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THE ROLE OF OVARIAN HORMONES, AGE AND MAMMARY GLAND DEVELOPMENT IN POLYOMAVIRUS MAMMARY TUMORIGENESIS

By

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ABSTRACT

THE ROLE OF OVARIAN HORMONES, AGE AND MAMMARY GLAND DEVELOPMENT IN POLYOMAVIRUS MAMMARY TUMORIGENESIS

By

Rachel Mae Heindel

Polyomavirus (Py) infection of adult athymic female mice causes a high incidence of mammary adenocarcinomas. In this thesis, I have examined the role of ovarian hormones, age and mammary gland development at the time of infection in Py infection and tumorigenesis. Ovary intact mice were infected at 3, 6, 10, 20 and 30 weeks of age with Py A2. In addition, mice were ovariectomized (OVX) at 5, 9 and 19 weeks of age and infected 1 week later. Mice were also OVX at 3 weeks, 1 week or 48 hours before or 1 week after infection at 6 weeks of age. To determine the effect of proliferation and differentiation on tumor induction, eight or 10 week old mice that were OVX and treated with 1 ug 17-beta estradiol (E) and 1 mg progesterone (P) for 3 or 1 weeks before infection and late pregnant mice were also infected. OVX-saline treated mice served as controls. All mice were palpated for 14 weeks

post infection (pi) for tumors or killed at 10 days pi to assess the levels of viral genomes, early protein and middle T kinase activity in their mammary glands. Mammary tumor incidence and number were significantly decreased and mammary tumor latency was significantly increased with increasing age at the time of infection and by OVX at least 1 week before infection. In addition, longer periods between OVX and infection further decreased tumor induction. Late pregnant mice with fully differentiated mammary glands remained susceptible to Py transformation. The severe effect of OVX on tumorigenesis suggests a positive role for ovarian hormones in this process and the target cell population may be influenced by age and mammary development state. No significant differences were observed in the levels of viral genomes (high in all groups) or in viral early proteins and middle T kinase activity (low in all groups) in mammary glands at 10 day pi. However, the levels of all three were high in mammary tumors and correlated with transformation.

To my parents

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LITERATURE REVIEW

Breast cancer is a major killer of North American women and there has been an alarming and significant increase in incidence for which there is no explanation (1). The National Cancer Institute has predicted that one in nine women can expect to develop breast cancer in her lifetime (2). While the etiology of this disease has not been defined, there is solid epidemiological evidence for an important role of ovarian hormones in mammary cancer development (3). A woman's total lifetime exposure to the ovarian hormones estrogen and progesterone has been implicated in the etiology of breast cancer (4). This hypothesis takes into account her age at menarche, her age at first full-term pregnancy, her total parity and her age at menopause. In addition to natural exposure to ovarian hormones, women who have taken oral contraceptives for long periods of time, starting at young ages (<25 years) have an elevated risk for developing breast cancer (5-7). A model that attempts to explain the risk increases associated with total lifetime exposure to ovarian hormones has been presented (5). This model emphasizes that early menarche and older age at first full term pregnancy result in higher levels of mammary proliferation due to longer hormone exposure and correlate with higher breast cancer risk. It is this proliferative response of the mammary epithelium to

ovarian hormones that either creates a more susceptible gland for transformation or induces a longer period of proliferation that may act to expand a transformed cell population. Therefore, it is apparent that this disease requires further investigation into the mechanisms of neoplastic transformation of the breast and the role of ovarian hormones in this process.

Several experimental models have been investigated to identify mechanisms of mammary neoplasia and the role of ovarian hormones. A major model system involves the use of chemical carcinogens to induce mammary tumors in rodents. N-nitroso-n-methylurea (NMU), 3-methylcholanthrene (3-MC or MCA) and 7,12-dimethylbenz(a)anthracene (DMBA) have been used extensively to induce mammary tumors in rodents since their carcinogenic properties were first described (8-12). Pre-cancerous ductal and alveolar dysplasias are induced by the carcinogens and ovarian hormones are critically required for tumor initiation and promotion (8, 9, 12-14).

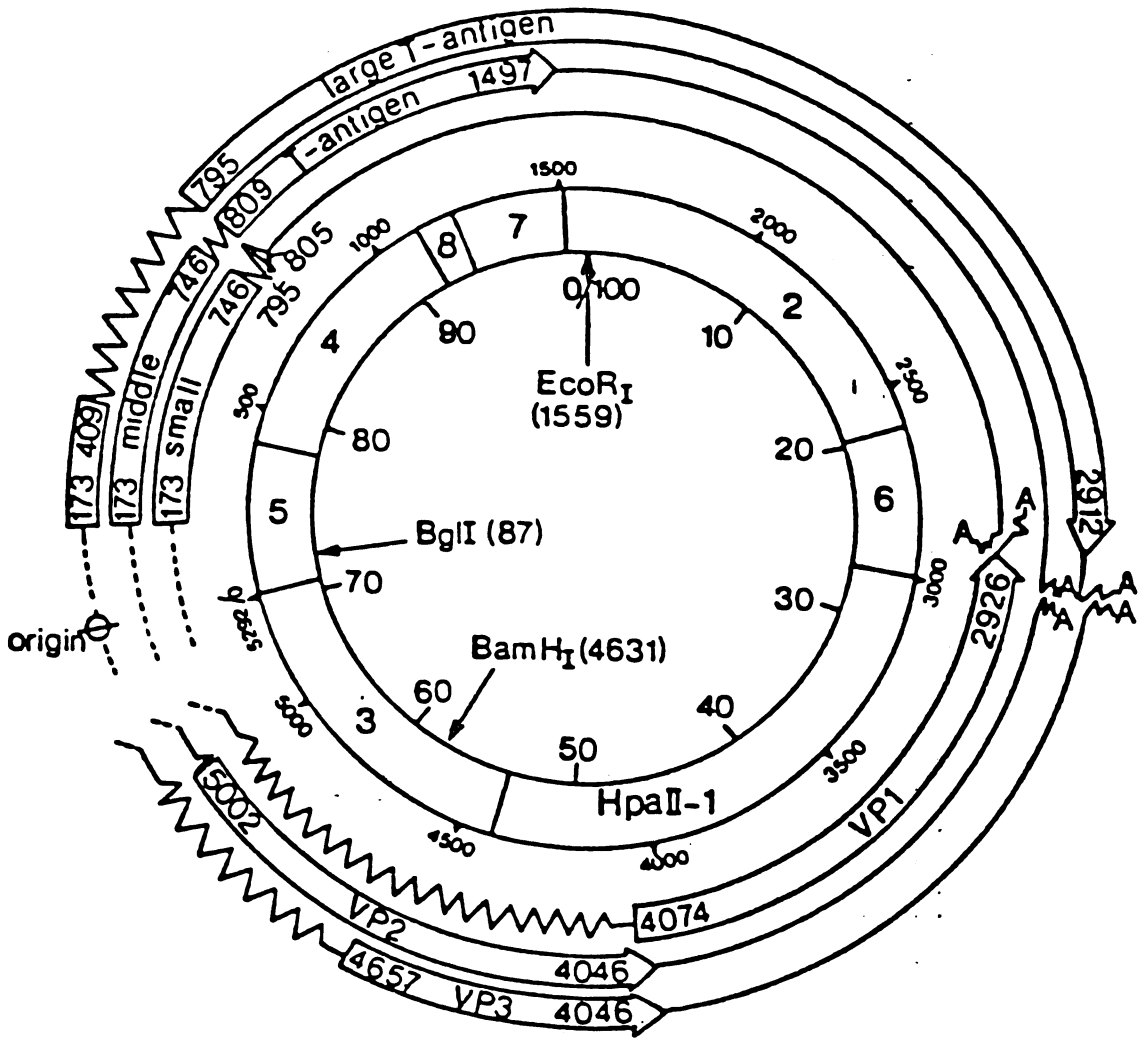
In addition to chemical carcinogens, the mouse mammary tumor virus (MMTV) has also been studied as a model of mammary tumorigenesis (15). MMTV infected mice develop mammary tumors, but only after undergoing several pregnancies, suggesting that ovarian hormones are also critically required for tumorigenesis in this model. Tumors are preceded by the development of alveolar dysplasias and in contrast to carcinogen induced tumors, MMTV induced tumors are ovarian hormone independent for growth (16). Further research has

identified glucocorticoids (17, 18), progestins (19, 20), prolactin (21) and androgens (20), as the positive regulating molecules of the long terminal repeat (LTR) of MMTV. The LTR controls gene expression of MMTV and steroid hormones and prolactin are therefore important in regulating the oncogenic potential of MMTV. Thus in this model, hormones can interact directly with the oncogenic agent as well as act to expand the transformed cell population.

Mouse polyomavirus (Py) is an important model system used in the study of virally induced tumors in mice and cell transformation in tissue culture. Py is a tumorigenic DNA virus whose natural host is the mouse. Its genome is comprised of 5292 base pairs with two coding regions, early and late, and a non-coding, enhancer-origin region that functions as the control region for viral transcription and replication (see Figure 1). The early region codes for three proteins, small, middle and large T antigens. Large T antigen controls viral DNA replication and gene expression. Middle T antigen also plays a role in viral gene expression and DNA synthesis, as well as in capsid assembly. The function of small T antigen, which is essentially a fragment of middle T antigen, is poorly understood. The late region codes for the three viral capsid proteins, VP1, VP2 and VP3.

Recently it has been reported that infection of adult athymic, immuno-incompetent Balb/c mice with Py results in a high incidence of mammary tumors with a short latency period (23). Several aspects of the Py mammary tumor model are

Figure 1. Physical map of the polyomavirus genome. The outer circles depict the two coding regions of the polyomavirus genome, early (top half) and late (bottom half), each of which produces one transcript that is differentially spliced (jagged lines represent the introns) to produce three gene products. The early region codes for three proteins, small T, middle T and large T antigens. The late gene products are the capsid proteins VP1, VP2 and VP3. The non-coding enhancer/origin region is located between these two coding regions on the left side of the map. The enhancer region is located between nucleotides 5021 and 5262, on the late side of the origin. This figure is taken from Soeda et al. (22).


large T-antigen
1497
795
809
t-antigen
746
795 805
746
173 middle
173 small
173 409
origin
EcoR$_I$
(1559)
BglI (87)
BamH$_I$(4631)
HpaII-1
VP1
VP2
VP3
4074
4046
4046
4657
5002
2912
2926
A
1500
2000
2500
3000
3500
4000
4500
5000
500
1000
0/100
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70
80
90
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2
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7
8

Figure 1.

intriguing and hold promise for providing some unique insights into the mammary neoplastic process. Recently, the histopathogenesis of Py-induced mammary tumors has been characterized in 6 week old infected athymic female mice (24). By 2 weeks post infection (pi), the mammary glands exhibit a hyperplastic appearance, similar to that observed during early to mid-pregnancy, characterized by moderate to extensive ductal sidebranching. However, the mammary glands eventually return to a normal state associated with the non-pregnant condition. Ductal dysplasias are frequently observed and are associated with the mammary adenocarcinomas, suggesting either a precancerous nature of the ductal dysplasias or that they are in fact, microscopic tumor foci. In addition, stromal abnormalities are often observed in association with the dysplasias and frank tumors. Similar morphological structures occur in humans, such as hyperplasia and atypia of ducts and lobules, and are associated with an increased relative risk for breast cancer in humans (25) Therefore, the Py model allows the study of early stages of tumor development that may be similar to those occurring in human breast cancer.

Recent studies have revealed that ovarian hormones play an important role in the induction of mammary tumors by Py (26, 27). In this thesis, I have examined the effect of ovariectomy at various times before and after Py infection and I will present evidence that suggests that ovarian hormones are important at the time of infection for Py mammary tumor induction. These observations suggest that ovarian hormones

play an early role in the tumorigenic process. Thus, this model system may provide unique insights into the role of ovarian hormones in mammary carcinogenesis. Interestingly, Py induced tumors appear to be ovarian hormone-independent for continued growth (24, 26), similar to approximately 50% of human breast cancers. Therefore, the Py tumor model also provides an opportunity to investigate potential treatments of hormonally unresponsive mammary cancers.

Many studies have examined the involvement of oncogenes in human breast cancer and these findings have been recently reviewed (28). The oncogenes that have been found to be amplified or overexpressed in human breast tumors include myc, myb, Ha-ras and neu. While these oncogenes are upregulated in some human tumors, their involvement in the etiology of this disease is unclear. To elucidate the role of oncogenes that are implicated in human breast cancer, many lines of transgenic mice have been created that overexpress these oncogenes and induce tumors in their mammary glands (29-34). To determine if any of these implicated oncogenes are involved in the polyoma system, the expression of these various oncogenes has also been recently investigated in infected athymic female mice (24). This study found that with increased time post infection, the level of c-myc gene expression was slightly elevated. However, no increases were observed in Ha-ras, c-neu, int-1 or int-2 gene expression, indicating that transcriptional upregulation of these proto-oncogenes does not appear to be a key step underlying Py

oncogenesis with the possible exception of c-myc.

The tumorigenic property of polyomavirus has been linked to the action of middle T antigen (Figure 2). The mechanism behind the neoplastic transformation induced by polyoma has been suggested to be mediated by the interaction of middle T antigen with $pp60^{c\text{-}src}$ which activates c-src's tyrosine kinase and phosphorylates middle T antigen (35). The activated middle T/c-src complex can then associate with phosphatidylinositol-3 (PI-3) kinase (36-41). Although the metabolites of the PI-3 kinase are not cleaved by a known phospholipase, this PI-3 kinase is suggested to play an important role in growth control since middle T mutants that are PI-3 kinase deficient have decreased transformation capabilities (40, 42). In addition to its association with $pp60^{c\text{-}src}$, middle T antigen has also been reported to associate with other protein kinases such as c-fyn and c-yes, both tyrosine kinases, and can activate c-raf, a serine-threonine kinase (43-46).

Middle T antigen has also been shown to activate the protein kinase C (PKC) pathway (47, 48). This property of middle T may be mediated by the interaction with phospholipase C-gamma (49), activating the PI signal transduction pathway downstream of PI kinase. Middle T antigen can also associate with a protein phosphatase, pp2A (50). By interacting with pp2A, middle T antigen may interfere with the regulation of important proteins responsible for signal transduction and cell cycle control. These perturbations caused by the

Figure 2. Polyomavirus mediated signal transduction. Polyomavirus middle T antigen (mT) has been shown to associate with a variety of cellular proteins and the consequences of these interactions may result in neoplastic transformation. Py mT binds to c-src and activates c-src's tyrosine kinase activity which results in autophosphorylation of c-src and phosphorylation of mT. The phosphorylated mT/c-src complex can now associate with phosphatidylinositol-3 kinase (PI3-K). PI3-K phosphorylates phosphatidylinositol (PI) on the 3 position, however, no known phospholipase can cleave this metabolite. The mT/c-src complex has been shown to activate phospholipase C-gamma (PLC) and protein kinase C (PKC). Also, the transcriptional activators AP1 and c-ets, are activated by middle T. In addition to c-src, mT can also associate in complexes with c-fyn, c-yes and c-raf, other cellular kinases. The consequences of these interactions have not been determined. Middle T has also been shown to associate with a cellular protein phosphatase, pp2A. Collectively or individually, the perturbations that may be caused as a result of mT interacting with these various cellular proteins may lead to cellular transformation.

Polyoma middle T antigen transformation pathway(s)

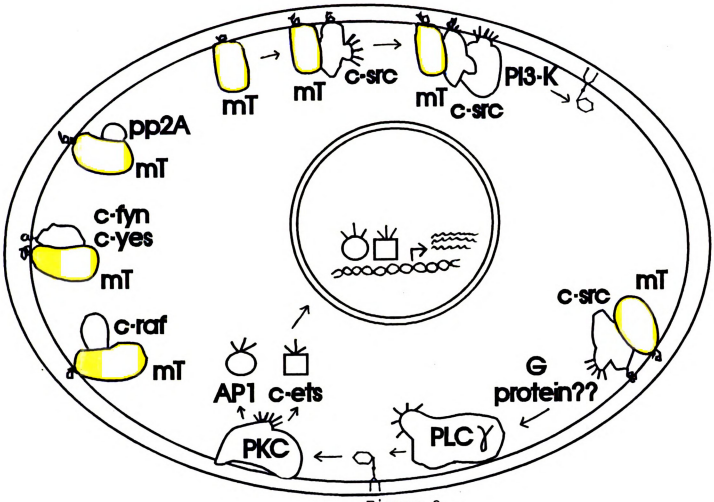


Figure 2.

interaction of middle T antigen with cellular proteins may play a role in Py transformation. It is also noteworthy to mention that small T antigen, which is identical to the amino terminus of middle T antigen except for 4 amino acids at its carboxyl terminus can also associate with pp2A (50). The role of small T in Py infection and transformation is not known but it has been shown to cooperate with middle T in the transformation of cells in culture by increasing foci growth (51). Therefore, small T antigen may play a role in Py transformation as well.

It has been shown that polyomavirus replication, which is bi-directional and mediated by large T antigen, is strongly coupled to viral transcription (52). Studies by de Villiers et al. (53) and Veldman et al. (54) demonstrated that polyoma replication requires an enhancer. Indeed, the mutation of individual nucleotides within the enhancer simultaneously affected both viral DNA replication and transcription, suggesting that these two molecular processes may share a common mechanism (55). Due to this link, the study of the polyoma enhancer region which is located between nucleotides 5020 and 5265 has been facilitated, since enhancer function can be measured in terms of the amount of viral DNA that has been replicated. The Py enhancer has been previously implicated in the control of organ specific viral replication in the mouse (56, 57).

Previous studies that examined viral DNA replication in different organs of both the neonate as well as the adult

mouse, revealed an organ specific pattern of polyoma replication. After infection with Py, neonate mice exhibit high levels of viral DNA replication throughout most of the mouse with the exception of the brain and blood (56, 58, 59). This stage of viremia, which peaks at 7-10 days post infection (pi) is followed by almost complete viral clearance by the host immune system. Infection of adult normal mice results in very low levels of viral replication and clearance of the virus by the immune system of the mouse. However, when adult athymic, immuno-deficient mice are infected, moderate levels of replication occur, resulting in the accumulation of high copy numbers of viral genomes in long-term infections (59). The three major sites of Py replication in the athymic adult mouse are the mammary gland, skin and bone.

From these results, four broadly defined organ specific patterns of Py replication have been identified in the mouse. They include organs with 1) high levels of replication in neonates and moderate levels in adults (i.e. mammary gland, skin and bone), 2) high levels of replication in neonates and very low levels in adults (i.e. kidney and to a lesser extent, the liver and lung), 3) higher levels of replication in adult females compared to adult males (i.e. mammary gland) and 4) low levels of replication in both neonates and adults (i.e. brain and blood) (59).

These age related, organ-specific viral replication patterns observed in neonatally infected and adult infected mice have led our laboratory to examine the effect of the non-

coding enhancer region within the polyoma genome as a possible site controlling these patterns. The Py enhancer has been segmented into domains and sub-domains, based primarily upon restriction enzyme recognition sites. Very generally, it has been split into two domains, A and B (Figure 3). The effects of enhancer rearrangements on the pattern of replication of the A2 genome in neonatally infected Balb/c mice were previously investigated (56, 57). These studies demonstrated that rearrangements within the enhancer, involving deletions of the B domain and duplications of the A domain of Py, severely decreased viral replication in the kidney, liver and lung of the neonate mouse. However, these rearrangements did not greatly affect replication in the mammary gland, skin and bone of either neonate or athymic adult mice (57). These studies suggested that the B domain is important for viral replication in all organs at neonatal infection. In terms of the mammary gland, these results suggest that either the B domain has no mammary control element or that the duplications within the A enhancer domain can compensate for the B deletion. In either case, a major control of Py replication in the mammary gland lies outside of the B enhancer domain and it is the A domain that is implicated in this control.

The sites of polyoma tumorigenesis also show an age-, organ- and sex-specific pattern. The oncogenicity of wild-type strains of polyomaviruses was extensively studied in the 1960's and has been recently reviewed (60). Infection of neonatal mice with the wild type strain A2 results in a

Figure 3. Physical map of the enhancer region of the polyomavirus A2 genome. The polyomavirus enhancer has been divided into two very general domains, A and B. PEA1, the mouse homologue to AP1, and PEA3, the mouse homologue to c-ets, both bind to multiple sites within the A and B domains. This figure was designed by Larry G. Martin.

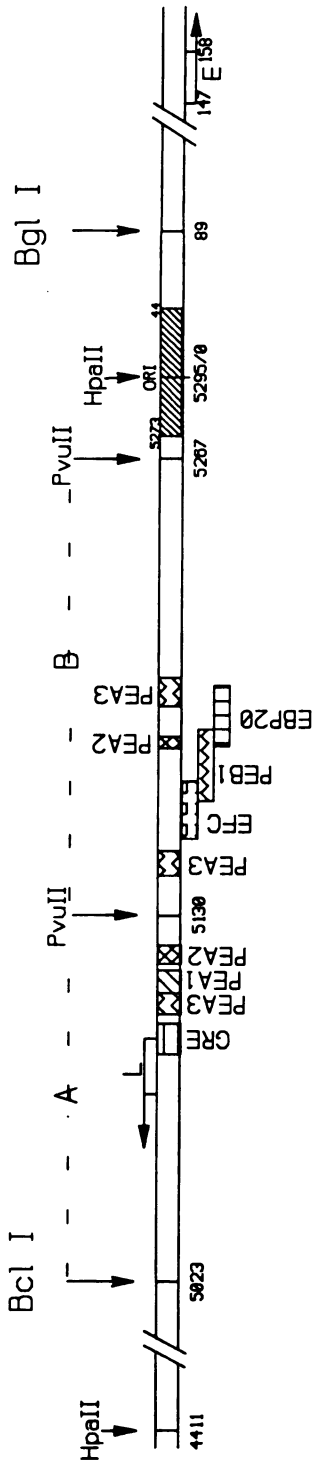

Bcl I
Bgl I
HpaII
PvuII
PvuII
HpaII
A
B
L
GRE
PEA3
PEA1
PEA2
PEA2
PEA3
EFC
PEB1
EBP20
PEA3
ORI
4411
5023
5130
5207
5272
5295/0
89
147
158

Figure 3.

variety of tumors of both epithelial and mesenchymal origin. The major types include tumors of the mammary gland, skin, bone, salivary glands and kidney (sarcomas). This review described over 30 different cell types that are susceptible to infection and approximately 23 sites for tumorigenesis by polyomavirus in neonatal mice. While mice that are neonatally infected with Py are susceptible to the virus and develop these tumors later in life, infection of normal adult mice results in rapid immune clearance of the virus and no tumors develop.

However, infection of adult athymic female mice with Py results in the induction of mainly mammary adenocarcinomas and osteosarcomas (23, 61). Mammary adenocarcinomas arise in a very short period after infection (6 weeks) and at a very high incidence (96%) in ovary intact athymic female Balb/c mice. Infection of adult athymic male mice results mainly in osteosarcomas; but no mammary tumors are induced (23). The basis for the lack of mammary tumor development in males is not known but it is intriguing that the virus does replicate in the male mammary gland, albeit at lower levels (59). However, a threshold level of organ specific viral replication may not be all that is required for organ specific tumorigenesis. Since Py does not cause tumors in all organs in which it replicates, Py may require the cells to possess additional characteristics for transformation. It may be that these characteristics are present only in the adult female mammary gland and are regulated by ovarian hormones. The

control of this mammary-tropic viral replication and sex-specific mammary tumorigenesis remains to be elucidated.

It is possible that the organ-specificity as well as the sex-specificity of Py replication and tumorigenesis are mediated by transcription factors that bind to enhancer sequences. As discussed above, in vivo experiments with B enhancer deletion mutants suggested that the major control of Py replication in the adult mammary gland lies outside of the B enhancer domain and implicated the A domain (57). These age dependent, organ-specific viral replication patterns have led to the ongoing examination of the Py enhancer region for specific sequences that control these patterns (62). Within the A enhancer domain, there are known binding sites for the transcriptional activators PEA1 and PEA3 which are the mouse homologues to AP1 and c-ets, respectively (see Figure 3). Therefore, the binding of these activators to the A enhancer domain of Py may control viral replication and gene expression in the mouse mammary gland. The influence of ovarian hormones on the activity of these factors may help to explain the sex-specific pattern of Py mammary tumorigenesis.

Recent cell culture studies have shown that Py middle T antigen activates PEA1 (47) and PEA3 (63). This is taken as evidence that middle T antigen induces a signal transduction pathway which activates protein kinase C as described above. PEA3 has recently been shown to cooperate with the PEA1 components, c-Fos and c-Jun, to activate transcription through their binding sites in the Py enhancer (64). PEA1 can exist

as a combination of protein complexes comprised of c-Fos, b-Jun and/or c-Jun dimers (65). Both estrogen and progesterone are able to increase expression of the human c-fos gene and consequently, AP1 (66, 67). c-fos gene expression is induced by estrogen in HeLa cells and by progesterone in T-47D human breast cancer cells. If PEA1 is also induced in the mouse mammary gland in response to ovarian hormones, this might explain the ability of Py to replicate to higher levels in the female mammary gland. However, this thesis will show that ovariectomy does not decrease the level of Py genomes in the mammary gland but clearly affects the transformation of the gland. It remains plausible that an ovarian hormone regulated factor(s) is required for Py transformation of the mammary gland. It may be that an indirect factor (i.e. cellular metabolic state, proliferative status, etc.) is upregulated in the ovary intact gland and makes the gland immediately susceptible to Py transformation which is reflected in short latencies and high mammary tumor incidence.

Indeed, the activation of PEA1 and PEA3 by middle T antigen in the mammary gland might play a major role in the neoplastic cascade induced by the virus. A positive feedback loop may be created by the middle T induced signal transduction cascade, with PEA1 and PEA3 increasing the expression of not only Py genes but also cellular genes that may play a crucial role in transforming the mammary epithelium. It may be that the ovary intact female mammary gland may contain a factor(s) that mediates the organ-tropism

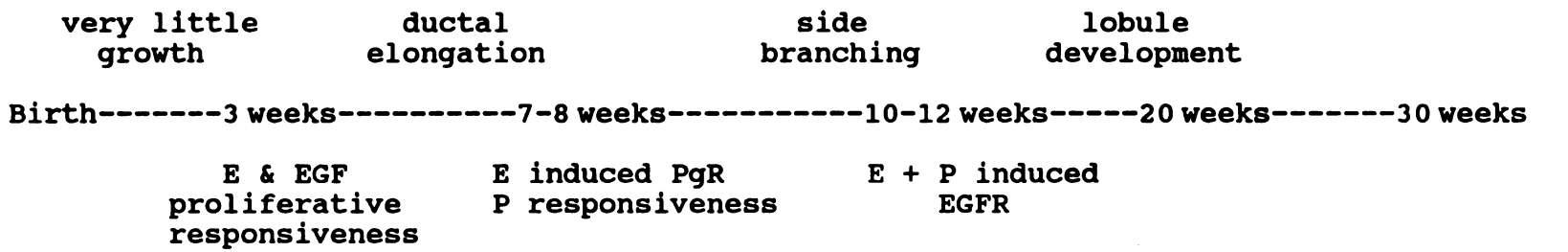
of viral replication and tumorigenesis. This factor may not be present or may not function optimally in the male mammary gland.

In order to understand the possible basis for sex-related patterns of tumorigenesis it is necessary to understand the differences in the development of mammary glands of male and female mice. These differences have been reviewed (68) and are presented in Figure 4. During the first half of gestation, mammary gland development is similar for both sexes. At 13-15 days of gestation, androgen secretion begins in the male embryo and the growth of the mammary epithelium is halted and the primary duct detaches from the nipple region, leaving only a rudimentary gland. The male gland does not undergo any further development without hormonal manipulation. By contrast the epithelial rudiment of the female continues to grow and branch; this embryonic growth is hormone independent. The rudimentary gland remains quiescent and unresponsive to estrogen and progesterone until puberty at 3-4 weeks of age with the onset of ovarian cycles (68). At this time, the epithelium proliferates in response to estrogen and extensive ducts develop and elongate. It is also at this age that the epithelium can respond to epidermal growth factor (EGF) which induces proliferation and ductal elongation (69). Therefore, at this age, the mammary gland is very proliferative in response to estrogen and EGF, but is not yet responsive to progesterone (70). The mammary epithelium continues to grow until it reaches the boundaries of its stromal fat pad by 8-10

Figure 4. Mouse mammary gland development. In both sexes during early gestation, the mammary epithelial bud is formed and begins to grow. However, unlike the female primary duct that continues to grow and branch until birth, the male epithelial growth is halted at mid-gestation by the increase in circulating androgens. The epithelium regresses and the primary duct detaches from the nipple. At birth, the female mammary gland remains rudimentary and very little growth occurs until puberty at approximately 3 to 5 weeks of age. At this time, the ductal epithelium elongates in response to estrogen (E) and epidermal growth factor (EGF). At 7 to 8 weeks of age, the mammary gland now responds to E by inducing epithelial progesterone receptors (PgR) which confer responsiveness to progesterone (P). P induces sidebranches at this age. At 10 weeks of age, EGF receptors (EGFR) can be induced by E and P and more extensive sidebranches are induced. Increased sidebranching occurs as the mouse ages.

Mammary Gland Development

Female Mice:



Male Mice:

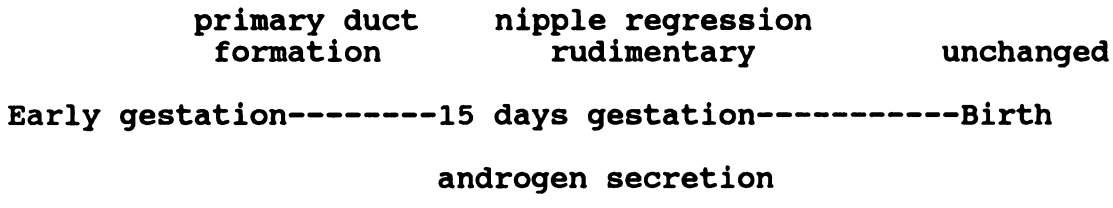


Figure 4.

weeks. At 10 weeks of age, the gland begins to develop sidebranches as a result of the induction of progesterone receptors by estrogen and the acquisition of responsiveness to progesterone (70-72). EGF receptors are inducible by estrogen and progesterone at 10 weeks of age and confer a different responsiveness to EGF which can now induce extensive sidebranching to create a more differentiated gland (73). By 20 and 30 weeks of age, more extensive sidebranching occurs (74). With the onset of pregnancy, in response to the elevated levels of estrogen, progesterone, EGF and placental hormones, extensive epithelial proliferation and morphogenesis take place that produce a fully differentiated gland (74). After parturition, with the onset of lactation, the mammary gland becomes refractory to estrogen and progesterone (75).

Therefore, as the female mouse ages, the mammary gland progresses through different morphological states that have varying degrees of proliferation and differentiation. During the early growth stages from puberty until about 10 weeks of age, the gland is very proliferative, especially at the terminal end buds (76). As the gland begins to develop sidebranches at 10 weeks of age, it becomes more differentiated, as these sidebranches are the structures from which lobuloalveoli will form if the mouse becomes pregnant (77). The proliferative end buds are present from puberty until the epithelium reaches the boundaries of the stromal fat pad between 8 and 10 weeks of age. It is at this time that the end buds regress and the proliferative activity of the

gland decreases (76, 78). At later ages, more sidebranching and lobule development occurs creating a more differentiated gland (74).

One specific difference between male and female mammary glands that may determine sex-related differences in tumor development may be differences in ovarian hormone and growth factor receptor expression. The ontogeny of estrogen, progesterone and EGF receptors and mammary gland responsiveness to these factors has been described for the female mouse (69-71, 79). Estrogen receptors are present in the female mouse mammary gland at 3 days of age but the gland does not respond to estrogen until at least 3-5 weeks of age. At 3-5 weeks of age, the mouse mammary gland proliferates in response to estrogen treatment, but it is not until 7 weeks of age that the gland produces progesterone receptors in response to estrogen. The inability to induce progesterone receptors before this age suggests a difference in the response to estrogen or function of the estrogen receptors at different developmental stages. Progesterone receptors are present in low concentrations in the stromal cells of the mouse mammary gland at 5 weeks of age but are not responsive to progesterone until 7 weeks of age. The responsiveness to progesterone has been shown to correlate with the presence of the estrogen-inducible progesterone receptors in the mammary epithelium (70). EGF receptors are also present in immature animals (80) but are not inducible by estrogen and progesterone until 10 weeks of age (73). Thus, the ability of the female mammary

gland to respond to ovarian hormones could account for the difference in tumorigenesis between males and females. If a sequential developmental process, such as the acquistion of hormone receptors, that is influenced by ovarian hormones and growth factors must occur to create a susceptible gland for Py tumorigenesis, then the male mammary gland may not provide an optimal target for Py transformation.

It is possible that the rudimentary development of the mammary gland in male mice is not sufficient to create optimal conditions for mammary tumorigenesis similar to that of the females. One might expect that by increasing the number of target epithelial cells, the chance for transformation by the virus will also increase. In this regard, it is of interest that Py infection of the neonate mouse is associated with high levels of replication in the mammary gland (56, 59) and that mammary tumors do appear in both sexes later in adulthood (60). Thus, it is unlikely that the size of the gland alone is a sufficient explanation for the lack of mammary tumors in adult athymic male mice. A possible explanation for mammary tumor induction in neonatally infected males may be that the neonate mouse tissues which contain high numbers of stem cells, may permit a sufficient level of replication and gene expression for subsequent mammary tumorigenesis or have a higher number/ratio of susceptible cells than adult male mice. This could be explained by the presence of 1) a factor that is ovarian hormone independent in the neonate and subsequently hormonally regulated in the adult or 2) a unique

characteristic in the neonate male and female as compared to the adult female mammary gland. Thus, by infecting the male mouse neonatally, the virus may escape the sex specificity for mammary tumor development that is present in the adult mouse.

Clearly, there is something about the adult female mouse mammary gland that allows the production of tumors by Py. Previous studies have examined the effect of age and proliferative status on the induction of mammary tumors by chemical carcinogens and have observed age dependencies for initiation (11, 81-83). This age effect is inherent in the tissue "age" and not the animal age or its accompanying hormonal milieu as donor mammary glands from young rats are equally susceptible to DMBA when transplanted into young or old rats (11). Whereas, older donor mammary glands remain less susceptible to DMBA tumor induction, even when transplanted into young animals. It has been shown that young animals have more terminal end buds that are highly proliferative (84) and the presence of these proliferative end buds in rats correlates with the ability of carcinogens to induce mammary tumors, suggesting that they are the target sites for transformation (82, 85-89). Sharkey and Bruce (76) have demonstrated the targeting effect of ionizing radiation, DMBA and NMU to the terminal end buds of 7 week old mice by quantitating nuclear aberrations. Younger animals had higher tumor incidence and shorter latency periods than older animals that do not have the end buds and have more differentiated mammary glands that are generally much less proliferative.

In this regard, high levels of cellular proliferation are also observed in intralobular terminal ducts, the human equivalent to the mouse terminal end bud (90). This study also identified increased growth fractions and longer S phases in the terminal ducts of younger women. If rapidly proliferating cells are the targets for carcinogenic agents, then younger animals and women may be more susceptible to initiation events. Indeed, increased breast cancer incidence has been observed in young women (ages 10-20 years) who were exposed to repeated fluoroscopic chest examinations and ionizing radiation during the atomic bombing of Japan compared to their older counterparts (91, 92).

The age effect on carcinogenesis may also be due to the process of differentiation that occurs as the animal ages (82, 86, 88, 89, 93). Several studies have shown protective effects of full term pregnancy, lactation and chorionic gonadotropin, a placental hormone, on carcinogen induced mammary tumors, suggesting that more differentiated cells are less susceptible to initiation and/or promotion of neoplastic foci (94-96). The target cell population for the carcinogen is decreased in late pregnant and lactating mammary glands that are fully differentiated and less proliferative (89). However, when carcinogens are given before or at mid-pregnancy, high mammary tumor incidence is induced, which may be due to high proliferation rates at these stages of development (97).

In this regard, I have infected ovary intact or

ovariectomized athymic mice with Py at various ages to determine if the stage of mammary gland development and hormonal responsiveness influences the level of Py genomes, early viral protein and/or middle T associated kinase activity and mammary tumorigenesis. If the cells in which Py can replicate and produce middle T antigen differ due to the state of the gland, one may expect different responses to Py at various ages and developmental states. This study may provide clues about the nature of ovarian hormone- and age-related influences on mammary tumorigenesis.

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Chapter 2

The role of ovarian hormones, age and mammary gland development state in polyomavirus mammary tumorigenesis.

ABSTRACT

Polyomavirus (Py) infection of adult athymic female mice causes a high incidence of mammary adenocarcinomas. I have examined the role of ovarian hormones, age and mammary gland development at the time of infection in Py tumorigenesis. Ovary intact mice were infected at 3, 6, 10, 20 and 30 weeks of age with Py A2. In addition, mice were ovariectomized (OVX) at 5, 9 and 19 weeks of age and were infected 1 week later. To establish the time at which ovarian hormones are required for tumorigenesis, mice were OVX at 3 weeks, 1 week or 48 hours before or 1 week after infection at 6 weeks of age. To determine the effect of proliferation and differentiation on tumor induction, eight or 10 week old mice that were OVX and treated with 1 ug 17-beta estradiol (E) and 1 mg progesterone (P) for 3 or 1 weeks before infection and late pregnant mice were also infected. OVX-saline treated mice served as controls. All mice were palpated for 14 weeks post infection (pi). Mammary tumor incidence and number were significantly decreased and mammary tumor latency was significantly increased with increasing age at the time of infection. In addition, OVX at older ages (10 and 20 weeks)

drastically reduced or eliminated tumor induction. Since OVX at least 1 week before infection significantly reduced tumorigenesis, ovarian hormones are important at the time of infection. In addition, longer periods between OVX and infection further decreased tumor induction. Late pregnant mice with fully differentiated mammary glands remained susceptible to Py transformation, suggesting that the decrease in Py tumorigenicity in older animals is not simply due to differentiation but may be due to the loss of proliferation or certain target cell types that are present in young mice. The drastic effect of OVX on tumorigenesis suggests a positive role for ovarian hormones in the poloymavirus carcinogenic process and it may be that the target cell population is influenced by both age and mammary development state.

INTRODUCTION

Mouse polyomavirus (Py), a DNA tumor virus, is an important model system used in the study of virally induced tumors in mice and cell transformation in tissue culture. Previous studies that examined viral DNA replication in different organs of both the neonate as well as the adult mouse revealed an age- and organ-specific pattern of Py replication (1).

The sites of Py tumorigenesis in adult athymic mice also show an age-, organ- and sex-specific pattern (2, 3). Infection of adult athymic, immuno-incompetent Balb/c female mice results in a high incidence of mammary tumors with a short latency period (3, 4). Athymic male mice do not develop mammary tumors (3). However, the induction of other types of tumors does occur but is somewhat less frequent than the mammary tumors and the basis for this difference is not known. Although it has been shown that ovarian hormones are important at or around the time of Py infection for mammary tumors to develop in female mice (3, 4), the role of the ovarian hormones in this process is not known. It is possible that an appropriate state of mammary development and/or ovarian hormone responsiveness of the gland may be required for Py induction of tumors. Therefore, the purpose of the present study was to define the effect of ovarian hormones on Py mammary tumorigenesis and to investigate the transformation susceptibility of the mouse mammary gland to Py at various

ages and different developmental states.

I report here that ovarian hormones are required at least one week before Py infection for high levels of mammary tumor induction. In addition, older animals (20 and 30 weeks of age) are less susceptible to Py tumorigenesis than their 6 and 10 week old counterparts. Ovariectomy decreased tumor incidence when performed one week before Py infection at 6 and 10 weeks of age and eliminated tumor induction at 20 weeks of age. This study has examined the ability of Py to induce tumors in fully differentiated, late pregnant mammary glands of athymic mice. These mice developed mammary tumors at a high incidence, suggesting that the decrease in mammary tumor incidence in older mice is not simply due to a more differentiated mammary gland and may be related to the loss of proliferative target cells or another type of "differentiation" that occurs as the nulliparous mouse ages.

MATERIALS AND METHODS

Animals:

All athymic mice were obtained from Life Sciences, Inc. and were housed in our vivarium in a sterile laminar flow rack and given sterile water and rodent chow (Purina) ad libitum. Mice were infected subcutaneously with 10^6 plaque forming units (pfu) of the wild type strain A2 of Py.

Treatments:

Female mice were infected with Py at 3, 6, 10, 20 and 30 weeks of age to assess the influence of different stages of mammary gland development on mammary tumor induction. In addition, 5, 9 and 19 week old mice were OVX before infection at 6, 10 and 20 weeks of age, respectively. Two mice from each group were killed on the day of infection by cervical dislocation and the mammary glands were removed and wholemounts were prepared and stained as previously described (5) to assess the state of morphological development at the time of infection. To examine the role of ovarian hormones at the time of Py infection, mice were bilaterally ovariectomized (OVX) at 3 weeks, 1 week or 48 hours before or 1 week after infection at 6 weeks of age. Ovary intact mice served as controls.

To determine the effect of increasing differentiation on

Py mammary tumor induction, 8 or 10 week old mice were OVX and given daily subcutaneous injections of 1 ug 17-beta estradiol (Sigma) + 1 mg progesterone (Sigma) in 5% gum arabic (Sigma) for 3 weeks or 1 week, respectively before infection. Ten week old, ovary intact and age-matched and OVX-saline treated mice served as controls. In addition, 8 week old mice were mated with normal Balb/c male mice from our own colony for timed pregnancies. All mice were infected with Py at late pregnancy, 18-19 days gestation, and allowed to deliver and nurse their progeny. All late pregnant mice were infected by 13 weeks of age. Two mice from each group were killed on the day of infection by cervical dislocation and the mammary glands were removed to determine the extent of morphological development at the time of infection as described above.

All mice were palpated weekly for the appearance of tumors. These mice were killed by cervical dislocation when moribund or when tumor load exceeded more than 10% of their body weight. Tumor incidence, number and latency were assessed as of 14 weeks post infection (pi). Mammary glands and mammary tumors were removed at the time of death and were analyzed for viral genome level, early protein production and middle T associated kinase activity and these results are presented in Chapter 3.

Parameter and statistical calculations:

Tumor incidence was determined by dividing the number of mice that developed tumors by the total number of mice in an experimental group. Average mammary tumor number per mouse was determined by dividing the total number of mammary tumors that appeared in an experimental group by the number of mice in the group. Average mammary tumor latency was calculated by assigning each tumor with the week pi that it was first palpable and determining the mean for all tumors that appeared within an experimental group.

All statistical analyses were performed using the SYSTAT (SYSTAT, Inc.) statistical software package on a Packard Bell 386SX-II computer. Tumor incidence data were analyzed for significance by Chi square analysis. Average mammary tumor number per mouse and average mammary tumor latency data were analyzed for significance by Kruskal-Wallis tests, followed by Mann-Whitney analyses with Bonferroni p value adjustments for the number of comparisons.

RESULTS

Effect of age and hormonal responsiveness on tumor induction.

To examine the effect of various ages that are characterized by various degrees of mammary epithelial development and ovarian hormone responsiveness on the induction of mammary tumors by Py, female mice were infected at 3, 6, 10, 20 and 30 weeks of age. In addition, 5, 9, and 19 week old mice were OVX and then infected at 6, 10, or 20 weeks of age, respectively, to determine the effect of ovarian hormone withdrawal at these ages on Py tumorigenesis. Wholemount analysis of mammary glands taken from these mice on the day of infection depict the expected morphology for that age and ovarian status (Figure 5). These wholemounts illustrate that each age is morphologically different. As the mouse ages or if it is OVX, the proliferative end buds are lost. Increasing age creates a more differentiated gland with respect to the amount of sidebranching that was present.

Mammary tumor incidence of the ovary intact mice groups is shown in Figure 6a. This figure clearly demonstrates that after 10 weeks of age, the susceptibility of the mouse mammary gland to Py tumorigenesis is significantly decreased (55%; $p<0.05$) as compared to that of younger mice (96%). This same age influence was also observed in the average number of mammary tumors that were induced (Figure 6b), with

Figure 5. Mammary gland wholemount analysis of ovary intact and ovariectomized mice at various ages. Mammary glands were removed from ovary intact mice at 3, 6, 10, 20 or 30 weeks of age and from mice that were ovariectomized (OVX) for 1 week before infection at 20 weeks of age. The bulbous, proliferative end buds of the young glands (a and b) are lost as the epithelium reaches the boundaries of the stromal fat pad by 10 weeks of age (c). Sidebranching begins at 10 weeks of age and becomes more extensive with increasing age (d and e). Panel f demonstrates the regression that occurs when mice are OVX for 1 week. The ducts become thin and sidebranching is diminished. LN, lymph node. Magnification x 42.

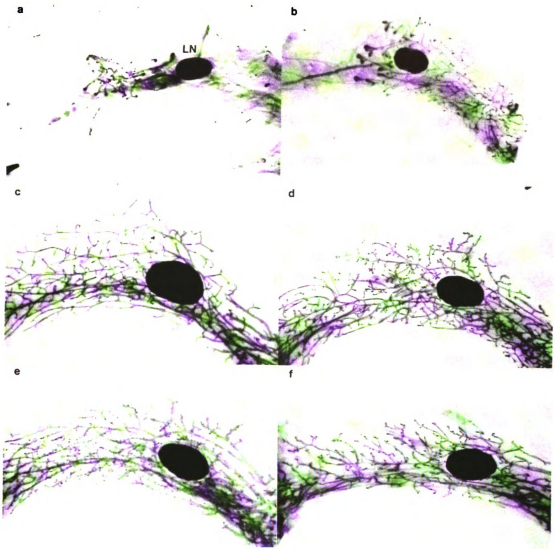


Figure 5.

Figure 6. Effect of age on mammary tumor induction. Ovary intact mice at 3, 6, 10, 20 and 30 weeks of age were infected with polyomavirus and followed for mammary tumor induction. All statistical comparisons were made versus the 6 week old mice, with a p value < 0.05 constituting significance (*). (a). Mammary tumor incidence, n values represent the number of mice in each group. (b). Average mammary tumor number per mouse, n values represent the number of mice. (c). Average mammary tumor latency, n values represent the number of mammary tumors.

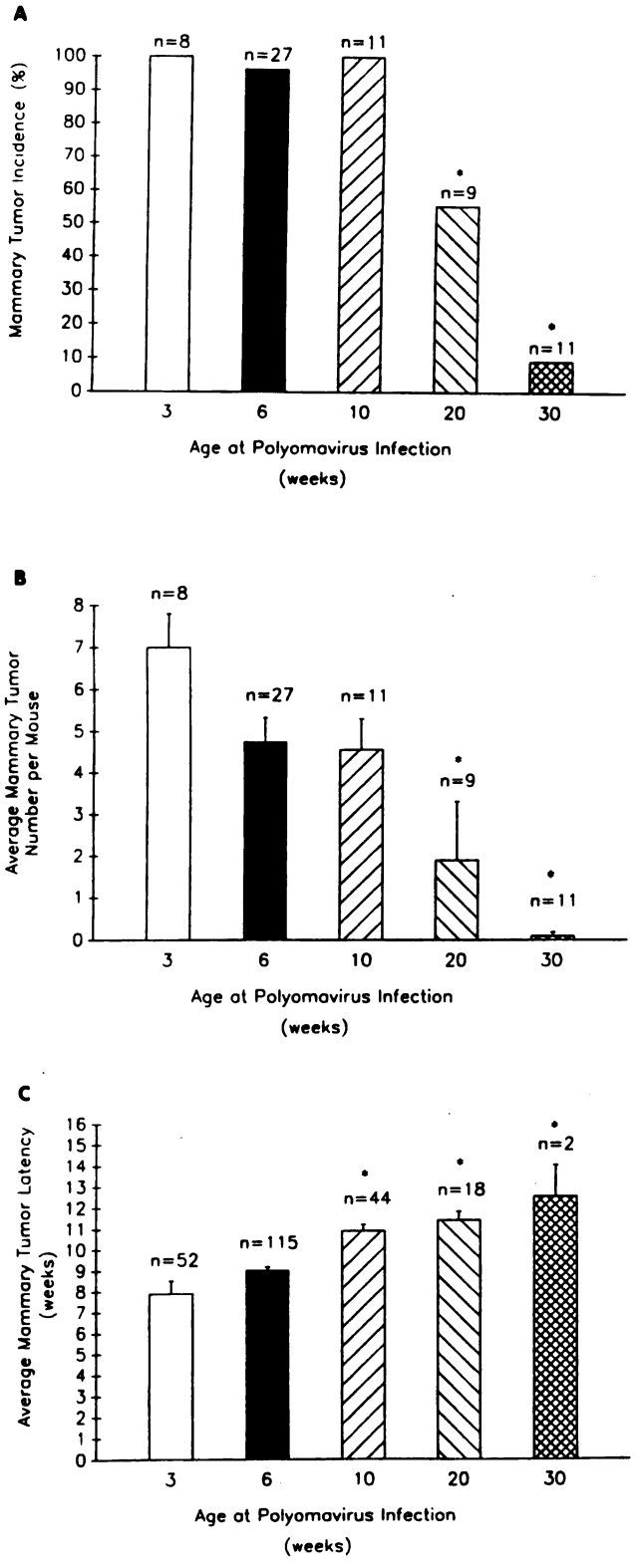

A
Mammary Tumor Incidence (%)
n=8
n=27
n=11
n=9
n=11
Age at Polyomavirus Infection
(weeks)
B
Average Mammary Tumor Number per Mouse
n=8
n=27
n=11
n=9
n=11
Age at Polyomavirus Infection
(weeks)
C
Average Mammary Tumor Latency (weeks)
n=52
n=115
n=44
n=18
n=2
Age at Polyomavirus Infection
(weeks)

Figure 6.

significant decreases apparent in the 20 and 30 week age groups (2 and 0.18, respectively) as compared to the 6 week group (4.3; $p<0.0125$). Significantly longer average mammary tumor latencies were observed in mice infected at 10 weeks of age and older (1.9, 2.4 and 3.5 weeks, respectively; $p<0.0125$) (Figure 6c). These results may reflect the changes that are occurring developmentally and hormonally within the mammary gland between these ages. The mammary gland is vigorously growing at 6 weeks of age as the end buds proliferate and penetrate the fat pad. However, by 10 weeks of age, the end buds have reached the boundaries of the fat pad, they have regressed and the gland is less proliferative than that of younger mice. If tumor latency is determined by the rate of target cell proliferation, then the decrease in proliferation that occurs as the mouse ages (6) correlates well with the increase in tumor latency and may determine the lower tumor incidence and number that was observed in these older groups as well.

The effect of OVX at these various ages on Py mammary tumorigenesis was also determined. Figure 7a illustrates that OVX 1 week before infection at 6, 10 or 20 weeks of age significantly reduced (61% and 11%; $p<0.05$), and in the case of the 20 week OVX group, eliminated mammary tumor induction by Py as compared to the ovary intact, age-matched controls (96%, 100% and 56%, respectively). Similarly, OVX 1 week before Py infection at these various ages also significantly

Figure 7. Effect of ovariectomy at various ages on mammary tumor induction. Mice were ovariectomized (OVX) at 5, 9 or 19 weeks of age before polyomavirus infection at 6, 10 and 20 weeks of age, respectively and were monitored for mammary tumors. All statistical comparisons were made versus age-matched, ovary intact controls, with a p value < 0.05 constituting significance (*). (a). Mammary tumor incidence, n values represent the number of mice in each group. (b). Average mammary tumor number, n values represent the number of mice per group. (c). Average mammary tumor latency, n values represent the number of mammary tumors. Statistical analyses were not performed on the 20 week OVX group because no tumors were induced.

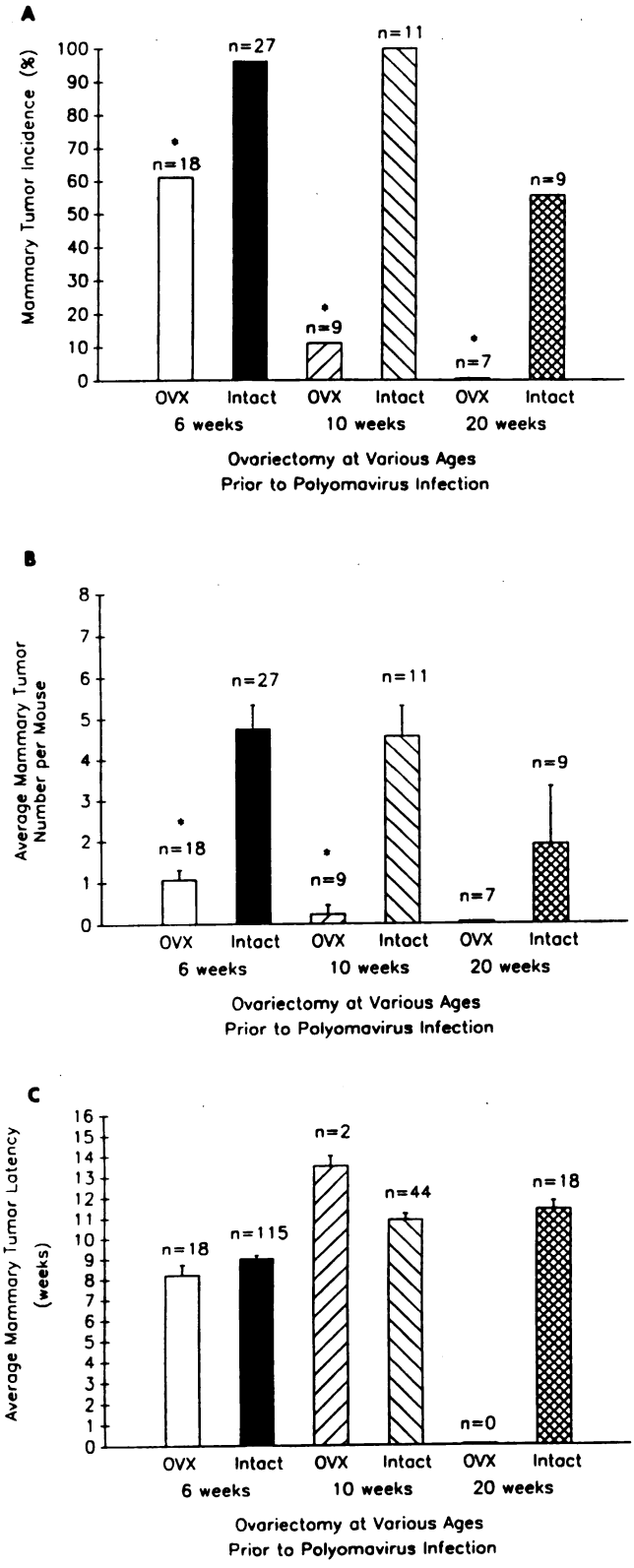

A
Mammary Tumor Incidence (%)
100
90
80
70
60
50
40
30
20
10
0
n=18
n=27
n=9
n=11
n=7
n=9
OVX
Intact
OVX
Intact
OVX
Intact
6 weeks
10 weeks
20 weeks
Ovariectomy at Various Ages
Prior to Polyomavirus Infection
B
Average Mammary Tumor Number per Mouse
8
7
6
5
4
3
2
1
0
n=18
n=27
n=9
n=11
n=7
n=9
OVX
Intact
OVX
Intact
OVX
Intact
6 weeks
10 weeks
20 weeks
Ovariectomy at Various Ages
Prior to Polyomavirus Infection
C
Average Mammary Tumor Latency (weeks)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
n=18
n=115
n=2
n=44
n=0
n=18
OVX
Intact
OVX
Intact
OVX
Intact
6 weeks
10 weeks
20 weeks
Ovariectomy at Various Ages
Prior to Polyomavirus Infection

Figure 7.

decreased ($p<0.017$) the average number of mammary tumors per mouse by at least 4 fold (Figure 7b). It is intriguing that between 6 and 10 weeks of age, tumor induction appeared to become more sensitive to the withdrawal of ovarian hormones than at the younger ages. However, OVX only had an effect on average tumor latency when performed at 20 weeks of age (Figure 7c). These results indicate a progression to strict ovarian hormone dependence of Py for the induction of mammary tumors in older aged mice.

Effect of ovariectomy at 6 weeks of age.

The next set of experiments was designed to examine the role of ovarian hormones on the induction of mammary tumors. Female mice were OVX at 3 weeks, 1 week or 48 hours before or 1 week after infection at 6 weeks of age. The animals were palpated weekly to monitor the appearance of mammary tumors. The results are shown in Figure 8a. In mice that were OVX at 3 weeks or 1 week before infection, the incidence of mammary tumors was significantly decreased (25% and 61%, respectively) as compared to the ovary intact controls (96%; $p<0.05$). However, no difference was observed in mice that were OVX 48 hours before or 1 week after infection. Average mammary tumor number was also significantly decreased by 4 fold ($p<0.017$) in mice that were OVX at least 1 week before infection (Figure 8b) while OVX at other times before or after infection had no

Figure 8. Effect of ovariectomy at 6 weeks of age. Mice were ovariectomized (OVX) at 3 weeks, 1 week or 48 hours before or at 1 week after infection with polyomavirus A2 at 6 weeks of age. Ovary intact, 6 week old mice served as controls. All mice were followed for mammary tumor incidence, average mammary tumor number per mouse and average mammary tumor latency. All statistical comparisons were made versus the intact mice, with a p value < 0.05 constituting significance (*). (a). Mammary tumor incidence, n values represent the number of mice in each group. (b). Average mammary tumor number per mouse. (c). Average mammary tumor latency, n values represent the total number of mammary tumors.

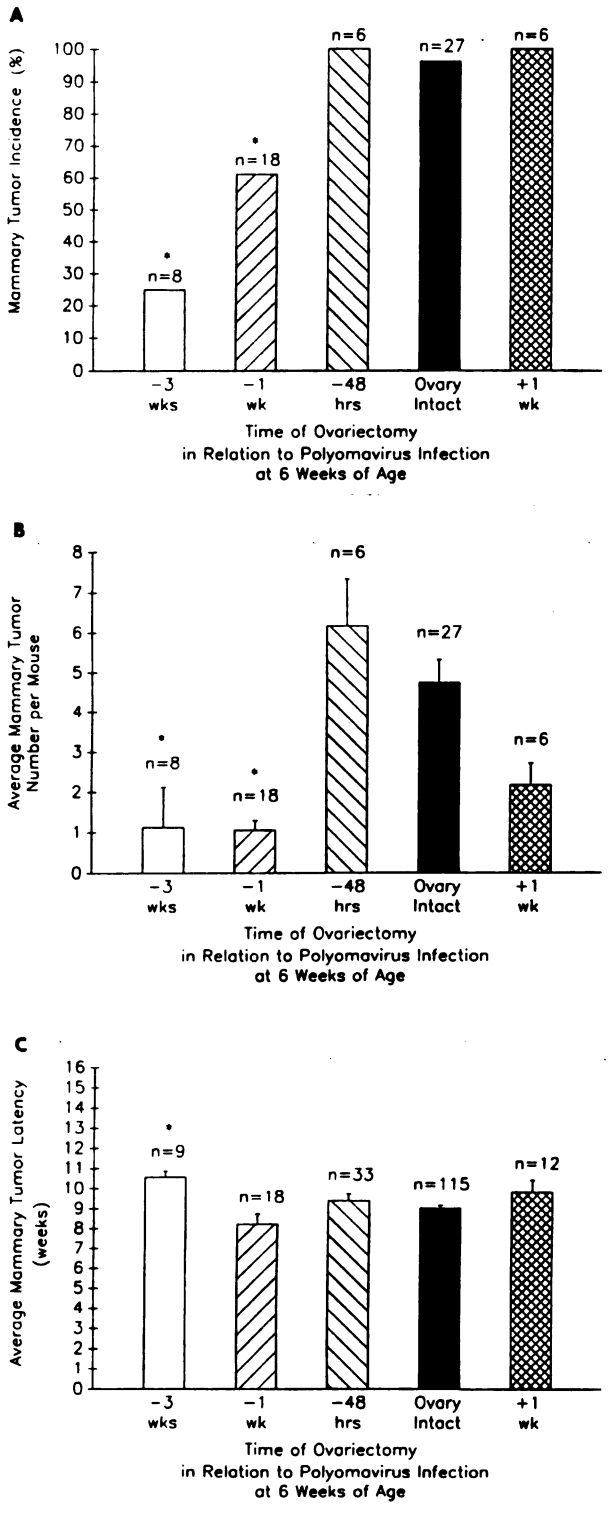

A
Mammary Tumor Incidence (%)
100
90
80
70
60
50
40
30
20
10
0
n=8
n=18
n=6
n=27
n=6
−3 wks
−1 wk
−48 hrs
Ovary Intact
+1 wk
Time of Ovariectomy in Relation to Polyomavirus Infection at 6 Weeks of Age
B
Average Mammary Tumor Number per Mouse
8
7
6
5
4
3
2
1
0
n=8
n=18
n=6
n=27
n=6
−3 wks
−1 wk
−48 hrs
Ovary Intact
+1 wk
Time of Ovariectomy in Relation to Polyomavirus Infection at 6 Weeks of Age
C
Average Mammary Tumor Latency (weeks)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
n=9
n=18
n=33
n=115
n=12
−3 wks
−1 wk
−48 hrs
Ovary Intact
+1 wk
Time of Ovariectomy in Relation to Polyomavirus Infection at 6 Weeks of Age

Figure 8.

effect. However, average mammary tumor latency was only significantly increased when mice were OVX 3 weeks before infection (Figure 8c). This increased latency for tumor appearance is reflected as well in tumor incidence and number for this group. These results indicate that ovarian hormones are required at the time of infection for Py mammary tumor induction.

Effect of different proliferative and differentiated mammary states on tumor induction.

To determine the effect of increasing differentiation on Py induced mammary tumorigenesis, 8 or 10 week old mice were OVX and given daily injections of 1 ug 17-beta estradiol (E) + 1 mg progesterone (P) in 5% gum arabic for 3 weeks or 1 week, respectively before infection. One week of E + P is expected to create an early pregnant-like, proliferative gland while 3 weeks of E + P should create a late pregnant-like, differentiated mammary gland. Ten week old, ovary intact and age-matched and OVX-saline treated mice served as controls. In addition, 8 week old mice were mated with normal male mice, infected with Py at 18-19 days gestation and allowed to deliver and nurse their progeny.

Mammary gland wholemounts taken from a subset of these mice on the day of infection are shown in Figure 9 and demonstrate the morphological changes that were induced by

Figure 9. Mammary gland wholemount analysis of ovariectomized, ovarian hormone treated mice and late pregnant mice. Mammary glands were removed on the day of infection from a subset of mice that were ovariectomized (OVX) at 8 or 10 weeks of age and given daily injections of 1 ug 17-beta estradiol (E) + 1 mg progesterone (P) for 3 weeks or 1 week, respectively, before infection. The mammary glands of late pregnant mice were also removed on the day of virus infection. Ten week old, ovary intact and OVX-NaCl treated mice served as controls. Panels a and b demonstrate the increase in sidebranching that occurs after 1 week and 3 weeks of E + P injections, respectively. Panel b represents a slightly more differentiated gland, similar to that created during early-pregnancy. Panels c and d show the effect of the length of OVX for 1 or 3 weeks, respectively. Note the greater extent of ductal thinning and diminished sidebranching in the longer OVX gland (d). Panel e demonstrates the extent of lobuloalveolar development that occurs in the late pregnant mouse mammary gland, conferring complete functional differentiation. Panel f depicts the mammary gland of an ovary intact 10 week old mouse. LN, lymph node. Magnification x 42.

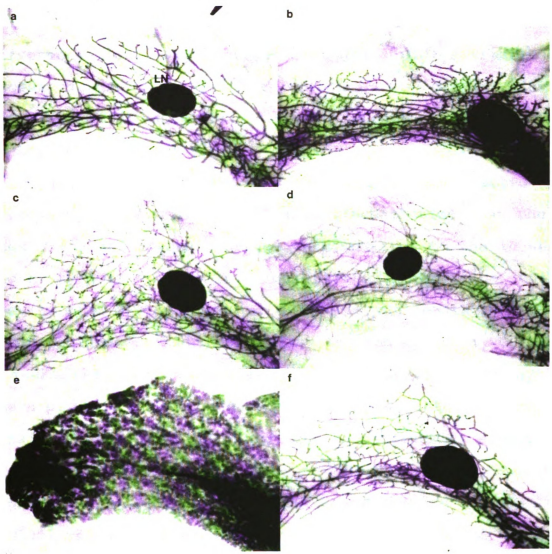
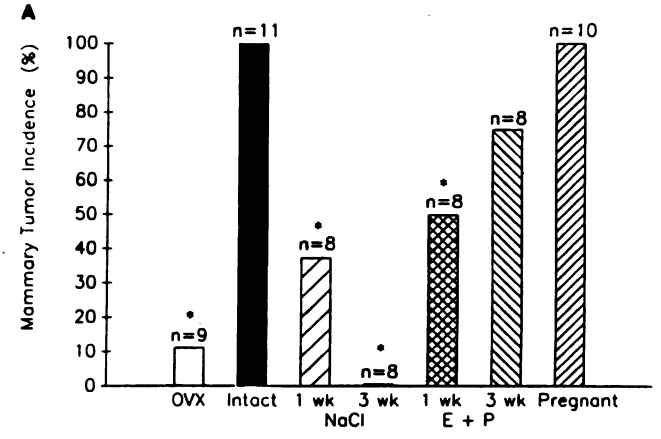


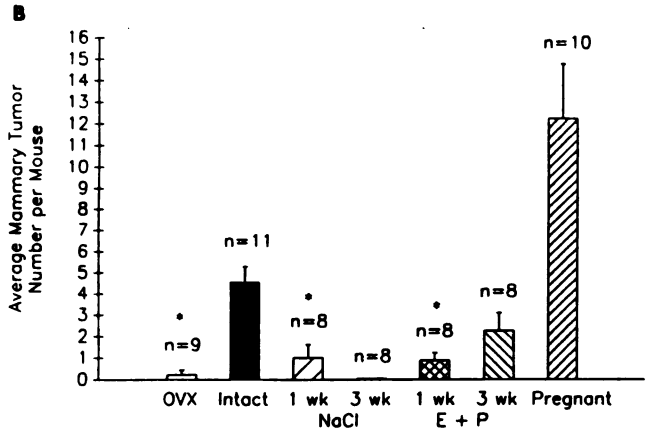
Figure 9.

OVX, length of E + P administration and pregnancy. In this study, 3 weeks of E + P treatment induced only a mid-pregnant like gland, suggesting that the response to exogenous hormones is different in athymic mice than in normal mice. Regardless, the mammary glands of the groups examined are different from each other in their extent of epithelial proliferation and differentiation. With increased length of E + P treatment, increased sidebranching occurs, creating a more differentiated gland. In addition, a fully differentiated gland was achieved in the late pregnant mice.

Tumor incidence was not significantly affected by increasing amounts of epithelial differentiation when the 3 week E + P and pregnant groups were compared to the 10 week old ovary intact controls (Figure 10a). In addition, these results also demonstrated the effect of the length of OVX on tumor incidence. Among the saline treated controls, OVX for 3 weeks before infection eliminated tumor induction by 14 weeks pi while mice that were OVX for 1 week before infection remained susceptible to Py mammary tumorigenesis, albeit at significantly lower levels (38%; $p<0.05$). Tumor incidence was significantly lower in the 1 week E + P treated group (50%: $p<0.05$) which is interesting in that this length and dose of treatment, which induces a highly proliferative gland in normal mice, did not cause such a response in the athymic mice (Figure 9), again suggesting that athymic mice have a less efficient response to exogeneous hormone treatment. Thus,

Figure 10. Effect of mammary gland proliferation and differentiation on tumor induction. Mice were ovariectomized (OVX) at 8 or 10 weeks of age and given daily subcutaneous injections of 1 ug 17-beta estradiol (E) + 1 mg progesterone (P) for 3 weeks or 1 week, respectively, before infection with polyomavirus. Late pregnant mice were also infected with polyomavirus. Ten week old, ovary intact and OVX-NaCl treated mice served as controls. All mice were monitored for mammary tumor induction. All statistical comparisons were made versus the 10 week old ovary intact controls, with a p value < 0.05 constituting significance (*). (a). Mammary tumor incidence, n values represent the number of mice in each group. (b). Average mammary tumor number per mouse, n values represent the number of mice. Statistical analyses were not performed on the 3 week OVX-NaCl treated group because no tumors were induced. (c). Average mammary tumor latency, n values represent the number of mammary tumors.


A
Mammary Tumor Incidence (%)
100
90
80
70
60
50
40
30
20
10
0
n=9
n=11
n=8
n=8
n=8
n=8
n=10
OVX
Intact
1 wk
3 wk
1 wk
3 wk
Pregnant
NaCl
E + P
Ovarian Hormone Status and Mammary Gland Differentiation


B
Average Mammary Tumor Number per Mouse
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
n=9
n=11
n=8
n=8
n=8
n=8
n=10
OVX
Intact
1 wk
3 wk
1 wk
3 wk
Pregnant
NaCl
E + P
Ovarian Hormone Status and Mammary Gland Differentiation

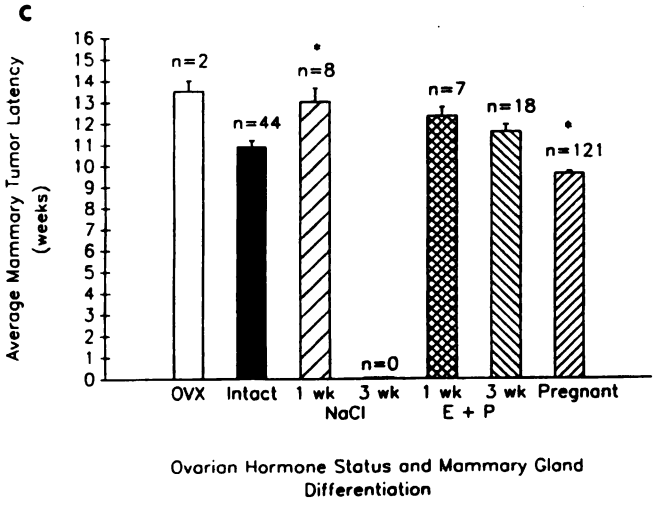

C
Average Mammary Tumor Latency (weeks)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
n=2
n=44
n=8
n=0
n=7
n=18
n=121
OVX
Intact
1 wk
3 wk
1 wk
3 wk
Pregnant
NaCl
E + P
Ovarian Hormone Status and Mammary Gland Differentiation

Figure 10.

these glands are more like OVX mammary glands than hormone treated glands.

The average number of mammary tumors determined for these experimental groups followed the same pattern as that seen in mammary tumor incidence (Figure 10b). Interestingly, average mammary tumor latency (Figure 10c) was significantly shorter by 1 week in the pregnant infected group than the ovary intact nulliparous mice ($p<0.01$), while tumor latency was increased in the OVX-saline treated and 1 week and 3 week E + P treated animals (1.1, 5 weeks and 1.4 weeks respectively; $p<0.01$). Collectively, these data indicate that a fully differentiated mammary gland such as that created in late pregnancy is equally susceptible to Py tumorigenesis as that of the ovary intact, nulliparous controls, suggesting that increased epithelial differentiation is not the reason for decreased Py tumor induction in older aged mice.

Induction of other tumors.

Table 1 describes the incidence of skin and bone tumors. Older and OVX mice that do not have a high incidence of mammary tumors are able to live longer and will develop skin and osteosarcomas at later stages following Py infection. Therefore, these treatments that lower mammary tumor incidence may be desirable to create a skin or bone tumor model using the Py system.

Table 1. Skin and bone tumor induction by 14 weeks post infection in athymic female mice following polyomavirus A2 infection.

	Skin Tumors	Bone Tumors
Ovary Intact Females:		
3 weeks	75% (6/8)	0% (0/8)
6 weeks	44% (12/27)	7% (2/27)
10 weeks	64% (7/11)	0% (0/11)
20 weeks	44% (4/9)	0% (0/9)
30 weeks	18% (2/11)	9% (1/11)
Ovariectomized (OVX) Females:		
OVX -3 weeks } infected at 6 weeks of age	100% (8/8)	50% (4/8)
OVX -1 week	67% (12/18)	11% (2/18)
10 week OVX	78% (7/9)	56% (5/9)
20 week OVX	71% (5/7)	14% (1/7)

DISCUSSION

If ovarian hormones play a role in Py mammary tumor induction, then one might expect mice that are infected at various ages that are characterized by different mammary developmental and ovarian hormone and growth factor responsive states will exhibit different susceptibilities to Py transformation. This study has investigated the developmental and ovarian hormone responsive states required by Py for mammary tumorigenesis. From the results presented here, there is an age effect on Py mammary tumor induction, with increasing age resulting in decreased susceptibility to Py transformation. In addition, OVX at all ages examined decreased mammary tumor induction, with the most pronounced effects observed at 10 and 20 weeks of age. The effects of ovarian hormones on tumor promotion at these older ages were not examined, but the present findings suggest that Py is more dependent upon ovarian hormones at older ages for mammary tumor induction. The effect of ovarian hormones after the time of infection at these older ages warrants further investigation. Interestingly, when mammary tumor-bearing mice that were infected with Py at 6 weeks of age are ovariectomized, the tumors do not regress, indicating that they are not ovarian hormone dependent for growth (4, 7). Based on the arguments presented above, the ovarian hormone dependency of Py tumors that are induced in older mice might be different and should also be determined.

The drastic effects of OVX at older ages on Py mammary tumor induction suggests that a transition occurs between 6 and 10 weeks of age that confers more ovarian hormone dependency for Py tumorigenesis. This is interesting in regard to the hormonal responsiveness and morphological changes that occur in the mammary gland between these ages. At approximately 7 weeks of age, the gland acquires the ability to induce progesterone receptors in response to estrogen which confers progesterone responsiveness and consequently, induces sidebranches in response to progesterone (8-10). Increased sidebranching represents a more differentiated gland, as these are the structures from which lobuloalveoli will develop (11). As the epithelial end buds reach the end of the stromal fat pad at approximately 8 to 10 weeks of age, these highly proliferative structures regress (6, 12). EGF has also been shown to induce proliferation in the epithelium at 5 weeks of age, independently of estrogen and may actually mediate estrogen effects at this age (13). Therefore, young mouse mammary glands may remain more proliferative due to EGF. Indeed, ovarian hormone independent epithelial growth does occur in young mice to a greater extent than in older mice (14). In contrast, the older mice may have mammary glands that are more ovarian hormone dependent for proliferation and these differences may reflect their increased sensitivity to ovarian hormone withdrawal and decreased tumor susceptibilities to Py that were identified in this study.

While this study demonstrated no effect of OVX at 48 hours before or 1 week after Py infection on mammary tumor profiles, an effect was observed when mice were OVX at least 1 week before Py infection. The tumor incidence results presented here may at first, appear to conflict with the results of Berebbi et al. (4) which showed that in mice ovariectomized at 3 weeks of age, the presence of estrogen was critical at a stage between 10 to 21 days pi for the subsequent development of mammary tumors. Indeed, the length of time between ovariectomy and Py infection was found to influence mammary tumor induction, which may explain the requirement for estrogen in their study. By removing the ovaries at such an early age, the development of mammary gland hormonal responsiveness and/or development may have been arrested in Berebbi et al.'s study (4). Thus a specific treatment period with estrogen may have been required for the acquisition of an appropriate hormonal responsiveness or state of development in these mice. The lack of an effect of OVX at 48 hours before and 1 week after Py infection in the present study suggests that ovarian hormones do not function as promoting agents for Py tumorigenesis in mice that are infected at 6 weeks of age.

The fact that fully differentiated mammary glands present in the late pregnant mice infected in this study remained susceptible to Py tumorigenesis indicates that the decrease in tumorigenicity that was observed in older mice is not simply due to increasing differentiation of the mammary gland. These

results may suggest the loss of a certain cell type or population that is present in the young mouse that is the primary target for Py transformation. In addition, it may be that this population or another target cell type is also present in the late pregnant mouse mammary gland, conferring the high tumorigenicity that was observed in this study. While chemical carcinogen models have shown protective effects of late pregnancy/lactation exposure on subsequent tumor induction (15-17), the mice infected in this study may have had a high level of proliferation remaining in the gland that allowed tumor induction. Alternatively, the mechanism of Py transformation may be such that a pregnant gland and its functionally differentiated cell types remain susceptible to tumor induction, while older mice have another type of "age" acquired differentiation that is less susceptible to Py.

It is noteworthy that the wholemount analysis presented here (Figure 9) of epithelial development following 3 weeks of E + P injections to OVX mice was expected to produce a late pregnant-like gland, however, only an early-pregnant state of development was attained, suggesting a different response to exogenously administered ovarian hormones. Decreased effectiveness of exogenously administered ovarian hormones has been previously observed in athymic mice (20).

In this study, skin tumors and osteosarcomas were induced in the mice in addition to mammary adenocarcinomas. It is curious that skin and bone tumors appear to occur less frequently than mammary tumors. This may be due to the

observation that when 6 week old, ovary intact mice were infected, they developed mammary tumors very rapidly after infection and became moribund before the development of other types of tumors which appear to have much longer latencies. Some of the older mice groups that did not develop mammary tumors, did develop skin and bone tumors. Therefore, this study has defined conditions to induce primarily skin and bone tumors, which may provide an interesting model in which to study these types of tumors. It is worth mentioning, that while this study observed both osteosarcomas and skin tumors, those of Berebbi et al. (3, 4) and Demengeot et al. (18) found only osteosarcomas, even though the same strains of mice and virus were used. It is difficult to reconcile these differences of organ tropic tumorigenesis but it may be that these two A2 strains may be different in their tumorgenicity of the skin, as both strains do replicate to high levels in the skin (1, 18). Alternatively, there may be discrete differences between the two types of Balb/c mice that were used in these two separate studies.

It is clear that the stage of mammary gland development and degree of hormonal responsiveness at the time of Py infection affect mammary tumorigenesis in athymic female mice. Previously, age effects have also been demonstrated for chemical carcinogen mammary tumor initiation (21-24). These age effects correlate with the levels of proliferation in young glands and the loss of proliferation in terminal end buds in older mice and rat mammary glands as they age and

differentiate (24-28). The mammary cells in which Py replicates and transforms have not been identified, however, only adenocarcinomas are induced following viral infection, suggesting that it is the epithelium that is the target for Py transformation. If proliferative cells are the targets for Py, older mice would be less likely to develop mammary tumors. While in late pregnancy, the gland is functionally differentiated, the level of proliferation probably remains high until lactation. This may explain the induction of mammary tumors following Py infection of late pregnant mice. However, it may be that the functional differentiation that occurs during pregnancy and age induced differentiation may result in very different cell type populations that have different susceptibilities to Py transformation. These possibilities cannot be discerned by the present study but it is imperative to identify the target cells for Py in order to understand the differences in mammary cell types, their transformation potential and the role of ovarian hormones in this process.

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Chapter 3

Polyoma viral genome and early protein levels and middle T antigen associated kinase activity in the mammary gland and tumors of athymic mice infected at different ages and mammary gland development states.

ABSTRACT

Polyomavirus (Py) infection of adult athymic female mice causes a high incidence of mammary adenocarcinomas. I have examined the role of ovarian hormones, age and mammary gland development at the time of infection in Py infection. Ovary intact mice were infected at 3, 6, 10, 20 and 30 weeks of age with Py A2. In addition, mice were ovariectomized (OVX) at 5, 9 and 19 weeks of age and were infected 1 week later. In addition, mice were OVX at 3 weeks, 1 week or 48 hours before or 1 week after infection at 6 weeks of age. To determine the effect of proliferation and differentiation on infection, eight or 10 week old mice that were OVX and treated with 1 ug 17-beta estradiol (E) and 1 mg progesterone (P) for 3 or 1 weeks before infection and late pregnant mice were also infected. OVX-saline treated mice served as controls. Mice were killed at 10 days post infection (pi) to assess the levels of viral genomes, early viral proteins and middle T kinase activity in their mammary glands. While no significant differences were observed in the levels of viral genomes,

which were high in all groups or in early viral protein and middle T kinase activity levels (low in all groups) in mammary glands at 10 days pi, these levels were very high in mammary tumors. These results suggest that the level of viral genomes in the mammary glands at 10 days pi is not indicative of the tumorigenicity of Py. However, the levels of genomes, early proteins and middle T kinase activity correlated with Py transformation and may reflect a threshold level of gene expression that is required for transformation.

INTRODUCTION

Mouse polyomavirus (Py), a DNA tumor virus, is an important model system used in the study of virally induced tumors in mice and cell transformation in tissue culture. Previous studies that examined viral DNA replication in different organs of both the neonate as well as the adult mouse revealed an age and organ specific pattern of Py replication. After infection with Py, neonate mice exhibit high levels of viral genomes throughout most of the mouse with the exception of the brain and blood (1-3). This stage of viremia, which peaks at 7-10 days post infection (pi) is followed by almost complete viral clearance by the host immune system to establish a persistent stage of Py infection, with some mice developing tumors later in life (4). Py infection of normal adult mice results in a quick viral clearance and no tumors are induced. However, when adult athymic mice are infected with Py, moderate levels of viral DNA replication occur in three major sites, the mammary gland, skin and bone (3).

The level of viral genomes was previously analyzed in the mammary glands of Py infected athymic female mice and found to be variable, though relatively high at all stages of the infection (3). However, when viral gene transcription was analyzed, a higher level of gene expression was found to correlate with the presence of mammary tumors. This study suggested that the levels of viral replication and gene

expression may determine the ability of Py to transform.

The oncogenicity of Py has been linked to the action of middle T antigen and its activation of a signal transduction cascade that is similar to that which is induced by platelet derived growth factor (PDGF) (5). Middle T has also been shown to activate protein kinase C (6, 7). These functions of middle T may culminate in the activation of important transcriptional activators PEA1 and PEA3 (6, 8), both of which are able to bind the Py enhancer and increase viral replication and gene expression. In addition, cellular genes may be overexpressed in Py infected cells and this overexpression as well as other, yet undefined, actions of middle T may lead to neoplastic transformation. Interestingly, when oncogenes that are suspected to be involved in human breast cancer were analyzed for overexpression in the Py model, a slight increase was only observed in c-myc expression, indicating that activation of other proto-oncogenes does not appear to be a key step in Py transformation (9).

Similar to viral replication patterns, the sites of Py tumorigenesis in adult athymic mice also show an organ- and sex-specific pattern (3, 10-12). Recent studies have revealed that ovarian hormones play a role in this oncogenic process of the female mouse mammary gland (12-13). Although it has been shown that ovarian hormones are required at or around the time of Py infection (12) for mammary tumors to develop, the role of the ovarian hormones in Py tumorigenesis is not known. It

is possible that an appropriate state of mammary development and/or ovarian hormone responsiveness of the gland may be required for Py to induce mammary tumors. Therefore, the purpose of the present study was to define the effect of ovarian hormones on viral replication, early protein expression and middle T antigen associated kinase activity in relation to Py mammary tumorigenesis. Mice that were infected at various ages which are characterized by different developmental and ovarian hormone responsive states were investigated.

While ovarian hormones, age and mammary gland development state influence mammary tumor induction (12, 13 and this thesis, Chapter 2), I report here that the levels of viral genomes, early proteins and middle T kinase activity in the mammary gland are not influenced by these factors, at least at the levels that can be detected by the methods used herein. However, in almost all cases tested, viral genome, early protein and middle T kinase activity levels were very high in Py induced mammary tumors.

MATERIALS AND METHODS

Animals:

All athymic mice were obtained from Life Sciences, Inc. and were housed in our vivarium in a sterile laminar flow rack and given sterile water and rodent chow (Purina) ad libitum. Mice were infected subcutaneously with 10^6 plaque forming units (pfu) of the wild type strain A2 of Py.

Treatments:

Female mice were infected with Py at 3, 6, 10, 20 and 30 weeks of age to assess the influence of different degrees of ovarian hormone responsiveness and mammary epithelial development on the levels of Py genomes, early viral proteins and middle T kinase activity. In addition, 5, 9 and 19 week old mice were OVX before infection with Py at 6, 10 and 20 weeks of age, respectively. To examine the role of ovarian hormones at the time of Py infection, mice were bilaterally ovariectomized (OVX) at 3 weeks, 1 week or 48 hours before or 1 week after infection at 6 weeks of age. Six week old, ovary intact mice served as controls.

To determine the effect of increasing differentiation on the levels of Py genomes, early viral proteins and middle T kinase activity in the mammary gland, 8 or 10 week old mice were OVX and given daily subcutaneous injections of 1 ug 17-

beta estradiol (Sigma) + 1 mg progesterone (Sigma) in 5% gum arabic (Sigma) for 3 weeks or 1 week, respectively before infection. Ten week old, ovary intact and age-matched and OVX-saline treated mice served as controls. In addition, 8 week old mice were mated with normal Balb/c male mice from our own colony for timed pregnancies. All mice were infected with Py at late pregnancy, 18-19 days gestation and allowed to deliver and nurse their progeny. All late pregnant mice were infected by 13 weeks of age.

The effect of age, ovarian status and different developmental and hormone responsive states of the mouse mammary gland on viral genome and early protein levels and middle T kinase activity were compared to the tumor incidence profiles of these mice that were determined in Chapter 2.

Viral genomes, early proteins and middle T kinase activity in polyoma induced mammary tumors.

Mammary tumors that were induced in the experimental groups examined in this study in Chapter 2 were analyzed for the levels of viral genomes, early proteins and middle T kinase activity as described below.

Viral genome, early protein and middle T kinase assays.

Two mice from each group were killed at 10 days post infection (pi) and their mammary glands were removed. In addition, mammary tumors that were induced at later times pi were also removed at the time of death. All mammary glands and mammary tumors were analyzed for the levels of viral genomes, early proteins and middle T antigen associated kinase activity. All lymph nodes were removed from the mammary glands prior to the extractions to reduce the amount of non-mammary DNA and protein.

Total DNA was extracted as previously described (9) and analyzed for the level of viral genomes by dot blot analysis following standard protocols (14). Five ug of total RNase-treated DNA was blotted onto a Hybond-N membrane (Amersham) and hybridized at 65° C with a random-primed, ^{32}P-labeled probe that represented the entire polyoma A2 genome. A polyomavirus transformed FR3T3 cell line which contains approximately 1 viral genome per host cell was used as a control. Following autoradiography, the levels of viral genomes were quantitated using a beta scanner (Ambis). Representative samples from each group are shown.

The level of viral early proteins was assessed in mammary gland and tumor protein extracts by Western blot analysis following standard protocols (14). Mammary glands from uninfected mice and wildtype Py A2 and mutant Py 18-5 (small and middle T minus) infected NIH3T3 cells served as controls.

Fifty ug of total protein was denatured and electrophoresed on a 10% SDS-polyacrylamide gel. The gel was blotted onto PVDF membrane (Dupont) and small, middle and large T antigens were detected using a polyclonal rat anti-polyoma T antigen ascites serum, a goat anti-rat horseradish peroxidase labelled secondary antibody (Kirkegaard and Perry Laboratories) and the Lumiglo chemiluminescent detection system (Kirkegaard and Perry Laboratories).

In addition, the associated kinase activity of middle T antigen within mammary gland and tumor protein extracts was analyzed as previously described (15, 16). The middle T antigen/protein complexes were immunoprecipitated from 500 ug of protein extract using a polyclonal rat anti-Py T antigen ascites fluid that was prepared in Norwegian brown rats (Harlan). The immunoprecipitated complexes were assayed for kinase activity in vitro using [gamma-^{32}P]ATP (New England Nuclear). The radiolabeled complexes were denatured and electrophoresed on a 10% SDS-polyacrylamide gel and autoradiography of the dried gel was performed at -70° C. Representative samples from each group are shown.

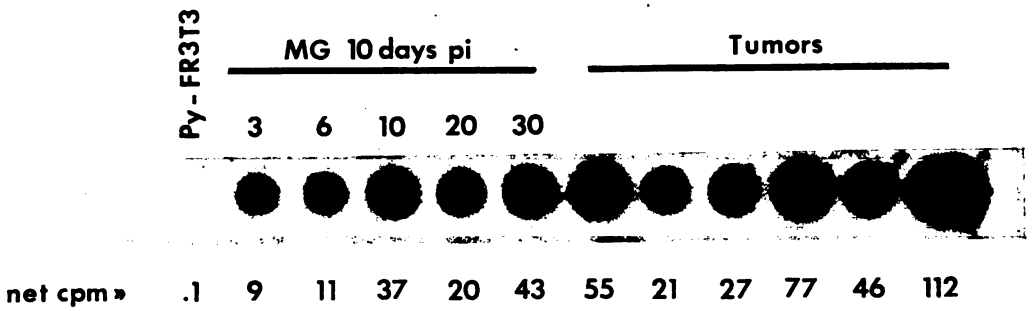
RESULTS

To examine the role of ovarian hormones in Py mammary tumorigenesis, female mice were infected at different ages with Py to assess the influence of different stages of mammary gland development and different degrees of ovarian hormone responsiveness on the levels of Py genomes, early viral proteins and middle T kinase activity.

While mammary tumor induction by Py was influenced by age and ovarian hormone status (Chapter 2), the level of viral genomes was not. As shown in Figure 11, increased age at the time of Py infection did not significantly influence the ability of the virus to replicate to high levels in the mammary glands of ovary intact mice. As compared to the Py transformed FR3T3 cell line that contains approximately 1 viral genome copy per host cell genome, the mammary glands of these mice that were taken at 10 days pi exhibited viral genome levels that ranged from 100 to 400 copies per cell. In addition, OVX at these various ages or for different lengths before or after Py infection had no significant effect on the level of viral genomes in the mammary gland (data not shown). Mammary tumors from these mice also had very high levels of viral genomes, ranging from 200 to over 1,000 copies per host cell.

The expression of the viral early proteins, small, middle and large T antigen was examined in mammary glands and mammary

Figure 11. Level of viral genomes in mammary glands and mammary tumors. (a). Ovary intact mice at 3, 6, 10, 20 and 30 weeks of age were infected and their mammary glands were removed at 10 days post infection (pi). In addition, mammary tumors were removed from mice at later times post infection. A polyomavirus transformed FR3T3 (Py-FR3T3) cell line which contains approximately 1 viral genome per host cell genome was used as a control. DNA was isolated from the cell line, mammary glands and tumors and 5 ug was transferred to a Hybond-N membrane. The blot was hybridized with a ^{32}P-labeled probe that represented the entire polyomavirus A2 genome and autoradiographed to detect viral sequences. Net counts per minute (cpm) were determined by beta-scanning and demonstrate the high level of viral genomes per cell in both mammary glands and mammary tumors.



net cpm »	.1	9	11	37	20	43	55	21	27	77	46	112

Figure 11.

tumors from mice that were infected at various ages and at late pregnancy. Western analysis of mammary protein extracts at 10 days pi revealed that the levels of Py early viral proteins were undetectable (Figure 12a). However, all mammary tumors that were examined expressed high but variable levels of T antigens (Figure 12a and b).

When the level of middle T associated kinase activity was determined in the mammary glands of mice at various ages and ovarian status at 10 days pi, it was undetectable in all mice (Figure 13). However, the level of middle T kinase activity in mammary tumors was again high but variable.

Figure 12. Level of viral early protein expression in mammary glands and mammary tumors. Ovary intact mice at 3, 6, 10, 20 and 30 weeks of age and late pregnant (P) mice were infected and their mammary glands were removed at 10 days post infection (pi). In addition, mammary tumors were removed at later times post infection. Uninfected (U) mammary glands and polyoma A2 (large T +, middle T + and small T +) and 18-5 (large T +) infected NIH3T3 cells served as controls. Proteins were extracted and 50 ug of total protein was denatured and loaded onto a 10% SDS-polyacrylamide gel and electrophoresed, blotted onto PVDF membrane and detected using a polyclonal rat anti-polyoma T antigen ascites serum, a goat anti-rat horseradish peroxidase labeled secondary antibody and Lumiglo chemiluminescent detection system. (a). Western analysis of mammary glands at 10 days pi and mammary tumors. (b). Western analysis of additional mammary tumors. The viral early proteins, large, middle and small T antigen are indicated by arrows.

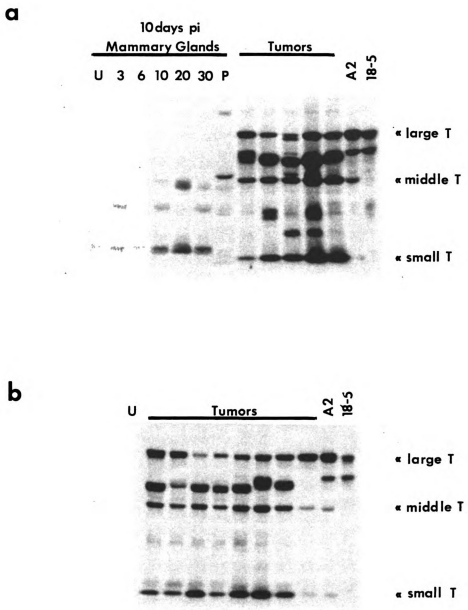

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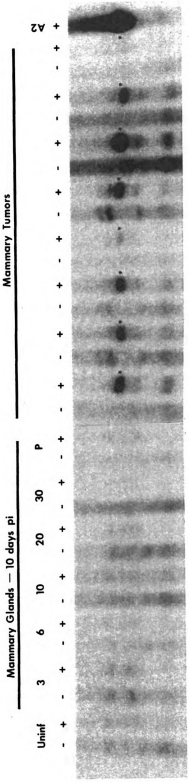
Figure 12.

Figure 13. Level of middle T antigen associated kinase activity in mammary glands and mammary tumors. Mammary glands of ovary intact mice that were infected at 3, 6, 10, 20 or 30 weeks of age or at late pregnancy (P) were removed at 10 days post infection (pi). In addition, mammary tumors were removed from mice at later times post infection. Uninfected (Uninf) mammary glands and polyoma A2 infected NIH3T3 cells (A2) served as controls. Polyclonal rat anti-polyoma T antigen ascites serum (+) was used to immunoprecipitate middle T antigen protein complexes from 500 ug of protein extract. Preimmune rat serum (-) was used as a control for each immunoprecipitation. The immunoprecipitated complexes were assayed for kinase activity in vitro using [gamma-^{32}P]ATP. The radiolabeled complexes were denatured and electrophoresed on a 10% SDS-polyacrylamide gel. The level of middle T associated kinase activity was determined by autoradiography of the dried gel. *, radiolabeled middle T antigen.


Mammary Glands — 10 days pi
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3
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Figure 13.

DISCUSSION

This study has concentrated on the analysis of polyomavirus genome level, early protein expression and middle T antigen associated kinase activity in the mammary glands and mammary tumors of mice that were infected at various ages that differ in mammary epithelial development and their response to ovarian hormones and growth factors. The purpose of this study was to explore the link between viral replication/gene expression and tumorigenesis.

Although Py mammary tumor induction has been shown to be influenced by ovarian hormones, age and perhaps mammary gland development stage, the level of viral genomes remained high in the mammary glands. This finding suggests that viral genome copy number at 10 days pi is not indicative of the mammary gland's susceptibility to Py mammary tumor formation. However, the possibility exists that a threshold level of replication is required <u>in addition</u> to another factor(s). Indeed, organ-tropic replication of the virus may not be all that is required for organ-tropic tumorigenesis. It is possible that only cells with certain transcription factors will replicate the virus but since polyoma does not cause tumors in all tissues in which it replicates, the cells may need to express other factors that are targeted by middle T antigen. It is possible that this additional factor(s) required for Py mammary tumorigenesis is present in high amounts in young female mouse mammary glands and allows rapid

and high mammary tumor induction.

In contrast, the levels of Py early T antigen protein expression and middle T kinase activity were undetectable in whole mammary gland extracts at 10 days pi. However, as shown for the first time in this study, high levels of viral replication, early viral protein and middle T antigen associated kinase activity correlate with mammary tumors. This observation supports the model of Py tumorigenesis that occurs through the continual activation of the signal transduction pathway(s) by middle T, leading to increased kinase activity and uncontrolled cell proliferation.

It may be that Py gene expression is affected by age, ovarian hormones and/or mammary gland development state within the virus' target cell populations. More than likely, a dilution problem exists within the assays used in this study. At the time post infection that the infected mammary glands were examined, the ratio of infected cells to uninfected cells is probably fairly low. Therefore, any effects on kinase activity/gene expression that may occur due to age or ovarian hormone status in the target cells may be diluted out by the high percentage of uninfected cells that are present when the proteins are extracted. Indeed, a threshold level of transgene expression was required in neu transgenic mice mammary glands for mammary tumors to be induced (17). The level of neu expression was high in the mammary tumors, but was lower in the surrounding normal tissue. Therefore, T antigen immunocytochemical analysis of Py infected mammary

glands from the various age and ovarian hormone status groups that were analyzed in this present study might resolve this issue.

Overall, this study has shown that the levels of viral genomes, early proteins and middle T antigen kinase activity were not significantly affected by age, ovarian hormone status or mammary gland differentiation, at least as detected by the assays used herein. However, high levels of viral genomes, early proteins and middle T kinase activity were observed in the mammary tumors that Py induced in these mice and these levels may be reflective of the mechanism by which Py transforms the mouse mammary gland.

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