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Review

Immortalization protocols used in cell culture models of human breast morphogenesis

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Abstract. Defining the key players in normal breast differentiation is instrumental to understanding how morphogenesis becomes defective during breast cancer progression. During the past 2 decades much effort has been devoted to the development of technologies for purification and expansion of primary human breast cells in culture and optimizing a relevant microenvironment, which may help to define the niche that regulates breast differentiation and morphogenesis. In contrast to the general property of cancer, normal human cells have a finite lifespan. After a defined number of population doublings, normal cells enter an irreversible proliferation-arrested state referred to as replicative senescence. To overcome this obstacle for continuous long-term studies, replicative senescence can be bypassed by treatment of cells with chemical agents such as benzopyrene, by radiation or by transfection with viral oncogenes or the gene for human telomerase (human telomerase reverse transcriptase, hTERT). A drawback of some of these protocols is a concurrent introduction of chromosomal changes, which sometimes leads to a transformed phenotype and selection of a subpopulation, which may not be representative of the tissue of origin. In recent years, we have sought to establish immortalized primary breast cells, which retain crucial characteristics of their original in situ tissue pattern. This review discusses various approaches to immortalization of breast-derived epithelial and stromal cells and the application of such cell lines for studies on human breast morphogenesis.

Key words. Breast morphogenesis; differentiation; immortalization; cell lines.

Introduction

The normal human breast comprizes a branching ductal lobular system lined by an inner layer of polarized luminal epithelial cells and an outer layer of myoepithelial cells separated from the fibroblast-rich stroma by a basement membrane [1]. Disruption of the breast tissue architecture, including the balance between luminal epithelial and myoepithelial cells, is one of the earliest changes seen in breast cancer formation [2] and commonly used by pathologists to classify tumors into subtypes [3]. The luminal epithelial cells have received much attention in the past as the functionally active cell with tissue-specific milk secretion, and as the most likely target cell for carcinogens [4] (reviewed in [1]). This is supported by the fact that the majority of human breast carcinomas express luminal epithelial markers, and as such, most probably are derived from progenitor cells within the luminal epithelial compartment [5, 6]. Myoepithelial cells are present in normal and premalignant breast and in the major-

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ity of carcinoma in situ lesions [7] but only in a minority of invasive breast cancers [8]. In general, myoepithelial tumors are of low malignancy, with the exception of malignant myoepithelioma, which is an aggressive, but rare tumor [8–10]. However, myoepithelial cells have recently attracted more attention along with the recognition of these cells as functionally active in branching morphogenesis and tumor suppression [11–13]. Taking into account the phenotypic and functional difference between luminal epithelial- and myoepithelial cells, it is of utmost importance to unravel the cellular relationship between these two lineages in the normal human breast and their roles in tissue morphogenesis, as this is a prerequiste for understanding breast cancer formation and progression. Furthermore, the breast epithelium is highly dependent on interactions with the surrounding stroma, including the fibroblasts [1, 14]. Understanding the subtleties of morphogenic signalling between cell-cell and cell-stroma will in our opinion, for the major part, rely on well-characterized immortalized breast cell lines and an appropriate cell culture assays that can capture critical aspects in breast structure and function. Studies on mouse mammary glands have provided important information regarding some aspects of mammary gland morphogenesis [15]. However, unlike human breast epithelial cells, mouse mammary cells exhibit a remarkable capacity for cell proliferation in culture and relatively often undergo spontaneous immortalization, a phenomenon almost never seen in primary human breast cells. The discussion of mouse mammary cells has been intensively covered by others [15–18] and is beyond the scope of this review.

A finite lifespan of normal breast cells

During the past 2 decades, the preferred sources of cells for studying human breast differentiation and morphogenesis have been reduction mammoplasties and milk from the lactating gland. This has provided information to an extent where cell-cell and cell-matrix interactions have proved to be important in patterns of the spatial organization of the normal and cancereous breast [13, 19–25]. However, like every other model system, there are advantages and disadvantages of employing primary epithelial cells for culture studies. The major advantage is that the cells do indeed represent the tissue of origin. Furthermore, the cells are cultured for a short period of time, and therefore have limited propensity to undergo transformation as sometimes seen in the long-term culture of immortalized cell lines [26]. The drawbacks, however, include limited access to biopsy material and a finite lifespan of the explanted cells, which may hamper long-term studies. Finally, primary cells may display inter-biopsy variations which may lessen the reproducibility of some of the morphogenic events in culture.

As first described by Hayflick [27], normal cells proliferate in culture until they reach a state where proliferation ceases. This state is named replicative senescence and is an indicator of aging [27, 28]. However, recent studies suggest that early senescence and premature cell death in culture may be attributed to inadequate culture conditions [29]. Indeed, routine tissue culture methods impose a state of oxidative stress on cells which can cause either premature senescence, cell death or adaptation [30]. Consequently, cells that do not adapt may enter growth arrest or die, and only those cells which are capable of adapting to particular culture conditions will survive for longer periods [30]. Forsyth et al. [31] compared the lifespan of fetal vs. adult human lung fibroblasts cultured at ambient (21%) and physiological oxygen concentrations (2–5%), respectively. Interestingly, the growth in low oxygen extended the lifespan of both fetal and adult strains. This is supported by the fact that when cells are grown at low O_2, more cell doublings are possible [32]. The significance of O_2 levels applies to breast epithelium as well. Thus, when breast epithelial cells are cultured at physiological O_2 levels, the expression of the cdk (cyclin dependent kinase) inhibitor p16 is reduced, resulting in delayed senescence [33, 34].

Apparently, however, there is a distinct difference between primary breast epithelium and fibroblasts in terms of growth arrest and senescence in culture [35]. Whereas normal fibroblasts reach the plateau of senescence after approximately 50 population doublings, the isogenic breast epithelial cells can emerge from the first growth plateau [termed selection or mortality stage 0 (M_0)] at a relatively high frequency (1 out of 10^4–10^5 cells). The postselection cells undergo further rounds of doublings before entering the second growth plateau or agonescence [previously termed senescence or mortality stage 1 (M_1)] after ~75 population doublings (see fig. 1. in [35]). Cells that reach agonescence are phenotypically different from cells during selection. These cells have both a high proliferation rate and high death indexes, with no net increase in cell number [35, 36]. At the molecular level the postselection cells have low or undetectable expression of p16 [35]. Surprisingly, postselection cells exhibit chromosomal instability, which is reminiscent of the chromosomal changes observed in premalignant and malignant breast cancer [36, 37]. Recently, Holst et al. [36] have addressed the question whether the postselection epithelial cells originate as a result of proliferation arrest at the first growth plateau or whether these cells already preexist in the breast gland and are thus also present in preselection cell population. They showed that hypermethylation of the p16 promoter occurs in focal patches of the histologically normal breast [36], which indicates that cells with methylated p16 are already present in the preselection phase and that these cells could be more adapted to pass through selection than p16-expressing cells. Irrespective

of source, however, with few exceptions (see below) normal human breast cells will eventually die in culture if no measures are taken to immortalize them.

Spontaneous immortalization of normal breast cells

Established cell lines provide – at least in theory – an almost unlimited supply of cells with similar genotype and phenotype, allowing them to be used for complex continuous long-term studies. Spontaneous immortalization of cultured normal human breast cells is an extremely rare event and has been observed in epithelial cells only. In the few reported cases, the tissue source was not fully normal [38–40]. The HMT-3522 cell line [38] was established from a sample from fibrocystic breast disease and has been widely used as a representative model for normal breast epithelial cells [41]. Like primary luminal epithelial cells, the HMT-3522 cell line form almost phenotypically normal acinus-like structures and growth arrest when cultured in a three-dimensional reconstituted basement membrane (rBM) matrix [23]. A tumorigenic counterpart (designated T4-2) was established by the withdrawal of epidermal growth factor from the culture medium of the parental cell line [42]. In contrast to the parental line, the T4-2 form disorganized, continuously growing colonies in response to rBM stimuli [41]. Interestingly, when treated with function blocking antibodies against either β1-integrin or epidermal growth factor receptor (EGFR), T4-2 cells growth arrest and restore normal tissue morphogenesis [41, 43]. As such the HMT-3522 series is an example of how spontaneous immortalized cells can serve as a model for normal morphogenesis and tumor progression.

Another example is the MCF-10A cell line [39], which was also derived from human fibrocystic breast tissue, and which exhibited immortality after extended cultivation in medium containing low calcium concentration. In this regard, it has previously been demonstrated that decreasing the calcium concentration in the culture media from 1.05 mM to $<$0.06 mM increases the in vitro lifespan of normal breast epithelial cells [44], possibly due to the inhibition of differentiation [45]. MCF-10A has the characteristics of normal breast epithelium by the following criteria: (i) lack of tumorigenicity in nude mice; (ii) three-dimensional growth in collagen; (iii) growth in culture that is controlled by hormones and growth factors; (iv) lack of anchorage-independent growth; and (v) dome formation in confluent cultures [39]. Like the HMT-3522 series, the MCF10A cell line has been extensively used as breast cancer progression model. Thus, the MCF10 AneoN and MCF10AneoT cell lines were created by stable transfection of MCF10A with the neomycin-resistance gene and either the HRAS gene or the mutated T-24 HRAS gene, respectively [46]. These cell lines have been widely used as representatives for breast cancer progression [47, 48]. Finally, Shay et al. [40] established a breast epithelial cell line from adjacent normal tissue from a breast cancer patient with Li-Fraumeni syndrome (harboring a germline mutation in p53). Interestingly, a comparison between epithelial cells and fibroblasts from this patient revealed that only the epithelial cells exhibit spontaneous immortalization [40], indicating an inherent difference between epithelial and stromal cells.

These cell lines have provided invaluable data in the context of breast morphogenesis and breast cancer progression. However, as mentioned earlier, spontaneous immortalization of human breast cells is an extremely rare and unpredictable event which precludes experiments aimed at reproducible immortalization of separate subpopulations of individual cell types of the breast, i.e. luminal or myoepithelial-derived cells.

Ectopic immortalization protocols

To overcome the pre- or postselection growth-arrest barrier, researchers have applied different methods such as carcinogenic agents [49, 50], radiation [51] and viral oncogenes [52, 53], which are discussed below. Carcinogenic agents such as benzoapyrene (BaP) have been widely used to immortalize normal breast epithelial cells [49]. BaP is activated by metabolizing enzymes such as cytochrome P450, which yields intermediate metabolites that are chemically more reactive than the initial compound. Long-term treatment with agents such as BaP results in transformation of cells as evidenced by chromosomal abnormalities, anchorage-independent growth in soft agar and tumorigenicity in nude mice [54]. Also, BaP has been used to establish a series of immortalized cell lines from a finite lifespan human mammary epithelial cell culture (184 HMEC). Initially, these cell lines (termed 184A1 and 184B5) [34, 49] showed little or no telomerase activity, and their telomeres continue to shorten. When the mean telomerase restriction fragment (TRF) length decreases to $<$3 kbp, cell growth becomes slow and heterogeneous, with very low colony-forming efficiencies. These early passages are termed conditional immortal because only a subpopulation of the cells is able to sustain continuous growth [55]. Gradually, these cells show changes to a more uniform growth accompanied by increasing expression of telomerase activity and stabilization of telomere length [55]. These changes are referred to as conversion. In contrast to agonescence or crisis, conversion is characterized by a low labeling index as measured by ^{3}H-thymidine incorporation followed by gradual recovery of moderate proliferation, which indicates that most cells within the population are able to reenter the proliferative phase. This implies that instead of expansion of single mutant cells (selection), the con-

version is dependent on epigenetic changes occurring in culture and accompanied by loss of p16 and p57 expression, suggesting that these genes are possible targets of BaP-induced changes [34]. In prolonged culture, BaP immortalized 184 cell lines develop resistance to transforming growth factor-β (TGF-β) mediated growth arrest, which is commonly seen in established cancer cell lines [34]. The studies on the 184 HMEC series have provided valuable information regarding the cellular changes that occur during selection, agonescence and conversion to the fully immortalized phenotype. However, as with many other protocols even though BaP and other chemical agents have proved successful for establishment of various cell lines, the usefulness of these cell lines for studies on differentiation and normal morphogenesis is questionable, due to the fact that these agents may lead to a complete transformed cellular phenotype [50, 54].

Another consequence associated with early events in BaP-induced immortalization of breast epithelial cells is overexpression of genes such as S100P, a calcium-binding protein and ZNF217, an alternatively spliced form of kruppel-like transcription factors [56, 57]. S100P is overexpressed (4- to 10-fold) in BaP-transformed cell lines and in breast cancer cell lines such as T47D, but not in normal breast epithelial cells. S100P shows homology with the mts-1 gene (metastasis-associated gene), which has been postulated to be involved in p53 sequestration, tubulin depolymerization and G1-S transition [56]. The relevance of ZNF217 is emphasized by the fact that the gene is amplified in breast cancers, and transfection of the ZNF217 oncogene into normal breast epithelial cells results in immortalized cell lines which exhibit patterns of conversion similar to breast cell lines immortalized after exposure to chemical carcinogens [57].

An alternative method of achieving immortalization is by radiation. Radiation has been shown to be sufficient for immortalization of breast epithelial cells [58]. However, immortalization by radiation occurs relatively infrequently and results in morphological transformation of cells (reviewed in [58]) and formation of tumors in nude mice [59]. Thus, similar to carcinogenic transformation, immortalization with radiation may result in a completely transformed phenotype. The availability of isogenic normal and radiation-transformed cells has allowed comparative gene expression analysis that has resulted in identification of a gene that is transcriptionally downregulated during breast cell transformation [60]. This gene, called normal epithelial specific 1 (NES1), is expressed in normal but not radiation-transformed breast epithelial cells [60]. Likewise, NES1 is downregulated in the majority of breast- and prostate-derived cancer cell lines [60, 61].

It has has recently been shown that radiation leads to aberrant morphogenesis of breast epithelial cells in culture [62]. In this study, radiated single cells gave rise to colonies exhibiting aberrant expression of proteins such as E-cadherin, β-catenin and connexin 43 which are necessary to establish polarity and this resulted in disrupted cell-cell and cell-stroma interactions and compromized acinar organization [62]. Moreover, it has been shown that radiation of stromal tissue can promote tumorigenesis of non-irradiated epithelial cells [63], which underscores the importance of the microenvironment in maintaining normal breast architecture.

A number of viral oncogenes, including simian virus-40 (SV40) large T-antigen, adenovirus E1A and E1B, and polyoma T-antigen, have also been used to immortalize human cells [52, 64–66]. In particular, the SV40 large T-antigen has been widely applied for transfection of different cell types [67]. Transfection of cells with SV40 large T-antigen generates cells with extended lifespan as a result of binding and inactivation of protein products of the p53 and retinoblastoma (Rb) genes [68]. However, for the cells to become fully immortalized, additional changes are needed. These additional changes are thought to happen only in a subpopulation of cells expressing the viral oncogenes (ranging from 1 in 10^5 to 1 in 10^9 cells) [69]. Recent data demonstrated that SV40-induced immortalization is accompanied by loss of DNA damage response, karyotypic instability and, in some cases, tumorigenicity [70]. Although cells immortalized by SV40 large T-antigen are under severe selection during the initial growth phase, these cell lines have provided important information about breast differentiation and cancer progression. Thus, Bartek et al. [71] transfected luminal epithelial cells cultured from milk using SV40 large T-antigen. Of the 17 established cell lines, 5 passed through a crisis period as characterized by cessation of growth. When characterized by immunohistochemical staining, 14 cell lines showed some features of luminal epithelial cells, and of these, 7 cell lines resembled the common luminal epithelial cell type in the profiles of keratins expressed such as keratins 7, 8,18 and 19 [71]. Others [53] have shown that primary breast epithelial cells immortalized with SV40 large T-antigen can give rise to a cloned epithelial-like cell line with stem cell characteristics, as evidenced by differentiation to myoepithelial-like cells in culture. When cultured on floating collagen, these cells showed branching morphogenesis with sac-like structures resembling acini in vivo [72]. Kao et al. [73] have shown that only a subpopulation of breast epithelial cells are susceptible to immortalization by SV40. These authors characterized two breast epithelial cell types in culture by morphology and marker expression: type I cells, which express luminal epithelial markers and stem cell characteristics, i.e. the ability to differentiate into other cell types and to form budding structures on rBM matrix, and type II cells, which show a myoepithelial phenotype and form spherical organoids in rBM [73]. Whereas none of the transfected type II cell clones became immortal, 2 of 9 type I cell clones did so [73]. With regard to stromal

cells, immortalization of breast fibroblasts using SV40 large T-antigen alone has been unsuccessful [74]. SV40 large T-antigen has been used to immortalize other cell types such as human proximal tubule epithelial cells. These cell lines retain their differentiation capacity as seen by their ability to form electrically resistant monolayers with apical microvilli, tight junctional complexes and expression of numerous markers for proximal tubule cells [75]. In contrast, preadipocytes show aberrant differentiation after immortalization with SV40 large T-antigen [76]. In these cells, SV40 large T-antigen inhibited the differentiation of preadipocytes into adipocytes due to the ability of SV40 T-antigen to inactivate the p300/CBP (cAMP-response element-binding protein) transcription factor, known to be indispensable for adipocyte differention [76]. Thus, although transfections with SV40 large T-antigen have proved successful for complete immortalization of a number of cell types, most breast cell types need additional changes for complete immortalization.

Immortalization protocols based on hTERT and HPV

Most immortalization protocols discussed so far confer altered geno- and phenotype to the immortalized cells, limiting their application in studies on normal differentiation and morphogenesis. Introduction of other immortalization protocols, including the human telomerase and E6 and E7 oncogenes from human papilloma virus (HPV)-16 have helped to bypass some of the problems encountered by earlier protocols and as such allowed cells to some extent to retain their original phenotypic traits.

hTERT

The discovery of the telomerase enzyme and its subunits has resulted in major progress in studies on cellular proliferation, immortalization and neoplastic transformation [77]. Telomerase is a ribonucleoprotein that has a catalytic subunit with reverse transcriptase activity (hTERT) which synthesizes and maintains the telomeres at the end of chromosomes, helping cells to escape replicative senescence as a result of telomere shortening [78]. Ectopic introduction of the hTERT and subsequent telomerase activation have been shown to extend the lifespan and in some cases immortalize different cell types. Thus, transfection with hTERT is sufficient for immortalization of some human fibroblasts and retinal pigment epithelial cells [79]. However, a number of studies have demonstrated that introduction of hTERT alone is not sufficient for complete immortalization of human breast epithelial cells [36, 70, 80–83]. We have recently transduced a human breast fibroblast cell strain with hTERT with the result of extended lifespan as evidenced by continuous growth for 25 passages before entering senescence. These cells retain the normal phenotype by marker expression and by the ability to contract hydrated collagen gel in an in vitro assay [83]. So far, we have not been able to establish epithelial cell lines with hTERT alone [unpublished]. This is in agreement with studies done by others showing that the expression of hTERT alone significantly extends the lifespan of human fibroblasts, whereas breast epithelial cells require expression of hTERT together with inactivation of the Rb pathway to become immortal [80]. Another possible difference between fibroblasts and epithelial cells is that hTERT may induce premalignancy in fibroblasts. Thus, it has been shown recently that prolonged culture of hTERT-immortalized human embryonic lung fibroblasts leads to a premalignant phenotype as evidenced by loss of contact inhibition and increased sensitivity to ras-induced transformation [84]. Until recently, human fibroblasts were believed to lack hTERT expresssion. However, low transient telomerase expression has now been found in some presenescent human embryonic lung fibroblasts in their transit through the S-phase [85]. This indicates that regulation of telomerase is a dynamic process where active maintenance of telomere is necessary for the cell to continue through the cell cycle [85]. However, the periodic expression of hTERT fails to stabilize overall telomere length [85]. Furthermore, data have shown that early passage hTERT-transfected human breast epithelial cells continuously decrease the length and density of telomeres even in the presence of telomerase activity, with a significant number of cells staining positive for the senescence-associated β-galactosidase [82]. The inability of hTERT to immortalize breast epithelial cells has been linked to upregulation of p16 in culture. It has been suggested that p16 is at least partially responsible for driving cells into senescence. This is supported by the fact that cells which progress through the selection phase lack p16 expression [34]. Recently, Beausejour et al. [86] used lentiviruses to express hTERT in senescent human breast epithelial cells and breast fibroblasts. Expression of telomerase did not reverse the senescence dependent arrest. However, cells with low levels of p16 at senescence resumed robust growth upon p53 inactivation. In contrast, cells with high levels of p16 at senescence failed to proliferate upon p53 inactivation. These data indicate that the senescence response to telomere dysfunction is reversible and is maintained primarily by p53. Thus, p16 provides a dominant second barrier to the unlimited growth of human cells. It has been argued that the senescence-like state in hTERT transfected cells is induced by inadequate culture conditions [29]. This might explain why breast epithelial cells cultured on a feeder layer escape the p16-dependent growth arrest and are more easily immortalized with hTERT [87].

To summarize, hTERT transfection has proved successful for some cell types; however, most cell types, including breast epithelial cells, are usually refractory to this im-

mortalization protocol, at least when cultured under standard conditions.

E6 and E7 oncogenes from HPV16

HPVs are associated with cervical carcinomas and benign genital warts [88]. Transfection of the HPV genome into keratinocytes, a natural host for these viruses, leads to efficent immortalization [89]. It has also been shown that DNA from HPV is highly efficient for immortalization of different cell types, including human breast epithelial cells [90]. The ability of HPV DNA to immortalize cells has been attributed to the actions of E6 and E7 oncogenes and their competence to manipulate cell cycle regulators such as p53 and pRb, respectively [91]. As mentioned earlier, primary breast epithelial cells have two growth plateaus in culture, selection and agonescence [35]. Comparison of pre- and postselection cells has revealed that breast epithelial subtypes show widely differing susceptibility to transfections with E6 and E7. Postselection cells are easily immortalized with E6 alone, whereas preselection cells are refractory to E6 immortalization. Preselection cells exhibit prolonged lifespan when transfected with E7 but only rarely give rise to immortalized cell lines [92]. In contrast, when applied together, E6 and E7 immortalize the majority of all breast epithelial cells in preselection cultures. These epithelial cells are anchorage dependent and do not form tumors in nude mice [90, 92]. The major concern in this respect is that while immortality is achieved by inactivation of p53 and pRb pathways, these may not be the only affected molecules, and other cellular functions may be altered, which can result in genetic instability. We and others have shown that transfection of distinct cell types using HPV16-E6E7 results in established cell lines that bypass crisis and retain most of the differentiation pattern seen in their tissue of origin. We have established a panel of early passage breast epithelial cell lines using E6 and E7 together [5, 6]. Under our culture conditions we only rarely observed crisis as frequently seen in breast epithelial cells immortalized with other agents such as SV40, hTERT, radiation and chemical carcinogens. It has recently been shown that the E6 gene (but not the E7 gene) can induce telomerase expression in human keratinocytes [93, 94]. This concurs with our data, as we have observed induction of telomerase activation in our E6/E7 immortalized epithelial cell lines [5]. It is therefore likely that E6/E7 immortalization is at least partially achieved by E6 induction of hTERT (possibly via inactivation of p53) together with E7 inactivation of Rb. Interestingly, breast-derived fibroblasts [83] and breast microvascular endothelial cells [unpublished] do not activate telomerase expression upon transfection with E6 and E7. These cells exhibit extended lifespan but eventually enter senescence.

In our hands, E6/E7-transfected breast epithelial cells retain much differentation capacity for at least for 35–40 passages (~120 population doublings). We cannot exclude that prolonged culture of E6/E7 transfected epithelial cells may result in increased genomic instability, as has been suggested by others [95, 96]. However, if it is kept in mind that prolonged culture of E6/E7-immortalized cell lines can promote chromosomal changes – a phenomenon also seen in postselection primary breast epithelial cells – we may conclude that these cell lines can be used at least in early passages for studies on breast morphogenesis.

E6- and E7-immortalized cell lines as a model for breast morphogenesis

Recently, we and others have provided evidence that the stem cells of the mouse and human mammary gland may be contained within the luminal epithelial lineage [5, 97–100] (reviewed in [101]). Thus, we have shown previously in primary cultures that a subset of the luminal epithelial cells convert to myoepithelial cells in culture, suggesting the existence of a progenitor cell [99]. In more recent studies, we identified a candidate precursor cell type in the luminal epithelial compartment [5]. Using cell surface markers and immunomagnetic sorting, we isolated and subsequently E6/E7 immortalized two distinct luminal epithelial cell populations from primary cultures of reduction mammoplasties. One subline co-expressed sialomucin (MUC1) and epithelial specific antigen (ESA), whereas another subline derived from a suprabasal subpopulation and expressed ESA but essentially no MUC1. Both cell lines maintained a luminal epithelial phenotype as evidenced by expression of the tight junction proteins clau-din-1 and occludin, and by generation of a high transepithelial electrical resistance on semipermeable filters. In clonal cultures, the $MUC1^+/ESA^+$ epithelial cell line was luminal epithelial restricted in its differentiation repertoire, whereas the suprabasal-derived $MUC1^-/ESA^+$ epithelial cell line was able to generate itself as well as some $MUC1^+/ESA^+$ epithelial cells and Thy-$1^+/\alpha$-smooth muscle $actin^+$ myoepithelial cells. When cultured within a three-dimensional rBM, the $MUC1^+/ESA^+$ epithelial cell line formed acinus-like spheres. In contrast, the $MUC1^-/ ESA^+$ epithelial cell line also formed elaborate branching structures resembling uncultured terminal duct lobular units (TDLU) by both morphology and marker expression. Similar structures were obtained by inoculating the extracellular matrix-embedded cells subcutaneously in nude mice [5]. To narrow down the identity of a candidate stem cell population, we performed an analysis of the markers expressed by the $MUC1^+/ESA^+$ and $MUC1^-/ESA^+$ cell lines. Cytokeratin 19 was identified as a distinctive trait expressed by the $MUC1^-/ESA^+$, as opposed to the MUC^+/ ESA^+ epithelial cells [5]. It has previ-

ously been shown that CK19 is expressed only in a subpopulation of luminal epithelial cells within the TDLU [102]. We have confirmed the limited expression of CK19 in vivo but also demonstrated that some of the $CK19^+$ cells have suprabasal location [5]. Recent evidence indicates that CK19 is expressed by diverse stem cell compartments. Thus, CK19 is one of the earliest keratins expressed in the human embryo [103], and whereas the fetal breast contains homogenous expression of CK19, CK19-negative luminal epithelial cells arise in adulthood [104]. Studies of other organs, including liver, pancreas, skin, testes and prostate, have shown that the stem cell compartments express CK19 [105–110]. This does not imply, however, that all $CK19^+$ cells are progenitor cells. For instance, the entire basal layer of the skin is $CK19^+$, and this by far exceeds the expected number of stem cells. It should be mentioned that we cannot rule out the possibility that the $MUC1^-/ESA^+/CK19^+$ cells may be downstream of the ultimate stem cell of the breast. Nevertheless, $CK19^+$ cells are capable of generating a fundamental epithelial component of the breast [5]. In addition, cells with similar characteristics could be sorted out from primary cultures. This confirmed that the immortalized cells were likely representatives of normal cells. However, the limited yield of these cells also underscored the crucial significance of the use of immortalization for experimental purposes.

We have recently shown that primary myoepithelial cells are necessary for correct polarization of luminal epithelial cells in a three-dimensional collagen assay [6, 13]. We found that laminin-1 and not other extracellular matrix molecules could replace the myoepithelial cells in this assay. Interestingly, the E6/E7-immortalized $Thy\text{-}1^+/\alpha$-smooth muscle $actin^+$ myoepithelial cells were positive for laminin-1 expression and could also facilitate correct polarization of luminal epithelial cells [13]. In contrast, some primary cancer-derived myoepithelial cells as well as a cancer-derived myoepithelial cell line failed to polarize luminal epithelial cells unless they expressed laminin-1 [13]. Most recently, we have generated a bona fide myoepithelial cell line [unpublished] using E6/E7 as evaluated by morphology as well as a significant number of specific markers. Further studies based on these immortalized cell lines with preserved phenotypes may lead to additional insights into aspects of breast morphogenesis in the near future.

Others have reached the conclusion that immortalization of breast epithelial cells with E6/E7 does not lead to severely aberrant behavior [92, 111]. Also, breast epithelial cells transfected with E7 alone show normal polarization when grown in three-dimensional culture [112]. These observations are not unique for cells of the human breast. Thus, it has been shown that endocervical cells transfected with E6/E7 appeared normal with regular stratification [113]. Likewise, human pancreatic epithelial cells transfected with E6/E7 remain polarized on collagen gels and refrain from growth in soft agar [114]. Normal human bronchial epithelial cells transduced with DNA from HPV16 give rise to cell lines with epithelial morphology similar to their primary counterparts [115]. Furthermore, human bone marrow endothelial cells immortalized with E6/E7 essentially maintain an apparent normal phenotype [116]. Finally, some studies have benefitted from the combination of transfection protocols. Primary endometrial cells are particularly difficult to culture and enter senescence within two passages, and this has complicated experimental modelling of the tissue [117]. When transfected with E6/E7, however, endometrial cells exhibit extended lifespan but eventually enter senescence [117]. In contrast to breast epithelial cells [5], but like fibroblasts and breast endothelial cells, endometrial cells do not activate telomerase upon E6/E7 transfection. However, the combination of E6/E7 and hTERT transfection has led to successful immortalization of the endometrial glandular cells. These cells retained a normal phenotype as evidenced by lack of gross chromosomal abnormalities, responsiveness to sex steroid hormones, formation of glandular structure in three-dimensional culture, and the absence of transformed phenotype in the soft agar assay or in nude mice [117].

In summary, even though immortalization protocols based on the expression of oncogenes like E6 and E7 should be used cautiously, we find it reasonable to interpret data related to differentiation, in particular when comparisons with primary cultures and the tissue of origin are made.

Immortalized breast epithelial stem cells and cancer

In addition to studying normal breast morphogenesis, the purpose of generating immortal cell lines has been to study cancer evolution. In this respect, the concept of adult stem cells has become particularly pertinent to cancer biology. Thus, evidence is emerging that characterization of stem cells could provide insights into cancer biology because tumors might contain cancer stem cells that drive cancer cell proliferation (reviewed in [101, 118, 119]). Using a model where human breast cancers were grown in immunocompromised mice, Al-Hajj et al. [120] found that only a minority of breast cancer cells could form new tumors. Surface marker expression, enabled them to isolate a tumorigenic $CD44^+/CD24^{-/low}$ cell population. Further enrichment of tumorigenic activity was achieved by isolating the ESA^+ subset of $CD44^+/CD24^-$ cell population. Interestingly, the $ESA^+/CD44^+/CD24^{-/low}$ tumorigenic cell population continued to generate phenotypic heterogeneity, indicating the inherent plasticity of the cancer stem cells. Therefore, after having observed that our E6/E7-immortalized $ESA^+/MUC1^-$ isolated suprabasal cells exhibited stem-cell-like characteristics

in culture, we sought to establish whether this cell population contained any characteristics that might link normal adult stem cells and breast cancer cells. In the majority of breast carcinomas, the neoplastic epithelial cells stain positive for cytokeratin 19 [21]. Although it could be argued that CK19$^-$ luminal epithelial may have CK19 induced along with malignant transformation, so far all reported transformations of nonmalignant CK19$^-$ breast cell lines have resulted in CK19$^-$ tumor cells [48, 121].

To date, we have established three ESA$^+$/MUC1$^-$ cell lines which all express stem-cell-like characteristics as evidenced by their ability to differentiate into luminal-like and myoepithelial-like cells. We believe that these epithelial stem-cell-like cell lines will provide valuable information in future studies on cancer progression. The potential use of these stem cell lines will hopefully lead to further resolution of the precursor cell compartment to capture the true identity of the actual stem cell and the first and second degree bipotent progenitor cells. This could ultimately result in identification and isolation of cell types showing different degrees of susceptibility to breast cancer development.

Concluding remarks

During the past 2 decades, we have experienced tremendous progress in cell culture technology, including introduction of various immortalization protocols that have been invaluable tools for studies on cell fate and cancer progression. Furthermore, we have seen improvements in culturing cells in microenvironments approaching in vivo-like conditions, rendering current culture assays more physiologically correct than previous assays.

Whereas primary cultures are indispensable for direct comparison between tissues and immediately derived cells, immortalization protocols are necessary for long-term studies and for gaining information about rare cell types – such as human breast epithelial stem cells, which can be difficult to expand in primary culture. Immortalization is important whenever a link between normal and cancer is sought for – in particular when it comes to human cells, where no autologous host for in vivo experiments is available. We have established a number of human breast cell lines based on 'spontaneous' as well as induced immortalization. These cell lines have been invaluable in studies on breast morphogenesis and in dissecting lineage evolution in the normal human breast as well as in the search for the cellular origins of breast cancers. In particular, the combination of established cell lines with primary cultures and three-dimensional culture assays has proved powerful. In the future there will be an increased demand for well-characterized breast cell lines that can capture the cellular plasticity found within the human breast gland, with a view to understanding the plethora of tumor histologies based on the classical hypothesis that tumors are caricatures of normal tissue renewal [122]. In this respect, it remains an enormous challenge to improve culture conditions and to develop new 'minimal' immortalization protocols, leaving as much of the cellular profile intact as possible. In this regard, it will be necessary to fine-tune the immediate cellular microenvironment, including adjustment of serum-free media, oxygen tension and the three-dimensional extracellular scaffold. Furthermore, there is an increased demand for understanding the heterotypic interactions between different cell types in the breast. This necessitates long-term cell culture assays using well-defined cell lines with minimal disturbance. An attractive choice is to develop conditional immortal cell lines – including temperature-sensitive mutants – which offer the possibility of producing human cell lines upon demand and which can revert to their original phenotype whenever needed. Under all circumstances it is becoming increasingly clear that long-term sophisticated physiologically correct model systems are needed for studies on tissue morphogenesis and cancer in complex organs such as the human breast.

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