The Effects of Tamoxifen on Mammary Organoids from Young and Old MMTV-c-*neu* Mice

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Dedication

This thesis is dedicated to my mother, Mary Ann Troness, and aunt, Margaret “Peggy” Lambert, both survivors of breast cancer.

Chapter Abstracts:

Chapter 1: The History of Endocrine Therapy and Tamoxifen for the Treatment and Prevention of Breast Cancer

The link between the ovary and the mammary gland was first established in 1896 by George Beatson when he demonstrated that the removal of the ovaries could lead to the regression of breast cancer in patients. This discovery led to identification and characterization of estrogen, its receptor, and their connections to the development of breast cancer. Compounds that could block estrogen signaling were also identified, leading to the discovery of tamoxifen. Clinical trials of tamoxifen demonstrated its effectiveness for preventing tumor recurrence following the removal of primary tumors. These studies also demonstrated a reduction in contralateral breast cancer, leading to the hypothesis that tamoxifen can prevent the development of breast cancer. Trials testing tamoxifen as a chemopreventive demonstrated that tamoxifen-treatment leads to a long-lasting reduction in estrogen receptor (ER)-positive breast cancers. These findings led to tamoxifen becoming the first drug approved for the prevention of breast cancer.

Chapter 2: The Effects of Tamoxifen on Tamoxifen-sensitive and Resistant MMTV-c-*neu* Mouse Mammary Organoids

Introduction:
Tamoxifen has proven to be an effective chemopreventive in both clinical trials and in mouse models of breast cancer. These studies have demonstrated that a relatively short duration of tamoxifen-treatment can result in a long-lasting reduction in tumor incidence. However, this effect was only observed when mice were treated when they were younger while older mice did not benefit from tamoxifen. The mammary epithelium is made up of basal and luminal cell populations that contain differentiated cells as well as stem-like progenitor cells from which the differentiated cells are believed to arise. Aging is associated with significant changes in mammary epithelial cell numbers, gene expression patterns, and in the activity of mammary progenitor cells. We hypothesized that tamoxifen's failure to act as a chemopreventive in older MMTV-*c-neu* mice is the result of an intrinsic change in the response of mammary epithelial cells to tamoxifen that occurs with age. To test this hypothesis, mammary epithelial cell organoids were isolated from young and old MMTV-*c-neu* mice, treated with tamoxifen, and the resulting cell populations were quantified, examined for progenitor activity, and assayed for cell death signaling.

Methods:
MMTV-c-*neu* mouse mammary organoids were isolated from young, 10 to 15 weeks-of-age, and old, 29 to 34 weeks-of-age, mice. Organoids were cultured in estrogen-free conditions with or without tamoxifen for 10 days. Cell growth assays were performed by assessing sulforhodamine B-retention in secondary culture of organoid cells, cultured for 2, 4, and 6 days. Adherent (2D) colony-forming assays were performed for 6 days in the presence of growth-arrested NIH-3T3 feeder cells with the total organoid cell population or FACS-sorted luminal and basal populations. Non-adherent (3D) colony-forming assays were performed with sorted epithelial, luminal, and basal populations for 7 days in Matrigel. The proportions of mammary cell populations, their EdU retention, and Annexin V labeling were assessed by flow cytometric analysis. RT-qPCR was performed to assess the transcription of *Esr1*, *Cdkn1a*, *Bcl2*, and *Bax* in sorted luminal and basal populations.

Results:
Tamoxifen increased the growth and colony-forming efficiency of mammary organoid cells from young and old MMTV-c-*neu* mice. Tamoxifen caused significant reductions in the proportions of the basal and CD61-positive luminal cell populations in organoids from young and old mice. The proportion of the CD61-negative luminal cell population among all organoid cells was not significantly changed by tamoxifen but tended to decrease in young mouse organoids while it tended to increase in old mouse organoids. EdU-labeling was significantly reduced in the basal populations of both young and old organoids. 3D colony-forming assays showed an increase in luminal-like colony-formation among sorted luminal cells from old, but not young, mouse organoids. The frequency of solid colonies was not changed in the luminal or basal populations of young or old mouse organoids. Assessments of apoptosis showed that annexin V labeling was unchanged within the organoid cell populations. *Esr1* transcription did not change in the luminal cell populations of young or old mouse organoids. *Cdkn1a* transcription tended to increase in the luminal population of young mouse organoids. *Bax* and *Bcl2* transcription were both decreased in the luminal population of young mouse organoids.

Discussion:
Tamoxifen induced a significant increase in the number of colony-forming cells within the luminal populations of young and old MMTV-*c-neu* mouse mammary organoids. The CD61-negative population was relatively unchanged by tamoxifen, suggesting that this change resulted from either CD61-negative progenitor cells making up a greater proportion of the cell population following tamoxifen-treatment or from luminal cells within this population gaining the ability to form colonies. The basal and CD61-positive luminal populations were significantly reduced in both young and old mouse organoids. EdU-labeling was significantly reduced in the basal population but there were no signs of increased apoptosis in any cell population. Earlier time points may need to be examined to determine the mechanisms behind the changes in cell populations. Tamoxifen produced different effects in young and old mouse organoids, namely within the luminal population. The CD61-negative luminal cell population tended to decrease with tamoxifen in young organoids but tended to increase with tamoxifen in old organoids. Likewise, 3D colony-forming activity was only stimulated in organoids from old mice. HER2/*neu* mammary tumors are believed to arise from the luminal progenitor population, some of which may reside in the CD61-negative luminal cell population. The difference in response to tamoxifen between young and old organoids may indicate that the CD61-negative luminal cell population is the target population for tamoxifen's chemopreventive effect. By reducing this population, tamoxifen may reduce tumor incidence in young MMTV-*c-neu* mice however, changes in this population that occur with age appear to lead to it acquiring a resistance to this effect. Further examination of this population's ability to form tumors and the effects of tamoxifen on this population may reveal more details of tamoxifen's chemopreventive mechanism.

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Chapter 1

The Discovery of Estrogen and its Role in Breast Cancer Development

The connection between breast cancer and estrogen was first demonstrated in 1896 by George Beatson (1). Beatson reported his treatment of a woman with inoperable, metastatic cancer originating from her right breast. With no other treatments available, Beatson hypothesized that the growth of breast cancer is linked to ovarian functions and chose to remove the patient's ovaries and uterine tubes. Extraordinarily, the patient reported significant reduction in symptoms just three weeks after the surgery and the tumors were visibly in remission. With this initial success, oophorectomy became a common palliative treatment for breast cancer, utilized until the 1960s (2). However, only a third of all breast cancer cases responded to treatment (3).

With the effectiveness of oophorectomy limited to a subset of patients, considerable research was devoted to determining which cases of breast cancer would respond to treatment. A significant clue to answering this question came in 1923 with the discovery of estrogen by Edgar Allen and Edward Doisy (4). Allen and Doisy gave subcutaneous injections of extracts from the ovarian follicles of hogs to castrated rats and mice and then assessed the influence of the extracts on the histology of the vaginal epithelium. Cyclical changes in the vaginal epithelium, following the stages of estrus, had previously been observed and these changes could be ablated with the removal of the ovaries (5). 40 to 48 hours after injection, all of the treated mice were in full estrus. Additional isolations of the components of the ovarian extract found that the active component was located outside of the follicular cells, suggesting that the active component was a hormone. Additional injections of the isolated hormone showed that it could induce growth in the genital tract and mammary gland, stimulate mating behavior in mice, and prematurely induce puberty.

The characterization of the physiological effects of estrogen in the mammary gland, vagina, and uterus continued through the 1920s and 30s. In 1936, Antonio Lacassagne

demonstrated the effects of large, weekly doses of exogenous estrogen on the development of reproductive tissues and mammary cancer in males and females of various mouse strains (6). Treating mice with a synthetic estrogen, estrone benzoate, stimulated cell division in the vaginal and uterine epithelium of female mice and stimulated the expansion of the mammary epithelium in both female and male mice.

Treating males from different strains of mice demonstrated the influence of estrogens on mammary tumor development (6). The strains examined included a strain with a high rate of carcinogenesis, where 72% of females develop mammary cancer after a year, and another that showed a low rate of carcinogenesis, with only 2% of females developed mammary tumors. In males from the strain that experienced high rates of carcinogenesis, estrogen stimulated the development of the mammary epithelium and tumors appeared between the 4^{th} and 10^{th} months of treatment. In males from the strain with that developed tumors at a lower rate, the growth of the mammary epithelium was slower and tumors didn't appear until after 12 months of treatment. Lacassagne concluded that the different responses observed in these two strains were due to differences in each mouse models' sensitivity to estrogen and also speculated that a therapeutic treatment that could antagonize estrogen action could prevent the development of tumors.

With estrogen identified as the hormonal signal from the ovaries and its effects on various organs and breast cancer development characterized, efforts turned to identifying why some tissues are responsive to estrogen while others are not. This was achieved with the development of an *in vivo* assay for estrogen-retaining tissues in 1959 (7). By injecting low-doses of tritium-labeled hexoestrol into young goats and sheep, Glascock and Hoekstra demonstrated that estrogens are retained within responsive tissues, such as the vagina, uterus, mammary gland, and pituitary gland at much higher concentrations than in non-responsive tissues; like the brain, lungs, heart, and skeletal muscle (7). These findings were reiterated with the use of tritium-labeled estradiol (8).

The first assays for estrogen-retention in cases of human cancer were reported by Folca and colleagues in 1961 (9). Patients with metastatic breast cancer were given injections of tritium-labeled hexoestrol 6 hours before undergoing bilateral adrenalectomy and oophorectomy. The concentration of hexoestrol was assessed in samples of striated muscle, blood and tumor tissue then correlated with the patients' response to endocrine ablation. Of the 10 patients examined, 4 showed improvement following surgery. Tumors from these 4 patients showed noticeably higher hexoestrol concentrations compared to tumors from unresponsive patients, suggesting that responsiveness to endocrine ablation was dependent on the estrogen receptor content of the tumor.

This method was later adapted into an *in vitro* assay that could determine the estrogen receptor content of slices of tumor tissue and was used to correlate the retention of tritiated-estradiol in tumors with their responsiveness to endocrine ablation (10). Block and associates examined 274 patients, of whom 133 had tumors that tested positive for the estrogen receptor (10). 58% of patients with estrogen receptor-positive tumors responded to endocrine ablative therapies, while only 5% of patients with estrogen receptor-negative tumors responded.

The localization of estrogens to responsive tissues and tumors reinforced the hypothesis that these tissues carried a receptor that facilitates the interaction between circulating estrogens and the cell. Early efforts to isolate and characterize the estrogen receptor were performed in 1966 by Toft and Gorski (11). In a sucrose gradient, tritium-labeled estradiol-17β became bound to a putative estrogen receptor with a sedimentation coefficient of 9.5S. Subsequent tests showed that the 9.5S complex had the characteristics of a protein enzyme. The addition of the synthetic estrogen, diethylstilbestrol, reduced tritiated-estradiol-17β binding and suggested that the two molecules compete for receptor binding. The addition of the non-estrogenic isomer, estradiol-17α, only inhibited tritiated-estradiol-17β binding by one tenth, showing that the receptor was highly specific to estradiol-17β. Furthermore, the addition of pronases or trypsin, but not DNase or RNase, removed estradiol binding at the 9.5S peak, suggesting that the receptor was a protein.

Additional characterization by Jensen and associates determined the signaling kinetics of the estradiol-bound receptor (12). In addition to the 9.5S estradiol-receptor complex, an estradiol-receptor complex with a sedimentation coefficient of 5S was discovered. Autoradiographic examination of the 9.5S complex's localization determined that the 9.5S complex could be localized to the nucleus or to the extranuclear space. When uteri were exposed to tritiated-estradiol, either *in vivo* or *in vitro* at 37°C, the nucleus showed the highest autoradioactivity. However, by exposing uteri *in vitro* to tritiated-estradiol at 2°C, large amounts of radioactivity were observed in the extranuclear space. The formation of the 5S complex was also slowed when uteri were exposed to estradiol at 2°C. Examination of the binding capacity of the estrogen receptor showed that increasing doses of tritiated-estradiol decreased the maximum binding capacity of the 9.5S complex receptor. However, the amount of 5S complex increased with the reduction in 9.5S complex, suggesting that the formation of the 5S complex is dependent on the 9.5S complex. This relationship was confirmed by heat-treating the supernatant fraction, causing the dissociation of estradiol from the 9.5S receptor complex. When nuclei were exposed to this fraction, the formation of the 5S complex was not observed. These results suggested that estradiol-17β first binds to the estrogen receptor in the cytosol and then moves into the nucleus before the estradiol-receptor complex is transformed.

The Discovery of Anti-estrogens

While the estrogen receptor was being characterized, the first substances that could antagonize estrogen signaling were also identified. In the mid-1950s, Dr. Leonard Lerner discovered MER25, the first synthetic, non-steroidal anti-estrogen (13). Originally examined in a study to develop synthetic estrogens, MER25 was found to have no estrogenic or other hormonal activity but instead blocked the effects of estradiol in the reproductive tissues of mice, rats, rabbits, and chickens. MER25 also prevented pregnancy in rats treated post-coitus. This finding drew considerable attention to anti-estrogens for possible use in "morning-after" pills, a lucrative

prospect in the 1950s. This line of research led to the discovery of other non-steroidal anti-estrogens; such as clomiphene, nafoxidine, and tamoxifen, all of which were found to be contraceptives in animal models (15-17). However, trials in women showed that anti-estrogens did not act as contraceptives in humans, rather they stimulated ovulation and enhanced fertility. While this was a breakthrough in the field of fertility, much of the interest in anti-estrogens disappeared as the market for fertility drugs was far smaller than the market for contraceptives (17-21).

As treatments for breast cancer, these anti-estrogens proved to be as effective as other endocrine therapies but concerns over their long-term toxicities caused the interest in their development to wane. Much of this work was performed in the DMBA-induced mammary cancer model in Sprague-Dawley rats. The majority of DMBA-induced tumors are dependent on estrogen and prolactin and regress following endocrine ablation, either through ovariectomy or hypophysectomy, or with the administration of a prolactin antagonist (22-24). MER25, clomiphene and nafoxidine were able to limit tumor growth in DMBA-induced rat mammary tumors (25-27). Clinical trials in women with advanced breast cancer found that 39.2% of patients responded to clomiphene while trials using nafoxidine saw that 31% of patients responded (28, 29). Similar response rates were recorded for patients treated with other forms of endocrine therapy. Patients' responsiveness to nafoxidine correlated with estrogen receptor expression and nafoxidine was shown to prevent estradiol-17β retention in slices of DMBA-induced rat mammary tumor and human breast cancers (30, 27).

The potential for long-term toxicities of anti-estrogens raised concerns over their usefulness as cancer therapies. MER25 was originally derived from triparanol, a drug developed to lower circulating cholesterol for treating coronary heart disease (31). In studies with rats, the inhibition of cholesterol biosynthesis by triparanol also caused the accumulation of desmosterol, a precursor to cholesterol, in many tissues (32). This alteration in cholesterol synthesis was associated with cataract formation in women taking triparanol (33). Triparanol was removed from

the market and the legal issues faced by its manufacturer, the Merrell Company, discouraged further development of any drugs that could affect desmosterol levels. Bloom and Boesen reported a single patient who developed cataracts after 3 years of nafoxidine treatment as well as findings from other researchers that associated nafoxidine treatment in dogs, cats and rats with cataract formation (34). Ultimately, the clinical trials of nafoxidine and other anti-estrogens were halted due to concerns over the drugs' toxicities.

Discovery and Development of Tamoxifen

Tamoxifen was first identified in 1967 by Michael Harper and Arthur Walpole as part of a project to develop anti-estrogens as contraceptives (16). In these studies, tamoxifen was shown to inhibit embryo implantation in rats and mice treated post-coitus. Interestingly, the effects of tamoxifen within the reproductive tract were different between the two species. In rats, tamoxifen only caused vaginal cornification at doses that far exceeded the required dose needed for contraception. Likewise, tamoxifen could block the effects of estradiol on vaginal cornification and uterine weight. However, in mice, tamoxifen stimulated vaginal cornification even at low dosages. These results suggested that tamoxifen could act as a partial-agonist/antagonist to estrogen in rats but was a full agonist in mice. Importantly, unlike other anti-estrogens, tamoxifen lowered circulating cholesterol levels without increasing desmosterol concentrations in the serum or liver.

Following the findings that tamoxifen could oppose estrogen action without affecting desmosterol concentrations; early clinical trials were performed to examine tamoxifen's effectiveness in advanced breast cancer. Cole, Jones, and Todd tested the effectiveness of 10mg of tamoxifen, either once or twice daily, at inhibiting breast cancer growth among 46 post-menopausal women (35). 22% of patients showed a definitive response to tamoxifen treatment with significant reductions in tumor size. This response rate was similar to that of other hormone therapies. However, tamoxifen was better tolerated than other treatments and relatively few

patients withdrew from the trial due to complications from side effects. Just as in the studies with rats, desmosterol levels were not affected by tamoxifen. Likewise, a clinical trial by Ward saw definitive responses in 36% and 40% of patients who received 10mg and 20mg of tamoxifen, respectively, twice daily and side effects among these patients were similarly mild (36).

While tamoxifen showed effectiveness in these early trials, there was initially little interest in developing tamoxifen further. Like other anti-estrogens, tamoxifen did not act as a contraceptive in women and was found to instead induce ovulation (37, 38). Furthermore, despite having a better side effect profile, tamoxifen was only as effective as other endocrine treatments and there was little interest in simply adding another drug to the repertoire of endocrine treatments already available. Meanwhile, the focus of cancer research had turned toward cytotoxic chemotherapies and a therapy that was only effective in a limited number of cancer patients was of little interest.

Continued research on tamoxifen examined its mechanism of action. In the DMBA-induced rat mammary tumor model, the majority of established tumors regressed or stopped growing following tamoxifen treatment (39-41). Tamoxifen could also prevent the development of tumors if DMBA and tamoxifen were administered together (41). Because the growth of DMBA-induced mammary tumors is dependent on estrogen and prolactin, it was hypothesized that tamoxifen could cause tumor regression by either reducing the concentrations of circulating hormones or by interfering with estrogen signaling. Doses of tamoxifen that were sufficient to cause tumor regression were shown to have no effect on the plasma concentrations of either estradiol or prolactin (40-42). Likewise, no differences in the concentrations of either hormone were detected between tumors that regressed and those that continued growing following tamoxifen treatment (40). These findings were reiterated in post-menopausal patients with advanced breast cancer (43).

While the concentrations of prolactin or estradiol were not affected by tamoxifen, tamoxifen was shown to oppose the effects of estrogen and directly interfere with the binding of

estradiol to estrogen receptors in mammary tumors and other target tissues. In ovariectomized mice, tamoxifen could prevent increases in uterine weight and plasma prolactin concentration stimulated by exogenous estradiol (41, 42, 44).These effects could be mitigated with the addition of estradiol to the tamoxifen-treatment (41). Examinations of estradiol binding sites in the cytosols of DMBA-induced tumors showed an estrogen-specific binding site that sediments at 8S in a sucrose gradient and another, non-specific binding site at 4S (45). Tamoxifen blocked estradiol binding to the 8S receptor in both DMBA-induced tumors and human breast tumors (40, 41, 46, 47). However, non-specific binding to the 4S complex was unaffected (46, 47). These findings matched earlier examinations of tamoxifen's effects on estradiol binding to receptors in the rat uterus, which suggested that the two compounds compete for a single binding location (48). Because tamoxifen acts on the estrogen receptor, it was predicted that the estrogen receptor content of a tumor would correlate with the tumor's responsiveness to treatment. In DMBA-induced tumors, tumors that retained higher amounts of estradiol before treatment showed greater response to tamoxifen (49). Similarly, postmenopausal women with breast cancer were more likely to respond to tamoxifen if the estrogen receptor content of their tumors was high or if they previously responded to hormone therapy (50). The combined results of these studies indicated that tamoxifen's anti-carcinogenic effect primarily results from the competitive inhibition of estrogen signaling within the tumor.

Interestingly, despite inhibiting estradiol's effects, tamoxifen's affinity for the estrogen receptor was only about 2% that of estradiol's (48). An explanation for this paradox came with the examination of tamoxifen's metabolism. In both animals and women, tamoxifen was found to be metabolized into several metabolites (51, 52). These included the major metabolites, N-desmethyltamoxifen, metabolite Y, and metabolite Z, as well as 4-hydroxytamoxifen which was found to be present in the serum at lower concentrations (53-55). All of these metabolites could compete with estradiol at the receptor and exert anti-estrogenic effects on tissues (54-56). N-desmethyltamoxifen, metabolite Y and metabolite Z were found to have similarly low affinities

for the estrogen receptor as tamoxifen while 4-hydroxytamoxifen's affinity was similar to that of estradiol (54-56). 4-hydroxytamoxifen could exert anti-uterotrophic effects when administered at lower concentrations than tamoxifen and significantly contributed to the blockade of the uterine estrogen receptor despite its serum concentration being relatively low in rats given tamoxifen (54-57). However, the metabolism of tamoxifen is not a requirement for its anti-estrogenic effect, as demonstrated by its ability to prevent epithelial cornification when administered to rats intravaginally and its ability to cause cell death in estrogen-dependent cell lines *in vitro* (58, 59). Furthermore, when administered alone to DMBA-treated rats, 4-hydroxytamxoifen could not prevent the appearance of tumors to the same extent that tamoxifen could (60). Thus, the metabolism of tamoxifen gives rise to other anti-estrogenic compounds but tamoxifen's anti-carcinogenic activity results from the combination of it and the products of its metabolism.

Tamoxifen as an Adjuvant Cancer Therapy

With evidence showing its effectiveness in patients with advanced breast cancer, other treatment strategies using tamoxifen were explored. In the 1970s, cytotoxic chemotherapy was tested as an adjuvant therapy following the surgical removal of the primary tumor (61, 62). The belief was that chemotherapy could eliminate any remaining micro-metastases thereby preventing cancer recurrence. *In vitro* studies of tamoxifen's effects on MCF-7 cells, a cell line derived from an estrogen-dependent breast cancer, showed that tamoxifen could be cytotoxic to estrogen-dependent cancer cells and could potentially be effective in adjuvant therapy (63, 59). Tamoxifen was also believed to have less severe side effects compared to chemotherapies, making it attractive as a potential therapy.

The first clinical trials of tamoxifen as an adjuvant therapy following the removal of primary tumors demonstrated that 1 or 2 year courses of tamoxifen could significantly reduce tumor recurrence and improve patient survival (64). These trials showed that tamoxifen had an immediate effect on tumor recurrence but a delayed effect on survival, suggesting that longer

treatment durations could be beneficial to patients (65). This observation was reinforced by rodent models of breast cancer, where DMBA-treated rats showed a significantly increased delay in tumor appearance when they were treated with 4-hydroxytamoxifen for 6 months rather than 1 month (60). Subsequent trials examined tamoxifen's effects with various durations of therapy. The cumulative results from trials of 1, 2, and 5 years of adjuvant tamoxifen showed that tumor recurrence rates were reduced by 18%, 25%, and 42%, respectively, providing strong evidence that patients benefit most from longer durations of treatment (66). Extending therapy to 5 years did not affect patients' tolerance for treatment, with most patients leaving the studies within the first year of treatment. However, 5 years of tamoxifen therapy was associated with at least a 2-fold increase in the incidence of endometrial cancer (66, 67). Despite this, no increases in mortality due to endometrial cancer were observed and the overall mortality rate was significantly reduced. Extending tamoxifen therapy beyond 5 years provided no further reductions in breast cancer recurrence and, in some cases, recurrence was higher than in patients who halted treatment after 5 years (67). Likewise, the incidence of negative events, such as endometrial cancer and thromboembolism, tended to increase after 5 years, resulting in an overall reduction in patient mortality. Based on these findings, 5 years became the standard duration for adjuvant tamoxifen therapy. These studies also correlated the benefit of tamoxifen to the ER status of the primary tumors. Patients with ER-positive tumors, treated with tamoxifen for 5 years, saw a 50% reduction in tumor recurrence and a 28% reduction in mortality while patients with ER-negative tumors showed only a 6% reduction in both tumor recurrence and mortality.

The clinical trials also demonstrated tamoxifen's potential as a preventive of breast cancer. Cuzick and Baum reported the results of a 2-year trial of tamoxifen where the incidence of new breast cancers, in the breast contralateral to the original cancer, was 70% lower in tamoxifen-treated women (68). Tamoxifen had been shown to prevent the development of DMBA-induced mammary tumors in the earliest assays of tamoxifen's anti-tumor effects (41). The clinical findings led to further studies in rodents that showed that long-term tamoxifen

treatments could prevent the development of mammary tumors in both carcinogen-treated rats and mouse strains predisposed to developing mammary cancer (39, 41, 69, 70). The combined results from multiple clinical trials showed that 5 years of adjuvant tamoxifen could reduce the incidence of contralateral breast cancer by 39% and raised interest in examining tamoxifen as a breast cancer preventive in high-risk women (71).

While tamoxifen had potential as a breast cancer preventive, concerns over possible long-term toxicities, especially those related to cardiac and bone health, prohibited its administration to otherwise healthy women. Evidence suggested that estrogen plays a role in controlling plasma cholesterol concentrations. Bilateral oophorectomy in premenopausal women was associated with a significant increase in cholesterol concentrations and a doubling of the risk of coronary heart disease (72, 73). However, the increase in coronary heart disease risk could be attenuated by estrogen therapy (73). This improvement in cardiac health was associated with an increase in the plasma concentrations of high-density-lipoprotein (HDL) cholesterol relative to the concentrations of low-density-lipoproteins (LDL) (74, 75, 76, 77). In postmenopausal women, adjuvant tamoxifen reduced the concentrations of HDL and LDL cholesterol but the final ratio of HDL to LDL cholesterol was favorable to cardiac health (78, 79). Furthermore, the cumulative effects of tamoxifen on lipids and lipoproteins were consistent with an estrogenic effect despite tamoxifen's anti-estrogenic effects in other tissues (79).

Tamoxifen also appeared to have estrogenic effects in bone. Bone density loss, leading to osteoporosis, accelerates following menopause or oophorectomy. The administration of exogenous estrogens was shown to maintain bone density in the radii, metacarpals, and vertebrae of women who had undergone bilateral oophorectomy, implicating estrogens as regulators of bone maintenance (80). In oophorectomized rats, rather than having an anti-estrogenic effect in bone, tamoxifen maintained bone density in the femur while bone density in untreated, oophorectomized rats decreased (81). Likewise, tamoxifen-treatment could prevent increases in body weight resulting from oophorectomy. These findings were reiterated in breast cancer

patients where 2 years of tamoxifen treatment reduced the decrease in radius bone density and stimulated an increase in vertebral bone density (82). Concentrations of osteocalcin and alkaline phosphatase were also decreased, suggesting that bone turnover and remodeling were reduced by tamoxifen (82). With its apparent anti-estrogenic effects in the uterus and breast and estrogenic effects in bone and on lipid concentrations, tamoxifen became the first selective estrogen receptor modulator, a class of chemicals defined as having either estrogenic or anti-estrogenic effects depending on the target tissue. A preliminary toxicity trial reiterated these findings in women only at risk of developing breast cancer and showed the feasibility of clinical trials to test tamoxifen as a preventive (83).

Clinical Trials of Tamoxifen as a Chemopreventive

Tamoxifen's potential as a chemopreventive was examined in four large clinical trials, the National Surgical Adjuvant Breast and Bowel Project P-1 (NSABP P-1), International Breast Intervention Study (IBIS-I), Royal Marsden Hospital Tamoxifen Randomized Prevention Trial (Marsden), and the Italian Randomized Tamoxifen Prevention Trial (Italian) (84-93). In each trial, women were given 20mg of tamoxifen daily for at least five years; the Marsden Trial treated participants for 8 years. However, the studies varied in their criteria for enrollment. The NASBP P-1, IBIS-I, and Marsden trials enrolled women at high risk of developing breast cancer as determined by their family history (84-89). The NASBP P-1 and IBIS-I trials also assessed each participant's risk by personal characteristics; such as their age at menarche, nulliparity or age at first full-term pregnancy, and their history of lobular carcinoma in situ, atypical hyperplasia, and benign lesions (84-87). The Italian trial only admitted participants who had undergone a total hysterectomy, resulting in a study population with a lower-than-average risk of developing breast cancer (90-93). The participants were subgrouped as high or low risk based on personal risk factors and whether they had undergone a bilateral or unilateral oophorectomy or had two intact ovaries. Subgroups of women who had undergone hysterectomy were also examined in the

NSABP P-1, IBIS-I, and Marsden trials (84, 86, 88). The influence of hormone replacement therapy (HRT) was examined in the IBIS-I, Marsden and Italian trials, where participants with histories of HRT were admitted and participants were allowed on HRT during the trial (86, 88, 90, 92).

Individual results from the studies varied in their conclusions; however the combined results of the four trials suggest that administering tamoxifen significantly reduces the incidence of breast cancer. Meta-analysis of the combined results from the four trials showed a 38% reduction in the incidence of invasive breast cancer in participants taking tamoxifen compared to participants taking placebo (94). Independently, the NSABP P-1 and IBIS-I studies observed 49% and 32% reductions, respectively, in invasive breast cancer occurrence in patients taking tamoxifen (84, 86). However, neither the Marsden nor the Italian studies observed any reduction in invasive breast cancer incidence (88, 90). The different results of these trials were attributed to the differences in study populations and criterion for entry in each trial, suggesting that tamoxifen may only be effective as a breast cancer chemopreventive in a subset of women.

The comparison of tumor characteristics between the tamoxifen and placebo groups gave evidence for the limitations of tamoxifen's effectiveness as a chemopreventive. In the NSABP P-1 and IBIS-I trials, participants taking tamoxifen showed reductions in the incidence of estrogen receptor-positive tumors by 69% and 31%, respectively (84, 86). However, neither study observed a reduction in estrogen receptor-negative tumors, leaving the reduction in ER-positive tumors as the sole factor accounting for the overall reduction in breast cancer with tamoxifen treatment (84, 86). Within their complete study populations, the Marsden and Italian trials did not show a significant effect of tamoxifen on the occurrence of estrogen receptor-positive tumors (88, 90). Tamoxifen did not have a significant effect on other characteristics of tumors, such as their size, grade, or nodal status (84, 86). These findings suggest that tamoxifen is only effective at preventing estrogen receptor-positive tumors and thus its benefits as a chemopreventive are dependent on the individual's risk of developing estrogen-dependent breast cancer.

Because tamoxifen's effectiveness is limited to ER-positive cancers, the differences in the criterion for participant selection among the trials could explain the disparities among the trials' results. The studies that observed a significant reduction in breast cancer occurrence with tamoxifen treatment, the NSABP P-1 and IBIS-I trials, admitted participants based on family history of breast cancer as well as personal history and characteristics that place them at a higher risk of developing breast cancer (84, 86). However, the Marsden trial selected participants based exclusively on family history of breast cancer (88). By selecting participants based only on family history, it was postulated that participants with genetic predispositions to breast cancer were selected at a higher rate in the Marsden trial compared to the NSABP P-1 or IBIS-I trials (88). Breast cancers caused by genetic risk factors, such as BRCA1 or BRCA2 mutations, tend to be hormone independent while risk factors related to personal characteristics, such as age at menarche, nulliparity or age at first live birth, and history of LCIS and atypical hyperplasia, are associated with the development of ER-positive tumors (95, 96, 97). Thus, the participants in the Marsden trial may have been more predisposed to developing ER-negative tumors, where tamoxifen was shown to not have a chemopreventive effect, compared to the participants of the NSABP P-1 and IBIS-I trials.

Similarly, tamoxifen did not have a significant effect on breast cancer incidence in the participants of the Italian trial, likely due to the trial's criterion for entry. All participants in the Italian study had undergone a hysterectomy and may have also had a bilateral or unilateral oophorectomy (90). In addition, the participants were not selected based on personal characteristics that might contribute to breast cancer development. As a result, the study population had a lower risk of developing breast cancer compared to the general population and a much lower risk of breast cancer than the participants in the NSABP P-1 and IBIS-I trials. Because tamoxifen's effectiveness as a chemopreventive is limited to ER-positive tumors, the effectiveness of tamoxifen within the subset of participants who were at high risk of developing ER-positive tumors may not have been apparent when the entire study population was examined.

Subgrouping participants found significant chemopreventive effects by tamoxifen within participants at higher risk of developing ER-positive breast cancer. Hormone replacement therapy is associated with increased risk of hormone receptor-positive breast cancer (97). When breast cancer incidence among participants who were on hormone replacement therapy during the trials was examined independently, an 87% lower incidence of breast cancer was observed in the tamoxifen-treated group (90). Given this finding, and findings from the NSABP P-1 trial, the researchers followed-up with additional analyses of subgroups of women on hormone replacement therapy and women at high risk of developing hormone receptor-positive cancer based on personal characteristics, such as height greater than 160cm, at least one functioning ovary, menarche before the age of 13, and not having a full-term pregnancy before the age of 24 (91, 92). With a median follow-up time of 81.2 months, women on hormone replacement therapy showed a 64% reduction in breast cancer risk while women at high risk showed an 82% reduction in breast cancer incidence with tamoxifen treatment (91, 92). These findings come in contrast to the original conclusions of the study and show that tamoxifen's chemopreventive effect is most apparent in women at high risk of developing ER-positive breast cancer.

Participant follow-up continued beyond the active treatment period and has given insight into the long-term effects of tamoxifen. The NSABP P-1 trial was unblinded at the end of the 5 year treatment period to give their participants the opportunity to begin tamoxifen-treatment or to transfer to the NSABP P-2 trial, however participants in the IBIS-I, Marsden and Italian studies remained blinded after treatment ended (84, 87, 89, 93). Cumulative results from the first 10 years of follow-up showed a reduction of all breast cancers by 33% and a reduction of ER-positive breast cancers by 44% (98). Independently, the IBIS-I trial, showed a reduction of ER-positive breast cancer by 44% in the 5 years following the study's active treatment period (87). After 13 years of follow-up, the Marsden trial observed a 39% reduction in the incidence of ER+ breast cancer (89). During the active-treatment period, a non-significant 23% reduction in ER+ breast cancer. However, during the post-treatment period, ER-positive breast cancers were

reduced by 51% among participants on tamoxifen, thus observing a significant effect of tamoxifen that was more apparent in the long-term than it was during the treatment period. The Italian trial observed a 76% reduction in breast cancer occurrence over an average of 9 years of follow-up in participants determined to be at high-risk of developing hormone receptor-positive breast cancers (93). During the active-treatment period, this group saw an 82% reduction in breast cancer incidence which continued into the 6-year post-treatment period.

The elucidation of the mechanisms underlying breast cancer development has led to the development of treatments that counter its development. In turn, the evaluation of these treatments has expanded their application and may help to further inform our understanding of the mechanisms behind cancer. By identifying the role of estrogen in cancer development and mapping its signaling in the breast, tamoxifen was developed to counter breast cancer growth. Further evaluation of tamoxifen has demonstrated its efficacy as a cancer preventive. This observation suggests that tamoxifen has a significant effect on the normal mammary epithelium. Further evaluation of tamoxifen's effects may shed light on how breast cancers develop, namely the cells which are susceptible to oncogenic transformation and the mechanisms that facilitate their transformation. In the following chapter, experiments utilizing mammary cell organoids from the MMTV-*c-neu* mouse model of breast cancer to evaluate the direct effects of tamoxifen on the mammary epithelium and the effects that aging has on tamoxifen's efficacy as a cancer preventive are described.

Chapter 2

The Effects of Tamoxifen on Young and Old MMTV-c-*neu* Mouse Mammary Organoids

Introduction

Tamoxifen and Breast Cancer Chemoprevention

Tamoxifen has been shown to reduce the occurrence of ER-positive breast cancers by 48% in clinical trials and was the first treatment approved for the prevention of breast cancer (94). However, the mechanism underlying tamoxifen's chemopreventive effect remains unknown. Studies using rodent models of mammary cancer have characterized tamoxifen's effect on tumor incidence and have offered clues to its mechanism. Tamoxifen acts as a chemopreventive in various rodent models of mammary cancer, including models with tumors induced by MMTV infection (99), carcinogen exposure (100, 101), p53-knockout (102), or the transgenic overexpression of oncogenes (103, 104). Notably, these include models that develop ER-negative tumor subtypes where tamoxifen is ineffective for reversing the growth of established tumors. However, tamoxifen can be effective at preventing the appearance of tumors in some of these models if treatment is started in animals at a relatively young age. The age-dependent effects of tamoxifen have not yet been explored but studying the different effects of tamoxifen on the mammary epithelial cells from young and old mice may offer insight into the mechanism of tamoxifen's chemopreventive action and inform new strategies for preventing ER-negative breast cancers in patients. Furthermore, short-term tamoxifen treatments have been shown to provide long-term reductions in tumor incidence that extended beyond the initial treatment period, implicating long-lived cell populations as the target of tamoxifen's chemopreventive effect. Together, these observations suggest a mechanism for the prevention of mammary tumor development where tamoxifen quickly alters long-lived mammary cell populations but that this effect depends on the age of the mammary epithelium. To evaluate this hypothesis, we isolated mammary epithelial organoids from young and old MMTV-*c-neu* mice and examined the

organoids for differences in the ways their cellular populations and activities were affected by tamoxifen.

Tamoxifen can prevent the development of ER-negative mammary tumors in transgenic mouse models if animals from those models are treated at a young age. MMTV-c-*neu* transgenic mice overexpress the growth factor receptor HER2/neu under the control of the MMTV promoter and develop HER2-positive, ER-negative tumors (105). NRL-TGFα mice express transforming growth factor alpha (TGFα) under the control of the NRL promoter and develop ER-positive, PR-negative tumors (106). Both models develop tamoxifen-resistant tumors starting at 24 weeks-of-age however, MMTV-c-*neu* and NRL-TGFα mice each showed 50% reductions in tumor incidence when tamoxifen was administered at 12 and 8 weeks of age, respectively (103, 104). However, tamoxifen failed to prevent tumor development if treatment began in later weeks.

Tamoxifen's effect on tumor incidence has been shown to persist beyond the treatment period. Meta-analysis of data from 9 prevention trials showed that women treated with tamoxifen for 5 years were less likely to develop breast cancer for at least another 5 years beyond the treatment period (98). This observation has been reiterated in mouse models. NRL-TGFα mice that were treated with tamoxifen for 60 days, beginning at 8 weeks-of-age, showed reduced tumor incidence until they reached 2 years-of-age (104). Likewise, a 3 month tamoxifen treatment in C3H/OUJ mice prevented tumor development in 80% of treated mice after 18 months while all control mice developed tumors (99). These observations indicate that tamoxifen has a significant, long-lasting effect on the normal mammary epithelium, likely in cell populations that are long-lived. Understanding how tamoxifen affects the various cell populations of the normal mammary epithelium may be the key to understanding how tamoxifen can produce a long-term reduction in cancer risk in both animal models and patients.

Histology and Development of the Mammary Gland

The mammary gland is a complex exocrine gland, formed by a bilayered secretory epithelium embedded within a stromal matrix. Starting at openings in the nipple, the epithelium forms a branching network of ducts that extends throughout the matrix. Alveolar buds, where milk synthesis takes place, also branch off of the ducts. The two epithelial layers are morphologically distinct and perform different functions. The outer, basal layer is primarily composed of myoepithelial cells that contract during lactation to move milk through the ducts. The inner, luminal cell layer is composed of the cells that line the ducts and alveoli. The ductal and alveolar luminal cells form separate subpopulations with different functions. Notably, ductal luminal cells provide hormone sensing for the epithelium while alveolar cells synthesize milk components during pregnancy. The matrix around the mammary epithelium contains stromal cells, including adipocytes, fibroblasts, endothelium and immune cells that support the mammary epithelium's functions (107, 108, 109).The structure of the mammary epithelium is highly dynamic and dependent on the concentrations of circulating hormones. Before puberty, the mammary epithelium is present as only a rudimentary ductal structure that undergoes isometric growth with the rest of the body. At puberty, increased concentrations of hormones stimulate the expansion of the epithelium into a branching network of ducts that fills the stromal matrix. Cyclical changes in hormone concentrations during the menstrual/estrus and pregnancy cycles induce significant changes in the epithelium. As hormone concentrations rise during the menstrual/estrus cycle, the mammary ductal network expands. Without pregnancy, the ducts retract as hormone concentrations fall and the cycle resets. With pregnancy, the ducts expand further, replacing much of the area occupied by stroma with ducts and milk-synthesizing alveoli. Suckling from infants stimulates lactation and maintains the hormone concentrations that maintain the lactation state. When infants are weaned, the loss of hormone signals leads to the involution of the expanded epithelium, characterized by the apoptosis of the alveolar cells and the remodeling of the epithelium back to its pre-pregnancy state.

The Epithelial Hierarchy of the Mammary Gland

The adult mammary epithelium contains multiple populations of stem-like, progenitor cells that facilitate the rapid expansion of the mammary gland during pregnancy. The identities and activities of these cells have been elucidated by determining the unique protein expression patterns of each cell population, their growth potential with *in vivo* and *in vitro* assays, and by performing lineage tracing to assess their growth in the intact mammary epithelium. Basal cells have been characterized as CD29-high, CD24-low, EpCAM-low, CD49f-high and are also positive for basal keratins 5 and 14 (110, 111, 112, 113). In *in vivo* growth assays, individual basal cells were transplanted into the mouse mammary stroma where the endogenous epithelium had been removed (111, 112). Basal cells were able to give rise to complete, functional mammary outgrowths containing both luminal and basal cells. Likewise, basal cells formed dense colonies, containing both basal and luminal cells, in non-adherent, 3-dimensional (3D) cultures (111, 112). In adherent, 2-dimensional (2D) cultures basal cells could form colonies containing either basal and luminal cells, or basal cells only (110). Together, these observations suggested that the basal cell population contains multi-potent stem cells as well as more differentiated, basal-restricted progenitor cells.

Luminal cells have been characterized as CD29-low, CD24-high, EpCAM-high, CD49f-low and express keratins 8/18 (110-113). Transplantation assays have shown that luminal cells capable of regenerating the mammary epithelium are very rare. However, when cultured in 3D conditions, luminal cells form acinar structures composed of a single layer of epithelial cells surrounding a lumen and in 2D cultures they form dense colonies (111, 112). In both conditions, these colonies only contained cells with luminal characteristics, suggesting that the luminal population contains luminal-restricted progenitor cells. These colony-forming, putative, luminal progenitor cells have been isolated from the rest of the luminal population using either CD61 or CD49b expression, however there is incomplete overlap in the expression of these two markers and a significant number of luminal progenitor cells express CD49b but not CD61 (113). Sca1

expression has also been used to separate the luminal population into Sca1-positive, ER-positive cells and Sca1-negative, ER-negative cells. Combining CD49b and Sca1 labeling divides the luminal population into four subpopulations; differentiated ductal cells (Sca1-positive, ER-positive, CD49b-negative), ductal progenitor cells (Sca1-positive, ER-positive, CD49b-positive), alveolar progenitor cells (Sca1-negative, ER-negative, CD49b-positive), and differentiated alveolar cells (Sca1-negative, ER-negative, CD49b-negative) (113).

Lineage tracing studies have assessed the growth and differentiation potential of cells within the intact mammary epithelium. In adult mice, the basal layer is maintained by populations of progenitor cells that reside within the basal population and produce more differentiated basal cells (114, 115, 116). Tracking these cells over multiple rounds of pregnancy has shown that the proportion of labeled basal cells within the population remains consistent, suggesting that these cells are both long-lived and capable of self-renewal (114, 116). There have been conflicting observations as to whether these cells, or other populations of cells within the basal population, are also able to give rise to luminal cells. Separate studies report that basal cells are capable of giving rise to clonal patches that are either composed of only basal cells or both luminal and basal cells, suggesting that the basal population contains populations of basal-restricted progenitor cells and multi-potent mammary stem cells (117, 118, 119). However, studies that only observed basal-restricted clonal patches during lineage tracing also demonstrated that labeled basal cells could regenerate a complete mammary epithelium, containing both basal and luminal cells, after being transplanted into the mouse mammary stroma (114, 116). These observations indicate that these basal progenitor cells are developmentally plastic and their activity is partially dependent on environmental cues. Thus, these cells may show basal-restricted growth in the context of the intact gland but become multi-potent following transplantation, where their normal environmental cues are lost. Similarly, other lineage tracing studies have observed basal cells that exclusively maintain the basal population in the resting mammary gland but may also contribute to the

alveolar luminal lineage when the epithelium expands during pregnancy, suggesting that activity of basal progenitor cells can also change depending on the hormonal environment (120).

Tracking the progeny of luminal cells has demonstrated that the ER-positive and ER-negative luminal cell populations are each self-sustaining. Labeling the cells for markers that are specific to the ER-negative or ER-positive populations demonstrated that the progeny of the ER-positive and ER-negative luminal cells remain within their respective populations (121, 122). The labeling within each population persisted through multiple rounds of pregnancy and involution. Furthermore, the proportion of labeled cells within each population remained steady between each pregnancy, suggesting that each cell population is maintained independently by populations of long-lived progenitor cells that are capable of self-renewal. Assessments of cell division kinetics also suggest that the basal, ER-positive luminal, and ER-negative luminal cell populations are maintained independently (123). Furthermore, assessing telomere lengths showed that differentiated luminal cells have longer telomeres than CD49b-positive, luminal progenitor cells, which have been hypothesized to give rise to differentiated luminal cells (123). This observation suggests that differentiated luminal cells maintain their own population and the primary role of the CD49b-positive luminal progenitor population is to facilitate the expansion of the mammary epithelium during pregnancy. Combined, these studies suggest a model where each population of the mammary epithelium; the basal, ER-positive luminal cell, and ER-negative luminal populations are primarily maintained independently by its own population of stem-like, progenitor cells. The basal population may also contain multi-potent stem cells that contribute to the luminal cell populations, either continuously or only during pregnancy, however the presence of such cells with this activity in the intact mammary gland remains controversial.

The Epithelial Hierarchy and Breast Cancer

Understanding the cellular architecture of the mammary epithelium has also shed light on the origins of cancer within the epithelium. Exposure to ionizing radiation results in a significant

increase in breast cancer risk that persists for many years, suggesting that the long-lived progenitor cell populations are the cells from which tumors arise (124). The differences between the various mammary epithelial cell populations may also explain the heterogeneity of breast cancer. Assessing the gene expression profiles of many different breast cancers has characterized 6 primary subtypes of breast cancer. Tumors defined as "Luminal A" show high expression of genes associated with luminal cells and ER with low expression of genes associated with HER2 and proliferation (125, 126). "Luminal B" tumors also show luminal-like characteristics but are highly proliferative and show lower ER and hormone receptor expression. "HER2-enriched" tumors show high expression of HER2 and its associated genes while showing low expression of luminal, basal, and hormone receptor-associated genes (125, 126). "Basal-like" tumors express genes associated with basal cells and are highly proliferative but tend to lack ER, progesterone receptor (PR), and HER2 expression (125, 126). The "claudin-low" subtype shows low expression of genes associated with cell-cell adhesion, such as claudin 3, 4 and 7, and high expression of mesenchymal genes (127). The "normal-like" subtype is characterized by high expression of genes associated with basal, adipose, and non-epithelial cells (125, 126).

The different characteristics of these subtypes are believed to be accounted for by a combination of the cell population from which the tumors arise and the nature of early genetic, or epigenetic, lesions that lead to cancer development (127). Selectively deleting *Brca2*, *Pten*, or *p53* in the ER-negative luminal or basal populations produced different subtypes of tumors depending on the targeted population. Tumors originating from the basal population consisted of adenomyoepitheliomas while tumors from the luminal population took on a variety of characteristics, including both ER-positive and ER-negative subtypes. Notably, the different types of deletion directed the subtype of tumor that developed, producing luminal, basal, or claudin-low type tumors from luminal cells at different frequencies depending on the deletion. Earlier comparisons of gene expression signatures showed that luminal and basal-like tumors showed genetic characteristics suggesting that they arise from differentiated luminal cells and luminal

progenitor cells respectively, but claudin-low tumors appeared to be most related to basal progenitor cells (113, 128-131). These findings suggest that many different cancer subtypes can arise from the luminal cell population and that luminal-derived tumors can show significant plasticity in their identity.

The Epithelial Hierarchy and Tamoxifen Chemoprevention

As a selective estrogen receptor modulator, tamoxifen can influence the mammary epithelium's development by altering estrogen signaling. ER in the mammary epithelium is exclusively expressed by a subset of luminal cells (132, 133). Signaling from estrogen is relayed through paracrine signals, such as amphiregulin, to the rest of the epithelium (134). Through these mediators, estrogen signaling stimulates proliferation in both the basal and luminal cell populations, leading to the expansion of the mammary epithelium during puberty and the estrus or menstrual cycles in adult hood (123, 135, 136). Tamoxifen competitively inhibits estrogen binding to ER, preventing its mitogenic effect in the mammary epithelium and influencing the activity of both ER-positive and negative cell populations. Tamoxifen also exerts pro-apoptotic effects through ER-independent mechanisms such as the activation of caspase-3 and c-Jun NH_2-terminal kinase (JNK) 1, the deactivation of phospho-Akt and other proteins involved in survival, and increasing the production of reactive oxygen species (137-140). Through both of these mechanisms, tamoxifen has the potential to influence all cell populations within the mammary epithelium. Particularly, tamoxifen may impact the luminal and basal progenitor cell populations from which ER-negative breast cancers are predicted to arise. By modulating estrogen signaling or by inducing apoptosis in these populations, tamoxifen may be able to prevent their transformation into cancer cells.

Within the normal mouse mammary epithelium, short-term tamoxifen treatments have been shown to have a significant effect on the proportions and activity of multiple cell populations (141). A single, 5mg dose of tamoxifen was shown to induce a transient increase in

both cell division and apoptosis within the mammary epithelium. 2 days after treatment, the basal population increased by 56% while the luminal population saw a slight decrease. However, 21 days after treatment, the basal cell population was reduced 40% and the differentiated luminal cell population was reduced 51%. By 56 days after treatment, both populations had returned to levels similar to control-treated mice. Tamoxifen also significantly affected progenitor cell activity, observed as reductions in the colony-forming efficiency of luminal cells 21 days after treatment. This study demonstrated that tamoxifen can significantly affect the normal mouse mammary epithelium in a way that may affect breast cancer development.

Age-related Changes in the Mammary Epithelium and Differences in the Effectiveness of Tamoxifen Chemoprevention

While Tamoxifen can alter mammary epithelial cell populations, there is evidence to suggest that tamoxifen's effects are not universal and instead depend on the age of the mammary epithelium. Studies in mouse models of ER-negative cancer show that the age of treated mice significantly influences tamoxifen's efficacy as a chemopreventive (103, 106). In these studies, mice that were treated with tamoxifen at younger ages showed reductions in tumor incidence while older mice did not. It is currently unknown how the effects of tamoxifen can change based on the age of the mammary gland that it acts on.

Aging has been linked to significant changes in the mammary epithelium in humans and in animal models. In humans and in the C57BL6/J and BALB/c mouse models, luminal cells in older subjects displayed more basal like characteristics than luminal cells from younger subjects (142-144). These characteristics included increased expression of basal-associated proteins like CD49f, K14, and YAP, decreased expression of luminal-associated proteins like K19, increased MAPK pathway activity, and the acquisition of basal-like cell adhesion properties. Mammary progenitor activity also differed between younger and older subjects. In mice, bi-potent progenitor cells increased in frequency while luminal progenitor cells decreased with age (142).

Bi-potent progenitor cells from older mice also showed reduced ability to repopulate the mammary epithelium when transplanted into cleared mouse mammary fat pads compared to cells from younger mice. In humans, the proportion of cKit-positive, luminal progenitor cells increased and colonies formed from these cells in non-adherent conditions showed increased cell proliferation and a bias towards basal cell differentiation (143, 144). Aging was also associated with shifts in the proportions of basal and luminal cells within the glands, however these changes appear to be species specific. In humans, the ratio of luminal to basal cells increased with age as fewer myoepithelial cells were present in the gland however, the luminal to basal cell ratio decreased with age in C57BL6/J and BALB/c (142, 143, 144). Likewise, there appear to be differences in aging between mouse strains. While aging was associated with changes in cell proportions in C57BL6/J and BALB/c mice, comparisons between younger and older FVB/N mice did not show significant differences in their cell populations, suggesting that genetic differences can alter the aging process (145).

Aging is associated with increased risk of breast cancer development and the changes in the mammary gland that occur with aging are believed to be linked to cancer progression. The basal-like characteristics acquired by aging luminal cells resemble the characteristics that human mammary epithelial cell lines acquire following their immortalization, suggesting that acquiring these traits is an early sign of oncogenic transformation (144). Likewise, increases in the proportions of certain progenitor cells, from which tumors are believed to arise, have been linked with increased cancer incidence in mouse models, like the MMTV-*c-neu* mice, and in women carrying *BRCA1* mutations (146, 128). Changes in the mammary epithelium such as increases in inflammatory signaling, changes in the mammary stroma, and changes in the mammary extra-cellular matrix that occur with age are hypothesized to underlie these changes in gene expression and cell population proportions by altering gene expression patterns through epigenetic modifications (147). These age-related changes in gene expression and cell proportions may also render mice and patients resistant to tamoxifen's chemopreventive effect. Thus, in both mouse

models of cancer and in women, the aging process may create a period of sensitivity to tamoxifen where the mammary epithelium has yet to undergo the changes associated with advanced age and another period of insensitivity that occurs after the epithelium has accumulated age-related changes that also lead towards tumorigenesis.

Examination of Tamoxifen's Effects in Young and Old Mouse Organoids

To better understand tamoxifen's direct effects on the mammary epithelium and how they may change depending on the age of the target, we examined the effects of tamoxifen on mammary organoids isolated from young and old MMTV-c-*neu* mice in estrogen-free conditions. Mammary organoids are composed of clusters of cells, isolated from mouse mammary glands by collagenase digestion, that contain both luminal and basal cells. Culturing these clusters of cells together maintains the interactions between the two cell types *in vitro* and better recapitulates the *in vivo* mammary epithelial environment. Using this model, we examined the effects of tamoxifen on mammary epithelial cells outside of the influence of any systemic factors that may affect tamoxifen's activity or any systemic effects that tamoxifen may have that could affect the mammary epithelium (141). The cell populations and activities of these organoids were examined following a 5 day tamoxifen treatment and the effects in the young and old mammary organoids, were compared. HER2 tumors are believed to arise from luminal progenitor cell populations (146, 148). Progenitor activity in the luminal and basal cell populations and the activation of cell death signaling pathways were assessed in luminal and basal cell populations and to assess the differences in responses between cell populations in young and old mouse mammary organoids. We hypothesized that the effects of tamoxifen on young and old mammary organoid cell populations would be different, namely on the luminal progenitor cell population where HER2/neu-induced tumors are thought to arise. In doing so, the mechanism underlying tamoxifen's ability to prevent tumor development in young, but not old, MMTV-*c-neu* mice, would be better characterized.

Methods

Mice, Organoids and Cell Isolation:

All studies were approved by the University of Minnesota's Institutional Animal Care and Use Committee. 10 to 15 week of age (young), and 29 to 34 week of age (old) female, MMTV-c-neu mice were euthanized by asphyxiation with CO_2 then left and right mammary glands, 2 through 5, were collected. The glands were stored in a 50:50 mixture of Dulbelco's Modified Eagles Serum (DMEM, GibCo) and F12 (GibCo) on ice. The mammary glands were diced three times on a McIlwain Tissue Chopper and placed in digest medium containing 50:50 DMEM/F12, 0.75 Units/mL Collagenase A, 1mg/mL Hyaluronidase, and 0.02mg/mL DNase. The digesting tissue was incubated in a shaking incubator for 1.5 hours at 37.5°C.

After digest, the mammary tissue was spun at 350 RCF for 5 minutes. The supernatant, containing floating adipocytes, was aspirated and the tissue pellet was resuspended in 2 mL of Hank's Balanced Salt Solution (HBSS) with 10mM HEPES. 8mL of 0.8%NH_4Cl with 0.1mM EDTA was added to the cell suspension to remove the red blood cells. The suspension was centrifuged again and the supernatant was removed. The organoids were resuspended in 6mL of HBSS with 5% Fetal Bovine Serum (FBS). The suspension was filtered through a 40μm filter and the flow-through was discarded. The filter was inverted over a collecting tube and the organoids were washed off of the filter with 6mL of HBSS with 5% FBS. The organoids were seeded on 10cm cell culture plates in 15mL of DMEM with 5% FBS. The plates were incubated for 5 hours at 37.5°C in 5% CO_2 to allow the mammary organoids to adhere before the media was replaced with 15mL of Phenol Red-free DMEM/F12 containing 5% charcoal-stripped FBS, 2mM L-glutamine, 0.5μg/mL hydrocortisone, 5μg/mL Human insulin, 1ng/mL Cholera toxin, 10pg/mL rhEGF, and 1μg/mL Gentamycin sulfate. The organoids were incubated at 37.5°C in 5% CO_2. 4-hydroxytamoxifen in ethanol was added to the plates to bring the final concentration to 2uM. Control plates were treated with an equal volume of ethanol.

Organoid Cell Collection

On the fifth day of incubation, the media was removed from the plates and 3mL of 0.25% trypsin-EDTA was added to each. The plates were incubated at 37.5°C in 5% CO_2 until the cells were resuspended. The trypsin reaction was halted with 3mL of HBSS containing 5% FBS. The suspension was centrifuged at 350 RCF for 5 minutes. The supernatant was removed and the cells were resuspended in HBSS with 5% FBS. The concentration of cells was calculated using a hemocytometer.

Sulforhodamine B (SRB) Growth Assays:

Organoid cells were resuspended and dilutions were prepared in EpiCult-B media, containing 5% FBS, to make cell suspensions with 10,000 cells/mL. Secondary cultures were prepared by adding 0.4mL of the suspension to each of 9 wells of a 48 well plate. The plates were incubated at 37°C with 5% CO_2. On the 2^{nd}, 4^{th}, and 6^{th} days of incubation, the media was removed from one well of each treatment. The wells were washed with phosphate-buffered saline (PBS) and the cells were fixed in a 50:50 solution of acetone and methanol for 1.5 minutes. The fixative was removed and the cells were allowed to air dry. After all rows were fixed, 0.5% SRB in 1% acetic acid was added to each well. The cells were stained for 10 minutes before the SRB solution was removed and the excess was washed out with 1% acetic acid. The wells were dried in an incubator at 40°C. The stain was solubilized with 700uL of 10mM solution of Tris buffer and the absorbance of each well was recorded using a SpectraMax M3 plate reader from Molecular Devices at 538nm.

Adherent (2D), Unsorted Colony-Formation Assays

24 hours before collecting the cultured organoid cells, 6-well plates with NIH-3T3 feeder cells were prepared. NIH-3T3 cells were treated with 15μg/mL mitomycin-C for 5 hours to arrest their growth. The media was removed and the cells were washed once with PBS. The cells were

treated with 3mL of 0.25% trypsin-EDTA and incubated at 37.5°C in 5% CO_2 for 10 minutes to suspend the cells. The trypsin reaction was halted with 3 mL HBSS with 5% FBS. The NIH-3T3 cells were centrifuged at 300 RCF for 10 minutes and the supernatant was removed. The pellet was resuspended in DMEM with 5% newborn calf serum (NBCS) and the concentration of NIH-3T3 cells was calculated with a hemocytometer. Cell suspensions containing 20,000 cells/mL were created in DMEM with 5% NBCS. 2mL of the suspension were added to each well of a 6-well plate and the plates were left to incubate at 37.5°C in 5% CO_2.

After collection, organoid cells were added to EpiCult-B media, containing 5% FBS, to make a suspension with 500 cells/mL. 2 mL of the suspension were added to 3 wells of a 6-well plate, containing growth-arrested NIH-3T3 feeder cells. After seeding the wells, the plates were incubated at 37°C in 5% CO_2. On the second day of culture, the media was removed and replaced with EpiCult-B media without FBS. On the sixth day of incubation, the media was removed and the wells were washed with PBS. The cells were then fixed with a 50:50 solution of acetone and methanol for 1.5 minutes and allowed to air dry. The colonies were stained with 0.5% SRB in 1% acetic acid for 10 minutes before the SRB solution was removed and the excess was washed out with 1% acetic acid. The wells were dried in an incubator at 40°C. The number of colonies in each well was then counted.

Antibody Labeling

Suspensions containing 1 x10^6 cells/50µL of HBSS with 5% FBS were prepared. 50µL of HBSS with 5% FBS, containing 0.2µg APC Anti-mouse EpCAM, 0.5µg anti-CD49f and 0.5µg anti-CD61 were added to each of the cell suspensions. The cells were incubated, in the dark, on ice for 1 hour. The cells were washed twice and resuspended in 1mL of HBSS with 5% FBS. 10µM BrdU was added to the cells just before being analyzed on a Sony SH800Z cell sorter.

EdU Flow Cytometry Assays

5-ethynyl-2'-deoxyuridine (EdU) assays were performed using a Click-iT EdU Flow Cytometry Assay Kit from Life Technologies according the manufacturer's instructions. In brief, before being collected on the fifth day of culture, the organoids were treated with 10µM of EdU. The plates incubated for 1.5 hours in at 37°C with 5% CO_2. The media was removed and 3mL of 0.25% trypsin-EDTA was added to each plate and the plates were incubated until all cells were suspended. 3mL of HBSS containing 5% FBS were added to the suspension to neutralize the trypsin reaction and the suspension was centrifuged at 350 RCF for 5 minutes. The supernatant was removed and the cells were resuspended in HBSS containing 5% FBS. The concentration of cells was calculated using a hemocytometer.

The cells were labeled with antibody against EpCAM, as described above, then resuspended in 4% paraformaldehyde in PBS. The cells were fixed at room temperature for 15 minutes then centrifuged and washed once with HBSS containing 5% FBS. The cells were resuspended in 0.1mL of Click-iT saponin-based permeabilization and wash reagent. 0.5mL of Click-iT reaction cocktail, containing 438µL PBS, 10µLof 100mM $CuSO_4$, 2.5µL of Alexa Fluor 488 azide solution, and 50µL of Reaction Buffer additive, were added to each tube. The cells were incubated in the dark for 30 minutes at room temperature. The cells were centrifuged again and washed twice with the saponin-based permeabilization and wash reagent. The cells were resuspended in 1mL of saponin-based permeabilization and wash reagent and analyzed on a Sony SH800Z Cell Sorter for EdU incorporation and EpCAM expression. EdU incorporation among the cells within the EpCAM-positive fraction was assessed. The EpCAM-positive population was divided into quartiles, each containing 25% of the population, and each was assessed for EdU incorportation.

2D Sorted, Colony-formation Assays

Additional 2D colony-formation assays were performed on sorted organoid cells. After the organoid cells were collected, they were labeled with antibodies against EpCAM, as previously described. The quartiles with the highest EpCAM expression (EpCAM-high) and the quartiles with the lowest EpCAM expression (EpCAM-low) were sorted and seeded into 2D colony-forming assays, as described above.

Annexin V

FITC-Annexin V was purchased from BioLegend (Cat# 640905) and the labeling procedure was performed according to the manufacturer's instructions. The cells were labeled with antibodies against EpCAM, CD49f, and CD61, as described above, then resuspended in Annexin V Binding Buffer from BioLegend to create a suspension with $1x10^6$ cells/mL. 5µL of FITC-Annexin V were added to 100µL of the cell suspension. The suspension was vortexed and incubated for 15 minutes at room temperature away from light. An additional 400µL of Annexin V Binding Buffer (Cat# 422201) was added to each suspension. 10µM BrdU was added to each suspension just before the collected cells were analyzed with a Sony SH800Z cell sorter.

Non-adherent (3D) Colony-formation Assay

Organoid cells were labeled with antibodies against EpCAM and CD49f, as described above, then sorted to make separate EpCAM-positive (epithelial), EpCAM-High/CD49f-Low (luminal), and EpCAM-Low/CD49f-High (basal) cell fractions. The concentrations of cells in the collected epithelial, luminal, and basal fractions were calculated with a hemocytometer. Suspensions of 500 cells/mL were made in EpiCult-B media, with 5% FBS, 5% Matrigel, and 10µM ROCK inhibitor. 2mL of the cell suspension were added to the wells of 6-well, ultra-low adhesion plates. The plates were incubated at 37°C in 5% CO_2 for 7 days before the colonies were

fixed by adding 200µL of 20% paraformaldehyde. The number of colonies in each well was counted and their morphology was recorded.

RT-qPCR

mRNA was isolated from luminal and basal populations, sorted from cultured mammary organoids as described above. mRNA isolation from each fraction was carried out using an RNeasy Plus Micro Kit from Qiagen, following the manufacturer's procedures. After being sorted, the cells from each fraction were lysed in 350µl Buffer RLT Plus with 10µl β-mercaptoethanol added per 1mL of Buffer RLT Plus. The samples homogenized by vortexing for 1 minute, then were stored as -80°C. The samples were later thawed and RNA extraction was carried out using the provided RNeasy MinElute spin columns as specified by the manufacturer. The concentration of the isolated RNA was determined using a Nanodrop ND-1000. cDNA synthesis was performed using an AffinityScript cDNA Synthesis Kit from Agilent Technologies. 140ng of RNA were added to each reaction mixture, containing 10µl of first strand master mix, 3µl of oligo(dT) primer, 1µl of Affinity Script RT/RNase Block enzyme mixture, and RNase-free H_2O to bring the final reaction volume to 20µl. Mixtures without RT/RNase were prepared as controls. The mixtures were incubated using a Eppendorf Mastercycler Thermo Cycler Model 5332, beginning with a 5 minute incubation at 25°C to allow primer annealing, followed by 15 minutes at 42°C for cDNA synthesis, and finally 95°C for 5 minutes to terminate the reaction. The samples were stored at -20°C until RT-qPCR was performed.

RT-qPCR was performed using a Brilliant II SYBR Green qPCR Master Mix kit from Agilent Technologies. 20µl qPCR reaction mixtures were created according to the manufacturer's instructions. Reaction mixtures were prepared in triplicate with 0.5µl of cDNA, 10µl of Brilliant II SYBR Green qPCR master mix, 8.9µl HPLC water and 0.6µl of primer solution containing 5µM of both forward and reverse primers in each reaction mixture. The RT-qPCR reactions were performed with a Rotor-Gene RG-3000 from Corbett Research. All expression data was

normalized to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA expression. Samples were assayed for estrogen receptor α (*Esr1*),cyclin-dependent kinase inhibitor 1A (*Cdkn1a*),B cell leukemia/lymphoma 2 (*Bcl2*), and Bcl2-associated X protein (*Bax*) mRNA expression. The primers for assaying *Gapdh* mRNA expression were: forward primer: 5'-TCAACAGCAACTCCCACTCTTCCA-3', and reverse primer: 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'. Conditions for RT-qPCR for *Esr1* were 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 95°C for 10 seconds, 59°C for 15 seconds for primer annealing, and 72°C for 13 seconds for elongation. The primers for assaying *Esr1* mRNA expression were: forward primer: 5'-TGCACCATTGACAAGAACCGGA-3', reverse primer: 5'-AGCACCCATTTCATTTCGGCC-3'. Conditions for RT-qPCR for *Esr1* were 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 95°C for 10 seconds, 59°C for 15 seconds for primer annealing, and 72°C for 16 seconds for elongation. The primers for assaying *Cdkn1a* mRNA expression were: forward primer: 5'-TGTCTGAGCGGCCTGAAGATT-3', reverse primer: 5'-ACCAATCTGCGCTTGGAGTGAT-3'. Conditions for RT-qPCR for *Cdkn1a* were 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 95°C for 10 seconds, 58.5°C for 15 seconds for primer annealing, and 72°C for 10 seconds for elongation. The primers for assaying *Bcl2* mRNA expression were: forward primer: 5'- GAG GAA CTC TTC AGG GAT GGG -3', reverse primer: 5'- GGC CAT ATA GTT CCA CAA AGG C -3'. Conditions for RT-qPCR for *Bcl2* were 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 95°C for 10 seconds, 56°C for 15 seconds for primer annealing, and 72°C for 22 seconds for elongation. The primers for assaying *Bax* mRNA expression were: forward primer: 5'- CCA GCT CTG AAC AGA TCA TGA AGA CAG GG -'3, reverse primer: 5'- GCT GTC CAG TTC ATC TCC AAT TCG CC-3'. Conditions for RT-qPCR for *Bax* were 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 95°C for 10 seconds, 61.7°C

for 15 seconds for primer annealing, and 72°C for 18 seconds for elongation. All cycles were followed by a dissociation run to record the amplicon's melt profiles.

Statistics

All statistics were performed in R: A Language and Environment for Statistical Computing (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/). Normality of data was determined using a Shapiro-Wilk normality test. Comparisons between measurements in control and tamoxifen-treated organoids were performed using paired t-tests or Wilcoxon signed rank test, in cases where the data was not normally distributed. Comparisons between measurements from young and old organoids were performed using Welch two sample t-test. F test was used to determine if samples had equal variance.

Results

Tamoxifen Increases Cell Proliferation among Young and Old Mammary Organoids

Tamoxifen could exert its chemopreventive effect by altering cell proliferation within the mammary epithelium. To investigate differences in tamoxifen's effects on mammary organoids isolated from old and young MMTV-*c-neu* mice, the growth potential of cells from tamoxifen and control-treated, young and old mammary organoids was examined using sulforhodamine B (SRB)-binding assays. The growth of the cells was assessed on culture days 2, 4 and 6 (Fig. 1, 2).

On day 2, neither cells from young nor old mouse organoid cultures showed a significant difference in SRB-binding between their control and tamoxifen-treated cells, suggesting that both culture types contained similar numbers of cells. For cultures from young organoids, the average absorbance was 0.229 and 0.284 for cultures with control and tamoxifen-treated cells respectively (p-value =0.0814). For cultures from old organoids, the average absorbance was 0.234 and 0.282 for cultures with control and tamoxifen-treated cells, respectively (p-value = 0.2728).

By day 4, cultures containing tamoxifen-treated organoid cells showed significantly higher SRB-binding than cultures with control organoid cells. The average absorbance of young mouse organoid cell cultures were 0.508 and 0.767 for cultures with control and tamoxifen-treated cells, respectively (p-value = 0.001). The average absorbance of old organoid cell cultures were 0.472 and 0.811 for cultures with control and tamoxifen-treated cells, respectively (p-value = 0.033). The higher SRB staining in wells with tamoxifen-treated cells suggests that there were greater amounts of cell proliferation among the tamoxifen-treated organoid cells from both young and old mice.

By day 6, the average absorbance of tamoxifen-treated cell cultures was significantly higher than contol-treated cell cultures with young, but not old, mouse mammary organoid cells. The average absorbance of young mouse organoid cell cultures were 0.9 and 1.243 for cultures with control and tamoxifen-treated cells, respectively (p-value = 0.011). The average absorbance of old organoid cells were 0.894 and 1.228 for cultures with control and tamoxifen-treated cells, respectively. However, the difference in absorbance was not statistically significant (p-value = 0.199). Comparisons between the cultures with young and old mouse organoid cells point showed no significant differences at any time point for cells from organoids given either treatment. Together, these observations demonstrate that cells from tamoxifen-treated organoids undergo greater proliferation than control organoids.

Tamoxifen Increases the Colony-forming Efficiency of Cells from Young and Old Mammary Organoids in Adherent (2D) Colony-forming Assays

The increase in SRB absorbance in cultures with tamoxifen-treated cells indicated an increase in cell proliferation. This increase in proliferation could have resulted from an increase in the proportion of colony-forming cells among the organoids, namely an increase in the proportion of luminal progenitor cells which show the greatest colony-forming potential in 2D cultures of mammary epithelial cells (111). To test this possibility, cells from control and

tamoxifen-treated organoids were reseeded onto adherent 6-well plates with growth-arrested NIH-3T3 feeder cells to assess colony-forming efficiency (Fig 3, 4).

Both young and old mouse mammary organoid cells generated more colonies after being treated with tamoxifen. For young mouse organoid cells, the average number of colonies per well was 18.6 and 69.5 for control and tamoxifen-treated organoid cells, respectively (p-value = 0.027). For old mouse organoid cells, the average number of colonies in each well was 35.5 and 110.5 for control and tamoxifen-treated organoid cells, respectively (p-value = 0.027). On average, young mouse organoid cells generated 3.8-times more colonies after tamoxifen treatment while old mouse organoid cells generated 4.9-times more colonies. No difference in colony-forming efficiency was observed between young and old mouse organoid cells. These results suggest that the previously observed increase in growth potential following tamoxifen-treatment was due to an increase in the proportion of colony-forming cells within the organoids.

Tamoxifen alters the Proportions of Mammary Epithelial Cell Populations within Organoids

The majority of colony-forming cells found in the mammary epithelium are luminal progenitor cells (111). The different mammary epithelial cell populations can be separated based on differences in cell surface protein expression. To determine if the increase in colony-forming cells could be accounted for by changes in the proportions of the mammary organoid cell populations, control and tamoxifen-treated mouse mammary organoid cells were labeled with antibodies against EpCAM, CD49f, and CD61 and the populations were measured with flow cytometry. The measured populations included differentiated luminal cells (EpCAM-high, CD49f-low, CD61-negative), luminal progenitor cells (EpCAM-high, CD49f-low, CD61-positive), and basal cells (EpCAM-low, CD49f-high), as well as stromal cells (EpCAM-negative) (Fig 5).

Tamoxifen caused significant changes in the proportions of cell populations among both young and old MMTV-c-*neu* mouse mammary organoids. In organoids from young mice, tamoxifen significantly reduced the proportion of epithelial cells from an average of 53.31% of all cells in control-treated organoids to 39.2% in tamoxifen-treated organoids (p-value = 0.0001) (Fig 6). In organoids from old mice, tamoxifen reduced the average proportion of epithelial cells from 60.3% to 51.08% (p-value = 0.009).

The decrease in epithelial cells was associated with a significant decrease in the proportion of basal cells (Fig 7). In young mouse mammary organoids, the average proportion of basal cells within the total cell population decreased from 14.57% in control organoids to 7.82% in tamoxifen-treated organoids (p-value = 0.008). In old mouse organoids, tamoxifen reduced the average proportion of basal cells from 23.76% in control organoids to 15.02% in tamoxifen-treated organoids (p-value = 0.004). Between young and old organoids, old organoids tended to have a higher percentage of basal cells within the total cell population of control organoids (p-value= 0.083). Between tamoxifen-treated organoids, old mouse organoids contained significantly more basal cells than young mouse organoids (p-value = 0.009).

Tamoxifen significantly affected the proportion of luminal cells within the total cell population of young but not old mouse mammary organoids (Fig 7). In young mouse organoids, tamoxifen decreased the average percentage of luminal cells from 38.54% of all cells in control organoids to 31.27% (p-value = 0.016). The average percentage of luminal cells in old mouse organoids was similar in control and tamoxifen-treated organoids, 36.18% and 35.93%, respectively (p-value = 0.9).

Within the EpCAM-positive epithelial cell populations of both young and old mouse mammary organoids, tamoxifen skewed the distribution of cells to favor the luminal population (Fig 8). In young mouse organoids, the average percentage of luminal cells among the epithelial population increased from 72.7% to 80.24% with tamoxifen treatment, while the basal population significantly decreased (p-value = 0.017). In old mouse organoids, the average percentage of

luminal cells increased from 60.49% to 69.69% with tamoxifen treatment (p-value = 0.007). Between young and old organoids, the difference in their proportions of luminal and basal cells approached significance when their proportions in control organoids were compared (p-value = 0.06) but the difference was significant when tamoxifen-treated organoids were compared (p-value = 0.023).

Despite causing an increase in the proportion of colony-forming cells, tamoxifen was shown to significantly reduce the proportion of the EpCAM-high/CD49f-low/CD61-positive, luminal progenitor population in both young and old mouse mammary organoids (Fig 9). In young organoids, the average proportion of the CD61-positive luminal progenitor population within the total cell population was reduced from 20.88% in control organoids to 16.73% in tamoxifen-treated organoids (p-value = 0.003). In old organoids, the average proportion of the CD61-positive luminal progenitor population was reduced from 20.65% to 16.19% with tamoxifen treatment (p-value = 0.03). Within just the luminal population of old organoids, the average proportion of luminal progenitor cells was reduced from 57.53% to 47.55% in tamoxifen-treated organoids (p-value = 0.017) (Fig 10). However, in young organoids, the proportion of CD61-positive cells within the luminal population was not significantly changed by tamoxifen, with CD61-positive cells making up 55.14% and 53.6% of the luminal cells in control and tamoxifen-treated organoids respectively (p-value = 0.4).

The proportion of EpCAM-high/CD49f-low/CD61-negative luminal cells within the organoids was not significantly changed by tamoxifen in organoids from either young or old MMTV-c-*neu* mice (Fig 11). However, the proportions of CD61-negative luminal cells tended to decrease with tamoxifen in organoids from young mice, from 17.56% to 14.44% (p-value = 0.062), but tended to increase in organoids from old mice, from 15.41% to 19.65% (p-value = 0.12). Within the luminal population of old mouse organoids, the average proportion of CD61-negative cells significantly increased with tamoxifen-treatment, from 42.18% to 52.25% (p-value = 0.016). Within the luminal population of young organoids, the average proportion of CD61-

negative cells was largely unchanged, 44.64% and 46.16% in control and tamoxifen-treated organoids respectively (p-value = 0.1). Because the populations of CD61-positive luminal progenitor cells in young and old organoids were reduced, while the CD61-negative luminal cell population remained present, it is possible that the increase in colony-forming efficiency from tamoxifen results from an increase in the number of colony-forming cells among the CD61-negative luminal cell populations.

Tamoxifen alters the Proportion of Proliferating Cells among Mammary Organoid Cell Populations

Cell proliferation within the organoids was assessed to determine if the tamoxifen-induced increase in cell growth and colony-forming ability corresponded with an increase in the proportion of proliferating cells among the organoid cell populations. EpCAM-positive, mammary epithelial cells were assayed for proliferation based on their incorporation of EdU. The EpCAM-positive fraction was separated into quartiles, each containing 25% of the EpCAM-positive cell population to make 4 groups; EpCAM-high, EpCAM-med-high, EpCAM-med-low, EpCAM-low quartiles. EpCAM expression differs between luminal and basal cells, with luminal cells showing higher EpCAM expression than basal cells. Therefore, the EpCAM-high and EpCAM-med-high quartiles contain the majority of luminal cells while basal cells reside in the EpCAM-med-low and EpCAM-low quartiles. The proportion of EdU-positive cells in each quartile was assessed (Fig 12, Table 1).

Tamoxifen caused significant changes in the proportions of actively dividing cells among the EpCAM-positive fractions (Fig 13). In young MMTV-c-*neu* mouse mammary organoids, the percentage of EdU-positive cells in the EpCAM-low and EpCAM-med-low quartiles decreased from 27.90% and 22.38%, respectively, in control organoids to 9% and 10.13% in tamoxifen-treated organoids (p-value = 0.004 and 0.018 respectively). EdU incorporation in the EpCAM-high and EpCAM-med-high quartiles was unaffected by tamoxifen. In old mouse organoids, the

percentage of proliferating cells in the EpCAM-low quartile decreased from 19.88% in control organoids to 8.27% in tamoxifen-treated organoids (p-value = 0.016). The proportion of EdU-positive cells in the other quartiles was not significantly affected by tamoxifen.

Tamoxifen also affected the distribution of EdU-positive cells among the EpCAM-positive quartiles (Fig 14). In young mouse organoids, the EpCAM-high quartile increased from an average of 17.64% of the total EdU-positive population to 30% with tamoxifen treatment (p-value = 0.043). The EpCAM-low quartile decreased from an average of 35.04% to 21.34% of the EdU-positive population with tamoxifen-treatment (p-value = 0.01). In old mouse organoids, the EpCAM-high and EpCAM-med-high quartiles increased with tamoxifen-treatment from averages of 21.42% and 21.74% to 32.18% and 27.42% of the EdU-positive population, respectively (p-value = 0.003 and 0.007). The average proportion of EpCAM-low cells among the EdU-positive population decreased from 29.5% to 17.72% with tamoxifen treatment (p-value = 0.03). Between control organoids from young and old mice, the proportion of EpCAM-low cells in the EdU-positive population was significantly higher in young organoids, 35.04%, than in old organoids, 29.5% (p-value = 0.025). The proportion of EpCAM-med-high cells among the EdU-positive population was significantly lower in young organoids, 18.56%, than in old organoids, 21.74% (p-value = 0.008).

The EpCAM-med-low and EpCAM-low quartiles contain the majority of basal cells. The results from these EdU assays suggest that the reduction in the EpCAM-low/CD49f-high, basal population caused by tamoxifen resulted from a reduction in cell proliferation. Tamoxifen had less effect on cell division within the EpCAM-high and EpCAM-med-high quartiles, corresponding to the luminal cell population of the mammary organoids.

Tamoxifen Increases Colony-forming Efficiency in Isolated EpCAM-high and EpCAM-low Cells Indicating Changes in Progenitor Activity among the Luminal and Basal Populations

Mammary organoids contain populations of both luminal and basal progenitor cells. To determine which population displays increased progenitor activity, and if the increase in the proportion of colony-forming cells among tamoxifen-treated organoids was maintained if the luminal and basal populations were isolated from other cell populations, the EpCAM-high, luminal-enriched and EpCAM-low, basal-enriched populations were separated by FACS and seeded into 2D colony-forming assays.

In young MMTV-c-*neu* mouse mammary organoids, tamoxifen significantly increased the colony-forming efficiency of the EpCAM-high and EpCAM-low fractions. In cultures with EpCAM-high cells, the average number of colonies doubled from 41.5 to 88.56 colonies with tamoxifen treatment (p-value = 0.03) (Fig 15). In cultures with EpCAM-low cells, the average number of colonies also doubled from 10.7 to 21.3 with tamoxifen treatment (p-value = 0.004) (Fig 16).

In organoids from old MMTV-c-*neu* mice, greater variability in colony-forming efficiency was observed between cell fractions from different mice and the differences in colony-forming efficiency between tamoxifen and control treated fractions were not statistically significant. In the EpCAM-high fraction, an average of 50.06 colonies arose from control-treated cells while 96.33 colonies arose from tamoxifen-treated cells, however this difference only approached significance (p-value = 0.09) (Fig 15). In the EpCAM-low fraction no change in colony-forming efficiency was observed; an average of 53.9 colonies arose from control-treated cells while 52.2 colonies arose from tamoxifen-treated cells (p-value = 0.84) (Fig 16).

The EpCAM-high fraction is composed entirely of luminal cells, containing a heterogenous mix of colony-forming and non-colony forming cells. One possible explanation for the increase in colony-forming efficiency was that the non-colony forming luminal and basal cell populations were reduced, causing a relative increase in colony-forming cells. However, when the

luminal populations from control and tamoxifen-treated organoids were isolated, the increase in colony-forming efficiency was maintained, suggesting that the increase in colony-forming cells originates from within the luminal cell population. The EpCAM-low, basal cell population showed only modest colony-forming potential. This quartile contains a mix of luminal and basal cells. However, we previously demonstrated that tamoxifen reduced the basal cell population, suggesting that the increase in colony-forming cells within the EpCAM-low population came from a relative increase in the number of luminal cells within this quartile.

Tamoxifen alters the Non-adherent (3D) Colony-forming Potential of Sorted Luminal Cells from Old, but not young, MMTV-*c-neu* Mice.

To assess the effects of tamoxifen on luminal and basal progenitor cells within the organoids, antibodies against EpCAM and CD49f were used to isolate epithelial (EpCAM-positive), luminal (EpCAM-high, CD49f-low), and basal (EpCAM-low, CD49f-high) populations of control and tamoxifen-treated MMTV-*c-neu* mouse mammary organoids were sorted and cells were seeded into 3D cultures, the numbers and phenotypes of the resulting colonies were recorded (Fig 17, Table 2). Tamoxifen-treated organoid cells tended to show higher colony-forming efficiency, however statistically significant increases were only observed among cells from old mice. In organoids from young mice, no significant increases in colony numbers were observed between control and tamoxifen-treated organoid cells from any of the tested cell fraction (Fig 18). Colonies originating from luminal and basal progenitors can be identified based on their morphology. Colonies from luminal progenitors have a hollow, acinar morphology while colonies from basal progenitors have a solid morphology. When solid and hollow colonies were considered individually, no changes in the colony-forming efficiency of either type of colony were observed in any cell populations from young mice following tamoxifen-treatment. Among organoid cells from old mice, the average number of hollow colonies formed from sorted epithelial cells increased from 13.4 to 26 colonies with tamoxifen (p-value = 0.035) (Fig 19).

Colony formation in the luminal fraction increased from an average of 39.3 to 86.4 colonies with tamoxifen (p-value = 0.016). This was accounted for by a significant increase in the number of hollow colonies, which averaged 22.7 and 54.5 colonies in control and tamoxifen-treated organoid cell respectively (p-value = 0.032). Despite these changes, no significant differences in the ratios of hollow to solid colonies were observed between control and tamoxifen-treated organoid cells. No significant differences in colony-formation were observed when the results from young and old mouse organoids were directly compared.

Hollow colonies result from luminal progenitor cells. Tamoxifen caused an increase in the number of hollow colonies that formed from the epithelial and luminal populations from old mouse organoids. This finding reiterates earlier findings which suggest that the increase in colony formation results from an increase in the number of luminal cells capable of forming colonies.

Tamoxifen did not increase the Proportion of Cells that Bind Annexin V within the Organoids

Tamoxifen caused significant reductions in the total number of epithelial cells among the mammary organoids. This was most notable in the luminal CD61-positive and basal cell populations where the proportions of each population within the total population were reduced by approximately 20% and 40%, respectively, in organoids from both young and old mice that were treated with tamoxifen. To test if apoptosis had a role in this reduction of cells, control and tamoxifen-treated organoid cell populations were assayed for annexin V binding. No observable increase in annexin V-binding resulted from tamoxifen-treatment in either the luminal or basal populations within young or old MMTV-c-*neu* mouse mammary organoids, suggesting that apoptosis is not continuously operating to alter the cell populations in tamoxifen-treated organoids (Fig 20).

Tamoxifen altered the Transcription of Genes Related to Apoptosis in Young but not Old MMTV-c-*neu* Mouse Mammary Organoids

RT-qPCR assays were performed to test for changes in gene expression among sorted luminal and basal cells that may shed light on the mechanism underlying the increase in colony-forming cells caused by tamoxifen. The expression of *Esr1* transcription was measured to gauge the status of fully differentiated, non-clonogenic luminal cells. Transcripts of *Cdkn1a*, coding for p21, were measured to assess the activation of cell cycle arrest mechanisms. *Bax* and *Bcl2* transcription was measured to assess the activation of apoptosis pathways.

In the luminal fractions from young and old organoids, no significant changes in *Esr1* transcription were observed with tamoxifen treatment (Fig 21). In the basal fraction of old organoids, *Esr1* transcription increased by an average of 170% with tamoxifen-treatment (p-value = 0.003). No significant differences in *Esr1* transcription were measured between cell fractions in organoids from young and old mice.

No statistically significant changes in *Cdkn1a* transcription were detected in young or old mouse organoids treated with tamoxifen (Fig 22). However, *Cdkn1a* transcription tended to increase among luminal cells from young mouse mammary organoids. Average *Cdkn1a* transcription increased 180% with tamoxifen-treatment, but this change only approached significance (p-value = 0.057). Transcription in the young basal population and in both luminal and basal cell populations from old mouse organoids did not show any consistent changes with tamoxifen. These results suggest that the reduction in cell division among basal cells caused by tamoxifen is independent of p21 activation. However, they suggest that the luminal cell population in organoids from young mice may be more sensitive to tamoxifen than the luminal cell population in organoids from old mice.

Tamoxifen significantly affected *Bax* transcription in young, but not old, organoids (Fig 23). In the luminal fraction of young organoids, tamoxifen-treated cells showed only 54% the transcription of Bax compared to transcription in control-treated luminal cells (p-value = 0.006).

Between cell populations, the average transcriptional level of *Bax* in control-treated organoids was 260% higher in luminal cells compared to basal cells (p-value = 0.03).

The difference in *Bcl2* transcription between control and tamoxifen-treated organoids only approached significance in the luminal fraction of young organoids (Fig 24). Expression in the other cell fractions was unaffected. In young organoids, tamoxifen decreased average *Bcl2* transcription in the luminal fraction by 30% but this change failed to reach significance. (p-value = 0.125). The *Bcl2* transcription in the luminal fraction of control-treated organoids was on average 380% higher than in the basal fraction but this difference also failed to reach significance (p-value = 0.06). The significant reduction in *Bax* transcription, while *Bcl2* transcription was less effected, suggests that tamoxifen may have stimulated survival signals in the luminal population of young organoids.

Discussion

Tamoxifen causes an increase in colony-forming activity among luminal cells

Tamoxifen is clinically effective for preventing ER-positive breast tumors, reducing tumor incidence by 48% (94). Mouse studies have demonstrated that tamoxifen may also be effective for preventing ER-negative cancers if administered before the transformation of normal mammary cells into cancer cells (103, 106). Furthermore, the effects of tamoxifen on tumor incidence have been shown to extend beyond the treatment period in both clinical trials and studies of mouse models, suggesting that tamoxifen affects long-lived cell populations (98, 99, 106). The experiments here examined the direct effects of tamoxifen on mammary epithelial cells, isolated as organoids from young and old MMTV-*c-neu* mice in hormone-free conditions with the purpose of identifying the direct, age-dependent effects of tamoxifen on mammary epithelial cells that may shed light on the mechanism behind tamoxifen's chemopreventive effects. Other studies demonstrated that treating 12 week old MMTV-c-*neu* mice with tamoxifen prevented mammary tumor development while treatments beginning at 24 weeks did not (103). The results

here demonstrate that, in a hormone-free environment, tamoxifen causes significant changes in various mammary organoid cell populations, altering their proportions within the organoids, their proliferation, and apoptosis signaling pathway activity. Furthermore, different responses to tamoxifen were observed between organoids isolated from young and old MMTV-*c-neu* mice, suggesting a target population of cells for tamoxifen's chemopreventive effect.

Initial tests of progenitor activity showed that 2D colony-forming efficiency was increased in cells from tamoxifen-treated organoids from both young and old MMTV-*c-neu* mice. The proportions of different cell populations were assessed to identify changes in the cell populations that may have accounted for the relative increase in colony-forming cells. Within the total cell populations of both young and old organoids, the total number of epithelial cells was reduced. This reduction was characterized by reductions in the basal and CD61-positive luminal cell populations while the proportion of CD61-negative luminal cells was relatively unchanged. The majority of colony-forming cells within the mammary epithelium are luminal progenitor cells that are enriched within the CD61-positive luminal cell population (111). However, colony-forming assays of sorted cells demonstrated that the increase in colony-forming efficiency resulting from tamoxifen was maintained after the EpCAM-high, luminal cells were isolated away from other cell populations, suggesting that tamoxifen increased the proportion of colony-forming cells within the luminal population.

These results suggest there was an increase in the proportion of colony-forming cells among the CD61-negative, luminal cell population as a result of tamoxifen-treatment. Transplantation studies with mouse mammary luminal cells have shown that luminal cells maintain a measure of plasticity and can be induced to form mammary outgrowths when challenged with an unusual environment (113). Furthermore, while the CD61-positive luminal cell population is enriched in progenitor activity, not all luminal progenitors show CD61 expression and up to 50% of luminal progenitor cells may be CD61-negative (113). This population may be relatively resistant to tamoxifen's deleterious effects, causing a relative

increase in the proportion of CD61-negative, luminal progenitor cells within the organoids while other luminal cell populations decline as a result of tamoxifen.

Mechanisms underlying the changes in cell populations

To uncover the mechanism underlying the reductions in the basal and CD61-positive luminal population caused by tamoxifen, the mammary organoid cell populations were evaluated for markers of cell cycle inhibition and apoptosis. Organoid cell populations that were separated by EpCAM expression and labeled for EdU showed that significantly fewer cells within the EpCAM-low, basal population were in S phase among tamoxifen-treated organoids. However, transcriptional levels of *Cdkn1a*, which codes for the cyclin-dependent kinase inhibitor p21, in the basal population were not significantly changed by tamoxifen. Transcription in the luminal population tended to be elevated, however this trend failed to achieve statistical significance and the number of luminal cells in S phase was not significantly changed by tamoxifen-treatment. Assessments of apoptosis did not suggest that the luminal or basal populations in these organoids were undergoing greater amounts of cell death due to tamoxifen. Tamoxifen-treatment did not increase annexin-V binding in any cell population in young or old organoids. The transcription of *Bax* and *Bcl2*, two regulators of apoptosis, tended to be reduced within the luminal cells from young organoid cells. The balance between Bax and Bcl2 expression is a regulator of apoptosis. With transcription in both of these genes decreasing, it is unclear how tamoxifen may have influenced apoptosis in the luminal cells. Likewise, luminal cells did not show any additional signs of apoptosis, suggesting these changes in gene expression may not have affected the luminal cell population in young mouse mammary organoids. The transcription of these genes in the basal population of organoids from young mice and in any cell population in organoid from old mice was unchanged.

Tamoxifen has been shown to induce cell cycle arrest and apoptosis in both estrogen-dependent and independent breast cancer cell lines (137-140, 149-152). Cell cycle arrest and

apoptosis in estrogen-dependent MCF7 cells was characterized by increases in p21 and decreases in Bcl2 expression 48 hours after treatment with 15μM tamoxifen (151). In ER-negative MDA-MB-231 cells, 5 μM tamoxifen induced apoptosis after 24hrs through the activation of caspase-3-like proteases and JNK 1 pathways, demonstrating its potential to cause cell death through off-target mechanisms (137). Likewise, 5 μM tamoxifen can reduce Bcl2 expression in MDA-MB-231 cells; however expression was unaffected in other ER-negative cell types or in Jurkat cells treated with 10 μM tamoxifen (139, 140). In these other studies, tamoxifen-stimulated apoptosis was observed within a few days of treatment. In our studies reported here, organoid cells were analyzed on the 5^{th} day of treatment. The apoptotic response of the cells may have already run its course, leaving the proportions of the cellular populations altered. Likewise, apoptosis and cell cycle arrest may occur by different mechanisms in different cell types, as shown by other studies where Bcl2 expression was reduced in some cell types but not others. It may also be possible that apoptosis and cell cycle arrest occur in the normal mammary cell populations through different mechanisms besides changes in Bax, Bcl2, or p21 expression.

Different responses to tamoxifen between young and old mammary organoids suggest a mechanism for the prevention of cancer

Between organoids from young and old MMTV-*c-neu* mice, differences were observed in the way their respective luminal populations responded to tamoxifen. In both young and old organoids, tamoxifen caused significant reductions in the number of CD61-positive luminal progenitor cells within the total cell population while the proportion of CD61-negative luminal cells was not significantly changed. However, the proportions of CD61-negative cells within the total cell population tended to decrease in organoids from young mice and increase in organoids from old mice. These slight changes contributed to other observed differences in the responses of organoids from young and old mice to tamoxifen. When the proportion of all luminal cells within the total cell population was measured, the luminal population was significantly reduced by

tamoxifen in organoids from young, but not old, MMTV-*c-neu* mice. Likewise, when the proportions of CD61-positive and CD61-negative cells were measured in the luminal cell population, significant changes resulting from tamoxifen were only observed in organoids from old mice, with the CD61-negative population making up a significantly greater proportion of the luminal cell population. Differences were also observed in tamoxifen's effect on colony formation by luminal cells in 3D growth conditions. When sorted luminal and epithelial cell populations were seeded into 3D colony-forming assays, old mouse organoid cells produced more hollow, luminal colonies than their control organoids. In young mouse organoids, tamoxifen did not increase the number of hollow colonies formed from the epithelial and luminal populations. Transcription changes in the *Bax* gene resulting from tamoxifen were restricted to the luminal population of organoids from young mice. Likewise, the transcription of other genes, such as *Cdkn1a* and *Bcl2*, tended to change in the luminal population of organoids from young, but not old, mice with tamoxifen-treatment.

These observations suggest that the CD61-negative luminal cell population in organoids from young mice was far more sensitive to the negative effects of tamoxifen than the CD61-negative luminal cell population in organoids from old mice. Tamoxifen appears to have had an inhibitory effect on CD61-negative luminal cells from young organoids, resulting in a reduced luminal cell population with no apparent increase in 3D colony-forming efficiency. Meanwhile, tamoxifen appears to have had a stimulatory effect on CD61-negative luminal cells from old organoids, where there was a significant increase in 3D colony-forming efficiency and a shift in the proportions of cells within the luminal cell population to favor the CD61-negative, luminal cell population.

Tamoxifen has been shown to prevent breast cancer development in younger, 12 weeks-of-age MMTV-c-*neu* mice but caused a modest increase in cancer development in MMTV-*c-neu* mice treated at 24 weeks-of-age (103). However, clinical trials of tamoxifen as a chemopreventive have not shown that tamoxifen impacts the occurrence of ER-negative tumors

(94). In the MMTV-*c-neu* mouse model, tumor development progresses in a stepwise manner which mimics tumor progression in humans. Thus, like in mice, tamoxifen's effectiveness may depend on the age at which patients are treated. The effects of aging on the mammary epithelium may result in separate stages of life; an early stage where the mammary epithelium is sensitive to tamoxifen and tamoxifen is effective at preventing tumor development and a later stage where the mammary epithelium is insensitive to tamoxifen and treatment does not affect tumor development.

Tamoxifen-resistance in the 24 weeks-of-age mice was hypothesized to have resulted from the presence of undetected tumor cells within the mammary epithelium (103) Furthermore, tamoxifen-treated mice showed a modest increase in tumor development suggesting that tumor growth could be stimulated by tamoxifen (103). The increase in the number of 3D colony-forming cells in old, but not young, mouse mammary organoids observed here may have resulted from a similar stimulation of progenitor-like activity among luminal progenitor cells that are on the path towards transformation into tumor cells. Aging is associated with significant changes in the mammary epithelial structure and microenvironment that in turn influences cell fate decisions and activity within the mammary epithelium (147). Assays of activity by older mammary cells show that the effects of aging are retained by cells even after transplantation, suggesting alterations to mammary cell epigenetics (143). The key to ensuring the effectiveness of tamoxifen as a chemopreventive may be to administer tamoxifen to patients before these age-related changes take place. Otherwise, tamoxifen-resistant cells within the epithelium may persist despite tamoxifen treatment and become the cells from which tumors derive.

Between organoids from old and young MMTV-*c-neu* mice, the proportion of basal cells within the epithelial cell population tended to be higher in organoids from old mice. An increase in the proportion of basal cells with age has also been observed in C57BL/6 and BALB/c mice but not in FVB/N mice, the strain that is the background for the model of MMTV-*c-neu* mice used here (142, 145). The overexpression of *Her2/neu* may interact with aging mechanisms to influence the aging process. Studies have demonstrated that mammary epithelial cells are

dependent on signaling from their microenvironment to make cell fate decisions that direct the activity of progenitors and determine their differentiation towards the basal or luminal lineages (153). *Her2/neu* overexpression in these mice is also associated with an increase in the proportion of luminal progenitor cells, demonstrating that *Her2/neu* expression is a factor influencing cell fate decisions (146, 148). *Her2/neu* overexpression, combined with the changes in mammary epithelial cells and in the mammary microenvironment that occur with age, appears to alter cell fate decisions in a way that biases the epithelium towards basal cell differentiation. However, this bias may also lead to the incomplete differentiation of luminal cells, as aging has also been associated with the acquisition of basal-like characteristics by luminal cells, which may promote their oncogenic transformation (142, 143, 144).

The increase in the proportion of basal cells in C57BL6/J and BALB/c mice was associated with an increase in bi-potent, basal progenitor cells and inflammatory signaling within the mammary epithelium (142). *Her2/neu* overexpression has been found to stimulate inflammatory signals, IL-1a and IL-6, which also lead to an expansion of mammary progenitor cell populations in pre-neoplastic mammary epithelial tissue and stimulates cancer cells to acquire more stem cell-like properties (154). Inflammation has also been shown to promote tamoxifen-resistance within breast cancer cells by down-regulating ERα expression and promoting breast cancer malignancy (155). The activation of pro-inflammatory pathways by *Her2/neu* transgene expression may also promote tamoxifen resistance in older MMTV-*c-neu* mice (103).

In the context of breast cancer prevention in patients, tamoxifen was effective for preventing ER-positive but not ER-negative tumors (94). This difference in tamoxifen's effectiveness may in part be due to differences in the mechanisms for preventing these tumor types. Tamoxifen's mechanism against ER-positive tumors may be to disrupt estrogen signaling while its mechanism against ER-negative tumors appears to involve a broader influence on the microenvironment and cell fate decisions. Resistance to tamoxifen's effects may result from changes in the mammary microenvironment, such as increases in inflammation, which occur with

aging and direct the epithelium towards a more oncogenic phenotype (147). While current evidence suggests that the aged mammary microenvironment alters mammary epithelial cell epigenetics, it is unclear if such changes can be reversed (147). Tamoxifen may be more effective as a chemopreventive against ER-negative tumors with the addition of other drugs that modify the mammary microenvironment, such as anti-inflammatories, to a state that resembles the younger microenvironment where tamoxifen is more potent.

The gene signatures of *Her2/neu* mammary tumors also suggest that they arise from the luminal progenitor cell population (148). Likewise, this population has been shown to be long-lived, suggesting that they may harbor mutations that can lead to their transformation into cancer cells (121, 122, 124). The results presented herein suggest that tamoxifen significantly affects the CD61-positive, luminal progenitor cell population of both young and old MMTV-c-*neu* mice. However, the increase in colony-forming efficiency in adherent cultures suggests that other luminal progenitor cells, either from existing luminal progenitor population or cells that acquired progenitor-like activity, become more prevalent within the epithelium. This effect appears greater in old MMTV-*c-neu* mouse mammary organoids where a shift in the proportion of luminal cells towards CD61-negative cells and an increase in colony-forming activity in 3D were observed following tamoxifen-treatment. Clinical studies and studies in mouse models show that tamoxifen causes a long-lasting reduction in breast cancer incidence that extends beyond the treatment period (98, 99, 106). Assessments of the repopulation potential of basal mammary progenitor cells from ovariectomized mice have shown that the loss of estrogen signaling has a persistent effect on basal progenitor cells that reduces their mammary repopulating capacity following their transplantation into the mammary fat pads of mice with intact ovaries (156). By disrupting estrogen signaling, tamoxifen may have a similar effect on the remaining luminal progenitor cells, possibly through epigenetic modifications within the cells, that leads to the long-term reduction in breast cancer incidence. Further examination of the progenitor cells that survive tamoxifen-

treatment will need to be done to fully understand how tamoxifen causes a long-term reduction in breast cancer risk.

Figures

Young MMTV-c-*neu* Mouse Organoid Cell SRB Growth Assays

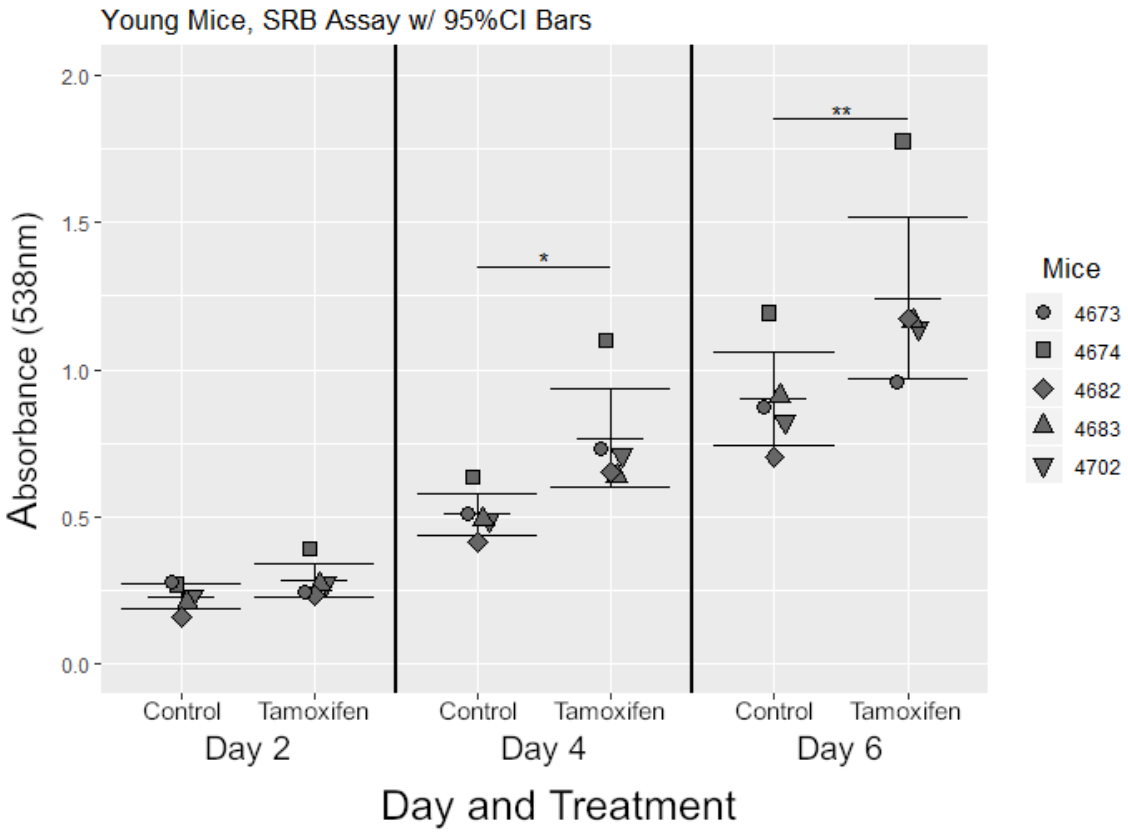

Figure 1. Tamoxifen-treatment led to an increase in cell proliferation among cells from young MMTV-c-*neu* mouse mammary organoids. At day 2 of culture, the difference in SRB content between wells containing cells from control and tamoxifen-treated organoids was not significantly different (paired t-test of log transformed data, p-value = 0.0.0814). At day 4 and 6 of culture, the differences in SRB content between wells with control and tamoxifen organoid cells were significantly different (paired t-test of log transformed data, day 4 * p-value = 0.001, day 6 ** p-value = 0.011).

Old MMTV-c-*neu* Mouse Organoid Cell SRB Growth Assays

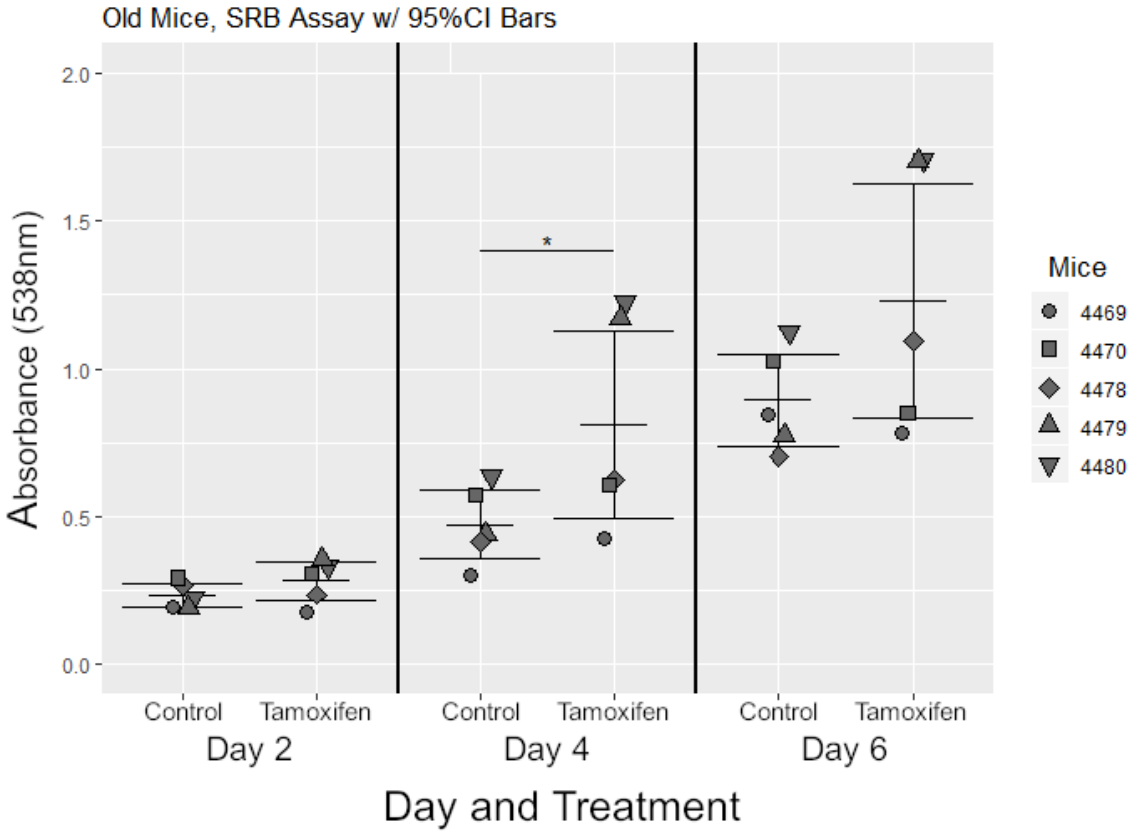

Figure 2. Tamoxifen-treatment led to an increase in cell proliferation among cells from old MMTV-c-*neu* mouse mammary organoids. At day 2 and 6 of culture, the differences in SRB content between wells containing cells from control and tamoxifen-treated organoids were not significantly different (paired t-test of log transformed data, day 2 p-value = 0.2728, day 6 p-value = 0.199). At day 4 of culture, the difference in SRB content between wells with control and tamoxifen-treated organoid cells were significantly different (paired t-test of log transformed data, day 4 * p-value = 0.033)

Representative Images of Colony-forming Assays

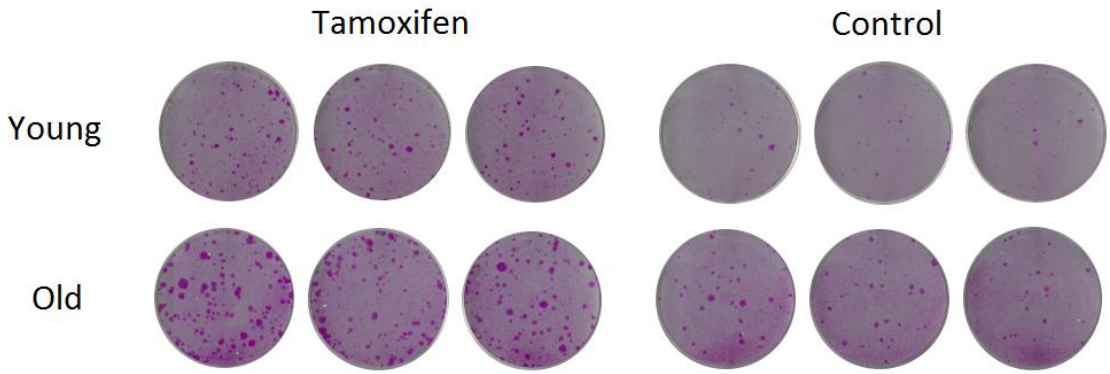

Figure 3. Shown are representative images of 2D colony-formation assays with wells containing cells from young and old mouse mammary organoids treated with control or tamoxifen. More colonies were formed by mammary organoid cells that were treated with tamoxifen than by control organoid cells.

2D Colony-forming Assays within the Total Cell Population

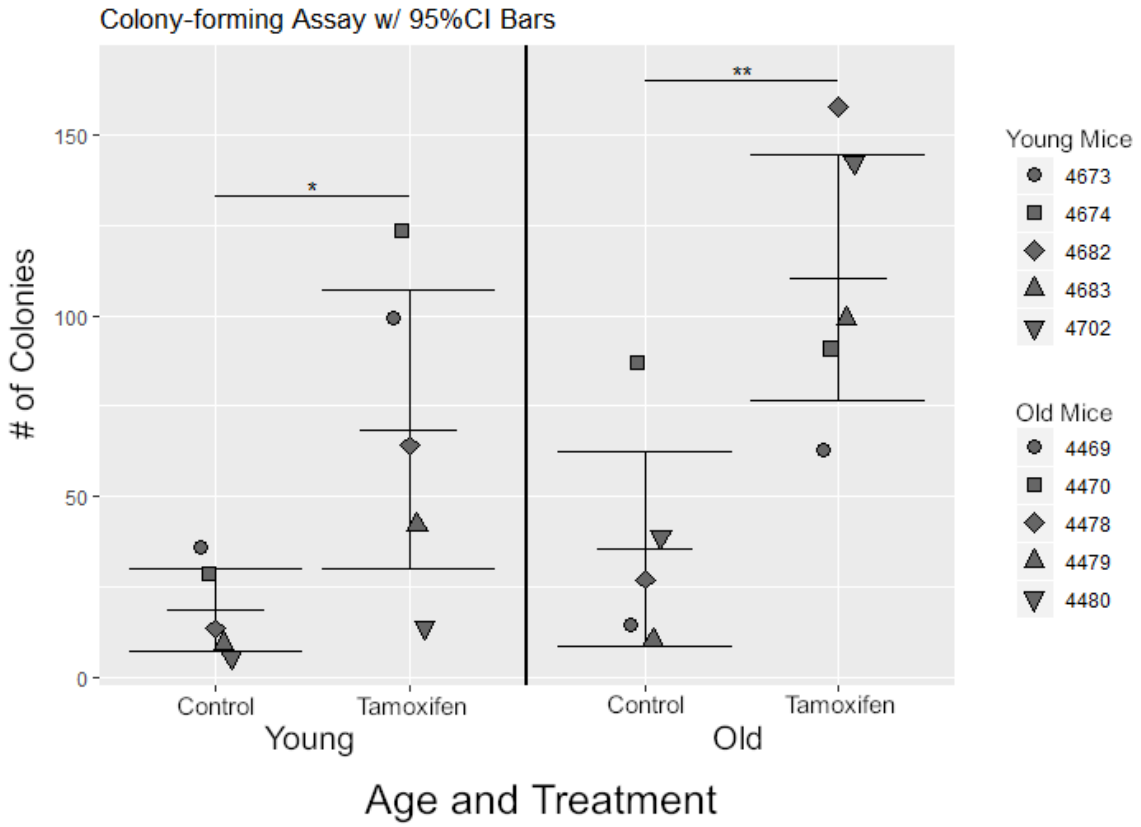

Figure 4. More colonies were formed by mammary organoid cells that were treated with tamoxifen than by control organoid cells. The number of colonies in wells containing cells from tamoxifen-treated organoids, from either young or old mice, was significantly higher than in wells containing control organoid cells (paired t-test, young * p-value = 0.0268, old ** p-value = 0.027).

Flow Cytometric Gating of Mammary Organoid Cell Populations

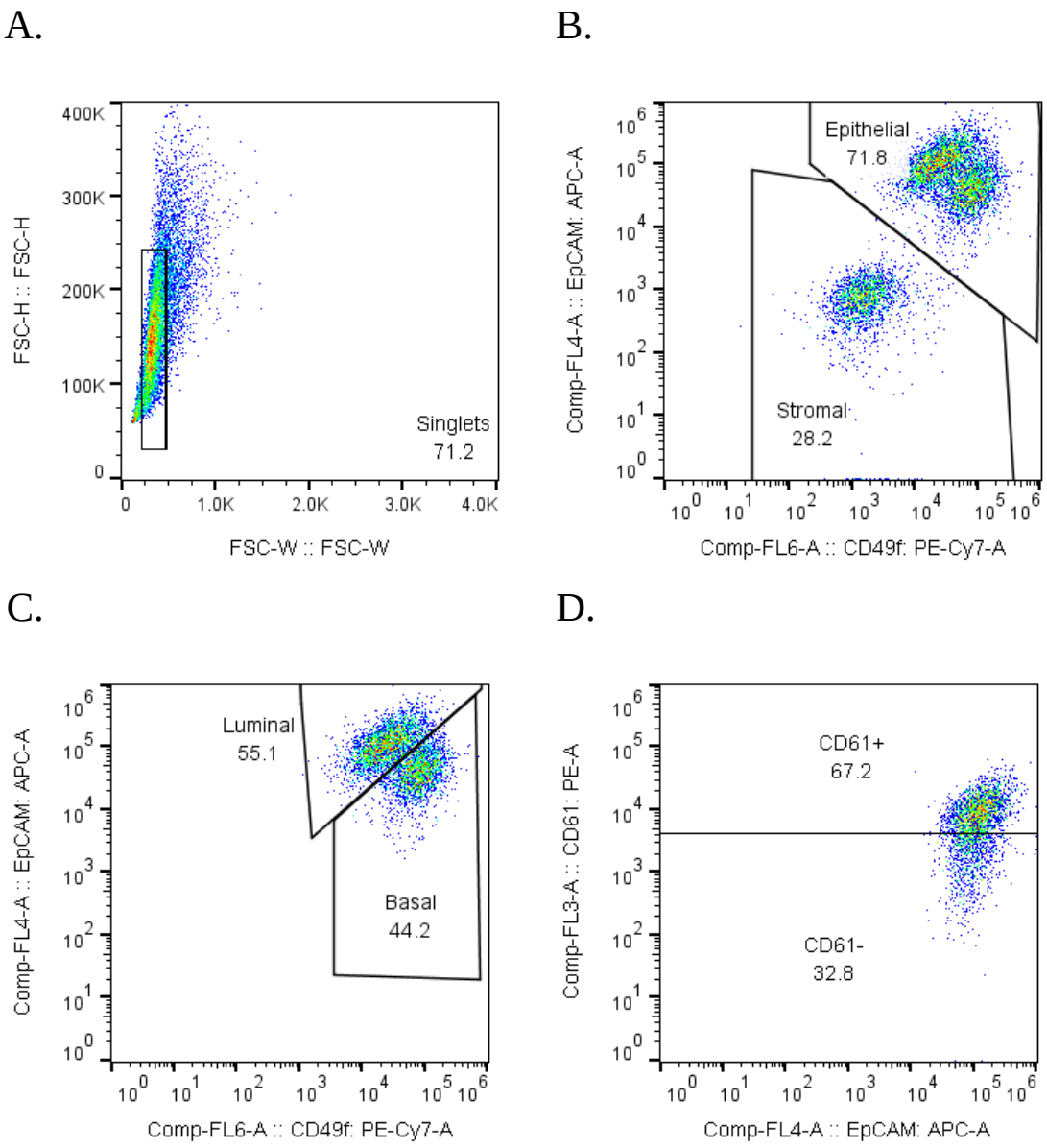

Figure 5. Representative images of the flow cytometric gating strategy for identifying epithelial (EpCAM-positive), basal (EpCAM-low, CD49f-high), CD61-negative luminal (EpCAM-high, CD49f-low, CD61-negative), and CD61-positive, luminal progenitor-enriched (EpCAM-high, CD49f-low, CD61-positive) cells. (A) Single cells were separated from cell fragments and doublets, (B) epithelial and stromal cells were separated, (C) epithelial cells were separated into luminal and basal cells, and (D) luminal cells were separated into CD61-positive, luminal progenitor-enriched and CD61-negative populations.

Proportion of Epithelial Cells within the Total Cell Population

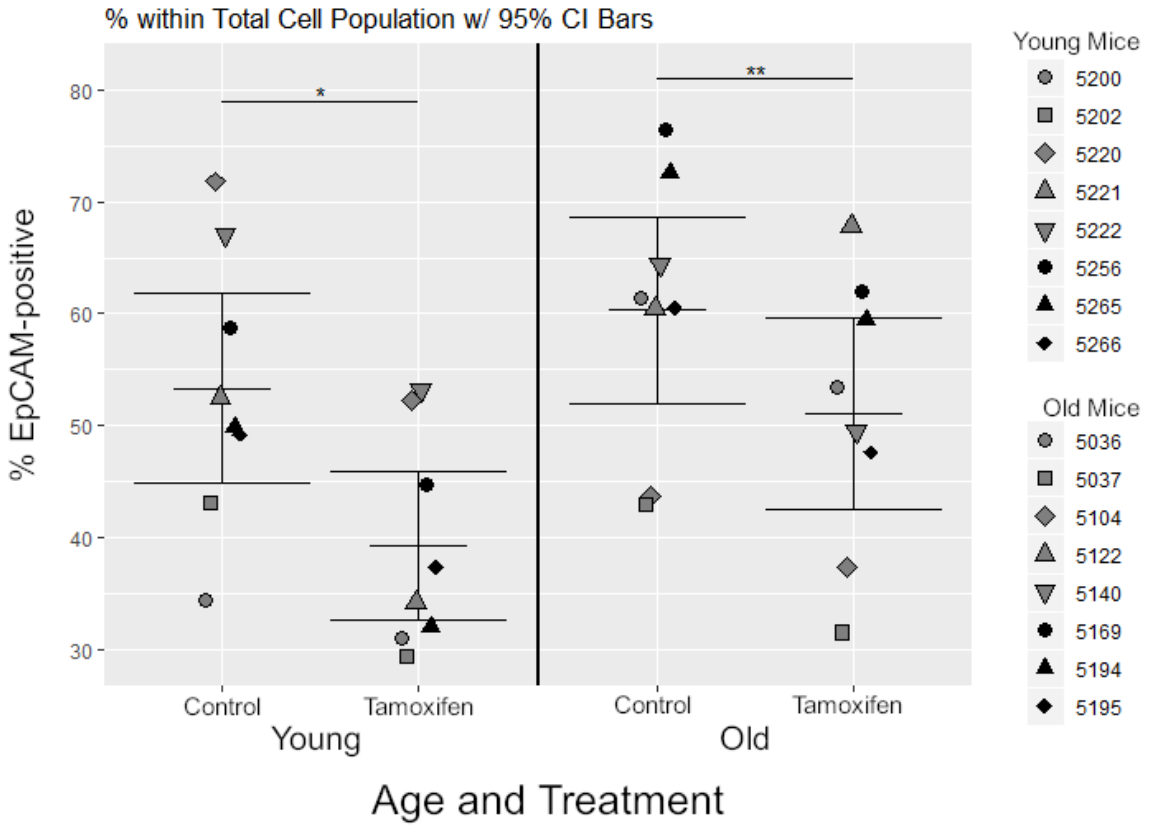

Figure 6. Tamoxifen significantly reduced the proportion of mammary epithelial cells within young and old MMTV-c-*neu* mouse mammary organoids. The proportions of EpCAM-positive cells within the total population of cells from tamoxifen-treated, young and old organoids were significantly lower than in control organoids (paired t-test, young * p-value = 0.0001, old ** p-value = 0.009). The difference in the proportion of EpCAM-positive cells in tamoxifen-treated organoids between organoids from young and old mice approached significance (Welch's t-test, p-value = 0.0505)

Proportion of Luminal and Basal Cell Populations within the Total Cell Population

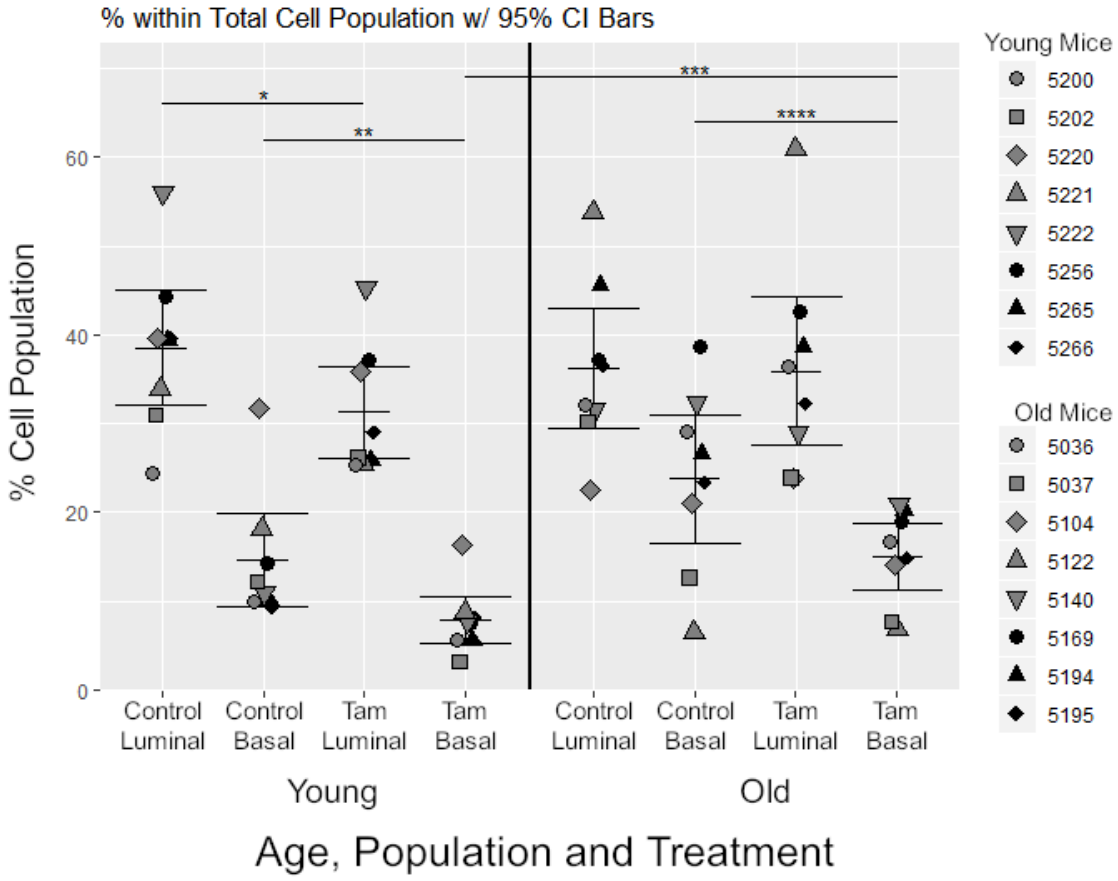

Figure 7. Tamoxifen-treatment reduced the proportion of luminal and basal cells within the total cell populations of young and old MMTV-c-*neu* mouse mammary organoids. The proportion of luminal cells within the total population of cells from young MMTV-*c-neu* mouse organoids was significantly lower in tamoxifen-treated organoids than in control organoids (paired t-test, * p-value = 0.01563). The proportion of basal cells within the total population of organoids cells was significantly lower in tamoxifen-treated organoids from young and old mice compared to their respective controls (paired t-test, young **p-value = 0.007813, old **** p-value = 0.004332). Between young and old mice, the proportion of basal cells within the tamoxifen-treated organoids was significantly different (Welch's t-test, *** p-value = 0.008843). Control organoids from old mice also tended to contain more basal cells than control organoids from young mice (Wilcoxon test p-value = 0.083).

Proportion of Luminal Cells within the Epithelial Cell Population

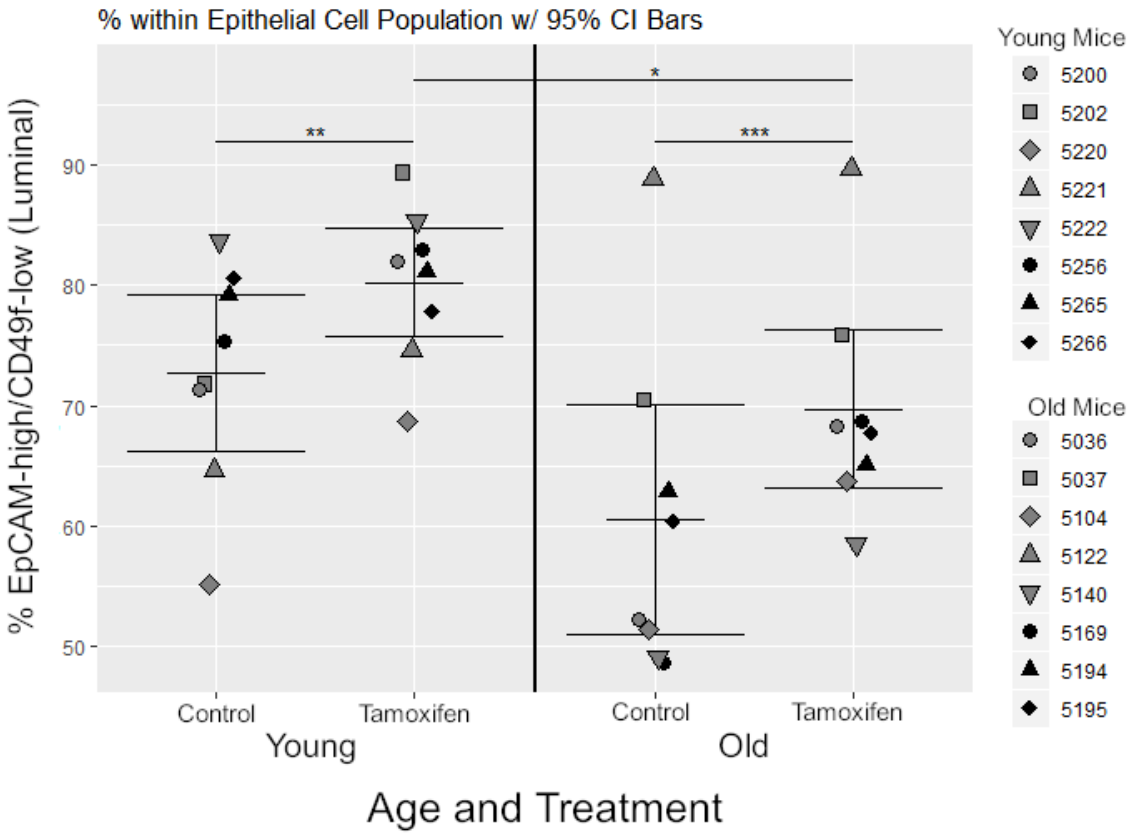

Figure 8. Tamoxifen-treatment increased the proportion of luminal cells within the mammary epithelial (EpCAM-positive) cell population of young and old MMTV-c-*neu* mouse mammary organoids. The proportions of luminal cells among the EpCAM-positive population of tamoxifen-treated young and old organoids were significantly higher compared to the proportions of luminal cells in their respective control organoids (paired t-test, young ** p-value = 0.01685, old *** p-value = 0.005852). Between organoids from young and old mice, the proportion of luminal cells within the tamoxifen-treated organoids was significantly different (Welch's t-test, p-value = 0.02271).

Proportion of CD61-positive Luminal Cells within the Total Cell Population

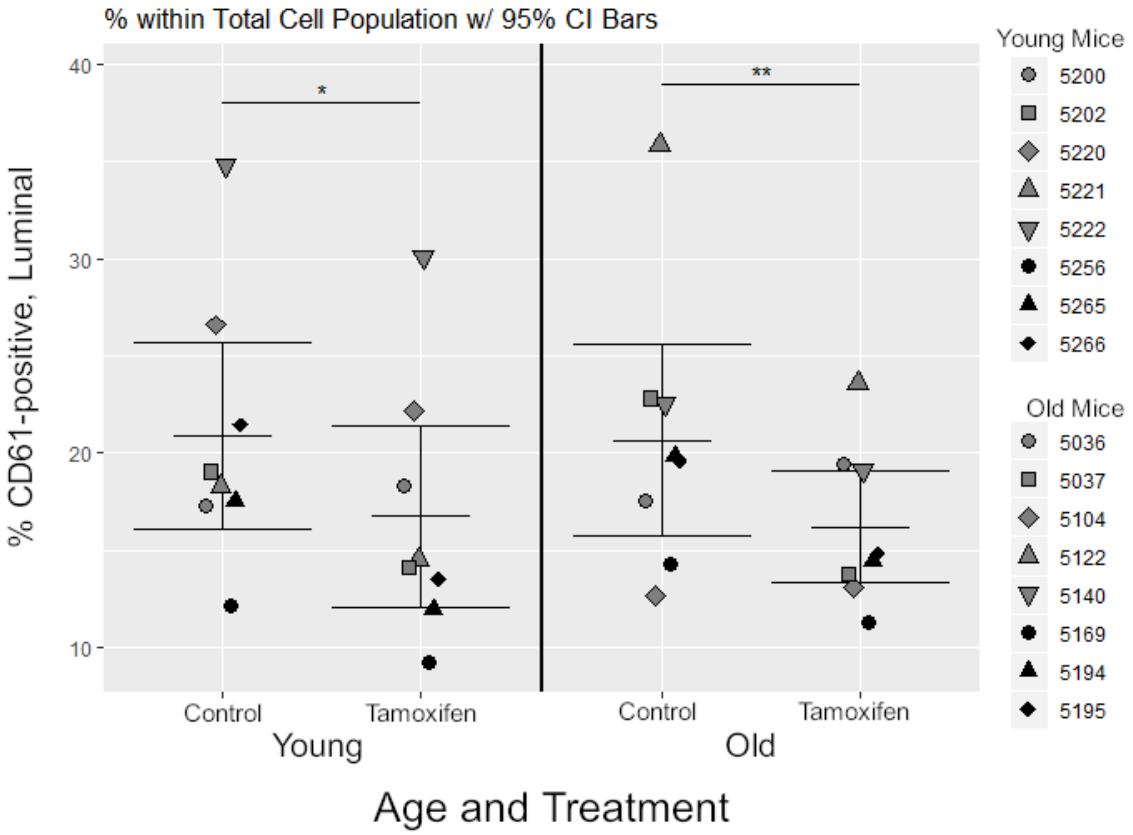

Figure 9. Tamoxifen-treatment significantly reduced the proportions of CD61-positive luminal cells in the total cell populations of young and old MMTV-c-*neu* mouse mammary organoids. Significantly fewer CD61-positive luminal cells were among the tamoxifen-treated organoids from young and old mice compared to the proportion of CD61-positive luminal cells among control organoids (paired t-test, young * p-value = 0.002523, old ** p-value = 0.02936). These results indicate a significant reduction in the proportion of CD61-positive luminal progenitor cells among the organoids following tamoxifen-treatment.

Proportion of CD61-positive Luminal Cells within the Luminal Cell Population

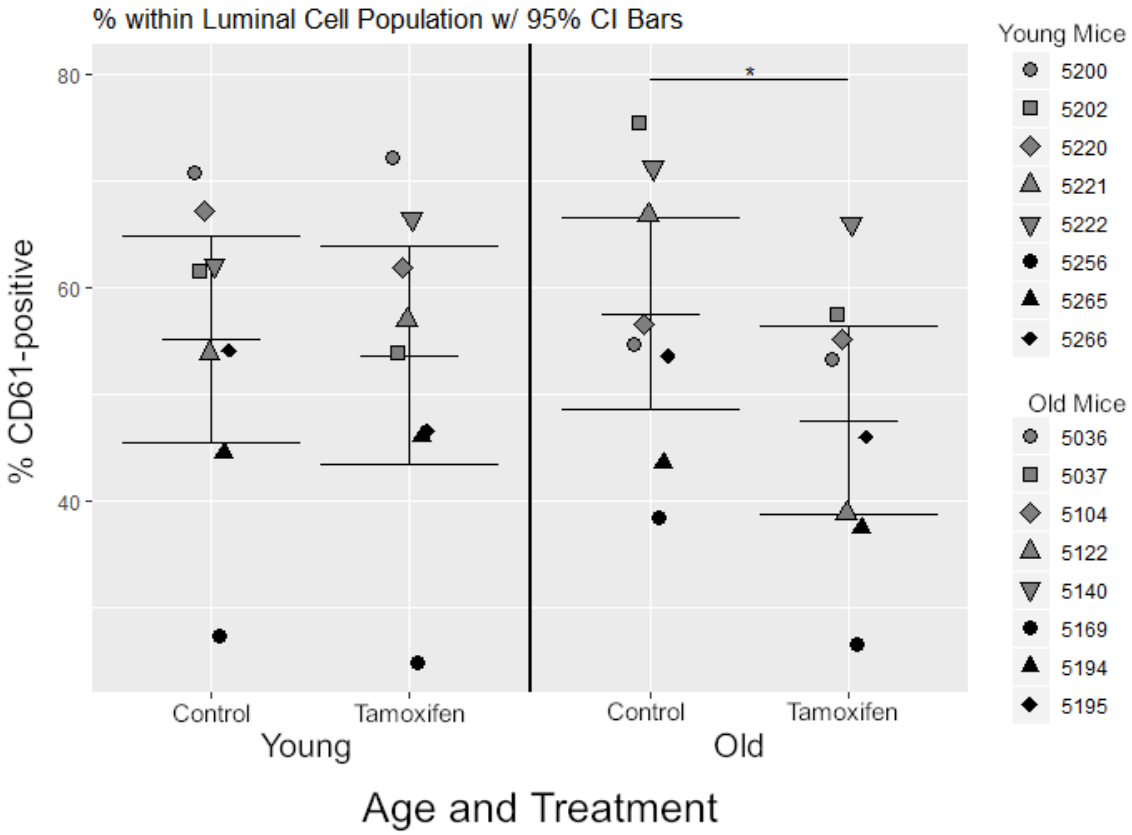

Figure 10. Tamoxifen reduced the proportion of CD61-positive luminal cells within the luminal cell population of old MMTV-c-*neu* mouse mammary organoids but did not affect the proportion in young mouse organoids. The average proportion of CD61-positive luminal cells among the organoids from old mice was significantly lower in tamoxifen-treated organoids than in control organoids (paired t-test, p-value = 0.01724).

Proportion of CD61-negative Luminal cells within the Total Cell Population

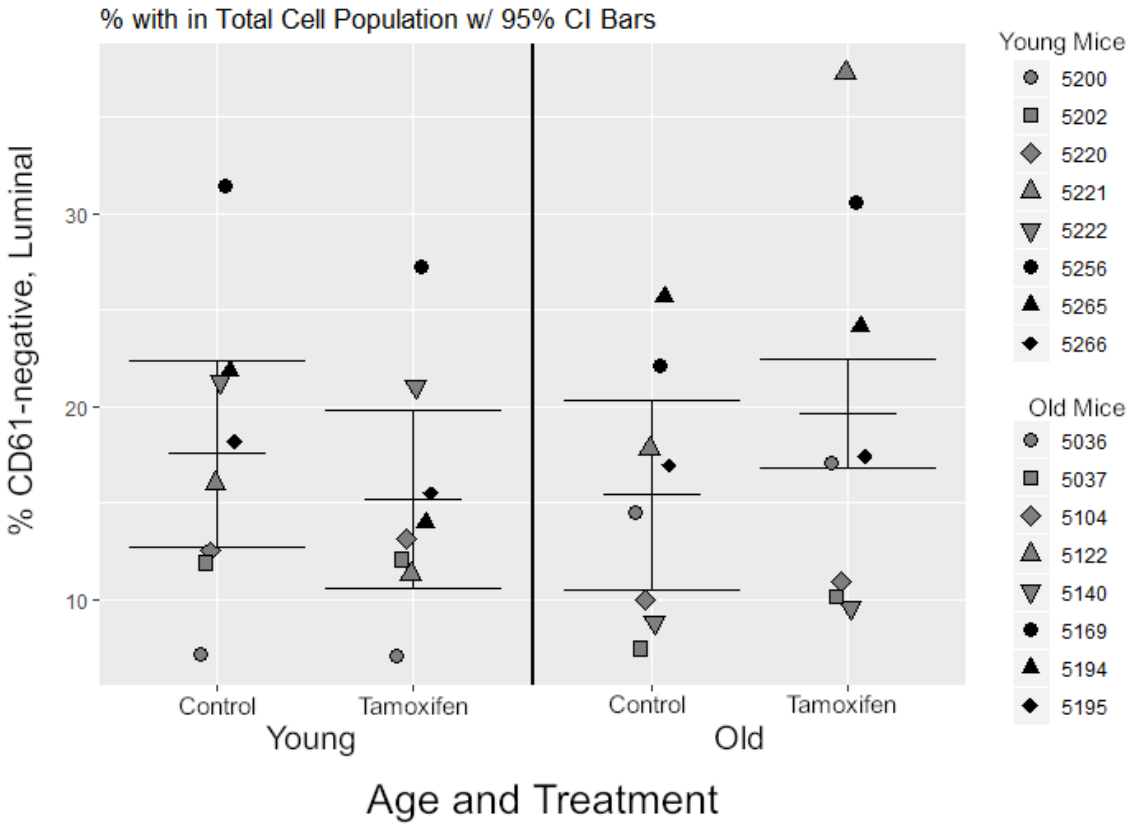

Figure 11. The proportions of CD61-negative luminal cells within the total cell population of tamoxifen-treated MMTV-c-*neu* mouse mammary organoids tended to be lower in young mouse organoids but tended to be higher in old mouse organoids compared to their respective control organoids. However, these trends in young and old mouse organoids failed to achieve statistical significance. (p-values = 0.06189, 0.1217 in young and old organoids respectively).

Flow Cytometric Gating for EpCAM-positive, EdU-positive Cells

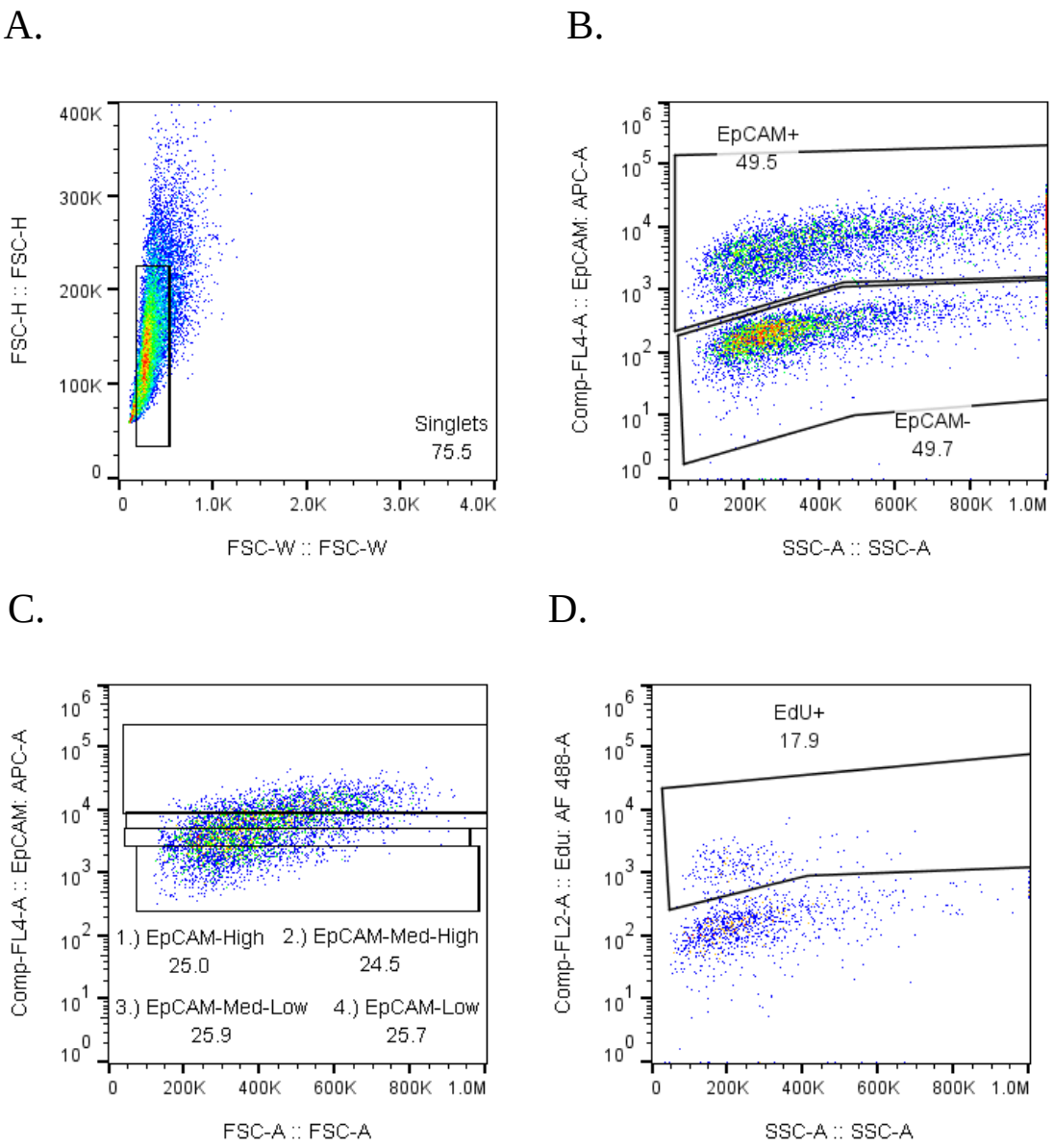

Figure 12. Representative images of the flow cytometric gating strategy for assessing EpCAM-positive and EdU-positive cells collected from MMTV-c-*neu* mouse mammary organoids. (A) Single cells were isolated from cell fragments and doublets, (B) EpCAM-positive and negative cells were separated from each other, or (C) the EpCAM-positive cell population was separated into quartiles, (D) EdU-positive and EdU-negative cells within each quartile were separated.

Average Proportion of EdU-positive Cells within Each EpCAM-positive Quartile

	Young, Control	Young, Tam	Old, Control	Old, Tam
EpCAM-High	13.36%	13.80%	13.83%	15.81%
EpCAM-Med-High	14.40%	10.96%	14.48%	13.35%
EpCAM-Med-Low	22.38%	10.13%*	18.48%	11.08%
EpCAM-Low	27.90%	9%**	19.88	8.27%***

Table 1. The average percentages of the EdU-labeled cells within each EpCAM-positive quartile of young and old MMTV-c-*neu* mouse mammary organoids treated with either control or tamoxifen. Asterisks denote cell quartiles in tamoxifen-treated organoids that were significantly lower than their control counterparts. In young mouse organoids, the EpCAM-med-low and EpCAM-low quartiles showed a significant reduction in EdU-labeling with tamoxifen-treatment (paired t-test, p-values = 0.018 and 0.004 respectively). In old mouse organoids, the EpCAM-Low quartile showed significantly reduced EdU-labeling with tamoxifen-treatment (paired t-test, p-value = 0.16)

Proportion of EdU-positive Cells within each EpCAM-positive Quartile

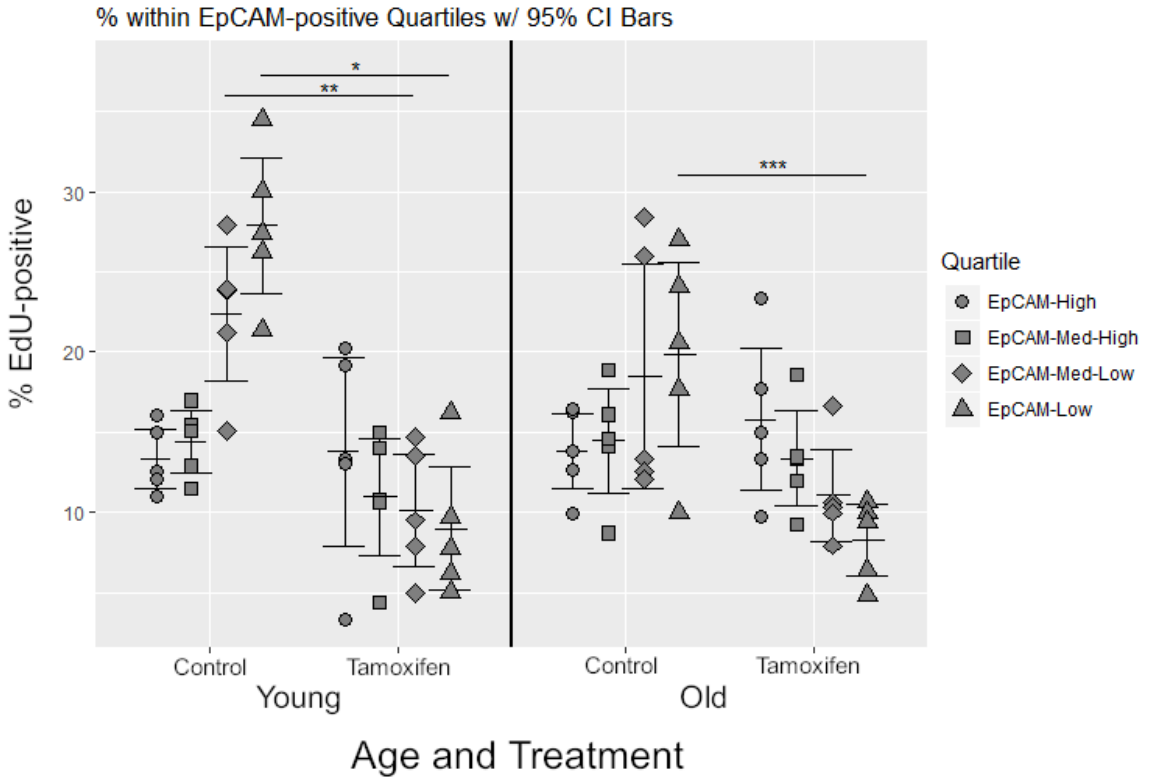

Figure 13. Tamoxifen-treatment significantly reduced EdU-labeling in the EpCAM-low quartiles, which is a basal cell-enriched cell fraction, in organoids from young and old mice. The percentage of EdU-positive cells among the EpCAM-positive fractions of mammary organoids from young and old MMTV-*c-neu* mice were measured. The percentage of EdU-positive cells within the EpCAM-med-low fraction was significantly lower in tamoxifen-treated organoids from young mice compared to their control organoids (paired t-test, *p = 0.018). The percentage of EdU-positive cells within the EpCAM-low quartile was significantly lower in both young and old mouse organoids treated with tamoxifen compared to their control organoids (paired t-test, **p = 0.004, ***p = 0.016).

Proportion of each EpCAM Quartile within EdU-positive Cell Population

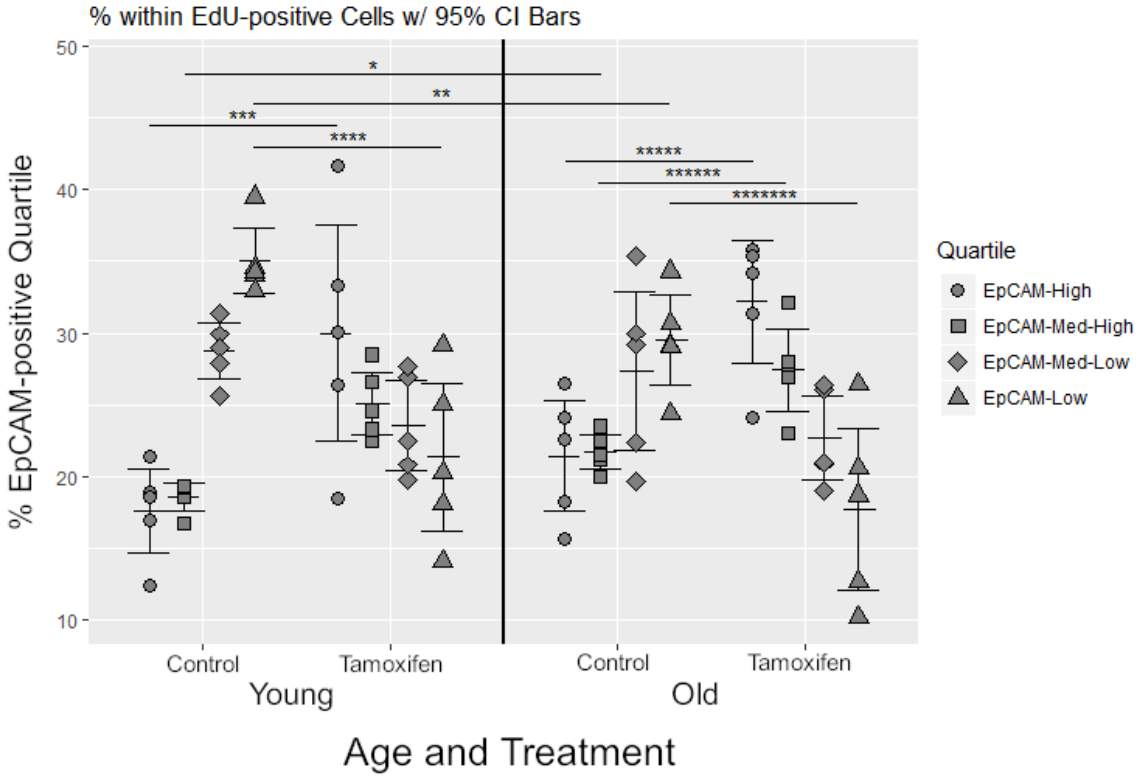

Figure 14. Tamoxifen-treatment altered the proportions of each cell population among the EdU-positive cell fraction. Tamoxifen increased the proportion of EpCAM-high, luminal cells among the dividing cells and decreased the proportion EpCAM-low, basal cells. Among the mammary organoids from young mice, the percentages of EpCAM-high and EpCAM-low cells among the dividing, EpCAM-positive/EdU-positive, population of tamoxifen-treated organoids were significantly different compared to the percentages in the control-treated organoids; the percentage of EpCAM-high cells increased while the percentage of EpCAM-low cells decreased (paired t-test, ***p = 0.043, ****p = 0.01, respectively). Among the mammary organoids from old mice, the percentages of EpCAM-high, EpCAM-med-high, and EpCAM-low cells among the dividing, EpCAM-positive/EdU-positive, population of tamoxifen-treated organoids were significantly different compared to the percentages in the control-treated organoids; the percentage of EpCAM-high and med-high cells increased while the percentage of EpCAM-low cells decreased (paired t-test, *****p=0.003,******p=0.007, *******p = 0.03, respectively). Between the young and old mice, there were significant differences in the proportions of EpCAM-med-high and EpCAM-low cells among the EpCAM-positive/EdU-positive among the cells from control-treated organoids (Wilcoxon rank sum test, *p = 0.008; Welch's t-test, **p = 0.025).

2D Colony-forming Assays with Sorted EpCAM-high Cells

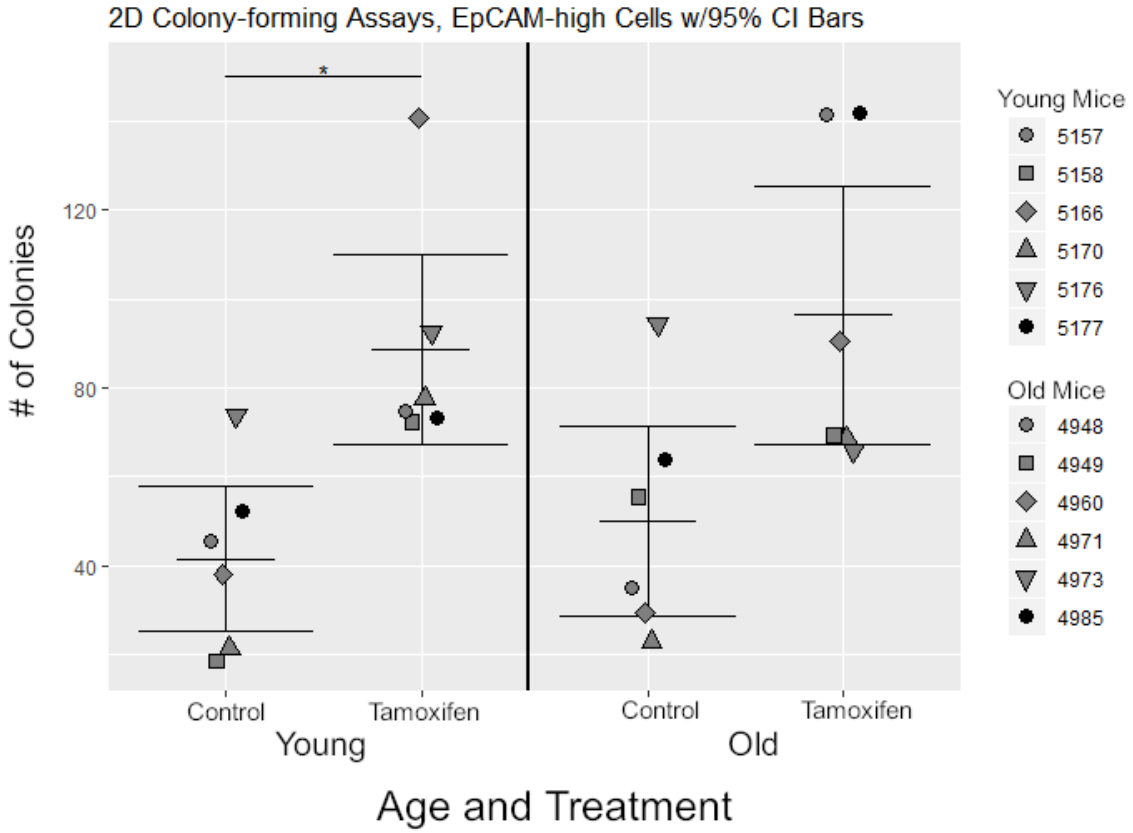

Figure 15. The number of colonies formed from sorted EpCAM-high cells was significantly higher among cells taken from tamoxifen-treated organoids than cells from control organoids. The number of colonies formed from cells from tamoxifen-treated mouse mammary organoids from young mice was significantly higher compared to the number of colonies formed from control-treated organoids (Wilcoxon signed rank test, $p = 0.03125$). The difference between the number of colonies formed from cells from tamoxifen and control-treated mouse mammary organoids from old mice only approached significance (Wilcoxon signed rank test, $p = 0.09375$).

2D Colony-forming Assays with Sorted EpCAM-low Cells

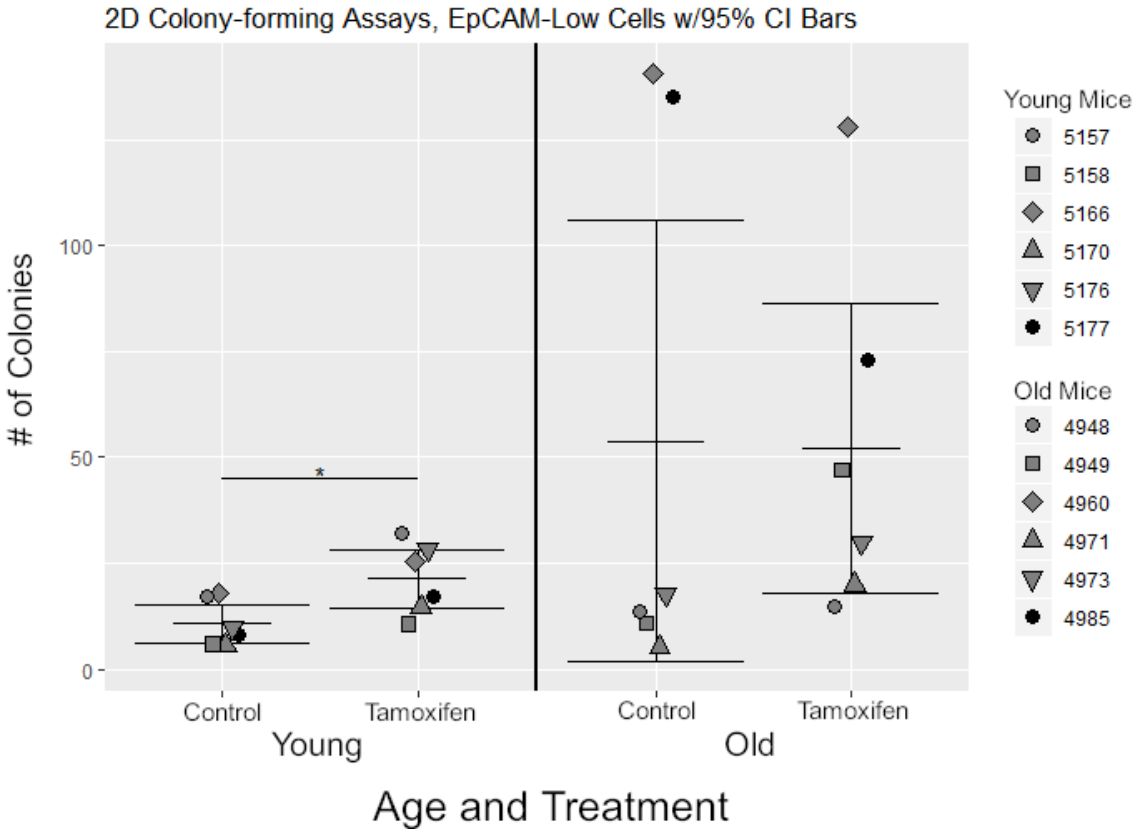

Figure 16. The number of colonies formed from EpCAM-low cells increased with tamoxifen-treatment among cells from young, but not old, mouse mammary organoids. The number of colonies formed from cells from tamoxifen-treated mouse mammary organoids from young mice was significantly higher compared to the number of colonies formed from control organoid cells (paired t-test, *p = 0.004).

Representative Images of Hollow and Solid 3D Colonies

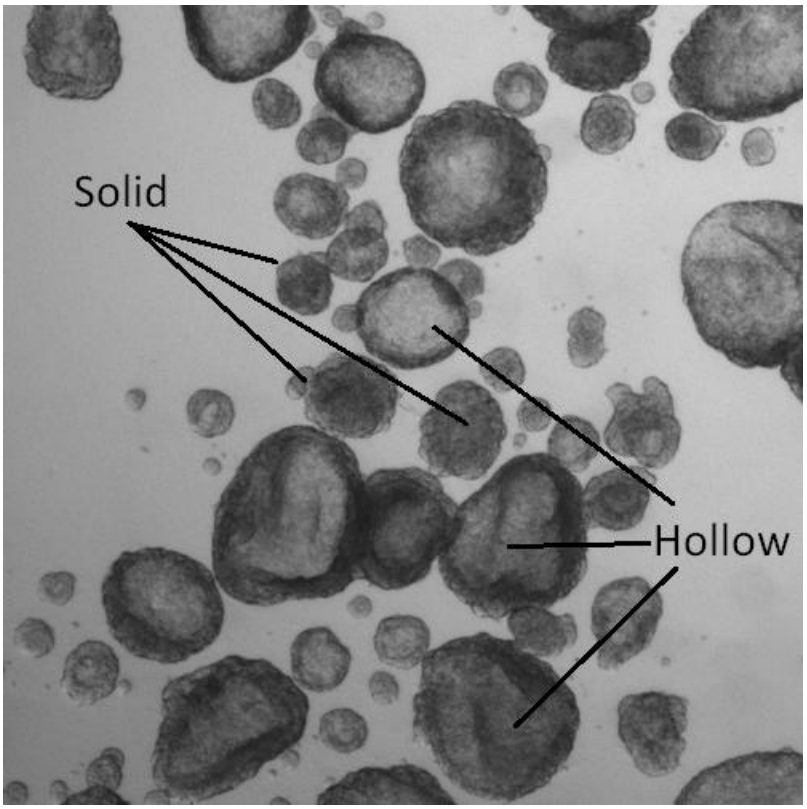

Figure 17. Representative picture of hollow and solid colonies formed in non-adherent (3D) culture conditions. Hollow colonies represent colonies derived from luminal progenitor cells. Solid colonies represent colonies derived from basal progenitor cells.

3D Colony-forming Assays Colony Counts

		Colony Type and Treatment					
		All		Hollow		Solid	
Cell Fraction	Age	Control	Tam	Control	Tam	Control	Tam
Epithelial	Young	16.6	71.7	5.6	39.4	11	32.3
	Old	31.3	47	13.4	26*	17.8	21
Luminal	Young	38.2	53.2	21.1	37.1	17.1	16.1
	Old	39.3	86.4***	22.7	54.5***	16.6	31.9
Basal	Young	18.6	35.8	3.4	7.6	15.2	28.2
	Old	27.3	34.5	12.1	6.8	15.3	27.7

Table 2. Tamoxifen increased the number of hollow, luminal-like colonies formed from old mouse organoid cells. Tamoxifen caused statistically significant increases in the average number of hollow colonies formed by the epithelial and luminal cell population of old mouse mammary organoids (paired t-test, p-values = 0.016* and 0.032*** respectively). When all colonies were counted together, the luminal population of old mouse organoids treated with tamoxifen produced more colonies that the luminal population from control organoids (paired t-test, p-value = 0.035).

3D Colony-forming Assays with Young Mammary Organoid Cells

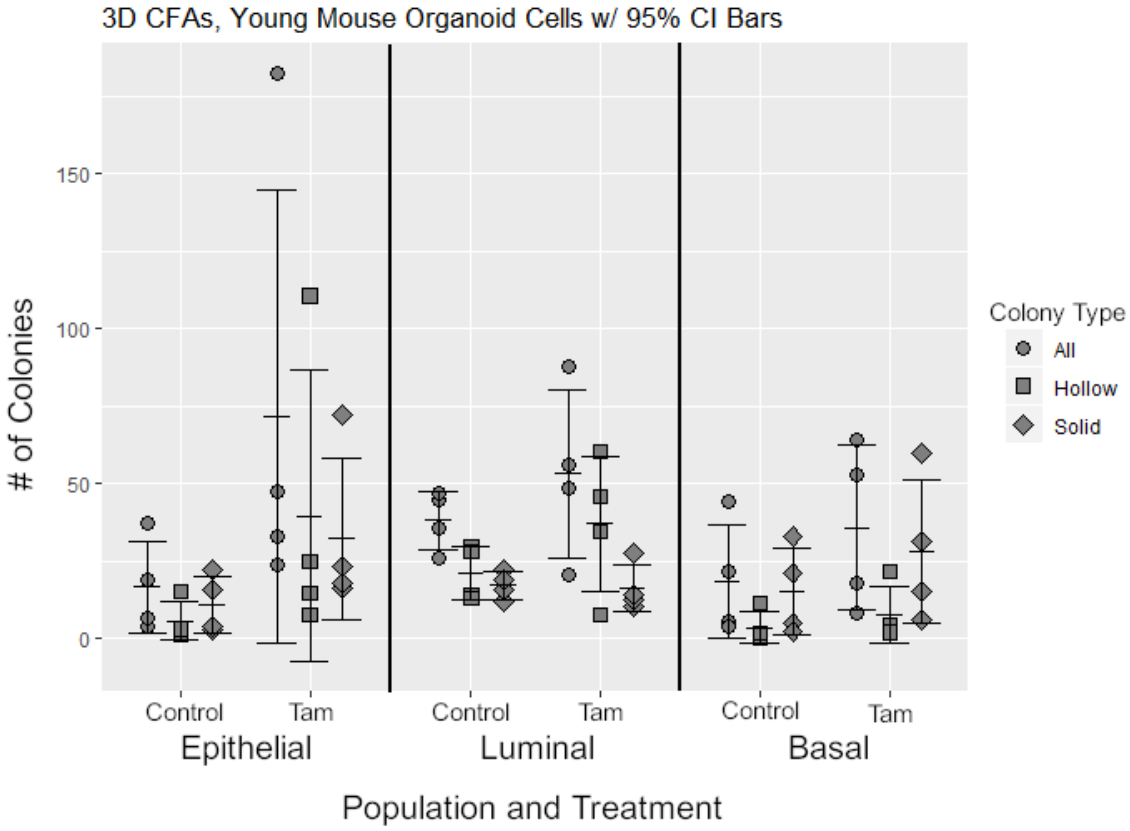

Figure 18. Tamoxifen did not cause any statistically significant changes in the number of colonies formed in 3D, of either hollow or solid morphology, that formed from any cell population in young mouse mammary organoids.

3D Colony-forming Assays with Old Mammary Organoid Cells

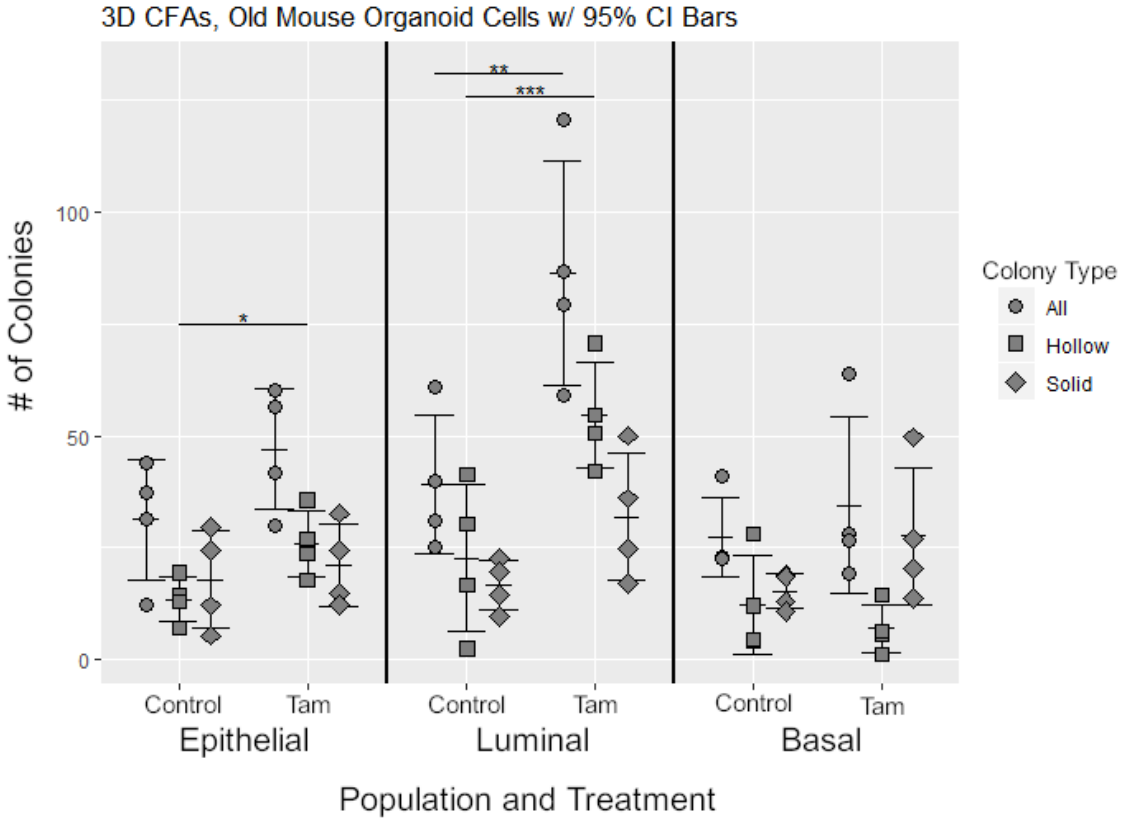

Figure 19. Tamoxifen caused significant changes in the number of hollow colonies that formed from the epithelial and luminal cell populations of old mouse mammary organoids in 3D cultures. In wells seeded with EpCAM-positive (epithelial) cells, the number of hollow colonies was significantly higher in wells with cells from tamoxifen-treated organoids than in wells with cells from control organoids (paired t-test, *p = 0.035). The total number of colonies present in wells seeded with EpCAM-high/CD49f-low (luminal) cells from tamoxifen-treated organoids was significantly higher than in wells with luminal cells from control organoids (paired t-test, **p = 0.0155). The number of hollow colonies present in wells seeded with luminal cells from tamoxifen-treated organoids was significantly higher than in wells with luminal cells from control-treated organoids (paired t-test, ***p = 0.032).

Proportion of Apoptotic Cells within Mammary Organoids

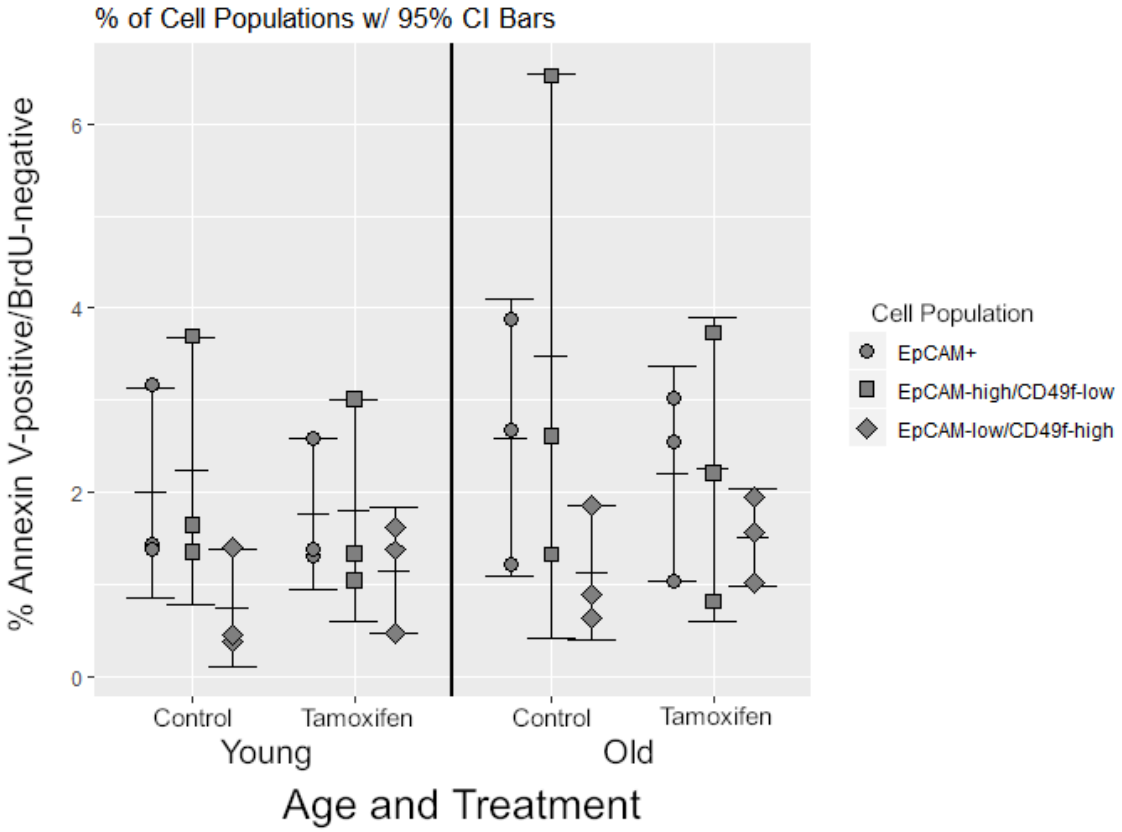

Figure 20. The percentage of Annexin V-positive/BrdU-negative, apoptotic cells within the EpCAM-positive (epithelial), EpCAM-high/CD49f-low (luminal), or EpCAM-low/CD49f-high (basal) fractions was not significantly changed by tamoxifen treatment in either young or old mouse mammary organoids.

RT-qPCR of *Esr1* Transcription

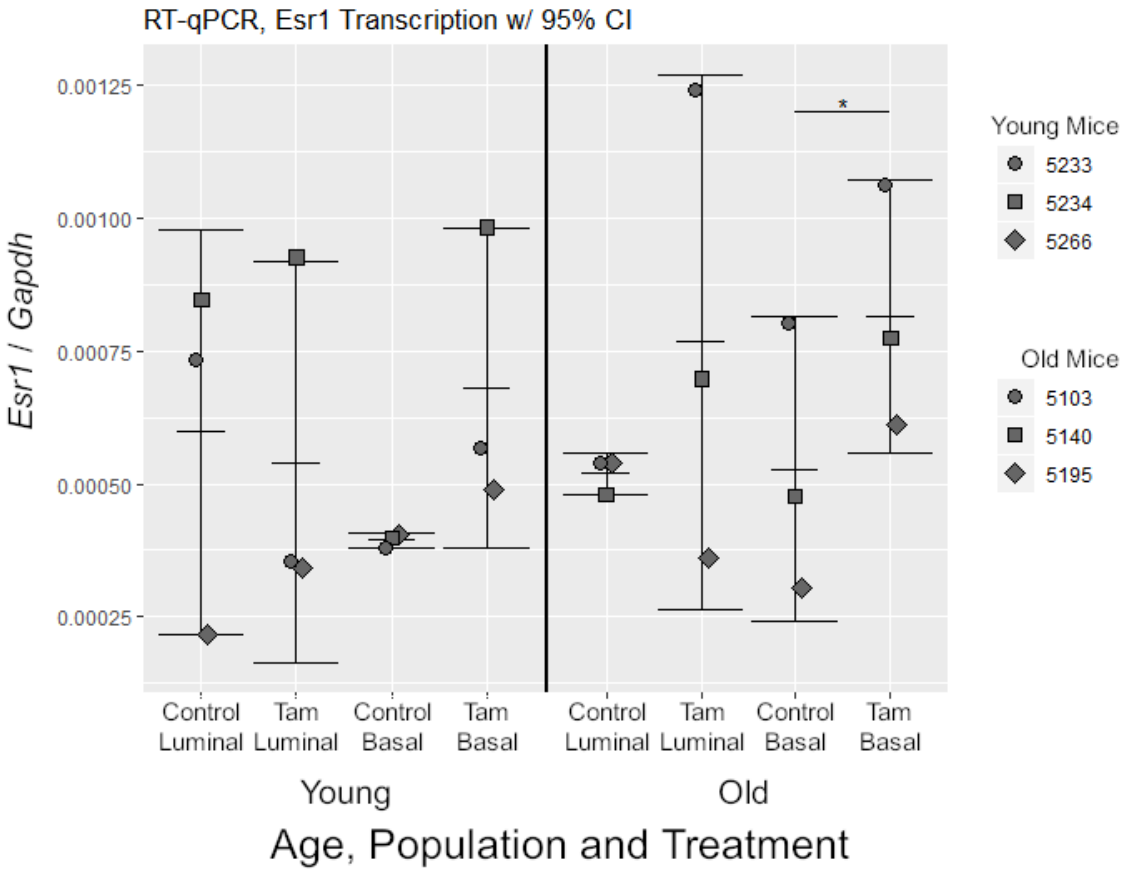

Figure 21. A significant increase in the transcription of *Esr1*, relative to *Gapdh* transcription, was only observed in the basal population of old mouse mammary organoids following tamoxifen-treatment. The transcription of *Esr1* among basal cells from old mouse organoids was significantly higher in cells from tamoxifen-treated organoids than in cells from control organoids (paired t-test, *p = 0.003). *Esr1* transcription in the luminal cell population of old mouse organoids or the luminal and basal populations of young mouse organoids was unchanged by tamoxifen.

RT-qPCR of *Cdkn1a* Transcription

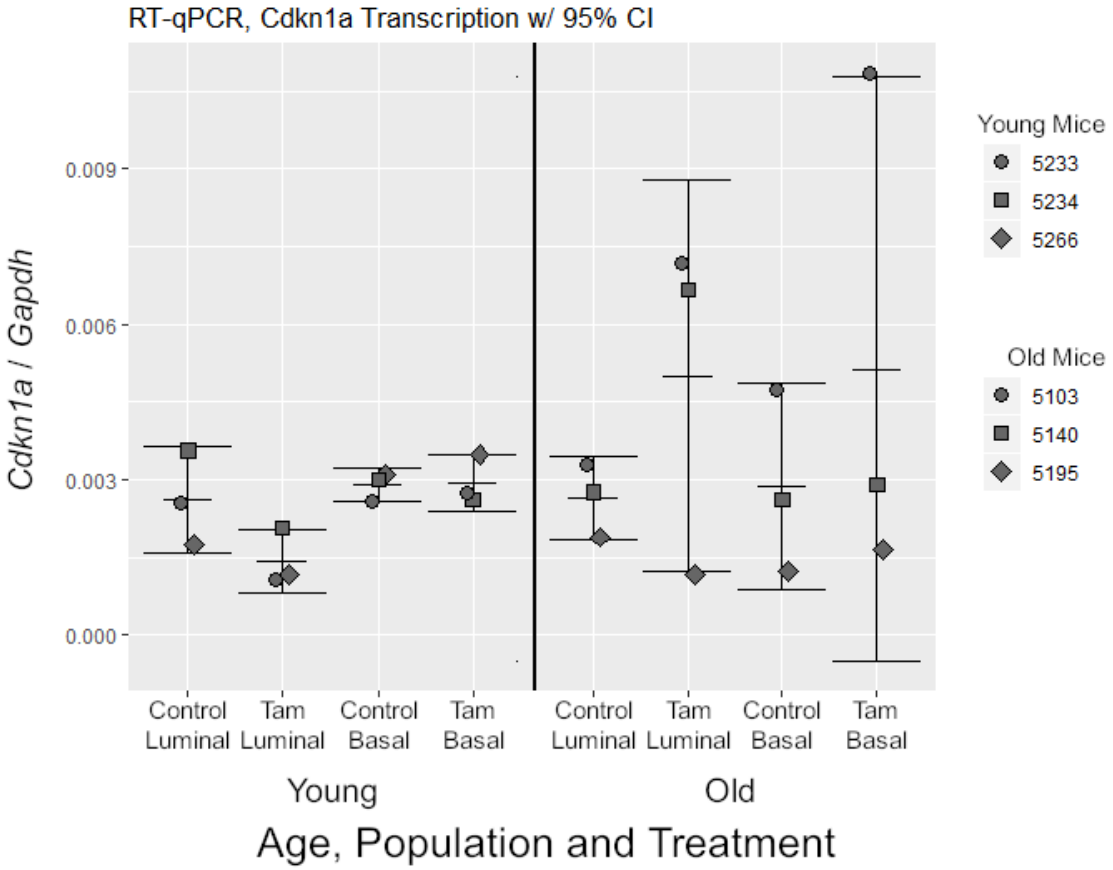

Figure 22. The transcription of *Cdkn1a* relative to *Gapdh* transcription in the luminal and basal fractions of young and old mouse mammary organoids was not significantly different between cells from control and tamoxifen-treated organoids. However, the difference in the transcription of *Cdkn1a* in the luminal cells from young mouse organoids approached significance between cells from tamoxifen-treated organoids those from control-treated organoids (paired t-test, p = 0.057).

RT-qPCR of *Bax* Transcription

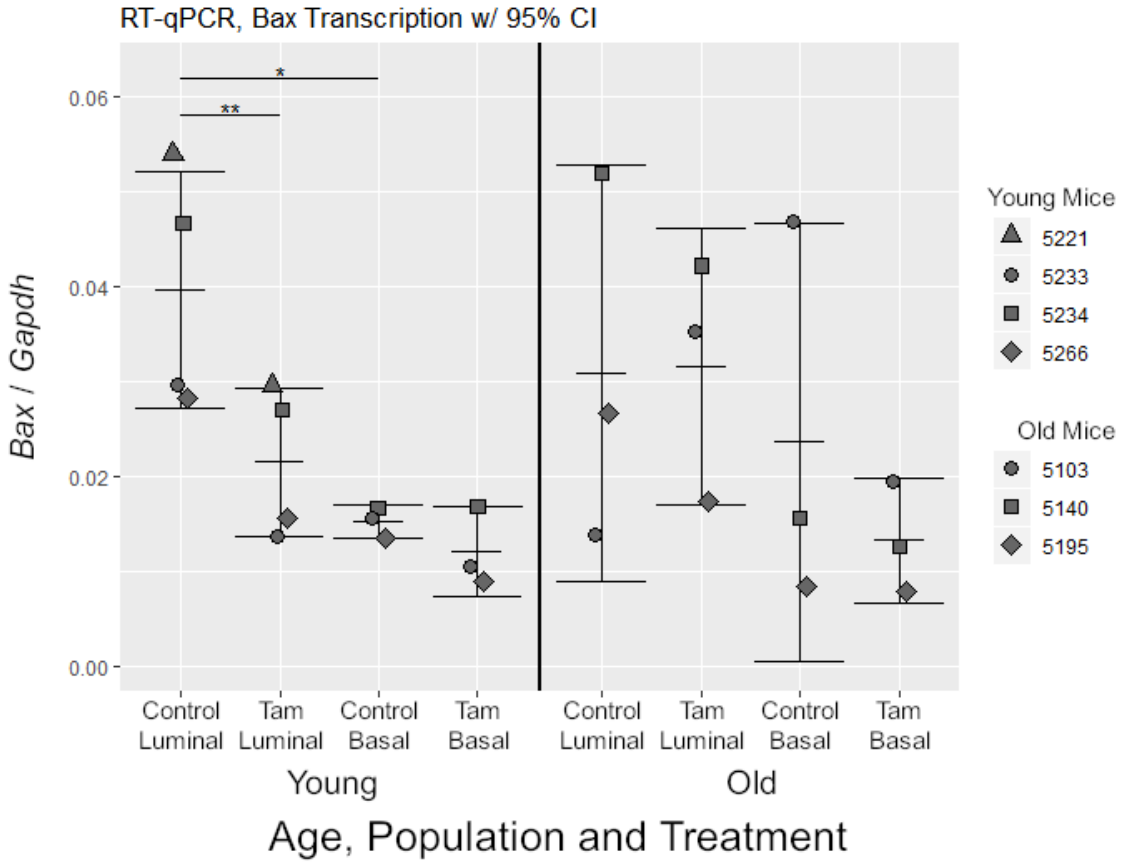

Figure 23. Tamoxifen significantly reduced *Bax* transcription in the luminal cell populations of young mouse mammary organoids but not in old mouse organoids. The transcription of *Bax* among luminal cells from young mouse organoids was significantly lower in cells from tamoxifen-treated organoids than in cells from control organoids (paired t-test, **p = 0.006). The transcription of *Bax* between luminal and basal cells from control, young mouse mammary organoids was significant different (Welch's t-test, *p = 0.03).

RT-qPCR of *Bcl2* Transcription

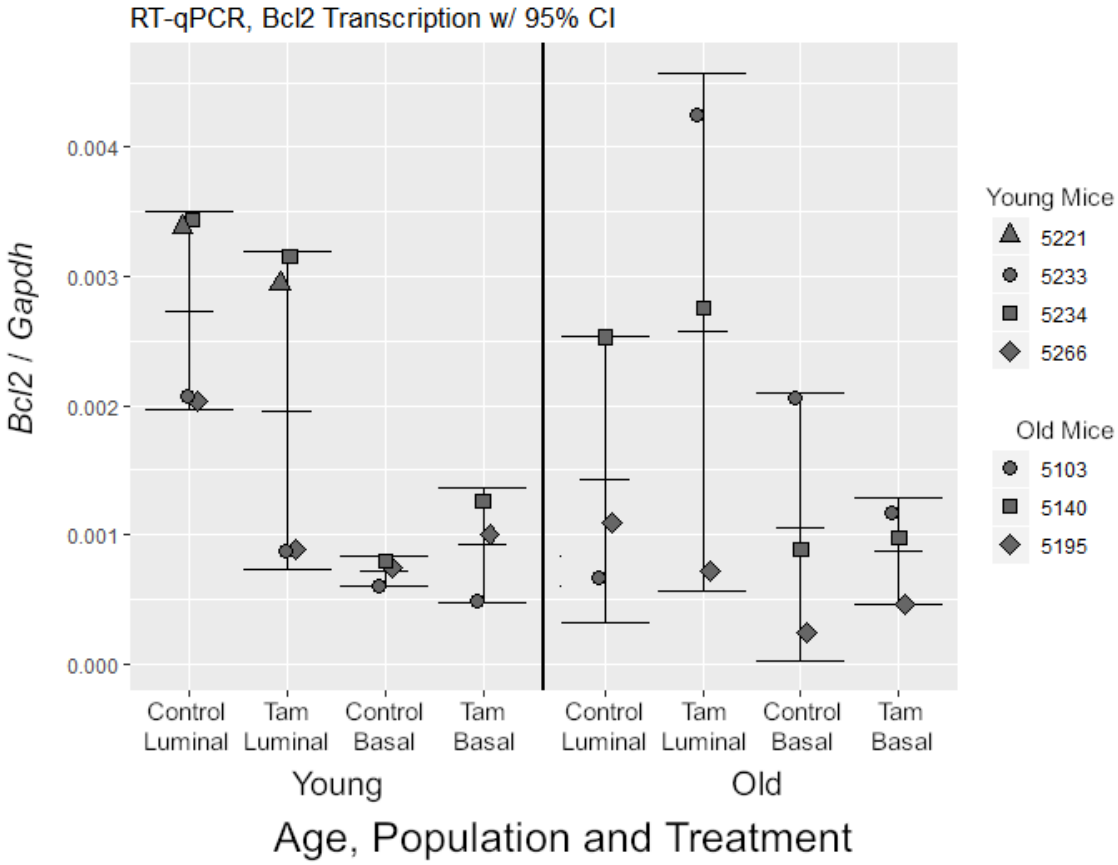

Figure 24. No significant changes were observed in *Bcl2* transcription between cell populations from control and tamoxifen-treated organoids from either young or old mice. However, the difference in the transcription of *Bcl2* between luminal and basal cells from control-treated young mouse organoids approached significance (paired t-test, p = 0.057).

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