DISSERTATION

DEVELOPMENT AND UTILIZATION OF A MACAQUE-BASED MAMMOSPHERE CULTURE TECHNIQUE FOR BREAST CANCER RESEARCH

Submitted by

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ABSTRACT

DEVELOPMENT AND UTILIZATION OF A MACAQUE-BASED MAMMOSPHERE CULTURE TECHNIQUE FOR BREST CANCER RESEARCH

Human breast cancers are thought to commonly arise from progressive neoplastic changes to the adult stem cells within the normal mammary gland. Research in this area of breast carcinogenesis currently relies heavily on the acquisition of mammary gland stem cells from the tissues of rodents and humans. While a great deal of information has been gained utilizing these models, there remain large gaps in our knowledge of breast cancer due to certain limitations with these species. The relevance of rodents as models for human breast cancer has been brought into question by notable differences between rodents and humans with regard to genetics, biology and mammary gland carcinogenesis. In contrast, the utility of human-derived samples is limited by ethical concerns and by the restricted availability of mammary tissues from women.

Macaque monkeys are closely related to humans phylogenetically and these animals develop mammary gland tumors that are comparable to human breast cancers. Furthermore, mammary gland tissues can be easily collected from any demographic of animal. Despite their potential, only minimal breast cancer work has been undertaken in the macaques to date and research techniques common to both rodents and human are lacking for these species.

This dissertation describes the optimization of a commonly-used mammary gland stem cell isolation technique, mammosphere culture, for the rhesus macaque (*Macaca mulatta*) and provides validation as to the reliability, relevance, and usefulness of this assay for human studies. Data obtained from this research demonstrated that the mammosphere culture technique is highly

reproducible between homologous macaque tissue samples. This work also found that mammary gland biopsies collected from different anatomical locations on the same monkey share comparable mammosphere-forming ability and mammosphere-differentiation ability (collectively, the mammosphere potential). Finally, these initial studies identified macaque mammospheres to have proliferative and differentiating properties that are nearly identical to those described for human mammospheres.

This dissertation also describes a series of macaque studies performed using the optimized mammosphere culture technique. In the first study, mammary gland tissues were obtained from female macaques in different reproductive demographics and the mammosphere potential of these animals was compared. The results suggested that the mammosphere potential of nulliparous mammary glands is significantly greater than that of multiparous mammary glands and that this difference is likely due to greater ratios of mammary gland stem cells within the nulliparous mammary gland. These data also suggested that there are differences in the mammosphere potential of mammary glands collected from animals at different stages of the reproductive cycle. An additional study comparing the mammosphere potential of young-multiparous and multiparous macaques collected during the menses stage of the menstrual cycle supported the parity-related findings of the first study. Data from the second study also identified significantly larger ratios of senescent cells in the mammosphere cultures of multiparous macaques as compared to young-nulliparous macaques. Finally, a study comparing the effects of ionizing radiation on mammospheres derived from young-nulliparous and multiparous macaques was performed. This last study found that stem cell-like cells of the young-nulliparous mammary gland were more resistant to the lethal effects of ionizing radiation than were those of the multiparous gland.

The findings of these three studies are notable in that young-nulliparous girls are known to have a higher susceptibility to radiation-induced breast cancer than are multiparous women and these studies provide the first direct evidence as to the potential mechanistic reasons behind this observation. Specifically, as macaques appear to be relevant models for the study of the human breast, these data suggest that the increased susceptibility of young-nulliparous girls to radiation-induced carcinogenesis could arise from: 1) higher number of mammary stem cells within the breast; 2) a decreased predilection of these stem cells to undergo senescence; and 3) a decreased sensitivity of these stem cells to the lethal effects of ionizing radiation.

In summary, macaque mammospheres appear to be relevant models for the study of the human breast. Use of this model allows for the study of mammary gland tissues from some demographics of interest (e.g., prepubescent individuals) that are impossible to investigate utilizing human tissues. The mammosphere culture techniques and data described in this dissertation serve as a foundation toward the use of macaques in future breast cancer research projects and other study data from this dissertation has provided novel insight as to the increased risk of radiation-induced breast cancers in young women.

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DEDICATION

This work is dedicated to my children Kennedy, Bonnie and Colter for all the lessons they teach me and for their never ending curiosity which pushes me daily to never stop searching for answers. This work is also dedicated to my parents Kenny and Becky Wilkerson for inspiring a solid work ethic in me, for encouraging me to learn on my own, and for giving me the courage and confidence to attempt feats well-beyond my perceived abilities. First and foremost, however, this work is dedicated to my wife Nicole. She is the only member of the 'Wilkerson Team' who was actually recruited into the position and is truly our team's Most Valuable Player. I am eternally grateful to her for keeping our family functioning and happy for all of these years while I pursued my professional goals.

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Chapter 1: Review of the Mammary Gland, Breast Cancer, and Breast Cancer-Research Models

Introduction

Breast cancer remains a prominent cause of morbidity and mortality to women in the United States despite advances in diagnosis and treatment. This chapter reviews the background on human breast cancer in addition to information on the development, morphology and physiology of the mammary gland as each of these topics relates to contemporary theories on breast cancer carcinogenesis. It provides background on radiation-induced breast cancer and introduces some current technologies and animal models that are relevant to the ongoing study of breast cancer. Finally, this chapter outlines my research interests and describes how I have incorporated the use of the aforementioned technologies and research models into this dissertation on breast cancer carcinogenesis.

Mammary Gland

Human mammary gland morphology. The mature human breast consists of approximately 15-25 lobes that originate and radiate out from the nipple as illustrated in Figure 1. These lobes are embedded within abundant fibrous and fatty tissue stroma. At the most basic anatomic level, the lobes of the lactating breast are composed of large numbers of glandular alveoli and branching ducts and ductules that function to produce and transport milk respectively. Each lobe generally functions as a distinct glandular unit with all alveoli, ductules, and ducts that comprise an individual lobe feeding into a single secretory pore within the nipple.[110, 214]

At the microscopic level, the glandular portion of the breast can be subdivided and described utilizing a variety of anatomical terms to denote functionality and/or location. With relevance to this discussion, the structural components of the breast will be divided into the following subunits, starting with the most externally located structures and proceeding inward extralobular ducts, intralobular ducts, intralobular ductules and alveoli (Figure 2). Likewise, the stromal component of the breast will be divided into the intralobular stroma and interlobular stroma for this review (Figure 2).

The extralobular ducts are tubular structures that are lined by 1-2 layers of columnar to low cuboidal epithelial cells, known as the ductal epithelium.[110, 214] The ductal epithelial cells, in turn, overlie a basal layer of myoepithelial cells and a basement membrane which is surrounded by interlobular stroma. Interlobular stroma is similar to dermal stroma, and is best characterized as a poorly-cellular stroma composed primarily of adipose tissue,

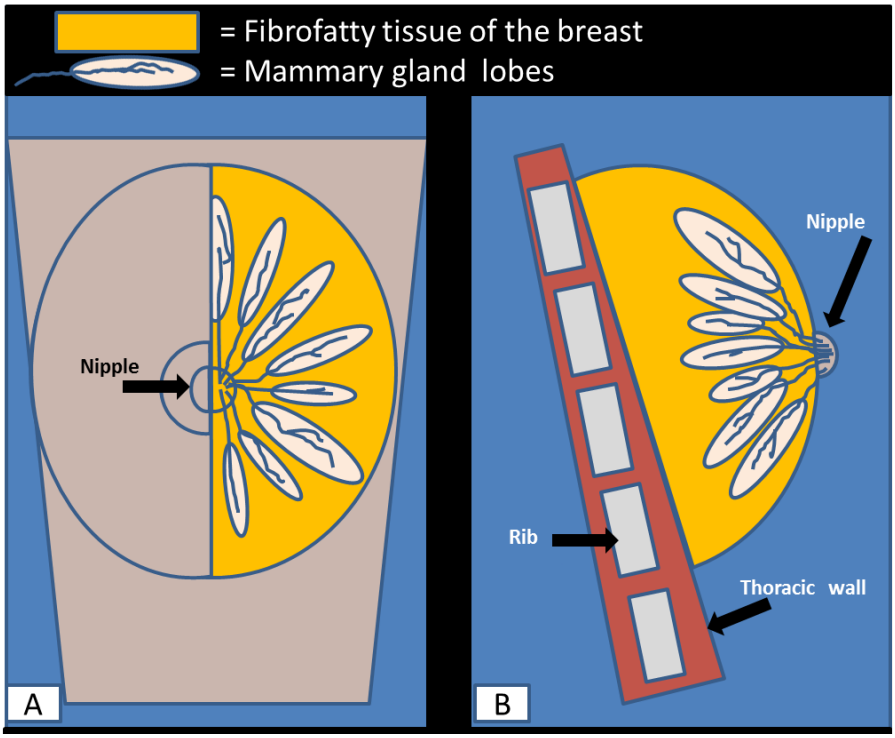

Figure 1. Illustration of the gross anatomy of the human breast from the anterior (A) and lateral (B) views demonstrating the radial distribution of the glandular lobes from the nipple and the distribution of the fibrofatty tissue within the organ.

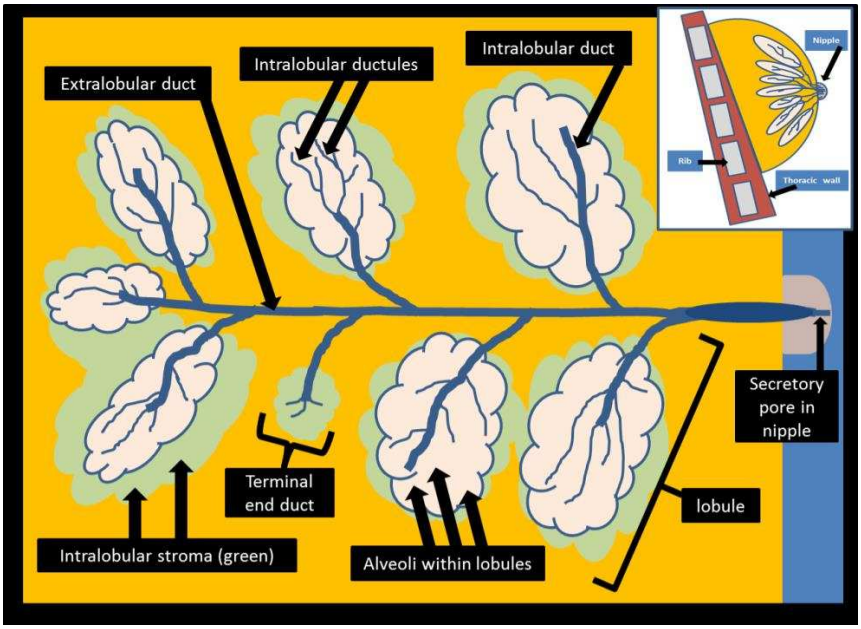

Figure 2. Illustration of the subgross anatomy of an individual breast lobe. Constituent parts of the mammary gland lobe include secretory pore, extralobular ducts, intralobular ducts, intralobular ductules and alveoli. Lobes are subdivided into lobules which are invested within an intralobular stroma (green). The fibrofatty material comprising the bulk of the breast (yellow) is interlobular stroma. Inset is the distribution of lobes within the breast (from Figure1).

loose fibrous connective tissue, collagen and elastin fibers.[110, 214] Most of the tissue comprising the non-lactating human breast is interlobular stroma.

At the level of the lobular subdivisions, both the lobules and the ductal system become enveloped within a highly cellular form of stroma that lacks a fatty component, and which is referred to as intralobular stroma (Figure 2). Endothelial cells, leukocytes and large numbers of fibroblasts comprise the bulk of the cellular components within the intralobular stroma.[110, 214] The junction at which the ducts are invested in intralobular stroma is the point where the ducts are considered to be intralobular ducts. Besides being invested in intralobular stroma and being slightly smaller in caliper than the extralobular ducts, the intralobular ducts are essentially indistinguishable from the extralobular ducts under standard microscopic examination. In the post-pubescent female, most of the intralobular ducts are further subdivided into the highly branched, thinly-lumened structures known as the intralobular ductules, which eventually terminate as clusters of spherical alveoli. At the microscopic level, the ductules generally have smaller lumens than ducts and are lined by only a single cell layer of ductal epithelium, but otherwise have a layer of myoepithelium and a basement membrane similar to what was described earlier for the ducts (Figure 3).[110, 214]

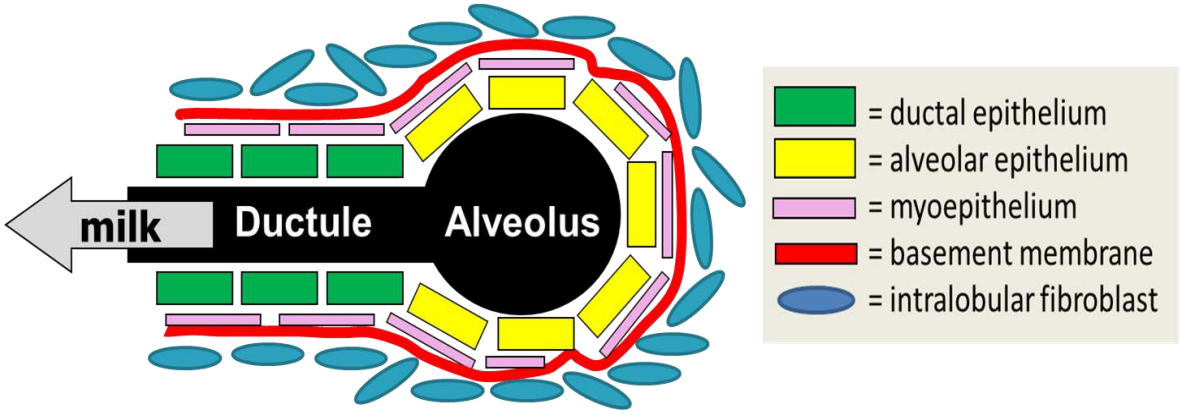

Figure 3. Cellular composition of the mature ductule and alveolus. Note the contact of the alveolar luminal cells with the basement membrane through the gaps in the myoepithelial cell layer.

The alveolar lumens are lined by a single layer of cuboidal to columnar epithelial cells, called alveolar epithelium, which overlie a basal myoepithelial cell layer and basement membrane, similar to what is seen in the ducts/ductules. Unlike the ducts and ductules, however, the myoepithelial cells of the alveoli frequently have small gaps between adjacent cells allowing for areas of direct contact between the alveolar epithelium and the basement membrane (Figure 3).[110, 214, 221]

The functional unit of the breast is the considered to be all of the alveolar and ductal components within the intralobular stroma of one lobule and this structure has collectively been termed the terminal duct-lobular unit (TDLU). In the non-pregnant, parous female, the majority of TDLUs contain around 40 alveoli that are situated on numerous intralobular branching ductules and ducts.[110, 214, 219, 221] These have been referred to as Type 2 TDLUs or Lobules type 2/3 by different investigators.[110, 219, 221] In contrast to mature parous females, the breasts of mature nulliparous females are primarily composed of TDLUs that are less developed. These immature TDLUs are recognized to have shorter ducts, smaller numbers of intralobular ductules, and typically have 10 or fewer alveoli per lobule. These TDLUs have, in turn, been referred to as Type 1 TDLUs, Lobules type 1, or terminal end ducts (TEDs).[110, 214, 219-221] An example of a TED is illustrated in Figure 2.

While the aforementioned patterns of breast development are accurate generalities, it is also important to understand that marked variations in glandular development can occur between breasts, and even between lobes/lobules within the same breast. Developmental homogeneity within the breast has been primarily correlated to the number of full-term pregnancies a woman has undergone and, to a lesser extent, advanced age.[110, 214, 219, 220] To that, multiparous women tend to have less developmental variability between the individual lobes of their breasts than do nulliparous women and, likewise, aged nulliparous women have less variability than do younger nulliparous women.

Human mammary gland development and physiology. The mammary gland is a unique organ in that the majority of morphologic and physiologic development occurs outside of the womb, with the most significant of these changes taking place throughout puberty and pregnancy. Each human mammary gland begins development in utero as a placode of ectodermal cells that extend thin ductular structures into the underlying mesenchyme to form the breast bud.[110, 214] Shortly after parturition, the breast bud comes to rest as a collection of short, blunt-ended ducts with only small numbers of branching ductules and alveoli; similar in histological appearance to what was described of the TEDs in the adult breast.[110, 221]

To this point, the development and morphology of the male and female breast buds in humans are identical and remain so until puberty. In males, the breast tissue typically never progresses beyond this stage and eventually undergoes glandular regression due to androgen secretions.[110, 214] In females, the onset of puberty begins to transform the epithelial ductular structures of the immature mammary gland into the individual lobes of the mature breast, while the fibroblastic components of the breast bud begin to differentiate into the intralobular and

interlobular stroma of the adult mammary gland.[76, 110, 214, 221] With increased amounts of systemic estrogen, the fibrous and fatty tissues of the interlobular stroma are the first components of the female breast to expand. This is followed shortly thereafter by extension and differentiation of the glandular components of the breast from the apical aspects of the adolescent TEDs.[76, 214] The invasion of the glandular network into the fibrofatty stroma and extralobular branching of the ducts are primarily driven by systemic estrogen while intralobular ductular branching and alveolar formation are largely driven by systemic progesterone.[26, 76, 108, 110, 214, 221] In conjunction with expansion of the ductal elements of the breast, a glove of hormonally-responsive intralobular stroma is also formed around the individual TDLUs. This stroma, in turn, produces additional paracrine factors recognized to be essential for complete maturation of human TDLUs.[110, 214] Continued development and maturation of the female breast is recognized to occur for at least 10 years following the onset of puberty.[214]

Even after puberty, other recurring morphological and physiological changes occur within the constitutive cell populations of the breast in accordance with the various stages of the menstrual cycle. In the typical 28-day menstrual cycle (with onset of menses denoted as Day 0), the menses stage (Days 0-3) is generally recognized to be the stage with the lowest proliferative activity in the mammary gland.[86, 156, 214, 272] In contrast, the stage of the menstrual cycle with the highest proliferative ability remains a subject of debate. At least one study suggests the highest proliferation rates occurs in the early-follicular (proliferative) stage of the cycle (Day 3-7)[178] while other studies have identified the late-follicular stage (Day 8-14)[97, 214] and the luteal stage (Day 15-20)[170, 214, 272] to have the highest amount of cellular growth. While these results appear to contradict one another, it is proposed that most of the confusion generated by this research has occurred due to measurements of different epithelial cell subpopulations within the mammary gland.[214] In short, what is evident from the combined results of these studies is that both the alveolar and ductal epithelial populations of the human mammary gland undergo profound proliferative changes throughout the menstrual cycle. Further support as to a cyclic variation within the mammary gland epithelium is provided by observations that the number and pattern of cells immunoreactive for sex hormone receptors is highly variable with the stage of the menstrual cycle. Per this parameter, the early-follicular stage of the cycle has the highest number of estrogen receptor (ER)-positive epithelial cells while the late-follicular stage of the cycle has the highest number of progesterone receptor (PR)-positive epithelial cells.[41, 214]

Pregnancy is another period of significant change to the mammary gland, with the most notable modifications occurring in nulliparous females undergoing a first full-term pregnancy. In nulliparous women,

pregnancy-associated breast development first begins with the transformation of large numbers of TEDs into Type 2 TDLUs.[110, 214, 217, 218, 221] This occurs through the extension and branching of ductules into the intralobular stroma and through alveolar formation due to rising concentrations of progesterone. Later, as systemic levels of prolactin increase, the Type 2 TDLUs differentiate into their lactating phenotype, known as Lobule type 3/4 or Type 3 TDLUs through the proliferation of small ductules and alveolar epithelium (alveologenesis). Ultimately, each typical Type 3 TDLUs will contain 80 or more alveoli.[26, 108, 110, 214, 219-221] While differentiation of the TEDs and Type 2 TDLUs occur to some extent in all pregnancies, these processes do not occur evenly throughout the breast. Consequently, not all available TDLUs within the breast are utilized for lactation and some TEDs are maintained throughout each pregnancy, although with each successive pregnancy fewer and fewer TEDs are present within the breast.[26, 108, 110, 214] At weaning, the systemic prolactin levels drop and the breast undergoes involution of large numbers of alveoli and intralobular ductules through apoptosis, phagocytosis, and desquamation of cells into their respective lumens. Following involution, the majority of Type 3 TDLUs typically assume a Type 2 TDLU morphology.[110, 214, 221]

Cellular constituents of the mammary gland. The bulk of the mammary gland is composed of populations of terminally-differentiated myoepithelial and luminal epithelial cells that are thought to be highly limited in their proliferative capacity. However, within the breast there also exist at least some persistent and highly proliferative cells, as confirmed by the dynamic ability of the mammary gland to repeatedly lactate and involute through multiple pregnancies.[123, 270] These proliferative cells are recognized to be populations of adult stem cells and progenitor cells.[139, 185, 208]

Per the most-widely accepted definition, adult stem cells are multipotent, proliferative cells that are capable of both symmetrical and asymmetrical cell division (Figure 4). Through symmetrical division, adult stem cells produce two exact copies of themselves and thereby maintain or promote the proliferative capacity of the organ. Through asymmetrical division, adult stem cells produce one stem cell and one daughter-cell. The daughter-cells, in turn, undergo either partial differentiation to serve as proliferative progenitor cells or terminal differentiation to serve as the individual functional units of the organ.[123, 139, 146, 243]

The existence of an adult stem cell in the mammary gland, the mammary gland stem cell (MSC), has been firmly established for both rodents and humans utilizing in vivo methodologies. Specifically, murine transplantation studies have produced fully functional glands from a single mammary gland cell[230, 243] while human X-chromosome,

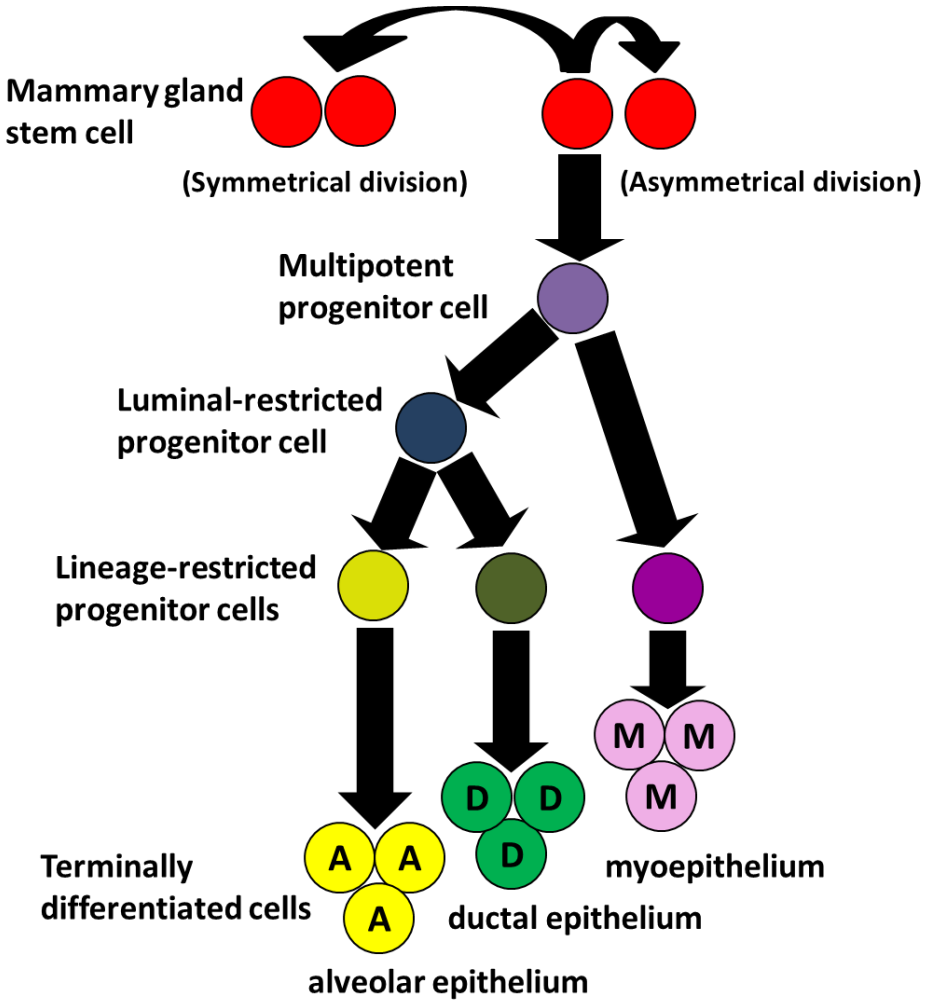

Figure 4. Symmetrical and asymmetrical division of the mammary gland stem cell and the proposed cellular hierarchy present within the glandular cells of the human breast. Nine distinct cell populations have been identified.

gene-inactivation studies of the breast have established that individual TDLUs are clonal in origin.[262] In addition, in vitro work has further substantiated the existence of MSCs. In these studies, individual proliferative cells obtained from the rodent and human mammary gland have been demonstrated to be capable of producing large, mixed-colonies composed of ductal epithelium, alveolar epithelium and myoepithelium when grown as cell cultures.[71, 139, 230, 243]

In addition to MSCs, a variety of other proliferative cell populations, the progenitor cells (PCs), have also been identified to exist within the mammary gland. While the exact number and organization of these cell populations is still debated, current research suggests that there are no less than five unique mammary gland PC-types which can be arranged into a hierarchy as depicted in Figure 4.[20, 139, 243, 270, 271] One of these PC populations,

known as multipotent progenitor cells (MPCs), is able to produce mixed-colonies composed of ductal epithelium, alveolar epithelium and myoepithelium similar to those seen with the MSCs. Unlike the MSCs, however, this population is unable to self-renew and is therefore more limited in its proliferation capacity. As such, MPCs form only small outgrowths when transplanted into cleared rodent mammary gland-fat pads or when plated as cell cultures. Another of these PC populations has been shown to be capable of forming bilineage colonies composed of both ductal epithelium and alveolar epithelium. These cells are referred to as the luminal-restricted progenitor cells. The remainder of human PCs are more restricted in their colony-forming abilities and only produce a single differentiated-cell type, that being either alveolar epithelium, ductal epithelium, or myoepithelium. These three PC-types, in turn, are collectively referred to as lineage-restricted progenitor cells.

There is evidence that while MSCs appear to be responsible for initiating breast development and maintaining the proliferative capacity of the mammary gland, the PC populations likely contribute to the bulk of the routine glandular proliferation. This is proposed to be the case, in that MSCs are estimated to make up only 0.01-0.03% of the cells within the human breast and therefore appear too limited in distribution to account for all of the diffuse proliferative changes that occur with pregnancy and throughout the menstrual cycle.[139, 243, 270] Other research suggests that while MSCs are currently recognized to be long-lived cells within the mammary gland, it is likely that in vivo these cells persist, for the most part, as non-proliferative cells in a state of cellular quiescence.[17, 20, 139, 208, 238, 243, 270, 271, 282]

Breast Cancer

Breast cancer incidence rate and classification. The term "breast cancer" is frequently used to describe a multitude of different benign and invasive neoplasms affecting the breast, although for clinical and epidemiological purposes the use of the term breast cancer is often reserved to refer to more invasive forms of the disease.[297] Accordingly, the use of the term "breast cancer" in this review will be limited to refer to the more refined definition of invasive disease. As men account for only around 1% of all breast cancer cases worldwide there is much less is known about the disease in men and, as such, this review of will focus only on mammary gland disease as it occurs in women.[111, 213]

As per the National Cancer Institutes-Surveillance, Epidemiology and End Results (NCI-SEER), it is estimated that within the United States approximately 1 in 8 women will be diagnosed with breast cancer during her lifetime and 1 in 35 will die as a direct result of the disease.[111] Human breast cancers are frequently classified by

their histological appearance, their hormone-receptor status, and as to whether or not they overexpress the Human Epidermal Growth Factor Receptor-2 (HER-2) gene. More than 99% of the histologically-confirmed cases of breast cancer within the United States over the last decade have been diagnosed as carcinomas. Over 97% of these carcinomas have, in turn, been characterized to be of an epithelial (luminal) phenotype with only a small percentage classified as myoepithelial (basal) in origin. Of the luminal carcinomas, approximately 71% were identified as infiltrating duct carcinomas, 11% were mixed infiltrating duct-lobular carcinomas and 8.5% were lobular carcinomas. The remaining 9.5% of carcinomas were classified into a number of other small categories.[111, 270] Around 70% of human breast cancers have been identified to be hormonally-responsive neoplasms, possessing either ER, PR or, more typically, both.[27, 169, 192, 270] Notably, breast cancers that lack hormone-receptor expression tend to be more clinically and histologically aggressive in nature than tumors demonstrating ER and/or PR positivity. Finally, overexpression of the HER-2 genes has been identified in approximately 30% of human breast cancers and, when found, is frequently associated with more invasive forms of the disease.[192, 250]

Breast cancer cellular composition. Human breast cancers, like most other solid tissue tumors, are frequently comprised of a phenotypically and/or genotypically heterogeneous mixture of neoplastic cell types.[62, 68, 270, 280] While most breast cancers contain large numbers of neoplastic cell types that are incapable of significant independent proliferation, these tumors also typically contain at least a small population of cancer cells that are highly proliferative.[5, 62, 198, 271, 280] Investigations into the proliferative cell populations of breast cancers have, in turn, shown that the bulk of the proliferative neoplastic cells are fairly limited in their ability to differentiate. This has been best demonstrated in transplantation experiments where the tumors formed by these proliferative cells were identified to be composed of only one (or a few) of the cell-types found within the original cancer.[5, 6, 44, 62, 68, 72, 146] However, there are also other, smaller populations of proliferative cells identified within most breast cancers that have the ability to produce tumors in mice which fully recapitulated all of the heterogeneous cell types found within their tumors-of-origin. In light of these proliferative and multipotent differentiation capabilities, this last cell population has been termed the cancer stem cells (CSCs).[5, 6, 62, 68, 299]

Cancer stem cells in breast cancer. Cancer stem cells are currently of great interest to breast cancer research for a number of reasons. First, as per their proliferative and multipotent differentiation capabilities, the CSCs are proposed to represent the archetype cell for their respective cancers and, to that, are thought to be the primary neoplastic cell population responsible for the overall growth and maintenance of the cancers from which

they are derived.[5, 36, 68, 72, 105] Second, in that research has suggested that many CSCs are resistant to anchorage-dependent apoptosis (anoikis) and could be capable of surviving as single cells in vivo, it is also proposed that CSCs play a significant role in cancer metastasis.[68, 127] Third, as there are data to suggest that CSCs are more resistant to the effects of irradiation and/or chemotherapeutics as compared to the more-differentiated cells of the breast cancer, the CSCs have been likewise proposed to be the primary cell population responsible for cancer recurrence.[6, 12, 68, 174, 198, 282, 293, 299] Finally, there are breast cancer studies which have correlated an increased ratio of CSCs to a higher (more malignant) histological grade.[43, 79, 84, 196, 271] In short, this information suggests that increased knowledge of the CSCs in general is likely to be beneficial toward developing effective preventative, diagnostic, prognostic and therapeutic methodologies for breast cancer patients in the future. To that, identifying the normal mammary gland cell populations which most-commonly give rise to the CSCs and elucidating the mechanistic changes that govern the formation of the CSCs is likely to provide valuable insight toward these goals.[36, 68, 72, 79, 105, 196, 237]

Although the origin of the mammary gland cancer CSCs is currently unknown, there is evidence to suggest that CSCs often arise as a result of neoplastic transformation of the MSCs. In women it has been demonstrated that ductal carcinomas most frequently arise within the TEDs of the breast, the lobule-type known to contain the highest numbers of MSCs.[27, 154, 216, 220] Rodent studies have likewise identified the terminal end buds (TEBs), the anatomical structure of the rodent mammary gland with the highest concentrations of MSCs, to be the primary site of carcinogenesis.[49, 115, 220, 222, 223] As is further described in the *Breast cancer risk factors* section below, lifestyles associated with decreased numbers of TEDs/MSCs (e.g., multiparity) and lifestyles associated with a decreased time-of-retention of undifferentiated-TEDs/MSCs (e.g., early age at first full-term pregnancy) are also both associated with a decreased risk for developing breast cancer. Additional support for an MSC-origin to the CSCs is the finding that MSCs and the breast cancer CSCs share a number of cell surface markers that are not readily identified on most of the more-differentiated cell types within the normal mammary gland.[6, 62, 71, 84, 108, 128, 147, 153, 270] Finally, the finding that MSCs and breast cancer CSCs tend to show similar patterns of expression in cell signaling pathways such as Notch, WNT, hedgehog, Bmi-1 and TGF-beta also lends credence to the proposal that MSCs are a common cell-of-origin for the CSC.[5, 27, 72, 84, 123, 152-154, 230, 282]

Neoplastic transformation of the MSC has, in turn, been proposed to involve an initiation event followed by sequential promotion and progression stages as is classically described for the multistage process of carcinogenesis of more differentiated cells.[259] Critical to the discussion of MSC carcinogenesis, however, is that the normal cellular

fate of MSCs is recognized to be highly dependent on paracrine communication with the glandular and stromal cells of the immediate niche. That is to say, signaling factors excreted by the cells of the niche determine if MSCs will remain quiescent, undergo symmetric cell division, or undergo asymmetric division with differentiation.[26, 123, 139, 270] As such, while genetic mutations to the MSCs are thought to be the most likely initiating event toward neoplastic transformation, it has also been proposed that initiation of the MSCs could arise as a result of genetic and/or epigenetic alterations occurring to the cells constituting the niche. Studies which have demonstrated the transforming effect of the aberrant niches on both CSCs and normal stem cells are highly supportive of this position.[139]

Regardless of how initiation occurs, the initiated MSCs are hypothesized to follow one of three main carcinogenesis pathways:[71, 74, 133, 158, 160, 209, 260] 1) an initiated MSC may produce daughter-cells that are capable of undergoing terminal-differentiation down only one line of its multipotent capabilities; 2) an initiated MSC may produce daughter-cells that are incapable of undergoing complete terminal differentiation down any lineage; or 3) an initiated MSC may undergo only symmetrical division. Examples of the potential pre-neoplastic and neoplastic end-products for each of these pathways are provided below. Collectively, these three pathways are thought to be able to account for essentially all of the phenotypical manifestations of breast cancer as it is currently known to occur in humans. The fact that MSCs are generally recognized to be long-lived provides additional support to these hypothesized carcinogenesis pathways, as significant periods of time would likely be required for the initiated MSC to acquire the multiple changes needed to achieve complete carcinogenesis.[44, 71]

Per the first pathway, a MSC is initiated in such a way that when it undergoes asymmetrical division only one type of terminally-differentiated cell is produced, regardless of the needs of the tissue. Additional alterations to the initiated MSC, or its niche cells, over time could promote the initiated MSC to acquire other neoplastic properties, such as a loss of contact-inhibition, which would thereby result in tumor formation of the MSC progeny. Further alterations to the MSC or niche could then lead to the production of cells with an increased neoplastic potential and so on. In this scenario, clonal-cell aggregates from each pre-neoplastic/neoplastic step of the MSC carcinogenesis process accumulate within the tissue leading to a breast cancer composed of a highly heterogeneous cell population. As such, this pathway provides a means by which to account for the well-established biologic continuum of benignancy-to-malignancy that is frequently identified within many breast cancers.[6, 18, 99, 105, 146, 160, 209,

[238, 280] This proposed mechanism also helps address a common question as to how the sex hormone-receptor negative MSCs and MPCs might be able to produce breast cancers that are predominantly hormonally responsive.[27]

Per the second pathway, a MSC is initiated in such a way as to produce daughter cells that are unable to undergo complete terminal differentiation. As with the first pathway, additional alterations occurring to the initiated MSC or the niche over time could then lead to tumor formation, although in this scenario the neoplasm would be comprised of PCs or PC-like cells with more proliferative potential. As these tumors could theoretically be derived from any PC population within the cellular hierarchy, this pathway can account for the variation in histologic appearance, sex-hormone status and biological aggressiveness that exists between breast cancers. Similar to what was described above, further alterations occurring within the initiated MSC or niche over time could ultimately result in the development of PCs with greater neoplastic potential and to the formation of a breast cancer comprised of a highly heterogeneous cell population. While all of the alterations leading to breast cancer in this pathway could occur within a single MSC, another possible outcome is that one of the partially-transformed PCs derived from the MSC could, itself, become further altered. In this instance, the PC would then ultimately become the cell-of-origin for the CSC within the breast. This is mentioned as a possibility in that some breast cancer CSCs have been observed to have variable expression of the MSC-associated cell-surface markers CD24, CD29, CD44, and CD133, and the early PCs (e.g., MPCs and luminal-restricted PCs) have been proposed as a possible alternative cell-of-origin.[27, 79, 114, 123, 196]

Per the third pathway, a MSC is initiated in such a way as to only undergo symmetrical cell division. Additional mutations to the MSCs or the niche could then promote the development of MSC-rich tumors while further malignant transformation could ultimately result in the formation of a CSC with only marginal differentiating capabilities. In this pathway all of the neoplastic transformation could occur within a single cell, although it is equally possible that any one of the cells in the pools of identically-initiated MSCs could ultimately become a breast cancer CSC. With increased numbers of initiated stem cells, the third pathway might be expected to produce tumors with higher rates of promotion and progression than tumors originating through the first two pathways. Additionally, in that the CSCs of this scenario have minimal potential for differentiation, it might also be anticipated that the aggressive and metastatic potential of cancers originating via this pathway would be greater as well. This is stated as such, in that that most of the neoplastic cells originating by the third pathway would be CSC-like cells and theoretically retain anchorage independence and the capacity for marked proliferation similar to that of the MSC.

Finally, it is plausible that breast cancers derived directly from the MSCs would be more likely to be sex hormone-receptor negative. Consistent with these lines of thought, highly aggressive and rapidly metastasizing breast cancers have frequently been identified to contain higher ratios of CSCs and these breast cancers are commonly negative for sex hormone receptors.[27, 79, 196, 297]

Breast cancer risk factors. Only 5-10% of breast cancers diagnosed within the United States have been identified to be hereditary in nature.[213] Mutations within the breast cancer susceptibility genes BRCA-1 and BRCA-2 are the two most commonly identified genetic alterations in people and, by some estimates, can increase the risk of breast cancer in women by as much as 40 percent over those of the general population.[27, 297] Of note here though, is the fact that BRCA-related breast cancers are much more likely to have a myoepithelial phenotype and/or lack sex hormone receptors than are non-hereditary forms of the disease.[27, 155, 297]

With regard to the most-common (hormonally-responsive), non-hereditary forms of breast cancer in women, there are a number of factors that have been associated with an increase in the lifetime risk for developing the disease. These factors include: increased-age at first pregnancy; nulliparity; early menarche; late menopause; total-attained-number of menstrual cycles; oral contraceptive use; estrogen-progesterone hormone therapy following menopause; total-attained age; obesity; and exposure to radiation.[7, 26, 27, 38, 82, 112, 131, 132, 140, 162, 164, 175, 199, 220, 224, 258, 294, 297] Furthermore, primiparous women have also been estimated to have an approximate 10% higher long-term risk of developing breast cancer than multiparous women.[7, 162] Of all these factors, increased-age at first pregnancy is widely recognized to be the most significant risk factor related to the development of breast cancer in the general population of women within the developed world.[6, 27, 65, 130, 140, 172, 220, 270]

While the details of just how each of these risk factors plays into breast cancer carcinogenesis is currently unknown, it is notable that many can be directly linked to prolonged exposure to sex hormones. To expand on this point, the risk factors of early-menarche, late-menopause and total-attained-number of menstrual cycles, all increase the number of endogenous sex hormone fluctuations that a woman experiences over her lifetime. On the other hand, oral contraceptive use and hormonal therapy in post-menopausal women both have the potential to artificially increase the total-attained-number of menstrual cycles through exogenous mechanisms. Irrespective of the source, sex hormones are recognized mitogens that are known to contribute to the malignant transformation of DNA-damaged cells.[27, 38, 130, 213] This occurs as sex hormones directly, or indirectly, stimulate cells to increase their rates of proliferation to such a point that some mutated cells are able to bypass the cellular safeguards of DNA repair and/or

avoid apoptosis.[46, 69, 88, 92, 103, 115, 116] Through this process, the misrepaired or unrepaired DNA mutations then become "fixed" within the genome leading to formation of "initiated" cells. Sex hormones can also contribute to the progression of carcinogenesis by stimulating the initiated cells to proliferate and produce pools of mutated progeny which, along with the originally-initiated cell, are then available for further neoplastic transformation.[213]

The data above also suggest that maturation of the breast through pregnancy, especially an early-life pregnancy, is likely to reduce the overall risk of breast cancer formation in women. These human data are supported by numerous studies in rats and mice which have also demonstrated a protective effect of pregnancy on the mammary gland. In these experiments the mammary glands of parous rodents, and rodents that were experimentally "matured" through the use of exogenous hormones, were shown to be less susceptible to the effects of chemical carcinogens than were the glands of nulliparous animals.[27, 101, 176, 220, 234, 249, 256] Although just how pregnancy confers protection remains uncertain, some hypotheses propose that the reduced risk is, at least in part, due to decreased numbers of MSCs within the breast.[27, 65, 213, 215, 270] As previously discussed, the nulliparous breast is comprised of large numbers of TEDs which are proposed to contain large numbers of MSCs.[154, 216, 220] During a woman's first pregnancy the majority of the TEDs within the breast are recruited to terminally-differentiate into a lactational phenotype. With each successive pregnancy, more and more TEDS are recruited from the reserves, leading to smaller numbers of MSCs within the breast as a whole. While the ratios of MSCs between nulliparous and multiparous women have not yet been directly compared, some studies performed in mice have demonstrated significantly more functional MSCs in nulliparous mice as compared to multiparous animals.[235] Likewise, studies in the rat have also documented that cells obtained from the parous mammary gland have less overall potential for proliferation than do those acquired from nulliparous glands and suggests that lactation may decrease the numbers of MSCs in the mammary gland of this species as well.[27]

Radiation-induced Breast Cancer

Ionizing radiation as a carcinogen. Exposure to ionizing radiation is a well-established cause of breast cancer in women.[4, 35, 47, 135, 142, 213, 271] Historically, the majority of the carcinogenic effects of radiation on mammary gland cells have been thought to occur as a result of direct genetic damage.[159, 160, 245] More recently, however, it has been appreciated that epigenetic modifications of the genome also play into the development of many radiation-induced cancers.[48, 66, 115, 159, 160, 261, 271] These epigenetic changes are found to occur through the direct exposure of DNA to radiation but also can result from cellular signaling between radiation-affected and unaffected cells within

the same microenvironment through a process that has been termed the non-targeted "bystander effect".[48, 66, 159, 160, 226]

Regardless of which type of radiation-induced damage is initially sustained, exposures to ionizing radiation followed by, or in conjunction with, factors that promote cellular proliferation are now understood to be significantly more oncogenic than are exposures to ionizing radiation alone.[115, 213, 245] This is supported by epidemiological studies which have identified an increased risk of breast cancer in women who were exposed to radiation and were likewise identified to have elevated levels of endogenous hormones.[115] Additionally, rodent studies have also demonstrated that the incidence of radiation-induced mammary tumors could be increased if irradiation of animals was followed by exogenous hormonal stimulation.[213] The exacerbation of radiation-induced damage through increased proliferation is believed to occur by the same mechanisms as previously described in the *Breast Cancer Risk Factors* section above.

Ionizing radiation and risk factors for breast cancer. Utilizing data obtained from the medical establishment and atomic bomb survivors, it has been well-established that the risk of developing breast cancer after radiation exposure is inversely correlated with age.[4, 35, 47, 135, 142, 257] The population of females most frequently reported to be at the greatest risk are those individuals who were exposed to radiation at less than 20 years of age. Likewise, the linear dose response to ionizing radiation has also been determined to be most pronounced within this same demographic of women.[213]

In addition to a decreased-age-at-exposure, other risk factors have also been identified to be significant with respect to the development of radiation-induced breast disease in humans. In particular, women who are exposed to radiation and then remain nulliparous or give birth to their first child late-in-life are known to be at greater risk than are similarly-exposed women who undergo a first full-term pregnancy at a younger age.[32, 35, 141, 172, 213] A decreased risk of radiation-induced breast cancer has also been independently associated with both multiparity and prolonged lactation where radiation exposure occurred at a young age, and either the multiparity or the prolonged lactation-history occurred before a woman reached 20 years of age.[141]

Interestingly, these risk factors for radiation-induced breast cancer are similar in nature to many of those described for the hormonally-responsive breast cancers in the general population. As such, a role for MSCs in radiation-induced disease is also possible in that, with age, and with each successive pregnancy, fewer and fewer MSCs are thought to be present within the breast.[117, 220, 282] Furthermore, as previously described with hormonally-

responsive breast cancer in the general population, the risk factors described here also implicate exposure to sex-hormones as critical to the development of radiation-induced disease. Taken together, these risk factors could be interpreted to suggest that the incidence of radiation-induced breast cancer is correlated with: 1) the number of MSCs present within the breast at the time of exposure; 2) the duration of time for which MSCs are maintained as undifferentiated cells following irradiation; and 3) the number of proliferative events (e.g., menstrual cycles) a woman undergoes following irradiation.[220, 282] In support of a MSC-origin to radiation-induced breast cancer, one rat study has demonstrated that radiation-induced mammary cancers in rats most commonly arise in the TEBs, an area known to be high in MSCs.[49] In support of the idea that a reduction in the MSC numbers is related to a decreased risk of mammary gland cancer, another rodent study has shown that hormonally-induced terminal differentiation of the mammary gland immediately following irradiation resulted in a decreased incidence of tumor formation.[115]

While it cannot be said with certainty that MSCs are the cell-of-origin for most radiation-induced breast cancers, there are aspects of MSC biology that make them an attractive candidate toward this hypothesis. MSCs are currently thought to be long-lived and have a high proliferative potential. The longevity of these cells provides the intrinsic means by which further progressive and promotional oncogenic events of the initiated cells could occur, as radiation-induced breast disease is appreciated to often takes decades to manifest itself.[27, 146] The high proliferative potential of the MSCs, in turn, provides the intrinsic methods by which mutations could be fixed into the genome and then passed on to progeny.

Additional information to suggest that MSCs may be common cells-of-origin for radiation-induced breast disease comes from studies looking at the response of these cells to irradiation. Research utilizing human breast cancer cell lines and mouse mammary cell lines has shown that the MSCs, as well as some MPCs, are more resistant to radiation-induced death, apoptosis, and senescence than are the more-differentiated cell types within these cell cultures.[27, 40, 125, 198, 293] These results have been used to propose that MSCs are more resistant to the lethal effects of radiation than are other cell populations of the breast and therefore are more likely to accumulate radiation-induced mutations. Finally, as previously discussed, MSCs are known to be highly influenced with regard to proliferation and differentiation by the cells comprising their niches. To that, it has been proposed that MSCs may be more prone to carcinogenesis through non-targeted, bystander effects originating from irradiated niches than would other, more-differentiated, cells of the mammary gland.[26, 27, 123, 139, 270]

Animal Models in Breast Cancer Research

Historical perspective and future needs of laboratory animal models for breast cancer research. Relevant animal models of human breast cancer are crucial to understanding the complex interplay of genetic, epigenetic, and molecular alterations that lead to the neoplastic transformation of mammary gland cells. To date, the bulk of this work has been accomplished through the use of mouse models and, to a lesser extent, rat models. The importance of the rodent contributions to the overall understanding of the cellular mechanisms associated with mammary gland cancer cannot be overstated. However, the relevance of some rodent-based research findings to the human condition have been brought into question based on two main issues: 1) the distant phylogenetic relationship between rodents and man; and 2) the fact that the majority of spontaneously-occurring rodent mammary gland tumors do not morphologically or physiologically resemble the most common forms of breast cancer in women. [33, 100, 255]

With the creation and use of genetically modified animals and other contemporary research techniques, investigators have been able to produce mammary tumors in rodents that are more representative of the human disease. However, the failure to recognize molecular changes in humans that have been identified as critical toward the initiation and/or progression of mammary cancer in some genetically-altered mice continues to raise concerns as to the overall utility of these models toward the study of human breast cancer.[33, 100]

While rodents will almost certainly remain essential toward the study of breast cancer, the use of adjunct animal models are likely to prove useful in areas of research where the rodent models have shown to lack relevance. Consequently, the ideal adjunct breast-cancer animal models would have a closer phylogenetic relationship to humans, they would have a reproductive morphology and physiology more similar to women, and they would develop spontaneous mammary gland disease that is more representative of human breast dysplasias and cancers.

Genetics and relatedness of nonhuman primate animal models. The rhesus macaque (*Macaca mulatta*) monkey has been found to share ~93% of its total genetic sequence identity with humans.[95] Furthermore, the rhesus macaque is identified to have ~97% average orthologous similarity to humans at both the nucleotide and amino acid sequence levels. In contrast, the orthologs of humans and laboratory mouse (*Mus musculus*) share only ~85% and ~88% average nucleotide and amino acid sequence similarity, respectively.[42, 95, 276]

The rhesus macaque and cynomolgus macaque (*Macaca fascicularis*) are closely-related nonhuman primate species and both have been widely utilized in reproductive physiology research.[1, 25, 122, 166, 189, 190, 244, 279]

These two monkey species can interbreed to produce fertile offspring and by some genetic analyses demonstrate only 0.34-0.40% sequence divergence between their genomes.[295] This is notably similar to the 0.31% sequence divergence that has been identified to exist between the Chinese-origin and Indian-origin rhesus macaque-subspecies, and is likewise similar to the 0.40% variation that can exist between individual humans.[295] Due to their marked interrelatedness and the fact that the morphology and physiology of the rhesus and cynomolgus macaque mammary glands have been identified to be nearly identical, the generic term "macaque" will be utilized throughout the remainder of this review when referring to mammary gland information pertinent to both species.[50, 56, 291] Differences between the two species will be directly stated, where known to exist, and the generic term "macaque" will not be utilized herein to refer to other species of monkey from the genus *Macaca*.

Morphology of the human, macaque and rodent mammary gland. At the gross anatomic level, humans and macaques both have one pair of mammary glands overlying the thorax, with the bulk of the glandular tissue within each mammary gland located cranial and lateral to the nipple.[56] Rodents, by comparison, have numerous pairs of mammary glands organized into chains along the ventrum that extend from the thorax to the pelvis, with the largest of these glands located adjacent to the inguinal area.

At the subgross level, the macaque and human mammary glands are once again similar while, as before, both are conspicuously different from those of rodents. In humans and macaques, each mammary gland is composed of multiple lobes that are largely independent of adjacent lobes with each lobe communicating to the external environment through its own individual duct and secretory pore.[56] In contrast, mice are described to have unilobular mammary glands as there is less physical and physiological separation between the individual lobules and all ductules within a single mammary gland converge into a single large duct prior to joining the secretory pore.[253]

At the microscopic level, there are also distinct differences in the rodent gland as compared to those of the human and macaque.[56, 263] In particular, while macaques and humans have large distinct clusters of alveoli at the distal aspects of the TDLUs, rodents lack TDLUs and instead have small glandular arrangements known as alveolar buds located over the entire ductal system.[27, 270] Furthermore, the prominent intralobular layer of fibroblastic cells that surround and provide critical molecular signals to the TDLUs of humans and macaques is not present in rodent species.[110, 237] Finally, the cytokeratin profile and hormonal expression of the macaque and human mammary gland are also identified to be highly similar to one another, while rodent glands have been specifically noted to be substantially different with regard to these parameters.[45, 50, 53, 55, 263]

Development and physiology of the human, macaque and rodent mammary gland. Studies looking into the development of the macaque mammary gland have identified the response of proliferating and differentiating cells to exogenous hormones to be nearly identical to those of the human, whereas there are notable differences to the mammary gland development in mice.[55] As one example, the role of prolactin on the mammary gland in humans and macaques appears to be almost wholly limited to the initiation and maintenance of lactogenesis, while in mice, prolactin is known to be essential to early developmental steps of the gland.[129, 134, 279] Additionally, the expression of ER and PR within the cells of the developing macaque mammary gland are nearly indistinguishable from what is currently known to be the case for women.[13, 56, 110, 288]

Even beyond the developmental stages, there remain stark differences between the mammary glands of rodents as compared to those of humans and macaques. This is proposed to be in large part due to the marked physiologic variations in the type of female reproductive cycles that exists between rodents and those of humans and macaques.[244, 279] Specifically, mature rodents undergo a 4-5 day estrus cycle throughout which they retain a functional corpus luteum. As a result of the intact corpus luteum, rodents also maintain significant levels of serum progesterone throughout their entire cycle. Humans and macaques, in contrast, both share a 25-35 day menstrual cycle in which the corpus luteum undergoes regression in the absence of pregnancy. Resultantly, the progesterone levels in these species tend to fluctuate to a greater extent than in rodents.[41, 253] Additionally, macaques and women are known to have equivalent serum levels of circulating estrogen throughout the phases of the menstrual cycle and both have been labeled as "high-level estrogen secretors".[21] Female rodent levels of systemic estrogen, on the other hand, are generally considered low, reaching just 10-16% of the serum concentrations found in macaques and humans.[179, 253] Finally, the proliferative responses of the macaque mammary gland to pharmacological estrogen and progesterone compounds is essentially identical to what is known to occur in women and provides yet another example of the similarities in mammary gland physiology between the two.[52, 54, 289-291]

Consistent with the similarities between the human and macaque reproductive cycles and sex hormone levels, macaques have been identified to have profound proliferative cyclic changes to both the ductal and alveolar mammary gland epithelium throughout the menstrual cycle.[41, 244] This is in contrast to those of the rodent estrus cycle where proliferation is currently recognized to be almost entirely limited to the alveolar epithelium.[237] Further analysis of the macaque mammary glands have also demonstrated significant differences in the expression of ER and PR throughout the reproductive cycle expression that are similar to those reported to women.[41]

With regard to lactogenesis, the morphological and physiological changes that occur in the macaque TDLUs throughout pregnancy, lactation, and involution are essentially identical to those of humans.[56, 291] In contrast, most changes to the rodent gland during lactogenesis are focused on the alveolar bud structures and are more limited with regard to ductal proliferation.[56, 291] Further research has also shown that the increase in glandular tissue and volume of milk produced, on a per-body-weight basis, is highly similar between humans and macaques.[56] Other studies have identified the composition of milk from humans and macaques to be highly similar, while the milk mice produce is known to be substantially higher in protein.[157, 225]

Spontaneous mammary gland disease in nonhuman primates. A number of case reports and reviews have documented the occurrence of spontaneous mammary gland lesions in a variety of nonhuman primates with most describing hyperplastic and neoplastic changes remarkably similar to those identified in humans.[9, 15, 59, 93, 98, 113, 161, 180, 240, 284, 291, 296] Rhesus macaques and cynomolgus macaques account for most of these cases, with a variety of baboons (genus *Papio*) and lesser numbers of other monkey species making up the remainder of the reports. The numbers of cases identified for each species is largely in line with the popularity of these animals as research models and, as such, macaques are not necessarily thought to be predisposed to mammary gland disease over other monkey species.

With regard to macaques in particular, a number of reports have also noted the occurrence of spontaneous ductal hyperplasias similar to those found in humans. While spontaneous malignant neoplasms in the macaque mammary gland have been documented more frequently than have hyperplasias, this is thought to be due to a reporting bias. This view is supported by the observation that hyperplasias of the macaque mammary gland have been identified with much more frequency than have cancers when groups of older study-animals were systematically examined for such lesions.[50, 252, 266] Specifically, the occurrence of ductal dysplasia, to include both the common and atypical form, has been reported to be around 3% in juvenile macaques in one study, and ranges between 6-40% in breeding-aged macaques.[50, 252, 266, 291] The variation in the data obtained from the older monkeys is due, in large part, to the fact that while some studies examined only a few histologic slides of the mammary gland for evidence of hyperplasia, other studies evaluated entire mammary glands as whole-mount preparations. Research projects examining human breast tissue from age-demographics of women that are analogous to the aforementioned macaque studies are rare. However, one project utilizing reduction mammoplasty tissues identified ductal dysplasia to be present in ~20% of women.[75] In comparison, the occurrence of spontaneous mammary gland dysplasias in

rodents is highly variable with regard to the rodent strain examined and, unlike humans and macaques, the dysplasias in these species tend to be alveolar in origin.[33, 107]

The majority of the spontaneously-occurring, macaque mammary gland cancers identified in the literature were classified as infiltrative ductular carcinomas (IDC) (n=17) and ductal carcinoma in situ (DCIS) (n=11), while a smaller number (n=3) were classified as lobular carcinoma in situ (LCIS).[59, 60, 113, 143, 240, 291] There was also a single case of squamous cell carcinoma and a single case of malignant myoepithelioma of the mammary gland described, while the remainder of the cases were reported simply as either carcinoma (n=4) or adenocarcinoma (n=1). These findings are consistent with those of the human disease, in that nearly all of the cancers were classified as carcinomas, and also in the fact that ductal disease predominated over lobular disease in these animals. This is in contrast to findings in the mouse and rat, where alveolar (lobular) carcinomas and fibroadenomas are the most common spontaneous tumors of the mammary gland, respectively.[33, 100, 107, 255]

Additional findings from macaques that correlate well with the human data include an increased incidence of mammary gland lesions with age, and the observation that metastasis of mammary cancer to the lymph nodes is common.[59, 60, 113, 143, 240, 291] Of note here is that while the incidence of mammary gland cancer in rodents is also identified to increase with age, the lung is the primary site of mammary gland metastasis in rodents. Finally, in some of the macaque case reports, the mammary gland carcinomas were treated surgically and/or medically. In these cases, the treated macaques were noted to have a similar disease progression, recurrence and prognosis as might have been expected of humans with similar forms of the disease.[59, 113, 240]

In the most exhaustive examination of spontaneously-occurring, macaque mammary gland cancers to date, two D.V.M. pathologists and one M.D. pathologist jointly characterized the mammary gland lesions of 35 monkeys and identified a number of similarities between the macaque and human conditions.[291] Akin to what has been found in humans, some macaque mammary cancers demonstrated a continuum from atypical ductal hyperplasia to DCIS to IDC. Furthermore, with regard to DCIS, four out of the five major morphological classes of human DCIS were also identified within the 35 macaques examined here. The IDCs of these monkeys also correlated well histologically with those seen in humans, were describable using human classification schemes, and ranged from low- to high-grade morphology. Further examination of these lesions utilizing immunohistochemical stains yielded even more similarities between macaque and human mammary gland disease and included: 1) a significantly increased expression of the proliferation marker Ki67/MIB1 in cancerous tissues over hyperplastic lesions; 2) expression of

sex steroid receptors in 60% of carcinomas; 3) expression of HER-2 in 40% of carcinomas; 4) selective loss of sex hormone receptors and increased expression of the HER-2 in higher grade carcinomas; and 5) loss of E-cadherin expression in LCIS lesions.[233, 250, 291] Of note here is that, in contrast to these macaque findings, the vast majority of spontaneously-occurring rodent mammary tumors do not match up well with the human classification schemes, are hormone independent, and lack relevant HER-2 expression (other than a few transgenic animal models).[33, 107, 108, 200]

In addition to its other contributions, the aforementioned study on spontaneously-occurring macaque mammary gland cancer was also significant in that it was the first published report to propose an incidence rate for mammary gland cancer in these species. While historically, mammary gland cancer has been considered a rare disease of macaques, this group reported a 6.1% incidence rate based on data obtained from a population of aged, control animals located at a single institution. Although this number is lower than the ~12% incidence rate of breast cancer found within the general population of the United States, this number is in agreement with the 4-8% incidence rate proposed to be the case for lower-risk populations of women.[87] The comparison of the incidence rates in these macaques to those of the lower-risk populations of women was reasoned to be appropriate, in that the animals utilized in this study were not obese, and nearly all of these animals were either ovariectomized or multiparous (all three factors associated with a lower incidence rate of breast cancer in women). To date, there have been no other reports utilizing similarly aged-macaque populations to challenge the incidence rates proposed by this group. The reasons as to the anecdotal underestimation of spontaneously-occurring mammary gland disease in macaques prior to the publication of this paper are proposed to be multifactorial and include: 1) most macaques that are utilized in research are young animals and therefore most are not alive past middle-age when the incidence of mammary gland disease has been shown to be highest for these animals; 2) many of the macaques that are kept for lengthy research projects are ovariectomized in order to minimize hormonal fluctuations (an intervention that is also likely to reduce incidence of mammary gland disease); 3) the vast majority of macaques that are kept for long periods of time are breeding-colony animals and the females in these colonies are often multiparous and are very young when bred for the first time (both factors of which are likely reduce their incidence of mammary gland disease); and 4) historically, most macaques that are kept as breeding animals never undergo exhaustive post-mortem examinations to include a detailed inspection of the mammary glands.[251, 291]

Hormone-induced mammary gland disease in nonhuman primates. Numerous hormonal-replacement studies in macaques have demonstrated equivalent physiological responses to those identified in women, while at

the same time, many of these same studies have also produced large numbers of mammary gland lesions in the macaque.[51, 52, 54, 55, 59, 83, 90, 244, 251, 252, 265, 266, 287, 289-291] Of the studies for which the data have been best characterized, long-term hormone therapy in macaques has produced hyperplastic lesions with incidence rates 3-4 times those found in controls.[252, 266] Other research has documented DCIS and ductal carcinoma incidence rates between 9-15% in hormonally-treated animals. [252, 265] The fact that some of these studies utilized hormone doses similar to those routinely prescribed to women, and that the macaque cancer incidence rates then met or exceeded those of the general human population, lends further credence to the proposal above that macaques may have lower spontaneous incidence rates of mammary gland disease due to a decreased risk-exposure. The hyperplastic and neoplastic lesions identified in these macaques were almost entirely ductal in origin, similar to what was described above in the spontaneously-occurring mammary gland cancers of macaques. Additionally, when cancerous lesions were identified in these animals, nearly all of these were reported to be carcinomas. Finally, in these reports, most cancer metastases were found within the lymph nodes and the majority of the mammary gland cancers that were examined using immunohistochemical techniques were identified to be ER-positive.[83, 291]

Animal models of radiation-induced breast carcinogenesis. Of the numerous carcinogens utilized toward the study of breast cancer in laboratory animals, ionizing radiation is one of the few that is also known to be a significant cause of cancer in people.[30] Additionally, unlike most chemical carcinogens, ionizing radiation has also been shown to produce a wide spectrum of pre-neoplastic and malignant mammary gland lesions in some animal models that are comparable to progressive stages of human breast disease.[2, 30, 81, 108, 115, 151, 174, 185, 232, 264]

Rodents have contributed greatly to the understanding of radiation-induced disease in general. However, the use of these animal models toward the study of radiation-induced, human breast carcinogenesis is proposed to be less than ideal for several reasons.[115, 245] At the forefront of these concerns is the fact that most radiation-induced mammary gland tumors of rodents are phenotypically different from the most common radiation-induced cancers of humans. Similar to what was described for the spontaneously-occurring mammary gland cancers, rats frequently develop fibroadenomas and adenocarcinomas of the mammary gland following irradiation, while most mouse models of radiation-induced disease develop alveolar-centric cancers. Also similar to what was previously described, only a very few strains of rodents routinely express sex hormone receptors within their respective, radiation-induced mammary gland tumors.[100, 115, 181, 255] Radiation-induced breast cancer in humans, by contrast, is most often identified to be hormonally-responsive ductal carcinomas.

Another issue with the use of rodents in radiation-based research is that the data as to the rodent demographics with the highest-risk of developing radiation-induced mammary gland disease is not entirely consistent with the human findings. As an example, some studies have reported adult rats to be more susceptible to the development of radiation-induced mammary gland cancers than are younger animals.[115, 213] This has led researchers to speculate that there may be significant differences in the cell populations and/or molecular pathways susceptible to radiation-induced disease between rodents and humans.[30, 117] Specifically, it has been proposed that radiation-induced mammary gland carcinogenesis in the rodent species is likely associated with the TEB-to-alveolar transition phase of mammary development, while the ductular-expansion phase of mammary development is proposed to be the most radiosensitive phase in women. The predisposition of the rodent alveoli to radiation induction is further supported by studies in which pregnant and/or lactating rats were found to be significantly more susceptible to mammary gland cancer development than were other demographics of animals.[115, 213]

One last issue with the use of rodents in radiation-based research arises from the observation that, unlike humans, parity does not appear to be protective of radiation-induced mammary gland disease in the rodent.[115, 213] This information is especially disconcerting in light of the fact that parity is well-recognized to be protective of mammary gland cancers induced by a variety of chemical carcinogens.[27, 65, 101, 115, 176, 220, 234, 249, 256] The full implications of these findings are unknown, but again suggest that different cell populations and/or physiologic mechanisms may underlie mammary gland disease in rodents as compared to humans.

The rhesus macaque has been used with some frequency in radiation-based research,[11, 16, 30, 37, 64, 78, 109, 184, 186, 187, 204, 241, 269, 286, 292] although little is known about the long-term effects of irradiation on the macaque mammary gland due to several factors. First, much of the macaque-based radiation research to date has been focused on the acute effects of high-dose, total body irradiation and, as per their design, these studies have not contributed any information as to the long-term effects of radiation on the macaque mammary gland.[16, 184] Second, even where macaques have been kept for longer periods of time following exposure to high-dose, total body irradiation, there are essentially no mammary gland cancers reported for these animals. Critical to the interpretation of this information, however, is the knowledge that much of this long-term data was acquired from follow-up studies on macaques that survived an initial high-dose, total body irradiation experiment[29, 30, 109, 286, 292] while other long-term data has come from high-dose, total body irradiation research that concentrated on individual organ systems other than the mammary glands.[11, 64, 186, 187] In short, the data as to the long-term radiosensitivity of the mammary gland

may be confounded by the fact that information was only acquired from the individual animals that were most resistant to the effects of acute, high-dose total body irradiation and also by the fact that none of these long-term projects were mammary gland-specific research. This last piece of information being important as essentially nothing is described in these studies as to the means by which the mammary glands were examined for pre-neoplastic/neoplastic disease and it is plausible that mammary lesions could have been overlooked if a thorough gross dissection was not performed or if the microscopic examination of the mammary glands was based only on a few histological slides rather than whole-mount sections. Finally, even where long-term macaque studies have been performed using lower doses of radiation that may have been more conducive to mammary carcinogenesis,[37, 78, 204, 241, 269] there are only a few cases of mammary gland cancers reported.[37, 78] Importantly though, most of these studies utilized focused irradiation techniques that greatly limited secondary exposure to the breast and there was essentially nothing mentioned in these reports as to the means by which the organ systems, outside their own interests, were examined for neoplastic growths.[204, 241, 269]

What is known about rhesus macaques and irradiation is that the acute response of these animals to high-dose total body irradiation has been identified to be essentially identical to those of the human with regard to the gastrointestinal and hematopoietic systems.[16, 109] Furthermore, it has also been reported that the long-term, excess relative risk for cancer development following X-irradiation is similar between humans and macaques.[16, 109] Finally, while the numbers are few, there are at least two reports related to the development of mammary gland cancer in the rhesus macaque following irradiation. In the first case report, a single animal was exposed to fractionated total-body irradiation at 0.25 Gy every-other-week for over two years (20 Gy total). The animal then developed mammary gland ductal carcinoma with lymph node metastasis within a year of completing irradiation.[37] In the second case report, a single rhesus macaque was administered routine estrogen injections for a three year period prior to having a radioactive silver (110Ag) disk implanted in the subcutaneous region of the skin overlying the mammary gland. Three years following the implantation, the animal developed a carcinosarcoma of the mammary gland adjacent to the site of disk. Of note here is that none of the other monkeys entered in this same estrogen trial study were exposed to radiation and none of the other animals developed breast cancer.[78]

In summary, in spite of their potential limitations, rodents continue to be the primary animal models utilized for the study of radiation-induced mammary disease of humans due to the fact that no better animal models have yet been identified. While information related to radiation-induced mammary gland cancer in macaques is

minimal at this time, there is no definitive data to suggest that macaque mammary glands are resistant to radiation-induced carcinogenesis. To that, investigations into macaques as a radiogenic model of human breast cancer are warranted given the similarities between macaques and humans with regard to genetics, reproductive physiology, and mammary gland-disease and due to their similar acute and long-term responses to irradiation.[245]

Acquisition of Mammary Gland Stem Cells and Progenitor Cells for Research

Enrichment and isolation of MSCs and PCs. Due to the possibility that MSCs and/or PCs are common cells-of-origin for breast cancer CSCs, there is currently much interest in acquiring these cell populations from the mammary gland for study. Through the use of fluorescence-activated cell sorting (FACS), many different human and murine mammary gland cell populations have been isolated and examined. The sorting of these cells has come primarily through the use of a variety of cell-surface markers, namely CD24, CD29, CD31, CD44, CD45 and CD49, although a number of other cell-identifying qualities have also been utilized such as: aldehyde dehydrogenase activity; retention of the label bromodeoxyuridine; and efflux of Hoechst 33342 dye.[6, 27, 71, 96, 127, 147, 230, 232, 239, 243, 254] While several independent studies using different FACS-gating techniques have purported to isolate populations of MSCs, there have been major phenotypical and physiological discrepancies described between cells isolated by the different groups.[2, 79, 108, 127, 144, 150, 206, 230, 243, 293] Based on these findings, it has been proposed that different subpopulations of MSC-like cells may coexist within the breast and that the subpopulations acquired using these techniques likely represent different states of activation of the MSCs at the time of isolation.[79, 293] Accordingly, there is currently no consensus as to definitive FACS biomarkers for human or murine MSCs and, at best, FACS can only be said to "enrich" for MSC-like cells.[230] While the use of these cell sorting techniques is useful toward the isolation of highly specific cell populations from the breast for study, it is also possible that this level of specificity could limit our understanding of breast carcinogenesis as a whole. This is because breast cancer CSCs have been demonstrated to exhibit a variety of surface markers and thus breast cancer CSCs may be derived from more than one subpopulation of MSCs and/or even some early PC-populations.[71, 79] As such, if research becomes too focused on just one, or a few, subpopulations of proliferative cells then it is entirely possible that biomedical research could miss out on relevant data toward carcinogenesis if the FACS-selected cell populations are not inclusive of all of the MSC/PC-types that are readily susceptible to neoplastic transformation.

A cell culture method for the acquisition of MSCs and PCs from mammary tissues that is less limited in its selection of proliferative cell-types than FACS has been developed and is likely to prove useful toward breast cancer

research.[71] The nonadherent mammosphere culture technique (hereafter referred to as "mammosphere culture") utilizes the stem cell properties of anchorage-independence, apoptosis resistance and high proliferation index to isolate MSCs, and some less-differentiated (early) PCs, from the bulk of the mammary epithelium.[71, 74] In short, primary mammospheres are created as mammary gland tissue is mechanically and enzymatically dissociated into single-cell suspensions, and then placed into serum-free media within ultralow attachment cell-culture plates. In the absence of serum and with no surface to attach to, the more-differentiated mammary epithelial cell types in the plates undergo anchorage dependent apoptosis (anoikis), while the MPCs, and some early PCs, persist and proliferate to form free-floating spherical colonies. Secondary mammospheres can then be created from these primary cultures by mechanically and enzymatically dissociating the primary mammospheres back into single cell suspensions, and then allowing the cells to form spheres once again in serum-free media within ultralow attachment plates. This same process can then be repeated to create tertiary mammospheres and so on, as desired. The rationale behind creating the secondary mammosphere cultures and later-stage cultures is that some research suggests that with each passage of the sphere-forming cells there are higher ratios of MSCs in the cultures as the PCs undergo senescence due to their limited proliferative ability.[71, 74, 155]

Another possible advantage of mammosphere cultures over FACS-based methods is that the cell suspensions used for mammosphere cultures do not require the addition of antibodies, reagents or dyes to obtain the cells-of-interest. While most of these cell-markers are generally considered to have negligible physiologic effects on the cells to which they are applied, there are minimal experimental data to confirm these assumptions. As such, there is a possibility that at least some of these reagents could confound study results through unidentified alterations in cell function.[79, 139]

Mammosphere cultures in research. The use of mammosphere cultures toward the isolation of MSCs and PCs has been well characterized using human breast tissues.[71, 74, 139, 155, 185, 226, 270] In these studies, mammospheres were routinely grown and examined as both primary and secondary cultures. The identity of the cells comprising individual spheres was largely determined by growing the cells in a variety of culture conditions, and then examining the outgrowths for differentiation utilizing immunohistochemical staining methodologies. One study found that around 68% of primary mammospheres obtained from humans were able to undergo multi-lineage differentiation, and thus, were proposed to be of MSC or MPC origin, while the remaining spheres were identified to be more lineage-restricted in nature.[71] Nearly all (around 98%) secondary mammospheres in this study were

identified to be capable of multi-lineage differentiation.[71] Additional work demonstrated that when single cell isolates were plated in low cell densities, the vast majority of primary and secondary spheres in these cultures were clonal in origin.[43, 71, 74, 85] These same studies, and others, further demonstrated that most mammospheres derived from low-density platings contained, on average, 1.3 sphere-reforming cells (the MSC) per sphere while the remainder of the cells comprising these spheres appeared to lack characteristics of MSCs.[71, 79] Together, this information suggests that dissociated cell suspensions produced between the primary and secondary mammosphere cultures provide a means by which highly-enriched populations of MSCs can be obtained.[71, 74]

Mammosphere culture techniques have also been utilized to evaluate the murine mammary gland with some frequency. The results from most of these studies are comparable to those of humans with regard to: 1) the ratios of the MSCs identified within the mammary gland (0.02-0.07%); 2) the clonal identity of low-density derived mammospheres; and 3) the numbers of multi-lineage and lineage-specific sphere types found in primary and secondary cultures.[26, 138, 146, 147, 172, 236, 243]

Summary and Research Outline

Breast cancer remains a prominent cause of cancer-related morbidity and mortality of women in the United States and relevant animal models are still needed toward the study of this disease. Macaques are more similar to humans than are laboratory rodents with regard to genetics, mammary gland morphology and reproductive physiology. Additionally, macaques have been shown to spontaneously develop pre-neoplastic lesions and mammary gland cancers similar to those of humans whereas most rodent models do not. Finally, the incidence rate of mammary gland disease within some populations of research-macaques has been identified to be similar to those of the low-risk human population in the United States.

Mammary gland stem cells and, to a lesser extent PCs, continue to attract attention as possible cells-of-origin for breast cancer CSCs. Research animals that can provide MSCs and PCs that are most similar to those of humans are arguably the models most likely to advance the science in this area. I propose, based on the cumulative information provided above, that carcinogenesis studies utilizing macaque-derived MSCs and PCs are likely to be more relevant to human breast disease than are identical studies in which the MSCs and PCs are acquired from laboratory rodents.

An ideal modeling system for breast cancer carcinogenesis would, amongst other factors, be one that could: 1) obtain cells-of-interest from a wide range of age-demographics; 2) allow longitudinal studies within individuals;

and 3) reduce the number of experimental confounders. The collection of mammary gland tissue from humans is limited by ethical concerns and it is notable that the vast majority of the human tissues utilized for research are obtained as a byproduct of reduction mammoplasties. As such, collection of mammary gland from adolescent females, a life-stage of great interest to carcinogenesis research, is rarely performed and longitudinal studies of breast changes in individual women are all but impossible.[22] The clinical and family histories of women undergoing reduction mammoplasty procedures are frequently incomplete and other variables, such as diet and lifestyle choices (e.g., smoking), that may affect cancer risks are largely unknown.[22] Finally, as the different phases of the menstrual cycle are appreciated to lead to significant morphologic changes in the cells comprising the human mammary gland,[86, 97, 156, 170, 178, 214, 272] it is plausible that variations in the collection-time between individuals, with regard to the phase of the menstrual cycle, could confound comparative studies. In contrast, mammary gland biopsies can be easily collected from macaques at any age and biopsies can also be collected from the same animal at multiple time points throughout its life. Furthermore, as the medical histories and pedigree of these animals are generally well known, confounders such as numbers of pregnancies and familial histories of cancer can be accounted for while other research variables, such as diet and lifestyle choices, are essentially not an issue. Lastly, the timing of mammary gland biopsy collection to specific stages of the menstrual cycle in the macaques is easily accomplished and, in theory, can further reduce variation between the biopsy samples. Based on this information, I propose that carcinogenesis studies utilizing macaque-derived MSCs and PCs have the possibility of producing more significant data with regard to human breast cancer than identical studies in which MSCs and PCs are acquired from highly-variable human tissue sources.

There are currently no published data to indicate that MSCs/PCs have yet been isolated from any nonhuman primate species. I therefore undertook an aim to develop and validate a mammosphere culture technique for the rhesus macaque with the goal of obtaining MSCs and PCs for use in biomedical research. I describe this process in detail in Chapter 2. In Chapter 3, I describe a cytology method that I validated in the rhesus macaque to aid timing of mammary gland collections to a specific stage of the menstrual cycle.

As fundamental differences are known to exist between the nulliparous and multiparous mammary gland, I undertook an aim to quantify, characterize and compare the MSCs/PCs collected from both nulliparous and multiparous macaques. I describe the findings from these studies in Chapter 4.

Ionizing radiation is an established cause of breast cancer in women. Despite the fact that macaques have been described to be similarly susceptible to the effects of radiation as humans, basic data as to use of these animals for studies on radiation-induced breast cancer is lacking. Therefore, I undertook an aim to examine the effects of ionizing radiation on MSCs/PCs collected from both nulliparous and multiparous female macaques which is also described in Chapter 4.

In conclusion, there is evidence to suggest that macaques are relevant animal models for the study of human breast cancer and that use of these animals could open up avenues of research that are currently inaccessible using tissues derived from either humans or rodents. The work included in this dissertation represents the development of novel experimental techniques in macaques that are applicable toward the study breast cancer and also provides baseline data on the macaque mammary gland that are essential to the continued use of these animals in research. This work also represents the first descriptive and comparative experiments performed on mammary gland stem cell populations from any nonhuman primate species and, beyond the information gained from these studies, serves as a foundation on which future nonhuman primate-breast carcinogenesis studies can be based.

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Chapter 2: Development and Validation of a Novel Model for Breast Cancer Research

Introduction

Breast cancer remains a significant cause of mortality for women in the United States with the majority of these deaths attributable to recurrence or metastasis.[6, 111] Most breast cancers are composed of highly heterogeneous neoplastic cell populations.[6, 62, 68, 73, 270] Contemporary evidence suggests that a small subpopulation of the neoplastic cells within breast tumors known as the cancer stem cells (CSCs) represent the archetype cell for each cancer. In light of their multipotent potential, the CSCs are considered to be the cell population most likely responsible for cancer recurrence and metastasis.[6, 44, 62, 73, 74, 146, 280, 299] Hypotheses as to the origin of the CSCs propose that these cells frequently arise from mutations to the mammary stem cells (MSCs) and/or multipotent progenitor cells (MPCs) that exist within the normal mammary gland.[73, 196, 209, 260] This has sparked interest into the acquisition of these two particular cell populations for study. While there are currently no methods available by which to obtain pure populations of MSCs or MPCs, a number of cell markers and functional assays have been utilized to sort cells obtained from mammary gland digests to enrich for these specific cell populations.[67, 79, 84, 96, 147, 196, 206, 230, 243]

A cell culture technique, commonly called mammosphere culture, has also been extensively utilized to obtain similarly-enriched cell populations from the mammary gland from both humans[67, 71, 73, 85, 196, 206, 231] and rodents.[147, 208, 293] Briefly, single-cell suspensions obtained from digested mammary glands are placed in specialized, serum-free media and are cultured in ultralow attachment plates to take advantage of the fact that MSCs and some MPCs are identified to be anchorage independent cell types.[68, 74] Under these conditions, the MSCs and some MPCs proliferate as free-floating spheres (mammospheres). In contrast, the vast majority of the other, more-differentiated, mammary cell types acquired from the tissue digests undergo anoikis (anchorage-dependent apoptosis). Mammosphere studies have demonstrated that when these initial (primary) mammospheres are dissociated back into single-cell isolates and allowed to proliferate under the same conditions, secondary mammospheres are formed. These secondary mammosphere cultures have, in turn, been identified to be composed of populations of cells that are even more highly enriched for stem cell-like cells than are the primary mammosphere cultures.[71]

Historically breast cancer research has relied heavily on rodent models for the study of breast cancer. Rodents may not be ideal research models however, as spontaneously-occurring mammary gland cancers in these species rarely resemble the most-common forms of human breast cancer.[33, 100, 255] Equally disconcerting is the fact

that even when rodents can be manipulated through experimental techniques to develop cancers more representative of the human disease, there still exist significant genetic, physiologic, and morphologic differences between humans and rodents.[33, 100] These differences have brought into question the relevance of diagnostics and treatments developed in rodent models that are to be utilized for human breast disease. Human breast tissues, typically obtained as byproducts of reduction mammoplasties, have also been utilized with some frequency in the study of breast cancer. However, there are limitations to the usefulness of the human-derived tissues as well. These include the lack of availability of samples and the fact that there is an inherent variability between individual donors as a result of environmental, genetic, and life-style differences.[22] Other limitations associated with the use of the human derived tissues are the limited demographic age-range of the women undergoing reduction mammoplasty and, logically, the inability to perform longitudinal carcinogenesis studies on the individuals themselves.[22]

Rhesus macaques (*Macaca mulatta*) have mammary glands that are morphologically and physiologically similar to humans.[56, 288] Additionally, although female rhesus macaques do undergo a seasonal period of reproductive inactivity, during their reproductively-active times of the year these animals undergo a regular menstrual cycle that is similar in length and form to that of the human.[10, 31, 50, 63, 244, 267, 279] Recent investigations suggest that some macaque species have breast cancer incidence rates similar to those of certain human populations.[291] It was also reported in these same studies that the preneoplastic and neoplastic mammary gland lesions of most macaques were comparable to those of humans. As macaques are common research animals, large in size and long-lived, they are likely to prove useful toward the study of breast cancer. Despite their potential, the utilization of these animals in breast cancer research is still limited, in large part, due to the fact that little work has been done to validate commonly used research techniques for these species.

This study describes the modification of the mammosphere culture technique for use in the rhesus macaque and also describes the first mammosphere studies performed using nonhuman primate tissues. One major aim of this project was to optimize a mammosphere culture technique for the rhesus macaque. The hypothesis being that viable and reproducible mammosphere cultures could be obtained from this species. Minor goals associated with this first aim were to collect mammary gland weights for the species and also to investigate whether or not the mammosphere potential was similar throughout the entire macaque mammary gland. A second major aim of this study was to validate the rhesus macaque mammosphere culture as a relevant assay by which to study the human breast. The hypothesis being that rhesus macaque mammospheres have morphological and functional characteristics similar to

those described for human-derived mammospheres. A third major aim of the study was to investigate what effect that age, parity-status, and stage of the menstrual cycle may have on mammosphere formation and function. The hypothesis being that at least some of these parameters would have an effect on the numbers and/or function and/or morphology of mammospheres produced in this species.

Materials and Methods

Animal model. Biological specimens were collected from 41 female, Indian-origin, rhesus macaques. Thirty-nine of these animals (Animal #s 1-39 in Table 1) originated from the breeding colony at The University of Texas, MD Anderson Cancer Center, Keeling Center for Comparative Medicine and Research (KCCMR) in Bastrop, TX. Throughout the timeframe of this project, the KCCMR breeding colony maintained an ongoing census of approximately 600 adult female macaques. The KCCMR breeding colony has been a closed colony since 1983 and no outside animals have contributed to the genetic lines since that time. The colony has been documented through serological means to be Specific Pathogen Free (SPF) for Cercopithecine Herpesvirus 1, Simian Immunodeficiency Virus, Systemic T-lymphotrophic Virus and Simian Retroviruses 1, 2, and 5 since 1991. The final two animals included in this study (Animal #s 40 and 41 in Table 1) were originally procured from an external source and were maintained at the KCCMR, in a study setting outside of the breeding colony, for approximately 2 years prior to their euthanasia. These two animals were seropositive for Cercopithecine Herpesvirus 1 at the time of their death but were clinically normal and were SPF otherwise. Signalment and medical history were collected for each study animal. A Body Condition Score (BCS) was also assigned to each study animal based on published BCS parameters for the rhesus macaque.[58] The BSC was a consensus score agreed upon by at least two individuals (veterinarians and/or veterinary technicians) experienced in nonhuman primate work. Information pertaining to each animal utilized in the study is presented in Table 1. The KCCMR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACI) and all animals utilized in this study were housed in full compliance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals* (ILAR).[182] All tissue collection methods used for this study were reviewed and approved through The University of Texas, MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC).

Mammary gland biopsies. In vivo mammary gland biopsies were collected from a total of four animals utilizing techniques similar to those previously described in the macaque.[244] In brief, the animals were anesthetized

Table 1. Macaques collected for the mammosphere studies represented a wide range of demographics. Definition of table terms. *Animal #:* order processed in the study. *Process:* N=necropsy; B=biopsy. *Age*: rounded to nearest quarter year. *Wt (kg):* Body weight in kg. *BSC:* body condition score (as detailed in text). *Class* (parity): N=nulliparous; P=primiparous; M=multiparous. *Stage* (reproductive status per uterus/ovary histology): M=menses phase; F=follicular phase; L=luteal phase; I=inactive phase; P=pregnant or peri-parturient; Cy=polycystic ovaries. *Lact*: (lactating, per gland histology for necropsies or per clinical evidence of lactation for biopsies): Y=yes; N=no. *Fibrous* (qualitative amount of fibrous connective tissue within the mammary gland histologically): 0=minimal; 1=mild; 2= moderate; U=unknown (histology not available). *Infants*: number equals number of live births. *Mam wt (g)*: average weight of each gland for necropsied animals or weight of biopsy for biopsied animals in grams; U=unknown. Animals listed as "opportunistic" were euthanized as part of study that was unrelated to this project.

Animal #	Process	Age (yr)	Wt (kg)	BCS	Class	Stage	Lact	Fibrous	Infants	Mam wt (g)	Relevant reproduction history	Cause of death/euthanisia
1	N	2.25	2.87	3	N	I	N	U	0	1.79	N/A	opportunistic
2	N	17	9.68	3.5	M	P	Y	U	13	10.77	Dam and full-term fetus died at parturition	parturition
3	N	14	5.76	3	M	L	N	U	4	5.18	None	trauma
4	N	13	8.95	4	M	P	Y	0	10	11.62	Dam died 1 day postpartum	parturition
5	N	16.5	6.3	3.5	M	I	N	0	12	5.61	None	cancer
6	N	2.5	2.8	3	N	I	N	0	0	2.14	N/A	trauma
7	N	19.25	5.3	2.5	M	I	N	2	10	4.64	None	cancer
8	B	19.5	6.12	3.5	M	U	Y	U	7	0.532	Biopsy 1 month post-weaning	biopsy technique (alive)
9	B	4	5.25	3	P	U	N	U	1	0.398	Biopsy 3 months post-weaning	biopsy technique (alive)
10	N	22.5	6.26	3	M	P	Y	0	11	11.79	Dam died 1 month post-abortion	cancer
11	N	4.5	5.6	3.5	P	P	Y	U	1	U	Dam died carrying second trimester fetus	cardiac disease
12	N	11	5.61	2.5	M	I	Y	0	7	9.53	Foster dam	amyloidosis
13	N	14	4.99	2.5	M	P	Y	0	8	6.74	Abortion of second trimester fetus	liver abscess
14	N	21.25	5.79	2.5	M	I	N	2	12	2.9	None	cancer
15	N	17.5	6.32	3.5	M	F	N	2	12	6.46	None	arthritis
16	N	22.5	5.5	3	M	P	Y	1	16	7.24	Dam and full-term fetus died at parturition	parturition
17	N	6	4	2	P	I	N	2	1	U	None	arthrtitis
18	B	19.75	5.55	3	M	U	Y	U	9	0.776	Biopsy 1 month post-weaning	biopsy technique (alive)
19	B	22.25	5.89	3.5	M	U	N	U	11	1.012	Biopsy 5 months post-weaning	biopsy technique (alive)
20	N	4.25	3.58	2.5	N	L	N	0	0	2.79	N/A	enteritis/arthritis
21	N	12	9.23	4	M	P	Y	1	8	10.98	Dam died 1 day postpartum	trauma
22	N	12.75	7.31	4	M	P	Y	0	8	13.42	Dam died carrying first trimester fetus	trauma
23	N	21	5.49	2.5	M	F	N	1	13	7.08	None	cancer
24	N	19	6.01	3	M	F	N	2	12	4.36	None	amyloid/arthritis
25	N	18	5.76	2.5	M	I	N	2	12	6.12	None	cancer
26	N	8	5.09	3	N	F	N	2	0	2.95	N/A	opportunistic
27	N	3.5	4.44	3	N	F	N	1	0	3.22	N/A	opportunistic
28	N	16	6.65	3.5	M	L	N	2	7	6.84	None	cancer
29	N	19	5.07	2.5	M	P	Y	0	11	6.47	Dam died carrying first trimester fetus	cancer
30	N	20	5.77	3	M	I	N	2	2	5	None	liver abscess
31	N	8	6.8	3.5	M	L	N	0	2	8.12	None	opportunistic
32	N	22.5	5.2	2.5	M	I	N	U	16	4.81	None	opportunistic
33	N	16.5	6.25	3	M	F	N	U	9	4.66	None	opportunistic
34	N	17	5.6	3	M	F	N	2	7	5.92	None	opportunistic
35	N	17	5.5	3	M	M	N	1	11	6.51	None	amyloid/enteritis
36	N	18.75	12	5	M	Cy	N	0	8	U	polycystic ovaries	pneumonia
37	N	10.75	4.3	2.5	M	I	Y	0	4	6.15	None	enteritis
38	N	19.75	6.45	3	M	F	N	1	13	7.8	None	arthritis
39	N	8.75	4.4	2.5	M	L	N	0	4	4.73	None	enteritis
40	N	4.5	5.05	2.5	N	I	N	2	0	3.31	N/A	opportunistic
41	N	5.5	6.15	3	N	L	N	0	0	4.18	N/A	opportunistic

and the skin of the chest was shaved and surgically prepped for the biopsy procedure. Using aseptic surgical technique, a 2-3 cm incision was made in the skin over the craniolateral aspect of one mammary gland. The underlying mammary gland tissue was dissected free from the adjacent soft tissue structures and ligated with suture at the proximal and distal ends. The biopsy was removed and placed into an ice-chilled solution of commercial media (Complete Epicult-B Basal Medium [Human]; StemCell Technologies, Vancouver, BC, Canada)

supplemented with 5% fetal bovine serum (Fetal Bovine Serum-Advantage; Atlanta Biologicals, Flowery Branch, GA) (EB/FBS). The surgical rent in the mammary gland, subcutaneous tissue, and skin were closed with surgical suture. Animals were recovered 3-7 days in the veterinary clinic before being returned to their breeding groups.

Necropsy-derived tissues. Reproductive tract tissues and mammary glands were opportunistically collected at necropsy from select animals that died or that were euthanized at the KCCMR due to illness, trauma, or that were euthanized as part of IACUC-approved research projects unrelated to this study. Uterine and ovarian tissues collected at necropsy were immersion-fixed in 10% neutral buffered formalin (NBF) (LabChem, Pittsburg, PA) for a minimum of 72 hours. Following fixation, the tissues were routinely processed for histologic examination by the KCCMR Histology Laboratory and paraffin-embedded sections were cut at 4 μm and stained with hematoxylin and eosin (H&E; Poly Scientific R and D, Bay Shore, NY). The uterine and ovarian morphology was assessed by two boarded veterinary pathologists to collectively determine the specific reproductive stage for each animal (Table 1) using histologic parameters as previously described.[10, 31, 267] For the collection of mammary tissue, one or both glands were dissected free of the adjacent skin, muscle and adipose tissue and then individual glands were weighed prior to further processing. A portion of the mammary gland tissue was immersion-fixed in NBF and processed for histologic examination, similar to that described for the uteri and ovaries. The remainder of the mammary gland tissue was collected into an ice-chilled solution of EB/FBS and was utilized for studies involving mammosphere optimization, validation, and characterization. Mammary tissues utilized for the mammosphere culture techniques had the initial digestion processes begun within one hour of death and the majority of these tissues were processed within 30 minutes of death.

Optimization study design. While only animals with healthy-appearing mammary gland tissues were selected for inclusion in the study, there was a wide range of demographic variables (e.g., age, parity, reproductive stage, disease state, etc.) across study subjects. In order to minimize the confounding effects of utilizing animals from varied demographics to compare different mammosphere culture techniques, a within-subject approach toward the optimization of the technique was performed. In this approach, multiple aliquots of homogenized breast tissue (defined below) were acquired from one animal. A number of these aliquots were then utilized to explore a single modification to the tissue digestion or cell culture technique. For each experiment all of the variables associated with the procedure remained constant except one (e.g., trypsin digestion time), so that the most productive modification of that one single aspect of the technique could be identified and incorporated into the final protocol. For most

subjects included in the study, there were enough aliquots of mammary tissue acquired from a single animal to allow for exploration of multiple modifications to the mammosphere culture protocol. All experiments investigating the mammosphere culture techniques were performed using at least two aliquots of mammary tissue per modification (e.g., two tissue aliquots from a single animal were utilized to explore each trypsin digestion-time of interest). Also, other than the initial experiments conducted using Animal #s 1-4, each modification to the digestion/dissociation technique was investigated using tissues derived from a minimum of two different animals. As not all aliquots of tissue could be processed simultaneously, intensive efforts were made to rotate the processing order of the individual aliquots in attempts to minimize any effects that time-of-processing may have on the outcome of the experiments. An illustrative example of this within-subject approach is provided in Figure 1.

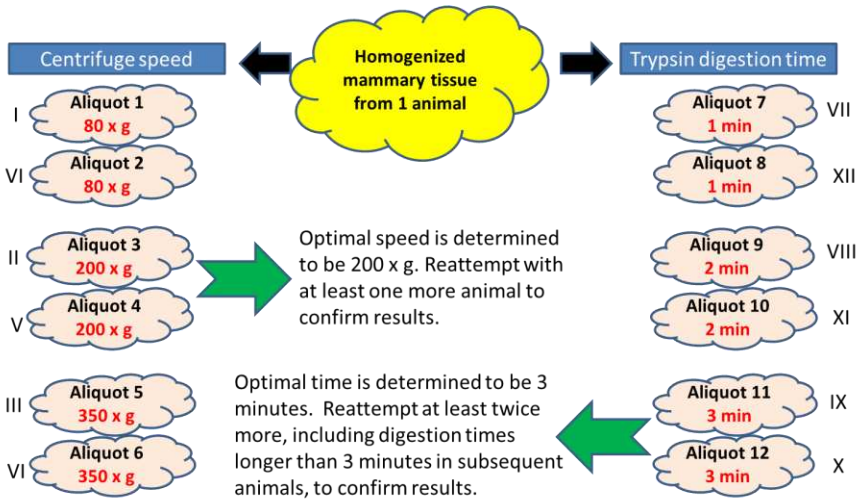

Figure 1. Within-subject approach toward the optimization of the mammary gland digestion and single-cell dissociation. In this example 12 equally-weighted aliquots of homogenized mammary tissue are obtained from one animal. Six aliquots (#s 1-6) are utilized to investigate the effect that various centrifugation speeds have on overall production (all other procedural variables remained constant). The other six aliquots (#s 7-12) are utilized to investigate the effect that variations in trypsin digestion time have on production (all other procedural variables remained constant). Each of these experiments were then repeated using tissues from additional animals to confirm/refute these results. The Roman numerals to the outside of each aliqout represent a hypothetical processing order for each aliqout.

Mammary gland morphology and physiology is dynamic and could vary with anatomic location.[196] Based on this possibility, homogenized mammary gland tissues, rather than individual mammary biopsies acquired from various locations, were utilized in the optimization experiments in efforts to minimize any confounding effects associated with regionalization. The process of mammary gland tissue homogenization is described here in brief. Mammary gland tissues were aseptically collected and trimmed of lymph nodes, muscle, and excess adipose tissue.

The remaining mammary tissue was minced into small pieces (approximately 0.5-2.0 mm^3) in a glass petri dish using scalpel blades. The minced mammary gland pieces were mixed well in attempts to evenly distribute the tissues within the petri dish. Individual aliquots of predetermined weights were collected from the homogeneous mixture for use in the optimization experiments.

The metrics by which modified culture techniques were determined to be either an enhancement or a diminishment of the process are detailed in the *Results* section below. Once a modification to the technique was identified to be advantageous toward mammosphere production, this modification was incorporated into the protocol for all subsequent experiments. When modifications to the technique demonstrated no appreciable improvement, the procedural variation that produced the most rapid results or that was least costly (i.e., utilized the smallest volume of enzymatic solution) was selected for use in the final protocol. This pattern of optimization was continued throughout the study until such time that no relevant improvement in the success of the protocol could be readily detected.

Basic methodology. Animal #s 1-3 were utilized to establish the aseptic mammary gland collection technique, weighing technique, and biopsy technique. Animal #s 2 and 3 were also cultured as mammospheres using protocols and laboratory-prepared solutions as described for the recovery of human mammospheres.[71] This was done as a proof-of-concept study to demonstrate that mammospheres could be readily recovered from the rhesus macaque mammary gland. Published protocols describing the initial digestion methods for human and rodent mammary gland tissue are highly varied within and between species.[43, 67, 71, 85, 106, 147, 153, 196, 206, 230, 243] Therefore, the six most-commonly described initial digestion methods were explored using tissues from Animal # 4. In these experiments multiple 800 mg aliquots of mammary tissue were placed in 25 ml of the laboratory-prepared enzymatic solutions described above and processed as follows: 1) digestion in aluminum foil-covered flasks rotated overnight (16 hours); 2) digestion in aluminum foil-covered flasks rotated for 8 hours; 3) digestion in sealed flasks rotated for 8 hours; 4) digestion in unsealed conical vials for 5 hours with physical agitation every 20 minutes; 5) digestion in sealed conical vials on a vial rotator for 2 hours; and 6) digestion in unsealed conical vials for 2 hours with physical agitation every 20 minutes. The cells derived from the various digestion processes were then cultured as mammospheres using methods as previously described.[71] The use of a mechanical grinder (Stomacher® 3500 series, Seward Lab Systems Inc., Port Saint Lucie, FL), in conjunction with the digestive enzymes, was also explored as a possible method for improving mammary gland digestion.

Mammary gland digestion and single-cell dissociation. A number of different mammosphere protocols originating from both the human and rodent literature were explored in this study in efforts to identify the optimal digestion and single-cell dissociation (digestion/dissociation) technique for the rhesus macaque mammary gland. Commercially-available, human-specific enzymatic solutions and medium were utilized for all mammosphere culture attempts following Animal # 4. Modifications to the mechanical digestion process, digestion times and enzymatic solution concentrations were made throughout these experiments; however, all tissues were processed in the same order and utilized the same enzymatic solutions. The only exception to the prior statement is that Animal #s 1-9 did not include a red blood cell-lysis step. The procedural order and enzymatic solutions utilized for all digestion/dissociation experiments is outlined in the optimized primary mammosphere protocol provided in Figure 2. The cells were confirmed to be present as a single-cell suspension, using techniques as previously described.[71] Total cell numbers and cell viability were determined by the trypan blue exclusion assay using a 0.4% Trypan Blue Solution (Sigma Life Sciences, St Louis, MO) and a hemocytometer. Cells obtained from the digestion/dissociation process were resuspended in a complete mammosphere medium (CMM). The CMM consisted of a commercially-available cell culture medium supplemented with heparin, hydrocortisone, L-glutamine and a penicillin-streptomycin-amphotericin solution as detailed in Figure 2.

Human and rodent mammary gland digestion/dissociation protocols often fail to provide specific ratios of tissue weight to digestion solution. As this information was critical to the optimization efforts here, a quantity of mammary tissue that could be routinely acquired via biopsy from all demographics of female rhesus macaques, 400 mg, was ascertained from the initial methodology and biopsy studies in this project. In turn, the digestion/dissociation optimization procedures undertaken in this project were performed utilizing homogenized aliquots of mammary gland approximately 400 mg in weight, except where specifically stated otherwise. Experiments were performed to identify an optimal initial digestion time for macaque mammary gland tissues and also to identify any differences in digestion times that might exist between the animals from the various demographics. Only the duration of the digestion time varied in these experiments and multiple digestion times ranging from 2-6 hours were explored for each animal. Additional experiments explored the effects of a number of other variables on the protocol including: 1) addition of 5% fetal bovine serum (FBS) to the initial-digestion solution; 2) centrifuge speed of the initial tissue digest; 3) trypsin exposure time; 4) trypsin volume; 5) cell-screen size; 6) use of red blood cell lysis solution; 7) DNase volume; 8) pipette size; and 9) pipette brand.

Rhesus Macaque Primary Mammosphere Culture Protocol

(300-600 mg of homogenized mammary gland processed in a 15 ml conical vial)

Note: Technique can be modified to accommodate larger volumes of tissue as described in the text.

1) Add 300-600 mg of tissue to the initial digestion solution: 7 ml Complete Epicult®-B Basal Medium (Human) (StemCell Technologies (SCT), Vancouver, BC, Canada); 0.7 ml Collagenase-Hyaluronidase Solution (SCT); 70 µl of 10^{-4} M Hydrocortisone solution (SCT); 70 µl 100 X Penicillin-Streptomycin-Amphotericin Solution (MP Biomedicals, Solon, OH); 70 µl 100 X L-glutamine Solution. (GlutaMAX®; Life Technologies, Carlsbad, CA). Cap vial and agitate.
2) Loosen lid and place into 37ºC, 5% CO_2 cell-culture incubator. Optimal digestion times typically vary between 3.0-4.5 hours. At 20 minutes remove the vial and inspect for completeness of digestion (see text). If not digested, tighten lid and thoroughly agitate sample back into solution, loosen lid and replace in incubator. Repeat agitation every 20 minutes until digestion is complete.
3) Once digested, add 7 ml of 1 X HBSS (Sigma Life Science, St. Louis, MO) + 2% FBS (Fetal Bovine Serum-Advantage; Atlanta Biologicals, Flowery Branch, GA) (HBSS/FBS) and centrifuge at 200 x *g* for 5 minutes. Collect the pellet.
4) Add 5 ml of 37ºC, 0.25% trypsin-EDTA solution (Life Technologies) to the pellet and pipette up and down for 3 minutes using a 10 ml pipette (Fisher Scientific, Waltham, MA).
5) Add 5 ml HBSS/FBS and centrifuge 350 x *g* for 5 min. Collect the pellet.
6) Add 2 ml 37ºC, Dispase solution (SCT) and 200 µl DNase 1 (SCT) to the pellet and pipette up and down for 1 minute with a 10 ml pipette.
7) Add 5 ml HBSS/FBS, filter solution through a 70 µm cell strainer and flush strainer with another 5 ml HBSS/FBS. Filter this solution through a 40 µm cell strainer (both Fisher Scientific) and flush strainer with another 5 ml HBSS/FBS. Centrifuged 350 x *g* for 5 min. Collect the pellet.
8) Add 3 ml RBC Lysis solution (SCT) agitate pellet into suspension and let stand for 3 minutes.
9) Add 5 ml HBSS/FBS and centrifuge 350 x *g* for 5 min. Collect the pellet.
10) Resuspend pellet in 1-5 ml of a premade solution of complete mammosphere medium (CMM): 500 ml of Complete MammoCult™ Medium [Human](SCT) supplemented with 1 ml Heparin Solution (SCT); 5 ml of 10^{-4} M Hydrocortisone solution; 5 ml 100 X Penicillin-Streptomycin-Amphotericin Solution; 5 ml 100 X L-glutamine solution.
11) Confirm single-cell suspension and perform cell counts.
12) Plate the cells at the desired concentration using CMM in ultralow attachment plates (Corning, Corning, NY).
13) Seal the plate with paraffin tape (Parafilm M®, Fisher Scientific).
14) Incubate in a 37ºC, 5% CO_2 cell-culture incubator.
15) On day 3 of the incubation process, remove paraffin tape from culture plates. At this same time 80% of the original media is exchanged for fresh CMM in 100k cultures, 2 ml of fresh CMM is added to each well of the 10k well cultures. No media is added or exchanged for the 1k cultures.

Figure 2. Optimized protocol for the digestion and dissociation of rhesus macaque mammary gland tissues into single-cell suspensions and the initial plating protocols for the cells derived from these processes. The optimized conditions recommended within Figure 2 are based on experimental results as detailed within the text.

Following the optimization of the procedure for 400 mg of tissue, studies were then performed to expand the use of this protocol to other weights of mammary tissues. For some experiments, the volume of the digestion/enzymatic solutions utilized remained constant while the weights of the mammary gland aliquots varied. In other experiments, the volume of digestion/enzymatic solutions identified to be optimal for the digestion/ dissociation of 400 mg of tissue was doubled, tripled, and quadrupled for the processing of 800 mg, 1200 mg, and 1600 mg aliquots of mammary gland tissue, respectively.

Primary mammosphere cultures. Primary mammosphere cultures were produced using methodologies similar to those previously described.[71] In brief, on the same day (Day 0) the mammary gland tissue was digested, aliquots of the single-cell suspension were diluted with CMM to create 1 x 10^3 viable cells/ml (1k) and 1 x 10^4 viable cells/ml (10k) suspensions (collectively referred to as low-density mammosphere cultures). Other aliquots of the single-cell suspensions were diluted with CMM to create a 1 x 10^5 viable cells/ml (100k) suspension (high-density mammosphere cultures). Five ml aliquots of each suspension were plated into individual wells of flat bottom, 6-well ultralow-attachment plates (Corning, Corning, NY). The cell-suspensions were then grown for 7 days in a 37ºC, 5% CO_2 cell-culture incubator. The mammosphere-forming efficiency (MFE), defined as the average number of mammospheres (≥ 40 μm in diameter) formed in each well divided by the number of viable cells initially plated per well (expressed as percentage), was calculated for each of the suspensions produced.

Unpublished studies performed in our lab have demonstrated that the use of a conditioned media can promote the growth of rodent-derived mammospheres and therefore the use of conditioned media was also investigated for macaques. Conditioned media, in this case, referring to fresh media supplemented with media obtained from previously-grown mammosphere cultures. Toward the creation of a conditioned media here, all high-density mammosphere cultures (starting with Animal # 11) had 80% of the original media replaced with fresh media on Day 3. For this exchange, the 5 ml of media and cells/spheres from each well were collected into a single conical vial (Polypropylene Conical Tube; BD Falcon, Franklin Lakes, NJ) and centrifuged at 80 x *g* for 1 minute. Four ml of the supernatant was collected and refrigerated at 4ºC for later use (described below). Four ml of fresh CMM was then added to the spheres/cells and each 5 ml aliquot was plated back into a single well of a 6-well ultralow-attachment plate and incubated until Day 7. At Day 7 the mammospheres and media were again collected and centrifuged and the bulk of the supernatant was again retained. The supernatant collected from the high-density primary mammosphere cultures on both Day 3 and Day 7 was combined and centrifuged at 350 x *g* for 10 minutes

and then filtered with a 0.2 μm syringe filter (Fisher Scientific, Waltham, MA). The filtered media collected from each animal, hereafter termed "conditioned media-1" (CM1), was utilized in other aspects of the research project for that same animal.

Optimization experiments performed on the primary cultures included: 1) addition of 2 ml fresh CMM at Day 3; 3) 80% replacement of the original media with fresh CMM at Day 3; and 3) use of plastic paraffin tape (Parafilm M®, Fisher Scientific) to seal the culture plates.

Secondary, tertiary, and quaternary mammosphere cultures. Secondary mammospheres were created from the 10k and/or 100k primary mammospheres as detailed in the optimized secondary mammosphere protocol provided in Figure 3. In brief, primary mammospheres were collected using a 40 μm cell strainer and transferred into conical vials where they were dissociated into single-cell suspensions using a trypsin-EDTA solution. As before, single-cell suspension was confirmed and the total number of viable cells was determined. Five ml aliquots of 1k suspensions and/or 10k suspensions were then plated into individual wells of 6-well ultralow-attachment plates and grown for 7 days in a 37°C, 5% CO_2 cell-culture incubator. Tertiary mammospheres were created from some 10k secondary mammosphere cultures utilizing a similar approach. Likewise, quaternary mammospheres were created using 10k tertiary mammospheres for some animals in the study. The MFE was calculated for each of these suspensions.

Importantly, two independent assays have suggested that the vast majority of human mammospheres have a single-cell origin (are clonal) when the spheres are derived from cultures plated at 1 x 10^3 cell/ml or less.[71] Likewise, other studies have proposed that nearly all mammospheres are clonal in origin when the cell cultures are plated at densities of up to 1000 cells per cm^2.[84] To that, the 6-well plates (surface area/well=9.2 cm^2) and 96-well plates (surface area/well=0.26 cm^2) prepared as 1k cultures for this project were plated with a 1 x 10^3 viable cells/ml suspension at maximum volumes of 5 ml and 0.2 ml, respectively. These cell densities match, or are lower than, those proposed to promote mammosphere clonality for human spheres.

Optimization experiments performed on these cultures included: 1) addition of 2 ml fresh media at Day 3; 2) 80% replacement of the original media with fresh media at Day 3; 3) supplementation of CMM with CM1; 4) use of plastic paraffin tape to seal the culture plates; 5) timing of the primary mammosphere incubation period; and 6) trypsin concentration used for primary mammosphere dissociation.

Rhesus Macaque Secondary Mammosphere Culture Protocol

(5-10 ml of 100k mammospheres or 30-60 ml of 10k mammospheres)

Note: The same protocol is also utilized for later generations of spheres (i.e., tertiary, quaternary, etc.)

1) Collect primary mammospheres on days 6-8 into a conical vial and centrifuge at 80 x *g* for 1 minute. Remove supernatant.
2) Add 2 ml of 37ºC, 0.05% trypsin-EDTA solution to the pellet and pipette up and down using Pasteur pipettes with fire-polished tips for 10-20 minutes until dissociation is confirmed (see text).
3) Following dissociation, add 5 ml of 1 X HBSS + 2% FBS (HBSS/FBS).
4) Filter solution through a 70 µm cell strainer and flush strainer with another 5 ml HBSS/FBS. Centrifuged 350 x *g* for 5 min. Collect the pellet.
5) Resuspend pellet in 1-5 ml of a premade solution of complete mammosphere medium (CMM).
6) Confirm single-cell suspension and perform cell counts. If small numbers of spheres persist, the solution can be passed through a 40 µm cell strainer to remove the intact spheres. If large numbers of spheres persist, the solution can undergo a second trypsin digestion of 5-10 minutes.
7) Plate the cells at the desired concentration using CMM supplemented with conditioned media-1 (see text) at a 4:1 ratio (CMM/CM1) in ultralow attachment plates.
8) Seal the plate with paraffin tape.
9) Incubate in a 37ºC, 5% CO_2 cell-culture incubator.
10) On day 3 of the incubation process, remove paraffin tape from culture plate. At this same time, 2 ml of fresh CMM/CM1 is added to each well of the 10k well cultures. No media is added to the 1k cultures.
11) Replace plate in incubator and leave undisturbed until mammosphere harvest at day 6-8.

Figure 3. Optimized protocol for the dissociation of rhesus macaque mammary mammospheres into single-cell suspensions and the initial plating protocols for the cells derived from these processes. The optimized conditions recommended within Figure 3 are based on the experimental results as detailed within the text.

Mammosphere size and cellular composition. Images of mammospheres in media were obtained using an inverted light microscope (Olympus IX51 microscope, DP70 camera and DP Controller software Version 2002, Olympus Corporation, Waltham, MA) and were measured utilizing imaging software (cellSens imaging software Version 1.5, Olympus Corporation). Additionally, images of individual cells in media that were derived from dissociated 10k mammospheres and images of formalin-fixed, paraffin-suspended, intact spheres were obtained and measured using a standard light microscope and its associated software (Olympus BX41 microscope, DP25 camera, cellSens imaging software Version 1.5, Olympus Corporation).

Mammospheres derived from single cells. Studies have demonstrated that human mammospheres can be produced from a single cell.[71, 85] Experiments were undertaken here to demonstrate that similar results could be obtained for rhesus macaque mammospheres. Primary 10k mammospheres from 4 animals were dissociated into single-cell suspensions. Each of these suspensions were then diluted and plated into 96-well plates using CMM

supplemented with CM1 at a 4:1 ratio (CMM/CM1) to obtain wells which contained single cells. Eleven plates were made for each animal and single-cell dilution was confirmed independently by two investigators. Only those wells confirmed to contain single cells were included in the experiment. A second set of 11 plates were made for each animal using media that lacked CM1. All plates were sealed with paraffin tape for three days. On Day 7, the wells were examined for mammosphere formation and the MFE of each suspension was calculated.

Two-dimensional mammosphere differentiation on collagen. In humans, the ability of low-density spheres to differentiate into both the luminal epithelial and myoepithelial lineages has been used as evidence to support a mammary gland stem cell and/or early progenitor cell origin to these structures.[71] To demonstrate that a similar differentiation capacity existed in the low-density spheres obtained from the rhesus macaque mammary gland, the spheres and media from some of the 1k and/or 10k mammosphere cultures were harvested at Day 7. Following collection, the mammosphere suspensions were centrifuged at 80 x *g* for 1 minute. The media supernatant was removed and the mammospheres were resuspended in 5 ml of complete RPMI 1640 media (Mediatech, Manassas, VA) supplemented with 5% FBS, 1% Penicillin-Streptomycin-Amphotericin Solution (MP Biomedicals) and 1% L-glutamine Solution (GlutaMAX; Life Technologies) (RPMI/FBS). The newly-created mammosphere suspension was transferred into a single well of a standard 6-well tissue culture plate (BD Falcon) or the contents were divided equally into the individual wells of a single multichambered-chamber slide (Fisher Scientific). Prior to deposition of the spheres into the plates/slides, the plates and chambered slides used for these experiments were coated with a bovine collagen solution (StemCell Technologies) as per the manufacturer's instructions. These spheres were then allowed to grow for 5-10 additional days in a 37ºC, 5% CO_2 cell-culture incubator. Cultured this way, the spheroid colonies became adherent to the surface of the wells/slides and the cells of the spheroid colonies underwent differentiation as the colonies spread over the surface of these vessels. At the end of the incubation period, the media was removed and the adherent cells were prepared for visualization with either crystal violet stain (Sigma Life Science) using standard technique or immunohistochemical (IHC) methods as described below. Spheres attached to the surface of the wells were assessed for bilineage (epithelial and myoepithelial) differentiation. The mammosphere differentiation-potential (MDP), defined as the number of bilineage spheres/well divided by the total-number of spheres/well (expressed as a percentage), was the metric used to quantify this assay.

Mammosphere-derived colony forming units. Previous mammosphere-based studies have investigated the growth and differentiation-potential of single cells derived from the dissolution of primary mammospheres.[71, 196]

Similar, although not identical, experiments were performed here. For these experiments 100k primary mammospheres were dissociated into single-cell suspensions as described for generational growth above. However, instead of plating these cells under mammosphere-culture conditions, the cells were diluted with RMPI/FBS into 1 x 10^3 viable cells/ml suspensions. These suspensions were plated in collagen-coated, standard 6-well culture plates or chambered slides as described above for the spheres. The cells were then incubated undisturbed for 3-21 additional days in a 37ºC, 5% CO_2 cell-culture incubator. The resultant cultures were observed directly, stained with crystal violet stain or fixed with NBF for approximately 30 minutes prior to being processed via IHC methods (described below). Individual outgrowths containing more than 50 cells were counted as colonies and these colonies were also assessed for bilineage differentiation. The colony forming efficiency (CFE), defined as the average number of colonies formed in each well divided by the number of viable cells initially plated per well (expressed as percentage), was calculated for each of the suspensions. Additionally, the colony differentiation-potential (CDP), defined as the number of bilineage colonies/well divided by the total-number of colonies/well (expressed as percentage), was also utilized to quantify this assay.

Three-dimensional modeling of mammosphere growth and differentiation. Macaque mammospheres were embedded within basement membrane extract (BME), as previously described.[70] This assay was used to evaluate the three-dimensional growth characteristics of the macaque spheres, similar to what has been undertaken for human and rodent mammospheres.[67, 70, 71, 85] In brief, a commercial BME (BD Matrigel Matrix, High Concentration, Growth Factor Reduced; BD Biosciences, Bedford, MA) was obtained and subsequently diluted with RPMI to standardize the BME to a 12 mg/ml protein concentration. Spheres and media from mammospheres cultures were collected in a conical vial and placed in ice for approximately 15 minutes. The vials were then centrifuged at 80 x *g* for 1 minute and the majority of the supernatant media was removed. A 100 µl aliquot of the mammospheres in media was resuspended in 250 µl of ice-cold BME and then approximately 50-100 µl of the BME-mammosphere suspension was pipetted onto the surface of a well of a standard 6-well tissue culture plate so as to form a convex round "bleb". Frequently, multiple blebs were plated on the surface of the same well and aligned so that the blebs did not make contact with one another. The blebs were then warmed in a 37ºC, 5% CO_2 cell-culture incubator until polymerized. Three ml of RPMI/FBS was added to the wells to completely submerge the blebs. The plates were then incubated for 3-28 additional days in a 37ºC, 5% CO_2 cell-culture incubator. The RPMI/FBS was changed out completely every 6-8 days until the completion of the incubation process.

Mammospheres in BME were observed and imaged directly within the plates using an inverted light microscope or were prepared for histologic examination. Spheres were assessed for the presence or absence of significant ductular-like and/or alveolar-like structures extending from the main sphere body, hereafter referred to as "budding". The mammosphere budding-potential (MBP), defined as the number of budding spheres/bleb divided by the total number of spheres/bleb (expressed as a percentage), was the metric used to quantify this assay.

For histologic examination of the BME-mammosphere blebs, the media was removed from the wells and NBF was added to the wells to completely submerge the blebs. After approximately 1 hour, the formalin was removed and the blebs were carefully lifted intact from the surface of the well using a flat chemistry spatula. A single bleb was encased within 2% high melting-point agarose (Difco Agar Noble; BD Medical Supplies, Franklin Lakes, NJ) which, after solidifying, was embedded in paraffin for histologic processing. Paraffin-embedded serial sections were cut at 4 μm and placed on charged glass slides. The slides were prepared for visualization with H&E stain or periodic acid-Schiff stain (PAS; American MasterTech, Lodi, CA) using standard technique or IHC methods as described below.

Lactogenic differentiation of mammospheres and conditioned media-2. Human- and rodent-derived mammospheres have been demonstrated to produce the milk protein, β-casein, under specific culture conditions.[71, 196, 230] Similar experiments were performed here on BME-encased mammospheres that were cultured in a media supplemented with prolactin (#L4021; Sigma-Aldrich, St Louis, MO) as previously described.[230] However, also crucial to the lactogenic differentiation of macaque mammospheres was the addition of a second substance to the media termed "conditioned media-2" (CM2). Conditioned media-2 was created using the adipose tissue, muscle, and lymph nodes trimmed from the necropsy-derived mammary tissue. Briefly, these three tissues were minced and homogenized on Day 0 in a manner similar to that described above for the mammary gland. An aliquot of this material was mixed with an aliquot of the mammary gland homogenate from the same animal at a ratio of approximately 5:1. Between 1-2 g of this mixed homogenate was then placed in a 100 x 20 mm polystyrene cell culture dish (Corning) with 25 ml of RPMI/FBS and incubated for 5 days in a 37ºC, 5% CO_2 cell-culture incubator. On Day 5, the media was collected and centrifuged at 350 x g for 10 minutes. The resulting supernatant was filtered with a 0.45 μm syringe filter (Fisher Scientific) and was frozen at -80ºC until needed for lactogenic differentiation. As needed, the prolactin-containing media was supplemented with CM2 at a ratio of 4:1 and then 3 ml of the CM2-prolactin media was added to each well to completely submerge the mammosphere-containing blebs. The CM2 was

only utilized in an autologous fashion for these experiments, that is, both the CM2 and mammospheres were derived from the same animal. The CM2-prolactin media was replaced every 5-8 days throughout the incubation process. Mammospheres were prepared for histologic examination (as above) and processed using IHC methods as described below.

Morphologic comparisons. Following the optimization process, mammospheres were cultured from animals representing a variety of different demographics and these spheres were observed for differences in morphology. Specifically, these observations were made on free floating spheres in media and also on spheres that underwent two-dimensional and three-dimensional differentiation. Observations were also made on the colonies of cells produce in the colony forming unit studies.

Quadrant and reproducibility studies. The mammosphere forming potential from different regions of the mammary gland was examined and compared in a number of individual animals. These experiments were also used to examine the reproducibility of the mammosphere culture assay. In these experiments, entire mammary glands were dissected from the animals and individual glands were divided into quadrants (with the teat serving as the quadrant convergence point) based on their anatomical origin: quadrant 1 = craniolateral quarter; quadrant 2 = caudolateral quarter; quadrant 3 = caudomedial quarter; and quadrant 4 = craniomedial quarter. As there was appreciably less mammary gland tissue caudal to the level of the teat in most animals, tissues from the medial aspect of quadrant 2 and the lateral aspect of quadrant 3 were collected as one unit (quadrant 2/3). Three mammary biopsies were acquired from each of the quadrants 1, 2/3, and 4 for both the left and right gland (total = 18 biopsies/animal). Each biopsy was individually minced and processed using the optimized mammosphere protocol. The total number of viable cells recovered per mg of tissue weight (TVC) and the MFE of primary mammosphere cultures were used to assess the individual biopsies.

Immunohistochemical protocols. Plates, chambered slides, and histologic sections of paraffin-embedded BME-mammosphere blebs from a number of animals were processed with antibodies to CD10 (putative bilineage progenitor cell marker and myoepithelial cell marker),[71] smooth muscle actin (SMA; myoepithelial cell marker),[71] cytokeratin 18 (CK18; differentiated epithelial cell marker),[67, 71] estrogen receptor-alpha (ER), progesterone receptor (PR) and β-casein (BC) to assess differentiation of the spheres. These antibodies had been previously validated for use in nonhuman primates and each antibody was confirmed to work here utilizing formalin-fixed, paraffin-embedded macaque mammary gland sections as positive controls. Sections processed without primary antibody

were utilized as negative controls. All formalin-fixed, paraffin-embedded tissue sections utilized in this study were deparaffinized in xylene and hydrated by graded alcohols to water as is standard histologic procedure. Formalin fixed plates and chambered slides were washed twice with 1 x PBS prior to processing.

For CD10, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water followed by a water wash. Antigen retrieval was performed with 10mM Citrate Buffer (pH 6.0) (Polysciences Warrington, PA) in a microwave oven. After cooling and a water wash, non-specific antibody binding was blocked by incubating slides with a blocking reagent (#BS966M; Biocare Medical, Concord, CA). Slides were drained and incubated with CD10 primary monoclonal antibody (sc#80021; Santa Cruz Biotechnology, Dallas, TX) at a 1:100 dilution for 1 hour at room temperature (RT) and then buffer washed. Slides were incubated with biotinylated rabbit-anti-rat IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 30 minutes at RT and then buffer washed. Slides were incubated with streptavidin horseradish peroxidase (SA-HRP) (Biocare Medical, Concord, CA) for 30 minutes at room temperature and then buffer washed. Slides were incubated with 3,3'Diaminobenzidine (DAB) chromogen (Dako Carpinteria, CA) and monitored for stain development. The slides were washed, hematoxylin counterstained, dehydrated, cleared and coverslipped for viewing.

For the CK18, SMA, ER and PR staining protocols, the peroxidase block, antigen retrieval, and non-specific antibody binding blocking steps were identical. For the BC staining protocol the peroxidase block and antigen retrieval were identical but the non-specific antibody binding blocking step was replaced by a 15 minute Tween and Albumin incubation as the blocking reagent contains casein which could interfere with interpretation of BC expression. The remaining steps of each IHC procedure was similar to that described for CD10 other than the specific antibodies and developing reagents required for those protocols. The reagents and specific protocol requirements for these processes are provided. The CK18 protocol included: 1) CK18 monoclonal antibody (#ab55395; Abcam, Cambridge, MA); 1:250 dilution; 1 hour RT; 2) biotinylated rabbit-anti-mouse F(ab)'(Accurate Chemical and Scientific, Westbury, NY); 1:250 dilution; 15 minutes RT; and 3) SA-HRP; 30 minutes RT. The SMA protocol included: 1) SMA polyclonal antibody (#ab5694; Abcam); 1:100 dilution; 1 hour RT; 2) Envision plus labeled polymer, anti-rabbit-HRP (Dako); 30 minutes RT; and 3) DAB and monitored for stain development. The ER protocol included: 1) ER alpha polyclonal antibody (#sc-542; Santa Cruz Biotechnology); 1:500 dilution; 2 hour RT; 2) Envision plus labeled polymer, anti-rabbit-HRP (Dako); 30 minutes RT; and 3) DAB and monitored for stain development. The PR protocol included: 1) PR monoclonal antibody (#ab2764; Abcam); 1:100 dilution; overnight at

4°C; 2) Envision plus labeled polymer, anti-MOUSE-HRP (Dako); 30 minutes RT; and 3) DAB and monitored for stain development. The BC protocol included: 1) β-Casein monoclonal antibody (#MA1-46056; Thermo Fisher); 1:100 dilution; overnight at 4°C; 2) Envision plus labeled polymer, anti-MOUSE-HRP; 30 minutes RT; and 3) DAB and monitored for stain development.

Statistical analysis. Significant results are expressed as mean and standard deviation. Statistical significance for all data was set at $P<0.05$. A univariate analysis of variance (ANOVA) with post-hoc Tukey HSD test was used to compare the mammary gland weights and the MFE of mammary gland quadrants. Demographic comparisons of the sphere/cell sizes, MFE, MDP, CFE, and CDP along with the paraffin-tape study were performed using a t-test (two sample assuming equal variances). Correlations between the individual variables associated with the optimally-processed glands were performed using the Pearson correlation coefficient. These assays, along with the intraclass correlation coefficient (ICC) for mammary gland MFE and the principal component analysis (PCA) of the optimally-processed glands, were performed using IBM SPSS version 22 (SPSS Inc., Chicago, IL) software.

Results

Mammary glands. The four tissue aliquots acquired through the in vivo biopsy technique ranged in weight from 398-1012 mg (Table 1). The remaining tissues in the study were acquired via necropsy. On gross examination, nulliparous mammary glands were typically organized into fairly distinct lobes while the glandular tissue of multiparous animals was more diffusely dispersed across the ventral thorax. Histologically, glandular tissue was identified to be homogenously distributed throughout all lobes in nulliparous animals. In nonlactating multiparous animals, the mammary gland tissue cranial to the level of the teat tended to have the densest and most homogenously distributed glands while mammary tissue caudal to the level of the teat occasionally had glandular regions that were interdigitated with fibrofatty tissues. Nonlactating multiparous glands also commonly had appreciably more fibrous connective tissue and interlobular stroma within the individual glandular units themselves as compared to nulliparous glands. The average weight of each mammary gland was determined for most necropsied animals (Table 1). For animals with a BCS between 2.5 and 4.0, the average weight of the gland was calculated as a percentage of total body weight (%TBW) and compared across demographics. Statistically significant differences in %TBW were identified between nulliparous (0.069 ± 0.003), nonlactating-multiparous (0.093 ± 0.006) and lactating-multiparous (0.145 ± 0.010) animals. The lactating demographic included pregnant, postparturient, and nursing females. The nonlactating-multiparous and nulliparous demographics included animals from all other

aspects of the reproductive cycle (i.e., luteal, follicular and menses stages of the menstrual cycle, and inactive phase). No differences in %TBW were identified between the stages/phases of the reproductive cycle for nonlactating-multiparous or nulliparous animals although only small numbers of subjects per reproductive phase were available for comparison.

Digestion/dissociation optimization. The results of the individual experiments are summarized in Table 2 and the optimized digestion/dissociation protocol obtained from this work is provided in Figure 2. The metrics by which a digestion/dissociation technique modification was assessed included: 1) Total number of viable cells/mg of tissue (TVC); 2) MFE of the tissue-derived, single-cell suspension (primary MFE); and 3) MDP of 10k primary mammospheres. Digestion/dissociation optimization was primarily guided by the TVC, in that the higher the TVC obtained using a particular modification, the higher the total number of mammospheres that could be ultimately recovered. With regard to MFE, it was found that tissue-derived, single-cell suspensions from multiparous glands typically produced less than one sphere per 5 ml well when plated as 1k primary mammospheres. As impractically

Table 2. Summary of the digestion/dissociation optimization study results. For each box, the underlined black variable(s) represent(s) the condition found to be most productive. Red (non-underlined) variables were attempted but not optimal. *Animal #*: identification of animal (corresponds to the Animal # in Table 1). *Timing*: each number represents the time (in hours) each aliquot of tissue was initially digested with collagenase solution. For Animal # 4 a variety of digestion methods were explored with the "s" in 2s and 8s referring to "sealed" containers. *Tryp (min):* time of the trypsin digestion in minutes. *Cent (x g):* numbers represent centrifuge speeds (expressed as gravity) used in the initial collection. *FBS*: the addition or omission of fetal bovine serum to the digestion solution; Y=Yes; N=No. *Cell screen*: the use of 40 µm (40), 70 µm screen (70), or both (both) screen types. *Grinder*: the use or omission of a mechanical grinder in tissue processing; Y=Yes; N=No. *Tryp (ml):* volume of trypsin used in ml. *RBC lysis*: the use or omission of a red blood cell lysis solution; Y=Yes; N=No. *Tissue mass (g)*: tissue volume in grams that were digested similarly to a 400 g aliquot of tissue. *Pipette*: entries denote where studies were performed looking at the effect of different sizes and brands of pipettes (see text for results). *DNase*: volume of DNase utilized in µl. *Ratio study*: numbers are the volumes of tissue in grams. Digestion of the 800 gram aliquot of tissue was explored using both a 15 ml conical vial (800^{15}) and a 50 ml conical vial (800^{50}).

Animal #	Timing (hr)	Tryp (min)	Cent (x *g*)	FBS	Cell screen	Grinder	Tryp (ml)	RBC lysis	Tissue mass (g)	Pipette	DNase	Ratio Study
4	**2**, 2s, **5**, 8, 8s, 16	1, **3**, 6										
6	2, **3**, **4**, 5, 6											
7	2, 3, **4**, **5**, 6		80, **200**, 350	Y, **N**						**size**		
10	3, **3.5**, **4**, 4.5, 5		80, **200**, 350	Y, **N**	40, 70, **both**	Y, **N**				**size**		
11	3, **3.5**, **4**, 4.5, 5		80, **200**, 350			Y, **N**						
12	3, **3.5**, **4**, 4.5, 5				40, 70, **both**		**5**, 10	**Y**, N				
13	**3**, **3.5**, 4, 4.5, 5				40, 70, **both**		2, **5**, 10					
14	3, 3.5, 4, **4.5**, **5**											
15							2, **5**, 10		**300**, **500**, 800			
16									**300**, **600**, 800	**brand**		
17								**Y**, N	**300**, **600**, 800	**brand**	**200**, 400	
19										**brand**	**200**, 400	
20	3, **3.5**, **4**, 4.5, 5		80, **200**, 350									
23												**800**, **1200**, 1600
25												800^{15}, **800^{50}**
28												**1200**, 1600
29												800^{15}, **800^{50}**, **1200**, 1600

high numbers of 1k cultures would have had to be routinely plated for accurate MFE quantification, 10k primary mammosphere cultures, rather than 1k cultures, were utilized to determine the primary MFE for study subjects. Experiments directly comparing the primary MFE from 10k and 1k cultures found that 10k cultures (multiparous Animals #7=0.017, #10=0.014, #15=0.023; nulliparous Animals #6=0.388, #20=0.484, #26=0.222, #27=0.633) produced slightly higher MFEs than 1k cultures (#7=0.015, #10= 0.012, #15=0.020; #6=0.366, #20=0.448, #26=0.200, #27=0.607). However, the individual 10k and 1k MFE values were significantly correlated to one another and both plating densities were found to be equally sensitive at detecting inefficient protocol modifications. The use of 10k cultures was therefore deemed to be acceptable toward the optimization process. Ultimately, there was minimal variation identified in the MFE from any one animal, regardless of the digestion/dissociation modification, and this metric was only occasionally useful toward optimization here. There was even less variation in the MDP from any one animal, regardless of the digestion/dissociation modification, and this metric was only rarely useful toward protocol selection. The individual instances where the MFE and/or MDP metrics were useful toward the optimization of the digestion/dissociation processes are specifically mentioned below.

Basic methodology experiments utilizing Animal # 4 tissues suggested that initial-digestion times of greater than five hours decreases the TVC obtained from tissue and also lowers the MFE of the viable cells. Similar to what has been previously described,[171] the overnight (16 hour) digestion process also produced larger ratios of spheres that were solely myoepithelial in nature and thereby decreased the MDP. Detrimental effects to the TVC and MFE were also identified when the digestion process was performed within sealed containers. Subsequent timing experiments performed in 8 different animals (2 nulliparous, 1 lactating-primiparous, 2 nonlactating-multiparous, and 3 lactating-multiparous) did not identify a single optimal digestion time, even within a particular demographic of animals. Instead, the optimal digestion time for individual animals most often ranged between 3.0-4.5 hours. Retrospective histologic examination of the mammary glands suggested that longer digestion times are frequently associated with higher amounts of fibrous tissues within the mammary gland. Rather than corresponding to a specific time, optimal tissue digests were identified to correlate well with a fairly-distinct set of physical characteristics. Specifically, at the digestion times that were most productive approximately 25-33% of the original tissue volume was still present in the vials as thinly-glandular strands of fibrous material suspended within a homogeneous, cloudy-chylous, light pink solution. Additionally, the majority of the solids within the optimally-digested tissues generally took more than 10 seconds to fully settle back out of the suspension following agitation.

Tissue digests collected prior to, or after, this optimal digestion point consistently resulted in lower TVC. Tissue digests collected well after the optimal digestion point also tended to have a decrease in MFE and a few demonstrated a decrease in the MDP similar to that described above for the 16 hour-digestion process.

Experiments performed looking at the addition of FBS to the initial-digestion solution demonstrated no appreciable advantage and, conversely, the addition of FBS was noted to slow down the digestion process. Centrifuge rates of 200 x *g* were identified as optimal for the initial tissue digest. Tissue digests centrifuged at 80 x *g* produced lower TVC while those centrifuged at 350 x *g* had a notably lower MFE. Trypsin digestion of the tissue was identified to be optimal using 5 ml of 0.25% trypsin for 3 minutes. Smaller volumes or decreased exposure times led to lower TVC while increased exposure times resulted in lower TVC and MFE. Recovery of single cells from the tissue digests was found to be most productive when cells were passed sequentially through 70 μm and 40 μm cell screens rather than when passed through either screen alone. The addition of a red blood cell lysis step proved useful toward quantification of TVC and thereby increased the MFE without appreciably affecting MDP. A DNase volume of 200 μl was identified to be optimal for the standard sized tissue aliquots. Increased volumes of DNase did not provide any advantage to TVC recovery except in cases where cells lysis was excessive, such as overexposure to enzymatic solution or as a result of poor pipetting technique.

The use of a mechanical grinder toward mammary tissue digestion was not incorporated into the final protocol as this device created large amounts of cellular lysis and negatively affected the reproducibility of the TVC between samples. Tissues were found to be optimally dissociated in the trypsin step of the protocol when a 10 ml pipette was utilized. The use of smaller pipette sizes (e.g., 5 ml) or the use of micropipettes typically led to an increase in cell lysis. Certain brands of disposable pipettes were also found to increase the incidence of cell lysis due to the presence of jagged projections at the pipette opening. Finally, pipetting technique was also identified to affect the TVC and reproducibility. The optimal technique involved keeping the tip of the pipette at least 1 cm from the bottom of the conical vials and pipetting at a less-than-maximum speed to minimize lysis of the cells.

Tissue-mass studies found that 300, 500, and 600 mg aliquots of tissue processed using the same volume of digestion/enzymatic solutions as used for 400 mg aliquots yielded comparable TVC, MFE, and MDP. In contrast, 800 mg tissue aliquots processed the same way had a substantial decline in TVC. In ratio studies, the volume of digestion/enzymatic solutions that were identified to be optimal for 400 mg of tissue produced comparable results for 800 mg (processed in 50 ml conical vials) and 1200 mg tissue aliquots when the volume of digestion/enzymatic

enzymes was doubled or tripled, respectively. However, when the volume of digestion/enzymatic enzymes was quadrupled for 1600 mg aliquots or when the 800 mg aliquots were processed in a 15 ml conical vial, rather than a 50 ml conical vial, the TVC were appreciably lower than those identified in 400 mg aliquots.

Mammosphere culture optimization. A number of variables were explored to optimize the growth, maintenance, and generational capabilities of the mammosphere cultures. The optimized culture conditions were primarily guided by changes in the MFE and the resultant protocols for plating primary and secondary spheres are presented in Figure 2 and Figure 3, respectively.

High-density primary mammosphere cultures (Animal #s 7 and 10) were identified to be most proliferative when approximately 80% of the original CMM was replaced with fresh CMM on day 3. Conversely, the addition of 2 ml of fresh media at day 3 was found to be superior to the 80% media-replacement method for both primary and secondary 10k mammospheres (Animal #s 7, 11, and 12). Primary and secondary 1k mammosphere cultures (Animal #s 11, 12, and 20) were identified to be most proliferative when no changes were made to the media throughout their 7-day incubation process.

Other experiments (Animal #s 12, 14, 15, and 20) demonstrated that supplementation of CMM with CM1 at a ratio of 4:1 increased the numbers of 10k and 1k secondary mammospheres an average of 16.2% and 28.4%, respectively, within the 6-well plates. In studies performed using 1k secondary spheres (Animal #s 13, 14, 15, and 20) grown in 96-well ultralow attachment plates (Corning), it was identified that the sealing of the plates with paraffin tape between days 0-3 of the incubation process resulted in a greater consistency in the MFE between individual wells. Specifically, the MFE of the wells around the periphery (edge) of the plate was statistically higher in paraffin-sealed plates as compared to plates incubated without the use of paraffin. In contrast, no statistical difference in the MFE was identified in the centrally-located wells between paraffin-sealed and unsealed plates. As a result of the increased consistency, the overall MFE for each paraffin-sealed plate was also statistically greater than that of unsealed plates. Greater MFE-consistency between wells was also appreciated to occur when paraffin tape was utilized to seal mammosphere cultures grown in 6-well culture plates. The use of paraffin tape beyond Day 3 did not provide any appreciable advantage and, unexpectedly, seemed to inhibit further sphere growth in some cultures. No appreciable differences in the MDP, MBP or budding morphology were identified for secondary mammospheres acquired using CM1 and/or paraffin tape as compared to mammospheres derived from cultures lacking these modifications.

Viable cell recovery (VCR), defined as the total number of viable cells recovered from the dissociation of a mammosphere culture divided by the number of viable cells originally plated for that culture, was the primary metric utilized to gauge the productivity of the dissociation process. Dissociation of primary, and later generational, spheres into single-cell suspensions with trypsin required variable amounts of time ranging between 10-20 minutes (typically 12-15 minutes). Dissociation of spheres was identified to be most productive at the time point when no, or only very minimal, cellular material could be visualized within the Pasteur pipette used for this process. Dissociation of primary mammospheres at Day 7 was identified to produce a 41.2% higher VCR, on average, than sphere dissociations performed at Day 10 (Animal #s 7-10 and 20). Of note here is that Day 10 spheres often failed to fully disassociate even when pipetted for 20 minutes. In other experiments (Animal #s 9 and 10), the use of a 0.05% trypsin-EDTA solution for the dissociation process produced a 54% higher VCR, on average, than when a 0.25% trypsin-EDTA solution was utilized. No differences in secondary MFE or MDP was identified between the Day 7 and Day 10 dissociations although the use of a 0.25% trypsin-EDTA solution did appreciably lower the secondary MFE as compared to disassociations utilizing a 0.05% solution.

Mammosphere culture validation. Tissues from 18 animals (Table 3) were processed using the optimized mammosphere protocols (Figure 1 and Figure 2). Differences in mammosphere potential were readily apparent between the multiparous and nulliparous cohorts for most of the parameters examined in this study. Only three nulliparous animals (2 follicular-phase; 1luteal-phase) were available for comparison however. In efforts to minimize the effects of a potential confounding variable (reproductive stage), most of the comparisons made between the nulliparous and multiparous animals below utilized only the data collected from the eight multiparous animals that were in either the follicular phase (n=5) or luteal phase (n=3) of the menstrual cycle.

The TVC, MFE of primary 10k mammospheres, and MFE of secondary 1k mammospheres (derived from 100k primary cultures) were obtained for 18 animals (Table 3). The TVC for the follicular/luteal multiparous animals (13.26×10^3 cells/mg $\pm$ 3.23×10^3 cells/mg) were significantly lower than those of the three nulliparous subjects (17.49×10^3 cells/mg $\pm$ 2.62×10^3 cells/mg). The MFE for primary 10k mammospheres (primary MFE) was 0.024% ± 0.003% for follicular/luteal multiparous animals and 0.446% ± 0.208% for nulliparous subjects. The MFE for secondary 1k mammospheres (secondary MFE) was 0.511% ± 0.089 % for follicular/luteal multiparous animals and 2.144% ± 0.378% for nulliparous subjects. Both of the multiparous MFEs were statistically lower than those of the nulliparous MFEs.

Table 3. Macaques of various demographics were processed utilizing the optimized mammosphere techniques. *Animal #*: identification of animal (corresponds to the Animal # in Table 1). *Age*: rounded to nearest quarter year. *Class* (parity): N=nulliparous; P=primiparous; M=multiparous. *Stage* (reproductive status): M=menses phase; F=follicular phase; L=luteal phase; I=inactive phase; P=pregnant; Cy=polycystic ovaries. *TVC*: total number of viable cells recovered (x 10^3) per mg of tissue processed. *1°MFE*: primary mammosphere forming efficiency (MFE, %; see text) of a tissue-derived, single-cell suspension plated as 10K primary mammospheres. *1°MBP*: primary mammosphere budding potential (MBP, %; see text) of 10k primary mammospheres. *2°MFE*: secondary MFE of primary 100k mammosphere-derived, single-cell suspension plated as 1k secondary mammospheres. *2°MBP*: secondary MBE of 1k secondary mammospheres. *2°MDP*: secondary mammosphere differentiation potential (MDP, %; see text) of 1k secondary mammospheres. *CFE*: colony forming efficiency (CFE, %; see text) of a 100k primary mammosphere-derived, single-cell suspension plated at 3 x 10^3 cells/well. *CDP*: colony differentiation potential (CDP, %; see text) of the colonies obtained from the CFE.

Animal #	Age (yr)	Class	Stage	TVC	1°MFE	1°MBP	2°MFE	2°MBP	2°MDP	CFE	CDP
17	6	P	I	9.28	0.021	61.7	0.384	56.7	92.8	1.93	14.7
20	4.25	N	L	15.26	0.484	91.6	2.186	87.2	100	9.53	18.2
23	21	M	F	11.2	0.021	65.3	0.392	59	96.1	2.07	11.3
24	19	M	F	12.37	0.024	67.4	0.506	55	100	2.55	13.2
26	8	N	F	16.82	0.222	82.4	1.746	84.7	98.6	8.09	12.2
27	3.5	N	F	20.38	0.633	88.9	2.5	82.1	100	8.85	11.2
28	16	M	L	9.24	0.023	66.9	0.64	65.3	93.6	3.57	16.3
29	19	M	P	17.46	0.084	79.9	0.972	80.9	85.1	5.2	12.9
30	20	M	I	8.86	0.011	67.2	0.366	44.3	95.2	1.76	10.9
31	8	M	L	18.23	0.02	71.2	0.584	72.9	100	3.04	12.2
32	22.5	M	I	11.79	0.019	57.9	0.468	52	97.9	2.42	13.7
33	16.5	M	F	15.16	0.027	62.6	0.478	66.2	96.4	2.73	17.9
34	17	M	F	9.87	0.025	71.7	0.592	70.7	95.1	3.09	15.5
35	17	M	M	15.32	0.017	42.7	0.302	38.4	88.6	1.59	9.8
36	18.75	M	Cy	22.73	0.059	65.7	0.572	72.4	88.6	3.1	10.3
37	10.75	M	I	9.56	0.016	53.1	0.396	29.7	83.6	1.74	7.9
38	19.75	M	F	13.26	0.022	69	0.486	57.6	92.7	2.78	14.6
39	8.75	M	L	16.74	0.028	75.2	0.406	69.7	93.2	2.15	17.1

Representative images of Day 7, primary 10k mammospheres in culture media were acquired from three multiparous (Animal #s 23, 24, and 28) and two nulliparous animals (Animal #s 26 and 27) and the diameters of all spheres ≥ 40 μm were measured (multiparous mean= 61.8 μm ± 23.0, range= 40-194 μm, [n=186]; nulliparous mean = 68.0 μm ± 21.2, range= 40-174, [n=305]). The average number of individual cells per sphere was calculated using two different methods. First, single-cells in media that had been dissociated from Day 7, primary 10k spheres were imaged and their diameters were measured (multiparous mean = 12.6 μm ± 2.0; [n=39]; nulliparous mean = 11.2 μm ± 2.1 [n=56]). As no statistical differences were identified between multiparous and nulliparous animals

with regard to their average sphere or cell sizes, these numbers were combined and the average sphere was calculated to contain approximately 162 cells. Second, the diameter of individual formalin-fixed, paraffin-embedded, Day 7, primary 10k spheres was identified on H&E stained slides (multiparous mean= 83.4 μm ± 31.0 [n=16]; nulliparous mean= 89.4 μm ± 33.6 [n=24]) and the number of cell nuclei present within each cross-section of the formalin-fixed spheres above were quantified. The diameter of each sphere was then utilized to determine the average diameter of the cells within each sphere (multiparous mean = 14.6 μm ± 1.4; nulliparous mean = 14.3 μm ± 1.8). As above, the multiparous and nulliparous numbers were combined and the average sphere was calculated to contain approximately 214 cells per this method.

Generational studies produced quaternary mammospheres from 4 of the 6 subjects processed using optimal culture conditions (Table 4). Notably, the two animals that failed to produce quaternary spheres (Animal #s 25 and 32) were highly multiparous and both were in the inactive-phase of their reproductive cycle at the time of tissue collection. The secondary MFE of the luteal-phase multiparous animals (mean MFE of 0.495% or ~1 mammosphere forming unit (MFU)/202 cells plated) in this study was identified to be lower than that of the nulliparous animals (mean MFE of 1.65% or ~1 MFU/61 cells plated). Furthermore, the MFE for all multiparous animals decreased to a greater extent through the three generations than did the nulliparous MFEs. A marked decrease in the MFE for all generations of spheres in both cohorts was also identified when CM1 was omitted from the mammosphere culture media.

Table 4. Generational study identified differences in the MFEs between nulliparous and multiparous animals. *Animal #*: identification of animal (corresponds to the Animal # in Table 1). Animal #s 27 and 28 were cultured once using the optimized culture conditions and once in media lacking CM1 (-CM1). *Age*: rounded to nearest quarter year. *Class* (parity); N=nulliparous; M=multiparous. *Stage* (reproductive status): F=follicular phase; L=luteal phase; I=inactive phase; *Infants*: number equals number of live births. *2°MFE:* mammosphere-forming efficiency (MFE), between primary 10k and secondary 1k mammosphere cultures. *3°MFE:* MFE between secondary 10k and tertiary 1k mammosphere cultures. *4°MFE:* MFE between tertiary 10k and quaternary 1k mammosphere cultures.

Animal #	Age (yr)	Class	Stage	Infants	2^{o}MFE	3^{o}MFE	4^{o}MFE
25	18	M	I	12	0.32	0.09	0
26	8	N	F	0	1.41	1.01	0.94
27	3.5	N	F	0	1.88	1.17	1.4
27 (-CM1)	3.5	N	F	0	0.92	0.36	0.3
28	16	M	L	7	0.47	0.27	0.09
28 (-CM1)	16	M	L	7	0.29	0	0
31	8	M	L	2	0.52	0.43	0.25
32	22.5	M	I	16	0.38	0.21	0

The primary 10k mammospheres of Animal #s 26, 27, 31 and 32 that were utilized in generational studies were also utilized in the studies examining the formation of secondary mammospheres from a single cell. On average, 73% (range 66-83%) of the plated wells were identified to contain single cells. Under optimal culture conditions, the two nulliparous animals produced 10 (# 26; MFE =1.31%) and 15 (# 27; MFE 1.84%) mammospheres (mean MFE of ~1.58%). Cultured the same way, two multiparous animals produced 4 (# 31; MFE 0.51%) and 3 (# 32; MFE 0.42%) mammospheres (mean MFE of ~0.47%). When cultured without CM1, the two nulliparous animals produced 4 and 9 spheres (mean MFE of ~0.78%), and the multiparous animals produced 3 and 1 spheres (mean MFE of ~0.26%). Notably, the MFEs for the secondary spheres in this study were similar to the secondary 1k MFEs derived from the same four animals in the generational study, as reported above. Collectively these data differed by less than 3%. This is significant, in that these findings suggest that the majority of the mammospheres derived from 1k cultures are likely to be clonal in origin, similar what has been described in human mammosphere studies.[71, 84] Spheres acquired from the animals in this study (n=27) were encased in BME and allowed to undergo differentiation. These spheres demonstrated morphological budding patterns and bilineage staining properties at Day 21 that were similar to the small secondary 1k spheres, as described below.

Bilineage differentiation of spheres on collagen-coated plates was confirmed using IHC methods for all generations of spheres although the MDP was most often assessed using crystal violet stain in this study (Figure 4, A-D). A minimum of 89 secondary 1k mammospheres were assessed for each subject at Days 7-9 of the plating process and the MDP for 18 animals is reported in Table 3. The secondary MDP for follicular/luteal multiparous and nulliparous animals were close in number, although statistically different, at 95.9% ± 2.86% and 99.5% ± 0.80%, respectively. Regardless of demographic, the majority of the spheres that attached to collagen underwent a significant morphologic change by Day 5 of the plating process. The cells within the spheres themselves transitioned from rounded cells with distinct margins to a more elongate, poorly-defined cell population. Additionally, most spheres had two distinct populations of cells extending from their base. Epithelial-like cells were clear and rounded with distinct cellular edges and stained positive with CK18. Myoepithelial-like cells, in contrast, were elongate-spindle shaped cells with indistinct margins and were often refractile. Myoepithelial-like cells within the center of the colonies typically stained well with SMA although the elongate cells within the spheres, directly adjacent to the spheres, and along the leading edge of colonies generally stained more intensely with CD10 rather than SMA. In most subjects, a small number of spheres produced only epithelial-like or myoepithelial-like colonies around their

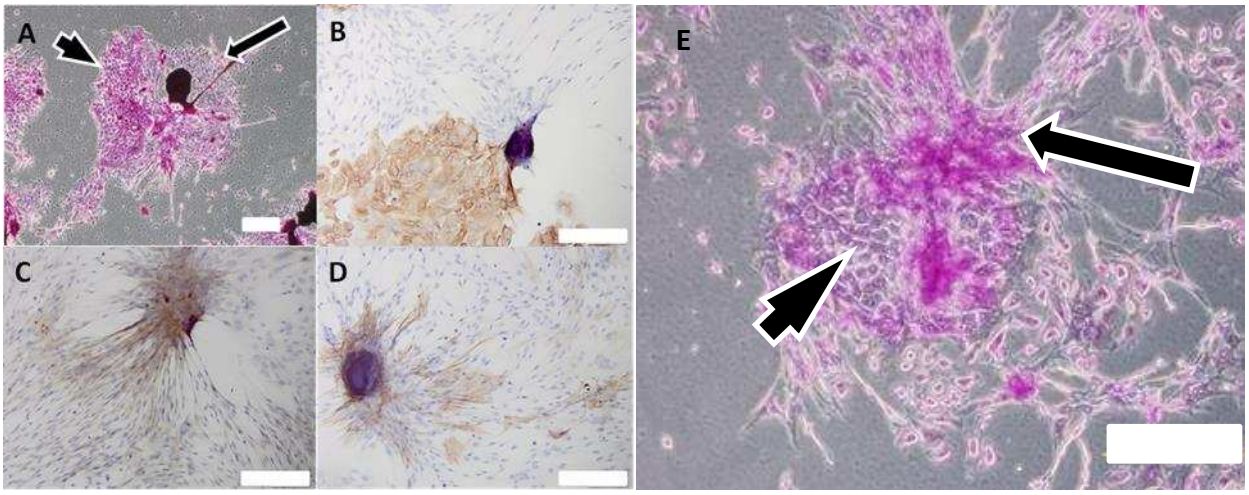

Figure 4. Spheres and single cells plated on collagen-coated wells show bilineage differentiation. A-D Secondary 1k mammospheres on collagen-coated wells. A is a crystal violet stain of mammosphere with epithelial (arrowhead) and myoepithelial (arrow) differentiation at 4x magnification. B-D are immunohistochemistry stains at 10x magnification: B=anti-cytokeratin 18 (CK18); C=anti-CD10; D=anti-smooth muscle actin (SMA). E is a crystal violet stain of a bilineage colony formed from a primary mammosphere-derived single-cell suspension. Epithelial (arrowhead) and myoepithelial (arrow) differentiation of the colony at 10x magnification. Scale bars = 200 μm.

base while, less frequently, other spheres failed to produce any appreciable colony growth. Small numbers of individual epithelial-like cells, far removed from the base of the sphere, stained positive with ER and PR for all subjects in which these assays were attempted.

The colony forming unit assay was identified to be optimally plated using 3 x 10^3 viable cells/35 mm well. At this cell density, the numbers of colonies produced for both nulliparous and parous individuals allowed for the acquisition of a reliable CFE estimate when 6 wells were plated. The CFE and CDP of cells derived from the dissociation of primary 100k mammospheres were obtained for 18 animals (Table 3). A minimum of 256 colonies were then evaluated for each subject between Days 10-14 of the plating process. Nulliparous cultures were most often evaluated around Day 10 as the colonies in these cultures were numerous, grew quickly and tended to overrun one another by Day 14. The CFE for follicular/luteal multiparous animals (2.75 ± 0.50%) was statistically lower than nulliparous animals (8.83 ± 0.72%). Bilineage differentiation of the colonies was confirmed using CK18 and SMA IHC although the CDP was most often assessed using crystal violet stain alone. The morphology of the epithelial-like and myoepithelial-like cells produced using this assay was similar to those described for the collagen-plated spheres. However, unlike the colonies derived from the collagen-plated spheres, most of the colonies formed here were uniformly myoepithelial-like in nature. Bilineage colonies (Figure 4, E) typically represented less than 15% of the total colonies and epithelial-like colonies were only rarely identified for most subjects. No statistical

differences were identified in the CDP between the follicular/luteal multiparous and nulliparous subjects which averaged 14.8% and 13.9%, respectively.

The ability of BME-embedded mammospheres to undergo budding was confirmed for all generations of spheres. The MBP of 10k primary and 1k secondary mammospheres for 18 animals at Day 21 of the plating process are reported in Table 3. A minimum of 48 spheres were assessed for each subject. Budding was subjectively assessed by direct visualization of the BME-embedded spheres in culture (Figure 5, A-C). Spheres observed to produce no projections, or only small numbers of short ductular-like projections, from the main sphere body by Day 21 were counted as non-budding spheres. Spheres were classified as budding on Day 21 if they were identified to have any of the following characteristics: 1) small numbers of individualized, elongated ductular-like projections (DLPs); 2) numerous DLPs of any length; 3) DLPs capped with alveolar-like structures; or 4) secondary-body formation (defined below). The primary MBP for follicular/luteal multiparous animals was 68.7% ± 3.99% and

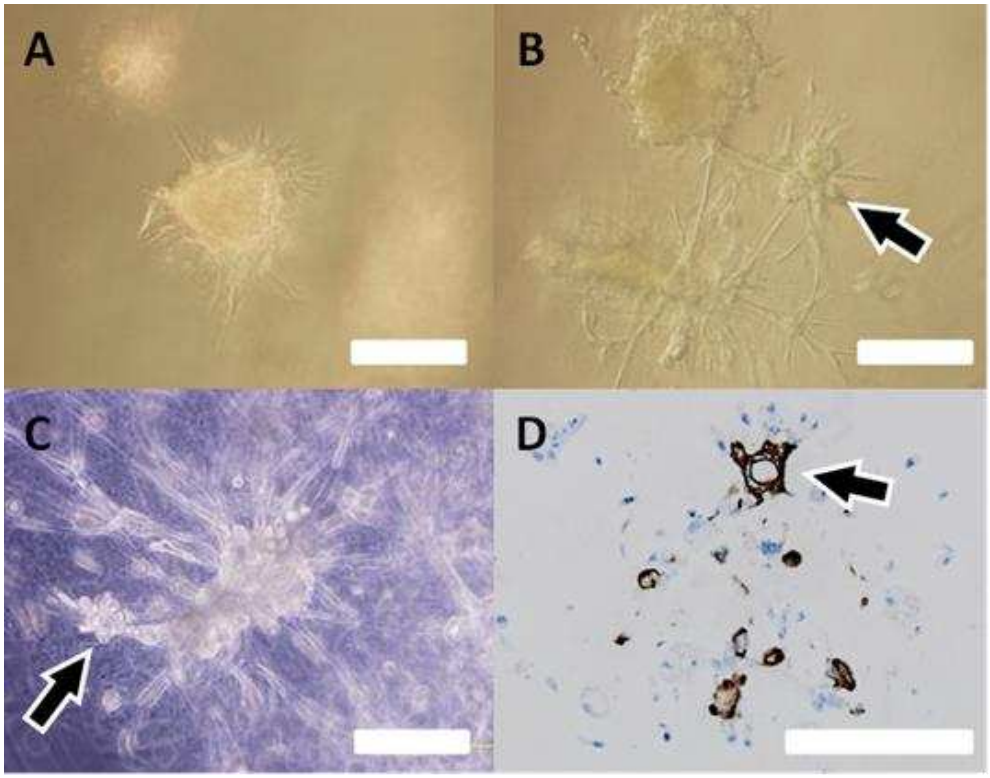

Figure 5. Spheres placed in BME underwent budding differentiation and, with the addition of prolactin and CM2, produced a β-casein-containing fluid (milk). Images A-C are secondary mammospheres in basement membrane extract at 10x magnification: A=early budding; B=late budding with alveolar-like structures (arrow) at the end of ductular-like projections; C=late budding of mammospheres supplemented with prolactin and CM2. This sphere has a secondary body from which a ductular-like projection, capped with an alveolar-like structure (arrow) is evident. Image D is anti-β-casein staining of alveolar-like structures, similar to those highlighted in image C at 20x magnification. Scale bars = 200 μm.

87.6% ± 4.73% for nulliparous subjects. The secondary MBP for follicular/luteal multiparous animals was 64.7% ± 6.63% and 84.7% ± 2.55% for nulliparous subjects. The primary and secondary MBPs for multiparous animals were both statistically lower than those of nulliparous subjects.

The morphological assessment of the mammospheres, as described below, was based on observations made using secondary 1k mammospheres although these observations were essentially identical to those identified for the primary 10k spheres. Most of the individual BME-encased spheres underwent growth and differentiation over time. At Day 0 of the BME-plating process (Figure 6, row A), spheres were typically composed of densely-packed, rounded cells. Most cells within these spheres stained positive for CD10 and cells along the periphery of the sphere commonly stained strongly for CK18. Staining with SMA was more variable but was usually weak or absent for cells within these spheres. No cells could be definitively identified to be positive for ER or PR in these spheres although occasionally very weak staining for one or both of these receptors was appreciated. Mammospheres that were cultured for 7 days in mammosphere media, prior to being harvested for BME-differentiation, typically had a thin layer of PAS-positive extracellular material (matrix) between the individual cells along the periphery of the sphere at Day 0 of the BME-embedding process. When spheres from the same animal were cultured for 10 days in mammosphere media prior to being harvested, the volume of PAS-positive material between the peripheral cells at Day 0 of the BME-embedding process was subjectively more pronounced. This observation is notable in that mammospheres cultured in media for 10 days were consistently more resistant to trypsin dissociation than mammospheres cultured for 7 days, as previously discussed above. In turn, it is possible that an increased volume of intercellular matrix within mammospheres over time could be associated with an increased resistance to trypsin dissociation.

By Day 5 of the BME-plating process (Figure 6, row B), the individual cells within spheres had enlarged and the cell numbers increased for many spheres as well. Cells were typically more angular with distinct margins and the cells stained diffusely for CK18. Staining with CD10 usually diminished or was absent at this time point and strong SMA staining started to become apparent in random cells throughout the spheres. No cells were identified to be positive for ER or PR in these spheres. Abundant PAS-positive material was often apparent between the individual cells throughout the sphere and this material also formed a thin shell around the outside of the sphere. Most of the spheres identified as non-budding spheres maintained this morphology throughout the 21 day incubation process. Rarely, some non-budding spheres would fail to undergo this initial change or would only express CK18.

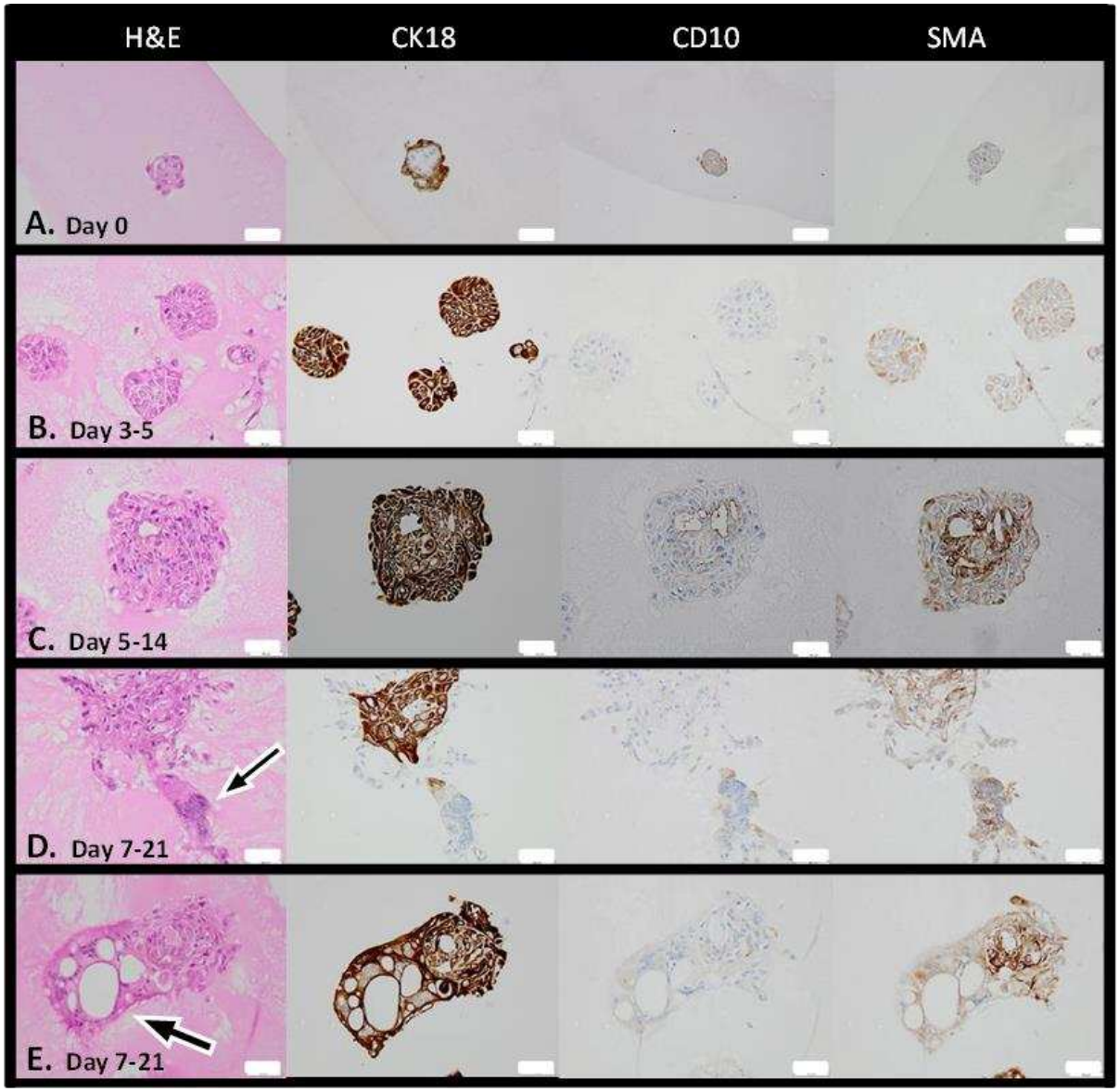

Figure 6. The budding morphology of mammospheres over time often followed a predictable pattern of maturation that could be somewhat altered through the addition of prolactin and CM2. Serial sections of secondary mammospheres plated in basement membrane extract for 0-21 days (different time periods represented by rows A-D) at 10x magnification. Sections from each time period were stained with: hematoxylin and eosin (H&E); anti-cytokeratin 18 (CK18); anti-CD10 (CD10); or anti-smooth muscle actin (SMA). Row C: cavitation of the central aspect of the sphere. Row D: numerous small ductular projections extending from the main sphere body and one highly-cellular, expansive projection (arrow on H&E). Row E (mammosphere was supplemented with prolactin and CM2): vacuolated cytoplasm of the cells in the secondary body of the mammosphere (arrow on H&E) that are extending from the main sphere body. Scale bars = 100 μm

Between Day 5 and Day 21 of the BME-plating process, most spheres continued to proliferate and differentiate. As early as Day 5, some spheres underwent a central cavitation (Figure 6, row C). In these spheres, CK18 staining remained diffuse throughout the main sphere while SMA staining was most intense surrounding the edge of the central cavity. Staining with CD10 was minimal to absent in the main sphere body. Budding spheres

typically had at least a few projections from the main sphere body by Day 7 although these varied in appearance. Commonly, numerous thin branching DLPs originated from all surfaces of the sphere and this appearance was referred to here as "coronal budding" (Figure 5, A and C; Figure 6, row D). In other spheres, only a few, elongate DLPs were present (Figure 5, B). For both of these growth patterns, a thin centralized lumen could frequently be visualized within larger DLPs. Additionally, large, oblong-cylindrical growths that lacked a lumen and that were highly cellular along their leading edge, were also identified to be extending from some spheres (Figure 6, row D). With time, many of these larger projections expanded, to form cellular masses confluent with, and nearly as large as, the main sphere body. At this point, each of the large growths was referred to here as a "secondary body" and DLPs were commonly observed to be originating from these formations (Figure 5, C; Figure 6, row E). Lumen-containing, alveolar-like structures that lay atop DLPs were typically identified in approximately 2-7% of the budding spheres for most animals. The only notable exception to this being that in two early-pregnant animals (Animal #s 13 and 29) alveolar budding was identified to occur in 9 and 13% of budding spheres. In actuality, many of the individual budding spheres exhibited a combination of the morphological growth patterns described above and essentially every possible combination of these variations was identified within the BME-plated spheres for each animal examined. Other than for the alveolar budding mentioned above, no variation in morphological growth pattern was appreciated to exist between the different demographics of animals. As mammosphere size has previously been associated with variations in differentiation potential,[70, 153] sphere size was compared to variations in budding morphology (cavitation, coronal budding, elongate ductal budding, alveolar formation, and secondary-body extrusion) in three animals. Small spheres (~40-60 μm in diameter) were never recognized to undergo cavitation or produce secondary-body extrusions, but otherwise the other budding variations were identified with similar frequency throughout spheres of all sizes. Finally, while the overall budding for secondary 1k mammospheres was typically slightly lower than the primary 10k mammospheres, the budding of secondary spheres was noted to be subjectively, but consistently, more exuberant than that of the primary spheres from the same animal.

Collectively, IHC-stained spheres derived from a number of animals suggested that budding projections, as a whole, tend to stain predominantly with CD10 in the early stages of the process. As these projections aged and expanded, a diffuse SMA staining predominated these structures. CK18 staining was usually only identified in fully matured projections or within the older, more organized, regions of the individual immature projections. Essentially all budding spheres exhibited some regions of CK18 and SMA staining and therefore budding was considered to be

indicative of bilineage potential for this assay. Small numbers of cells in the distal aspects of the mature projections stained positive for ER, although no ER staining was ever observed within the main sphere body. No cells were identified to be PR positive for any of the animals. In budding spheres, abundant, dense PAS-positive material was identified between the individual cells and around the periphery of most main sphere bodies, secondary bodies, and budding projections.

Lactogenic differentiation of BME-encased mammospheres was accomplished using the spheres from two multiparous animals. These mammospheres were incubated in media containing prolactin and CM2, as detailed above. In these spheres, the cells comprising the alveolar-like formations and small volumes of liquid material present within the lumens of these structures stained positive for β-casein (Figure 5, D). No cells or material within the main sphere body or secondary body of these spheres were identified to stain positive for β-casein. Notably, the spheres plated using this technique consistently developed a diffuse foamy gross appearance between days 7 and 21 (Figure 5, C). Histologically, the cells in these spheres and projections were identified to have variably-sized clear intracellular vacuoles (Figure 6, row E). Other than their vacuolar nature and the β-casein staining of the alveolar structures, the morphological variability of these BME-encased spheres resembled those of spheres plated without the use of CM2-prolactin. The other staining properties of these spheres were similar to those plated without the use of CM2-prolactin with the exception that more cells within the mature projections stained positive for ER and there were small numbers of cells identified to be PR-positive.

The quadrant studies were performed using four animals (Animal #s 26, 27, 30 and 31) which provided 24 unique mammary gland quadrants by which to assess reproducibility of the mammosphere culture technique. The intraclass correlation coefficient (ICC) for the three biopsies from each of the 24 quadrants was identified to be 0.778 for TVC and 0.914 for primary MFE. In three animals, no statistical differences in the MFE were identified between any of the quadrants. In Animal # 30, however, although the MFEs for quadrants 1 and 4 were similar to one another, the left and right quadrant 2/3 MFEs were significantly lower than those of quadrants 1 and 4. Notably, Animal # 30 was in the inactive phase of the reproductive cycle while the other three animals were actively cycling.

Principle component analysis (PCA) was performed utilizing 17 of the animals (polycystic ovary Animal # 36 was not included) and the 11 variables listed in Table 3. Three principal components were identified to represent approximately 78% of the variance between the subjects. Principal component (PC)-1 entailed Age, Class (parity status), TVC, primary and secondary MFE, and CFE and accounted for ~52% of the variance. The TVC, primary

and secondary MFE, and CFE for PC-1 were positively correlated with one another. Primary and secondary MBP, secondary MDP and CDP comprised PC-2 (~14% of the variance) and all four variables were positively correlated with one another. Stage (reproductive phase) was the only variable identified for PC-3 (~12% of the variance). The corresponding vector diagram of the PC-1/PC-2 data is provided in Figure 7.

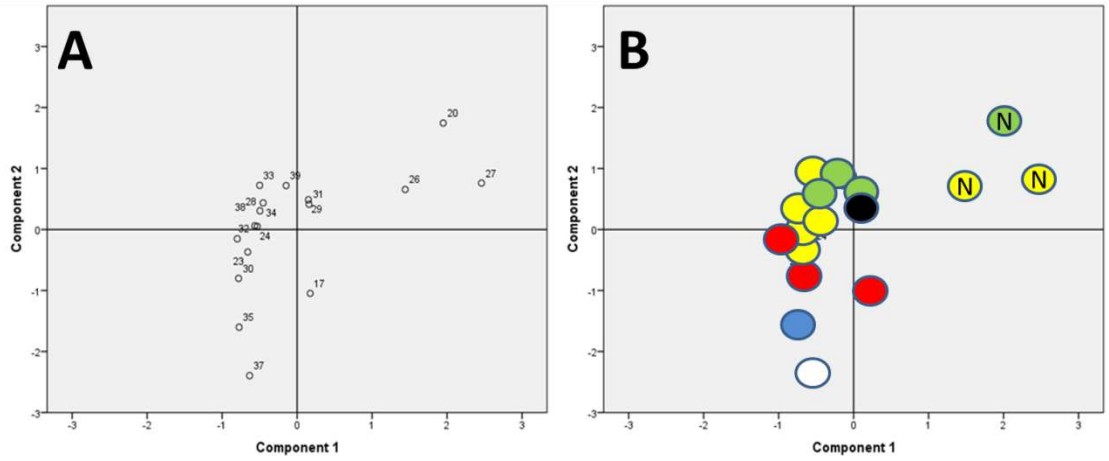

Figure 7. The mammosphere potential of macaques is stratified by parity status, reproductive stage and possibly age. A: vector diagram of PC-1/PC-2 data from animals processed utilizing the optimized techniques. Numbers within the vector diagram correspond to the *Animal #s* listed in Table 3. B: same vector diagram as "A" but with colored-circle overlay to highlight the reproductive demographics. Reproductive demographics are represented by circles of different colors: menses phase = blue; follicular phase = yellow; luteal phase = green; inactive phase = red; pregnant = black; white = lactating; circles containing "N" are nulliparous animals.

Animals toward the right side of the vector diagram collectively tended to exhibit a higher growth potential (primary and secondary MFE and CFE) while animals toward the top of the diagram collectively exhibit higher differentiation potential (primary and secondary MBP and CDP). The three nulliparous animals occupy the upper right quadrant of the graph and are distinctly removed from the parous individuals. The 8 year-old nulliparous animal (Animal # 26) is positioned below and to the left of the younger nulliparous animals and, notably, is situated well to the right of the similarly-aged, parous subjects within this data set (Animal #s 17 and 31). Most of the parous subjects aligned within an elongated central cluster although the sole lactating animal and sole menses-stage animal (Animal #s 37 and 35, respectively) are positioned well below the other animals. The main cluster of parous animals is somewhat stratified by "Stage" with the luteal-phase animals positioned higher than all but one of the follicular-phase animal. Likewise, the follicular-phase animals are positioned higher than all but one of the inactive-phase individuals. The nulliparous luteal-phase animal (Animal # 20) is similarly positioned higher than the two follicular-

phase nulliparous animals. Finally, there is also a weak trend for the data to be stratified by age. In short, the younger animals within each particular reproductive Stage tend to be located to the right of older animals.

Discussion

This work represents the first mammosphere culture technique formally developed and optimized for use in any species of nonhuman primate. This work further represents the initial validation process of the rhesus macaque mammosphere culture as an assay toward the study of the human breast. Data collected in these studies suggest that the mammosphere potential of mammary glands is affected by the reproductive state of the donor and this information could open up new avenues of research within mammosphere-based research as a whole. Likewise, the novel culture modifications incorporated into the mammosphere protocols produced here and the observations made regarding the longitudinal morphologic development of the spheres are also likely to be of general benefit to mammosphere research. Finally, the mammary gland weights derived from this work and the data demonstrating the physiologic homogeneity of mammary gland throughout a large portion of the organ could prove useful to a variety of studies involving macaques.

The mammosphere culture technique in general has been criticized as a research assay in that, due to the numerous steps associated with its use, there is high potential for variability in the data obtained between individual investigators.[194, 231] Information obtained throughout the optimization processes suggest that this criticism is not without merit. With regard to the digestion/dissociation of mammary tissues in particular, overt changes to the protocol (e.g., duration of enzymatic digestions), as well as seemingly-minor variations to the process, affected the TVC obtained from homologous tissue aliquots. When this information is considered in light of the fact that the possibility for “minor” modifications exists at essentially every step of the process (e.g., digestion vessel size, centrifugation speed, pipette size, cell-screening technique, etc.) it can be appreciated that TVC data may not be comparable between studies if a detailed standardized protocol is not followed. Although just such a protocol is provided above, at least two other factors must also be taken into account with regard to the potential for TVC variability. The first is that no universally-applicable time point was identified to be optimal for halting the initial-digestion step in all macaque tissues. The other factor is that the physical appearance of what defines an “optimally-digested tissue” is largely subjective in nature. As such, if marked differences in tissue digestion occur between individual laboratories due to disparities in the perceived optimal processing time, it is likely that there will be significant variations in the TVC of homologous samples. Ultimately, good internal consistency may be the best that

can be expected of studies requiring highly-reproducible TVCs (i.e., studies comparing the mammosphere potential between whole glands). Notably, the MFE was identified to be substantially more stable in regard to most modifications of the digestion/dissociation process than was the TVC. This finding is significant because, unlike the TVC which has limited research applications, the MFE is likely to be critical for most mammosphere-related studies.

Although MFE was not overtly sensitive to variations in the digestion/dissociation technique, there were a number of potential sources of MFE variability identified in these studies. Central to the issue of MFE reproducibility is the standardization as to what constitutes a mammosphere. Consistent with some previously utilized standards,[67, 147] all tightly-associated, free-floating, spheroid cellular growths, ≥40 μm in diameter were counted as mammospheres for this project. Growths composed of loosely-associated cells or growths that were elongate-amorphous in shape were not counted as mammospheres.

Even more critical to MFE variability between samples, however, was the discovery that the MFE could be influenced by a number of factors associated with culture conditions. The first of these factors was plating density. In one study, the single-cell suspensions derived from the mammary glands of seven macaques were plated as 10k suspensions and also as 1k suspensions. The average MFE of the 10k cultures for these seven subjects was identified to be approximately 10% higher than that of their 1k cultures. This observation is similar to those noted in previous mammosphere studies[70, 71, 85] and in other types of sphere-forming assays (e.g., neuronal and prostate sphere cultures).[194] In these reports, the increased sphere formation was suggested to occur as a result of cell aggregation, with larger numbers of "aggregate spheres" expected in cultures plated at higher densities. Beyond cell aggregation, however, is the possibility that the differences between the MFEs for the 1k and 10k cultures may also be related to the concentration of pro-mammosphere growth factors within the culture media.[85] As per this mechanism, the stem/progenitor cells that are cultured in high-density plates would be exposed to greater concentrations of cellular growth factors and, in turn, would be more proliferative (i.e., have a higher MFE) than would similar cells from low-density plates. The observation that cells plated as 10k cultures, 1k cultures, and single cells all consistently had higher MFEs when they were supplemented with CM1 is evidence that growth factor concentration can influence the MFE.

Other potential sources of MFE variability were also identified to be associated with plating conditions. Early experiments identified notably smaller numbers of spheres within the corner wells of the 6-well plates and

within the perimeter (edge) wells of 96-well plates as compared to the center wells. Sealing the culture plates with paraffin tape, as recommended in at least one mammosphere protocol,[34] essentially eliminated the regional inconsistencies and, in turn, increased the average MFE. With the knowledge that stem cells typically reside in niches of the body with extremely low oxygen tension,[67, 149] the increased MFE and consistency within the sealed plates is proposed to be due to the creation of a hypoxic environment through the use of paraffin tape. In support of the proposal that hypoxia may be responsible for the increased mammosphere proliferation, some research has found that hypoxic environments promote the maintenance of stem cells in an undifferentiated state and thereby prompt an increase in stem cell production.[102, 149, 195, 205] Furthermore, other work has also demonstrated that hypoxia leads to an increase in the clonal growth of mammary epithelial cells.[208] Beyond promoting the stem cells to remain in an undifferentiated state, hypoxia may also promote mammosphere proliferation in these studies by reducing the incidence of cellular senescence in the MSCs/MPCs. This proposal is based on the observation that increased oxygen tension has been associated with an increase in senescence for a variety of different cell cultures and that increased senescence has been directly correlated with a decrease in the MFE of mammosphere cultures.[67]

The other potential source of MFE variability related to plating conditions involves the types of plate used for the mammosphere cultures. Specifically, in a study of four different animals, 96-well plates and 6-well plates were both plated with 1k mammosphere cultures. The average MFE of the 96-well plates in this project was identified to be ~41% greater than that of the 6-well plates. It was subsequently discovered that the cells within the 96-well plates, but not the 6-well plates, tended to aggregate in groups along the periphery of the wells. A significant concave meniscus was identified within the media of the individual wells of the 96-well plates and is proposed to be the cause of the peripheral displacement and aggregation of the cells. Despite the fact that these cells were plated within the recognized standards of clonal density, it was considered likely that many of the 96-well "mammospheres" were actually derived from the aggregation of individual cells rather than being a clonally derived outgrowth.

Collectively these observations suggest that mammosphere cultures that are processed similarly, but not identically, have a high potential for variability. In efforts to decrease this variability, a detailed macaque mammosphere culture protocol has been developed and is described above. Furthermore, two recommendations aimed at further increasing the reproducibility of this assay are also included here. First, whenever TVC reproducibility is desired, similarly weighted aliquots of mammary gland should be used for the comparisons, or at

the very least, the ratio of tissue to enzymatic solution should be standardized between samples within a study. Second, efforts should to be made within individual studies to reduce the variability of the subjective components of the mammospheres process. This can be achieved by having each subjective step of the process assigned to a single investigator or to a consistent group of multiple investigators who each evaluate the step and then come to consensus. Specifically, this information pertains to: the decision as to the completeness of the initial-digestion process; the viable cell counts; and the mammosphere counts.

In line with these recommendations, the reproducibility experiments in this project were performed by a single investigator and intensive efforts were made to limit any inconsistencies in the processing of the individual samples. Additionally, the viable cell counts of the initial digests were performed in triplicate and large numbers of primary mammosphere wells were plated so as to increase the accuracy of the primary MFE estimates for each sample. Executed in this manner, the correlation between the individual samples collected from each quadrant was good for TVC and excellent for the primary MFE. The finding that TVC was more varied was not unexpected as the samples utilized for these experiments were biopsies and it is plausible that the ratios of glandular and fibrous tissue varied with location. In spite of the numerous potential sources of variability, the MFE data collected in this study demonstrated that the mammosphere culture assay can provide highly reliable and reproducible data if conscientious efforts are made with regard to sample processing. These data also identified the MFE to be consistent within the individual mammary gland quadrants of this species. Additional analyses found no statistical differences in the MFE of quadrants 1 and 4 for any of animals and suggest that individual biopsies taken from these quadrants are likely to be representative of this region in general. This is significant, in that individual biopsies can be repeatedly collected from quadrants 1 and 4 from the same animal over time thereby allowing for longitudinal, mammosphere-related studies to be performed. The in vivo biopsies collected as part of this project were found to provide ample, viable tissues toward mammosphere culture and this information further supports the use of these animals in longitudinal studies. Notably, all of the animals utilized for the in vivo biopsy procedures remained active breeder animals within the colony and there was no appreciable decrease in milk production of the biopsied glands as assessed clinically in subsequent pregnancies.

Data obtained from this study were compared against similarly processed tissues, cells, and spheres derive from humans in efforts to validate the rhesus macaque mammosphere culture as a relevant assay toward human research. A variety of data related to morphological parameters were collected. The TVCs for nearly all of the

rhesus macaque mammary tissues fell within the TVC ranges reported for human breast (5.0 - 20.0 x 10^3 cells/mg)[106] suggesting that the mammary glands of these species are similar in cellular composition. Macaque mammospheres were found to be similar to those of humans with regard to: mean sphere diameter and size-range (mean=75 μm; range = 40-110 μm);[67] mean diameter of the cells comprising the spheres (9-15 μm);[67] and the numbers of cells within each sphere (150-300 cells/sphere).[71] Furthermore, undifferentiated macaque mammospheres were identified to have strong epithelial (CK18) and weak myoepithelial immunostaining (macaque=SMA; human=cytokeratin-14) comparable to that of humans.[67] Putative markers for bilineage progenitor cells[71] (macaque=CD10; human=epithelial specific antigen[67]) and abundant extracellular material[71] were also identified to be diffusely distributed throughout the spheres for both species.

Data pertaining to the differentiation potential of the mammospheres were also compared between the two species. In the only human MDP study that could be identified, human tertiary mammospheres had comparable rates of differentiation (100%)[71] to those of the secondary mammospheres utilized in this study. No human studies were identified which directly evaluated the MBP. However, with regard to three-dimensional culture techniques, the macaque and human mammospheres shared similar properties with regard to: formation of ductular- and alveolar-like structures;[67, 71, 85] immunostaining with both epithelial and myoepithelial markers;[71] and secretion of β-casein-positive material within the lumens of the alveolar-like structures.[71, 196] Per the CDP assay, macaques were identified to produce colonies with bilineage differentiation (immunostained with CK18 and SMA) similar to what has been reported for the human.[71, 196] This parameter, however, was not entirely consistent between the two as the macaque CDP was notably lower than what is described in the human study (65-71%).[71] While it is possible that there are general differences in the types of cells that typically form mammospheres between the two species (i.e., more human mammospheres may be derived from MSCs), it is proposed that this discordance is more likely a result of culture methodology. First, the media that was used in the human CDP study contained growth factors known to promote stem cell maintenance while the media used in the macaque studies favored cellular differentiation. This probably accounts for at least part of the CDP discrepancy as similar variations in media have been noted to alter the differentiation ability of other sphere-forming assays.[194] Another point of consideration is that the single cells utilized in the macaque CDP studies were derived from 100k primary mammospheres while those in the human study were derived from 20k primary mammospheres. This difference is important in that most primary mammosphere cultures have at least a small percentage of the "mammospheres" that are formed by cells other than

MSCs/MPCs through the aggregation and subsequent proliferation of linage-specific progenitor cells and/or more-differentiated cell types.[70, 71] Formation of these "aggregate spheres" is known to be promoted through an increase in cell-to-cell contact and therefore high-density mammosphere cultures are more likely have higher percentages of aggregate spheres as compared to low-density mammosphere cultures.[70] Furthermore, as the aggregate spheres often lack cells with bilineage potential, it is also logical that high-density mammosphere cultures are more likely have a lower CDP as compared to low-density mammosphere cultures. To that, an experiment utilizing mammary tissues from two macaques found that the CDP of 100k primary cultures was ~31.7% lower, on average, than the CDP of cells derived from 10k primary cultures. These results support the contention that differences in the cell-densities of the suspensions used to create the CDP assays could have also contributed to the discrepancy between the macaque and human CDP values, as noted above.

The proliferative capacities of the mammospheres were also compared between the humans and macaques. Notable here, is that none of the human studies utilized for these comparisons described the reproductive status of the donors. However, with the knowledge that all the human samples in these studies were derived from reduction mammoplasties and the mean age for reduction mammoplasties has been reported to be around 38 years of age,[242] it is plausible that a large percentage of the tissue donors in these studies were parous individuals. This information is important because most of the proliferative parameters in the macaque differed significantly between nulliparous and multiparous animals. While the differences between macaque demographics are discussed in more detail below, what is important to the discussion here is that many of the comparisons to human data are made with reference to the parity-status of the macaque.

In studies using comparable methodologies, the human primary MFE (0.013%)[196] and secondary MFE (0.4%)[71] were similar to those of the parous macaques but were substantially lower than those of nulliparous macaques. As with humans, macaques produced mammospheres that were capable of being passaged over multiple generations. Some of the human generational studies reported a marked loss in MFE with each generation[85, 196] which was similar to what was identified for parous animals. Interestingly, other studies identified more consistent MFEs,[71] or even increases in the MFEs,[67] between the successive generations and this was comparable to data from the nulliparous animals. Utilizing the secondary MFE values acquired from the generational study and comparing them to the average number of cells/sphere in the macaque, each parous mammosphere was calculated to contain ~1 cell capable of reforming a sphere (mammosphere forming unit (MFU)). Using these same methods, the nulliparous

mammospheres were calculated to contain ~3 MFUs per mammosphere. These numbers were consistent with the ranges (1- 4 MFUs/mammosphere) identified in human studies.[71] In the current project it was also demonstrated that macaque mammospheres could be derived from singly-plated cells which is similar to what has been reported for human mammospheres.[67, 71] No human studies were identified that reported the CFE and therefore no comparisons could be made.

Collectively, these studies support the proposition that mammospheres derived from macaques are comparable to those of humans. While not all macaque data aligned perfectly with every human study, there were inconsistencies in methodology that may account for these discrepancies. The proposal that methodology is critical to mammosphere potential is supported by the fact that data reported between the human mammosphere studies are varied, and at times contradictory to one another, where different methods have been used to process these cultures. In turn, macaque mammospheres appear to be a relevant surrogate model to human mammospheres where human tissue is unavailable or toward areas of research that are not accessible using human tissues. Examples of studies where macaque mammospheres might prove useful include investigations into the effects of carcinogens on the mammary gland stem cells of young females and any longitudinal study of the breast where repeated biopsies from the same individual are required.

The morphological observations made on the macaque mammospheres suggest that this assay produces populations of spheres that are highly variable with regard to their individual differentiation potential. Specifically, by Day 5 of the differentiation process in BME, notable variations in the morphology of individual spheres became apparent that persisted throughout the entire time spheres were maintained in culture. While these variations might have been predicted of the primary 10k mammospheres, with the knowledge that some spheres within primary cultures are invariably of a non-stem cell/non-bilineage progenitor cell origin (as discussed above with CDP), it was surprising to find nearly identical results in the 1k secondary spheres. The 1k results were unexpected because mammospheres plated at, or below, 1k have been proposed to be primarily clonal in origin; and also because secondary mammospheres are proposed to be comprised of cell populations that are more homogeneous than those of the primary spheres.[71, 85] Regardless, the morphological results of this study are consistent with reports which collectively propose that the mammosphere-forming cells obtained via this assay are, in actuality, a highly heterogeneous population of proliferative bilineage cell types.[67, 70, 153, 194] While this lack of specificity has incited criticism from some,[70, 194] it could be argued that an assay that is broader in its selection of proliferative cell types is

likely to be useful toward cancer research. That is to say, as we do not yet know the cellular origins of the various forms of breast cancers, it may be preferable, at least initially, to collect a wider variety of proliferative cell types for study rather than use assays that are too selective and risk missing entire populations of cells that are prone to carcinogenesis.

Beyond budding variability, the morphological observations of macaque mammospheres also identified aspects of proliferation and differentiation that may prove useful toward future research. First, if CD10 is truly a marker of bilineage progenitor cells as previously suggested,[71] then it appears that most macaque spheres in culture are predominantly composed of relatively undifferentiated cells within a mantle of epithelial-like cells. It also appears that as if the leading edges and less-organized areas of the budding projections are also predominated by undifferentiated cells. Expression of SMA and/or CK18 was typically only appreciated within the more matured (presumed, based on cellular morphology) aspects of the budding projections and there were areas of some budding projections that were negative for all three markers. Notably, ER staining and PR staining (following lactogenic differentiation) were commonly identified to occur in the parts of the budding projections that lacked CD10, SMA, and CK18 expression. The significance of the expansive, highly-cellular extrusions that were associated with the formation of secondary-bodies, and the secondary-bodies themselves, is unknown. Both of these structures frequently had strong CD10, SMA, and CK18 expression with each marker demonstrating distinct regions of preferential staining. No ER or PR staining was identified within these structures. Whether these structures are representative of in vivo physiological processes (e.g., expansion of the terminal end ducts associated with mammary gland development) or are artifacts of the in vitro plating process still needs to be explored in future studies.

Lactogenic differentiation of BME-encased macaque mammospheres was accomplished through the addition of prolactin to the culture media, as previously described,[71, 196, 230] but also required a novel modification to the technique. Initially, a number of experiments that incorporated prolactin use at various stages of mammosphere development failed to promote lactation in the macaque mammospheres. Based on the knowledge that the mammary gland niche provides hormones and other growth factors that can promote lactogenic differentiation,[127, 145, 226, 277] CM2 was created and added to the media. The addition of CM2 was successful at stimulating lactation (β-casein staining) within the alveolar-like structures of the budding spheres but also, unexpectedly, induced intracytoplasmic vacuoles within most cells of these spheres. Both prolactin and CM2 appeared to be essential to lactation, as the

addition of prolactin alone did not appreciably alter the spheres while the addition of CM2 without prolactin resulted in vacuolation of the spheres without lactation.

As to the reason CM2 was required for lactogenic differentiation in the macaque mammospheres but was not in the human experiments is unknown but it is proposed to be related to the differences in methodology that were utilized to produce the mammospheres themselves. In the macaque studies, the 1k mammospheres were first grown in culture and then were encased within BME. In the human studies,[71, 196] single-cell suspensions were plated into BME which then formed into mammospheres. In an experiment comparing the differences of the BME-encased spheres produced by these methodologies, cells acquired from one subject were used to produce mammospheres using the human technique and also to produce mammospheres using the macaque technique. Morphological analysis of the two sphere populations after 21 days of incubation identified similar immunostaining patterns but found that the mammospheres derived from the macaque-method contained a denser and more angular population of cells than those derived from the human-method. Furthermore, the spheres derived from the human-method had notably more PAS-positive extracellular matrix within the main sphere body than did the spheres derived using the macaque-method. While these investigations are only preliminary, the initial observations suggest distinct morphological differences in the mammospheres produced using these two methods and this may account for the additional growth factors needed in the spheres produced using the macaque technique. Further studies investigating the differences in the spheres formed using these two methodologies are warranted.

The nature of the CM2-induced vacuoles has yet to be elucidated but the histologic appearance suggests that the material is likely lipid. One mouse study has identified "oil-filled adipocytes" within "stromal" mammospheres.[70] However, these vacuoles were present without any stimulation and the primary mouse mammospheres of this study may not be appropriate for comparison to the secondary macaque spheres used here.[70] In macaques, secretory fat vacuoles are commonly identified within normal mammary epithelial cells[56] and one study has identified myoepithelial vacuoles in the immature ducts of young animals undergoing mammary gland development.[288] This last observation is of particular interest and further investigation into the possible in vivo significance of the mammosphere vacuoles is warranted. In the future, lactogenic-differentiated mammospheres will be collected as frozen samples, in addition to being preserved in formalin, to allow for a more complete histological examination of the vacuoles.

Statistically significant differences were identified for most of the mammosphere growth and differentiation parameters between nulliparous and multiparous follicular/luteal-phase animals in this study. These results are similar to those of another study between nulliparous and multiparous macaques which compared the mammosphere potential of biopsies that were collected from animals in the menses phase of the cycle (Chapter 4). Consistent with these macaque data, other research has identified the mammary glands of nulliparous mice to contain significantly higher numbers of stem cells as compared to the glands of parous animals.[235] Information obtained from the PCA supported the parity-related differences in the macaque and furthermore suggested, albeit weakly, that the phase of the reproductive cycle may also play a role with regard to mammosphere potential. Although ultimately no significant differences in mammosphere potential were identified with regard to the phase of the cycle, it should be noted that a major limitation of this study was the low number of subjects available for analysis from each of the various demographics. Regardless of the lack of statistical significance to the trend above, there is literature to suggest that mammosphere potential is likely to vary with the phase of the reproductive cycle. Specifically, there are significant differences in the proliferative capacity of the macaque mammary gland in vivo that are associated with distinct stages of the menstrual cycle.[50, 244] Additionally, murine studies have found that single-cell isolates collected from mammary glands at different stages of the estrus cycle have significantly different in vitro proliferative potential.[121] Based on this information, and the study trend above, it is proposed that additional research into the effects of the menstrual cycle on mammospheres potential is warranted.

While direct comparisons in mammosphere potential between the individual stages of the reproductive cycle was not possible due to the low numbers of optimally-processed animals in the study, there were some observations made with regard to reproductive stage that bear mentioning. The first is that, other than the lactating animal, the menses stage monkey is the multiparous subject that was furthest removed from the main cluster of animals. Taken by itself, this observation is easily dismissed as individual variation amongst animals. However, the data obtained from this animal were found to be highly consistent with data obtained from the biopsies of six, similarly-processed, multiparous, menses-stage animals in another study (Chapter 4). In light of this information, it is proposed that the macaque gland may have overall less mammosphere potential during the menses-stage than at the two other stages (i.e., follicular and luteal stages) of the active menstrual cycle.

Other observations with regard to the reproductive cycle involve the inactive-phase animals. Little is known about the inactive-phase of the macaque reproductive cycle other than that during this reproductive phase the

blood levels of the sex hormones are typically very low. However, even these hormone levels are variable as some animals experience irregular or anovulatory menstrual cycles throughout the inactive-phase.[50, 279] Consistent with a low hormonal state, the inactive-phase animals included in this study tended to have lower mammosphere potential than the follicular- or luteal-phase animals. This was illustrated in the vector diagram but is also exemplified in the generational studies where the two inactive-phase animals were the only subjects that failed to produce quaternary spheres. Consistent with the reports of hormonal variability in the inactive-phase of the cycle, the four subjects comprising the inactive-phase cohort were also found to be more divergent on the vector diagram than were the animals within the follicular- or luteal-phase cohorts. Furthermore, it is also notable that an inactive-phase animal was the only subject in the quadrant studies that was identified to have significant differences in the MFE between any of its mammary gland quadrants. Per these observations, and with the knowledge that there is no stage of the normal human reproductive cycle that correlates with the inactive phase, the use of mammary gland tissues from animals in the inactive-phase of the cycle should likely be avoided where reproducible, human-relevant research data are required.

Although mammary gland was acquired from only one pregnant animal in the formal study utilizing the optimized technique, a number of pregnant/early post-partum animals were utilized in the developmental and optimization aspect of this project. The general observation for these subjects was that there was little consistency as to the mammosphere potential between any two animals in this particular reproductive stage. This information is not particularly surprising as pregnancy is a highly dynamic state but, rather, serves as a reminder that if tissues from pregnant animals are to be utilized in studies, precise timing of the pregnancies will likely be required to maintain reproducibility between subjects.

There was some study data which suggested that age may also play a role in the mammosphere potential of mammary gland cells. The position of the various-aged nulliparous subjects on the vector diagram is particularly supportive of this possibility, while the age-comparisons between multiparous subjects within each reproductive stage are also intriguing. However, what must be appreciated with regard to the multiparous animal-comparisons is that breeding-colony monkeys were used as the tissue donors in this study and older monkeys also tended to have produced larger numbers of offspring (greater parity). In short, it is essentially impossible to deduce from these data what influence age may have contributed to the change in mammosphere potential for the multiparous subjects.

In review, parity status is almost certainly related to mammosphere potential in the macaque and there is information to suggest that the reproductive stage and age may also influence the proliferation and/or differentiation capabilities of the mammospheres as well. As such, demographic information to include age, parity-status and reproductive stage should ideally be obtained of every macaque donor used for mammosphere cultures to allow for appropriate comparisons between subjects and between studies. Furthermore, it is proposed that if similar information can be obtained from human and rodent donors utilized for mammosphere cultures it is possible that mammosphere data can be better standardized across species.

The results obtained from this study have a number of limitations in addition to those previously mentioned. First is the issue that the majority of the tissues utilized for this project were derived from animals that were euthanized due to disease, trauma or as part of unrelated research studies. As such, it is possible that any of the factors associated with the death of these animals may have affected the mammosphere potential of the mammary glands utilized for this work. Future studies utilizing tissues collected from clinically-healthy subjects will be beneficial at validating the results obtained from this project.

Information provided above suggest that mammospheres derived from 1k cultures are more likely to be of a clonal origin and therefore may provide a more accurate, overall assessment of the mammosphere potential for any given single-cell suspension as compared to mammospheres derived from 10k sphere cultures. To that, the mammosphere potential of secondary, and later generational, macaque mammospheres was evaluated utilizing mammospheres derived from 1k mammosphere cultures alone. However, the mammosphere potential of primary macaque mammospheres was most commonly assessed using mammospheres obtained from 10k cultures and this fact constitutes another limitation to the interpretation of the data reported here. The use of 10k-derived, rather than 1k-derived, primary mammospheres in this study was deemed necessary for two reasons. The first issue, as previously discussed in the *Results* section, was that large numbers of primary 1k mammospheres could not be efficiently obtained from the mammary gland tissue-digests of multiparous animals. The second, and more problematic issue was that even when multiparous, primary 1k mammospheres were obtained, these spheres were small and ragged in appearance and they had minimal ability to undergo attachment or differentiation as compared to the multiparous, primary 10k spheres or the nulliparous, primary 1k spheres. Experiments using macaque tissues suggest that the decreased proliferation and viability of the multiparous 1k mammospheres is largely due to insufficient concentrations of pro-mammosphere growth factors within the culture media. Specifically, when

primary 1k mammosphere cultures from two of the multiparous macaques in this study were supplemented with CM1 from another multiparous animal, these 1k cultures produced larger numbers of more viable-appearing spheres. These CM1-supplemented spheres had MDP and MBP values that were nearly identical to those of the primary 10k cultures from the same two animals. Additionally, in these same experiments the difference between the MFEs for the 1k and 10k cultures was identified to be only 5.2%, rather than the 13.3% when CM-1 supplementation was withheld. This last finding is significant in that it suggests that the use of 1k primary cultures may actually underestimate the primary MFE potential for multiparous macaques. In light of the similarities in the differentiation potential between the primary 10k and 1k spheres, in conjunction with the previous data that demonstrated a significant correlation between the primary 10k and 1k MFEs, the use of 10k cultures was considered to be acceptable, albeit not ideal, for the comparison studies here.

Notably, a number of experimental techniques aimed at obtaining viable primary 1k mammospheres cultures from these animals were considered prior to the decision to utilize the primary 10k cultures for this work. Unfortunately, it was not possible to supplement the individual primary 1k cultures with their own CM1 at Day 0 of the plating process as CM1 is not collected from the primary 100k sphere cultures until Day 3. Additionally, experiments showed that supplementation of macaque primary 1k cultures with CM1derived from their own 100k cultures at Day 3 of the incubation process only marginally improved mammosphere viability. The possibility of routinely using CM1 from other animals to improve the viability of primary 1k cultures was rejected as the biologically-active components of the CM1 may vary between subjects and could have confounded the comparison studies. In that the deficiency in pro-mammosphere growth factors within the 1k cultures is likely a direct result of the ratios of proliferative to non-proliferative cell types within the mammary gland single-cell suspensions, consideration was also given to experimental techniques that could specifically concentrate proliferative cell types (e.g., fluorescence-activated cell sorting (FACS)). However, this approach was also rejected as this would have required the selection of specific cell phenotypes that may not be similarly expressed across the different demographics of animals and, as such, could have confounded comparisons as to the overall mammosphere potential between these populations. In short, culture supplementation with CM1 and the use of proliferative-cell concentration techniques are both likely to provide further insight into the macaque mammosphere potential in future research projects although neither approach was perceived to be superior to the 10k mammosphere culture technique in regard to the initial assessment of the single-cell suspensions derived from the mammary gland.

There are some limitations to the interpretation of the morphologic studies of the BME-encased spheres as the variation in budding morphology of these spheres was assumed to be due to differences in the outgrowth potential of single cells. As previously discussed, it is likely that at least a small percentage of the spheres in these studies were derived from the aggregation and proliferation of two or more cell types.[70, 71, 231] Furthermore it is possible that some spheres, the larger spheres in particular, could be the byproduct of two or more spheres that became adjoined during the mammosphere culture process. The fact that only large spheres were identified to form secondary bodies is intriguing in this regard and it is plausible that formation of this specific budding structure may require the influence of two or more progenitor cell types. The proposition that secondary body-formation may be dependent on the contributions of two or more cell types is supported by the observation that no secondary bodies were identified in any of the mammospheres that were derived from a single cell plated in a 96-well plate. The results of the single-cell studies cannot be considered definitive, however, as only a small number of spheres were available for BME-encasement. Larger studies of a similar nature are warranted to investigate this proposition further.

Data from the morphological comparisons of BME-encased mammospheres in this study suggested that, other than in a few early pregnant females, there was minimal variation in the differentiation-potential between the different demographics of animals. There are limits to the interpretation of this work, however, in that the characterization of budding morphology was based on a few overt features of the budding spheres as a whole. Future studies which utilize a greater number of parameters to assess differences in budding morphology may identify variations in morphology between the demographics that were not appreciated here.

Finally, the ability of the mammospheres to be formed from a single cell, have sustained generational growth, and undergo bilineage differentiation was proposed as evidence that the macaque mammosphere cultures represent a population of cells that are enriched for MSCs. However, in vivo transplantation of mammosphere-derived cells into mice is generally considered the gold standard by which stem cell enrichment is validated.[67, 71, 196, 230, 231] As these experiments have not yet been performed, there are limitations in the interpretation as to the stem cell-enriched nature of the macaque mammosphere cultures in general. Studies are currently in progress to address this deficiency.

The first major aim of this project was achieved as a rhesus macaque mammosphere technique was developed and demonstrated to be highly reproducible. The mammosphere potential of the mammary gland was also

demonstrated to be similar throughout a large, well-defined region of the organ and the first reported mammary gland weights were obtained for this species. The second major aim of this project was achieved insomuch as the comparisons between the human and macaque mammospheres did not identify any major differences that would preclude the use of macaque mammospheres as models for human research. As with most validations of this kind, however, minor differences were identified for some assays and are proposed to be methodological in nature. Future work is proposed to explore these discrepancies in more detail. Finally, the third major aim was achieved in that significant differences in mammosphere potential were identified to be associated with parity-status in the macaque. Trends in the data, experimental observations, and comparisons with other studies suggest that mammosphere potential may also be affected by the stage of the menstrual cycle and further work is proposed in this area. There was minimal data to associate mammosphere potential with age although the numbers of subjects available for these analyses were low. The possibility that age could affect mammosphere potential cannot be excluded based on the results of this study.

In conclusion, a mammosphere culture technique was optimized for the rhesus macaque. The resultant protocols, along with outcomes of the individual optimization experiments, are described here in detail in efforts to promote the use and advancement of this technique. The assay was demonstrated to be highly reproducible and small biopsies acquired from the cranial aspect of the mammary gland were found to be representative of the region in general. Rhesus macaque mammospheres were identified to have properties similar to those of human mammospheres and, as such, are likely to be a relevant study model for the human breast. A study utilizing tissues from 18 animals demonstrated that the mammosphere potential of mammary glands is likely affected by the reproductive state of the animal and it is suggested that this information be taken into consideration when utilizing the mammosphere culture technique in research.

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Chapter 3: Vaginal Cytology of the Rhesus Macaque

Introduction

Rhesus macaques (*Macaca mulatta*) are widely used as models for the study of human reproductive biology and disease as a result of their genetic, morphologic, and physiologic similarities to humans.[10, 31, 63, 77, 201, 244, 267, 279] Of particular importance to the current study is the fact that the menstrual cycle of sexually-active female rhesus macaques is generally recognized to be comparable in length (i.e., 25-35 days) and form (i.e., menstruation) to that of women. Despite overall similarities, one notable difference between rhesus macaques and humans is breeding seasonality. Rather than being reproductively active year round, rhesus typically only exhibit regular ovarian cycling and sexual receptivity for one 2-6 month period annually.[50, 63, 77, 94, 274, 278, 279]

Research studies that involve sexually-intact macaques may require monitoring of the ovarian cycle in order to synchronize the collection of study samples to one specific stage of the cycle. This is performed to minimize the difference in sex hormone concentrations between the individual study animals, as varying hormone levels have the potential to confound data interpretation.[50, 244, 279] Ovarian cycle stage can be monitored with high accuracy by to evaluating progressive changes in the serum levels of the sex hormones. Unfortunately, reliable assessment of ovarian cycle stage in these animals usually requires that blood be collected from each subject 2-3 times per week over several months duration.[177, 202, 274, 278, 279] This degree of diligence is required as sex hormone levels vary significantly between individual subjects and there are currently no established hormone concentration standards that accurately identify one particular stage of the macaque ovarian cycle. Serial monitoring of sex hormone levels is undesirable for large studies as it can be extremely laborious to acquire the requisite number of blood samples. Additionally, the results of hormone assays are often not immediately available. Finally, as both physical and psychosocial stress can lead to disruption of the normal reproductive cycle in nonhuman primates (NHPs),[3, 94, 148, 188, 202, 274, 279] it is plausible that the stresses associated with repeated blood draws in macaques could confound reliable study data collection. Novel approaches toward the timing of the ovarian cycle that are less work-intensive and that are less stressful to the individual animals and their social groups are desirable and are likely to be useful to many facets of research involving these species.[183, 279]

Routine monitoring of vaginal cytology has been investigated as a less stressful and less work-intensive means to evaluate and pinpoint stages of the ovarian cycle. Unfortunately, previous attempts to demonstrate that this

technique can accurately identify each of the six most commonly-defined stages of the macaque menstrual cycle (i.e., menses, early-follicular, late-follicular, ovulatory, early-luteal and late-luteal stages) have not been encouraging. This is because studies have found that certain stages of the macaque cycle are indistinguishable from one another by vaginal cytology alone and, importantly, these "indistinguishable stages" tend to be the stages that are typically of greatest interest to researchers (i.e., late-follicular, ovulatory and early-luteal stages).[10, 63, 124, 165, 173, 177, 183, 201, 281] However, if a researcher is able to utilize tissue samples that are synchronized to one of the other three stages of the macaque cycle (i.e., menses, early-follicular, late-luteal stages) then the use of vaginal cytology to manage synchronization of sample collections may still be a viable option.

Synchronization to the menses stage of the cycle via vaginal cytology is an attractive option for research projects that have no need for their study samples to be collected during either the follicular or luteal stage. This is because previous work in macaques has suggested that the vaginal cytology of the menses stage is distinct from the other stages of the active cycle.[63, 173, 183] Furthermore, in that this stage is also associated with vaginal bleeding (menstruation), daily cageside observations to inspect the perineum of the animals, and/or daily vaginal swabbings can serve as minimally invasive means by which macaques can be presumptively identified to be within the menses stage of the cycle.[77, 165, 183, 202, 244, 274, 278, 279]

While the identification of vaginal-derived blood in regularly-cycling NHPs may serve as a presumptive diagnosis of the menses stage of the cycle, there are other causes of vaginal bleeding in these species that should be ruled out if the research results might be affected by variable hormone levels between study animals. Anovulatory cycles are documented to occur in many NHP species and these cycles can result in vaginal bleeding that is similar in character to the menses stage of a normal ovulatory cycle.[63, 77, 165, 173, 274] Anovulatory cycles are potentially problematic for studies, in that the animals experiencing these cycles typically lack a fully-functional corpus luteum and therefore have hormone levels that are notably different from ovulating animals .[63, 202, 274, 278, 279] Other common reproductive tract pathologies of NHPs such as vaginitis, leiomyomas, ovarian cysts, and endometriosis, can also potentially produce vaginal bleeding that might be confused with menstruation.[39, 57, 60, 126, 183, 283, 298] Finally, for any female animals that are cohoused with sexually-intact males, there is also the potential for pregnancy-associated vaginal bleeding to be confused with menstruation. This could present itself as either non-pathologic gestational hemorrhage ("spotting") or early to mid-term abortions.[173]

Comprehensive vaginal cytology-studies are described in the rhesus macaque which utilized vaginal smears prepared with Shorr stain[63] and hematoxylin and eosin (H&E) stains[173] although a review of the literature found that most macaque vaginal cytology studies have utilized classical Papanicolaou (Pap) stains.[51, 124, 165, 177, 183, 244] Pap stains are also recommended in industry-standard guidelines as they reliably produce high quality cytologies.[268] However, Pap stains are relatively time-consuming and complicated to prepare[19, 120, 183] and are therefore unsuitable for certain research situations where short turnaround times are required. There is consequently a need to identify a rapid staining technique which can produce vaginal cytologies that are of Pap stain diagnostic quality.

Romanowsky-type stains are the most popular stains in veterinary medicine[19, 120, 207]and are frequently utilized to process the vaginal cytologies of dogs[14, 197, 203, 227-229] and laboratory rodents.[61] Additionally, at least one specific Romanowsky-type stain has been reported to be equivalent to the classical Pap stain with regard interpretation of vaginal cytologies in the baboon.[136] A modified procedure for Diff-Quik® (a Romanowsky-type stain) has been demonstrated to produce rapid cytological specimens of comparable quality to those of Pap stains for a wide variety of tissue types in domestic veterinary species.[120] In light of these previous successes, it is plausible that the modified-Diff-Quik® (MDQ) staining might also serve as a means by which rapid and reliable vaginal cytology results can be obtained for the rhesus macaque.

The current project evaluates the utility of a novel, rapid-staining (MDQ) technique for use in rhesus macaque vaginal cytology. The first aim of this project was to use MDQ-stained vaginal cytologies to define the cytologic parameters of the menses, follicular, periovulatory, and luteal stages of the menstrual cycle in reproductively-active rhesus macaques, with the hypothesis that MDQ vaginal cytology can be utilized to accurately and reliably identify the menses stage in reproductively-active rhesus macaques. An additional aim of this project was to identify the MDQ vaginal cytology parameters that were characteristic of the rhesus macaque: 1) during the reproductively-inactive period of the year; 2) when pregnant; 3) when undergoing anovulatory cycles; 4) following abortions; 5) when affected by reproductive tract disease. The hypothesis of this aim was that MDQ vaginal cytology characteristics can be used to reliably differentiate the menses stage from: 1) animals in the inactive stage of the reproductive cycle; and, 2) animals with other potential causes of vaginal bleeding. Successful completion of these aims is expected to result in the identification of specific vaginal cytology parameters by which future tissue collections in the rhesus macaque can be reliably and efficiently synchronized to the menses stage of cycle.

Materials and Methods

Animal model. Biological specimens were acquired from a total of 53 female, Indian-origin, rhesus macaques. In vivo vaginal swabbings were collected from nine clinically-healthy pregnant animals that ultimately carried their infants to term; and from five animals that had undergone a spontaneous abortion. Additionally, vaginal swabbings and ovarian and uterine tissues were opportunistically collected at necropsy from 39 animals of reproductive age. These macaques were animals that either died or were euthanized due to illness or trauma, or were euthanized as part of other research projects unrelated to the current study. All of the pregnant animals, abortive animals, and 37 of the necropsied animals originated from the breeding colony at The University of Texas, MD Anderson Cancer Center, Keeling Center for Comparative Medicine and Research (KCCMR) in Bastrop, TX. Throughout the timeframe of this project, the KCCMR breeding colony maintained an ongoing census of approximately 600 adult female macaques. The KCCMR breeding colony has been a closed colony since 1983 and no outside animals have contributed to the genetic lines since that time. The colony has been documented through serological means to be Specific Pathogen Free (SPF) for Cercopithecine Herpesvirus 1, Simian Immunodeficiency Virus, Systemic T-lymphotrophic Virus and Simian Retroviruses 1, 2, and 5 since 1991. The final two animals which provided necropsy-derived specimens were animals originally procured from an external vendor, although they had been maintained at the KCCMR for approximately 2 years prior to euthanasia. These final two animals were seropositive for Cercopithecine Herpesvirus 1 at the time of their death but were clinically normal and were SPF otherwise. Signalment and medical history were collected for each of the study animals. The KCCMR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACI) and all animals utilized in this study were housed in full compliance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals* (ILAR).[182] All tissue collection methods used for this study were reviewed and approved through The University of Texas, MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC).

Vaginal swabbings and vaginal impression smears. In vivo vaginal swabbings from the pregnant and abortive animals were performed as the animals were maintained under anesthesia in the KCCMR veterinary clinic for routine physical and ultrasound examinations. Ultrasound examination was utilized to diagnose animals as "pregnant" and fetal measurements were utilized to estimate gestational age. Ultrasound examination was also utilized to confirm the diagnosis of "abortion" in animals that had previously been diagnosed as pregnant. The

gestational ages of the abortive pregnancies were determined from the results of the previous ultrasound examination in which the dams had been determined to be pregnant. The abortive animal swabbings utilized in this study were all collected within 8 hours (range 1-8 hours) of the time that the abortive animals had been noted as having bloody discharge from the vagina. Necropsy-derived vaginal swabbings were collected within 1 hour of the death of the animal with many of the swabbings collected immediately following euthanasia. Vaginal swabbings were collected in a manner similar to that previously described for NHPs. [10, 51, 124, 136, 173, 177, 183] A cotton-tipped applicator (swab) was moistened slightly with 0.9% saline solution and then gently inserted into the vagina until it made light contact with the cervix and then the swab was retracted approximately half that distance. The swab was rolled against the vaginal walls with slight pressure to obtain a smear that was representative of the vaginal vault midway between the cervix and vaginal opening. After removal, the swab was rolled along a glass slide to create multiple linear impression smears. A minimum of two swabs and slides were prepared for each animal. The smears were allowed to air-dry thoroughly at room temperature. The slides were prepared for cytologic examination utilizing a modified Diff-Quik® (EK Industries, Joliet, IL) protocol.[120] This technique used the same three staining solutions as the standard Diff-Quik® technique but increased the exposure to each solution to 20 dips/solution and included an additional xylene step (5 dips) at the end of the process once the slides had dried.

Necropsy-derived tissue collections and histologic assessment. The uterine and ovarian tissues collected at necropsy were immersion-fixed in 10% neutral buffered formalin (LabChem, Pittsburg, PA) for a minimum of 72 hours. Following fixation, the tissues were routinely processed for histologic examination by the KCCMR Histology Laboratory and paraffin-embedded sections were cut at 4 μm and stained with hematoxylin and eosin (H&E; Poly Scientific R and D, Bay Shore, NY). The uterine and ovarian morphology was assessed by two boarded veterinary pathologists who collectively determined the specific reproductive stage for each animal using histologic parameters as previously described.[10, 31, 267]

Vaginal cytologic assessment. Review of the vaginal impression smears was performed by a boarded veterinary pathologist and two certified medical technologists. The mean score of the three examinations was reported for each parameter. Similar to previous studies, [10, 51, 63, 177, 183] the vaginal smears were examined under light microscopy to qualitatively assess the number of erythrocytes, leukocytes, and bacteria, and also the amount of mucus and cell clumping present throughout each slide. Each of these parameters was then scored between 0 (lowest number/amount) and 3 (highest number/amount) for each slide. Differential cell-counts of 300 cells were also

performed on the vaginal epithelial cells of the impression smears. This was done using a back and forth meandering approach in a minimum of three separate regions per slide. Individual cells were classified as either parabasal, intermediate, or superficial cells based on morphologic parameters as previously described for NHP vaginal cytologies [10, 51, 124, 163, 173, 183, 244] and as detailed in Figure 1. Similar to other macaque vaginal cytology studies,[51, 244] a maturation value of the vaginal epithelial cell population was calculated for each subject as follows: (Parabasal cell number x 0.2) + (Intermediate cell number x 0.6) + (Superficial cell number x 1.0) /300 = Maturation Value (MV).

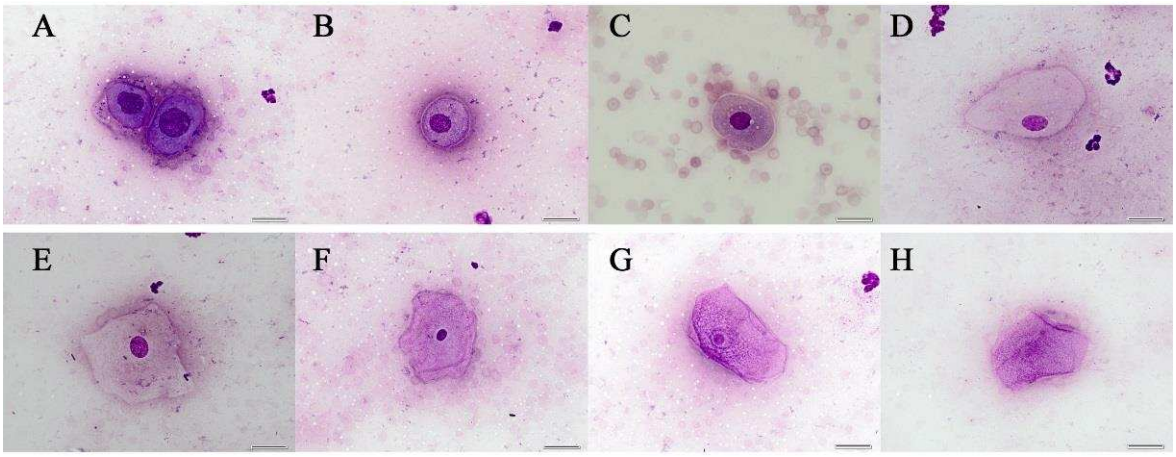

Figure 1. MDQ-vaginal cytology cellular morphology allows identification of distinct cell types. Image A are *parabasal cells.* These cells have rounded edges, basophilic cytoplasm and contain a rounded, centralized nucleus composed of ropy to clumped chromatin. The diameter of the nucleus of these cells is approximately one-third or more the size of the cell diameter. Image B and C are *small intermediate cells*. These cells are round (Image B) to ovoid and can have angular projections (Image C). The cytoplasm is slightly basophilic to eosinophilic. The nucleus is round to ovoid and has ropy to clumped chromatin. The diameter of the intermediate cell nucleus is notably less than one-third the size of the cell diameter. Image D and E are *large intermediate cells*. These cells have angular margins, pale cytoplasm and ovoid nuclei with ropy to clumped chromatin. *Superficial cells* are keratinized cells with angular margins. Image F is a *superficial cell* with a small pyknotic nucleus. Image G is a *superficial cell* with a pyknotic nucleus surrounded by a perinuclear halo. Image H is an *anucleate superficial cell*. MDQ-images at 100 x magnification were collected from animals in the menses stage of the cycle (note background erythrocytes and occasional polymorphonuclear leukocytes). Scale bars = 25 µm.

Vaginal cytology comparisons and statistical analysis. As previously described, the histological characteristics of the reproductive organs of necropsied animals, and the ultrasound findings of pregnant and abortive animals, were utilized to define the reproductive stage of each study subject. The MDQ vaginal cytologies collected from animals in the menses, early-follicular, late-follicular, periovulatory, early-luteal, and late-luteal phases of the active menstrual cycle were quantitatively and qualitatively compared to one another. The MDQ vaginal cytologies from menses stage animals were also quantitatively and qualitatively compared to MDQ vaginal cytologies collected from animals in the inactive stage of the cycle, pregnant animals, abortive animals, one

anovulatory menstruation animal and one animal with polycystic ovaries. Statistical comparisons of the various reproductive stages were performed using a t-test (two sample assuming equal variances) through IBM SPSS version 22 (SPSS Inc., Chicago, IL) software. Statistical significance for all data was set at $P<0.05$.

Results

Ovarian and uterine histological assessment. Results of the uterine and ovarian histological evaluation for each necropsy-derived study animal are provided in Table 1. There were 28 animals identified to be in a reproductively active stage of the menstrual cycle at the time of their death. These animals were classified into the

Table 1. Vaginal cytologies obtained from various demographics of rhesus macaques. Definition of table terms. *Animal #:* order processed in the study. Asterisks (*) indicate the two study animals that did not originate from the KCCMR breeding colony. *Age*: rounded to nearest whole year. *Class* (parity): N=nulliparous; P=primiparous; M=multiparous. *Stage* (reproductive status per uterus/ovary histology): M=menses stage; Fe=follicular stage (early); Fl=follicular stage (late); PO=periovulatory stage; Le=luteal stage (early); Ll=luteal stage (late); AO=anovulatory menstruation; I=inactive phase; Cy=polycystic ovaries. *MV*: calculated Maturation Value of the vaginal epithelial cell population (see text). *RBC*: red blood cell/erythrocyte qualitative count. *WBC*: white blood cell/leukocyte qualitative count. *Bacteria*: qualitative bacterial cell count. *Clumping*: qualitative assessment of the amount of cell clumping. *Mucus*: qualitative assessment of the amount of mucus present. For all qualitative counts/assessments a score of 0=lowest number/amount and 3=highest number/amount. Animals listed as "opportunistic" were euthanized as part of a study unrelated to this project.

Animal	Age (yr)	Class	Stage	Infants	MV	RBC	WBC	Bacteria	Clumping	Mucus	Cause of death/euthanisia
1	14	M	Ll	4	87.2	0	0.33	1.33	0.33	0	trauma
2	17	M	I	12	33.6	0	0.67	1.33	2.61	3	cancer
3	19	M	I	10	64.8	0	2	2.67	3	2.33	cancer
4	11	M	I	7	99.7	0	1.67	3	2.67	2	amyloidosis
5	21	M	I	12	100	0	1	2.33	3	0.67	cancer
6	15	M	M	7	51.8	2.33	3	1.33	2	2	trauma
7	18	M	Fl	12	99.1	0	2	3	3	2	arthritis
8	6	P	I	1	81.2	0	1	2.33	2	0.33	arthrtitis
9	4	N	Ll	0	90	0	0.33	1.33	1.67	1	enteritis/arthritis
10	21	M	Fe	13	66	0	2.33	3	1	3	cancer
11	19	M	Fl	12	98	0	3	2	3	1.67	amyloid/arthritis
12	18	M	I	12	74.4	0	2.67	1.33	2.67	3	cancer
13	8	N	Fl	0	99.6	0	2.33	2	2	1	opportunistic
14	4	N	Fl	0	100	0	3	3	3	2	opportunistic
15	16	M	Ll	7	71.2	0	0	2	1.33	2	cancer
16	20	M	AO	2	88.9	2	0.67	3	2	1.67	liver abscess
17	11	M	M	4	71.2	1.33	2	2.33	2.33	2	cancer
18	8	M	Ll	2	81.6	0	1.67	2	2	2	opportunistic
19	23	M	I	16	98.8	0	2	2	1.67	2.33	opportunistic
20	17	M	Fl	9	99.2	0	3	3	3	3	opportunistic
21	17	M	PO	7	99.6	0	1	2.33	1.33	1	opportunistic
22	17	M	M	11	60.4	3	3	2.33	1.33	2	amyloid/enteritis
23	19	M	Cy	8	100	0	3	3	3	2	pneumonia
24	11	M	I	4	100	0	0.33	2.67	3	1.67	enteritis
25	20	M	Fe	13	73.6	0	2	3	1.67	2	arthritis
26	9	M	Le	4	97.6	0	0.67	2	1	1	enteritis
27*	5	N	I	0	30.5	0	0.33	0.67	2	2	opportunistic
28*	6	N	Le	0	99.5	0	0.33	2	0.67	1	opportunistic
29	13	M	M	7	64.8	3	3	1.33	2	2.33	opportunistic
30	8	P	M	1	72.6	3	2	1.33	0.33	1	opportunistic
31	7	N	Le	0	74.6	0	0	3	1.33	2	opportunistic
32	9	N	Fe	0	86.3	0	0.33	1	0	1	opportunistic
33	7	N	M	0	55.3	3	2	1.33	0.33	2	opportunistic
34	7	M	Fl	2	98.6	0	3	2	2.67	2.67	opportunistic
35	10	M	Fe	2	96.5	0	2.67	2	2	3	opportunistic
36	12	M	PO	2	99.3	0	2	3	3	0.67	opportunistic
37	8	M	Ll	2	75.3	0	1	3	0	2	opportunistic
38	6	N	Le	0	98.9	0	2.67	2.67	2.33	3	opportunistic
39	6	N	Le	0	99.4	0	0.33	2	3	1	opportunistic

following cohorts: 6 menses stage; 6 late-follicular stage; 5 early-luteal stage; 5 late-luteal stage; 4 early-follicular stage; and 2 periovulatory stage. The histologic parameters use to define the individual stages of the active menstrual cycle were similar to those previously described.[10, 31, 267] A brief summary of the defining characteristics of each stage is provided. The menses stage animals had ovarian follicles that were small or undergoing atrophy and a corpus luteum (produced during the previous menstrual cycle) that was also undergoing atrophy; the uterine endometrium was undergoing hemorrhage with cellular degeneration and sloughing. The early-follicular stage animals had several developing secondary and/or early-tertiary ovarian follicles and a largely-atrophied corpus luteum; the endometrium was densely cellular, moderately thickened, and contained elongate, straight, narrow glands. The late-follicular stage animals had tertiary and/or Graafian follicles in at least one ovary; the cellularity of endometrium was less dense than the early-follicular stage, markedly thickened, and the glands often became coiled/tortuous toward the end of this stage. The periovulatory stage animals had a recently-ruptured Graafian follicle in the ovary, that was either cavitated or blood-filled (corpus hemorrhagicum); the cellularity and thickness of the endometrium was similar to that of the late-follicular stage and it had coiled/tortuous glands. The early-luteal stage animals had a developing corpus luteum in one ovary; the cellularity and thickness of the endometrium was similar to that of the late-follicular stage and it had coiled/tortuous glands that contained small amounts of proteinaceous fluid. The late-luteal stage animals had a well-developed corpus luteum in one ovary; the cellularity and thickness of the endometrium was similar to that of the early-follicular stage and it had coiled/tortuous glands that contained abundant proteinaceous fluid.

There were nine necropsied macaques identified to be in the reproductively inactive phase of the cycle at the time of their death. The defining histological characteristic of these animals was the lack of follicle formation and the absence of a corpus luteum in the ovaries. One necropsied macaque was identified to be undergoing anovulatory menstruation. Histologically, this animal lacked a corpus luteum in the ovaries; the endometrium had mild hemorrhage and sloughing of the surface cells, but the endometrium was thin and densely cellular. One necropsied macaque was diagnosed with polycystic ovaries by the presence of numerous fluid-filled cysts throughout both ovaries.

General findings of MDQ-vaginal cytology. The vaginal cytology findings for each of the necropsied macaques are provided in Table 1. The MV, qualitative erythrocyte counts, qualitative leukocytes counts, qualitative cell clumping volumes and the identification of specific cells types (e.g., parabasal cells, degenerate cells) were

found to be the most useful parameters for the identification of specific menstrual cycle stages and reproductive states. The qualitative bacterial count was highly variable within all cohorts, was not indicative of any particular stage or state, and therefore is not discussed further in this report. The qualitative assessment of mucus volume was also highly variable within most of the cohorts, was of minimal diagnostic utility, and therefore is only discussed in the few states/stages where this parameter was determined to statistically significant. The leukocytes identified within the smears of this study were almost exclusively polymorphonuclear cells (neutrophils) except where specifically mentioned otherwise below.

MDQ-vaginal cytology and comparisons across stages of the active menstrual cycle. Summaries of the changes to the vaginal cytology throughout the menstrual cycle of the rhesus macaque are provided in Figure 2. The vaginal cytology of the menses stage was the most distinctive stage of the active menstrual cycle due to the presence of abundant erythrocytes. The stage was also unique in that the menses vaginal cytologies were the only cytologies of the active cycle in which degenerate epithelial cells were present. In addition to the presence of the occasional degenerate cell, the epithelial cell population of the menses stage was characterized by high numbers of intermediate cells, moderate numbers of parabasal cells and small to moderate numbers of superficial cells. In that similar ratios of vaginal epithelial cells were only identified in the early-follicular stage vaginal cytologies, the menses stage had a

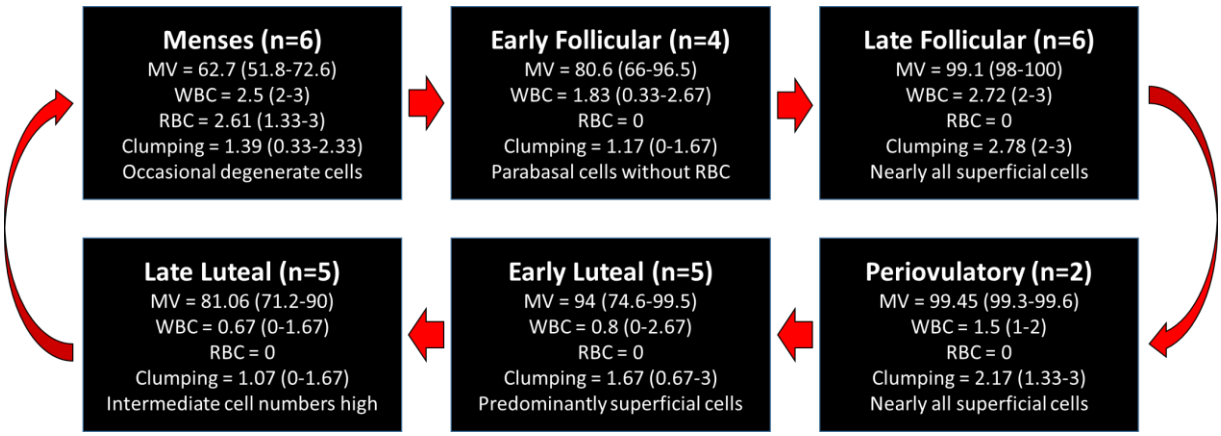

Figure 2. MDQ vaginal cytology identified morphological characteristics that were distinct for several stages of the rhesus macaque menstrual cycle. The flow diagram represents the progressive changes in the vaginal cytology throughout the menstrual cycle. Each box is titled with the Stage of the cycle and the individual parameters utilized to characterize the stages are provided in rows below the title. The final row under each Stage describes other features common to that stage. For each parameter, the mean score for all animals in that Stage are provided, as are the ranges (in parentheses) where applicable. Parameters: *MV*=calculated Maturation Value of vaginal epithelial cell population (see text). *WBC*: white blood cell/leukocyte qualitative count. *RBC*: red blood cell/erythrocyte qualitative count. *Clumping*: qualitative assessment of the amount of cell clumping. For all qualitative counts/assessments a score of 0=lowest number/amount and 3=highest number/amount.

MV that was significantly lower than every stage of the active cycle except the early-follicular stage. The menses stage was even further distinguished from the two luteal stages of the cycle in that the qualitative leukocyte counts of the menses stage were significantly greater than those of either the early-luteal or late-luteal stages.

Early-follicular stage vaginal cytologies had MVs and qualitative leukocyte counts that were statistically similar to every other stage of the active cycle. Despite this fact, early-follicular vaginal cytology was distinct from the vaginal cytology of all other stages of the active cycle due to the presence of small to moderate numbers of parabasal cells in the absence of erythrocytes.

Late-follicular vaginal cytology had statistically higher cell-clumping values than did the vaginal cytologies of the menses, early-follicular and late-luteal stage. The late-follicular stage was also distinct from these three stages in that the late-follicular vaginal cytology was comprised of high numbers of superficial cells, very few intermediate cells, and almost no parabasal cells. These differences resulted in late-follicular MVs that were statistically greater than those of the menses and the late-luteal stages; and late-follicular MVs which were also typically greater, although not significantly, than the MVs of early-follicular vaginal cytologies.

In contrast to the comparisons of the previous three stages, the late-follicular vaginal cytology was essentially indistinguishable from the vaginal cytologies of the periovulatory and early-luteal stages in that all these stages were dominated by superficial cells that frequently formed large clumps. Differentiation between these three stages was also confounded by the fact that the MVs and cell-clumping values for these stages were also statistically similar. Although the early-luteal stage had statistically lower qualitative leukocyte counts than did the late-follicular or periovulatory stages, the ranges of this parameter between the three stages markedly overlapped one another. Likewise, although the periovulatory-stage had statistically lower mucus volumes than did the late-follicular stage, the mucus volume values overlapped between the two stages. As per the overall similarities between the these stages, and the fact that leukocyte counts and mucus volumes could not be relied upon for precise stage identification, the vaginal cytologies from the late-follicular, periovulatory, and early-luteal stages were all classified within a single, cytologically-distinct “periovulation phase”.

The late-luteal stage vaginal cytology had a MV, and qualitative leukocyte counts, that were significantly lower than those of the late-follicular stage. However, the late-luteal stage vaginal cytology was most distinguishable from the vaginal cytologies of the periovulation phase, the menses stage and the early-follicular stage by the presence of high numbers of intermediate cells that often equaled, or exceeded, the numbers of

superficial cells. The general lack of parabasal cells in this stage was also useful for differentiating the late-luteal stage from the early-follicular stage (the stage with which the late-luteal stage was most likely to be confused). Illustrative examples of the histologic and vaginal cytology findings associated with the menses stage, early-follicular stage, periovulation phase, and late-luteal stage are provided in Figure 3.

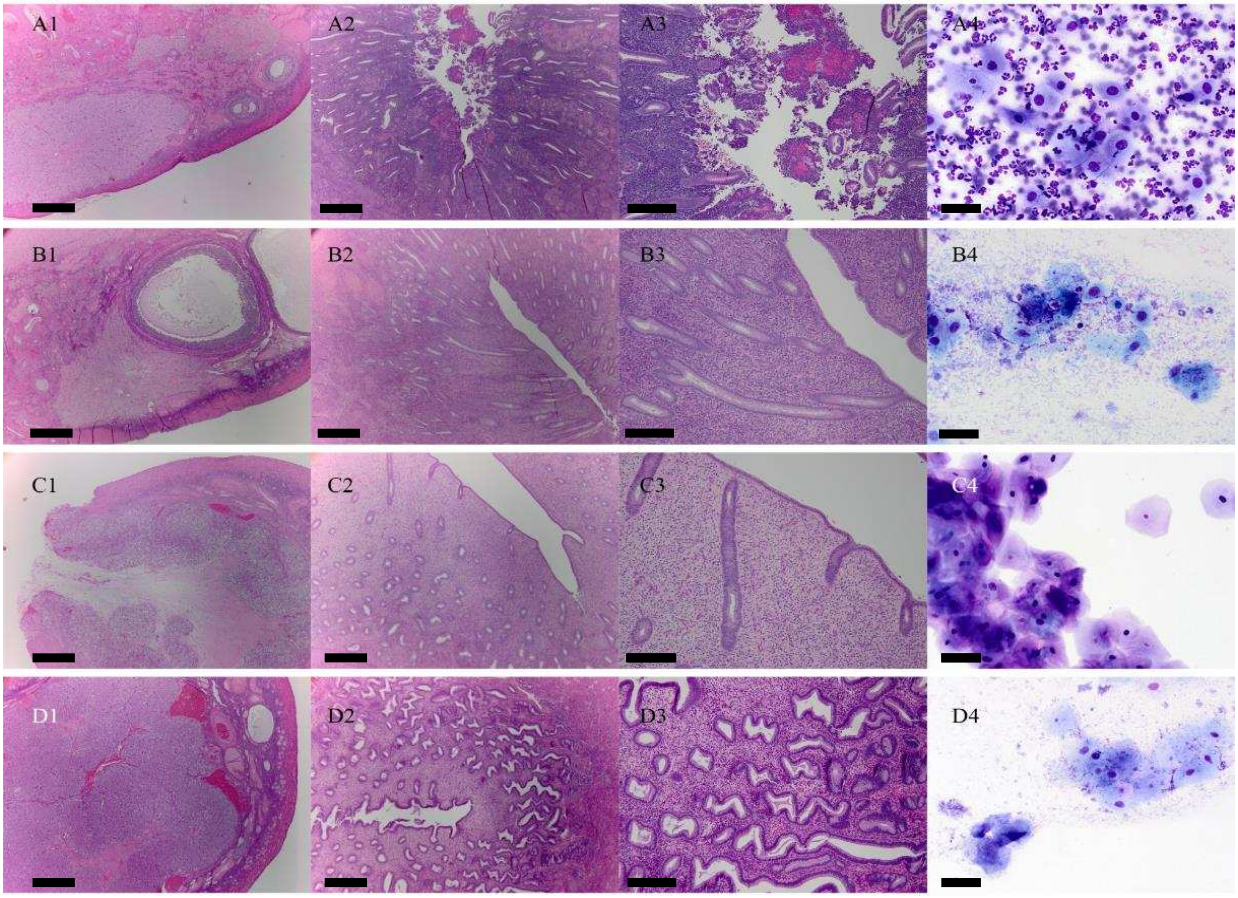

Figure 3. The MDQ vaginal cytology is distinct for three stages and one phase of the rhesus macaque menstrual cycle. Column 1 is histology of the ovary at 4 x magnification (scale bars = 500 µm). Columns 2 and 3 are histology of the uterus at 4 x (scale bars = 500 µm) and 10 x (scale bars = 200 µm) magnification, respectively. Column 4 is images of the typical MDQ vaginal cytology for each stage at 50 x magnification (scale bars = 40 µm). *Row A: Menses stage of the cycle*. A1-A3: Ovarian and uterine histology as previously described in the text. A4: Vaginal cytology contains abundant erythrocytes within a mixture of parabasal, and intermediate cells with only small-moderate numbers of superficial cells present; there is typically moderate to marked numbers of leukocytes (neutrophils predominately) present. *Row B: Early-follicular stage of the cycle*. B1-B3: Ovarian and uterine histology as previously described in the text. B4: Vaginal cytology lacks erythrocytes and consists of a mixture of parabasal, intermediate and superficial cell types; low to moderate numbers of leukocyte are present. *Row C: Periovulatory phase of the cycle* (collectively, the late-follicular, periovulatory, and early-luteal stages). C1-C3: Ovarian and uterine histology as previously described in the text. C4: Vaginal cytology is composed almost entirely of superficial cells and these cells often form large clusters (clumping); leukocyte numbers are highly variable throughout this phase. *Row D: Late-luteal stage of the cycle*. D1-D3: Ovarian and uterine histology as previously described in the text. D4: Vaginal cytology contains abundant intermediate cells which frequently outnumber the superficial cells; parabasal cells are rare; leukocyte numbers are typically low.

MDQ-vaginal cytology from animals outside of the normal active-menstrual cycle. Vaginal cytologies collected from the nine rhesus macaques identified to be in their annual period of sexual inactivity ("inactive (I)"; Table 1) varied widely in their cytologic parameters (MV=30.5-100; WBC=0.33-2.67; Clumping 1.0-3.0). Importantly, however, erythrocytes were notably absent from all of these vaginal smears. Consistent with these findings, the vaginal cytologies obtained from animals in this cohort resembled every stage of the active-menstrual cycle except the menses stage. Inactive-phase cytologies were also distinct from the menses stage cytologies in that the inactive cytologies had statistically lower quantitative leukocyte counts and statistically greater amounts of cell clumping.

The single animal identified to be undergoing an anovulatory menstruation (Animal # 16) had a qualitative erythrocyte count that fell within the ranges of the menses stage animals from ovulatory cycles. However, the qualitative leukocyte count (0.67) of that vaginal cytology was lower than any of the true menses stage vaginal cytologies. Furthermore, the cytology of the anovulatory menstruation animal had high ratios of superficial cells that resulted in a MV that was greater in value (88.9) than any of the cytologies from the menses stage animals.

The vaginal cytology from the polycystic ovaries-animal (Animal # 23) had no erythrocytes. Additionally, the vaginal cytology of this animal was consistent with the periovulation phase of the cycle with a MV (100) and qualitative cell clumping value (3.0) that were greater than any of the menses stage animals.

MDQ-vaginal cytology of pregnancy and abortion. Vaginal cytologies were collected from nine pregnant animals. Six animals were in their first-trimester of pregnancy and three animals were in their second-trimester of pregnancy. The results of these evaluations are presented in Figure 4. Erythrocytes were notably absent from all of the pregnant animal vaginal cytologies. The mucus volume and qualitative leukocyte counts of pregnant animals were significantly lower than the mucus volume (mean=1.8 (range= 1.0-2.33)) and leukocyte counts of the menses stage animals. While at least a few parabasal cells were identified in all of the pregnant animal cytologies, the ratios of the vaginal epithelial cells were highly variable between the individual subjects. A summary of the predominate cell types for the nine pregnant animals is as follows: 1) two first-trimester vaginal cytologies were predominated by parabasal cells; 2) four first-trimester and two second-trimester vaginal cytologies were predominated by intermediate cells; and 3) one second-trimester vaginal cytology was predominated by superficial cells. Consistent with these findings, the MV of the second-trimester pregnancies was significantly higher than the

MVs of both the menses stage animals and the first-trimester pregnancies, while the MVs of the menses stage and the first-trimester pregnancies were statistically similar.

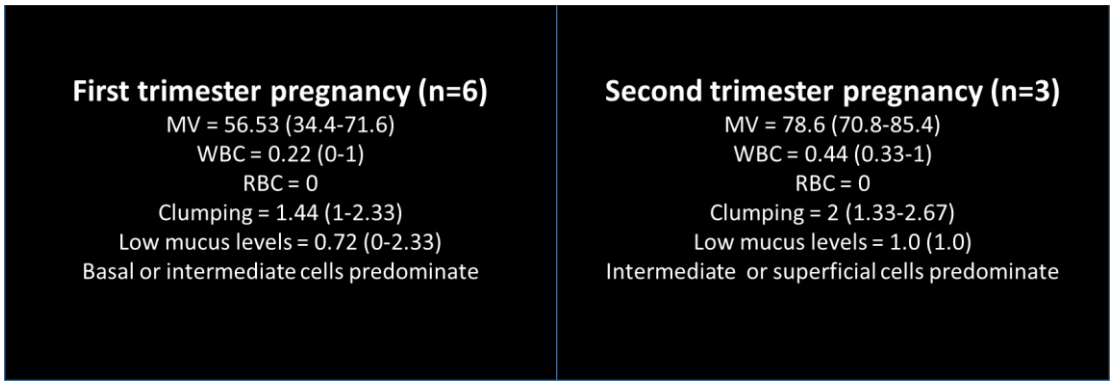

Figure 4. The MDQ vaginal cytology of pregnant macaques is distinct from the vaginal cytology of menses stage macaques. The *Left Box* is the collective findings of the vaginal cytology from six first-trimester pregnancies. The *Right Box* is the collective findings of the vaginal cytology from three second-trimester pregnancies. For each parameter, the mean score for all animals in that trimester are provided, as are the ranges (in parentheses) where applicable. Parameters: *MV*= calculated Maturation Value of vaginal epithelial cell population (see text). *WBC*: white blood cell/leukocyte qualitative count. *RBC*: red blood cell/erythrocyte qualitative count. *Clumping*: qualitative assessment of the amount of cell clumping. For all qualitative counts/assessments a score of 0=lowest number/amount and 3=highest number/amount.

Vaginal cytologies were also collected from five animals that were identified to have undergone spontaneous abortions. Four of these animals were in their first-trimester of pregnancy and one animal was in its second-trimester of pregnancy. The first-trimester abortions (Figure 5) had qualitative erythrocyte counts, qualitative leukocyte counts and cell-clumping values that were statistically similar to those of actively-cycling menses stage animals. However, first-trimester abortive vaginal cytologies did have some features that differentiated them from menses stage vaginal cytologies. First, the MV of abortive vaginal cytologies was significantly higher than those of the menses stage. This was due to the fact that abortive vaginal cytologies were dominated by superficial and intermediate cells with only small numbers of parabasal cells identified. Second, the numbers of degenerate epithelial cells were subjectively greater in abortive smears as compared menses smears. Third, the overall cell staining qualities of the abortive vaginal cytologies was generally poor. Finally, macrophages/foamy macrophages were commonly identified in the abortive vaginal cytologies while these cells were rare in all other vaginal cytologies, including those of the normal menses stage.

Figure 5. The MDQ vaginal cytology of first trimester-abortive macaques is distinct from the vaginal cytology of menses stage macaques. *Image on left* is representative of vaginal cytology as identified in four first-trimester abortions at 50 x magnification (scale bars = 40 µm). There are large numbers of neutrophils and degenerate cells; large to moderate numbers of erythrocytes; and moderate numbers of macrophages. Intermediate and superficial cells are the prominent vaginal epithelial cells identified and these cells tend to be pale staining as compared to vaginal cytology derived from other animals. The *Right Box* is the collective findings of the vaginal cytology from four first-trimester abortions. For each parameter, the mean score for all animals in that trimester are provided, as are the ranges (in parentheses) where applicable. Parameters: *MV*= calculated Maturation Value of vaginal epithelial cell population (see text). *WBC*: white blood cell/leukocyte qualitative count. *RBC*: red blood cell/erythrocyte qualitative count. *Clumping*: qualitative assessment of the amount of cell clumping. For all qualitative counts/assessments a score of 0=lowest number/amount and 3=highest number/amount.

The vaginal cytology of the sole second-trimester abortion also had qualitative erythrocyte counts (3.0) and a cell clumping value (1.33) that fell within the ranges of the vaginal cytologies of the menses stage of the cycle. However, this vaginal cytology was different from those of the menses stage animals in that it had a qualitative leukocyte count (1.66) that was lower than any of the menses stage vaginal cytologies and it was also dominated by superficial cells which resulted in an extremely high MV (98.9). Finally, although the second-trimester abortive animal had much smaller numbers of macrophages than did the first-trimester abortive animals, the numbers of macrophages in the second-trimester vaginal cytologies was still subjectively greater than what was seen in those of the menses stage vaginal cytologies.

Discussion

This is the first study to directly compare reproductive tract histology and vaginal cytology from various stages of the reproductive cycle in the rhesus macaque. This is also the first study to utilize a Romanowsky-type stain (MDQ) for the evaluation of vaginal cytology in any macaque species. Finally, this study is the first to explore the possibility of using MDQ-stained vaginal cytologies to definitively identify the menses stage of the ovulatory menstrual cycle (hereafter referred to simply as “menses stage”) in the rhesus macaque.

There is currently no "gold standard" method by which macaques are determined to be within a specific stage of the active reproductive cycle. Previous macaque studies that examined the vaginal cytologies of multiple menstrual-cycle stages identified their study subjects to be within specific reproductive stages through the use of one or more of the following diagnostic methods: hormonal assays;[177] ovarian laparotomy examination;[63, 177] rectal palpation;[63, 165] and timed-collection practices (based on paired-menstruation dates).[10, 63, 173, 183, 244] The current macaque study, by contrast, is the first to utilize established histologic parameters of the reproductive tract[10, 31, 267] to define the stage of the vaginal smears collected from various stages of the active menstrual cycle. Given the accuracy with which these cytologies were able to be characterized to a specific menstrual cycle stage, the cytologic descriptions of this report are likely to serve as a resource by which future vaginal cytology studies investigating specific stages of the macaque cycle can be compared. One potential limitation of these data however is that all the vaginal smears of the active menstrual cycle were collected from animals that were euthanized due to disease, trauma or as part of a research protocol of unrelated to the current study. Therefore, while the cytology descriptions as a whole are thought to be highly representative of the individual stages from which they are derived, it is plausible that some of the individual animals within this study could have individual cellular characteristics, as reported in Table 1, that contradict the results of other vaginal cytology studies.

The MDQ stained vaginal cytologies of this study identified overall relative changes to the vaginal epithelial cell populations throughout the menstrual cycle that were highly comparable to the changes identified in previous macaque studies that utilized Pap-,[165, 177, 183] Shorr-,[10, 63] and H&E-stained[173] vaginal cytologies. Specific observations regarding the individual stages that were made in both the current study and previous studies included: 1) cell clumping was most prominent in the late-follicular stage;[10, 177] 2) the late-follicular, ovulatory, and earl-luteal stages of the active cycle were essentially indistinguishable;[173, 177, 183] 3) the menses stage was distinct from other stages as per the presence of erythrocytes;[63, 177, 183] 4) the menses stage had the lowest mean MV of all stages;[173, 177, 183] 5) the early-follicular and late-luteal phases were cytologically distinct from the other stages;[63] and 6) the early-follicular and late-luteal stages had mean MVs that were nearly identical in value.[10]

Only one previous vaginal cytology study was identified to contain any information that varied, to any real extent, from the what was observed in the MDQ study.[10] In that Shorr-stained study, the MVs obtained from both follicular and both luteal stages were identified to be slightly lower (5-10 points/stage) than the MVs reported in the current study. A review of the data suggests that the MV discrepancy between these two studies is likely related to

differences in the Shorr and MDQ stains with regard to their cut-off points for the differentiation of the intermediate and superficial cell types. However, it is also plausible that at least some the difference in the MVs may be accounted for by a cell-characterization bias that potentially existed between the two research groups conducting these studies. Regardless of the MV differences, the Shorr and MDQ studies were comparable with regard to the general, as well as several specific, cytologic changes throughout the cycle, as detailed above.

It warrants mentioning that all of the Pap-stained vaginal cytology studies[165, 177, 183] and one of the Shorr-stained studies[10] used for comparative purposes in this report were performed in cynomolgus macaque (*Macaca fascicularis*, a.k.a. *Macaca irus*) monkeys. The use of cynomolgus data was deemed necessary for the Pap-stain comparisons of this report in that there are no rhesus Pap-stain vaginal cytology studies identified which have compared more than two stages of the menstrual cycle. The comparison of the MDQ-stained rhesus macaque vaginal cytologies to the Pap-stained and Shorr-stained cynomolgus macaque vaginal cytologies constitutes a potential limitation to the comparative value of this report. However, this cross-species comparison is proposed to be both appropriate and relevant for two reasons. First, cynomolgus macaques have a reproductive physiology that is identified to be "nearly identical" to that of the rhesus macaque.[279] Second, all of the macaque vaginal cytologies studies that were reviewed for this report identified similar changes to the vaginal epithelial cell populations across the menstrual cycle, regardless of the cytology stain-type and regardless of the macaque species.[10, 63, 124, 165, 173, 177, 183, 244]

Collectively, the information presented in the comparative discussions above suggest that MDQ-stained vaginal cytologies are equivalent to Pap-, Shorr-, and H&E-stained vaginal cytologies with regard to their ability to identify and differentiate the individual stages of the macaque menstrual cycle.

The results of this study, as well as other studies, suggest that the presence of abundant erythrocytes[63, 177, 183] and occasional degenerate cells[63] on vaginal cytology are highly specific parameters that can be used to diagnose the menses stage in actively cycling macaques. The results of the current study also found that these same two parameters can be used to distinguish the vaginal cytologies of the menses stage from those of pregnant animals, and animals within the inactive phase of the reproductive cycle. However, caution is warranted in utilizing either of these two parameters as the sole means of diagnosing the menses stage given that: 1) degenerate cells were not identified in every menses stage vaginal cytology of this study; 2) erythrocytes were associated with the vaginal cytology of anovulatory menstruation-animals in this, and a previous,[63] study; 3) erythrocytes have previously been

reported in the vaginal cytologies in some pregnant animals[173]; and 4) erythrocytes and degenerate cells were associated with the vaginal cytologies of the abortive animals in this study.

Collectively this information suggests that the presence of erythrocytes on vaginal cytology (with or without the presence of degenerate cells) is a sensitive, though nonspecific means, of identifying animals in the menses stage of the active cycle. As such, every vaginal cytology containing erythrocytes is a potential menses stage cytology, however other vaginal cytologic parameters must also be assessed and certain donor-animal related information must be known before a menses stage diagnosis can be made with any appreciable degree of certainty. At a minimum, all menses-like vaginal cytologies (MLVCs) must be evaluated for the presence of macrophages and must have their MVs assessed. Donor-related information that is important to the accurate characterization of MLVCs includes the age, reproductive history, and menstruation cyclicity-patterns of the donor as well at the date on which the vaginal smears were collected.

In this study, macrophages were only rarely seen in the menses stage cytologies but were readily identified within most abortive animal cytologies. As such, it is suggested that no MLVC identified to have more than the rare macrophage should be diagnosed as menses stage. For MLVCs that are questionable as to whether there are "rare" or "low" numbers of macrophages present on the slide, the use of additional vaginal cytology parameters may prove helpful in differentiating between abortive and menses stage cytologies. Specifically, any MLVCs with poor staining characteristics and/or the moderate to high numbers of degenerate cells should not be given a diagnosis of menses stage, as data from this study suggest that both of these features are more consistent with abortive animal vaginal cytologies. Finally, this study and a previous study have found that the mean MV of menses stage cytologies is lower that the MVs of most abortive cytologies and some anovulatory menstruation cytologies.[63] Given this information and the findings of the current and previous studies,[173, 183] it is suggested that no MLVC with a MV greater than 75 should be diagnosed as menses stage.

While some anovulatory menstruation cytologies can be ruled out as being menses stage based on their high MV values, there have been other anovulatory menstruation cytologies identified to have MVs and vaginal epithelial cell populations that were similar to those of menses stage animals.[63] Given the potential similarities between the vaginal cytologies of some menses stage and anovulatory menstruation animals, the use of other diagnostic modalities, such as serial hormone analysis, would likely be of benefit in differentiating these two reproductive states. However, as previously discussed, the use of such methods is not always a viable option for

every research project. For research situations where the use of additional diagnostic modalities is limited, a practical means of minimizing confusion between anovulatory menstruation and the menses stage cytologies is to only collect vaginal smears from the animals that are most likely to be undergoing ovulatory menstrual cycles. Donor-related information is important to this process in that previous studies have found: 1) between 93 to 97% of the vaginal bleeding-events that occur in mature, reproductively-sound, rhesus macaques during the breeding season are associated with ovulatory menstrual cycles[274, 278]; 2) anovulatory cycles occur most frequently in rhesus macaques during the inactive phase of the year;[63] and 3) anovulatory cycles are commonly associated with macaques that have irregular menstrual cycling patterns or have prolonged intervals between consecutive menstrual events.[63, 77, 278, 279] Based on these findings, it is proposed that MLVCs can be diagnosed as being menses stage cytologies with a high degree of certainty if the vaginal-swabs are obtained from rhesus macaques during the middle third of their breeding season and the animals used for these studies meet certain criteria. These criteria include: 1) the study animals must be undergoing regular menstruation events (~25-35 days in duration); 2) parous study animals must have recently produced offspring; and 3) nulliparous study animals must be of an age (~43 months old[285]) where most animals are expected to be undergoing regular ovulatory menstrual cycles.

Although no erythrocytes were identified in the pregnant animal vaginal cytologies of this study, gestational-associated spotting is occasionally identified in pregnant macaques and a previous study has also reported erythrocytes to be a feature of some pregnant macaque cytologies.[173] This information, in conjunction with the finding that the vaginal epithelial cell populations of some pregnant animals in this study resembled those of menses stage animals, makes it plausible that spotting-pregnant animals could be confused with menses stage animals if vaginal cytology is used as the sole means of diagnosis. It is therefore suggested that any animal producing a MLVC should undergo a thorough physical exam to include the use of diagnostic modalities (e.g., ultrasound examination) which can ensure study animals are not pregnant and can also rule out reproductive tract disease with a relatively high degree of accuracy.

The only animal identified to have a reproductive tract disease in this study (polycystic ovaries), had a vaginal cytology that was wholly inconsistent with that of the menses stage. A previous report of another macaque with polycystic ovaries also reported comparable vaginal cytology findings.[177] In that no other reproductive tract diseases (i.e., leiomyomas, endometriosis, and vaginitis) were identified in this study and there is no literature as to the expected vaginal cytologies of animals with these conditions, no definitive conclusions can be made as to the

ability of vaginal cytology to differentiate menses stage animals from animals affected by reproductive tract disease. It therefore remains possible that macaques with hemorrhagic forms of reproductive tract disease could produce vaginal cytologies that are consistent with those of the menses stage. To that, a thorough physical examination is suggested to be the likely best method of ruling out reproductive tract disease for any animals producing MLVCs.

In summary, this is the first study to provide descriptions of rhesus macaque vaginal cytologies from six histologically-defined menstrual cycle stages and three histologically-defined reproductively inactive states. Descriptions of vaginal cytologies were also obtained from pregnant animals and abortive animals in this study. While all of these descriptions have provided additional information to the field, the descriptions of the histologically-defined menstrual cycle stages are proposed to be the most significant for future studies as per the accuracy with which each stage was able to be characterized. Comparisons of the current study results to those of previous vaginal cytology studies identified the MDQ-staining technique to be capable of producing macaque vaginal cytologies that are of equivalent diagnostic utility to those produced using the Pap-, Shorr-, and H&E-staining techniques. As MDQ-staining requires less processing time than do the other three staining methods, this finding is likely to be of benefit to any future vaginal cytology studies where a decreased time of vaginal cytology processing is desired or required. In support of the two hypotheses of this study, the menses stage vaginal cytologies were found to have characteristics that made them distinct from the vaginal cytologies of: 1) all other stages of the active-menstrual cycle; 2) inactive phase animals; 3) pregnant animals; 4) abortive animals; 5) an anovulatory-menstruation animal; and 6) an animal with reproductive disease. These findings, along with the results of previous vaginal cytology studies, were then utilized to formulate a systematic approach by which the menses stage animals can be readily distinguished from animals in other reproductive stages/states. Ultimately, the results of this study suggest that the menses stage of the rhesus macaque menstrual cycle can be identified with a relatively high degree of accuracy when MDQ vaginal cytology is paired with certain pieces of information regarding medical and reproductive history.

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Chapter 4: Proliferation, Differentiation, and Radiosensitivity of Mammary Epithelial Cells Derived from Multiparous and Nulliparous Rhesus Macaques

Introduction

There is evidence to suggest that many breast cancers may arise as a result of neoplastic alterations to the mammary stem cells (MSCs) and/or the multipotent (bilineage) progenitor cells (MPCs) that exist within the normal mammary gland.[71, 74, 133, 146, 158, 160, 209, 260] In efforts to facilitate research into this area of breast carcinogenesis, a cell culture technique known as mammosphere culture has been developed. This culture technique is capable of obtaining highly enriched populations of MSCs/MPCs from mammary gland tissues through the formation of free-floating, spherical cell colonies (mammospheres).[67, 71, 73, 85, 147, 196, 206, 230, 231, 243]

Beyond simply enriching for MSC/MPCs, mammosphere cultures have a number of additional properties which make them useful toward other aspects of mammary gland research. First, it has been demonstrated that when single-cell isolates are plated at low cell-densities in a mammosphere culture, most of the spheres in these cultures originate from the clonal proliferation of a single MSC or MPC.[22, 71, 85, 147, 230, 243] In turn, mammosphere-forming ability has been utilized as a metric by which to estimate the ratio of MSCs/MPCs in mammary gland tissues as well as in cell cultures of mammary gland origin.[71, 230, 243] Second, mammospheres of a MSC or MPC origin are capable of forming in vitro and in vivo bilineage-outgrowths comprised of both epithelial and myoepithelial components.[71, 206, 230, 243] In this way mammosphere cultures provide another means by which to easily obtain and study the two principle cell populations of the mammary gland. Finally, most clonally-derived mammospheres contain only a few MSC/MPC-like cells capable of future mammosphere production (referred to here as mammosphere forming units (MFU)) while the bulk of the cells that make up these spheres are more phenotypically-restricted mammary gland cell types such as lineage-specific (epithelial or myoepithelial) progenitor cells and non-proliferative cells.[71, 206, 230, 243] This cellular arrangement is similar to the hierarchical organization of the normal mammary gland[20, 139, 243, 270, 271] and, therefore, intact mammospheres can potentially serve as an in vitro research model by which to study the physical and molecular interactions of the various cell populations comprising the mammary gland.[71, 206, 230, 243]

Macaque monkeys have mammary glands that are developmentally, morphologically, and physiologically comparable to those of humans.[56, 288] Macaques have also been identified to spontaneously develop preneoplastic and neoplastic mammary gland lesions that are highly similar to those of humans.[291] In that further understanding of macaque mammary gland tissues is likely to be of benefit toward breast cancer research, the mammosphere culture

technique has recently been optimized for use with tissues obtained from the rhesus macaque *(Macaca mulatta)* (Chapter 2). The optimization study found that macaque mammospheres contain cells with MSC/MPC-like properties and also identified these spheres to have morphologic and physiologic characteristics that were comparable to mammospheres derived from human breast tissues.

Exposure to ionizing radiation is an established cause of breast cancer in women.[4, 35, 47, 135, 142, 213, 271] Data obtained from the medical establishment and atomic bomb survivors has shown that the lifetime risk of developing breast cancer after radiation exposure is inversely correlated with age.[4, 35, 47, 135, 142, 257] The population of females most frequently reported to be at the greatest risk are those individuals who were exposed to radiation at less than 20 years of age. Other studies have also found that women who are exposed to radiation and then remain nulliparous, or who give birth to their first child late in life, are at greater risk of developing breast cancer than are similarly-exposed women who undergo a first full term pregnancy at a younger age.[32, 35, 141, 172, 213]

These relative-risk findings are of great interest with regard to the possible role of MSCs in radiation-induced breast cancer. This is because the overall numbers of MSCs in the breast are proposed to decrease with age and with each successive pregnancy, as the MSCs undergo terminal differentiation throughout breast maturation.[220, 282] Collectively, the relative-risk data and MSC observations have been interpreted to suggest that the risk of radiation-induced breast cancer may be associated with the number of MSCs present within the breast at the time of irradiation and/or the duration of time for which MSCs are maintained as undifferentiated cells following irradiation. An alternative interpretation of these data, however, is that with age and/or pregnancy the breast may undergo biological alterations, even beyond those of MSC-terminal differentiation, that render "matured" MSCs and/or MPCs less susceptible to radiation-induced neoplastic change.[27, 220]

Identifying the mechanisms behind the increased sensitivity of young-nulliparous females to radiation-induced breast cancer could ultimately also lead to insights into breast carcinogenesis as a whole as it is known that women who undergo an early-life full-term pregnancy also have a greatly diminished lifetime risk of developing the most-common spontaneous forms of non-hereditary breast cancer.[6, 27, 65, 130, 140, 172, 220, 270]

Given the similarities in the risk factors for radiation-induced and spontaneous breast cancers, in conjunction with the knowledge that MSCs/MPCs are suspected to play a role in breast carcinogenesis, a logical first step toward understanding the parity-related risk factors associated with breast cancer is to look for differences in the numbers, functionality, and radiosensitivity of the MSC/MPC populations in young-nulliparous and

multiparous females. While ideally this work would be performed using human breast tissues, studies of this nature are extremely unlikely as most human tissues are obtained as byproducts of reduction mammoplasties and young-nulliparous breast tissue is seldom collected.[22] Although rodent tissues can be easily collected from any demographic of animal, comparative studies using rodent tissues are not ideal in that there are significant developmental, physiological, and morphological differences known to exist between the mammary glands of humans and rodents.[33, 56, 100, 129, 253, 279] Additionally, it is appreciated that the most common, spontaneous and radiation-induced forms of mammary gland neoplasia in rodents are derived from different populations of mammary gland cells than are most human cancers.[30, 33, 100, 181, 255] As such, rodent studies comparing differences in the nulliparous and multiparous mammary glands may not fully model the cellular mechanisms behind human breast carcinogenesis. Moreover, it has been proposed that data obtained from rodent studies could even confound our understanding of some aspects of the human disease.[30, 115, 213]

In light of the aforementioned limitations to human and rodent mammary tissues, an animal model known to have mammary glands highly similar to those of humans, the rhesus macaque, was utilized in the current study to compare the mammosphere potential between the nulliparous and multiparous mammary gland. For these comparisons, in vivo mammary gland biopsies were collected from seven multiparous and seven young-nulliparous animals with the biopsy procedure for each animal synchronized to occur during the menses-stage of the menstrual cycle. The synchronization of the biopsy procedure to one particular stage of the menstrual cycle was undertaken here as previous macaque work has suggested that the mammosphere potential of the gland may be affected by the stage of the menstrual cycle (Chapter 2). Following collection, the tissues were processed as mammosphere cultures and then comparisons were made between the proliferative ability, differentiating capabilities and the radiosensitivity of the cells derived from the young-nulliparous and multiparous mammary glands.

The first major aim of this study was to quantify the number of cells capable of forming mammospheres for both the young-nulliparous and multiparous mammary glands. The hypothesis being that the young-nulliparous mammary gland would have a greater total number of cells with mammosphere forming ability than would the multiparous gland. The second major aim of this study was to compare the proliferative and differentiation capabilities of the cells comprising the nulliparous and multiparous mammospheres. The hypothesis being that the mammospheres derived from the young-nulliparous mammary gland would be composed of cells with greater proliferative and differentiation abilities than would mammospheres derived from the multiparous gland. The third

major aim of this study was to compare the effects of irradiation on the proliferative and differentiation abilities of the cells derived from the nulliparous and multiparous mammospheres. The hypothesis being that the cells derived from the nulliparous and multiparous mammospheres would demonstrate significant differences in their proliferative and/or differentiation abilities in response to irradiation.

Materials and Methods

Animal model. Study animals were selected from approximately 600 adult and juvenile, Indian-origin female rhesus macaques (*Macaca mulatta*) within a breeding colony at The University of Texas, MD Anderson Cancer Center, Keeling Center for Comparative Medicine and Research (KCCMR) in Bastrop, TX. The KCCMR colony has been a closed colony since 1983 and has been documented through serological means to be Specific Pathogen Free (SPF) for Cercopithecine Herpesvirus 1, Simian Immunodeficiency Virus, Systemic T-lymphotrophic Virus and Simian Retroviruses 1, 2, and 5 since 1991. In vivo mammary gland biopsies were collected from seven multiparous and seven nulliparous animals. All subjects were given a physical exam and were identified to be clinically healthy and in a nonlactating state prior to their inclusion into the study. All study animals had a Body Condition Score (BCS) that ranged between 2.5 and 3.5 out of a maximum score of 5, as determined by the consensus of two veterinarians utilizing published BCS parameters for the rhesus macaque.[58] The multiparous females used for this study were housed in single-male/multi-female (harem) breeding groups. The nulliparous animals used for this study were group-housed exclusively with other females. The husbandry of all animals utilized in this study was in full compliance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals* (ILAR)[182] and the KCCMR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACI). All tissue collections for this study were reviewed and approved through The University of Texas, MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC).

Mammary gland biopsy synchronization and collection. Biopsy collections were begun during the approximate midpoint of the breeding season for this colony and each biopsy was collected during the presumed menses stage of the menstrual cycle. All study animals had observable vaginal bleeding (interpreted as menstruation) occurring at the time of the biopsy procedure and each animal had the mammary biopsy procedure performed within 1-3 days of the onset of vaginal bleeding. Two vaginal cytology slides for each study animal were prepared for examination utilizing a modified Diff-Quik® (EK Industries, Joliet, IL) protocol.[120] The cellular

morphology was confirmed to be consistent with the menses stage of the menstrual cycle as previously described (Chapter 3).

All of the study animals had also been identified to undergo repeated menstrual cycles at intervals consistent with those described of ovulatory menstrual cycles in the rhesus macaque (25-35 days).[77, 274] This was done in efforts to decrease the possibility of acquiring mammary gland tissues from animals during an anovulatory menstrual cycle. The multiparous animals utilized in the study were observed to have had at least one prior episode of vaginal bleeding that occurred less than 33 days (range 27-32 days) prior to the date on which the mammary gland biopsy was collected. Similarly, the nulliparous animals used in the study were observed to have had at least two consecutive episodes of vaginal bleeding separated by less than 35 days (range 28-34 days), prior to the date on which the mammary gland biopsy was collected. A physical examination and ultrasound examination was also performed on each study animal to further ensure that no other external or internal causes of vaginal bleeding (e.g., trauma, reproductive disease, pregnancy, early-term abortion) were present.

In vivo mammary gland biopsy collection was performed as previously described (Chapter 2). In brief, each animal was anesthetized and aseptic surgical technique was utilized to collect a mammary gland biopsy from the craniolateral quadrant of the left mammary gland. The biopsy was placed in an ice-cold solution of commercial media (Complete Epicult-B Basal Medium [Human]; StemCell Technologies, Vancouver, BC, Canada) supplemented with 5% fetal bovine serum (Fetal Bovine Serum-Advantage; Atlanta Biologicals, Flowery Branch, GA) and then immediately transferred to the laboratory for processing. Animals were recovered for 3-7 days in the veterinary clinic before being returned to their breeding groups.

Initial processing of mammary gland tissues. All biopsies collected for the study had the initial digestion process begun within 30 minutes of collection (Day 0). A general outline of the procedures and work flow from this study are provided in Figure 1. A small portion of the biopsy was collected in 10% neutral buffered formalin for histologic examination and the remainder of the biopsy was weighed. Biopsies less than 600 mg were processed as a single sample while biopsies greater than 600 mg were divided into two similarly-weighted aliquots and processed separately. The mammary tissues obtained from each study animal were digested and dissociated into single-cell suspensions (Figure 1, A) utilizing optimized protocols and materials identical to those previously described for the rhesus macaque (Chapter 2). A standardized ratio of enzymatic solution to tissue weight was utilized to process all tissue aliquots. For the mammary gland biopsies that were larger than 600 mg and had to be initially processed as

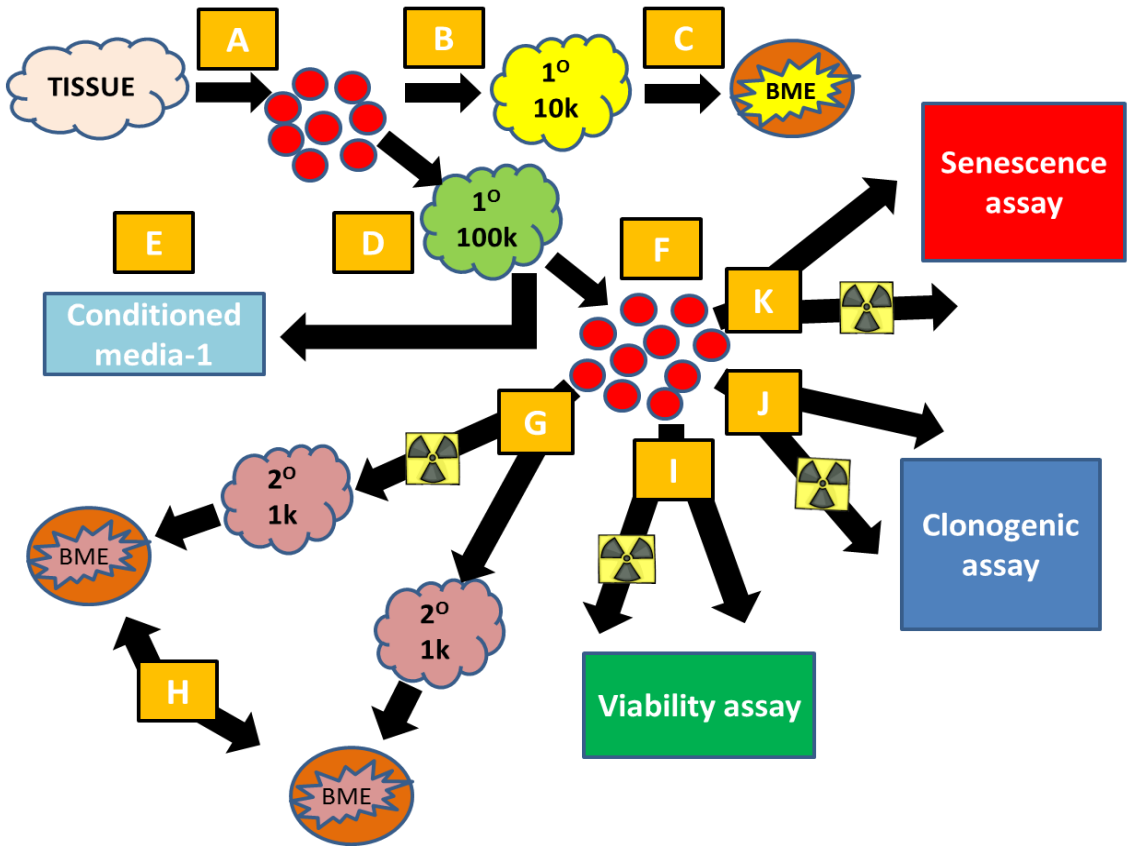

Figure 1. Schematic illustration of experimental procedures. A) Mammary gland tissue digested into single-cell suspension. B) 1 x 10^4 viable cells/ml plated and grown as spheres for 7 days (1° 10k). C) Resultant 1° 10k spheres counted and then plated in basement membrane extract (BME) and grown for 14 days; after 14 days spheres were evaluated for budding morphology. D) 1 x 10^5 viable cells/ml plated and grown as primary spheres for 7 days (1° 100k). E) Conditioned media-1 collected from the 1° 100k cultures at Day 3 and Day 7. F) 1° 100k spheres dissociated back into single-cell suspensions. G) Irradiated and non-irradiated cells plated at 1 x 10^3 viable cells/ml and grown as secondary spheres for 7 days (2° 1k). H) Resultant 2° 1k spheres counted then plated in BME and grown for 14 days; after 14 days spheres were evaluated for budding morphology. I) Irradiated and non-irradiated cells plated at 1 x 10^4 viable cells/ml and grown as secondary mammospheres for 4 days; after which the spheres were assessed for viability. J) Irradiated and non-irradiated cells plated at 3 x 10^3 viable cells/well and grown as colonies on collagen-coated plates for 14 days; after which the survival fraction of each well was assessed. K) Irradiated and non-irradiated cells plated at 1 x 10^4 viable cells/ml and grown as secondary spheres for 2 days; after which the cells were assayed for senescence. Additional details for each process/assay are provided in the text.

two separate aliquots, the cells derived from each digestion/dissociation process were combined into one vessel prior to cell counts or further processing.

The cells obtained from the digestion/dissociation process were resuspended in a complete mammosphere media (CMM). The CMM consisted of a commercially-available cell culture medium (Complete MammoCult Medium [Human]; StemCell Technologies, Vancouver, BC, Canada) supplemented with heparin solution and

hydrocortisone (both StemCell Technologies), penicillin-streptomycin-amphotericin solution (MP Biomedicals, Solon, OH) and L-glutamine solution (GlutaMAX; Life Technologies, Carlsbad, CA) as previously described (Chapter 2). The cells were confirmed to be present as a single-cell suspension, using techniques as previously described.[71] Cell viability and cell counts were performed in triplicate for each sample, using the trypan blue exclusion assay (0.4% Trypan Blue Solution; Sigma Life Sciences, St. Louis, MO) and a hemocytometer.

Primary mammospheres and conditioned media-1. On Day 0, the single-cell suspension created from mammary gland digestion/dissociation for each of the 14 animals was diluted with CMM to create 1 x 10^4 viable cells/ml (10k) suspensions and 1 x 10^5 viable cells/ml (100k) suspensions. Five ml aliquots of the 10k suspension (Figure 1, B) and 100k suspension (Figure 1, D) were plated into the individual wells of flat-bottom, 6-well ultralow-attachment plates (Corning, Corning, NY). A minimum of six wells were prepared for each suspension. The edges of the 10k and 100k plates were sealed with paraffin tape (Parafilm M, Fisher Scientific, Waltham, MA) and plates were placed in a 37ºC, 5% CO_2 incubator. At Day 3, the paraffin was removed from all plates and 2 ml of fresh CMM was added to the 10k cultures while the 100k cultures had approximately 80% of original media replaced with fresh media. The "conditioned media" removed from the 100k cultures was retained for later use as described below. The unsealed 10k and 100k plates were replaced in the incubator and allowed to grow undisturbed for 4 more days. On Day 7 the bulk of the cultured media was once again collected from the 100k suspensions and retained. The cultured media collected from the primary 100k mammospheres on Day 3 and on Day 7 was combined and processed into conditioned media-1 (CM1) (Figure 1, E) through centrifugation and filtration as previously described (Chapter 2).

On Day 7, the average number of primary 10k (1º10k) mammospheres (≥ 40 μm in diameter) formed in each well was determined for all 14 animals. The mammosphere-forming efficiency (MFE), defined as the average number of mammospheres formed in each well divided by the number of viable cells initially plated per well (expressed as percentage), was also calculated for each of the suspensions produced. The intact 1º10k spheres were then collected and encased in a standardized 12 mg/ml protein concentration of basement membrane extract ((BME): BD Matrigel Matrix, High Concentration, Growth Factor Reduced; BD Biosciences, Bedford, MA) (Figure 1, C) using techniques as previously described (Chapter 2). The BME-encased spheres were submerged in 3 ml of RPMI 1640 media (Mediatech, Manassas, VA) supplemented with 5% FBS, 1X L-glutamine Solution and 1X Penicillin-Streptomycin-Amphotericin Solution (RPMI/FBS) and incubated for 14 days. On Day 21, the

mammosphere budding potential (MBP), defined as the percentage of individual mammospheres that underwent "budding" differentiation, as previously described (Chapter 2), was calculated for each animal (Figure 1,C). Mammospheres in direct contact with other spheres or in contact with the bottom of the well were not included in these counts. The same previously described parameters (Chapter 2) were also utilized to characterize the morphology of each budding sphere as well.

Also on Day 7, the average diameter of the nulliparous and multiparous mammospheres (≥ 40 μm) in the primary 100k (1°100k) mammosphere cultures was determined from representative samples (Olympus IX51 microscope, DP70 camera and DP Controller software Version 2002, cellSens imaging software Version 1.5, Olympus Corporation, Waltham, MA). The 1°100k mammospheres from 12 animals (6 multiparous and 6 nulliparous) were collected using a 40 μm cell screen (Fisher Scientific) and then dissociated into single-cell suspensions (Figure 1, F) over a 20 minute period using 0.05% trypsin-EDTA solution (Life Technologies) and techniques as previously described (Chapter 2). Viable cell recovery (VCR), defined as the total number of viable cells recovered from the dissociation of a mammosphere culture divided by the number of viable cells originally plated for that culture (expressed as percentage), was calculated for the 1°100k mammosphere cultures of each animal.

Irradiation. On Day 7, the single-cell suspensions derived from the dissociation of the 1°100k mammospheres were diluted into 10k suspensions using CMM supplemented with CM1 at a 4:1 ratio (CMM/CM1). Five ml aliquots of these single-cell suspensions were placed into 15 conical vials. The vials were then either mock-irradiated (0 Gy) or irradiated with 2 Gy, 4 Gy, 8 Gy or 20 Gy of X-ray radiation using a cabinet X-ray irradiator (RS 2000 X-ray Biological irradiator,160 kV at a dose rate of 268 cGy/min; Rad Source Technologies, Inc. Suwanee, GA). Irradiation occurred within 1 hour of the time the cells were initially dissociated.

Secondary mammospheres. On Day 7, CMM/CM1 was used to dilute a portion of the 0 Gy and 4 Gy irradiated, single-cell suspensions into 1 x 10^3 viable cells/ml (1k) suspensions. Five ml aliquots of each suspension were plated into the individual wells of 6-well ultralow-attachment plates (Figure 1, G) for 12 study animals (six from each cohort). A minimum of 1 plate (6 wells) was prepared for each radiation dose. The plates were sealed with paraffin tape and placed in a 37°C, 5% CO_2 incubator. Paraffin was removed from the plates at Day 10 and otherwise the cultures were allowed to grow undisturbed for 7 days in the incubator. On Day 14, the average number of secondary 1k (2°1k) mammospheres (≥40 μm in diameter) formed in each well, and the MFE, were determined

for each animal and then these spheres were then encased in BME, as described above for the 1°10k cultures. The BME-encased 2°1k spheres were incubated for an additional 14 days prior to being evaluated for budding differentiation and budding morphology (Figure 1, H).

Viability assay. On Day 7, 200 µl aliquots of the 0 Gy, 2 Gy, 4 Gy, 8 Gy and 20 Gy irradiated, 10k suspensions were plated into the individual wells of a flat-bottom, 96-well ultralow-attachment plate (Corning) for 12 study animals (six from each cohort). For each animal, a minimum of 8 wells were plated with each radiation dose. These plates were sealed with paraffin tape and were allowed to grow undisturbed for 72 hours in a 37°C, 5% CO_2 incubator. On Day 10, 20 µl of 10X alamarBlue® cell viability reagent (Life Technologies) was added to the cell suspensions and the plates were incubated overnight in a 37°C, 5% CO_2 incubator. The fluorescence intensity (570 nm excitation/585 nm emission) of the individual wells was obtained the following morning using a plate reader (Victor^{3}V multilabel counter, PerkinElmer Life and Analytical Sciences, Shelton, CT) (Figure 1, I).

Clonogenic assay. On Day 7, RPMI/FBS was used to dilute a portion of the 0 Gy, 2 Gy, 4 Gy, 8 Gy and 20 Gy irradiated, single-cell suspensions into 1k suspensions. Three ml aliquots of each suspension were plated into the individual wells of standard 6-well culture plates (Sigma-Aldrich) for 12 study animals (six from each cohort). Each of these plates had been previously coated with a bovine collagen solution (StemCell Technologies) as per the manufacturer's instructions. For each animal, one plate (6 wells) of 0 Gy, two plates of 2 Gy, four plates of 4 Gy, eight plates of 8 Gy, and eight plates of 20 Gy irradiated cells were cultured (Figure 1-J). The plates were then placed in a 37°C, 5% CO_2 incubator for 14 days. The original media was exchanged for fresh media on Day 14 and the wells were examined for colony growth and differentiation on both Day 17 and Day 21. On Day 21, the cells were fixed with a formalin solution and stained with crystal violet stain (Sigma-Aldrich) or immunostained with cytokeratin-18 (CK18) or α-smooth muscle actin (SMA) using methods and reagents identical to those previously described (Chapter 2). Growths were scored as a colony if an independent group of cells was composed of 50 or more cells. The total number of colonies/well was determined for each subject at all radiation doses. The colony forming efficiency (CFE), defined as the total number of colonies/well divided by the number of viable cells initially plated/well (expressed as a percentage), was also calculated for each radiation dose. The individual colonies were further qualified as being epithelial colonies, myoepithelial colonies, or bilineage colonies (composed of both myoepithelial and epithelial cells) using parameters as previously described (Chapter 2). The total number of bilineage-colonies/well, in addition to the bilineage-colony forming efficiency (bCFE), was calculated for each

subject at all radiation doses as well. Finally, the colony differentiation-potential (CDP), defined as the number of bilineage-colonies/well divided by the total number of colonies/well (expressed as percentage), was also calculated for each radiation dose.

Senescence assay. On Day 7, two 3 ml aliquots of the 0 Gy, 4 Gy, and 8 Gy irradiated 10k suspensions were plated into the individual wells of 6-well ultralow-attachment plates for 9 study animals (five multiparous and four nulliparous animals). These plates were then sealed with paraffin tape and placed in a 37°C, 5% CO_2 incubator. On Day 9, the two wells for each radiation dose were collected into a single conical vial and centrifuged at 80 x *g* for 1 minute. The supernatant was removed and the cells were fixed and stained for β-galactosidase expression using a staining kit (Senescence β-Galactosidase Staining Kit; Cell Signaling Technology, Danvers, MA) as per the manufacturer's instructions. Following an overnight incubation in a 37°C, 5% CO_2 incubator, the cells and solution were placed into a dual-chambered, cytospin apparatus (Double cytology funnel, Fischer Scientific) and centrifuged at 200 x *g* for 8 minutes. Individual cells and cells that were part of 2-cell clusters and 3-cell clusters were then scored for the presence (senescence) or absence of blue dye within the cell cytoplasm (Figure 1-K). No clusters of 4 or more cells were evaluated in this process. Two cytospin preparations were acquired for each animal at each radiation dose. Both preparations were each reviewed independently by two boarded veterinary pathologists. The percentage of senescent cells reported for each cytospin preparation was the average of the two pathologists' scores.

Cytokine analysis of conditioned media-1. Conditioned media-1 was collected from five nulliparous and five multiparous animals and was frozen at -80°C. On the day of the assay, the CM1 was thawed and vortexed. The concentration of the cytokines GM-CSF, IFNγ,IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), MIP-1α, MIP-1β and TNFα were assessed using a cytokine assay kit (Nonhuman Primate Cytokine kit; Millipore Corp., Billerica, MA). The assay was run overnight according to the manufacturer's instructions and analyzed using the Luminex 200 system (Bio-Rad Corp., Hercules, CA). Acquisition gates were set at 8,000–15,000. Sample volume was 25 µl and all samples were run in duplicate. Manufacturer supplied cytokine standards were run on each plate and 50 events per bead were acquired. Mean fluorescence intensity was analyzed using the BioPlex manager software version 5.0 (Bio-Rad Corp.) and concentration values were generated using a standard curve. The minimum detectable concentrations in pg/ml for each cytokine was as follows: IFNγ, TNFα and IL-8 (0.1); IL-8 (0.2); IL-2, IL-6 and IL-10 (0.3); IL-1β and IL-13 (0.4); IL-8 (1.8); MIP-1α (3.5); MIP1β (4.5); and IL-12p40 (10.5).

Statistical analysis. Significant results are expressed as mean and standard deviation. Statistical significance for all data was set at $P<0.05$ unless otherwise indicated. Direct comparisons of differences between multiparous and nulliparous parameters were performed using a one-way ANOVA. Comparisons within the individual cohorts and across different radiation doses were performed using a repeated-measures ANOVA. All statistical tests were performed using IBM SPSS (version 22) software.

Results

Mammary gland biopsies. All 14 biopsies were identified to consist of grossly and microscopically normal-appearing nonlactating mammary gland tissue. The multiparous biopsies were thicker than the nulliparous biopsies and this difference was subjectively identified to be the result of increased amounts of adipose, fibrous connective tissue and interlobular stroma within the multiparous glands. The morphological differences identified between the two cohorts in this study were similar to what has been previously described (Chapter 2). Due in large part to the heterogeneous nature of the glandular tissue distribution in the multiparous macaque mammary gland (Chapter 2), the biopsy weights often varied greatly between the multiparous subjects (Table 1). Despite the variation, the weight of the average multiparous biopsy (725.8 mg ± 259.7) was statistically greater than that of nulliparous animals (456.3 mg ± 75.4). No correlation was identified between the biopsy weights and any of the metrics utilized to assess the mammosphere proliferation/differentiation potential (MFE, MBP, VCR, CFE and bCFE) for either cohort.

The body weight of each study animal was used to extrapolate an "approximate-whole mammary gland weight" for one gland in each animal (~Mam wt (mg); Table 1). This calculation was based on data previously obtained from the same breeding colony which identified the mean weights of the individual mammary glands in nulliparous and nonlactating-multiparous rhesus macaques to be 0.069 percentage of total body weight (%TBW) and 0.093 %TBW, respectively (Chapter 2). Utilizing this measurement, the average biopsy weight of the nulliparous animals was found to represent a slightly greater, though nonsignificant, percentage of the approximate-total mammary gland weight for these animals (14.8%) as compared to the average biopsy weight of the multiparous animals (12.3%).

The time required for the biopsies to obtain the optimal digestion point in the initial digestion step of the process varied by subject. Nulliparous digestion times ranged from 3.33 to 3.67 hours and multiparous digestion times ranged from 3.33 to 4.33 hours. The mean digestion times were found to be slightly, although not

Table 1. Macaque, mammary biopsy and initial-digestion data for parity cohorts. *Animal #*: order in which the animals were processed for the study. *Parity* (reproductive status): M=multiparous; N=nulliparous. *Infants*: number of live births recorded for each animal. *Age (yr)*: age of the animal (in years). *Body wt (kg)*: total body weight of the animal in kg. *~Mam wt (mg)*: approximate weight of one whole mammary gland (in mg) for each animal based on calculations as described in the text. *Biop wt (mg)*: weight of the biopsy (in mg) collected for each animal. Asterisk (*) denotes that sample was divided into two similarly-weighted aliquots and processed separately through the initial digestion and dissociation steps. *% Gland*: weight percentage of the biopsy as compared to the approximate weight of one whole mammary gland. *Fibrous* (qualitative amount of fibrous connective tissue within the mammary biopsy as determined histologically): 0=minimal; 1=mild; 2= moderate. *Digest time (h)*: time in hours required for the initial enzymatic digestion of the biopsy sample to obtain the "optimal digestion point", as described in Chapter 2. *TVC* (Total Viable Cell count): number of viable cells/mg of mammary tissue identified in the single-cell digests. *%VC* (Percentage of Viable Cells): percentage of viable cells to nonviable cells identified in the single-cell digests.

Animal #	Parity	Age (yr)	Infants	Body wt (kg)	~Mam wt (mg)	Biop wt (mg)	% Gland	Fibrous	Digest time (h)	TVC	%VC
1	M	6.58	2	5.41	5030	1222*	24.2	1	3.67	9625	90.2
2	M	7.58	2	5.96	5540	487	8.7	0	3.33	16,428	95.1
3	M	13.67	9	8.31	7730	525	6.8	1	3.67	8788	84.4
4	M	11.58	6	6.01	5580	788*	14.1	2	4	15,291	93.7
5	M	20.83	13	6.39	5940	588	9.9	2	4.33	12,755	94
6	M	13.92	9	8.04	7470	873*	11.7	1	3.67	10,423	86.2
7	N	3.83	0	4.65	3210	348	10.8	1	3.67	13,948	88.6
8	N	3.67	0	3.64	2510	427	17	1	3.33	14,066	90.3
9	N	3.83	0	4.44	3060	480	15.7	0	3.33	19,062	96.5
10	N	3.83	0	4.08	2810	447	15.9	1	3.67	16,051	89.7
11	N	4.75	0	4.91	3390	567	16.7	2	3.67	21,146	92.1
12	N	3.83	0	5.37	3700	397	10.7	1	3.33	20,592	92.3
13	M	14.00	9	5.83	5420	598	11	2	4	16,327	92.2
14	N	3.92	0	4.53	3130	528	16.9	2	3.67	15,221	90.1

significantly, longer for multiparous biopsies (3.81 ± 0.32 hours) as compared to nulliparous biopsies (3.52 ± 0.18 hours). When the data from both cohorts were analyzed collectively, increased digestion times were significantly correlated with greater amounts of fibrous tissue, increased age, and increased number of infants (Table 1). There was no correlation identified between digestion time and biopsy weight, or between digestion time and any of the metrics utilized to assess the mammosphere proliferation/differentiation potential for either cohort.

The total number of viable cells/mg of tissue (TVC) obtained from the digestion and dissociation of the nulliparous mammary gland biopsies (17,155 ± 3060) was statistically greater than the TVC of the multiparous glands (12,805 ± 3257). The percentage of viable cells to nonviable cells (%VC) that was initially obtained from the tissue digests was similar between the two cohorts with nulliparous %VC ranging between 88.6 and 96.5% (mean 91.4%) and multiparous %VC ranging between 84.4 and 95.1% (mean 90.8%). There was no correlation identified for the TVC or %VC values for either cohort with regard to time of digestion, degree of biopsy fibrosis, or weight of the biopsy sample (Table 1). Notably, the three large multiparous biopsies that had to be processed as two similarly-

weighted aliquots had TVCs and %VCs that fell inside the range of the other four multiparous animals that were processed as a single aliquot.

Primary mammospheres. Nulliparous animals produced statistically greater numbers of 1°10k spheres/well (270.6 ± 78.8 [mean 1° MFE =0.541%]) than did multiparous animals (8.7 ± 3.8 [mean 1° MFE =0.017%]). Nulliparous animals also had a statistically higher MBP of the 1°10k spheres (1° MBP =65.7 ± 5.3%) as compared the multiparous animals (1° MBP =49.6 ± 10.3%). The morphology of the 1° budding spheres in this study was similar to those previously described for non-pregnant female macaques (Chapter 2) and there was no variation in the morphological growth patterns of the spheres identified between the two cohorts.

The average diameter of the nulliparous 1°100k mammospheres (60.7 ± 20.2 μm; range 40-122 μm [n =354]) and multiparous 1°100k mammospheres (54.7 ± 18.5 μm; range 40-148 μm [n =231]) were not statistically different from one another. Although the confluent nature of the 1°100k mammosphere cultures prevented accurate quantification of the spheres, the nulliparous 1°100k cultures were subjectively more-densely populated when observed under the microscope. This observation was supported by the finding that the VCR of the nulliparous 1°100k mammospheres (31.3 ± 4.0%) was statistically greater than the VCR of the multiparous 1°100k spheres (16.4 ± 1.0%).

Secondary mammospheres. As presented in Figure 2,A, the nulliparous animals produced statistically greater numbers of 2°1k spheres/well than did the multiparous animals at both the 0 Gy (nulliparous (N) =68.2 ± 24.0 [mean 2° MFE =1.36%]; multiparous (M) =17.8 ± 3.1 [mean 2° MFE =0.36%]) and 4 Gy (N =40.0 ± 10.6 [mean 2° MFE =0.80%]; M =11.0 ± 1.8 [mean 2° MFE =0.22%]) radiation doses. The average number of 2°1k spheres/well in the 0 Gy radiation-dose cultures was statistically higher than those of the 4 Gy radiation-dose cultures for both nulliparous and multiparous animals. However, there was no statistically significant interaction identified between the radiation dose and cohort, indicating that the nulliparous and multiparous animals both had similar rates of change (similar slopes) between the 0 Gy and 4 Gy radiation doses.

As presented in Figure 2,B, there were no statistically significant differences between the nulliparous and multiparous animals with regard to the MBP of 2°1k spheres (2° MBP) at either the 0 Gy (N =45.3 ± 7.5%; M =36.6 ± 11.3%) or 4 Gy (N =35.9 ± 9.9%; M =31.2 ± 4.5%) radiation doses. There was a significant decrease in the 2° MBP between the 0 Gy and 4 Gy radiation doses for both nulliparous and multiparous animals, although as before, the rate of change between the 0 Gy and 4 Gy radiation doses was similar between the nulliparous and multiparous

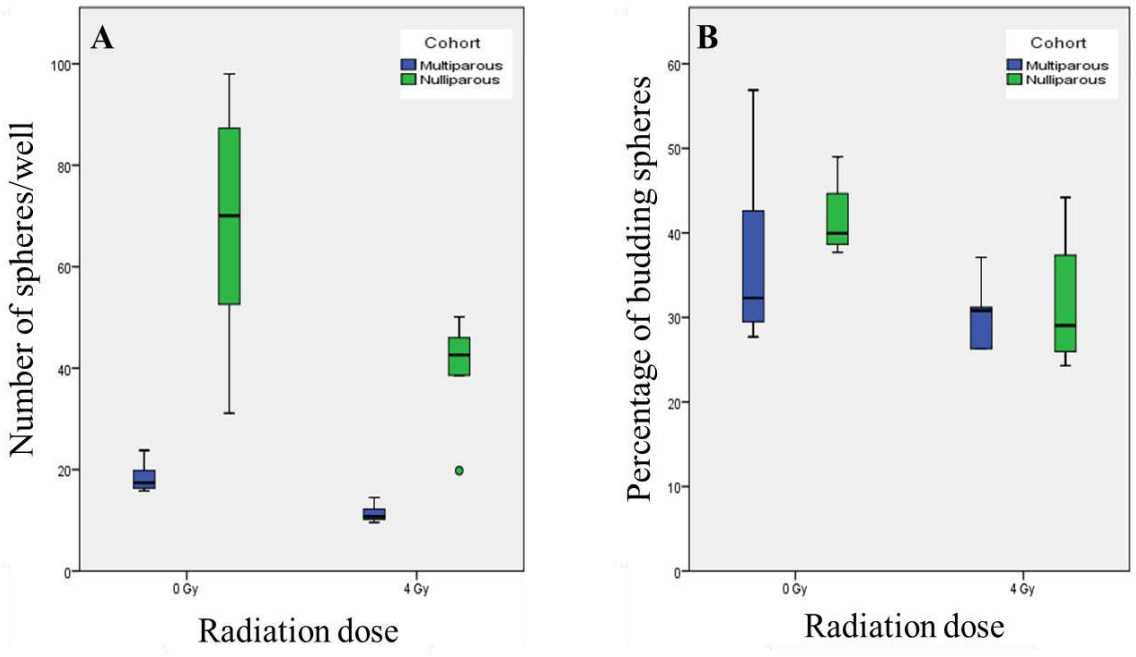

Figure 2. Secondary 1k (2º1k) nulliparous mammospheres have significantly greater MFE, but not MBP, than do 2º1k multiparous mammospheres at 0 Gy and 4 Gy. **A**, Numbers of 2º1k mammospheres produced by the multiparous and nulliparous single-cell suspensions exposed to 0 Gy and 4 Gy irradiation. The x-axis represents the multiparous and nulliparous mammosphere cultures that were derived from single-cell suspensions exposed to 0 Gy and 4 Gy irradiation. The y-axis represents the number of mammospheres formed/well for six multiparous and six nulliparous animals. The mammospheres derived from these experiments were then utilized for the experiments described in Figure 2,B. **B**, Percentage of the basement membrane extract (BME)-encased 2º1k mammospheres that underwent "budding" differentiation for multiparous and nulliparous animals. The x-axis represents the multiparous and nulliparous, 0 Gy and 4 Gy mammospheres obtained from the Figure 2,A experiments that were encased in BME. The y-axis is the percentage of the BME-encased spheres identified to have undergone budding differentiation after 14 days in culture for six multiparous and six nulliparous animals.

subjects. The budding morphology of the 2º spheres was similar to those previously described for non-pregnant female macaques (Chapter 2) and no variation in the morphological growth patterns of the spheres was identified between the two cohorts or between the 0 Gy and 4 Gy radiation doses for either cohort.

Viability assay. Figure 3 shows the results of the cell viability assay for the nulliparous and multiparous cells plated under conditions that promoted mammosphere formation. The curves were based on the percentage of fluorescence absorbance of the samples within the wells. An increase in absorbance for this assay is correlated with greater numbers of viable cells. The 0 Gy culture wells for each subject was normalized to 100% absorbance and then the percentage absorbance for the 2 Gy, 4 Gy, 8 Gy, and 20 Gy mammospheres culture wells was calculated based on the values obtained for the 0 Gy samples. Both cohorts demonstrated a significant decrease in the percentage absorbance (i.e., decrease in the number of viable cells) with each increasing radiation dose. However,

there were no statistically significant differences in percentage absorbance identified between the nulliparous and multiparous animals for any radiation dose and the slopes of the two curves were similar.

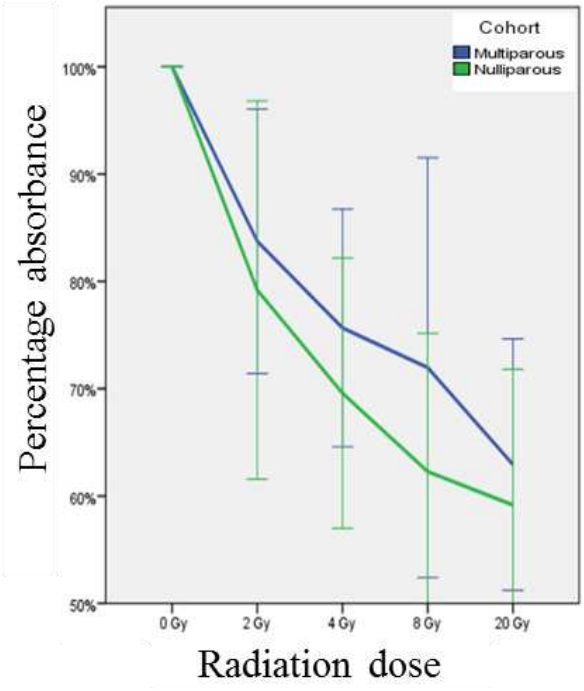

Figure 3. Nulliparous and multiparous mammosphere-derived cells have similar radiation survival curves when grown under mammosphere forming conditions. Radiation response curves of single-cell suspensions exposed to increasing doses of radiation and then plated under conditions that promoted the formation of mammospheres. The x-axis represents the multiparous and nulliparous mammosphere cultures derived from single-cell suspensions exposed to 0 Gy, 2 Gy, 4 Gy, 8 Gy, and 20 Gy irradiation. The y-axis represents the percentage of viable cells present at each radiation dose for six multiparous and six nulliparous animals. The percentage of viable cells for this assay was based on the average fluorescence absorbance of the samples at each radiation dose as compared to the 0 Gy dose of radiation which was normalized to represent 100% cell viability. Error bars represent ± one standard deviation.

Clonogenic assay. Culture viability for this assay was confirmed by the observation that there was a general increase of the colony sizes in the 0 Gy, 2 Gy, 4 Gy, and 8 Gy cultures for subjects between the tenth and fourteenth day of the plating process. Figure 4 shows survival curves for the nulliparous and multiparous cells plated under conditions that promoted the formation of adherent cell colonies. For the total colony survival curves (Figure 4,A), the total number of colonies/well present at the 0 Gy dose of radiation for each subject was designated to be 100%. The total-colony survival fraction, defined as the total number of colonies/well for a particular radiation dose divided by the total number of colonies/well at the 0 Gy dose (expressed as a percentage), was calculated for each subject at the 2 Gy, 4 Gy, 8 Gy, and 20 Gy radiation doses. The curves derived from this assay were typical of

radiation survival curves and both the nulliparous and multiparous animals demonstrated a statistically significant decrease in the total number of colonies/well with each increasing radiation dose. There were, however, no statistical differences identified between the nulliparous and multiparous total-colony survival fractions at any radiation dose and the slopes of the two curves were similar.

For the bilineage colony survival curves (Figure 4,B), the number of bilineage-colonies/well present at the 0 Gy dose of radiation for each subject was designated to be 100% and the survival fraction of bilineage-colonies was then calculated at each radiation doses, as above, for each subject. Similar to the total-colony survival fraction results, there were no significant differences identified between the nulliparous and multiparous bilineage-colony survival fractions at any radiation dose and both cohorts demonstrated a statistically significant decrease in the number of bilineage colonies/well with each increasing radiation dose between 2 Gy and 20 Gy. Unlike the total-

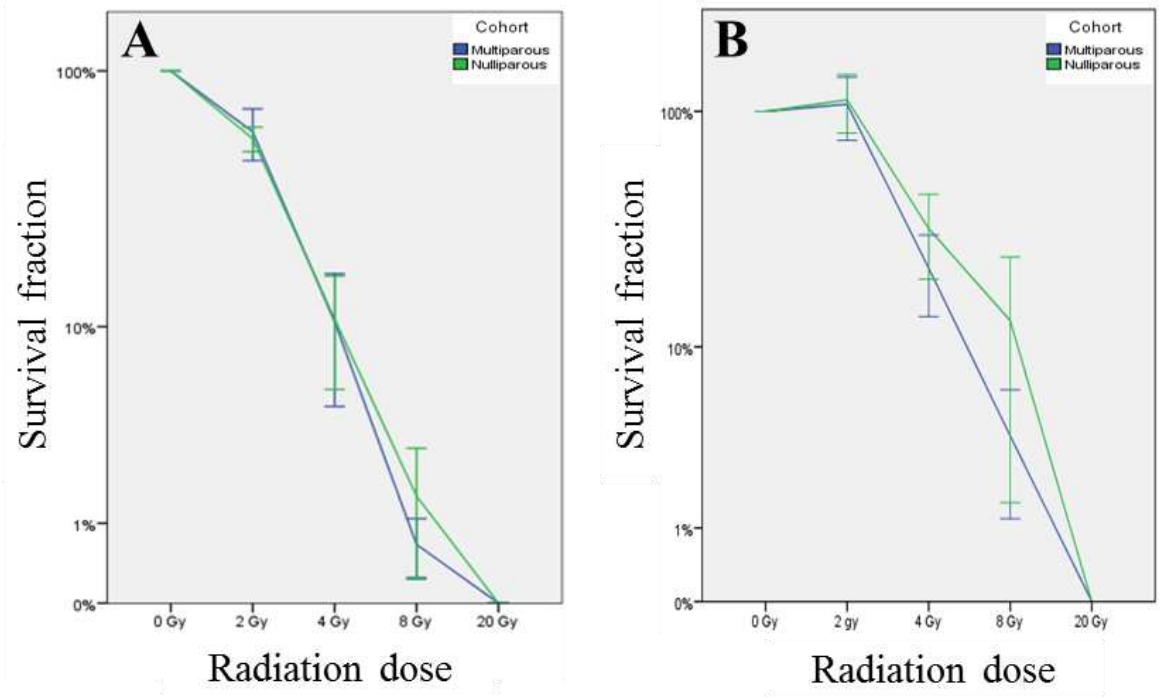

Figure 4. Nulliparous and multiparous mammosphere-derived cells have similar radiation survival curves when grown under conditions that form adherent colonies. Radiation response curves of single-cell suspensions exposed to increasing doses of radiation and then plated under conditions that promoted the formation of adherent colonies. For both graphs, the x-axis represents the multiparous and nulliparous cell cultures derived from single-cell suspensions exposed to 0 Gy, 2 Gy, 4 Gy, 8 Gy, and 20 Gy irradiation. **A**, the y-axis represents the total number of cell colonies/well present at each radiation dose divided by the total number of cell colonies/well at the 0 Gy dose of radiation (total-colony survival fraction). **B**, the y-axis represents the number of bilineage-cell colonies/well present at each radiation dose divided by the number of bilineage-cell colonies/well at the 0 Gy dose of radiation (bilineage-colony survival fraction). Both graphs are expressed as a logarithmic function of radiation dose. Error bars for both graphs represent ± one standard deviation.

colony survival fraction results, the numbers of bilineage colonies at the 2 Gy radiation dose were slightly elevated, although statistically equivalent, to those of the 0 Gy radiation dose for both cohorts.

Although the total-colony survival curves were similar between the cohorts, the mean CFE for the nulliparous animals (0 Gy =5.1%; 2 Gy =2.80%; 4 Gy =0.51%; 8 Gy =0.069%; and 20 Gy =0%) was statistically greater than the mean CFE of multiparous animals (0 Gy =2.0%; 2 Gy =1.12%; 4 Gy =0.20%; 8 Gy =0.012%; and 20 Gy =0%) at all radiation doses other than 20 Gy. Likewise, the mean bCFE for the nulliparous animals (0 Gy =0.45%; 2 Gy =0.47%; 4 Gy =0.13%; 8 Gy =0.047%; and 20 Gy =0%) was statistically greater than the mean bCFE of multiparous animals (0 Gy =0.28%; 2 Gy =0.28%; 4 Gy =0.06%; 8 Gy =0.009%; and 20 Gy =0%) at all radiation doses other than 20 Gy.

The percentage of colonies/well with bilineage potential, the CDP, was identified to vary with radiation dose and also by cohort (Table 2; "% Bilineage (CDP)"). Specifically, the CDP was statistically greater with each increasing radiation dose within each cohort and there were statically significant differences in the CDP identified between the multiparous and nulliparous cohorts at the 0 Gy, 2 Gy, and 8 Gy radiation doses. Additionally, the rate

Table 2. The ratio of bilineage colonies varies significantly with increasing doses of radiation within each cohort and also between the multiparous and nulliparous cohorts. *Dose*: radiation dose used for each clonogenic assay plate. *Parity*: M =multiparous; N =nulliparous. *% Myoepithelial*: percentage of total colonies/well identified to be composed of only myoepithelial-like cells. *% Epithelial*: percentage of total colonies/well identified to be composed of only epithelial-like cells. *% Bilineage (CDP)*: percentage of total colonies/well identified to be composed of both myoepithelial-like and epithelial-like regions (the CDP). The numbers in the three previous columns represent the mean percentage of colonies ± one standard deviation. Superscript "a" ([a]) indicates a statistically significant difference in the CDP within the same cohort between radiation doses. Superscript "b" ([b]) indicates a statistically significant difference in the CDP between cohorts at the same radiation dose. *NCDP/MCDP*: the mean nulliparous CDP value (NCDP) of one particular radiation dose divided by the mean multiparous CDP value (MCDP) of the same radiation dose, expressed as a percentage. NA =not applicable.

Dose	Parity	% Myoepithelial	% Epithelial	% Bilineage (CDP)	NCDP/MCDP
0 Gy	M	84.7 ± 3.1	1.3 ± 0.7	14.0 ± 3.4[a,b]	NA
0 Gy	N	90.4 ± 2.5	1.0 ± 1.0	8.6 ± 2.2[a,b]	61.43
2 Gy	M	73.3 ± 4.1	2.0 ± 1.3	24.7 ± 3.2[a,b]	NA
2 Gy	N	81.5 ± 2.3	1.9 ± 1.2	16.6 ± 1.7[a,b]	67.21
4 Gy	M	67.2 ± 4.4	2.5 ± 1.0	30.3 ± 4.5[a]	NA
4 Gy	N	71.1 ± 4.3	2.6 ± 1.0	26.3 ± 3.7[a]	86.80
8 Gy	M	35.4 ± 6.0	3.0 ± 2.5	61.6 ± 7.7[a,b]	NA
8 Gy	N	27.2 ± 3.7	3.2 ± 1.6	69.6 ± 3.4[a,b]	112.99

(slope) of increase for the nulliparous CDP was identified to be statistically greater than that of the multiparous CDP between the 2 Gy and 4 Gy radiation doses as well as between the 4 Gy and 8 Gy radiation doses (Figure 5). Consistent with this last finding is the observation that although the multiparous CDP was greater than the nulliparous CDP at the 0 Gy, 2 Gy, and 4 Gy radiation doses, the mean CDPs of the cohorts become more similar in value with each increasing radiation dose, until, at the 8 Gy radiation dose the mean nulliparous CDP exceeded that of the mean multiparous CDP (Table 2; "NCDP/MCDP" and Figure 5).

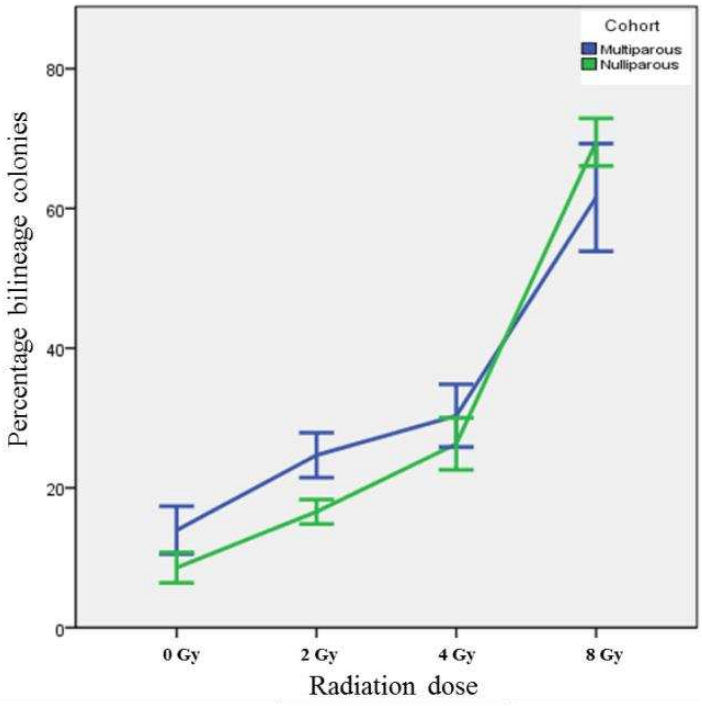

Figure 5. The slope of the nulliparous CDP curve is greater than the slope of the multiparous CDP curve between 2 Gy and 8 Gy irradiation. The x-axis represents the multiparous and nulliparous cell cultures derived from single-cell suspensions exposed to 0 Gy, 2 Gy, 4 Gy, and 8 Gy irradiation and then plated under conditions that promoted the formation of adherent colonies. The y-axis is the percentage of bilineage colonies present within each well for each cohort. The values on the y-axis correspond to the "% Bilineage (CDP)" in Table 2.

Senescence assay. A minimum of 132 cells per subject were evaluated for β-galactosidase expression at each radiation dose although for most subjects there were enough cells present on the slides to allow each radiation dose to be evaluated using a total 400 cells (200 cells per each cytospin preparation). As illustrated in Figure 6, the percentage of β-galactosidase-positive (senescent) cells was statistically higher for multiparous than nulliparous

animals at the 0 Gy, 4 Gy, and 8 Gy radiation doses. There were, however, no statistically significant differences in the percentage of senescent cells between any of the radiation doses for either cohort.

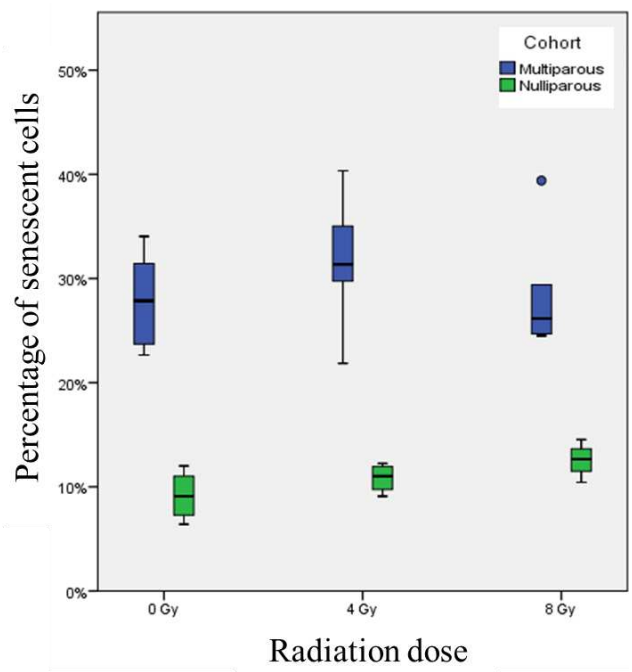

Figure 6. The mammosphere-derived cells of multiparous animals contain greater ratios of senescent cells than do the mammosphere-derived cells of nulliparous animals, regardless of radiation dose. The x-axis represents the multiparous and nulliparous mammosphere cultures derived from single-cell suspensions exposed to 0 Gy, 4 Gy, and 8 Gy irradiation. The y-axis is the percentage of cells that stained positive for β-galactosidase expression, two days post-irradiation.

Cytokine assay. Of the 14 cytokines evaluated in this study only interleukin-6 (IL-6) and interleukin-8 (IL-8) were identified to be present in the CM1 in concentrations adequate for analysis. The concentrations of both IL-6 and IL-8 were statistically higher in the multiparous CM1 (IL-6 =12.1 ± 2.8 pg/ml; IL-8 =233.8 ± 49.4 pg/ml) as compared to the nulliparous CM1 (IL-6 =7.8 ± 2.1 pg/ml; IL-8 =142.0 ± 33.4 pg/ml).

Discussion

This work compares the proliferative and differentiation potential of the cell populations within the mammary glands of young-nulliparous and multiparous primates. Mammosphere culture methodologies were utilized for this study as mammosphere forming ability has been correlated with the MSC and/or MPC composition of mammary glands in humans and rodents[71, 230, 243] and the MSCs/MPCs are of particular interest due to their potential role in breast carcinogenesis.[71, 74, 133, 146, 158, 160, 209, 260] Individual cells derived from the mammospheres in

this study were identified to have both mammosphere-forming and bilineage-colony forming potential. It is therefore considered likely that the macaque mammosphere cultures are enriched for MSCs or, at the very least, MPCs. However, in that no xenotransplantation or FACS-based studies have yet been completed by which to estimate the percentage of macaque mammospheres that are of a MSC and/or MPC origin, the individual cells capable of mammosphere formation and/or bilineage-colony formation in this study have been intentionally referred to using the more generic term of "mammosphere forming units" or "MFUs".

The results of a previous research project suggested that mammary glands of young-nulliparous rhesus macaques have greater numbers of MFUs, and that the mammospheres derived from these MFUs have greater mammosphere potential (proliferation and differentiation capabilities), than do those of nonlactating-multiparous macaques (Chapter 2). However, it is notable that most of the mammary glands used in that project were opportunistically collected from animals euthanized due to illness, trauma, or as part of unrelated research projects, and it is possible that the conclusions of that project could have been confounded by factors related to the cause of death in these animals. Furthermore, in that the tissue collection process of that initial project occurred over a time span of several years, it is also possible that the results of that project could have been influenced by slight variations in tissue processing techniques that unknowingly occurred between the individual samples. The current research study was undertaken to reexamine the mammosphere potential between nulliparous and multiparous cohorts using a study design aimed at reducing the number of potential confounding variables. Specifically, the current project utilized a synchronized, in vivo biopsy procedure to collect tissue samples for the mammosphere assays. This approach allowed for the acquisition of mammary gland tissues from clinically healthy monkeys rather than relying on tissues derived from animals that were euthanized for health or research-related reasons. This study design also allowed for the acquisition of mammary gland tissue over a relatively short period of time (six weeks) which was thought to likely improve the between-subject consistency with regard to the techniques and enzymatic solutions utilized to process the individual samples. Finally, this approach allowed for the collection of mammary gland tissues from a defined stage (menses stage) of the menstrual cycle.

While the current study design minimized many of the potential variables that may have confounded results in the earlier project, there were still some limitations associated with this biopsy collection process that merit discussion. The first limitation is that, per the study design, it was not possible to randomize the subjects included in this study. This was due to the fact that one criterion for inclusion was that each animal had to be actively

reproductively cycling at the time of the collection process. As such, animals that had not yet begun to cycle regularly or multiparous animals that were already pregnant by the middle of the breeding season were excluded from the study. In essence, this study design selected against animals that tended to cycle either early or late in the breeding season. Randomization of subjects was further skewed in that most of the multiparous biopsies were collected earlier in the study than nulliparous biopsies in efforts to ensure that there would be enough regularly-cycling multiparous subjects available for inclusion in the study. This was deemed necessary as the multiparous females, unlike the nulliparous animals, were housed with breeding males and with each passing week greater numbers of these animals became pregnant. Six of the seven multiparous biopsies were therefore obtained during the first two weeks of the biopsy collection process while six of the seven nulliparous animals were collected between the third and fifth weeks of the collection process. While it is possible that differences in the timing of the biopsy procedures over the 6-week collection period could have influenced the mammosphere potential between individual animals, this was considered unlikely given that the biopsy collection process occurred at approximately the midpoint of the typical 24-week breeding season for this colony. Supportive of the proposition that there was minimal variation in the mammosphere potential of the colony subjects over the 6-week collection period is the finding that the one multiparous biopsy (Animal #13) and the one nulliparous biopsy (Animal #14) that were collected during the sixth week of the study had cell-isolation values (e.g., %VC and VCR) and mammosphere potential values (e.g., MFE and MBP) that were comparable to those of the other animals in their respective cohorts.

A second limitation related to the current study-design is that, although efforts were made to collect biopsies only from animals in the menses stage of an ovulatory menstrual cycle, it is possible that some incidences of vaginal bleeding could have been menses associated with anovulatory cycles.[63, 77, 165, 173, 274] This possibility is important in that animals undergoing anovulatory menstrual cycles often have sex hormone levels that are markedly different from animals undergoing an ovulatory cycle.[63, 202, 244, 274, 278, 279] In that differences in sex hormone levels have been associated with variations in mammary gland physiology and morphology,[50, 244] it is possible that some of the outlying data obtained from these studies could have resulted from the use of tissues collected from animals experiencing an anovulatory cycle. Although the ovulatory status of individual menstrual cycles in macaques can be confirmed by analyzing the blood sex-hormone concentrations of individual animals 2-3 times per week over several months duration,[202, 274, 278] this approach was not attempted here. This is because over 600 animals were considered as candidates for inclusion in the study and it was logistically unfeasible to acquire the requisite number of blood

draws required for sex-hormone monitoring. Additionally, the routine removal of female animals from their breeding groups for multiple blood draws/week was undesirable as stresses associated with this process is likely to have decreased the fecundity rate of the breeding colony as a whole.[3, 94, 148, 188, 202, 274, 279]

Anovulatory cycles do occur in macaques but have been reported to represent only between 3-8% of all menstrual cycles occurring during the breeding season in reproductively-sound, actively-cycling females that are housed under conditions similar to those of the KCCMR.[274, 278] As to the question of reproductive soundness, all of the multiparous females included in the study had delivered a live infant in the breeding season prior to the onset of this project; and all of the nulliparous animals included in the study were of an age at which ovulatory menstrual cycles are expected to be occurring in the vast majority of individuals housed under conditions similar to those of the KCCMR.[285] The reproductive soundness of most animals was likewise confirmed by a follow-up analysis which found that all of the study animals that remained part of the breeding colony (10 animals total; three multiparous and one nulliparous study animals were sold prior to this analysis) produced offspring during the breeding season immediately following this project. Based on this information, in addition to the information collected on each subject as to their cycling status and vaginal cytology, it is considered likely that most of the animals included in this study were undergoing ovulatory menstrual cycles at the time of mammary gland collection.

In the current study, the mean biopsy weight of the multiparous animals was found to be significantly greater than those of the young-nulliparous animals. While the usefulness of biopsy weight as a sole metric by which to compare the mammary glands between the two cohorts is questionable, the individual biopsy weights were essential measurements toward calculating the TVC for each mammary biopsy. Consistent with previous comparisons made using mammary tissues from luteal/follicular-stage macaques (Chapter 2), the TVC of the nulliparous glands in this study were significantly greater than those of the multiparous glands. These results, in conjunction with the histological observations of the biopsies, suggest that the nulliparous mammary biopsies in this study contained larger ratios of glandular cells to stromal elements than did the multiparous mammary biopsies.

The current study also identified a statistically higher 1^{o} MFE in young-nulliparous subjects as compared to multiparous subjects; a finding that is once again similar to those of previous experiments (Chapter 2). Additionally, the young-nulliparous subjects were identified to have a statistically higher 1^{o} 100k VCR than multiparous subjects. Collectively, the 1^{o} MFE and VCR data suggest that the nulliparous biopsies have greater ratios of the glandular cells that are capable of mammosphere formation as compared to multiparous biopsies.

Possible limitations with regard to the accuracy of the 1° MFE data of this study warrant discussion at this time. The 1° MFE calculations for this study were based on mammosphere cultures plated at 10k, rather than 1k, cell densities. This is important in that while most mammospheres derived from rodent and human cell cultures plated at 1k densities (or lower) originate from the clonal proliferation of a single MSC and/or MPC,[22, 71, 85] there is little information as to frequency of clonal sphere formation in mammosphere cultures plated at higher (i.e., 10k) densities. As such, it is possible that some of the spheres produced within 10k cultures may not have been true mammospheres but rather "aggregate spheres". Aggregate spheres being defined here as spheres derived from the aggregation and subsequent proliferation of two or more lineage-specific progenitor cells and/or more-differentiated cell types rather than being spheres formed through the proliferation of a single MSC or MPC alone. The presence of aggregate spheres is a potential study confounder in that if aggregate spheres represent a substantial percentage of the spheres in a mammosphere culture this can lead to an overestimation of the MSC/MPC populations within the single-cell isolates being evaluated. In previous macaque studies (Chapter 2), and in experiments performed using four representative animals from the current study, the mean 1° MFE of 10k primary cultures was identified to be higher than those of 1k primary cultures by around 10.0% and 11.3%, respectively. In turn, it is possible that presence of aggregate spheres in the 10k cultures may have led to overestimations of the MFE in this study. However, what must also be appreciated is that the use of 1k density mammosphere cultures toward MFE calculations has been found to be problematic for primary mammosphere cultures derived from multiparous macaque glands in an earlier project (Chapter 2). As previously reported, the primary 1k cultures from multiparous macaque glands produced extremely low numbers of small, poorly-differentiating mammospheres which confounded attempts to accurately quantify the MFE or budding potential of these cultures. Equally important is that this same project also found that the 1k cultures may actually underestimate the true MFE of the multiparous mammary gland by as much as 8% if the 1k cultures are not provided with growth factors (CM1) obtained from other mammosphere cultures. When the above observations as to the possible MFE-overestimations of 10k cultures and the possible MFE-underestimations of 1k cultures are considered in tandem, it appears likely that 10k mammosphere cultures provide estimates of the true 1° MFE of cell isolates to within 3-11% of their actual values.

In spite of the imperfect nature of the 10k mammosphere assay to quantify the 1° MFE, it is proposed that the study data above still supports the conclusion that the mammary glands of young-nulliparous menstruating macaques have significantly greater ratios of cells with mammosphere potential than do the glands of multiparous

menstruating macaques as per the marked differences in the mean 1° MFEs between the two cohorts. Additionally, the 1° MFE results are supported by similar findings of a second metric, the VCR, which also was used to assess the primary mammosphere potential of the biopsies. Future research studies, utilizing other types of assays aimed at quantifying the 1° MFE, are likely to prove useful toward further refinement of the 1°MFE estimates in individual macaque mammary glands.

The number of cells with mammosphere forming ability (CMFA) within each biopsy can be estimated using the TVC, 1° MFE, and the biopsy weight obtained from the individual subjects. As per these estimates, the mean CMFA of the young-nulliparous biopsies (41.2×10^3 cells) is approximately 24 times greater than that of the multiparous biopsies (1.7×10^3 cells). However, the relevance of the biopsy-CMFA as a metric for comparison between the cohorts is uncertain as the mean biopsy weight of the nulliparous animals represented a slightly greater percentage of the mean approximate-total mammary gland weight as compared to the biopsy weights of multiparous animals. In attempts to account for any discrepancies in the representative nature of the biopsy-CMFA, a whole-gland CMFA for each animal can be extrapolated using the TVC, 1° MFE, and the approximate-whole mammary gland weight for each subject. As per these calculations, the mean CMFA of the young-nulliparous glands ($29.3 \times 10^4 \pm 11.5 \times 10^4$ cells) is approximately 23 times greater than the mean CMFA of the multiparous glands ($1.28 \times 10^4 \pm 0.33 \times 10^4$ cells) for the study subjects. Collectively, the results of the biopsy-CMFA and whole-gland CMFA analyses suggest that the mammary glands of young-nulliparous animals have statistically greater numbers of cells with mammosphere forming ability than do multiparous animals.

In that the biopsy-CMFA and whole-gland CMFA data were both calculated using the 1° MFE for each animal, there are inherent limitations to the accuracy of these results, as previously discussed. In addition to this first limitation, however, it must also be appreciated that the whole-gland CMFA data was extrapolated using an approximate mammary gland weight for each animal and that the whole-gland CMFA was also calculated with the assumption that the 1°MFE was equivalent throughout the entire gland. In defense of using an approximate mammary gland weight to determine the whole-gland CMFA for this study is that: the mammary gland %TBW calculation used in this study was derived from data collected from animals originating from the same breeding colony; the variation in the mammary gland %TBW for each cohort was minimal (one standard deviation representing less than 6.5% of the value of the mean %TBW for either of the two cohorts); and the %TBW was identified to be significantly different between multiparous and nulliparous animals in this colony (Chapter 2). In

support of the assumption that the 1° MFE is equivalent throughout the entire gland, is the fact that previous experiments found no significant differences in the 1° MFE of breast biopsies obtained from different quadrants of the mammary gland that were collected from actively-cycling monkeys during the breeding season (Chapter 2).

The validity of the whole-gland CMFA data reported for the multiparous animals in this project is also supported by the finding that these CMFA ranges are in agreement with the whole-gland CMFA obtained from a menstruating multiparous macaque that was processed in a previous study (1.07×10^4 cells) (Chapter 2). While only a single menstruating monkey was obtained in that study, the whole-gland CMFA data from this animal are particularly valuable in that actual weight of the mammary gland was known and, also, because the tissues used to calculate the whole-gland CMFA of that subject were obtained from throughout the entire gland. While the above arguments favor the inclusion of the whole-gland CMFA data in this report, it is the opinion of the author that the whole-gland CMFA be considered only a rough estimate and caution is warranted in its interpretation.

In spite of the acknowledged limitation to the accuracy of these CMFA metrics, it is proposed that the vast divide in whole-gland CMFA and biopsy-CMFA values between the two cohorts are of sufficient magnitude to reasonably conclude that the mammary glands of young-nulliparous menstruating macaques have significantly larger CMFAs than do those of multiparous menstruating macaques. Additional studies in which large numbers of whole glands are collected and analyzed for mammosphere potential in menstruating monkeys will prove essential to further refining the quantification of the whole-gland CMFA for this species.

The 1° MBP, 2° MFE (at 0 and 4 Gy), CFE (at 0, 2, 4 and 8 Gy) and bCFE (at 0, 2, 4 and 8 Gy) were significantly greater for the young-nulliparous animals as compared to the multiparous macaques. These results are similar to those previously found using non-irradiated tissues of luteal/follicular-stage macaques, although in that project the 2° MBP was also identified to be significantly greater for nulliparous females (Chapter 2). While 2° MBP for the animals in the current study were not found to be significantly different between the cohorts, the mean 2° MBP of nulliparous animals (0 Gy= 45.3%; 4 Gy= 35.9%) did trend higher than the mean 2° MBP of multiparous animals (0 Gy= 36.6%; 4 Gy= 31.2%). Collectively, these data suggest the mammospheres and sphere-derived cells obtained from young-nulliparous glands have a greater proliferative and differentiation potential over time, and/or throughout subsequent plating processes, as compared to the spheres and sphere-derived cells obtained from multiparous glands.

The CDP was also found to be significantly different between the nulliparous and multiparous cohorts although prior to attempting interpretation of these results it is perhaps useful to review background information related to the CDP. Previous work using human and rodent tissues has found that most primary mammospheres of a MSC/MPC origin contain between 1-4 MFUs per sphere.[71, 230, 243] Moreover, the vast majority of the cells comprising these mammospheres are of more-restricted phenotypes, such as lineage-specific progenitor cells and non-proliferative differentiated cells, which are incapable of independent mammosphere formation.[71, 230, 243] In addition to being capable of forming mammospheres, the individual MFUs derived from dissociated mammospheres have also been shown to have the ability to form bilineage adherent colonies when plated in permissive environments such as that of the clonogenic assays.[71, 230] In contrast, the mammosphere-derived cells that are of more restricted phenotypes are incapable of independent bilineage colony formation and these cells either form colonies of a single phenotype or fail to form colonies at all. Notably, aggregate spheres (as previously described) as well as spheres which are derived from the proliferation of linage-specific progenitor cells frequently lack any MFUs[70] and therefore the cells obtained from the dissociation of these spheres have minimal ability to form mammospheres or bilineage colonies.

In light of the above information, it can be appreciated that if single-cell isolates derived from mammosphere cultures of two different cohorts are compared, the cohort with the greatest ratios of MFUs will also be the cohort with the highest CDP. There are three potential ways that one cohort might have a CDP that is larger than that of another cohort. First, if one cohort has some inherent mechanism by which it can maintain greater numbers of MFUs per primary mammosphere than does the other cohort, this would result in a larger CDP for that cohort. Second, if the two cohorts have similar numbers of MFUs per mammosphere but one cohort produces smaller mammospheres (comprised of lesser numbers of cells) on average than the other cohort, this would also result in a larger CDP. Finally, if a large percentage of the "mammospheres" in one cohort originate from a source other than the clonal replication of MSCs or MPCs (e.g., aggregate sphere formation), it is expected that this cohort would have a lower CDP than a second cohort in which more of the mammospheres were derived from MSCs/MPCs.

In the current study, the mean multiparous CDP was identified to be significantly higher than the mean nulliparous CDP for the 0 Gy and 2 Gy cultures; a result consistent with the trend seen in an earlier experiment using non-irradiated macaque tissues (Chapter 2). Given that there is no significant difference in the mean

mammosphere size between the two study cohorts, and previous macaque studies suggest that that primary mammospheres of multiparous subjects typically contain smaller numbers of MFUs (1/sphere) than nulliparous subjects (3/sphere) (Chapter 2), the most plausible interpretation of these results is that a greater percentage of primary 100k multiparous mammospheres are derived from MSCs and/or MPCs as compared to primary 100k nulliparous mammospheres. Mechanistically, these CDP differences could occur if the nulliparous lineage-specific progenitor cells have a greater ability to proliferate on their own to form lineage-specific mammospheres than do the multiparous lineage-specific progenitor cells. Alternatively, these CDP differences could occur if the primary 100k nulliparous mammosphere cultures have a greater propensity to form aggregate spheres than do the primary 100k multiparous mammosphere cultures.

The results from other studies potentially provide some insight as to which of these two mechanisms is most likely responsible for the CDP differences identified above. Previous research using mammosphere cultures have found that an increase in cell-plating density is associated with an increase in the formation of aggregate spheres.[70, 85] Additional experiments conducted using macaque tissues also found that that single-cell isolates derived from 100k mammosphere cultures had CDP values that were ~31% lower than those derived from 10k mammosphere cultures when each were plated at identical cell-densities in a clonogenic assay (Chapter 2). The most straightforward interpretation of the macaque CDP experiments, and one that is consistent with the aforementioned mammosphere studies, is that there are greater ratios of aggregate spheres present in high-density (100k) mammosphere cultures. Of final importance to this discussion is that the VPC results of the current study found that nearly twice as many nulliparous, as multiparous, cells survived in the 100k primary cultures over a 7 day period. This last piece of information is remarkable in that although the 100k primary mammosphere cultures of both cohorts were initially plated at identical cell densities, the nulliparous cultures had a greater total number of cells in their culture plates, as compared to the multiparous cultures, within just a few days of the plating process. Viewed another way, the nulliparous 100k cultures were essentially higher-density platings than those of the 100k multiparous cultures. In light of the above information and these findings, it is plausible that the cells of the nulliparous primary mammospheres cultures formed aggregate spheres in greater ratios than did the cells of the multiparous primary cultures. While it also remains possible that the CDP differences between the cohorts could be due to an increased potential of the nulliparous lineage-specific progenitor cells to clonally proliferate as spheres, there are no study data or literature in direct support of this mechanism. The possibility of aggregate sphere

formation as the primary mechanism responsible for the differences in CDP identified in this study will be explored as part of future research projects.

The 2º MFE, 2º MBP, cell viability, survival fraction and budding morphology data utilized to compare the radiosensitivity between the nulliparous and multiparous cohorts produced a mixture of expected and unexpected findings. As anticipated, the 2º MFE, 2º MBP, cell viability and total-colony survival fraction values decreased significantly with each increasing radiation dose for both cohorts. Similarly, there was a significant decrease in the bilineage-colony survival fraction values with each increasing radiation dose between 2 Gy and 20 Gy for both the nulliparous and multiparous subjects, as was expected; although there was also radioresistance of the bilineage colonies to the 2 Gy radiation dose by both cohorts that had not been predicted. Most unexpected, however, was the finding that there were no statistically significant differences in radiosensitivity between the nulliparous and multiparous cohorts for any of the aforementioned metrics. Finally, while previous experiments (Chapter 2) suggested that there would be no appreciable differences in the budding morphology of the spheres between the cohorts, the fact that there were also no differences in budding morphology between the 0 Gy and 4 Gy-irradiated spheres within either cohort was surprising.

Similar to the other metrics, the CDP data also identified changes within each cohort in response to irradiation. In particular, the CDP was identified to be significantly increased with each increasing radiation dose between 0 Gy and 8 Gy for both cohorts. One mechanism to possibly account for this observation is that irradiation could have stimulated the transformation of linage-specific progenitor cells and/or non-proliferative differentiated cells into bilineage-competent progenitor cells (dedifferentiation). This proposal is not completely unprecedented as radiation has been identified to drive dedifferentiation of hematopoietic cell populations in vivo[89, 167, 168] and has also been shown to reprogram breast cancer cells into breast cancer stem cells in vitro.[275] While the slight, nonsignificant, increase in the bCFE between the 0 Gy and 2 Gy cultures for the nulliparous cohort offers some support for dedifferentiation, the significant reduction in the bCFE of both cohorts at all radiation doses beyond 2 Gy does not. Collectively, these study data and the fact that there is no literature identified which directly supports the rapid dedifferentiation of normal cell populations under in vitro conditions makes this mechanism appear unlikely. Furthermore, based on the current understanding of dedifferentiation as it applies to multistep principle of carcinogenesis,[104] it appears doubtful that a single dose of radiation administered to a population of normal

mammary gland epithelial cells would be capable of consistently dedifferentiating enough cells over just one cell-culture generation to cause the profound CDP changes identified in this study.

A more simplistic mechanism, and one that can account for the CDP changes in both cohorts throughout all radiation doses, is that the MSCs and/or MPCs may be more radioresistant than are the more phenotypically-restricted cell populations in these cultures. A comparison of the total-colony survival curves and bilineage-colony survival curves, whose data are derived from the same clonogenic assay as the CDP metric, are supportive of this proposal. Prior to discussing this comparison, however, it is important to first understand a few specific aspects the survival curves themselves. First, the vast majority of the colonies formed in the clonogenic assays were comprised of a single phenotype of cells and therefore most of these colonies were likely derived from the more phenotypically-restricted cell phenotypes. In turn, it can be appreciated that the radiation-induced changes identified in the total-colony survival curves were largely dependent on the radiosensitivity of the more-restricted cell phenotypes within these assays. In contrast, only the colonies containing both epithelial and myoepithelial cells were quantified in the bilineage-colony survival curves. As such, it can be appreciated that the radiation-induced changes identified in the bilineage-colony survival curves is mostly dependent on the radiosensitivity of the MFUs within these assays. The finding that total-colony survival fractions were significantly lower than the bilineage-colony survival fractions at the 2 Gy, 4 Gy, and 8 Gy radiation doses for both cohorts (Figure 4,A and 4,B) suggests that there is an overall greater radioresistance of the MFUs as compared to the more-phenotypically restricted cell types. In addition to these results, there is also abundant literature in support of the proposition that stem cells/early-progenitor cells (i.e., MPCs) are more radioresistant than are other proliferative cell populations in mammary gland epithelial cultures[12, 40, 175, 293] and breast cancer cell lines.[6, 68, 198, 275, 282, 299]

Unlike any of the previous metrics, the CDP data identified significant differences in radiosensitivity between the cohorts. Specifically, the slope of nulliparous CDP response to irradiation was found to be significantly greater than that of the multiparous CDP between the 2 Gy and 8 Gy radiation doses. These differences are especially intriguing when it is appreciated that the trends of the bilineage-colony survival curves largely mirrored the CDP changes but those of the total-colony survival curves did not. In short, these observations collectively suggest that the CDP variability between the cohorts is primarily due to differences in the radiosensitivity of the MFU populations of the cultures and, furthermore, that the MFUs of nulliparous subjects are more resistant to the effects of increasing radiation doses than are those of the multiparous subjects. Previous work with mammary

epithelial cell lines[12, 40, 175, 293] and breast cancer cell lines[6, 68, 198, 275, 282, 299] have suggested that gradients of radiosensitivity are likely to exist between the stem and progenitor cell populations. In turn, it is plausible that the CDP differences of this study could occur if the mammosphere cultures of the two cohorts were comprised of different ratios of the same proliferative cell populations and/or if there were proliferative cell populations that were unique to the mammosphere cultures of only one of the two cohorts.

There is at least one aspect of the study data which supports the proposition that the two cohorts vary in regard to the proliferative cell populations that comprise their secondary mammospheres cultures. Specifically, when the 2º MFE data of each cohort is compared to other mammosphere-potential metrics within their respective cohorts there are notable differences between the nulliparous and multiparous subjects. For the nulliparous subjects, the 2º MFE demonstrated a statistically positive correlation with the 2º MBP at both 0 Gy and 4 Gy irradiation doses. Stated another way, the individual nulliparous cultures that produced the greatest number of secondary mammospheres also had the highest number of mammospheres capable of budding, even when exposed to 4 Gy radiation. This suggest that most nulliparous secondary mammospheres originate from a highly radioresistant population of cells with good differentiation potential; a finding consistent with what might be expected if most nulliparous mammospheres were of a MSC origin. The multiparous subjects, by contrast, had no significant correlation between the 2º MFE and 2º MBP at 0 Gy and had a statistically negative correlation between the 2º MFE and 2º MBP at 4 Gy. As the secondary multiparous mammospheres were less consistent with regard to differentiation potential and radiosensitivity, it is plausible that the cellular origin of the multiparous mammospheres is more heterogeneous in nature than that of the nulliparous spheres. Stated another way, the multiparous secondary mammosphere properties are consistent with what might be expected if these mammospheres were frequently derived from an assortment of progenitor cell populations of variable radiosensitivity rather than being of a primary MSC origin.

While it is possible that the differences in radiosensitivity between the nulliparous and multiparous subjects could be entirely due to variable ratios of proliferative cell populations common to both cohorts, it is also not unreasonable to think that there may exist novel progenitor cell populations within the multiparous macaque mammary glands as a result of the maturation/lactation process.[220] In support of this contention, studies have found that parous rodent glands contain a mammosphere-competent population of bilineage proliferative cells, termed parity-induced mammary epithelial cells (PIMECs), which are not present within nulliparous rodent glands.[24, 172, 273]

As to date, there have been no studies which have attempted to look for a similar parity-induced cell population within monkey mammary glands, it remains a possibility that a PIMEC-like population could also exist in the glands of parous macaques. The potential existence of macaque PIMECs, along with the prospect that these cells could contribute to CDP radioresistance-differences between the nulliparous and multiparous subjects,[220] warrants investigation and will be explored as part of future research projects.

As to why some of the clonogenic assay-derived metrics (CDP and bilineage-colony survival curves) suggested differences in radiosensitivity between the cohorts, but the 2° MFE, 2° MBP, cell viability or budding morphology metrics did not, is potentially due to the different culture conditions used for these assays. Specifically, the fact that the latter metrics were all derived from cells grown as secondary mammospheres under presumed hypoxic conditions (paraffin-wrapped culture plates) could have confounded the results of these assays. Previous experiments have found that normal mammary cells,[198] as well as breast cancer-cell lines,[125] grown under conditions that promote mammosphere formation are more radioresistant than are the same cell populations grown as adherent (monolayer) colonies. Additionally, other studies have shown that hypoxic conditions can increase the radioresistance of cancer cells in vivo[191, 193, 210, 246] as well as increase the radioresistance of cancer cells grown as spheroid colonies in vitro.[91, 212, 247, 248] Finally, there are data from the current study that also support the proposition that cells plated under hypoxic mammosphere culture conditions (2° MFE) are less affected by irradiation than are cells plated under adherent colonies in normoxic conditions (CFE/bCFE). In particular, the current study found that while the average 4 Gy 2° MFE values were around 60% of the value of the average 0 Gy 2° MFE, the average 4 Gy CFE and bCFE values were only around 10% and 25.2% of value of the average 0 Gy CFE and bCFE, respectively. In light of this information, it is proposed that differences in radiosensitivity between the mammary gland-derived cells of the two cohorts likely exist, as supported by the CDP data, but that the detection of these differences in the 2° MFE, 2° MBP, and cell viability assays are possibly being obscured by radiation-blunting culture conditions.

As there were no differences in the budding morphology identified between cohorts, or even between the individual radiation doses within either cohort, it is proposed that there may be limitations to the sensitivity of this metric even beyond those imposed by the mammosphere culture conditions. In particular, it is possible that the BME-medium used for the MBP assay may artifactually limit the differentiation potential of the spheres to only a few morphologic manifestations and/or the parameters currently being used to qualify the budding morphology of spheres may lack the specificity required to appreciate subtle differences between the cohorts.

Xenograft experiments, in which cells derived from primary mammospheres are irradiated and then immediately transplanted into the fat pads of immunodeficient mice, are likely to be an effective method for determining whether mammosphere culture conditions and/or the use of BME-medium are obscuring the detection of differences in radiosensitivity between cohorts. Pilot studies using xenograft transplantation to compare multiparous and nulliparous macaque mammosphere-derived cells for differences in radiosensitivity are currently funded and underway.

Senescence, as generally defined, is a permanent, non-proliferative state that is identified in proliferative somatic cell populations undergoing certain types of stress.[8, 80, 119] A number of cellular modifications are associated with the senescent phenotype[8, 125, 137, 211] and some of these changes have been exploited toward the identification of individual cells and cell cultures undergoing senescence. On a general level, senescent cells are known to increase their secretion of IL-6 and IL-8 as part of a senescence-associated secretory phenotype (SASP). The SASP has been proposed to be a physiologic alteration that senescent cells undergo to maintain their non-proliferative cellular state and also to promote their own in vivo clearance through the stimulation and/or activation of inflammatory cells.[8, 137, 211] Additionally, senescent cells are also known to have an increase in senescence-associated β-galactosidase activity. While this alteration is of undetermined mechanistic importance to the senescence phenotype, the intracellular accumulation of β-galactosidase has been used to distinguish senescent cells from other non-proliferative cell populations such as apoptotic cells or cells within the quiescent phases of the cell-cycle.[8, 125, 137, 211] Immunohistochemical assay kits capable of identifying intracellular β-galactosidase have previously been utilized to detect senescence in cells within intact mammospheres[125] as well as in individual cells acquired from the dissociation of mammospheres.[67]

In the current study the CM1 obtained from multiparous primary mammosphere cultures had significantly higher concentrations of IL-6 and IL-8 as compared to the CM1 derived from young-nulliparous primary cultures. Likewise, the β-galactosidase assay performed on non-irradiated (0 Gy) cells derived from the early secondary mammosphere cultures identified β-galactosidase staining in a significantly larger percentage of multiparous cells as compared to the cells of young-nulliparous animals. Collectively these data suggest that cells derived from multiparous mammary glands are more prone to undergo senescence than are cells acquired from young-nulliparous glands. Moreover, the cytokine analyses suggest that the senescence differences are true of the cells obtained

directly from the mammary gland while the β-galactosidase assay suggest that these differences also persist in the cells comprising the mammospheres as well.

Senescence is reported to occur in vitro when cell cultures undergo stresses such as when cells are not provided with the proper substratum, when media exchanges are prolonged, or when cultures are maintained in environments with inappropriately high oxygen levels.[137, 211] In that the mammosphere cultures of the two cohorts were processed identically and no correlation was identified between the senescence metrics and any of the biopsy parameters (e.g., biopsy weight, digestion time, %VC, etc.), it is considered unlikely that in vitro stresses played a significant role in the increased incidence of senescence in the multiparous animals.

An increased incidence of in vivo senescence of various cells types has been positively correlated with advancing age in mice, monkeys, and humans.[118, 137] As increased age was found to be statistically positively correlated with β-galactosidase expression in the multiparous individuals of this study, it is possible that age may be the primary factor related to the differences in senescence identified between the two cohorts. What must also be appreciated here, however, is that the biopsies used in this study were obtained from animals in an active breeding colony and age is also significantly positively correlated with parity in the multiparous subjects. This information is important as increased parity was even more highly associated with β-galactosidase expression ($P<0.01$) than was increased age ($P<0.05$) in the multiparous subjects. While no literature can be identified in support of the proposal that increased parity is associated with an increase in cellular senescence, there is likewise no evidence to the contrary. In turn, it is possible that with each pregnancy the macaque mammary gland may undergo some form of maturation that increases the overall susceptibility of these cells to enter the senescence state upon being stressed.

It is interesting that increased senescence was highly correlated with increased parity in the multiparous macaques of this report given that the numbers of rodent PIMECs, as previously described, were also identified to increase with each successive pregnancy.[24, 172, 273] Although no PIMEC-like cells have yet been identified within parous macaque glands and none of the aforementioned rodent studies looked for any differences in senescence between the parous and nulliparous mammary cells, it is tempting to speculate that parous macaques might also have a PIMEC-like population that is prone to undergo senescence which could account for the differences in β-galactosidase expression between the animals of the multiparous cohort. This possibility further validates the previously mentioned need for investigations into the potential existence of PIMEC-like cells within the parous macaque mammary gland.

An increased incidence of senescence in human mammosphere-derived cells has previously been associated with a decrease in the MFE of mammospheres cultures.[67] This finding is of importance as it suggests that the differences identified in the MFEs between the two cohorts of this study is not necessarily due to a difference in the numbers of MSCs/MPCs between the cultures. Rather, this information suggests that the MFE differences of the cohorts could be due, in part or in whole, to a propensity for the MSCs/MPCs of multiparous cultures to undergo senescence with a greater frequency than those of nulliparous cultures. Unfortunately, none of the assays performed in this study were capable of determining if the MFUs, or the more-differentiated cell types, were the population of cells most prone to senescence. However, this information is crucial to understanding the full implications of the MFE differences identified between the cohorts and future experiments utilizing technologies such as Fluorescent Activated Cell Sorting (FACS) are warranted to help determine the specific proliferative-cell populations most prone to senescence in each of these cohorts.

The lack of a significant increase in β-galactosidase expression within either study cohort in response to increasing doses of radiation was unexpected. This is because β-galactosidase assays had previously been utilized to compare the incidence of senescence within various populations of mammosphere-derived cells[67] and had also been utilized to document increases in cellular senescence associated with exposure to ionizing radiation in a variety of cell types.[119, 125] The lack of significant findings was most surprising, however, in that pilot experiments using multiparous macaque mammospheres suggested that notable increases in β-galactosidase staining would likely be evident at radiation doses greater than 4 Gy. In retrospect, it appears as if the vast majority of the senescent-prone cells in the current study were stimulated into senescence prior to irradiation through the stress of the mammosphere dissociation process (background senescence). Furthermore, in that there was no appreciable trend identified between irradiation and β-galactosidase expression in the multiparous cultures of the current study, it is believed that the results of the pilot experiments are best accounted for by random sample variation. Compared to the multiparous cultures, the nulliparous cultures showed less variation in the levels of senescence between individual subjects and had an overall lower incidence of background senescence. Likely as a result of these two properties, there was an appreciable, though nonsignificant, trend for β-galactosidase expression to increase with increasing radiation dose within the nulliparous cultures (Figure 6). While the trend of nulliparous cultures is consistent with the proposition that irradiation can stimulate senescence within mammosphere-derived cells, the experimental design of this study was suboptimal for comparing radiation-induced senescence in mammospheres. Experiments which utilize confocal-

microscopy imaging to evaluate intact, size-matched, irradiated mammospheres are likely to be more effective at evaluating differences in radiosensitivity between the cohorts, and these techniques will be attempted in future studies.

Ideally, this study would have been able to identify differences in the overall mammosphere potential, senescence, and radiosensitivity as a function of both age and parity. Unfortunately, the use of tissues from breeding colony animals confounds this distinction as age and parity are highly correlated. In only one assay, β-galactosidase expression, was there any difference noted between age and parity within the multiparous cohort. Additional information crucial to deciphering the individual roles that age and parity may play in mammosphere potential, senescence, and radiosensitivity will have to come from future studies comparing: 1) variably- aged nulliparous animals; 2) parity-matched animals of different ages; 3) and age-matched parous and nulliparous animals. As these data are currently lacking, the most that can be definitively stated at this time with regard to age and parity is that there are appreciable differences in the mammary gland-derived cells of the young-nulliparous and older multiparous macaques.

The first aim of this project was achieved and, as expected, the 1º MFE, VCR, and CMFA data showed that during the menses-stage of the menstrual cycle the mammary glands of young-nulliparous monkeys contains larger ratios and larger numbers of cells capable of in vitro mammosphere formation than do the multiparous mammary glands. It is possible that the ability of the nulliparous cohort to form greater numbers of mammospheres than multiparous cohorts is simply due to increased numbers of MFUs within the glands of these subjects. This conclusion is consistent with the results of some rodent studies which have identified nulliparous mammary glands to have significantly greater numbers of MSC-like cells than those of similarly-aged parous animals.[28, 235] However, there are also some study data to suggest that there may also be innate differences in the proliferative cell types that constitute the MFU populations between the two cohorts. In turn, future studies are needed to determine: 1) if a PIMEC-like proliferative cell population exist in parous macaque glands; 2) if the proliferative cell populations of the two cohorts are made up of different ratios of the same types of proliferative cells; or 3) if equal ratios of same proliferative cell populations exist between the two cohorts, but the multiparous MFUs are more prone to undergo senescence than are the nulliparous MFUs.

The second aim of this project was likewise achieved. Specifically, the 2º MFE and CFE/bCFE study data demonstrated that the cells derived from primary mammospheres of young-nulliparous monkeys maintain a higher

proliferative potential in subsequent cell-culture assays as compared to the cells derived from multiparous mammospheres. Additionally, the 1º MBE study results demonstrated that the primary mammospheres derived from young-nulliparous primates have greater in vitro differentiation potential as compared to the multiparous mammospheres. Although the 2º MBE was not significantly different between the cohorts, there was a trend for the nulliparous subjects to have greater numbers of budding mammospheres as compared to multiparous subjects. Future studies utilizing greater numbers of subjects and/or xenotransplantation experiments may prove useful to determining whether nulliparous secondary mammospheres routinely maintain a statistically greater differentiation-potential (2º MBE) over time as compared to multiparous spheres. Future studies to explore differences in the MFU populations between the two cohorts, as mentioned above, are also likely to provide valuable insight into the mechanisms behind the variation in mammosphere potential and differentiation.

The third aim of this study was achieved as there were significant differences in radiosensitivity identified between the cohorts as per the CDP metrics. These CDP results were also supported by similar, although nonsignificant, changes to the data from the bilineage-survival colony assay. Finally, the analysis of the CDP, CFE, and bCFE data for both study cohorts indicated that the MFUs are likely more radioresistant than are the other, more-phenotypically restricted, cell types typically obtained from the dissociation of mammospheres. Collectively, the results of these experiments suggest that greater ratios of the young-nulliparous MFUs are capable of continued proliferation in the clonogenic assays following irradiation, as compared to the multiparous MFUs. Unexpectedly, there were no significant differences in radiosensitivity identified between the cohorts in any of the assays that utilized intact mammospheres or which examined cells that were maintained within mammosphere culture conditions following irradiation. As previously discussed, the lack of differences in radiosensitivity between the cohorts in these latter assays is likely to have been a research artifact related to the culture conditions in which these particular experiments were performed. Xenotransplantation studies will be used in the future to reexamine the radiosensitivity of the mammosphere-derived cells between the two cohorts.

Differences in the overall incidence of senescence were also identified between the two cohorts of this study. The increased incidence of senescence in multiparous cells likely played at least some part in the decreased mammosphere production, proliferation, and differentiation rates of the multiparous cohort. The increased incidence of senescence of the multiparous cells is perhaps most intriguing, though, in that senescence has been proposed to be

a mechanism by which the body minimizes tumorigenesis of initiated cells[8, 125, 137, 211] and at least one study has associated increased senescence with a decreased risk of mammary cancers in rodents.[23]

In summary, the MSCs and MPCs (MFUs) of the human breast are thought to be common cells-of-origin for breast cancers. Human mammosphere cultures have previously been utilized to study the MFUs and, recently, macaque mammosphere cultures have been demonstrated to produce mammospheres and MFUs which are comparable to those described in humans. The current study identified a number of differences between multiparous macaques and young-nulliparous macaques that are of potential interest to breast cancer research with regard to parity-related risk factors. First, multiparous macaque mammary glands were identified to have lower ratios and numbers of MFUs than young-nulliparous macaque glands. Second, the proliferative and differentiation potentials of the multiparous mammospheres were identified to be lower than those of young-nulliparous mammospheres. Third, the MFUs derived from multiparous mammospheres were identified to be more radiosensitive than the young-nulliparous MFUs, as per some assays. Finally, the multiparous mammosphere cultures were identified to have higher rates of senescence than the mammosphere cultures of young-nulliparous cultures. Collectively, the findings that young-nulliparous mammary glands have increased numbers of potential cellular targets (MFUs) for mutagenesis, and that these cells are more prone to proliferate (i.e., lower incidence of senescence) following their removal from a homeostatic niche (i.e., mammary gland or mammospheres) or after irradiation, could be reasonably expected to make nulliparous subjects more susceptible to radiation-induced cancers as compared to multiparous subjects. This expectation is consistent with what is known to be the case in humans and, as such, the data from this study has potentially provided the first direct insight as to the cellular mechanisms responsible for the increased risk of breast carcinogenesis in young nulliparous women exposed to radiation. In addition to these findings, the current study is also novel in that it demonstrated, for the first time, the feasibility and utility of the macaque mammosphere model toward breast cancer research.

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Chapter 5: Research Summaries, Ongoing Studies, and Future Directions

Introduction

The overarching goal of the work comprising this dissertation was to develop novel methods by which to explore parity-related risk factors in breast carcinogenesis. Viewed through a broad lens, this project can be considered an overall success. However, when inspected at a more refined level, there are numerous questions and limitations related to the study results that have yet to be overcome. The results, conclusions, and limitations for each aspect of this research project are provided in detail within their respective chapters. It is the goal of the current chapter to provide a summary of the research advances achieved throughout this project and, where applicable, address the setbacks to the research process. The ongoing studies related to this work will also be discussed, as will the plans for future experiments utilizing the methods that were developed in this project.

Research Summary

The first arm of this project sought to modify, optimize, and validate an established in vitro research model (mammosphere culture assay) for use in the rhesus macaque (*Macaca mulatta*). This task was undertaken in that while both rodent-based and human-based mammosphere research have contributed to the general knowledge of breast biology and carcinogenesis, there remain critical questions as to these aspects of research that are arguably best explored using nonhuman primate (NHP) models. The initial work of this project produced an exceedingly-detailed macaque mammosphere protocol that was demonstrated to be highly reproducible. Through the optimization process, two novel modifications, the use of conditioned media and hypoxic plating conditions, were identified and added to the classical mammosphere protocol. Experiments showed that in vivo mammary gland biopsies collected from macaques could be utilized for the production of mammosphere cultures and also demonstrated, through mammosphere culture results, the physiologic/morphologic homogeneity of biopsies collected from throughout the rhesus macaque mammary gland. Finally, the first-ever reported rhesus macaque mammary gland weights were collected in this project.

Following this initial work, macaque mammospheres were validated to have similar properties to the mammospheres identified in other species. Specifically, it was shown that macaque mammospheres: 1) could be generated from a single cell; 2) have the ability to replicate over several generations; 3) have bilineage (epithelial

and myoepithelial) potential; and 4) contain cells that, on their own, have the bilineage potential. In short, this work suggested that the macaque mammospheres contain mammary stem cell-like cells.

Macaque mammospheres were also validated to be relevant models for human breast research by demonstrating that rhesus macaque mammospheres are comparable to human mammospheres. Most significantly, macaque and human mammospheres were shown to be similar with regard to: 1) size of the spheres; 2) numbers of mammary stem cell-like cells per sphere; 3) immunogenic staining patterns; 4) proliferative potential; 5) differentiation potential; 6) bilineage potential; and 7) lactogenic potential.

Another arm of this project utilized the optimized macaque mammosphere protocol to explore the proliferative and differentiation potential (collectively referred to as mammosphere potential) of mammary epithelial cells acquired from rhesus macaques of various demographics. Through these studies it was demonstrated that cells derived from the nulliparous animals have an overall greater mammosphere potential than do cells derived from multiparous animals. While not as definitively conclusive, there were also differences in mammosphere potential noted between animals in different phases of the reproductive cycle. Finally, these studies also produced a few results to suggest that age may also play a role with regard to mammosphere potential.

An additional aspect of these demographic comparisons was a study to look for differences in the morphological manifestations of mammospheres suspended in basement membrane extract. Other than in a few pregnant animals, there were essentially no appreciable differences identified in morphology between the cohorts. However, this study did produce a highly-comprehensive review as to the morphological and physiological transformation of mammospheres over time. Through this work a number of novel observations related to mammosphere growth and differentiation were identified that are likely to be relevant and useful to mammosphere-based research across all species. Finally, a novel conditioned media (created using the stromal elements of the mammary gland) was also utilized in these experiments, and where it was shown to be capable of altering the morphological and physiological growth potential of the spheres.

In that the results of the earlier studies suggested that the mammosphere potential of macaque mammary glands is directly associated with the phase of the reproductive cycle, the third arm of this research project sought to identify a minimally-invasive method by which to synchronize tissue collections. Specifically, a novel rapid-staining technique for vaginal cytology was evaluated for its ability to definitively identify the menses stage of the ovulatory menstrual cycle. The results of this study found that, on its own, vaginal cytology lacked the specificity required for

the accurate diagnosis of the menses stage. In spite of this setback, the research literature collectively suggested that synchronization of tissue collections to the menses stage could be achieved with relatively high accuracy in study animals that: 1) are regularly-cycling; 2) have vaginal bleeding during breeding season; 3) are clinically healthy otherwise; 4) produce a vaginal cytology that is consistent with the menses stage. Beyond identifying a supportive role for vaginal cytology in the synchronization of tissue collections, this study was also determined to be a success in that it showed a rapid-staining technique could produce vaginal cytologies that were equivalent in their stage-diagnostic abilities to those of more labor-intensive stains.

The final arm of this project utilized the cumulative information gained from the previous work to perform a study comparing the mammary epithelial cell populations of young-nulliparous and multiparous animals with regard to mammosphere potential and radiosensitivity. The impetus for this work was that young-nulliparous girls are appreciated to have a higher lifetime risk of breast carcinogenesis following irradiation as compared to parous women. This study found that, as compared to multiparous macaques, the mammary epithelial cell populations derived from young-nulliparous animals had: 1) greater proliferation potential; 2) greater differentiation potential; 3) lower overall rates of cellular senescence; and 4) less radiosensitivity. These collective findings were taken to suggest that the mammary glands of young-nulliparous macaques have larger numbers of mammary stem cell-like cells than do the glands of multiparous macaques. Given that mammary stem cells, and progenitor cells with mammary stem cell-like properties, are thought to be common cells-of-origin for many breast cancers, the findings of the young-nulliparous macaques were concluded to be consistent with those of a population at an increased risk of breast cancer initiation. Importantly, this conclusion is similar to what is known to be the case in young-nulliparous girls. In short, it is suggested that this study may have provided the most informative insight, to date, as to the possible mechanisms (i.e., increased numbers of cellular targets, increased resistance to cellular senescence) involved with the increased risk of radiation-induced cancers in young-nulliparous girls.

Ongoing Research and Future Directions

The research performed as part of this dissertation utilized rhesus macaque mammary tissues exclusively. However, it was always considered likely that the techniques and general information acquired from this project would also be applicable to other closely-related primate species as well. The initial results of a grant-funded research project aimed at investigating the protective effects of phytoestrogens on cynomolgus macaque (*Macaca fascicularis*) mammary glands have supported this proposition. In short, the author and his collaborators from the

primate research centers of Wake Forest University and Bogor Agricultural University (Indonesia), have found that the mammary gland weights and the rates of mammosphere growth/differentiation of the cynomolgus macaque fall within the parameters identified for the rhesus macaque. With the continued collection and processing of samples from this ongoing project, it is expected that the staining characteristics of the mammospheres will also be demonstrated to be similar between the two species. In short, it appears as if the methods and parameters developed for this study are likely to be useful to a wide range of projects involving NHPs.

The initial successes of the macaque mammosphere project have subsequently led to the procurement of additional grant funds for use in future macaque mammosphere studies. Some of these funds have been targeted toward further validation of the macaque mammosphere model. The validation studies will involve xenotransplantation experiments in which mammospheres, and mammosphere-derived cells, will be placed in the cleared-fat pads of immune-deficient mice with the goal of producing rhesus macaque mammary outgrowths in the mice. Xenotransplantation experiments are currently considered to be the "gold-standard" by which mammospheres are confirmed to contain mammary stem cells. Although the data from this project collectively supports the proposal that the bulk of proliferative cells responsible for the formation of macaque mammospheres are mammary stem cells, the author of this dissertation has taken a conservative approach and will refer to these proliferative cell populations as mammary stem cell-like cells and/or mammosphere forming units (MFUs) until such time the xenotransplantation studies can be completed.

Additional funds from the current grant award will be used toward macaque mammosphere characterization. This process will involve the use of fluorescent-activated cell sorting (FACs) modalities to identify cell-surface markers associated with the mammosphere-derived cells. The purpose of these experiments is twofold at this time. The first goal of these studies is to attempt to identify cell surface markers that have previously been identified in the mammosphere cultures of other species. Identification of these specific cell-surface markers in the macaque mammospheres would further validate this model, and especially so if the cell-surface markers identified to be characteristic of human mammospheres can also be identified in the macaque mammospheres. The second goal of these studies is to characterize and compare the cells obtained from young-nulliparous and multiparous macaque mammosphere cultures. Any difference identified between the two cohorts is likely to be of some benefit to the overall understanding of the mammary gland maturation process. However, one specific objective of this study will be to look for a proliferative population of cells in the multiparous cultures that are equivalent to the parity-induced

mammary epithelial cells (PIMECs) identified within the mammary gland of parous rodents. In the studies mentioned above, which directly compared the multiparous and young-nulliparous animals, there were some data to suggest that the cell populations responsible for mammosphere formation may be variable between the cohorts and the existence of a PIMEC-like population in the multiparous animals was proposed as a possibility to explain the observed differences.

The remaining funds from the current grant award will be utilized to expand on the previous research which looked for differences in radiosensitivity between the mammary epithelial cell populations of multiparous and young-nulliparous macaques. In the initial study, differences in radiosensitivity were identified between the two cohorts in some of the assays in which the irradiated cells were allowed to undergo differentiation on collagen-coated plates. However, in that same study there were no significant differences identified between the two cohorts when the irradiated cells were maintained under culture conditions that promoted mammosphere formation. While these observations have been proposed to be an artifact of the mammosphere culture conditions, what is still not known, is if the results of the collagen-coated plates are actually representative of the in vivo differentiation-potential of mammary gland cells. In the upcoming studies, xenotransplantation of irradiated cells, as well as xenotransplantation of mammospheres derived from irradiated cells, will be used to assess differences in the post-radiation proliferation and differentiation potentials between the multiparous and young-nulliparous macaques. These studies will additionally look for neoplastic features (breast carcinogenesis) within the mammary gland outgrowths and these results will likewise be compared between the cohorts.

The genetic and morphologic similarities between humans and macaques, in addition to the fact that macaque mammary tissues can be readily obtained from essentially every demographic of animal, endow macaque mammosphere cultures with the potential to provide information about certain aspects of breast biology and carcinogenesis that is difficult, or even impossible, to assess utilizing either human or rodent mammosphere assays. Furthermore, given the fact that in vivo biopsies can be utilized for mammosphere cultures, and the mammosphere potential of biopsies has been recognized to be homogeneous throughout the gland, it is plausible that the mammosphere potential of individual animals could be monitored over time through the collection of multiple biopsies. This within-subject longitudinal comparison is an especially attractive study-design here in that this approach can minimize the confounding variables that are often associated with using a genetically diverse research model such as the macaques.

In light of this potential, future macaque studies on breast carcinogenesis are of interest and could be designed so that individual animals have multiple biopsies collected at specific time points. The mammosphere-cultures derived from each of these biopsies could then be exposed to radiation (or another carcinogen). After a period of in vivo or in vitro growth, the cells collected from different time points could be compared to look for any differences in their susceptibility to undergo breast carcinogenesis. These comparisons could be performed across different stages of the menstrual cycle or across different phases of the reproductive cycle (i.e., pregnancy, lactation). Furthermore, studies of this nature could also be performed to look for differences in the susceptibility of the mammary epithelial cells to undergo breast carcinogenesis in nulliparity, primiparity and multiparity.

Other future projects of interest involve studies into the use of conditioned media to alter mammosphere potential, and/or possibly alter the susceptibility of the mammospheres to breast carcinogenesis. Two different types of conditioned media were utilized in the experiments of this dissertation. Conditioned media-1 (CM1) was mammosphere media that was collected from first-generation mammospheres grown at high densities. The CM1 was utilized in an autologous fashion in the previous experiments to promote the growth of low density, second-generation mammospheres. Conditioned media-2 (CM2) is a conditioned media that was made from the stromal elements of the gland that were collected from necropsied animals. The CM2 was utilized in an autologous fashion in the previous experiments to promote lactational differentiation of the mammospheres suspended in basement membrane extract. The previous studies on cellular senescence have identified significant differences in the cytokine levels of the CM1 between multiparous and young-nulliparous animals. As the mammosphere potentials of these two cohorts have also been identified to be significantly different, it is plausible that if CM1 acquired from the nulliparous mammosphere cultures was supplemented into the multiparous mammosphere cultures that there might be alterations to the mammosphere potential of the multiparous cohort. Initial experiments related to these types of studies could simply look for differences in proliferation and differentiation potential in the mammosphere cultures of one cohort (e.g., nulliparous) to which CM1 from another cohort (e.g., multiparous) had been added. Additional studies could also be performed using FACS to look for up-regulation or down-regulation of cell-surface markers that coincide with the addition of the "foreign" CM1. As there are a multitude of cohorts that could be used for this process (e.g., menses stage-nulliparous animals, luteal stage-multiparous animals, lactating primiparous animals, first-trimester pregnancies, second-trimester pregnancies, etc.) the number of possible permutations for these studies are seemingly endless. Similar experiments could also be performed through the addition of foreign CM2 to the

mammospheres suspended in basement membrane extract. It is considered likely that through this process, information related to the changes of the mammary gland epithelium throughout growth, development and lactation would be identified which could enhance the scientific knowledge of mammary gland biology in general. Furthermore, these same types of studies could also be performed on cells exposed to radiation (or another carcinogen) to identify if the CM1 or CM2 from any particular cohort is protective of carcinogen-induced neoplastic alterations. This work is particularly intriguing, in that if a cohort can be identified to have CM1 and/or CM2 that is protective, it may be possible to identify the molecular mechanisms responsible for these actions and utilize it as a preventative or therapeutic treatment for cancers.

In closing, the cumulative work comprising this dissertation is considered an overall success in that it has established a basic foundation for future NHP mammosphere work and has additionally identified novel information related to macaque biology, mammary gland biology, and possibly breast carcinogenesis. The results of the initial macaque mammosphere study have been deemed worthy of additional financial support by a granting body and further validation, characterization, and investigation of the mammosphere assay will begin in the near future. Finally, even prior to the publication of these results, this work has inspired investigators to undertake the use of mammosphere methods in their research. It is my sincere hope that the information contained in this dissertation will continue to motivate others to explore the use of these novel research methods in future mammary gland related-studies.

Appendix

Abbreviations and Definitions of Terms for the Metrics Utilized to Gauge Mammosphere Potential

bCFE (bilineage-colony forming efficiency): used to assess the ability of single cells to form bilineage colonies (containing both myoepithelial and epithelial components) of greater than 50 cells on collagen-coated plates (two-dimensional bilineage proliferation). Calculated as the average number of bilineage colonies formed in each well divided by the number of viable cells initially plated per well (expressed as percentage). For this project bCFE was only evaluated on cells obtained from the dissociation of primary mammospheres.

Biopsy-CMFA (numbers of cells with mammosphere forming ability in individual biopsies): used to assess the number of cells within each biopsy that are capable of forming mammospheres in mammosphere culture. Calculated using the TVC, 1^{o} MFE, and the biopsy weight obtained from the individual subjects.

CDP (colony differentiation-potential): used to assess the differentiation abilities of colonies formed by single cells on collagen-coated plates (two-dimensional differentiation). Calculated as the number of bilineage colonies/well divided by the total-number of colonies/well (expressed as percentage). For this project CDP was only evaluated on cells obtained from the dissociation of primary mammospheres.

CFE (colony forming efficiency): used to assess the ability of single cells to form colonies of greater than 50 cells on collagen-coated plated (two-dimensional proliferation). Calculated as the average number of colonies formed in each well divided by the number of viable cells initially plated per well (expressed as percentage). For this project CFE was only evaluated on cells obtained from the dissociation of primary mammospheres.

MBP (mammosphere budding-potential): used to assess the ability of individual mammospheres to produce ductal and alveolar outgrowths (budding) from the main sphere body when suspended in basement membrane extract (three-dimensional differentiation). Calculated as the number of budding spheres divided by the total number of spheres (expressed as a percentage) within a single "bleb" of basement membrane extract. For this project MBP was evaluated on spheres obtained directly from the single cell isolates derived from mammary gland tissues (1^{o} MBP) as well as from spheres obtained from the single cell isolates derived from primary mammospheres (2^{o} MBP).

MDP (mammosphere differentiation-potential): used to assess the bilineage differentiation abilities of individual mammospheres on collagen-coated plates (two-dimensional differentiation). Calculated as the number of bilineage spheres/well divided by the total-number of spheres/well (expressed as a percentage). For this project MDP was only

evaluated on secondary mammospheres.

MFE (mammosphere-forming efficiency): used to assess the mammosphere forming abilities of single cells plated under mammosphere culture conditions. Calculated as the average number of mammospheres (≥ 40 μm in diameter) formed in each well divided by the number of viable cells initially plated per well (expressed as percentage). For this project MFE was evaluated on cells obtained directly from the dissociation of mammary gland tissues (1^{o} MFE) as well as from cells dissociated from primary mammospheres (2^{o} MFE), secondary mammospheres (3^{o} MFE), and tertiary mammospheres (4^{o} MFE).

TVC (total number of viable cells/mg of tissue): used to assess the number of viable cells obtained from the digestion and dissociation of mammary gland tissues and mammospheres. Calculated using the trypan blue exclusion assay as observed using a hemocytometer.

VCR (viable cell recovery): used to assess the number of cells recovered from the dissociation of intact mammospheres. Calculated as the total number of viable cells recovered from the dissociation of a mammosphere culture divided by the number of viable cells originally plated for that culture (expressed as a percentage). For this project VCR was utilized to quantitate the number of cells obtained from the dissociation of primary mammospheres to estimate the numbers of mammospheres produced in the 100k cultures and to compare the effectiveness of the mammosphere dissociation process at Day 7 and Day 10 of primary mammosphere growth.

Whole-gland-CMFA (numbers of cells with mammosphere forming ability in one mammary gland of individual animals): used to estimate the number of cells that are capable of forming mammospheres in mammosphere culture within one mammary gland of individual subjects. Calculated using the TVC, 1^{o} MFE, and the approximate-whole mammary gland weight for each subject.