DISSERTATION

PROGRESS TOWARDS AN UNDERSTANDING OF RADIATION-INDUCED MAMMARY CANCER USING A MURINE MODEL

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY REBEKAH HENDERSON KLINGLER ENTITLED PROGRESS TOWARDS AN UNDERSTANDING OF RADIATION-INDUCED MAMMARY CANCER USING A MURINE MODEL BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PROGRESS TOWARDS AN UNDERSTANDING OF RADIATION-INDUCED MAMMARY CANCER USING A MURINE MODEL

Theoretically, any exposure to ionizing radiation (IR) results in an increased risk of developing breast cancer. We have used a mouse model of radiation-induced breast cancer to study the effects of genetic background and molecular mechanisms of carcinogenesis. The BALB/c mouse strain is susceptible to radiation-induced mammary cancer while other laboratory strains are not. In this dissertation telomere-specific FISH was used to show that mammary epithelial cells derived from BALB/cByJ mice develop significantly more telomere-DSB fusions after IR exposure compared to those derived from C57BL/6J mice. The kinetics of telomere instability follow the same kinetics as the strain-specific genomic instability observed in earlier studies.

An experimental system involving transplantation of cells from the breast of one mouse and regenerated in another mouse has been used extensively to demonstrate the genetic susceptibility of the BALB/c mouse to radiation-induced mammary cancer. The numbers of cells necessary for successful transplantation suggests that the cell capable of regenerating a mammary gland is a rare cell, perhaps a pluripotent stem cell. In this dissertation detailed protocols were created for isolation and tissue culture of murine mammary stem cells as mammospheres grown at high density, clonal density, and grown in basement membrane extract. A great deal of size variation was found in each culture of mammospheres. To test the hypothesis that only large mammospheres contained true stem cells the self-renewal capacity of specific sizes of mammospheres was tested using serial passaging. The data suggest that cells derived from larger mammospheres are capable of

more passages than small mammospheres. Additionally, mammospheres were dissociated and tested for the presence of multiple cell lineages, as expected for pluripotent cells. Finally, we developed an assay to assess the radiation response of mammospheres derived from five strains of inbred mouse related to the BALB/c model of radiation-induced mammary cancer. These data show that mammary stem cells are more resistant to the killing effects of IR than fibroblasts derived from the same strains of mice. The data also show varying radiation sensitivities between genetically distinct mouse strains.

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All of my friends, family and loved ones

DEDICATION

This dissertation is dedicated to my amazing husband Michael Henderson for his unwavering love and support over the last six years regardless of how crazy I acted.

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Introduction

The earliest documented cases of breast cancer are found in the "Edwin Smith Papyrus" an Ancient Egyptian textbook on trauma surgery which dates back to 1600 B.C. [1]. Currently, breast cancer is the most commonly diagnosed invasive cancer among women in the United States [2]. Though the incidence of female breast cancer is decreasing, approximately one in eight women will still develop invasive breast cancer at some point in their lifetime [3]. Advances in clinical diagnostic techniques over the last 20 years have allowed for earlier detection and enhanced treatment of breast cancer resulting in a steady decline in mortality rates. However, even with these advances one out of every 35 women in America will die from breast cancer [3]. Advances in diagnostics and treatment modalities are vital for helping the people currently combating cancer, but even more important are advances in our understanding of how and why cancers develop; only these answers can lead to preventative measures.

Though breast cancer has been recognized as a clinical disease for hundreds of years, the underlying reasons why certain individuals develop the disease remains unclear. In the search for a cause, many risk factors have been identified; these include attained age, geographic location, early age at menarche, low parity (number of live births), late age at first birth, late age at menopause, obesity, height, mammographic density, bone density, diet and

alcohol consumption [4-11]. Exposures to exogenous estrogens, chemical carcinogens and ionizing radiation (IR) can also increase an individual's risk of developing breast cancer [12]. The risk conferred by each of these factors is still hotly debated and seems to be highly individualized. It has been suggested that this individualized susceptibility stems from the unique genetic background that each of us possesses [13].

1.1 GENETIC PREDISPOSITION TO BREAST CANCER:

Most of what we understand about the pathogenesis of breast cancer comes from studies of hereditary breast cancers. Hereditary breast cancers often present early in life and are frequently multifocal or bilateral, while sporadic cancers are usually unilateral and appear at a more advanced age [14]. Pedigree analysis of families with a high incidence of breast cancer reveals that women who have one first-degree relative with breast cancer are twice as likely to develop breast cancer themselves as the general population [15]. Additionally, monozygotic twins of breast cancer patients have a higher rate of breast cancer then dizygotic twins or other siblings; this strongly suggests that in some cases, inherited genetic factors are more important than lifestyle factors or environmental exposures [15, 16].

Genetic analysis of familial breast cancer clusters has lead to discovery of the BRCA1 and BRCA2 "breast cancer susceptibility genes." A "breast cancer susceptibility gene" is a genetic mutation that increases an individual's susceptibility or predisposition for developing breast cancer, the mutation is found throughout the body, not only in the tumor [17]. Carriers of mutations in either BRCA1 or BRCA2 have a 45-87% chance of developing breast cancer during their life [18]. Mutations in TP53 and PTEN have also been identified in familial breast cancers [19, 20]. Mutations of this kind, which impart very high risk of developing disease, are considered "high penetrance" gene mutations or alleles (versions of a

gene) [18, 21]. High penetrance alleles are rare in the population, but confer very high risk to the carriers, regardless of lifestyle factors or environmental exposures [21].

Recently many linkage studies have been done in search of other high risk breast cancer alleles, but these studies suggest that additional high penetrance alleles are unlikely [22]. This implies that much of the familial aggregation of cancer is due to more common moderate risk alleles [22]. Moderate risk alleles are said to confer incomplete penetrance, meaning that some carriers will develop breast cancer while others will not. A few of these moderate risk alleles have been found in genes like ATM, NBS1, CHEK2, BRIP1, LKBI, PALB2, CASP8, and TP53 [4, 21-23]. Moderate or incomplete penetrance alleles are more common in the population than high penetrance alleles, but the risk associated with these alleles is lower and can be modified by lifestyle factors and environmental exposures. Although high and moderate penetrance alleles associated with familial breast cancers confer a high degree of risk, familial breast cancer only accounts for 5-10% of all breast cancer cases [3]. The remaining ~90% of breast cancer cases are considered "sporadic".

Even in carriers of high penetrance alleles we find a large variation in penetrance, latency and the clinical course of breast cancer. It has been proposed that these variations are caused by the specific genetic background of the carrier [13, 20, 24]. The term genetic background refers to the "genetic make-up" or the specific combination of alleles at all loci across the genome, with each of us carrying many mutated genes throughout our genomes. It is obviously not feasible to research the effects of all possible mutations at all possible loci in the genome; however, some of these mutations are relatively common in the general population. Single Nucleotide Polymorphisms (SNPs) are, by definition, mutations which are seen in at least 1% of the study population [13, 24]. Determining the effect of these SNPs is complicated by the fact that more than nine million SNPs have been identified in the human genome, and that the majority of SNPs have no effect on protein expression or

function [25, 26]. The risk conferred by any given genetic mutation depends on the location of the mutation within the gene, the specific type of mutation, the effect of the mutation on protein function, and the presence of other mutations or alleles in the genome which can modify risk [18]. It has been suggested that each functional polymorphism involved in breast cancer risk has a very small impact on its own, or low penetrance, and that it is the combined effect of all the low penetrance alleles in an individual's genome that determines their susceptibility to breast cancer and their response to therapy [13, 24].

Though many genetic association studies have been done over the last decade in search of low penetrance alleles that alter susceptibility to breast cancer, they have been largely unsuccessful [26, 27]. These studies basically fall into two categories; one is genetic screening of breast cancer patients for common genetic variants in potential breast cancer susceptibility genes. The second looks at specific variant alleles or SNPs which have known functional consequences and screens breast cancer patients for those mutations [15]. These studies have had little success most likely due to the diverse nature of breast cancer and the genetic variability of the women in the study populations [27]. In addition, all of these studies are very limited in the number of genes or mutations they can screen. Breast cancers can vary in morphology, clinical behavior, and molecular alterations, etc. but it is treated as a single disease. It is possible that subcategories of breast cancer may have common underlying genetic susceptibility factors but this has yet to be explored in any detail.

The search for candidate low penetrance breast cancer susceptibility alleles are also limited in scope by what is currently known about carcinogenesis. For instance, we know that one of the defining features of cancer is abnormal growth, thus, many studies have focused on genes known to be involved in cell cycle control, proliferation and differentiation including PTEN, TP53, MTHFR [4], CHEK2 [28], FGFR2 [29], CDKN2A [30], IGFBP3, CASP8, TGFβ1, and PGR [15]. Proteins involved in metabolic pathways are important to

the study of carcinogenesis because of the carcinogenic effects of many compounds that we are exposed to on a daily basis. So, some studies have looked for correlations between breast cancer risk and the genes known to be involved in metabolism including NQO1 [31], SOD2 [15], CYP1A1, CYP2D6, CYP19, GSTP1, ADH, and LKB1 [4]. Most of these studies have not shown significant correlations between the allele analyzed and breast cancer risk. One of the major problems with epidemiological studies like these is that when looking for the effect of a single low penetrance allele, it is usually associated with a very small amount of increased risk on its own, and modified by exposure to environmental toxins, lifestyle, and the genetic background of the individual. It is extremely difficult to identify these small increases in risk with broad epidemiological studies.

One area of research that has had a bit more success has looked for low penetrance alleles in genes involved in the DNA repair pathways. Our DNA is subject to damage all the time from both endogenous sources like products of normal metabolism (ROS, etc) and exogenous sources like UV, IR and chemical toxins [32]. If this damage goes unrepaired, mutations result. Thus, mammals have developed five different DNA repair pathways to deal with the constant insults. Nucleotide excision repair recognizes and repairs bulky DNA lesions including pyrimidine dimers [33]. Base excision repair corrects DNA damage that does not distort the helical structure of the DNA, like oxidized bases or incorporation of uracil [33]. Mismatch repair corrects mismatched base pairs after DNA replication by being able to discriminate between new and old strands of DNA [33]. DNA double stranded breaks (DSBs) are repaired by either homologous recombination (HR) or non-homologous end-joining (NHEJ). HR is used in post-replication phases of the cell cycle and uses the DNA sequence from the sister chromatid to repair the break, usually without any DNA sequence loss. DNA DSBs are also repaired via the error prone NHEJ pathway, which ligates broken

DNA ends together after processing the ends with nucleases. This results in alteration of the DNA sequence.

More than 70 human genes have been found to be directly involved in DNA repair pathways [14]. Severe mutations in these DNA repair pathways are associated with clinical diseases. For example defects in nucleotide excision repair results in Xeroderma pigmentosa [26]. Defective mismatch repair can cause hereditary non-polyposis colorectal cancer [26]. Defects in the HR proteins NBS1 and ATM cause Nijmegen Breakage Syndrome and Ataxia-telangiectasia respectively [4]. The mutations that result in these clinical diseases are usually large deletions or severe mutations that cause drastic changes in protein function. It has been hypothesized that minor genetic variants and SNPs in DNA repair genes result in low penetrance alleles, which are not serious enough to cause severe clinical diseases but can alter susceptibility to cancer [7, 34]. Many studies have searched for low penetrance breast cancer susceptibility alleles within the DNA repair pathways. Studies looking at polymorphisms in genes involved in nucleotide excision repair have found a few alleles that increase breast cancer susceptibility of carriers up to 2.6 fold, particularly when the individuals are exposed to environmental carcinogens like polycyclic aromatic hydrocarbons, cigarette smoke, or exogenous estrogen [4, 35-37]. A few polymorphisms in genes associated with base excision repair have also been shown to increase risk of breast cancer, especially in African-American women or women who have had severe adverse reactions to radiation therapy [4, 38, 39]. Mutations in genes involved in HR have obviously been linked to breast cancer susceptibility, as the high or moderate penetrance alleles in BRCA1, BRCA2, ATM and NBS1 are all associated with familial breast cancers, as of yet data on low penetrance alleles of these genes is very thin [4, 40]. Finally, polymorphisms in the NHEJ proteins Ku70 and XRCC4 have been associated with increased risk of breast cancer [41]. It

is hypothesized that a reduction in the DNA repair capacity can result in increased risk for developing cancer, as many mutations are required for carcinogenesis.

1.2 MECHANISMS OF CARCINOGENESIS:

Though the specific cellular and molecular mechanisms remain unclear, it is commonly accepted that tumorigenesis is a multi-step process that involves accumulation of genetic alterations over time leading from a normal cell to malignancy [42]. It has been suggested that there are six characteristics that any cell must obtain in order to become malignant, these are limitless replicative potential, the ability to evade apoptosis, growth signal self-sufficiency, insensitivity to anti-growth signals, sustained angiogenesis, and tissue invasion and metastasis [42]. The number of mutations required for transformation seems to vary from three to 12 mutations depending on the form of cancer being studied and the type of cell being transformed [43]. Though scientists disagree on the mechanisms involved, it is commonly held that carcinogenesis is an evolutionary process combining genetic change and clonal selection [44]. There are currently two prevailing theories as to origin of tumors, the "classical model" of carcinogenesis and the "cancer stem cell hypothesis." In the "classical model" of carcinogenesis a stochastic event causes an irreversible genetic mutation in a terminally differentiated cell. This mutation is considered an initiating event which puts the cell on the path towards cancer. In the "classical model" all cells are equally susceptible to transformation [19]. In 1976, Peter Nowell suggested that cancer was a result of the clonal evolution of a single cell which acquired genomic instability, and that the instability made it possible for the cell lineage to gain more and more mutations, subject to selective pressures, and eventually became neoplastic [43, 45]. In the "classical model" of carcinogenesis all of the cells within a fully developed tumor are equally malignant [19].

Competing with the "classical model" of carcinogenesis is the "cancer stem cell hypothesis." In the "cancer stem cell hypothesis" the cancer-initiating event takes place in a tissue stem cell as opposed to a differentiated cell [19]. The accumulation of mutations via clonal evolution still occurs, however it is believed that many fewer mutations are necessary to transform a stem cell because they already possess many of the features found in tumor cells [46]. Not only are the tumor-initiating cells different in these two models, the pathology of the fully developed tumor is different as well. In the "cancer stem cell hypothesis" the mature tumor is primarily made up of differentiated cells which are not tumorigenic themselves. In addition there is a small subset of cells that are fully tumorigenic; these are called the "cancer stem cells." The "cancer stem cells" are responsible for the cellular heterogeneity of the tumor, because they possess multi-lineage differentiation potential. The "cancer stem cells" are also responsible for tumor progression, metastasis, resistance to cancer treatments, and subsequently tumor recurrence [47, 48].

It is very likely that both the "classical model" of carcinogenesis and the "cancer stem cell hypothesis" are viable mechanisms for human carcinogenesis. It is probable that if we learn to identify the mechanism involved in the carcinogenesis of individual tumors, we will be able to tailor treatment modalities to fit the mechanism, target the tumor-initiating cell, and more effectively treat breast cancers [17].

1.3 "CANCER STEM CELL HYPOTHESIS" AND BREAST CANCER:

Tissue stem cells are found in fully developed organisms. They have the ability to self-renew and the capacity to differentiate into all of the specific cell types found in that particular tissue [49]. In tissues where stem cells have been identified, there is a specific stem cell to differentiated cell hierarchy, the stem cell produces undifferentiated progenitor cells which create the lineage specific progenitors or "transiently amplifying population"

capable of extensive proliferation, in response to specific growth signals. The lineage specific progenitor cells produce the terminally differentiated cells of the tissue, which are usually incapable of proliferation [49]. The key feature of a stem cell is its ability to self-renew, or divide and produce another stem cell with the exact same proliferative and differentiation potential as the parental stem cell [49]. The number of stem cells in a given tissue is usually carefully regulated [50]. Self-renewal via asymmetric division results in one daughter cell that remains a stem cell and a second daughter that becomes a committed progenitor cell. This type of cell division maintains the stem cell number in the tissue [51]. Self-renewal can also occur by symmetrical division, in which both daughter cells are stem cells; this increases the total number of stem cells within the tissue [51]. It has been hypothesized that in cancers involving the "cancer stem cell hypothesis" mechanism of carcinogenesis, the initiating mutation results in disruption of this normally tightly regulated stem cell self-renewal pathway [51].

The mammary gland is an extremely dynamic tissue. It must undergo changes during pregnancy, lactation and involution with each pregnancy [52]. In addition to the vast restructuring involved in pregnancy, the non-pregnant mammary gland is not truly "resting" either, it undergoes cycles of proliferation and apoptosis with each menstrual cycle [53]. Understanding of the dynamic nature of the mammary gland has led to the suggestion that mammary stem cells are responsible for directing the changes and proliferation events that occur regularly in the mammary gland. The dynamic nature of the mammary gland has also led to the hypothesis that in many breast cancers the tumor-initiating cell is a mammary stem cell. Breast cancer is an incredibly diverse disease in terms of pathological features, clinical course and responsiveness to treatments [54]. It has been suggested that this diversity is explained by the "cancer stem cell hypothesis" mechanism of carcinogenesis.

The most compelling data supporting the role of the stem cell as the tumor-initiating cell in many breast cancers are the effects of pregnancy on breast cancer risk. It has been shown that women who complete their first pregnancy before 20 years old have half the risk of developing breast cancer, and women completing their first pregnancy after the age of 35 have increased risk, when compared to women who have never been pregnant [4, 55, 56]. The protective effect caused by early pregnancy is seen universally among women with different ethnic backgrounds and from different geographical locations, which suggests that the effect is not due to environmental or socioeconomic factors but from biological changes in the breast tissue caused by pregnancy [56]. One caveat to this protection is that for the first 5- 7 years after giving birth women are actually at higher risk of developing breast cancer [4]. This has been associated with the extreme exposure to estrogens during pregnancy and it has been suggested that if there are cells that are already initiated for tumorigenesis, the exposure to estrogens and the huge amount of proliferation required for pregnancy can result in progression of the initiated tumor cells, resulting in increased risk immediately following pregnancy [4]. It is hypothesized that the protection given by pregnancy is due to permanent differentiation of the stem cells within the breast, which reduces their susceptibility to the tumor promoting effects of estrogen exposure [7].

Estrogen is known to cause proliferation of mammary cells. Proliferation is the key factor in tumor promotion as cells that do not divide are not a threat in terms of tumor development. In addition to the effects of estrogen exposure to parous women, there are many other risk factors that have been associated with breast cancer, that are directly related to endogenous or exogenous estrogen exposure. Women born to mothers who had unusually low estrogen levels (toxemia) have reduced risk for developing breast cancer [6]. On the other hand women whose mothers had elevated levels of estrogen during pregnancy have

four-fold increased risk [6]. In addition, lifestyle risk factors like alcohol consumption, height and obesity are thought to increase risk by increasing endogenous levels of estrogen.

Though the involvement of mammary stem cells in breast carcinogenesis has been hypothesized for many years it was not possible to directly test for this involvement until very recently. Techniques are now becoming available to enrich mammary cell isolations for stem cells, and also culture them *in vitro* as nonadherent mammospheres. Using these newly available techniques a few groups have started to look for cancer stem cells in actual human mammary tumors. Ginestier et al. used the ALDH1 marker, thought to have a role in the early differentiation of stem cells and known to be highly expressed in hematopoietic and neural stem cells, to isolate both normal mammary stem cells and cancer stem cells from human mammary tissue samples [48]. Li et al. used mammosphere formation efficiency as a method to show that mammary stem cells from human tumor samples are more resistant to chemotherapy than the differentiated cells of the tumor [57]. The field of mammary stem cell research is extremely new; it will be very interesting to see where this research takes the study and treatment of breast cancer.

1.4 THE MECHANISM OF RADIATION CARCINOGENESIS:

Carcinogenesis, by any mechanism, is a multi-step process of clonal evolution involving initiation, promotion and progression [58]. Low linear energy transfer (LET) IR, such as X-rays or gamma-rays, is a tumor-initiating agent [59-61]. X- and γ-rays deposit energy though discrete ionization events which are distributed randomly throughout the cellular space they traverse [62]. If these ionization events occur in close proximity to DNA the energy deposited can result in single stranded DNA breaks, double stranded DNA breaks (DSBs), sugar and base modification, oxidative damage to bases, inter-strand DNA cross-linking, DNA-protein cross-linking, and locally multiply damaged sites (LMDSs) [32, 62]. It

is generally accepted that irradiation with 1Gy X-ray will cause approximately 20-40 DNA DSBs, 1,000 single-strand breaks, 1,000 damaged bases, and 150 DNA-protein cross-links per diploid mammalian cell [63]. The ability of IR to cause these structural aberrations puts it into a class of exogenous factors which are considered clastogens, virtually all of which are mutagenic and carcinogenic [62]. The mutations most commonly induced by IR in mammalian cells are large-scale deletions and chromosomal rearrangements; both of which are seen at very high frequencies in breast cancer [62].

Though IR can cause many kinds of DNA damage; the DNA DSBs are commonly believed to be the most detrimental to the cell, as a single unrepaired DNA DSB is sufficient to induce cell death [63, 64]. Studies have shown that DNA repair machinery can mend radiation-induced DSBs rapidly, with a half-time of ten minutes or less [65]. In mammalian cells DNA DSBs occurring in G_1 are repaired via the NHEJ pathway of DNA repair, which is an error-prone method of rejoining chromosome ends [66, 67]. DNA DSBs occurring in G_2/M are repaired by both NHEJ and HR, which uses the sequence from the sister chromatid to repair the break without any loss of sequence. In order for repair to occur the sites of DNA DSBs must be in close proximity in both space and time [62]. Radiation-induced DSBs have three repair options; first, they can reconstitute without any morphological change to the chromosomes; this would not be seen using cytogenetic analysis but may still result in mutation, especially deletions. Second, the DNA end can rejoin illegitimately with the broken end of another chromosome; this can result in mutation and/or cytogenetically visible aberrations. Third, the DSB can remain "open" leading to a terminal deletion at mitosis; these cells will often die due to DNA loss prior to the second mitosis [68]. The misrepair of radiation-induced DNA DSBs which leads to chromosomal aberrations has been associated with the NHEJ repair pathway because it is the primary repair pathway and is inherently error-prone [69, 70]. Cells containing unbalanced chromosome aberrations, such as dicentric

chromosomes and terminal deletions, or possessing substantial DNA loss are not expected to survive or contribute to the post-irradiation cell population. However, it has been suggested that some dicentrics enter a break-bridge-fusion cycle where the dicentric breaks during anaphase and forms another dicentric in the following cell cycle; little DNA is lost in this process but it can greatly increase the genomic instability observed. Cells containing balanced aberrations such as reciprocal translocation often do survive because little DNA has been lost [69]. Depending on the specific aberrations and mutations resulting from misrepair, these cells may have the potential to develop into cancer [69].

1.5 RADIATION-INDUCED MAMMARY CANCER IN HUMANS:

It is well accepted that IR can cause breast cancer [71, 72]. Over the last 70 years or so, many epidemiological studies have looked at the relationship between IR and breast cancer. These data come from survivors of the atomic bombings, occupational exposures and medically exposed individuals [69]. The vast majority of our understanding of radiation-induced breast cancer comes from studies of the survivors of the atomic bombings [73-75]. The Life Span Study clearly shows a strong linear dose response for the induction of breast cancer by IR [73-75]. These data in combination with data on the low sporadic incidence of breast cancer in Japan suggest that 1/3 of all breast cancer cases seen in the Life Span Study were caused by exposure to radiation [73, 74].

The study of breast cancer induction in occupationally exposed individuals is difficult because of the low number of females in industries where IR exposure is likely. The one area where women dominate the field is radiation technology. Recently, a very large study was completed to determine if these women were at increased risk for developing breast cancer compared to the general population. This study was the "Survey of US Radiologic Technologists" cohort which analyzed data from 56,436 women who administered medical

X-rays [76]. These data showed that there was a correlation between increased risk and increased attained age and height. There was also a three-fold increase in risk of breast cancer for women who had worked prior to 1935, and a two-fold increased risk in women who had worked before 1940 [76]. The study also determined that there was no increased risk for women who began working later in the 1940s or if they had begun working after 17 years of age. The decreased risk associated with working later than 1940 may be related to increased protective measures taken by hospitals and radiation workers, which decreased the annual exposure to workers from about 100mSv to about 25mSv per year [76]. Since then protective measures and exposure limits have become even more stringent [76].

Finally, there is a fair amount of evidence from medically irradiated women. These data come from patients with scoliosis or tuberculosis who were monitored by frequent chest X-rays or fluoroscopy, radiotherapy patients treated for benign disorders as infants or children, women of child bearing age who receive X-rays for benign breast disease or post-partum mastitis, childhood cancer survivors who were treated with radiotherapy, and finally survivors of adult cancers that were treated with radiotherapy [77]. Data from all of these medically irradiated populations support the linear no-threshold model for risk of breast cancer [77].

All of these studies have agreed that ionizing radiation can cause breast cancer, especially if exposure occurs before the age of 20, and that risk fits a linear no-threshold dose response curve [73-75]. This means that any exposure to radiation results in increased risk of developing breast cancer. In today's society most people are not exposed to large amounts of environmental or occupational sources of radiation, so the effects of IR used for medical purposes have become the major focus of radiation carcinogenesis studies. In terms of breast cancer, there are three main medical sources of radiation exposure, Computed Tomography (CT), chest X-rays and radiation therapy treatment for existing breast cancer or other types of

cancer near the breast tissue. CT scans have been used with increasing frequency since 2004 to test for coronary artery disease and to monitor lung health in individuals with cystic fibrosis. It is assumed that there is an increased risk of breast cancer associated with CT scans however because this is a relatively new technology no quantitative data are available. A few studies have attempted to predict risk associated with this technology, they have concluded that there is increasing risk of developing breast cancer associated with increasing number of CT scans and with decreasing age of the individual [78-80].

The risk of developing breast cancer from the low doses of IR used in X-rays and mammograms is controversial [81]. Modern mammograms usually involve two pictures of each breast with each picture releasing about 0.1-0.2 rads (1-2 x 10^{-3} Gy) [3]. The exposure to IR from mammograms is well below the yearly exposure dose from naturally occurring background sources. Additionally, most women do not begin screening until after the age of 40, which is when the risks are decreased [3]. It has been suggested that carriers of BRCA mutations begin screening regimen at 25 years of age; however this increases their exposure to mammographic radiation. Studies of risk associated with mammogram screening of BRCA carriers have shown that the radiation exposure from mammograms is unlikely to cause significant increase in breast cancer in this population [82]. But carriers of BRCA mutations already have an incredibly high risk of developing breast cancer. It is possible that some women carry low penetrance alleles for breast cancer for which even the low doses of IR from mammograms provide significant increased risk. One study looked at the relationship between four polymorphisms found in DNA repair genes (XRCC3 codon 241; NBS1 codon 185; XRCC2 codon 188; and BRCA2 codon 372). They reported a positive dose-response relationship between number of chest x-rays and mammograms a woman was exposed to and breast cancer risk among women who carried two or more of the less common alleles for these DNA repair genes [81]. This is a very new area of research, but it

suggests that there may be a subset of the population who are genetically predisposed to radiation-induced mammary cancer, for which any IR exposure will be harmful.

Mammographic screening results in earlier detection and more effective treatment of the existing breast cancer, both of which lead to increased patient survival. But this increased survival has also led to an increased risk of second cancers [83]. Second cancers are distinct from cancer recurrence, which is when the original tumor redevelops after the therapy meant to cure it. Second cancers come from a different initiating event. Women with breast cancer have 3-4 fold increased risk of developing new breast cancer in the contralateral breast. This increased risk maybe a result of the same hormonal and genetic factors that resulted in the first cancer, but it may also be due to chemotherapy and/or radiation therapy [84].

Radiotherapy results in 60-80Gy total dose to the target area [85, 86]. However, studies have shown that the contralateral breast also receives a significant dose, varying from 1-10% of the total dose depending on a variety of factors including direct irradiation, scatter off of the collimators and filters, and leakage through the head of the machine [84, 87]. This exposure is a major concern, but it has only been recently that studies have attempted to determine if a significant proportion of secondary tumors are actually related to the treatment of the original tumor. Broeks et al. showed that 24% of women in the study group who developed contralateral breast cancer after therapy carried a mutation in BRCA1, BRCA2, ATM or CHEK2, suggesting that these women may be predisposed to the damaging effects of IR to a degree where radiation therapy may be more harmful then it is helpful [84].

A slightly different problem associated with radiation therapy for breast cancer is that 5-7% of all patients develop severe adverse side effects of radiation therapy in the normal tissues surrounding the target area [32]. It has been hypothesized that the occurrence and severity of the adverse reactions to radiation therapy are also influenced by genetic susceptibility [32]. It has also been suggested that the women who develop secondary tumors

after radiation therapy may be restricted to this genetically defined radiosensitive subpopulation, unfortunately current data on the subject is extremely scarce [84].

1.6 BALB/c MOUSE MODEL OF RADIATION-INDUCED MAMMARY CANCER:

Although radiation exposure certainly increases a woman's risk for developing breast cancer, the pathology of radiation-induced breast cancer appears indistinguishable from that of spontaneous breast cancers; making elucidation of the mechanisms involved in radiation-induced carcinogenesis just as problematic as that of spontaneous breast cancers [71]. In human studies, it is impossible to control the circumstances under which breast cancer develops. As such many researchers use animal models of disease, where genetics and environmental exposures are much easier to control. The mouse is the animal of choice for cancer research for several reasons; most importantly that mice and humans have basically the same genes and signaling pathways [88]. Studies have clearly shown that the molecular events which occur in human breast cancer also occur in mouse mammary carcinogenesis [89]. Additionally, cancer has such a long latency; using animals with relatively short lifespan is highly beneficial [90]. There are also a large number of inbred mouse strains available, which allows the effect of specific mutations on cancer development to be explored in a way that will never be possible in human studies.

It is commonly accepted that inbred mouse strains exhibit differential susceptibility to toxins and carcinogens, and that this variation is due to the genetic differences between the strains. A differential response of BALB/c and C57BL mice to the effects of IR was first shown by Kallman in 1962. He showed that BALB/c mice were more sensitive to the lethal effects of IR than C57BL mice exposed to similar doses and dose rates [91]. Although both strains of mice show a linear dose response to radiation-induced death, the LD_{50} (dose required to cause death in 50% of treated animals) for C57BL mice was significantly higher

than that for the BALB/c mice at all dose rates tested. Whole body irradiation leading to lethality is a result of damage to the hematopoietic cells in the bone marrow and/or damage to crypt cells of the intestines. The research done by Kallman suggests that these tissues are significantly more sensitive, or less capable of repairing the damage caused by radiation, in BALB/c mice then they are in C57BL mice [91]. As these are inbred mouse strains, the differential response to radiation must be caused by differences in genetic backgrounds.

Sub-lethal radiation exposure has been shown to result in a wide variety of tumors over the life of the exposed individual. For the BALB/c mouse, the mammary gland is one of the most sensitive tissues to the damaging effects of IR. Like human IR-induced mammary cancer, the incidence of mammary tumors in BALB/c mice exposed to IR fits a linear dose response curve with increasing total dose or dose rate resulting in increased tumor formation [92]. Mouse mammary tumorigenesis is difficult to study in the exposed individual due to extremely long latency. It is commonly accepted that cellular proliferation is necessary for carcinogenesis but most studies on the effects of potential carcinogens on mammary tissue use adult virgin female mice; in these animals the mammary gland is fully developed and minimal growth occurs in absence of pregnancy [93]. To shorten the latency period hormone stimulation (pregnancy or pseudo-pregnancy) has been used [93].

In an attempt to simplify the study of murine mammary tumorigenesis, DeOme developed a system of mammary transplantation [94]. In this system, tissue or cells from an adult mouse are injected into the gland-free (cleared) fat pad of a three-week old recipient mouse. Upon puberty, the normal physiological development of the recipient mouse causes proliferation and differentiation of the transplanted mammary cells. In three-week old mice the mammary ductal tree is confined to a very small area near the lymph node that can be cleanly excised prior to injection of the donor cells [94]. Upon transplantation, untreated donor cells will result in a fully functional mammary gland within 10-16 weeks that is

basically indistinguishable from a normal, untouched gland. Using mixed populations of green fluorescent and cyan fluorescent protein expressing donor cells, Stingl et al. showed that the mammary outgrowths resulting from transplantation are clonally derived from single donor cells [95].

The ability of donor cells to produce fully developed and functional mammary glands upon transplantation strongly suggests the presence of a mammary stem cell. Only a stem cell would have the ability to produce all three kinds differentiated cells required for mammary gland development (luminal epithelial, alveolar epithelial and myoepithelial cells). That the mammary stem cell is the basis for the mammary transplantation assay was definitively shown a few years ago. Individual putative mammary stem cells were injected into the cleared fat pads of recipient mice and were shown to produce mammary outgrowths [95, 96]. This transplanted mammary gland could then be dissociated and transplanted again suggesting the donor cell had self-renewal capacity [95, 96]. The transplanted mammary gland was followed through pregnancy to verify that it could in fact produce all of the cells required for a functional mammary gland [96].

Using the mammary transplantation system Ethier et al. showed that in BALB/c mice, mammary tumors were preceded by ductal dysplasias which are precancerous lesions [97, 98]. Ductal dysplasias are areas of abnormal ductal growth including abnormalities in branch number, branch pattern and increased cellularity of the ducts and end buds [97]. Ullrich et al. showed that after exposure to 1Gy γ-radiation mammary cells derived from BALB/c mice frequently produced ductal dysplasias and eventually mammary tumors upon transplantation, while cells from C57BL/6 mice rarely produced abnormal outgrowths [99]. It was determined that this difference in ductal dysplasia frequency was due to the inherent susceptibility of the BALB/c mammary cells to the transforming effects of IR [99]. The fact that these studies are based on data obtained using the mammary transplantation assay

suggests that the ductal dysplasias and eventual mammary tumors are actually a result of radiation-induced damage to the mammary stem cells. It is reasonable to assume that if normal mammary gland development results from the proliferation and differentiation of a mammary stem cell, then so does abnormal mammary gland development. Unfortunately, at the time of these studies, there was no way to isolate or manipulate mammary stem cells specifically to test this hypothesis.

A reduction in the lethal effects of radiation exposure is achieved by reducing the rate at which it is delivered. This dose rate effect has generally been attributed to the ability of exposed cells to repair the DNA damage caused by radiation [100]. C57BL mice exhibit a very pronounced dose rate effect, suggesting that as the dose rate is decreased the exposed cells are more able to repair the damage caused by radiation. BALB/c mice show a very small dose rate effect [91]. Taken together, these observations suggest that cells derived from BALB/c mice have a DNA repair defect when compared to those derived from C57BL mice. To test this hypothesis primary kidney fibroblasts were exposed to 50Gy γ-radiation and the ability of the cells to repair the DNA damage was determined by measuring DNA joining over time using the FAR assay [100]. It was determined that cells derived from C57BL/6 and other "wild-type" strains were able to repair most of their DNA within four hours of irradiation, while cells from SCID mice were severely deficient in their repair abilities and BALB/c cells showed intermediate repair kinetics.

SCID (severe combined immunodeficiency) mice are immunodeficient because of defective V(D)J recombination which results in improper differentiation of both T and B lymphocytes [101]. They have been shown to carry a truncating mutation in the *Prkdc* gene which encodes the protein DNA-PKcs (DNA-dependent protein kinase catalytic subunit). The mutation results in degradation of the protein and seems to be responsible for their

deficient DNA repair capacity. Additionally, *in vitro* radiation studies have isolated radiosensitive clones with altered DNA-PKcs function [102].

To determine if the radiation sensitivity observed in BALB/c mice was due to altered DNA-PKcs, protein expression was analyzed. Western blotting of whole kidney cell extracts showed that SCID cells lack DNA-PKcs protein, while C57BL/6, C3H/HeJ, A/J, DBA2/J, and SWR/J cells all had very high levels of expression. BALB/c cells had intermediate levels of DNA-PKcs expression [100, 103]. This intermediate phenotype is similar to the intermediate repair observed earlier. To determine if DNA-PKcs expression levels were tissue specific, analysis was performed on tissue from the brain, heart, liver, kidney, mammary, and spleen from C57BL/6 and BALB/c mice. Western blot analysis showed that out of all tissues tested, the mammary gland had the lowest level of DNA-PKcs expression, and that this reduced expression was even more pronounced in mammary tissue derived from BALB/c mice [103]. These data suggest that the extremely low levels of DNA-PKcs expressed in the mammary tissue may be responsible for the specific susceptibility of BALB/c mice to mammary carcinogenesis.

To further characterize DNA-PKcs in these mouse strains, the kinase activity of the protein was assayed. Again, SCID cells had very low levels of DNA-PK kinase activity, while C57BL/6 and other "wild-type" cells had very high levels of kinase activity. Again, BALB/c cells had intermediate levels of kinase activity [100]. The relationship between mammary tumorigenesis, reduced DNA repair, reduced expression of DNA-PKcs and reduced kinase activity are still being investigated. However, sequence analysis showed that the DNA-PKcs from BALB/c mice contains two single nucleotide polymorphisms (SNPs) [103]. These polymorphisms are M3844V in the phosphatidylinositol 3-kinase domain; and R2140C in a leucine zipper domain [103]. It was hypothesized that these two SNPs in *Prkdc* are responsible for the reduced DNA-PKcs protein expression, reduced DNA repair capacity

and increased susceptibility to radiation-induced mammary cancer observed in the BALB/c mice.

To address the role of DNA-PKcs in the radiation-induced mammary carcinogenesis seen in BALB/c mice this lab recently created two congenic strains of mice. Congenic strains are developed by breeding mice from the two parental strains, in this case C57BL/6J and BALB/cByJ to create F_1 hybrid pups. In the case of B6.C-*Prkdc*^{BALB}, pups carrying the BALB/c variant allele for *Prkdc* were then backcrossed to C57BL/6J mice for ten generations. This was followed by brother-sister mating for another ten generations to create an inbred mouse strain. The results of this breeding scheme are mice that contain 99.9% C57BL/6J genome as well as the variant *Prkdc*^{BALB} allele. C.B6-*Prkdc* is the complementary strain containing the BALB/cByJ background genome and the common *Prkdc* allele. Use of these two strains in conjunction with their parental strains allows us to determine the effect of a single locus, in this case *Prkdc* [104].

In addition to creating the mouse strains congenic for *Prkdc*, Dr. Ramaiah performed some preliminary experiments to determine the radiation sensitivity of the congenic strains compared to the parental strains. The B6.C-*Prkdc*^{BALB} mice allow us to determine if *Prkdc*^{BALB} is **sufficient** to confer susceptibility to radiation damage. The C.B6-*Prkdc* strain which has the susceptible background with the resistant *Prkdc* allows us to determine if *Prkdc*^{BALB} is **required** to confer susceptibility to radiation-induced damage and cancer. Using primary kidney fibroblasts Dr. Ramaiah found that *Prkdc*^{BALB} is required and sufficient to decrease DNA-PKcs protein expression. C57BL/6J and C.B6- *Prkdc* express equal quantities of DNA-PKcs protein, while BALB/cByJ and the congenic strain B6.C- *Prkdc*^{BALB} express very little protein [104]. Dr. Ramaiah was also able to determine that *Prkdc*^{BALB} is required and sufficient to decrease clonogenic survival in BALB/c mouse kidney fibroblasts, by using a colony formation assay. The results suggest that cells derived from

B6.C-*Prkdc*^{BALB} are susceptible to cell killing by IR to the same degree as the BALB/cByJ parental strains, and that cells derived from C.B6-*Prkdc* are resistant to IR to the same magnitude as the C57BL/6J parental strain. Together these data suggest that both DNA-PKcs protein expression and survival after radiation exposure seem to follow the *Prkdc* allele, at least in primary kidney fibroblasts [104].

1.7 RATIONAL OF DISSERTATION:

Breast cancer is the most commonly diagnosed invasive cancer among women in the United States [2]. Recent advances in clinical diagnostic techniques have allowed for earlier detection and enhanced treatment of breast cancer resulting in a steady decline in mortality rates [3]. However, the specific cellular and molecular mechanisms involved in mammary carcinogenesis remain unclear. It has been suggested that individual susceptibility to breast cancer comes from the combined effect of all the low penetrance alleles in an individual's genome in addition to the effects of exposure to carcinogens [13].

Any exposure to ionizing radiation results in increased risk of developing breast cancer. We believe that there is a population of women who are genetically predisposed to the damaging effects of IR, for which any exposure to IR, via X-rays, mammograms, or radiation therapy, will be dangerous. Currently, it is impossible to identify these women prior to radiation exposure. In an attempt to elucidate the mechanisms involved in radiation-induced mammary cancer and the role of genetic susceptibility our lab has been utilizing the BALB/c mouse model. These mice are specifically genetically susceptible to radiation-induced mammary cancer [92]. We have previously shown that the mammary cells derived from BALB/c mice are inherently susceptible to the transforming effects of IR [99]. We have shown that this genetic predisposition to breast cancer is related to two SNPs in the *Prkdc* gene of the BALB/c mice compared to the resistant control strain C57BL/6 [103].

Finally, we have created two congenic mouse strains in an attempt to determine if this variant *Prkdc* gene is required and sufficient to cause the BALB/c radiation sensitive phenotype [104].

The methods used to determine that the BALB/c mice are susceptible to developing IR-induced ductal dysplasias and mammary cancers involve the mammary gland transplantation assay. This system relies on the hormonal stimuli of a pubescent recipient mouse to produce a mammary gland outgrowth from donor cells. It has recently been definitively determined that these mammary gland outgrowths result from the growth of a mammary stem cell from the donor mouse. It follows that if the transplanted normal mammary outgrowth results from the growth of a stem cell, then so does aberrant mammary outgrowth that result from transplantation of irradiated BALB/c mammary cells. Until recently it was not possible to isolate or target mammary stem cells specifically. In this dissertation we have developed laboratory techniques to isolate, culture, and propagate mammary stem cells *in vitro* as non-adherent mammospheres. In order to enhance our ability to identify true mammary stem cells *in vitro* we have attempted to create mammosphere size criteria based on the ability of the stem cell to self-renew and differentiate. Finally, we have used these new techniques to develop a mammosphere colony formation assay to test the theory that the stem cell is the target cell of radiation in BALB/c model of IR-induced mammary cancer.

LITERATURE CITED:

1. ACS. The History of Cancer. [web source] 2002 [cited 2009 2/2/09]; Available from: www.cancer.org/docroot/CRI/content?CRI_2_6x_the_history_of_cancer_72.asp

2. Brody JG, Moysich KB, Humblet O, Attfield KR, Beehler GP, Rudel RA. Environmental pollutants and breast cancer: epidemiologic studies. Cancer 2007; 109: 2667-711.

3. Society AC. Breast Cancer Detailed Guide. 2008 9/4/08 [cited 2009 1/22/09]; Available from: www.cancer.org

4. Dumitrescu RG, Cotarla I. Understanding breast cancer risk -- where do we stand in 2005? J Cell Mol Med 2005; 9: 208-21.

5. Vogel VG. Breast cancer prevention: a review of current evidence. CA Cancer J Clin 2000; 50: 156-70.

6. Davis DL, Axelrod D, Bailey L, Gaynor M, Sasco AJ. Rethinking breast cancer risk and the environment: the case for the precautionary principle. Environ Health Perspect 1998; 106: 523-9.

7. Hsu HM, Wang HC, Chen ST, Hsu GC, Shen CY, Yu JC. Breast cancer risk is associated with the genes encoding the DNA double-strand break repair Mre11/Rad50/Nbs1 complex. Cancer Epidemiol Biomarkers Prev 2007; 16: 2024-32.

8. Michels KB, Mohllajee AP, Roset-Bahmanyar E, Beehler GP, Moysich KB. Diet and breast cancer: a review of the prospective observational studies. Cancer 2007; 109: 2712-49.

9. Su Y, Simmen FA, Xiao R, Simmen RC. Expression profiling of rat mammary epithelial cells reveals candidate signaling pathways in dietary protection from mammary tumors. Physiol Genomics 2007; 30: 8-16.

10. Tamimi RM, Cox D, Kraft P, Colditz GA, Hankinson SE, Hunter DJ. Breast cancer susceptibility loci and mammographic density. Breast Cancer Res 2008; 10: R66.

11. Vachon CM, Sellers TA, Carlson EE, Cunningham JM, Hilker CA, Smalley RL, Schaid DJ, Kelemen LE, Couch FJ, Pankratz VS. Strong evidence of a genetic determinant for mammographic density, a major risk factor for breast cancer. Cancer Res 2007; 67: 8412-8.

12. Allen N, Robert Newton, Amy Berrington de Gonzalez, Jane Green, Emily Banks, and Timothy J. Key. The Causes of Cancer. In: Shelby MKaP, editor. Introduction to the Cellular and Molecular Biology of Cancer. 4 ed. New York: Oxford University Press, Inc.; 2005. p. 25-44.

13. Briollais L, Wang Y, Rajendram I, Onay V, Shi E, Knight J, Ozcelik H. Methodological issues in detecting gene-gene interactions in breast cancer susceptibility: a population-based study in Ontario. BMC Med 2007; 5: 22.

14. Dufloth RM, Arruda A, Heinrich JK, Schmitt F, Zeferino LC. The investigation of DNA repair polymorphisms with histopathological characteristics and hormone receptors in a group of Brazilian women with breast cancer. Genet Mol Res 2008; 7: 574-82.

15. Consortium TBCA. Commonly studied single-nucleotide polymorphisms and breast cancer: results from the Breast Cancer Association Consortium. J Natl Cancer Inst 2006; 98: 1382-96.

16. Peto J, Mack TM. High constant incidence in twins and other relatives of women with breast cancer. Nat Genet 2000; 26: 411-4.

17. Bertucci F, Birnbaum D. Reasons for breast cancer heterogeneity. J Biol 2008; 7: 6.

18. Kotsopoulos J, Olopado OI, Ghadirian P, Lubinski J, Lynch HT, Isaacs C, Weber B, Kim-Sing C, Ainsworth P, Foulkes WD, Eisen A, Sun P, Narod SA. Changes in body weight and the risk of breast cancer in BRCA1 and BRCA2 mutation carriers. Breast Cancer Res 2005; 7: R833-43.

19. Kakarala M, Wicha MS. Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. J Clin Oncol 2008; 26: 2813-20.

20. Blackburn AC, Hill LZ, Roberts AL, Wang J, Aud D, Jung J, Nikolcheva T, Allard J, Peltz G, Otis CN, Cao QJ, Ricketts RS, Naber SP, Mollenhauer J, Poustka A, Malamud D, Jerry DJ. Genetic mapping in mice identifies DMBT1 as a candidate modifier of mammary tumors and breast cancer risk. Am J Pathol 2007; 170: 2030-41.

21. Thompson A, Brennan K, Cox A, Gee J, Harcourt D, Harris A, Harvie M, Holen I, Howell A, Nicholson R, Steel M, Streuli C. Evaluation of the current knowledge limitations in breast cancer research: a gap analysis. Breast Cancer Res 2008; 10: R26.

22. Driver KE, Song H, Lesueur F, Ahmed S, Barbosa-Morais NL, Tyrer JP, Ponder BA, Easton DF, Pharoah PD, Dunning AM. Association of single-nucleotide polymorphisms in the cell cycle genes with breast cancer in the British population. Carcinogenesis 2008; 29: 333-41.

23. Baynes C, Healey CS, Pooley KA, Scollen S, Luben RN, Thompson DJ, Pharoah PD, Easton DF, Ponder BA, Dunning AM. Common variants in the ATM, BRCA1, BRCA2, CHEK2 and TP53 cancer susceptibility genes are unlikely to increase breast cancer risk. Breast Cancer Res 2007; 9: R27.

24. Onay VU, Briollais L, Knight JA, Shi E, Wang Y, Wells S, Li H, Rajendram I, Andrulis IL, Ozcelik H. SNP-SNP interactions in breast cancer susceptibility. BMC Cancer 2006; 6: 114.

25. Hansen LL, Madsen BE, Pedersen K, Wiuf C. Conflicting results in SNP genotype assessment. Biotechniques 2007; 43: 756, 8, 60 passim.

26. Bau DT, Mau YC, Ding SL, Wu PE, Shen CY. DNA double-strand break repair capacity and risk of breast cancer. Carcinogenesis 2007; 28: 1726-30.

27. Tamimi RM. Single nucleotide polymorphisms and breast cancer: not yet a success story. Breast Cancer Res 2006; 8: 108.

28. Weiss JR, Moysich KB, Swede H. Epidemiology of male breast cancer. Cancer Epidemiol Biomarkers Prev 2005; 14: 20-6.

29. Garcia-Closas M, Hall P, Nevanlinna H, Pooley K, Morrison J, Richesson DA, Bojesen SE, Nordestgaard BG, Axelsson CK, Arias JI, Milne RL, Ribas G, Gonzalez-Neira A, Benitez J, Zamora P, Brauch H, Justenhoven C, Hamann U, Ko YD, Bruening T, Haas S, Dork T, Schurmann P, Hillemanns P, Bogdanova N, Bremer M, Karstens JH, Fagerholm R, Aaltonen K, Aittomaki K, von Smitten K, Blomqvist C, Mannermaa A, Uusitupa M, Eskelinen M, Tengstrom M, Kosma VM, Kataja V, Chenevix-Trench G, Spurdle AB, Beesley J, Chen X, Australian Ovarian Cancer Management G, Kathleen Cuningham Foundation Consortium For Research Into Familial Breast C, Devilee P, van Asperen CJ, Jacobi CE, Tollenaar RA, Huijts PE, Klijn JG, Chang-Claude J, Kropp S, Slanger T, Flesch-Janys D, Mutschelknauss E, Salazar R, Wang-Gohrke S, Couch F, Goode EL, Olson JE, Vachon C, Fredericksen ZS, Giles GG, Baglietto L, Severi G, Hopper JL, English DR, Southey MC, Haiman CA, Henderson BE, Kolonel LN, Le Marchand L, Stram DO, Hunter DJ, Hankinson SE, Cox DG, Tamimi R, Kraft P, Sherman ME, Chanock SJ, Lissowska J, Brinton LA, Peplonska B, Klijn JG, Hooning MJ, Meijers-Heijboer H, Collee JM, van den Ouweland A, Uitterlinden AG, Liu J, Lin LY, Yuqing L, Humphreys K, Czene K, Cox A, Balasubramanian SP, Cross SS, Reed MW, Blows F, Driver K, Dunning A, Tyrer J, Ponder BA, Sangrajrang S, Brennan P, McKay J, Odefrey F, Gabrieau V, Sigurdson A, Doody M, Struewing JP, Alexander B, Easton DF, Pharoah PD. Heterogeneity of breast cancer associations with five susceptibility loci by clinical and pathological characteristics. PLoS Genet 2008; 4: e1000054.

30. Debniak T, Gorski B, Huzarski T, Byrski T, Cybulski C, Mackiewicz A, Gozdecka-Grodecka S, Gronwald J, Kowalska E, Haus O, Grzybowska E, Stawicka M, Swiec M, Urbanski K, Niepsuj S, Wasko B, Gozdz S, Wandzel P, Szczylik C, Surdyka D, Rozmiarek A, Zambrano O, Posmyk M, Narod SA, Lubinski J. A common variant of CDKN2A (p16) predisposes to breast cancer. J Med Genet 2005; 42: 763-5.

31. Fowke JH, Shu XO, Dai Q, Jin F, Cai Q, Gao YT, Zheng W. Oral contraceptive use and breast cancer risk: modification by NAD(P)H:quinone oxoreductase (NQO1) genetic polymorphisms. Cancer Epidemiol Biomarkers Prev 2004; 13: 1308-15.

32. Bourguignon MH, Gisone PA, Perez MR, Michelin S, Dubner D, Giorgio MD, Carosella ED. Genetic and epigenetic features in radiation sensitivity Part I: cell signalling in radiation response. Eur J Nucl Med Mol Imaging 2005; 32: 229-46.

33. Gorbunova V, Seluanov A, Mao Z, Hine C. Changes in DNA repair during aging. Nucleic Acids Res 2007; 35: 7466-74.

34. Bau DT, Fu YP, Chen ST, Cheng TC, Yu JC, Wu PE, Shen CY. Breast cancer risk and the DNA double-strand break end-joining capacity of nonhomologous end-joining genes are affected by BRCA1. Cancer Res 2004; 64: 5013-9.

35. Terry MB, Gammon MD, Zhang FF, Eng SM, Sagiv SK, Paykin AB, Wang Q, Hayes S, Teitelbaum SL, Neugut AI, Santella RM. Polymorphism in the DNA repair gene XPD, polycyclic aromatic hydrocarbon-DNA adducts, cigarette smoking, and breast cancer risk. Cancer Epidemiol Biomarkers Prev 2004; 13: 2053-8.

36. Lee SA, Lee KM, Park WY, Kim B, Nam J, Yoo KY, Noh DY, Ahn SH, Hirvonen A, Kang D. Obesity and genetic polymorphism of ERCC2 and ERCC4 as modifiers of risk of breast cancer. Exp Mol Med 2005; 37: 86-90.

37. Bernard-Gallon D, Bosviel R, Delort L, Fontana L, Chamoux A, Rabiau N, Kwiatkowski F, Chalabi N, Satih S, Bignon YJ. DNA repair gene ERCC2 polymorphisms and associations with breast and ovarian cancer risk. Mol Cancer 2008; 7: 36.

38. Moullan N, Cox DG, Angele S, Romestaing P, Gerard JP, Hall J. Polymorphisms in the DNA repair gene XRCC1, breast cancer risk, and response to radiotherapy. Cancer Epidemiol Biomarkers Prev 2003; 12: 1168-74.

39. Wang L, Bhattacharyya N, Rabi T, Wang L, Banerjee S. Mammary carcinogenesis in transgenic mice expressing a dominant-negative mutant of DNA polymerase beta in their mammary glands. Carcinogenesis 2007; 28: 1356-63.

40. Lu J, Wei Q, Bondy ML, Li D, Brewster A, Shete S, Yu TK, Sahin A, Meric-Bernstam F, Hunt KK, Singletary SE, Ross MI, Wang LE. Polymorphisms and haplotypes of the NBS1 gene are associated with risk of sporadic breast cancer in non-Hispanic white women <or=55 years. Carcinogenesis 2006; 27: 2209-16.

41. Fu YP, Yu JC, Cheng TC, Lou MA, Hsu GC, Wu CY, Chen ST, Wu HS, Wu PE, Shen CY. Breast cancer risk associated with genotypic polymorphism of the nonhomologous end-joining genes: a multigenic study on cancer susceptibility. Cancer Res 2003; 63: 2440-6.

42. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100: 57-70.

43. Merlo LM, Pepper JW, Reid BJ, Maley CC. Cancer as an evolutionary and ecological process. Nat Rev Cancer 2006; 6: 924-35.

44. Beckman RA, Loeb LA. Genetic instability in cancer: theory and experiment. Semin Cancer Biol 2005; 15: 423-35.

45. Nowell PC. The clonal evolution of tumor cell populations. Science 1976; 194: 23-8.

46. Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea--a paradigm shift. Cancer Res 2006; 66: 1883-90; discussion 95-6.

47. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, Song E. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 2007; 131: 1109-23.

48. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007; 1: 555-67.

49. Kalirai H, Clarke RB. Human breast epithelial stem cells and their regulation. J Pathol 2006; 208: 7-16.

50. Al-Hajj M, Becker MW, Wicha M, Weissman I, Clarke MF. Therapeutic implications of cancer stem cells. Curr Opin Genet Dev 2004; 14: 43-7.

51. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res 2005; 7: 86-95.

52. Dontu G, Al-Hajj M, Abdallah WM, Clarke MF, Wicha MS. Stem cells in normal breast development and breast cancer. Cell Prolif 2003; 36 Suppl 1: 59-72.

53. Clayton H, Titley I, Vivanco M. Growth and differentiation of progenitor/stem cells derived from the human mammary gland. Exp Cell Res 2004; 297: 444-60.

54. Dimri G, Band H, Band V. Mammary epithelial cell transformation: insights from cell culture and mouse models. Breast Cancer Res 2005; 7: 171-9.

55. Siwko SK, Dong J, Lewis MT, Liu H, Hilsenbeck SG, Li Y. Evidence that an early pregnancy causes a persistent decrease in the number of functional mammary epithelial stem cells--implications for pregnancy-induced protection against breast cancer. Stem Cells 2008; 26: 3205-9.

56. Tu Y, Jerry DJ, Pazik B, Smith Schneider S. Sensitivity to DNA damage is a common component of hormone-based strategies for protection of the mammary gland. Mol Cancer Res 2005; 3: 435-42.

57. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, Hilsenbeck SG, Pavlick A, Zhang X, Chamness GC, Wong H, Rosen J, Chang JC. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 2008; 100: 672-9.

58. Knowles LMFaMA. What is Cancer? In: Shelby MKaP, editor. Introduction to the Cellular and Molecular Biology of Cancer. New York: Oxford University Press Inc.; 2005. p. 1-24.

59. UNSCEAR. Sources, Effects, and Risks of Ionizing Radiation. The 1988 Report to the General Assembly with Annexes. New York; 1988.

60. UNSCEAR. Sources and Effects of Ionizing Radiation. 1994 Report to the General Assembly, with Scientific Annexes. Annex B Adaptive Responses to Radiation in Cells and Organisms. New York; 1994.

61. Rabes HM, Demidchik EP, Sidorow JD, Lengfelder E, Beimfohr C, Hoelzel D, Klugbauer S. Pattern of radiation-induced RET and NTRK1 rearrangements in 191 post-chernobyl papillary thyroid carcinomas: biological, phenotypic, and clinical implications. Clin Cancer Res 2000; 6: 1093-103.

62. Cornforth MN. Radiation-Induced Damage and the Formation of Chromosomal Aberrations. In: Heokstra JANaMF, editor. DNA Damage and Repair. Totowa, NJ: Humana Press Inc.; 1998. p. 559-85.

63. Bedford JS, Dewey WC. Radiation Research Society. 1952-2002. Historical and current highlights in radiation biology: has anything important been learned by irradiating cells? Radiat Res 2002; 158: 251-91.

64. Boulton SJ, Jackson SP. Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing. Embo J 1998; 17: 1819-28.

65. Ward JF, Limoli CL, Calabro-Jones PM, Aguilera J. An examination of the repair saturation hypothesis for describing shouldered survival curves. Radiat Res 1991; 127: 90-6.

66. Smith GCM, Jackson SP. The DNA-dependent protein kinase. Genes Dev 1999; 13: 916-34.

67. Martin M, Genesca A, Latre L, Jaco I, Taccioli GE, Egozcue J, Blasco MA, Iliakis G, Tusell L. Postreplicative joining of DNA double-strand breaks causes genomic instability in DNA-PKcs-deficient mouse embryonic fibroblasts. Cancer Res 2005; 65: 10223-32.

68. Savage JR. Insight into sites. Mutat Res 1996; 366: 81-95.

69. Academies NRCotN. Health Risks from Exposure to Low Levels of Ionizing Radiation. Beir VII: Phase 2 ed: National Acadamy of Sciences; 2006.

70. Morris T, Thacker J. Formation of large deletions by illegitimate recombination in the HPRT gene of primary human fibroblasts. Proc Natl Acad Sci U S A 1993; 90: 1392-6.

71. UNSCEAR. Sources and Effects of Ionizing Radiation. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation to the General Assembly: Volume 2:Effects. New York; 2000 2000.

72. Loree J, Koturbash I, Kutanzi K, Baker M, Pogribny I, Kovalchuk O. Radiation-induced molecular changes in rat mammary tissue: possible implications for radiation-induced carcinogenesis. Int J Radiat Biol 2006; 82: 805-15.

73. Thompson DE, Mabuchi K, Ron E, Soda M, Tokunaga M, Ochikubo S, Sugimoto S, Ikeda T, Terasaki M, Izumi S, et al. Cancer incidence in atomic bomb survivors. Part II: Solid tumors, 1958-1987. Radiat Res 1994; 137: S17-67.

74. Tokunaga M, Land CE, Tokuoka S, Nishimori I, Soda M, Akiba S. Incidence of female breast cancer among atomic bomb survivors, 1950-1985. Radiat Res 1994; 138: 209-23.

75. Land CE, Tokunaga M, Koyama K, Soda M, Preston DL, Nishimori I, Tokuoka S. Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950-1990. Radiat Res 2003; 160: 707-17.

76. Doody MM, Freedman DM, Alexander BH, Hauptmann M, Miller JS, Rao RS, Mabuchi K, Ron E, Sigurdson AJ, Linet MS. Breast cancer incidence in U.S. radiologic technologists. Cancer 2006; 106: 2707-15.

77. Ronckers CM, Erdmann CA, Land CE. Radiation and breast cancer: a review of current evidence. Breast Cancer Res 2005; 7: 21-32.

78. de Gonzalez AB, Kim KP, Samet JM. Radiation-induced cancer risk from annual computed tomography for patients with cystic fibrosis. Am J Respir Crit Care Med 2007; 176: 970-3.

79. Hurwitz LM, Reiman RE, Yoshizumi TT, Goodman PC, Toncheva G, Nguyen G, Lowry C. Radiation dose from contemporary cardiothoracic multidetector CT protocols with an anthropomorphic female phantom: implications for cancer induction. Radiology 2007; 245: 742-50.

80. Einstein AJ, Henzlova MJ, Rajagopalan S. Estimating risk of cancer associated with radiation exposure from 64-slice computed tomography coronary angiography. JAMA 2007; 298: 317-23.

81. Millikan RC, Player JS, Decotret AR, Tse CK, Keku T. Polymorphisms in DNA repair genes, medical exposure to ionizing radiation, and breast cancer risk. Cancer Epidemiol Biomarkers Prev 2005; 14: 2326-34.

82. Goldfrank D, Chuai S, Bernstein JL, Ramon YCT, Lee JB, Alonso MC, Diez O, Baiget M, Kauff ND, Offit K, Robson M. Effect of mammography on breast cancer risk in women with mutations in BRCA1 or BRCA2. Cancer Epidemiol Biomarkers Prev 2006; 15: 2311-3.

83. Sachs RK, Shuryak I, Brenner D, Fakir H, Hlatky L, Hahnfeldt P. Second cancers after fractionated radiotherapy: stochastic population dynamics effects. J Theor Biol 2007; 249: 518-31.

84. Broeks A, Braaf LM, Huseinovic A, Nooijen A, Urbanus J, Hogervorst FB, Schmidt MK, Klijn JG, Russell NS, Van Leeuwen FE, Van 't Veer LJ. Identification of women with an increased risk of developing radiation-induced breast cancer: a case only study. Breast Cancer Res 2007; 9: R26.

85. Sachs RK, Brenner DJ. Solid tumor risks after high doses of ionizing radiation. Proc Natl Acad Sci U S A 2005; 102: 13040-5.

86. Chang-Claude J, Popanda O, Tan XL, Kropp S, Helmbold I, von Fournier D, Haase W, Sautter-Bihl ML, Wenz F, Schmezer P, Ambrosone CB. Association between polymorphisms in the DNA repair genes, XRCC1, APE1, and XPD and acute side effects of radiotherapy in breast cancer patients. Clin Cancer Res 2005; 11: 4802-9.

87. Bernstein JL, Langholz B, Haile RW, Bernstein L, Thomas DC, Stovall M, Malone KE, Lynch CF, Olsen JH, Anton-Culver H, Shore RE, Boice JD, Jr., Berkowitz GS, Gatti RA, Teitelbaum SL, Smith SA, Rosenstein BS, Borresen-Dale AL, Concannon P, Thompson WD. Study design: evaluating gene-environment interactions in the etiology of breast cancer - the WECARE study. Breast Cancer Res 2004; 6: R199-214.

88. Berns JJaA. Animal Models of Cancer. In: Selby MKaP, editor. Introduction to the Cellular and Molecular Biology of Cancer. 4 ed. New York: Oxford University Press, Inc.; 2005. p. 317-36.

89. Cardiff RD. Validity of mouse mammary tumour models for human breast cancer: comparative pathology. Microsc Res Tech 2001; 52: 224-30.

90. Frame S, Crombie R, Liddell J, Stuart D, Linardopoulos S, Nagase H, Portella G, Brown K, Street A, Akhurst R, Balmain A. Epithelial carcinogenesis in the mouse: correlating the genetics and the biology. Philos Trans R Soc Lond B Biol Sci 1998; 353: 839-45.

91. Kallman RF. The effect of dose rate on mode of acute radiation death of C57BL and BALB/c mice. Radiat Res 1962; 16: 796-810.

92. Ullrich RL, Storer JB. Influence of gamma irradiation on the development of neoplastic disease in mice. III. Dose-rate effects. Radiat Res 1979; 80: 325-42.

93. Medina D. Mammary tumorigenesis in chemical carcinogen-treated mice. I. Incidence in BALB-c and C57BL mice. J Natl Cancer Inst 1974; 53: 213-21.

94. Deome KB, Faulkin LJ, Jr., Bern HA, Blair PB. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 1959; 19: 515-20.

95. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ. Purification and unique properties of mammary epithelial stem cells. Nature 2006; 439: 993-7.

96. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. Generation of a functional mammary gland from a single stem cell. Nature 2006; 439: 84-8.

97. Ethier SP, Adams LM, Ullrich RL. Morphological and histological characteristics of mammary dysplasias occurring in cell dissociation-derived murine mammary outgrowths. Cancer Res 1984; 44: 4517-22.

98. Medina D. Preneoplastic lesions in murine mammary cancer. Cancer Res 1976; 36: 2589-95.

99. Ullrich RL, Bowles ND, Satterfield LC, Davis CM. Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation. Radiat Res 1996; 146: 353-5.

100. Okayasu R, Suetomi K, Yu Y, Silver A, Bedford JS, Cox R, Ullrich RL. A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse. Cancer Res 2000; 60: 4342-5.

101. Bosma GC, Davisson MT, Ruetsch NR, Sweet HO, Shultz LD, Bosma MJ. The mouse mutation severe combined immune deficiency (scid) is on chromosome 16. Immunogenetics 1989; 29: 54-7.

102. Priestley A, Beamish HJ, Gell D, Amatucci AG, Muhlmann-Diaz MC, Singleton BK, Smith GC, Blunt T, Schalkwyk LC, Bedford JS, Jackson SP, Jeggo PA, Taccioli GE. Molecular and biochemical characterisation of DNA-dependent protein kinase-defective rodent mutant irs-20. Nucleic Acids Res 1998; 26: 1965-73.

103. Yu Y, Okayasu R, Weil MM, Silver A, McCarthy M, Zabriskie R, Long S, Cox R, Ullrich RL. Elevated Breast Cancer Risk in Irradiated BALB/c Mice Associates with Unique Functional Polymorphism of the Prkdc (DNA-dependent Protein Kinase Catalytic Subunit) Gene. Cancer Res 2001; 61: 1820-4.

104. Ramaiah L. Of Mice, Genes, and Radiation: The Genetics of Non-Hereditary Breast Cancer Explored using the Common Laboratory BALB/c Mouse. Fort Collins, CO: Colorado State University; 2008.

Chapter 2:

Telomere Dysfunction in the BALB/c Mouse Model

Chapter 2 is part of a recently published article in *Cancer Research* [11].

SUMMARY:

BALB/c mice have been shown to be genetically susceptible to radiation-induced mammary carcinogenesis, presumably due to a variant allele of the *Prkdc* gene which encodes the protein DNA-dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PKcs has been shown to be involved in DNA double strand break repair as well as telomere end-capping. In the BALB/c model of radiation-induced mammary carcinogenesis it has been hypothesized that genomic instability is an early step in the transformation process. In this chapter the role of telomere dysfunction in the overall genomic instability is explored. It was determined that after exposure to IR mammary epithelial cells derived from BALB/cByJ mice develop elevated levels of telomere dysfunction compared to cells derived from C57BL/6 mice and that this telomere dysfunction followed the same kinetics as the general genomic instability observed previously.

INTRODUCTION:

BALB/c mice have been shown to be genetically susceptible to radiation-induced mammary carcinogenesis, presumably due to a variant allele of the *Prkdc* gene which encodes the protein DNA dependent protein kinase catalytic subunit (DNA-PKcs).

DNA-PKcs is a critical component of the mammalian double strand break repair pathway, non-homologous end-joining (NHEJ). *Prkdc*^{BALB} results in decreased protein expression, decreased DNA-PK kinase activity, and reduced DNA repair capacity [1]. In 1976, Peter Nowell suggested that cancer was a result of the clonal evolution of a single cell which acquired genomic instability, and that the instability made it possible for the cell lineage to acquire increased mutations, subject to selective pressures, and eventually became neoplastic [2]. The existence of genomic instability in most human cancers suggests that it is a key factor in carcinogenesis, however, it is unclear if genomic instability plays a causal role in carcinogenesis or if it is the effect of some other causal agent [3].

In the BALB/c model of radiation-induced mammary carcinogenesis it has been hypothesized that genomic instability is an early step in the transformation process. This is supported by a study where mammary epithelial cells from BALB/c and C57BL/6 mice were isolated, irradiated *in vitro* with 3Gy γ-irradiation, and then analyzed for cytogenetic aberrations every three to four population doublings for 35 days [4]. The data obtained in this study showed that both populations start with a high level of both chromosome and chromatid aberrations [4]. The frequency of aberrations decreases with each cell cycle and by population doubling eight to 12 they reach background levels [4]. From that point on C57BL/6 cells maintained aberration frequencies of unirradiated control cells. Interestingly, cells from BALB/c mice showed increasing frequency of both chromosome and chromatid aberrations beginning at about population doubling number 16 and increasing until population doubling number 28, which was the endpoint of this study due to the replicative lifespan of these cells in culture [4]. It was further demonstrated that in the BALB/c mouse model, this radiation-induced cytogenetic instability can arise *in vivo* in mammary cells that are left *in situ* for one, four or 16 weeks prior to extraction and culturing. Under these conditions the mammary cells exhibited high levels of cytogenetic abnormalities at all time

points assayed [5]. These data make a strong argument for the involvement of radiation-induced genomic instability in the BALB/c model of radiation-induced mammary carcinogenesis.

Telomeres, first described by Muller in 1938, are unique structures at the ends of linear chromosomes [6]. We now know that telomeres consist of short tandem repeats which end in a 3' single-stranded G-rich overhang, that is roughly 100 bases long [6, 7]. In vertebrates the telomeric repeat sequence is $(TTAGGG)_n$ [8]. The number of repeats varies by species, and in humans is estimated to be 5-10kb, while in mice they are roughly 50kb in length [8]. It is well accepted that if telomeres are sufficiently short or dysfunctional in some other way, it can lead to chromosomal instability [8]. In fact, in human breast cancer telomere crisis has been shown to occur at the transition between ductal hyperplasia and ductal carcinoma *in situ* and coincide with a large increase in observed genomic instability in the tissue [9].

It was originally believed that long telomere sequence was sufficient to protect chromosomes from aberrant fusions and loss of genetic material; however, it has become clear that this block of telomere sequence combines with telomere associated proteins, to achieve its protective functions [6]. As part of this DNA–protein complex in mammals, the terminal single stranded overhang is looped back and invades the duplex telomeric DNA, creating a "t-loop" [6]. The structure of the "t-loop" is maintained by the proteins TRF1 and TRF2 [8]. Interestingly, in addition to these and other telomere associated proteins, proteins normally involved in DNA repair pathways have been shown to be involved in the creation or maintenance of telomeres [6]. It has previously been shown that the NHEJ proteins Ku70 and Ku80 are able to bind to telomeric DNA [6].

In studies using DNA repair deficient mouse cells, it was determined that cell lines expressing normal DNA repair capacity never developed spontaneous telomeric-fusions [6].

However SCID cells showed telomere fusions at a rate significantly higher than background levels [6]. As discussed in the previous chapter, SCID cells carry a truncating mutation in the coding region of *Prkdc* that results in degradation of the DNA-PKcs protein and severely deficient DNA repair capacity. These results suggest that DNA-PKcs plays an important role at mouse telomeres, in some way protecting the ends from fusion [6]. Importantly, the fluorescence *in situ* hybridization (FISH) signals between these proficient and deficient cell lines were equally strong, suggesting that the problem with the telomere was not loss of telomeric sequence but improper capping [6].

To investigate the end-capping of telomeres, Bailey et al. used dominant-negative TRF2 mutant cells [10]. The uncapped telomeres are identified as DSBs and "repaired" via NHEJ, resulting in covalent end-to-end ligations that preserve telomeric DNA at the ligation site [10]. Importantly, these fusions were chromatid-type fusions, suggesting that they occurred only after DNA replication [10]. This suggests that in order to replicate the DNA, the structure and protein associations which protect the telomere must be disassembled to allow replication and then reassembled properly to protect the chromosome through the rest of the cell cycle [10]. The fact that none of the chromatid-chromatid fusions were between sister chromatids suggests that the processing of replicated telomeres is different for leading and lagging strand telomeres, and that in this particular model one is defective while the other method is functional [10]. Using Chromosome Orientation-FISH (CO-FISH), Bailey et al. were able to determine that in SCID cells, which have deficient DNA-PKcs, 100% of telomeric fusions were leading-to-leading strand fusions [10]. This implies that DNA-PKcs is specifically required for postreplicative processing of the leading strand telomeres.

DNA-PKcs protein is completely degraded in SCID cells, resulting in a null phenotype; however, in cells derived from BALB/c mice the protein level is decreased but still present. Thus, the Bailey lab set out to determine if the BALB/c variant *Prkdc* had any

effect on telomere function or stability. In these experiments SCID and BALB/c $p53^{-/-}$ fibroblasts were analyzed for telomere aberrations after exposure to 1 or 2Gy γ-irradiation. CO-FISH was again used to show the involvement of leading strand telomeres in the resulting aberrations [11]. As shown earlier with SCID cells, the telomeric uncapping phenotype seen in BALB/c cells is expressed as telomere-DSB fusions, not as spontaneous telomere-telomere fusions [11]. Spectral karyotyping (SKY) was combined with telomere CO-FISH (SKY-CO-FISH) in order to paint the chromosomes and illustrate the presence of chromosomal translocation breakpoints at the site of the telomere-DSB fusions. In addition, SKY analysis determined that the end-capping problem was not chromosome specific [11].

Short telomeres have been shown to cause chromosomal instability which can lead to breast cancer in humans [12]. The BALB/c mouse model has been shown to develop radiation-induced genomic instability as an early step in the development of mammary cancer. It was therefore hypothesized that the telomere end-capping problem found in cells derived from BALB/c mice, as a result of defective DNA-PKcs, was the mechanism of induction of radiation-induced genomic instability.

It was previously established that mammary epithelial cells isolated from BALB/c mice develop radiation-induced genomic instability after about 16 population doublings in culture, while those from C57BL/6 mice do not [4]. However, because the earlier telomere studies were carried out using fibroblasts, the first analysis of BALB/c cells vs. C57BL/6 cells was also done using fibroblasts, this time, primary mammary fibroblasts. There was a significant increase in the frequency of telomere-DSB fusions in cells derived from BALB/c mice as compared to those from C57BL/6 mice [11]. Interestingly, the frequency of telomere-DSB fusions observed in BALB/c derived cells exceeded that of dicentric chromosomes, which suggests that after insult, telomere-DSB fusions are relatively common events in DNA-PKcs deficient cells [11]. BALB/c mice are specifically susceptible to

radiation-induced mammary cancer, but it has yet to be determined if the telomere dysfunction observed in DNA-PKcs deficient cells is also seen in the mammary epithelial cells.

RESULTS:

It was hypothesized that telomere dysfunction was the mechanism of genomic instability in the mammary epithelial cells derived from BALB/c mice. To test this hypothesis primary mammary epithelial cells were isolated from 12-week old virgin female BALB/cByJ and C57BL/6 mice. These cells were irradiated *in vitro* and plated for culture. Cells were examined for telomere dysfunction every three population doublings from 0-18. Doubling times were previously determined to be roughly 30 hours for BALB/cByJ, and 38 hours for C57BL/6 cells [4]. Cells were incubated with BrdU for 24 hours and with Colcemid for 6 hours prior to harvesting.

As discussed earlier, BALB/c mammary epithelial cells develop radiation-induced genomic instability after about 16 population doublings [4]. However the mechanisms responsible for this instability are unknown. Here cells were analyzed every three population doublings for telomere-telomere and telomere-DSB fusions. Importantly, telomere-telomere fusions remained baseline for cells derived from both C57BL/6 and BALB/cByJ mice, suggesting that the uncapping phenotype observed in BALB/cByJ-derived cells is expressed only after insult and not as spontaneous telomere-telomere fusions. However, as shown in Figure 2.1, the frequency of telomere-DSBs increased around population doubling 12 and remained elevated for the remainder of the experiment for BALB/cByJ-derived cells while remaining at baseline levels for C57BL/6-derived cells. These data show that after exposure to IR mammary epithelial cells derived from BALB/cByJ mice develop elevated levels of telomere dysfunction compared to cells derived from C57BL/6 mice. It also shows that the

timing of increased telomere-DSB fusions coincides with the increase in overall chromatid aberrations as shown by Ponniaya et al. [4]. The presence of telomere-DSB fusions but not telomere-telomere fusions suggests that the telomere uncapping phenotype does not cause the delayed instability seen in BALB/c mammary epithelial cells but certainly contributes to the ongoing genomic instability.

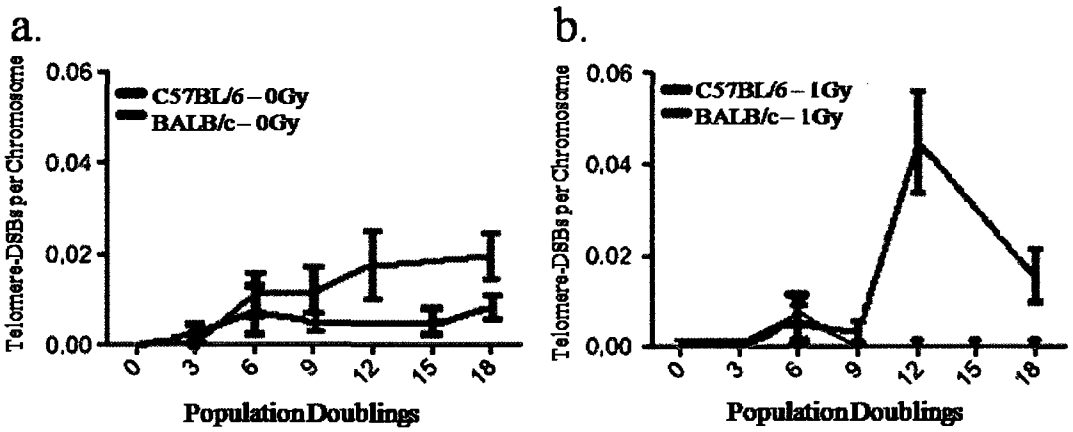


Figure 2.1. Ongoing telomere instability is present in BALB/cByJ mammary epithelial cells. Elevated levels of telomere-DSB fusion, indicative of ongoing telomere dysfunction, are evident in BALB/cByJ but not in C57BL/6. **a.** this ongoing telomere instability can be seen in unirradiated BALB/cByJ cells as a gradual increase in telomere-DSB fusion compared with C57BL/6. **b.** the progeny of irradiated BALB/cByJ cells show a striking increase in telomere-DSB fusion compared with C57BL/6 at 12 PDs. Adapted from Williams et al., 2009 [11].

DISCUSSION:

In 1941, McClintock first described the breakage-fusion-bridge cycle, where broken chromosomes are fused together to "repair" the damage, but during mitosis the different chromosomes are pulled apart, eventually breaking the DNA again [13]. This results in daughter cells that contain newly broken chromosomes, which again fuse together, beginning the cycle over. In this model the breakage-fusion-bridge cycle occurs with each cell cycle and creates long term genomic instability. It has been shown that cells expressing dysfunctional DNA-PKcs have problems properly capping the leading strand telomeres after

DNA replication, and that these improperly capped telomeres are identified as DSBs by the cells' DNA repair machinery. In mammary epithelial cells from BALB/cByJ mice that have been exposed to IR, the DSBs caused by the radiation often combine with the uncapped telomeres resulting in misrepair and a telomere-DSB fusion.

The timing of telomere-DSB fusions formed after irradiation seems to coincide well with the chromatid instability observed in previous studies. This suggests that the telomere uncapping problem does not cause the chromosome aberrations but instead may help drive forward the ongoing genomic instability observed in these cells. If the telomere capping problem were causative of genomic instability on its own, then the cells from BALB/cByJ mice and other cells with defective DNA-PKcs would exhibit inherent instability. This does not seem to be the case, possibly because in unperturbed cells the uncapped telomeres are simply fused to other uncapped telomeres, and in the breakage-fusion-bridge cycle they just are broken apart again, leaving plenty of telomere sequence behind. In addition, after IR exposure, it would seem that the telomere aberrations, especially telomere-DSB fusions, would simply remain a very frequent event, rather than initially be frequent and then decline to background levels after a few cell divisions. At this time it is clear that telomere dysfunction plays an important role in the ongoing genomic instability observed in BALB/cByJ-derived mammary epithelial cells after exposure to IR but the mechanisms responsible for the delayed instability remain to be elucidated.

MATERIALS AND METHODS:

Detailed protocols can be found in Appendix A.

Animals:

The animals used in these experiments were 12 week old virgin female BALB/cByJ and C57BL/6J mice obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Colorado State University Laboratory Animal Resources Painter Center.

Cell Dissociation:

Five 12-week-old mice of each strain were euthanized via charged tank CO_2 euthanasia and mammary glands number four and five were surgically removed as previously described [4]. Mammary epithelial cells were isolated as previously described with slight alterations [4]. Briefly, glands were minced with scalpels and dissociated in a 50ml conical centrifuge tube with M-199 medium supplemented with 200 units/ml Collagenase Type III (Worthington #4183) and ~4.7 units/ml Neutral Protease (Dispase Type 1; Worthington). Tissue was incubated for two hours in a shaking water bath at 37°C and 125 rpm, until very cloudy and homogeneous. Cells were then washed five times in DMEM containing 5% FBS and centrifuged at 1500 rpm for 5 minutes. Fibroblasts were then removed by plating cell suspension on a tissue culture plate with DMEM containing 5% FBS for 60 minutes and allowing fibroblasts to attach. The supernatant containing the epithelial cells was collected and pelleted. The cells were resuspended in 1-2ml Mouse Primary Cell Media and counted with a Coulter Counter (Beckman Coulter Z-2 Series, Fullerton, CA). Each cell suspension was then split into three separate tubes, one for the 0Gy control, one for 0.5Gy and the last for 1.0Gy irradiation. After irradiation cells were seeded onto collagen-coated 60mm dishes at a density of approximately $1.3x10^5$ cells/dish for control cells and $2.6x10^5$ cells/dish for irradiated cells.

Irradiation:

γ -ray exposures were delivered at a dose rate of 3.9 Gy/min in a calibrated, sealed source Mark 1 ^{137}Cs γ-irradiator (J.L. Sheperd and Associates).

Cell Culture:

Cells were maintained under 10% CO_2 at 37°C. Cells were plated in Mouse Primary Cell Media with 5% FBS and allowed to attached for 12-24 hours. Then media was replaced with fresh Mouse Primary Cells Media containing 2% FBS. Media was changed every three days. Plating efficiency is 30-50% depending on the isolation. Cells were passaged when epithelial colonies were growing but not yet touching. All populations were maintained in culture for 29 days, which was approximately 21 population doublings for BALB/cByJ cells and 18 populations for C57BL/6J cells.

Cytogenetic Analyses:

Mammary epithelial cells derived from each mouse strain were harvested every three population doublings for the duration of the experiment as described previously [11]. Briefly, for each time point cell cultures were treated with 10μM BrdU for 24 hours and 0.04μg/ml Colcemid for six hours. Cells were then harvested using the passaging protocol and treated for making metaphase spreads. Slide preparation, telomere CO-FISH, and image analysis were performed by Dr. Eli Williams as previously described [11].

LITERATURE CITED:

1. Okayasu R, Suetomi K, Yu Y, Silver A, Bedford JS, Cox R, Ullrich RL. A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse. Cancer Res 2000; 60: 4342-5.

2. Nowell PC. The clonal evolution of tumor cell populations. Science 1976; 194: 23-8.

3. Bau DT, Fu YP, Chen ST, Cheng TC, Yu JC, Wu PE, Shen CY. Breast cancer risk and the DNA double-strand break end-joining capacity of nonhomologous end-joining genes are affected by BRCA1. Cancer Res 2004; 64: 5013-9.

4. Ponnaiya B, Cornforth MN, Ullrich RL. Radiation-induced chromosomal instability in BALB/c and C57BL/6 mice: the difference is as clear as black and white. Radiat Res 1997; 147: 121-5.

5. Ullrich RL, Davis CM. Radiation-induced cytogenetic instability in vivo. Radiat Res 1999; 152: 170-3.

6. Bailey SM, Meyne J, Chen DJ, Kurimasa A, Li GC, Lehnert BE, Goodwin EH. DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. Proc Natl Acad Sci U S A 1999; 96: 14899-904.

7. Bailey SM, Cornforth MN, Ullrich RL, Goodwin EH. Dysfunctional mammalian telomeres join with DNA double-strand breaks. DNA Repair (Amst) 2004; 3: 349-57.

8. Bouffler SD, Blasco MA, Cox R, Smith PJ. Telomeric sequences, radiation sensitivity and genomic instability. Int J Radiat Biol 2001; 77: 995-1005.

9. Chin K, de Solorzano CO, Knowles D, Jones A, Chou W, Rodriguez EG, Kuo WL, Ljung BM, Chew K, Myambo K, Miranda M, Krig S, Garbe J, Stampfer M, Yaswen P, Gray JW, Lockett SJ. In situ analyses of genome instability in breast cancer. Nat Genet 2004; 36: 984-8.

10. Bailey SM, Cornforth MN, Kurimasa A, Chen DJ, Goodwin EH. Strand-specific postreplicative processing of mammalian telomeres. Science 2001; 293: 2462-5.

11. Williams ES, Klingler R, Ponnaiya B, Hardt T, Schrock E, Lees-Miller SP, Meek K, Ullrich RL, Bailey SM. Telomere dysfunction and DNA-PKcs deficiency: characterization and consequence. Cancer Res 2009; 69: 2100-7.

12. Shen J, Terry MB, Gurvich I, Liao Y, Senie RT, Santella RM. Short telomere length and breast cancer risk: a study in sister sets. Cancer Res 2007; 67: 5538-44.

13. McClintock B. The Stability of Broken Ends of Chromosomes in Zea Mays. Genetics 1941; 26: 234-82.

Chapter 3:

Isolation and *In Vitro* Growth of Murine Mammosphere Cultures

SUMMARY:

In this chapter the development and optimization of protocols for the isolation, propagation and *in vitro* growth of murine-derived mammospheres is discussed. Techniques were developed for high density suspension cultures, growth in basement membrane extract, and clonal density suspension cultures. The growth characteristics of mammospheres grown under each of these conditions are also described.

INTRODUCTION:

The existence of mammary stem cells in mice has been hypothesized since DeOme first developed the mammary gland transplantation system in the 1950s [1]. This system takes advantage of the normal physiological development of the recipient mouse at puberty to cause growth and differentiation of the transplanted mammary cells. Transplantation studies in mice have consistently estimated the stem cell frequency to be between 1:1000 and 1:2000 mammary cells, this seems to be fairly consistent between mouse strains, although results vary between laboratories [2, 3].

Stingl et al. (2006) recently showed that the mammary outgrowths derived from these transplantation studies are clonal by mixing and transplanting mammary cells expressing

either green fluorescent (GFP) or cyan fluorescent protein (CFP). The majority of the resulting outgrowths were GFP^+ or CFP^+, but very few expressed both proteins [3]. Recently two groups have shown definitive proof that there are mammary stem cells by transplanting one single cell into a cleared fat pad and verifying its ability to produce a mammary outgrowth [3, 4]. This transplanted mammary gland could then be dissociated and transplanted again into recipient mice. These experiments showed that the stem cell can undergo at least 10 symmetrical self-renewal divisions [3, 4]. Finally, Shackleton et al. followed the single cell derived mammary gland through pregnancy to verify that it could in fact produce all of the cells required for a functional mammary gland [4]. Together, these studies have proven that in the mammary gland transplantation assay the donor cell population contains stem cells, one of which survives and proliferates to create the resulting mammary tree.

Using the mammary transplantation system Ullrich et al. showed that mammary cells derived from BALB/c mice are inherently susceptible to the carcinogenic effects of ionizing radiation [5]. Exposure to 1Gy of γ-radiation resulted in mammary tumors in 12% of the recipient mice and 40% incidence of ductal dysplasias, which are believed to be precancerous lesions [5]. It follows that if stem cells are responsible for the development of normal mammary gland outgrowths resulting from these mammary transplantation experiments, then stem cells are also responsible for the development of abnormal outgrowths. We therefore hypothesize that the mammary stem cells derived from BALB/c mice are more susceptible to the damaging effects of ionizing radiation then mouse strains that are less susceptible to breast cancer.

Although the existence of mammary stem cells has been accepted since DeOme first developed the mammary cell transplantation system, it has only been in the last few years

that researchers have tried to identify and isolate these stem cells and culture them *in vitro*. Utilization of mammary stem cells in research is still in its infancy, far behind the identification and use of other stem cells, especially hematopoietic stem cells. At this time, there are a number of labs trying to isolate and propagate mammary stem cells *in vivo* and *in vitro,* but it is still at a stage where the techniques used are highly individualized. The vast majority of mammary stem cell research is devoted to trying to identify and isolate the specific stem cells, either *in situ* in a mammary gland, or by using Fluorescence Activated Cell Sorting (FACS) to isolate the cells with stem-like properties.

Different researchers have used different cell surface markers in combination with cytometric sorting to separate out populations of cells that contain stem cell-like properties. FACS analysis of bone marrow cells treated with Hoechst dye show a small population of cells which efflux the dye very efficiently; this distinct population has been termed the "side population." It has been shown that the cells from this "side population" are capable of repopulating the hematopoietic system and are thus hematopoietic stem cells. This has been adapted for use with other tissues, however in mouse mammary cells the dye is toxic and thus limits its usefulness as an isolation technique. Some have tried pulse labeling with BrdU during periods of growth and then looking for BrdU labeling at a much later time. This is based on the hypothesis that stem cells are usually quiescent and will retain the BrdU much longer than more differentiated cells [6]. Still other groups have tried to use FACS for specific cell surface markers such as Sca-1, $CD24^{-}$, $CD24^{+}$, $CD44^{+}$, $CD29^{hi}$, PrP^{medium}, and $CD49f^{+}$ to name a few [2-4, 6-9]. None of the methods developed to date result in a pure mammary stem cell population. They all result in a population that has been enriched for stem cells, when compared to whole mammary cell isolations, but also containing many progenitor and differentiated cells.

Dontu et al. developed a technique based on the neural stem cell work by Renyolds and Weiss, who were able to grow and propagate neural stem cells as nonadherent neurospheres [10, 11]. Dontu et al. expanded this technique to work with human mammary stem cells isolated from reduction mammoplasties [10]. The principle behind this technique is that differentiated cells require extracellular interactions to survive, while stem cells can survive and proliferate under anchorage-independent conditions [10]. They were able to show that if single cells from mammary glands were cultured under anchorage-independent conditions, the vast majority of the cells died from anoikis, a specific kind of apoptosis resulting from lack of cellular interactions [12]. However, small subsets of the mammary cells were able to survive and in fact proliferate to produce small spheres of cells, which they termed "mammospheres" [10].

Although Hoechst dye is relatively toxic to murine mammary cells, Welm et al. used Hoechst dye efflux to sort the "side population" from mouse mammary epithelial cells, and showed that these cells were capable of repopulating a mammary gland upon transplantation into a recipient mouse [6]. Using human mammary cells, Dontu et al. showed that only cells from the "side population" were capable of mammosphere formation [10]. Thus, the cells capable of mammosphere formation and the cells capable of mammary gland formation are found in the same subpopulation of mammary epithelial cells.

Dontu et al. showed very clearly that a single mammary cell can produce a mammosphere [10]. First, they plated a single cell in each well of a 96 well plate and determined that one in 250 cells could generate a mammosphere. In addition, they plated many different concentrations of cells and were able to show that plating cells at 1,000 cells/ml also gave a mammosphere formation efficiency of four mammospheres. They therefore speculated that if cells were seeded at 1,000 cells/ml, then the resulting

mammospheres were indeed clonal in origin because the mammosphere formation efficiency was the same as that for plating a single cell per well. To further test that mammospheres generated after seeding cells at 1,000 cells/ml were clonally derived, they transfected cells with retroviral vectors capable of expressing either red or green fluorescent proteins and then mixed equal ratios of red and green cells. The mixed samples were then seeded at 1,000 cells/ml and allowed to form mammospheres. Upon analysis they determined that under these plating conditions, the vast majority of resulting mammospheres expressed only red or green fluorescent protein, not both. This suggests that the mammospheres were derived from single cells, because mammospheres resulting from aggregation would be primarily mixed colonies expressing both red and green proteins [10]. Finally, they took samples from three different mammosplasty patients, and mixed the cells at equal ratios. These mixed cells were then plated at 1,000 cells/ml and allowed to form mammospheres. Individual mammospheres were then collected, and PCR genotyping was performed. These analyses showed that 83% of the mammospheres resulting from these experiments were genotypically homogeneous, and thus clonal in origin [10]. These data show that if human mammary cells are plated at a density of 1,000 cells/ml the resulting mammospheres will be clonally derived.

Analysis of the mammospheres showed that they were enriched for cells possessing the functional characteristics of stem cells [10]. One of the main defining features of the adult stem cell is its ability to self-renew. Most cells produce two identical daughter cells upon division which go on to terminally differentiate; stem cells can perform asymmetrical division, where one daughter cell can go on to differentiate and the other remains a stem cell [10]. Using mammospheres derived from human cells, Dontu et al. showed that mammospheres could be passaged at least five times, suggesting that they possess self-renewal capacity and very long if not limitless replicative potential [10]. They reasoned that

if mammospheres were produced by progenitor cells then the plating efficiency and size of the mammosphere would both decrease with each passage. However, they found very consistent size and plating efficiency at each passage. These data suggest that the cells that produce the mammospheres are capable of self-renewal.

The second defining feature of stem cells is the ability to produce all of the cell types that make up the specific tissue. For the mammary gland the stem cell must be able to produce the luminal epithelial cells and the myoepithelial cells. The luminal epithelium consists of ductal epithelial cells, which line the ducts of the mammary gland, and alveolar epithelial cells which are capable of milk production. The myoepithelial cells are contractile cells that line the outside of the ductal tree; these cells contract to move the milk through the gland. Analysis of mammosphere populations has shown that 68% of cells derived from primary human mammospheres and 98% of cells derived from secondary mammospheres are capable of producing differentiated colonies containing both luminal and myoepithelial cell lineages [10]. In comparison, only 8% of human primary mammary epithelial cells were capable of producing bi-lineage colonies [10]. Dontu et al. also showed that the majority of bipotent cells derived from mammospheres were able to differentiate along the third lineage, alveolar epithelial cells. Finally, they used basement membrane extract cultures to induce differentiation of the mammary cells to produce mammary gland structures *in vitro*. They determined that one out of 250 cells derived from mammospheres could form combined ductal and acinar development, similar to the fully developed mammary gland. These data show that the cells derived from mammospheres are enriched for cells capable of differentiation along all three mammary cell lineages, suggesting that they are stem cells or very high progenitor cells.

The main goal of this project was to determine the effects of radiation on mammary stem cells derived from the BALB/c mouse. It was hypothesized that the mammary stem cells are the tumor-initiating cells in the BALB/c model of radiation-induced mammary cancer. Development of the murine mammosphere tissue culture system was predicted to allow the characterization and manipulation of the stem cell population. This chapter will discuss the development of the murine mammosphere tissue culture system, including isolation of mammary cells for high density mammosphere cultures, the *in vitro* growth characteristics of high density mammospheres grown in suspension cultures and in stationary cultures, as well as isolation and growth characteristics of clonally derived mammosphere suspension cultures.

RESULTS:

At the onset of this project, there was very little data published on how to grow mammospheres derived from murine mammary tissue. Thus, prior to testing the effects of radiation on mammospheres derived from BALB/c mice, the isolation and tissue culture procedures needed to be established. The basic mammary stem cell isolation protocol was obtained from Stem Cell Technologies and used with adaptations.

The number of cells plated after isolation is a crucial detail. Most groups count with a Hemocytometer using Trypan Blue exclusion as an indicator of viability, but none of the literature contained information on the size of the cells being isolated or the purity of the resulting population. To determine the size of the mammary cells obtained using the stem cell isolation protocol, a Coulter Counter was used. Multiple samples were counted with gates set in overlapping intervals covering 2μm-20μm. The resulting histograms consistently showed only two peaks, one at 4μm and one at 6μm, as seen in Figure 3.1.

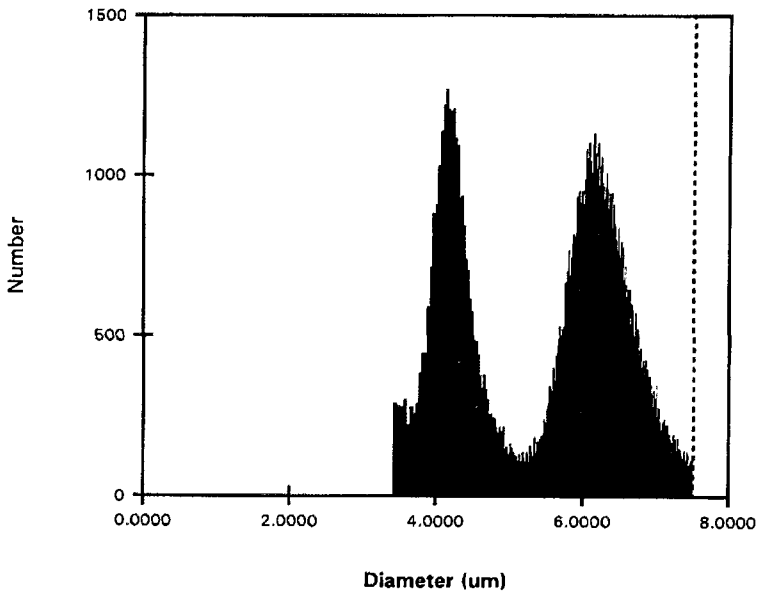


Figure 3.1. Coulter Counter histogram from mammary cell isolation.

The peaks at 4μm and 6μm changed in magnitude relative to each other depending on the isolation. It was determined that the peak at 6μm contains cells derived from the lymph node. If the lymph nodes are removed very cleanly prior to tissue dissociation only the 4μm peak is visible, an example is shown in Figure 3.2. One advantage for using an automated counting system like the Coulter Counter is the ability to differentiate between cells that are 4μm and those that are 6μm, which is nearly impossible using a hemocytometer. The histogram also provides a good indication of how completely the lymph nodes were removed and thus the purity of the isolated cell population.

The density at which to plate mammary cells for mammosphere formation depends on the desired end point. High density mammosphere cultures can provide qualitative information on quality of the isolation and they are necessary for "conditioning" media required for clonal density cultures, in addition to a variety of other endpoints where clonality is not necessary. For high density mammosphere cultures 1-3 x 10^6 cells are seeded onto 60mm Ultra-Low Attachment plates. Although plating this many cells results in some

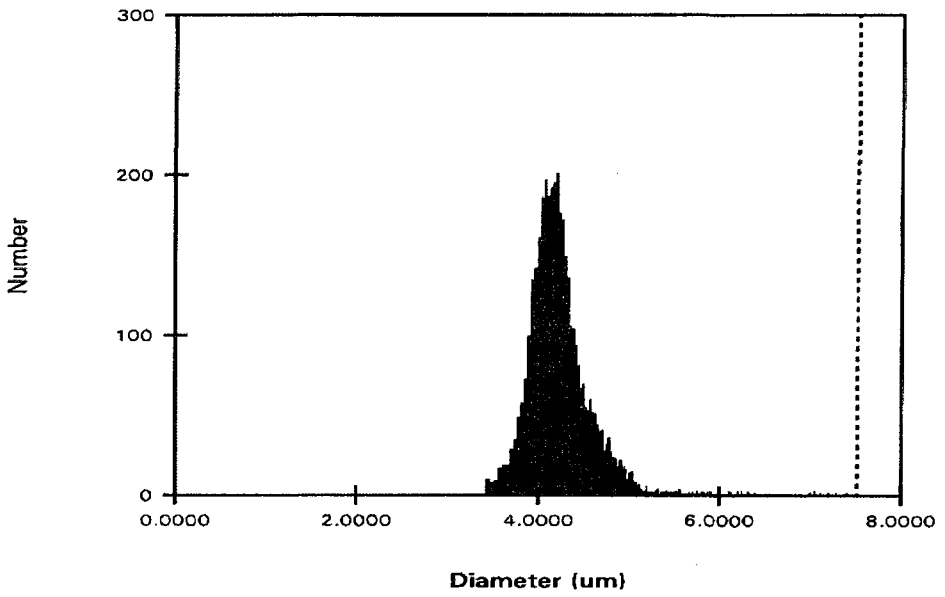


Figure 3.2. Coulter Counter histogram of mammary cell isolation after complete removal of lymph nodes.

aggregation, if too few cells are plated the resulting mammospheres will be significantly growth retarded. The mammary cells seem to require some secreted factors which have yet to be identified. This is highlighted in later discussions of clonally derived mammospheres, which require media that has been used for high density mammosphere cultures for two days prior to use in clonal cultures.

Quality of mammosphere growth is a very subjective topic and there are no published guidelines. It was therefore important to create guidelines that could be used to assess the fitness of mammosphere cultures. For these mammosphere cultures, media should be changed every four days. If the isolation and media supplementation protocols are followed carefully, the resulting high density mammosphere suspension cultures should develop according to the following semi-quantitative guidelines. Upon plating, the cell suspension should be filled primarily with single cells. These are high density cultures so a little clumping does not matter. On day one, the cells should have settled to the bottom of the

plate. Sometime there will be small clumps visible, but usually it is not possible to tell if the cells are alive or dead. By day two, clumps should definitely be forming, though there will still be a lot of single cells; these are most likely in the process of dying or already dead. Day four after isolation there should be a large population of obvious mammospheres. There will still be single cells, but many will have broken down into random debris. On day four, mammospheres should start looking symmetrical and have deliberate spherical structure, no longer just clumps of cells. There should also be many mammospheres approaching or surpassing 100μm in diameter. On day five the mammospheres should look very round, and hopefully will have had a growth spurt in response to the fresh media. Due to the media change, there should be very few single cells in the plate. By day eight there should be a few mammospheres that have grown to at least 200-250μm. If these large mammospheres are not seen then something is wrong with the culture. Figure 3.3 shows a representative sample of a high density culture of murine mammospheres that is growing very well on day seven. It is important to point out the many different sizes of mammospheres shown in Figure 3.3. This

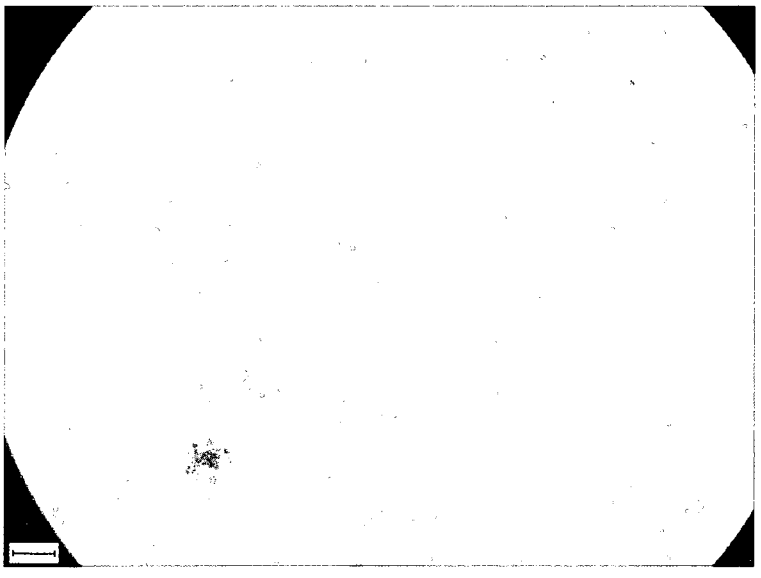


Figure 3.3. Scale bar = 100μm; 4x magnification. Representative photograph of high density mammosphere suspension cultures. Taken on day 7.

size variation was seen in all healthy high density non-adherent mammosphere cultures throughout the entire project. Interestingly, there was nothing in the published literature that discussed this huge variety of mammosphere sizes. It was hypothesized that the size of the mammosphere was determined by the identity of the mammosphere-initiating cell. It was further hypothesized that only the large mammospheres were produced by mammary stem cells and that the smaller sizes were derived from various progenitor cells and differentiated cells.

Figure 3.4 below shows some representative photos of high density mammospheres of known size. Again they illustrate how symmetrical the majority of the mammospheres should be and the size variation observed in the high density mammosphere cultures.

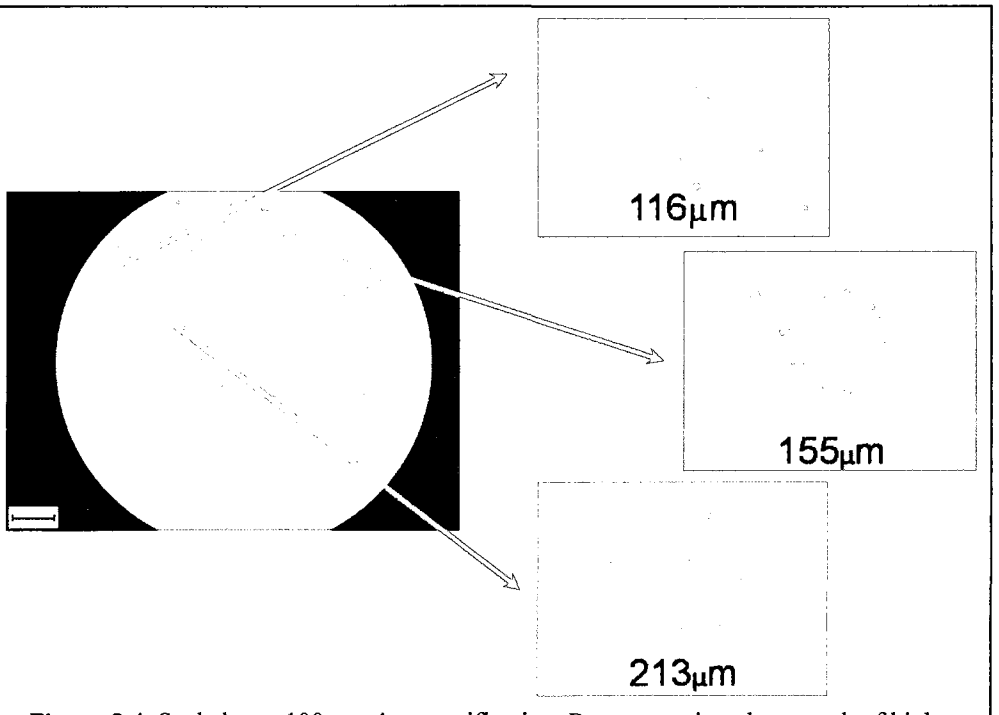


Figure 3.4. Scale bar = 100µm; 4x magnification. Representative photograph of high density mammospheres of known size.

Figure 3.5 shows a photograph of mammospheres that have been growing continuously for 15 days. Though there is no definitive proof, it is hypothesized that this photograph depicts mammospheres that are in the process of clearing the lumen, as the mammary ductal system

does. The mammospheres are capable of clearing the lumen and also filling it with milk when stimulated with prolactin. Although this was never attempted during the work discussed here, the timing correlates well with the work done by Dr. Joan Brugge (Presented at the AACR annual meeting, 2007).

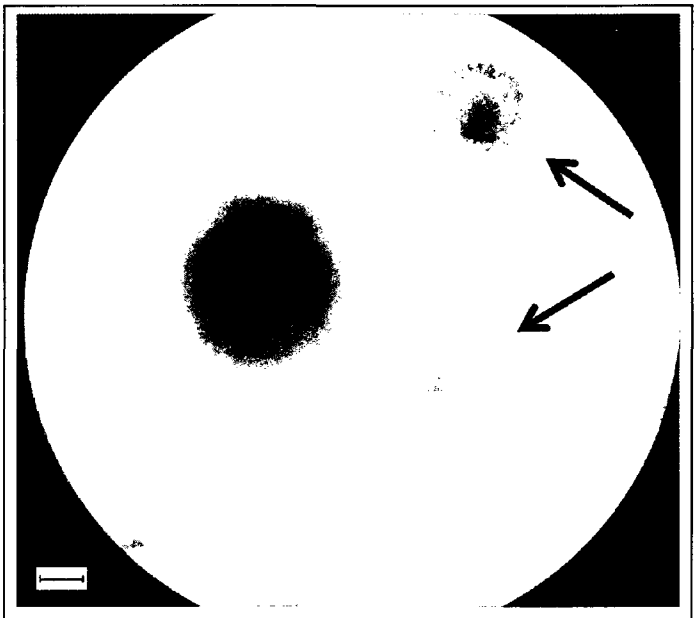


Figure 3.5. Scale bar = 100μm; 4x magnification. Photograph of high density suspension culture, taken on day 15. Arrows show mammospheres in the process of clearing the lumen.

Very little was known about the growth kinetics of murine mammospheres, and it is impossible to track the growth of any single mammosphere from day to day when using suspension cultures, however this type of growth curve assay becomes possible if mammospheres are grown in basement membrane extract (BME). Commercially available BME is solubilized basement membrane that has been extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. This tumor is rich is extracellular matrix proteins, particularly laminin, collagen IV, heparan sulfate proteoglycans, and entactin/nidogen. It is a three-dimensional gelatinous substance that surrounds cells in nutrients and allows growth and differentiation in a more natural environment than tissue culture plastic or mammosphere

suspension cultures. By seeding mammary cells in BME it is possible to grow mammospheres in a semisolid setting. This prevents many of the problems associated with suspension cultures of mammospheres, particularly aggregation. In this stationary gel, the cells are able to form colonies, which are likely to be clonal even when plating at much higher density then clonal suspension cultures allow. Figure 3.6 shows some examples of primary mammospheres grown in BME for 14 days.

Figure 3.6. Scale bar = 100μm; 4x magnification. Photograph of mammospheres grown in BME for 14 days.

To determine the growth kinetics of individual mammospheres single mammary cells were plated in BME in a 24-well plate immediately after isolation. The cultures were watched carefully for mammosphere formation, after seven days it was decided that any viable stem cell had had sufficient time to produce a visible mammosphere. For this experiment a "mammosphere" was considered any cell aggregate larger than 25μm. On day seven a map of each well was created and the location of each mammosphere within the well was recorded. Each mammosphere was numbered and its diameter was measured. Every 24-hours the diameter of each of the mammospheres was measured from day seven to day 21. The growth of 220 individual mammospheres was recorded in this experiment. Figure 3.7 shows the entire set of data collected from this experiment. In this graph, each line represents

the size of one specific mammosphere on each day. These data show that 73% of mammospheres that were small at the first measurement did not grow through the remainder of the experiment. Mammospheres are very dynamic structures, Figure 3.7 highlights this fact by showing that the size of any particular mammosphere can increase, decrease or remain the same from day to day as opposed to increasing constantly. This variation in growth comes mainly from reorganization of the cells within the mammosphere, which sometimes results in a smaller, probably more tightly packed cellular organization. Additionally, some of the variation is due to human error. In this experiment it was possible to locate the same mammosphere from one day to the next; however it was not possible to measure the diameter of the mammosphere from exactly the same location each day and this resulted in slight variation in size because the mammospheres are not prefect geometric spheres.

In traditional colony formation assays, cells that are capable of producing colonies consisting of at least 50 cells are scored as viable colonies. When using the mammosphere tissue culture system as opposed to colony growth on plastic, it is not possible to determine the number of cells in the sphere by looking at it. Therefore it was important to determine what size a mammosphere must reach in order to be considered a colony. Attempts to determine cell number within each size of mammosphere were made, but were largely unsuccessful. As such, mammosphere formation data was collected based on diameter, with the plan of later determining which particular sizes of spheres to include in the data analysis.

A number of groups have stated scoring anything $\geq$60μm in diameter as a mammosphere [13, 14]. If this criterion is followed only 67 of the total 220 spheres were able to reach a diameter of 60μm by 21 days of growth. If the criterion for a mammosphere is that it reaches 60μm by day 12 (the day used in later clonal suspension culture

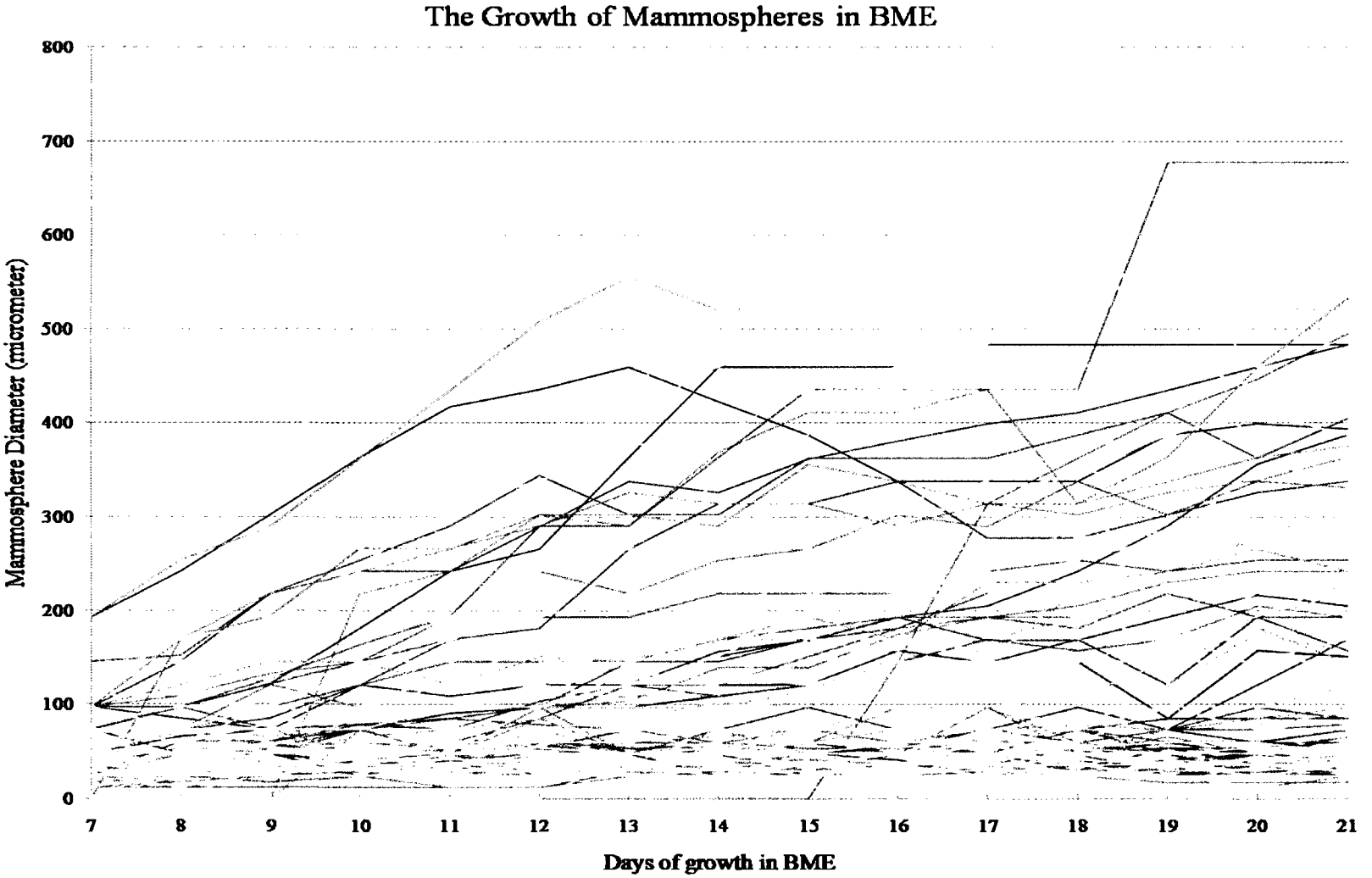


Figure 3.7. Graph of the diameter of mammospheres grown in BME for 21 days. Individual mammospheres were measured each day from day 7 to day 21. Each line represents the growth of a single mammosphere.

experiments) there are only 59 spheres. If a colony must grow to 100μm then only 49 of 220 spheres can be counted as mammospheres. Similarly, if colony growth requires a mammosphere >200μm or >250μm this limits the number of mammospheres to 36 and 29 respectively. Figure 3.8 shows the average diameter of all spheres scored as "mammospheres" based on these different size requirements. Figure 3.8 depicts that as the required size of the mammospheres increases, so does the average size of the mammospheres scored. However, when this happens the total number of mammospheres scored as colonies decreases, therefore to obtain significant data, it is very important to determine what counts as a colony prior to performing a colony formation assay.

All of the work to this point has shown that there is variation in the sizes of mammospheres that can grow. This variation is not discussed in any of the published literature, but it is found in every single isolation, in high density and clonal density suspension, and in BME cultures. It has been hypothesized that this size variation is connected to the identity of the mammosphere-initiating cell. To test this hypothesis the frequency of mammospheres belonging to each size group was determined. A plating efficiency assay, or mammosphere formation efficiency assay, was performed and the number of resulting mammospheres for each size group was counted. Rather than simply counting everything above 40 or 60μm as suggested in the literature; diameter size was divided into five groups; 50-99μm, 100-150μm, 151-200μm, 201-250μm and ≥251μm. In this experiment different concentrations of cells were plated, to determine if the size variation was simply due to agregation. It was hypothesized that if large mammospheres were a result of agregation of many smaller mammospheres then plating higher cell concentrations would result in an increased plating efficiency of large mammospheres. Figure 3.9 shows that although there is some variation, there is a definite trend showing decreasing plating

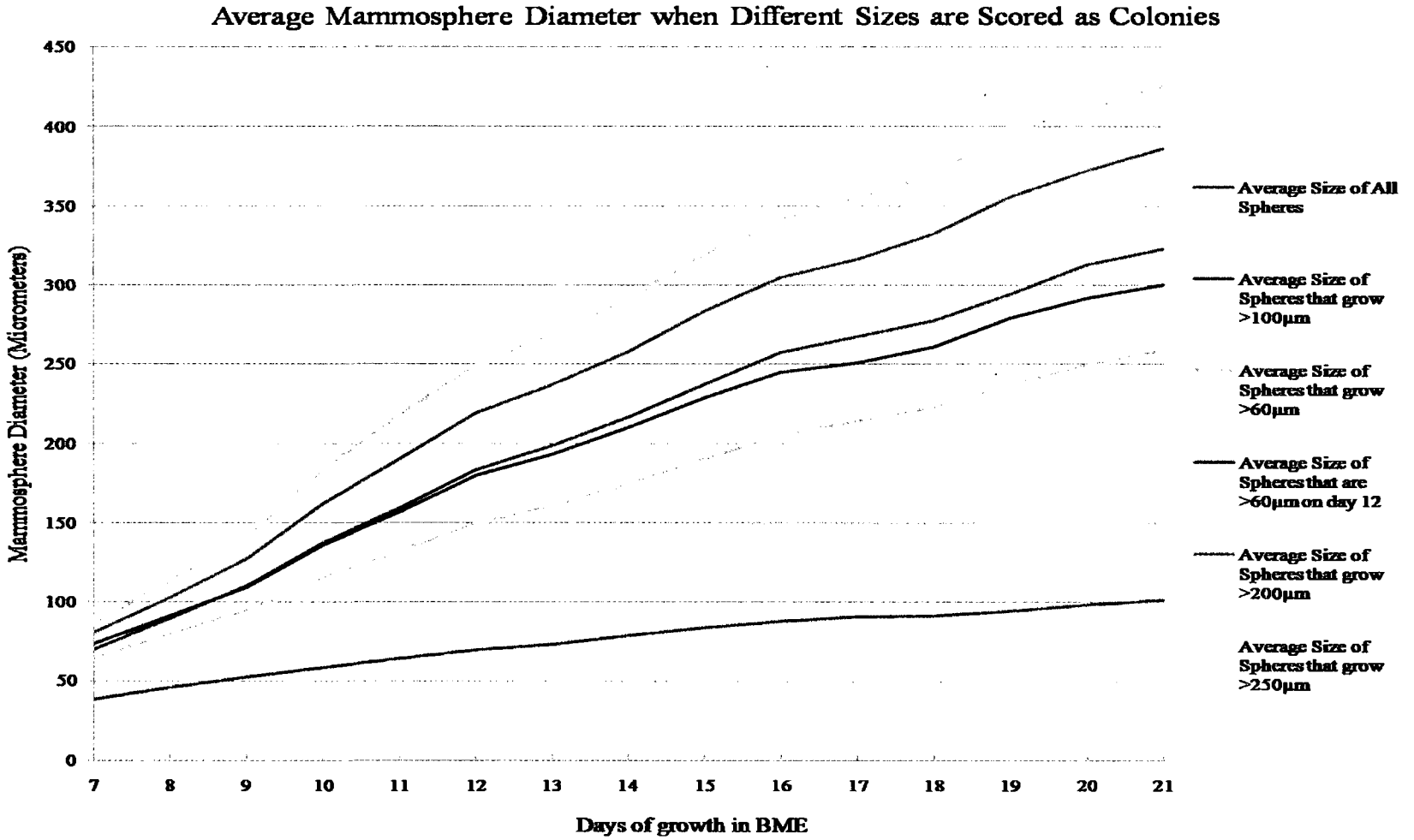


Figure 3.8. Individual mammospheres were measured each day from day 7 to day 21. The data shown here represents the average mammosphere size on each day when different guidelines are set for what counts as a mammosphere. It illustrates the difference in data obtained depending on the criteria used for the experiment.

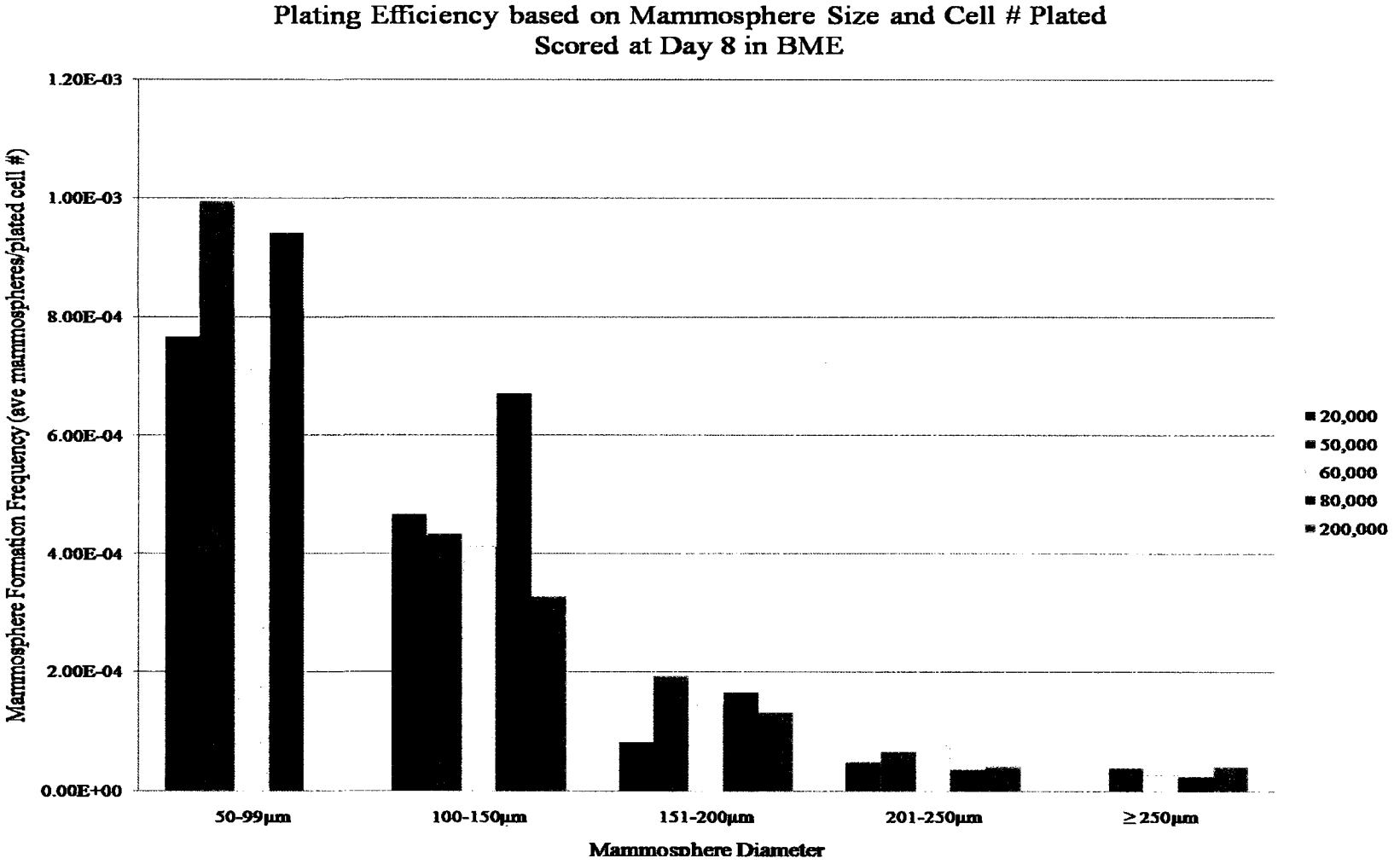


Figure 3.9. Plating efficiency for mammosphere growth based on diameter after 8 days of growth in BME. Bars represent number of single cells plated in triplicate per well of a 24-well plate. These data show that the plating efficiency is consistent regardless of number of single cells seeded. The data also show that mammospheres 50µm-150µm in diameter are very frequent in mammosphere populations while larger sizes occur much less frequently.

efficiency with increasing mammosphere diameter. The plating efficiency for mammospheres of each particular size group is consistent regardless of the number of cells seeded. It is abundently clear that mammospheres ranging from 50-150μm in diameter are far more frequent than are the mammospheres of the larger size groups. It would seem that the smaller sizes of mammospheres occur too frequently to actually represent the stem cell population, which is thought to be very rare.

After establishing protocols for the isolation and tissue culture of high density murine mammospheres, it was necessary to establish cultures of mammospheres grown at clonal density. These clonal mammospheres would be used to show that, like the human mammospheres used by Dontu et al., the murine mammospheres possess the stem cell-like functional characteristic of self-renewal and multi-lineage differentiation capacity. These clonal mammospheres would also be used to determine the radiation response of mammary stem cells by creating a radiation dose response curve. Data for a dose response curve comes from colony formation assays, which assess the ability of single cells to survive radiation exposure and proliferate to produce a colony, or in this case a mammosphere. It is commonly accepted that at least 100 colonies must grow in the unirradiated control plates in order to obtain statistically significant results from irradiated plates.

At first mammary cells isolated using the high density mammosphere isolation protocol were plated at the clonal density of 1,000 cells/ml using 1x mammosphere media, as suggested by Dontu et al. [10]. The results however were not very promising. At best this method resulted in five to ten mammospheres around 60μm in diameter per plate and no larger sized mammospheres were observed. Using mammary cells directly from isolation was not resulting in a high enough plating efficiency to use for a colony formation assay. However, Stem Cell Technologies had just developed the EasySep® Mouse Mammary Stem

Cell Enrichment Kit. The EasySep® kit involves negative selection of mouse mammary epithelial cells by removing hematopoietic and endothelial cell using biotinylated monoclonal antibodies. The biotinylated antibodies are then bound to magnetic nanoparticles and the cell suspension is passed through a series of magnets. Through this series of antibody labeling and magnetic separation the hematopoietic and endothelial cells are removed from the cell suspension leaving only epithelial cells. The epithelial cell population is then labeled with antibodies for CD24 and CD49f which mark putative stem cells. The cells expressing both proteins are then sorted from the total population using cytometric sorting. However it was decided that since the mammospheres would be grown in 60mm tissue culture dishes and not from single cells in 96-well plates, enrichment steps with the magnet would be used but the cytometric sorting would not because any unwanted differentiated cells would die from anoikis during the culturing processes. Again, the resulting cells were seeded at 1,000 cells/ml in 60mm Ultra-Low Attachment plates. This time mammospheres were grown in mixed conditioned media. Mixed conditioned media contains one part mammosphere media that has been used for culturing high density mammospheres for two days mixed with one part mammosphere media containing double the supplements to make up for any depletion or degradation of supplements in the conditioned media. Using the EasySep® enrichment kit in combination with mixed conditioned media resulted in improved plating efficiency. In fact, under these culturing conditions 1,000 cells/ml resulted in too many mammospheres per plate and aggregation became a problem.

It was determined that the optimal culturing procedure for growing clonal murine mammospheres involved seeding cells at 1,500 cells in 10ml of mixed conditioned media per 60mm dish, and allowing mammospheres to grow completely undisturbed for 12 days. This limits the amount movement in the media, which causes the mammospheres to swirl towards

the center of the plate and aggregate. Culturing cells for 12 days allows for sufficient mammosphere growth, without requiring fresh media.

Prior to beginning the work with clonal mammosphere cultures, it was hypothesized that the size of a mammosphere was directly related to the mammosphere cell of origin. However, it was not possible to prove that the large mammosphere sizes were not simply a result of the aggregation of many smaller mammospheres, until clonal mammosphere cultures were analyzed. Using the adapted EasySep® Mouse Mammary Stem Cell Enrichment kit protocol and mixed conditioned media, cultures of clonal mammospheres still developed in the range of mammosphere sizes that were previously observed in the high density suspension cultures. The clonal mammospheres grow slower than the high density cultures, probably due to the involvement of aggregation high density suspension cultures. For example, if clonal mammospheres were grown for 12 days the largest mammospheres would be around 200-250μm in diameter. If instead high density mammospheres were grown for that long, it is likely that some mammospheres would be >600μm in diameter. There is no denying that aggregation participates in the formation of mammospheres cultured at high density, just that aggregation is not the only reason for the size variation observed. Examples of different sizes of mammospheres found in clonal suspension cultures are shown in Figure 3.10.

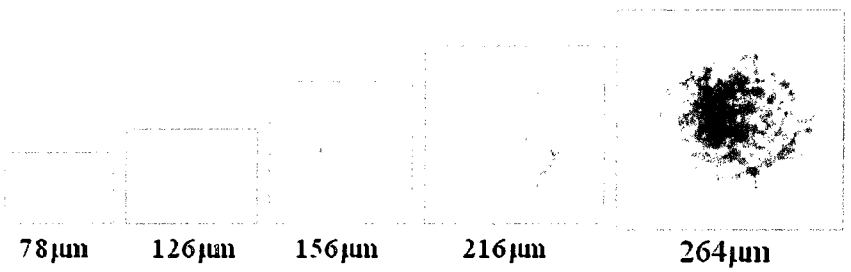


Figure 3.10. 4x magnification. Representative photographs showing the size variation seen in 1° mammospheres grown at clonal density. Taken on day 12.

DISCUSSION:

In this chapter the optimization of the protocol published by Stem Cell Technologies for the isolation of murine mammary stem cells has been discussed. For the isolation to be successful all steps must be performed carefully and quickly with cells kept on ice whenever possible. It was determined that if the lymph nodes are removed completely prior to dissociation of the mammary tissue, the resulting mammary cells will have a diameter of 4μm after the isolation. These mammary cells can then be seeded at $1\text{-}3x10^6$ cells/plate to produce high density suspension mammosphere cultures. The development of a semi-quantitative guideline for determining the health status of these high density mammosphere cultures was discussed in detail. Though somewhat subjective, these guidelines are very important for determining if something went wrong with the cell isolation or if there are problems with the media. The high density mammospheres that result from the isolation and tissue culture procedures developed in this chapter can be serially passaged using the protocol developed by Dontu et al. with great success [10]. However, the mammary cells as well as every reagent used in this project are extremely sensitive and must be mixed, stored and used exactly as stated in the Materials and Methods or the mammospheres will not grow properly.

Throughout the development of the isolation and tissue culture protocols a wide variety of different mammosphere sizes was observed. This was intriguing as no other labs using mammospheres had published anything concerning this variety of sizes. It was determined that as long as the isolation protocol produced viable cells, and all of the supplements required for the mammosphere media were functional; the mammospheres resulting after about a week of growth would vary in size from very small clumps around

40μm in diameter to large symmetrical mammospheres many hundreds of micrometers in diameter.

To determine the growth kinetics of murine mammospheres mammary cells were grown in BME and the diameter of individual mammospheres was measured every 24 hours for 14 days. The results show that although single mammary cells are capable of the proliferation required to form a mammosphere, not all spheres continue to grow. It was hypothesized that this size variation was due to the identity of the mammosphere initiating cell, and not simply a result of cellular aggregation. This hypothesis was tested by seeding different numbers of mammary cells in BME and then determining the mammosphere formation efficiency for mammospheres of different sizes. The results show that the plating efficiency for each particular size group of mammospheres was consistent regardless of cell number plated. These data suggest that the size of the mammosphere is not a result of aggregation of many smaller mammospheres, but of some innate ability of the cells within the mammosphere to proliferate. It was hypothesized that only the large mammospheres were derived from stem cells, and that the smaller sizes observed were derived from more differentiated cells. Taken together, the data presented here highlight the importance of developing criteria for what size spheres should be counted as mammospheres.

The last section of this chapter deals with the development of protocols to isolate and culture clonally derived murine mammospheres. The isolation procedure was adapted from the newly available EasySep® Mouse Mammary Stem Cell Enrichment Kit developed by Stem Cell Technologies. This isolation technique in combination with the use of mixed conditioned media has been shown to produce clonal mammospheres, in all the varieties of sizes seen in high density cultures. These clonal mammospheres can then be used for a variety of different endpoint. In the following chapters we attempt to create size criteria

which can be used to ascertain which mammospheres came from a true stem cell, as well as to analyze the effects of ionizing radiation on the mammary stem cells derived from different mouse strains.

MATERIALS AND METHODS:

Detailed protocols can be found in Appendix A.

Animals:

The majority of the animals used in these experiments were 8-12 weeks old although a few ranged in age from 6 weeks to 9 months old. All were virgin female mice bred at CSU. All mice were maintained in the Colorado State University Laboratory Animal Resources Painter Center.

Mammary Cell Isolation for High Density Mammosphere Cultures:

This protocol was adapted from one published in the product literature for Epicult-B media by Stem Cell Technologies. Briefly, mice were euthanized via charged tank CO_2 euthanasia and mammary glands number four and five were surgically removed. Tissue was then minced with scalpels and enzymatically dissociated into organoids in a 15ml conical centrifuge tube with Epicult-B basal medium supplemented with 300 units/ml Collagenase Type III, 100 units/ml Hyaluronidase, Epicult-B Supplement, Antibiotic/Antimycotic, GlutaMAX, and 5% FBS. Tissue was incubated at 37°C and 5% CO_2 until the tissue was very cloudy and homogeneous (45 minutes -1.5 hours). Cells were then washed with 10ml cold Hanks Balanced Salt Solution with 2% FBS (HBSS with 2% FBS) and pelleted at 1460 rpm (450 x g) for 5 minutes. The organoids were then dissociated into single cells using Trypsin followed by Dispase II and DNase 1 with intermittent washes of HBSS with 2%

FBS. Cells were then resuspended in 1x mammosphere media and counted using a Coulter Counter (Beckman Coulter Z-2 Series, Fullerton, CA). Cells were plated at 1-3 x 10^6 cells onto 60mm Ultra-Low Attachment plates (Corning #3261).

Tissue Culture of High Density Mammosphere Suspension Cultures:

Mammospheres were grown in Ultra-Low Attachment plates at 37°C in humidified 5% CO_2 atmosphere. The day of isolation is day zero. These cultures can be monitored for growth every day, it will increase the amount of aggregation but for high density cultures it does not matter. See the Results section for a detailed description of growth characteristics. Mammospheres should be grown in 5ml 1x mammosphere media. Add 2-3ml fresh media on day three to counteract the effects of all the dead cells. The media should be changed every four days. Mammospheres can be passaged after day eight.

Tissue Culture of Mammospheres Grown in BME (BME "On-Top" Culture Assay):

This protocol was adapted from "Three-D culture models of normal and malignant breast epithelial cells" by Lee et al., 2007 [15]. The volumes we used were directly taken from this protocol. Briefly, BME was thawed overnight at 4°C in beaker filled with ice water. Then working on ice at all times, 24-well tissue culture plates were coated with 120µl BME per well. BME was allowed to dry for 15-30 minutes at 37°C. Then cells were plated by carefully pipetting the media down the side of the well. Cells were allowed to settle and attach to the matrix for 10-30 minutes then the 300µl top coat of media and BME was added. Cultures were incubated at 37°C and 5% CO_2 for duration of the experiment. Media was replaced every 2 days by removing the old media with a P1000 and replacing it with 500µl of fresh media containing 10% BME.

Fixing Cultures of Mammospheres Grown in BME:

This protocol is adapted from "Indirect Immunofluorescent staining of MCF-10A acini cultured in Matrigel (detailed)" from Brugge.med.harvard.edu. Briefly, media was removed using a P1000 and wells were rinsed twice with 500µl PBS. BME was then fixed using 500µl 1:1 methanol:acetone for 10 minutes at -20°C. Fixative was removed with a P1000 and BME was allowed to air dry for 2-3 minutes then rinsed again. Fixed cultures coated with a thin layer of PBS, wrapped in parafilm and stored in the refrigerator for up to 2 weeks.

Mammary Cell Isolation for Clonal Density Mammosphere Cultures:

This protocol uses the EasySep® Mouse Mammary Stem Cell Enrichment Kit from Stem Cell Technologies (Cat #19757). The protocol is basically the same as what comes with the kit; however only the negative selection steps were used we did not use the cytometric sorting. After negative selection mammary epithelial cells were counted using a Hemocytometer and 1,500 cells were plated onto each 60mm Ultra-Low Attachment plate with 10ml 1:1 conditioned media: fresh 2x media. Cells were incubated completely undisturbed for 12 days prior to scoring for mammosphere formation.

LITERATURE CITED:

1. Deome KB, Faulkin LJ, Jr., Bern HA, Blair PB. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 1959; 19: 515-20.

2. Li Y, Rosen JM. Stem/progenitor cells in mouse mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 2005; 10: 17-24.

3. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ. Purification and unique properties of mammary epithelial stem cells. Nature 2006; 439: 993-7.

4. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. Generation of a functional mammary gland from a single stem cell. Nature 2006; 439: 84-8.

5. Ullrich RL, Bowles ND, Satterfield LC, Davis CM. Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation. Radiat Res 1996; 146: 353-5.

6. Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM, Goodell MA. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol 2002; 245: 42-56.

7. Sleeman KE, Kendrick H, Ashworth A, Isacke CM, Smalley MJ. CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. Breast Cancer Res 2006; 8: R7.

8. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005; 65: 5506-11.

9. Woodward WA, Chen MS, Behbod F, Rosen JM. On mammary stem cells. J Cell Sci 2005; 118: 3585-94.

10. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003; 17: 1253-70.

11. Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 1996; 175: 1-13.

12. Dontu G, Liu S, Wicha MS. Stem cells in mammary development and carcinogenesis: implications for prevention and treatment. Stem Cell Rev 2005; 1: 207-13.

13. Liao MJ, Zhang CC, Zhou B, Zimonjic DB, Mani SA, Kaba M, Gifford A, Reinhardt F, Popescu NC, Guo W, Eaton EN, Lodish HF, Weinberg RA. Enrichment of a population of mammary gland cells that form mammospheres and have in vivo repopulating activity. Cancer Res 2007; 67: 8131-8.

14. Grimshaw MJ, Cooper L, Papazisis K, Coleman JA, Bohnenkamp HR, Chiapero-Stanke L, Taylor-Papadimitriou J, Burchell JM. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. Breast Cancer Res 2008; 10: R52.

15. Lee GY, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods 2007; 4: 359-65.

Chapter 4:

Self-Renewal and Differentiation Capacity of Mammosphere-Initiating Cells

SUMMARY:

Transplantation studies have shown that not every primary mammosphere contains an actual stem cell. The studies included in Chapter 3 have shown that murine mammary cells consistently produce mammospheres ranging in size from ~40μm-300μm in diameter. This size range was consistent in high density suspension cultures, BME cultures, and clonal suspension cultures. Here the hypothesis that only large mammospheres are produced by stem cells was tested by assessing the self-renewal and differentiation capacity of mammosphere initiating cells. The results showed that large mammospheres are capable of more passages than small mammospheres, and that only 19% of primary mammospheres contained both luminal and myoepithelial cell lineages.

INTRODUCTION:

The existence of mammary stem cells has been suspected since the 1950's when DeOme first developed the mammary tissue transplantation system [1]. More recent work using serial dilutions of mammary cells in single cell suspension has suggested that the mouse mammary gland contains stem cells at a frequency of 1:1,000 - 1:2,000 mammary epithelial cells [2, 3]. This number varies due to inter-lab differences, but seems to be fairly

constant between strains of mice [3]. Thus, out of the millions of cells isolated from the mouse mammary gland, stem cells capable of regenerating an entire mammary gland make up only 0.05-0.1% of the total cells isolated. As discussed in detail in Chapter 3, techniques have recently become available to enrich mammary cell isolations for these stem cells, but to date, there is still no way to specifically obtain only the stem cells. The resulting population of cells has a higher percentage of stem cells than the original isolation, but also consists of progenitor cells and differentiated cells. For example following extensive cell sorting for a putative stem cell population, Shackleton et al., transplanted single mammary cells into recipient mice, and found 5.9% of transplanted cells were capable of mammary gland regeneration [4].

Dontu et al. created a technique to enrich isolated populations for human mammary stem cells by growing them as nonadherent mammospheres [5]. They have extensively characterized these mammospheres and successfully shown that the cells which create the mammospheres have stem-like properties. They have also shown that the cell population capable of producing mammospheres is the same cell population capable of producing a mammary gland upon transplantation [6]. However, depending on the isolation, anywhere from 0.1-5% of the primary mammary cells can form primary mammospheres. Thus, a significant proportion of the resulting primary mammospheres are not derived from stem cells, but rather more differentiated cells. In fact, Moraes et al. transplanted single mouse-derived primary mammospheres into the cleared fat pad of 3 week old recipient mice and found that only 15-33% of these individual mammospheres were capable of regenerating a mammary tree depending on the experiment [7]. They suggested that the remaining mammospheres were derived from downstream progenitor cells as opposed to true stem cells [7].

There are two characteristics that define an adult stem cell; the ability to self-renew, and the ability to produce all of the cell types that make up the particular tissue. Self-renewal is a property that only stem cells posses. It is the ability to undergo a cell division in which one or both of the daughter cells retains the same capacity to differentiate and proliferate as the parental stem cell. Self-renewal can occur through symmetrical cell division, in which both daughter cells are stem cells, this type of division results in an increase in the total number of stem cells in the tissue [8]. Self-renewal can also occur through asymmetrical cell division, in which one daughter cell remains a stem cell and the other becomes a committed progenitor cell. Asymmetrical cell division is a way to maintain the total number of stem cells in the tissue. The ability of mammary stem cells to self-renew has been clearly demonstrated through serial transplantation studies in mice [8]. Transplantation of cells into a recipient mouse and the development of a fully functional mammary gland is the only definitive way to show that a certain cell was a true mammary stem cell. However, serial passaging of mammospheres has been shown to be the second best method of identifying mammary stem cells when combined with analysis of differentiation potential.

Dontu et al. created the mammosphere tissue culture system and showed that human mammary stem cells could be maintained and expanded in culture by serial passaging of mammospheres [5]. They showed that passaging mammospheres at 10-14 day intervals resulted in a consistent plating efficiency of one mammospheres per 250 cells at each passage, for at least five passages. They reasoned that if mammospheres were produced by progenitor cells, which have limited proliferative capacity, then the mammosphere plating efficiency and mammosphere size would decrease with each passage as the progenitors became more and more differentiated. Instead, they observed very consistent mammosphere size and plating efficiency from passage to passage. In addition, they showed that some of

the stem cells underwent symmetrical cell division, by determining that upon passaging one to four new mammospheres were produced for each original mammosphere [5].

The ability of mammosphere-derived cells to form another mammosphere after passaging suggests that the mammosphere cell of origin is capable of self-renewal and thus a stem cell. However in addition to possessing the capacity to self-renew the mammary stem cell must be able to produce luminal epithelial cells and myoepithelial cells. The luminal epithelium consists of ductal epithelial cells, which line the ducts of the mammary gland, and alveolar epithelial cells which are capable of milk production. The ductal and alveolar cells differentiate from a common luminal progenitor cell. The myoepitheial cells are contractile cells that line the outside of the ductal tree; these cells contract to move the milk through the gland (Figure 4.1).

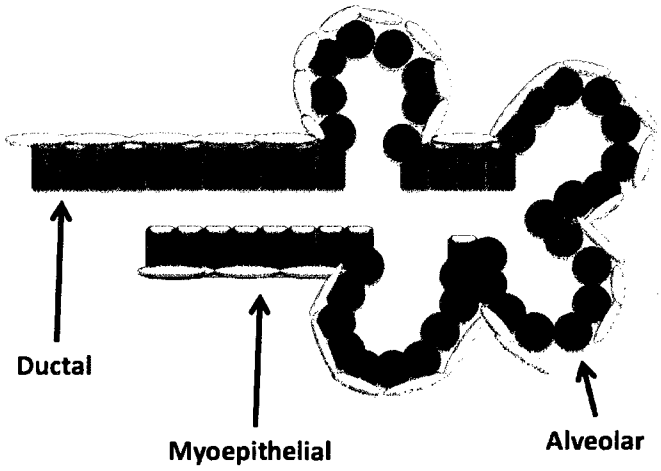


Figure 4.1. Depicts the location of the ductal epithelial, alveolar epithelial and myoepithelial cells in the mammary gland ductal tree structure.

The culturing of mammary cells as mammospheres greatly enriches the cell population for stem cells or high progenitor cells capable of producing all of the cell types necessary for the development of the mammary gland; however only a relatively small fraction of primary mammospheres are derived from stem cells [7]. Although most groups

who have tested the differentiation capacity of mammospheres have assayed the ability of single mammosphere-derived cells to produce multi-lineage colonies when grown on collagen substrate, review of the available literature suggests that in primary mammospheres at least, the majority of the cells are differentiated, and therefore do not require growth on collagen to induce differentiation [5, 9].

It is evident from transplantation studies that not every primary mammosphere contains an actual stem cell. Murine mammary cells consistently produce mammospheres ranging in size from ~40μm-300μm in diameter after about 10 days of culturing. This size range was consistent in high density suspension cultures of mammospheres including passaged cultures. This size variation was also observed in BME cultures, and finally in clonal suspension cultures. It was hypothesized that this size variation was indicative of the identity of the mammosphere-initiating cell. To test this hypothesis, size groups based on mammosphere diameter were created. These size groups were 60-99μm, 100-150μm, 151-200μm, 201-250μm and ≥251μm. The self-renewal capacity of mammospheres from each size group was tested via serial passaging. Additionally, mammospheres from each size group were stained with luminal and myoepithelial specific markers to test the differentiation potential of the mammosphere-initiating cell. The goal of this work was to determine which size categories of mammospheres contained stem cells, as opposed to progenitor cells or more differentiated cells. These data would then be used to develop criteria by which primary mammospheres derived from stem cells can be identified and sorted from those mammospheres derived from more differentiated progenitor cells, without any further culturing or manipulation. These criteria would result in greater mammary gland transplantation efficiencies, which would in turn increase the amount of useful results for any given experiment while decreasing the number of mice necessary.

RESULTS:

High Density 1° Mammospheres – To test the hypothesis that the size of the mammosphere was determined by the cell of origin, specific sizes of mammospheres were serially passaged in order to assess the self-renewal capacity of the mammosphere-initiating cell. It was assumed that the mammospheres within a certain size group would have similar characteristics and be derived from cells at the same stage of differentiation. In these serial passaging experiments, groups of around 20 individual primary (1°) mammospheres for each size group were removed from suspension cultures and dispersed into single cells. Cells were plated into a single well of a 24-well Ultra-Low Attachment plate and allowed to grow. Under these conditions, secondary (2°) mammospheres grew extremely quickly and needed to be passaged every two to three days. Like passaging entire plates of mammospheres, passaging groups of high density 1° mammospheres resulted in 2° mammospheres that varied greatly in size. Table 4.1 shows examples of the sizes of mammospheres generally observed at each passage.

Table 4.1. The sphere sizes observed at each passage (p1-p4) in relation to the original mammosphere size (μm)

Size at passage → / Size of 1° ↓	<99	100-150	151-200	201-250	>251
< 99	p1 p2 p3	p2			
100-150	p3	p1 p2 p4	p3		
151-200	p3	p3 p4	p1 p4	p3	
201-250	p3	p4	p3	p1 p3 p4	p2 p4
>251	p3	p3	p4	p3	p1 p2 p4

Serial passaging of groups of high density 1° mammospheres was repeated multiple times and consistently showed that all groups measuring ≥100μm in diameter were capable of at least five passages. Groups of 1° mammospheres measuring 60-99μm were capable of three passages. However, plating large cell numbers into a single well of a 24-well plate resulted in a considerable amount of aggregation, making it very difficult to definitively determine if the small 2° spheres were actually growing or simply aggregating. In addition, the aforementioned results suggest that all mammospheres are capable of multiple passages, but this seemed unlikely, thus the experimental design was altered to use clonal primary mammospheres.

The differentiation capacity of mammospheres, grown at high density, from each size group was also tested. In the first set of experiments, 20 primary mammospheres from each size group were picked out of suspension cultures. These mammospheres were then dispersed and attached to collagen-coated coverslips. Cells were stained for cytokeratin-18 (K18), a luminal epithelial specific cytoplasmic protein, and alpha-smooth muscle actin (α-SMA), a myoepithelial specific cytoplasmic protein. According to the hypothesis that only large mammospheres were derived from stem cells, cells derived from the smaller sized mammospheres would only express one protein or the other, but not both. Additionally, larger mammospheres, formed by stem cells, would contain some cells expressing either K18 or α-SMA, indicative of differentiation. We found however, that all sizes of mammospheres contained both differentiated luminal and myoepithelial cells. Examples are shown in Figure 4.2. As shown in Figure 4.2 mammospheres from all size groups contain both luminal epithelial and myoepithelial cell types. This would suggest that the diameter of a mammosphere is not specifically related to the cell of origin. There are three possibilities why all size groups contained both cell types. First, it is possible that within a particular size

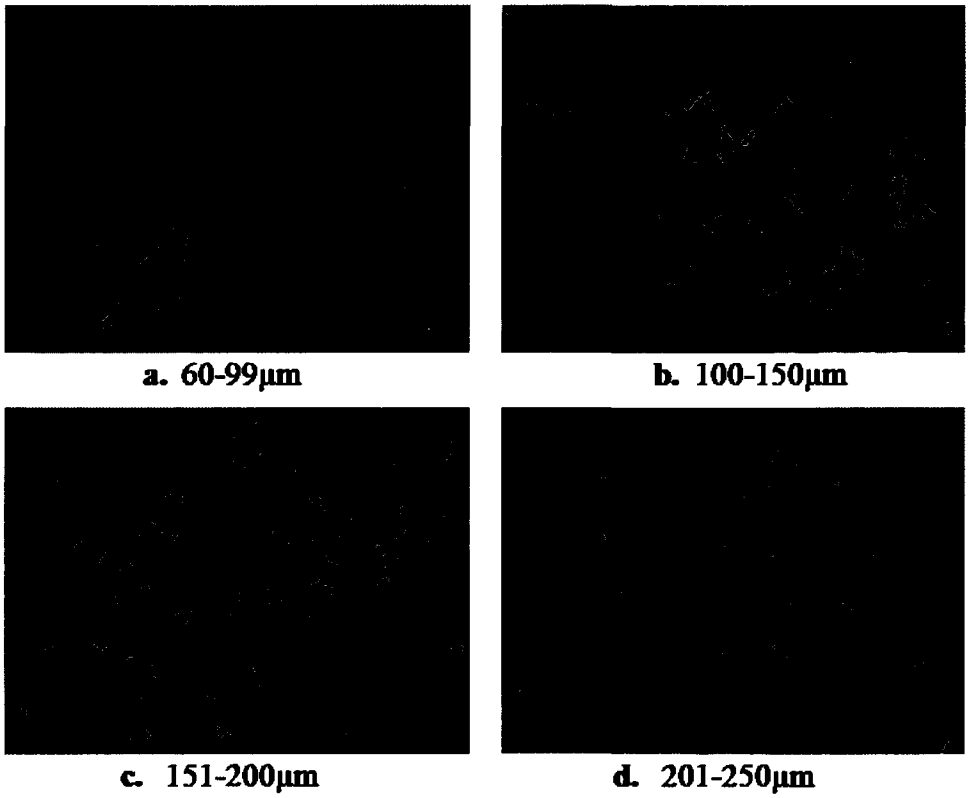


Figure 4.2. 10x magnification. Representative images of differentiated cells derived from 20 high density mammospheres from each size group.
Red = K18 = luminal epithelial; Green = α-SMA = myoepithelial

group some spheres are all luminal (red) while others are all myoepithelial (green) and by staining 20 dispersed mammospheres at one time, both lineages are observed. Second, the mammospheres used here were from high density cultures, so it is possible that progenitor cells from different lineages aggregated to create mammospheres containing both cell types. Finally, it is possible that size has no relation to the mammosphere cell of origin and that some of the mammospheres from each size group do contain a stem cell. To definitively determine if mammospheres of a particular size were created by a stem cell, it was necessary to use cells derived from individual clonal mammospheres.

Clonal 1° Mammospheres – Serial passaging of high density 1° mammospheres passaged as groups did not provide clear results as to the size of mammosphere that

originated from a stem cell. Based on the results of Dontu et al. serial passaging of individual 1° clonal mammospheres should result in growth of one to four new mammospheres at each passage [5]. This decreased cell number and decreased 2° mammosphere number was expected to limit the problems associated with aggregation observed in the high density passaging experiments. In these experiments individual 1° mammospheres grown at clonal density were removed from suspension cultures, dispersed into single cells, and plated for regrowth. It was determined that although the clonal mammospheres grew to all the size groups observed in the high density mammosphere cultures (examples shown in Figure 4.3); they behaved very differently when passaged. The biggest problem was that the clonal mammospheres have less densely packed cells making up the sphere. As a result, the exposure time for Trypsin was extremely different than those previously determined for high density cultures. Even when exposed to Trypsin for less than one minute the majority of the cells died.

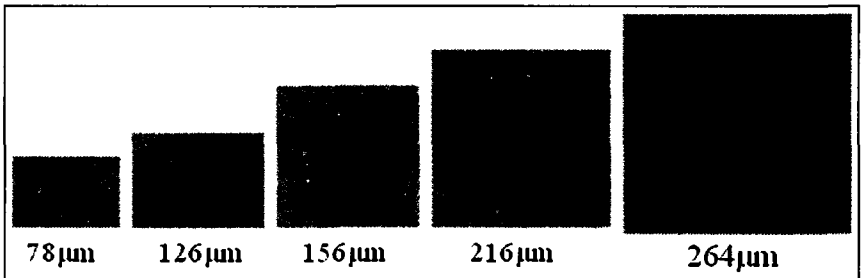


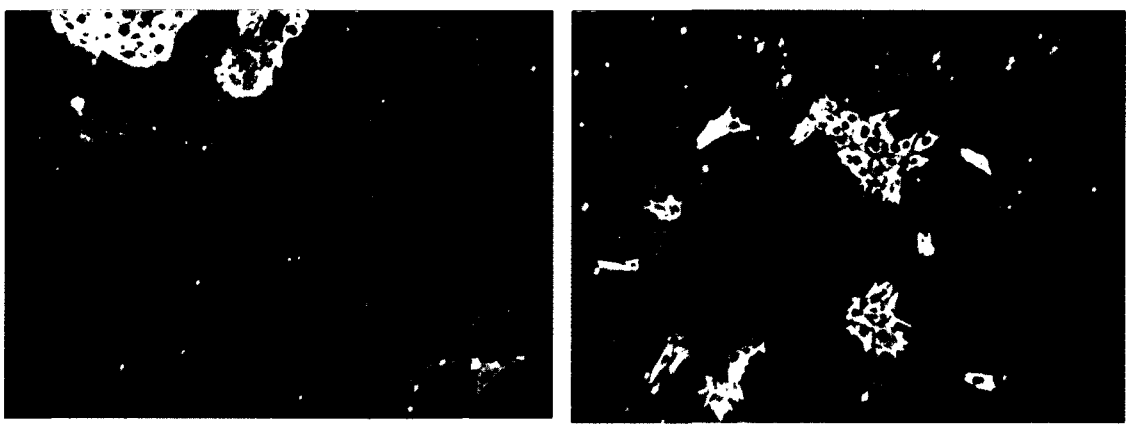
Figure 4.3. 4x magnification. Representative photographs showing the size variation seen in 1° mammospheres grown at clonal density. Photographs taken on day 12.

We were able to determine trypsinisation times closely enough to work well for the differentiation experiments which follow however; they were not perfected for serial passaging. To complicate matters further, when 1° mammospheres were plated into 24-well plates, the resulting 2° mammospheres seemed to have a different cellular density and require different trypsinisation conditions for each passage.

When clonal 1° mammospheres were passaged, again many different sizes of 2° mammospheres were seen in each well. Of note is the fact that the larger the 1° mammosphere, the more 2° mammospheres that were produced. We hypothesize that the variety observed is a result of individual cells from each mammosphere having reached different degrees of differentiation, and thus having different capacities for proliferation. For instance, the most differentiated cells in the mammosphere probably only produce extremely small 2° mammospheres, ones that would not be able to regenerate into a 3° mammosphere. In an attempt to further limit the bias created by aggregation, individual 2° mammospheres were picked out of the wells and dispersed and plated into their own wells at each passage. At the end of the experiment the data was compiled to determine which 1° mammosphere the surviving mammospheres had originated from. In this manner it was determined that 1° clonal mammospheres ranging in size from 150μm-420μm were capable of at least three passages. It was found that mammospheres smaller than 150μm could only be passaged one to two times before being incapable of producing a mammosphere larger than 60μm.

Although the experiments used to determine the self-renewal capacity of different sizes of mammospheres need to be repeated; the differentiation potential of the mammosphere-initiating cells of mammospheres derived from clonal culture conditions was tested. Again, mammospheres of a particular size were picked out of suspension cultures, dispersed into single cells and grown on collagen-coated coverslips for two days prior to staining for cell lineage. Surprisingly, under these conditions cells derived from primary clonal mammospheres did not stain positive for either K18 or α-SMA, these data suggest that the primary clonal mammospheres do not contain fully differentiated luminal epithelial or myoepithelial cells as suggested [5, 9]. Comparison between earlier experiments and the current ones showed two major differences; in earlier experiments high density

mammospheres were used but for the later experiments clonal mammospheres were used. The second difference was that in the earlier experiments mammospheres were collected on day eight, and the clonal mammospheres were collected on day 12. It was hypothesized that this extended time in tissue culture, and not the cell density, was resulting in mammospheres containing cells that did not express luminal or myoepithelial cell markers. To test this hypothesis cells derived from day eight high density and clonal mammospheres were dispersed and grown on collagen for two days before staining, Figure 4.4 shows representative images of both. These data show that if clonal mammospheres are grown for eight days in suspension and then two days on collagen, both luminal and myoepithelial cells express lineage specific proteins. These data suggest that the density at which mammospheres are grown has little effect on their ability to produce differentiated cells of either mammary cell lineage. Figure 4.4 shows that both mammospheres grown at high density and those grown at clonal density for eight days contain differentiated luminal and myoepithelial cells. If however, they are grown for 12 days in suspension, followed by two



a. Day 8 high density 1° mammospheres on collagen for 2 days

b. Day 8 clonal 1° mammospheres on collagen for 2 days

Figure 4.4. 10x magnification. Representative images of differentiated cells derived day 8 mammospheres. **a** - sample of cells derived from high density mammospheres. **b** – sample of cells derived from clonal mammospheres. Both images clearly show both differentiated luminal and myoepithelial cells. Red = K18 = luminal epithelial; Green = α-SMA = myoepithelial

days on collagen, the cells do not express of K18 or α-SMA. Together these data suggest that extending the mammosphere culture for four days drastically changes the extent of differentiation of the component cells.

We hypothesized that cells derived from day 12 clonal mammospheres were in a less differentiated state then those derived from day eight clonal mammospheres, and thus would require additional time in differentiating tissue culture conditions. To test this hypothesis a time course experiment was performed to determine how long cells derived from day 12 clonal mammospheres would need to be grown on collagen before expressing lineage specific proteins. Clonal day 12 mammospheres from many plates were collected, dispersed into single cells, and then plated onto collagen coated coverslips. Every 24 hours after plating samples were fixed and stained for differentiation. At every time point the coverslips contained sufficient cell numbers for analysis. At 24 hours after plating a few of the cells stained positive for K18, but no α-SMA$^+$ cells could be found. Results were similar for the 48 hour time point. At the 72 hour time point, again there were distinct K18$^+$ cells, and it seemed as though some cells were starting to express α-SMA, but it was extremely hard to tell if this was simply background staining or true protein expression, an example can be seen in Figure 4.5a.

Figure 4.5. 10x magnification. Representative images of differentiated cells derived from day 12 clonal density mammospheres. Grown on collagen to induce myoepithelial differentiation for 72 hours (a), 96 hours (b), 120 hours (c). These images show increased expression of α-SMA with increased time on collagen. Red = K18 = luminal epithelial; Green = α-SMA = myoepithelial

At the 96 hour time point, the K18 expression was still strong but again the α-SMA expression was questionable. It definitely seemed like a few of the cells were staining positive but the majority of the cells were still not expressing either protein (Figure 4.5b). After 120 hours on collagen some very clear α-SMA expressing cells were seen, as shown in Figure 4.5c. It is interesting to note that though some cells were definitely expressing α-SMA, there was a huge amount of cell to cell variation in the amount of expression. Figure 4.6 shows the same image of the 120 hour time point after being enhanced for printing, to illustrate the variation in α-SMA expression between cells.

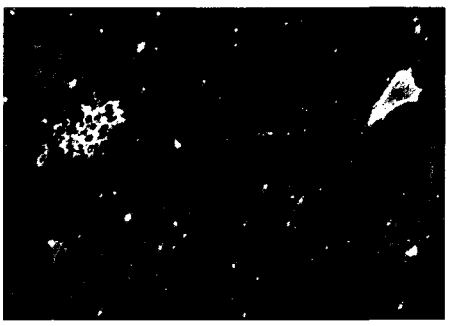


Figure 4.6. 10x magnification. Representative images of differentiated cells derived from day 12 clonal density mammospheres. Grown on collagen to induce myoepithelial differentiation for 120 hours. This is the same image as Figure 4.5c but has been enhanced to show the variation in α-SMA expression between cells. Red = K18 = luminal epithelial; Green = α-SMA = myoepithelial

At this point it was clear that primary clonal mammospheres contained cells that required at least five days of growth under differentiation promoting conditions in order to express the lineage specific cellular proteins K18 and α-SMA. It was now possible to test the hypothesis that the size of a particular clonal mammosphere was directly related to the mammosphere cell of origin. A total of 81 individual mammospheres were collected, dispersed into single cells, attached to collagen, and stained for differentiation potential. In order to reduce the amount of α-SMA protein expression variation observed after five days, cells were grown on collagen for eight days prior to staining. Figure 4.7 shows some representative pictures of mixed lineage colonies produced by individual clonal mammospheres. There are no pictures of the smallest size group because the cells tended to

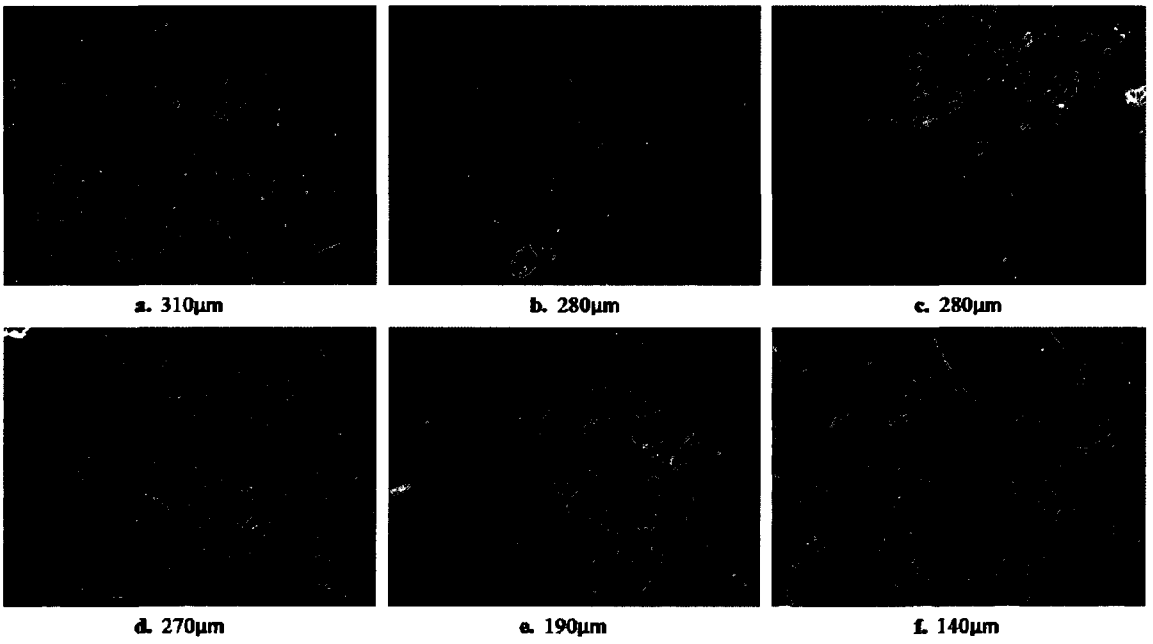


Figure 4.7. 10x magnification. Representative images of differentiated cells derived from individual clonal mammospheres. Red = K18 = luminal epithelial; Green = α-SMA = myoepithelial

be spread much farther apart and it was difficult to find a good field of view which showed expression of both proteins. Of the 81 mammospheres analyzed only 15 expressed both K18 and α-SMA. Interestingly, this is 19% of the tested mammospheres, which coincides perfectly with the work mentioned earlier in this chapter by Moraes et al. who found that 15-33% of mammospheres were capable of developing into a mammary gland upon transplantation [7]. Unfortunately, there does not seem to be any correlation between mammosphere size and its ability to produce luminal and myoepithelial cell lineages, results are shown in Table 4.2 below.

Table 4.2. The number of mammospheres tested for differentiation per size group, and the number of colonies from each size group that expressed both K18 and α-SMA

	60-99μm	**100-150μm**	**151-200μm**	**201-250μm**	**≥ 251μm**
Total spheres scored	18	27	21	6	9
Colonies expressing K18 + α-SMA	5	3	2	0	5

DISCUSSION:

In this chapter, mammospheres of different sizes were tested for self-renewal and differentiation capacity in an attempt to determine which sizes of mammospheres were derived from stem cells as opposed to more differentiated cells. It was determined that only 19% of the clonal mammospheres tested contained cells capable of differentiating into both cellular lineages required for mammary gland development. This correlates extremely well with the work of Moraes et al., who show that only 15-33% of mammospheres are actually capable of producing a mammary gland upon transplantation into a recipient mouse [7]. We had anticipated finding that the smaller sized mammospheres contained only one cell type. One very interesting observation was that all single lineage colonies contained only myoepithelial cells. We did not find a single luminal-only colony out of 81 tested mammospheres, and cannot think of any explanation for this result, except that maybe the culturing conditions caused selection for myoepithelial cells at the expense of luminal cells.

The results showed that some mammospheres from each size group contained cells that could differentiate into both luminal epithelial and myoepithelial cells when attached to collagen. It is possible that the smaller mammospheres which were capable of multi-lineage differentiation were produced by common progenitor cells, as opposed to stem cells. It is possible to distinguish between stem cells and common progenitor cells by assessing the self-renewal capacity of the mammospheres. The results of the serial passaging experiments suggest that the small mammospheres (60-150μm) are capable of only one to two passages which suggest that they may have been produced by progenitor cells. Unfortunately the protocol for serial passaging clonally derived mammospheres needs to be optimized and more data need to be obtained before finalizing the mammosphere size criteria.

Trypsin timing is crucial to the success or failure of the serial passaging experiments and at this time there is still a lot of work to be done to determine the optimal passaging conditions for clonally derived mammospheres. In addition, aggregation of single cells as well as mammospheres was a problem. Unfortunately, plating a single cell per well is not realistic given the fact that each mammosphere has hundreds of cells, and that significant data can only be obtained by passaging many mammospheres of each size. The amount of aggregation was limited in the experiments using clonal 1° mammospheres by plating cells derived from larger mammospheres in multiple wells of a 24-well plate. This certainly helped, but also severely limited the number of 1° mammospheres that could be followed for multiple passages.

Finally, when plating at very low cell densities like that for passaging clonal mammospheres, the debris in the 24-well plates was overwhelming. Figure 4.8 shows some examples of the type of debris observed. It was determined that the clear looking debris comes from the pipette tips, presumably residual plastic from manufacturing. Though it is very distracting, the cells do not appear to attach to it. The filaments seen, particularly in Figure 4.8b, are extremely problematic. They appear in almost every well, and their origin could not be determined. Sometimes they are in the plate before it is even used. Using only plugged pipette tips and media and PBS/FBS that had been spun at 3,000 rpm for 10 minutes and transferred to new tubes before use, helped to limit the amount of filaments and random debris in the wells, but it did not resolve the problem. We even tried manually picking out each piece of debris after plating the cells, but found that forceps could not be used, because they caused scratching of the plate surface. Picking out the debris with a pipette in the same way that each mammosphere was picked did actually work, however it was extremely time

consuming, to the point where it was not a viable option. At this time I do not know how other labs were able to successfully perform similar experiments.

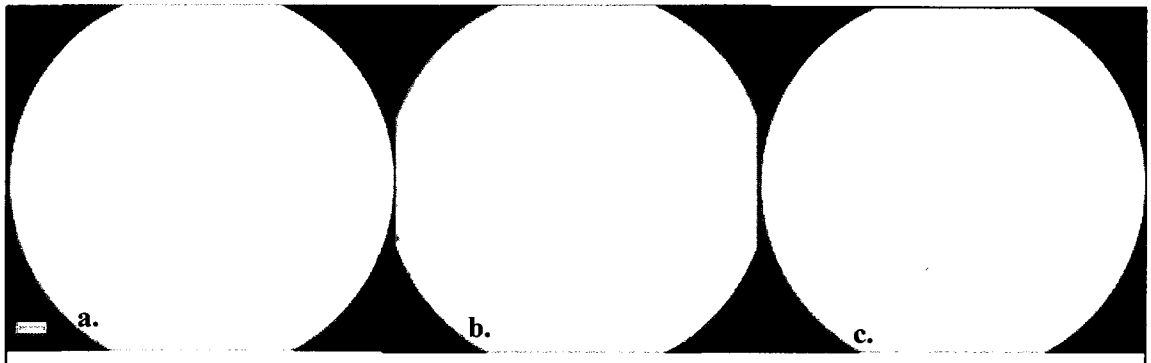


Figure 4.8. Size bar = 100μm; 4x magnification. Representative photographs of debris and filaments found in the 24-well plates.

We would still like to determine if there is a correlation between 1° mammosphere size and the presence of a true stem cell. However, for future work we recommend a different approach. We believe that useful results may be easier to obtain if clonal mammospheres were picked and put into groups (maybe 10 mammospheres for each size group) and trypsinized as a group to reduce the amount of cell death. Dispersed cells could then be plated at clonal densities in 60mm Ultra-Low Attachment plates. Mammospheres would need to grow for 12 days in 10ml of mixed conditioned media, exactly the same procedure as for growing 1° clonal mammospheres. Then after 12 days of growth, the plates could be scored for mammosphere formation efficiency, taking note of the resulting sizes. This procedure could be repeated until passaging was not possible or sufficient results were obtained. This approach would require determining the cell number in each size of mammosphere, in order to know how many plates to use for 2° growth.

MATERIALS AND METHODS:

Detailed protocols can be found in Appendix A.

Animals:

The animals used in these experiments were 8-12 week old virgin female mice bred at CSU. All mice were maintained in the Colorado State University Laboratory Animal Resources Painter Center.

Mammosphere cultures:

High density mammosphere cultures and clonal mammosphere cultures were performed as described in Chapter 3. Cultures were maintained for 8-12 days depending on experiment.

Picking Mammospheres Out of Suspension Cultures:

As the size of individual mammospheres was vital to our goal, we developed methodology for manually removing specific mammospheres from suspension cultures while still maintaining aseptic conditions. Briefly, a dissecting microscope fitted with an ocular reticle was placed in the tissue culture hood and exposed to UV for at least 30minutes. After which a plate of mammospheres was placed in the hood. I followed and wearing a plastic shower cap, shoulder-length latex gloves and a surgical mask. Cap and gloves were extensively exposed to UV and sprayed with ethanol before each use. Using a P10 or P20 Pipetteman, individual mammospheres were manually removed from suspension. Mammospheres were kept in small volumes of mammosphere media, on ice in 1.7ml microcentrifuge tubes until use.

Serial Passaging of Mammospheres:

Only round and symmetrical mammospheres were chosen for any experiment. All passaged cells were plated into 24-well Ultra-Low Attachment plates (Corning, #3473).

High Density - Groups or single mammospheres were picked from culture as described above. Mammospheres were passaged in 250µl room temperature 0.05% Trypsin-EDTA and agitated using a fire polished Pasteur pipette, times varied according to size and number of spheres (for roughly 20 mammospheres of each size the times were as follows: 60-99µm = 4mins; 100-150µm = 12mins; 151-200µm = 18mins; 201-250µm = 20mins; ≥250µm = 30mins). After the required time, 1ml cold rinse media was added and cells were pelleted at 800 rpm, for 5 minutes at 4°C. Mammospheres were plated in 500µl 1x mammosphere media. Mammospheres were grown for variable lengths of time (2-7 days usually) to allow sufficient growth for the next passage (usually waited until most spheres were >150µm). For the next passage, all mammospheres from each well were picked out and passaged as a group.

Clonal Density - Single mammospheres were picked from clonal cultures as described above. Mammospheres were passaged using 250µl cold 0.05% Trypsin-EDTA, and agitated by flicking the tube or pipetting with a fire polished Pasteur pipette specific conditions are provided in Appendix A Table A.1. After dispersion, 1ml cold PBS with 10% FBS was added and cells were pelleted at 800 rpm, for 5 minutes at 4°C. Cells were plated in 500µl mixed conditioned media (see Chapter 3). PBS and media were centrifuged at 3,000 rpm for 10 minutes prior to use in order to remove any debris. To limit sphere formation due to aggregation the following numbers of wells were used (60-120µm = 1 well; 130-150µm = 2 wells; 170-200µm = 3 wells; 201-250µm = 4 wells; 251-300µm = 5 wells; >300µm = 6 wells). Mammospheres were grown for 12 days, with 250-500µl fresh mixed conditioned

media added per well every 4 days. For the next passage, individual mammospheres >60μm in size were picked out of the well and passaged (all the 2° mammospheres resulting from dispersion of a single 1° mammosphere were passaged individually).

Dissociating Groups of Mammospheres for Differentiation on Collagen:

Mammospheres were picked out of suspension cultures as described above. Small numbers of mammospheres were dissociated following the serial passaging procedures described above, while whole plates of mammospheres were passaged using the protocol described in Chapter 3. After dissociating the mammospheres cells were plated on collagen-coated coverslips with Mouse Primary Cell Media with 5% FBS and allowed to attach.

Dissociating Individual Clonal Mammospheres for Differentiation on Collagen:

Individual mammospheres were picked out of clonal suspension cultures as described above. Mammospheres were passaged using 250μl cold 0.05% Trypsin-EDTA (kept on ice), and agitated by flicking the tube gently to avoid creating bubbles, or by pipetting with a fire polished pipette. After the prescribed time 1ml cold PBS with 10% FBS was added and cells were pelleted at 800 rpm for 5 minutes at 4°C.

Cells were resuspended in 200-250μl Mouse Primary Cell Media with 5% FBS. Cells were then very carefully plated directly onto pre-rinsed collagen-coated coverslips. Cells were allowed to attach for about 3 hours and then 1ml of media was added. The next day, media was removed with a P1000 and cells were rinsed with PBS to remove dead cells. Media was replaced with 2ml Mouse Primary Cell Media with 2% FBS. The media was changed every 3-4 days.

Collagen Coated Cover Slips:

Collagen solution was prepared as described in Chapter 2. Using forceps single autoclaved glass coverslips were aseptically transferred into 35mm tissue culture dish. Plates with coverslips were coated with use 680µl of solution per plate. Before plating cells, plates were rinsed with Hank's Balanced Salt Solution w/out Mg^{++} or Ca^{++}. For plating individual mammospheres, collagen coverslips were rinsed and then transfered into a clean, dry 35mm tissue culture plate. This helped to make sure the cells attach to the coverslip and not the tissue culture plate.

Fixation of Mammary Cells for Immunofluorescence:

Cells were rinsed twice with PBS and then fixed with add 100% cold methanol (for 35mm dish use 1ml). Cells were incubated at -20°C for 10 minutes then methanol was removed and cells were allowed to air dry for 10 minutes. All fixed cells were stored at -20°C.

Immunofluorescence (IF):

There is general agreement in the field of mammary cell culture that cytokeratin 18 (K18) is a specific marker of luminal epithelial cells and that alpha–smooth muscle actin (α-SMA) is a specific marker for myoepithelial cells. The antibodies used here were as follows – K18 – (mouse monoclonal [C-04] to cytokeratin 18 (Biotin conjugated) – Abcam, Cambridge MA; #ab27553); secondary – (Streptavidin – Alexafluor 594, Invitrogen #532356); α-SMA – (Rabbit polyclonal to alpha smooth muscle actin – Abcam, Cambridge Ma; #ab5694); secondary – (Goat anti-rabbit Alexafluor 488, molecular probes #A11034). DAPI = VectaShield Mounting Medium with 4', 6-Diamidino-2-phenylindole (Vector Lab, Burllingame, CA). Cells were stained with 1:100 K18 primary antibody for 30 minutes, then

1:100 Streptavidin secondary antibody for 30 minutes. Cells were then blocked with 5% milk for one hour. This was followed by 1:50 α-SMA primary antibody for one hour and 1:100 FITC secondary antibody for one hour. Coverslips were then rinsed and mounted onto slides with VectaShield mounting medium.

Analysis and Imaging:

Image analysis was performed using Photometric Coolsnap ES2 on a Zeiss Axioskop 2 Plus Microscope and Metavue 7.1 Software at 10x or 20x magnification.

LITERATURE CITED:

1. Deome KB, Faulkin LJ, Jr., Bern HA, Blair PB. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 1959; 19: 515-20.

2. Li Y, Rosen JM. Stem/progenitor cells in mouse mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 2005; 10: 17-24.

3. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ. Purification and unique properties of mammary epithelial stem cells. Nature 2006; 439: 993-7.

4. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. Generation of a functional mammary gland from a single stem cell. Nature 2006; 439: 84-8.

5. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003; 17: 1253-70.

6. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007; 1: 555-67.

7. Moraes RC, Zhang X, Harrington N, Fung JY, Wu MF, Hilsenbeck SG, Allred DC, Lewis MT. Constitutive activation of smoothened (SMO) in mammary glands of transgenic mice leads to increased proliferation, altered differentiation and ductal dysplasia. Development 2007; 134: 1231-42.

8. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res 2005; 7: 86-95.

9. Liao MJ, Zhang CC, Zhou B, Zimonjic DB, Mani SA, Kaba M, Gifford A, Reinhardt F, Popescu NC, Guo W, Eaton EN, Lodish HF, Weinberg RA. Enrichment of a population of mammary gland cells that form mammospheres and have in vivo repopulating activity. Cancer Res 2007; 67: 8131-8.

Chapter 5:

Effects of Ionizing Radiation on Mammospheres Derived from Mice of Different Genetic Backgrounds

SUMMARY:

The work presented in this chapter draws upon the protocols developed in previous chapters to determine the radiation dose response of mammospheres derived from different strains of mice. The dose response of mammospheres was determined using a mammosphere formation assay. The results suggest that mammosphere-initiating cells are much more resistant to radiation than kidney fibroblasts derived from the same mouse strains. Mammosphere formation results in the same dose response relationship between strains that has been previously observed using other cell types.

INTRODUCTION:

The process of carcinogenesis is believed to occur in a lineage of cells through the acquisition of a series of mutations over time [1]. There is evidence that at least in some breast cancers, there is a specific "tumor-initiating cell" or "cancer stem cell" [2-6]. This cell is capable of self-renewal and of regenerating the phenotype of the original tumor upon transplantation. There is also evidence that the tumor-initiating cell shares many of the cellular properties of normal tissue stem cells, such as the capacity to self-renew, ability to

produce a variety of differentiated cells, and anchorage independence, to name a few [7]. This has led many to hypothesize that tissue stem cells are in fact the tumor-initiating cells, or "cancer stem cells". This hypothesis is very attractive when applied to the BALB/c model of radiation-induced mammary carcinogenesis [8].

It has previously been shown that the mammary epithelial cells from BALB/c mice are inherently more susceptible to the damaging effects of IR than are those from C57BL/6 mice. These data were obtained experimentally by using the mammary transplantation system first developed by DeOme in the late 1950's, and then looking for the development of precancerous ductal dysplasias and eventually mammary tumors [9, 10]. Transplantation of untreated donor cells results in a fully developed and functional mammary gland [9]. Recently, Stingl et al. showed that these mammary outgrowths are clonally derived from donor cells by injecting mixed populations of green fluorescent and cyan fluorescent protein expressing cells and showing that the resulting outgrowths expressed only one color. They also injected individual putative stem cells into recipient fat pads and showed that a single stem cell was capable of regenerating the entire mammary gland structure and function [11, 12].

Using this transplantation system it was previously shown that only irradiated BALB/c cells will develop ductal dysplasias and tumors, irradiated C57BL/6 cells will not, nor will unirradiated BALB/c cells [8]. Additionally, these studies showed that about 2,500 cells needed to be injected into each recipient to cause growth in 50% of the recipient mice. These data suggest that 1:2,500 cells are capable of regenerating a mammary gland, which correlates well with the predicted frequency of mammary stem cells [8, 11, 13]. If normal mammary gland development after transplantation requires the presence of a viable mammary stem cell, it follows that any abnormal growth is also a result of that stem cell. In

the BALB/c model of radiation-induced mammary carcinogenesis, development of ductal dysplasias only occurs if the donor cells are irradiated BALB/c cells. The resulting mammary outgrowths posses all the cell lineages required for the mammary gland, and are functional, however some show abnormal growth and eventually develop tumors. It is hypothesized that this abnormal growth is directly related to radiation-induced damage to the mammary stem cells. The technology is now available to test the effects of IR on mammary stem cells *in vitro*.

In this chapter the use of the nonadherent mammosphere tissue culture system to determine the effects of IR on mammary stem cells and their immediate progeny will be discussed. This system has been used to assess the radiation sensitivity of mammary stem cells derived from different strains of mice (BALB/cByJ, C57BL/6J, SCID, B6.C-*Prkdc*^{BALB} and C.B6-*Prkdc*). It is hypothesized that the stem cells derived from SCID mice will be the most sensitive to cell killing (shown by inability to form mammospheres in culture). It is also anticipated that stem cells derived from C57BL/6J mice will be the least sensitive to IR, and that BALB/cByJ will fall in between these two. These results are predicted based on the DNA repair capacity of other cell types derived from these mice. B6.C- *Prkdc*^{BALB} and C.B6-*Prkdc* are mouse strains congenic for *Prkdc*; their sensitivity to radiation is unknown.

RESULTS:

The goal of the entire spectrum of work included in this dissertation was to determine the effects of IR on mammary stem cells derived from different strains of mice, in an attempt to determine if the stem cell is the target of radiation in the BALB/c model of IR-induced mammary cancer. In all of the following experiments, mammary stem cells were isolated directly from mammary tissue, dissociated into single cells, enriched for the stem cell

population, then exposed to γ-radiation and plated. After 12 days of undisturbed growth each plate was scored for mammosphere growth and the size of each mammosphere. In all cases, cells were exposed to 0, 2, 4, or 8Gy γ-radiation and plated in at least three replicate plates, two to three individual experiments were performed for each mouse strain represented.

Figure 5.1 shows the resulting dose response curves for mammospheres derived from all five strains of mice, BALB/cByJ, C57BL/6J, SCID, B6.C-*Prkdc*^{BALB} and C.B6-*Prkdc*.

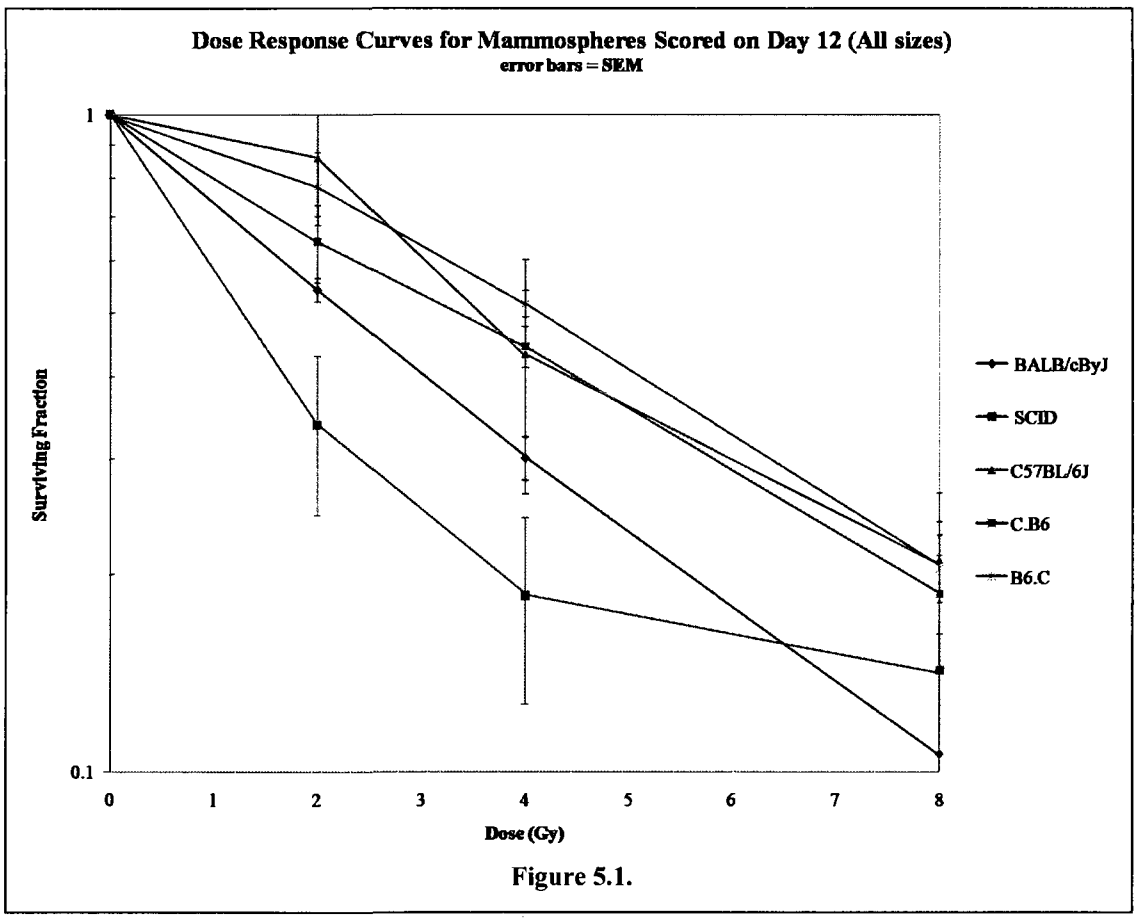


Figure 5.1.

Mammospheres included in these data are any colonies that reached 60μm in diameter or larger. As expected, mammospheres derived from SCID mice are the most sensitive to cell killing, especially at the lower doses. This is shown more clearly in Figure 5.2, which shows only the data from "control mouse strains", that being SCID, BALB/cByJ and C57BL/6J. Figure 5.2 clearly shows the separation in the dose response curves of SCID, BALB/cByJ

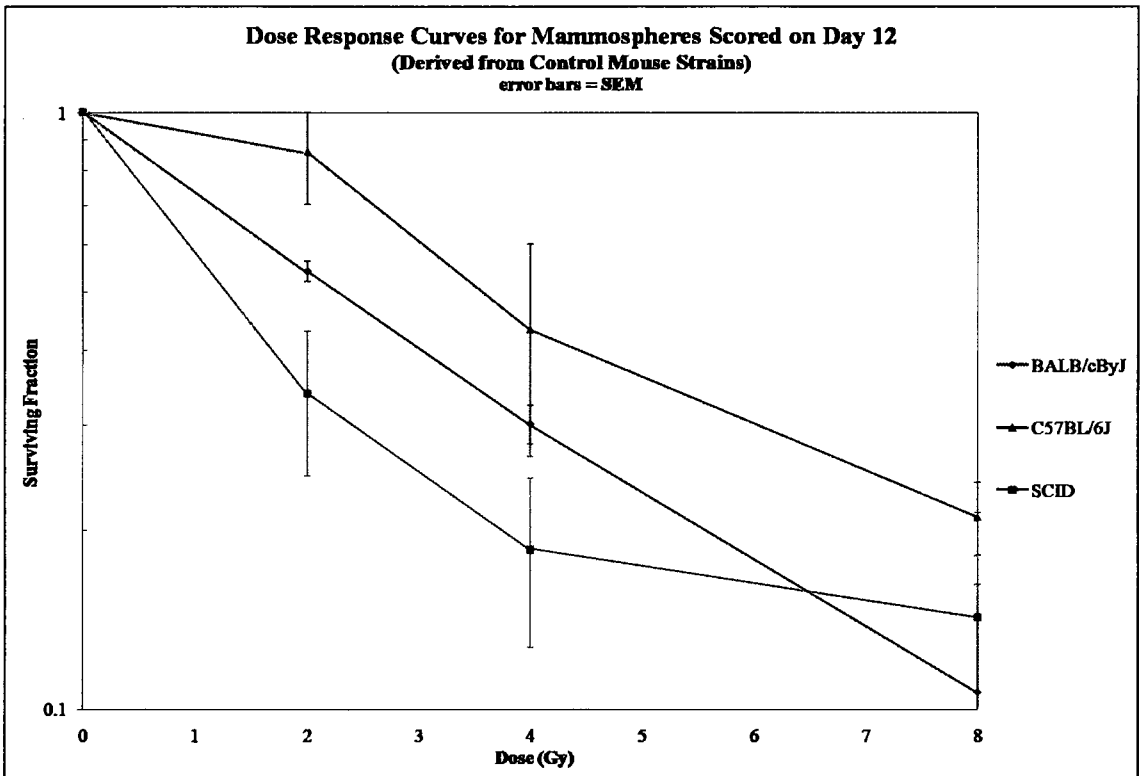


Figure 5.2.

and C57BL/6J-derived mammospheres. Note the different shapes of the survival curves between the strains, and also that although the error bars are fairly large, in most cases they do not overlap. We found these data particularly noteworthy because they prove that our mammosphere colony formation assay works as designed. In addition, these data suggest that the mammary stem cells derived from different strains of mice maintain the same radiation response relationship as do other cells derived from those strains, SCID cells are most sensitive, C57BL/6J cells are the most resistant, and BALB/cByJ cells show intermediate sensitivity. It is particularly interesting to note that although the dose response relationships between strains remains the same, the IR doses required to see this effect are significantly higher than those used in similar experiments with fibroblasts derived from the same strains. In those studies exposure to 4-5Gy γ-IR resulted in roughly 10% survival [14]. Here cells have been exposed to 8Gy, and have still not reached 90% cell killing. This

highlights the fact that the sensitivity of the target cells is extremely different depending on the tissue and cell type being studied. In this case, it supports the hypothesis that stem cells are particularly resistant to radiation-induced cell killing as compared to other cell types; however strain differences still definitely exist. Figure 5.3 shows the dose response curves for mammospheres derived from the congenic mouse strains B6.C- *Prkdc*^{BALB} and C.B6- *Prkdc*.

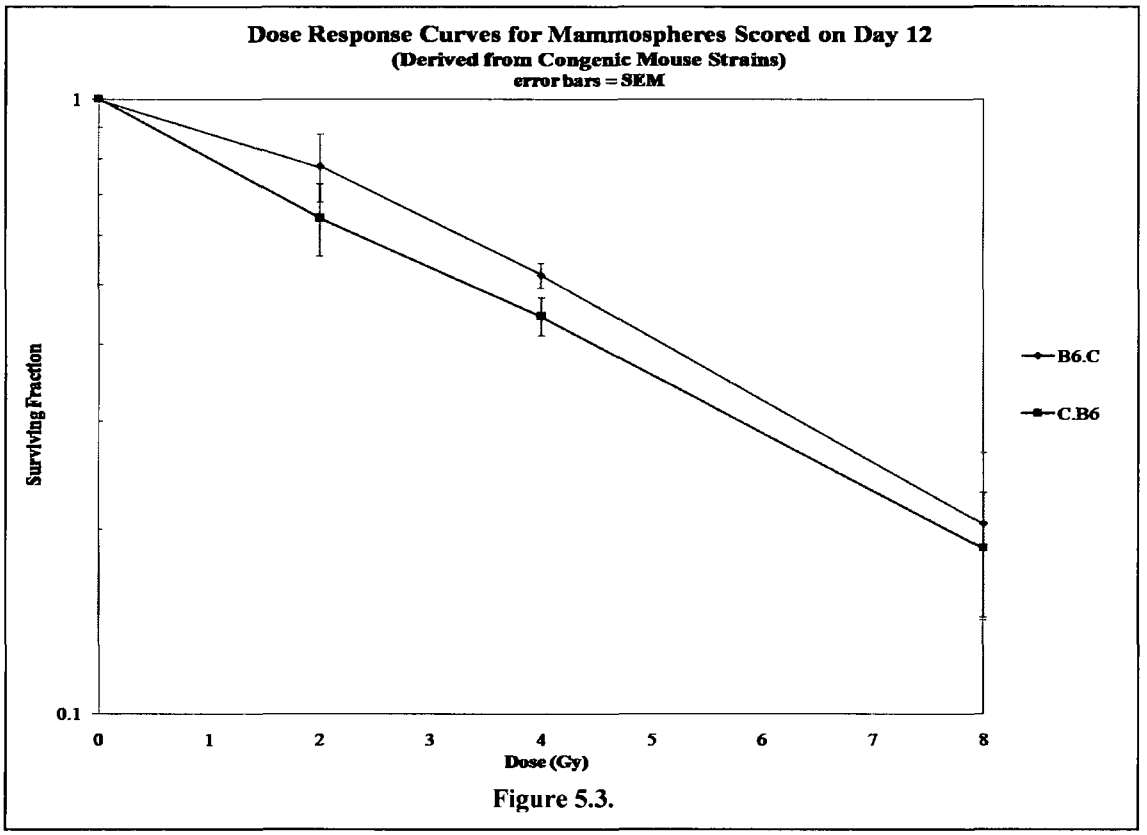


Figure 5.3.

As a reminder, C.B6- *Prkdc* has the background genome from BALB/cByJ and the *Prkdc* gene from C57BL/6J mice. B6.C- *Prkdc*^{BALB} is the exact opposite; it carries the resistant C57BL/6J background genome with the variant *Prkdc*^{BALB} allele. These are newly created inbred mouse strains which have not yet been fully characterized for DNA repair capacity or sensitivity to IR. The data in Figure 5.3 suggest that in terms of mammosphere formation efficiency, mammary stem cells derived from these two strains of mice react very similarly.

Figure 5.3 shows the surviving fraction data when all colonies capable of reaching 60μm in diameter are counted. If you look back to Figure 5.1, which shows the same data, but includes all 5 mouse strains, you see that the B6.C- *Prkdc*^{BALB} and C.B6- *Prkdc* lines fall in basically the same place as dose response curve for mammospheres derived from C57BL/6J mice. Thus, if all mammospheres are included in the analysis, it suggests that *Prkdc*^{BALB} allele is neither required nor sufficient to cause radiation-induced cell killing in mammary stem cells grown as mammospheres. In other words, based on these data, it would be predicted that both strains of congenic mice would be resistant to the other effects of radiation as well (IR-induced genomic instability, ductal dysplasias, mammary cancers, etc.).

Next the effect of IR on mammosphere colony size and morphology was examined. Other researchers have reported counting mammospheres as anything that grew larger than 40 or 60μm in diameter, since these were on the very small side of our size categories all colonies that grew larger than 60μm were counted [2, 15]. One possible effect of IR was abnormal mammosphere growth so the size group 60-99μm was divided into "symmetrical" and "asymmetrical" categories. The Materials and Methods section provides some photographic examples of some of the abnormal colonies that were counted as part of the "60-99μm asymmetrical" size group. In addition, it was hypothesized that only mammospheres that grew larger than 100μm would actually contain stem cells. Figure 5.4 shows an example of the data collected for SCID-derived mammospheres, when it was broken down into surviving fractions based on which mammospheres were counted as colonies. For mammospheres derived from SCID mice, if sensitivity is determined by only including colonies larger than 100μm, the mammary stem cells would be considered very sensitive to killing or at least severely growth retarded, to the point where additional

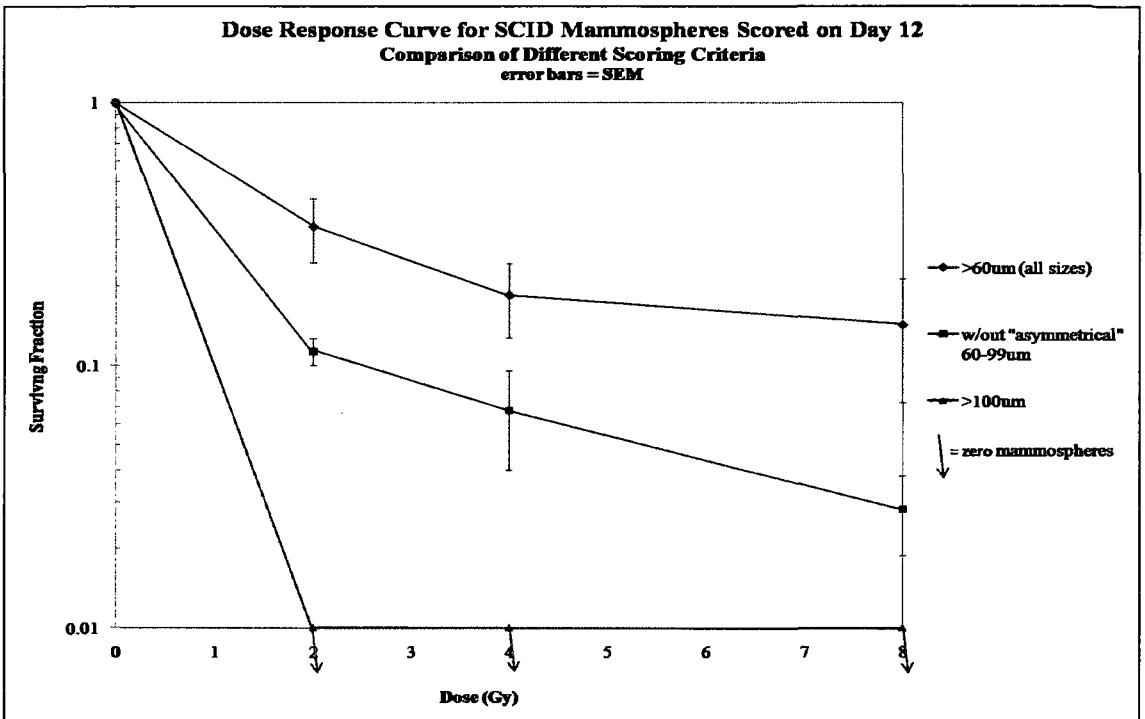


Figure 5.4.

experiments using much smaller dose than 2Gy would be required. If, however all colonies are included in the analysis, cells could easily be exposed to doses larger than 8Gy to increase the cell killing.

Figure 5.5 shows the dose response data for all five mouse strains if the "asymmetrical" 60-99μm size group is excluded from analysis. That is to say that only well formed, round, symmetrical mammospheres were counted as colonies. In these data, a very clear separation of the dose response curves is observed for each strain of mice except for the two congenic strains. Again, there is a very sensitive response from SCID-derived mammospheres, a very resistant response from C57BL/6J-derived mammospheres, and an intermediate response from the BALB/cByJ-derived mammospheres. Of extreme interest here is that although the two congenic strains show basically the same dose response relative to each other, the response compared to the parental mouse strains is intermediate.

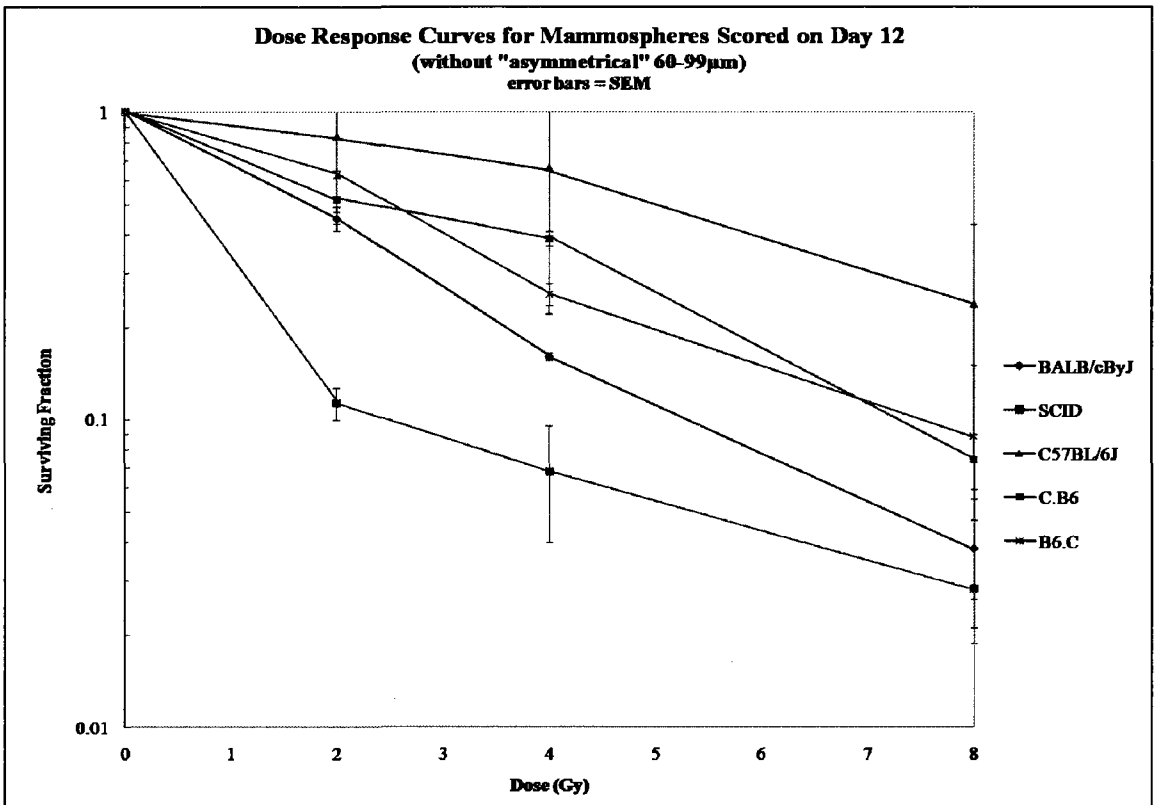


Figure 5.5.

If only mammospheres reaching 100μm in diameter are included, as shown in Figure 5.6, there is again a clear separation of mammospheres derived from "control" mouse strains, but this time both congenic strains follow a dose response most like BALB/cByJ cells.

DISCUSSION:

The data presented here represent two to three independent mammosphere formation experiments for each strain, and the error bars shown in each graph represent the standard error. The error bars are particularly large because of the variation in plating efficiency from one experiment to the next, which ranged from 0.96% - 4.9% depending on experiment. The variation in plating efficiency is due to variations in the mammary cell isolations. Each mammary gland has slightly different characteristics and the dissociation proceedure is very

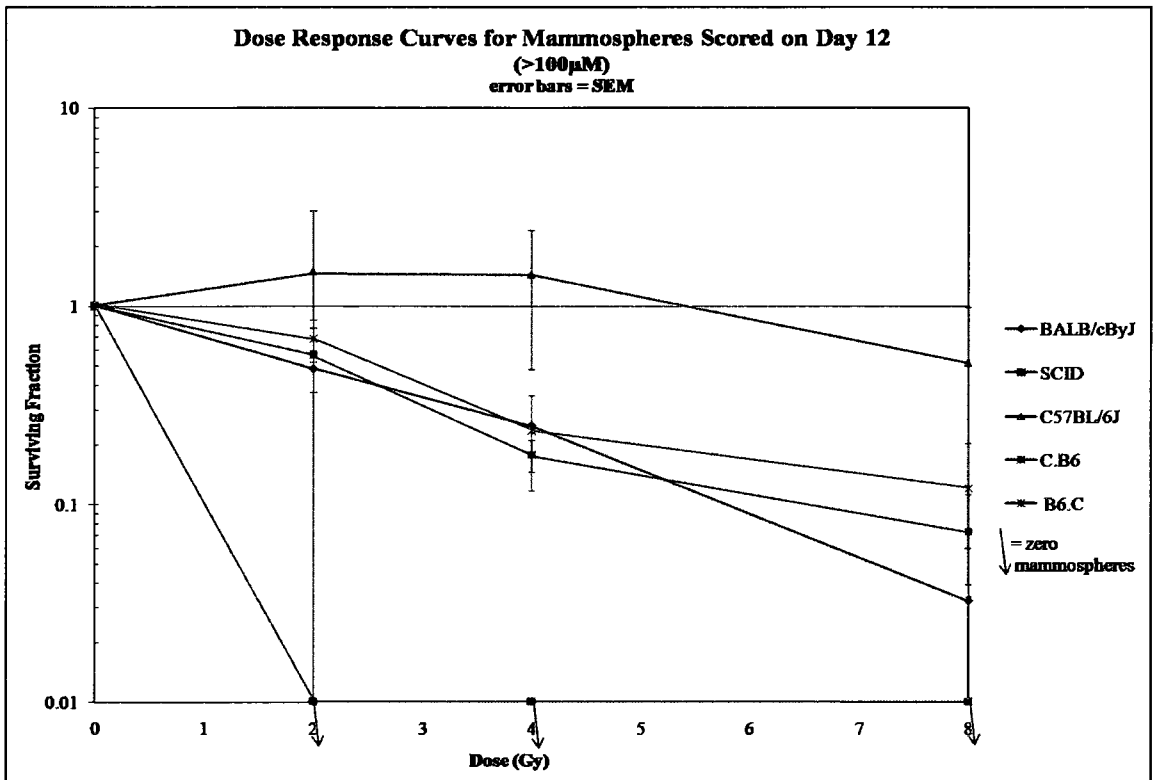


Figure 5.6.

subjective, this results in variation in the number of viable cells from one isolation to the next. Techniques that assess viability of cells during counting, such as Trypan Blue exclusion, do not reduce this variability because they only show dead cells, not ones that will die later in response to the stress of the isolation procedure. With regard to the inherent variability of this mammosphere culturing system, it is suggested that experiments using mammosphere formation efficiency be repeated as many times as possible.

The results shown in this chapter provide evidence that mammary stem cells, grown as nonadherent mammospheres, are relatively resistant to radiation-induced cell killing as compared to other cell types. Although the majority of cells are in single cell suspension following the isolation protocol it is possible that some of the mammosphere-initiating cells were irradiated as small clumps of cells as opposed to single cells. If this occured, the cells would seem more resistant to IR because a larger dose is required to kill all the cells within a

clump then it does to kill one single cell. However, it is extremely unlikely that the degree of cell clumping would be different between cells derived from different strains of mice. Therefore, the dose response relationships between mammospheres derived from different strains of mice is probably accurate. The results show that although stem cells as a group are resistant to cell killing (noted by the use of relatively high doses of IR), they still maintain the dose response relationships between strains that are observed when using other cell types. Regardless of how the data are analyzed, mammospheres from SCID mice are most sensitive to IR, C57BL/6J are most resistant and BALB/cByJ are intermediate in their response.

The mammospheres derived from the congenic mouse strains C.B6- *Prkdc* and B6.C-*Prkdc*^{BALB} did not respond as expected. The data shows very different trends in sensitivity to IR depending on what is counted as a mammosphere colony. We had predicted that the radiation sensitivity of the congenics would follow the *Prkdc* allele, or in other words that B6.C-*Prkdc*^{BALB} would be sensitive to radiation to the same magnitude that the parental BALB/cByJ strain, and that C.B6-*Prkdc* would be resistant to radiation to the same extent as the C57BL/6J parental strain. However, at this time the dose response relationship between the congenic strains and the parental strains remains elusive.

MATERIALS AND METHODS:

Detailed protocols can be found in Appendix A.

Animals:

The animals used in these experiments were 24-27 week old BALB/cByJ and C57BL/6J virgin female mice purchased from Jackson Laboratory (Bar Harbor, ME). 20-23 week old C.B-17 SCID virgin female mice purchased from Taconic. 8-12 week old C.B6N12F10 and

B6.CN10F10 virgin female mice bred at CSU. All mice were maintained in the Colorado State University Laboratory Animal Resources Painter Center.

Mammary Stem Cell Isolation:

Isolation of mammary stem cells for clonal mammosphere cultures was performed as described in Chapter 3. Briefly, mammary cells were dissociated into single cell suspension and then enriched for stem cells using the EasySep® Mouse Mammary Stem Cell Enrichment Kit from Stem Cell Technologies. After counting, cells were aliquoted into the bottom of 15ml conical tubes as follows: 0Gy + 2Gy = 1,500 cells; 4Gy = 2,500 cells; and 8Gy = 5,000 cells. Cells were then irradiated and plated.

Irradiation:

γ -ray exposures were delivered at a dose rate of 3.9 Gy/min in a calibrated, sealed source Mark 1/69A ^{137}Cs γ-irradiator (J.L. Sheperd and Associates).

Mammosphere Tissue Culture:

After irradiation, 10ml of mixed 1:1 conditioned: 2x mammosphere media was added to each tube of cells and mixed vigorously to provide maximum cell dispersion. Cells were then carefully plated onto 60mm Ultra-Low Attachment plates (Corning, NY), and placed in the incubator at 37°C and 5% CO_2. Mammospheres were allowed to grow undisturbed for 12 days and then scored for mammosphere growth.

Scoring Mammosphere Growth:

Mammospheres grown in suspension cultures at clonal densities were scored for growth by measuring the diameter of each mammosphere and manually counting the number of mammospheres per plate. However, due to the nature of mammosphere growth, scoring had to occur while mammospheres were still in suspension. This causes a problem with condensation on the cover of the plate, and since mammospheres were to be used for future experiments it was important to maintain sterility while scoring for growth. As such scoring was performed using the aseptic techniques described in Chapter 4 for picking mammospheres out of suspension cultures.

For colony formation assays like this it is vital to score all colonies and to count each colony only once, this is difficult in suspension, so a colored grid printed on overhead plastic was developed which could be placed under the tissue culture dish during counting. The grid plastic served two purposes, one, it helped to keep track of exactly where in the plate we were counting. It also helped by making it easier to slide the plate slowly without causing the media inside to move.

Colony formation is usually considered the ability of a cell to form a colony of $\geq$50 cells. However, for mammospheres it is impossible to tell how many cells are in the 3-dimensional sphere when looking at it, therefore diameter was used as a measure of colony size. The size of each mammosphere was recorded by including it in a size group. The size groups were 60-99μm "symmetrical", 60-99μm "asymmetrical", 100-150μm, 151-200μm, 201-250μm, and $\geq$251μm. By recording that detailed information it was possible to analyze the data in different ways as new information became available. Figures 5.7 - 5.9 show examples of how the mammospheres were scored for colony growth.

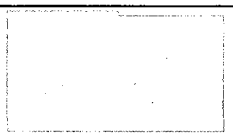


Figure 5.7. – Examples of symmetrical mammosphere growth. Size is determined by measuring the diameter of the mammosphere

Figure 5.8. – Examples of "asymmetrical" mammospheres.

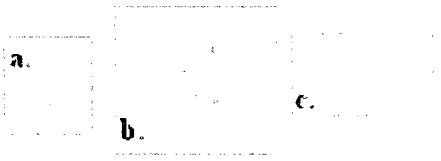


Figure 5.9. – Examples of mammosphere aggregation. **a.** 2 mammospheres, measure the diameter of each as separate colonies. **b.** Large mammosphere with two small ones attached, again measure the diameter of each. **c.** Two mammospheres that have grown almost completely together, do not measure length of oval, but measure width of opposite ends to estimate size of the individual spheres within.

LITERATURE CITED:

1. Nowell PC. The clonal evolution of tumor cell populations. Science 1976; 194: 23-8.

2. Grimshaw MJ, Cooper L, Papazisis K, Coleman JA, Bohnenkamp HR, Chiapero-Stanke L, Taylor-Papadimitriou J, Burchell JM. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. Breast Cancer Res 2008; 10: R52.

3. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005; 65: 5506-11.

4. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, Hilsenbeck SG, Pavlick A, Zhang X, Chamness GC, Wong H, Rosen J, Chang JC. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 2008; 100: 672-9.

5. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, Song E. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 2007; 131: 1109-23.

6. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007; 1: 555-67.

7. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res 2005; 7: 86-95.

8. Ullrich RL, Bowles ND, Satterfield LC, Davis CM. Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation. Radiat Res 1996; 146: 353-5.

9. Deome KB, Faulkin LJ, Jr., Bern HA, Blair PB. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 1959; 19: 515-20.

10. Medina D. Preneoplastic lesions in murine mammary cancer. Cancer Res 1976; 36: 2589-95.

11. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ. Purification and unique properties of mammary epithelial stem cells. Nature 2006; 439: 993-7.

12. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. Generation of a functional mammary gland from a single stem cell. Nature 2006; 439: 84-8.

13. Li Y, Rosen JM. Stem/progenitor cells in mouse mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 2005; 10: 17-24.

14. Ramaiah L. Of Mice, Genes, and Radiation: The Genetics of Non-Hereditary Breast Cancer Explored using the Common Laboratory BALB/c Mouse. Fort Collins, CO: Colorado State University; 2008.

15. Liao MJ, Zhang CC, Zhou B, Zimonjic DB, Mani SA, Kaba M, Gifford A, Reinhardt F, Popescu NC, Guo W, Eaton EN, Lodish HF, Weinberg RA. Enrichment of a population of mammary gland cells that form mammospheres and have in vivo repopulating activity. Cancer Res 2007; 67: 8131-8.

Chapter 6:

Discussion

It has been hypothesized that an individual's susceptibility to breast cancer results from a combination of carcinogen exposure and genetic predisposition [1]. For many years, our lab has been using the BALB/c mouse model of radiation-induced mammary carcinogenesis. These mice are genetically predisposed to develop mammary cancer following exposure to IR at higher frequencies than resistant strains [2]. We have previously shown that the mammary epithelial cells from BALB/c mice are inherently susceptible to radiation-induced genomic instability. We hypothesized that this genomic instability is an early event in the carcinogenic process [3]. In this dissertation we first used telomere-specific FISH to analyze the role of telomere dysfunction in the radiation-induced genomic instability seen in BALB/c mammary epithelial cells compared to those derived from C57BL/6 mice. It was found that although there are a significant number of telomere-DSB fusions after IR exposure, the kinetics of this particular type of instability follow the same kinetics as the overall genomic instability observed previously [3]. We therefore conclude that telomere dysfunction is involved in genomic instability but is not responsible for causing it. Following the conclusion of these experiments we became aware of successful attempts to isolate mammary stem cells and culture them *in vitro*.

The mammary gland transplantation system, which was used previously to show that the mammary cells derived from BALB/c mice are more susceptible to the transforming

effects of IR then those derived from C57BL/6 mice, suggests that it is in fact the mammary stem cells specifically that are susceptible to transformation by IR exposure [4]. In order to investigate that possibility techniques were developed and optimized for culturing high density and clonal density nonadherent mammospheres. Techniques were also optimized for growing mammospheres in basement membrane extract, a nutrient rich semi-solid gel, which allowed growth curve analyses on individual mammospheres.

A great deal of size variation was observed in each culture of mammospheres. This size variation could potentially be due to the presence or lack of a true mammary stem cell. To test the hypothesis that only mammospheres capable of reaching a certain size contained true stem cells we attempted to characterize the self-renewal capacity of specific sizes of mammospheres. These data are important for future studies because stem cells are the only cells capable of self-renewal. Though the stem cell hierarchy for the murine mammary gland is not well defined, it is believed that the stem cells go through asymmetrical self-renewal to produce high progenitor cells. These high progenitors are then able to produce both the luminal progenitor cells and the myoepithelial progenitor cells. However, the high progenitors should not have self-renewal capacity [5]. Growth of a mammary gland or serial passaging are the only ways to differentiate between the stem cell and these high progenitor cells.

Characterization of multi-lineage differentiation potential was used to test specific sizes of mammospheres for the presence or absence of stem cells. In the end, a protocol was created for this assay; however the results argue that the hypothesis that only larger mammospheres possess true stem cells capable of multi-lineage differentiation appears to be incorrect. The data show that though only 19% of the mammospheres are capable of producing both myoepithelial and luminal epithelial cell lineages, there is no correlation with mammosphere size.

Finally, we developed a mammosphere formation efficiency assay to assess the radiation response of mammospheres derived from five strains of inbred mouse related to the BALB/c model of radiation-induced mammary cancer (BALB/cByJ, C57BL/6J, SCID, C.B6-*Prkdc* and B6.C-*Prkdc*^{BALB}). These data show that mammary stem cells are more resistant to the killing effects of IR than are differentiated cells derived from the same mice. The data also show that although the stem cells are more resistant to cell killing than kidney fibroblasts, they maintain the radiation sensitivity relationships between mouse strains observed previously [6]. Mammary stem cells derived from SCID mice are more sensitive to radiation-induced cell killing than those derived from C57BL/6J mice. Mammary stem cells derived from BALB/cByJ mice show sensitivity to radiation that is intermediate compared to SCID and C57BL/6J mice. These data were consistent regardless of how the mammosphere colonies were scored.

The dose response of mammospheres derived from the congenic mouse strains C.B6-*Prkdc* and B6.C-*Prkdc*^{BALB} was also determined. These data are different depending on what is scored as a true colony. Based on the lineage differentiation potential experiments, that the most accurate way to analyze these data are to include all symmetrical mammospheres $\geq$60μm but exclude the asymmetrical looking 60-99μm colonies. When this method is used, the dose response curves for both of the congenic strains fall intermediate between the parental BALB/cByJ and C57BL/6J strains.

The dose response of the congenic mice remains questionable, but the data obtained on the sensitivity of mammospheres derived from the control mouse strains is sufficient to warrant further analysis. This mammosphere formation assay can be greatly expanded to assess the development of genomic instability, differential gene expression in stem cells between strains of mice, the capacity of stem cells and their immediate progeny to repair

DNA, etc. We believe that the results shown here are promising enough to attempt differentiation experiments using BME. Mammary cells can be grown in three-dimensional BME cultures and induced to differentiate and produce mammary ductal-acinar structures *in vitro* [7]. Using this system it may be possible to culture irradiated mammary cells derived from BALB/c mice and produce ductal dysplasias *in vitro*. If this is the case it would open up a whole new field of experimentation with this model of radiation-induced mammary carcinogenesis.

LITERATURE CITED:

1. Briollais L, Wang Y, Rajendram I, Onay V, Shi E, Knight J, Ozcelik H. Methodological issues in detecting gene-gene interactions in breast cancer susceptibility: a population-based study in Ontario. BMC Med 2007; 5: 22.

2. Ullrich RL, Storer JB. Influence of gamma irradiation on the development of neoplastic disease in mice. III. Dose-rate effects. Radiat Res 1979; 80: 325-42.

3. Ponnaiya B, Cornforth MN, Ullrich RL. Radiation-induced chromosomal instability in BALB/c and C57BL/6 mice: the difference is as clear as black and white. Radiat Res 1997; 147: 121-5.

4. Ullrich RL, Bowles ND, Satterfield LC, Davis CM. Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation. Radiat Res 1996; 146: 353-5.

5. Visvader JE, Lindeman GJ. Mammary stem cells and mammopoiesis. Cancer Res 2006; 66: 9798-801.

6. Ramaiah L. Of Mice, Genes, and Radiation: The Genetics of Non-Hereditary Breast Cancer Explored using the Common Laboratory BALB/c Mouse. Fort Collins, CO: Colorado State University; 2008.

7. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003; 17: 1253-70.

Appendix A:

Detailed Materials and Methods

Mammary Epithelial Cell Isolation:

1. Prepare dissociation solution immediately prior to use in M-199 basal medium (15ml for 1 mouse, 20ml for 2 mice).

   200 units/ml Collagenase Type III (Worthington #4183)

   ~4.7 units/ml Dispase I (Neutral Protease; Worthington)

   - Filter sterilize with 0.45μm filter.
   - Transfer to 50ml conical centrifuge tube and store at 4°C until use.

2. Charge CO_2 chamber for 5 minutes and then let CO_2 settle for 2 minutes.

3. Put **200μl** cold M-199 (no collagenase or dispase) on glass Petri dish and keep covered.

4. Euthanize mouse with CO_2. Pin out on corkboard. Swab with Betadine solution, and spray with 70% ethanol. Place in animal hood on sterile blue autoclave paper. Swab again with 70% ethanol. Open abdomen with a "Y" incision to access the mammary glands. Remove lymph nodes and discard then remove mammary glands #4 and #5 and place on Petri dish.

   - *Switch dishes and tools between strains*

5. Cover dish and transfer to tissue culture hood. Mince tissue with sharp razor blades in a scissor motion until pieces are tiny ~ $1mm^3$ **(no longer than 2 minutes)**.

6. Transfer mammary tissue to the dissociation solution.

7. Incubate tubes in shaking water bath at 125 rpm and 37°C for 1-3 hrs. E**very 30 minutes remove tubes and shake 8-10 times.**

   - *Digest until most clumps are broken up and solution is homogenized and cloudy.*

8. Centrifuge cells at 1500 rpm and 4°C for 5 minutes.

9. Aspirate supernatant and break up pellet by flicking tube.

10. Resuspend cells in 10mls cold rinse media and transfer to a 15ml tube.

11. Repeat steps 8-10 for a total of 5 rinses.

12. After the last rinse aspirate supernatant and resuspend cells in ~5ml rinse media. Plate cells onto a 100mm normal tissue culture dish and incubate at 37° and 5% CO_2 to allow fibroblasts to attach to the plate.

13. Incubate for 45 minutes and then monitor fibroblast attachment every 10-15 minutes. Usually 60-75 minutes is sufficient.

14. Carefully collect the suspension of unattached epithelial cells. Place the suspension into a 15ml tube.

15. Centrifuge cells at 1500 rpm and 4°C for 5 minutes.

16. Aspirate supernatant. Resuspend mammary epithelial cells in 1-2ml of Mouse Primary Cell Media with 5% FBS.

17. Count cells using the Coulter Counter:

    - 20µl + 180µl media + 9.8ml Isoton solution = 1:500 dilution

    - Set gates at 4.5-8µm, average 3 counts

18. Plate cells at $1.2x10^6$ cells per 60mm RINSED collagen-coated dish in 5ml Mouse Primary Cell Media with 5% FBS.

    - *Plates must be coated ahead of time and stored in the freezer. Before use warm plates to room temperature.*

    - *Immediately before plating rinse the plates with 5ml Hank's Balanced Salt Solution (no Mg^{++} or Ca^{++}).*

19. Incubate mammary epithelial cells under 10% CO_2 at 37°C.

Mouse Mammary Epithelial Cell Culture:

After plating mammary epithelial cells, allow >6 hours for cells to attach, usually overnight. Then using a pipette, very carefully remove media and rinse cells twice with Hank's Balanced Salt Solution (with Mg^{++} and Ca^{++} ; Hyclone #SH30030). Replace media with Mouse Primary Cell Media containing 2% FBS. Change media every 3 days.

- *Passage cultures while they are growing but before the colonies touch.*

Passaging Mammary Epithelial Cells:

- *When colonies are growing but not yet touching*

1. Rinse cells with warm Hank's Balanced Salt Solution (HBSS) (Hyclone #SH30030).
2. Add 0.5ml COLD TrypLE (Gibco) to rinsed 60mm dish.
3. Incubate at Room Temperature for 5-10 minutes. Observe cell detachment.
    - *Want most cells to balling up, even those in the large monolayers and clumps.*
    - *Often monolayer cells will show edges curling up and this may be the maximum detachment you see for the layers.*
4. Immediately add 5ml warm HBSS with 5% FBS and triturate vigorously to loosen cells. Place cell suspension in 15ml tube.
5. Use cell scraper to remove remaining cells.
6. Add 5ml more HBSS with 5% FBS to plate and rinse vigorously to remove remaining cells from the dish and transfer to 15ml tube.
7. Spin down cells 1500 rpm, 5 min, 4°C.
8. Resuspend cells in Mouse Primary Cell Media with 2% FBS.
    - *Typically split 1:3*
9. Plate cells onto room temperature, **rinsed** collagen-coated plates with 5ml Mouse Primary Cell Media with 2% FBS.

Collagen-Coated Plates:

For 5 - 60mm plates:

9.4ml 3xdH2O

0.125ml 1N sterile HCl (Sigma #H9892, store at room temperature)

0.5ml collagen solution (PureCol – INAMED 2586-C02-0206, store 4°C)

Total volume = 10ml

1. Use 2ml of diluted collagen solution to each 60mm dish.
    - *Adjust volume for other sized plates according to surface area.*
2. Swirl plate to ensure even coating and completely covered bottom.

3. Arrange plates under tissue culture hood with lids off, hood vent on, lights off – NO UV.
4. Dry overnight.
   - *takes ~6-8 hours*
   - *flasks can be coated but take far longer to dry*
5. Once dry, return plates to sterile plastic wrap, seal, and store at -20°C.

Before use:

1. Thaw to room temperature.
2. Immediately prior to use (don't let them dry out before plating cells) - rinse plates with HBSS modified (without Mg^{++} or Ca^{++}; Hyclone #SH30031).

Rinse Media:
Any unsupplemented media containing 5% FBS (We usually use expired DMEM).

Mouse Primary Cell Media:
Ham's F12 basal media (JRH #51651 or Hyclone # SH30010.03 pH6.9)

Supplement with:

Ingredient	Stock conc.	Working conc.	for 250ml
Insulin	2 mg/ml	5 µg/ml	0.625 ml
Hydrocortisone	0.5 mg/ml	1 µg/ml	0.5 ml
CholeraToxin	125 µg/ml	0.1 µg.ml	0.2 ml
Apotransferrin	2.0 mg/ml	5 µg/ml	0.625 ml
Antibiotic/antimycotic	1x	100x	2.5ml
FILTER – 0.22µm filter then add EGF and FBS			
EGF	10 µg/ml	10 ng/ml	0.250 ml
FBS	1x	5%	12.5 ml
FBS	1x	2%	5 ml

- *Final product has a 10 day to 14 day shelf life maximum.*
- *Avoid heating and reheating media.*
- *May choose to equilibrate media in the incubator prior to use because it has very little buffering capacity (loosen bottle top and put in incubator for 1-2 hours).*

Mouse Primary Cell Media Supplement Stock Preparation:

1. *Insulin* 2mg/ml stock–Sigma, #I-5500
   - Add 50ml of 0.005N HCl to 100mg powder (mix 250µl of 1N HCL to 50ml ddH_2O)
   - Stir well to dissolve
   - Filter thru 0.45µm low protein binding filter (i.e. PES) or wait to filter once in media
   - Aliquot into 0.625ml aliquots
   - Store @ -20°C—DO NOT FREEZE THAW

2. *Hydrocortisone* 0.5mg/ml stock– Sigma #H0135
   - To a 1mg bottle, Add 1ml 100% Ethanol & add 1ml sterile H_2O (bring up under sterile conditions).
   - Aliquot in 0.5ml aliquots and store @ -20°C. (does not mention anything about filtering so wait until have it mixed with media to filter)
   - AVOID FREEZE THAWS ALTHOUGH DOES NOT REALLY FREEZE

3. *Cholera Toxin* 125µg/ml stock - Sigma # C-8052
   - For a 0.5mg bottle, add 4ml of sterile HBSS (can use sterile water also)
   - Aliquot using sterile conditions into 0.2ml aliquots
   - REFRIGERATE - DO NOT FREEZE
   - Stores up to 1 year once hydrated, 3 years lyophilized; can filter using 0.2µm filter or filter when filtering media.

4. *Apotransferrin* 2mg/ml stock - Sigma #T5391
   - Add 5ml sterile media to 10mg bottle.
   - Aliquot in 0.625ml aliquots and store @ -20°C.
   - Can filter sterilize for stability or just filter when filtering media.
   - AVOID FREEZE THAW.
   - Stable at 4°C for 5-10 days. AVOID REPEATED HEATINGS TO 37°C.

5. *Antibiotic-antimycotic* 100x stock – Gibco #15240
   - 100ml bottle, aliquot into 2.5ml aliquots and store @ -20°C.
   - Can filter sterilize or just filter when filtering media
   - AVOID FREEZE THAW. Stable at 37°C for 3 days

6. *EGF* 10µg/ml stock – Sigma #E4127
   - Add 10ml sterile media with 10% FBS to 0.1mg bottle.
   - Aliquot in 125µl aliquots and store @ -20°C
   - DO NOT FREEZE THAW
   - DO NOT STERILE FILTER-prepare under sterile conditions and add to media **after** it has been sterile filtered, even with low protein binding filter get too much loss of EGF.

Harvest Technique for CO-FISH:

1. Add BrdU to media in plates - 24 hours before harvest
    - *should be one cell cycle if known*
    - final concentration 10μM (stock = 10^{-2} molar, so use **1μl per ml** media)
2. Add Colcemid to media in plates - 6 hours before harvest
    - **20μl per ml** media (0.04μg/ml) (KaryoMax - Gibco #15212-012)
3. Collect cells:
    - *Use epithelial cell passaging protocol*
    - *Collect cells into 15ml **Polystyrene** tube (cannot be PE).*
4. Centrifuge @ 1000 rpm for 5 mins.
5. Aspirate media
6. Resuspend pellet in 75mM KCl using plastic pipette (add drop-wise to 2ml, then resuspend pellet and add 3ml more directly to tube).
    - 75mM KCl = 5.59g KCl + 1L dH_20 (does not need to be sterile)
    - *Do not keep for longer than 2 months.*
7. Mix well by flicking and let sit for 15 minutes (fibroblasts can be vortexed, but delicate cells just need to be flicked).
8. Mix cells again
9. Prefix – add ~1ml fixative using Pasteur pipette
    - Fix = 3:1 methanol: acetic acid
    - *Always mix fresh*
10. Mix well
11. Centrifuge @ 1000 rpm for 5 mins
12. Aspirate supernatant
13. Resuspend in fix using Pasteur pipette (add drop-wise to 2ml, then resuspend, then top off to 5ml)
14. Incubate at room temperature for 10 minutes
15. Cells can be frozen at this time -20°C
    - Prior to making slides repeat fix steps 2 more times.
    - For storage >1 year go through all 3 fixes and store in 100% methanol.

Mammary Cell Isolation for High Density Mammosphere Cultures:

This protocol was adapted from one published in the product literature for Epicult-B media by Stem Cell Technologies.

Dissociation of Mouse Mammary Tissue:

1. Charge CO_2 chamber for 5 minutes and then let CO_2 settle for 2 minutes.
2. Put **200µl** of DMEM/F12 media on glass Petri dish and keep covered.
3. Euthanize mouse with CO_2. Pin out on corkboard. Swab with Betadine solution, and spray with 70% ethanol. Place in animal hood on sterile blue autoclave paper. Swab again with 70% ethanol. Open abdomen with a "Y" incision to access the mammary glands. Remove lymph nodes and discard then remove mammary glands #4 and #5 and place on Petri dish.
4. Cover dish and transfer to tissue culture hood. Mince tissue with sharp razor blades in a scissor motion until there are no larger big chunks **(no longer than 2 minutes)**.
5. Transfer mammary tissue to the Dissociation solution.
6. Incubate the **15ml** tubes at 37°C and 5% CO_2, until the tissue is broken down.
    - *In our hands this takes 45 minutes – 1.5 hours but the protocol recommends 6 hours.*
    - *The **cap** MUST be **loosened** completely to allow gas exchange or all the cells will die. Stable 5% CO_2 is required for the digestion to work properly, maybe by altering the pH of the dissociation.*
    - ***Every 15 minutes** - close cap and flick tube ~10 times (hard) then loosen cap again. Monitor the extent of tissue dissociation.*
    - *In general, the dissociation should go until there is no change in the way the liquid or tissue pieces look between flicks. The media should be very and cloudy, and the tissue pieces should look broken down and fibrous.*

- ***Fatty glands*** *- Will develop a thick layer of fat that settles on the top of the media, distinct tissue chunks will be visible at early time points, but at later times the pieces will break down completely and form a homogeneous layer. If this occurs, the digestion is very close to completion, it should be checked every 2-3 minutes. If the layer is completely homogeneous then the digestion should be stopped immediately, it may even be over-digested. When fatty glands are digested the media will look cloudy faster, and will reach a point where it is completely opaque. If you cannot see through the media at all, stop the reaction regardless of time, some stem cells may be lost in the tissue pieces, but this will prevent the single cells from being killed*

- ***Lean glands*** *- The media should still turn from transparent to cloudy, though it may never reach the point where it is completely opaque. These digestions will not really develop a fatty layer, though it doesn't hurt to look for one. For very lean tissue the most important aspect is the characteristics of the tissue pieces. When the digestion is near completion the pieces will look stringy and fibrous not solid like at the beginning of the digestion.*

7. After incubation period, add 10ml of cold Hanks Balanced Salt Solution with 2% FBS (HBSS with 2% FBS) Centrifuge at 1460 rpm (450 x g) for 5 minutes @ 4°C. Discard supernatant.

    - *The pellet should contain clumps of epithelial cells, stromal cells and lymphocytes (epithelial organoids).*

8. Warm up 0.25% Trypsin and Dispase (37°C waterbath)

Generation of Single Cell Suspension from Mouse Mammary Tissue:

9. Add **3ml** of pre-warmed 0.25% trypsin-EDTA to the organoids.

10. Pipette with a P1000 for 1-2 minutes. The sample should become very stringy due to lysis of dead cells and the release of DNA.

    - *Use plugged pipette tips, there are often still chunks of tissue that get stuck and splash media up into the pipette.*

11. Add 10ml of HBSS with 2% FBS and spin at 1460 rpm (450 x g) for 5 minutes @ 4°C.

12. Remove as much supernatant as possible. The cells may be a big stringy mass floating in the HBSS with 2% FBS.

13. Add **2ml** of pre-warmed Dispase II and **400 units** DNase 1.

    - *The DNase 1 can be omitted for high density mammospheres, the cells just stick together more. If DNase is not used, increase the dilution used for counting to 1:500 to avoid clogging the Coulter Counter.*

14. Pipette the sample for 1 minute using a P1000. The sample should now be cloudy, and stringy.

15. Add 10ml of HBSS with 2% FBS.

16. Filter the cell suspension through a 40μm cell strainer into a 50ml centrifuge tube.

17. Centrifuge at 1460 rpm (450 x g) for 5 minutes @ 4°C.

18. Resuspend sample in 10ml **mammosphere** media.

Counting:

Coulter Counter: 200μl cells + 9.8ml Isoton = 1:50 dilution

Gate Coulter Counter to 3.5μm-7μm

- *For examples of histograms see Figures 3.1 + 3.2*

Plating:

Plate 1-3 x 10^6 cells on 60mm Ultra-Low Attachment plates (Corning #3261)

- *Plating too many cells results in decreased growth after 2-3 days, adding 2-3ml fresh mammosphere media helps to reduce this problem.*
- *Plating too few cells results in growth retardation.*
- *Do not use 100mm dishes, they are too cumbersome and get contaminated easily.*
- *FBS exposure for longer than ~48 hours destroys the coating on these plates.*

Preparation of Reagents for Mammary Cell Isolation for Mammosphere Cultures:

1. Dissociation Media:

   Mix Epicult-B Basal Medium with **300 units/ml** Collagenase Type III (Wothington #4183) and **100 units/ml** Hyaluronidase (Sigma #H-3506). Filter-sterilized with **0.45µm** PES filter. To the tube add Epicult-B Supplement, Antibiotic/Antimycotic, GlutaMAX, and 5% FBS. Store on ice until use.

   - *Units of enzyme activity are VITAL pay attention!*
   - *Original protocol calls for hydrocortisone, but seems to work fine without*
   - *Supplements should be used at a final concentration of 1x*
   - *For single mouse use 3ml for two mice use 5ml, DO NOT TRY MORE THAN 2 mice per 15ml tube, DO NOT increase size of tube.*
   - *If glands from more than 2 mice are needed use more tubes, keep close track of digestion start times for each!*

2. Hank's Balanced Salt Solution w/ Hepes (HBSS) and 2% FBS (need ~40ml per TUBE)
3. Dispase Solution (need 3ml per TUBE of tissue)

   Mix **5mg/ml** Dispase II (Roche #165859) with Hank's Balanced Salt Solution w/Hepes (no FBS). Filter-sterilized with **0.45µm** PES filter. Store on ice until use.

4. DNase 1 (need **400 units** DNase 1 **per TUBE** of tissue)
5. 0.25%-Trypsin-EDTA (need 3ml **per TUBE** of tissue)

Reagent Stock Preparation for Mammary Cell Isolation:

1. *EpiCult-B* - (Stem Cell Technology #05601):
   - 100ml Epicult media + 1ml Epicult supplement
   - Media should be stored at 4°C – has a shelf life of 1 year
   - Supplement - before use thaw on ice and aliquot then stored at -20°C.
   - Do Not freeze thaw. Stable at 4°C for 2 weeks
     - *We have never kept it at 4°C.*

2. *FBS* –
   - Choose a brand and lot # and stick with it
   - it is only used for isolation and rinse media so it is not likely to make any difference what brand or lot is used.

3. *Hank's Balanced Salt Solution w/ Mg^{++} and Ca^{++}* (Hyclone # SH30015)
   - Comes as a powder (can buy premade liquid but must still add Hepes and filter)
   - add 0.35g/L Sodium Bicarbonate
   - add 10mM Hepes = 2.383g/L Hepes
   - Filter sterilize with 0.22μm filter.
   - Store at 4°C

4. *DNAse 1* – (Sigma Aldrich #D4513)
   - Need 400 units per tube of tissue
   - Want stock solution ≥20,000 units/ml reconstituted with sterile PBS
   - Aliquot and freeze, do not freeze-thaw
   - Seems to lose activity fairly fast so don't make too much at a time
   - It is not necessary for the isolation but makes counting more difficult due to increased sticky DNA.

Mouse Mammosphere Media:

For **30ml** 1:1 DMEM/F12 basal media

- *Final product has a 10 day to 14 day shelf life maximum*
- *Avoid repeated heating*

Supplement	[Stock]	[**1x** Working]	**1x** Volume	[**2x** Working]	**2x** Volume
EGF	10μg/ml	20ng/ml	60μl	40ng/ml	120μl
B27	50x	1x	600μl	2x	1200μl
bFGF	25μg/ml	20ng/ml	24μl	40ng/ml	48μl
Heparin	50mg/ml	4μg/ml	2.4μl	8μg/ml	4.8μl
Antibiotic	100x	1x	300μl	2x	600μl
GlutaMAX	100x	1x	300μl	2x	600μl

DO NOT FILTER

Mouse Mammosphere Media Supplement Stock Preparation:

1. *DMEM/F12* 1x liquid media – Hyclone #SH30023
   - Store at 4°C protected from light

2. *EGF (Epidermal Growth Factor)* 10μg/ml stock – Sigma # E4127 or E1257
   - Store lyophilized at 4°C
   - Add 10ml sterile media with 10% FBS to 0.1mg bottle

- Make aliquots (60µl for 30ml of media) and store at -20°C
- Do Not freeze thaw
- Solution stable for 14 days after thawing

3. *B-27 Supplement* 50x stock – Gibco # 17504
   - 10ml bottle; aliquot into 600µl aliquots
   - Store @ -20°C protected from light
   - Stable for 1 year at -20°C
   - Do Not freeze thaw.
   - Assume stability at 4°C is 14 days like other supplements

4. *Heparin Sodium Salt* 50mg/ml stock – Sigma # H1027
   - Store lyophilized chemical at room temperature in desiccator
   - 100mg Heparin + 2ml dH_20 = 50mg/ml
     - *Our current Heparin is 164units/mg – seems to work well*
   - Filter through a 0.2µm filter to sterilize and increase stability
   - Aliquot (we use 50µl aliquots though smaller is probably better)
   - Store at 4°C for 2 years

5. *bFGF (Fibroblast Growth Factor-Basic)* 25µg/ml stock – Sigma #F0291
     - *NOTE: BD brand is not as good, it has a much shorter shelf life. If you use it – it has very different reconstitution guidelines!*
   - 25µg lyophilized powder
   - To the 25µg bottle of bFGF add 1ml of the 20mM Tris (pH 7.0)
   - Make 25µl and 72µl aliquots (for 30ml + 90ml media)
   - Store at -20°C for 6 months
   - Store at 4°C for 14 days
   - Do Not freeze thaw

6. *Antibiotic-Antimycotic* 100x stock – Gibco #15240-062
   - 100ml bottle
   - Filter sterilize using 0.45µm filter, and aliquot (300µl, 600µl, 900µl)
   - Store at -20°C for 1 year
   - Do Not freeze thaw.
   - Assume stability at 4°C is 14 days, stable at 37°C for 3 days

7. *GlutaMAX* 100x stock – Gibco #35050-061
   - 100ml bottle- Substitute for L-Glutamine, makes up for the spontaneous breakdown during incubation or media storage.
   - Aliquot (300µl, 600µl, 900µl)
   - Store @ -20°C shelf life 24 months
   - Freeze thaws OK

Tissue Culture of High Density Mammosphere Suspension Cultures:

Mammospheres should be grown in Ultra-Low Attachment plates only. Grow at 37°C in humidified 5% CO_2 atmosphere. The day of isolation is day 0. These cultures can be monitored for growth every day, it will increase the amount of aggregation but for high density cultures it does not matter. See Chapter 3 for a detailed description of growth characteristics. Mammospheres should be grown in 5ml 1x mammosphere media. Add 2-3ml fresh media on day 3 to counteract the effects of all the dead cells. The media should be changed every 4 days.

Media Changing Protocol for High Density Mammosphere Suspension Culture:

- *Media should be changed every 4 days*
- *Mammospheres need to be handled more gently the larger they grow or the spheres will break apart.*
- *Ultra-Low Attachment plates CAN be reused but they must be treated very carefully. If anything (pipette etc.) touches the surface it will scratch the coating and cells will stick.*

For cultures containing very small mammospheres (in culture 1-7 days):

1. GENTLY collect media from plate and put it in a centrifuge tube
2. Rinse plate with fresh mammospheres media OR rinse media, and add it to the centrifuge tube.
3. Centrifuge for **5 minutes** at **500 rpm** and **4°C**.
4. Carefully aspirate media.
5. Resuspend pellet by flicking tube (GENTLY)
6. Gently add the appropriate volume of fresh mammospheres media to the tube.
7. Mix gently but thoroughly
8. If the Ultra-Low Attachment plate is to be reused, rinse it vigorously with sterile PBS at least 2x.
9. Gently pipette cells from tube onto the plate.

For cultures containing decent sized mammospheres (in culture for 7+ days):

1. GENTLY collect media from plate and put it in a centrifuge tube
2. Rinse plate with fresh mammospheres media OR rinse media, and add it to the centrifuge tube.
3. Centrifuge for **2 minutes** at **500 rpm** and **4°C**.
4. Carefully aspirate media.
5. Resuspend pellet by flicking tube (GENTLY)
6. Gently add the appropriate volume of fresh mammospheres media to the tube.
7. Mix gently but thoroughly
8. If the Ultra-Low Attachment plate is to be reused, rinse it vigorously with sterile PBS at least 2x.
9. Gently pipette cells from tube onto the plate.

To Harvest Conditioned Media for Clonal Mammospheres:

- *No sooner than day 7, spheres should have reached ~250μm before being used for conditioned media*
- *High density cultures can be used for 16 days, and then we discard them.*
- *It is best NOT to passage these cultures*
- *If mammospheres are growing well and there are a lot in the plate, we add 8-10ml of media for making conditioned media, it limits the number of plates necessary.*

1. GENTLY collect media from plate and put it in a centrifuge tube
   - *DO NOT USE RINSE MEDIA*
2. Centrifuge for **2 minutes @ 500 rpm** and **4°C**.
3. Carefully remove the media and transfer it to a new 15ml centrifuge tube.
4. Replace media on mammospheres and put them back into culture
5. Centrifuge the conditioned media again, for 5 minutes @ 1460 rpm and 4°C.
6. Very carefully transfer the media to a sterile bottle for storage. Keep track of the amount of media collected.
7. The shelf life of the conditioned media is unknown
   - *We have tried to use it within 4 days.*

Passaging Mammospheres from 60mm Plates of High Density Suspension Cultures:

- *Adapted from Dontu et al., 2003*
- *Use 0.05% Trypsin-EDTA (Hyclone – SH30236.01), TrypLE takes much longer, did not bother working out timing.*
- *Within 1-3 days should see very good 2° sphere formation*
- *MUST USE fire polished pipette – P1000 is too harsh, mammospheres just get shredded.*

1. Collect mammospheres and transfer to a 15ml centrifuge tube
2. Rinse plate with 5ml of mammospheres media OR rinse media to remove any sticking cells. Add to centrifuge tube.
3. Centrifuge at 4°C and 800 rpm for 5 minutes.
4. Aspirate media and flick tube to resuspend pellet.
5. Add 2ml 0.05% Trypsin (ROOM TEMPERATURE).
6. Pipette gently with a 9" fire polished Pasteur pipette for 10 minutes.
7. Add 10ml cold rinse media and mix well.
8. Put cells through a 40μm cell strainer (collect in a 50ml centrifuge tube)
9. Centrifuge at 4°C and 800 rpm for 5 minutes.
10. Aspirate media and flick tube to resuspend pellet
11. If Ultra-Low Attachment plate is to be reused – rinse with sterile PBS at least 2x.
12. Resuspend cells in appropriate volume of fresh mammospheres media and plate.

Making Fire Polished Pasteur Pipettes:

- *Perfectly polished glass Pasteur pipettes are absolutely vital for successful passaging*
- *We did try P1000 and different gauge needles - these all resulted in shredding of the mammospheres and cells and very poor recovery.*
- *Use cotton plugged pipettes whenever possible to limit contamination of samples.*

1. First examine pipette for any blemishes, cracks etc, if they are extensive the pipette should not be used at all.

2. Pass pipette through an open flame 2-5 times, rotating while it passes, in order to melt the sharp edges created during manufacturing. All sharp edges, points and cracks must be rounded out while still maintaining a large opening

   - *We use a P10 tip as a guide as the majority of mammospheres can fit through this tip.*

3. Once cooled, the pipettes should be analyzed again for imperfections

4. Autoclave for sterility.

5. Immediately prior to use a sterile fire polished pipette must be carefully analyzed again for any sharp spots or cracks resulting from sterilization or storage.

6. It should then be passed quickly through a flame, to remove any cotton fibers picked up from the padding in the storage box.

Basement Membrane Extract:

BME is now commercially available from three companies, Matrigel™ is most well known and is available from BD Biosciences (#356231), Cultrex is available from Travigen (#3433), and Geltrex is available from Invitrogen (#12760). All are isolated from the EHS mouse sarcoma, and Geltrex and Cultrex are actually from the same laboratory and just sold by two different trade names. All brand seem to work equal however, there is significant lot-to-lot variation, so each new lot must be extensively tested prior to experimental use. To limit the amount of variation always use "growth factor reduced" BME. Protein concentrations tend to vary from 12-18mg/ml, the lower concentrations result in softer gel, this seems to increase differentiation of mammary cells compared to higher protein concentrations which result in more symmetrical mammospheres. BME should be stored at -80°C for long term storage, once thawed try NOT to refreeze, if necessary aliquot into useful sizes for media changes only and store at -20°C.

BME "On-Top" Culture Assay:

This protocol was adapted from "Three-D culture models of normal and malignant breast epithelial cells" by Lee et al., 2007. The volumes we used were directly taken from this protocol. The published protocol recommends changing media every 2 days, and only culturing the cells for a total of 4 days, we have maintained cultures for as long as 21 days using the same protocol. Store all plates, tips and tubes in the freezer, and work on ice at all times. The BME itself must be kept on ice at all times, even holding the tube will cause the BME to start solidifying after a few minutes. Once it has solidified it cannot be reverted back to liquid form.

1. Thaw appropriate amount of BME over night at 4°C in beaker filled with ice water
   - *DO NOT LET THE ICE MELT*
2. ON ICE - Coat wells of the pre-chilled plate with BME
   - 120µl per well
   - Spread evenly with pipette tip
   - *Be very careful not to make bubbles or touch the bottom, this makes little scratches in the BME gel which allows cells to grow down to the plastic and differentiate.*
   - *Works well to put individual drops in different places around the well and then spread between them.*
3. Incubate for 15-30 minutes at 37°C to allow BME to gel. DO NOT OVERDRY.
4. While the BME is incubating prepare the cells – the appropriate cell number for each well in 250µl mammosphere media.
5. Plate the cells by carefully pipetting the media down the side of the well.
6. Incubate at 37°C to allow cells to settle and attach to the matrix for 10-30 minutes.
7. While cells are attaching chill mammosphere media. When COLD add appropriate volume of BME (10% of total media volume). Mix well but GENTLY (no bubbles!).
8. Gently add 300µl of the mixture onto each well (pipette the mixture down the side of the well to avoid disturbance of cells or gel).
9. Incubate cultures at 37°C and 5% CO_2 for duration of the experiment.
10. Replace media every 2 days.

BME "On Top" Media Changing Protocol:

Media should be changed every 2 days for the duration of the experiment. For a 24-well plate use 500µl of mammosphere media and 50µl BME for each well. It works best to have individual aliquots for each well. When a large volume of BME is thawed, aliquot the leftovers into 50µl aliquots in 1.7ml centrifuge tubes. Store these at -20°C to use for media changes. We did attempt ½ volume media changes but this is really not an option for experiments running more than a few days because the media tends to evaporate.

1. Thaw the appropriate number of BME aliquots in ICE WATER.
    - *DO NOT LET THE ICE MELT!!!!*
    - *The BME will change from cloudy white to clear when it thaws. DO NOT attempt to flick the tube to see if it is thawed- you will create bubbles.*
2. Using frozen pipette tips add 500µl COLD mammosphere media to the tube of BME and pipette up and down. Mix thoroughly but avoid creating bubbles.
3. KEEP ON ICE
4. Take plate out of the incubator and remove old media.
    - *Can aspirate CAREFULLY by tipping the plate and not putting the tip anywhere near the BME. It is very easy to suck off the BME by accident and lose the cells.*
    - *Can also use a P1000 and suck off the old media using the same technique. This is more easily controlled and risk of sucking off BME is reduced. This is particularly recommended for wells that have been in culture for long periods of time (the layers build up and come off more easily).*
5. Carefully mix the BME-cold media mixture again.
6. Pipette the mixture down the side of the well gently.
7. Put plate back in the incubator.

Fixing BME (24-well plate):

This protocol is adapted from "Indirect Immunofluorescent Staining of MCF-10A Acini Cultured in Matrigel (detailed)" from Brugge.med.harvard.edu

- *Need to be extremely careful with rinses so don't mess up gel*
- *DO NOT STAIN with crystal violet – too much background*
- *Should be stable in refrigerator for 2 weeks – size does not change*
- *Wrap plates in Parafilm to prevent drying out*

1. Remove media with P1000
2. Rinse carefully with 500µl cold PBS 2x (put it down the side of the well very carefully so as not to disrupt the BME)
3. Remove with P1000 again
4. Add 500µl methanol:Acetone (1:1) MAKE FRESH!!
5. Put in freezer -20°C for 10 minutes
6. Remove alcohol with P1000
7. Let air dry for 2-3 minutes
8. Put on layer of PBS 500µl to rinse off alcohol
9. Remove PBS with P1000
10. Add final layer of PBS for storage 200-500µl per well

Mammary Cell Isolation for <u>CLONAL</u> Density Mammosphere Cultures:

- *This protocol uses the EasySep® Mouse Mammary Stem Cell Enrichment Kit from Stem Cell Technologies (# 19757). The protocol is basically the same as what comes with the kit; however we only use the magnet enrichment part and stop prior to the cytometric sorting.*
- *The set up is exactly the same as for high density mammospheres*
- *We prefer to use only one single mouse for these isolations, the cells are less clumpy*
- *We have been using conditioned media produced from mammospheres derived from syngeneic mouse strains as those to be grown in clonal cultures.*
- *The quality of the conditioned media is VITAL, if the high density cultures are not growing wonderfully, don't use them and wait for good media before doing the clonal isolation.*

1. Follow the High Density Isolation protocol until step #17 – (the last spin).
2. Resuspend cells in 10ml HBSS with 2% FBS.
3. Count cells using Coulter Counter.
4. Plate 1-3 x10^6 cells onto 1 Ultra-Low Attachment plate (as isolation control).
   - *If there are enough cells from the isolation a control high density plate is very helpful. It is the only way to know if the isolation went well and the mammospheres are growing.*
5. Mix 3ml of HBSS w/ 2% FBS and 200-300 units/ml DNase 1.
   - *DNase is very important for this procedure!*
6. Centrifuge cells again. Aspirate media. Resuspend at **1x10^8cells/ml** (=100,000cells/µl) (or lowest **volume of 200µl** total). In Hank's 2% FBS with 0.1mg/ml DNase in a 5ml Polystyrene round-bottom tube.
7. Spin down tube of EasySep® Negative Selection Enrichment Cocktail before use. Add **50µl/ml** of cells. (0.05µl/µl) (for 200µl = 10µl)
8. Mix well. Incubate ON ICE for 15 minutes.
9. Add EasySep® Biotin Selection Cocktail at **100µl/ml** of cells. (0.1µl/µl) (for 200µl = 20µl)
10. Mix well. Incubate ON ICE for 15 minutes.
11. Mix EasySep® Magnetic Nanoparticles (pipette 5x to get uniform suspension). Add **50µl/ml** of cells. (0.05µl/µl) (for 200µl = 10µl)
12. Mix well. Incubate ON ICE for 15 minutes.
13. Bring cell suspension to a **total volume of 2.5ml** by adding HBSS with 2% FBS. (for 200µl = 2,260µl)
14. Mix well by **gently** pipetting 2-3 times.
15. Place tube (without cap) into the magnet. Let set for 5 minutes.
16. Pick up magnet and in one continuous motion invert the magnet and tube – pouring off supernatant into a NEW tube. Leave inverted for 2-3 seconds, then return to upright position. DO NOT SHAKE OR BLOT ANY DROPS FROM TUBE.

(The magnetically labeled unwanted cells will remain bound inside the original tube held by the magnet.)

17. Remove original tube from magnet and add another **2ml** of HBSS with 2% FBS.
18. Mix well by gently pipetting 2-3 times.
19. Place tube (without cap) into the magnet. Let set for 5 minutes.
20. Pick up magnet and in one continuous motion invert the magnet and tube – pouring off supernatant into the tube containing the 1st set of cells. Leave inverted for 2-3 seconds, then return to upright position. DO NOT SHAKE OR BLOT ANY DROPS FROM TUBE. Volume should be about 4.5ml total.
21. Centrifuge tube of desired cells at 350 x g for 5 minutes.
22. Discard supernatant and resuspend cells to a **total volume of 2.5ml** in HBSS with 2% FBS.
23. Place tube (without cap) into the magnet. Let set for 5 minutes.
24. Pick up magnet and in one continuous motion invert the magnet and tube – pouring off supernatant into a NEW tube. Leave inverted for 2-3 seconds, then return to upright position. DO NOT SHAKE OR BLOT ANY DROPS FROM TUBE.
    (The magnetically labeled unwanted cells will remain bound inside the original tube held by the magnet.)
25. Centrifuge tube of desired cells at 350 x g for 5 minutes.
26. Discard supernatant and resuspend cells in **2ml** of **Hank's 2%FBS with 0.1mg/ml DNase 1**.
27. Count cells again using Hemocytometer.
    - *Coulter Counter does NOT work for this step, it seems to shred the cells*
28. For 0Gy plate - 1,500 cells onto each 60mm Ultra-Low Attachment plate
29. Use **10ml 1:1 conditioned media: fresh media with 2x Supplements** to improve growth. DO NOT TOUCH for 12 days. On day 12 score for mammosphere formation.

Picking Mammospheres Out of Suspension Cultures:

As the size of individual mammospheres was vital to our goal, whole plates of mammospheres could not be used. As such, we developed methodology for manually removing specific mammospheres from suspension cultures while still maintaining aseptic conditions. Briefly, a dissecting microscope fitted with an ocular reticle was placed in the tissue culture hood and exposed to UV for at least 30 minutes. After which a plate of mammospheres was placed in the hood. I followed and wearing a plastic shower cap, shoulder-length latex gloves and a surgical mask (Figure A.1.). Cap and gloves were extensively exposed to UV and sprayed with ethanol before each use. This technique has about 98% success rate for being aseptic. Using a P10 or P20 Pipetman, I manually chose and removed the individual mammospheres used in the passaging experiments. Mammospheres were kept in small volumes of mammosphere media, on ice in 1.7ml microcentrifuge tubes until use.

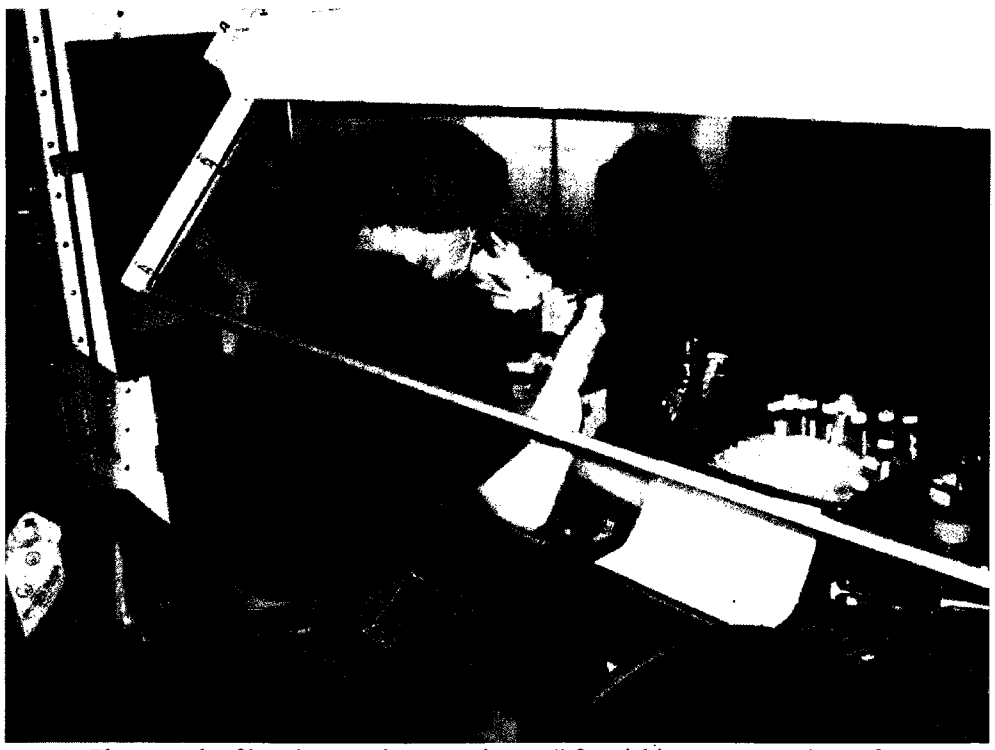


Figure A.1. Photograph of hood set up for "aseptic gear" for picking mammospheres from suspension

Experimental Design for Clonal Immunofluorescence Experiments:

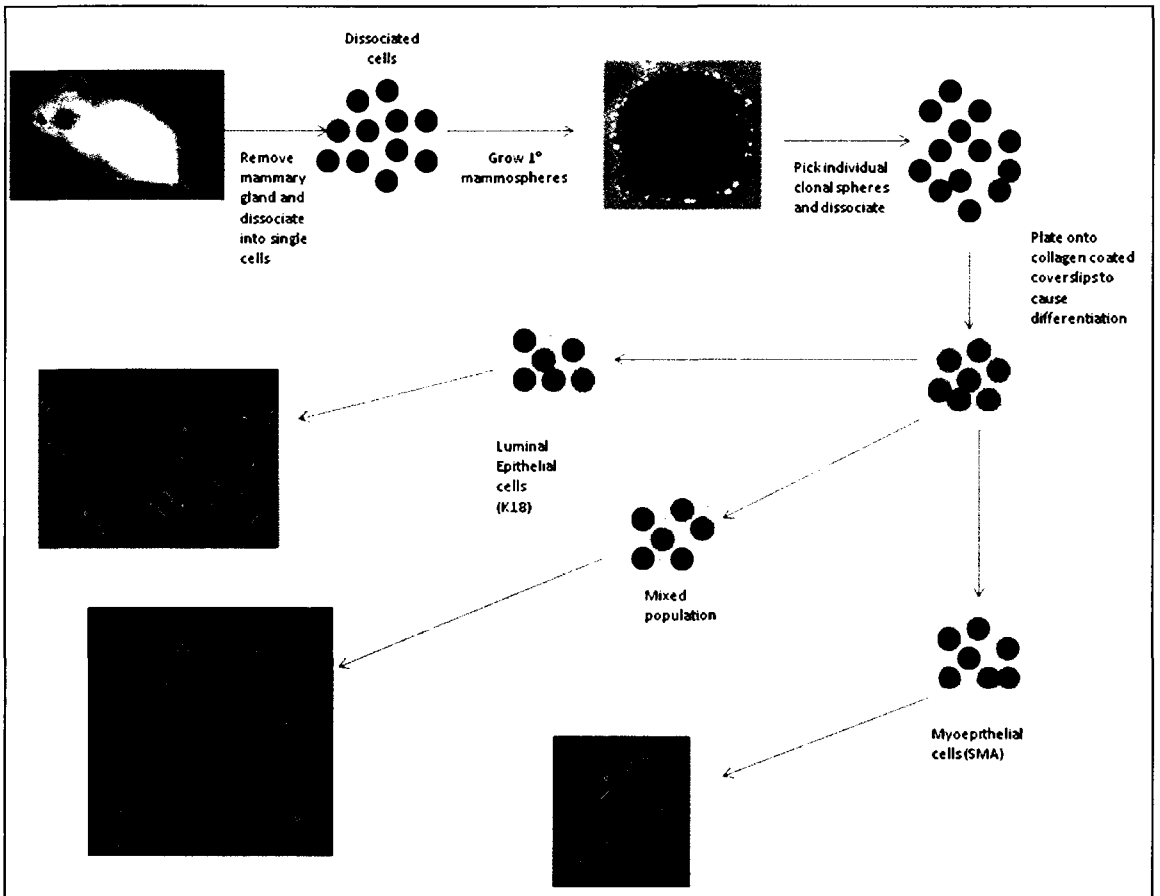


Figure A.2. Flow chart of the experimental design of clonal IF experiments. Mammary cells are isolated from mouse mammary tissue and dissociated into single cells. Cells are grown as non-adherent mammospheres. Mammospheres are collected and dissociated into single cells. Cells are plated onto Collagen-coated coverslips and allowed to grow and differentiate. Coverslips are fixed and then stained for K18 (red) and α-SMA (green). It is hypothesized that three types of colonies will be observed, all red, all green and mixed red and green.

Dissociating Individual Clonal Mammospheres for Differentiation on Collagen:

Table A.1. Size of 1° clonal mammosphere and corresponding time required for dissociation.

Size	Trypsin	Size	Trypsin	Size	Trypsin	Size	Trypsin
60μm	Flick 20 sec	120μm	Flick 30 sec	180μm	45 sec	300μm	1:10
70μm	Flick 20 Sec	130μm	Flick 35 sec	190μm	50 sec	310μm	1:10
80μm	Flick 25 Sec	140μm	Flick 40 sec	210μm	50 sec	330μm	1:15
90μm	Flick 25 Sec	150μm	30 sec	230μm	55 sec	360μm	1:20
100μm	Flick 30 sec	160μm	35 sec	270μm	1 min	420μm	1:40
110μm	Flick 30 sec	170μm	40 sec	280μm	1 min		

Fixation of Mammary Cells for Immunofluorescence:

Coverslips should be fixed with 100% methanol. However it is essential that the methanol be tested before beginning a project because it seems to degrade while on the shelf. Once a bottle that works for immunofluorescence is found it should be put in usable sized containers (we did 500ml bottles wrapped in foil) and stored at -20°C.

To fix mammary cells remove tissue culture media. Rinse 2x with PBS (the same PBS as used for the IF steps – it is absolutely vital to test all new batches/lots of PBS, it has a huge impact on if the IF works or not). Remove PBS and add 100% cold methanol (for 35mm dish use 1ml). Incubate at -20°C for 10 minutes. Aspirate methanol and air dry for 10 minutes. Store frozen.

Immunofluorescence (IF):

The antibodies used here were as follows – K18 – (mouse monoclonal [C-04] to cytokeratin 18 (Biotin conjugated) – Abcam, Cambridge MA; #ab27553); secondary – (Streptavidin – Alexafluor 594, Invitrogen #532356); α-SMA – (Rabbit polyclonal to alpha smooth muscle actin – Abcam, Cambridge Ma; #ab5694); secondary – (Goat anti-rabbit Alexafluor 488, molecular probes #A11034). DAPI = VectaShield Mounting Medium with 4',6-Diamidino-2-phenylindole (Vector Lab, Burlingame, CA).

- *Once the antibodies arrive at 4°C, they need to be aliquoted and frozen for long term storage.*

- *Diluting the antibodies prior to storage results in poor activity – AVOID if possible*
- *Quality of PBS is VITAL!!! Test each new batch before using it. Poor quality PBS can result in poor staining and drastically increased background staining.*
- *Perform incubations in a humidified chamber so that slides do not dry out.*

1. Thaw coverslips for 10 minutes
2. Rehydrate in PBS for 10 minutes
3. Rinse 2x with PBS
4. **Primary Antibody** (100µl) - 1:100 **K18** for **30 minutes**
5. Wash with PBS 3x
    - *After this step all incubations should be done in the dark to prevent bleaching of the secondary antibodies.*
6. **Secondary Antibody** (100µl) - 1:100 **Streptavidin** for **30 minutes**
7. Wash with PBS 3x
8. Block with **750µl** of 5% milk (spun 800 rpm; 5 minutes) for **1 hour**
9. Rinse quickly with PBS 3x
10. **Primary Antibody** (100µl) - 1:50 **α-SMA** for **1 hour**
11. Wash with PBS 3x
12. **Secondary Antibody** (100µl) - 1:100 **FITC** For **1 hour**
13. Rinse with PBS quickly
14. Rinse with PBS for **10 minutes**
15. Rinse with PBS quickly
16. Rinse well with H_2O, and carefully dry back of coverslip with a Kimwipe
17. Mount with 5µl DAPI