

## Review Article

# The Role of Stem Cells in Parity Induced Protection against Breast Cancer

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## Abstract

Parity (childbearing) significantly decreases a woman's risk of breast cancer. Several factors within the mammary gland are postulated to contribute to parity-induced protection including (but not restricted to), a reduction in the number of mammary stem cells (MaSCs). This review will explore whether the protection afforded by parity is specific to certain breast cancer subtypes or is influenced by hormone receptor status. We will discuss how additional reproductive factors such as multiple births, a young age at first full term birth, and breastfeeding can also impact protection. We specifically assess whether the beneficial effects of early childbearing are mediated by changes in the MaSC population, which are thought to be abundant in the young breast. Finally we provide an update on the in-vivo work being performed in mice to directly investigate the effect of parity on MaSC and then discuss whether these findings provide evidence for the MaSCs being key mediators of parity-associated protection against breast cancer.

## ABBREVIATIONS

**MaSC:** Mammary stem cell; **ER:** estrogen receptor; **DCIS:** ductal carcinoma in-situ; **LCIS;** lobular carcinoma in-situ; **RR;** relative risk; **TEB:** terminal end bud.

## INTRODUCTION

In a passing comment in his book "The diseases of workers", the father of occupational medicine, Italian physician Bernardino Ramazzini, stated that nuns had a higher risk of breast cancer than the rest of the population. This comment, made in 1714, has become arguably the most important epidemiological finding relating to breast cancer risk to date. It would remain an anecdotal observation until large case controlled epidemiological studies performed in the 19<sup>th</sup> century validated nulliparity, or a lack of childbearing, as a risk factor for the development of breast cancer. Women who had children were shown to have a reduction in breast cancer risk, and thus deemed protected [1,2]. A previous epidemiological study in 1926 correlated breast cancer risk and reproductive history in a cohort of women from the UK and found that early age at marriage (which at the time correlated well with age at first birth), increasing number of births and breastfeeding for longer than 12 months were all associated with a lower risk of breast cancer [3]. This study was repeated using a US cohort and found similar associations [4]. Recently both studies were

reassessed using modern statistical methods, which validated the findings of the earlier studies [5]. This review will explore the evidence for parity-associated protection against breast cancer and if it is specific to a certain stage or subtype of cancer. We will assess whether parity protects against initiation, progression or metastasis and also whether it protects against all cancer subtypes or only the estrogen driven tumours. In addition to parity protecting against breast cancer, the age at which a woman bears her first child significantly impacts on her protection. We will discuss why parity associated protection is stronger in younger mothers and the relationship that MaSCs may play in mediating this.

## Does parity reduce breast cancer risk and is it specific to certain subtypes of cancer?

Even though the protective effect of parity has been known for over half a century, relatively little is known about whether it protects against initiation and/or progression. In order to ascertain whether parity protects against initiation or progression of breast cancer we will begin by reviewing whether parity protection has been shown to be specific to the development of early lesions, the progression of breast cancer to invasive lesions and or metastasis. As pregnancy is associated with alterations in steroid hormone levels, we will also assess whether protection is specific to hormonally regulated breast cancers.

## Current methods for classifying histological grade, metastatic burden and molecular subtype of breast cancer

Histological grading of breast cancer ranges from 0 (non-invasive or *in situ*) to IV (metastatic). *In situ* carcinomas can be either lobular (LCIS) or ductal (DCIS), with the latter being more common. DCIS can be further subdivided into comedo, cribriform, micropapillary, papillary and solid types [6]. Invasive carcinomas can take the form of infiltrating ductal, invasive lobular, mucinous, tubular, medullary and papillary as well as ductal/lobular histological types. Infiltrating ductal (IDC) accounts for 70-80% of breast cancer [7] and is sub-divided into grades I, II and III depending on differentiation status which is assessed by mitotic index and nuclear pleomorphism (that is, the size and shape of the nucleus) [8]. Breast cancer is also classified according to the expression of hormone receptors, estrogen receptor (ER) and progesterone receptor (PR), and growth factor receptors to ascertain the suitability of particular adjuvant therapies. Patients who are ER<sup>+</sup>PR<sup>+</sup> positive are much more likely to respond to the ER antagonist Tamoxifen than those who are ER<sup>+</sup>PR<sup>-</sup> [9]. Tamoxifen has been shown to halve annual recurrence of ER<sup>+</sup> breast cancer and reduce mortality rate by a third [10], thus patients responding to this treatment are associated with more favourable outcomes.

## Epidemiological evidence suggests parity is not specific to histological subtype

Most epidemiological studies correlating parity with breast cancer generalize to overall incidence although some studies have determined whether parity protects against a particular grade or molecular subtype of breast cancer. A number of case-control studies of varying sizes and across different ethnicities have been completed to assess whether the protective effect of parity was similar for DCIS as well as invasive breast cancer [11-18]. Collectively, these studies show a decrease in breast cancer risk with increasing parity for both DCIS and invasive breast cancer, and that parity-associated protection was not restricted to either [11,12,15-18]. Similarly, a number of these studies showed that increased age at first full term birth, correlated with increasing breast cancer risk for both DCIS and invasive breast cancer [13,14,16-18]. However, this was not the case for all studies with Trentham-Dietz and associates showing that increased age at first birth affected only invasive breast cancer risk [15]. As it has been suggested that DCIS is a precursor for invasive breast cancer [16,19], these studies indicate that parity is able to protect against the early stages of breast cancer development and thus also prevent the incidence of invasive breast cancer.

In addition to assessing the effects of parity on the histology and grade of breast cancer, some studies have reported an increase in local metastasis (axillary lymph node) with increasing births [20-23]. This is in contrast to the observation that multiple births decrease the overall relative risk of breast cancer, as will be discussed in further detail below. However, these studies are limited by small samples size and papers reporting on larger cohorts of breast cancer patients have not been able to validate these findings [24-26]. Larger studies with multivariate analysis, which allows the relationships of age and tumour size to be corrected for, are required. It would also be informative to see if the increase in node involvement in parous women is restricted

to pregnancy associated breast cancers (immediately after pregnancy), which are known to be of poorer prognosis than non-pregnancy associated breast cancers. There is no conclusive evidence to our knowledge that parity specifically decreases the metastatic capability of breast cancers.

## Parity may protect only against ER positive breast cancers

A number of studies have determined if parity protection is dependent on hormone receptor status and have collectively found that parity protection is restricted to ER<sup>+</sup> cancers. In 2004, Althuis and colleagues reviewed 12 epidemiological studies that had reported hormone receptor status among their cases and controls and found nulliparity to be associated only with the risk of developing ER<sup>+</sup> cancer [27] which represent approximately 75% of all breast cancers. Similarly, Ma et al., found that parity protection was restricted to ER<sup>+</sup>PR<sup>+</sup> breast cancer, based on analysis from eight studies published between 1995 and 2005 (5 of which were also included in the Althuis review) [28]. Bao and associates also found that parity protection was restricted to ER<sup>+</sup>PR<sup>+</sup> breast cancers when compared to ER<sup>+</sup>PR<sup>-</sup> subtypes, but only in postmenopausal cases [29].

## Additional reproductive factors influence parity-associated protection

To deduce the mechanisms of parity associated protection against breast cancer, several studies have assessed the impact of additional reproductive factors such as multiple births, duration of breastfeeding and birth spacing. By exploring these different aspects of reproduction and how they modulate breast cancer risk, the mechanisms of protection, such as changes to the architecture of the breast and duration of pregnancy hormone exposure, could be identified. Ever breastfeeding versus never breastfeeding has been shown consistently to reduce breast cancer risk [12,30,31], and an increased duration of breastfeeding offers more protection [30-34]. In fact, the reduction in breast cancer risk found to be offered by breastfeeding is 4.3-4.5% for every 12 months of breastfeeding [35,36], which, unlike childbearing itself, does not appear to be influenced by hormone receptor status [12,37]. In these studies it was also found that the more cumulative breastfeeding a mother performed over her lifetime, the lower the risk of breast cancer, which is consistent with findings showing that an increasing number of births further reduces breast cancer risk [38-40]. Lambe et. al. demonstrated that the reduction in risk of breast cancer was approximately 10% for each additional full-term birth [41] and more recently Tamakoshi and associates showed that in women who had 4 or more pregnancies, their risk of breast cancer was reduced by 66% (compared to that of single pregnancy) [42]. The spacing between pregnancies also modulates breast cancer risk. In 2009, a study showed that a 1-3 year interval between successive births significantly increased the number of cases of breast cancer compared to intervals of less than one year and greater than 3 years [43]. Whilst this is an intriguing finding, the underlying mechanism is not at all obvious.

## The young mammary gland is particularly sensitive to reproductive events

Cumulatively, these additional modulators of breast cancer risk indicate that the exposure to steroid hormones may play a

role in reducing the risk of breast cancer. As menses is suspended for the duration of pregnancy and lactation, it can be inferred that the more full-term pregnancies experienced, the longer the period of breastfeeding and the shorter the interval between births, the less menses a woman will complete and therefore the less time that the mammary gland is exposed to fluctuating steroid hormones. This is reinforced by studies showing that a later age at menarche and earlier age of menopause is also associated with a decrease in breast cancer risk [5,31,34,44]. Of particular interest in terms of parity is the observation of a later age at menarche resulting in a decrease in breast cancer risk. Like a late menarche, the earlier a woman has her first child the more protected she is against breast cancer. A first full term birth before the age of 20 reduces the risk of breast cancer by 50% compared to nulliparous women [1]. This has been validated by numerous studies over the past three decades [32,45-48]. Therefore it is clear that the timing of the influence on growth and development of the mammary gland by steroid hormones is important and adolescence represents a critical time point in programming developmental consequences within the mammary gland.

### **Why is the young breast susceptible to both cancer promoting and protective events?**

The young breast is thought to be highly sensitive to cancer promoting and protective events. Epidemiological evidence exists for an increase in the risk of breast cancer following carcinogen exposure during adolescence, and numerous experimental studies in animals investigating breast cancer risk following administration of a carcinogen at different time points support these findings.

### **Epidemiological studies show the young breast is particularly at risk of carcinogenic insult**

The epidemiological dataset that has provided the strongest support for the young breast being particularly carcinogen sensitive is the Hiroshima and Nagasaki atomic bomb survivors. Numerous investigators have assessed this cohort, collectively referred as the Life Span Study (LSS), for their risk of breast cancer. They have stratified the cohort based on sex, age at exposure, age at diagnosis, radiation dose and reproductive history [49-52]. Consistently, the studies show an increase in excess relative risk of breast cancer with decreasing age at exposure, with women who were less than 20 years of age far more likely to develop breast cancer than those exposed after 20 years of age [52,53]. Exposure to medical radiation in adolescence has also been associated with breast cancer risk. X-ray exposure in adolescence to monitor scoliosis increased breast cancer incidence later in life [54]. Similarly, the relative risk (RR) of breast cancer following x-ray examination of tuberculosis patients was highest in those women treated between 15 and 19 years of age (RR= 2.26 compared to 0.76 for women 30 to 39 years of age) [55]. The increased risk of breast cancer due to radiation exposure to treat Hodgkin's lymphoma was highest in women receiving radiation before the age of 20 [56]. However, in this study the cases were more likely to have a family history of benign breast disease, an earlier menarche, higher nulliparity, older age at first full-term birth and shorter duration of breast feeding compared to controls. As mentioned above, all of these factors contribute to

an increase in breast cancer risk, which raises concerns over the validity of these results. Another study assessed breast cancer risk following radiation treatment for Hodgkin's lymphoma and found that girls exposed to radiation between 10 and 16 years of age had a higher risk of cancer than those exposed under 10 years of age (using COX regression models) [57]. When they re-analysed the same cohort using Poisson's regression, which takes into account the age specific increase in breast cancer risk that all women experience, they found no statistically significant increase in risk in exposure during this time [58]. Furthermore, no reproductive histories were provided for cases or controls in either analysis and thus one must assume that the data were not controlled for these variables on breast cancer risk. Similar inconsistencies in the contribution of age of radiation exposure to breast cancer risk was observed in two other case-control studies looking at the risk of breast cancer to the contralateral breast following radiation therapy for primary breast cancer. An assessment of a cohort from Connecticut found that the risk of breast cancer in the contralateral breast was higher in women treated for breast cancer before 45 years of age. However, in a cohort from Denmark of similar size, there was no effect of age at exposure on risk of breast cancer in the contralateral breast [59,60]. Base-line incidence, mortality and 5-year survival rates for breast cancer are similar between US and Danish women [61] and both studies used similar radiation doses to the contralateral breast in their subjects and matched their cases and controls for cancer grade. Additionally they both used conditional logistical regression to analyse their data sets. The Danish study did have a lower representation of women under 45 years of age at diagnosis, as well as women with regional spread and carcinoma, which differs from the adenocarcinomas and infiltrating breast cancers observed in the US study. Whether this contributed to the differences in risk assessment is uncertain. In addition to the studies of radiation exposure and breast cancer risk, one epidemiological study has assessed the risk of breast cancer following exposure to a chemical carcinogen, Dichlorodiphenyltrichloroethane (DTT). Fifty years after exposure to DTT, it was found that women were 5 times more likely to develop breast cancer than the average population, but only if they were exposed before 14 years of age [62].

### **Experimental evidence in rodents of a critical period of increased carcinogenic vulnerability in the mammary gland**

Experimental exposure to radiation and chemical carcinogens in rodents has shown a similar relationship between age of exposure and risk of breast cancer development. The rodent is a good model system for the assessment of carcinogen exposure on the mammary gland as the glandular architecture is similar to that of the human [63] and following exposure to carcinogens, the histology of malignant lesions largely mirrors that of the human [64,65]. A period of increased sensitivity to carcinogen-induced mammary carcinogenesis was identified as 7 to 55 days following radiation or the administration of a chemical carcinogen 7,12-Dimethylbenz(a)anthracene (DMBA) [66,67]. As rats usually undergo puberty around 45 days of age, both studies indicate that the period of increased susceptibility to carcinogens occurs during puberty. In an attempt to explore this period of



susceptibility, the Russo group characterized the development of the mammary gland during this time and identified terminal end buds (TEBs), club-like structures on the leading end of a growing epithelial duct, were at their highest frequency at 20 days of age [67,68]. TEB numbers then gradually decreased until 46 days, when they dramatically decrease due to the onset of estrogen signalling from the ovary, causing differentiation into terminal ducts (TDs). Following administration of DMBA during puberty, instead of differentiating into TDs, the TEBs undergo hyperproliferation and develop into adenocarcinomas [68,69].

### Do stem cells mediate the increased susceptibility to carcinogens experienced during puberty?

The overlap of the period of increased susceptibility to carcinogen insult and high TEB frequency has led to speculation that stem cells may account for the increased susceptibility of the young breast/mammary gland. Early morphological analysis of the developing mammary gland suggested MaSCs were found in the TEBs [70], and more recent studies using transgenic approaches to label stem cells have supported this [71,72]. However, as yet, no direct evidence correlating high TEB frequency and high MaSC numbers has been provided. Furthermore there is little evidence to suggest that MaSCs are particularly more sensitive to carcinogenic stress than other more differentiated cells, although again, this has not yet been investigated extensively.

### Experimental evidence of TEBs housing MaSCs

A detailed assessment of the cellular morphology of the TEB identified the outermost layer of the TEB as being comprised of 'cap cells' which, due to their cuboidal structure, nuclear morphology and lack of cellular adhesions, appeared to be stem cells [70]. Cap cells may also give rise to both myoepithelial cells (due to their close proximity to this cell layer) and luminal cells (an explanation as to the purpose of the cap cells migrating into the medulla of the TEB), giving functional validation to their identity as stem cells. The location of the stem cells in the TEBs has been confirmed in more recent studies that have utilized transgenic mouse models to mark expression of known stem cell markers. Bai and associates [71] generated a transgenic mouse where GFP was expressed under the promoter of *s-SHIP*, whose expression in the hematopoietic system is restricted to putative stem cells [73]. In the mammary gland *s-SHIP-GFP* was first observed in these mice at 4 weeks of age in the outer layer of TEBs, and was then lost in the post-pubertal mammary gland only to re-appear in the alveolar buds during the early stages of pregnancy, no doubt to contribute to the extensive expansion of glandular tissue required at this time [71]. Importantly, this GFP positive population represented a subset of the basal  $\text{Lin}^-\text{CD24}^+\text{CD49}^{\text{fhi}}$  population, which has been shown in various studies to contain the MaSC [74,75]. Similar results were shown in a study using the histone 2B-GFP (H2b-GFP) gene under the control of the mammary gland basal marker Keratin 5 (K5) [76]. Briefly, the H2B-GFP gene is regulated by a tetracycline responsive element (TRE) as well as a tetracycline controlled transcription activator (tTA) which itself is under control of the K5 promoter. Thus wherever K5 is expressed, the tTA can bind to the TRE and allow transcription of the H2B-GFP fusion gene. When the synthetic form of tetracycline, doxycycline, is administered, the tTA can

no longer bind to the TRE which turns off transcription of the H2B-GFP and thus the GFP signal is lost over time by dilution of the protein into cell progeny. In this study, 3 week old mice were treated with doxycycline for 12 weeks and assessed for GFP expression [72]. Not surprisingly, GFP+ cells were restricted to the tips of TEBs after 12 weeks of doxycycline treatment.

### Stem cells in hematopoietic system and skin are resistant to carcinogenic insult and repair their DNA using error-prone methods

Whilst support exists for TEBs housing MaSCs, at least during puberty, the correlation between high TEB frequency and stem cell number has never been directly assessed, and thus the theory connecting high stem cell numbers and increased carcinogen sensitivity during puberty remains speculative. However one cannot rule out the stem cells as being the cause of increased susceptibility of carcinogenesis. In the hematopoietic system, conflicting results exist on the sensitivity of hematopoietic stem cells to radiation-dependent apoptosis [77-81], and the result appears to be dependent on the specific cell subset being analysed [79,80,82]. One study that compared the radiosensitivity of hematopoietic stem and progenitor cells (HSPCs) to that of common myeloid and granulocyte progenitors found that HSPCs were more resistant to radiation-induced apoptosis and had fewer  $\gamma\text{H2AX}$  foci, indicating fewer DNA double-stranded breaks [79]. Furthermore, HSPCs were more likely to repair DNA double-strand breaks induced by radiation with non-homologous end joining (NHEJ), known to be error-prone [83,84]. Hair follicle stem cells have also been shown to be resistant to radiation-induced apoptosis and, similar to the HSPCs, repaired their DNA damage by NHEJ [85]. This indicates that while the stem cells in other organ systems are less vulnerable to radiation-induced apoptosis, their mechanism of evading elimination results in error-prone DNA repair that may lead to the generation of oncogenic gene translocations.

### Evidence to support MaSCs as being more sensitive to carcinogenesis is controversial

A limited number of studies have been completed in the mammary gland to ascertain whether MaSC are more or less prone to DNA damage compared to more mature progeny. MaSCs characterized by  $\text{Lin}^-\text{CD24}^+\text{CD49}^{\text{fhi}}$  expression have lower levels of reactive-oxygen species (ROS) than progenitors [86], which would suggest that they are less likely to be targets of the deleterious effects on DNA by ROS [87]. Two studies have found that MaSCs do not exhibit more DNA damage than progenitors as measured by  $\gamma\text{H2AX}$  staining following radiation exposure [88,89]. Despite this, Woodward and associates showed a decrease in MaSC numbers following radiation exposure, while Insinga and colleagues actually saw an expansion of MaSCs. The two studies used different methods to isolate MaSCs (FACS and cell surface markers vs label-retention), but a third study that used the same isolation method as Woodward and associates (FACS and cell surface markers) also found an increase in MaSCs following radiation exposure [90], so the discrepancies in the findings are not due to isolation methods.

Whilst Woodward and associates did not see an increase in MaSCs, they did observe an increase in progenitor cells, shown

previously to be Sca-1 positive [91,92], following radiation exposure. Sca-1<sup>+</sup> progenitors isolated from the iCommaDβgeo cell line (an immortalized pre-neoplastic mammary cell line with epithelial stem/progenitor characteristics) have since been shown to be radioresistant, exhibit no change in mammosphere forming ability and no increase in γH2AX staining compared to more putative cells, however this time no expansion in progenitor numbers was observed [93]. These results indicate that it is the progenitors in the mammary gland that are more resistant to carcinogenic insult, however as yet no further analysis has been reported in relation to whether their resistance is conferred through error-prone DNA repair mechanisms, as has been shown in the hematopoietic system.

### Does parity lead to a decrease in stem cell number or function?

There have been significant advances in MaSC isolation over the past decade. The use of cell surface markers and FACS have allowed investigators to assess stem cell characteristics on a more purified population and begin to ask questions about what regulates their numbers and activity. As mentioned above, MaSC are thought to be enriched in the basal population of Lin<sup>-</sup>CD24<sup>+</sup>CD29<sup>hi</sup> or Lin<sup>-</sup>CD24<sup>+</sup>CD49<sup>hi</sup> (CD29 and CD49f are used interchangeably as these represent similar populations). Single cells from this subset can re-epithelialize cleared mammary fat pads and have serial transplantation capability [74]. CD61 can separate the luminal progenitors (CD61<sup>+</sup>) from the mature luminal cells (CD61<sup>-</sup>), whilst Sca-1 and c-kit in combination can further differentiate ER<sup>+</sup> mature luminal cells (CD24<sup>+</sup>Sca-1<sup>+/-</sup>c-kit<sup>-</sup>) from ER<sup>+</sup> (CD24<sup>+</sup>Sca-1<sup>+</sup>c-kit<sup>+</sup>) and ER<sup>-</sup> (CD24<sup>+</sup>Sca-1<sup>-</sup>c-kit<sup>+</sup>) luminal progenitors within the CD24<sup>+</sup> population [92]. Some of the studies investigating the effects of parity on MaSC number and function were undertaken whilst the above markers were being validated and so the early studies were more restricted in their isolation methods. However, the emerging studies in the area are now using these markers to assess the impact of parity on MaSCs and have largely found similar results.

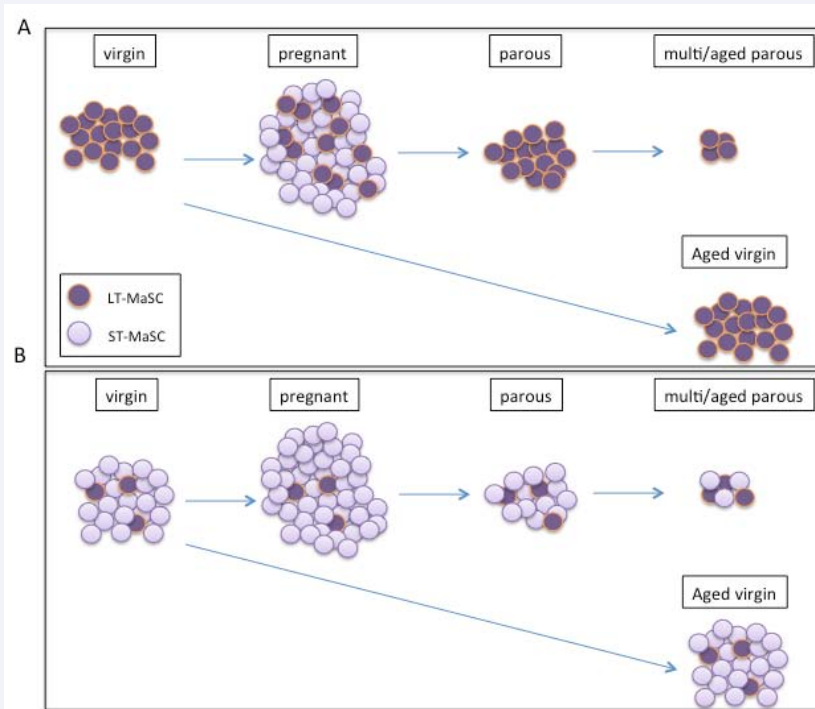
### Early but not late parity decreases MaSC numbers

There have been four attempts to determine if parity leads to a decrease in MaSC number or activity. From these studies, it appears that early, but not late pregnancy can reduce the number of MaSC [94-97], thereby supporting the epidemiological findings of early pregnancy being the most protective reproductive factor against breast cancer. The studies that assessed stem cell activity in animals experiencing a late pregnancy found no change in MaSC activity [95,96] but they assessed mice that were mated at 9-10wks of age. At this age, mice are only 3 weeks post sexual maturity, and so could still be considered quite young. To mimic the effects of older pregnancies in women, mice should be mated at ages closer to the decline of reproductive function. The other limitation of these studies was the use of non-fractionated mammary glands [96] or epithelial cell enriched fractions [95] rather than isolated MaSCs. Two studies that used young mice (5-6weeks of age) showed a decrease in MaSC activity [94,97]. However the contribution to this decrease by non stem cells cannot be determined since the studies used either non-fractionated mammary gland [94] or MaSC enriched fractions

in combination with Matrigel, which affords stem cell activity to a non stem cell population [98]. Thus the effect of parity on MaSCs will remain elusive until a direct comparison of MaSC number and activity is made from mice undergoing early and late pregnancy using the same method of MaSC isolation. In addition to completing limiting dilution mammary stem cell transplants with these isolated cells (in the absence of matrigel) serial transplants are required.

### Assessment of MaSCs during reproductive cycles provide evidence for short- and long-term MaSCs

In addition to these transplant studies, which are considered the gold standard for MaSC analysis, a recent lineage tracing study claimed that a population of label-retaining cells with high MaSC activity is completely depleted during pregnancy [72]. This finding is unexpected given what we know about the mammary gland and its ability to regenerate epithelial structures required for successive pregnancies, which would imply that some cells with repopulating potential must survive the involution process. One explanation could be that they are marking short-term stem cells, which are induced to differentiate at early pregnancy and then lose their characteristic stem cell marker expression. This would assume that their remains a pool of long term MaSC, which acts as a reservoir to facilitate the differentiation and repopulation required in successive pregnancies. The idea of short- and long-term stem cells in the mammary gland is supported by studies assessing MaSC number and activity during pregnancy. Asselin-labat and colleagues reported a 24-fold increase in the number of Lin<sup>-</sup>CD24<sup>+</sup>CD29<sup>hi</sup> cells at day 12.5 of pregnancy, which they confirmed as MaSCs by demonstrating mammary repopulation activity in primary fat pad transplants. However, this expanded subset of cells had limited serial transplantability [99]. Furthermore, a transgenic mouse that expresses luciferase and GFP under the control of the constitutive CMV/β-actin promoter was used to assess MaSC numbers over the course of pregnancy and lactation using flow cytometry. A similar expansion of MaSCs was reported, which was not as extensive during a second round of pregnancy in the same mice [100]. MaSC are also expanded during the reproductive cycle. MaSC are increased during the diestrus stage of the estrus cycle of the mouse [101]. The diestrus stage is characterized by high serum progesterone levels and elevated estrogen levels [102-104], which may underlie the changes. To confirm this, ovariectomized mice were treated with steroid hormones, resulting in combined estrogen and progesterone treatment, but neither alone induced the same changes observed in diestrus [101]. However, to discern if this is the same population of MaSC expanded at pregnancy, serial transplants need to be completed. Should these MaSC, expanded at diestrus, show similar regenerative restrictions as those increased at pregnancy, these studies would provide collective evidence for the presence of hormone sensitive short-term MaSC within the mammary gland. Whether these short-term MaSC arise from more primitive long-term MaSC in response to steroid hormones, and are then removed during involution (Figure 1a), or whether they exist pre-programmed to their specific fate alongside long-term MaSCs (Figure 1b) is unknown. In addition, it is unclear if parity is conferring protection by reducing one or both of these cell subsets. The answers to these issues may



**Figure 1** Two theories on the origins of long term (LT) and short term (ST) MaSC through out reproductive cycle. (A) Long-term MaSC differentiate into short-term stem cells which expand modestly at diestrus and rapidly with pregnancy, but these short-term MaSCs are exhausted and removed at involution. Over time or successive pregnancies, more long-term MaSCs are induced to differentiate into the short-term stem cells thus reducing the overall pool of long-term stem cells also. (B) Short-term MaSCs and long-term MaSCs co-exist, short-term MaSCs contributing to expansion at diestrus and pregnancy, long-term MaSCs required as an emergency back up only. Overtime, only the short-term MaSC reduce in numbers, and long-term MaSC numbers remain stable.

provide information on the mechanisms by which MaSCs can influence the risk of breast cancer.

### Parity-identified mammary epithelial cells are long-term repopulating cells but not true stem cells

Another subset of cells influenced by parity are long-term alveolar progenitors. These cells known as parity-identified mammary epithelial cells (PI-MECs) and were identified using a *Lox/Cre/LacZ* system where Cre recombinase was under the control of whey acidic protein (WAP) that is expressed late in pregnancy [105] and considered a marker of differentiation. When the WAP is expressed, the Cre recombinase removes the lox-flanked stop codon upstream of LacZ, allowing for the expression of LacZ in the cell and their progeny. The primary function of PI-MECs is to contribute to alveologenesis in consecutive pregnancies [106] and they can be found within the parous mammary gland for up to one year [107]. These cells differ from the short term MaSCs identified by Asselin-Labat and colleagues [99], not only in their ability to contribute to further alveologenesis, but by their contribution to luminal and myoepithelial lineages in secondary mammary fat pad transplant assays [108]. Although able to self-renew and avoid apoptosis, these cells are not true stem cells, since they are unable to give rise to all the mammary epithelial cell lineages. The degree to which they contribute to mammary gland expansion with successive pregnancies will be important in understanding the rate of turnover and use of more primitive stem cells in the mature organ.

### Parity-dependent lineage skewing may explain decrease in breast cancer risk

Instead of decreasing MaSCs, is it possible that parity skews the lineage differentiation to favour progeny with less tumorigenic potential. In human mammary epithelial cells, a decrease occurs in myoepithelial cells with an increase in luminal progenitors, indicating a preferential skewing of lineage differentiation [109]. The authors propose that this underpins the increased breast cancer risk with age as luminal progenitors with increasing age have been implicated as a cancer cell of origin in some cancers [110]. This speculation is justified given data from the hematopoietic system. With age there is an increase in myeloid derived cells and a decrease in cells of lymphoid origin [111-113]. The basis for changes in lineage differentiation is proposed to be due to changes in the hematopoietic microenvironment. Decreases in expression of adhesion molecules, decrease in bone mass and an increase in adipose tissue could all contribute (reviewed in [114]). One study found that aged HSC had an increase in methylation at genomic regions associated with lymphoid and erythrocyte lineage specificity, which was leading to this observed lineage skewing [115]. The promotion of differentiation into cells of a myeloid lineage may underlie the decrease in adaptive immunity and the increase in myeloid cancers with age [116,117]. It will be interesting to see if further work in the mammary gland can validate the findings of Garbe and associates and that MaSC share this property with stem cells from other compartments.



## Parity may not reduce the risk of cancer through direct effects on MaSC

It is possible that the decreased risk of breast cancer in parous women is not due to stem cells, but to changes in the number of ER+ mature luminal cells [118]. Support for this idea comes from research using flow cytometry and immunohistochemistry to show that CD24+ Sca-1+ cells (ER+ luminal cells) are decreased in parous mice [97]. While not implicated as cancer cells of origin, Sca-1 positivity confers radio-resistance [89,93] and identifies tumour-initiating cells in BalbC-neuT mammary tumours [119]. However, it is not clear if these are luminal cells, as the studies were performed on isolated Sca-1+ total epithelial or Sca-1+ side-population subsets. The decrease in ER+ luminal cells may be directly responsible for the decrease in breast cancer risk, or it may induce these effects via the MaSC population. The work by Meier and colleagues has shown that the decrease in ER+ luminal cells is coincident with a decrease in Wnt4 signalling [120,121], leading to a decreased proliferation potential of the parous MaSC [97]. Further studies into the effect of parity on ER+ cells may shed light on why parity protection is specific to ER+ breast cancer.

## CHALLENGES FOR THE FUTURE

There are several challenges to overcome when studying parity-associated protection against breast cancer. First, whilst CD24 and CD29/CD49f consistently enrich for cells with stem cell characteristics, the population they isolate is still heterogeneous. Advances in cell lineage tracing techniques have revealed new MaSC enriching markers, but these have yet to be validated extensively. Second, while FACs is irrefutably accepted as the optimal method for MaSC isolation, techniques to validate isolated MaSC functionally are less stringent. The gold-standard method for assessing MaSC function is the mammary fat pad assay to measure repopulation capacity. This method needs to be used correctly by completing multiple transplants at several cell dilutions, followed by serial transplantation. However this assay necessitates the dissociation of the MaSC from their microenvironment, which in other systems, is known to play a critical role in stem cell maintenance. Furthermore the injection of isolated cells into a cleared fat pad may stimulate cells to repopulate the glandular architecture, when they would have otherwise remained quiescent or showed little repopulating activity [122]. Another common method used to assess MaSC repopulation is the colony-forming assay or mammosphere assay. As these are *in vitro* assays, completely devoid of normal vascularization or systemic hormonal and growth factor influence, any results from such studies must be interpreted with caution. Advances in 3D culture systems such as those introduced by Tanos and associates [123] that allow the preservation of intact tissue architecture *ex-vivo* and maintain complex signalling relationships otherwise interrupted in normal 3D methods, may prove to be a superior model to assess the relationship of different mammary cell populations following extrinsic or intrinsic stimuli.

## CONCLUSION

Parity-associated protection against breast cancer is one of the most important reproductive factors influencing breast cancer risk. The protection afforded by parity has not been

shown to be specific to cancer subtype but it is strongest in women having their first full-time birth before 20 years of age. The time of greatest protection against breast cancer from parity appears to coincide with a period of increased susceptibility to carcinogenic insult shown by epidemiological evidence in humans and experimental evidence in rodents. There is speculation that the high number of stem cells during this time underlies the increase in risk of transformation, as they are believed to be particularly sensitive to carcinogen insult. Thus an early parity will reduce this large pool of carcinogen sensitive cells. Despite major advances in the field isolating MaSCs and evidence to show early but not late parity reduces MaSC numbers, direct evidence for MaSCs being highest in young women or rodents has not yet been provided. A more thorough assessment of MaSC numbers with age as well as carcinogen sensitivity needs to be performed in order to conclusively link MaSCs as being the mechanism of parity-associated protection against breast cancer.

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