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Breast Cancer beyond the Age of Mutation

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Abstract

Age is the greatest risk factor for breast cancer, but the reasons underlying this association are unclear. While there is undeniably a genetic component to all cancers, the accumulation of mutations with age is insufficient to explain the age-dependent increase in breast cancer incidence. In this viewpoint, we propose a multilevel framework to better understand the respective roles played by somatic mutation, microenvironment, and epigenetics making women more susceptible to breast cancer with age. The process of aging is associated with gradual breast tissue changes that not only corrupt the tumor-suppressive activity of normal tissue but also impose age-specific epigenetic changes that alter gene expression, thus reinforcing cellular phenotypes that are associated with a continuum of age-related tissue microenvironments. The evidence discussed here suggests that while the riddle of whether epigenetics drives microenvironmental changes, or whether changes in the microenvironment alter heritable cellular memory has not been solved, a path has been cleared enabling functional analysis leading to the prediction of key nodes in the network that link the microenvironment with the epigenome. The hypothesis that the accumulation of somatic mutations with age drives the age-related increase in breast cancer incidence, if correct, has a somewhat nihilistic conclusion, namely that cancers will be impossible to avoid. Alternatively, if microenvironment-driven epigenetic changes are the key to explaining susceptibility to age-related breast cancers, then there is hope that primary prevention is possible because epigenomes are relatively malleable.

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Introduction

Phenotypes of aging tend to be tissue specific. For example, with age, the skeletal muscle does not regenerate well, cognitive impairments in the brain are not uncommon, and in many epithelial tissues, including breast, there is an increased incidence of carcinomas. Indeed, more than 80% of all breast cancers in the US are diagnosed in women aged over 50 [1, 2]. Although aging is generally associated with loss of function in tissues, age-related cancers may be paradoxical examples of gains of function in that there is uncontrolled growth and the appearance of novel functions, such as invasion and metastasis [3]. A long held and dominant view has been that progressive accrual of mutations in oncogenes and tumor suppressors with age accounts for the increased cancer incidence [4]. While some cancers indeed show an expo-

nential increase in incidence with age, consistent with the accumulated mutation hypothesis, the vast majority of breast cancers are age-related, whose incidence rates slow after the age of 50 [5]. Breast cancer has a bimodal distribution with respect to age with peaks at 50 and 70 years. There is undeniably a genetic component to all cancers, but mutation alone is insufficient to explain the age-dependent increases of breast cancer incidence. What is known of aging in human breast has been mainly the domain of pathologists who utilized normal tissues as controls for breast cancer studies. In order to develop a functional understanding of the effects of aging, we have successfully used a combination of primary cell culture, bioengineering, and histology [6–8]. Based on an emerging understanding of the impact of tissue microenvironment on tumor genesis, and our approach to understanding consequences of aging in human mammary epithelia, we propose an alternate hypothesis. The increased incidence of age-related breast cancers results from the gradual loss of function changes at the level of tissue structure and organization that corrupt tumor-suppressive activity of normal tissue architecture. These changes also drive epigenetic states that alter gene expression, thereby altering normal stem and somatic cell functions. These alterations lead to tissue-level phenotypes that make breast epithelia susceptible to transformation.

In this viewpoint, we aim to summarize the theoretical background of prevailing constructs, and expand the discussion of accumulation of somatic mutation and age-dependent breast cancer incidence based on the evidence that tissue microenvironments and epigenetic states strongly influence tumor genesis.

Aging and Breast Tissue Fitness

The term 'breast cancer' represents a diverse group of diseases, which are commonly classified as either luminal A and B, triple-negative/basal-like, or *HER2*-positive subtypes based on their expression of hormone receptors, *HER2* amplification, and other biochemical and molecular markers. A full 80% of all breast cancers in women over 50 are the luminal subtypes [9]. There are no particular patterns of gene mutations in these age-related cancers, but rather they have the greatest transcriptional diversity, and their transcriptomes exhibit age-specific expression patterns [10, 11]. Increasing age correlates with shifting gene expression patterns in a number of healthy human tissues including mammary epithelia [6, 12–14], but the sources and functional consequences of

those changes are largely unknown. Age-dependent transcriptomes could be explained by mutational, epigenetic, and microenvironmental changes.

Tissue microenvironments, defined as the combinations of cell-cell, cell-ECM, and cell-soluble factor interactions surrounding each cell, exchange information with cells via a combination of physical, chemical, and electrical signals, frequently activating or deactivating the same pathways triggered by oncogenes [15–17]. Deleterious mutations can be amplified throughout a phenotypically normal epithelia, and only participate in the tumorigenic process in discrete locations where, presumably, additional deleterious events took place [18]. The influence of microenvironment can be so profound as to make frankly malignant cells behave in a phenotypically normal manner, so long as the normal tissue structure remains intact. Thus, situations that challenge normal tissue architecture could unleash predisposed cells. Steady age-related decline in breast tissue fitness may explain why luminal subtype breast cancers carry the burden of risk of recurrence as far out as 10–20 years following the initial diagnosis [19].

Aging is a gradual process, but the change to our tissues is not subtle, even from a superficial perspective. The epidemiologist Malcolm Pike suggested that 'breast tissue age' was a predictor of breast cancer risk that is distinct from chronological age [20]. This conceptually reasonable model mainly considered low-resolution changes such as hormones and childbirths; however, this model could not account for the cell- and molecular-level changes that arise with age in breast tissue. The breast consists of the gland that is a branching pseudostratified epithelium, which is surrounded by the stroma, composed of ECM, adipose cells, endothelial cells, fibroblasts, and blood cells. The vast majority of breast cancers originate in the epithelium. There are a number of systemic changes that occur in the transition into and during menopause, such as a loss of estrogen production. Hormone changes are coincident with other changes in breast tissue, such as decreased connective tissue, increased adipose, and discontinuities in the basement membrane that maintains normal polarity of the epithelium [21–23] (fig. 1). When we examined human mammary epithelia at high definition, aging was found to be associated with the accumulation of mammary epithelial progenitor cells, which are putative cells-of-origin for breast cancers, and with the presence of fewer myoepithelial cells, which can suppress malignant tumor-forming cells [6]. Thus, during the aging process, the population of cells potentially targeted for transformation is increased, and there is a

Fig. 1. A cartoon showing archetypical age-related states of breast tissue. With advanced age, there is an increase in fat and a decrease in connective tissue in the stromal that surrounds the pseudostratified mammary epithelia. The mammary epithelium is a branching, bilayered tissue, but the diagram shown is meant to convey the age-related changes that can arise at cellular resolution. With age, multipotent progenitors accumulate, luminal cells lose lineage fidelity and take on characteristics of the more basal myoepithelial cells. The proportions of tumor-suppressive and contractile myoepithelial cells themselves are also diminished with age. Reduction of myoepithelial cells might help explain putative discontinuities in the basement membrane that are thought to arise with age because those cells produce many key basement membrane components.
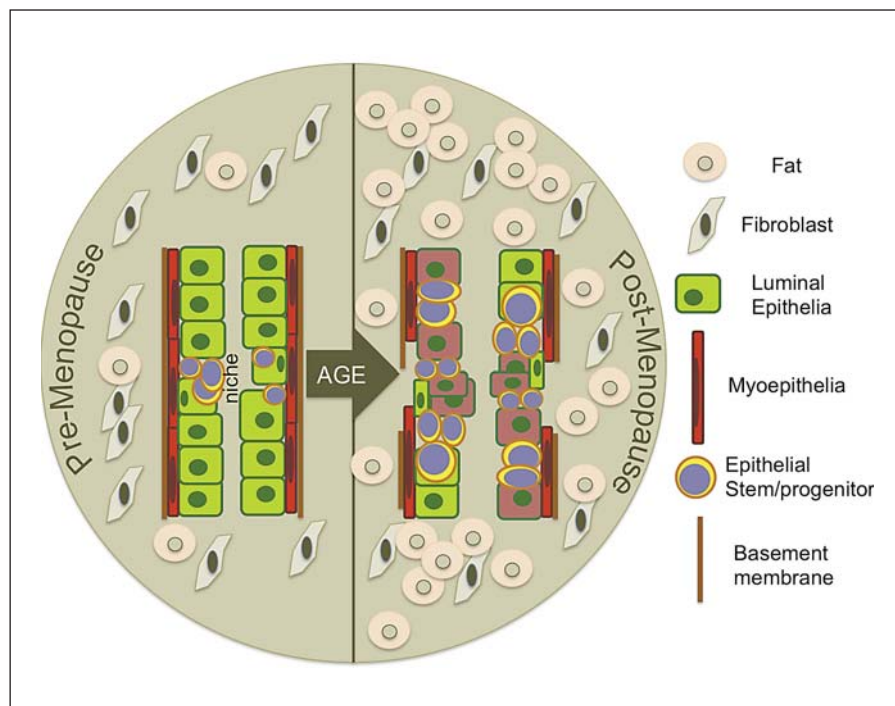
simultaneous decrease in tumor-suppressive cells, which suggests a cell- and tissue-level mechanism that leads to increased susceptibility to malignant progression. The shape of the curve that best describes the rate of these changes will require more sample accumulation, and whether there is a biological age of breast tissue that is distinct from a chronological age remains an open question.

The concept of dynamic reciprocity posits that a cell's gene expression is modified by the microenvironment, and the cells in turn modify the microenvironment still further, creating cycles of information feedback [24]. Because many microenvironment molecules, such as ECM and growth factors that tightly bind to ECM, have very long half-lives, we expect that the dynamic and reciprocal communication leads to gradual changes rather than rapid ones, which is consistent with the pace of aging. Such information cycles would explain the age dependence of tissue transcriptomes. The transcriptome changes that accompany aging essentially alter the internal wiring diagram of cells; thus, we should expect functional consequences. For most human tissues, establishing links between age-related gene expression and specific functional behaviors has not been possible, but using primary culture systems, we have had success with normal human mammary epithelial cells (HMEC). Mammary progeni-

tor cells are tasked with continually renewing the various epithelial lineages throughout a woman's adult life. By exposing these progenitor cells to a range of engineered microenvironments, we learned that after menopause, they become less sensitive to tissue biomechanical cues that otherwise induce young progenitors to differentiate into tumor-suppressive myoepithelial cells [7]. Reduced sensitivity to differentiation cues provides one explanation for the accumulation of progenitors with age and the loss of myoepithelial cells, but more broadly, the evidence that the parameters that govern conversations between cells and microenvironments change with age sheds light on how successive homeostatic states might be established in mammary epithelia. A second interesting finding was that the same mechanically activated transcription factors could be triggered in both younger and older progenitors, although at different mechanical thresholds, and they would initiate distinct differentiation responses, suggesting that the genetic information being read by the transcription factors was somehow different [7]. Maintaining healthy breast tissue requires the well-choreographed production of the correct proportions of differentiated cells, the organization of the cells into functional higher-order structures, and maintenance of communication between the epithelia and stroma. Such choreography relies on the individual cells accessing the correct

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Table 1. Summary of studies that addressed, directly or indirectly, the accumulation of somatic mutations with age in different tissues

Species	Tissue	Gene analyzed/database	Whole-genome analysis	Accumulation of somatic mutations with age	Ref.
Healthy tissues					
Mouse	Brain; liver; spleen; heart; small intestine	*lacZ*	No	Yes	31–33
Human	Blood	HPRT	No	Yes	34
Cancer tissues					
Human	Leukemia		Yes	Yes	35
	Colorectal cancer	TCGA ICGC; CLL	Yes	Yes	35
	Uterine corpus endometrial cancer		Yes	Yes	35
	Pancreatic cancer		Yes	No	35
	Ovarian adenocarcinoma	TCGA	Yes	No	35
	Breast cancer	Original sample set COSMIC	Yes	No	36

Single-locus analysis suggested that mutations do accumulate with age. The only genome-wide studies were performed in tumors where accumulation seems to be tissue specific. Leukemia and colorectal cancer show a correlation with the accumulation and exponential increase of cancer incidence with age. Uterine corpus endometrial, pancreatic, ovarian, and breast cancer do not show a correlation with the accumulation of mutations with increased age. TCGA = The Cancer Genome Atlas; ICGC = International Cancer Genome Consortium; CLL = chronic lymphocytic leukemia; COSMIC = Catalogue Of Somatic Mutations In Cancer.

information in their genomes based on cues from their microenvironment, and then responding as self-organizing collectives [25]. Based on transcriptome analyses of multiple human tissues, aging changes the internal wiring of cells, which must be traceable to corruption of the basic genetic information, resulting in tissues with suboptimal function. The prevailing dogma would suggest that genomes are corrupted by mutational changes. However age-related epigenetic states may provide a better explanation for the changes that arise in breast.

Testing the Convention: Accumulation of Somatic Mutations with Age and Cancer Development

A decline in DNA repair efficiency, accumulation of somatic mutation, cellular mosaicism, and increasing senescent cells and epigenetic alterations are thought to be hallmarks of aging [26]. The foundation of the concept that age-related cancers are caused by accumulated mutations is that aging is not an acute, but rather a time-dependent process [27, 28]. The gradual accumulation of somatic mutations is due to failures in the removal of mutations over time, which leads to the loss of the integrity and stability of the genome, so that malignant transformation occurs. This is why a large number of somatic mutations in tumors are detected [11, 28, 29], although it

is not yet known whether the genomic instability observed in tumors is a cause or a consequence of tumor development.

The available data do not show a clear picture that mutations accumulate with age (table 1). Accumulations were observed in a number of tissues using *lacZ* transgenic mouse models [30–32], and the *HPRT* (hypoxanthine phosphoribosyltransferase 1) gene on the X chromosome in clones of human T lymphocytes [33]. While these studies indicated that the accumulation of mutations with age occurs at those loci, they did not consider the phenomenon on a genome-wide scale. In addition, the accumulation of mutations in both cases was not reported to coincide with cancer incidence. Because human tissues present a number of challenges to studying the normal aging process, a common approach also has been applied to make a comparison of the number of somatic mutations among tumors from various age groups [34, 35]. If the majority of somatic mutations in cancers are accumulated during the normal aging process, then cancers from older individuals are likely to have more somatic mutations than those from younger subjects. However, it must be noted that the accumulation of mutations with age seems to occur in a tissue-specific manner. Thus, chronic lymphocytic leukemia, uterine corpus endometrial carcinoma, and colorectal cancer all have shown an association between the number of somatic mutations

with age, but pancreatic cancer did not; presumably because it is not a self-renewing tissue [34]. It is suggested therefore that the accumulation of mutations is correlated with the rate of cell proliferation rather than strictly with age. There is no correlation between age and the number of mutations in luminal-subtype breast cancers even though the breast epithelium is a self-renewing tissue [34, 35]. However, breast epithelia undergo monthly cycles of proliferation and can expand as much as ten-fold in preparation for lactation; thus, there are ample opportunities for cell proliferation, and there is a distinct window of increased breast cancer risk for several years after childbirth [36]. These data suggest that the majority of the somatic mutations in the breast and ovarian genomes are introduced after the cancer initiation step. In addition, a general shortcoming of experiments to detect accumulation of somatic mutations in tissues were performed with different individuals rather than longitudinally from the same person over time, and thus the possibility of individual genome variation cannot be ruled out.

There are a great number of mutations detected in tumors, but the majority of them, known as passenger mutations, have no deleterious effect on the cells. Multiple mutations are thought to be required for initiation and development of solid tumors [37–39], but not all tumors have a driver gene mutation [40]. Three errors – epigenetic and/or genetic errors – are minimally required to gain a malignant phenotype in the absence of passenger errors in otherwise normal HMEC [41]. Indeed, cancers appear to require much more than mutations in order to develop. Stoker et al. [42] provided a powerful demonstration of this principle when they showed that the vast majority of cells in chickens that were infected with and expressing the *v-src* oncogene did not form tumors, but that a wound-healing environment was required for the tumors to form from infected cells. Loss-of-function mutations in the gene encoding *BRCA1* (breast cancer 1, early onset) carries an 80% lifelong risk for breast and ovarian cancers. Although *BRCA1* is expressed in every tissue of the human body, there is only a slightly increased cancer incidence in other tissues, and the effect is mainly seen in breast and ovary, which exhibits the tissue-specific nature of cancer development and the ability of most tissue microenvironments to suppress cancers even in the presence of a mutated tumor suppressor [43]. Thus, genetic mutation alone is insufficient to understand tumor development in terms of aging and, at least in the breast, genetic mutations are unlikely to be the major factor that increases susceptibility to cancer with age.

Linking Microenvironments to Age-Related Epigenetic States

That normal primary HMEC isolated directly from breast tissues can be grown for multiple passages on plastic dishes in low-stress media and still retain transcriptome, biochemical, and functional phenotypes characteristic of specific lineages and chronological age that are largely consistent with in vivo [6] suggests that aging phenotypes are metastable. Metastability denotes an extended equilibrium that can be changed in an energy-dependent manner. Epigenetics provides a reasonable mechanism for biological metastability. Broadly defined, epigenetics is heritable changes in gene expression or cellular memory not encoded by the underlying DNA sequence. The major epigenetic phenomena identified are DNA methylation, chromatin remodeling, histone modification, long noncoding RNAs and microRNAs. Determination of whether epigenetics drive microenvironmental change or, rather, changes in the microenvironment alter heritable cellular memory is a classic chicken and egg situation. It is clear that perturbation of one can lead to alteration of the other. Importantly, deregulation of either the microenvironment or epigenetic states can lead to oncogenesis. There are few reports showing that microenvironments have an impact on epigenetic states. For example, mesenchymal cells placed in embryonic versus adult tissue microenvironments [44] or in tumor core versus periphery regions [45] show specific patterns of DNA and histone modifications. Moreover, patterns of histone modification and DNA methylation in carcinoma cell lines are different in 2D versus 3D cultures versus xenografts [46–48] (table 2). There is a general paucity in this area of study, perhaps due to the relatively few systems available where cells are analyzed in situ with their relevant stroma, especially in the context of chronological aging.

Microenvironment-imposed epigenetic changes that drive susceptibility to age-related breast cancer have been largely unexplored because the studies addressing the role of aging and cancer interrogate the differences between young and old patients who already have cancer, rather than focusing on changes that occur normally during aging and subsequent consequences for breast tissue function. There appears to be a role for age-related epigenetic states in breast cancer etiology, as we have shown that immortal transformation of HMEC from younger women more often results in basal subtypes seen in younger cancer patients, whereas immortalized postmenopausal HMEC exhibited the luminal subtypes that are most often seen in postmenopausal patients [49]. It will be crucial to

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Table 2. Summary of studies that have addressed effects of the microenvironment on epigenetic states or of epigenetic perturbations on microenvironments

Microenvironmental perturbation	Epigenetic effect	Ref.
Exposure of melanoma cells to embryonic microenvironment	Methylation of *Lefty B,* inhibitor of *Nodal*	44
Laser-dissected tumor captured both center and peripheral cells	Hypermethylation of *p16INK4a* in center cells only	45
Growth of E-cadherin-deficient carcinoma cells in 2D vs. 3D	Loss of E-cadherin hypermethylation in 3D	46
Growth of CP70 ovarian cancer cell line in 2D vs. 3D	Decrease in H3 acetylation, increase in H3K27 methylation in 3D	47
Growth of patient glioma stem cells in 2D vs. xenograft	Epigenetic pattern of GSCs grown in xenograft more closely mimic parental tumor	48

Epigenetic perturbation	Microenvironmental effect	Ref.
DNA methylation of *Cav-1*	Increased number of stromal cells	56
DNA methylation of *TIMP3*	Breast cancer/increased progenitor pool	59

GSCs = Glioma stem cells.

understand whether there is a connection between aging microenvironments and epigenetic states because some age-related epigenetic changes are thought to promote cancers [50].

Perhaps the longest studied epigenetic phenomenon is DNA methylation, which involves the addition of a methyl group to the cytosine pyrimidine ring of DNA. DNA methylation functions to repress transcription by inhibition of transcription factor binding and through recruitment of co-repressor complexes. Whereas global DNA methylation decreases with age, resulting in overall genomic hypomethylation, there are CpG island-containing regions that are hypermethylated with age [51]. It is well known that altering cellular microenvironments will alter gene expression patterns, causing some genes to be expressed and others to be turned off [52]. Because persistent transcriptional repression of a given CpG-containing promoter can favor increased methylation, whereas persistently active promoters favor unmethylated states [53], there is good reason to suspect that distinct microenvironments beget distinctive patterns of methylation. For example, the caveolin-1 deficient mouse model has been used as a model of accelerated aging as animals lacking the expression of the *Cav-1* gene have a significantly diminished lifespan and exhibit signs of premature aging, such as increased beta-amyloid production and neurodegeneration [54]. In the mammary gland, *Cav-1*-deficient mice exhibit increased stromal cells that can function similar to breast cancer-associated fibroblasts [55]. Indeed, loss of expression of *Cav-1*, although context-dependent, has been observed in luminal subtype

breast cancers, and several cases were attributed to DNA methylation-related silencing, suggesting that loss of *Cav-1* in the stroma promotes both aging and a tumor-permissive microenvironment [56, 57].

Extracellular metalloproteases are critical components of the mammary microenvironment that serve to facilitate the remodeling of the tissue at the interface of the stroma and the epithelium. These metalloproteases are regulated in part by tissue inhibitors of metalloproteases (TIMPs) that are expressed in both the breast stroma and epithelium [58]. It has been demonstrated that *TIMP3* is often hypermethylated in breast cancer, although the effects of this silencing appear to be context-dependent, as it is thought to both promote and prevent cancer genesis. Interestingly, when a *TIMP3* null mutation is coupled with loss of *TIMP1* in mice, they exhibit an increased pool of stem/progenitor cells in their mammary glands, even in older mice where these cells normally are in decline [59]. These results suggest that epigenetic alteration of *TIMP3* in the stroma can alter the microenvironment to provide an increased progenitor pool that, perhaps, increases the probability that molecular insults may lead to oncogenesis. It should be noted, however, that much of our understanding of the interplay between microenvironment and epigenetics is derived from mouse models, which do not fully capture the complexity of mammary gland biology observed in humans.

Taken together, these examples illustrate how epigenetic states might arise from age-related changes in the breast microenvironment. The importance of identifying the specific environmental and microenvironmental

drivers of epigenetic states cannot be understated because that information will have a broad impact well beyond understanding age-related breast cancers.

Conclusions

From our viewpoint, aging causes a gradual loss of function at the levels of the breast microenvironmental structure and tissue organizational features that normally suppress tumor formation. The tissue changes not only corrupt the tumor-suppressive activity of normal tissue, but the effects of the changes are reinforced by consequent epigenetic changes that alter gene expression. Thus, the spectrum of normal cellular functions is gradually altered in the cells that maintain tissue integrity. While the evidence discussed here suggests that the riddle of whether epigenetics drives microenvironmental changes, or whether changes in the microenvironment alter heritable cellular memory has not been solved, a path has been cleared enabling functional analysis leading to the prediction of key nodes in the network that link the microenvironment with the epigenome. The hypothesis that accumulation of somatic mutations with age drives the age-related increase in breast cancer incidence, if correct, has a somewhat nihilistic conclusion, namely that cancers will be impossible to avoid. Alternatively, if microenvironment-driven epigenetic changes are the key to explaining the tissue-level changes that make older women more susceptible to breast cancer, there is hope that primary prevention is possible. Whereas genomes are intractable to change, there is translational promise for preventing (or altering) epigenomes with chemoprevention, nutrition, stress, and exercise [60, 61].

Finally, a new tool set for dissecting the cellular and molecular mechanisms of age-related breast cancers needs to be developed, for this is a situation in which the most common models for aging research are unlikely to be useful. Yeast, flies, and worms do not have mammary glands. In addition, three relevant limitations of rodent models are (i) major tumor-suppressive barriers are absent in mice, (ii) the tumor incidence curves differ by inbred strain and do not mimic human populations, and (iii) there is a striking paucity of luminal-type mammary tumor mouse models, which is the subtype principally concerned with aging. There is some hope that genetically diverse murine cohorts will better model population-level disease patterns [62], and there may be promise for the *STAT1* knockout mouse model, which has a characteristic, slow-growing, estrogen receptor-expressing, luminal subtype [63]. Although humans are notoriously challenging experimental systems, it does not represent an intractable problem. We need to consider how to develop human model systems that can better address this issue, such as the establishment of cell culture systems, using normal healthy tissues serially collected from the same individual over a span of time. To understand age-related cancers, which represent the majority of human cancers, we need to determine conclusively the relative roles played by accumulated mutations as well as altered microenvironments and epigenetic states in different tissues. Achieving this goal will require the selfless establishment of biobanking programs that span multiple investigator generations, whose aim will be to collect tissue samples longitudinally from healthy volunteers. We believe that through literature review and a critical appraisal of the respective roles of somatic mutation, environmental and epigenetic influences, we lay out new conceptual issues that future research on breast cancer, beyond the age of mutation, needs to address.

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