DISSERTATION

CHARACTERIZING *IN VITRO* PROPAGATION AND RADIATION RESPONSE OF MURINE MAMMARY STEM CELLS

Submitted by

Tonya Sirisalee Magers

Department of Environmental and Radiological Health Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2009

COLORADO STATE UNIVERSITY

December 3, 2008

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TONYA SIRISALEE MAGERS ENTITLED CHARACTERIZING *IN VITRO* PROPAGATION AND RADIATION RESPONSE OF MURINE MAMMARY STEM CELLS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Eugene J. Ehrhart

Thomas E. Johnson

Susan M. Bailey

Michael M. Weil

Advisor: Robert L. Ullrich

Department Head/Director: Jac A. Nickoloff

ABSTRACT OF DISSERTATION

CHARACTERIZING *IN VITRO* PROPAGATION AND RADIATION RESPONSE OF MURINE MAMMARY STEM CELLS

Stem cells in breast tissue may be sensitive to known carcinogens (i.e. ionizing radiation), which impact their susceptibility to transformation. The involvement of mammary stem cells in tumorigenesis could explain the heterogeneity and molecular complexity of breast cancer. However, the involvement and the underlying mechanisms of such targets have yet to be fully elucidated. This study was designed to investigate mammary stem cells as plausible targets of radiation-induced damage in radiation-induced mammary carcinogenesis.

We utilized an *in vitro* system (mammospheres) that was developed for the detection of mammary stem cells. We expanded the applicability of this *in vitro* assay through the development of a methodology and novel size criteria to address specific radiation biology endpoints. We applied the methodology and size criteria to analyze the effects of ionizing radiation (IR) on the survival of mammary stem cells derived from mice carrying one mutated copy of *Atm*.

Our results demonstrated that mammary stem cells derived from *Atm*-ΔSRI heterozygous mice ($Atm^{(+/\Delta SRI)}$) do not exhibit increased radiation sensitivity compared to their wildtype littermates ($Atm^{(+/+)}$). In fact, mammary stem cells derived from *Atm*-ΔSRI heterozygous mice exhibited increased radioresistance. To our knowledge, this is the first study to examine the radiation response of mammary stem cells as mammospheres using *Atm* heterozygous mice carrying a known missense mutation found in human A-T.

These studies demonstrated the proof of principle for this model development and the utility of this methodology. Our improved methodology has expanded the feasibility and the applicability of this model to examine numerous functional *in vitro* endpoints. We believe the methodology described here will facilitate investigating the radiation response of mammary stem cells and their progeny, and key components involved in early events of the carcinogenic process in murine model systems.

Tonya Sirisalee Magers
Department of Environmental and Radiological Health Sciences
Colorado State University
Fort Collins, CO 80523
Spring 2009

ACKNOWLEDGEMENTS

To Dr. Robert L. Ullrich: for your enthusiastic encouragement, and for being an extremely supportive mentor. Thank you for not letting me give up, for giving me the freedom to develop my own skills and independence as a scientist, and for teaching me to trust my instincts.

To Rebekah H. Klingler: for being a wonderful friend, for collaborating on the mammary stem cell project, for getting me up in the wee hours of the morning, for helping me with my dissertation, and giving me support to finish this Ph.D.

To Dr. Lila Ramaiah: for being a wonderful friend who helped me through this graduate experience by mentoring me not only scientifically, but also emotionally.

To Christine Battaglia: for being a great listener and wonderful friend, and for making life easier through your uncanny ability to get things ordered and prepared in a timely fashion.

To Scott Pearson: for being my sounding board.

To Dr. Ron Carsten: for being a good lab mate, and for helping me with my dissertation.

To Greg Wilkerson: for collaborative discussions, for creating the stem cell club, and for brightening our long lab workdays.

To Julie Asmus: for being a great listener and friend, and an incredible asset to the ERHS department.

To my committee Members: for their guidance over the years and always being available for questions.

DEDICATION

I dedicate this dissertation to my husband, Jeremiah D. Magers, my father, Preda Sirisalee, my brother, Paul Sirisalee, my family and my pug, Maelee. I could not have accomplished all that I have without their love, endless support, and encouragement, especially during the six years I spent in graduate school. I would also like to dedicate this dissertation to my mother, Sasi Sirisalee and my aunt, whose untimely death to cancer, has lead me down this path.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Page

Page

CHAPTER 1: INTRODUCTION

Cancer is a complex genetic disease. The inherent nature of cancer has made it extremely problematic to address due to the diversity of cancer and genetic variation among the human population. Tumor heterogeneity makes every cancer phenotypically distinct in which most diagnostic and treatment options are not tailored to undertake such diversity. Despite numerous advances in the prevention, diagnosis and treatment of the disease, controlling cancer remains a major hurdle. There is increasing evidence to support the cancer stem cell hypothesis, which states some cancers are derived from aberrant stem cells [1-5]. Any cell can acquire the replicative and multipotency capacity of normal stem cells to produce a heterogeneous population of tumorigenic and non-tumorigenic cells [6-8]. These cells are known as cancer stem cells (CSCs). The cancer stem cell hypothesis refers to a set of behaviors (i.e. stem cell properties) acquired by aberrant cells (CSCs) that are responsible for tumor formation [9, 10]. Conventional therapies may eradicate mature differentiated cells while sparing cancer stem cells; they do not effectively treat a heterogeneous population resulting in treatment failure. If this is true, the cancer stem cell hypothesis suggests new prevention and treatment modalities are required to properly address and eliminate these cells.

Breast cancer research suggests tissue stem cells are a plausible target for carcinogenesis [11-13]. The involvement of mammary stem cells in tumorigenesis could explain the heterogeneity and molecular complexity of breast cancer. It has been hypothesized that stem cells derived from genetically susceptible subpopulations may be predisposed to transformation and could account for the large portion of sporadic breast cancers observed [14, 15]. A better understanding of the genetic factors that influence

the initiating carcinogenic events in stem cells could be the key to elucidating how the tumorigenic phenotype behavior displayed in cancer stem cells is responsible for tumor formation.

Polymorphic variation in genes associated with stem cell activity may lead to increased risk for developing breast cancer. The combination and interaction of such allelic variants and breast carcinogens (e.g. ionizing radiation) may perpetuate the occurrence of sporadic breast cancer. Ascertaining particular mutations and genes may give us a better understanding of specific polymorphic variants that are important and how they affect susceptibility to breast cancer. The etiology of breast cancer suggests increased cancer susceptibility is associated with mammary stem cells whose replicative role to maintain the breast tissue during development predisposes them to carcinogenesis. However, the underlying mechanisms of such targets have yet to be fully elucidated. Stem cells in breast tissue may be sensitive to known carcinogens that impact their susceptibility to transformation. We hypothesize that mammary stem cells are a critical target of radiation-induced damage and play a role in the initiation of radiation-induced mammary cancer. We have developed a methodology to investigate the radiation response of mammary stem cells by characterizing their *in vitro* behavior.

Increased susceptibility to breast cancer

Breast cancer is the most prevalent type of cancer in women (22% of all cancers) [16] and represents 15% of all female cancer deaths [17, 18]. The overall number of breast cancer deaths in 2007 as compared to 1990 has declined by 25% due to a 2% annual decrease in the death rate [18, 19]. Despite the recent advances in the field of

cancer research, breast cancer in the US claims the lives of more than 44,000 women annually [19]. There are numerous risk factors associated with breast cancer. These include gender, age, race, genetic predisposition, reproductive factors (early menarche, older age at menopause, older age at first full-term pregnancy), exposure to exogenous hormones (oral contraception and hormone replacement therapy, high tissue density, lifestyle risk factors (alcohol consumption, obesity, smoking, diet, and physical activity) [16, 20, 21], and exposure to ionizing radiation [22-24]. Many of these risk factors have been largely identified with epidemiological studies of breast cancer and yet only a few clearly demonstrate an association of increased risk for breast cancer.

Unlike other risk factors, genetic predisposition has been shown to play a definitive role in breast cancer susceptibility. Rare, highly penetrant, heritable mutations associated with familial breast cancer susceptibility have been identified through epidemiologic and family pedigree studies [25]. Heritable mutations have been localized to moderately and/or highly penetrant genes for breast cancer such as *BRCA1*, *BRCA2*, *TP53, ATM,* and *PTEN* [26, 27]. These well-characterized genetic mutations all impart a high cancer risk (high penetrance). However, only a small percentage (5-10%) of the population is affected (low prevalence) [28]. Most sporadic breast cancers cannot be attributed to high penetrance mutations so other genetic and environmental factors are likely contributors. The nature of the interactions between genetic and environmental factors that manipulate susceptibility to cancer is unclear. It has been speculated that many low penetrance mutations and genetic variants (polymorphic genes), such as single nucleotide polymorphisms (SNPs), are highly prevalent throughout the population, and that their additive effects impart susceptibility to cancer. The additive effect of these

weakly expressing genes, combined with environmental factors, may be responsible for the majority of sporadic cancers observed [20, 29].

Mammary gland and stem cells

The mammary gland is a remarkable organ that differs from other organ systems in that it undergoes morphological changes in response to hormones. It is generally believed that mammary stem cells are responsible for the ability of the mammary gland to differentiate and regenerate [30, 31]. Extensive developmental changes that are induced by hormonal stimulation during puberty, pregnancy, and involution govern the constant turnover of cells in the mammary gland (Figure 1.1a-d) [11]. Mammary stem cells respond to growth stimuli by dividing and differentiating to allow the mammary gland to expand during puberty and pregnancy and then contract during involution [31, 32]. Only the stem cell can accommodate such development changes through its replicative and multilineage potential. The mammary gland is an organized bilayered epithelial organ that consists of tree branching hollow structures (Figure 1.2a-b) [33]. Three cell lineages are present within the mammary gland: myoepithelial cells, ductal epithelial cells, and alveolar epithelial cells (Figure 1.2b) [34, 35]. The branching structures consist of an inner layer of luminal epithelial cells surrounded by an outer layer of myoepithelial cells that is nestled in a stromal fat pad [36]. The basic structure and cell organization of the mammary gland is similar in both human and rodents, in which only the number and location of glands varies. In the rodent mammary gland, hormonal stimulation causes the ducts to branch and invade the stromal fat pad. The tips of the ducts develop into "club-like structures" called terminal end buds (TEBs) (Figure 1.2a) that are comprised of an

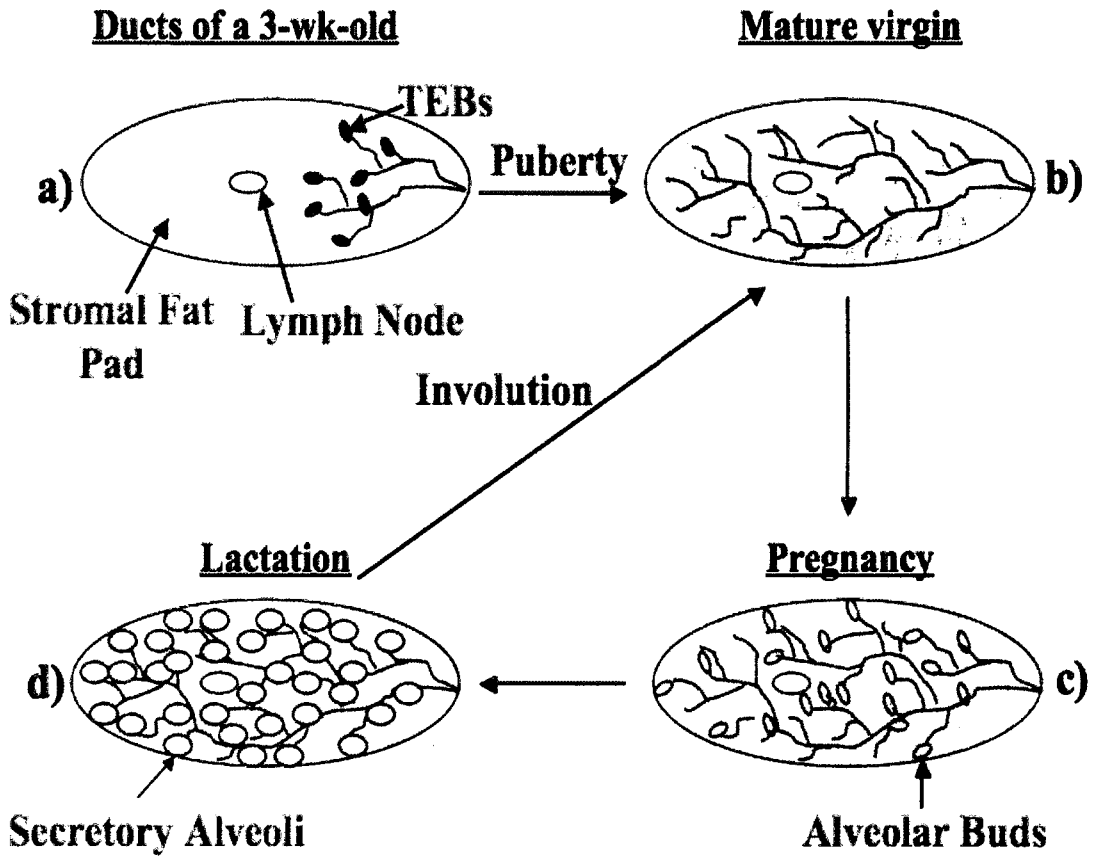

Figure 1.1: Schematic diagram depicting developmental stages of a rodent mammary gland. a) 3-wk-old mammary gland: Tips of the ducts resemble club-like structures (TEBs). b) Mature virgin: Hormonal stimulation during puberty causes ducts to invade and branch to the edges of the stromal fat pad. The cells in the TEBs differentiate into luminal and myoepithelial cells c) Pregnancy: Increased proliferation leads to additional branching and the production of alveolar buds at the end of the branches. d) Lactation: Alveolar buds grow and differentiate into secretory alveoli that are filled with milk. Once pregnancy and lactation have elapsed, the branching structures involute by apoptosis. (Adapted by permission from Macmillian Publishers Ltd.: [Nature Reviews Molecular Cell Biology],[37], copyright (2005) and [Nature Reviews Cancer], [13], copyright (2003)).

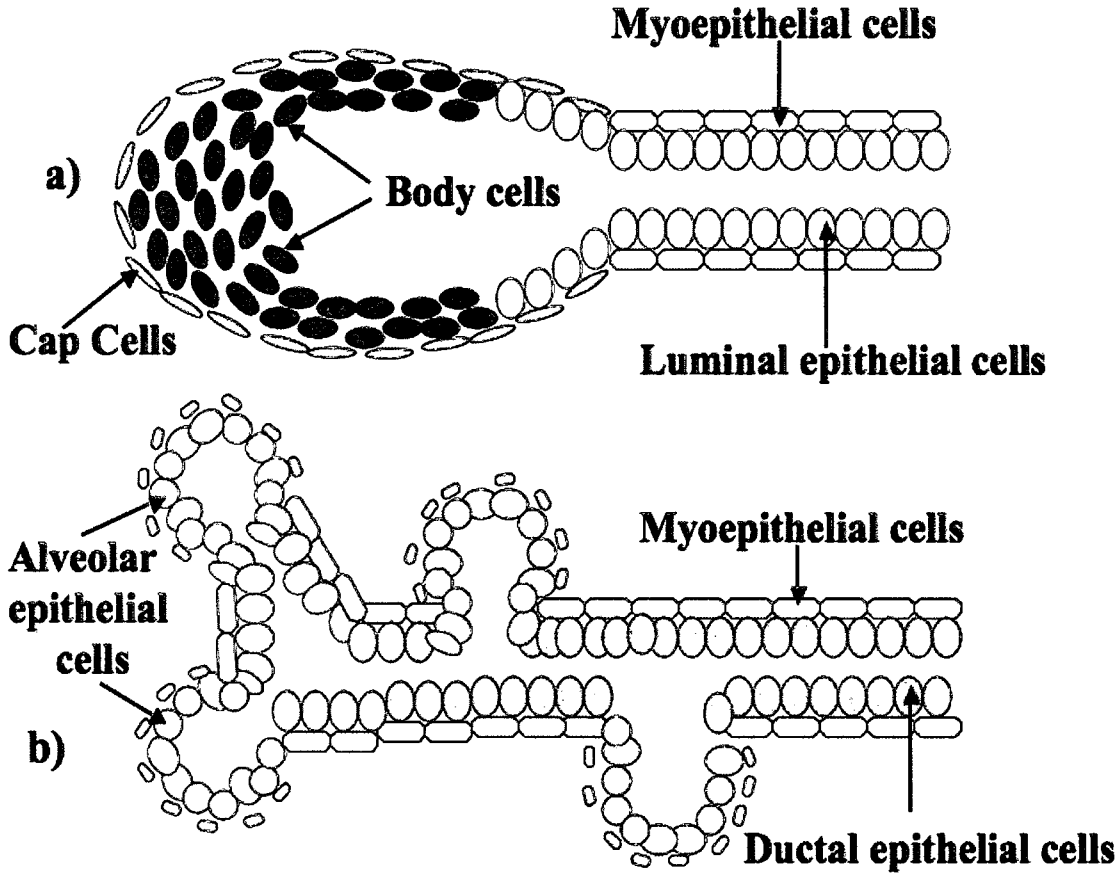

Figure 1.2: Schematic diagram of mammary gland ducts before puberty and during pregnancy. a) Terminal end buds (TEBs): This structure is comprised of an inner layer of body cells, which give rise to luminal cells that lines the ducts, and an outer layer of cap cells that give rise to myoepithelial cells. The proliferative and differentiation capacity of the cells at TEBs suggest stem cells reside here. b) Differentiation of the TEBs during pregnancy to form alveoli for lactation. (Adapted by permission from Macmillian Publishers Ltd.: [Nature Reviews Cancer], [13], copyright (2003) and Woodward *et al.*, Journal of Cell Science, 2005)

inner cell layer mass of body cells surrounded by a layer of cap cells [37, 38]. As the ducts elongate within the stromal fat pad, it is generally believed that the body cells give rise to the luminal epithelial layer whereas the cap cells give rise to the myoepithelial cell layer [13]. A tree branching structure forms and reaches the outer edges of the mammary fat pad during puberty. There is increased proliferation within the mammary gland during pregnancy and lactation that results in additional side branching and the formation

of secretory alveoli at the end of the branches (Figure 1.1c-d and Figure 1.2b). The secretory alveolar structures produce milk, which is pumped out by the contraction of the myoepithelial cells. Once pregnancy has elapsed, the mammary gland proceeds to involute by apoptosis and eventually resembles a structure similar to a nulliparous animal (e.g. mammary gland after puberty). Only stem cells have multipotency potential and retain the ability to regenerate the mammary gland. The replicative and differentiation potential of stem cells maintain these morphological changes during successive cycles of development that occur throughout the animal's lifetime. Based on the multipotency of the TEBs during development, it is speculated that stem cells reside there [38-40].

Stem cells are generally defined by their ability to self renew and generate cells of a particular cell lineage through differentiation dependent on tissue type [6, 41]. The role of adult stem cells is to maintain the tissue by continued cell replacement, thus maintaining tissue homeostasis. The mammary stem cell hierarchy has been hypothesized to include an undefined number of stem cells, which have the ability to self-renew by symmetric or asymmetric division [41, 42]. Symmetric division produces two daughter stem cells that are thought to generate additional progeny and cell lineage progenitors whereas asymmetric division produces a daughter stem cell and an additional progenitor cell that leaves the niche to divide and differentiate (Figure 1.3a-b). It has been hypothesized that the mammary gland is organized in a stem cell hierarchy that includes stem cells, progenitor cells and cell-lineage specific progenitors (Figure 1.4). The number of stem and progenitor cells that make up the hierarchy is unclear, but evidence indicates that there are stem cells physically located throughout the gland at all stages of development [43]. It is generally believed that the mammary gland is

comprised of two distinct cell lineage progenitors that include the luminal epithelial and myoepithelial [44, 45]. During pregnancy and lactation, hormonal stimulation results in the differentiation of the progenitors into the ductal epithelial, alveolar epithelial, and the myoepithelial cells.

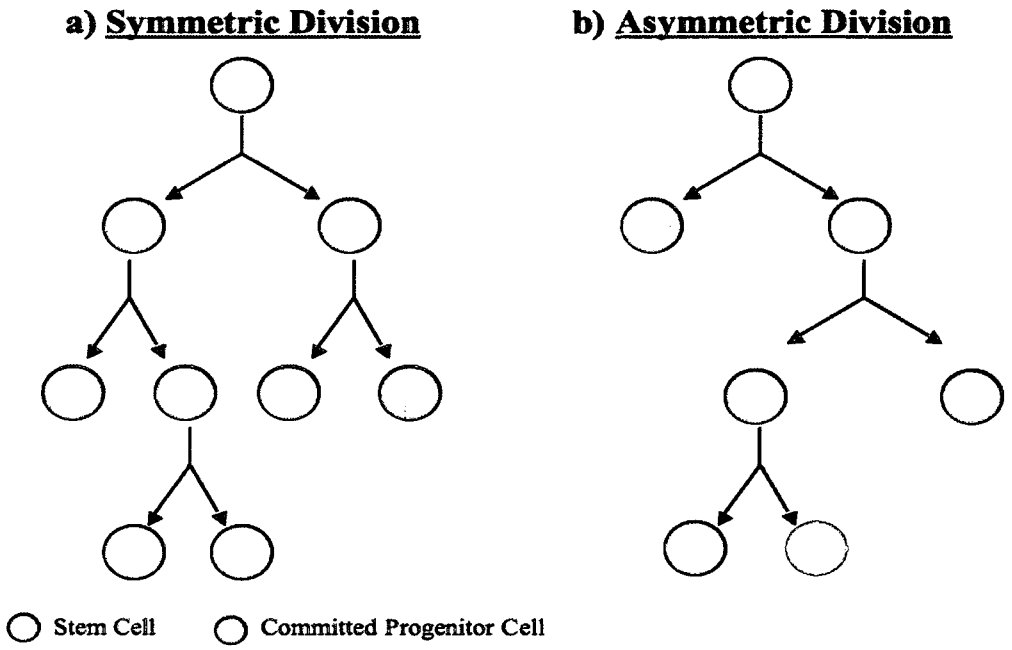

Figure 1.3: Schematic diagram of stem cell division. a) Symmetric division produces two daughter stem cells or two differentiated (progenitor) cells. b) Asymmetric division produces a daughter stem cell and an additional differentiated (progenitor) cell that leaves the niche. (Adapted from Morrison, Shah, & Anderson, Cell, 1997 and Morrison & Kimble, Nature, 2006.)

There is controversy surrounding the definition of a stem cell. Functional criteria (e.g. self-renewal capacity, replicative potential, differentiation potential into other cell lineages, etc.) may be used to distinguish stem cells. However, it has been suggested that stem cells should rather be defined according to their niche and that the niche provides functional criteria for the stem cells [36]. The stem cell niche is defined as a

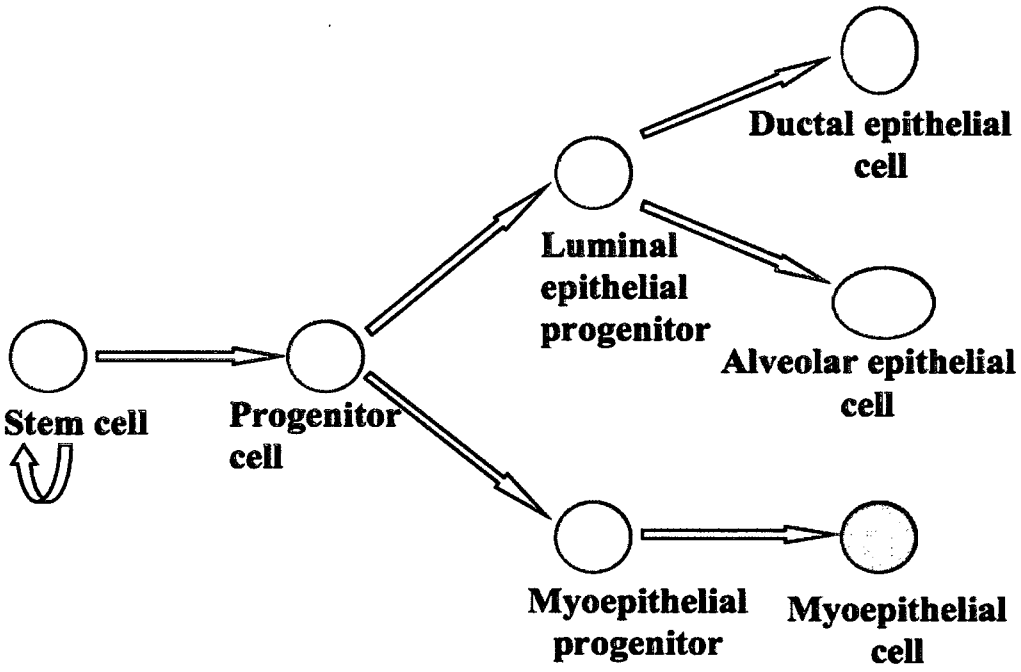

Figure 1.4: Model of mammary stem cell hierarchy. (Adapted from Stingl *et al.*, J Mammary Gland Biol. Neoplasia, 2005 and Visvader & Lindeman, Cancer Research, 2006)

'microenvironment' that preserves stem cells and their properties (i.e. self-renewal and differentiation capabilities) for an indeterminate period of time until they are needed [46-49]. Niches have been identified for many organ systems that are known to contain stem cells and require a constant turnover of cell proliferation (e.g. intestine, bone marrow, hair). The stem cell niche serves as a protective environment that directs and controls stem cell activity. Although the exact location of the stem cell niche in the mammary gland is still undefined [47], the niche operates to mediate signaling between cells and maintain tissue homeostasis by controlling stem cell quiescence and division. It is suspected that quiescence of the stem cells is regulated by cell adhesion via cell-cell interactions, extracellular matrix (ECM), and integrins [50]. Based on other stem cell models, mammary stem cell quiescence is generally believed to be maintained in the

niche through these growth inhibitory adhesions [33]. This would suggest that once stem cells leave the niche, they begin to actively divide and become more committed progenitors to produce the cell lineages within the mammary gland. Stem cells are locked into their niche by adjacent stromal cells which position the stem cells to receive and relay intra- and intercellular signals until they are ready to leave the niche and properly differentiate [48]. Many of the cell signaling pathways (e.g. Notch, Wnt/β-catenin, Hedgehog, PTEN) that are involved in the functional regulation of stem cells utilize the niche as a medium to control stem cell activity and prevent uncontrollable proliferation [49, 51, 52]. The mammary gland must undergo extensive reconstruction during reproductive cycles. This entails reorganization of the stem cells and their progeny to accommodate such changes [32]. The ability of the stem cells to accommodate such developmental changes can be attributed to the niche and its ability to maintain tissue homeostasis [36, 47].

Experimental evidence for mammary stem cells

It has long been suspected that mammary stem cells existed based on the unique nature of the mammary gland. The ability of the mammary gland to undergo developmental changes (puberty, pregnancy, lactation, and involution) has suggested that stem cells are responsible for maintaining tissue homeostasis during these changes. Compelling evidence that supports the existence of stem cells has been derived from *in vivo* mouse mammary gland transplantation studies, X chromosome inactivation studies, and retroviral tagging [32, 53-56]. The tissue fragment transplantation technique has successfully demonstrated the ability of the transplanted mammary tissue fragments to

generate mammary outgrowth similar to the original tissue [30]. This technique was further refined by dissociating the mammary tissue into single cells before injection into the cleared fat pad, which resulted in a more effective means of generating a mammary outgrowth [55]. Any portion of the mammary gland can regenerate the ductal tree upon transplantation, indicating that stem cells are located throughout the mammary gland and have the ability to give rise to three distinct and separate cell progenitors that can produce lobular and ductal phenotypes [56]. It is believed that a population of stem cells resides in the TEBs based on serial transplantation studies. These studies identified morphologically distinct cells that exhibited stem cell properties and were present at all developmental stages [43]. X chromosome inactivation involves the permanent marking of the X chromosome after random inactivation of the maternal or paternal X chromosome. The permanent markings of the X chromosome involve methylation changes in CpG islands [57]. Clonal analysis involves analyzing the human X chromosome-linked androgen receptor gene from tissue samples, which has polymorphic CAG repeat regions and three *HhaI* and two *HpaII* restriction sites [54, 57]. The unmethylated active X chromosome will be degraded by these restriction enzymes whereas the restriction sites that were methylated will reveal the inactivated X chromosome [54]. Analyzing the methylation of polymorphic DNA markers on X-linked genes can reveal tissue clonality. X chromosome inactivation studies have demonstrated that nonmalignant and malignant human breast tissue is clonal in origin based on the permanent marking of the X chromosome during random inactivation [54]. This would suggest that the initiating events occurred in the same cell (i.e. the stem cell). Mouse mammary tumor virus (MMTV) retroviral tagging studies indicate that mammary

outgrowths are clonal in origin (derived from a single epithelial stem cell) and remain clonal after subsequent transplantations [53]. This suggests that stem cells have the self-renewal capacity to regenerate additional mammary outgrowths and give rise to distinct cell lineage progenitors with limited proliferative capacity [53]. Thus, a stem cell hierarchy exists within the mammary gland and has different cell lineage capabilities. While these studies imply that a stem cell component exists within a mammary gland, identification and purification techniques remain problematic, preventing proper characterization of the role of the stem cell in the mammary gland.

Mammary stem cell candidate populations: Methods of Identification

Isolation and purification techniques are relatively new for the mammary gland and many of these techniques have been based on other well-characterized stem cell systems. Current knowledge concerning the identification of prospective stem cells has been derived mainly from the hematopoietic system [58-60]. A small, distinct population of cells that has the ability to efflux Hoechst dye was first identified in hematopoietic cells and termed the "side population" (SP) cells [60]. This particular characteristic is attributed to increased transporter activity, which is considered a hallmark of stem cells. It is believed that this property allows stem cells to protect themselves against harmful toxins and other damaging agents to prevent/minimize the accumulation of mutations throughout their lifetime. SP cells have the ability to recapitulate the whole hematopoietic system by contributing to the myeloid and lymphoid lineages, thus constituting a highly enriched population of hematopoietic stem cells [60]. Stem cell antigen-1 (Sca-1) is an additional cell surface marker identified within the SP cell

population and has subsequently been utilized to identify an enriched population of mammary stem and progenitor cells [61].

The mammary gland contains SP cells, which are also enriched with Sca-1 positive cells and are capable of generating mammary outgrowths with all three cell lineages [61]. The ability to efflux Hoechst dye has been attributed to the breast cancer resistance protein-1 (BCRP-1), which is a member of the ATP binding cassette (ABC) transporter superfamily [58] and is considered an early differentiation marker in human mammary gland responsible for the SP phenotype [62]. Many attempts have been made to characterize the population of SP cells by utilizing other markers such as lineage-specific and steroid receptor markers. SP cells lack luminal and myoepithelial markers, but are able to produce both cell lineages and are considered an enriched population of undifferentiated cells [62]. SP cells identified in both human and mouse mammary glands are able to produce luminal and myoepithelial cell lineages upon transplantation, suggesting that SP cells represent a candidate stem cell population [62, 63]. Markers for quiescence and asymmetric division have been shown to be properties of SP cells [39]. This would suggest that SP cells are comprised of a population of stem and progenitor cells with self-renewal and differentiation potential; however, it has not been proven that using Sca-1 marker (to isolate SP cells) results in a pure population of stem cells, especially in human breast tissue. Some of these markers rely on particular stem cell characteristics (i.e. increased transporter activity, steroid receptor status, cell-lineage specificity) that are believed to be associated only with stem cells. It is possible that these characteristics are not limited to just stem cells, but may include progenitor and other committed cell-lineage progenitor cells. The lack of definitive cell surface markers

(molecular signature) in which most methods can only isolate an enriched population has made it extremely difficult to characterize the true properties of mammary stem cells.

Other stem cell systems have demonstrated that some stem cells remain quiescent to maintain their proliferative capacity throughout the lifetime of the organism, but it is a characteristic not shared by all stem cells. This characteristic has been used to identify potential stem cells using bromodeoxyuridine (BrdU) label retention method. As cells replicate, BrdU is incorporated into the DNA and eventually the label is lost as the cell actively divides or dies. Rapidly proliferating cells are suspected to lose their label quickly. Cells that are slowly proliferating, known as label-retaining cells (LRCs) will retain the BrdU label and are believed to represent the stem cell population. Several studies have attempted to use the BrdU label retention method to identify mammary stem cells with conflicting results. LRCs have been identified in the mouse mammary gland [61], but other studies suggest there is a considerable amount of turnover in the mammary gland in which LRCs traverse the cell cycle [64]. It appears that within the mammary stem cell hierarchy certain cell types retain the quiescent characteristic depending on their differentiation status. However, the correlation between mammary stem cells and label retaining cells is not yet clear.

Several studies have attempted to distinguish stem cells within human breast tissue, but results are somewhat conflicting. The mouse model has proven to be an excellent alternative for the isolation and characterization of mammary stem cells in which the properties identified can be compared to human. An enriched population of stem and progenitor cells capable of self-renewal and regeneration of a mammary gland can be isolated using Fluorescence Activated Cell Sorting (FACS) and limiting dilution

technique in which a single cell can generate a mammary outgrowth [65]. Using this technique, the frequency of mammary stem cells in single cell suspensions was estimated to be 1 per 1,400 dissociated cells. This was determined by comparing the relationship between the number of cells transplanted and the proportion of mammary outgrowths that occurred in the cleared fat pad. A single mammary stem cell can reconstitute a functional mammary gland (clonally derived) *in vivo* with luminal and myoepithelial cell lineages without the need of additional cells to support growth [66]. In addition, mouse mammary stem cell transplantation experiments support the hypothesis that mammary stem cells are the targets for mutagenic transformation yielding a cancer stem cell [66, 67]. An increased number of stem cells were observed in pre-malignant tissue derived from mammary tumor-prone MMTV-*wnt-1* transgenic mice and serial transplantation resulted in hyperplastic outgrowths [66]. This would suggest that defective signaling pathways responsible for controlling stem cell activity lead to malignancies by increasing the number of stem cells (i.e. cancer stem cells). According to recent advancements in our understanding of the mouse mammary tumorigenesis model, the identification of mammary stem cells has important implications in mammary carcinogenesis.

The lack of a suitable set of markers to specifically isolate a population of stem cells has lead to alternative methods to characterize the properties of stem cells. Many approaches involve characterization of stem cells *in vivo*. The ability to culture and maintain stem cells as spheroids using an *in vitro* system was first shown with neural stem cells [68]. Neural stem cells form clusters termed "neurospheres" that are comprised of stem and progenitor cells in various stages of differentiation. Using a similar approach, mammary stem cells were cultivated and enriched in an

undifferentiated state as "nonadherent mammospheres" using human breast tissue [69]. Mammospheres contain cells that retain the ability to reproduce a functional ductal-acinar structure *in vitro*, capacity for self-renewal and differentiation, and are clonally derived. Unlike other methods devised to isolate mammary stem cells, this *in vitro* system provides the opportunity to directly assess self-renewal capacity and differentiation potential in order to examine multiple endpoints.

Role of stem and progenitor cells in carcinogenesis

Tumorigenesis is thought to arise through the acquisition of multiple mutations over a period of time [70, 71]. Tumor development involves the acquisition of genetic alterations (e.g. point mutations, deletions, chromosomal rearrangements) that selectively allow the cell to proliferate and accumulate additional mutations [29]. The combination of genetic alterations may enhance the progression to malignancy. The effect of particular mutations may influence the proliferative capacity of the target cells resulting in aberrant growth. It is generally believed that several mutations are required to promote carcinogenesis. It is unclear whether mature (i.e. differentiated cells) or primitive (i.e. stem cells) cells are more susceptible to transformation for certain tissues or if susceptibility varies depending on differentiation status at all. It has been suggested that stem and progenitor cells may represent important cellular targets in which they already possess the characteristics needed to initiate carcinogenesis [6, 8, 9, 72, 73]

The acquisition of stem cell properties through mutagenesis may be responsible for the genetic and phenotypic heterogeneity seen in some types of cancers. There are two models of carcinogenesis that have been developed to elucidate the functional

heterogeneity observed in some cancers and to illustrate that not every cell has the ability to maintain the malignant tissue [6]. The stochastic model predicts that any cell has the potential to transform, but transformation occurs at a low probability due to strict genomic integrity mechanisms that prevent cells from proliferating uncontrollably [74, 75]. The tumor is composed of a phenotypically heterogeneous population of cells, which retain the abilities to self-renew and proliferate extensively. The genetic alterations responsible for the malignancy should be present in the majority of the cells and therapeutic treatments address the bulk of the tumor cells. In contrast, the hierarchy model suggests that tumor heterogeneity arises from a small subset of cells capable of tumor initiation [19, 74-76]. The tumor is phenotypically heterogeneous, but contains functionally distinct subset of cells that are capable of self-renewal, proliferation, and regeneration of the tumor upon transplantation. The tumor-initiating cells are biologically and functionally distinct from the bulk of the tumor. Failure of existing therapeutic treatments to eradicate this distinct subset of cells may explain tumor reoccurrence. Based on these two models, identification and characterization of tumor-initiating cells responsible for malignancy require different approaches; tumor-initiating cells that follow the hierarchy model can be isolated and separated from the bulk of the tumor whereas the stochastic model predicts these cells will always be present in any sorted cell fraction. The diversity of cancer is represented by both of these models in that they are not mutually exclusive. Cancers that follow the hierarchy model require new treatment strategies to effectively target the cells responsible for maintaining the tumor, which may be spared with traditional therapies.

Recent studies of hematopoietic, colon, neuronal, and mammary malignancies

have implicated a subpopulation of cells are responsible for maintaining the malignant tissue through the proliferation of tumorigenic and non-tumorigenic cells [3, 5, 77, 78]. The small subset of cells that have the capacity for self-renewal and maintain the malignant tissue are defined as cancer stem cells (CSCs)[9]. The cancer stem cell hypothesis refers to the cellular origin of the tumor and the cellular component (CSCs) that drives tumorigenesis [76]. Properties associated with cancer stem cells are not limited to self-renewal and extensive proliferative capacity, but the most important property is the ability of these cells to differentiate into the tissue-specific cell lineages. Cancer stem cells have been identified and characterized in several organ systems [2, 79, 80]. It has been demonstrated that within some solid human breast tumors there are a subset of cells identified with unique cell surface markers (i.e. CD44 and CD24), which drive tumorigenesis upon transplantation and are responsible for the phenotypic heterogeneity observed within the tumor [3]. Similar results have been shown in some brain tumors in which the cancer stem cells identified contribute to the heterogeneity of the tumor [78, 79]. It is unclear whether the stem or progenitor cells are the more likely target of transformation in the cancer stem cell model, mainly because the stem cell hierarchy is not well characterized for many systems and it is difficult to distinguish between the two cell types.

Most therapeutic treatments are not designed to eradicate tumors derived from a small, aggressive subset of cells with stem cell activity and likely fail because they do not target the right cells. Tumor reoccurrence is likely related to conventional therapies only targeting malignant cells with limited proliferative capacity while the cells with self-renewal capabilities remain. There may be differences in sensitivity of these cancer stem

cells that certain treatments do not address [81, 82]. Without further characterization of cancer stem cells, diagnostic and treatment strategies cannot effectively target these cells. Traditional therapies may fail to account for differences in sensitivity to treatment [82]. Radiation therapy may prove to be an effective treatment option to the eradication of cancer stem cells. Radiation therapy does not require identifying cancer stem cells (unlike chemotherapy) and does not discriminate between tumorigenic and non-tumorigenic cells. Few studies have attempted to address the radiation sensitivity of cancer stem cells and the effectiveness of such treatment. Human breast cancer-initiating cells have been shown to display radiation resistance to single acute and fractionated doses of ionizing radiation [83]. Glioblastoma cancer stem cells displayed reduced sensitivity to radiation-induced apoptosis, and preferential checkpoint response and DNA repair that lead to the radioresistance observed [80]. It has been argued that these results are limited and may not accurately depict cancer stem cells within the tumor because these studies examine an enriched population identified with cell surface markers (not necessarily a pure population) by utilizing an *in vitro* system. It is not currently possible to discern the effect of radiation on cancer stem cells; this will require functional radiation biology assays devised to properly examine these cells in their natural environment. Additional research may provide possible therapeutic targets aimed to selectively sensitize cancer stem cells if they are indeed radioresistant.

Mammary stem cells and breast cancer

Research in breast cancer biology has suggested that stem cells are a plausible target for initiation in the carcinogenic process and may play a role in some cancers [11-13]. It has been hypothesized that genetically susceptible subpopulations may be

predisposed to transformation of stem cells and could account for the large portion of spontaneous cancers observed [14, 15]. Properties of stem cells include self-renewal capacity, replicative and differentiation potential, active telomerase expression, anti-apoptotic pathways, increased membrane transporter activity, and the ability to migrate and metastasize [76]. Stem cells have a long life span in which they continue to divide as needed [41]. Stem cells are an attractive target for mutational transformation based on their properties (i.e. ability to expand and proliferate) and the potential to acquire mutations over a long period of time. Stem and progenitor cells may represent an important target for transformational events as acquiring stem cell-like properties are the key to a tumorigenic phenotype.

Breast cancer is considered highly heterogeneous, which may indicate that different molecular mechanism may contribute to the complexity observed [19]. It has been suggested that initiation can occur in different cell types and influences the pathology of breast cancer. Five major classification groups have been identified among breast cancer cases that include luminal A and B, HER2/ER$^-$ (often referred to as ERBB2), basal-like, and normal breast-like subtypes [84, 85]. Each group contains a unique molecular gene signature that is distinguished by their distinct gene expression. Gene expression arrays have proven to be a useful prognostic marker in diagnosis and treatment of breast cancer [86-88]. It has been argued that the different breast cancer subtypes are a reflection of the target cell type and their susceptibility towards transformation [85]. This may explain the different subtypes of breast cancer observed and their molecular signature. Recent studies of acute myeloid leukemia (AML) and mammary carcinogenesis have suggested that specific mutations were clonal in origin

and arose from a common progenitor [7, 67]. This would suggest heterogeneity of the tumor may arise from transformation of cells at different stages of development that result from mutations that activate different oncogenic pathways. Additional studies in the hematopoietic system have demonstrated that AML arose from leukemia initiating cells, which were similar to normal stem cells, and suggest that the stem cells are the target of transformation [2]. It is possible that mutational events occur in the stem cell, but the mutations drive tumorigenesis in the downstream progeny and influence the path of differentiation; thus, ultimately impacting the heterogeneity in the phenotype. Recent studies have implicated that the target cell type and certain mutational events contribute to the heterogeneity of the tumor [89] and this needs to be specifically addressed in the diagnosis, prevention, and treatment of breast cancer.

Radiation-induced mammary carcinogenesis

Exposure to ionizing radiation (IR) is a well-established cancer risk factor, and is known to cause cancer in almost any tissue or organ in the body [90]. We are constantly exposed to radiation from a variety of sources, such as natural background radiation (radon), occupational exposures, or medical procedures. Occupational and medical exposures are typically low linear energy transfer (LET) radiation (X-rays and γ-rays) and exposures are usually limited to low dose and/or low dose-rates. There is much uncertainty regarding the cancer risk in genetically susceptible populations exposed to ionizing radiation [91].

Ionizing radiation induces a broad spectrum of DNA damage that includes single strand and double strand breaks (SSBs & DSBs), cross-linking and nucleotide base

changes [92-94]. DNA double strand breaks are considered to be the most biologically important type of lesion induced by ionizing radiation [25, 95, 96]. It is likely that defects in genes associated with recognition or repair of DNA damage hold the most importance for radiation sensitivity and for radiation-induced cancer [25]. This phenomenon is illustrated by genetic disorders such as ataxia-telangiectasia (*ATM*), Nijmegen breakage syndrome (*NBS*), and Fanconi's anemia (*FA-A* to *FA-C*). All of these disorders have defects in DNA repair pathways, which lead to increased radiosensitivity [97].

Epidemiological studies of medically exposed populations (i.e radiologic technologists, patients that received multiple fluroscopies as treatment for pulmonary tuberculosis, therapeutic treatment for acute postpartum mastitis and benign breast diseases, mantle radiotherapy for Hodgkins's lymphoma, etc.) and atomic bomb survivors clearly demonstrate that exposure to ionizing radiation (IR) can increase the risk of breast cancer [22-24, 98]. Much of the data concerning risk of developing breast cancer is derived from the Life Span Study (LSS) of atomic bomb survivors, which indicates higher excess relative risk (ERR) correlate with exposure at a younger age (<35) [22, 99]. The radiation effect on breast cancer incidence rates suggests an "early onset" effect that may be due to genetically susceptible subpopulations among the atomic bomb survivors [100]. Mortality risks among U.S. radiologic technologists have been shown to be 40-60% greater if workers began working at ages younger than 25 years [23]. Recognized risk factors that have been shown to influence the risk of radiation-induced breast cancer include radiation dose, age at exposure, and age at first term pregnancy (e.g. younger age reduces excess risk) [101]. It has been postulated that the

age at radiation exposure may significantly influence breast cancer risk due to susceptibility of undifferentiated cells during the development of the breast tissue [101]. Stem cells may be susceptible to radiation-induced damage [76], especially during mammary gland development (i.e. puberty) when stem cells are at their highest number [32]. Increased breast cancer incidence occurring 30 years after radiation exposure for atomic bomb survivors [98] would suggest stem cells were responsible for increased breast cancer susceptibility [76]. However, the underlying cellular and molecular mechanisms of radiation-induced breast cancer remain unclear.

Elucidation of underlying cellular and molecular mechanisms requires identification of target cells, an understanding of their radiation response and cell-cell interactions, which may govern progression in the carcinogenic process. The target cells for breast carcinogenesis are believed to be stem cells, but only recently has the opportunity to directly study mammary stem cells become available. There is increasing evidence to suggest that, particularly for genetically susceptible subpopulations, stem cells play a role in some cancers; therefore new strategies are needed for breast cancer prevention and treatment.

The radiation response of mammary stem cells has yet to be characterized due to past difficulties in their identification and isolation. Stem cells may possess unique mechanisms to prevent or minimize radiation-induced damage. Protective mechanisms for genomic integrity (in normal non-irradiated cells) may be maintained through the stem cell niche. Susceptibility of stem cells to radiation-induced carcinogenesis may be derived from IR compromising the cell-cell interactions within the niche. Human mammary epithelial cells demonstrate characteristics of neoplastic progression after

exposure to IR in which organizational integrity and cell polarity is no longer intact [102]. Recent studies suggest that mouse mammary stem cells are radiation resistant and this radioresistant response is mediated through certain cell signaling pathways (i.e.Wnt/β-catenin) [103, 104]. It has been argued that these studies lack a proper microenvironment to evaluate the response of stem cells to various endpoints, including radiation [36]. A better understanding of the DNA damage response and repair processes of stem cells is likely to aid in elucidating mechanisms responsible for the susceptibility of stem cells to radiation-induced carcinogenesis.

Ataxia-Telangiectasia and ATM

Individuals afflicted with the rare autosomal recessive disorder ataxia-telangiectasia (A-T) have a heightened sensitivity to ionizing radiation and a predisposition to cancer [97]. Additional clinical hallmarks that characterize A-T include progressive cerebellar ataxia, oculomotor apraxia, frequent infections, choreoathetosis, telangiectasias of the conjunctivae, and immunodeficiency [105, 106]. At least one third of A-T patients develop cancer, particularly B-cell and T-cell origin, non-Hodgkin's lymphoma, Hodgkin's lymphoma, and several types of leukemia [107]. The cellular phenotype of A-T displays characteristics such as chromosomal instability, radiation sensitivity, and defective cell cycle arrest [108].

The mutated gene attributed to the A-T phenotype is referred to as *ATM* (A-T mutated) and encodes a 350-kDa protein that is known to be involved in the cellular response to DNA damage [109]. It is a member of the phosphatidylinositol-3 (PI3) kinase family that encompass additional proteins (e.g. DNA-Pkcs, ATR, mTOR) involved in

processing double strand breaks (DSBs) [110]. There is a cascade mechanism that involves the sensing of DNA DSBs and regulation of specific DNA repair pathways, apoptosis, and cell cycle checkpoints during different stages of the cell cycle, which is mediated by ATM [111-113]. ATM is responsible for the activation of these processes through phosphorylation of different targets such as p53, MDM2, Chk1/Chk2, BRCA1, and SMC1 [111]. Although the exact mechanism of DNA damage response and repair is not fully understood, exposure to ionizing radiation results in the rapid intermolecular autophosphorylation and dimer dissociation of ATM that induces a conformational change to release the protein and allow for the recruitment of repair proteins [114]. Evidence from ATM-deficient cells has demonstrated significant defects in the activation of cell cycle checkpoints, particularly the G1 checkpoint, which is dependent on the activation of the p53 pathway by ATM [112]. ATM is responsible for activating and directing p53 to DSBs through phosphorylation of p53 directly and phosphorylation of additional substrates that stabilize p53 [112]. ATM-deficient cells display unique characteristics that hint to the pivotal, central role of ATM in response to radiation-induced damage. However, the mechanisms that elicit this particular phenotype have yet to be fully elucidated.

The majority of A-T patients are considered compound heterozygotes in which individuals inherit two different *ATM* mutations [106]. It is predicted that at least 85% of these mutations results in a truncated protein [110]. Low to non-existent levels (reduced expression) of the ATM protein are produced and usually undetected with immunoblotting. Less than 15% of these mutations are "missense" mutations, which produce a full or near-full length protein with varying degrees of stability and protein

expression level, and include small in-frame deletions/insertions [109]. It is suspected that these two classes of *ATM* mutations may lend some insight into the identification of important allelic variants that confer increased breast cancer susceptibility.

Although *ATM* mutations impart a high cancer risk, the prevalence of A-T is very low with an incidence of 1:40,000 to 1:100,000 live births [105]. The frequency of A-T carriers (i.e. individuals with one mutated ATM allele) within the population is estimated to be 1% of U.S. population (white) [115]. Previous studies have suggested that *ATM* mutations predispose individuals to certain types of cancer (e.g. breast, pancreas, stomach, ovary, and chronic leukemia) [115]. Limiting radiation exposure for *ATM* heterozygotes may have important implications for decreasing their overall lifetime risk of cancer. Breast cancer is the most common type of cancer observed among female blood relatives of A-T patients [116] and is considered to carry the most significant relative risk for *ATM* heterozygotes [117]. Relative risk for breast cancer has been estimated to be 2.23 for A-T carriers compared with the general population, but is considerably higher (4.94) for carriers younger than 50 years of age [118]. There is little evidence to suggest increased risk to *ATM* heterozygotes for any other cancers, due to the lack of significant power and sample size [118, 119]. Despite conflicting evidence surrounding the association between increased risk of breast cancer for *ATM* heterozygotes, A-T heterozygotes could account for a large portion of the sporadic cancers (5.4-8%) and 9-18% of all breast cancer patients in the US [115, 120].

The relationship between *ATM* mutations (i.e. absence/presence of a protein) and risk for breast cancer has yet to be fully understood. Results from several epidemiological studies of A-T families confirmed that *ATM* mutations in obligate female

carriers confer an increased risk for breast cancer, which was relative to age (<44 years of age) [115, 119, 121, 122]. Though some studies of A-T families have indicated an increased relative risk for breast cancer [123, 124], studies of breast cancer patients fail to confirm this finding [125-127]. However, much of the controversy stems from the different *ATM* mutations (i.e. truncating and missense) identified in these large cohorts of familial A-T and breast cancer cases and whether these mutations are responsible for the significant differences in the degree of breast cancer risk.

It has been argued that the mutational spectrum which confers risk may differ for breast cancer and A-T patients [125]. Several studies among patients with breast cancer have concluded that classical ATM mutations (i.e. truncating mutations) do not confer genetic predisposition to breast cancer [125-127]. In contrast, germline ATM-truncating mutations that are considered "A-T disease causing" have been shown to be associated with an increased relative risk of breast cancer for heterozygotes [128]. These particular studies focused on truncating mutations that were either previously identified in A-T patients or detected using a protein truncation test (PTT) assay, and failed to screen for missense mutations. A protein truncation test assay detects only chain-terminating mutations that cause premature translation termination [129]. Detection of protein truncation involves analyzing the *ATM* transcript by reverse transcription PCR (RT-PCR) to amplify the target sequence, *in vitro* transcription-translation, and analysis of the protein by SDS-PAGE [127]. Although truncating mutations are considered to be the most prevalent mutation among A-T patients, the significance of this mutation may be an overestimation that is likely attributed to the assay utilized, and lends bias in the detection of only protein truncations rather than missense mutations or short in-frame

insertions/deletions. Several missense mutations have been identified in both breast cancer cases and obligate ATM heterozygotes, which demonstrate these mutations appear frequently, and may infer breast cancer risk [124, 130-132]. There is additional evidence to support that some missense mutations act in a dominant-negative manner in which the mutant protein loses not only its function, but also prevents the normal protein from functioning properly [133-135]. Therefore, the mutant ATM protein affects the interactions and/or pathways of ATM (from normal allele) in heterozygous individuals. However, the underlying mechanisms are still unclear. Missense and truncating mutations identified in *ATM* heterozygotes may impart distinct phenotypes for cancer susceptibility in which the degree of penetrance varies and possibly explains the differences in cancer risk observed [105, 136]. Are there two clinically distinct classes of ATM mutations that exist among the general population? If so, then *ATM* mutations that confer susceptibility to breast cancer may not necessarily include mutations that are commonly associated with an A-T phenotype. This would suggest that breast cancer susceptibility might depend on the functional consequences incurred by truncating and missense mutation and their effect on the phenotypic outcome.

Much interest has turned to examining the functional consequences of breast cancer-associated ATM mutations and their impact on cancer predisposition [133, 134]. The degree of penetrance for these mutations may rely on the level of protein (e.g. haploinsufficiency), loss of heterozygosity (LOH), and its impact on the interactions and/or pathways of *ATM*. Varying degrees of breast cancer risk could be attributed to the combination of the mutation and environmental factors to influence the incidence among the population. Thus, exposure to ionizing radiation may greatly impact the overall

breast cancer risk for ATM heterozygotes.

ATM heterozygotes are suspected to share a similar, but milder phenotype compared to A-T patients [111]. ATM-deficient cells display increased radiation sensitivity, chromosomal instability, and defective cell cycle checkpoints [105]. A-T carriers may be predisposed to radiation-induced breast carcinogenesis as compared to the general population. Cell lines derived from ATM heterozygotes carrying truncating mutations demonstrated increased levels of micronuclei (MN) formation after ionizing radiation (IR) (chromosomal instability), intermediate radiosensitivity, and resistance to early-onset apoptosis after IR [137, 138]. A similar cellular phenotype was observed in breast cancer patients carrying missense ATM mutations [138]. However, an additional study reported variability in the cellular response to IR of ATM heterozygotes that carried either missense or truncating mutations [139]. The radiation sensitivity of cells with missense mutations exhibited heightened radiation sensitivity compared to normal cells and those carrying truncating mutations; both mutations showed no differences in the ability to phosphorylate downstream ATM targets and induce normal progression into the cell cycle [139]. Although several studies have produced promising insight into the functional consequences and radiation response of particular ATM sequence variants [140-142], it is still unclear how these mutations lead to increased susceptibility to breast cancer.

Another useful model for genetic susceptibility has been provided through inbred strains of mice. Mouse models of A-T have served to enhance our knowledge concerning breast cancer-associated *ATM* mutations and radiation-induced mammary carcinogenesis. Attempts have been made to replicate the human A-T phenotype using the mouse model,

and have resulted in mice that exhibit all the clinical phenotypic characteristics of human A-T except for neurodegeneration [108]. Atm-deficient mice (Atm $^{-/-}$) display growth retardation in which they are smaller in weight and size as compared to wild-type and heterozygous littermates [108]. These mice are highly susceptible to thymic lymphomas (4 months of age) and leukemias, and display similar features to human A-T cells such as increased chromosomal instability, heightened sensitivity to IR, and defective cell cycle arrest [107, 108]. However, in this model mammary cancer is never observed.

Some of the research has shifted to addressing the ramifications of certain *ATM* mutations that may affect predisposition to cancer and increase radiation sensitivity using *Atm* heterozygotes mice ($Atm^{+/-}$). The majority of these studies use mice carrying truncating mutations that essentially expressed 50% of the ATM protein and displayed no A-T phenotype. $Atm^{+/-}$ mice exhibit increased sensitivity to IR that resulted in reduced life span and premature greying [143]. In these studies, a wide spectrum of tumors was observed, but breast cancer was rarely observed. However, the rare occurrence of breast cancer may be attributed to small number of mice (i.e. 24 $Atm^{+/-}$ mice) used in the study. Some studies have shown increased susceptibility to ductal dysplasias, a precursor to mammary carcinogenesis, after IR and/or chemical carcinogen [144, 145]. It is suspected that the phenotype observed in $Atm^{+/-}$ mice may be a result of haploinsufficiency in which the controlled environment and diet of the animals influences the susceptibility to breast cancer [143, 145]. Although *Atm* heterozygous mice exhibit a cellular phenotype that would seem to confer increased susceptibility to mammary carcinogenesis, results are conflicting and appear to be dependent on the mutations and its functional consequences.

Data from humans and mice suggest that breast cancer susceptibility is governed

by particular mutations carried by heterozygous individuals and that missense mutations might confer this phenotype [131, 135, 146-148]. Only recently has a mouse model been developed to examine the effects of *ATM* missense mutations [149]. Mice referred to as *Atm*-ΔSRI harbor a mutation that corresponds to the human 7636del9 mutation commonly found in A-T patients [149]. This mutation is a nine-nucleotide in-frame deletion (7636del9) in exon 54, which is located in the FAT domain upstream of the kinase domain, and results in a protein with three amino acids deleted (SRI: 2556-2558). Similar characteristics such as growth retardation, occurrence of thymic lymphomas, increased radiation sensitivity and chromosomal instability were observed in the *Atm*-ΔSRI as compared to $Atm^{-/-}$ mice, except that *Atm*-ΔSRI mice exhibited a greater life span. After exposure to IR the mutant Atm protein appears to be less stable than the wildtype in which it is unable to phosphorylate downstream targets (p53). Approximately 9% of *Atm*-ΔSRI heterozygous mice develop tumors (3-fold increase over wildtype littermates), but only 2% are mammary tumors (without exposure to IR) [148]. This would suggest that additional factors (i.e. IR) compound the occurrence of mammary cancer. Radiation sensitivity of *Atm*-ΔSRI heterozygotes was intermediate as compared to wild-type and *Atm*-ΔSRI homozygous mice. The kinase activity in heterozygotes is indistinguishable from wildtype, and further investigation revealed the mutant ATM acts in a dominant negative manner to compromise the kinase activity of the wildtype after irradiation [135]. Although these studies yield promising results to link missense mutations as breast cancer-associated ATM mutations, the exact nature of susceptibility to radiation-induced mammary carcinogenesis has yet to be fully elucidated in *ATM* heterozygotes.

Rationale for Dissertation

Ascertaining particular mutations and genes may give us a better understanding of which polymorphic variations are important and how they affect susceptibility to breast cancer. The interaction between allelic variants and environmental carcinogens (e.g. ionizing radiation) may account for the occurrence of some sporadic cancers. Research in stem cell biology has implicated stem cells as a likely target for the early carcinogenic event [11-13]. Stem cells are an attractive target based on their properties (i.e. tendency to proliferate) and the potential to acquire mutations over a long period of time [6]. It has been hypothesized that stem cells derived from genetically susceptible subpopulations may be predisposed to transformation [14, 15]. However, the underlying mechanisms and plausible targets have yet to be fully elucidated.

Previous studies in the Ullrich laboratory have utilized mouse models to examine early cellular events in the carcinogenic process, as well as the progression toward cancer [150-153]. These studies used an *in vivo* cell dissociation model in which dissociated cells derived from mammary glands of irradiated donors were injected into non-irradiated recipients and allowed to replenish their cleared fat pads. Recipients were assayed for specific lesions involved in the carcinogenic process. The mammary outgrowths are clonally derived from mammary stem cells, but radiation responses were difficult to adequately characterize because there were no established methods to isolate mammary stem cells. Specific defects in DNA repair proteins have been shown to increase radiation sensitivity and radiation-induced genomic instability in mammary tissue [152, 154, 155]. Stem cells may be radiosensitive, and thus susceptible to radiation-induced damage.

The radiation response of mammary stem cells has yet to be characterized due to past difficulties in their identification and isolation. It has been hypothesized that stem cells have unique mechanisms to prevent or minimize radiation-induced damage in order to maintain their genomic integrity. A better understanding of the DNA damage response and repair processes of mammary stem cells is likely to elucidate contributing factors to susceptibility of breast tissue to radiation-induced carcinogenesis. Characterization of the radiation response of mammary stem cells will also provide new insight into prevention and treatment strategies.

An *in vitro* system analogous to a neural stem cell assay was developed for the detection of mammary stem cells [68, 69]. Mammary stem cells form spheroid structures (mammospheres) when cultured in non-adherent cell culture conditions. This system provides the opportunity to directly examine the replicative and multilineage capacity of mammary stem cells and their progeny. Utilizing this in vitro assay, we hypothesized that mammary stem cells are a critical target of radiation-induced damage in radiation-induced mammary carcinogenesis.

The overall purpose of this project was to expand the applicability of this *in vitro* assay for specific radiation biology endpoints and demonstrate the utility of the methodology developed. The aims of this project were to 1) develop a methodology to isolate and propagate mammary stem cells, 2) establish novel size criteria through characterization of mammospheres, and 3) apply the methodology and novel size criteria to evaluate the radiation response of mammary stem cells as mammospheres. Using this *in vitro* assay required establishing a foundation for the isolation and *in vitro* propagation of mammospheres. We developed a methodology through the modification of

mammosphere culture techniques and adapted this assay to examine radiation cell survival. During the development of our methodology, we characterized the *in vitro* behavior of mammary stem cells and established novel size criteria that distinguished mammospheres originating from a stem/progenitor cell. Our criteria had two components: 1) serial passaging to assess self-renewal capacity and 2) lineage composition of mammospheres. We hypothesized that mammospheres capable of self-renewal and multilineage capacity indicated they arose from a stem or high progenitor cell. Mammospheres were categorized based on size and those size groups, which failed to meet our size criteria, were excluded. We applied the methodology and size criteria developed to analyze the effects of ionizing radiation (IR) on survival of mammary stem cells derived from mice carrying one mutated copy of *Atm*. Based on our results, we were able to demonstrate the utility of this methodology and size criteria. Our improved methodology has expanded the feasibility of mammospheres and the applicability of this model to examine numerous functional in vitro endpoints. We believe the methodology described here will facilitate investigating the radiation response of mammary stem cells and their progeny, and key components involved in early events of the carcinogenic process in murine model systems.

LITERATURE CITED

1. O'Brien, C.A., et al., *A human colon cancer cell capable of initiating tumour growth in immunodeficient mice.* Nature, 2007. **445**(7123): p. 106-10.
2. Bonnet, D. and J.E. Dick, *Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell.* Nat Med, 1997. **3**(7): p. 730-7.
3. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-8.
4. Ponti, D., et al., *Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties.* Cancer Res, 2005. **65**(13): p. 5506-11.
5. Lapidot, T., et al., *A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.* Nature, 1994. **367**(6464): p. 645-8.
6. Reya, T., et al., *Stem cells, cancer, and cancer stem cells.* Nature, 2001. **414**(6859): p. 105-11.
7. Krivtsov, A.V., et al., *Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9.* Nature, 2006. **442**(7104): p. 818-22.
8. Shipitsin, M. and K. Polyak, *The cancer stem cell hypothesis: in search of definitions, markers, and relevance.* Lab Invest, 2008. **88**(5): p. 459-63.
9. Clarke, M.F., et al., *Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells.* Cancer Res, 2006. **66**(19): p. 9339-44.
10. Lewis, M.T., *Faith, heresy and the cancer stem cell hypothesis.* Future Oncol, 2008. **4**(5): p. 585-9.
11. Dontu, G., et al., *Stem cells in normal breast development and breast cancer.* Cell Prolif, 2003. **36 Suppl 1**: p. 59-72.
12. Smith, G.H., *Stem cells and mammary cancer in mice.* Stem Cell Rev, 2005. **1**(3): p. 215-23.
13. Smalley, M. and A. Ashworth, *Stem cells and breast cancer: A field in transit.* Nat Rev Cancer, 2003. **3**(11): p. 832-44.
14. Liu, S., et al., *BRCA1 regulates human mammary stem/progenitor cell fate.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1680-5.
15. Foulkes, W.D., *BRCA1 functions as a breast stem cell regulator.* J Med Genet, 2004. **41**(1): p. 1-5.
16. Parkin, D.M., *International variation.* Oncogene, 2004. **23**(38): p. 6329-40.
17. Parkin, D.M., et al., *Global cancer statistics, 2002.* CA Cancer J Clin, 2005. **55**(2): p. 74-108.
18. www.cancer.org, *American Cancer Society. Cancer Facts & Figures 2008.* Atlanta: American Cancer Society, Inc., 2008: p. 1-68.
19. Kakarala, M. and M.S. Wicha, *Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy.* J Clin Oncol, 2008. **26**(17): p. 2813-20.
20. Dumitrescu, R.G. and I. Cotarla, *Understanding breast cancer risk -- where do we stand in 2005?* J Cell Mol Med, 2005. **9**(1): p. 208-21.
21. www.cancer.org, *American Cancer Society. Breast Cancer Facts & Figures 2007-2008.* Atlanta: American Cancer Society, Inc, 2007: p. 1-32.

22. Preston, D.L., et al., *Solid cancer incidence in atomic bomb survivors: 1958-1998.* Radiat Res, 2007. **168**(1): p. 1-64.
23. Doody, M.M., et al., *Breast cancer incidence in U.S. radiologic technologists.* Cancer, 2006. **106**(12): p. 2707-15.
24. Ron, E., *Cancer risks from medical radiation.* Health Phys, 2003. **85**(1): p. 47-59.
25. Academics, N.R.C.o.t.N., *Health Risks from Exposure to Low Levels of Ionizing Radiation BEIR VII PHASE 2.* Committee to Assess Health Risks from Exposure to Low Levels of Ionizing Radiation, 2006.
26. Houlston, R.S. and J. Peto, *The search for low-penetrance cancer susceptibility alleles.* Oncogene, 2004. **23**(38): p. 6471-6.
27. Oesterreich, S. and S.A. Fuqua, *Tumor suppressor genes in breast cancer.* Endocr Relat Cancer, 1999. **6**(3): p. 405-19.
28. Okobia, M.N. and C.H. Bunker, *Molecular epidemiology of breast cancer: a review.* Afr J Reprod Health, 2003. **7**(3): p. 17-28.
29. Balmain, A., J. Gray, and B. Ponder, *The genetics and genomics of cancer.* Nat Genet, 2003. **33 Suppl**: p. 238-44.
30. Deome, K.B., et al., *Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice.* Cancer Res, 1959. **19**(5): p. 515-20.
31. Farnie, G. and R.B. Clarke, *Breast stem cells and cancer.* Ernst Schering Found Symp Proc, 2006(5): p. 141-53.
32. Smith, G.H. and G. Chepko, *Mammary epithelial stem cells.* Microsc Res Tech, 2001. **52**(2): p. 190-203.
33. Woodward, W.A., et al., *On mammary stem cells.* J Cell Sci, 2005. **118**(Pt 16): p. 3585-94.
34. P.S. Rudland, R.B., D.G. Fernig, and J.A. Smith *Mammary stem cells in normal development and cancer.* Stem Cells, ed. C. Potten. 1997: Academic Press, San Diego, CA. 147-232.
35. Daniel, C.W. and G.H. Smith, *The mammary gland: a model for development.* J Mammary Gland Biol Neoplasia, 1999. **4**(1): p. 3-8.
36. LaBarge, M.A., O.W. Petersen, and M.J. Bissell, *Of microenvironments and mammary stem cells.* Stem Cell Rev, 2007. **3**(2): p. 137-46.
37. Hennighausen, L. and G.W. Robinson, *Information networks in the mammary gland.* Nat Rev Mol Cell Biol, 2005. **6**(9): p. 715-25.
38. Williams, J.M. and C.W. Daniel, *Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis.* Dev Biol, 1983. **97**(2): p. 274-90.
39. Clarke, R.B., et al., *A putative human breast stem cell population is enriched for steroid receptor-positive cells.* Dev Biol, 2005. **277**(2): p. 443-56.
40. Blanpain, C., V. Horsley, and E. Fuchs, *Epithelial stem cells: turning over new leaves.* Cell, 2007. **128**(3): p. 445-58.
41. Morrison, S.J., N.M. Shah, and D.J. Anderson, *Regulatory mechanisms in stem cell biology.* Cell, 1997. **88**(3): p. 287-98.
42. Morrison, S.J. and J. Kimble, *Asymmetric and symmetric stem-cell divisions in development and cancer.* Nature, 2006. **441**(7097): p. 1068-74.

43. Smith, G.H. and D. Medina, *A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland.* J Cell Sci, 1988. **90 (Pt 1)**: p. 173-83.
44. Visvader, J.E. and G.J. Lindeman, *Mammary stem cells and mammopoiesis.* Cancer Res, 2006. **66**(20): p. 9798-801.
45. Stingl, J., et al., *Epithelial progenitors in the normal human mammary gland.* J Mammary Gland Biol Neoplasia, 2005. **10**(1): p. 49-59.
46. Moore, K.A. and I.R. Lemischka, *Stem cells and their niches.* Science, 2006. **311**(5769): p. 1880-5.
47. Brisken, C. and S. Duss, *Stem cells and the stem cell niche in the breast: an integrated hormonal and developmental perspective.* Stem Cell Rev, 2007. **3**(2): p. 147-56.
48. Ohlstein, B., et al., *The stem cell niche: theme and variations.* Curr Opin Cell Biol, 2004. **16**(6): p. 693-9.
49. Rizvi, A.Z. and M.H. Wong, *Epithelial stem cells and their niche: there's no place like home.* Stem Cells, 2005. **23**(2): p. 150-65.
50. Amorino, G.P., et al., *Dominant-negative cAMP-responsive element-binding protein inhibits proliferating cell nuclear antigen and DNA repair, leading to increased cellular radiosensitivity.* J Biol Chem, 2003. **278**(32): p. 29394-9.
51. Dontu, G., et al., *Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells.* Breast Cancer Res, 2004. **6**(6): p. R605-15.
52. Liu, S., et al., *Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells.* Cancer Res, 2006. **66**(12): p. 6063-71.
53. Kordon, E.C. and G.H. Smith, *An entire functional mammary gland may comprise the progeny from a single cell.* Development, 1998. **125**(10): p. 1921-30.
54. Tsai, Y.C., et al., *Contiguous patches of normal human mammary epithelium derived from a single stem cell: implications for breast carcinogenesis.* Cancer Res, 1996. **56**(2): p. 402-4.
55. DeOme, K.B., et al., *Detection of inapparent nodule-transformed cells in the mammary gland tissues of virgin female BALB/cfC3H mice.* Cancer Res, 1978. **38**(7): p. 2103-11.
56. Smith, G.H., *Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype.* Breast Cancer Res Treat, 1996. **39**(1): p. 21-31.
57. Diallo, R., et al., *Monoclonality in normal epithelium and in hyperplastic and neoplastic lesions of the breast.* J Pathol, 2001. **193**(1): p. 27-32.
58. Zhou, S., et al., *Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo.* Proc Natl Acad Sci U S A, 2002. **99**(19): p. 12339-44.
59. Spangrude, G.J. and G.R. Johnson, *Resting and activated subsets of mouse multipotent hematopoietic stem cells.* Proc Natl Acad Sci U S A, 1990. **87**(19): p. 7433-7.
60. Goodell, M.A., et al., *Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo.* J Exp Med, 1996. **183**(4): p. 1797-806.
61. Welm, B.E., et al., *Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population.* Dev Biol, 2002. **245**(1): p. 42-56.

62. Clayton, H., I. Titley, and M. Vivanco, *Growth and differentiation of progenitor/stem cells derived from the human mammary gland.* Exp Cell Res, 2004. **297**(2): p. 444-60.
63. Alvi, A.J., et al., *Functional and molecular characterisation of mammary side population cells.* Breast Cancer Res, 2003. **5**(1): p. R1-8.
64. Smith, G.H., *Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands.* Development, 2005. **132**(4): p. 681-7.
65. Stingl, J., et al., *Purification and unique properties of mammary epithelial stem cells.* Nature, 2006. **439**(7079): p. 993-7.
66. Shackleton, M., et al., *Generation of a functional mammary gland from a single stem cell.* Nature, 2006. **439**(7072): p. 84-8.
67. Li, Y., et al., *Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15853-8.
68. Reynolds, B.A. and S. Weiss, *Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell.* Dev Biol, 1996. **175**(1): p. 1-13.
69. Dontu, G., et al., *In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells.* Genes Dev, 2003. **17**(10): p. 1253-70.
70. Knudson, A.G., *Two genetic hits (more or less) to cancer.* Nat Rev Cancer, 2001. **1**(2): p. 157-62.
71. Nowell, P.C., *The clonal evolution of tumor cell populations.* Science, 1976. **194**(4260): p. 23-8.
72. Al-Hajj, M. and M.F. Clarke, *Self-renewal and solid tumor stem cells.* Oncogene, 2004. **23**(43): p. 7274-82.
73. Pardal, R., M.F. Clarke, and S.J. Morrison, *Applying the principles of stem-cell biology to cancer.* Nat Rev Cancer, 2003. **3**(12): p. 895-902.
74. Wang, J.C. and J.E. Dick, *Cancer stem cells: lessons from leukemia.* Trends Cell Biol, 2005. **15**(9): p. 494-501.
75. Dick, J.E., *Breast cancer stem cells revealed.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3547-9.
76. Wicha, M.S., S. Liu, and G. Dontu, *Cancer stem cells: an old idea--a paradigm shift.* Cancer Res, 2006. **66**(4): p. 1883-90; discussion 1895-6.
77. Ricci-Vitiani, L., et al., *Identification and expansion of human colon-cancer-initiating cells.* Nature, 2007. **445**(7123): p. 111-5.
78. Singh, S.K., et al., *Identification of a cancer stem cell in human brain tumors.* Cancer Res, 2003. **63**(18): p. 5821-8.
79. Singh, S.K., et al., *Identification of human brain tumour initiating cells.* Nature, 2004. **432**(7015): p. 396-401.
80. Bao, S., et al., *Glioma stem cells promote radioresistance by preferential activation of the DNA damage response.* Nature, 2006. **444**(7120): p. 756-60.
81. Al-Hajj, M., et al., *Therapeutic implications of cancer stem cells.* Curr Opin Genet Dev, 2004. **14**(1): p. 43-7.
82. Croker, A.K. and A.L. Allan, *Cancer stem cells: implications for the progression and treatment of metastatic disease.* J Cell Mol Med, 2008. **12**(2): p. 374-90.

83. Phillips, T.M., W.H. McBride, and F. Pajonk, *The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation.* J Natl Cancer Inst, 2006. **98**(24): p. 1777-85.
84. Brenton, J.D., et al., *Molecular classification and molecular forecasting of breast cancer: ready for clinical application?* J Clin Oncol, 2005. **23**(29): p. 7350-60.
85. Stingl, J. and C. Caldas, *Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis.* Nat Rev Cancer, 2007. **7**(10): p. 791-9.
86. Sorlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10869-74.
87. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
88. Sorlie, T., et al., *Repeated observation of breast tumor subtypes in independent gene expression data sets.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8418-23.
89. Melchor, L. and J. Benitez, *An integrative hypothesis about the origin and development of sporadic and familial breast cancer subtypes.* Carcinogenesis, 2008. **29**(8): p. 1475-82.
90. Trott, K.R. and M. Rosemann, *Molecular mechanisms of radiation carcinogenesis and the linear, non-threshold dose response model of radiation risk estimation.* Radiat Environ Biophys, 2000. **39**(2): p. 79-87.
91. *UNSCEAR 2000. The United Nations Scientific Committee on the Effects of Atomic Radiation.* Health Phys, 2000b. **Volume II, 79**(Annex F-J): p. 2-551.
92. Ward, J.F., *Radiation mutagenesis: the initial DNA lesions responsible.* Radiat Res, 1995. **142**(3): p. 362-8.
93. Goodhead, D.T., *Initial events in the cellular effects of ionizing radiations: clustered damage in DNA.* Int J Radiat Biol, 1994. **65**(1): p. 7-17.
94. Goodhead, D.T., *The initial physical damage produced by ionizing radiations.* Int J Radiat Biol, 1989. **56**(5): p. 623-34.
95. Little, J.B., *Radiation carcinogenesis.* Carcinogenesis, 2000. **21**(3): p. 397-404.
96. Cornforth, M.N., *Radiation-Induced damage and the formation of chromosomal aberrations.* DNA Damage and Repair: DNA repair in higher eukaryotes, 1998. **2**: p. 559-585.
97. Thompson, L.H. and D. Schild, *Recombinational DNA repair and human disease.* Mutat Res, 2002. **509**(1-2): p. 49-78.
98. Little, M.P. and J.D. Boice, Jr., *Comparison of breast cancer incidence in the Massachusetts tuberculosis fluoroscopy cohort and in the Japanese atomic bomb survivors.* Radiat Res, 1999. **151**(2): p. 218-24.
99. Land, C.E., et al., *Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950-1990.* Radiat Res, 2003. **160**(6): p. 707-17.
100. Land, C.E., et al., *Early-onset breast cancer in A-bomb survivors.* Lancet, 1993. **342**(8865): p. 237.
101. Carmichael, A., A.S. Sami, and J.M. Dixon, *Breast cancer risk among the survivors of atomic bomb and patients exposed to therapeutic ionising radiation.* Eur J Surg Oncol, 2003. **29**(5): p. 475-9.

102. Park, C.C., et al., *Ionizing radiation induces heritable disruption of epithelial cell interactions.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 10728-33.
103. Woodward, W.A., et al., *WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells.* Proc Natl Acad Sci U S A, 2007. **104**(2): p. 618-23.
104. Chen, M.S., et al., *Wnt/beta-catenin mediates radiation resistance of Sca1+ progenitors in an immortalized mammary gland cell line.* J Cell Sci, 2007. **120**(Pt 3): p. 468-77.
105. Meyn, M.S., *Ataxia-telangiectasia, cancer and the pathobiology of the ATM gene.* Clin Genet, 1999. **55**(5): p. 289-304.
106. Ahmed, M. and N. Rahman, *ATM and breast cancer susceptibility.* Oncogene, 2006. **25**(43): p. 5906-11.
107. Khanna, K.K., *Cancer risk and the ATM gene: a continuing debate.* J Natl Cancer Inst, 2000. **92**(10): p. 795-802.
108. Rotman, G. and Y. Shiloh, *ATM: from gene to function.* Hum Mol Genet, 1998. **7**(10): p. 1555-63.
109. Lavin, M.F., et al., *ATM signaling and genomic stability in response to DNA damage.* Mutat Res, 2005. **569**(1-2): p. 123-32.
110. Lavin, M.F., et al., *Functional consequences of sequence alterations in the ATM gene.* DNA Repair (Amst), 2004. **3**(8-9): p. 1197-205.
111. Khanna, K.K., et al., *ATM, a central controller of cellular responses to DNA damage.* Cell Death Differ, 2001. **8**(11): p. 1052-65.
112. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.
113. Lee, J.H. and T.T. Paull, *ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex.* Science, 2005. **308**(5721): p. 551-4.
114. Bakkenist, C.J. and M.B. Kastan, *DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation.* Nature, 2003. **421**(6922): p. 499-506.
115. Swift, M., et al., *Incidence of cancer in 161 families affected by ataxia-telangiectasia.* N Engl J Med, 1991. **325**(26): p. 1831-6.
116. Swift, M., et al., *Breast and other cancers in families with ataxia-telangiectasia.* N Engl J Med, 1987. **316**(21): p. 1289-94.
117. Concannon, P., *ATM heterozygosity and cancer risk.* Nat Genet, 2002. **32**(1): p. 89-90.
118. Thompson, D., et al., *Cancer risks and mortality in heterozygous ATM mutation carriers.* J Natl Cancer Inst, 2005. **97**(11): p. 813-22.
119. Easton, D.F., *Cancer risks in A-T heterozygotes.* Int J Radiat Biol, 1994. **66**(6 Suppl): p. S177-82.
120. Swift, M., et al., *The incidence and gene frequency of ataxia-telangiectasia in the United States.* Am J Hum Genet, 1986. **39**(5): p. 573-83.
121. Inskip, H.M., et al., *Risk of breast cancer and other cancers in heterozygotes for ataxia-telangiectasia.* Br J Cancer, 1999. **79**(7-8): p. 1304-7.
122. Janin, N., et al., *Breast cancer risk in ataxia telangiectasia (AT) heterozygotes: haplotype study in French AT families.* Br J Cancer, 1999. **80**(7): p. 1042-5.

123. Athma, P., R. Rappaport, and M. Swift, *Molecular genotyping shows that ataxia-telangiectasia heterozygotes are predisposed to breast cancer.* Cancer Genet Cytogenet, 1996. **92**(2): p. 130-4.
124. Stankovic, T., et al., *ATM mutations and phenotypes in ataxia-telangiectasia families in the British Isles: expression of mutant ATM and the risk of leukemia, lymphoma, and breast cancer.* Am J Hum Genet, 1998. **62**(2): p. 334-45.
125. Laake, K., et al., *Screening breast cancer patients for Norwegian ATM mutations.* Br J Cancer, 2000. **83**(12): p. 1650-3.
126. Cavaciuti, E., et al., *Cancer risk according to type and location of ATM mutation in ataxia-telangiectasia families.* Genes Chromosomes Cancer, 2005. **42**(1): p. 1-9.
127. FitzGerald, M.G., et al., *Heterozygous ATM mutations do not contribute to early onset of breast cancer.* Nat Genet, 1997. **15**(3): p. 307-10.
128. Broeks, A., et al., *ATM-heterozygous germline mutations contribute to breast cancer-susceptibility.* Am J Hum Genet, 2000. **66**(2): p. 494-500.
129. Gilad, S., et al., *Predominance of null mutations in ataxia-telangiectasia.* Hum Mol Genet, 1996. **5**(4): p. 433-9.
130. Izatt, L., et al., *Identification of germline missense mutations and rare allelic variants in the ATM gene in early-onset breast cancer.* Genes Chromosomes Cancer, 1999. **26**(4): p. 286-94.
131. Teraoka, S.N., et al., *Increased frequency of ATM mutations in breast carcinoma patients with early onset disease and positive family history.* Cancer, 2001. **92**(3): p. 479-87.
132. Sommer, S.S., et al., *ATM missense mutations are frequent in patients with breast cancer.* Cancer Genet Cytogenet, 2003. **145**(2): p. 115-20.
133. Pylkas, K., et al., *Evaluation of the role of Finnish ataxia-telangiectasia mutations in hereditary predisposition to breast cancer.* Carcinogenesis, 2007. **28**(5): p. 1040-5.
134. Chenevix-Trench, G., et al., *Dominant negative ATM mutations in breast cancer families.* J Natl Cancer Inst, 2002. **94**(3): p. 205-15.
135. Scott, S.P., et al., *Missense mutations but not allelic variants alter the function of ATM by dominant interference in patients with breast cancer.* Proc Natl Acad Sci U S A, 2002. **99**(2): p. 925-30.
136. Gatti, R.A., A. Tward, and P. Concannon, *Cancer risk in ATM heterozygotes: a model of phenotypic and mechanistic differences between missense and truncating mutations.* Mol Genet Metab, 1999. **68**(4): p. 419-23.
137. Shigeta, T., et al., *Defective control of apoptosis and mitotic spindle checkpoint in heterozygous carriers of ATM mutations.* Cancer Res, 1999. **59**(11): p. 2602-7.
138. Gutierrez-Enriquez, S., et al., *Functional consequences of ATM sequence variants for chromosomal radiosensitivity.* Genes Chromosomes Cancer, 2004. **40**(2): p. 109-19.
139. Fernet, M., et al., *Cellular responses to ionising radiation of AT heterozygotes: differences between missense and truncating mutation carriers.* Br J Cancer, 2004. **90**(4): p. 866-73.

140. Bremer, M., et al., *Clinical radiosensitivity in breast cancer patients carrying pathogenic ATM gene mutations: no observation of increased radiation-induced acute or late effects.* Radiother Oncol, 2003. **69**(2): p. 155-60.
141. Angele, S., et al., *ATM haplotypes and cellular response to DNA damage: association with breast cancer risk and clinical radiosensitivity.* Cancer Res, 2003. **63**(24): p. 8717-25.
142. Mitui, M., et al., *Functional and computational assessment of missense variants in the ataxia-telangiectasia mutated (ATM) gene: mutations with increased cancer risk.* Hum Mutat, 2009. **30**(1): p. 12-21.
143. Barlow, C., et al., *Atm haploinsufficiency results in increased sensitivity to sublethal doses of ionizing radiation in mice.* Nat Genet, 1999. **21**(4): p. 359-60.
144. Weil, M.M., et al., *Radiation induces genomic instability and mammary ductal dysplasia in Atm heterozygous mice.* Oncogene, 2001. **20**(32): p. 4409-11.
145. Lu, S., et al., *Atm-haploinsufficiency enhances susceptibility to carcinogen-induced mammary tumors.* Carcinogenesis, 2006. **27**(4): p. 848-55.
146. Sommer, S.S., et al., *Elevated frequency of ATM gene missense mutations in breast cancer relative to ethnically matched controls.* Cancer Genet Cytogenet, 2002. **134**(1): p. 25-32.
147. Lavin, M.F. and K. Spring, *Upregulation of FasL and apoptosis in thymic lymphomas in Atm knock-in mice.* Toxicology, 2002. **181-182**: p. 483-9.
148. Spring, K., et al., *Mice heterozygous for mutation in Atm, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer.* Nat Genet, 2002. **32**(1): p. 185-90.
149. Spring, K., et al., *Atm knock-in mice harboring an in-frame deletion corresponding to the human ATM 7636del9 common mutation exhibit a variant phenotype.* Cancer Res, 2001. **61**(11): p. 4561-8.
150. Adams, L.M., S.P. Ethier, and R.L. Ullrich, *Enhanced in vitro proliferation and in vivo tumorigenic potential of mammary epithelium from BALB/c mice exposed in vivo to gamma-radiation and/or 7,12-dimethylbenz[a]anthracene.* Cancer Res, 1987. **47**(16): p. 4425-31.
151. Ullrich, R.L., et al., *Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation.* Radiat Res, 1996. **146**(3): p. 353-5.
152. Ponnaiya, B., M.N. Cornforth, and R.L. Ullrich, *Radiation-induced chromosomal instability in BALB/c and C57BL/6 mice: the difference is as clear as black and white.* Radiat Res, 1997. **147**(2): p. 121-5.
153. Ullrich, R.L. and C.M. Davis, *Radiation-induced cytogenetic instability in vivo.* Radiat Res, 1999. **152**(2): p. 170-3.
154. Yu, Y., et al., *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**(5): p. 1820-4.
155. Okayasu, R., et al., *A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse.* Cancer Res, 2000. **60**(16): p. 4342-5.

CHAPTER 2: MATERIALS AND METHODS

MOUSE STRAINS

The *Atm* mutant mice used in this study were originally obtained from Dr. Martin F. Lavin at Queensland Institute of Medical Research, Brisbane, Australia. Upon arrival, maintenance of the mice was carried out by Dr. Michael Weil at Colorado State University (CSU), Fort Collins, CO. Generation of the Atm^{tm1Mfl} (referred to as *Atm*-ΔSRI) has been previously described [1] and these mice have been maintained on a 129T2/SvEmsJ:C57BL/6J mixed background. The *Atm*-ΔSRI mice have been inbred upon arrival at CSU (unknown time). In addition, the *Atm*-ΔSRI mice on a 129T2/SvEmsJ:C57BL/6J mixed background were repeatedly backcrossed to a BALB/c substrain (carried out by Dr. Martin F. Lavin). The *Atm*-ΔSRI mice were backcrossed an unknown number of generations with BALB/cArc mice, which were obtained from Animal Resources Centre (Western Australia). Upon arrival at CSU, the *Atm*-ΔSRI mice were backcrossed an unknown number of generations with BALB/cByJ mice (Jackson Laboratory, Bar Harbor, ME) that were carried out by Dr. Michael Weil. All animals were maintained at the Colorado State University Laboratory Animal Resources Painter Center. Rebekah Klingler carried out regular maintenance of these *Atm* mouse strains, including breeding schemes, to generate *Atm* mutated heterozygous and wildtype mice. In this study, *Atm*-ΔSRI heterozygous mice and their wildtype littermates were used to isolate mammary stem cells.

GENOTYPING

To genotype *Atm*-ΔSRI heterozygous and their wildtype littermates, tail snips from mice were used to extract DNA for PCRs. Tail snips were harvested from 3-week old mice according to approved CSU IACUC protocols. DNA was extracted from the tail snip using DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA). PCR genotyping was carried out using the following primers, forward (5'TCTCATGTATCAATTGGCTGCTGC-3') and reverse (5'AATTGTTAACCAATTCTGGGTGGC-3') as previously reported [1].

PRIMARY MAMMARY EPITHELIAL CELL ISOLATION

Mammary glands (#4 and #5) were isolated from 8- to 12-week old virgin female *Atm*-ΔSRI and *Atm*-S2592C heterozygous and wildtype mice. Enzymatic dissociation of the glands were as previously described with modifications [2]. The glands were mechanically minced (in 200 µl of sterile serum free DMEM/F12) to 1-2 mm pieces on a glass Petri plate using two sterile scalpels. Minced tissue was transferred to a sterile 15 mL conical tube containing 3-8 mL of EpiCult®-B Basal Medium (StemCell Technologies, Vancouver, BC, Canada) containing collagenase (300 units/mL, Type II, Worthington Biochemical Corporation, Lakewood, NJ), hyaluronidase (100 units/mL, Sigma Aldrich, St. Louis, MO), 10 µl/mL of EpiCult®-B Supplement (StemCell Technologies), 10 µl/mL of 1X antibiotic/antimycotic (Invitrogen, Carlsbad, CA), and 5% FBS (Hyclone, Logan, UT). Tissue was enzymatically digested for 30 minutes to 1 hour in a 37°C incubator containing 5% CO_2. The cap of the 15 mL conical tube was loosened to allow for CO_2 gas exchange. Tissue was agitated every 15 minutes to aid in

the enzymatic digestion. Dissociated mammary organoids were washed by centrifugation (Thermo/Forma Centrifuge Model GP8R 5682, rotor 216; ThermoFisher Scientific, Waltham, MA) at 4°C (450 × g, 5 minutes) using 10 mL of ice-cold Hank's Balanced Salt Solution (Hyclone) containing 10 mM OmniPur N-2-Hydroxyethylpiperazine-N`-2-ethanesulfonic Acid (HEPES) (EMD Chemicals, Gibbstown, NJ), sodium bicarbonate (MP Biomedicals, Solon, OH), Mg^{+}, Ca^{++}, and 2% FBS (referred to as HF). The supernatant was removed by aspiration after every centrifugation. Mammary organoids were dissociated into single cells by gentle pipetting with a P1000 micropipettor using 2 mL of pre-warmed (37°C) 0.25% Trypsin-EDTA (Hyclone) for 1 minute. The cells were washed by centrifugation at 4°C (450 × g, 5 minutes) using 10 mL of ice-cold HF. Cell clumps were further dissociated by gentle pipetting with a P1000 micropipettor using 2 mL of pre-warmed (37°C) 5 mg/mL Dispase II (Roche Applied Science, Indianapolis, IN) and 1 mg/mL DNase I (Sigma Aldrich) for 1 minute. The cells were washed with 10 mL of ice-cold HF and filtered through a 40 μm cell strainer (BD Falcon, San Jose, CA) into a 50 mL conical tube to yield single cell suspension. The cells were centrifuged at 4°C (450 × g, 5 minutes) and the cell pellet was resuspended in 1-5 mL of mammosphere media before counting. Cell counts were obtained using a Bright Line Hemacytometer (Hausser Scientific, Horsham, PA) at 1:10 with trypan blue (Invitrogen). In addition, cell counts were obtained using Beckman Coulter Z-2 Series (Beckman Coulter, Fullerton, CA) (3.5-7 μm size range) at 1:50 or 1:500 dilutions and resulted in consistent cell size peaks at 4 μm and 6 μm. Cell counts were fairly similar between the hemocytometer and Coulter counter immediately after isolation and either method was used for plating cells at high density.

STEM CELL SUSPENSION CULTURES

A flow diagram illustrating the procedure for culturing non-clonal mammospheres is illustrated in Figure 2.1. Mammosphere culture conditions were adapted as previously described with modifications [3]. Single cell suspensions were plated into 60 mm low binding plates (Nunc, Rochester, NY) at a density of 200,000 cells/mL directly after isolation to yield conditioned media for clonal mammospheres. Cells were grown in serum free DMEM/F12 (Hyclone) media supplemented with 1X B27 (Invitrogen), 20 ng/mL basic fibroblast growth factor (bFGF) (Sigma Aldrich), 20 ng/mL EGF (Sigma Aldrich), 4 μg/mL heparin (Sigma Aldrich), 1X Glutamax (Hyclone), and 1X antibiotic/antimycotic (Invitrogen). Mammospheres were collected by gentle centrifugation (4°C, 52 × g, 5 minutes) at day four to change media. At day six, conditioned media was collected by gentle centrifugation twice (4°C, 52 × g, 2 minutes) and (4°C, 450 × g, 5 minutes) to ensure remaining cells were removed. Mammospheres used to provide conditioned media for clonal mammospheres were typically cultured for no longer than 12-15 days. Conditioned media that was not used immediately was stored at 4°C for no longer than two days.

ENRICHMENT OF STEM AND PROGENITOR CELL POPULATION

To derive clonal mammospheres for the mammosphere formation efficiency and cell survival assay, single cell suspensions were enriched with stem and progenitor cells after primary mammary epithelial cell isolation. Non-epithelial cells ($CD45^+/Ter119^+/CD31$) were depleted from the cell population using the EasySep® Negative Selection Mouse Mammary Epithelial Cell Enrichment Cocktail and Biotin Selection Cocktail (StemCell Technologies). The EasySep® Negative Selection kit

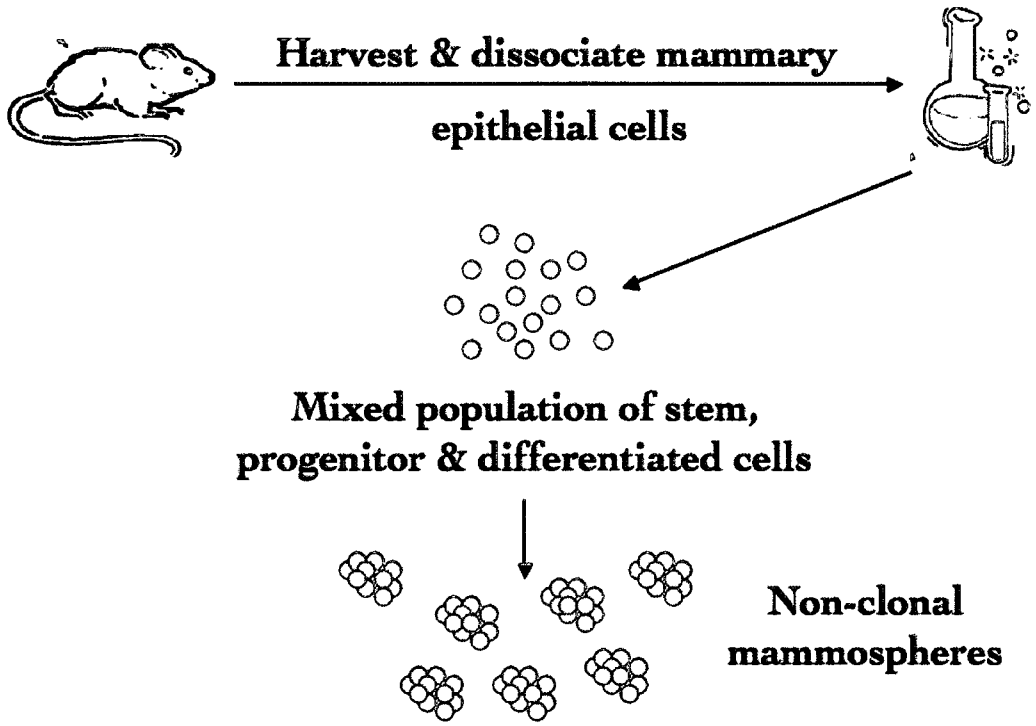

Figure 2.1: Flow diagram representation of non-clonal mammosphere cultures.

required the cell pellet to be resuspended in HF before counting. Cell counts were obtained using a Bright Line Hemacytometer (Hausser Scientific) at 1:10 with trypan blue (Invitrogen). The cells were centrifuged at 4°C (450 × g, 5 minutes) and the cell pellet was resuspended in HF supplemented with 0.1 mg/mL DNase I (referred to as HF medium). The cells were resuspended at a concentration of 1×10^8 cells/mL or in a minimum volume of 200 µl if the cell concentration was less than 2×10^7 cells. Single cell suspensions were transferred to a sterile 5 mL polystyrene round-bottom tube (Falcon). Before using the EasySep® Negative Selection Mouse Mammary Epithelial Cell Enrichment Cocktail, the tube was centrifuged. The single cell suspension was mixed with 50 µl/mL of cocktail and incubated on ice for 15 minutes. The cells were

then mixed with 100 µl/mL of EasySep® Biotin Selection Cocktail and incubated on ice for 15 minutes. Before using the EasySep® Magnetic Nanoparticles, the nanoparticles were gently pipetted (5×) with a P1000 micropipettor to ensure uniform suspension. The cells were mixed with 50 µl/mL of nanoparticles and incubated on ice for 15 minutes. HF medium was added to the cell suspension to bring the total volume to 2.5 mL. The cells were then mixed by gently pipetting (3×) with a P1000 micropipettor. The 5 mL polystyrene tube (without cap) was placed into the EasySep® Magnet for 5 minutes. In a continuous motion the magnet and tube was inverted for 2-3 seconds and the cell suspension was poured into a new sterile 5 mL polystyrene tube with no shaking or blotting of any additional drops of liquid around the rim of the tube. The original tube of the magnetically labeled unwanted non-epithelial cells was removed from the magnet and another 2 mL of HF medium was added to maximize cell recovery. The cells were then mixed by gently pipetting (3×) with a P1000 micropipettor. The 5 mL polystyrene tube (without cap) was placed into the EasySep® Magnet for 5 minutes. In a continuous motion the magnet and tube was inverted for 2-3 seconds again and the cell suspension was poured into the 5 mL polystyrene tube from the first separation (final volume 4.5 mL). The cells were centrifuged at 4°C (350 × g, 5 minutes) and the cell pellet was resuspended in 2.5 mL of HF medium after discarding the supernatant. The 5 mL polystyrene tube (without cap) was placed into the EasySep® Magnet for 5 minutes. As before, the cell suspension was poured into a new sterile 5 mL polystyrene tube and the cells were centrifuged at 4°C (350 × g, 5 minutes). The cell pellet was resuspended in 2-5 mL of HF medium before counting. Cell counts were obtained using a Bright Line

Hemacytometer (Hausser Scientific) at 1:10 with trypan blue (Invitrogen). Single cell suspensions were plated at the appropriate cell density to yield clonal mammospheres.

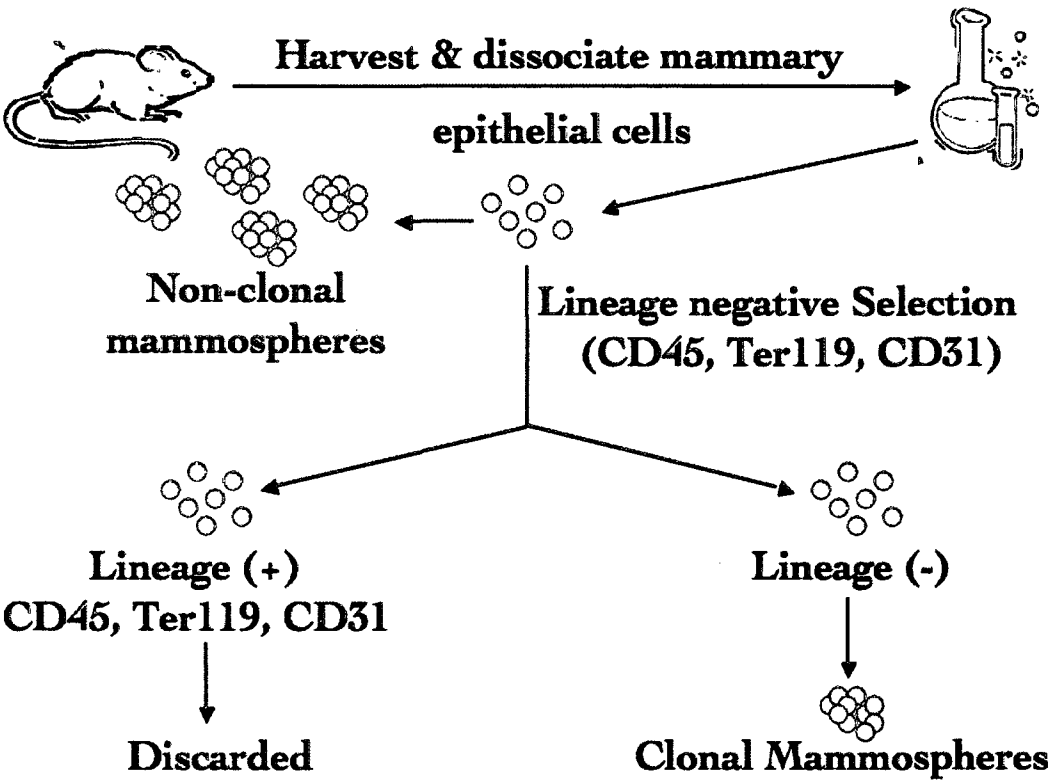

Figure 2.2: Flow diagram of modified enrichment procedure for mammary stem and progenitor cells.

MAMMOSPHERE FORMATION EFFICIENCY (MFE) USING SUSPENSION CULTURES

A representation of the modified enrichment procedure for mammary stem and progenitor cells is presented in Figure 2.2. Single cell suspensions were enriched with stem and progenitor cells after isolation using an EasySep® Mouse Mammary Stem Cell Enrichment Kit (Stem Cell Technologies). Single cell suspensions were plated in 60 mm low binding plates (Nunc) at a density of 1000 cells/mL (5000 cells) in primary cultures

to yield clonal mammospheres. Cells were grown in conditioned mammosphere media, which contained conditioned media collected from high-density plates and 2X mammosphere media (1:1). Only 5000 cells were plated in each 60 mm low binding plates with 10 mL of conditioned media and left untouched for 8-12 days. The mammospheres were not disturbed to ensure clonal growth and were scored at day eight and twelve.

HEMOCYTOMETER CELL COUNTS OF INDIVIDUAL MAMMOSPHERES

To determine the number of cells that comprised each mammospheres, individual mammospheres were picked and dissociated using a fire polished Pasteur pipette and 250 µl of cold 0.25% Trypsin-EDTA (Hyclone). Trypsinization times were customized for individual mammospheres based on diameter size for each passaging. The cells were washed by centrifugation at 4°C (140 × g, 5 minutes) using expired rinse media (e.g. DMEM/F12, McCoys, etc.) containing 10% FBS. The cells were resuspended in the remaining supernatant (~25 µl) after gently aspirating most of the media. Cell counts were obtained using a Bright Line Hemacytometer (Hausser Scientific).

SERIAL PASSAGING OF INDIVIDUAL CLONAL MAMMOSPHERES

For picking individual clonal mammospheres at day twelve, mammospheres were manually picked under sterile conditions in a tissue culture hood. Sterile conditions were maintained by placing the dissecting scope inside a tissue culture hood under ultraviolent light for 30 minutes before picking individual mammospheres. Mammospheres were measured using a 8 mm reticle and picked with a P10 micropipettor. Individual

mammospheres were placed in a 1.5mL centrifuge tube with 50 µl of mammosphere media on ice. Mammospheres were dissociated into single cells using a fire polished Pasteur pipette and 250 µl of cold 0.25% Trypsin-EDTA (Hyclone). Trypsin times were customized for individual mammospheres based on diameter size for each passaging. The cells were washed by centrifugation at 4°C (450 × g, 5 minutes) using expired rinse media (e.g. DMEM/F12, McCoys, etc.) containing 10% FBS. The cells were resuspended in 500 µl of 1:1 mixture of conditioned and 2X mammosphere media. Dissociated single cells from individual mammospheres were plated in individual wells of a 24-well low binding plate (Nunc) and allowed to form secondary mammospheres for 1-2 days. Mammospheres were serially passaged for at least five passages to determine which size groups originated from a sphere-initiating cell.

MAMMOSPHERE FORMATION ASSAY (MFE) USING 3-D MATRIX

Upon isolation, single cells were plated in reduced growth factor Cultrex Basement Membrane Extract (Trevigen, Gaithersburg, MD) and methods were adapted as previously described [4]. Cells were plated at a density of 20,000-200,000 cells/mL in individual wells of a 24-well plate in triplicate. At day eight the mammospheres were fixed with ice-cold methanol:acetone (1:1) at -20°C for 10 minutes. The mammospheres were categorized according to their diameter and the number of cells plated.

IMMUNOFLUORESCENCE

Immunocytochemistry to identify lineage composition of the mammospheres

A flow diagram representation of the modified immunofluorescence procedure is presented in Figure 2.3. Individual primary mammospheres were picked at day twelve

using a dissecting scope. Mammospheres were dissociated into single cells using a fire polished Pasteur pipette and cold 0.25% Trypsin-EDTA (Hyclone). The cells were washed by centrifugation at 4°C (450 × g, 5 minutes) using expired rinse media (e.g. DMEM/F12, McCoys, etc.) containing 10% FBS. The cells were resuspended in 50μl of 10% FBS in 1X PBS. The cells were deposited onto a glass slide using Shandon Cytospin® II Centrifuge (700 rpm, 7 minutes) and allowed to air-dry overnight. Cells were fixed with 100% ice-cold methanol (Fisher Scientific) at -20°C for 10 minutes. The cells were sequentially stained for Cytokeratin 18 (luminal) and α-Smooth Muscle Actin (myoepithelial) to identify lineage composition of the mammospheres. The antibodies used were mouse monoclonal [C-04] to Cytokeratin18-Biotin (Abcam, Cambridge, MA), Streptavidin Alexa Fluor 594 conjugate (Invitrogen), rabbit anti-α-Smooth Muscle Specific polyclonal (Abcam), and Goat anti-Rabbit AlexaFluor 488 (Invitrogen, Molecular Probes). All antibodies were used at a 1:100 dilution except for anti-α-SMA that was used at 1:50. Primary and secondary antibody staining was performed for 30 min for K18, 1 hr block with 5% milk, and 1 hr for SMA at RT in a dark, humidified chamber. Cells were washed three times with 1X PBS between primary and secondary antibody staining. Nuclei were counter-stained and coverslips were mounted with VectaShield mounting medium with 4',6-Diamidino-2-phenylindole (DAPI) (Vector Lab, Burlingame, CA). Images were captured using a Photometric Coolsnap ES2 on a Zeiss Axioskop 2 Plus Microscope and Metavue 7.1 software at 10X magnification.

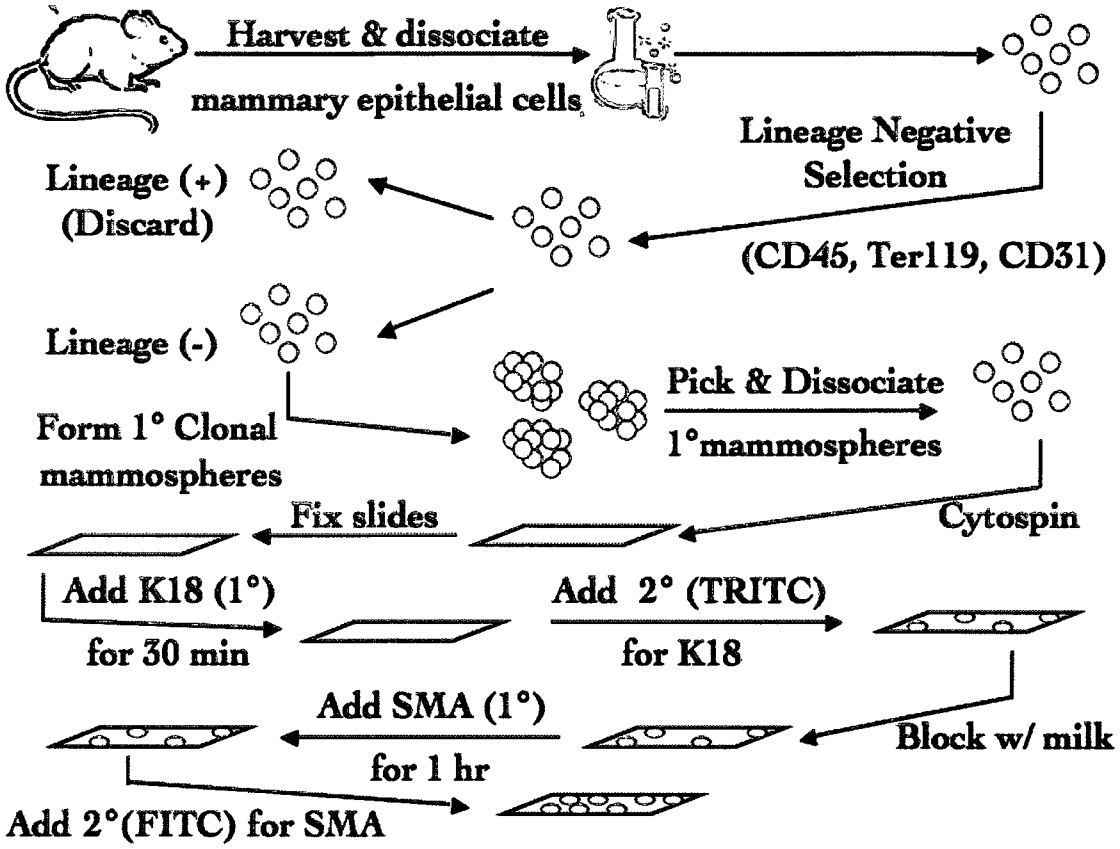

Figure 2.3: Flow diagram of immunofluorescence procedure to identify lineage composition of mammospheres.

IRRADIATIONS

Irradiations were carried out using J.L. Shepard 6000 Ci ^{137}Cesium Sealed Source Cabinet Beam Configuration (Model # Mark-I/69A, serial #1065). Doses used in this study were 0, 1, 3, and 5 Gy (position 3, dose rate of 3.9 Gy/minute).

CELL SURVIVAL ASSAY

A flow diagram of the modified cell survival assay procedure is depicted in Figure 2.4. Single cell suspensions were enriched with stem and progenitor cells after isolation using an EasySep® Mouse Mammary Stem Cell Enrichment Kit (Stem Cell

Technologies). Cells were aliquoted into 15 mL conical tubes and immediately irradiated (0, 1, 3, 5 Gy) at room temperature using a Shepherd 6000 Ci ^{137}Cs self-shielded cabinet irradiator. The cells remained on ice until plating. Single cell suspensions were plated in 60 mm low binding plates (Nunc) at a density of 1500 to 2000 cells (0 and 1Gy), 3000 cells (3 Gy), and, 4000 cells (5 Gy) in primary cultures to yield clonal mammospheres. Cells were grown in 10 mL conditioned mammosphere media (1:1). The mammospheres were not disturbed to ensure clonal growth and were scored at day twelve.

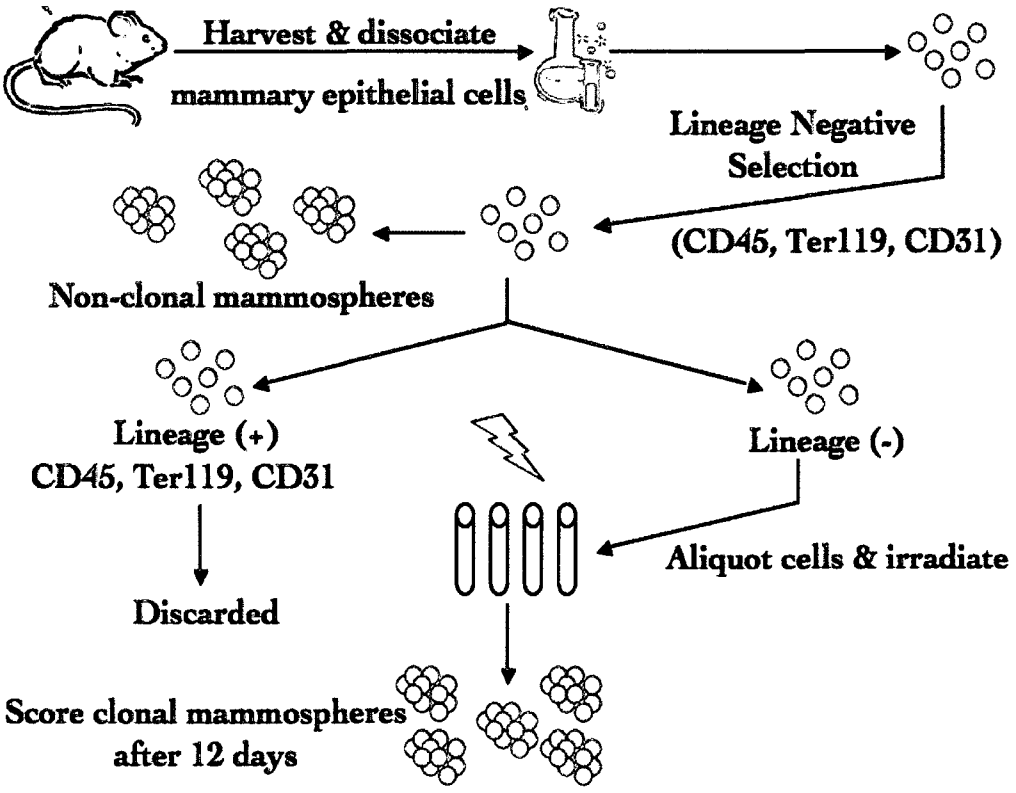

Figure 2.4: Flow diagram of modified cell survival assay procedure for mammary stem and progenitor cells.

LITERATURE CITED

1. Spring, K., et al., *Atm knock-in mice harboring an in-frame deletion corresponding to the human ATM 7636del9 common mutation exhibit a variant phenotype*. Cancer Res, 2001. **61**(11): p. 4561-8.
2. Stingl, J., J.T. Emerman, and C.J. Eaves, *Enzymatic dissociation and culture of normal human mammary tissue to detect progenitor activity*. Methods Mol Biol, 2005. **290**: p. 249-63.
3. Dontu, G., et al., *In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells*. Genes Dev, 2003. **17**(10): p. 1253-70.
4. Lee, G.Y., et al., *Three-dimensional culture models of normal and malignant breast epithelial cells*. Nat Methods, 2007. **4**(4): p. 359-65.

CHAPTER 3: DEVELOPMENT OF THE METHODLOGY TO ISOLATE MAMMARY STEM CELLS

Stem cells are generally defined by their multilineage capabilities and self-renewal capacity [1]. In the mammary gland, stem cells differentiate into the ductal epithelial, alveolar epithelial and myoepithelial cells that encompass the mammary gland structure [2]. Isolation and purification techniques are relatively new for mammary stem cells. Compelling evidence to support the existence of mammary stem cells was first demonstrated through *in vivo* mouse mammary gland transplantation studies, X-chromosome inactivation studies, and retroviral tagging studies [3-5]. Identification of prospective mammary stem cells has relied on distinguishing stem cells based on their properties that may change once stem cells are removed from the tissue and their particular niche. Despite recent advancements, a lack of definitive markers has limited proper characterization of mammary stem cells and ultimately, elucidating their putative role in carcinogenesis.

Detection of mouse mammary stem cells has relied on utilizing an *in vivo* model, the mammary gland transplantation assay. Transplantation studies have demonstrated that fragments of the mammary gland transplanted into a recipient mouse generate a fully functional mammary gland in which it is clonal in origin [3, 5, 6]. This assay was further improved by dissociating the mammary gland into single cells at limiting dilution before transplantation [7, 8]. This *in vivo* model is the most definitive assay to detect mammary stem cells based on their ability to generate a mammary outgrowth following transplantation, but lacks high-throughput capabilities and is not cost effective [9, 10]. The absence of a suitable model to analyze mammary stem cells and their progeny *in*

vitro has led to the development of a cell culture system to address investigating the properties of mammary stem cells that have evaded us in the *in vivo* model (transplantation assay) [2, 11]. The development of an *in vitro* cell culture model has facilitated the characterization of mammary stem cells derived from both human and rodent systems [12-15]. The ability to culture and maintain stem cells as spheroids was first shown with neural stem cells in which clusters formed that consisted of stem and progenitor cells in various stages of differentiation [16]. This approach was expanded to mammary stem cells, which formed similar 3-D structures in culture, known as mammospheres [11]. Mammospheres have been shown to contain stem and progenitor cells that differentiate into all the cell lineages in response to growth stimulus and produce a mammary tree structure similar to *in vivo* [11, 14]. Furthermore, mammospheres retain the ability to self-renew in cell culture [11]. Mammospheres are considered a biologically relevant *in vitro* system that closely mimics the mammary gland *in vivo* [14]. The ability to examine the behavior of mammary stem cells will provide a better understanding of how stem cells function in response to known breast cancer carcinogens (e.g. ionizing radiation) and their role in carcinogenic process.

Propagation of mammospheres has proven to be a powerful tool in the characterization of mammary stem cells and their progeny [17-19]. However, mammospheres are relatively new to the research field of stem cell biology and have yet to be fully utilized to explore numerous *in vitro* functional endpoints. Although the basic method to culture and maintain mammary stem cells (as mammospheres) has been established, the ability to fully expand this system for alternative endpoints such as radiation cell survival has not been explored. Unlike other cell types, the use of mammary

stem cells required establishing a foundation to optimize the isolation, establishment, and *in vitro* propagation of mammospheres. For our purposes, this entailed developing a methodology (i.e. isolation and cell culture protocols) that would allow us to utilize this system for future studies and to examine the radiation response of mouse mammary stem cells as mammospheres.

We first developed an isolation protocol that has been previously described in the literature with modifications [20]. Primary mammary epithelial cell isolation required removing the mammary glands from 8- to 12- week old virgin female mice and enzymatically dissociating the tissue. Single cell suspensions were obtained from dissociated mammary organoids and plated at high cell density ($1.5 - 3 \times 10^6$ cells per 60 mm plate). Enzymatic dissociation of the mammary glands resulted in a mixed population of stem, progenitor, and differentiated cells (epithelial and non-epithelial cells). Plating mammary epithelial cells directly after isolation required identifying cell sizes that belonged to the epithelial cell population rather than the non-epithelial cell population. Unlike other cell types, the cell size for mammary stem and progenitor cells has not been previously reported in the literature. Determining which cell sizes to count was needed to demonstrate consistent mammosphere formation efficiency (MFE) that could be affected by contaminating non-epithelial cells. Cell counts required identifying which particular cell sizes belonged to the stem, and progenitor cell population that appeared after enzymatic dissociation. Cell counts were first obtained using the Coulter counter and included cell size peaks observed at 4 μm and 6 μm. Similar cell counts were obtained using a hemacytometer. The cell sizes appeared to be consistent with previous studies using epithelial cells that are isolated in a similar manner, however, at

this point it was difficult to pinpoint the actual cell size of stem and progenitors cells since they were derived from a heterogeneous population. We aimed to develop an isolation protocol to culture mammospheres.

Mammospheres require strict cell culture conditions and media supplements to remain in an undifferentiated state and retain their replicative potential. Cells that were unable to adapt to the specific conditions set forth were selectively excluded and resulted in an enriched population of stem and progenitor cells. Mammosphere culture conditions were adapted as previously described with modifications [11]. Single cell suspensions were initially plated at high cell densities ($1.5 - 3 \times 10^6$ cells per plate) to ensure cell growth and viability. Utilizing mammospheres required devising unique guidelines to maintain the structural integrity of the mammospheres as they proliferated. Simply changing the media entailed determining the temperature, time, and centrifugation speed to maintain the mammosphere and allow the removal of unwanted dead cells. Furthermore, it was important to continually propagate mammospheres for further studies. Passaging mammospheres involved gently dissociating the spheroid using a fire polished Pasteur pipette to remove the cell layers and achieve single cell suspensions. Specific dissociation times and reagent concentrations were determined to ensure cell viability after subsequent passages. Therefore, new protocols (detailed in Chapter 2: Materials and Methods) were developed to ensure the maintenance and propagation of mammospheres in culture.

Radiation cell survival studies require plating at clonal densities to properly assess cell viability after irradiation. Previous studies reported plating 1000 cells per mL to achieve clonal densities [11]. However, this approach resulted in few mammospheres (2-

5) per plate and at least 50 to 100 mammospheres were needed for radiation cell survival studies. Depletion of non-epithelial cells from the mixed population produced an enriched population of mammary stem and progenitor cells. Single cell suspensions were then plated at a lower cell density (5000 cells) to yield clonal mammospheres. Clonal mammospheres were unable to proliferate without additional growth factors from other cells. Conditioned mammosphere media was obtained from high-density cell culture plates and combined with fresh media at 1:1 to ensure growth of clonal mammospheres. For our purposes, we modified the mammosphere culture technique in order to meet the requirements for the radiation cell survival assay.

Here we describe the development of a methodology to isolate and propagate mouse mammary stem cells. This involved establishing new cell culture techniques for further research applications. Using a modified mammosphere culture technique, we established basic cell culture protocols to properly characterize mammary stem cells *in vitro*. The aim of this study is to characterize mouse mammary stem cells and their radiation response as mammospheres.

LITERATURE CITED

1. Blanpain, C., V. Horsley, and E. Fuchs, *Epithelial stem cells: turning over new leaves.* Cell, 2007. **128**(3): p. 445-58.
2. Smith, G.H. and G. Chepko, *Mammary epithelial stem cells.* Microsc Res Tech, 2001. **52**(2): p. 190-203.
3. Kordon, E.C. and G.H. Smith, *An entire functional mammary gland may comprise the progeny from a single cell.* Development, 1998. **125**(10): p. 1921-30.
4. Tsai, Y.C., et al., *Contiguous patches of normal human mammary epithelium derived from a single stem cell: implications for breast carcinogenesis.* Cancer Res, 1996. **56**(2): p. 402-4.
5. Deome, K.B., et al., *Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice.* Cancer Res, 1959. **19**(5): p. 515-20.
6. Smith, G.H., *Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype.* Breast Cancer Res Treat, 1996. **39**(1): p. 21-31.
7. DeOme, K.B., et al., *Detection of inapparent nodule-transformed cells in the mammary gland tissues of virgin female BALB/cfC3H mice.* Cancer Res, 1978. **38**(7): p. 2103-11.
8. Smith, G.H. and D. Medina, *A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland.* J Cell Sci, 1988. **90 (Pt 1)**: p. 173-83.
9. Stingl, J., *Detection and analysis of mammary gland stem cells.* J Pathol, 2009. **217**(2): p. 229-41.
10. Stingl, J., et al., *Epithelial progenitors in the normal human mammary gland.* J Mammary Gland Biol Neoplasia, 2005. **10**(1): p. 49-59.
11. Dontu, G., et al., *In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells.* Genes Dev, 2003. **17**(10): p. 1253-70.
12. Liu, S., et al., *Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells.* Cancer Res, 2006. **66**(12): p. 6063-71.
13. Dontu, G., et al., *Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells.* Breast Cancer Res, 2004. **6**(6): p. R605-15.
14. Liao, M.J., et al., *Enrichment of a population of mammary gland cells that form mammospheres and have in vivo repopulating activity.* Cancer Res, 2007. **67**(17): p. 8131-8.
15. Matulka, L.A., A.A. Triplett, and K.U. Wagner, *Parity-induced mammary epithelial cells are multipotent and express cell surface markers associated with stem cells.* Dev Biol, 2007. **303**(1): p. 29-44.
16. Reynolds, B.A. and S. Weiss, *Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell.* Dev Biol, 1996. **175**(1): p. 1-13.
17. Grimshaw, M.J., et al., *Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells.* Breast Cancer Res, 2008. **10**(3): p. R52.

18. Ponti, D., et al., *Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties.* Cancer Res, 2005. **65**(13): p. 5506-11.
19. Farnie, G., et al., *Novel cell culture technique for primary ductal carcinoma in situ: role of Notch and epidermal growth factor receptor signaling pathways.* J Natl Cancer Inst, 2007. **99**(8): p. 616-27.
20. Stingl, J., J.T. Emerman, and C.J. Eaves, *Enzymatic dissociation and culture of normal human mammary tissue to detect progenitor activity.* Methods Mol Biol, 2005. **290**: p. 249-63.

CHAPTER 4: *IN VITRO* CHARACTERIZATION OF MAMMARY STEM CELLS AS MAMMOSPHERES TO ESTABLISH NOVEL SIZE CRITERIA

Utilizing mammospheres as an *in vitro* model for the study of mammary stem cells provides the opportunity to characterize a variety of endpoints using an enriched population that includes stem, progenitor and differentiated cells. The ability to culture and maintain mammary stem cells in an undifferentiated state as "nonadherent mammospheres" has been recently developed and some of the properties of mammospheres such as the cell lineage composition, self-renewal capacity and differentiation potential have been previously characterized [1]. However, mammospheres have yet to be fully utilized to explore *in vitro* functional endpoints. Unlike other cell types, use of mammary stem cells required the development of novel methods to assess growth kinetics and viability, which had not previously been explored. For our purposes, this entailed modifying the mammosphere cell culture technique to apply this model for the investigation of a variety of radiation biology endpoints such as cell survival, radiation-induced cytogenetic damage, and genomic instability.

Mammospheres have served as an important *in vitro* tool to study the properties of mammary stem cells derived from both human and rodent systems [2-5]. Mammospheres are highly enriched with progenitor cells capable of differentiation into multiple lineages and producing a mammary tree structure similar to *in vivo* [1, 2]. It is clear that mammospheres are a biologically relevant *in vitro* system and an alternative to *in vivo* assays in that mammospheres facilitate characterizing the properties of mammary stem cells [2, 4]. We have chosen to utilize mammospheres to characterize the radiation response of mammary stem cells as assessed by the cell survival assay. Most of the studies that have used mammospheres were limited in their application and choose only

to examine mammosphere formation efficiency and *in vitro* propagation after several passages as a property to distinguish stem cells, but not as an endpoint in the study [5, 6]. Assessing the viability of mammospheres after ionizing radiation (IR) entailed developing a unique methodology. The criteria used in standard clonogenic assays require scoring colonies of ≥50 cells to distinguish surviving cells with replicative capacity for radiation cell survival. However, due to the 3-D structure of mammospheres it was difficult to determine cell number; this required characterizing the mammospheres to properly assess their sensitivity to ionizing radiation. We developed novel size criteria to identify primary mammospheres containing true stem cells. Suspension cultures revealed variation in mammosphere sizes that has not been previously reported by other studies. This led us to question whether every mammosphere contains a stem cell. We hypothesized that determining the cell lineage composition of individual mammospheres should reveal which sizes contain a stem cell. Furthermore, the mammospheres that originated from a stem cell also have self-renewal capacity in which serial passaging would reveal their sphere formation capabilities. To our knowledge, this is the first demonstration of using size criteria to analyze the radiation response of mammary stem cells as mammospheres.

Here we describe the characterization of murine mammary epithelial stem cells and the establishment of novel criteria to assess the radiation response of mammary stem cells (as mammospheres). Endpoints such as mammosphere formation efficiency in suspension and 3-D matrix, lineage composition and serial passaging of individual mammospheres were ascertained to provide additional insight into the behavior of stem cells *in vitro*. We modified the mammosphere culture technique and developed a

methodology that would further expand the use of this *in vitro* system. Our results demonstrate the feasibility of using mammospheres to examine specific radiation biology endpoints such as cell survival and radiation-induced cytogenetic damage. The initial characterization and novel criteria will allow us to analyze the growth kinetics and viability of stem cells and their progeny after exposure to ionizing radiation.

RESULTS

Mammosphere Formation Efficiency (MFE)

We used primary mammary epithelial single cell suspensions to observe the plating efficiency in basement membrane extract (BME). A range of cell numbers (20,000-200,000 cells) was plated. Mammospheres began forming within 4-6 days, but were scored on day 8. The mammosphere formation efficiency was 0.1% ± 0.0005. Mammospheres were categorized according to their diameter (≤ 99, 100-150, 151-200, 201-250, ≥ 251 μm). Mammospheres with a diameter of ≤150 μm were observed much more frequently than mammospheres belonging to the larger size groups (Figure 4.1). Plating number did not appear to affect the size distribution of the mammospheres. We observed similar size distribution of mammospheres in both BME and cell culture suspensions.

It is possible that aggregation of cells could result in mammosphere formation in the absence of a stem and/or progenitor cell. It has been suggested that mammosphere formation involves cell aggregation and plating at low cell densities can demonstrate mammospheres that are clonal in origin [7]. To determine whether various sizes of

mammospheres were a consequence of cell aggregation, single cell suspensions were enriched for stem and progenitor cells after primary mammary epithelial cell isolation and plated at low cell density (500 cells/mL) in 60mm plates to yield clonal mammospheres [1]. Non-epithelial and hematopoietic cells were depleted from the heterogeneous population obtained through enzymatic dissociation of the mammary gland to increase the plating efficiency using an EasySep® Negative Selection kit. The frequency of mammosphere sizes observed in these clonal cultures was similar to non-clonal mammospheres grown in suspension and BME. The MFE was 1-2% for single cell suspensions plated at clonal density after negative selection for all mouse strains used in this study.

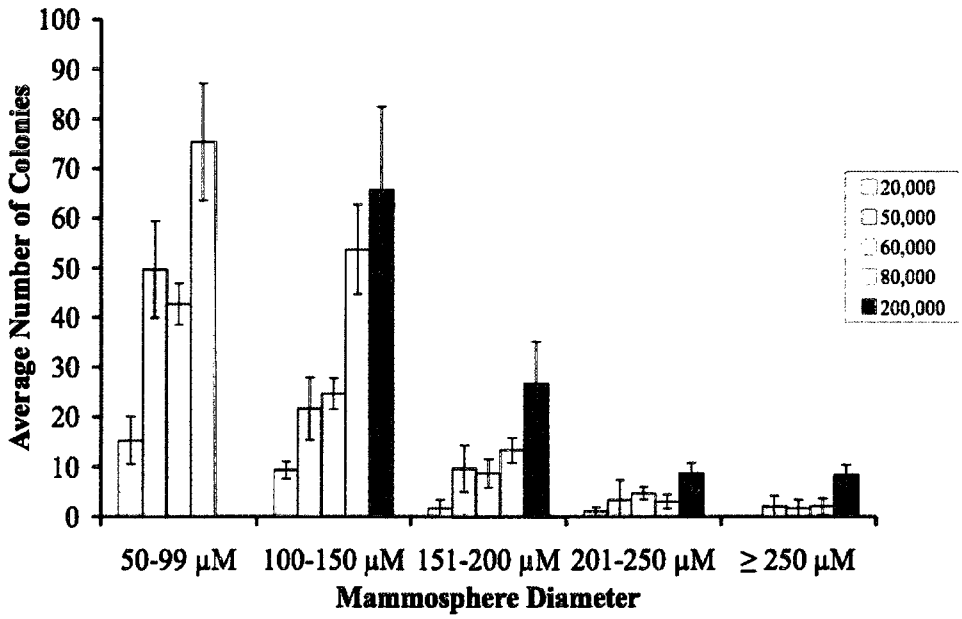

Figure 4.1: Size distribution of mammospheres grown in BME. 20,000-200,000 cells were plated in individual wells of a 24-well plate in triplicate. Mammospheres were categorized based on diameter size and scored on day 8. The error bars represent the standard deviation from the mean.

Development of novel criteria

Cell culture suspensions revealed variation in mammosphere size that has not been previously reported in the literature. However, it has been shown that not every mammosphere contains a true stem cell [1]. To investigate which mammospheres originated from a true stem cell, we assessed the lineage composition of mammospheres using indirect immunofluorescence to identify specific luminal and myoepithelial cell lineage markers and self-renewal capacity via serial passaging. Mammospheres were divided into five groups according to their diameter (≤ 99, 100-150, 151-200, 201-250, ≥251 μm) (Figure 4.2a-d). Twenty mammospheres for each size group were

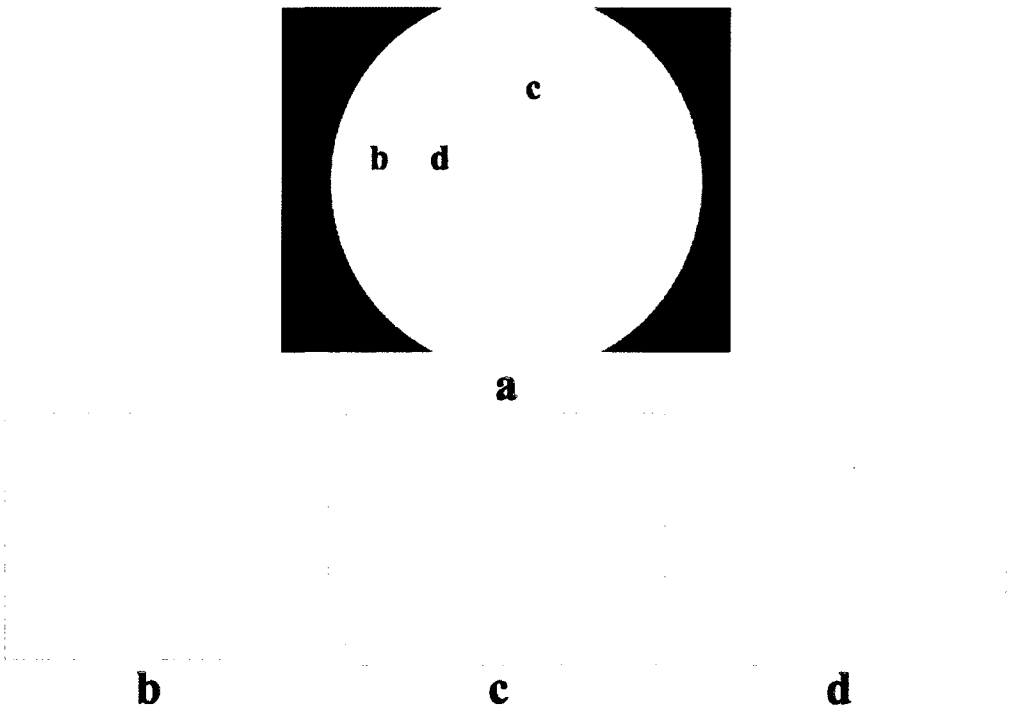

Figure 4.2a-d: Mammospheres in cell culture suspension at day eight. All images taken from the same cell culture suspension at 4X and b) - d) 4X magnification. a) Various sizes b) 116 μm c) 155 μm d) 213 μm

subsequently serially passaged to the fifth generation. Mammospheres for the 151-200 mm size group were contaminated and thus, excluded. The two larger size groups (201-250 and ≥ 251 μm) were able to form several mammospheres after each passaging. This indicates that these mammospheres contain sphere-initiating cells that have self-renewal capabilities. After two to three passages, the two smaller size groups (≤ 99 and 100-150 μm) were not able to form many mammospheres, however, a few were able to survive to the fifth generation. Individual mammospheres in each size group were also passaged to second generation and only the three larger size groups (151-200, 201-250, ≥ 251 μm) were able to form mammospheres.

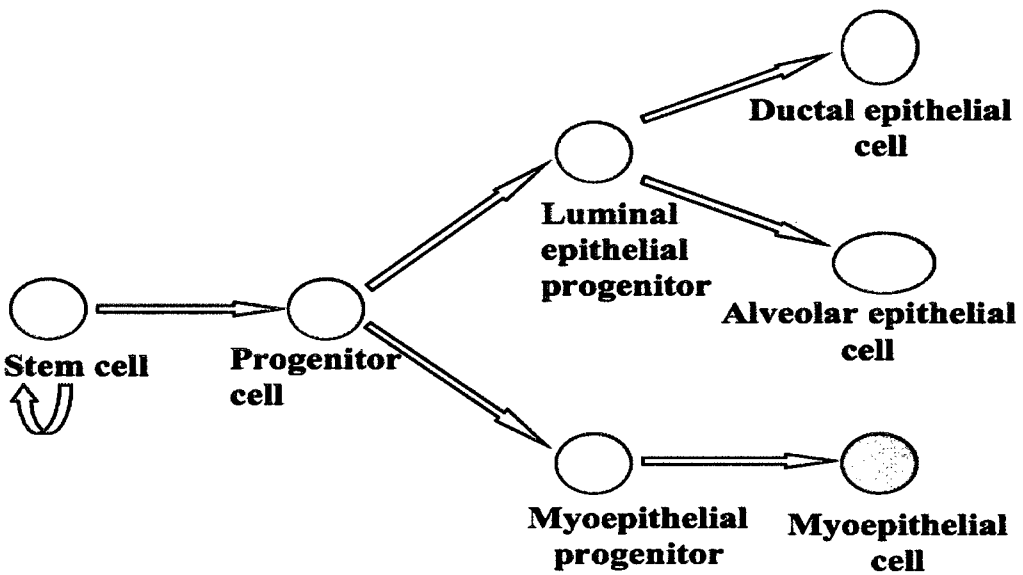

Figure 4.3: Model of mammary stem cell hierarchy. (Adapted from Stingl *et al.*, J Mammary Gland Biol. Neoplasia, 2005 and Visvader & Lindeman, Cancer Research, 2006)

Luminal epithelial and myoepithelial progenitors are derived from a common stem or progenitor cell, but how many distinct progenitors exist within the stem cell hierarchy is unknown (Figure 4.3) [8, 9]. If this stem cell hierarchy model is correct,

mammospheres that originated from a true stem cell should be composed of both luminal and myoepithelial cells, while mammospheres originating from committed progenitors or differentiated cells should be composed of either luminal or myoepithelial cells. To investigate the lineage composition of mammospheres, we used lineage-specific markers to identify luminal and myoepithelial cells. Lineage-specific markers used to identify luminal and myoepithelial cells were Cytokeratin 18 (K18) and α-Smooth Muscle Actin (SMA) respectively [1, 10, 11]. Twenty primary mammospheres were picked and dissociated for each size group except ≥250 μm, which were limited in number. Each size group expressed both luminal and myoepithelial cell lineages (Figure 4.4a-d), indicating that these mammospheres originated from a cell with multilineage capabilities (i.e. stem cell).

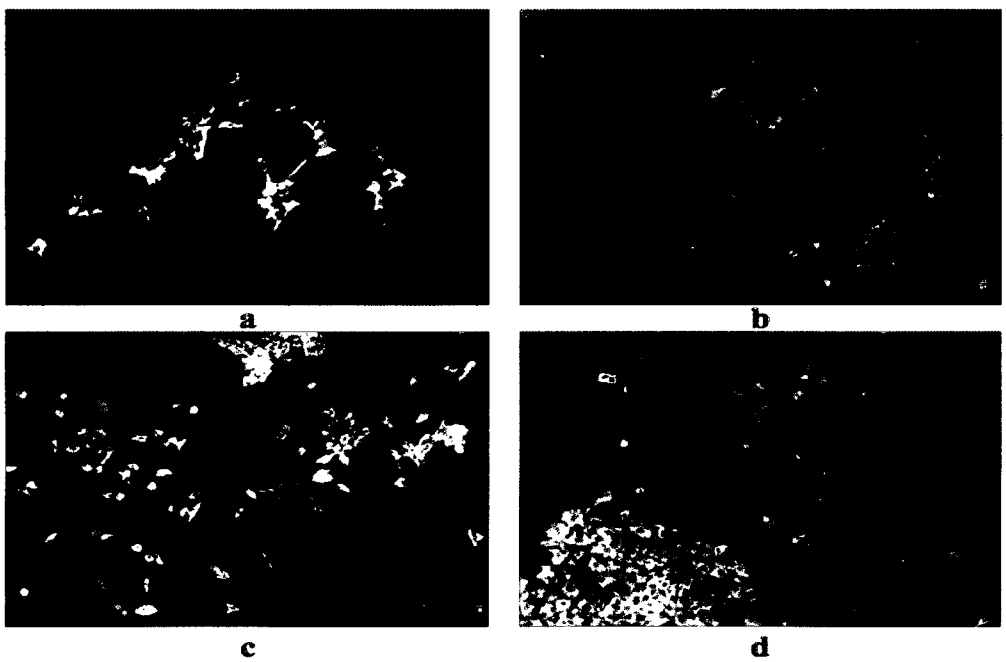

Figure 4.4a-d: Immunofluorescence images of primary mammospheres picked and dissociated for each size group (≥ 251 μm excluded). Luminal epithelial staining (K18) is displayed in red, myoepithelial (SMA) in green and nuclei in blue. a. ≤ 99 μm b. 100-150 μm c. 151-200 μm d. 201-250 μm. Results indicate all size groups originated from a cell that can produce luminal epithelial and myoepithelial cell types (based on positive staining for K18 and SMA).

DISCUSSION AND FUTURE DIRECTIONS

Using our modified mammosphere cell culture technique, we have demonstrated that the mammosphere formation efficiency (MFE) is similar to other published results, which estimate the number of stem cell is ~1 in 1,000 [2, 12, 13]. The MFE for non-clonal mammospheres was 0.1% ± 0.0005. For this study, we aimed to utilize mammospheres as a means to examine the radiation response of mammary stem cells; which required determining the MFE. Radiation cell survival studies require plating at clonal densities to properly assess cell viability after irradiation. Previous studies reported plating 1000 cells per mL to achieve clonal densities [1]. However, this approach resulted in very few mammospheres (2-5) per plate and at least 50 to 100 mammospheres were needed for radiation cell survival studies. The MFE obtained for clonal mammospheres was 1-2% after negative selection enrichment procedure, which according to the manufacturer's claims should result in ~90% enrichment. Depletion of non-epithelial cells through lineage specific-negative selection increases the MFE and the feasibility of examining radiation cell survival of mammospheres.

Previous studies utilizing mammospheres derived from humans and mice have failed to report the size variation observed in culture [1, 2, 14]. We do not know if this phenomenon is a result of using murine mammary stem cells, differences in cell culture technique and conditions, or plating density. We observed mammosphere size variation in cell culture suspension and in basement membrane extract. According to our size groups, the two smaller size groups (≤99 and 100-150 μm) appeared much more frequently when compared to the other size groups. We believe that size variation of the mammospheres indicates that not all spheres represent the progeny of stem cells. Studies

have shown transplantation of individual mammospheres into a clear fat pad of a recipient mouse resulted in only 15% engraftment success [15].

Our results also demonstrated that plating number did not affect the size distribution observed, which suggests that cell aggregation does not account for the difference in mammosphere size. To confirm this finding, single cell suspensions were depleted of non-epithelial cells through lineage specific-negative selection and plated at low cell density to achieve clonal mammospheres. The frequency of mammosphere sizes observed was similar to non-clonal mammospheres grown in suspension and BME. These results corroborate our finding that the size variation is not a result of cell aggregation. We suspect that the size variation is a result of mammospheres produced by stem and progenitor cells at different stages of differentiation. It is clear from previous studies that not every mammosphere contains a stem cell; but progenitor cells can also share this self-renewal and multilineage capability at limited capacity [1]. We hypothesize that progenitor cells have reduced replicative capacity compared to true stem cells. Primary mammary epithelial isolation produces a heterogeneous population of cells in which the stem cells represent a small percentage of the total population (1 in 1,000). If only a limited number of cells are plated (e.g. to achieve clonal densities), this may account for the random appearance of the size variation and may lead others to believe this phenomenon is due to cell aggregation.

Based on our results of the size variation observed, we developed novel size criteria to distinguish mammospheres that originated from a stem cell. Results showed that only the three larger size groups (151-200, 201-250, ≥251 μm) were capable of self-renewal capacity. These same size groups were comprised of both luminal and

myoepithelial cells. Although the two smaller size groups (≤99 and 100-150 mm) were able to form mammospheres for several passages, we believe mammosphere formation was due to cell aggregation as these spheres were small in size and did not appear to proliferate over time. Cell aggregation for the smaller sizes could have played a role in the positive staining for both luminal epithelial and myoepithelial cell lineages. We suspect that the positive staining of both cell lineages for all size groups was likely attributed to examining a mixed population of mammospheres whereas individual mammospheres contain clonally derived cells. It is highly unlikely that the two smaller size groups contain a stem/progenitor cell based their high frequency in suspension cultures and the assumption that stem cells represent 1 in 1000 cells.

Future studies should strengthen the criteria by assessing the self-renewal capability and cell lineage composition of primary clonal mammospheres (Figure 4.5). Examining clonally derived cells should confirm whether mammospheres originating from a stem/progenitor cells are distinguishable by size. We hypothesize that mammary stem cells are a target of radiation-induced damage. The criteria developed in this study will be vital for characterizing the radiation response of mammary stem cells and their progeny (as mammospheres) via cell survival assay.

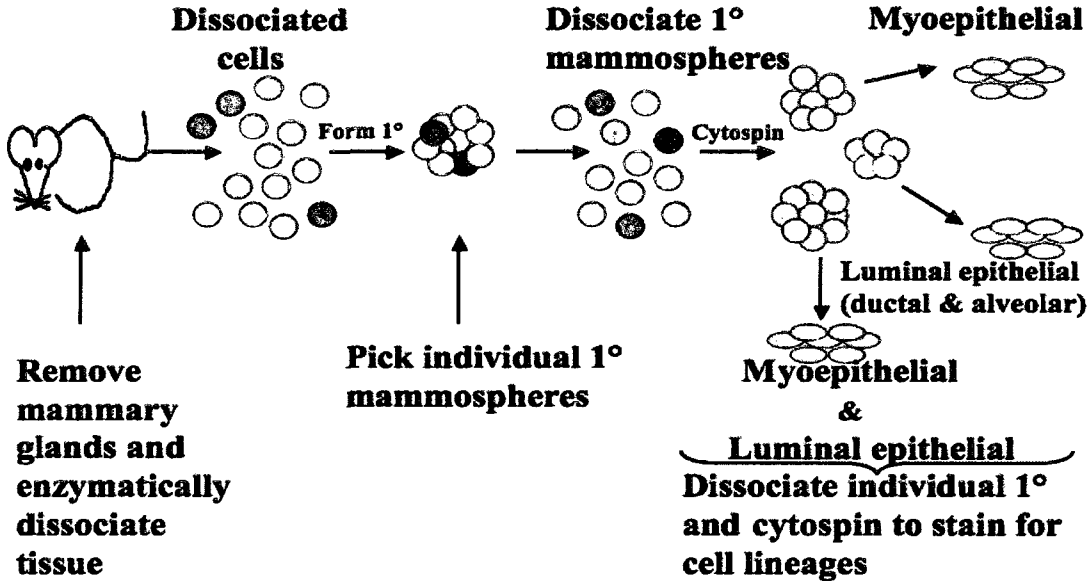

Figure 4.5: Experimental design to assess lineage composition of individual clonal mammospheres to establish size criteria.

LITERATURE CITED

1. Dontu, G., et al., *In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells.* Genes Dev, 2003. **17**(10): p. 1253-70.
2. Liao, M.J., et al., *Enrichment of a population of mammary gland cells that form mammospheres and have in vivo repopulating activity.* Cancer Res, 2007. **67**(17): p. 8131-8.
3. Dontu, G., et al., *Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells.* Breast Cancer Res, 2004. **6**(6): p. R605-15.
4. Liu, S., et al., *Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells.* Cancer Res, 2006. **66**(12): p. 6063-71.
5. Matulka, L.A., A.A. Triplett, and K.U. Wagner, *Parity-induced mammary epithelial cells are multipotent and express cell surface markers associated with stem cells.* Dev Biol, 2007. **303**(1): p. 29-44.
6. Farnie, G., et al., *Novel cell culture technique for primary ductal carcinoma in situ: role of Notch and epidermal growth factor receptor signaling pathways.* J Natl Cancer Inst, 2007. **99**(8): p. 616-27.
7. Stingl, J., *Detection and analysis of mammary gland stem cells.* J Pathol, 2009. **217**(2): p. 229-41.
8. Stingl, J., et al., *Epithelial progenitors in the normal human mammary gland.* J Mammary Gland Biol Neoplasia, 2005. **10**(1): p. 49-59.
9. Visvader, J.E. and G.J. Lindeman, *Mammary stem cells and mammopoiesis.* Cancer Res, 2006. **66**(20): p. 9798-801.
10. Pechoux, C., et al., *Human mammary luminal epithelial cells contain progenitors to myoepithelial cells.* Dev Biol, 1999. **206**(1): p. 88-99.
11. Asselin-Labat, M.L., et al., *Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation.* Nat Cell Biol, 2007. **9**(2): p. 201-9.
12. Stingl, J., et al., *Purification and unique properties of mammary epithelial stem cells.* Nature, 2006. **439**(7079): p. 993-7.
13. Shackleton, M., et al., *Generation of a functional mammary gland from a single stem cell.* Nature, 2006. **439**(7072): p. 84-8.
14. Chen, M.S., et al., *Wnt/beta-catenin mediates radiation resistance of Sca1+ progenitors in an immortalized mammary gland cell line.* J Cell Sci, 2007. **120**(Pt 3): p. 468-77.
15. Moraes, R.C., et al., *Constitutive activation of smoothened (SMO) in mammary glands of transgenic mice leads to increased proliferation, altered differentiation and ductal dysplasia.* Development, 2007. **134**(6): p. 1231-42.

CHAPTER 5: RADIATION RESPONSE OF MAMMARY STEM CELLS DERIVED FROM *ATM*-ΔSRI HETEROZYGOUS MICE

It has been hypothesized that stem cells derived from genetically susceptible subpopulations may be predisposed to transformation and could account for the large majority of spontaneous cancer cases [1, 2]. This holds important implications for assessing risk and minimizing exposure for individuals susceptible to specific carcinogens (e.g. ionizing radiation). Individuals afflicted with the rare autosomal recessive disorder ataxia-telangiectasia (A-T) have heightened sensitivity to ionizing radiation (IR) and increased predisposition to cancer [3]. However, the prevalence of A-T among the general population is very low [4]. The frequency of A-T carriers is estimated to be at least 1% [5, 6]. Exposure to IR could greatly impact and heighten the risk for *ATM* heterozygotes. Increased risk for radiation-induced breast cancer might be attributed to the susceptibility of stem cells (derived from A-T carriers) to radiation-induced damage.

Recent developmentments in breast cancer research have suggested that tissue stem cells are a plausible target for carcinogenesis [7-9]. The involvement of mammary stem cells (e.g. transformation) could explain the heterogeneity and molecular complexity of breast cancer. The etiology of breast cancer suggests increased susceptibility is associated with the replicative role of mammary stem cells to maintain the breast tissue during development, which predisposes them to carcinogenesis. Stem cells in breast tissue may be sensitive to known breast carcinogens (i.e. ionizing radiation) that impact their susceptibility. However, the underlying mechanisms of such targets have yet to be fully elucidated. We hypothesize that mammary stem cells are a target of radiation-induced damage and play a role in the initiation of radiation-induced mammary cancer.

There is considerable evidence to support *ATM* heterozygotes having an increased risk for breast cancer that is conferred by the *ATM* mutation they carry [10, 11]. A-T carriers are suspected to be susceptible to radiation-induced breast carcinogenesis in which they display a cellular phenotype of intermediate sensitivity to IR [12, 13]. Mammary stem cells may represent an important cellular target for radiation-induced damage in *ATM* heterozygotes.

It has been difficult to elucidate the impact of breast cancer-associated ATM mutations in heterozygous individuals. There are two distinct classes of *ATM* mutations (truncating and missense) that have been identified in A-T and breast cancer patients [14]. Mouse models of A-T have attempted to discern the discrepancies between these two *ATM* mutations. *Atm* heterozygous mice (*Atm* $^{+/-}$) have been shown to develop mammary tumors at a low frequency as a consequence of carrying a truncating *ATM* mutation[15-18]. Another mouse model was generated that carries a common human *ATM* missense mutation (7636del9) known as *Atm*-ΔSRI [19]. *Atm*-ΔSRI heterozygous mice develop mammary tumors, but at a low incidence (2%) [18]. The cell survival assay was used to determine the effect of radiation on spleen cells for wildtype (*Atm* $^{(+/+)}$), *Atm*-ΔSRI heterozygous, and homozygous mice. Spleen cells derived from *Atm*-ΔSRI heterozygous mice displayed an intermediate phenotype of radiosensitivity between the normal control (wildtype) and *Atm*-ΔSRI homozygous mice [18]. Similar results were observed for human and mouse ΔSRI heterozygotes in which the number of radiation-induced chromosome aberrations per metaphase was intermediate between values for normal controls and ΔSRI homozygotes. However, the radiation response of mammary epithelial cells derived from these heterozygous mice has yet to be elucidated.

We have developed a methodology to isolate and propagate mammary stem cells. We applied the methodology and size criteria developed to analyze the effects of ionizing radiation (IR) on survival of mammary stem cells derived from mice carrying one mutated copy of *Atm*. These studies demonstrate the utility of the methodology and size criteria developed. Utilizing a modified mammosphere technique we examined the radiation sensitivity of mammary stem cells derived from *Atm*-ΔSRI heterozygous mice. The ability to culture and maintain mammary stem cells in an undifferentiated state as mammospheres has been developed and demonstrated that mammospheres retain multilineage capacity to self-renew and differentiate [20]. We characterized the *in vitro* behavior of mammary stem cells and developed novel criteria, which discerned mammospheres originating from a stem cell, to directly assess the radiation response of mammary stem cells and their progeny. We utilized the size criteria developed and hypothesized that mammospheres ≥150 μm contain a stem and/or progenitor cell(s). The results described here are to demonstrate the proof of principle for this model development. Our results demonstrate that mammary stem cells derived from *Atm*-ΔSRI heterozygous mice ($Atm^{(+/\Delta SRI)}$) do not exhibit increased radiation sensitivity compared to their wildtype littermates ($Atm^{(+/+)}$). It appears that mammary stem cells derived from *Atm*-ΔSRI heterozygous mice exhibited increased radiation resistance. The size criteria failed to show differences of radiation sensitivity in mammospheres believed to be derived from a stem cell for both $Atm^{(+/\Delta SRI)}$ and $Atm^{(+/+)}$ mice. This suggests that increased cell killing is not a property of this particular *ATM* mutation in mammary stem cells. Previous studies have reported spleen cells derived from *Atm*-ΔSRI heterozygous mice exhibit intermediate radiation sensitivity compared to controls [18]. However,

radiation sensitivity may depend on the cell type in which *Atm*-ΔSRI mutation confers a different radiation response in other cell types. It is suspected that the *Atm*-ΔSRI mutation confers a dominant-negative effect in heterozygous mice in which the function of ATM after IR is impaired, but not detected using radiation cell survival assays. Future studies would need to examine additional endpoints such as downstream targets of ATM in response to IR, chromosomal instability, and genomic instability via γ-H2AX foci formation. To our knowledge, this is the first study examining the radiation sensitivity of *Atm* heterozygous mice carrying a known missense mutation found in human A-T patients through assessing the radiation response of mammary stem cells as mammospheres.

RESULTS

Cell survival assay

Atm-ΔSRI mutation was maintained on two backgrounds, 129T2/SvEmsJ:C57BL/6J (referred to as a mixed background) and BALB/cByJ mixed background (referred to as BALB/c *Atm*-ΔSRI). Mammospheres derived from *Atm*-ΔSRI heterozygous mice are referred to as *Atm* $^{(+/\Delta SRI)}$ and *Atm*-ΔSRI wildtype littermates are referred to as *Atm* $^{(+/+)}$. Similar abbreviations are used for BALB/c *Atm*-ΔSRI (i.e. BALB/c *Atm* $^{(+/\Delta SRI)}$ and BALB/c *Atm* $^{(+/+)}$).

Single cell suspensions were enriched with stem and progenitor cells after using an EasySep® Mouse Mammary Stem Cell Enrichment Kit (Stem Cell Technologies). Cells were aliquoted and immediately irradiated at 0, 1, 3, 5 Gy at room temperature using a Cesium 137 source. Single cell suspensions were plated at a density of 1500 to

2000 cells (0 and 1 Gy), 3000 cells (3 Gy), and 4000 cells (5 Gy) in triplicate. The cells were plated at low cell density to ensure clonal mammospheres and scored twelve days later. The relative cell survival of mammary stem cells derived from *Atm*-ΔSRI heterozygous mice ($Atm^{(+/\Delta SRI)}$) and their wildtype littermates ($Atm^{(+/+)}$) after exposure to 0, 1, 3, and 5 Gy is illustrated in Figures 5.1-5.4. The cell survival of mammary stem cells was determined by analyzing the formation of clonal mammospheres after irradiation. Clonal mammospheres that were > 60 μm were scored as colonies, measured, and then categorized by their size. The plating efficiency (PE) for each dose was determined by dividing the number of colonies (i.e. mammospheres) by the number of cells plated. To determine the surviving fraction, mammosphere counts were normalized using the PE of the corresponding unirradiated control.

Based on our results described in Chapter 4, we hypothesized that mammospheres <150 mm did not contain stem cells and perhaps these mammospheres represent committed progenitors and differentiated cells. We determined the surviving fractions for these two different cell populations: all mammospheres and mammospheres ≥ 150 μm. We believed that mammospheres ≥ 150 μm represented mammary stem and high progenitor cells. Furthermore, we suspected that mammospheres ≥ 150 μm might exhibit increased radiation sensitivity, which might not be observed within a mixed population of stem, progenitor, committed progenitor and differentiated cells (i.e. all mammospheres).

Mammospheres derived from $Atm^{(+/\Delta SRI)}$ mice exhibited increased radiation resistance as compared to $Atm^{(+/+)}$ mice (Figures 5.3 and 5.4). In addition, comparing the surviving fraction for all mammosphere sizes and mammospheres ≥ 150 μm showed no difference in dose response for both $Atm^{(+/+)}$ and $Atm^{(+/\Delta SRI)}$ mammospheres (Figure 5.1

and 5.2). Mammospheres derived from BALB/c *Atm* $^{(+/\Delta SRI)}$ mice exhibited increased radiation resistance as compared to BALB/c *Atm* $^{(+/+)}$ mice (Figures 5.7 and 5.8). However, comparison of the surviving fraction for mammospheres derived BALB/c *Atm* $^{(+/+)}$ and BALB/c *Atm* $^{(+/\Delta SRI)}$ showed a difference in dose response based on mammosphere size (Figures 5.5 and 5.6). In addition, mammospheres that were ≥150 μm displayed an increased cell killing from 3 to 5 Gy for BALB/c *Atm* $^{(+/\Delta SRI)}$ mice (Figure 5.6). Results were compiled from two independent experiments. Additional experiments may need to confirm this data.

Our results indicate that mammary stem cells derived from *Atm*-ΔSRI and BALB/c *Atm*-ΔSRI heterozygous mice are radioresistant. Our novel criteria did not appear to distinguish differences in radiation sensitivity among the mammosphere size groups for the mouse strains used in this study. Furthermore, higher radiation doses are needed to examine the radioresistance of mammary stem cells.

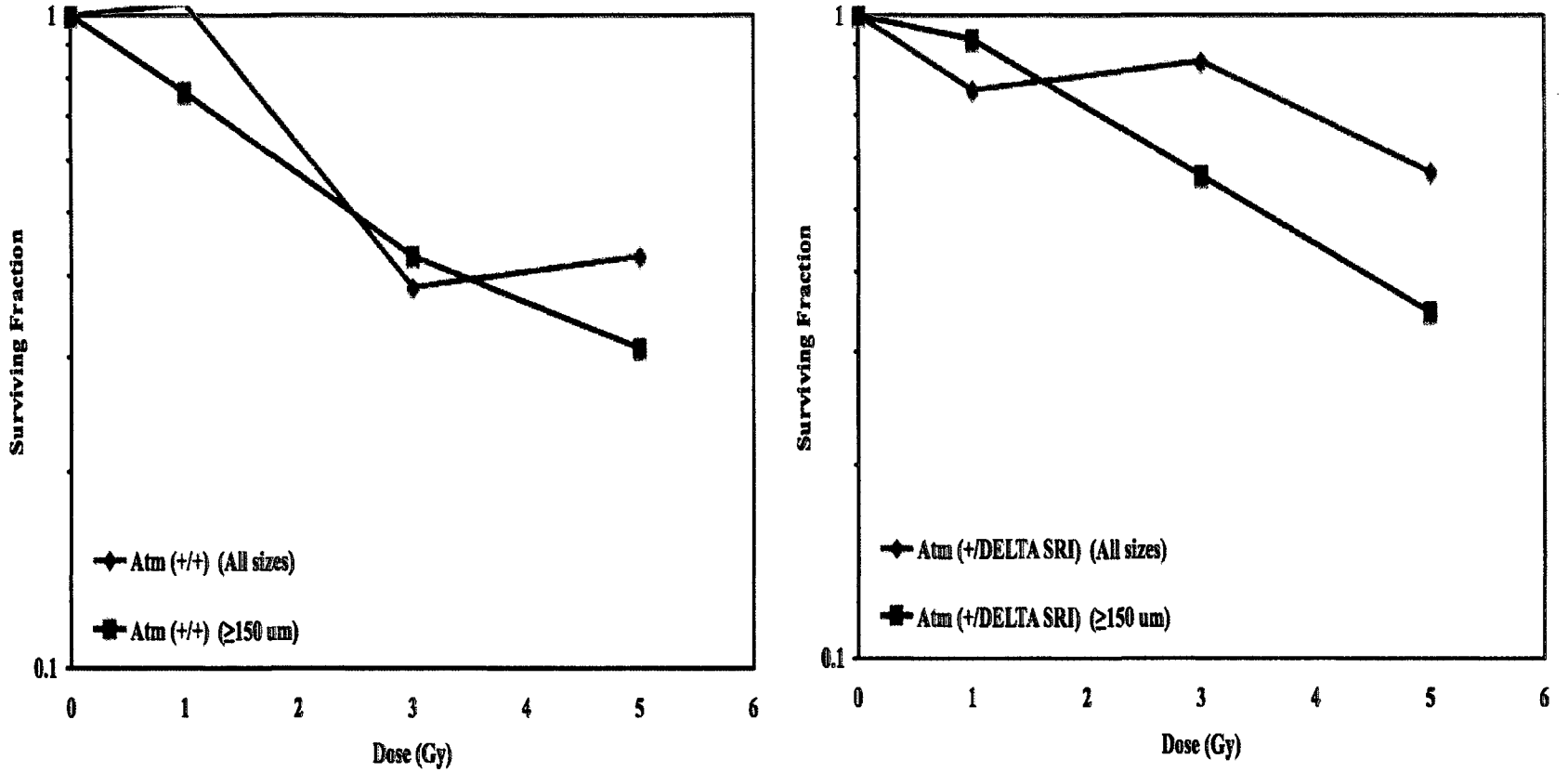

Figure 5.1 and 5.2: Cell Survival for *Atm* $^{(+/+)}$ shown in Figure 5.1 (Left). Cell Survival for *Atm* $^{(+/\Delta SRI)}$ shown in Figure 5.2 (Right). Clonogenic cell survival assay of mammospheres derived from *Atm* $^{(+/\Delta SRI)}$ (*Atm*-ΔSRI heterozygous mice) and *Atm* $^{(+/+)}$ (*Atm*-ΔSRI wildtype littermates). Cells were irradiated as single cell suspensions and plated to form mammospheres. Surviving fraction was determined by normalizing the plating efficiency for each dose to the corresponding unirradiated control. Two surviving fractions were calculated for two different cell populations based on size criteria: all mammospheres and mammospheres ≥ 150 μm. No difference in dose response between all mammosphere sizes and mammospheres ≥ 150 μm for both strains.

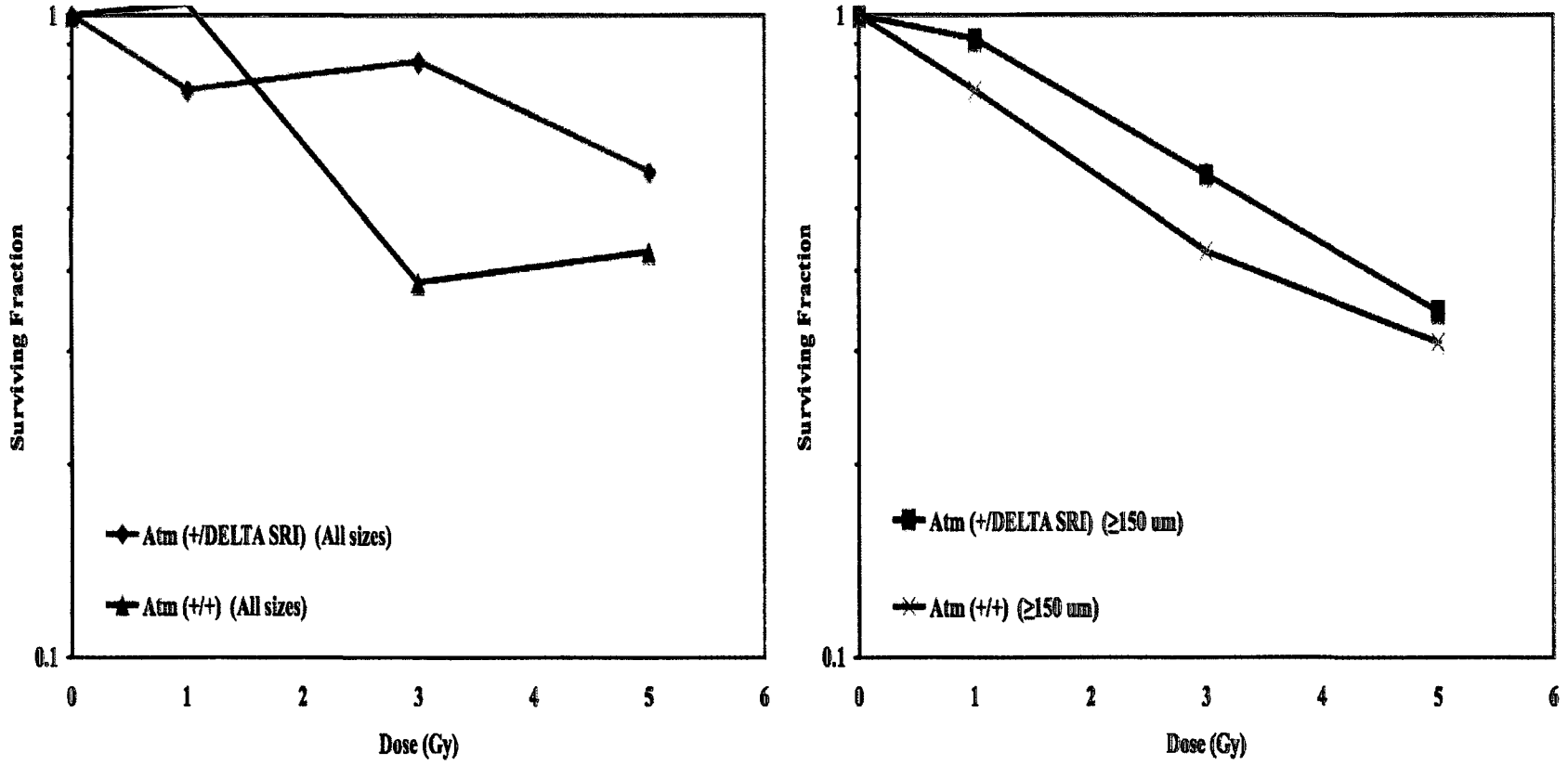

Figure 5.3 and 5.4: Cell Survival comparing all mammosphere sizes derived from *Atm* $^{(+/+)}$ and *Atm* $^{(+/\Delta SRI)}$ shown in Figure 5.3 (Left). Cell Survival comparing all mammosphere ≥ 150 μm derived from *Atm* $^{(+/+)}$ and *Atm* $^{(+/\Delta SRI)}$ shown in Figure 5.4 (Right). Clonogenic cell survival assay of mammospheres derived from *Atm* $^{(+/\Delta SRI)}$ (*Atm*-ΔSRI heterozygous mice) and *Atm* $^{(+/+)}$ (*Atm*-ΔSRI wildtype littermates). Cells were irradiated as single cell suspensions and plated to form mammospheres. Surviving fraction was determined by normalizing the plating efficiency for each dose to the corresponding unirradiated control. Two surviving fractions were calculated for two different cell populations based on size criteria: all mammospheres and mammospheres ≥ 150 μm.

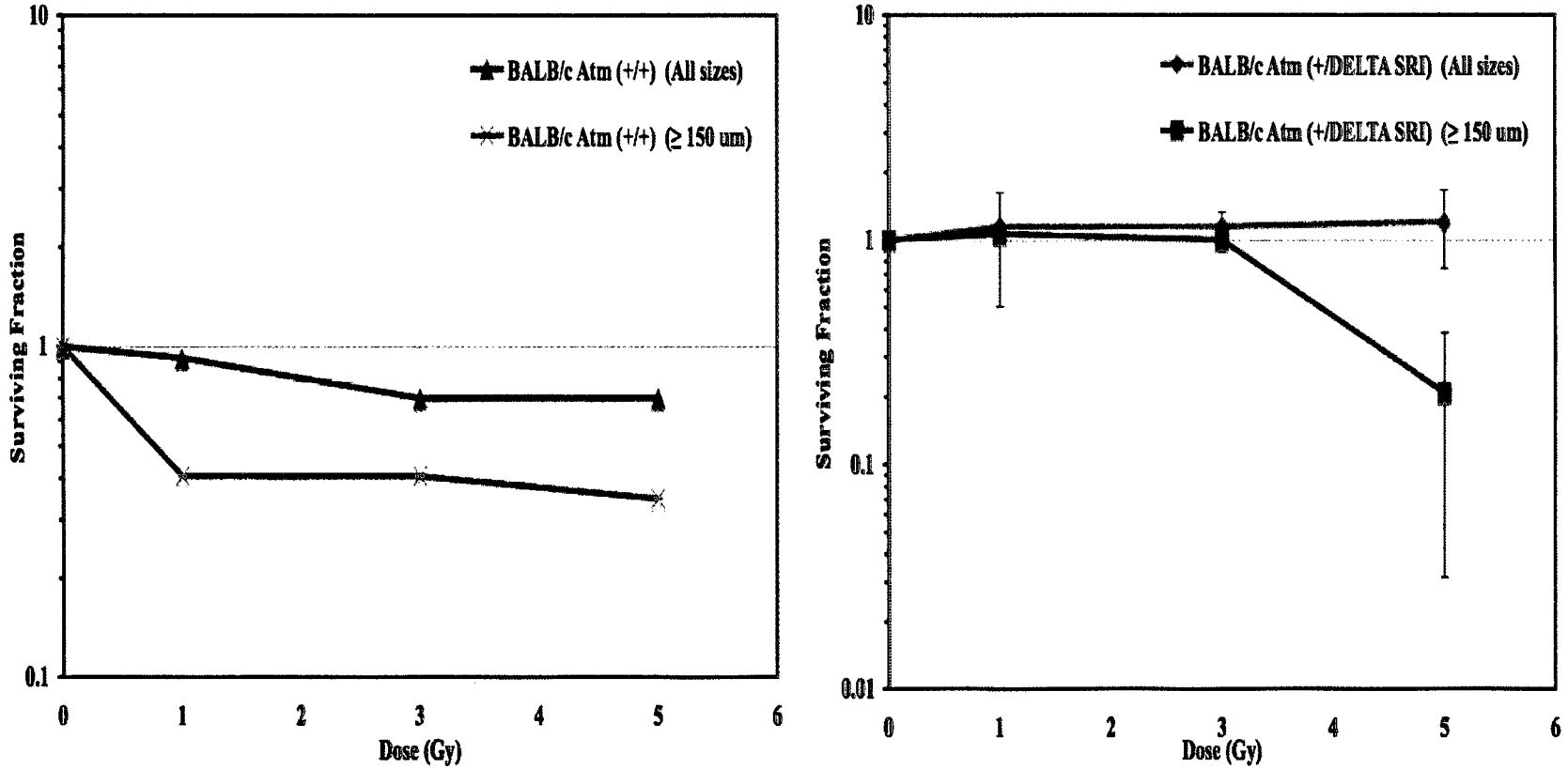

Figure 5.5 and 5.6: Cell Survival for BALB/c *Atm* $^{(+/+)}$ shown in Figure 5.5 (Left). Cell Survival for BALB/c *Atm* $^{(+/\Delta SRI)}$ shown in Figure 5.6 (Right). Clonogenic cell survival assay of mammospheres derived from BALB/c *Atm* $^{(+/\Delta SRI)}$ (BALB/c *Atm*-ΔSRI heterozygous mice) and BALB/c *Atm* $^{(+/+)}$ (BALB/c *Atm*-ΔSRI wildtype littermates). Cells were irradiated as single cell suspensions and plated to form mammospheres. Surviving fraction was determined by normalizing the plating efficiency for each dose to the corresponding unirradiated control. Two surviving fractions were calculated for two different cell populations based on size criteria: all mammospheres and mammospheres ≥ 150 μm. Mammospheres displayed increased cell killing from 3 to 5 Gy for BALB/c *Atm* $^{(+/\Delta SRI)}$.

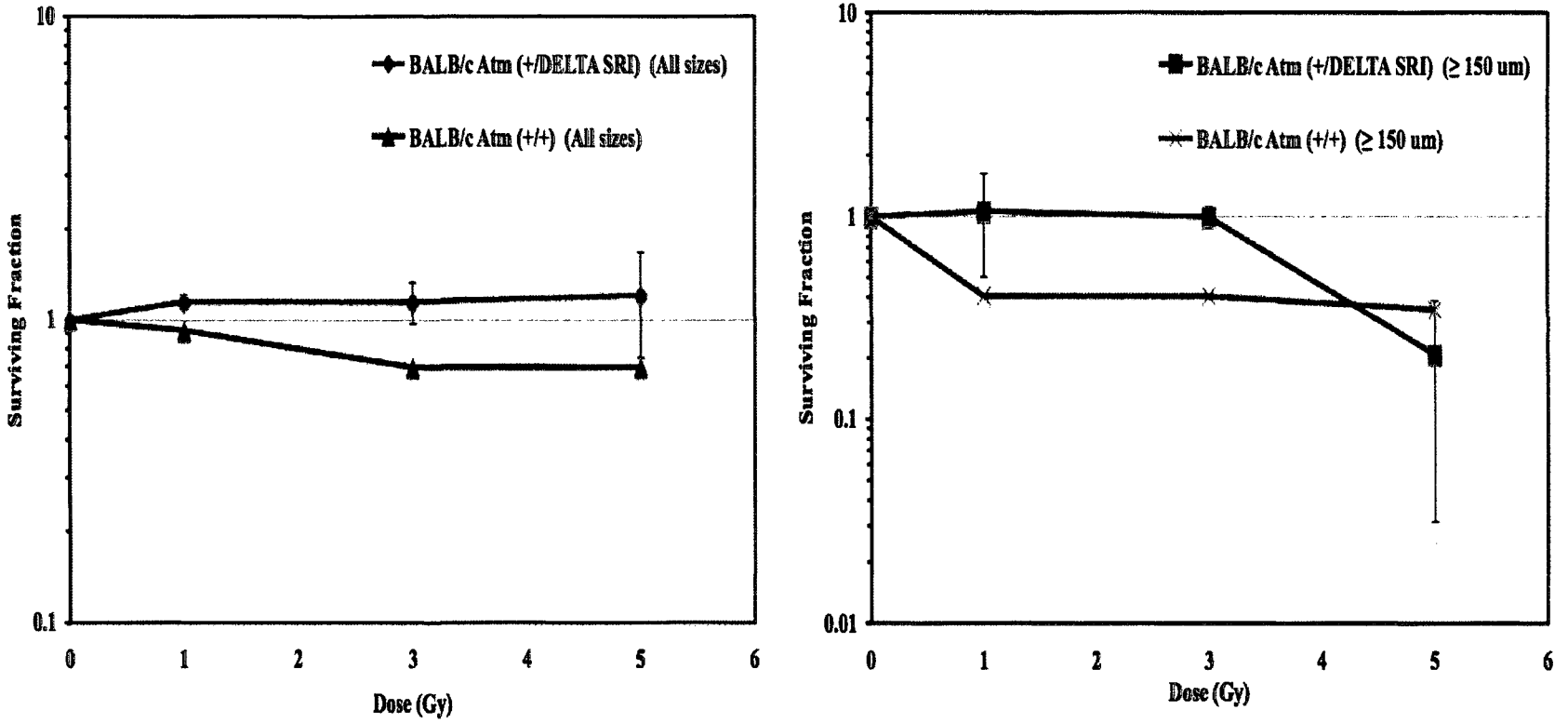

Figure 5.7 and 5.8: Cell Survival comparing all mammosphere sizes derived from BALB/c *Atm* $^{(+/+)}$ and BALB/c *Atm* $^{(+/\Delta SRI)}$ shown in Figure 5.7 (Left). Cell Survival comparing all mammosphere ≥ 150 μm derived from BALB/c *Atm* $^{(+/+)}$ and BALB/c *Atm* $^{(+/\Delta SRI)}$ shown in Figure 5.8 (Right). Clonogenic cell survival assay of mammospheres derived from BALB/c *Atm* $^{(+/\Delta SRI)}$ (BALB/c *Atm*-ΔSRI heterozygous mice) and BALB/c *Atm* $^{(+/+)}$ (BALB/c *Atm*-ΔSRI wildtype littermates). Cells were irradiated as single cell suspensions and plated to form mammospheres. Surviving fraction was determined by normalizing the plating efficiency for each dose to the corresponding unirradiated control. Two surviving fractions were calculated for two different cell populations based on size criteria: all mammospheres and mammospheres ≥ 150 μm. Mammospheres displayed increased cell killing from 3 to 5 Gy for BALB/c *Atm* $^{(+/\Delta SRI)}$ as compared to mammospheres derived from BALB/c *Atm* $^{(+/+)}$ in Figure 5.8.

DISCUSSION AND FUTURE DIRECTIONS

The *Atm*-ΔSRI mice were developed to investigate the functional consequences that a specific missense mutation may confer on breast cancer susceptibility. *Atm*-ΔSRI heterozygous mice are hypothesized to be susceptible to radiation-induced mammary cancer, which has not been determined [18]. We hypothesized that mammary stem cells are critical targets of radiation-induced damage. We developed a methodology to characterize the *in vitro* behavior of mammary stem cells, which allowed us to examine their radiation response as mammospheres. To investigate the utility of this methodology we analyzed the effects of radiation on the cell survival of mammospheres derived from *Atm*-ΔSRI heterozygous mice and their wildtype littermates. Novel size criteria were developed to discern which mammospheres originated from a true stem cell in order to analyze the effect of IR on stem cells and their progeny. Our results demonstrate that mammary stem cells derived *Atm*-ΔSRI heterozygous mice displayed increased radiation resistance compared to their wildtype littermates.

Intermediate radiation sensitivity is one of the cellular features of *ATM* heterozygotes [12, 13]. We originally hypothesized that the *Atm*-ΔSRI mutation would impart intermediate sensitivity for heterozygous mice and perhaps increased radiation sensitivity for heterozygotes on the BALB/c mixed background. Spleen cells derived from *Atm*-ΔSRI heterozygous mice display intermediate sensitivity to radiation compared to wildtype and *Atm*-ΔSRI homozygous mice [18]. Our results demonstrate that mammospheres derived from *Atm*-ΔSRI heterozygous mice do not appear radiation sensitive compared to their wildtype littermates, but in fact are radioresistant. For both

heterozygous and wildtype strains of *Atm*-ΔSRI, results suggest that there may be differences in the radiation response for mammospheres ≥150 μm, which was not observed when all mammosphere sizes are included. We cannot infer whether these strains of mice are sensitive or resistant to radiation because our size criterion is not complete. Radiation sensitivity for these strains of mice may be dependent on the cell type used in radiation cell survival studies. This study requires additional independent experiments to strengthen the data. Mammary stem cells appear to be radioresistant and require higher doses (e.g. 8 and 10 Gy) for additional cell survival studies. We are unable to explain why the reproductive capacity of mammospheres seems to be unaffected even at 5 Gy. Few studies have examined the radiation response of mammary stem cells as mammospheres. Recent studies have suggested that mouse mammary stem cells are radiation resistant, but used clinically relevant doses (2 Gy) [21, 22]. A clear understanding of the DNA damage response and repair processes of stem cells is likely to impact elucidating the susceptibility of stem cells to radiation-induced carcinogenesis. Stem cells are suspected to have unique mechanisms to prevent or minimize radiation-induced damage. Although it has been suggested that mammary stem cells may be resistant to radiation [21, 22], this relationship has yet to be elucidated.

ATM plays an important central role in response and repair to radiation-induced damage. The absence of ATM protein leads to defective cell cycle arrest, defective double strand break repair, chromosomal instability, and increased radiation sensitivity [23-25]. ATM is required for the activation of cell cycle checkpoints, DNA damage response and repair processes, telomere maintenance, transcriptional activity, and apoptosis [26]. Activation of these processes is facilitated through phosphorylation of

different targets such as p53, MDM2, Chk1/Chk2, BRCA1, and SMC1 by ATM [26]. ATM has been shown to interact and recruit BRCA1 in response to radiation-induced damage [27]. *BRCA1* is an important breast cancer susceptibility gene in which high penetrance mutations lead to breast and ovarian cancer [28]. Although the relationship between ATM and BRCA1 has not been fully elucidated, BRCA1 has been shown to regulate differentiation of mammary stem and progenitor cells [1]. It has been argued that BRCA1 may function as a breast stem cell regulator [2]. BRCA1 may play an important role in preventing radiation-induced damage in stem cells. The interaction between ATM and BRCA1 suggests an alternative role for ATM in which ATM maintains the genomic integrity in stem cells through BRCA1. The impact of *ATM* mutations may affect protecting stem cells from radiation-induced damage.

It is suspected that the *Atm*-ΔSRI mutation confers a dominant-negative effect in heterozygous mice, but results remain inconclusive [29]. The impact of this mutation may affect the activation of the p53 pathway in which DNA damage-induced apoptosis is delayed or defective. The radiation sensitivity (i.e. cell killing) may be dependent on cell type and/or tissue specific. Mammary epithelial stem cells may not be sensitive to radiation; however, additional endpoints are needed to examine the involvement of stem cells in the carcinogenic process.

Future studies should include additional independent cell survival experiments to confirm the results among the *Atm*-ΔSRI strains. The relative cell survival should be compared to $Atm^{-/-}$ mice to determine whether the function of ATM impacts radiation sensitivity of mammary stem cells. *Atm*-ΔSRI mutation may not lead to increased cell killing in mammary stem cells, but perhaps there are other effects induced after IR.

Examining additional endpoints such as *ATM* phosphorylation of downstream targets, chromosomal aberrations, and induction of apoptosis may help to uncover the consequences of *Atm*-ΔSRI mutation in mammary stem cells. Analyzing these endpoints may also reveal defective and/or delayed induction of particular pathways mediated by ATM that lead to ongoing genomic instability. Although mammary stem cells are not predisposed to cell killing, the effects of *Atm*-ΔSRI mutation may still confer increased breast cancer susceptibility through other mechanisms. These studies demonstrate the utility of methodology and size criteria developed. To our knowledge, this is the first study examining the radiation sensitivity of *Atm* heterozygous mice carrying a known missense mutation found in human A-T patients, through assessing the radiation response of mammary stem cells as mammospheres.

LITERATURE CITED

1. Liu, S., et al., *BRCA1 regulates human mammary stem/progenitor cell fate.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1680-5.
2. Foulkes, W.D., *BRCA1 functions as a breast stem cell regulator.* J Med Genet, 2004. **41**(1): p. 1-5.
3. Thompson, L.H. and D. Schild, *Recombinational DNA repair and human disease.* Mutat Res, 2002. **509**(1-2): p. 49-78.
4. Meyn, M.S., *Ataxia-telangiectasia, cancer and the pathobiology of the ATM gene.* Clin Genet, 1999. **55**(5): p. 289-304.
5. Swift, M., et al., *Incidence of cancer in 161 families affected by ataxia-telangiectasia.* N Engl J Med, 1991. **325**(26): p. 1831-6.
6. Swift, M., et al., *The incidence and gene frequency of ataxia-telangiectasia in the United States.* Am J Hum Genet, 1986. **39**(5): p. 573-83.
7. Dontu, G., et al., *Stem cells in normal breast development and breast cancer.* Cell Prolif, 2003. **36 Suppl 1**: p. 59-72.
8. Smith, G.H., *Stem cells and mammary cancer in mice.* Stem Cell Rev, 2005. **1**(3): p. 215-23.
9. Smalley, M. and A. Ashworth, *Stem cells and breast cancer: A field in transit.* Nat Rev Cancer, 2003. **3**(11): p. 832-44.
10. Swift, M., et al., *Breast and other cancers in families with ataxia-telangiectasia.* N Engl J Med, 1987. **316**(21): p. 1289-94.
11. Concannon, P., *ATM heterozygosity and cancer risk.* Nat Genet, 2002. **32**(1): p. 89-90.
12. Shigeta, T., et al., *Defective control of apoptosis and mitotic spindle checkpoint in heterozygous carriers of ATM mutations.* Cancer Res, 1999. **59**(11): p. 2602-7.
13. Gutierrez-Enriquez, S., et al., *Functional consequences of ATM sequence variants for chromosomal radiosensitivity.* Genes Chromosomes Cancer, 2004. **40**(2): p. 109-19.
14. Gatti, R.A., A. Tward, and P. Concannon, *Cancer risk in ATM heterozygotes: a model of phenotypic and mechanistic differences between missense and truncating mutations.* Mol Genet Metab, 1999. **68**(4): p. 419-23.
15. Barlow, C., et al., *Atm haploinsufficiency results in increased sensitivity to sublethal doses of ionizing radiation in mice.* Nat Genet, 1999. **21**(4): p. 359-60.
16. Weil, M.M., et al., *Radiation induces genomic instability and mammary ductal dysplasia in Atm heterozygous mice.* Oncogene, 2001. **20**(32): p. 4409-11.
17. Lu, S., et al., *Atm-haploinsufficiency enhances susceptibility to carcinogen-induced mammary tumors.* Carcinogenesis, 2006. **27**(4): p. 848-55.
18. Spring, K., et al., *Mice heterozygous for mutation in Atm, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer.* Nat Genet, 2002. **32**(1): p. 185-90.
19. Spring, K., et al., *Atm knock-in mice harboring an in-frame deletion corresponding to the human ATM 7636del9 common mutation exhibit a variant phenotype.* Cancer Res, 2001. **61**(11): p. 4561-8.
20. Dontu, G., et al., *In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells.* Genes Dev, 2003. **17**(10): p. 1253-70.

21. Woodward, W.A., et al., *WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells.* Proc Natl Acad Sci U S A, 2007. **104**(2): p. 618-23.
22. Chen, M.S., et al., *Wnt/beta-catenin mediates radiation resistance of Sca1+ progenitors in an immortalized mammary gland cell line.* J Cell Sci, 2007. **120**(Pt 3): p. 468-77.
23. Xu, B., et al., *Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation.* Mol Cell Biol, 2002. **22**(4): p. 1049-59.
24. Kuhne, M., et al., *A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity.* Cancer Res, 2004. **64**(2): p. 500-8.
25. Scott, D., *Chromosomal radiosensitivity and low penetrance predisposition to cancer.* Cytogenet Genome Res, 2004. **104**(1-4): p. 365-70.
26. Khanna, K.K., et al., *ATM, a central controller of cellular responses to DNA damage.* Cell Death Differ, 2001. **8**(11): p. 1052-65.
27. Gatei, M., et al., *Role for ATM in DNA damage-induced phosphorylation of BRCA1.* Cancer Res, 2000. **60**(12): p. 3299-304.
28. Balmain, A., J. Gray, and B. Ponder, *The genetics and genomics of cancer.* Nat Genet, 2003. **33 Suppl**: p. 238-44.
29. Khanna, K.K., *Cancer risk and the ATM gene: a continuing debate.* J Natl Cancer Inst, 2000. **92**(10): p. 795-802.

CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

To our knowledge, this is the first study to examine the radiation response of mammary stem cells as mammospheres using *Atm* heterozygous mice carrying a known missense mutation found in human A-T patients. These mice carry a common *ATM* missense mutation (7636del9) identified in ataxia-telangiectasia (A-T) patients [1]. Individuals afflicted with this rare genetic disorder are extremely sensitive to radiation and are susceptible to cancer [2]. It is suspected that *ATM* heterozygotes are susceptible to radiation-induced breast cancer that is conferred by certain breast cancer-associated *ATM* mutations. *Atm*-ΔSRI mice were created to investigate the impact of this particular missense mutation in mammary carcinogenesis [1]. *Atm*-ΔSRI heterozygous mice develop mammary tumors at a low incidence (2%) and cells from these mice display intermediate sensitivity to radiation as assessed by radiation cell survival studies and induction of chromosome aberrations [3]. However, the radiation response of mammary epithelial cells has yet to be elucidated. We hypothesized that mammary stem cells derived from *Atm*-ΔSRI heterozygous mice would exhibit increased radiation sensitivity compared to mammospheres from their wildtype littermates. However, our results suggest mammary stem cells derived from *Atm*-ΔSRI heterozygous mice are radioresistant.

We suspect that mammary stem cells derived from *Atm*-ΔSRI heterozygous mice may have unique mechanisms to prevent or minimize radiation-induced damage in order to maintain their genomic integrity. ATM is known to be involved in the cellular response to DNA damage [4]. There is a cascade mechanism that involves the sensing of DNA DSBs and regulation of specific DNA repair pathways, apoptosis, and cell cycle

checkpoints during different stages of the cell cycle, which is mediated by ATM [5-7]. ATM is responsible for the activation of these processes through phosphorylation of different targets such as p53, MDM2, Chk1/Chk2, BRCA1, and SMC1 [5]. Previous studies have demonstrated that cells derived from *Atm*-ΔSRI heterozygous mice display intermediate sensitivity to radiation compared to wildtype and *Atm*-ΔSRI homozygous mice [3]. However, stem cells may respond differently to the effects of ionizing radiation as compared to differentiated cells. *Atm*-ΔSRI mutation may not lead to increased cell killing in mammary stem cells, but perhaps there are other effects induced after IR. The manner in which the *Atm*-ΔSRI mutation affects DNA repair pathways in stem cells may differ from differentiated cells has yet to be elucidated.

We developed a methodology and size criteria to analyze the effects of ionizing radiation on survival of mammary stem cells. We utilized an *in vitro* system analogous to a neural stem cell assay, which was developed for the detection of mammary stem cells [8, 9]. Mammary stem cells form spheroid structures (mammospheres) when cultured in non-adherent cell culture conditions. Mammospheres retain their replicative and multilineage capacity *in vitro*, which provides the opportunity to directly examine mammary stem cells and their progeny. During the development of our methodology, we characterized the *in vitro* behavior of mammary stem cells and established novel size criteria. We suspected that not every mammosphere originated from a stem and/or progenitor cell. The size criterion was established to distinguish mammospheres that arose from a stem and/or progenitor cells. Our criteria consisted of two components: serial passaging to assess self-renewal capacity and lineage composition of individual mammospheres (based on size). We hypothesized that mammospheres capable of self-

renewal and multilineage capacity indicated they arose from a stem or high progenitor cell. We believed that utilizing this size criterion would facilitate in the analysis of mammary stem cells and their progeny after exposure to ionizing radiation.

The size criteria failed to show any differences in radiation sensitivity for mammospheres derived from *Atm*-ΔSRI heterozygous mice. However, we believe the size criteria needs to be improved and refined for future studies. Our results suggested that mammospheres ≥ 150 μm originated from a cell (i.e. stem and/or high progenitor cell), capable of self-renewal and multilineage capacity. However, these studies utilized non-clonal mammospheres whereas clonal mammospheres were used for radiation cell survival studies. Therefore, the components of the size criteria need to address the self-renewal and multilineage capacity of clonal mammospheres. Future studies should include serial passaging of individual clonal mammospheres to discern the self-renewal capabilities and analyzing lineage composition (i.e. luminal and myoepithelial cell lineages). In addition, transplantation of individual mammospheres into cleared fat pads of recipient mice would reveal whether our size criteria accurately identifies stem cells *in vitro* by demonstrating clonal reconstitution of a functional mammary gland *in vivo*. These future studies would strengthen the importance of the criteria for analyzing mammary stem cells *in vitro*.

Future studies are needed to follow up on the results described. Radiation cell survival studies should include additional independent experiments for *Atm*-ΔSRI heterozygous and *Atm* homozygous mice. Examining the radiation response of mammary stem cells derived from *Atm* homozygous mice may give insight into the role of ATM after exposure to IR and the impact of the ATM mutations. In addition, radiation cell

survival studies should include higher radiation doses to examine the radioresistance of mammary stem cells.

We have demonstrated that the methodology developed can be used to examine radiation stem cell survival. We believe that this *in vitro* system can be utilized to examine additional radiation biology endpoints such as cytogenetic damage, γ-H2AX foci formation, and DNA repair after radiation exposure. Mammospheres can be utilized to investigate cytogenetic damage derived from a clonally derived cell (i.e. stem cells and their progeny), mammary stem cells response to radiation-induced damage via γ-H2AX foci formation, and DNA repair mechanisms as compared to differentiated cells.

There are numerous advantages for using mammospheres as compared to other *in vitro* assays. Mammospheres can grow under non-adherent cell culture conditions and do not require the presence of an irradiated fibroblast layer [10]. Unlike other methods, mammary stem cells can be cultured and propagated without the need for cell surface markers to identify them [8]. The replicative and differentiation potential of mammary stem cells (mammospheres) can be analyzed using suspension cultures (mammospheres), 2-D and 3-D cultures (collagen and ECM), and *in vivo* transplantation animal models. Our methodology and size criterion allows us to pinpoint specific individual mammospheres that originated from real stem cells. Thus, providing a way to directly analyze stem cell properties, stem cells, and the progeny derived from that cell. Combining the mammosphere assay with the size criteria developed would help to focus studies in the investigation of the mechanisms that control stem cell renewal and differentiation.

The utility of the methodology and size criteria can be utilized for various mechanistic studies. Mechanistic studies can use mammospheres and the size criteria to manipulate stem cells *in vitro* and test the cancer stem cell hypothesis by examining stem cell susceptibility to transformation. Viral vectors and/or siRNA can be introduced to mammosphere cultures to disrupt genes involved in self-renewal and differentiation pathways. The susceptibility of luminal and myoepithelial cell lineages to transformation can be investigated using our size criteria, which distinguishes lineage composition of mammospheres based on size. Growth kinetics, mammosphere formation efficiency, and mammosphere size can be utilized as endpoints to examine how certain genes associated with breast cancer lead to tumorigenesis. Transformation studies can test the utility of size criteria by examining increased proliferation of mammospheres and apoptosis pathways (clearing of the lumen). Initiation (i.e. target cell) of ductal dysplasia could be elucidated using mammospheres derived from irradiated mice and analyzed in a 3-D matrix (i.e. BD Biosciences Matrigel™). Utilizing mammospheres may provide a better understanding of the behavior of mammary stem cells *in vitro* and the characterization of key components that are involved in early events in the carcinogenic process in murine model systems.

In conclusion, we have described the application of an *in vitro* mammary stem cell model employing mammospheres to examine the relationship between stem cell susceptibility and radiation-induced damage. We have developed a methodology for the proper identification and characterization of an enriched population of mammary stem and progenitor cells. Using this methodology, we have optimized this *in vitro* system through the establishment of novel criteria that will serve to enhance our understanding

of the response of stem cells *in vitro*. Furthermore, we have illustrated that not every mammosphere may contain a stem cell, which shows the importance of our criteria. We have demonstrated the applicability of our methodology and novel criteria to characterize the radiation response of mammary stem cells and their progeny derived from *Atm*-ΔSRI heterozygous mice. Although this *in vitro* system poses some limitations, our methodology has improved and expanded the feasibility to examine clonally derived cells in which further studies may build upon the behavior of stem cells and their role in breast cancer susceptibility.

LITERATURE CITED

1. Spring, K., et al., *Atm knock-in mice harboring an in-frame deletion corresponding to the human ATM 7636del9 common mutation exhibit a variant phenotype*. Cancer Res, 2001. **61**(11): p. 4561-8.
2. Meyn, M.S., *Ataxia-telangiectasia, cancer and the pathobiology of the ATM gene*. Clin Genet, 1999. **55**(5): p. 289-304.
3. Spring, K., et al., *Mice heterozygous for mutation in Atm, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer*. Nat Genet, 2002. **32**(1): p. 185-90.
4. Lavin, M.F., et al., *ATM signaling and genomic stability in response to DNA damage*. Mutat Res, 2005. **569**(1-2): p. 123-32.
5. Khanna, K.K., et al., *ATM, a central controller of cellular responses to DNA damage*. Cell Death Differ, 2001. **8**(11): p. 1052-65.
6. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases*. Genes Dev, 2001. **15**(17): p. 2177-96.
7. Lee, J.H. and T.T. Paull, *ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex*. Science, 2005. **308**(5721): p. 551-4.
8. Dontu, G., et al., *In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells*. Genes Dev, 2003. **17**(10): p. 1253-70.
9. Reynolds, B.A. and S. Weiss, *Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell*. Dev Biol, 1996. **175**(1): p. 1-13.
10. Stingl, J., *Detection and analysis of mammary gland stem cells*. J Pathol, 2009. **217**(2): p. 229-41.

Appendix I

List of Abbreviations

°C	degrees Celsius
γ	gamma
α	alpha
µg	microgram
µl	microliter
µm	micron
3-D	three-dimensional
× g	G-force
ABC	ATP-Binding Cassette
α-SMA	alpha Smooth Muscle Actin
AML	acute myeloid leukemia
AT	ataxia-telangiectasia
ATM	ataxia telangiectasia mutated
Atm-ΔSRI	Atm^{tm1Mfl}
Atm (+/ΔSRI)	*Atm*-ΔSRI heterozygous mice
Atm (+/+)	*Atm*-ΔSRI wildtype littermates/mice
ATP	Adenosine-5'-triphosphate
ATR	ataxia-telangiectasia-related protein
BALB/c *Atm* (+/ΔSRI)	BALB/c *Atm*-ΔSRI heterozygous mice
BALB/c *Atm* (+/+)	BALB/c *Atm*-ΔSRI wildtype littermates/mice
BCRP-1	breast cancer resistance protein-1
bFGF	basic fibroblast growth factor
BME	basement membrane extract
BRCA1/2	breast cancer susceptibility allele 1/ 2
BrdU	5'-bromo-2'-deoxyuridine
Ca^{++}	Calcium
Chk1/Chk2	CHK1/2 checkpoint homolog
Ci	Curie
CO_2	carbon dioxide
Cs	cesium
CSCs	cancer stem cells
CSU	Colorado State University
DAPI	4',6-diamidino-2-phenylindole
DMEM/F12	Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent Protein Kinase catalytic subunit
DNase I	Deoxyribonuclease I

DSBs	double-strand breaks
ECM	extracellular matrix
EGF	epidermal growth factor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ER	estrogen receptor
ERR	excess relative risk
FA-A to FA-C	Fanconi's anemia
FACS	Fluorescence Activated Cell Sorting
FAT	focal adhesion targeting
FBS	fetal bovine serum
G1	Gap 1 (cell cycle)
g	gram
Gy	gray
HER2	human epidermal growth factor receptor 2
HF	Hank's Balance Salt Solution with 2% FBS
HT	heterozygotes
H2AX	histone H2A, member X
IACUC	Institutional Animal Care and Use Committee
IR	ionizing radiation
kDa	kilodalton
K18	Cytokeratin 18
LET	linear energy transfer
LOH	loss of heterozygosity
LRCs	label-retaining cells
LSS	Life Span Study
mg	milligrams
mL	milliliter
mm	millimeter
mM	millimolar
mTOR	mammalian Target of Rapamycin
MDM2	Mdm2 p53 binding protein homolog (mouse)
MFE	mammosphere formation efficiency
Mg^{+}	Magnesium
MMTV	mouse mammary tumor virus
MN	micronuclei
ng	nanogram
nM	nanomolar

NBS1	nibrin/Nijmegen breakage syndrome
p53/TP53	tumor protein p53
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	plating efficiency
PI3	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog
PTT	protein truncation test
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
TEBs	terminal end buds
Sca-1	Stem cell antigen-1
SDS-PAGE	sodim dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
SMC1	structural maintenance of chromosomes-1
SNP	single nucleotide polymorphism
SP	side population
SSBs	single-strand breaks
UV	Ultraviolet light
Wnt-1	wingless type-1
WT	wildtype