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REVIEW

Breast cancer epigenetics: normal human mammary epithelial cells as a model system

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Abstract DNA hypermethylation and histone modifications are two critical players involved in epigenetic regulation and together play an important role in silencing tumor-suppressor genes in all cancers, including breast cancer. One of the major challenges facing breast cancer researchers is the problem of how to identify critical genes that are epigenetically silenced early in cancer initiation as these genes provide potential early diagnostic and/or therapeutic targets for breast cancer management. This review will focus on compelling evidence that normal Human Mammary Epithelial Cells (HMECs) that escape senescence in culture mimic genetic and epigenetic events occurring in early breast cancer, and provide a valuable system to delineate the early steps in epigenetic deregulation that often occur during transition of a normal breast cell to a premalignant cell. In particular, this model system has been used to investigate the relationship between gene silencing, DNA methylation, histone modifications, and polycomb association that may occur early in oncogenic transformation.

Keywords Human mammary epithelial cells · Breast cancer · DNA methylation · Histone modifications · Biomarkers

Abbreviations

bp	base pairs
BMI-1	B lymphoma Mo-MLV insertion region
BRCA1	breast cancer 1, early onset
CDKN2A	cyclin dependent kinase inhibitor 2A
ChIP	chromatin immunoprecipitation
Cox-2	cyclo-oxygenase 2
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EZH2	enhancer of zeste homolog 2
H	histone
HDAC	histone deacetylase
HMEC	human mammary epithelial cells
HMT	histone methyltransferase
K	lysine
kb	kilobases
MBD	methyl-C binding domain
MeCP2	methyl CpG binding protein 2
PcG	polycomb group
PRC	polycomb repressive complex
Rb	retinoblastoma 1
SUZ12	suppressor of zeste 12

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Introduction

Classic molecular biology research concentrates on the role that direct genetic alterations, such as insertions, deletions, and mutations have on DNA and gene expression. However, it is now recognized that epigenetics also plays a major role in carcinogenesis. Breast cancer is the leading cause of cancer death among women aged 20 to 59 in the United States, and it is estimated that over 472,000 people worldwide died from breast cancer in 2001 [1, 2]. Female breast cancer incidence rates have continued to increase [3], and so, a greater understanding of the initiating molecular mechanisms involved in cancer initiation is required if we are to make significant progress in combating breast cancer. One of the current challenges is the identification of new

biomarkers to lessen the burden of aggressive breast cancer by permitting early diagnosis and predicting the clinical behavior of the disease. This review aims to provide the reader with a comprehensive summary of the research to date in the field of breast cancer epigenetics, with a special focus on the use of human mammary epithelial cells as a model system to identify novel epigenetic biomarkers.

Epigenetics

Epigenetics is commonly defined as a heritable and reversible change in gene expression that is not accompanied by a change in the DNA sequence [4]. There are two major epigenetic modifications: those occurring at the DNA level (DNA methylation) and those occurring at the chromatin level (chromatin remodeling). DNA methylation is an enzyme-driven chemical change to the DNA sequence that most commonly occurs at CpG dinucleotides in mammals. Chromatin remodeling occurs via the modification of histone residues by enzymes primarily on the N-terminal tails and ultimately affects the interaction of DNA with chromatin modifying proteins. Both DNA methylation and histone modifications are associated with silencing critical tumor suppressor genes and activating oncogenes involved in cancer. Figure 1 highlights the epigenetic modifications that commonly occur across a typical tumor suppressor gene promoter in normal and cancer cells.

DNA methylation

The CpG dinucleotide is depleted in the genome in comparison to other dinucleotides; however, there are regions ranging from 200 bp to several kb in length that are up to five times richer in CpG density. These regions, known as CpG islands, typically span the promoter region and first exon of approximately 60% of all genes. The majority of these CpG islands are unmethylated in the normal cell (Fig. 1a), which correlates with active gene transcription, in comparison to the bulk of the genome which is methylated [5, 6]. In a cancer cell, however, these methylation states are often reversed: global levels of DNA methylation become hypomethylated (reduced) in conjunction with DNA hypermethylation (increased) of the CpG islands associated with promoters of housekeeping genes and tissue-specific genes (Fig. 1b) [7, 8]. CpG methylation is characterized by the addition of a methyl group to the 5-carbon on the cytosine base via a covalent bond (Me-C). This is achieved through the actions of DNA methyltransferase enzymes such as DNA methyltransferase 1 (DNMT1), which is responsible for the maintenance of methylation during DNA replication [9–11], and DNMT3a and 3b, which establish de novo methylation patterns [12]. DNTM3L, which is related to

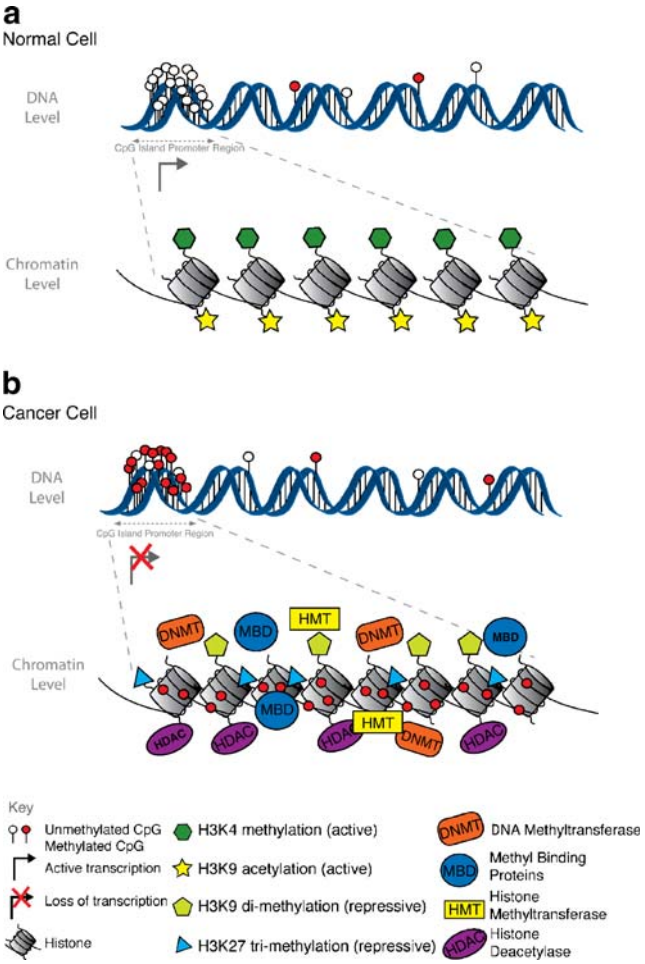

Fig. 1 Epigenetic modifications occurring in a tumor-suppressor gene during the transition from a normal cell to a cancer cell. **a** In the normal cell, actively expressing tumor-suppressor genes are characteristically unmethylated at the CpG island-associated promoter region, and are associated with the active histone 3 lysine 4 (H3K4) methylation and histone 3 lysine 9 (H3K9) acetylation chromatin marks. **b** In the cancer cell, tumor-suppressor genes are silenced, which is associated with extensive DNA hypermethylation (caused by DNMT1) of the CpG island-associated promoter region. Repressive proteins, such as histone deacetylases (HDAC) and histone methyltransferases (HMT) which deacetylate H3K9, and methylate H3K9 and histone H3 lysine 27 (H3K27) residues, respectively, are recruited in conjunction with methyl-binding proteins (MBD), which cooperatively work to enforce the silenced, compacted state of the chromatin

DNMT3a and 3b in sequence, stimulates de novo DNA methylation by interacting with DNMT3a [13], and this activation has recently been shown to be dependent on specific histone modifications [14]. DNA methylation-induced gene silencing is thought to occur by either blocking transcription factor binding [15, 16] and/or by binding specific proteins such as methyl CpG binding protein 2 (MeCP2) and histone de-acetylases (HDAC) which restricts the accessibility of the DNA component of chromatin, preventing transcription from occurring [17].

Gene silencing of tumor suppressor genes is frequently associated with hypermethylation of the CpG island-associated promoter region, but it is unknown what the initial trigger is. There are two conflicting proposals; firstly that DNA hypermethylation triggers gene silencing in cancer [18, 19] and, secondly, that prior gene silencing promotes the recruitment of the DNA methylation machinery and subsequent DNA hypermethylation [20–23]. Recently, antisense RNA has been shown to function in triggering heterochromatin formation, independent of DNA methylation, resulting in transcriptional silencing of tumor suppressor genes [24]. An understanding of the molecular events associated with DNA hypermethylation has grown over the last 10–20 years; however, less is known about the process leading to DNA hypomethylation and its association with cancer initiation and progression. It is well known that neoplasia is characterized by global demethylation relative to the normal cell, in conjunction with regional hypomethylation and hypermethylation of specific genes [25–27]. Hypomethylation changes of the genome typically affect the intergenic and intronic regions of the DNA, particularly at repeat sequences and transposable elements, and is believed to be involved in genome rearrangement and the chromosome instability pathway in neoplasia (reviewed in [28]) ultimately resulting in increased gene transcription [29, 30].

Chromatin remodeling

Chromatin consists of nucleosomes, each containing 147 bp of DNA wrapped around an octomer of the core histone proteins H3, H4, H2A, and H2B [31]. The tertiary structure of chromatin and the activity of the associated gene is directly mediated by numerous modifications of the chromatin components. Specifically, the chromatin structure around unmethylated CpG island promoters in normal cells permits proteins that promote transcription to access the gene, whereas a methylated CpG island in a cancer cell is associated with tightly compacted chromatin which prevents protein access and subsequent gene expression, as shown in Fig 1b [32].

Chromatin histone residues undergo posttranslational modifications at the N-terminal tails, which alters the ability for the DNA to interact with nuclear proteins. There are currently eight described classes of histone modifications, acetylation, lysine methylation, arginine methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deamination, and proline isomerization, which function to regulate various processes such as transcription, repair, replication, and condensation (reviewed in [33]). These modifications are directed to histone residues by various histone-modifying enzymes, and enzymes that act to remove the modification have also been identified. Recently, a new nomenclature system has been proposed to bring consistency and simplicity to the three main chromatin-modifying enzymes; the Lysine Demethylases (K-Demethylases), Lysine Acetyltransferases (K-Acetyltransferases), and Lysine Methyltransferases (K-Methyltransferases) [34].

With regard to transcriptional control, histone acetylation is primarily associated with activation (Fig. 1a) [35], whereas lysine methylation correlates with both activation and repression, depending on the lysine residue that has been modified. For example, methylation of histone 3 lysine 4 (H3K4) is associated with active and open chromatin (Fig. 1a), whereas histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27) methylation are associated with inactive and compacted chromatin (Fig. 1b) [36, 37]. The recent discovery of bivalent domains, those possessing both active (H3K4 methylation) and repressive (H3K27 methylation) modifications, has complicated the demarcation between active and repressive marks, and have thus been proposed to poise genes in a "transcription-ready state" which permits expression plasticity [38].

In addition to repressive histone modifications, the chromatin in a cancer cell is bound by a myriad of repressive histone-associated protein molecules, which enforce the compacted and silent state of the gene (Fig. 1b). These include HDACs, which deacetylate lysine residues prior to their subsequent methylation by histone methyltransferases (HMTs), DNMTs and methyl-C binding (MBD) proteins [29]. More recently, the Polycomb Group (PcG) proteins, which were initially described to play an essential role in long-term gene silencing in *Drosophila*, have also been shown to be involved in gene silencing in mammalian cancer cells (reviewed in [39]). The PcG proteins can cooperatively work together or with enzymes such as HDACs and HMTs to modify the histone tails. PcG proteins are classified into two groups, Polycomb Repressive Complex 2 (PRC2), which is involved in the initiation of gene repression, and Polycomb Repressive Complex 1 (PRC1) that acts as a "maintenance" complex. EZH2 (Enhancer of Zeste homolog 2), a member of PRC2, catalyses the addition of methyl groups to H3K27 (H3K27 trimethylation). EZH2 also interacts with DNMTs and is essential for DNA methylation of EZH2-target promoters [40], suggesting that there is a direct link between PcG-mediated gene repression and DNA methylation.

Epigenetic regulation of genes in breast cancer

Traditional molecular biology has concentrated on identifying genetic biomarkers that are silenced in breast cancer, but more recently, focus is directed to discovering new biomarkers that are epigenetically silenced early in carcinogenesis. Interestingly, almost half of the tumor suppressor genes that cause familial cancers via mutations can also be inactivated in association with promoter hypermethylation in sporadic cancers [4]. Table 1 summarizes a list of over 100 individual

Table 1 Genes that are epigenetically regulated by DNA hypomethylation or hypermethylation in breast carcinogenesis

Gene	Official gene name	Hypo/Hyper	Reference
ARH1	LDLRAP1 (low density lipoprotein receptor adaptor protein 1)	Hypo	[97]
		Hyper	
BCSG1	Synuclein (breast cancer-specific protein 1)	Hypo	[54]
CAV1	Caveolin 1	Hypo	[98]
		Hyper	[99]
CDH3	Cadherin 3, P-cadherin	Hypo	[55]
NAT1	N-acetyltransferase 1	Hypo	[100]
SAT2	Spermidine/spermine N1-acetyltransferase family member 2	Hypo	[101]
UPA	Plasminogen activator, urokinase	Hypo	[56]
14–3–3sigma	Stratifin (SFN)	Hyper	[102, 103]
ABCB1	ATP-binding cassette, sub-family B, member 1	Hyper	[104]
AK5	Amnionless homolog	Hyper	[105]
AMN		Hyper	[105]
APC	Adenomatous Polyposis Coli	Hyper	[106]
BCL2		Hyper	[104]
BRCA1	Breast Cancer 1, early onset	Hyper	[49, 50]
CALCA	Calcitonon-related polypeptide alpha	Hyper	[104]
CCND2	Cyclin D2	Hyper	[107]
CDCP1	CUB domain containing protein 1	Hyper	[108]
CDH1	Cadherin 1, E-cadherin	Hyper	[109]
CDH13	Cadherin 13, H-cadherin	Hyper	[110]
CDKN1C	P57	Hyper	[111]
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16, p14ARF)	Hyper	[104]
CEBPD	CCAAT/enhancer binding protein	Hyper	[112]
CLCA2	Chloride channel, calcium activated, family member 2	Hyper	[113]
CST6	Cystatin E/M	Hyper	[114, 115]
Cx26	Connexin 26	Hyper	[116]
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	Hyper	[104]
DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein	Hyper	[117]
DAL1	Erythrocyte membrane protein band 4.1-like 3	Hyper	[118]
DAPK	Death Associated Protein Kinase 1	Hyper	[104]
DCC	Deleted in colorectal carcinoma	Hyper	[105]
DLC1	Deleted in liver cancer 1	Hyper	[119]
DSC3	Desmocollin 3	Hyper	[120]
ESR1	Estrogen Receptor 1	Hyper	[104]
ESR2	Estrogen Receptor 2	Hyper	[104]
FHIT	Fragile histidine triad gene	Hyper	[121]
FOXA2	Forkhead box A2	Hyper	[105]
GPC3	Glypican 3	Hyper	[122]
GREM1	Gremlin 1	Hyper	[123]
GSTP1	Glutathione S-transferase pi	Hyper	[104, 124]
HIC-1	Hypermethylated in cancer 1	Hyper	[125]
HOXA5	Homeobox A5	Hyper	[126]
HOXD11	Homeobox D11	Hyper	[105]
HRAS	Harvey rat sarcoma viral oncogene homolog	Hyper	[104]
HS3ST2	Heparan sulfate 3-*O*-sulfotransferase 2	Hyper	[127]
HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4	Hyper	[104]
hTERT	Telomerase reverse transcriptase	Hyper	[104, 128]
ID4	Inhibitor of DNA binding 4	Hyper	[129]
IGFBP3	Insulin-like growth factor binding protein 3	Hyper	[130]
KLK10	Kallikrein-related peptidase 10 (NES1)	Hyper	[131, 132]
KLK6	Kallikrein-related peptidase 6	Hyper	[133]
LAMA3	Laminin, alpha 3	Hyper	[134]
LAMB3	Laminin, beta 3	Hyper	[134]
LAMC2	Laminin, gamma 2	Hyper	[134]
LATS1/LATS2	Large tumour suppressor, homolog 1/2	Hyper	[135]

Table 1 (continued)

Gene	Official gene name	Hypo/Hyper	Reference
MCJ		Hyper	[104]
MGMT	*O*-6-methylguanine-DNA methyltransferase	Hyper	[104]
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	Hyper	[104]
MYOD1	Myogenic differentiation	Hyper	[104]
PAX5	Paired box 5	Hyper	[136]
PCDH10	Protocadherin 10	Hyper	[137]
PGR	Progesterone receptor	Hyper	[138]
PLAGL1	Pleiomorphic adenoma gene-like 1	Hyper	[139]
PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	Hyper	[140]
PTGS2	Prostaglandin-endoperoxide synthase 2 (Cox-2)	Hyper	[104]
RAD9	RAD9 homolog A	Hyper	[141]
RAR-β	Retinoic acid receptor, beta	Hyper	[142]
RARRES1	Retinoic acid receptor responder (tazarotene induced) 1	Hyper	[143]
RASSF1A	Ras associated domain family 1	Hyper	[144]
RBP1	Retinol binding protein 1	Hyper	[145]
RIZ1	PR domain containing 2, with ZNF domain (PRDM2)	Hyper	[146]
RNR1	Mitochondrially encoded 12S RNA	Hyper	[142]
ROBO1	Roundabout, axon guidance receptor, homolog 1	Hyper	[147]
RUNX3	Runt-related transcription factor 3	Hyper	[123, 148]
SCGB3A1	Secretoglogin, family 3A, member 1 (HIN-1)	Hyper	[149]
SERPINB5	Serpin peptidase inhibitor, clade B, member 5	Hyper	[150]
SFRP1	Secreted frizzled-related protein 1	Hyper	[151, 152]
SIM1	Single-minded homolog 1	Hyper	[105]
SLIT2	Slit homolog 2	Hyper	[153]
SOCS1	Suppressor of cytokine signalling 1	Hyper	[142]
SRBC	Protein kinase C, delta binding protein (PRKCDBP)	Hyper	[154]
SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	Hyper	[155]
SYK	Spleen tyrosine kinase	Hyper	[156]
TDH	L-threonine dehydrogenase	Hyper	[105]
TFF1	Trefoil factor 1	Hyper	[142]
TGF-β R2	Transforming growth factor β receptor 2	Hyper	[142]
THBS1	Thrombospondin 1	Hyper	[142]
TIMP3	TIMP metallopeptidase inhibitor 3	Hyper	[58]
TMEFF2	Transmembrane protein with EGF-like and two follistatin-like domains 2	Hyper	[123]
TMS1	PYD and CARD domain containing (PYCARD)	Hyper	[157]
TNFRSF12	Tumour necrosis factor receptor superfamily, member 25	Hyper	[142]
TPM1	Tropomyosin 1	Hyper	[158]
TSC1	Tuberous sclerosis 1	Hyper	[159]
TSC2	Tuberous sclerosis 2	Hyper	[159]
TSLC1		Hyper	[118]
TSPAN-2	Tetraspanin 2	Hyper	[105]
TWIST1	Twist homolog 1	Hyper	[160]
TYMS	Thymidylate synthetase	Hyper	[142]
WIF1	WNT inhibitory factor 1	Hyper	[161]
WRN	Werner syndrome	Hyper	[162]
WT-1	Wilms tumour 1	Hyper	[163]
XT3	Solute carrier family 6, member 20 (SLC6A20)	Hyper	[105]

candidate genes that have been reported to be commonly hypo- and hypermethylated in breast cancer (see http://www.pubmeth.org [41] for a more detailed summary).

Of these genes, BReast CAncer 1, early onset *(BRCA1)* is the most commonly mutated gene in familial breast and ovarian cancers, with 63 distinct germ line mutations being identified in a study of more than 100 patients [42]. However, somatic mutations of *BRCA1* are virtually undetectable in sporadic breast tumors and are rarely detected in sporadic ovarian tumors [43–48]. Instead, studies have linked inactivation of *BRCA1* by promoter hypermethylation as a common event in sporadic breast and ovarian cancer [49, 50].

Another example of a genetically and epigenetically regulated gene is cyclin-dependent kinase inhibitor 2A (*CDKN2A, p16^{INK4A}*)[51]. *p16^{INK4A}* is responsible for maintaining the retinoblastoma (Rb) protein in an active and non-phosphorylated state in the cyclinD-Rb pathway. Cell cycle control by this pathway is often lost in human cancer, typically by disruption of *p16^{INK4A}* function via deletion [52], point mutations [52], or promoter hypermethylation [53]. Loss of *p16^{INK4A}* transcription associated with aberrant CpG island methylation has been shown to occur frequently in breast cancer cell lines (33%), as well as in primary breast tumors (31%) [53]. In addition to DNA hypermethylation, DNA hypomethylation of genes including *Synuclein* [54], *P-cadherin* [55], and *Urokinase Plasminogen Activator* (UPA) [56] has also been associated with breast cancer. There are clearly many more genes that are deregulated in breast cancer, and the challenge is to dissect which genes are critically relevant.

Cell lines versus primary cells: the need for a better system

One of the biggest obstacles in dissecting the early molecular events in cancer initiation is that once a tumor is large enough to detect, the genes responsible for this initiation have already undergone aberrant epigenetic suppression or activation. In particular, this is a challenge in the identification of early epigenetic biomarkers that are associated with cancer initiation. Traditional laboratory studies typically make use of readily available cultured cancer cell lines for gene discovery, but genomic methylation patterns of cultured tumor cells can often differ significantly from those found in primary tumors as culture conditions promote and accelerate further epigenetic changes [53, 57–60]. It has been proposed that the excessive levels of hypermethylation seen in some cancer cell lines is a consequence of the culturing process, rather than a reflection of the cell of origin [53, 60–63]. Smiraglia et al. [64] compared hypermethylation levels between cancer cell lines and primary malignancies and, found that in all cases, the cancer cell lines exhibited significantly higher levels of CpG island hypermethylation than their respective primary tumor. Specifically, colon cancer cell lines exhibited a fivefold increase in hypermethylation, and in the most extreme case, head and neck squamous cell carcinoma cell lines showed a 93-fold increase [64]. In contrast, Ueki et al. [65] have demonstrated concordant aberrant methylation in 96% of examined pancreatic carcinomas before and after in vitro culturing, and concluded that the methylation events occurring in the cell lines was a true reflection of the methylation events in the corresponding tumors [65]. Nonetheless, identification of DNA methylation changes that occur early in primary tissue are critically important, and if studies performed with cultured cells cannot be reproduced in primary samples, then the observations may not be relevant in the clinical setting. What is needed are cell model systems for identifying genes that are aberrantly epigenetically regulated and that more accurately reflect the events occurring in early carcinogenesis.

Normal human mammary epithelial cells: a model system to study breast cancer

Human mammary epithelial cells (HMECs) isolated from disease-free breast tissue provide a unique and informative system to study early events in breast tumorigenesis. Experimental transformation of HMECs, by the introduction of activated oncogenes, has been used to investigate the transition from a premalignant to a malignant state [66, 67], as well as studying the tumorigenic potential of such immortalized cells when implanted into immunocompromised mice [68]. Spontaneously immortalized breast epithelial cells derived from fibrocystic breast disease tissue has also been used to model the transition from preinvasive to invasive phenotypes [69]. However, using immortalized cells as a model system to study breast carcinogenesis fails to facilitate the identification of the very early molecular aberrations involved in disease initiation. This is because such transformed cells fail to account for the significant preexisting genetic and epigenetic changes that exist in the cells prior to transformation [70]. What is needed is a naturally occurring cell model system, which accurately reflects the critical events associated with cancer initiation, prior to malignant transformation, in order to gain a better understanding of the molecular mechanisms involved.

When grown in serum-free medium, HMECs do not follow the normal paradigm of senescence typical of human fibroblasts. A subpopulation of HMECs often undergo an initial growth phase, followed by a transient growth plateau termed “selection” (Fig. 2a) [71, 72]. The majority of HMECs at selection resemble human mammary fibroblasts in senescence, in that both cell populations have a low proliferation and death index, express senescence-associated β-galactosidase [73] and have maintained genomic integrity and a normal 2N/4N DNA content ratio [74]. However, unlike fibroblasts, small clusters of refractile HMECs with a basal mammary epithelial phenotype [75, 76] (termed post-selection or variant cells) spontaneously emerge from the selection stage to undergo further proliferation for another 20–40 generations before entering a second growth plateau crisis (Fig. 2a) [72, 77]. The cell morphology is different between pre- and post-selection HMECs (Fig. 2b) [77, 78], and spontaneous immortalization has never been observed

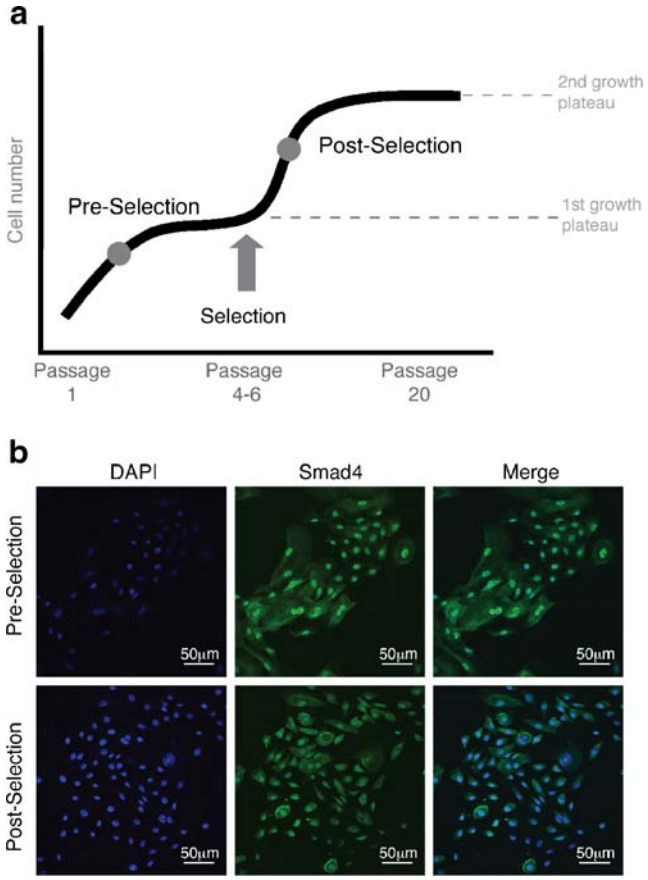

Fig. 2 The human mammary epithelial cell (HMEC) model system. **a** Schematic diagram showing the two phases of growth that HMEC undergo when grown in serum-free medium. The first phase is termed "preselection", after which growth temporarily ceases at the "selection" growth plateau. A subpopulation of cells escape the first growth plateau and continue to proliferate throughout the second growth phase known as "post-selection" before entering a second growth plateau. Approximate passage numbers are shown along the base of the diagram. **b** Smad4 immunostaining of patient Bre-38 pre- and post-selection HMECs, which highlights the differences in morphology between the two cell populations. Preselection cells display predominantly Smad4 nuclear staining (intact TGF-β signalling pathway), whereas post-selection cells show predominant cytoplasmic localization (disrupted TGF-β signalling pathway). Blue signal corresponds to 4′,6-diamidino-2-phenylindole (DAPI), and the green signal represents Smad4 expression

in cells that were not mutagenized or virally transduced during pre- or post-selection growth phases [70], unlike the human breast epithelial cell line described by Rizki et al. [69]. By studying genomic integrity and telomere function of pre- and post-selection HMECs, Romanov et al. [74] found that HMECs in the first growth plateau are fundamentally different from those at the second growth plateau. Post-selection cells have many of the hallmarks of cell crisis and exhibit many types of chromosomal abnormalities that are seen in the earliest lesions of breast cancer. Such abnormalities include a variety of translocations, deletions, other rearrangements, telomeric associations, polyploidy, and aneuploidy [74]. The total number of abnormalities in post-selection cells accumulates rapidly as the HMECs approach the second growth plateau, typically occurring in the final 10–20 population doublings of culture. Both the percentage of abnormal metaphases and the number of abnormalities per metaphase increase with population doublings, and in the final passages, polyploidy can be as high as 25% [74]. This increase in chromosomal abnormalities was shown to be independent of patient age and total proliferative potential of the cells [74]. It has been suggested that post-selection HMECs are in a state similar to telomere-based crisis, as they emerge from selection with eroding telomeric sequences until a critical length is reached at the second growth plateau [74].

Epigenetic regulation of genes in human mammary epithelial cells

Post-selection HMECs exhibit many preneoplastic characteristics, including transcriptional silencing of the $p16^{INK4A}$ tumor suppressor gene and overexpression of cyclo-oxygenase 2 (*Cox-2*) [62, 74, 77, 79–81]. Epigenetic deregulation of $p16^{INK4A}$ occurs commonly in premalignant lesions and rare foci of morphologically normal epithelial cells exhibiting $p16^{INK4A}$ methylation have been identified in vivo in disease-free breast tissue [79]. It has been postulated that these foci are cancer precursors, which can promote malignancy with additional epigenetic and/or genetic changes [82]. We and others have also previously shown that $p16^{INK4A}$ silencing in post-selection cells is associated with DNA hypermethylation of the CpG island promoter, and this occurs during early selection [62, 74, 77, 79–81].

Using the HMEC system to dissect the molecular events, Reynolds et al. [83] found that PcG proteins were involved in $p16^{INK4A}$ silencing. They showed that *SUZ12* and *EZH2* mRNA levels were upregulated four- and ninefold, respectively, in post-selection HMECs when compared to preselection HMECs. In support of this study, we also found that both *EZH2* and *BMI-1* (a member of PRC1) are overexpressed in post-selection HMECs and in breast tumor cells (Fig. 3). Interestingly, we found that $p16^{INK4A}$ which is methylated in post-selection HMECs, is enriched in tri-methylated H3K27 histones in preselection HMECs (Fig. 4) [84], a mark typically associated with chromatin repression. Tri-methylated H3K27 has recently been postulated to act as a "mark" in normal cells on unmethylated CpG islands for subsequent recruitment of DNMTs, which results in de novo methylation in cancer cells [85]. Polycomb-mediated silencing in post-selection HMECs has also been implicated in *HOXA9* methylation silencing, and this is also associated with SUZ12 and tri-methylated H3K27 enrichment, together with DNMT1, DNMT3a, and DNMT3b [83].

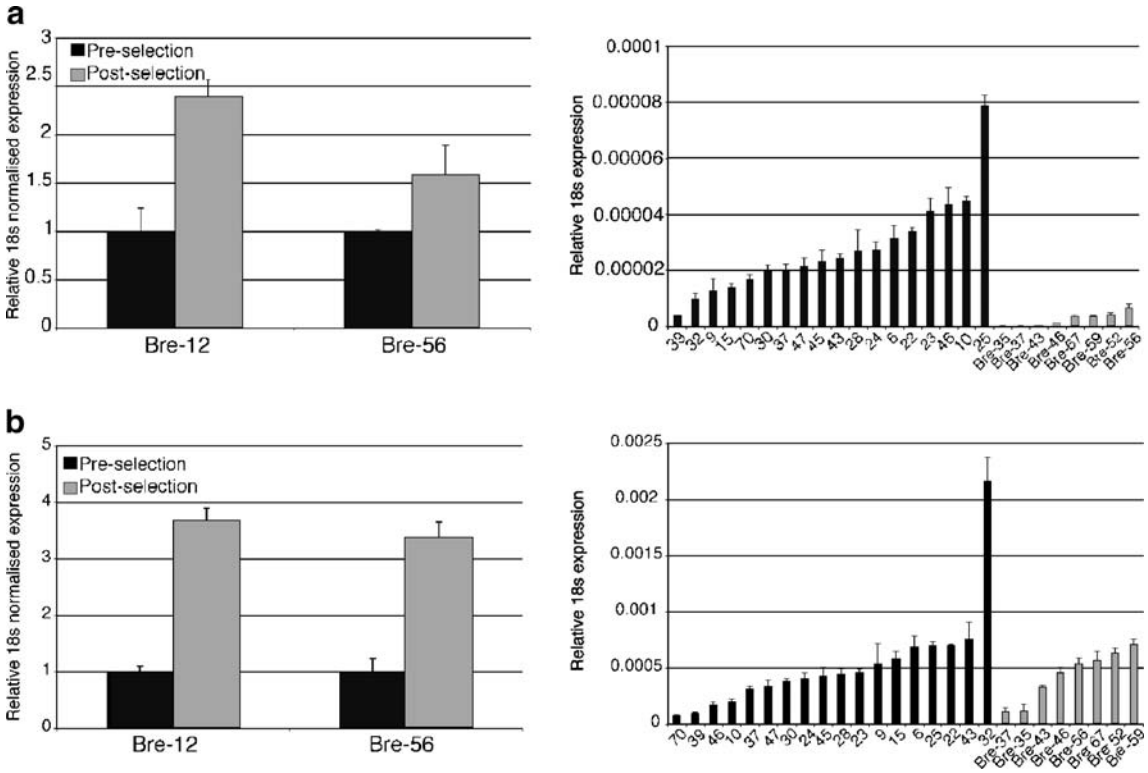

Fig. 3 Expression of Polycomb (PcG)-associated genes in breast cancer. mRNA levels of **a** *EZH2* and **b** *BMI-1* were determined by quantitative RT-PCR in pre- and post-selection HMECs (patients Bre-12 and Bre-56) as well as 18 breast tumors (tumors 39, 32, 9, 15, 70, 30, 37, 47, 45, 43, 28, 24, 6, 22, 23, 46, 10 and 25) and eight normal breast tissue samples (Bre-35, Bre-37, Bre-43, Bre-46, Bre-67, Bre-59, Bre-52, Bre-56). Expression levels were normalized to 18S rRNA and made relative to preselection HMECs (**a**)

The HMEC system has also been used to demonstrate concordant epigenetic silencing of an entire signaling pathway [84]. Our recent study showed that members of the TGF-β signaling pathway, namely, *TGF-β2*, the receptors *TGF-βR1* and *TGF-β2R2*, and the activator *THBS1*, are concordantly epigenetically suppressed in post-selection HMECs. This suppression was associated with a marked decrease in Smad4 nuclear staining (Fig. 2b and [84]), which indicates that the signaling pathway was also functionally disrupted in the cells that escape selection. Interestingly, we identified that the epigenetic suppression of these genes seen in the post-selection HMECs were also suppressed in breast cancer cells (MDAMB453). In both HMECs and breast cancer cells, the suppression was due to deacetylation and methylation of H3K9 residues, independent of DNA methylation [84]. Despite being unmethylated, the expression of the *TGF-β* pathway genes in MDAMB453 cells could be induced with epigenetic drug treatment [84] indicating epigenetic-based therapies will be important in activating gene pathways suppressed in cancer. Interestingly, the *TGF-β* pathway-associated genes were also enriched for tri-methylated H3K27 antibody in the preselection HMECs, despite the fact that they do not become methylated following selection [84]. Indeed, Ohm et al. [38] and Widschwendter et al. [86] have previously reported that many epigenetically silenced genes in cancer are also enriched for tri-methylated H3K27 in stem and progenitor cells, even in the absence of DNA hypermethylation. This supports the growing observation that polycomb-mediated silencing either potentiates hypermethylation in tumor cells or heterochromatin formation in the absence of hypermethylation [87].

Are post-selection HMECs derived from stem or cancer progenitor cells?

Stem cells have enormous replicative potential and capacity for self-renewal and give rise to many different cell lineages. The recent purification and prospective isolation of mouse mammary stem cells [88, 89] have now opened the door to the suggestion that breast stem cells, stem-like cells, or progenitor cells that are equivalent to cancer stem cells, are likely to play a critical role in initiating breast tumors. The discovery that post-selection HMECs can exist in vivo [79] has created much debate as to the true identity

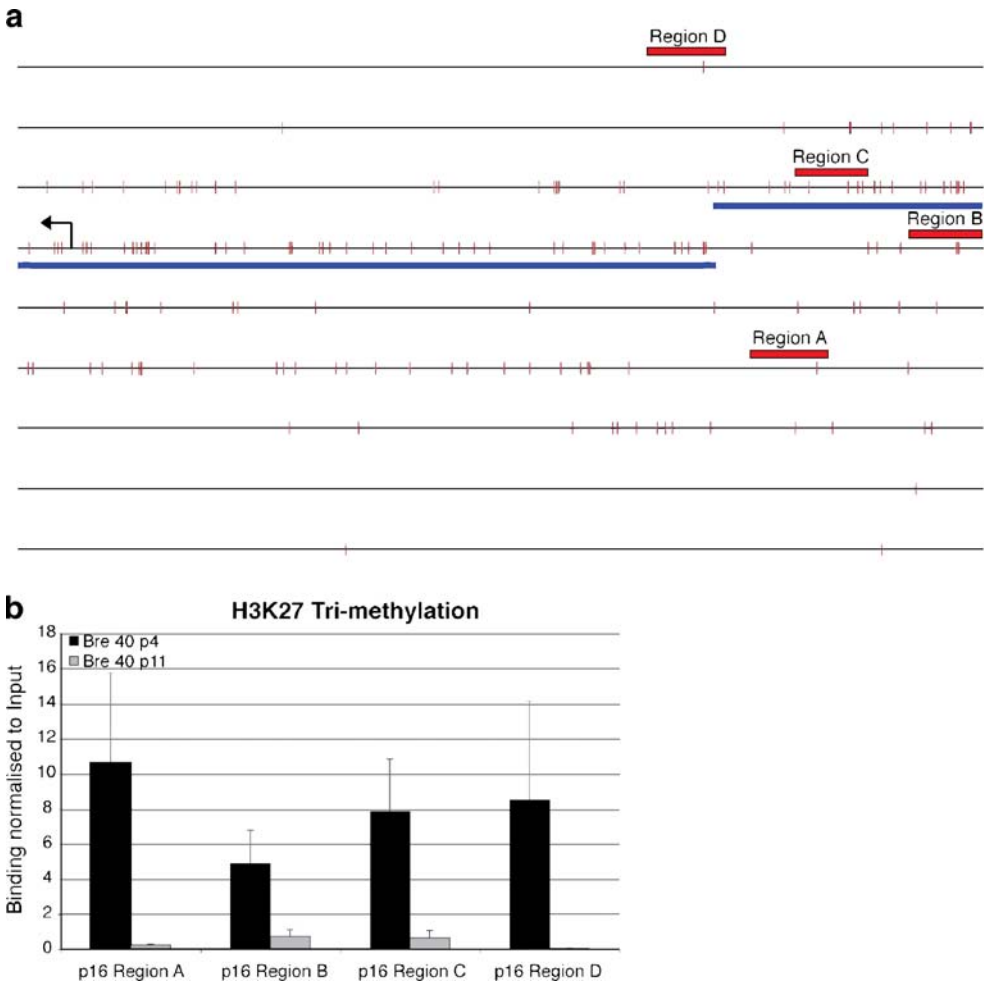

Fig. 4 Preselection HMECs are enriched for H3K27 tri-methylation. **a** Chromatin immunoprecipitation (ChIP) PCR primers were designed to four different regions (Regions A-D) spanning the $p16^{INK4A}$ CpG island-associated promoter region. **b** Chromatin from patient Bre-40 pre- (passage 4) and post-selection (passage 11) HMECs was immunoprecipitated with H3K27 tri-methylated antibody (Upstate). The amount of immunoprecipitated DNA was quantified by real-time PCR and was calculated as a ratio of immunoprecipitated DNA to the total amount of input DNA

of these cells that overcome "selection." That is, are the relatively homogenous population of cells that possess selective growth advantage in culture and many of the hallmarks associated with cancer actually enriched in stem or cancer progenitor cells? Studies in mice and *Drosophila* have shown that the PcG proteins Bmi-1 and E(z) (the homolog of EZH2), respectively, are involved in the maintenance of stem cells. Specifically, BMI-1-mediated suppression of $p16^{INK4A}$ and $p19^{ARF}$ has been shown to promote neural stem cell self-renewal and neural development, as well as being essential for the generation of self-renewing adult hematopoietic stem cells [90, 91], whereas E(z), has been shown to be critical in maintaining epigenetic patterns of pluripotent stem cells [92, 93]. We have shown that both *BMI-1* and *EZH2* are overexpressed in post-selection HMECs and in breast tumors (Fig. 3), which lends support to the proposal that these cells share some of the features of stem or progenitor cells. Only with further characterization of cell surface markers, and subsequent cell sorting experiments and in vivo studies to isolate potential progenitor cells will a definitive conclusion be made as to the nature of origin of these cells.

Conclusions and future challenges

This review presents a summary of research that supports normal human mammary epithelial cells as a potential resource to identify early epigenetic aberrations that may be involved in breast cancer initiation. A small population of HMECs emerge from selection and approach a second growth arrest exhibiting many cancer-related chromosomal abnormalities. A defining feature of these cells is that they exhibit epigenetic silencing of critical tumor suppressor genes, which therefore become very attractive therapeutic targets as both DNA methylation and histone modifications can be reversed by drug regimes [94, 95]. The identification that post-selection cells can exist in vivo supports the proposal that these cells may resemble a stem or progenitor cell population, or perhaps premalignant lesions before morphological aberrations take place. Despite this evidence, the proposal that post-selection cells may resemble breast cancer precursors has been criticized in the literature [96]. The identification of cell surface markers and subsequent cell sorting experiments will allow the preexisting post-selection cells to be isolated both in vitro and in

vivo for further characterization as well as addressing this very topical question. Nevertheless, HMECs are clearly the closest model system that researchers currently have access to that possess the potential to delineate the early epigenetic events that may be occurring in the initiation of breast carcinogenesis.

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