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REVIEW

Zero to one: normal derived human ER+ cells in culture-proliferating

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Cell culture technology is used to model structural and functional properties of human organs under normal and pathological conditions "in a dish". The most obvious reason to culture human breast-derived cells is our fundamental desire to understand and ultimately treat breast cancer. Highly reproducible serum-free formulations for long-term propagation of normal human breast epithelial cells have existed for more than three decades and have served to complement the insight gained from a vast number of established breast cancer cell lines. The unspoken dichotomy in the experimental approach, however, has lied in the puzzling fact that normal-derived cells show a more myoepithelial expression profile, while breast cancer cells show more of a luminal profile making these difficult to compare experimentally. Moreover, normal estrogen receptor positive (ER+) luminal cells, thought to be equivalents to the most frequent form of human breast cancer, the ER+ subtype, completely fail to grow under standard culture conditions. One might choose to ignore this fact since breast homeostasis relies on a stem cell hierarchy and stem cells reside in the myoepithelial compartment which, if given the right conditions, can differentiate into ER+ luminal cells. The problem with this is that myoepithelial cells in culture, for unknown reasons, fail to behave like myoepithelial cells in vivo. This review summarizes some of the progress that has been made in the field with regard to the ER+ luminal breast epithelial lineage, especially within a human context, and its relevance to human breast cancer.

Keywords: human breast; breast cancer; normal derived; estrogen receptor; immortalized luminal cells

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Introduction

Almost thirty years ago Pierce and Speers published the landmark review "Tumors as caricatures of the process of tissue renewal: prospects for therapy by directing differentiation" [1]. This hypothesis, that tumors reflect an aberrant differentiation regime, was developed based on the assumption that cancer stem cells emerge from normal stem cells with the capacity for terminal differentiation. Indeed, experimental evidence has been provided for a stem cell origin of cancer cells in tissues with rapid cellular turnover [2]. Whether this is also the case in tissues with relatively slow cellular turnover, such as in the human breast, remains an open question. It has been argued that the requirements for normal cells to be susceptible to tumor initiation are fulfilled if the cells divide asymmetrically, and if one of the

progeny remains long enough in the tissue to sustain and accumulate oncogenic mutations [3]. Aside from breast stem cells residing in the myoepithelial compartment [4], at least one other luminal cell type presumably stays long enough in the tissue to become classified as a candidate precursor cell, namely the label-retaining ER+ cell [5].

In line with this insight, breast cancer classification increasingly relies on taxonomy based upon normal cell phenotypes [6]. Furthermore, subtype-specific cell-of-origin hypotheses are integrated into emerging stem cell hierarchies of the human breast [7-9]. For these reasons, the ER+ cell in the normal human breast becomes increasingly important if we wish to understand the evolution of the most frequent type of human breast cancer i.e. the ER+ subtype. The generation of ER+ breast cancer still poses many questions in the field. Is ER+ breast cancer derived from an ER+ normal equivalent or a candidate precursor cell? At what level of differentiation within the hierarchy can ER+ breast cancer develop? In mice, ER+ tumors develop from both luminal and myoepithelial cells-of-origin if destabilized by oncogenic PI3K [10, 11]. However, tumors originating in mice are not similar to typical human luminal breast cancer [12, 13]. Questions such as these require normal cell culture models that can be sequentially transformed faithfully imitating a luminal breast cancer subtype, most importantly, by using human cells. Attempts to reach this goal have been hampered by the relatively low number of ER+ cells in the human breast [14], lack of surface markers for prospective isolation, as well as the reluctance of luminal cells in general and ER+ cells in particular, to proliferate in culture [15]. Here I will focus on contemporary insights in ER+ cells, drawing from rodent models only if relevant for the human context.

Estrogen receptor positive cells: A relay between systemic hormones and tissue specific function

Most organs in the human body are patterned and are fully developed at the time of birth. The human breast is also patterned embryonically with distinctly arranged hormone sensing cells however, ER+ cells are primarily active between the onset of puberty and menopause in females [16]. During this phase of life, the human breast is submitted to monthly cycles of circulating sex hormones only interrupted by pregnancies and subsequent lactation. These phases are largely choreographed through the ovarian hormone responsive ER+ and progesterone receptor positive (PR+) cells [17].

Understanding the relevance of ER+ cells requires a brief summary of their place in breast anatomy. Through the development of the breast, the bilayered epithelium consists of 15-25 ducts that originate from the nipple and end in terminal ductal-lobular units (TDLUs), the effective functional units of the breast. The epithelial compartment is segmented into ducts and lobules which act together to provide nutrients to the newborn. The outer layer is composed of myoepithelial cells which provide contractile capacity for milk ejection. The inner luminal layer is responsible for tissue specific milk secretion. Important markers for the myoepithelial layer include alpha smooth muscle actin (αSMA), cell surface antigen Thy1, transcription factor p63, and cytokeratins K5, K14 and K17. Luminal cells are marked by an epithelial cell adhesion molecule (EpCAM or CD326), a transmembrane glycoprotein sialomucin (MUC1 or MAM6), as well as the cytokeratins K7, K8, K18 and K19 [18-21]. The luminal epithelial compartment in both ducts and lobules is seemingly more heterogeneous than the myoepithelial compartment and is thought to be comprised of multiple cellular subsets with distinct functionality [6, 20, 22].

More comprehensive identification and characterization of the cells within the luminal lineage would allow us to gain a better understanding of their functional role in the tissue. There is good reason to assume that the ER+ (and PR+; see below) hormone sensing cells act as critical relay cells for the hormonal control of the cyclic cellular growth seen in this tissue. However, in the absence of cellular models, it has been difficult to unequivocally assess whether estrogen acts as a bona fide mitogen on its own under homeostatic conditions in the human breast.

There is extensive indirect evidence in humans for an effect of estrogen in the tissue, suggesting that it is an important player in breast epithelial growth and differentiation. This evidence is based on both clinical data as well as various mouse models [23-27]. Clinical evidence shows that rare genetic aberrations resulting in aromatase deficiencies, impairing estrogen biosynthesis, lead to the masculinization of the female external genitalia at birth due to the inability to convert androgens to estrogens [23, 24]. Initially, breast development is normal in these patients. However, affected females fail to undergo normal breast development at puberty. Estrogen replacement therapy for these patients initiates sexual maturation, highlighting the importance of estrogen and ER+ cells in breast development in humans. Similarly, ovariectomized or ER knockout mice fail to fill the fat pad with mammary ducts, showing that estrogen signaling is crucial for ductal epithelial growth during puberty [25-27]. Whereas these original studies associate estrogen and ER+ cells with growth, exactly how this is conveyed at the tissue level was a mystery due to the fact that ER+ cells themselves appear to be post-mitotic [14, 28]. However, meticulous double labeling experiments revealed that proliferation marker Ki67 and the ER are

expressed in different albeit often neighboring cells [29, 30]. This observation has since been confirmed by others [27, 31, 32]. This suggests that estrogen might acts in a paracrine manner by acting on ER+ cells which then exert proliferating signals to neighboring cells. Indeed, it has been shown that the EGFR ligand, amphiregulin (AREG) is one such mediator [33]. Upon hormone stimulation, AREG is released by ER+ cells and acts as a proliferative factor on neighboring cells. Aside from morphogenic properties during puberty, estrogen is also necessary for priming the gland for lobular and alveolar differentiation during pregnancy and lactation, at least in mice [32].

The majority of ER+ cells also express progesterone receptor (PR), which has also been shown to be a crucial factor for full mammary development in addition to estrogen [28]. However, the cellular proliferative response to progesterone is quite different from the proliferative response to estrogen [34]. PR knockout mice undergo normal ductal elongation during puberty, but lack secondary branches and display a complete absence of alveolar development upon pregnancy [35]. Progesterone signaling seemingly mediates the expansion of mammary alveoli into lobules (alveologenesis) during puberty and pregnancy in preparation for lactation [36]. Studies in ovariectomized mice show that RANKL, a downstream target of PR, mediates a growth signal to myoepithelial cells which express its receptor RANK to convey proliferative signals [37, 38]. However, it has to be pointed out, that the paracrine signaling of the RANK/RANKL pathway in mice is different in a human context as discussed below. Nevertheless, based on mice studies it is clear that estrogen, together with progesterone signaling depends on paracrine mechanisms to convey mitotic signals but it remains to be answered how estrogen and ER mediate these signals within the context of the human breast.

Understanding ER in humans: current and future models of ER+ cells in culture

Experimentally, the characteristics and function of normal ER+ cells have been studied immensely in the intact mouse mammary gland. However, for obvious ethical reasons, similar experiments cannot be performed in humans. Instead, experimental insight in human ER+ cells has been obtained to a limited extent from short-term cultures with primary normal tissue, but to a far larger extent from established cell lines, isolated entirely from breast cancer. It is important to emphasize that unlike the normal homeostatic breast gland, where ER is expressed in only a subset of luminal cells [14], ER+ breast cancer is composed of a much larger degree of ER+ luminal-like cells. This brings into question whether normal ER+ cells behave and respond to estrogen stimulation in the same way as ER+ cancer cells. Despite this difference, many conclusions made about the mode of action of estrogen and ER+ cells in the human breast have been deduced from data obtained on ER+ breast cancer cell lines. Moreover, although approximately 17 ER+ cell lines [39] are available from the American Type Culture Collection (ATCC) cell line repository, only a handful of cell lines are widely used, notably the cell lines MCF7 [40], T47D [41] and ZR-75-1 [42]. These ER+ cell lines have been isolated predominantly from late metastatic lesions of breast cancer. The MCF7 cell line, specifically, is by far the most widely utilized and was derived from a pleural effusion. This cell line has been extensively referenced for ER-related research, detailing the intricate binding of estrogen to its cognate receptor as well as the gene expression programs triggered by estrogen stimulation [43]. Although the binding properties of estrogen to the ER in MCF7 cells shows consensus in various laboratories [44], the gene expression programs initiated by estrogen in this cell line have shown variation in different studies [45, 46]. Multiple reasons may contribute to this variation, including the culture history of the cell line, varying culture conditions, duration of estrogen treatment, as well as bioinformatics used for analysis [47]. Whether ER+ cells in the normal gland will respond in a similar way as to MCF7 after hormone stimulation can, at the moment, only be speculated. This is due to the lack of appropriate culture models supporting proliferation and maintenance of lineage-characteristics of normal ER+ cells. Establishment of such a system will allow us to study for the first time the hormonal effect of both estrogen and progesterone in a normal context and more importantly, how they differ in ER+ breast cancer.

ER+ cells have been prospectively isolated from mouse mammary glands with a number of different protocols [48-50]. However, while it is tempting to extrapolate data gathered from mice to a human context, this is not always feasible or scientifically sound. Firstly, not all the markers in the mouse mammary gland are expressed in the human breast. For instance, humans have been shown to lack Sca-1, a widely used marker of differentiated luminal cells in the mice [48]. Similarly, prominin-1 is not restricted in humans to ER+ cells as in the mouse mammary gland, but rather presented on the apical surface of all luminal cells [51]. One might think such details are unimportant when it comes to gross physiology. However, we simply may not yet have seen the full picture of the species specific differences. Secondly, signal transduction pathways can differ between species, in part, due to the different distribution of ligands and their receptors as well as a sensitivity of signaling itself. For example, in the mouse mammary gland, one of the downstream effectors of progesterone, RANKL, triggers RANK signaling within the myoepithelial compartment [37,

[38]. Conversely, in the human breast RANK positive cells are mainly located in luminal progenitors [52]. Thirdly, while the pivotal role of ER in the human breast is quite obvious, an additional layer of complexity arises from potential epithelial-stromal signaling [53]. Unfortunately, mouse models do not accurately reflect the tissue context of the stroma in the human breast. The stromal compartment of the mouse mammary fat pad is predominantly composed of adipocytes and contains relatively few fibroblasts compared to the human breast. In humans, the TDLUs are characterized not only by their epithelial components but also by the presence of a specialized intralobular stroma [18, 55]. The fibroblast component develops together with the epithelium and presumably provides signaling cues throughout development and tissue homeostasis [56]. The TDLUs, in turn, are surrounded by a denser extra-lobular stroma composed predominantly of collagen. This is in contrast to mice in which epithelial structures grow into surrounding adipose tissue. Both the intra- and extra-lobular fibroblast-generated matrices provide secretory enzymes and cytokines that upon activation have morphogenic roles which contribute to epithelial development and homeostasis [56-58]. Whether the anatomical differences seen between human and mice translate to differences in the proliferative capacity with regard to estrogen or progesterone signals are less known. Not only are there obvious anatomical differences but also, estrogen specific signaling differences. For example, unlike human fibroblasts, mouse fibroblasts express ER [54]. Therefore, the stroma must be considered when comparing species with respect to relaying circulating levels of estrogen into tissue specific function. One could speculate that indeed the anatomical difference between intralobular and interlobular stroma in the human breast is provided by different fibroblast lineages, like it has been demonstrated for the different stromal compartments within the skin of mice [59, 60]. If correct, fibroblast compartmentalization may have a more significant impact on epithelial structure and function than previously thought, and may play a more decisive role in tumor initiation.

Clean separation of the epithelial cells is also of great importance. Cell sorting protocols and technologies have improved in the last two decades and in turn, have facilitated to a great extent the characterization of various cell types, including human breast epithelial cells. Magnetic-activated cell sorting (MACS) as well as fluorescence activated cell sorting (FACS) based strategies emerged as an efficient way for lineage separation. While the cell sorting technologies have themselves been improved significantly, the repertoire of lineage-specific surface markers in the human breast has expanded rather slowly. For the two lineages of the breast epithelium isolation was originally based on exclusive expression of epithelial membrane antigen (EMA or MUC1) for cells in the luminal compartment and the common acute lymphoblastic leukemia antigen (CALLA, or CDl0) for cells in the myoepithelial compartment [19, 61, 62]. Later, as a modification of the mouse lineage markers CD24 and CD49f [63], EpCAM and CD49f were adapted to enrich luminal and myoepithelial cells, respectively in the human breast [20]. Among the EpCAM+ cells, CD49f expression can be used as a marker to enrich the luminal progenitor populations (EpCAM+/CD49f^{hi}) from more mature luminal cells (EpCAM+/CD49f$^{low/-}$) [20, 64-66]. However, none of these markers facilitated the isolation of relatively clean populations of ER+ cells which have been done in mice. Instead, ER+ cells have been studied in short term culture systems made of either mixed cell cultures containing both luminal and myoepithelial cells at various differentiation stages or as organoids where physiological tissue architecture remains intact.

These initial attempts have shown that human ER+ cells were responsive to estrogen if excised from the tissue and placed in primary cell cultures [67]. Importantly, however, enriched populations of ER+ cells using this approach did not maintain their ER expression in culture. In a more recent study, organoid culture conditions maintained functionally relevant ER expression up to 5 days, where the hormone response was maintained as evidence by RANKL expression after progesterone stimulation, which then induced a proliferative response as expected [68]. With cells from mice, it has been shown that cultivation inside a three-dimensional basement membrane delays the loss of ER [69]. However, in our hands, this does not apply to human cells (unpublished observation). Another established non-malignant-derived ER+ cell line comes from the 184 series [70] which has been immortalized with benzo(a)pyrene and eventually fully transformed in severely immunodeficient NOG mice [71]. While such cell lines have provided important insights, the exact relations to normal cells in terms of ER signaling has remained obscure [72]. Progress was recently made in this area by our group by isolating ER+ cells and maintaining ER expression in cells culture from normal-derived human breast tissue [73]. These attempts were successful due to the utilization of markers to identify ER+ cells. By immunohistological identification of novel markers, which co-localized specifically with ER+ cells, the luminal lineage could be separated further by FACS into luminal progenitors and differentiated ER+ cells by using subpopulation-specific surface markers. The first initial attempt used carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6 or CD66c) which was shown to be associated with the luminal ER and PR cell populations in the normal breast tissue, predominantly in TDLUs [74]. Analysis of proliferative markers and an absence of proliferation in cultured cells suggested that CEACAM6 is predominantly

expressed in post-mitotic mature ER+ cell populations. A second attempt used the cell surface marker activated leukocyte cell adhesion molecule (ALCAM or CD166) which was also shown to be highly associated with ER expression and has since been used efficiently for the enrichment of ER+ cells by FACS [73]. Once isolated and in culture, ER expression fluctuated and therefore a marker to track these cells was needed. This was achieved by the antigen recognized by the Ks20.8 antibody, which acted as a surrogate marker with a longer half-life than the ER in culture. Importantly, Ks20.8 was shown to be highly associated with the majority (about 90%) of ER and PR expressing cells in ducts and lobules and therefore served as an excellent candidate to track these cells in culture [73].

Regardless of efforts to identify markers that allow isolation of these ER+ cells, ER expression of primary cells has been difficult to maintain in culture as these cells were shown to lose ER expression and undergo growth restriction [68, 69, 75]. The ability to circumvent these complications was deduced from previous observations made by Ewan et al [29, 30]. Specifically, these studies identified some of the critical components facilitating the growth inhibition *in vivo* of ER+ cells, namely TGFβ signaling. In mice, TGFβ signaling has been shown to influence mammary gland development [76]. ER and TGFβ signaling elicit distinctly opposing roles on mammary epithelial proliferation. While ER signaling promotes survival and proliferation within the luminal epithelial compartment, these cells were shown to be highly sensitive to TGFβ-induced cell cycle arrest. Around the time of puberty in mice, the rise of estrogen inhibits TGFβ signaling within the luminal compartment along the ductal epithelium resulting in an increase in proliferation [29, 76]. The ER and TGFβ signaling networks have been shown to interact directly though TGFβ-SMAD signaling components [30]. TGFβ depletion alone does not trigger epithelial proliferation but releases epithelial cells from growth restraint, while proliferative signals are still initiated and maintained by ovarian hormones or other proliferative stimuli. Upon estrogen stimulation, TGFβ signaling is uniformly repressed and ER- luminal cells proliferate [29]. Paradoxically, some ER+ cells increase TGFβ signaling in an autocrine manner. This results in the emergence of a population of non-proliferative ER+ cells amongst the proliferating luminal population [30]. This autocrine TGFβ signaling, shown to inhibit cell proliferation specifically in luminal ER+ cells, was seemingly responsible for the lack of success in culturing an ER+ luminal cell population. After isolating ER+ cells from normal human primary tissue using the lineage-specific markers ($EpCAM^{hi}/CD271^{low}/CD166^{hi}/CD117^{low}$), down-regulation of ER expression and growth restraint was released by small molecule inhibitors against TGFβR, presumably breaking an anti-proliferative autocrine loop [73]. More importantly, these prospectively isolated ER+ cells showed a proliferative response to estrogen suggesting an intact functional hormone sensing signaling program. For the first time, we could now begin to design experiments aimed at comparing these novel normal-derived ER+ cells with the cancer-derived ER+ cell lines previously available.

Developing a relevant model of normal ER+ cells for breast cancer research

Once established in primary culture, we wanted to establish stable ER+ cell lines to further pursue the ideal clean comparisons with MCF7 cells as envisioned above. Obviously, developing culture conditions designed to extend cellular life span in culture without having to integrate genetic alterations was preferred. However, eventually, such cells meet a stress-associated stasis barrier and replicative senescence due to critically shortened telomeres and are therefore subject to a limited number of cell divisions [77]. This limits the capacity for cell growth and thus material for experimentation. Hence, it was necessary to explore the possibilities for cell immortalization. Immortalization, in theory, establishes indefinite proliferation and generates essentially infinite material for experimentation [78]. Spontaneous immortalization does not occur readily in human cell cultures [79]. They have been, on rare occasion established "spontaneously" from patients with benign breast disease such as HMT3522 [80] and MCF10A [81]. However, due to the infrequency of these events immortalization strategies are employed. Previous successfully applied immortalization strategies have included introduction of viral oncogenes such as SV40 or E6/E7 [19, 82, 83], or of specific genes, such as hTERT [84], CCND1/c-MYC [85], shp16/c-MYC [77] and combinations thereof. While previously immortalized breast cells mostly displayed the desired luminal phenotype, they lacked relevant ER expression. Therefore, to immortalized the ER+ cells from normal primary tissue we employed a combination of hTERT and shp16, which has shown to be less aggressive compared to viral oncogenic immortalization, with the aim of maintaining the luminal phenotype and most importantly, ER expression. Indeed, upon selection for the relevant integrated vectors, normal-derived cell-lines emerged which could be kept long-term in culture [73, 86]. Single cell clones were generated from the parent populations of these ER+ immortalized cells and remained ER+ and for the most part, responsive to estrogen. However, these cells were prone to drifting towards a myoepithelial phenotype. Thus, while un-immortalized cells remain essentially luminal by our standard criteria, immortalized cells lines after selection quite often express myoepithelial markers such as αSMA, K14 and p63 along with the classical luminal markers. This was unacceptable given our initial aim of having a lineage

equivalent to MCF7. Additionally, it is worthwhile to mention that breast cancer, in general, differentiates along the luminal epithelial lineage [87]. In our hands, the most reliable luminal markers of the luminal epithelial lineage is K19 followed by MUC1 [73, 86]. However, certain subtleties should be particularly observed when dealing with cells in culture and for this reason, we take advantage of different staining patterns by numerous K19 antibodies. Whereas K19 antibodies seem to stain the luminal cells almost identically *in situ*, in cultured cells staining patterns differ when different K19 antibodies were applied. For example, we have previously used the A53-B/A2 clone of K19 antibody [19]. In later screens of our antibody library, we realized that this clone stains cultured cells much broader than other clones such as BA16 [87]. We have evidence that BA16 is limited to the near-mature luminal phenotype, while A53-B/A2 also stains intermediate or early stages of luminal differentiation. On the other hand, the myoepithelial lineage marker K14 has been used by us and others to demonstrate cells of the myoepithelial lineage in culture [20, 84]. One feature of K14 is that its expression is induced quite broadly in cells of the luminal epithelial lineage somewhat depending on the culture medium used. We found that it was expressed in most normal-derived cell-lines of the hTERT/shp16 transduced ER+ cells presumably as a result of a drifting phenotype (unpublished observation). Again this was unacceptable since, unlike K19 which is typically expressed in human breast cancers, K14 is quite rare in human breast cancer. To circumvent the culture-induced drift, we re-sorted the cells repeatedly with the cell surface marker CD146 [86]. Using this approach we obtained cell lines which were K19+/K14-, as well as MUC1+. Thus, we were successful in generating a normal derived ER+ immortalized cell line. In attempts to pursue a clean comparison to MCF7, we wanted to uniform culture conditions between the two cell lines. However, MCF7 cells did not readily adapt to the normal ER+ culture conditions TGFbR2i in Fridriksdottir *et al* [73]. After screening different conditions, we developed TGFβR2i-1 formulation, in which EGF was replaced by amphiregulin, and the two additives cholera toxin and hydrocortisone were omitted in the TGFb2i medium. These changes allowed instant serial-passage growth of both normal ER+ cells and MCF7 cells under identical conditions. To the best of my knowledge, hTERT/shp16 immortalized ER+ cells with this strict luminal profile have not been previously available which could be compared to the iconic MCF7 cancer cells under identical culture conditions. Interestingly we found that these cell lines were classified as progenitors based on expression arrays [86]. These cell lines also showed other interesting differences when compared to the MCF7 cell line. Nevertheless, we are now able to utilize these cells for a multitude of downstream applications.

Conclusion and Future prospective

As we have reviewed here ER+ luminal cells are detrimental to glandular growth in the human breast. How exactly the signal is translated from systemic ovarian hormone levels in the blood to proliferation in breast tissue is still largely unknown within a human context. With such large differences between the stromal compartments of mice and humans requires us to reevaluate the intralobular stroma with respect to a specific role in the TDLUs, and thus, as a potential partner in organoid cultures for the maintenance of luminal structure and function. However, cell models derived from normal breast tissue displaying intact functioning ER+ signaling networks have remained largely unexplored until now. “Zero to one” may sound overly enthusiastic, however, we have developed a robust reproducible protocol for ER+ cells proliferating in culture. A long line of important questions remains to be answered. For example, why do normal cells segregate into proliferating and resting ER+ cells, a feature which seems to be disrupted in breast cancer? What aspects of the stromal compartment are responsible for the separation these compartments? Is an aberrant stromal regime responsible for discrepancies shown in TGFβ signaling in cancer? Currently, we are pursuing cell polarity and the segregation of ER and proliferation as readouts in screens for early transformation. What is needed next is the enigmatic organoid culture allowing for both structural and functional differentiation. Hopefully, these novel cell lines can help answer some of the questions of how luminal ER+ breast cancer develops in hopes to design therapeutic or even prophylactic strategies against this prevalent subtype of breast cancer.

Conflicting interests

The authors have declared that no competing interests exist.

References

1. Pierce GB, Speers WC: Tumors as Caricatures of the Process of Tissue Renewal: Prospects for Therapy by Directing Differentiation. Cancer Research 1988; 48:1996-2004.
2. Owens DM, Watt FM: Contribution of stem cells and differentiated cells to epidermal tumours. Nat Rev Cancer 2003; 3:444-451.
3. Tomasetti C, Vogelstein B: Variation in cancer risk among tissues can be explained by the number of stem cell divisions. Science 2015; 347:78-81.
4. Eirew P, Stingl J, Raouf A, Turashvili G, Aparicio S, Emerman JT *et al*: A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability. Nature medicine 2008; 14:1384-1389.
5. Booth BW, Smith GH: Estrogen receptor-α and progesterone

receptor are expressed in label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands. Breast Cancer Research 2006; 8:R49-R49.

6. Santagata S, Ince TA: Normal cell phenotypes of breast epithelial cells provide the foundation of a breast cancer taxonomy. Expert review of anticancer therapy 2014; 14:1385-1389.
7. Skibinski A, Kuperwasser C: The origin of breast tumor heterogeneity. Oncogene 2015; 34:5309-5316.
8. Gross K, Wronski A, Skibinski A, Phillips S, Kuperwasser C: Cell Fate Decisions During Breast Cancer Development. Journal of developmental biology 2016; 4:4.
9. Visvader JE, Stingl J: Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes & development 2014; 28:1143-1158.
10. Van Keymeulen A, Lee MY, Ousset M, Brohee S, Rorive S, Giraddi RR *et al*: Reactivation of multipotency by oncogenic PIK3CA induces breast tumour heterogeneity. Nature 2015; 525:119-123.
11. Koren S, Reavie L, Couto JP, De Silva D, Stadler MB, Roloff T *et al*: PIK3CAH1047R induces multipotency and multi-lineage mammary tumours. Nature 2015; 525:114-118.
12. Dontu G, Ince TA: Of Mice and Women: A Comparative Tissue Biology Perspective of Breast Stem Cells and Differentiation. Journal of Mammary Gland Biology and Neoplasia 2015; 20:51-62.
13. Fridriksdottir AJ, Petersen OW, Ronnov-Jessen L: Mammary gland stem cells: current status and future challenges. The International journal of developmental biology 2011; 55:719-729.
14. Petersen OW, Hoyer PE, van Deurs B: Frequency and distribution of estrogen receptor-positive cells in normal, nonlactating human breast tissue. Cancer Res 1987; 47:5748-5751.
15. Arendt LM, St. Laurent J, Wronski A, Caballero S, Lyle SR, Naber SP *et al*: Human Breast Progenitor Cell Numbers Are Regulated by WNT and TBX3. PLoS ONE 2014; 9:e111442.
16. Russo J, Russo IH: Development of the human breast. Maturitas 2004; 49:2-15.
17. Brisken C, O'Malley B: Hormone Action in the Mammary Gland. Cold Spring Harbor Perspectives in Biology 2010; 2:a003178.
18. Ronnov-Jessen L, Petersen OW, Bissell MJ: Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiological Reviews 1996; 76:69-125.
19. Gudjonsson T, Villadsen R, Nielsen HL, Ronnov-Jessen L, Bissell MJ, Petersen OW: Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. Genes & development 2002; 16:693-706.
20. Villadsen R, Fridriksdottir AJ, Ronnov-Jessen L, Gudjonsson T, Rank F, LaBarge MA *et al*: Evidence for a stem cell hierarchy in the adult human breast. J Cell Biol 2007; 177:87-101.
21. Fridriksdottir AJR, Villadsen R, Gudjonsson T, Petersen OW: Maintenance of Cell Type Diversification in the Human Breast. Journal of Mammary Gland Biology and Neoplasia 2005; 10:61-74.
22. Shehata M, Teschendorff A, Sharp G, Novcic N, Russell IA, Avril S *et al*: Phenotypic and functional characterisation of the luminal cell hierarchy of the mammary gland. Breast Cancer Res 2012; 14:R134.
23. Conte FA, Grumbach MM, Ito Y, Fisher CR, Simpson ER: A syndrome of female pseudohermaphrodism, hypergonadotropic hypogonadism, and multicystic ovaries associated with missense mutations in the gene encoding aromatase (P450arom). The Journal of Clinical Endocrinology & Metabolism 1994; 78:1287-1292.
24. Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K: Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. The Journal of Clinical Endocrinology & Metabolism 1995; 80:3689-3698.
25. Daniel CW, Silberstein GB, Strickland P: Direct Action of 17β-Estradiol on Mouse Mammary Ducts Analyzed by Sustained Release Implants and Steroid Autoradiography. Cancer Research 1987; 47:6052-6057.
26. Bocchinfuso WP, Korach KS: Mammary gland development and tumorigenesis in estrogen receptor knockout mice. J Mammary Gland Biol Neoplasia 1997; 2:323-334.
27. Mallepell S, Krust A, Chambon P, Brisken C: Paracrine signaling through the epithelial estrogen receptor α is required for proliferation and morphogenesis in the mammary gland. Proceedings of the National Academy of Sciences of the United States of America 2006; 103:2196-2201.
28. Clarke RB, Howell A, Potten CS, Anderson E: Dissociation between Steroid Receptor Expression and Cell Proliferation in the Human Breast. Cancer Research 1997; 57:4987-4991.
29. Ewan KB, Shyamala G, Ravani SA, Tang Y, Akhurst R, Wakefield L *et al*: Latent Transforming Growth Factor-β Activation in Mammary Gland : Regulation by Ovarian Hormones Affects Ductal and Alveolar Proliferation. The American journal of pathology 2002; 160:2081-2093.
30. Ewan KBR, Oketch-Rabah HA, Ravani SA, Shyamala G, Moses HL, Barcellos-Hoff MH: Proliferation of Estrogen Receptor-α-Positive Mammary Epithelial Cells Is Restrained by Transforming Growth Factor-β1 in Adult Mice. The American journal of pathology 2005; 167:409-417.
31. Mueller SO, Clark JA, Myers PH, Korach KS: Mammary Gland Development in Adult Mice Requires Epithelial and Stromal Estrogen Receptor α. Endocrinology 2002; 143:2357-2365.
32. Feng Y, Manka D, Wagner K-U, Khan SA: Estrogen receptor-α expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. Proceedings of the National Academy of Sciences 2007; 104:14718-14723.
33. Ciarloni L, Mallepell S, Brisken C: Amphiregulin is an essential mediator of estrogen receptor α function in mammary gland development. Proceedings of the National Academy of Sciences 2007; 104:5455-5460.
34. Brisken C, Park S, Vass T, Lydon JP, O'Malley BW, Weinberg RA: A paracrine role for the epithelial progesterone receptor in mammary gland development. Proceedings of the National Academy of Sciences 1998; 95:5076-5081.
35. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr. *et al*: Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes & development 1995; 9:2266-2278.
36. Daniel AR, Hagan CR, Lange CA: Progesterone receptor action:

defining a role in breast cancer. Expert review of endocrinology & metabolism 2011; 6:359-369.

37. Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL *et al*: Progesterone induces adult mammary stem cell expansion. Nature 2010; 465:803-807.
38. Asselin-Labat M-L, Vaillant F, Sheridan JM, Pal B, Wu D, Simpson ER *et al*: Control of mammary stem cell function by steroid hormone signalling. Nature 2010; 465:798-802.
39. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T *et al*: A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer cell 2006; 10:515-527.
40. Soule HD, Vazguez J, Long A, Albert S, Brennan M: A human cell line from a pleural effusion derived from a breast carcinoma. Journal of the National Cancer Institute 1973; 51:1409-1416.
41. Keydar I, Chen L, Karby S, Weiss FR, Delarea J, Radu M *et al*: Establishment and characterization of a cell line of human breast carcinoma origin. European Journal of Cancer (1965) 1979; 15:659-670.
42. Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ, Joyce MJ: Establishment and Characterization of Three New Continuous Cell Lines Derived from Human Breast Carcinomas. Cancer Research 1978; 38:3352-3364.
43. Comsa S, Cimpean AM, Raica M: The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research. Anticancer research 2015; 35:3147-3154.
44. Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J *et al*: Genome-wide analysis of estrogen receptor binding sites. Nat Genet 2006; 38:1289-1297.
45. Kininis M, Kraus WL: A global view of transcriptional regulation by nuclear receptors: gene expression, factor localization, and DNA sequence analysis. Nuclear Receptor Signaling 2008; 6:e005.
46. Jagannathan V, Robinson-Rechavi M: Meta-analysis of estrogen response in MCF-7 distinguishes early target genes involved in signaling and cell proliferation from later target genes involved in cell cycle and DNA repair. BMC Systems Biology 2011; 5:138-138.
47. Cheung E, Kraus WL: Genomic analyses of hormone signaling and gene regulation. Annual review of physiology 2010; 72:191-218.
48. Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ: Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. The Journal of Cell Biology 2007; 176:19-26.
49. Kendrick H, Regan JL, Magnay FA, Grigoriadis A, Mitsopoulos C, Zvelebil M *et al*: Transcriptome analysis of mammary epithelial subpopulations identifies novel determinants of lineage commitment and cell fate. BMC genomics 2008; 9:591.
50. Asselin-Labat M-L, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ *et al*: Steroid Hormone Receptor Status of Mouse Mammary Stem Cells. Journal of the National Cancer Institute 2006; 98:1011-1014.
51. Raouf A, Zhao Y, To K, Stingl J, Delaney A, Barbara M *et al*: Transcriptome Analysis of the Normal Human Mammary Cell Commitment and Differentiation Process. Cell stem cell 2008; 3:109-118.
52. Nolan E, Vaillant F, Branstetter D, Pal B, Giner G, Whitehead L *et al*: RANK ligand as a potential target for breast cancer prevention in BRCA1-mutation carriers. Nature medicine 2016; 22:933-939.
53. Haslam SZ, Woodward TL: Host microenvironment in breast cancer development: Epithelial-cell–stromal-cell interactions and steroid hormone action in normal and cancerous mammary gland. Breast Cancer Research 2003; 5:208-215.
54. Shyamala G, Chou YC, Louie SG, Guzman RC, Smith GH, Nandi S: Cellular expression of estrogen and progesterone receptors in mammary glands: regulation by hormones, development and aging. The Journal of Steroid Biochemistry and Molecular Biology 2002; 80:137-148.
55. Morsing M, Klitgaard MC, Jafari A, Villadsen R, Kassem M, Petersen OW *et al*: Evidence of two distinct functionally specialized fibroblast lineages in breast stroma. Breast Cancer Research : BCR 2016; 18:108.
56. Atherton AJ, Anbazhagan R, Monaghan P, Bartek J, Gusterson BA: Immunolocalisation of cell surface peptidases in the developing human breast. Differentiation; research in biological diversity 1994; 56:101-106.
57. Atherton AJ, O'Hare MJ, Buluwela L, Titley J, Monaghan P, Paterson HF *et al*: Ectoenzyme regulation by phenotypically distinct fibroblast sub-populations isolated from the human mammary gland. Journal of Cell Science 1994; 107:2931-2939.
58. Atherton AJ, Warburton MJ, O'Hare MJ, Monaghan P, Schuppan D, Gusterson BA: Differential expression of type XIV collagen/undulin by human mammary gland intralobular and interlobular fibroblasts. Cell and tissue research 1998; 291:507-511.
59. Driskell RR, Lichtenberger BM, Hoste E, Kretzschmar K, Simons BD, Charalambous M *et al*: Distinct fibroblast lineages determine dermal architecture in skin development and repair. Nature 2013; 504:277-281.
60. Driskell RR, Watt FM: Understanding fibroblast heterogeneity in the skin. Trends in Cell Biology 2015; 25:92-99.
61. O'Hare MJ, Ormerod MG, Monaghan P, Lane EB, Gusterson BA: Characterization in vitro of luminal and myoepithelial cells isolated from the human mammary gland by cell sorting. Differentiation; research in biological diversity 1991; 46:209-221.
62. Pechoux C, Gudjonsson T, Ronnov-Jessen L, Bissell MJ, Petersen OW: Human mammary luminal epithelial cells contain progenitors to myoepithelial cells. Developmental biology 1999; 206:88-99.
63. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D *et al*: Purification and unique properties of mammary epithelial stem cells. Nature 2006; 439:993-997.
64. Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH *et al*: Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nature medicine 2009; 15:907-913.
65. Keller PJ, Arendt LM, Skibinski A, Logvinenko T, Klebba I, Dong S *et al*: Defining the cellular precursors to human breast cancer. Proc Natl Acad Sci U S A 2012; 109:2772-2777.
66. Keller PJ, Lin AF, Arendt LM, Klebba I, Jones AD, Rudnick JA *et al*: Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines. Breast Cancer Research : BCR 2010; 12:R87-R87.
67. Chambon M, Cavalie-Barthez G, Veith F, Vignon F, Hallowes R, Rochefort H: Effect of Estradiol on Nonmalignant Human Mammary Cells in Primary Culture. Cancer Research 1984;

44:5733-5743.

68. Tanos T, Sflomos G, Echeverria PC, Ayyanan A, Gutierrez M, Delaloye JF *et al*: Progesterone/RANKL is a major regulatory axis in the human breast. Science translational medicine 2013; 5:182ra155.
69. Novaro V, Roskelley CD, Bissell MJ: Collagen-IV and laminin-1 regulate estrogen receptor α expression and function in mouse mammary epithelial cells. Journal of cell science 2003; 116:2975-2986.
70. Stampfer MR, Bartley JC: Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proceedings of the National Academy of Sciences of the United States of America 1985; 82:2394-2398.
71. Hines WC, Kuhn I, Thi K, Chu B, Stanford-Moore G, Sampayo R *et al*: 184AA3: a xenograft model of ER+ breast adenocarcinoma. Breast cancer research and treatment 2016; 155:37-52.
72. Pearce VP, Sherrell J, Lou Z, Kopelovich L, Wright WE, Shay JW: Immortalization of epithelial progenitor cells mediated by resveratrol. Oncogene 2007; 27:2365-2374.
73. Fridriksdottir AJ, Kim J, Villadsen R, Klitgaard MC, Hopkinson BM, Petersen OW *et al*: Propagation of oestrogen receptor-positive and oestrogen-responsive normal human breast cells in culture. Nat Commun 2015; 6.
74. Balk-Moller E, Kim J, Hopkinson B, Timmermans-Wielenga V, Petersen OW, Villadsen R: A marker of endocrine receptor-positive cells, CEACAM6, is shared by two major classes of breast cancer: luminal and HER2-enriched. The American journal of pathology 2014; 184:1198-1208.
75. Brown DD, Dabbs DJ, Lee AV, McGuire KP, Ahrendt GM, Bhargava R *et al*: Developing in vitro models of human ductal carcinoma in situ from primary tissue explants. Breast cancer research and treatment 2015; 153:311-321.
76. Moses H, Barcellos-Hoff MH: TGF-β Biology in Mammary Development and Breast Cancer. Cold Spring Harbor Perspectives in Biology 2011; 3:a003277.
77. Garbe JC, Vrba L, Sputova K, Fuchs L, Novak P, Brothman AR *et al*: Immortalization of normal human mammary epithelial cells in two steps by direct targeting of senescence barriers does not require gross genomic alterations. Cell Cycle 2014; 13:3423-3435.
78. Hayflick L, Moorhead PS: The serial cultivation of human diploid cell strains. Experimental cell research 1961; 25:585-621.
79. Harris CC: Human Tissues and Cells in Carcinogenesis Research. Cancer Research 1987; 47:1-10.
80. Briand P, Petersen OW, Van Deurs B: A New Diploid Nontumorigenic Human Breast Epithelial Cell Line Isolated and Propagated in Chemically Defined Medium. In Vitro Cellular & Developmental Biology 1987; 23:181-188.
81. Soule HD, Maloney TM, Wolman SR, Peterson WD, Brenz R, McGrath CM *et al*: Isolation and Characterization of a Spontaneously Immortalized Human Breast Epithelial Cell Line, MCF-10. Cancer Research 1990; 50:6075-6086.
82. Bartek J, Bartkova J, Kyprianou N, Lalani EN, Staskova Z, Shearer M *et al*: Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus. Proceedings of the National Academy of Sciences of the United States of America 1991; 88:3520-3524.
83. Kao C-Y, Nomata K, Oakley CS, Welsch CW, Chang C-C: Two types of normal human breast epithelial cells derived from reduction mammoplasty: phenotypic characterization and response to SV40 transfection. Carcinogenesis 1995; 16:531-538.
84. Zhao X, Malhotra GK, Lele SM, Lele MS, West WW, Eudy JD *et al*: Telomerase-immortalized human mammary stem/progenitor cells with ability to self-renew and differentiate. Proceedings of the National Academy of Sciences 2010; 107:14146-14151.
85. Lee JK, Garbe JC, Vrba L, Miyano M, Futscher BW, Stampfer MR *et al*: Age and the means of bypassing stasis influence the intrinsic subtype of immortalized human mammary epithelial cells. Frontiers in Cell and Developmental Biology 2015; 3:13.
86. Hopkinson B, Klitgaard MC, Petersen OW, Villadsen R, Ronnov-Jessen L, Kim J: Establishment of a normal-derived estrogen receptor-positive cell line comparable to the prevailing human breast cancer subtype. Oncotarget (accepted) 2017.
87. Bartek J, Taylor-Papadimitriou J, Miller N, Millis R: Patterns of expression of keratin 19 as detected with monoclonal antibodies in human breast tissues and tumours. Int J Cancer 1985; 36:299-306.