also the transplanted cells themselves. Thus, the Cop rat could be resistant because preneoplastic mammary epithelial cells are able to present a fragment of a mutated protein via the major histocompatibility complex (MHC) that leads to their recognition and lysis by cytotoxic T-cells (CTLs). Since the NSD rat expresses a different set of MHC molecules from the Cop rat (RT-1^b and RT-1^a haplotypes respectively) (7,8), the NSD may be incapable of presenting the antigenic peptide fragment. In this case, no CTLs would recognize and lyse the preneoplastic cells, thus allowing them to develop into tumors. CTLs from a Cop × NSD F1 animal would also recognize and eliminate the transplanted Cop cells in an MHC-restricted manner. These F1 CTLs, however, would be unable to eliminate preneoplastic NSD cells, which would therefore develop into tumors in an F1 animal in this model. As outlined in section 1.4.2., several lines of evidence led us to believe that a mechanism for resistance involving the immune system was tenable.
Therefore, we present here experiments designed to show in a definitive manner whether elimination of preneoplastic cells by CTLs in the Cop rat could account for its resistance to mammary tumorigenesis. We crossed female Cop rats with a male athymic nude rat to produce F1 animals that were interbred to produce F2 rats. The F2 rats were treated with MNU to determine their susceptibility to mammary tumor development. Our experiments show that the T-cell component of the immune system does not play a role in Cop resistance.

2.3. Materials and Methods

Animals and Breeding.
Cop, WF, and Buf/N rats, purchased from Harlan Sprague Dawley (Madison, WI or Frederick, MD), were maintained on a 12 h light/dark cycle and were fed 6% fat Harlan Teklad animal diet and water ad libitum. A single nude male rat (Harlan Sprague Dawley, Madison, WI) was crossed with 8 female Cop rats to produce F1 animals. F2 animals were then produced by matings of the F1s. Nude rats were handled only in laminar flow hoods, and were maintained on sterilized Harlan Teklad diet with sterilized water ad libitum.

Carcinogen treatment.
We administered 50 mg/kg MNU (Ash-Stevens, MI) dissolved in acidified normal saline at a concentration of 10 mg/ml to 6 Buf/N, 24 F2 nude, and 26 F2 non-nude rats at 50 to 55 days of age. As well, we treated 15 Cop, 10 WF, 16 F2 nudes, and 20 F2 non-nude rats at 21 days of age with 50 mg/kg MNU as described by Thomson et al (9). Animals were
palpated weekly and sacrificed and necropsied when moribund or when tumors reached approximately 1 cm³. For the 50 day protocol, the study was terminated 8 months post-MNU for the nudes and non-nudes, and 7 months post-MNU for the Balb/N controls. For the 21 day protocol, the study was terminated 5-6 months post-MNU for all groups, except the nudes, which were sacrificed 8 months post-MNU. Portions of all tumors were fixed in 10% formalin for histopathological analysis.

*Flow cytometry analysis.*

Blood collected from the retro-orbital sinus was spun in 5 ml of washing buffer (1% BSA in PBS) in a clinical centrifuge at 1600 rpm. The supernatant was removed and the red blood cells were lysed in a standard red blood cell lysis buffer. The remaining white blood cells were washed in 5 ml washing buffer. The cells were then vortexed and 5 µl of rat serum was added followed by 1 µl of a FITC-labeled anti-CD-5 pan-T-cell specific antibody (Cedarlane, Ontario, Canada). The cells were left in the dark on ice for 30 min, washed in washing buffer, and resuspended in 400 µl washing buffer. Gated cells (10⁴/sample) were then counted on a FACscan flow cytometer (Becton Dickinson, CA).

**2.4. Results and Discussion**

Cop rats were bred with athymic nude rats in order to produce F2 animals that were either athymic (nude) or normal (non-nude), both phenotypes having a partial Cop background. The rationale for this experiment was that the mammary tumor incidences in these F2 animals will depend on whether there is a Cop rat resistance gene with a mode of
action that depends on T-cells, and whether the parental nude rat carries this resistance
gene. If such a T-cell-dependent gene exists, all athymic F2 animals should be susceptible
whether or not they carry the resistance gene, since these F2's would have no T-cells to
eliminate preneoplastic cells. In contrast, the incidence of tumors in the non-nude F2
littermates would depend on whether the parental nude rat carries the resistance gene. At
the time that this experiment was designed and performed, there was considerable
evidence that the resistance of the Cop rat was due to a single gene (2,4). Therefore, we
describe below several possible outcomes of our experiments based on a single gene
model (R for resistant, r for susceptible). Since it is now clear that resistance is due to at
least three loci, the implications for this experiment will be discussed below, in section 2.5.

If we assume that the nude rat is a susceptible strain (i.e., it does not carry the
resistance gene) and the Cop resistance gene is T-cell dependent, then the genotypes for
the F2 animals are as shown in Table 2-1. Since the resistance gene is T-cell dependent, a
normal immune system (Nu for normal, nu for nude athymic phenotype) is required for
resistance. There are sixteen possible genotypes in the F2 population. One quarter of the
rats will be nude (nu/nu) and therefore susceptible. Three quarters of the F2 rats will be
non-nude (Nu/Nu or Nu/nu), and, unlike the nudes, will show a distribution of resistance
and susceptibility. One quarter of the non-nude rats will have the RR genotype and will
therefore be resistant like the parental Cop strain. The non-nude rats that are heterozygous
at the resistance locus (Rr) are likely to be phenotypically similar to F1 animals. In the
past, tumorigenesis studies have shown that a resistant × susceptible mating results in F1
animals that are only 70-75% resistant, possibly because heterozygosity results in a lower
Table 2-1: Gametes of F1 (Cop × Nude) animals and genotypes of F2 animals from an F1 × F1 mating, assuming the parental nude rat does not carry the resistance gene.

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R refers to an allele that confers resistance  
r refers to an allele that confers susceptibility  
Nu refers to normal thymus (non-nude)  
nu refers to absence of thymus (nude)

Table 2-2: Gametes of F1 (Cop × Nude) animals and genotypes of F2 animals from an F1 × F1 mating, assuming the parental nude rat does carry the resistance gene.

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R refers to an allele that confers resistance  
Nu refers to normal thymus (non-nude)  
nu refers to absence of thymus (nude)
titer of the suppressor gene (2, 10) or because loss of the single functional copy of the suppressor gene occurs in a small proportion of cells (2). Finally, one quarter of the non-nudes will have the rr genotype and will therefore be susceptible. In summation, we would expect a mammary tumor frequency of approximately 40% for the non-nude F2's, compared to 100% for the nude F2's in this model.

If we assume the parental nude and Cop rats carry a T-cell dependent resistance gene, then the expected genotypes of the F2 animals are shown in Table 2-2. Since all non-nude rats will have the RR genotype, they will be resistant. The nude F2s will all be susceptible.

Finally, if T-cell immunity is not involved in resistance, the tumor incidences in the two groups should be identical and dependent on the phenotype of the parental nude.

There is some recent evidence to suggest that the resistance of the Cop rat may be a polygenic trait (11). The conclusions of the single gene models described above, however, are still generally valid if there is more than one resistance gene. The athymic nude F2s will still all be susceptible if resistance depends on T-cell immunity, while the non-nude rats will show a complex distribution of susceptibility/resistance phenotypes.

First, we showed by flow cytometry that the nude F2 rats were indeed athymic, since they lacked peripheral T cells (Figure 2-1a) whereas all non-nude littnermates had normal levels of T-cells (Figure 2-1b). We injected nude and non-nude F2 rats and susceptible Bu/ nude rats with 50 mg/kg MNU when the rats were 50 days of age, according to the standard protocol for mammary tumor induction (1). We also used a newly described protocol in which nude and non-nude F2 animals, resistant Cop rats, and
susceptible WF rats were injected at 21 days of age with 50 mg/kg of MNU. This protocol results in rapid mammary tumor development in susceptible rats (9). The results of these experiments are shown in Figures 2-2 and 2-3. Several of the tumors were selected for histopathological analysis and were shown to be adenocarcinomas, as previously described (3,9).

With both protocols, there was no significant difference in tumor incidences or average number of tumors per rat between the nude and non-nude littermates. This shows that T-cells are not involved in resistance. Furthermore, it is clear that in both experiments, the mammary tumor incidence and average number of tumors per rat in the F2 animals was essentially zero, whereas the susceptible control rats developed a high incidence of tumors. As outlined previously, if the parental nude had been either highly susceptible or of intermediate susceptibility to mammary carcinogenesis, the tumor incidences in the F2’s would have been much higher than those observed. Thus our results show that the parental nude rat is resistant to MNU-induced mammary tumorigenesis. The nude rat is derived from outbred hooded rats from the Rowett Research Institute (12) that have not been reported to be resistant. The nude rat is the fifth rat strain we now know to be resistant to chemically induced tumors, along with Cop, Wistar Kyoto (13), spontaneous hypertensive (14), and feral rats (4).

It is unclear whether the resistance of the nude rat, or any of the other resistant strains, is due to the same mechanism as in the Cop rat. Two pieces of evidence, however, support a common mechanism. First, the nude rats, like the Cop rats, are resistant to MNU-induced mammary tumors, indicating that neither of these strains is resistant as a result of a defect in the enzymes of drug metabolism, since MNU is a direct acting
Fig. 2-1. Flow cytometry profile using the pan T-cell specific antibody CD5 showing the absence of peripheral T-cells in a nude F2 rat (panel A) and the presence of T-cells in an F2 non-nude rat (panel B).
carcinogen. Second, we have recently shown that Cop rats are resistant to the development of chemically-induced hepatic preneoplastic lesions using a resistant hepatocyte protocol (15). The male nude rats from the breeding experiments outlined above were also resistant to the development of hepatic preneoplasia using the same protocol (data not shown), indicating that the resistance gene(s) of the nude rat may be similar to that of the Cop rat. Even if the resistance of the Cop and nude rats does not involve the same gene(s), our breeding experiments would have been able to determine if Cop rat resistance is T-cell dependent. The tumor incidence in the F2 nudes (4/16) would have been significantly higher than the tumor incidence in their non-nude littermates (3/48), as shown in Table 2-3.

Also of interest are several of the results of the experiments in which the rats were injected at 21 days of age. This protocol is reported to give rise to mammary tumors in a majority of treated Sprague Dawley rats within one month of treatment (9). While most of the susceptible WF rats we used as controls did develop tumors, they did not appear until 2-3 months after MNU treatment. It seems likely, therefore, that the latency of mammary tumor development in susceptible rats injected at 21 days of age is much more strain dependent than in the standard 50 day protocol. Furthermore, the 20% tumor incidence in the Cop rats treated at 21 days of age was much higher than expected. Since we have shown that Cop rats treated with MNU as neonates have a 28% incidence of mammary tumors (16), the present results suggest that Cop rats become resistant between 20 and 50 days of age. Since the developmental status of the mammary gland is known to be important in determining the susceptibility of the animal (3), it is possible the mammary
Fig. 2-2a Mammary tumor incidence in control BUF/N rats, nude F2 rats, and F2 non-nude littermates following treatment with 50mg/kg MNU at 50 d of age. Numbers in parentheses indicate number of rats with mammary tumors / total number in group. * significantly different (p<0.005) from BUF/N controls ($\chi^2$ analysis), † not significantly different from F2 non-nude rats ($\chi^2$ analysis).

Fig. 2-2b Average number of tumors in control BUF/N rats, nude F2 rats, and F2 non-nude littermates following treatment with 50mg/kg MNU at 50 d of age. Values shown are mean ± SEM. * significantly different from BUF/N controls by Wilcoxon rank sum test.
gland development in the Cop rats that developed tumors was somewhat delayed compared to their resistant littermates. Our findings show that it is unlikely that there is any T-cell involvement in Cop resistance to mammary tumorigenesis. Antibodies produced by B-cells would most likely have eliminated both Cop and WF cells in the F1 animals receiving transplants that were described in section 1.4.1. (5,6), suggesting that B cells are not responsible for resistance. Thus, these various studies indicate that the immune system is not involved in the resistance of Cop rats to mammary tumorigenesis.

2.5. Implications of a three gene model

As noted in section 2.2. and reviewed in section 1.4.1, since the paper that forms the basis for this chapter was published, it has become clear that there are at least three loci that are responsible for resistance in the Cop rat (18). It has been observed, however, that the effects of these loci are additive (18), meaning that a rat with all three loci is more resistant than a rat with two of the three loci, which is in turn more resistant than a rat that carries only one resistance locus. Therefore, if any of these loci require T-cells to confer resistance, it would be expected that all the F2 nude rats would be susceptible to mammary tumorigenesis since the locus requiring T-cells would essentially be non-functional, regardless of the genotype. Since we did not observe any difference in the incidence of mammary tumors between the nude and non-nude F2 rats and the incidences in these groups were essentially zero, our conclusions that T-cell immunity is not responsible for resistance and that the nude rat carries resistance genes are still valid.
Fig. 2-3a Mammary tumor incidence in control WF, Cop, nude F2 rats, and F2 non-nude littermates following treatment with 50mg/kg MNU at 21 d of age. Numbers in parentheses indicate number of rats with mammary tumors / total number in group. a significantly different (p<0.005) from B6/N controls ($\chi^2$ analysis), b not significantly different from F2 non-nude rats ($\chi^2$ analysis).

Fig. 2-3b Average number of tumors in control WF, Cop, nude F2 rats, and F2 non-nude littermates following treatment with 50mg/kg MNU at 21 d of age. Values shown are mean ± SEM. a significantly different from B6/N controls by Wilcoxon rank sum test.
Table 2-3: Gametes of F1 (Cop × Nude) rats and genotypes of F2 rats from an F1 × F1 mating, assuming the parental nude and Cop rats carry different resistance genes and that the Cop resistance gene is T-cell dependent.

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R₄ refers to a T-cell dependent allele carried by Cop rats that confers resistance (not carried by nude rats); Nu refers to normal thymus (non-nude); R refers to a non-T-cell dependent allele carried by nude rats that confers resistance (not carried byCop rats). Cells with squares around them indicate nude (athymic) F2 rats; shaded cells indicate susceptible rats.
2.6. Acknowledgements

The authors wish to thank Sam Kung for assisting with the flow cytometry and staining, Dr. Alan Medline for histopathological analysis, and Dr. Richard G. Miller for valuable discussions.

2.7. References


by the injection of sexually immature female rats with 1-methyl-1-nitrosoourea. *Carcinogenesis*, 16, 2407-2411.


CHAPTER 3

Resistance to mammary tumorigenesis in Copenhagen rats is associated with the loss of preneoplastic lesions

James E. Korkola and Michael C. Archer

(A version of: Carcinogenesis 20, 221-227, 1999)
3.1. Abstract

The resistance of Copenhagen (Cop) rats to mammary tumor development has recently been linked to three loci, but the genes have yet to be cloned and the mechanism of resistance is still largely unknown. In order to determine the cellular events associated with resistance, we prepared mammary whole mounts from Cop and susceptible Wistar Furth (WF) rats 0, 15, 30, 45, and 60 days after treatment with 50mg/kg N-methyl-N-nitrosourea (MNU). At 15 days, treated rats of both strains had significantly more undifferentiated structures (terminal end buds, TEBs) and significantly fewer differentiated structures (alveolar buds, ABs) than untreated rats. Treated Cop rats, however, had significantly more TEBs and fewer ABs than age-matched, treated WF rats. Histological analysis of preneoplastic lesions tentatively identified from the whole mounts showed that like WF rats,Cop rats developed early preneoplastic lesions (intraductal proliferations, IDPs) by 15 days post-MNU treatment. Unlike IDPs from WF rats, however, the IDPs in Cop rats then decreased in number until they were absent 60 days post-MNU treatment. Furthermore, they failed to progress into more advanced lesions such as ductal carcinomas in situ (DCIS). Finally, we found G→A activating mutations in codon 12 of the Ha-ras gene in 60% of IDPs from Cop rats and 75% of IDPs from WF rats. Our results show that resistance in Cop rats is not due to a target cell population for the carcinogen that is smaller than in susceptible rats or to the failure of the carcinogen to inhibit mammary gland differentiation. Furthermore, we have shown that Cop rats develop preneoplastic IDPs that harbor Ha-ras mutations but, unlike IDPs in susceptible strains, they fail to progress and ultimately disappear.
3.2. Introduction

As discussed in section 1.4.2., the pathogenesis of rat mammary tumors follows a well characterized developmental pathway from intraductal proliferations (IDPs) to ductal carcinomas in situ (DCIS) and finally to adenocarcinomas (1, 2). As reviewed in section 1.4.1., activation of the Ha-ras oncogene is found in the majority of N-methyl-N-nitrosourea (MNU)-induced rat mammary carcinomas (3), and may be the initiating event.

Previous results from our lab indicate that the resistance of the Cop rat to mammary tumorigenesis is not due to a defect in initiation, since mutant Ha-ras is present in the glands of treated Cop rats (4), but rather to suppression of the growth of preneoplastic cells. It is not known, however, whether this mutated Ha-ras allele is found only in sporadic cells that fail to grow, or whether the initiated cells are able to form IDPs or even DCIS. Furthermore, if these preneoplastic lesions are capable of forming, it is not clear at what stage their growth is inhibited or whether they are eventually lost. Thus, the goal of this study was to examine the cellular changes that take place in the mammary glands of Cop rats following MNU treatment in order to determine at what stage during carcinogenesis resistance occurs.
3.3. Materials and Methods

Animals and Carcinogen Treatment

Cop and Wistar Furth (WF) rats aged 6-7 weeks (Harlan-Sprague Dawley, Frederick, MD or San Diego, CA) were maintained on a 12 h light/dark cycle and were fed Harlan Teklad rodent diet (6% fat) and water ad libitum. After one week of acclimatization (i.e., at 7-8 weeks of age), they were randomized into 3 groups per strain. The first group of 5 rats were sacrificed prior to carcinogen treatment for whole mount analysis (day 0 controls). The second group of 16 rats were treated i.p. with 50 mg/kg MNU (Ash-Stevens, MI). The MNU was dissolved in 0.05% acetic acid in normal saline at a concentration of 10 mg/ml and used immediately. The third group of 16 rats received vehicle only. At 15, 30, 45, and 60 days post-MNU treatment, 4 rats randomly selected from each of these last two groups were sacrificed for whole mount analysis.

Whole Mount Analysis

The whole mount procedure we used has been described previously by Russo et al (5) and Thompson et al (2). Briefly, rats were anaesthetized using 75 mg/kg ketamine hydrochloride (Rogar/STB Inc., Ontario, Canada)/ 6 mg/kg xylazine (Haver, Ontario, Canada) in normal saline administered i.m., then killed by cervical dislocation. The pelts with the mammary glands still attached were fixed in 10% buffered formalin for 24 h. The glands were dissected from the pelts, defatted in acetone for 2 to 4 days, then hydrated through an ethanol series and left in distilled water overnight on a rocking platform. The
following day the glands were stained in 0.025% toluidine blue (Sigma, MO) for 2 h, rinsed in water and washed in methanol then 70% ethanol for 30 min each. The stained glands were fixed in 4% ammonium molybdate for 30 min, washed in distilled water, then stored in distilled water overnight. The following day, the glands were dehydrated through an ethanol series and stored in xylenes (a mixture of isomers) overnight. They were then sealed in plastic bags containing methyl salicylate, coded and scored for TEBs, alveolar buds (ABs), hyperplastic alveolar nodules (HANs), IDPs, DCIS, and tumors. Numbers of TEBs and ABs from untreated rats (day 0), untreated rats (day 15), and treated rats (day 15) were compared using a two way ANOVA followed by Tukey’s post test.

Histological Analysis

Any structures tentatively identified in the whole mounts as IDPs or DCIS were dissected from the glands. They were cleared in two changes of toluene, followed by two changes in molten paraffin wax before being embedded in paraffin wax. Sections (4μm) were mounted on slides coated with poly-L-lysine (Sigma, MO) and stained with haematoxylin and eosin (H&E) for histopathological analysis. Lesions were identified using the criteria of Russo et al (6) and Sakai and Ogawa (7). Briefly, IDPs were identified by their increased cross-sectional diameter, number of cells and number of cell layers compared to TEBs, as well as by infiltration of inflammatory cells. DCIS were identified by the above criteria as well as the presence of multiple lumenal spaces and a further size increase. For statistical analysis of the number of lesions per mammary gland chain, we performed a two-way ANOVA using strain and time as the variables followed by posthoc comparisons using Tukey’s test.
DNA Isolation and PCR Analysis of mutant Ha-ras

DNA for PCR amplification was prepared from paraffin embedded tissues by a modification of the method of Greer et al (8). Briefly, slides of serial sections from paraffin blocks known to contain IDPs from histological analysis were deparaffinized through two changes of xylenes, rehydrated in 100% ethanol followed by 75% ethanol, then placed in PBS. Lesions on the slides were located under a dissecting microscope, scraped off using a sterile pipette tip and placed in 200 µl of digestion buffer (200 µg/ml Proteinase K, 50 mM Tris-HCl, pH 7.5, 1% Triton-X 100, and 10 mM EDTA), and incubated at 37°C for 2 1/2 hours. After inactivating the Proteinase K by boiling for 10 minutes, the samples were concentrated by precipitation with ethanol using tRNA as a carrier, resuspended in 20 µl ddH₂O, and stored at -76°C prior to use.

For analysis of MNU-induced G➔A mutations at the second nucleotide of codon 12 in the Ha-ras gene, we used the PCR/liquid hybridization and gel retardation assay we have previously described (4, 9), with three modifications. First, we did not pre-digest the DNA with the restriction endonuclease Mnl I, which cleaves the normal but not mutant alleles. Second, the reaction was carried out in a total volume of 50 µl instead of 100µl, using 20 µl of the DNA solution described above. Third, we used Pfu polymerase (Stratagene) instead of Taq or Vent polymerases. Pfu eliminated the background we observed with Taq polymerase (9), presumably due to its higher fidelity and led to higher levels of amplification than Vent. Overnight digestion of 5 µl of the PCR mixture with 1U of the restriction endonuclease Xmn I (New England Biolabs) followed by labeling with a
20mer probe and separation on a 10% polyacrylamide gel (19:1) resulted in 53 and 18 bp bands for samples containing mutant \textit{Ha-ras} and a 71 bp band for wild-type \textit{Ha-ras}.

3.4. Results

We prepared whole mounts from the cervical-thoracic and abdominal-inguinal mammary chains of both resistant Cop and susceptible WF rats 0, 15, 30, 45, and 60 days post-MNU treatment. In preliminary counts prior to histological confirmation of coded samples from both mammary chains, we were able to detect more putative lesions in the abdominal-inguinal region. This was somewhat surprising, since it has been reported that the cervical-thoracic chain is less differentiated than the abdominal-inguinal chain (10), and is known to be more susceptible to tumor formation (11). The cervical-thoracic chain, however, has an associated muscle layer that stains very darkly with toluidine blue making the ductal tree difficult to visualize and analyze. Therefore, we decided to restrict our analysis to the abdominal-inguinal mammary chain. Thompson \textit{et al} (2) analyzed whole mounts only from the abdominal-inguinal region of Sprague Dawley (SD) rat mammary glands for similar reasons. An example of a mammary whole mount from a treated Cop rat 45 days post-MNU is shown in Fig. 3-1.

In susceptible rats, it has been observed that the mammary glands of treated animals are less differentiated than those of age-matched, untreated controls, the treated animals having a larger number of TEBs and fewer ABs (12). We found that there was no difference in the number of TEBs or ABs between the WF and Cop controls prior to
Figure 3-1. A mammary whole mount from the right abdominal-inguinal mammary gland region of a treated Cop rat, 45 days post-MNU. Arrow indicates lymph nodes; arrowheads indicate nipples.
carcinogen treatment (day 0). At 15 days post-MNU treatment, however, mammary glands from both Cop and WF rats had significantly more TEBs/mm² and significantly fewer ABs/mm² than the age-matched untreated rats (Fig.3-2A and 3-2B). Although there were no differences in the number of TEBs/mm² and ABs/mm² in untreated Cop rats compared to age-matched, untreated WF rats at either day 0 or 15, there were significant differences in both these parameters between the treated groups, with Cop rats being less differentiated than the WFs (Fig.3-2A and 3-2B).

An example of two putative IDPs in a whole mount from a Cop rat 15 days post-MNU treatment is shown in Fig. 3-3A. Compared to normal TEBs, these structures are clearly larger and stain more darkly and, indeed, both of these structures were confirmed to be IDPs histologically. A typical histological section of an IDP from a Cop rat is shown in Fig. 3-3B. The lesion is ~180 μm in diameter, has some areas 7-10 cell layers thick, and contains lumenal debris, all of which are characteristics of IDPs (6, 7). Two DCIS in a whole mount of a WF rat 45 days post-MNU are shown in Fig.3-3C. These structures still retain the club-like shape of TEBs and IDPs, but are significantly larger (~250 μm in diameter) than the IDPs. A histological section of a DCIS from a treated WF animal is shown in Fig. 3-3D. In addition to its larger size, the main characteristic that distinguishes this lesion from an IDP is the presence of distinct multiple lumenal spaces. As well, there is an inflammatory reaction in the stroma, which is also characteristic of these lesions (6).

We next quantified the IDPs and DCIS present in the abdominal-inguinal mammary chain of all of the rats sacrificed on days 15, 30, 45 and 60 days following MNU administration. We microdissected from the whole mounts, embedded in paraffin, and
Fig. 3-2. (A), number of TEBs/mm² and (B), ABs/mm² in Cop and WF rats from untreated (day 0) and untreated (day 15) and MNU-treated (day 15) animals. All bars not sharing the same letter differ significantly from each other (p<0.05). t=treated, u=untreated. To calculate the values, wholemounts from 4 or 5 animals were counted per time point, with 5-10 5mm² fields counted per wholemount.
sectioned suspected lesions from the 16 Cop and 16 WF rats. This analysis showed the presence of 36 IDPs, 1 DCIS, and 2 cysts in the Cop rats and 46 IDPs, 17 DCIS, 1 fibroma, 1 adenocarcinoma, and 3 cysts in the WF rats. The average diameter of the IDPs was 150 ± 6.1 μm for the Cop rats and 160 ± 12 μm for the WF rats with average cell numbers of 200 ± 12 and 210 ± 12 respectively (all values are means ± SEM). Anderson et al (13) classified terminal structures larger than 130 μm as abnormal in SD rats, in good agreement with our findings for IDPs. The average diameter of all the DCIS in WF rats was 370 ± 52 μm, whereas the one DCIS we observed in a Cop rat was 600 μm in diameter. We did not observe any IDPs in the untreated controls at any of the time points. Furthermore, we did not observe HANs in the treated rats at any time points, although we did observe cysts that arise from the same alveolar structures as HANs (13). In agreement with our observations, in rats treated with MNU, neither Sakai and Ogawa (16) nor Thompson et al (2) reported finding HANs, while Anderson et al (13) found fewer than 1 HAN per rat.

A time course for the appearance of histologically confirmed preneoplastic lesions (IDPs and DCIS) is shown in Fig. 3-4 for both strains. At 15 and 30 days post-MNU treatment the lesions were exclusively IDPs. There was an average of 2 DCIS in each of the WF rats at 45 and 60 days post-MNU. In contrast, we observed only one DCIS in all of the Cop rats and that appeared at day 60. It is clear that IDPs are present in the mammary glands of Cop rats 15 days post-MNU treatment. Moreover, the number of IDPs in Cop rats was not different from WFs at this time. Unlike the WF rats, however,
Fig. 3-3. (A) Example of an IDP from a Cop rat 30 d post-MNU treatment in a mammary whole mount (IDP indicated by solid arrow and a normal TEB indicated by open arrow; magnification, ~20X, alum carmine stain (see 4.4.3) and (B), an H&E stained section of an IDP from a Cop rat, 15 d post-MNU (arrow indicates an apoptotic cell; magnification, 250X). Examples of DCIS from a WF rat 45 days post-MNU treatment in (C), a mammary whole mount (magnification, ~12X) and (D), an H&E stained section (magnification, 125X).
the number of lesions in the Cop rats decreased at each subsequent time point until there
were significantly more preneoplastic lesions in the WF rats at 60 days post-MNU than in
the Cop rats (p<0.01). Furthermore, the number of lesions at this time in the treated Cop
rats was not significantly different from the untreated controls, which had no lesions. It
should be noted that Russo and Russo (1) have reported that 6-10 weeks post-DMBA
treatment, as many as 30 IDPs are present per gland pair in SD rats, whereas the greatest
number we observed was 2 per gland pair. It is likely that this difference is due to the
different carcinogens used, since others (6, 13) have reported that preneoplastic lesions
are less prevalent in the glands of MNU-treated rats than in those treated with doses of
DMBA that produce similar numbers of tumors.

We analyzed both Cop (Fig.3-5A) and WF (Fig.3-5B) DNA isolated from sections
of IDPs for G→A mutations at the second nucleotide in codon 12 of the Ha-ras gene.
Normal liver DNA was used as a negative control (lane 1, figs. 3-5A and 3-5B) and DNA
from an MNU-induced mammary tumor that contained a mutated Ha-ras allele was used
as a positive control (see lane 2, figs. 3-5A and 3-5B). Further controls included a sample
with no added DNA to show that there was no contamination of any components of the
reaction mixture (see lane 3, figs. 3-5A and 3-5B) and DNA from two TEBs isolated from
the wholemount of a 50 day old untreated WF rat (see lanes 4 and 5, Fig. 3-5B). Neither
of these TEBs contained a mutated Ha-ras allele. We found that Ha-ras was mutated in 6
of 10 IDPs from Cop rats (Fig. 3-5A, lanes 4-13) and in 6 of 8 IDPs from WF rats (Fig.3-
5B, lanes 6-13).
Fig. 3-4: Number of preneoplastic lesions (IDPs and DCIS) in COP and WF animals at various times following MNV treatment. n = 4 animals per time point.
Fig. 3-5. (A), *Ha-ras* analysis of IDPs from Cop rats. Lanes 1-3: negative control (normal liver DNA from a WF rat), positive control (mammary tumor DNA from an MNU-treated SD rat), and blank control (no DNA) respectively. Lanes 4-13: DNA samples amplified from sections confirmed to be IDPs. (B), *Ha-ras* analysis of IDPs from WF rats. Lanes 1-3: negative control (normal liver DNA from a WF rat), positive control (mammary tumor DNA from an MNU-treated SD rat), and blank control (no DNA) respectively. Lanes 4 and 5: DNA samples amplified from sections containing normal TEBs. Lanes 6-13: DNA samples amplified from sections confirmed to be IDPs.
3.5. Discussion

In order to further our understanding of the mechanisms underlying genetic resistance to breast cancer, we compared the cellular structure of the mammary glands of susceptible WF rats and resistant Cop rats before and after carcinogen treatment. TEBs contain the most rapidly proliferating cells in the mammary gland and these cells are thought to be the targets for the carcinogen (5). Thus, resistance could be caused by Cop glands having fewer TEBs. We showed, however, that untreated Cop rats had the same number of TEBs as untreated, age-matched WF rats prior to carcinogen treatment. Mammary gland differentiation has been shown to be inhibited in susceptible rats following DMBA treatment (12) but the state of differentiation following treatment of resistant animals is unknown. Different rates of mammary gland differentiation could affect the growth and progression of initiated cells. Our results show, however, that differentiation is inhibited in both strains following carcinogen treatment. At 15 days post-MNU treatment, the number of TEBs and ABs per mm² in both strains of treated rats was significantly different from untreated controls, the treated rats, as expected, being less differentiated. Unexpectedly, differentiation in the resistant Cop rats was inhibited to a greater extent by the carcinogen than in the susceptible WF rats. We conclude that resistance in Cop rats is not due to either a smaller target cell population for the carcinogen compared to susceptible rats or to the failure of the carcinogen to inhibit mammary gland differentiation.
We next measured the appearance of IDPs, the first clearly observable preneoplastic lesions that develop in the rat mammary gland (1, 5). In susceptible strains, a subpopulation of IDPs are thought to progress to form DCIS and ultimately adenocarcinomas (1, 5). Our results clearly show the presence of IDPs in the abdominal-inguinal mammary glands of both WF and Cop rats 15 days post MNU treatment. Indeed, at this time point, there were approximately the same number of IDPs in the Cop rats as in the WFs. The number of IDPs in the Cop glands, however, decreased with time until none could be detected at day 60. In contrast, the number of IDPs in WF mammary glands increased to day 30, then remained roughly constant to day 60. At this time the number of lesions was significantly higher than in Cop rats. Moreover, we observed an average of two DCIS in the mammary glands of WF rats on days 45 and 60, whereas we saw only one DCIS in 16 Cop rats, and that was detected at day 60. We conclude that the putative mcs genes discussed in section 1.4.1 do not prevent formation of IDPs in Cop rats, but rather cause the loss of IDPs and/or inhibit their development.

We have previously shown that DNA from whole mammary glands of Cop rats contains Ha-ras mutations 30 days post-MNU treatment (4). Since approximately 65% of IDPs from MNU-treated SD rats contain Ha-ras mutations (7), it seemed likely that presence of similar mutations in IDPs from Cop rats would account for our previous observations. Although we could not achieve amplification of DNA from all of our IDP sections, we were able to show that 6 of 10 IDPs from Cop rats and 6 of 8 IDPs from WF rats contained a mutated Ha-ras gene. These numbers are in good agreement with the results of Sakai and Ogawa (7) and indicate that the mutated Ha-ras genes that we
previously observed were probably present in IDPs. This notion is strengthened by our observations of similar kinetics for the appearance and disappearance of IDPs in this study and of mutant Ha-ras alleles in the previous study (4).

One of several mechanisms may account for the disappearance of IDPs in Cop rat mammary glands. First, it is possible that initiated cells within the preneoplastic lesions are removed by an immunosurveillance mechanism. We believe this is unlikely, however, since we have recently provided evidence that T-cell immunity is not involved in resistance to mammary tumorigenesis in Cop rats (14). Second, it is possible that there is a difference in the kinetics of cell proliferation and loss in the IDPs between susceptible and resistant strains. In order for growth of a lesion to occur, the proliferative rate must be greater than the rate of cell loss and, indeed, the proliferative rate in IDPs has been shown to be higher than in TEBs (1). If the increased proliferation rate in IDPs of Cop rats is not maintained such that cell death (apoptosis) then occurs at a higher rate, the lesion will shrink in size. Similarly, if the apoptotic rate in Cop lesions increases so that it becomes greater than the proliferative rate, then the IDP will be eliminated. This type of mechanism accounts for the inhibition of growth of micrometastases during the suppression of angiogenesis when the apoptotic rate increases sevenfold compared to non-inhibited tumors (15). We are currently measuring the proliferation and apoptotic rates to determine if these factors play a role in Cop resistance. A third potential mechanism to account for IDP loss is remodeling, a process that may account for the loss of preneoplastic foci in the liver (16). According to this mechanism, preneoplastic cells are not lost, but rather redifferentiate to yield a more normal phenotype. This reversion is thought to occur when the promoting
stimulus is withdrawn (16). Redifferentiated cells are capable of being promoted to form preneoplastic hepatic foci again if the promoting stimulus is restored (17). In the mammary gland the major stimulus for tumor development is provided by estrogenic hormones (18). The levels of these hormones in Cop rats, however, have been shown to be similar to those in other strains (19). Thus, if remodeling occurs, it is unlikely to be caused by a lack of promotional stimulus. Finally, it has been postulated that different subsets of IDPs may exist (1). The first set, known as IDP (i), are initiated but not promoted and do not progress to form tumors in susceptible animals. Instead, tumors ultimately form from a second set of IDPs that are initiated plus promoted (IDP (i+p)). One characteristic that distinguishes these two types of lesions is the inflammatory response, with IDP (i+p) having a more marked mast cell and lymphocyte infiltration in the surrounding stroma than IDP (i). Indeed, there appears to be a threefold increase in mast cells surrounding IDP (i+p) compared to IDP (i) (1). Furthermore, Russo has postulated that mast cells may play a role in promoting the growth of IDP (i+p), since these cells can release factors which stimulate cell division and angiogenesis (1). If lesions in Cop rats do not elicit a mast cell response, the IDPs may not be exposed to necessary factors that stimulate tumor formation. Studies investigating the possibility that there is a differential mast cell response in the IDPs of Cop rats compared to those of WFs using are presented in Chapter 4.

The formation of mammary tumors in the rat has been reported to follow a well-defined developmental pattern. IDPs arise from TEBs and progress to DCIS and eventually adenocarcinomas (1). Alternative mechanisms of tumor formation in proximal
regions of the gland via ductal hyperplasia (DH) and ductal alveolar hyperplasia (DAH) have been suggested (13). While most of the lesions we observed were at the periphery of the gland where TEBs are located, some IDPs and DCIS were found in regions in which alveolar buds were more prevalent than TEBs. We still classified these as IDPs and DCIS based on their histopathology, but it is possible that these are the same structures that Anderson et al (13) describe as DH and DAH. In support of this notion, Anderson et al (13) reported that many DAH and DH have large bulbous endings, much like IDPs, possibly leading to confusion in their classification. Neither Thompson et al (2) nor Sakai and Ogawa (7) observed DH or DAH in the glands of animals treated with MNU.

In summary, we have shown that resistance to mammary carcinogenesis in Cop rats is not due to either a target cell population that is smaller than in susceptible rats or to a failure of the carcinogen to inhibit mammary gland differentiation. Furthermore, our results show that Cop rats develop preneoplastic IDPs that harbor mutated Ha-ras alleles, but these fail to progress to form more advanced lesions such as DCIS and ultimately disappear.

3.6. Acknowledgements

The authors wish to thank Dr. Jose Russo for his valuable discussions and Diana Booth for assistance in the preparation and sectioning of samples.
3.7. References


Chapter 4

Changes in the proliferative and apoptotic indices and cyclin D1 levels in preneoplastic mammary lesions from resistant and susceptible rats

James E. Korkola and Michael C. Archer
4.1. Abstract

We have recently shown that resistance to mammary tumorigenesis in Cop rats is associated with loss of early preneoplastic lesions (IDPs). The cause of this disappearance, however, is unknown. We describe here experiments designed to examine several potential mechanisms that could be responsible for this phenomenon. We prepared mammary whole mounts from Cop and WF rats at 20, 30, and 37 days post-MNU treatment. In agreement with our previous findings, there were no differences in the number of lesions in Cop or WF mammary glands 20 or 30 days post-MNU, but at 37 days, there were significantly fewer lesions in the Cop glands. Furthermore, the lesions in the Cop glands were exclusively IDPs, whereas in the WF glands, more advanced lesions such as DCIS and tumors were also present. We estimated proliferation within the lesions based on BrdU incorporation, and apoptosis based on morphology within H&E stained sections. There were no significant differences in the labeling indices at either 20 or 30 days, but the BrdU labeling index in Cop IDPs at 37 days was significantly higher than in WF IDPs. This was at least partially offset by an increased apoptotic index within Cop IDPs relative to those in WF rats at this time. We also examined the infiltration of mast cells in proximity to the lesion, since mast cells have been implicated in the progression from IDP to DCIS. We found no differences in the number of these cells surrounding IDPs between Cop and WF rats at any time, indicating they are unlikely to be involved in Cop resistance. Finally, we measured cyclin D1 expression immunohistochemically. We observed high levels of expression in 4/5 DCIS and 3/3 tumors but only in 4/17 IDPs from WFs, and in no IDPs from Cop rats. Thus, cyclin D1 overexpression may be of importance in the transition from IDP to DCIS, and may be related to resistance in Cop rats.
4.2. Introduction

As discussed in chapter 3, we prepared mammary wholemounts from susceptible WF and resistant Cop rats at various time points to observe the cellular changes that occur in the two strains following MNU treatment. Both strains developed early preneoplastic lesions known as intraductal proliferations (IDPs), but only those in WF rats progressed into more advanced lesions such as ductal carcinomas in situ (DCIS) and adenocarcinomas. In contrast, the IDPs in Cop rats, which were equivalent in number to IDPs in the glands of WF rats at 30 days post-MNU, subsequently decreased with time until lesions were essentially absent from the mammary glands by 60 days post-MNU treatment.

It is unclear at this point what causes the disappearance of IDPs within Cop rats and hence resistance to mammary gland tumorigenesis. Several mechanisms could potentially explain this resistance. First, a decrease in the proliferation rate and/or an increase in the apoptotic rate in Cop IDPs compared to WF IDPs may lead to the disappearance of the Cop lesions. Second, mast cell infiltration within the lesions could be involved in resistance, since mast cells may promote the growth of some IDPs (see section 1.5.2.). Finally, as discussed in sections 1.1.2. and 1.3.1., overexpression of cyclin D1 within lesions could be of importance, since overexpression of this gene is known to be an important event in the progression of human breast preneoplasias to neoplasias (1). Overexpression of cyclin D1 has also been shown to occur in rat mammary tumors (2). Thus, the goal of this study was to examine these potential mechanisms for the
disappearance of IDPs in Cop rats and, therefore, resistance to mammary tumorigenesis.

4.3. Materials and Methods

Animals and Carcinogen Treatment

Cop and WF animals (6-7 weeks old) were purchased from Harlan Sprague Dawley (Madison, WI). Animals were maintained on a 12 h light/dark cycle and fed Harlan Teklad rat chow (6% fat) and water ad libitum. After one week of acclimatization the rats were given an i.p. injection of 50 mg/kg MNU dissolved in acidified normal saline. The rats for the final time point (37 days post-MNU treatment) were treated separately from the rats at the other two time points (20 and 30 days post-MNU treatment) and thus the data from these animals were analyzed separately. Five WF and five Cop rats were used for each time point.

BrdU treatment and Mammary Wholemount preparation

At 20, 30, and 37 days post-MNU treatment, 5 rats from each strain were randomly selected. The rats were given an i.p. injection of 50 mg/kg BrdU dissolved in PBS. Three hours later, they were killed and mammary wholemounts prepared, using the technique we described previously (see section 3.3.), with the exception that for some glands, alum carmine was used as the stain instead of toluidine blue.
Paraffin Embedding, Staining, and Immunohistochemistry

Putative lesions in the wholemounts were microdissected from the glands, cleared in xylenes (15 min.), processed through three changes of paraffin wax, and then embedded in paraffin wax for sectioning. Sections (4 µm thick) were placed on poly-L-lysine (Sigma, St. Louis, MO) coated slides and stained with H&E to identify lesions. Positive identification of IDPs, DCIS, and adenocarcinomas was based on the criteria we used previously (see section 3.4.) and as published elsewhere (3,4). Serial sections from confirmed lesions were then used for BrdU immunohistochemistry. For BrdU staining, the slides were deparaffinized in two consecutive changes of xylenes. Following deparaffinization and rehydration in two changes of 100% ethanol, the slides were immersed in 3% H₂O₂ in methanol for 20 min. The sections were then washed in tap water and distilled water, and then immersed in 20 µg/ml Proteinase K in 10 mM TrisHCl (pH 8.0) for 20 min at room temperature. The slides were washed in tap water and then placed in 4 N HCl for 20 min. Following washes in distilled water and PBS, an anti-BrdU antibody (Boehringer) was applied to the sections. The sections were then left overnight at 4°C in a humid chamber. The next day, the slides were washed in three changes of PBS, and then a goat anti-mouse antibody conjugated to horseradish peroxidase (Santa Cruz) was applied to the sections. The slides were incubated for 1 h in a humid chamber at room temperature. After incubation with the secondary antibody, the slides were washed in three changes of PBS then stained in a 0.5 mg/ml 3,3-diaminobenzidine solution in 50 mM
TrisHCl (pH 7.5) for 10-12 minutes. The slides were washed in PBS and running water, then counterstained in haematoxylin for 3 min. After a further wash in running water, the slides were dehydrated through an ethanol series, cleared in 2 changes of xylenes, and mounted using permount (Fisher). BrdU labeling index (an estimated measure of the proliferative index) was then determined by the number of BrdU positive cells divided by total cells in a lesion. Small intestine from BrdU-treated rats or livers from partially-hepatectomized rats were used as positive controls for staining. For negative controls, 1% normal sheep serum was applied to the section instead of the anti-BrdU antibody.

For cyclin D1 staining, the samples were deparaffinized and immersed in 3% H2O2 as above. The sections were washed in PBS, then microwaved in 10 mM Citrate buffer (pH 6.0) for 4 min at high power, which was sufficient to boil the samples for the final ~10s. The sections were then microwaved for 7 min at a lower setting, resulting in bursts of boiling every 30-45 s. The sections were left to cool in the buffer for 20 min, followed by washes in distilled water. A diluted anti-cyclin D1 antibody (Santa Cruz) was then applied to the sections, which were incubated overnight in a humid chamber. Following washes in PBS, the secondary goat anti-mouse IgG antibody (Santa Cruz) conjugated to horseradish peroxidase was applied and incubated as described for BrdU staining. DAB staining, haematoxylin counterstaining, and mounting were also as described for BrdU staining. Archival mammary tumor tissue was used as a positive control, since overexpression of cyclin D1 has been reported in mammary tumors (2).

For mast cell staining, samples were deparaffinized in xylenes, rehydrated in acetone followed by distilled water, then stained in 0.025% toluidine blue (Sigma,
St. Louis, MO) for 30 seconds. Slides were washed in distilled water, dehydrated in acetone, cleared in xylenes, and mounted using permount (Fisher). For the mast cell staining, some archival samples from the study described in Chapter 3 were also used (30 days post-MNU treatment).

Statistical Analyses

For comparison of numbers of lesions at 20 and 30 days post-MNU treatment, a one way ANOVA was used followed by post-tests. For the day 37 data, numbers of lesions were compared by the Mann-Whitney rank sum test. Analyses of BrdU labeling indices, apoptotic indices, and numbers of mast cells were done by ANOVA (20 and 30 days post-MNU rats) or Student’s t-test (37 days post-MNU). Correlations were determined using Pearson’s test.

4.4. Results

As discussed in Chapter 3, we have recently shown that both Cop and WF rats form preneoplastic lesions following MNU treatment, but unlike lesions in WF rats, those in Cop rats fail to progress into more advanced lesions and eventually disappear. We repeated this experiment, preparing mammary wholemounts from Cop and WF animals at 20, 30, and 37 days post-MNU treatment. Lesions developed in both strains as we had previously observed. At 20 and 30 days post-MNU treatment, the number of lesions in Cop rats was not different from the number in WF rats (Fig. 4-1a). By 37 days post-MNU, however, there were significantly fewer lesions in the glands of Cop rats (Fig. 4-1b).
Furthermore, we observed only IDPs in the glands of Cop rats, while more advanced lesions such as DCIS and tumors were also present in the glands of WF rats at 37 days, in agreement with our previous observations. To illustrate the striking differences apparent in the mammary glands of treated rats from the two strains, we show the same region of the inguinal mammary gland in Cop (Fig.4-2a) and WF (Fig.4-2b) rats 37 days post-MNU treatment. Multiple lesions, including DCIS, are clearly visible in the WF gland, while no lesions are obvious in the Cop gland.

We next stained samples using an anti-BrdU antibody to estimate the proliferative index in the IDPs and DCIS from both strains. An example of a lesion in the transition phase from IDP to DCIS (from a WF rat, 37 days post-MNU) stained for BrdU incorporation is shown in Fig.4-3. Cells positive for BrdU and, therefore, cells that are actively synthesizing DNA, have brown nuclei and appear as the brown or dark black cells in Fig.4-3. Note also the presence of mitotic figures, as indicated by the open arrow in Fig. 4-3. The labeling index was determined as the number of BrdU positive cells divided by the total number of cells in the lesion. The labeling indices in the Cop IDPs were not different from those in WF rats at either 20 or 30 days post-MNU treatment (Fig.4-4a). Surprisingly, at 37 days post-MNU, the BrdU labeling index was significantly higher in the Cop IDPs than in the WF IDPs (4-4b).

In the same lesions that we determined the BrdU labeling indices, we also counted apoptotic cells. We first attempted to label apoptotic cells using a TUNEL staining method that labels cells with DNA strand breaks. We found that all of the cells labeled
Fig. 4-1a Number of preneoplastic lesions in the mammary glands of treated Cop and WF rats at 20 and 30 days post-MNU treatment (n=5 animals per time point).

Fig. 4-1b. Number of preneoplastic lesions in the mammary glands of treated Cop and WF rats 37 days post-MNU treatment. (* significantly different, p<0.05; n=5 animals per time point).
Fig. 4-2. Appearance of the same region of the inguinal mammary gland of a (a) WF and (b) Cop rat at 37 days post-MNU treatment. Note the presence of lesions (see arrows) in the gland of the WF rat, while none are obvious in the gland of the Cop rat. Magnification ~7.5X.
using this method, even when we diluted the antibody to levels that gave faint staining in
the sections. This ubiquitous labeling was likely due to DNA degradation in our samples
that may have resulted from the numerous fixation, staining, destaining, clearing, and
dehydration steps during the wholemount and tissue embedding procedures. In view of
this problem, we identified apoptotic cells by their characteristic morphology (cell
shrinkage leaving a “halo” around the cell, chromatin condensation, and pyknotic nuclei)
(Fig.4-5). The apoptotic indices are shown in Fig. 4-6a and 4-6b. As with the BrdU
labeling indices, we found no significant differences between the Cop and WF rats at either
20 or 30 days post-MNU treatment. There was, however, a significant difference in the
apoptotic index at 37 days post-MNU, with the Cop rats showing higher indices than the
WF animals.

If the increased apoptotic rate within Cop lesions at 37 days is responsible for
maintaining the lesions in a steady state with respect to cell numbers, there must be a
positive correlation between BrdU labeling and apoptotic index within each lesion. A plot
of apoptotic index versus labeling index for IDPs from Cop rats 37 days post-MNU
treatment is shown in Fig. 4-7. There was a positive correlation between the two indices
by the Pearson correlation test (p<0.05).

We next determined the number of mast cells around a lesion per high powered
field of view, using the same magnification for each sample. Mast cells were visualized
using toluidine blue that stains mast cells metachromatically. Mast cell infiltration around a
lesion is shown in Fig. 4-8. We found no significant differences in numbers of mast cells
between the Cop IDPs and the WF IDPs at any of the time points (Fig. 4-9a,b).
Fig. 4-3. BrdU incorporation into a lesion from a WF rat (37 days post-MNU treatment). Arrows indicate BrdU positive cells, open arrow indicates mitotic figure. Magnification 250X.
Fig. 4-4. BrdU labeling indices in Cop and WF rats at (a) 20 and 30 days post-MNU treatment and (b) 37 days post-MNU treatment. (* significantly different than WF IDPs (p<0.05); D=DCIS, T=tumor)
Fig. 4-5. An example of an IDP from a WF rat with a cell undergoing apoptosis, as indicated by the arrow. Magnification 250X.
Fig. 4-6. Apoptotic indices in Cop and WF lesions at (a) 20 and 30 days post-MNU and (b) 37 days post-MNU. (* significantly different from WF IDPs, p<0.05; D=DCIS, T=tumor).
Fig. 4-7. Correlation between apoptotic indices and BrdU labeling indices for individual Cop IDPs, 37 days post-MNU. Pearson $r=0.5857$, $p<0.05$. 
Fig. 4-8 Example of mast cell infiltration around a tumor from a WF rat 37 days post-MNU treatment. Arrows indicate mast cells. Magnification 250X.
Fig 4-9. Number of mast cells around Cop and WF IDPs at (a) 20 and 30 days post-MNU treatment and (b) 37 days post-MNU treatment.
Finally, we determined cyclin D₁ expression in the lesions immunohistochemically (e.g., Fig.4-10). The staining, which was characterized as absent (-), very weak (+/-), weak (+), moderate (++) , and strong (+++), is summarized in Table 4-1. We observed significant cyclin D₁ staining in 9 out of 17 WF IDPs, with 4 out of 17 showing high levels of cyclin D₁. In contrast, only 2 out of 9 IDPs from Cop rats showed significant cyclin D₁ staining. Furthermore, we saw high levels of cyclin D₁ expression in 4/5 DCIS and 3/3 tumor samples.

4.5. Discussion

We have recently shown that preneoplastic mammary lesions known as IDPs develop in both resistant Cop and susceptible WF rats following treatment with MNU. The IDPs in the Cop rats, however, do not progress into more advanced lesions, but instead disappear with time. Here we investigate potential mechanisms which may cause the disappearance and/or removal of lesions in Cop rats. Rats were killed at various times following MNU treatment. Three hours prior to sacrifice, we administered BrdU.

We did not observe any differences in the labeling indices of the Cop IDPs compared to those in WF rats at either 20 or 30 days post-MNU treatment. This result was not surprising, since there were no significant differences in the number of lesions in the mammary glands of these two strains at these times, indicating that they were probably growing at similar rates. Furthermore, there were no differences in the apoptotic indices in IDPs between Cop and WF rats at these times. At 37 days post-MNU treatment, however,
Fig. 4-10. Example of a lesion from a WF rat showing high levels of cyclin D1 expression.

Brown cells indicate positive staining. Magnification 250X.
Table 4-1: Summary of cyclin D1 expression levels in individual IDPs from Cop and WF rats and DCIS and tumors from WF rats, 37 d post-MNU.

<table>
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<th>Cop IDPs</th>
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<th>WF tumors</th>
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the labeling index in IDPs was significantly higher in the resistant Cops compared to the susceptible WFs. This result was unexpected, since there were significantly fewer lesions in the Cop rats than in the WF rats at this time. Furthermore the lesions in Cop rats were decreasing in number at this time, in agreement with our previous findings. We did find, however, that the apoptotic index of Cop IDPs at 37 days post-MNU was higher than in IDPs of WF rats at this time. Thus, it seems that the higher proliferative index within Cop IDPs is at least partially offset by the increased apoptotic index. This notion is further supported by our finding that there was a positive correlation between the apoptotic indices and proliferative indices in Cop lesions at this time. It is unclear, however, whether the increased apoptosis is sufficient to cause the disappearance of IDPs in Cop rats, or merely maintains them in a steady state condition with respect to cell number. Indeed, the maintenance of lesions in a steady state by increased apoptosis has been demonstrated in a different system. In micrometastases that have been inhibited from growing by anti-angiogenic factors, there is no change in the proliferative index relative to non-inhibited tumors (5). There is, however, an increase in apoptosis relative to non-inhibited tumors that balances the proliferative rate, resulting in a steady state with respect to cell number within the micrometastases. To determine if increased apoptosis could lead to disappearance of the lesions, however, would require an accurate measurement of apoptosis over the same period of time as the proliferative labeling. Unfortunately, unlike proliferation in which pulse labeling with BrdU or tritiated thymidine can be used, there is no method currently to stain apoptotic cells that have accumulated over a period of time. Furthermore, it is possible that small, undetectable increases in the apoptotic rate relative
to the proliferative rate could have a large effect over a long period of time, leading to loss of lesions, but without a reliable measure, would be undetectable. Thus, while it is possible that the loss of preneoplastic lesions in the Cop rat may be due to the increased apoptosis, there currently is no method to test this hypothesis definitively.

As discussed in section 1.5.2., it has been postulated by Russo that there are two populations of IDPs (6,7). Initiated plus promoted IDPs (IDP [i+p]), are able to form more advanced lesions such as DCIS and tumors (6,7), whereas the IDPs that are only initiated (IDP [i]) are unable to progress. IDPs [i+p] can be distinguished from IDPs [i] by the infiltration of mast cells, which are three times more abundant around the former (6,7). Russo has postulated that these mast cells may be involved in the promotion of growth of the lesions, either by the secretion of mitogenic or angiogenic factors. We showed, however, that there were no differences in the numbers of mast cells surrounding IDPs between Cop and WF rats at any time point. Thus, it seems unlikely that mast cells play any role in the resistance of the Cop rat.

Overexpression of cyclin D1 has been reported in both human (8) and rat mammary tumors (2). Recently, it has been shown that cyclin D1 overexpression may be a critical early event in human mammary tumorigenesis, since overexpression of this gene is common in early lesions of the breast that form cancers, but not in those that form benign lesions (1). Cyclin D1 overexpression has not been investigated in preneoplastic mammary lesions of the rat. If cyclin D1 overexpression is an early event in tumorigenesis of the mammary gland, differences in the expression of this gene in WF versus Cop rats could account for the differences in susceptibility to mammary tumorigenesis seen in these rats.
By immunohistochemistry, we did not observe any cyclin D1 staining in IDPs at 20 or 30 days post-MNU treatment. We found cyclin D1 overexpression in IDPs at 37 days post-MNU treatment. This overexpression was more frequent in the IDPs of WF rats than in those of Cop rats. This was somewhat surprising, given the higher proliferation rate in the Cop IDPs, although it has previously been observed that cyclin D1 overexpression does not correlate with the proliferation rate in rat mammary tumors (2). Furthermore, 4/5 DCIS and all tumors from WF rats showed high levels of staining for cyclin D1. This result indicates that cyclin D1 overexpression may be of importance in the progression of IDPs to DCIS and adenocarcinomas. Indeed, we did not observe any Cop IDPs with high levels of expression of the gene, and we have only observed a single DCIS in a total of 31 MNU-treated Cop rats from this and our previous study.

We have previously speculated that remodelling of lesions may play a role in resistance (see section 3.5.). This notion is based primarily on results from the liver that show normal loss of preneoplastic lesions occurs by a process of redifferentiation or remodeling (9). Our lab has shown that Cop rats are resistant to liver carcinogenesis (10). Furthermore, we have recently shown that the rate of remodeling in Cop liver lesions is much higher than in lesions of susceptible rats. Remodeling is also likely to occur in the mammary gland, since reversion of chemically-induced mammary tumors is occasionally observed. We have no method however, to measure remodeling within the mammary gland. Since lesions are initially identified by their size relative to normal structures, a lesion that has begun to remodel and is shrinking would likely be excluded from our initial screening. As a result, we are unable to detect remodeling, and this hypothesis remains
In conclusion, we measured several parameters that could be potentially involved in resistance of the Cop rat to mammary tumorigenesis. We confirmed our previous finding that preneoplastic lesions in Cop rats form but fail to progress into more advanced lesions and instead disappear. We measured the proliferative and apoptotic indices within lesions of both WF and Cop rats. Surprisingly, we found that the labeling index was higher in Cop lesions than in WF lesions at a time when Cop lesions are disappearing. It appears, however, this is offset by an increased apoptotic index in the Cop lesions relative to WF lesions. It is unclear whether the increased apoptosis merely maintains the Cop lesions in a steady state with respect to cell number, or whether it leads to the disappearance of the lesions. We also measured mast cell infiltration around the lesions of both strains, but found no differences at any of the time points. Finally, we observed expression of cyclin D1 in a high percentage of IDPs from WF rats, but in a low percentage of IDPs from Cop rats. Cyclin D1 overexpression was evident in 4/5 DCIS and all tumor samples examined, indicating the potential importance of this event in the transition from IDP to DCIS. Furthermore, the overexpression of this gene may be of importance in relation to the resistant phenotype of the Cop rat.

4.6. Acknowledgements

The authors wish to thank Dr. Henry Thompson for discussions on BrdU staining of IDPs and Dr. Alan Medline for his help with identification and staining of mast cells.
4.7. References


Chapter 5

Discussion and Future Work
5.1. Overview

This thesis describes experiments designed to elucidate the phenotypic characteristics of resistance to mammary tumorigenesis in the Cop rat. The first goal was to determine if the immune system, specifically the T-cell component, is involved in resistance. The second goal was to examine the cellular changes that occur in the mammary glands of Cop rats in order to determine at what stage resistance occurs. The investigation of cellular changes in the mammary glands also allowed us to examine several potential resistance mechanisms.

Chapter 2 of this thesis describes the experiments designed to determine whether resistance to mammary tumorigenesis in Cop rats involves the immune system. Imnosurveillance has long been postulated to be a general mechanism for the elimination of any abnormal cells that have the potential to form tumors from an organism. There is evidence both for and against the hypothesis that the immune system can play an important role in susceptibility to tumorigenesis. For example, nude mice and rats which are athymic and therefore T-cell deficient, have not been shown to be more susceptible to tumorigenesis in general than non-nude animals (e.g. (1)). In contrast, studies in the 1960's found that neonatal thymectomy enhanced tumorigenesis in normal animals (2,3). These may be cases, however, in which the immune system plays an important role in susceptibility to a specific carcinogen. For example, perforin deficient mice, which have an impaired cytotoxic T-cell response, are more susceptible than wild-type mice to MCA-induced tumors but do not differ in their susceptibility to DMBA-induced skin tumors (4).
A number of tumor-specific antigens that may provide potential targets for recognition by the immune system have been identified. Of particular relevance to the MNU- and DMBA-induced mammary tumorigenesis model is the Ha-ras gene, which, as discussed in section 1.3.1., is activated by point mutation in ~80% of MNU-induced mammary tumors and up to 20% of DMBA-induced tumors (5). There is the potential that these mutated Ha-ras proteins may elicit an immune response since they differ from the wild-type protein. In support of this notion, a number of studies have shown that mutant p21ras peptides do indeed elicit an immune response (6-8). Interestingly, although immunization of mice with synthetic mutant ras peptides leads to formation of peptide-specific T cells, not every peptide was immunogenic in every strain of mouse, indicating that there may be a differential response depending on genetic background (7). Thus, some animals may be resistant to tumorigenesis initiated by a particular ras mutation, while others may be susceptible to the same mutation. These results suggested that Cop rats may be resistant to mammary tumorigenesis as a result of the elimination of mutant Ha-ras positive cells by the immune system, while other strains are susceptible to the same activating mutation.

F2 nude rats, derived from F1 rats produced by crossing Cop rats with nude rats, would be more susceptible to mammary tumorigenesis than their non-nude littermates if T cells were involved in resistance, since the nude rats lack the T cells that would be necessary to eliminate the preneoplastic cells. Treatment of such animals with MNU showed that they were resistant to mammary tumorigenesis. The finding that both nude and non-nude F2 animals are resistant to mammary tumorigenesis clearly shows that
resistance is not functioning through a T cell mediated mechanism. This result does indicate, however, that the parental nude strain is resistant to mammary tumorigenesis. The nude rat, which is derived from the Rowett hooded rat, is the fifth strain of rat shown to be resistant, along with Cop, Wistar-Kyoto, spontaneous hypertensive, and feral rats.

NK cells have also been postulated to play an important role in general immunosurveillance. For example, studies have found rats and mice with decreased NK activity are more likely to develop lung metastases than animals with normal levels of NK activity (9). Similarly, animals with increased NK activity have fewer lung metastases than those with normal levels of NK activity (9). NK cells may recognize tumors that have downregulated MHC I expression. Carrageenan has been reported to inhibit NK cell activity when administered to mice (10). To determine if NK cells are involved in Cop resistance, we administered carrageenan chronically to MNU-treated Cop rats. This regimen failed to induce tumors in the Cop rats. Unfortunately, this method does not eliminate NK cells completely, but instead partially inhibits their activity. A better alternative would have been treatment with an antibody such as anti-asialo GM1 that would have led to a greater decrease in NK cell number. Such an experiment, however, would have been prohibitively expensive due to the large amount of antibody required to treat groups of animals chronically. Thus, while the carrageenan experiment provided evidence that NK cells are not involved in Cop resistance, it was not definitive. Indeed, our finding that the nude rats are resistant to MNU-induced tumorigenesis may support a role for NK cells in resistance, since nude animals are known to have elevated levels of NK cells (9).
There are other components of the immune system that could play a role in the resistance of Cop rats to mammary tumorigenesis. Recently, there has been a great deal of interest in the innate immune system and its role in activating the immune response. The innate immune system differs from the acquired immune system in that its response is fairly non-specific, using germ-line encoded receptors for recognition of families of antigens such as carbohydrates that are commonly expressed on bacterial cell membranes (11). The innate immune response may also be induced when distress is detected within a tissue (11,12). For example, distress may occur because of necrotic cell death resulting from viral infection or overgrowth of a small preneoplastic lesion. Unlike apoptotic death in which the cell is usually phagocytosed, necrotic cell death leads to lysis of the cell, such that intracellular proteins that are not usually presented to the immune system become available to the 'professional' antigen presenting cells such as dendritic cells (12). The dendritic cells can then activate either T or B cell responses to proteins expressed by the abnormal or infected cells within the stressed tissue, leading to their removal. Alternatively, a stressed cell could upregulate proteins that mark the cell as stressed, such as heat shock proteins, that again could be recognized by dendritic cells, resulting in an immune response (12). Indeed, it has been postulated that tumors may escape immune recognition by inhibiting both the migration and activation of dendritic cells (13). Another cell type that may also be of importance in immune surveillance of tumors is the macrophage. In addition to a possible role in antigen presentation, these cells may have direct tumoricidal activity through their nitric oxide synthase pathway (14). In support of this idea, it has been shown that many tumors secrete substances such as transforming
growth factor-β or phosphatidylserine that inhibit the activity of nitric oxide synthase, rendering the macrophage less cytotoxic and thereby protecting the tumor cells (14). Reactive nitrogen intermediates from macrophages appear to kill cells by apoptosis, leading to speculation that tumor cells can also protect themselves from macrophage-induced cell death by overexpression of anti-apoptotic genes such as Bcl-2 (15). Finally, another component of the innate immune system that may be involved in immune surveillance of tumors is complement. It has been found that C3, a major effector of the complement system, can bind to some proteins found on the surface of malignant but not normal cells (16). Once bound, C3 will induce opsonization and a rapid macrophage and dendritic cell response (16). This in turn could lead to direct cytotoxicity as a result of macrophage activation or a T or B cell response through antigen presentation by the macrophages or dendritic cells. Thus, several components of the innate immune response have been shown to have potential anti-tumor activity, raising the possibility that one of these mechanisms may be involved in Cop resistance.

Chapters 3 and 4 of this thesis provide the first definitive evidence that resistance to mammary tumorigenesis in Cop rats occurs during the promotion-progression phase of carcinogenesis. Our lab had previously speculated that resistance in Cop rats developed during the post-initiation stage of carcinogenesis by preventing the sustained clonal expansion of cells harboring an activated Ha-ras oncogene (17). We have now shown that this interpretation is correct, since preneoplastic lesions (IDPs) that are likely to be clonally derived do develop in Cop rats following MNU treatment, but unlike IDPs of susceptible strains, do not progress into more advanced lesions such as DCIS and instead
disappear. Thus, it seems the putative Mcs genes of the Cop rat function by preventing the promotion/progression of IDPs into DCIS and/or cause their disappearance.

Our finding that preneoplastic lesions form but disappear from the mammary glands of resistant rats has important implications for human mammary tumorigenesis. It is thought that initiation occurs in the human breast during the period of time between menarche and the completion of the first full term pregnancy (18). Both resistant and susceptible women are likely to be exposed to carcinogens in the environment. Hence, it is likely that initiation may occur in the breasts of many women. Clonal expansion may occur in both resistant and susceptible women, with early, preneoplastic ductal tissue forming. Indeed, examination of normal breast tissue has shown that abnormal structures such as hyperplastic terminal ducts and lobules with nuclear atypism are rare but present, although they are less prevalent than in breast tissue isolated from cancer-associated breasts (18). Thus, preneoplastic lesions are likely to be present in women who will develop breast cancer as well as in those who will not. It is possible that a resistance mechanism exists in humans that is analogous to that in Cop rats. While lesions in some women will progress into tumors, lesions in other women will be prevented from progressing and instead will regress and/or disappear.

We have shown that the level of expression of the cyclin D1 gene may be of importance in the progression of IDPs to DCIS. In WF rats 37 days post-MNU treatment, we found all tumors and 4 out of 5 DCIS showed high levels of cyclin D1 staining. While 9 out of 17 WF IDPs stained positively for cyclin D1, only 4 had levels as high as observed in the DCIS. Interestingly, the number of IDPs that stained positively for cyclin D1 was
lower in Cop than in WF rats at the time when IDPs in Cop rats were disappearing from the glands despite the higher proliferation rate in the Cop rats. Overexpression of cyclin D1 has previously been observed in rat mammary tumorigenesis, and is thought to be of importance in tumor development (19). Furthermore, it has been shown that overexpression of cyclin D1 does not correlate with the proliferation rate within tumors (19), in agreement with our finding. Overexpression of cyclin D1 has been observed in a number of tumor types including breast cancer. This overexpression can result from amplification of the DNA, chromosomal rearrangement, point mutations, or retroviral integration (20). In the case of chemically-induced rat mammary tumors, the mechanism of overexpression is not known, although gene amplification does not seem to be responsible (19). The wild-type protein is rapidly degraded and thus overexpression may be a result of stabilization of the protein (19). Overexpression of cyclin D1 can be induced by overexpression of oncogenic ras proteins, with the concomitant downregulation of the cyclin dependant kinase (CDK) inhibitor, p27 (21). Overexpression of cyclin D1 in transgenic mice has been shown to lead to mammary hyperplasia and tumor formation (22). Furthermore, cyclin D1 overexpression can cooperate with oncogenic ras to transform cells (23). Thus, cyclin D1 overexpression seems to be of importance in the development of mammary tumors in both rodents and humans.
5.2. A Model for resistance to mammary carcinogenesis in Cop rats

The main objective of this thesis was to understand the mechanism by which Cop rats are resistant to mammary tumorigenesis. While we have a better knowledge of the cellular events associated with resistance, the molecular mechanism is still not clear. We are, however, able to formulate a model to explain resistance in the Cop rat based on previous findings and the advances described in this thesis.

Work done by Gould and co-workers has established that at least three genes are responsible for resistance (24). The first of these genes, \textit{Mcs-1}, functions in a gene dosage manner. Thus, a rat with one copy of this allele will be more susceptible than a rat with both copies of the gene present. This has led to speculation that \textit{Mcs-1} may not be a suppressor gene, but instead a defective susceptibility gene in the Cop rat. Furthermore, it has been observed that the actions of the three \textit{Mcs} genes are additive, with all three copies required for complete resistance. Therefore, in a Cop rat, tumor induction would require a cell to sustain an inactivating mutation in one of the three alleles as well as activation of an oncogene, likely \textit{Ha-ras}. If \textit{Mcs-2} or \textit{Mcs-3} is inactivated, then loss of the second allele would also be necessary. Obviously, the frequency of such an event would be vanishingly small. Instead, two populations of cells likely arise. The first population contains cells that have mutations in one of the \textit{Mcs} genes. These cells, however, lack an activating mutation, so would likely be unable to progress. The second population contains an activating mutation, such as oncogenic \textit{Ha-ras}. These cells would start to expand, forming IDPs. During promotion by prolactin and estrogens, overexpression of
activated Ha-ras may occur. Thus, it seems likely that overexpression of cyclin D1 results from the overexpression of Ha-ras in the mammary glands of MNU-treated rats, and that the overexpression of these two genes leads to transformation of the cells. What is unclear, however, is how Cop rats avoid this overexpression and whether it is of importance in Cop resistance. It has been observed that in response to ras activation, the tumor suppressor gene and CDK inhibitor p16\textsuperscript{\textit{NK4a}} can be induced, leading to G\textsubscript{i} arrest (25). Furthermore, it has been postulated that blockage of the G\textsubscript{i}-S transition by p16\textsuperscript{\textit{NK4a}} can lead to p53 mediated apoptosis in the cell (26). Mice lacking the p16 gene have been shown to be highly prone to carcinogen-induced tumorigenesis (27). Thus, one possible explanation for resistance in Cop rats is that oncogenic ras overexpression leads to p16\textsuperscript{\textit{NK4a}} mediated growth arrest and apoptosis in the mammary epithelial cells rather than overexpression of cyclin D1. If p16\textsuperscript{\textit{NK4a}} is induced in Cop IDPs along with p53, the apoptotic cell death could eliminate the preneoplastic cells and therefore prevent the progression into DCIS and tumors. This could explain why we observed a higher apoptotic rate in the IDPs from Cop animals compared to those of WF rats at 37 days post-MNU treatment. I am currently investigating this possibility by examining p16\textsuperscript{\textit{NK4a}} expression in Cop and WF IDPs by immunohistochemistry. A diagramatic representation of this speculative tumor development model is shown in figure 5-1.

Neonatal Cop rats, as discussed in section 1.4.1., are susceptible to mammary tumorigenesis induced by MNU. This raises the possibility that the Mcs genes may be developmentally regulated. Thus, the lesions induced by neonatal treatment could have progressed too far for the Mcs genes to prevent tumor formation by the time that they become active. Alternatively, inactivation of one of the Mcs genes in a mammary cell...
Figure 5-1. A model for the resistance of Cop rats to mammary tumorigenesis (Est= Estrogen, Prl=Prolactin)
followed by expansion into a focus of cells may occur. Since tumors form in these animals without any further carcinogen treatment, it is possible that the cells also acquire a second mutation that enables the growth of tumors. The frequency of tumors, however, is higher in animals given a second treatment of MNU, indicating that a second carcinogen exposure enhances tumorigenesis, presumably by activating an oncogene in the expanded pool of cells that has previously sustained damage to the Mcs gene(s).

As discussed in section 5.1., an alternate model is that the immune system is responsible for resistance in the Cop rat. Recognition of tissue distress may activate the innate immune system to eliminate preneoplastic lesions from the Cop mammary gland. In this model, it is possible that the Mcs genes could code for determinants required by the innate immune system for recognizing the stressed cells.

5.3. Future Work

Phenotypic characterization of this model is now virtually complete. Several candidate genes could be examined such as the CDK inhibitor p16 discussed in section 5.2., although it would be difficult to determine if the expression of such a gene was a cause or an effect of resistance. Furthermore, the potential role in resistance of the cyclin D1 gene in Cop rats could be examined, possibly by overexpressing the gene in Cop rats as a mammary-specific transgene to determine if this renders the animal sensitive to MNU-induced tumorigenesis. As well, it may be important to examine the infiltration of other inflammatory cells such as macrophages and dendritic cells in the Cop and WF IDPs. The
primary goal of the next phase of the project, however, should be to clone the \textit{Mcs} genes. When this is accomplished, we should be able to clearly define the mechanism by which the resistance occurs in the Cop rat. More importantly, we will be able to examine the involvement of the genes in humans to determine if they play a role in resistance to breast cancer.

There are several ways in which the \textit{Mcs} genes could be cloned. The classical approach, already in progress by Gould and co-workers (24), is to identify the genes using linkage analysis. This involves producing backcrosses and multiple intercross generations (F2, F3, F4, etc.) of rats from resistant Cop rats and a susceptible strain (WF). The resultant rats are then treated with carcinogen, and scored for tumor development. From animals that do not develop tumors, regions of Cop DNA are identified that are common to all resistant animals using DNA markers that are polymorphic between the Cop rats and WF rats, and genes are isolated from these regions and examined for their involvement in resistance. This approach is extremely laborious and costly. Furthermore, the number of genetic markers available in the rat is much smaller than in either mice or humans, making the cloning of the genes much more difficult since the distance between markers is large. Alternative approaches to the cloning of these genes, however, are available.

The first approach is to use retroviral tagging to isolate suppressor genes. A number of oncogenes that are activated as a result of retroviral integration have been identified from common integration sites in tumors. Identification of suppressor genes, however, is much less likely using this approach because inactivation of suppressor genes typically requires both alleles to be inactivated. The likelihood of such an event is low. If
F1 animals from a cross between Cop and WF rats are produced, then only one copy of the suppressor gene will be present at each locus. Therefore, inactivation of the suppressor gene is much more likely in these animals, since only one integration is necessary. These F1 rats have been shown to be almost as resistant as the parental Cop rats (24,28). The slightly lower resistance is likely due to the heterozygosity of the Mcs-l allele, since there is a gene dosage effect, as discussed in section 1.4.1. Tumors that develop following treatment with a retrovirus carrying the Ha-ras oncogene would be excised and DNA isolated from part of the tumor, while cell lines would be established from another part to provide a large pool of DNA. Probing the DNA with a retrovirus-specific probe should result in the identification of common integration sites. Common integration sites could then be cloned and cDNAs identified using genomic probes isolated from the common integration sites, as discussed below. Using this approach, the identification of suppressor genes that have become inactivated as a result of retroviral integration should be possible. A potential problem with this model is that the parental Cop rats also develop tumors when treated with retroviruses carrying activated Ha-ras. Thus, activation of an oncogene that cooperates with ras to overcome the activity of the suppressor genes seems to occur at a high frequency. Identification of common integration sites may therefore result in the cloning of oncogenes that can act in concert with activated ras to overcome the activity of the Mcs genes. Indeed, it is likely that a large number of the tumors from F1 animals would contain activating instead of inactivating mutations. However, it is still possible that the identification of suppressor genes will result from this approach, since there could be integrational hotspots within the suppressor genes. Furthermore, it is possible that tumors
develop in retrovirally-treated Cop rats as a result of integration into a suppressor gene, followed by loss of the second allele.

I have already tested the feasibility of the retroviral approach. Initially, I tested a retroviral construct (provided by Y. Ben-David, Sunnybrook Health Science Centre, Toronto) for its tumor forming ability in susceptible WF rats. This construct is a replication-defective retrovirus that contains the v-Ha-ras gene inserted between spleen focus forming virus (SFFV) long terminal repeat (LTR) arms packaged in an ecotropic packaging cell line. As discussed in section 1.5.1, it has previously been shown that injection of an amphotropic retrovirus carrying activated Ha-ras within Moloney LTRs into the mammary ductal tree via the nipple of rats resulted in mammary tumor formation (29). This injection into the nipple allows the direct exposure of the mammary epithelial cells to the retrovirus. I was able to induce mammary tumors in 4 out of 4 WF rats that were injected with the virus in this way. Following the success of the tumor induction in susceptible rats, I bred WF rats with Cop rats to produce F1 animals, which are heterozygous at the suppressor gene loci, as described above. These animals were subsequently treated with the virus. To date, 5 of 9 F1 animals have developed mammary tumors, and cell lines are being established from the tumors excised from these animals. A further 6 F1 animals have recently been treated with the retrovirus as well. The next step will be to determine whether or not common integration sites exist within these tumors. This will involve the isolation of clones that contain retroviral sequence from a genomic library. Next, a flanking piece of genomic DNA from the positive clone will be isolated and used as a probe to look for common integration sites in other tumors. Rearrangements
in multiple tumors detected using the genomic probe will indicate the presence of common integration sites. If common integration sites are identified, then the flanking probes will be used to probe Northern blots in order to detect transcriptional products. Probes that detect transcripts will be used to isolate the clones of interest from a Cop mammary cDNA library. This approach should result in identification of mammary tumor suppressor genes or oncogenes.

The second approach to cloning the *Mcs* genes involves the use of genetic suppressor elements (GSEs). GSEs are short, random pieces of cDNAs. When GSEs are introduced into a non-transformed cell line, they may lead to transformation by one of two mechanisms. First, the GSE may be a fragment of the antisense strand of a suppressor gene (30). Transcription of the GSE will result in expression of the antisense strand which will bind to the sense strand of the suppressor gene, thereby ‘knocking out’ the gene by preventing its translation. Second, small segments of a suppressor protein may be translated into protein. These fragments may in turn bind to the wild-type protein and inactivate it in a dominant negative fashion (30). Indeed, random fragments of the *p53* gene have been shown to block the activity of wild-type *p53* in a cell line, leading to its transformation (31). This approach has recently been used to clone a novel tumor suppressor gene known as *ING1* (32). Introduction of a GSE library by retroviral infection into a non-transformed cell line led to the formation of tumors when the infected cells were introduced into nude mice. Isolation of the GSE insert showed that an antisense strand to a gene was responsible for the transformation. Subsequent analysis revealed that the sense strand could suppress the growth of transformed cells in soft agar assays (32).
I propose that this method be used as a complimentary in vitro approach to cloning the suppressor genes from the Cop rat. This is particularly important since the retroviral approach may only lead to the identification of oncogenes. The first step will be to create a Cop rat mammary GSE library. In order to reduce the number of GSEs produced, Cop cDNAs could be enriched by PCR-based subtraction using WF cDNAs, such that cDNAs that are expressed only in the Cop mammary glands are isolated. I feel that the cDNAs from Cop animals should be isolated from the glands of carcinogen-treated Cop rats, since it is unclear whether the suppressor genes are active in normal mammary or not. The enriched cDNAs will then be randomly fragmented to produce a GSE library. Following production of the library and packaging into a retroviral vector, a Cop mammary epithelial cell line will be infected with the GSE library. The infected cells will then be injected into nude mice. As discussed above, tumor formation could occur as a result of inactivation of a suppressor gene either by introduction of an antisense cDNA strand or a dominant-negative cDNA fragment. Tumor development will allow the isolation of the GSE that caused transformation from the tumor cells. This GSE could then be tested in soft agar assays to confirm its transforming abilities. Following confirmation, isolation of a full length cDNA clone from a library will allow the sequencing of the suppressor gene. We expect that this approach should lead to the identification of at least one of the Meso genes.
5.4. Concluding Remarks

The Cop rat provides a unique opportunity to study resistance to mammary tumorigenesis. Identification of mammary tumorigenesis resistance genes in the rat will hopefully lead to identification of analogous genes in the human. If such genes are identified, they should be of fundamental importance in identifying individuals that are at low risk for developing the disease, as well as elucidating events involved in the tumorigenesis process and perhaps for the treatment of breast cancer.
5.5. References


Appendix

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