Chapter 15
An Integrated Human Mammary Epithelial Cell Culture System for Studying Carcinogenesis and Aging

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Abstract Experimental examination of the agents and processes that may propel or prevent human breast carcinogenesis can be facilitated by in vitro model systems of transformation, starting with normal cells, that accurately reflect the in vivo biology. Model systems that can replicate the types of alterations seen during in vivo progression offer the potential to understand the mechanisms underlying progression and to examine possible means of individualized prevention and treatment. To this end, we have developed an experimentally tractable human mammary epithelial cell (HMEC) culture system that has been used to examine the normal processes governing HMEC growth, differentiation, aging, and senescence and how these normal processes are altered during immortal and malignant transformation. Isogenic cells at different stages of multistep carcinogenesis were generated by exposing normal finite lifespan HMEC to a variety of oncogenic agents that may play an etiologic role in breast cancer. Examination of the molecular alterations present at each stage has indicated that this model is consistent with observed multistep carcinogenesis in vivo. We have seen that varying target cell type, and oncogenic agents used, can lead to multiple distinct molecular pathways of transformation, although the full diversity of human breast cancer cell types has not yet been generated in culture models. Using this integrated system, we have formulated a comprehensive model of the proliferative barriers normal HMEC must overcome to gain immortality and malignancy. Our data provide insights on acquisition of cancer-associated properties and suggest that the most crucial step in breast cancer progression involves the transition from a finite to an indefinite lifespan. For example, we see that genomic instability originates in finite lifespan HMEC when telomeres become critically short and engage in telomeric associations and is then maintained in resultant immortalized and malignant lines. Direct genomic targeting of the tumor-suppressive

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senescence barriers can produce lines lacking gross genomic errors, supporting the hypothesis that genomic instability is a mechanism to generate cancer-causing errors, but is not necessary per se. Immortalization through telomerase reactivation was also associated with acquisition of resistance to TGFβ growth inhibition and to oncogene-induced senescence (OIS) and with large-scale changes in gene expression and epigenetic marks. Being able to examine the progressive changes that fuel malignancy, starting with normal cells, provides an integrated perspective that can reveal novel information on the origins and consequences of individual cancer-associated aberrations.

15.1 Introduction

Human breast carcinomas exhibit great diversity in phenotypic expression, correlated with differences in clinical parameters [1–4]. The factors that contribute to transforming normal breast cells to cancer, and give rise to the observed diversity of breast cancer subtypes, are currently not well defined. The interplay between initial target cell, genomic alterations that overcome tumor-suppressive barriers and confer malignancy, and cell–stromal interaction is thought to be the main variable that influence the transition of normal cells to the different malignant phenotypes.

A wealth of recent information derived from direct examination of human breast tissues is providing new insights about the pathways and alterations associated with breast carcinogenesis and the extent of inter- and intra-tumor heterogeneity [5–9]. However, determining cause and effect relationships about factors and aberrations that may propel or halt human breast carcinogenesis is constrained by the limitations of in vivo human analyses. The use of animal model systems as in vivo models of human breast cancer also has limitations. Many significant differences in processes implicated in aging and carcinogenesis exist between human cells and cells derived from commonly used rodent model systems, for example, in regulation of telomerase activity and immortalization [10] and in the roles of the cyclin-dependent kinase inhibitors (CKI) $p16^{INK4a}$ and $p14^{ARF}$ [11]. Similarly, many significant differences in biological processes exist between epithelial and mesenchymal cells, for example, responses to chemical carcinogens and TGFβ, mechanisms of senescence, and expression of miRNAs [12–17]. Since the large majority of human cancers derive from epithelial cells, we believed that a full understanding of human carcinogenesis would require the ability to examine human epithelial cells in culture. Further, in order to understand deranged human cellular processes, we believed it would be necessary to have normal cells available for comparison. We therefore developed an experimentally tractable human mammary epithelial cell (HMEC) culture system that supports vigorous growth of normal HMEC of multiple lineages and has generated isogenic cultures that range from normal, to aberrant but still finite, to nonmalignant immortal, and to malignant immortal. Our extensive integrated system allows examination of the progressive changes that fuel malignancy, starting with normal cells, thereby providing a comprehensive perspective that can

offer insight on the origins, consequences, and interactions of individual cancer-associated aberrations. This in vitro system can also complement in vivo findings by supporting experimental evaluation of factors that may promote or inhibit malignancy at different stages in progression.

This review will describe the HMEC culture system we have developed and how it has been employed to gain an integrated overview of the central processes associated with human breast carcinoma development. To place the various cell cultures generated within the context of multistep carcinogenesis, we first review our model of the tumor-suppressive senescence barriers that need to be bypassed or overcome for malignant progression in cultured HMEC to proceed.

15.2 Senescence Barriers Encountered by Cultured HMEC

Based on our studies of normal HMEC grown under different culture conditions and exposed to various oncogenic agents (see below), we have generated a new model of the tumor-suppressive senescence barriers that prevent normal cells from becoming immortally and malignantly transformed [15, 18]. Figure 15.1 outlines the generation of our various cultures with respect to growth medium, oncogenic agents employed, and the senescence barriers, and Table 15.1 compares the phenotypes of HMEC and isogenic human mammary fibroblasts (HMF) arrested at distinct senescence barriers. We observe that cultured HMEC encounter at least two mechanistically distinct barriers to indefinite proliferation, stasis (stress-associated senescence) and telomere dysfunction due to telomere attrition. Finite lifespan HMEC are also vulnerable to oncogene-induced senescence (OIS). Some HMEC may cease growth as a consequence of terminal differentiation. Importantly, the model presented here is consistent with observations of in vivo breast cancer progression. We also note that the phenotype of senescent isogenic HMF resembles that of HMEC at stasis rather than at telomere dysfunction (Table 15.1).

Stasis is a stress-associated barrier mediated by the retinoblastoma (RB) pathway and is independent of telomere length and extent of replication [15]. The onset of stasis in cultured HMEC correlates with increased expression of p16, which prevents inactivation of RB [15, 18–20]. Cells at stasis express senescence-associated β-galactosidase (SA-βGal) activity and have a senescent morphology. The number of population doublings (PD) achieved prior to stasis varies with culture conditions; we have observed a range of ~10–60 PD [15, 18, 21, 22]. Molecular correlates that can identify stasis, in addition to p16 expression, include arrest in G1, low labeling index (LI), noncritically short telomeres, and normal karyotypes (Table 15.1) [15, 18, 20]. These parameters are consistent with an RB-mediated arrest and the absence of a DNA damage response (DDR). Stasis can be bypassed or overcome in cultured HMEC by multiple types of individual alterations (genetic and/or epigenetic) in pathways governing RB and does not require loss of p53 function [19, 22–25]. Overcoming stasis may correlate with hyperplasia/atypical hyperplasia in vivo, which can display clonal growth. Errors in the RB pathway (e.g., loss of p16

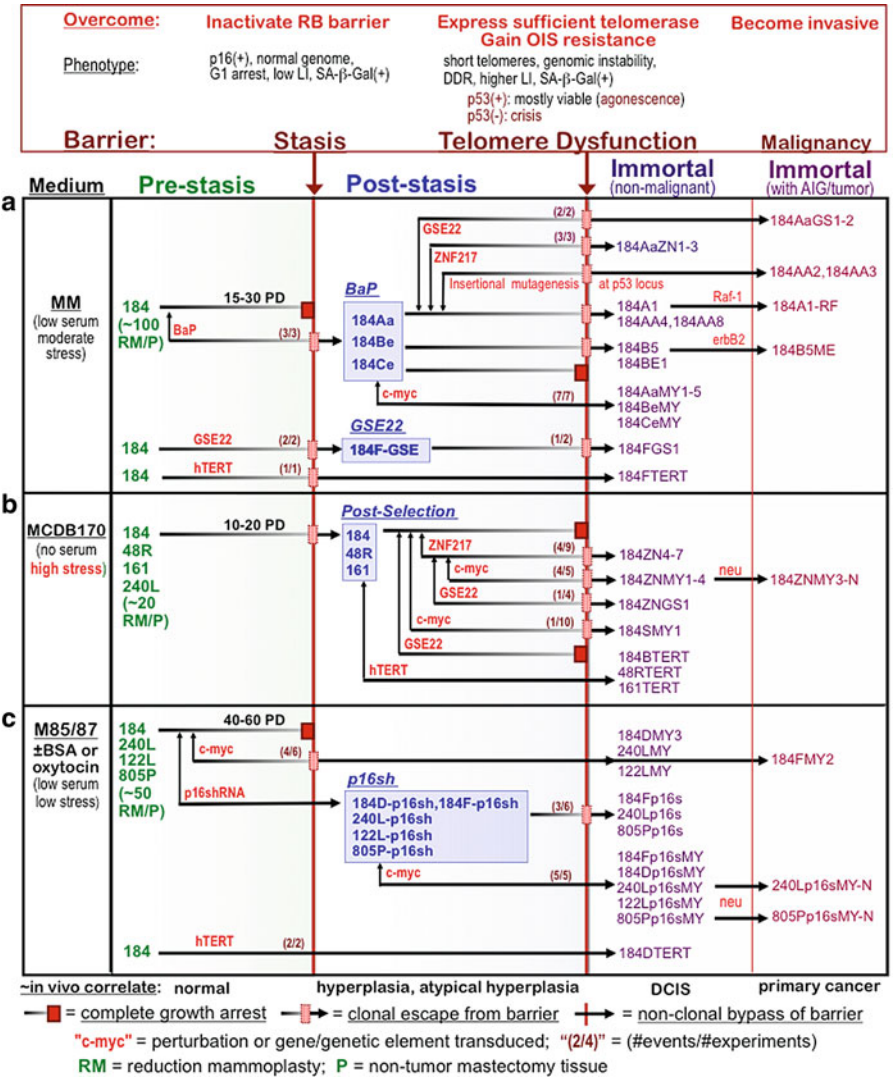

Fig. 15.1 Model of senescence barriers encountered by cultured HMEC and derivation of transformed HMEC cultures. Primary cultures obtained from reduction mammoplasties (RM) or non-tumor mastectomy tissues (P) were initiated in three different types of medium (panels **a, b, c**). All unperturbed cells grown in any serum-containing medium ceased proliferation at the stasis barrier (panels **a, c**). Exposure of pre-stasis cultures to various oncogenic insults (*red*) induced cells to overcome or bypass stasis and become post-stasis by different means. Further alterations were required to overcome the telomere dysfunction barrier, gain telomerase expression, and become immortal. Cells from post-selection post-stasis cultures all ceased proliferation at the telomere dysfunction barrier in the absence of additional oncogenic exposures (panel **b**). Nonmalignant immortal lines were no longer sensitive to OIS, and transduction of a number of different oncogenes conferred AIG (anchorage-independent growth) and/or tumorigenicity

Table 15.1 Molecular correlates of HMEC senescence barriers

Senescence Barrier	Morphology	SA-βGal	p16	p53	Arrest	LI (%)	Karyotype	Mean TRF	53BP1 foci	ser15P p53
Stasis (stress associated)	Senescent[a]	+	+	IND	Viable in G1	<2	Normal	~6–9 kb	+	∓
Telomere dysfunction: *agonescence*	Senescent	+	−	+	Mostly viable, all phases, some death	~15	Abnormal	~4–5 kb	+++	++
Telomere dysfunction: *crisis*	Growing, dying, debris	+	−	−	Eventual massive death	~40	Abnormal	<5 kb	ND	ND
OIS	Sick, dying, debris	−/?[b]	IND	IND	All phases	~15	IND	IND	ND	ND
Isogenic *fibroblast* senescence	Senescent	+	+	IND	Viable in G1	<2	Normal	~6 kb	+	−

IND senescence barrier independent of that property, *ND* not determined

[a]Pre-stasis HMEC arrested at stasis in serum-free MCDB 170 have a morphology showing abundant stress fibers

[b]Negative in pre-stasis HMEC, could not be determined in post-selection HMEC

The expression of 53BP1 foci and serine 15 phosphorylated p53 are markers of a DDR

expression by mutation or epigenetic silencing, mutated RB, overexpressed cyclin D1, mutated cdk4) are common in human carcinomas [19, 26–29]. Gross genomic aberrations are not common at this stage in vivo [30] and are not associated with overcoming stasis in vitro [15, 20].

Although neither cultured HMEC nor HMF express p21 at stasis, we postulate that stasis can also be enforced by p53-dependent p21 in response to DNA-damaging stresses such as oxidative damage or radiation. Other cell types, such as keratinocytes and foreskin fibroblasts, may be more vulnerable to DNA damage-inducing stresses in culture, express p21, and show greater evidence of a DDR at stasis. The presence of a DDR and telomeric damage foci in these cell types is not by itself evidence of senescence due to telomere erosion but can result from the decreased ability of telomeric ends to repair DNA damage [31]. HMEC in vivo may also experience p53-inducing stresses. This p53-dependent type of stasis arrest does not require critically short telomeres or genomic instability, and inactivation of p53 or p21 function may facilitate overcoming this barrier [32–34]. Reactivation of telomerase is neither necessary nor sufficient to overcome stasis; however, ectopic overexpression of hTERT prior to the onset of stasis in HMEC can bypass stasis and produce immortalization (Garbe and Stampfer, unpublished).

In post-stasis HMEC (cells that have bypassed or overcome stasis), ongoing replication in the absence of sufficient telomerase activity produces progressively shortened telomeres. Telomere dysfunction due to telomere attrition (i.e., replicative senescence) occurs when telomeres become critically short (mean TRF $\leq$ 5 kb), and uncapped telomeres elicit genomic instability and a DDR. Where wild-type p53 is present, most cells can mount a viable p53-dependent arrest; this barrier has been termed agonescence [18, 20, 35]. Karyotypic analysis of HMEC at agonescence has shown that virtually all metaphases exhibit gross chromosomal abnormalities, predominantly telomere associations [20, 36]. This result indicates that the p53-dependent senescence arrest due to telomere attrition does not occur as soon as one uncapped telomere is present [37, 38]. When p53 is nonfunctional the cells cannot mount a viable arrest, and crisis-associated massive cell death occurs [18]. Agonescence can be distinguished from stasis in HMEC by the presence of critically short telomeres and genomic instability, higher LI (~15 %), arrest at all phases of the cell cycle, and presence of a DDR (Table 15.1). HMEC at agonescence as well as at stasis display a senescent morphology and SA-βGal, so these properties do not readily distinguish between these two molecularly distinct senescence barriers. Crisis can be distinguished from agonescence by a higher LI (~40 %) and the absence of a viable arrest. Since most human epithelial and fibroblast cells induced to transform in culture had p53 function inactivated to overcome stasis (e.g., using viral oncogenes or inhibitors of p53 function), only crisis was observed in such cultures at the telomere dysfunction barrier.

The telomere dysfunction barrier can be overcome by the expression of sufficient telomerase to maintain stable telomere lengths. Overcoming telomere dysfunction may correlate with DCIS in vivo, which commonly displays short telomeres, genomic instability, and telomerase reactivation [30, 39–43].

Cultured finite lifespan HMEC are additionally vulnerable to OIS, which produces rapid growth inhibition and death [14]. The mechanism underlying OIS in HMEC is not yet fully delineated but, unlike many rodent and fibroblast cells, does not require functional p16 or p53 [14, 16]. The molecular correlates of OIS in HMEC differ from those seen in cells at stasis or telomere dysfunction and are consistent with a DDR (Table 15.1). HMEC that have attained immortality via reactivation of endogenous telomerase are no longer vulnerable to OIS and show gain of malignancy-associated properties when exposed to oncogenes such as Raf-1, Ras, or ErbB2 [14, 16, 44, 45]. HMEC immortalized by exogenous hTERT transduction retain some vulnerability to oncogene exposure [14, 46], but recent studies indicate that unlike finite HMEC they may maintain proliferative capacity [47]. Gaining resistance to OIS may be critical to acquiring malignant properties in vivo.

15.3 Normal HMEC in Culture

Normal and aberrant human mammary cells can be readily obtained from surgical discard tissues (reduction mammoplasties, mastectomies, benign tumors) and milk fluids. Most reduction mammoplasty material is from women in their late teens to early 30s, but tissues from older women are also available. These tissues show the range of age-appropriate pathologies, with increasing presence of mild to atypical hyperplasia with age. From mastectomies, non-tumor tissues are available that can provide material from older women. Peripheral mastectomy tissue is not assumed to be normal, as there may be microtumors within the tissue or field effects from the tumor or environmental exposures; similarly, tissues obtained from contralateral or prophylactic mastectomies are not considered normal. Milk fluids are valuable as a source of functionally differentiated cells. Our early studies developed methods to separate the epithelial cells from the bulk surgical tissues [48]. Epithelial organoids, free of surrounding mesenchymal tissue, were purified by enzymatic digestion, collected on filters, and stored frozen [48, 49]. The digestion process also yielded a single cell population in the filtrate from which isogenic HMF could be obtained for culture and comparison to the HMEC. Our HMEC Bank contains frozen organoids from ~300 individuals ranging in age from 11 to 91.

15.3.1 Pre-stasis Finite Lifespan HMEC

We have grown HMEC derived from reduction mammoplasties, milk, benign tumors, and non-tumor mastectomy tissues in three different types of media: serum-containing (MM and M85/M87A) or serum-free [MCDB 170 (commercial MEGM)] [15, 21, 22, 48, 50]. Depending upon the medium and culture conditions, active proliferation ceased at stasis after ~10–60 PD (Fig. 15.2). Our original medium,

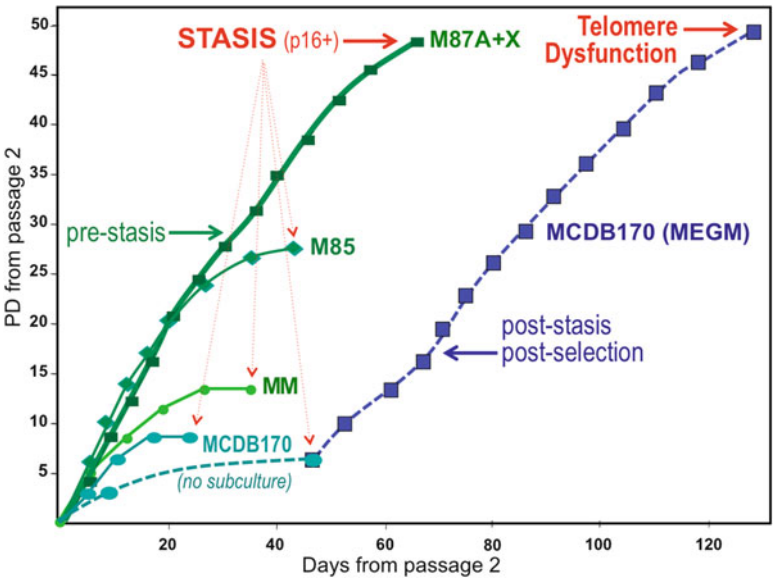

Fig. 15.2 Population doubling potential of pre-stasis HMEC in different media. Primary cultures from reduction mammoplasty specimen 184 were initiated from organoids in different media and subjected to partial trypsinizations. The number of PD in primary culture cannot be accurately determined; growth is shown starting from passage 2. Depending upon the medium, all proliferation stopped in HMEC grown in serum-containing media [MM, M85, and M87A with oxytocin (X)] after 10–50 PD beyond passage 2. The extensive proliferative potential in M87A+X supports generation of large batches of early passage pre-stasis HMEC from individual donors. HMEC initiated in serum-free MCDB170 (commercial MEGM) show rapid induction of p16 and cessation of growth. When cultures are allowed to sit without subculture for 2–3 weeks, post-selection post-stasis HMEC emerge and maintain growth to agonescence. If cultures are repeatedly subcultured, fewer to no post-selection cells may emerge

MM, supported ~15–30 PD, with HMEC with predominantly myoepithelial lineage markers present by second passage (p) [51]. Our more recent M85/M87A media will support long-term pre-stasis growth of ~60 PD. Early passage populations from reduction mammoplasty and non-tumor mastectomy tissues contain a mixture of cells with markers of myoepithelial, luminal, and progenitor lineages, but the luminal cells do not maintain active growth with long-term passage [15, 52]. HMEC grown in the serum-free MCDB 170 medium achieved only ~10–20 PD before stasis [22]. In media that support fewer PD, levels of p16 expression increase earlier. Although the mechanisms responsible for p16 induction have not been defined, it appears to reflect a cellular response to certain types of environmental stress. Virtually all cells express p16 at stasis in all media used [15, 19]. Figure 15.3a illustrates the gradually increasing p16 and SA-βGal expression and decreasing LI of HMEC growing in M85, and Fig. 15.3b, c illustrates expression of luminal lineage markers in M85-grown HMEC.

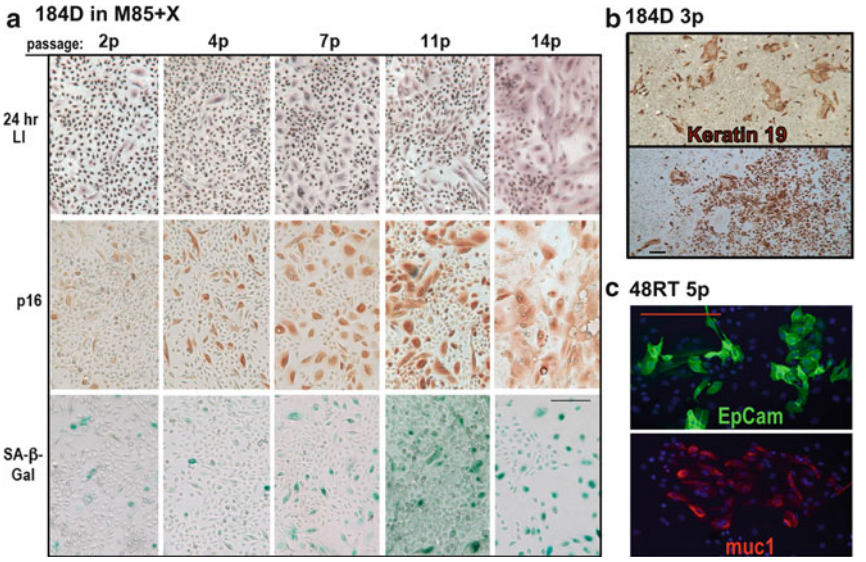

Fig. 15.3 Characterization of pre-stasis HMEC grown in M85 with oxytocin. (**a**) Expression of markers associated with proliferation (LI) and senescence (p16, SA-βGal) in pre-stasis 184 HMEC with increasing passage. Note the reciprocal relationship between the small cells with a positive LI and the larger, often vacuolated cells (senescent morphology) that are positive for p16 and SA-βGal and negative for LI. (**b**) Immunohistochemistry expression of luminal marker K19 in pre-stasis 184 HMEC. (**c**) Immunofluorescence expression of luminal markers EpCam and Muc1 in pre-stasis 48R HMEC. Size marker = 200 μm (Modified from Garbe et al. [15])

With the development of the M85/M87A media, it is now possible to generate and store frozen large quantities of normal HMEC. Using our protocol of repeated partial trypsinization of the primary organoid cultures [48–50], we can make large standardized HMEC batches from individual specimen donors at passages 2–5. These uniform batches permit reproducible large-scale and high-throughput experimentation with normal HMEC from multiple individuals of different ages. Currently, we have initiated cultures from ~50 individuals (reduction mammoplasty, non-tumor mastectomy, milk) of women ranging in age from 14 to 91.

The ability to grow heterogeneous HMEC populations in the M85/M87A medium has enabled us to experimentally examine potential differences in HMEC lineage composition and differentiation as a function of age. In recent studies [52], pre-stasis HMEC strains from 18 young (<30) and 18 older (>55) women were analyzed by FACS and immunofluorescence (IF) for lineage composition (Fig. 15.4). In cultured pre-stasis strains at 4p and in cells from uncultured dissociated organoids, increasing age was associated with a decline of myoepithelial cells and an increase of luminal cells that exhibited molecular features usually ascribed to myoepithelial cells (increased expression of integrin alpha 6 and keratin (K)14) (Fig. 15.4b, c). The tyrosine kinase receptor c-Kit has been postulated to be a marker

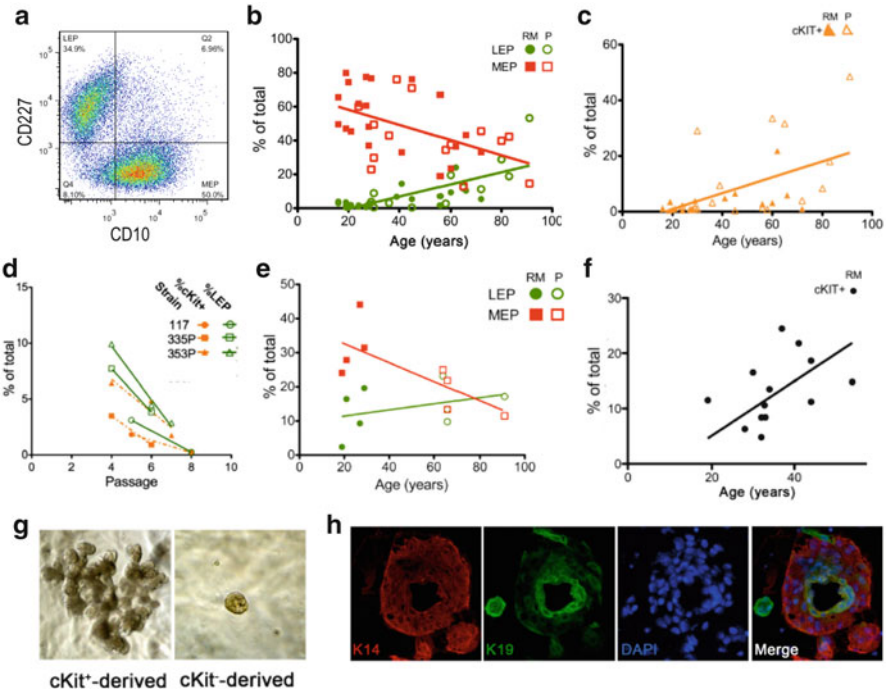

Fig. 15.4 Age-associated alterations in lineage markers in pre-stasis HMEC grown in M87A with oxytocin. (**a**) Representative FACS analyses of CD227 and CD10 expression in 240L HMEC at 4p. Myoepithelial cells (MEP) are CD10(+) CD227(−), while luminal cells (LEP) are CD10(−) CD227(+). (**b**) Linear regression showing changes in proportions of LEP and MEP in HMEC strains at 4p as a function of age ($n = 36$ individuals). LEP and MEP from RM-derived strains are shown with *filled circles* or *boxes* and from P-derived strains with *open circles* or *boxes*, respectively. (**c**) Linear regression of proportions of LEP and MEP in dissociated uncultured organoids as a function of age ($n = 8$ individuals). (**d**) Changes in proportions of LEPs and c-Kit(+) HMEC in three representative strains as a function of passage. (**e**) Linear regression of proportions of c-Kit(+) HMEC in strains at 4p as a function of age ($n = 36$ individuals). (**f**) Linear regression of proportions of c-Kit(+) cells in dissociated uncultured organoids as a function of age ($n = 11$). (**g**) Phase images of representative structures derived from c-Kit(+) (*left*) and c-Kit(−) (*right*) cells cultured in laminin-rich basement membrane for 14 days. (**h**) Immunofluorescence of a transverse frozen section that shows K14 (*red*) and K19 (*green*) protein expression in a duct of a c-Kit(+)-derived TDLU-like structure from 3D culture. Nuclei were stained with DAPI (*blue*); the three-color merged image is shown at *right* (Modified from Garbe et al. [52])

of luminal progenitors in humans [53]. With increasing age, the proportion of c-Kit-expressing HMEC increased in pre-stasis strains at 4p and in dissociated reduction mammoplasty samples (Fig. 15.4e, f). HMEC FACS enriched for c-Kit+ cells at 4p and cultured for three additional passages showed self-maintenance and multipotent differentiation. Primary and 4p c-Kit+ cells embedded in 3D laminin-rich ECM cultures gave rise to terminal ductal lobular (TDLU)-like structures that were composed of an inner core of K19-expressing luminal cells surrounded by K14-expressing myoepithelial cells, supporting the hypothesis that c-Kit+ cells are

progenitors bearing multipotent activity (Fig. 15.4g, h). However, similar to cells with luminal markers, the absolute percentage of c-Kit+ cells decreased with increasing passage (Fig. 15.4d). These data suggest the exciting possibility that the observed age-associated increase in luminal breast cancer may be connected to changes that occur normally with aging in the human breast. The significant age-dependent changes to the mammary epithelium that we observed could make older women more vulnerable to malignant progression and underlay the increased luminal breast cancer incidence in women >55 years. Myoepithelial cells are thought to be tumor-suppressive and progenitors are putative etiologic roots of some breast cancers. Thus, during the aging process, the potential target cell population may increase, while there is a simultaneous decrease in the cells thought to suppress tumorigenic activity.

Although normal HMEC can be obtained from reduction mammoplasties, growth in 2D on plastic does not recapitulate the complex normal in vivo situation, where cell structure and polarity and cell–cell and cell–matrix interactions play important biological roles [54–56]. The reduction of progenitor and luminal cell types with increasing passage likely reflects the limitations of the 2D culture conditions. Of note, despite growth for several passages on plastic dishes, placement of a heterogeneous HMEC population into constrained 3D conditions, such as micropatterned microwells [57] or laminin-rich ECM [49, 52], leads to 3D structures with correct lineage organization, with internally localized luminal cells surrounded by myoepithelial cells.

Pre-stasis HMEC remain genomically stable even when they reach stasis, consistent with the noncritically short telomeres at stasis and the absence of significant evidence of a DDR [15]. The proliferative arrest of cultured HMEC at stasis can be attributed to the rise in p16 expression, as transduction of shRNA to p16 (p16sh) into pre-stasis HMEC can allow them to bypass the stasis arrest [25, 58]. An outstanding question is how the HMEC perceive stress-inducing conditions and signal that information to promote induction of p16. HMEC arrested at stasis share a similar molecular profile regardless of their PD potential or growth media (Table 15.1), with one noticeable difference. HMEC grown in the serum-containing media have a typical senescent morphology of large flat vacuolated cells (Fig. 15.3a), whereas HMEC grown in serum-free MCDB 170 exhibit a more elongated morphology showing abundant stress fibers [15, 22, 50]. We believe this difference is due to the serum-free medium being more stressful for cultured HMEC, consistent with the early rise of p16 and the low PD potential of HMEC initiated in MCDB 170 [19, 22]. This difference in morphology at stasis may have led other investigators to consider this stasis arrest distinct and refer to it as “M0” [59, 60]. We do not yet know the molecular basis by which differentiated luminal cells may cease proliferation prior to stasis. Studies with HMEC from older women (below) suggest that p16sh transduction is not sufficient to maintain active growth to agonescence in portions of their normal population. Stasis arrest in vivo may also result from p53-inducing stresses such as radiation or DNA-damaging agents.

To enhance the usefulness of our HMEC resources, and as part of our studies on malignant progression, we have been characterizing the normal pre-stasis HMEC

for a variety of molecular properties, including gene transcript profiling, global promoter methylation, DDR, and lineage markers as a function of passage [15, 25, 52, 58]. These properties in the normal pre-stasis HMEC have been compared to HMEC that have overcome the stasis and/or telomere dysfunction senescence barriers and are described in more detail below. We have noted in these, and some additional assays [61], that as expected from human populations, interindividual differences can be seen. We also note, as referred to above and further illustrated in Fig. 15.7 below, that HMEC with luminal versus myoepithelial lineage markers may express significant biological differences. For example, EGFR is more highly expressed in late passage pre-stasis HMEC compared to early passage or milk-derived cells that have greater luminal cell representation, consistent with the greater dependence of basal versus luminal breast cancer subtypes on the EGF/MEK versus the IGF/PI3K signaling pathways [62–64]. These considerations become relevant when using normal HMEC as controls for cancer cells. Thus, it is important to examine cells from multiple individuals, and of different ages, to gain a more complete picture of normal HMEC physiology. Further, it may be of value to use lineage-enriched normal population for more accurate comparison to cancer cells with distinct lineage profiles [65].

15.4 Post-stasis Finite Lifespan HMEC

15.4.1 Generation of Post-stasis Finite Lifespan HMEC

Normal HMEC are capable of sensing stress-inducing environments and responding with an RB-mediated growth arrest. In vivo, stasis may serve to eliminate from the proliferative pool cells that have been damaged by stress exposures. Many types of errors in the RB pathway can produce post-stasis HMEC; consequently, post-stasis populations may exhibit significantly different biological properties. For example, a cell that overcame stasis by mutation of RB, a molecular hub, would have more profound alterations than a cell that only lost p16 expression. Bypassing or overcoming stasis is therefore one of the early stages at which the molecular alterations leading to malignancy can diverge. One of our long-term objectives has been to model the different types of pathways a normal HMEC may follow during transformation. We postulate that the combination of stasis-overcoming error and type of target cell affected establishes the initial pathway of cancer progression.

We have used several methods to obtain post-stasis cultured HMEC, focusing on perturbations that could play an etiologic role in human breast cancer. Although we have most commonly observed loss of p16 expression as the means used to overcome stasis, p16 loss can result from diverse mechanisms, and our different p16(−) post-stasis populations show distinct biological properties. Figure 15.1 charts the emergence of varying post-stasis populations from HMEC grown in the different media and subjected to differing oncogenic exposures.

Pre-stasis HMEC from over 150 individuals cultured in our serum-containing media have not shown any instance of a cell spontaneously overcoming the stasis barrier. However, early experiments that exposed primary cultures of specimen 184 HMEC grown in MM to the chemical carcinogen benzo(a)pyrene (BaP) resulted in the emergence of HMEC colonies that maintained growth after the bulk of the cultures ceased proliferation at stasis [23, 66]. Examination of three independently derived BaP-exposed post-stasis populations (initially called Extended Life) showed loss of p16 expression, associated with either mutations or promoter silencing at the p16 locus [19, 29]. BaP post-stasis cultures ceased growth after an additional 10–40 PD, with the exception of very rare cells that became immortal cell lines (see below). Later experiments with MM-grown 184 HMEC transduced with GSE22, a peptide that inhibits p53 function [67], also yielded clonal post-stasis populations. In two experiments, almost all cells ceased growth at stasis, but a few colonies maintained growth [18]. This result indicates that the loss of p53 per se was not sufficient to overcome stasis in these cultured HMEC; the GSE22 post-stasis population presumably sustained errors secondary to the p53 loss that enabled overcoming stasis. The GSE22 post-stasis HMEC exhibit a low level of p16 expression by immunohistochemistry (IHC).

Stasis can be readily bypassed by exposure to p16sh [25, 58]. Cells grown in M85 or M87A+X, from specimens 184, 240L, 805P, and 122L, have been transduced with p16sh at early passages, giving rise to p16sh post-stasis populations that maintained active growth until agonescence [58]. In several instances (see below) clonal immortal lines appeared around the period of agonescence. Recently, we have also observed clonal escape from stasis when c-Myc was transduced into M85/M87A+X-grown 184, 240L, and 122L HMEC [58].

When the HMEC are cultured in the stressful serum-free MCDB 170 medium, rare cells are able to overcome stasis in the absence of additional oncogenic exposures (Fig. 15.2) [22]. We originally called the emergence of these post-stasis cells "selection" and this class of post-stasis HMEC "post-selection." We now recognize that selection (what other labs later termed "M0") is a stasis arrest. Post-selection post-stasis cells express wild-type p53 that is present in a stable form [18, 68, 69] but show DNA methylation of the p16 promoter and absence of p16 expression, as well as nearly 200 other changes in promoter DNA methylation [19, 25]. Most of the differentially methylated regions (DMR) present in post-selection HMEC are also seen in breast cancer-derived HMEC [25], indicating that these changes are associated with malignant progression. A recent publication suggests that post-selection HMEC may be on a transformation pathway leading to metaplastic cancer [70]. Although pre-stasis populations may be heterogeneous with respect to a cell's ease in silencing p16 to become post-selection [71], our data indicate that post-selection cells are induced by growth in the stressful (oncogenic) serum-free MCDB 170 medium and are not present in the starting normal cultures. As mentioned above, we have never seen a post-stasis cell emerge from unperturbed normal pre-stasis HMEC grown in any of our serum-containing media. Additionally, the emergence of post-selection HMEC from pre-stasis HMEC grown in MCDB 170 can be reduced or eliminated by small changes in media composition or methodology. HMEC grown in MCDB 170 cease growth by passages 3–4, but after 2–3 weeks,

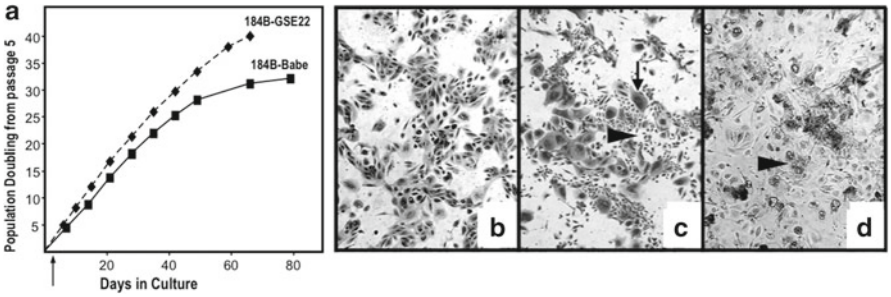

Fig. 15.5 Growth and morphology of post-stasis post-selection 184 HMEC with and without functional p53. 184 HMEC, batch B, were transduced with GSE22-containing or control (Babe) vectors at passage 5. (**a**) Growth curves of 184B-Babe and 184B-GSE22. Note the additional PD in the cultures lacking functional p53. We believe growth rates are similar ± p53, but the absence of p53-mediated growth inhibition allows more cells to continue to proliferate to crisis, leading to apparent faster growth of the population as cells near telomere dysfunction. (**b**) 184B-Babe at agonescence, 2 months after plating at passage 15, contains mostly large, flat cells with some vacuolization; the cell population can retain this morphology and viability for over a year. (**c**) 184-GSE22, 2 weeks after plating at passage 15, shows areas of small proliferating cells and many very large flat cells (*arrows*). (**d**) 184B-GSE22, 4 months after plating at passage 15, shows mostly large multinucleated, vacuolated cells and abundant cell debris. All photographs are at the same magnification (Modified from Garbe et al. [18])

small colonies of post-selection cells suddenly appear. Subculturing prior to the robust appearance of the post-selection cells greatly reduces the number of subsequent emergent colonies. We presume that the induction of the p16(−) cells is occurring during this time when the population is nonproliferative. The nature of the molecular mechanisms responsible for selection remains unknown.

As described further below, post-selection post-stasis HMEC display numerous aberrancies compared to normal pre-stasis cells. This is important to note since post-selection HMEC are being sold commercially as "normal primaries" although they are neither normal nor primaries. Since they have already acquired changes along the pathway to malignancy, they are not an accurate normal control for comparison to cancer cells. Given that it is now possible to grow large quantities of normal pre-stasis HMEC, we strongly recommend that studies aimed at understanding normal HMEC behavior use normal pre-stasis HMEC.

Post-selection p16(−) HMEC grow actively for an additional ~30–70 PD, depending on the individual. As they near agonescence, they exhibit a senescent morphology, SA-βGal activity, a DDR, and an increasing genomic instability [18, 20]. If p53 function is inactivated (e.g., using GSE22), cells continue to proliferate for an additional ~2–4 passages, with increasing evidence of cell death and debris characteristic of crisis (Fig. 15.5) [18]. The telomere dysfunction barrier is very stringent. We have never seen any unperturbed post-selection cell at agonescence spontaneously immortalize. We have also never seen any immortalization at crisis in post-selection HMEC with p53 function inactivated by GSE22, but rare immortalization at crisis using dominant-negative p53 constructs has been reported by others [72, 73]. This stringency is likely due to the molecular nature of this barrier;

cells that fail to maintain a G1 or G2 arrest with critically short telomeres will eventually die or become nonproliferative as a consequence of the genomic instability and mitotic catastrophes. Unlike an arrest based upon blocking cell cycle progression (e.g., elevated levels of CKIs at stasis), the widespread chromosomal derangements present at telomere dysfunction are not reversible. Overcoming this barrier also differs from overcoming stasis in that escaped cells will have acquired genomic abnormalities and may retain some degree of genomic instability [30].

Rare post-stasis cells likely preexist in some breast tissues; methylated p16 promoters in HMEC have been seen in apparently normal breast tissues in vivo [71]. These rare cells have been called vHMEC; the nature of the error(s) leading to the silencing of p16 in vHMEC in vivo is not known. The term vHMEC has also been used by others to refer to p16(−) post-stasis cells in culture that are specifically post-selection [70, 74].

15.4.2 Phenotypes of Post-stasis Versus Pre-stasis HMEC

We have begun molecular analyses to characterize and compare the different types of post-stasis HMEC cultures and post-stasis versus pre-stasis HMEC. These ongoing studies have thus far indicated that post-stasis HMEC have significant differences both from normal pre-stasis cells and among post-stasis types and that the post-selection type of post-stasis appears to be the most deviant from normal. Although not normal, for some experimental purposes post-selection or other post-stasis HMEC may be preferable, e.g., examining the requirements for and mechanisms of overcoming the telomere dysfunction barrier or assaying cells at different stages in progression.

By definition, unlike normal cells, post-stasis HMEC have lost their ability to respond to p16-inducing stresses with growth arrest and have overcome the first tumor-suppressive senescence barrier. Distinct from normal pre-stasis population, which contain cells with markers of multiple lineages [15], most post-stasis types examined have shown predominantly myoepithelial or basal lineage markers (e.g., K5/14, CD10, vimentin), although low levels of some luminal-associated markers can be seen and may increase with passage in post-selection populations (e.g., K8/18, Muc1) [15, 51]. Recent studies from the Kuperwasser lab have shown that post-selection HMEC can differentiate along an epidermal pathway [70]. However, our lineage studies were initially performed with post-stasis cells derived from younger women. Preliminary studies using HMEC from older women, and alternative methods of bypassing stasis, indicate that it is possible to obtain post-stasis HMEC in culture with markers of progenitor or luminal lineage. An important distinction between all post-stasis types and normal pre-stasis HMEC is the gradual increase in genomic instability as post-stasis cells approach the telomere dysfunction barrier, inserting potential unknown changes into these populations, whereas pre-stasis HMEC maintain a normal karyotype, even at stasis [15, 20].

As noted above, compared to pre-stasis cells, post-selection HMEC display a large number of DMR in addition to the p16 promoter locus. In contrast, the BaP

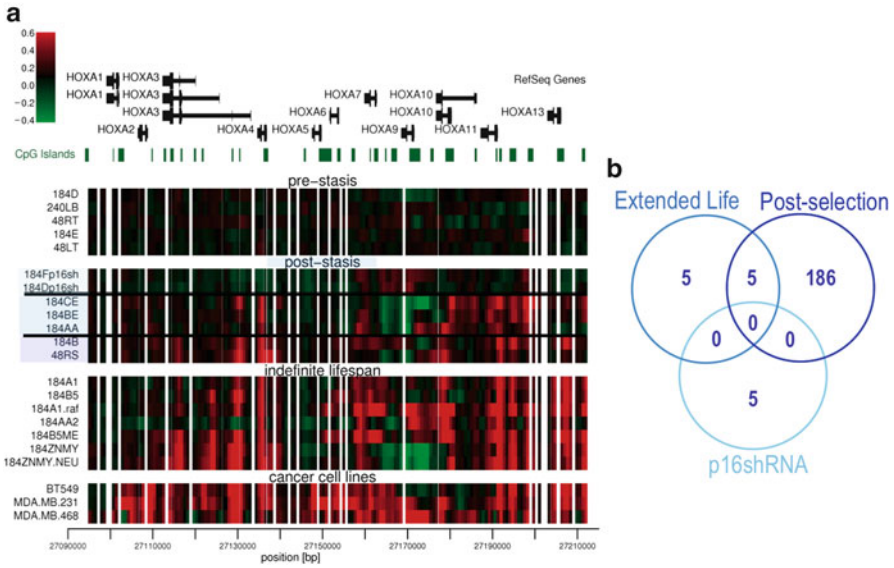

Fig. 15.6 DNA methylation changes in HMEC during malignant progression. (**a**) Progression of DNA methylation in the HOXA gene family cluster from finite lifespan HMEC to malignantly transformed breast cancer cells. *Top*, map of the RefSeq genes of the HOXA cluster followed by the location of CpG islands. *Bottom*, heat map of DNA methylation state of the HOXA gene family cluster based on the microarray data from a custom array with 11,328 probes. *Green*, hypomethylated sites; *red*, hypermethylated sites. Nucleotide position along chromosome 7 is shown below the heat map. Note the differences among the distinct post-stasis types. (**b**) Venn diagram illustrating common and different DMR among the distinct post-stasis types examined (Modified from Novak et al. [25])

and GSE22 post-stasis populations showed only ~10 DMR, including ones in the HOXA cluster also seen in post-selection and tumor-derived HMEC, while the p16sh post-stasis populations showed only ~5 DMR, which did not overlap with the other post-stasis cultures or tumor-derived cells (Fig. 15.6) [25]. Since most of the post-selection DMR are associated with breast cancer cells, post-selection HMEC may be further advanced along the pathway of malignant progression than post-stasis BaP, GSE22, or p16sh types.

Gene expression profiling has compared post-stasis post-selection BaP and p16sh types to normal pre-stasis HMEC and HMF [15, 58, 75] and immortal lines [75]. Gene transcripts from growing and senescent pre-stasis and post-selection HMEC show similarities as well as many differences; there are also a few interindividual differences (Fig. 15.7). Some genes predominantly expressed in pre-stasis HMEC (blue boxes) such as K19, EpCam (TACSTD1), and Prom1 (CD133) are luminal markers, with expression greatly reduced at the higher passages lacking luminal cells. Others, like Muc1, do not have obvious correlation with passage level. Some genes (pink box) appear to be more prevalent in myoepithelial cells, as they are low in the growing milk-derived 250MK luminal cells and reduced in most early passage pre-stasis cultures. The EGF receptor falls into this category, consistent with lower expression of the EGFR in luminal versus basal or metaplastic types

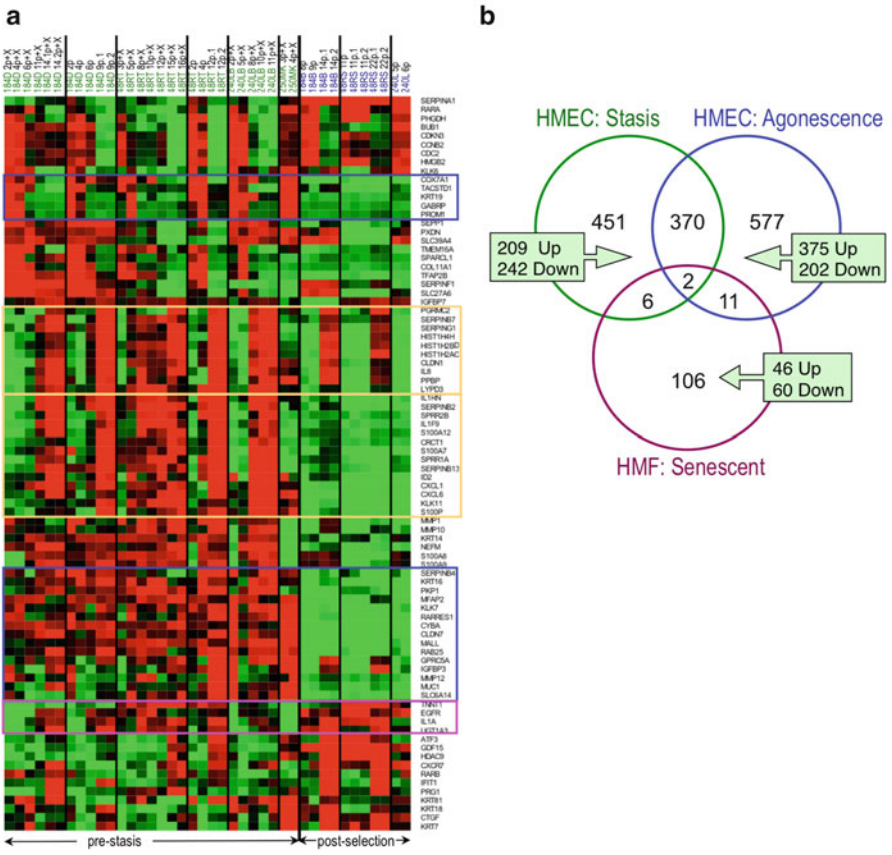

Fig. 15.7 Gene transcript profiles in pre-stasis and post-selection HMEC from different individuals. (**a**) Hierarchical clustering (by *rows*) of gene transcript profiles in growing and senescent pre-stasis and post-selection HMEC. Pre-stasis 184D, 48RT, and 240LB HMEC are shown in *columns* with increasing passage (p) up to stasis; (X) indicates growth in oxytocin. Post-selection 184B and 48RS HMEC are growing and agonescent populations. Genes shown are 77 selected for the greatest variance across all samples, plus a few selected lineage- or differentiation-associated genes. (**b**) Venn diagram of genes modulated at HMEC stasis using growing pre-stasis as baseline, at HMEC agonescence using growing post-selection as baseline, or at HMF senescence with growing fibroblast as baseline. Diagram depicts the number of genes unique to each group and the number that overlaps between and among the groups (Modified from Garbe et al. [15])

of breast cancers [62–64]. Many genes showed increased expression at higher passage levels (yellow boxes); these could be further separated into transcripts expressed at both stasis and agonescence (top yellow), mainly at stasis (lower yellow), and mainly at agonescence. Other genes were preferentially expressed in growing populations (turquoise box). Venn diagrams of transcripts differentially expressed in HMEC at stasis or agonescence and HMF at senescence, compared to their growing populations, illustrate these distinctions more clearly (Fig. 15.7b). The majority of genes modulated at stasis and agonescence were distinct, although there was also significant overlap. These data also highlight the nearly complete

lack of overlap between HMEC and HMF senescence-associated transcripts. Such results illustrate strong cell type specificity in biological processes associated with senescence and suggest caution in extrapolating properties of fibroblast senescence to epithelial cells. Preliminary studies comparing post-stasis BaP and p16sh cultures to pre-stasis and post-selection HMEC show significant differences among the post-stasis types, with the p16sh and BaP post-stasis cells more similar to normal pre-stasis HMEC than the post-selection cells.

Another major difference among post-stasis populations is their responses to overexpressed c-Myc [58]. Earlier work showed that transduction of c-Myc into post-selection HMEC did not have a significant effect on telomerase activity, as measured using the TRAP assay, and produced only one clonal immortalized line in ten experiments [58, 76]. In contrast, c-Myc transduced into three independent BaP post-stasis cultures, and all tested p16sh post-stasis populations, produced a rapid increase in TRAP activity and apparently uniform immortalization. We are currently investigating the molecular properties that might underlie this distinction.

Collectively, these data demonstrate how the molecular pathways associated with different types of transformed HMEC can diverge at the earliest stages of malignant progression, in still finite lifespan HMEC, when they bypass/overcome the stasis barrier.

15.5 Immortally Transformed HMEC Lines

15.5.1 Generation of Immortal HMEC Lines

The telomere dysfunction barrier can be overcome or bypassed by the expression of sufficient telomerase to maintain stable telomere lengths. Based on our experience and the reports of others, reactivation of sufficient telomerase in normal finite lifespan HMEC is difficult to achieve using agents thought to play a role in breast cancer etiology (i.e., not ectopic hTERT transduction or viral oncogenes) and may require multiple errors. This situation may reflect the fact that long-lived animals such as humans have evolved mechanisms for stringent repression of telomerase in normal adult non-stem cells, presumably for tumor suppression. In contrast, normal cells from short-lived mammals such as mice do not show such stringent telomerase repression and readily immortalize [10, 77]. We have postulated that telomerase reactivation and immortalization may be a rate-limiting step in human epithelial carcinogenesis and so believe that great caution should be exercised in extrapolating mechanisms of murine malignant progression to humans, since this critical barrier to malignancy is not present in the commonly used rodent cells. Overcoming telomere dysfunction may correlate with DCIS in vivo, which commonly display short telomeres and genomic instability, and may show telomerase reactivation [30, 39–43]. We have hypothesized that the genomic instability associated with agonescence and crisis can give rise to errors permissive for telomerase reactivation [18, 76] and that

the generation of breakage-fusion-bridge (BFB) cycles prior to immortalization may underlie some of the genomic instability seen in many carcinomas [30]. Additionally, the extensive genomic instability during telomere dysfunction may introduce unknown errors that can contribute to the ultimate cancer cell phenotype, including level of aggressiveness. Our hypotheses are consistent with recent publications indicating that many properties of invasive tumors are already present in their preinvasive DCIS lesions, such as tumor markers, gene expression profiles, gene methylation, PIK3CA mutations, and genomic errors [78–82].

Overcoming the telomere dysfunction barrier, with its associated genomic instability, is an additional point where molecular pathways to transformation may diverge. As described in more detail below, our studies have led us to speculate that at least two distinct sets of alterations may be involved in HMEC immortalization during malignant progression. One set of changes is needed for increased hTERT expression, and a subsequent set may be needed for maintenance of short stable telomeres.

We have generated a variety of immortally transformed lines, initially from specimen 184 and more recently from additional specimens, using various oncogenic agents (Fig. 15.1) [14, 18, 23–25, 58, 66, 83–87]. Most of these lines were derived from post-stasis cultures, although in a few instances (involving hTERT or c-Myc transduction) lines emerged following perturbations of pre-stasis populations. Our first immortal lines were obtained from the BaP post-stasis cultures, 184Aa and 184Be [23, 24, 66, 83]; extremely rare lines appeared at agonescence (184A1, 184AA4, 184AA8, 184B5, 184BE1) (Fig. 15.1, panel A). These cells had been exposed to BaP and likely harbor errors in addition to the loss of p16 expression. We hypothesize that rare errors produced by the genomic instability at agonescence can complement preexisting errors to allow telomerase reactivation. More frequent but still rare clonal lines appeared at agonescence following transduction of the breast cancer-associated oncogene ZNF217 into the BaP post-stasis 184Aa population (184AaZN1-3) [85], while inactivation of p53 in 184Aa using GSE22 produced frequent clonal immortalization at crisis (184AaGS1-2) [18]. Uniform immortalization was obtained following transduction of c-Myc into three different BaP cultures (184AaMY1, 184BeMY1, 184CeMY1) [58, 84].

No post-selection HMEC has been observed to spontaneously immortalize. Rare clonal lines appeared, usually around agonescence, following overexpression of ZNF217 (184ZN4-7) (Fig. 15.1, panel B) [84, 85]. One clonal line appeared in ten experiments where c-Myc was overexpressed (184SMY1) [58]. We hypothesize that rare errors generated by the genomic instability at agonescence may complement ZNF217 or c-Myc to allow telomerase reactivation. Overexpression of both c-Myc and ZNF217 in post-selection HMEC was able to produce immortal lines in repeat experiments (184ZNMY1-4, unpublished). Some of these lines immortalized early, prior to agonescence, and showed no chromosomal copy-number changes by comparative genomic hybridization (CGH) (Chin, Stampfer, Garbe, unpublished). However, Southern analysis of the retroviral insertion site indicates that these lines are also clonal.

More recently, we have targeted early passage pre-stasis cells grown in M85/M87A for transformation (Fig. 15.1, panel C) [58]. Our preliminary studies indicate that rare clonal lines may emerge following overexpression of c-Myc. If cultures are

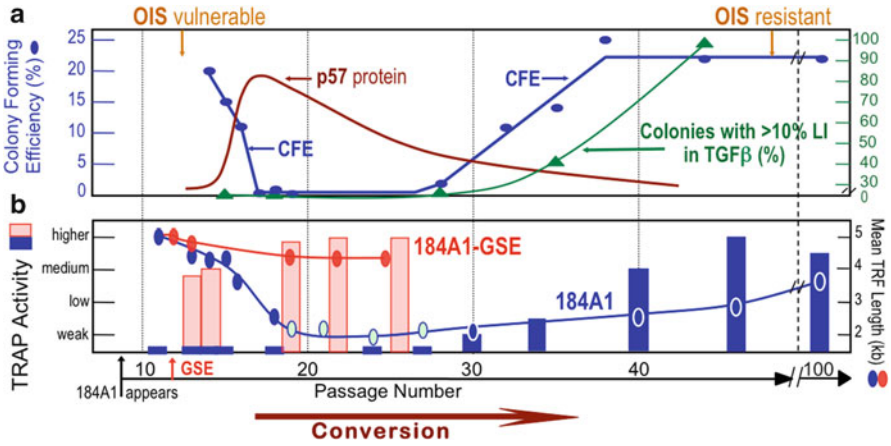

Fig. 15.8 Conversion of newly immortal p53(+) HMEC lines is associated with changes in many key properties. (**a**) The p53(+) 184A1 line undergoing conversion exhibits changes in growth capacity (CFE) and expression of p57 and becomes resistant to TGFβ growth inhibition and OIS. (**b**) 184A1 undergoing conversion exhibits changes in expression of telomerase activity and mean TRF length; *light blue ovals* indicate faint TRF signals. When pre-conversion 184A1 is transduced with GSE22, there is a rapid increase in telomerase activity associated with stabilization of TRF length (Adapted from Stampfer et al. [24, 83] and Nijjar et al. [88])

first transduced with shRNA to p16 and then c-Myc, apparently uniform immortalization occurs. In some instances, clonal lines have emerged around the time of agonescence from the cultures that received the p16sh alone. These new M85/M87A-derived lines are not yet well characterized. The ability of c-Myc to efficiently confer widespread, non-clonal immortalization to BaP and p16sh post-stasis populations provides a reproducible method of immortalization that can facilitate determining the mechanisms involved in HMEC immortalization and assaying agents that may prevent immortalization.

15.5.2 The Conversion Process During Immortalization

Observations of our immortally transformed lines possessing functional p53 led us to describe a process we called conversion [14, 24, 83–88]. Conversion has been most extensively studied in the first immortal line we derived, 184A1 (Fig. 15.8), but remains poorly understood. Tantalizing, but limited data, suggests that this little-known process could be involved in maintaining the short stable telomeres found in most carcinoma cells and in human epithelial cell lines immortalized by reactivation of endogenous telomerase activity [24, 83, 89].

Our basic observations have been that newly immortal clonal p53(+) HMEC lines, which have overcome agonescence and gained the potential to express telomerase, initially displayed little TRAP activity and had ongoing telomere erosion

with proliferation. When telomeres became extremely short (≤3 kb), the conversion process ensued. Expression of the CKI $p57^{Kip2}$ initially abruptly increased and then slowly declined, associated with initial slow-heterogeneous growth and then gradual reattaining of uniform good growth. Telomerase activity gradually increased, and the faint very short telomeres seen during conversion gradually became stabilized with a mean TRF of ~3–7 kb. Curiously, HMEC lines during conversion exhibit little evidence of telomere dysfunction and telomeric associations despite their extremely short mean TRF lengths and can emerge post-conversion with no or few additional gross genomic alterations [30, 58, 90]. We have speculated that the initial rapid rise in p57 levels, and associated growth inhibition, may provide protection from potentially catastrophic mitoses until sufficient telomerase becomes available to maintain telomeric ends. Associated with the increased telomerase activity, the immortal lines gradually developed the ability to maintain growth in the presence of TGFβ [86]. Inactivation of p53 function (using GSE22) in pre-conversion populations led to a rapid increase in endogenous TRAP activity, rapid reduction of existing p57 levels, gaining the ability to maintain growth in TGFβ, and lines with short, stable telomeres [24]. GSE22 transduction into the finite lifespan precursors of the immortal lines did not induce significant TRAP activity, indicating that abrogation of p53 function alone is not sufficient for telomerase reactivation in post-stasis HMEC. Therefore, functional p53 appears capable of repressing telomerase expression in newly immortal HMEC lines until conversion-associated change commence when the mean TRF declines to ≤3 kb.

Immortal HMEC lines that lack functional p53 (e.g., 184AA2, 184AA3) showed some initial TRAP activity that rapidly increased, showed no p57 expression, and quickly attained good uniform growth ± TGFβ. Their mean TRF length stabilized at ~4–5 kb and never declined to the very low levels seen in the p53(+) lines [24]. The rapid conversion and telomerase expression by p53(−) lines may explain why the process of conversion has not been commonly reported or investigated. Most in vitro immortalized human epithelial lines have been obtained using agents that inactivate p53. However, the majority of breast cancers express wild-type p53; a slower p53(+) conversion process may be relevant to early stage breast carcinogenesis in vivo. We have speculated that the low levels of telomerase expression coupled with extremely short telomeres could make newly immortal p53(+) breast cancers particularly vulnerable to therapeutic interventions targeting telomere dynamics.

In contrast to transduction of GSE22, ectopic overexpression of hTERT in p53(+) pre-conversion cells produces rapid full immortalization with TGFβ resistance but precludes conversion-induced reactivation of sufficient endogenous telomerase; the resultant populations did not exhibit short stable telomeres [86]. Similarly, when hTERT was transduced into post-stasis post-selection HMEC, rapid full immortalization with TGFβ resistance occurred, but the population also exhibited longer telomeres than 184A1 ± GSE22 [86]. Therefore, high telomerase expression by itself is sufficient to render these HMEC immortal and resistant to the growth inhibitory effects of TGFβ, although they remain sensitive to TGFβ induction of ECM-related molecules [91]. However, hTERT-induced immortalization did not lead to short stable telomeres.

A significant change associated with conversion is gaining resistance to OIS. Our post-conversion lines ± functional p53 are no longer vulnerable to OIS, but pre-conversion lines remained sensitive [14]. The TERT-immortalized lines from post-stasis and pre-conversion HMEC initially appear sensitive to OIS; SA-βGal is expressed and little proliferation is observed; however, growth can be maintained [47]. These results suggest that the process of endogenous telomerase reactivation during conversion is connected to changes in the pathways that govern OIS, a barrier relevant to in vivo human carcinogenesis [92–94].

Our current hypothesis is that conversion may reflect a need to alter chromosome conformation at the telomeres when cells transition from a finite state (no stable telomere length maintenance) to one where sufficient telomerase maintains short stable telomeres. Functional p53 may present a partial barrier to this process until very short telomeres provoke a structural change at the telomeric ends. As well studied in yeast, immortal cells can have "counting" mechanisms to maintain telomeres within a limited size range [95]. Since most human carcinoma cells, as well as our immortal HMEC lines, maintain telomeres within a short range (mean TRF~3–7 kb) [83, 89], some type of "counting" mechanism likely is involved. Short stable telomeres are not seen in normal telomerase-expressing human cells such as stem cells and lymphocytes [96], suggesting that active processes maybe required for conversion to the distinct telomeric state seen in the immortalized and cancer-derived cells. The longer mean TRF lengths of TERT-immortalized HMEC, and TERT-transduced pre-conversion 184A1 (lines that do not undergo conversion) [86] as well as their distinct OIS responses, indicate that lines immortalized by hTERT may not accurately reflect important biological properties and behaviors of carcinoma lines. Preliminary studies using the 184A1 line have shown that some of the epigenetic alterations seen in later passage 184A1 occur during the process of conversion and are not present in pre-conversion 184A1 populations or in later passage TERT-transduced pre-conversion 184A1 (Vrba, Novak, Stampfer, Futscher unpublished). Conversion may potentially represent a promising therapeutic target. If epigenetic alterations are required to allow stable telomere maintenance, interference with this process at the premalignant stage might prevent further progression.

15.5.3 Generation of Malignant HMEC Lines

Once the HMEC are immortally transformed and no longer vulnerable to OIS, the introduction of one or two oncogenes can further transform these cells towards malignancy (e.g., anchorage-independent growth, disorganized growth in Matrigel, growth factor independence, and/or tumorigenicity in immunosuppressed mice) (Fig. 15.1) [14, 44, 45, 97]. This property makes immortally transformed lines such as 184A1 and MCF10A useful for examining agents that can propel cells from the stage of nonmalignant immortal to malignancy and the mechanisms responsible for this transition [98–102]. The same oncogenes overexpressed in finite lifespan HMEC (both normal pre-stasis and abnormal post-stasis) do not confer malignancy

and commonly induce senescence. Thus, in marked contrast to normal or finite cells, nonmalignant immortally transformed lines have acquired the errors that allowed them to escape multiple tumor-suppressive senescence barriers and be only one oncogene away from malignancy. The acquisition of OIS resistance upon immortalization likely contributes to the observation that immortality is the most common alteration from normal associated with human carcinomas. Nonmalignant immortal lines, having undergone many significant transformations from the normal state, do not constitute accurate "normal" controls nor do they represent the starting point of early stage carcinogenesis. They can control for changes associated with immortality when comparing immortal malignant tumor lines with nonmalignant immortal cells. We view nonmalignant immortal lines as at a stage similar to abnormal telomerase (+) cells in DCIS, which recent studies have shown already possess many of the errors found in breast cancers [78–82]. Since the aggressive phenotype of breast cancers may be predetermined early, at the premalignant stage, a better understanding of early stage progression, i.e., the steps from normal pre-stasis HMEC to nonmalignant immortal cells, may offer new insight into both the mechanisms of carcinogenesis and possibilities for therapeutic intervention in this progression.

15.5.4 Phenotypes of Immortal Versus Finite Lifespans HMEC

As previously noted, immortal HMEC lines differ from finite lifespan HMEC in their ability to maintain growth in the presence of TGFβ and their resistance to OIS. Most immortal lines, having undergone the period of genomic instability during telomere dysfunction, also exhibit gross genomic errors and ongoing genomic instability [24, 30, 58, 90]. We have further compared immortal, post-stasis, and pre-stasis HMEC for DMR and gene transcript profiles (Figs. 15.6 and 15.9) [25, 58, 75]. As mentioned above, post-stasis HMEC vary widely in number of DMR. When representative immortal lines were examined, a total of ~500 DMR were observed, most of which are also found in breast tumor-derived cells [25]. DMR found in post-selection post-stasis HMEC were also seen in immortal lines that derived from BaP post-stasis cultures lacking these DMR. An unsupervised clustering of DMR (Fig. 15.9a) shows most nonmalignant immortal lines clustering with tumor-derived lines and post-selection post-stasis cultures. Notably, the non-clonal lines derived from Myc-immortalized BaP post-stasis cultures had fewer DMR than the finite post-selection cells, although still many more DMR than seen in their post-stasis precursors. Venn diagrams (Fig. 15.9b) illustrate the overlaps and distinctions in DMR among the normal to tumor cells. A good example of stepwise DNA methylation changes during HMEC transformation can be seen in the HOXA gene cluster, known to undergo aberrant methylation during breast carcinogenesis (Fig. 15.6a) [103, 104]. Methylation increases towards the 3′ end of the cluster as normal HMEC transition to malignancy, with HMEC at different stages in our transformation model showing appropriate intermediate levels of methylation. Altogether, our epigenetic data indicate that cancer-associated DMR can occur at the earliest stages of transformation,

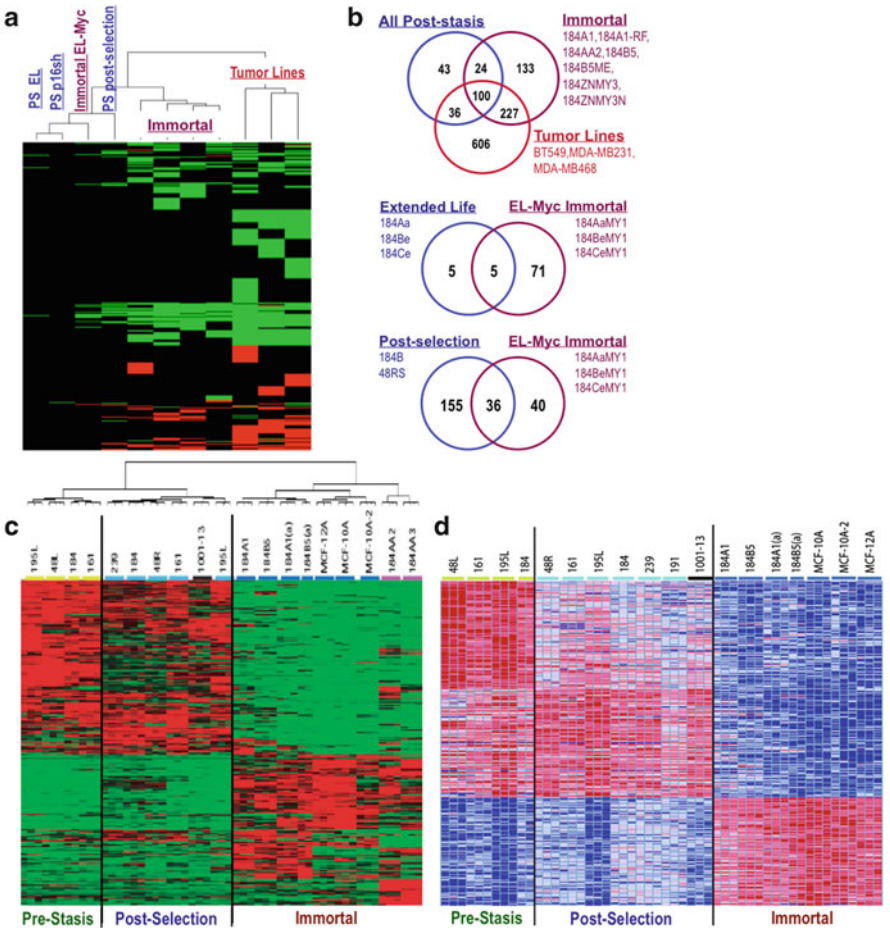

Fig. 15.9 Comparison of DMR and gene transcript profiles among finite (pre- and post-stasis) and immortal (nonmalignant and malignant) HMEC types. (**a**) Heat map showing clustering of DMR in post-stasis to malignant HMEC types, based on the microarray data from a custom array with 11,328 probes. *Red*, hypomethylated sites; *green*, hypermethylated sites. (**b**) Venn diagrams illustrating common and different DMR among post-stasis and immortal (nonmalignant and malignant) HMEC types. (**c**) Unsupervised clustering of gene transcript profiles of pre-stasis, post-selection, and immortalized HMEC. All genes (of 2,319) that changed expression in one or more samples were used to cluster the cell types and lines by overall similarity. Sample 1001-13 was HMEC, advertised as normal, obtained from Clonetics (Lonza). Samples of 184A1 and 184B5 designated by (**a**) were obtained from ATCC. (**d**) Supervised clustering of pre-stasis, post-selection, and immortalized HMEC. Gene expression values were normalized and characterized for the significance of overexpression in one group relative to other groups in the comparison. The top 200 genes (of 1,342) that are significantly overexpressed in one group are shown (**c** and **d** modified from Li et al. [75])

groups of DNA methylation changes can arise concurrently, and malignant progression is associated with progressive DMR changes. We are currently examining a larger range of our immortal HMEC to determine if specific epigenetic changes may correlate with specific parameters of the immortalization pathway.

Using both unsupervised and supervised clustering of gene transcript data, our immortal lines show similar expression as MCF10 and MCF12A and are clearly distinguished from the finite HMEC, although differences exist between pre-stasis and post-selection finite cells (Fig. 15.9c, d). HMEC advertised as normal that were obtained from Lonza/Clonetics (1001-13) sort cleanly with our aberrant post-selection post-stasis HMEC. Preliminary studies examining a greater range of our immortalized lines, post-stasis types (post-selection, BaP, and p16sh), and recent pre-stasis cultures (shown in Fig. 15.7a) are consistent with these earlier data.

Collectively, these data demonstrate that the transition to immortality is accompanied by major molecular alterations in gene expression, epigenetic marks, and other parameters associated with malignancy and show how nonmalignant immortal HMEC lines more closely resemble tumor cells than normal HMEC.

15.5.5 Lineage Characterization of Immortalized HMEC Lines

Human breast carcinomas exhibit a wide range of molecular properties, correlated with distinct clinical behaviors [1–4]. In general, we have seen that our different methods of inducing transformation can yield cell lines with significantly different properties; however, most in vitro immortalized lines thus far generated have lineage markers similar to the basal subtype of human breast cancers [1]. This subtype has a poor prognosis but represents only a minority of breast cancer. We now hypothesize that prior difficulty in developing a greater variety of transformed lines was due to (1) poor culture systems for growing normal human HMEC with luminal or progenitor properties, (2) use of cells from young reduction mammoplasty tissues, and (3) use of a limited number of oncogenic agents. We have begun characterization of lineage markers in some of our newly developed lines (Fig. 15.1, panel C). Lines from the younger women (184, 240L) again show a predominantly basal phenotype using FACS, IF, and IHC analyses, although variation is observed in CD24/CD44 ratios and EpCam expression [58]. The lines vary in expression of EMT- and stem cell-associated properties, gene transcript profiles, genomic errors, and other phenotypes [58] (Garbe, Vrba, Futscher, LaBarge, Stampfer, unpublished). Excitingly, our first experiments using HMEC from older women (805P, 122L) have yielded lines that express luminal and progenitor markers (Garbe, Stampfer, LaBarge, unpublished). Now that we can grow and FACS enrich progenitor and luminal cell populations, future studies can assess the relative contributions of target cell type and oncogenic agents employed in affecting the phenotype of resultant transformed lines.

The generation of transformed lines more representative of in vivo breast cancer types may enhance our understanding of the etiology and properties of a wider range of breast cancers. Thus far, a very limited number of nonmalignant immortal cell lines (mainly MCF10A, 184A1, MCF12A, 184B5, HMT-3522 S1) have been used in a large number of studies examining the transition from nonmalignant to

malignant immortal. However, these lines all exhibit a basal or claudin-low phenotype and may consequently not accurately reflect properties of the majority luminal breast cancer types. For example, basal versus luminal HMEC may differ in their relative usage of the EGF/MEK versus IGF/PI3K signaling pathways and in EMT-associated properties.

15.6 Integrated Analysis of HMEC Model System

The work reviewed thus far illustrates how we have developed a wide-ranging cell culture system for investigating HMEC transformation. Starting with pre-stasis HMEC, exposure to a variety of oncogenic agents has generated cells at different stages of multistep carcinogenesis, providing isogenic cultures for examining the molecular alterations associated with progression. Such an integrated system avoids many of the variability problems inherent in comparing normal and transformed cells not only from different individuals but also from different organ sites, tissue types, and/or species and facilitates focus on changes due to the process of transformation. Being able to analyze the range of alterations from normal finite lifespan pre-stasis cells to malignantly transformed cells provides a comprehensive overview that assists understanding how the many alterations associated with carcinogenesis collaborate molecularly and temporally to produce cancerous cells. Below, we provide some examples of how this integrated HMEC model of transformation offers insight into processes associated with human breast carcinogenesis.

15.6.1 Genomic Instability, Telomeres, and Telomerase Expression

Normal human epithelial cells retain a stable genotype in vitro and in vivo; however, carcinomas usually express genomic instability and aneuploidy. Our model system has allowed us to examine when the transition to genomic instability occurs during the process of transformation in culture [15, 20, 24, 30, 90]. Proliferative finite lifespan HMEC undergoing telomere erosion due to insufficient telomerase activity maintain genomic stability until telomeres become critically short, which then leads to uncapped telomeres and telomeric associations. Cells arrested at stasis have a normal karyotype and noncritically short mean TRF (>5 kb), but virtually all HMEC arrested at agonescence display abnormal metaphases, with a preponderance of telomeric associations, and a mean TRF <5 kb [20, 105]. These results are consistent with in vivo data that show normal karyotypes in atypical ductal hyperplasia but genomic instability, abnormal karyotypes, and short telomeres at the DCIS stage [30, 43]. We have consequently hypothesized that the genomic instability naturally encountered in finite cells with eroded telomeres may contribute to the errors that allow these cells to reactivate sufficient telomerase activity and become immortal. However, in the vast majority of instances, in the absence of preexisting

immortality-disposing errors, this genomic instability leads to cell death or proliferative arrest, providing an effective tumor-suppressive senescence barrier. The presence of short telomeres and genomic instability in a large percentage of DCIS argues against the proposition that the initial target cell for transformation in these cases already possessed sufficient telomerase activity.

We have further suggested that this inherent genomic instability resulting from eroded telomeres may be a significant contributor to the observed instability and resultant aneuploidy in breast cancer-derived cells. All our immortally transformed lines examined that encountered telomere dysfunction display CGH alterations and/or karyotypic abnormalities. Genomic alterations can be observed before and during the process of immortalization and conversion; however, once sufficient telomerase activity is present, the level of instability can decrease in p53(+) lines [24, 30, 105]. Presumably, telomerase allows telomere capping, preventing the formation of new telomeric associations, but the already present chromosomal derangements lead to ongoing cycles of BFB. In the absence of functional p53, lines that immortalized after undergoing genomic instability may show increasing instability [24]. Most of the genomic alterations generated by telomere dysfunction will be unrelated to the requirements of immortalization or carcinogenesis, but some could affect the clinical properties of a resultant malignant cell. It is therefore possible that the genomic instability in premalignant cells may be the source of many of the "passenger" mutations present in carcinomas, as well as of "driver" mutations that influence prognosis. This hypothesis is consistent with recent publications that suggest that the invasive phenotype of breast cancer is already genetically programmed at the preinvasive stages of disease progression [82].

Another possible corollary of our data is that the timing of telomerase reactivation during the period of telomere dysfunction may affect subsequent instability. Our immortal lines derived from cultures in the midst of telomere dysfunction contain many more genomic errors than lines derived before widespread instability ensued [24]. For example, the 184A1 line, which immortalized at ~7–8 p with a >5 kb mean TRF, has few errors, no BFB, and, unlike most in vitro transformed HMEC lines, can remain genomically stable upon passage. In contrast, the 184AA4 line, also derived from post-stasis 184Aa, immortalized at ~12–13 p when the population was experiencing telomere dysfunction, and exhibits numerous genomic errors and ongoing instability. Possibly, breast cancers with diploid karyotypes reflect cells that underwent immortalization prior to extensive telomere dysfunction-induced genomic instability.

We have recently begun examination of the CGH profiles and karyology of the non-clonal immortal lines generated by direct targeting of the stasis and telomere dysfunctional barriers using transduction of p16sh and c-Myc. The three lines thus far tested contained cells with normal karyotypes at early passage [58]. This result supports our model of the tumor-suppressive senescence barriers and the hypothesis that genomic instability functions to generate errors critical for transformation, but is not essential per se. If the stasis and telomere dysfunction senescence barriers are bypassed by direct targeting, generation of genomic errors may be unnecessary. We therefore believe that no specific mutator genes are required to account for the genomic instability seen in breast carcinomas, although mutations that do increase

instability could also be present and selected for during malignant progression, and contribute to this phenotype. Rather, development of genomic instability is inherent in the process of malignant progression, particularly at the stage of telomere dysfunction. The further development of aneuploidy has been proposed to result from dysfunctional telomeres interfering with the completion of cytokinesis [105].

15.6.2 Immortalization and Responses to TGFβ

Normal cultured HMEC are growth inhibited by TGFβ and show induction of ECM- and proteolysis-related molecules (e.g., fibronectin, collagen IV, laminin, type IV collagenase, uPA, and PAI-1) [13, 24, 86, 91]. Our studies comparing the responses to TGFβ of HMEC ranging from normal pre-stasis to transformed [13, 24, 86, 91, 106–108] have indicated that the expression of telomerase activity (from either endogenous reactivation or transduction of hTERT) is sufficient by itself to allow HMEC to maintain growth in TGFβ while also remaining responsive to TGFβ-mediated protein induction. These results were among the first to demonstrate that multiple TGFβ actions can operate via divergent pathways, since the effects on cell growth could be dissociated from stimulation of ECM components. Immortal and malignant lines can maintain growth in TGFβ, although some may exhibit a slightly decreased growth rate, likely reflecting the metabolic price exacted by the increased synthesis and secretion; we do not consider this reflective of a direct growth inhibition. The mechanism by which telomerase activity prevents TGFβ from inhibiting growth is still unknown; however, our results indicate that immortally transformed HMEC expressing hTERT do not require additional errors to become TGFβ growth resistant.

In contrast to normal HMEC, many human carcinomas, including breast, can maintain growth in the presence of TGFβ while retaining other metabolic responses, similar to our immortalized lines, although some carcinomas have lost all responsiveness [109]. However, only rare mutations in the TGFβ pathway have been found in breast cancers [109, 110]. Based on our in vitro data, we suggest that during in vivo carcinogenesis no additional errors beyond acquisition of immortality may be needed to confer resistance to TGFβ growth inhibition. During malignant progression in vivo, it could be beneficial to cancer cells to retain the capacity for TGFβ-inducible ECM- or EMT-related functions while avoiding the growth inhibition. However, it might be advantageous in some circumstances to avoid the growth inhibition prior to full immortalization or to forgo the additional metabolic expenditures, accounting for situations where mutations are observed and all responses to TGFβ are lost.

15.6.3 Immortalization and OIS

Another significant alteration associated with the process of telomerase expression and immortalization is the acquisition of resistance to OIS. For malignant

progression to proceed, the normal ability of cells to respond to inappropriate oncogenic expression by cessation of growth needs to be abrogated. We and colleagues have seen that ectopic overexpression of oncogenes such as Raf-1, Ras, and ErbB2 in finite lifespan HMEC can produce rapid growth inhibition, whereas similarly exposed nonmalignant immortal lines maintain growth and acquire malignancy-associated properties such as anchorage-independent growth and reduced growth factor requirements [14, 16, 44, 45]. The transition to OIS resistance is a critical alteration and emphasizes the importance of immortalization and particularly the conversion step in tumorigenesis. The mechanism by which HMEC and other human epithelial cells gain OIS resistance is currently unclear but differs from what has been reported for most fibroblast or rodent cells in lacking a requirement for functional p53, p16, ATM, or CHK2 [14, 16]. A curious and potentially important observation, although thus far not further explored, suggests the possibility that the differences in OIS responses of finite versus immortal HMEC may be based on different levels of expression of the oncogenes. We noted that basal and 4-HT-induced expression levels of the Raf-1:ER transgene, as well as phosphorylated MEK, were consistently reduced in post-conversion immortal 184A1 compared to finite HMEC or pre-conversion 184A1 [14]. When post-conversion 184A1-Raf-1:ER was sorted to obtain cells showing the highest levels of Raf-1, immediate assay showed decreased survival upon induction with 4-HT. However, when these sorted cells were amplified and reexamined, Raf-1 levels were again reduced. Conversely, when post-selection HMEC transduced with Raf-1:ER were exposed to low levels of 4-HT so that expression levels were similar to those seen in 184A1, not only were the cells not growth inhibited, they showed increased growth capacity in the absence of EGF. Possibly, abnormally high levels of oncogene expression can trigger OIS, while lower levels can confer malignant properties, and immortal HMEC may have a mechanism to prevent ongoing high-level expression.

Altogether, these examples emphasize the critical role the immortalization step plays in HMEC carcinogenesis.

15.7 Conclusions

The development of an extensive, integrated culture system for examining normal and aberrant HMEC behavior has allowed us to take a comprehensive overview of how the processes functioning in normal HMEC become subverted during transformation and the relationship of individual alterations incurred with resultant transformed phenotype. Several generic conclusions can be drawn from these studies.

First, a major caveat needs to be considered when evaluating these data. Most of this work was performed with cells growing in two dimensions on plastic substrates, whereas normal and aberrant epithelial cell processes in vivo involve complex interactions of polarized cells within three-dimensional organ systems. As others have elegantly shown [54–56], many important cellular behaviors will differ when cells are placed in culture environments that support cell polarity and provide ECM material and stromal interaction. In developing our HMEC culture system, we tried

to balance the goal of being amenable to widespread use with the goal of optimizing the system to reflect in vivo biology. We consequently focused on standard tissue culture technology in order to generate sufficient HMEC to support large-scale, reproducible investigation. HMEC cultures have subsequently been examined using 3D culture systems such as Matrigel or micropatterned wells [49, 52, 57], but such studies have thus far been limited. Importantly, normal HMEC placed in 3D environments have demonstrated appropriate self-organization, indicating that the needed lineage-specific properties have been retained. However, data obtained in 2D culture may not accurately reflect the in vivo biology. A situation where this issue may be most relevant involves the transition from a nonmalignant immortal cell (DCIS) to a malignant primary cancer cell. In vivo, this transition likely involves epithelial-stromal interactions, a hypoxic environment, and selection for errors that promote malignancy-associated properties, such as invasiveness and angiogenesis. Our in vitro selection is only for immortality and is unlikely to recapitulate the changes associated with this transition in vivo. A more accurate approximation of in vivo 3D biology can be expected to offer new and better insights into the processes underlying carcinogenesis and aging.

A common thread in our studies is the extent of diversity and heterogeneity among normal and abnormal cell types and how this manifests in significantly different molecular processes. In terms of understanding human carcinoma progression, HMEC and epithelial cells in general have many significant biological differences compared to HMF and fibroblasts in general. An outstanding example involves mechanisms of senescence. Among many distinctions, there was almost no overlap between genes modulated at HMEC senescence (stasis and telomere dysfunction) and genes modulated in senescent HMF; there were differences in molecules modulated by HMEC and HMF during OIS; normal HMEC are growth inhibited by TGFβ exposure, while isogenic HMF respond with a slight growth stimulation [13–16]. These distinctions are important in light of the common use of fibroblasts to study mechanisms of senescence, often with an implied assumption that the results obtained are generic to "cells." Our development of robust culture conditions for normal HMEC should encourage increased usage of human epithelial cells to understand what is distinct about their senescence mechanisms, which in turn play prominent roles in suppressing carcinogenesis.

Significant differences are found comparing human and rodent epithelial cells in mechanism relevant to carcinogenesis. Most important, rodents lack stringent repression of telomerase in adult cells and thus the crucial telomere dysfunction senescence barrier. They also differ in the relative roles of the CKIs $p19/14^{ARF}$ and p16 in stasis and immortalization. While rodent models offer the ability to perform in vivo experimentation, the critical errors required by human epithelial cells for immortalization will not be amenable for discovery using rodent models. The immortalization step presents a potentially valuable therapeutic target, since almost all breast cancers, regardless of subtype, exhibit telomerase reactivation and are dependent upon immortalization for malignant progression. Further, unlike signaling pathways where extensive redundancy contributes to development of therapeutic resistance, the use of alternate (ALT) pathways for telomere maintenance is

extremely rare in human epithelial cells and in breast cancers [111]. Efforts to clinically exploit the requirement for immortalization-promoting errors will be enhanced by the availability of human epithelial cell culture systems that support experimental examination of genomic, epigenomic, and gene expression alterations associated with immortalization.

Heterogeneity exists among normal HMEC in vivo and in vitro. Cells with luminal versus myoepithelial lineage markers may have differences, such as signaling pathway usage, that carry over to observed differences between luminal and basal tumor cell lines. Multiple types of progenitor populations are also present, with distinctions presumably based on epigenetic marks and other properties. Identification and characterization of the different normal HMEC types may be relevant for identification of the initial target cell types of the different breast cancer subtypes and how the properties of the target cell influence cancer progression and treatment. The ability to grow and FACS enrich these diverse lineages in our cultured pre-stasis HMEC can facilitate studies that assess the effects of various oncogenic exposures on differing initial target populations. Additionally, FACS-enriched normal populations may serve as more accurate normal controls for type-specific breast cancer cells than an unsorted heterogeneous population. Proliferative normal HMEC in culture can in some instances provide more relevant controls for proliferative cancer cells than comparisons of normal and tumor tissue in vivo. Normal HMEC in vivo have low proliferation rates, and properties associated with a proliferative state may be erroneously assessed to be tumor-specific based on examination of in vivo tissues.

During carcinogenesis, heterogeneity is amplified by driver and passenger alterations acting on the initial target cells, resulting in the diversity of breast cancer subtypes with corresponding diverse clinical parameters. Since a goal of personalized medicine is matching therapeutic modalities with the specific errors present in individual tumors, the accuracy with which experimental models in vitro match in vivo molecular parameters will influence the usefulness of such models for evaluating potential therapeutics. Such considerations underscored our use of oncogenic agents thought to play a role in breast cancer etiology in our HMEC transformation models. For example, most breast cancers express wild-type p53 and retain functional RB [112]. Loss of these key molecular hubs will have much greater consequences on a cancer cell's behavior than impairment of one sub-pathway, such as loss of p16. Immortalization of HMEC achieved by use of viral oncogenes SV40T or HPVE6 and E7 not only inactivates p53 and RB function but also produces many other undefined changes; cells transformed by such methods are unlikely to provide accurate models for exploring potential breast cancer type-specific therapeutics. Lines immortalized by ectopic overexpression of hTERT will lack the critical alterations associated with reactivation of endogenous telomerase, including, as discussed above, the process of conversion and related changes in telomere dynamics and OIS responses. Additionally, transformation systems employing hTERT and viral oncogenes are not amenable for understanding the mechanisms of HMEC immortalization during in vivo carcinogenesis or therefore examination of agents that might prevent this step in progression. By using pathologically relevant oncogenic agents, we have obtained transformed cells that share many of the properties seen during in

vivo breast carcinogenesis, such as retaining wild-type p53 and RB. By requiring cells to reactivate endogenous telomerase activity, we can examine the crucial immortalization step during cancer progression. Nonetheless, until recently, we have not been able to model most of the phenotypes observed in actual breast cancers.

Most published in vitro transformed HMEC lines have exhibited a basal, triple-negative, or claudin-low phenotype, while the majority of breast cancers belong to luminal subtypes. A large number of studies are being performed on a very limited set of immortalized lines that are not representative of most breast cancers and may not be reflective of most breast cancers' behavior. Consequently, our more recent efforts have been directed towards generating transformed lines more reflective of the range of breast cancers in vivo. These studies are currently in progress but indicate that using HMEC from older women as target cells, and employing additional agents to bypass stasis, can lead to transformed cells lines with luminal lineage markers.

The other main conclusion from our integrated model system is the crucial role of telomere dysfunction and the immortalization step in human breast cancer progression. The changes associated with overcoming telomere dysfunction support multiple aspects of tumor progression. In addition to the advantages provided by unlimited proliferative potential, the immortalization step also promotes genomic instability, changes OIS into oncogenic promotion of malignancy, and abrogates TGFβ-induced growth inhibition while leaving cells responsive to TGFβ-induced tumor promotion and EMT. Our comparisons of isogenic finite and immortal HMEC indicate that the transition from finite to immortal is associated with the greatest extent of changes in epigenomic marks and gene expression. The requirement of immortalization for malignancy and the lack of easy redundant alternatives to telomerase reactivation suggest that immortalization may be a valuable target for clinical intervention. While there has been significant effort to develop pharmacologic agents that could interfere with telomerase action, other errors necessary to attain or maintain immortalization could also be valuable targets. Our limited understanding of the mechanisms underlying human epithelial cell immortalization, and the absence of accurate rodent models of this step, has held up exploration of this possibility. Our development of reproducible methods for non-clonal immortalization using pathologically relevant agents may open up new way to explore potential novel therapeutics targeting this step.

The processes implicated in human epithelial cell senescence and carcinogenesis in vivo are complex, involving alterations both within a cell's genome and physiology, and in relationship to its immediate and whole-body environment. Many recent exciting publications that directly examine human breast tissues are providing large quantities of information about the pathways and derangements associated with breast carcinogenesis and illuminating the extent of inter- and intra-tumor heterogeneity during various stages of tumor development [5–9]. However, determining cause and effect relationships, identifying driver abnormalities among the hundreds of other changes, and testing potential therapeutics are constrained using only in vivo approaches. An in vitro HMEC model system, although also limited, offers an experimentally tractable approach to investigate the effects of individual perturbations in HMEC at different stages in transformation along distinct transformation

pathways. It can be expected that the closer such model systems reflect the processes occurring in vivo, the more accurate they will be for assessing potential clinical interventions. We have presented an overview of our integrated HMEC model system for such experimentation and highlighted some of the ways in which our comprehensive culture system has provided novel insight into these complex processes. Importantly, our model system starts with normal finite lifespan pre-stasis HMEC, allowing examination of the critical early stage changes that occur as normal cells transition to immortality. Ongoing improvements in HMEC model systems, including better modeling of 3D and microenvironmental conditions and of the range of pathways to and phenotypes of transformed cells, can greatly assist efforts to delineate the different pathways a normal HMEC can take to become malignant and enable investigation into potential therapeutic approaches to prevent malignant progression.

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