The Role of E2Fs in Mouse Mammary Gland Development

A Senior Honors Thesis

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by

April Sandy

The Ohio State University
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Project Advisor: Dr. Gustavo Leone, Department of Molecular Virology, Immunology, and Medical Genetics
Abstract:

The mammary gland is a highly organized structure that takes cues from a variety of cellular pathways and messengers to direct its development and changes—one of those classes of messengers is the E2F family of transcription factors. While loss of all three E2Fs that direct cellular proliferation results in loss of normal development, the specific roles of these factors are largely uncharacterized, especially in the mammary gland. Examining the effects of single and combinatorial deletions of the E2F family members will present a better picture of the roles of these factors in developing the structure of glands. The myc oncogene and E2F transcription factors are known to have ties to cancer, and the development of cancer in mammary glands is directly related to abnormal development, leading to oncogenic events. Therefore, a better understanding of the effect of E2Fs on development will inevitably give rise to a more insightful view of how cancer forms in the mammary gland.

Introduction:

Mouse mammary glands are complex structures that go through continuous cycles of development in the course of a mouse’s life. The mammary tissue is originally a simple duct system running through a fat pad under the mouse’s skin, which then undergoes drastic changes during and after pregnancy. The two primary components of the mammary gland are the epithelia, the branching ductal system, which develops into milk-secreting acini, and the surrounding stroma, which serves as a matrix for the proper growth and differentiation of the epithelia (Fig. 1A). In newborn mice, the epithelia is rudimentary and consists of a small tree of primary ducts ending in terminal end buds.
Sites of cellular proliferation and differentiation, the bulb-shaped terminal end buds extend the ducts outward from the nipple and grow until they reach the edges of the fat pad, at which time the buds disappear. Ductal elongation and branching continues at a slow rate from about 3-10 weeks of age. During pregnancy and early lactation, intense epithelial cell proliferation leading to increased ductal branching and lobulo-alveolar development causes the inter-ductal spaces to fill with milk-producing and -secreting structures (Fig. 1B). After lactation, secretory epithelial cells are gradually lost by cell death (apoptosis) and the mammary gland undergoes involution (1).

These main stages of mammary growth, differentiation, and apoptosis are controlled by complex interactions between hormones and growth factors, which are responsible for activating a network of signaling pathways within the epithelial cell. At this point, the particular target genes for the control of growth and differentiation have not been thoroughly characterized; however, it is safe to predict that transcription factors and components of the cell cycle machinery are mandatory mediators of these events. The retinoblastoma (Rb) tumor suppressor pathway plays a key role in the control of the cell cycle by directly binding and regulating E2F factors. Studies using tissue culture have led to the discovery of the E2F transcription factor family as an important effector of Rb function, impacting on cell proliferation, apoptosis, and differentiation. From these studies, a paradigm for Rb action in the control of cellular proliferation has emerged. In this view, cyclin dependent kinase activation results in the phosphorylation of Rb and the release of E2F family members from Rb-containing complexes, leading to E2F target activation and cell cycle progression (2).
Of the E2F family, E2F1, E2F2, and E2F3 are the transcriptional activators that help promote cellular proliferation. Consistent with an important role for these proteins in cellular proliferation, the combined disruption of *E2F1*, *E2F2*, and *E2F3* in mouse embryonic fibroblasts impedes cellular proliferation (3). A comparison of a developmentally normal mammary gland with one that is deleted for *E2F1*, *E2F2*, and *E2F3* shows that the triple knockout destroys normal mammary gland formation, as the gland shows a marked decrease in ductal branching throughout the fat pad. Consequently, it becomes of interest to determine which of the E2F transcription factors plays the most important role in mammary gland development, and how these factors work together to direct gland formation. Examining different combinations of E2F factor knockout glands will help give a more thorough picture of how morphological development is directed genetically.

Understanding the morphology and development of the mammary gland is imperative to a comprehensive understanding of breast cancer. By examining the effects of mutations and knockouts of specific genetic variables in the tissue, the events that lead to abnormal development can be better characterized. Early tumorigenesis characteristics are vital to prevention and comprehension of any cancer. By understanding the function of the E2F transcription factors involved in proliferation, E2F1, E2F2, and E2F3, their part in normal mammary development can be used to realize how they may take another role in cancer formation.

Because complete deletion of a gene can have undesirable effects of the subject being studied, such as impairment of embryological development and thus early death, it is often necessary to used conditional knockout systems in research models. This is the
case with \textit{E2F3}—deletion of this gene results in increased gestational mortality of mice (4). Since this poses a problem to studying the development of these mice, the tet-on expression system was used to conditionally delete \textit{E2F3}. This system makes the recognition of tet-o sequences in the DNA by a transcriptional activator, rtTA, dependent on the presence of doxycycline. With this method, the expression of a transgene is inducible and dependent on the presence of doxycycline (Fig. 2) (5). The \textit{E2F3} gene in the mouse DNA is flanked by loxP binding sites, which are recognized by cre recombinase. Cre is activated by the presence of doxycycline, at which time it can excise the area between the complimentary loxP sites and recombine the DNA, conditionally knocking out the \textit{E2F3} gene (6). Thus, \textit{E2F3} in the mouse can be artificially deleted after development of the mouse to ensure its survival and usefulness in the study. The tet-on system is also used to induce expression of the oncogene, \textit{c-myc}, to allow for maximum control of experimental conditions.

\textit{C-myc} is an oncogene that has a function in normal cells in cell cycle progression. Loss of \textit{myc} in mice causes embryonic death, indicating an important role for the gene in development. If nothing is wrong with the copy of this gene, cell cycles continue normally; however, when something goes awry with \textit{myc}, such as a mutation, it becomes stuck in a position that continually directs cells through the cycle, leading to increased proliferation. In response, tumor suppressor genes activate high levels of apoptosis to counteract the abnormal effects increased proliferation would have on the cell. Again, if this function occurs normally, all is well. A defect in the tumor suppressor gene in the same cell as the original defect can spell disaster in the way of cancer. Cellular proliferation will become unchecked by apoptosis, and tumorigenesis can occur (7). \textit{Myc}
was used in this study to examine the possible effects of mammary gland development abnormalities on early tumorigenesis possibilities. This relates closely to human cancers, as *myc* contributes to over 70,000 cancer deaths annually in the United States (8).

**Materials and Methods:**

**Induction of *myc* in mice:**

Because of the use of the tet-on inducible system in the mice, *myc* expression had to be induced with doxycycline. Mice were fed through the water supply at a concentration of 2mg/ml doxycycline in 5% sucrose water. The water was fed beginning at 5 weeks of age until time of harvest.

**Harvest of glands for carmine-stained whole mounts:**

Glands were harvested at approximately 30 days after induction of *myc* expression, and glands from position 9 or 4 were fixed on a slide in Carnoy’s solution (3 parts 95% ethanol:1 part glacial acetic acid) overnight. After fixation, glands were hydrated in consecutive changes of solutions of 70%, 50%, and 30% ethanol, followed by two washes of distilled water. Slides were then stained with Carmine overnight. After 24-48 hours, glands were dehydrated with consecutive changes of distilled water, 70%, 90%, 95%, 100%, and 100% ethanol. Slides were cleared in xylene and then mounted using Biomedia Permount and glass slide covers.
**Preparation of histological sections:**

Gland tissue from position 4 was harvested at the same time that whole mount glands were harvested, 30 days after addition of doxycycline. After harvest, glands were fixed in Neutral Buffered Formalin for several days at 4°C, then processed and embedded in paraffin sections. Thin 5µm sections were cut and placed on slides, where they were stained with Hematoxylin and Eosin (H&E).

**Immunofluorescent staining of mammary gland paraffin sections:**

From the paraffin sections of position 4 glands as described above, immunofluorescent staining with proliferation marker Ki67 was performed on unstained sections. The slides were deparaffinized using three washes in Accustain Xylene Substitute followed by washes in 100%, 95%, and 70% ethanol, and distilled water. Tissue sections were then placed in Dako Target Retrieval solution in a steamer for 45 minutes, cooled, and rinsed in PBS. A solution of 2% goat serum in IF Buffer was used to block the tissue, and then the primary antibody, Ki67, was diluted at 1:100 in Dako Antibody Diluent and applied to the tissue for one hour in a humidified chamber. After rinsing twice in PBS, a 1:500 dilution of Alexa conjugated secondary antibody goat-anti-mouse in Dako Antibody Diluent was applied for 45 minutes in a humidified chamber. Slides were rinsed once in PBS, once in TBS, and the nuclei were then counterstained with DAPI in TBS at a 1:150 dilution. Sections were rinsed once again in TBS and Biomega Anti-fade Mount was used to cover slip the slides.
Quantification of Ki67 positive cell counts:

At least six pictures of each gland were counted at a magnification of 40x for ductal cells stained positive for Ki67. Cells outside of the immediate ductal region were not quantified. Pictures were captured at different locations throughout the gland to gain the best homogenization of ductal morphology and staining.

Harvest of glands for β-galactosidase stained whole mounts and histological sections:

Glands were harvested approximately 30 days after induction of myc expression, using position 9 for the whole mount and positions 7-8 for histological sections. Whole glands were mounted on a slide placed in a fixative (4% paraformaldehyde, 0.1M phosphate buffer pH 7.3, and 25% glutaraldehyde) for 2 hours at 4°C. For histological sections, the gland was spread on a slide and placed in the same fixative for 1 hour at 4°C, and then was scraped off the glass with a blade and floated in a petry dish of fixative for an additional hour at 4°C. Following fixation, tissues were rinsed with PBS twice, and then staining media (0.8x PBS, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 0.8mg/ml X-gal, 0.1% deoxycholate, 0.2% NP-40, and 2mM magnesium chloride) was added and kept in the dark overnight at room temperature. After staining was visualized, tissues were rinsed in PBS overnight. Whole mount glands were then dehydrated in consecutive solutions of 70%, 90%, 95%, 100%, and 100% ethanol, and then cleared in Accustain Xylene Substitute before mounting. Histological sections were instead placed in formalin, embedded in paraffin, and cut for staining with H&E.
Regenotyping to confirm alleged genotype:

Upon harvest of each mouse, a small piece of the tail was clipped and DNA later extracted from the tissue. This DNA preparation was used to perform PCR techniques to reconfirm the original genotypes of the mice to ensure maximum accuracy of each gland.

Results:

Close examination of both the whole mount and histological sections of the mammary glands under a microscope revealed some interesting findings about the effects of the E2F transcription factors. Ductal branching complexity and progress through the fat pad was examined, along with terminal end bud presence and position. When E2F3 was deleted in the mammary gland in the absence of the myc oncogene, there seemed to be minimal difference compared with the presence of E2F3, indicating that deleting E2F3 alone is not sufficient to have a noticeable phenotype on mammary gland development (Fig. 3A). When myc was expressed, there was noticeably more ductal branching and development of the gland in both the E2F3 knockout and the wild type. These observations were backed by Ki67 staining, which showed much higher numbers of cells positive for proliferation in glands where myc was expressed in comparison to those that did not express the oncogene (Fig. 3B,C). Myc therefore does induce cellular proliferation, presumably by controlling continuation of the cell cycle.

Deletions of E2F2 manifested as more proliferation in ductal branching as compared to the control glands (not deleted for any E2F factors), both in the presence and absence of myc (Fig. 4). As is expected, there appeared to be more branching when myc was expressed, in terms of branch development and terminal end bud migration. When
both E2F2 and E2F3 were deleted, there seemed to be less development of the glands both with and without myc expression. Less branching of the ductal system was evident and remained in a more rudimentary state, as if normal development was impaired. This observed phenotype is logical, because if two of the three transcriptional proliferation factors are obliterated, less proliferation of the terminal end buds through the gland would be expected.

In the presence of myc, deleting E2F1 results in more branching of the ductal system as compared to the control (not deleted for any E2F factors); however, without myc, there appears to be no difference in gland maturation (Fig. 5). When a combinatorial deletion of E2F1 and E2F3 is examined, there does appear to be a significant decrease in developmental progress of the gland when myc is expressed, and a more slight decrease in the absence of the oncogene.

It is important to note that developmental phenotypes in the mammary glands vary from mouse to mouse. Even within mice of the same genotypic group, there was some degree of variability of ductal branching and progression. This can be expected because the complex nature of the mammary gland system. An enormous variety of growth factors, hormones, transcription factors, etc. work in concert to develop the gland through a myriad of pathways—clearly there is more than one variable here. Although there was some degree of variability from gland to gland, generalizations could still be observed and concluded from the work.

Deletion of E2F3 with the tet-on system was visualized with β-galactosidase staining of whole-mount and histological sections of mammary glands, and showed that the deletion is very efficient and uniform. When tet-o-cre was present, and thus E2F3
was deleted after doxycycline induction, all ducts in the gland uniformly stained blue (Fig 6A). Control glands proved that in the absence of tet-o-cre, no blue staining appeared, indicating E2F3 was not deleted (Fig. 6B). It seems that the whole ductal system was deleted, and that the deletion was complete throughout each duct. This ensures the effect of E2F3 deletion was present in the complete ductwork of the mammary gland, and thus the results observed were due to the effects of the conditional deletion. These results prove a more thorough deletion in the inducible system as compared to systems that were previously used. E2F3 conditionally deleted glands were previously harvested from mice under the influence of the wap-cre system, which is induced by two rounds of pregnancy rather than doxycycline. These glands demonstrated a more sporadic deletion of E2F3, as patches of blue ducts in the gland—not all ducts were uniformly deleted as in the tet-on system.

When stained with cellular proliferation markers, histological mammary gland sections proved that loss of E2F factors 1 and 3 resulted in less ductal proliferation and development. As was observed in whole-mounted glands, loss of both E2F1 and E2F3 resulted in three times less proliferation than was apparent in glands deleted for E2F1 alone. However, it is unusual to note the same trend was not observed in the deletion of E2F2 and E2F3 in glands, when compared to E2F2 deletion alone. Roughly similar numbers of cells positive for proliferation were observed in both categories—addition counts are currently being quantified to confirm these results (Fig. 7). The numbers of positive cells counted in ducts with loss of E2F1/E2F3 were rather similar to those obtained for E2F3 deleted glands; additionally, these numbers were also roughly similar to those obtained for the E2F2 deleted and E2F2/E2F3 deleted glands. Glands deleted for
E2F1 only showed a large increase in the numbers of proliferating cells, which could be due to the alleged role E2F1 plays in mediating apoptosis (9). Loss of this transcription factor could decrease the amount of apoptosis occurring in the epithelial cells, in turn increasing the total cellular proliferation.

A mammary tumor study is currently in process to study the effect of these same E2F deletions on tumorigenesis. Results from the mammary gland development study directly correlate with the tumor study because of the need for understanding how the transcription factors and oncogenes direct mammary development and thus diverge from a normal phenotype into a cancerous state. Identifying abnormalities and changes from normal gland development can help characterize pre-cancerous states and realize how cancer erupts in these mammary glands. In turn, the scope of these understandings can reach far beyond the immediate breast cancer of mice and help understand these same processes in humans. From the data collected so far, oncogenic mice with combinatorial E2F1/E2F3 or E2F2/E2F3 deletions have a delay in tumorigenesis as compared with those only deleted for E2F1 or E2F2, respectively (Fig. 8). The delay seems to be about one month. These findings seem logical, as we have observed that deletion of E2F1/E2F3 or E2F2/E2F3 in the virgin mammary gland leads to decreased cellular proliferation. Tumor data for E2F3 shows minimal difference in time-to-tumor numbers for presence or deletion of E2F3 in oncogenic mammary glands. Interestingly, the appearance of tumors occurs at about the same time for glands deleted for E2F3 alone, E2F1 alone, or a combination of E2F2/E2F3. More mice are being collected and analyzed to get more significant numbers for this cancer study.
**Discussion:**

The inducible tet-on expression system gives an efficient means to invoke oncogene expression and conditional E2F deletion in mammary glands, allowing otherwise lethal genotypes to be studied in a controlled fashion. Deletion is uniform and efficient, providing a sound model for this mammary gland study.

While it is known that these three E2F family members are important for cellular proliferation, not one transcription factor seems to be more important than the others. It appears the loss of a single E2F transcription factor is not sufficient to severely impair normal mammary gland development. Perhaps this is indicative of an elaborate system of signaling and messaging within the family members to complete their designated proliferative duties. The deletion of either E2F1, E2F2, or E2F3 in the mouse model did not have a strong phenotype, indicating the possibility of some kind of compensation mechanism occurring within this family of proliferation factors. Losing the activity of one factor likely causes upregulation of the other two, resulting in development that mimics that of a normal gland and thus lacks a significant phenotype. However, the loss of a combination of two transcription factors does result in a noticeable phenotype, most prevalent in the presence of the *myc* oncogene. This is suggestive of an important role of the E2F family members in interaction with myc, and thus in cancerous events. The combinatorial deletion phenotype also seemed to be more pronounced in the E2F1/E2F3 deletion than with the E2F2/E2F3 deletion. A western blot analysis of the protein expression levels in these mammary glands would be an essential future experiment to determine if compensation is the explanation for the results obtained.
While the tumor study data mentioned beforehand is preliminary and the study is still underway, the results seen so far present some interesting evidence. According to that data, mice deleted for E2F2 and E2F2/E2F3 develop tumors earlier than mice deleted for E2F1 and E2F1/E2F3. From this evidence alone, one would expect higher cell counts for Ki67 positive cells in mammary glands of the E2F2 group, presumably because these mice should have higher rates of cellular proliferation and thus less time for tumor formation. However, those results do not match those seen in the virgin mammary glands harvested for the developmental study, which show the E2F1 deleted group to have the highest rates of proliferation. This could be due to the low numbers of samples collected so far for the tumor study, since that project is still being conducted. Once more significant numbers have been achieved, the data will need to be reanalyzed to determine if the same pattern still prevails. Also, more slides should be stained for Ki67 and numerous more positive cell counts performed to ensure maximum accuracy.

Studying different developmental time points of the mammary gland would be an interesting continuation of this study to determine if the same results obtained in virgin glands holds true when different hormonal signaling pathways also have an effect on the mammary system. Pregnancy, lactation, and involution are important phases of mammary glands, and studying these various stages would help complete the picture of E2F transcriptional activator effect on gland development. Examining the changes in these stages could also give further insight into how cancer develops in oncogenic tissues. Furthermore, additional numbers of samples for some of the genotypic virgin categories should be harvested to account for differences in individual glands and create a better sense of the average development of each genotype.
There are many possible future experiments that could stem from this developmental work, some of which have already been mentioned. Another idea is to look at mammary gland development and the effect of E2F’s on that development in a system other than myc. Perhaps the use of a different oncogene could allow for a more thorough picture of E2F family function. Seeing similar results in another system could confirm the alleged roles of the different E2Fs in mammary gland development and tumorigenesis; in contrast, varied results could show specificity for each system and the interactions that occur in that particular setting. Additionally, staining histological sections for apoptosis markers would also add another dimension of the E2F story. Understanding how the transcription factors affect proliferation goes along with understanding their role in apoptosis, and perhaps this could give a clearer picture of the increased proliferation in E2F1 deficient glands.

Inducible expression of the *myc* oncogene and E2F3 deletion have allowed for a comprehensive study of mammary gland development. While the mission to determine which specific E2F transcription factor was the biggest mediator in development was not met, much important knowledge has been gained from this study about the action of E2F1, E2F2, and E2F3 in managing gland development in mice. Further studies to complete this picture would be useful to paint a comprehensive view of mammary glands and their journey into breast cancer through oncogenic events.


