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Propagation of oestrogen receptor-positive and oestrogen-responsive normal human breast cells in culture

Agla J. Fridriksdottir1,2,*, Jiyoung Kim1,2,*, René Villadsen1,2,*, Marie Christine Klitgaard1,2,3, Branden M. Hopkinson1,2, Ole William Petersen1,2 & Lone Rønnov-Jessen3

Investigating the susceptibility of oestrogen receptor-positive (ERpos) normal human breast epithelial cells (HBECs) for clinical purposes or basic research awaits a proficient cell-based assay. Here we set out to identify markers for isolating ERpos cells and to expand what appear to be post-mitotic primary cells into exponentially growing cultures. We report a robust technique for isolating ERpos HBECs from reduction mammoplasties by FACS using two cell surface markers, CD166 and CD117, and an intracellular cytokeratin marker, Ks20.8, for further tracking single cells in culture. We show that ERpos HBECs are released from growth restraint by small molecule inhibitors of TGFβ signalling, and that growth is augmented further in response to oestrogen. Importantly, ER signalling is functionally active in ERpos cells in extended culture. These findings open a new avenue of experimentation with normal ERpos HBECs and provide a basis for understanding the evolution of human breast cancer.
Understanding the taxonomy and evolution of breast cancer has always relied heavily on the use of normal cell types as reference (for review see ref. 1). Nevertheless, ever since the first protocol for cultivation of normal human breast epithelial cells appeared three decades ago2, it has become increasingly clear that there are no protocols that support propagation of oestrogen-receptor-positive (ER\textsuperscript{pos}) cells. Thus, along with the appreciation of epithelial cell lineages in the human breast, primarily the luminal lineage and the basal/myoepithelial lineage, it became evident that the fastest growing cells in culture are of basal origin3,4. Moreover, when it was revealed that ER\textsuperscript{pos} cells in situ accounted for an average of about 7% (mean 6.6%, ranging from 1.2 to 19.1% in a series of 15 normal breast samples) of the cells within the luminal epithelial lineage5, the chances of recovering these cells in culture without prospective isolation would in many cases be elusive. Thus, in culture medium that allowed luminal cells to be maintained after passaging, endogenous ER expression disappeared6,7. Likewise, even when employing freshly isolated small pieces of breast tissue, including the surrounding stroma, ER expression was eventually lost8,9. As a consequence of this, the comparison of cancer with ‘normal’, for example, the HMT-3522, MCF10A and 184B5 cell lines1,6,10, in cell-based assays has relied on normal cells lacking ER expression.

In an attempt to overcome the loss of receptor expression, ER has been ectopically introduced into such cell lines. This approach, however, has had a number of shortcomings, for example, instead of responding to oestrogen by increased proliferation as expected, the ER-transfected cells under standard culture conditions show growth inhibition11,12. Accordingly, most of our current knowledge of ER expression, regulation and action comes from breast carcinoma cell lines, whose relation to ER\textsuperscript{pos} normal breast cells at best remains speculative.

Here we first identify the ER\textsuperscript{pos} cells in situ and search for markers that allow their subsequent tracking in culture. We then describe culture conditions for primary ER\textsuperscript{pos} cells in the presence of small-molecule inhibitors of transforming growth factor-beta (TGF\beta) signalling. Importantly, these conditions also yield ER\textsuperscript{pos} cells from luminal ER\textsuperscript{neg} progenitors but not from basal cells. We envision that the present protocol will serve to bridge the existing gap of knowledge between normal human breast, which contain a small pool of ER\textsuperscript{pos} cells and the overwhelming ER expression found in the majority of breast cancers.

Results

Identification and isolation of normal ER\textsuperscript{pos} HBECs. To answer the long-standing question of whether loss of hormone receptors in culture is due to the loss of cells or the loss of receptor protein expression, it was necessary first to provide tools for cell tracking and sorting of the relevant cells. To unequivocally track ER\textsuperscript{pos} HBECs at the single-cell level we screened our antibody library for surrogate markers with a long half-life, for example, cytokeratin13, in culture. In situ staining of more than 30 reduction mammoplasties revealed a surprising pattern with a monoclonal antibody (clone Ks.20.8) originally raised against cytokeratin 20, a simple epithelial cytokeratin with a very restricted expression pattern and not expressed in normal human breast14. The lack of true cytokeratin 20 expression in normal breast was here confirmed with two specific antibodies (listed in Table 1). Instead, Ks.20.8 stained a subpopulation of luminal cells in a unique scattered pattern (Fig. 1a). Ks.20.8 antibodies from four different suppliers (Table 1) revealed similar staining patterns (Supplementary Fig. 1a). The characteristic staining pattern led us to speculate that it indeed represented ER\textsuperscript{pos} cells. While ER and Ks.20.8 apparently co-localized in acini as well as in ducts.

| Table 1 | List of antibodies used for immunostaining and/or FACS analysis. |
|---------|----------------------------------------------------------|
| Antibody | Clone | Company/Catalogue No. | Peroxidase | Fluorescence | FACS |
| AP28    | —     | Santa Cruz, sc-8976 | —          | 1:50       | —    |
| BC2L    | 124   | Dako, MO087 | —          | 1:25       | —    |
| CD975   | LN1   | NeoMarkers, MS-130-P | —          | 1:10       | —    |
| CD117   | 104D2 | Dako, M7140 | —          | 1:50       | —    |
| CD117   | K44.2 | Sigma, K0131 | —          | 1:50       | —    |
| CD117, PE | 104D2 | BD Biosciences, 332785 | —          | 1:20       |
| CD66    | 3A6   | BioLegend, 343002 | —          | 1:50       | —    |
| CD66, Alexa Fluor 488 | 3A6 | AbD Serotec, MCA1926A488 | — | 1:20       |
| CD271, APC | ME20.4 | Cedarlane, CL10019APC | — | 1:50       |
| CD326, PerCP/Cy5.5 | 9C4 | BioLegend, 324214 | — | 1:10-2:0 |
| TGF\beta | MM0056-4F14 | Abcam, ab78419 | — | 1:20       |
| E\textalpha | SP1 | Labvision, RM-9101-S | 1:25 | 1:10       | —    |
| E\textalpha | SP1 | Labvision, RM-9101-R7 | Prediluted, ready to use | — | — |
| E\textalpha | ID5 | Dako, M7047 | 1:100 | 1:25       | —    |
| GATA3   | HG3-31 | Santa Cruz, sc-268 | —          | 1:25       | —    |
| Keratin 8 | M20 | Abcam, ab9023 | 1:50 | 1:50       |
| Keratin 8 | TS1 | Novocastra, NCL-C8-KS1 | 1:50 | — |
| Keratin 20* | K20.8 | Dako, M7019 | 1:25 | 1:10       |
| Keratin 20* | K20.8 | Dako, IR777 | 1:10 | — |
| Keratin 20* | K20.8 | Abcam, ab8854 | 1:25 | — |
| Keratin 20* | K20.8 | Genemed, 61-0018-2 | 1:25 | — |
| Keratin 20* | K20.8 | Santa Cruz, SC-52320 | 1:25 | — |
| Keratin 20 | IT-Ks20.10 | Progen Biotechnik, 61054 | 1:25 | — |
| Keratin 20 | CK205 | Novocastra, NCL-C2-KS43 | 1:50 | — |
| Keratin 8/18 | NCL-5D3 | Abcam, ab90102 | 1:50 | — |
| Keratin 14 | LLO02 | NeoMarkers, MS-115-P | 1:300 | 1:25       |
| Keratin 15 | LHK15 | NeoMarkers, MS-106-P | 1:25 | — |
| Keratin 18 | M9 | Monosan, MON3006 | 1:100 | — |
| N-cadherin | 32 | BD Transduction Laboratories, 610920 | 1:10 | 1:25       |
| PR | SAN27 | Vector Laboratories, VP-1987 | 1:100 | 1:25       |
| PR | SP2 | Labvision, RM-9102-S | 1:10 | — |
| PR | PgR 636 | Dako, M356901-2 | 1:50 | 1:10       |
| 67-kDa laminin receptor | MLu5 | Abcam, ab3099 | 1:50 | 1:50       |

*Originally described to recognize cytokeratin 20, but keratin 20 is not expressed in normal human breast14.
enhancer-binding protein 2 beta (AP2β), a marker of luminal differentiation, GATA3 (ref. 16), a marker of cell survival/longevity, Bcl2, two TGFβ-mediated, epithelial–mesenchymal transition-related markers, N-glycan/CDw75 antigen17 and N-cadherin (reviewed in ref. 18), as well as the stem cell markers, ALCAM (CD166) (ref. 19) and the laminin receptor, 67LR (ref. 20; Supplementary Fig. 2).

For cell sorting purposes we found that CD166 and CD117 made good candidate surface markers of potential ERpos and ERneg cells, respectively—again as revealed by enhanced multi-colour immunofluorescence (Fig. 1c). The mutual exclusivity of CD117 and ER (Fig. 1c) confirms what has been reported by others11. Accordingly, we designed a fluorescence-activated cell sorting (FACS) protocol to first separate the basal cell population from the luminal cell population based on EpCAM (CD326) and NGFR (CD271) followed by sorting with CD166 and CD117 to further dissect the luminal compartment (Fig. 2a and Supplementary Fig. 3). This protocol yields three populations, the purity of which was assessed by staining smears with lineage and progenitor markers K14, K18 and K15 (ref. 22), ER–PR as well as the novel ERpos cell surrogate marker Ks20.8 (Fig. 2a).

As expected, we found that ERpos/PRpos HBECs were highly enriched in the CD166high/CD117low gate (Fig. 2a). While immunofluorescence staining for ER alone evoked a cytoplasmic background staining, which prevented reliable quantification, the smears turned out to be ideal for accurate assessment of the level of co-localization of ER–PR and Ks20.8. Indeed, up to 90% of Ks20.8pos cells were also ER–PRpos (Supplementary Fig. 4) and up to 87% of ER–PRpos cells were Ks20.8pos. The separation of the three subpopulations was further validated by quantitative reverse transcription–PCR (qRT–PCR), which confirmed a high ER expression (ESR1) in the CD166high cells compared with the other subpopulations (Fig. 2c). An additional panel of markers further distinguished the two luminal subpopulations from the basal cell population (Supplementary Fig. 5). Importantly, we found that known ER signalling-related genes such as trefoil factor family-1 (TFF1; ref. 23) and growth regulation by oestrogen in breast cancer 1 (GREB1; ref. 24) were highly expressed in CD166high cells as compared with other HBECs (Supplementary Fig. 5). The degree of separation in the CD166/CD117 FACS analysis was, however, somewhat biopsy dependent. In a series of six biopsies originating from women between 19 and 44 years old, five exhibited a similar separation between 19 and 44 years old, five exhibited a similar separation with 11–49% of the cells being CD166high cells of the other subpopulations (Fig. 2c). An additional panel of markers further distinguished the two luminal subpopulations from the basal cell population (Supplementary Fig. 5). Importantly, we found that known ER signalling-related genes such as trefoil factor family-1 (TFF1; ref. 23) and growth regulation by oestrogen in breast cancer 1 (GREB1; ref. 24) were highly expressed in CD166high cells as compared with other HBECs (Supplementary Fig. 5). The degree of separation in the CD166/CD117 FACS analysis was, however, somewhat biopsy dependent. In a series of six biopsies originating from women between 19 and 44 years old, five exhibited a similar separation between 11–49% of the cells being CD166high cells of the other subpopulations (Fig. 2c). While immunofluorescence staining for ER alone evoked a cytoplasmic background staining, which prevented reliable quantification, the smears turned out to be ideal for accurate assessment of the level of co-localization of ER–PR and Ks20.8.

ER protein is lost in culture. With the surrogate markers in hand we were now able to examine the behaviour of sorted ERpos HBECs in culture, potentially irrespective of ER expression. From the point of view that favourable conditions for luminal epithelial cells also apply to ERpos HBECs, we refined a protocol to permit ample colony formation of luminal cells at clonal density. As cell culture plastic we used Primaria, a substrate with a high content of nitrogen previously shown to promote adhesion of breast cells25. The growth medium 'FAD2' was modified from the point of view that favourable conditions for luminal epithelial cells also apply to ERpos HBECs, we refined a protocol to permit ample colony formation of luminal cells at clonal density. As cell culture plastic we used Primaria, a substrate with a high content of nitrogen previously shown to promote adhesion of breast cells25. The growth medium ‘FAD2’ was modified from...
but with less serum, that is, 5%, as in Liu et al.\textsuperscript{26} Under these conditions, we gauged for colony formation among the three FACS gated populations described above. When plated at a clonal density of 400 cells per cm\textsuperscript{2}, indeed colony forming luminal cells from the CD117\textsuperscript{high} gate were highly favoured over basal cells (Fig. 3a). However, it was also clear that the ERpos HBECs from the CD166\textsuperscript{high} gate entirely failed to form colonies under similar conditions. However, a scrutiny of cultures at a higher magnification revealed that CD166 high cells in fact did not disappear from the culture. Rather, they plated, survived and also stained with Ks20.8 as a testimony of their original identity, but they entirely refrained from growth and rapidly lost the ER protein (Fig. 3b). By comparison, other HBEC culture media, that is, M87A (ref. 28), MEGM or WIT-P-NC did not support plating of CD166\textsuperscript{high} cells. Therefore, we conclude that failure of culturing ERpos HBECs is caused by both lack of growth and loss of ER expression under conditions otherwise favouring propagation of luminal epithelial cells.

**Figure 2 | ERpos cells are purified and tracked by sequential CD326/CD271-CD166/CD117 FACS followed by multicolour staining and qRT-PCR.**

(a) Multicolour flow cytometry of uncultured HBECs incubated with CD326/CD271/CD166/CD117 and visualized pairwise (left diagrams) to recover luminal cells (CD326\textsuperscript{high}) and basal cells (CD271\textsuperscript{high}) and from the luminal gate CD166\textsuperscript{high} and CD117\textsuperscript{high} cells. Smears of sorted cells were stained (right panel) with either of the markers against basal cells, cytokeratin K14; luminal cells, cytokeratin K18; luminal progenitors, cytokeratin K15; Ks20.8 or ER–PR and counterstained with DAPI nuclear stain. Hormone receptor-positive cells are observed primarily among CD166\textsuperscript{high} cells. Scale bar, 50 μm. (b) Purity of sorted cells as determined by staining of smears followed by quantification of the percentage of cells stained with either of the markers cytokeratin K14, K18, K15, Ks20.8 or hormone receptors (ER–PR; 3 × 100 cells per slide, error bars indicate s.d.’s). (c) Heatmap representing qRT-PCR analysis of the relative gene expression of lineage markers in sorted basal cells (basal), CD117\textsuperscript{high} luminal cells (CD117) and CD166\textsuperscript{high} luminal cells (CD166) from six different biopsies. Data confirm lineage-specific transcriptional profiles of the three cell populations and restricts ER expression (ESR1) primarily to CD166\textsuperscript{high} luminal cells. Colour bar indicates the fold difference of the relative gene expression in log\textsubscript{2} scale.

**TGFβ inhibitors induce ER expression and release ERpos HBECs.** We noted that earlier in vivo studies had implicated TGFβ1 signalling in the restraint of ERpos mammary epithelial cells\textsuperscript{29,30}. Here we therefore examined three small inhibitor molecules of TGFβ signalling, RepSox, SB431542 and SD208, and combinations hereof for their ability to relieve a potentially negative regulation of ERpos HBEC growth in culture. We found that dual inhibition with SB431542 and RepSox, hereafter collectively termed TGFβR2i, recapitulated ER expression and stimulated ERpos HBEC colony formation in four out of four biopsies. Moreover, the cells maintained Ks20.8 reactivity, albeit more widespread than ER expression compared with what is seen
in situ (Fig. 4a). ER^{pos} cells from three different biopsies in low-density primary cultures in TGFβR2i (4,000 cells per cm^2) exhibited a clonal capacity of 0.54%, 0.33% and 0.43%, respectively. By comparison, control conditions resulted in small, mostly abortive ER^{neg} clones (Fig. 4b). In general, ER expression was particularly evident in the dense centre of proliferating colonies or at confluency. Two different sources of SP1 antibody gave similar results (Table 1). Moreover, staining was confirmed with the less-sensitive ER antibody 1D5 (ref. 31). The response to TGFβR inhibitors was reproducibly observed in 10 out of 10 biopsies.

In addition to ER, TGFβR2i induced the expression of keratin K8 and the luminal cell transcription factors forkhead box protein A1, FOXA1 and E74-like factor 5, ELF5 (for reviews, see refs 32,33) as revealed at the transcriptional level (Fig. 4c). This expression pattern was confirmed using another biopsy. TGFβR2i also upregulated the transcription of a number of genes known to be downstream targets of ER or modulators of ER activity, such as TFF1 (ref. 23) and insulin growth factor-binding protein 5 (IGFBP5; ref. 34; Supplementary Fig. 8a). Moreover, induction of ER protein expression to a significant degree was specific to RepSox as twice the concentration of SB431542 or replacement of RepSox with another ALK5 kinase inhibitor SD208 was insufficient to induce K8 and ER (data not shown). RepSox alone was not as effective in inducing ER or PR as in combination with SB431542 (Supplementary Fig. 8b). Likewise, while SB431542 alone was capable of inducing increased expression of ER at the mRNA level (Fig. 4c), this translated to the protein level to a significant degree only in the presence of RepSox (Fig. 4d and Supplementary Fig. 9). These findings suggest that ER expression is controlled through specific inhibition of TGFβ signalling. The presence of TGFβR in HBECs as well as the inhibition of phosphorylated SMAD2 concurrent with inhibitor-induced ER expression supported this (Supplementary Fig. 10 and Fig. 4d).

Indeed, TGFβR2i culture was a key to sustained ER protein expression as removal of TGFβR2i led to complete loss of ER protein expression as observed in four independent experiments. Thus, in a representative experiment the frequency of ER^{pos} cells...
was reduced from 33.7% (s.d. ± 5.0%; 3 × 100 cells) in continuous TGFβR2i culture to 10.7% (s.d. ± 0.5%) after 3 days and to 0% after 5 days without TGFβR2i.

We next tested whether TGFβR2i would induce de novo ER expression in other subpopulations of luminal cells, for example, in the much more frequent CD117<sup>high</sup>-derived HBECs. Consistent with the presumed progenitor status of CD117<sup>high</sup> cells in the human breast<sup>35</sup>, clones emerged from CD117<sup>high</sup> cells that gained ER and stained positively for Ks20.8. This was, however, the only additional source we found of ER<sup>pos</sup> HBECs, since TGFβR2i failed to induce ER in basal cells, in breast fibroblasts or in the established normal cell breast lines MCF10A or HMT-3522. Thus, both from preexisting ER<sup>pos</sup> HBECs and from ER<sup>neg</sup> progenitors, TGFβR2i readily provides growing colonies of ER<sup>pos</sup> HBECs.

**TGFβR2i allows growth of ER<sup>pos</sup> HBECs in serial subculture.** TGFβR2i appeared to support growth of ER<sup>pos</sup> HBECs also after passaging. To test this systematically, we plated CD166<sup>high</sup>-derived cells at a density of 6,400 cells per cm<sup>2</sup> in primary culture, and the cultures were subsequently passaged at a density of 4,000 cells per cm<sup>2</sup>. On passaging, ER expression was particularly evident in the dense centre of proliferating colonies. Continuous proliferation under these conditions was maintained for up to six passages, corresponding to 15 population doublings (exemplified in Fig. 5a). Importantly, however, in the absence of RepSox, the cells could not be expanded beyond fourth passage (Fig. 5a). The lifespan and ER expression (ranging from 21 to 77% in second- to fourth-passage cultures, Table 2) were somewhat biopsy dependent, and in general proliferation slowed between fourth and sixth passages, and at the same time down-regulation of ER expression was observed. While this narrowed the window of experimentation to up to fourth passage, the cells could easily and reproducibly be replaced with new cultures with similar luminal characteristics (Table 2). However, passage number could be increased by increasing seeding density. Thus, ER<sup>pos</sup> cells derived from three biopsies could be cultured for another two to three passages, that is, up to passage six (Fig. 5a). The lifespan of ER<sup>pos</sup> HBECs passed at low seeding density could be further extended by initial plating on 3T3 feeders, which lead to proliferation for more than 10 passages, corresponding to more than 25 population doublings (Fig. 5a).

Cells from a different sorting exhibited similar characteristics (Fig. 5a). We subsequently addressed whether TGFβR2i culture would allow for alternative sorting approaches to extend the lifespan of normal breast-derived ER<sup>pos</sup> cells. ER<sup>pos</sup> HBECs were successfully transduced with pBABE-neo-hTERT<sup>+</sup> and pLenti X2 hygro/shp16 constructs and have now been cultured for 4 months with weekly passages at 6,000 cells per cm<sup>2</sup>, exceeding 12 passages (Fig. 5a). Transduction of ER<sup>pos</sup> cells from a different biopsy and subsequent splitting at up to 1:4 supported the robustness of this approach. Of note, these long-term cultured cells exhibited a phenotype essentially similar to cultures with definitive lifespan, including a relatively high level of ER expression (Supplementary Fig. 11 and Table 2). Thus, while the proliferation of ER<sup>pos</sup> cells with definitive lifespan is somewhat slow as it took more than 100 days to generate more than 25 population doublings, the present protocol nevertheless allows a considerable expansion of the ER<sup>pos</sup> cell population extending from low-passage cultures with low seeding density through medium-passage cultures with high seeding density to high-passage cultures of hTERT/shp16-transduced cells, which provides a relatively wide window of experimentation. Of note, until senescence the cells maintain their expression of ER, Ks20.8 reactivity as well as—mainly in densely packed colonies—expression of PR (Fig. 5b). In conclusion, TGFβR2i readily supports serial subculture of a population of ER<sup>pos</sup> HBECs, that is, ER<sup>pos</sup> progenitors.

**ER<sup>pos</sup> progenitors respond to oestrogen.** As an ultimate test for a physiologically relevant ER expression we decided to assess the effects of added cognate ligand, that is, oestrogen (β-oestradiol). Accordingly, ER<sup>pos</sup> cells were exposed to 10<sup>−8</sup> M of β-oestradiol or vehicle. The effect of oestrogen was tested in the absence of epidermal growth factor (EGF) since the transcriptional activation of ER can be induced by EGF-induced MAP kinase activity<sup>38</sup>. As a first indication of a functional ER, a higher focal activity of PR protein, a downstream target of ER signalling, was seen in the presence of oestrogen (Fig. 6a).

![Figure 5](image-url) **Figure 5 | TGFβR2i allows efficient expansion of ER<sup>pos</sup> cells.** (a) Population doublings as a function of passage number calculated by continuous cell number recordings in triplicate cultures before confluency and plating at a fixed number of 4,000 cells per cm<sup>2</sup> per flask at each split. TGFβR2i allows proliferation for up to six passages, corresponding to 15 population doublings (open diamond). If RepSox is omitted, the cells cannot be expanded beyond fourth passage (cross). Initial plating on 3T3 feeders with quantification starting in passage three extends proliferation to more than 10 passages, corresponding to more than 25 population doublings (open circle). Cells from a different sorting (albeit followed for a shorter period) exhibit similar extended proliferative capacity (closed circle). hTERT/shp16-transduced CD166<sup>high</sup>/CD117<sup>low</sup> cells subsequently passaged at a fixed number of 6,000 cells per cm<sup>2</sup> at each split extended the proliferative capacity even further (closed triangle), and the cells have now been growing for more than 12 passages. hTERT/shp16-transduced CD166<sup>high</sup>/CD117<sup>low</sup> cells derived from a different biopsy, split at a ratio of 1:4, has so far been growing up to passage 9 (open square). (b) Even beyond 20 population doublings (passage 9), ER<sup>pos</sup> cells with definitive lifespan maintain ER and PR expression as shown by immunoperoxidase and haematoxylin staining (cells in ninth passage seeded at 4,000 cells per cm<sup>2</sup> and stained at day 5 with SP1 prediluted and SAN27, respectively). Scale bar, 50 μm.
cultures (passage 2–4) were exposed to oestrogen or vehicle, and the cell number was quantified. As seen in Fig. 6b, significantly higher cell numbers were recorded in cultures with oestrogen (Fig. 6b). Medium-passage cultures (passage 5–6) reached by high seeding density exhibited a similar response, albeit in general exhibiting a slower growth (Fig. 6b). Likewise, a proliferative response was observed in high-passage culture of hTERT/shp16-expressing cells. These data imply that the stromal microenvironment response was observed in high-passage culture of hTERT/shp16-expressing cells. Likewise, a proliferative response was observed in high-passage culture of hTERT/shp16-expressing cells. The prospective isolation and tracking of ER

### Table 2 | Frequency of ER

| Passage # | Per cent ER
|---|---|---|---|
| 2 | 41/21/24/38/42 | 100/100/100/100/100 | 44/47/100/ND/60 |
| 3 | 76*/27/21/39/29 | 100/100/100/100/100 | 44/47/100/ND/60 |
| 4 | 77*/26/27/38/51 | 100/100/100/100/100 | 44/47/100/ND/60 |
| 5 | 29*/72/39/30/20 | 100/100/100/100/100 | 44/47/100/ND/60 |
| 6 | 66/27/42/22 | 100/100/100/100/100 | 44/47/100/ND/60 |
| 7 | 54*/28/30/25/24 | 45/47/100/100/100 | 44/47/100/ND/60 |
| 8 | 57*/22/34/18/40 | 45/47/100/100/100 | 44/47/100/ND/60 |
| 9 | 52*/19/28/57 | 45/47/100/100/100 | 44/47/100/ND/60 |
| 10 | 20 | ND | ND | ND |

**Table 2** | Frequency of ER

| Passage # | Per cent K8
|---|---|---|
| 2 | 100/100/100/100/100 | 100/100/100/100/100 |
| 3 | 100/100/100/100/100 | 100/100/100/100/100 |
| 4 | 100/100/100/100/100 | 100/100/100/100/100 |
| 5 | 100/100/100/100/100 | 100/100/100/100/100 |
| 6 | 100/100/100/100/100 | 100/100/100/100/100 |
| 7 | 100/100/100/100/100 | 100/100/100/100/100 |
| 8 | 100/100/100/100/100 | 100/100/100/100/100 |
| 9 | 100/100/100/100/100 | 100/100/100/100/100 |
| 10 | 100/100/100/100/100 | 100/100/100/100/100 |

**Table 2** | Frequency of ER

| Passage # | Per cent K19
|---|---|
| 2 | 100/100/100/100/100 |
| 3 | 100/100/100/100/100 |
| 4 | 100/100/100/100/100 |
| 5 | 100/100/100/100/100 |
| 6 | 100/100/100/100/100 |
| 7 | 100/100/100/100/100 |
| 8 | 100/100/100/100/100 |
| 9 | 100/100/100/100/100 |
| 10 | 100/100/100/100/100 |

**Table 2** | Frequency of ER

| Passage # | Per cent P63
|---|---|
| 2 | 100/100/100/100/100 |
| 3 | 100/100/100/100/100 |
| 4 | 100/100/100/100/100 |
| 5 | 100/100/100/100/100 |
| 6 | 100/100/100/100/100 |
| 7 | 100/100/100/100/100 |
| 8 | 100/100/100/100/100 |
| 9 | 100/100/100/100/100 |
| 10 | 100/100/100/100/100 |

**Table 2** | Frequency of ER

**Discussion**

In spite of the fact that there are multiple protocols for enrichment, long-term cultivation and clonal growth of HBECs, none of them are able to isolate, track or support the growth of ER

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*ND, not done.

The numbers in black (roman font) indicate low-density split, ~4,000 cells per cm². Bold entries denote high-density split, ~8,000–20,000 cells per cm². Italics denote cells with extended lifespan on initial cultivation in BMY1AB and mouse feeders. The entries that are underlined indicate hTERT/shp16-transduced cells. The percentage of ER

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3 The prospective isolation and tracking of ER

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cells from normal breast tissue hold promises for the future comparisons between normal, benign and malignant ER

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cells, which will hopefully shed some light on the evolution and pathogenesis of the most frequent form of human breast cancer. Being able to isolate and track the cells, however, would be of limited value if the ER expression was lost on culture. It has been anticipated that ER

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normal cells cannot divide and that this is why HBECs rapidly lose ER expression in culture. We show here that ER

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cells can be released from growth restraint and sustained by TGFβR2i and that ER

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cells under these conditions can be expanded considerably. These findings may represent a paradigm shift in studying ER expression and function in the breast, in the future no longer relying exclusively on in vivo rodent models and human breast cancer cell lines. In addition, we believe the results answer the long-standing question of whether ER

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cells can self-renew, and further establishes that ER

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cells can be generated from ER

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progenitors, here represented by CD117

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luminal cells. Importantly, the response to TGFβR2i is specific to luminal cells only, as neither basal cells, fibroblasts nor ER

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normal breast cell lines are able to switch on ER in response to TGFβR2i. The fact that ER

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HBECs are here shown to be proliferating is in favour of the existence of ER

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progenitors. In general, however, most ER

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HBECs are considered to be close to the base in the hierarchy. As such, this is a potential therapy for breast cancer, which, if successful, will significantly impact the treatment of this disease.
The release of ER<sup>pos</sup> HBECs in culture by TGFβR2i is clinically relevant because it may help explain an enigmatic difference between the normal human breast and breast cancers. Thus, in the normal breast in vivo there is a strict dissociation between steroid receptor expression and proliferation<sup>15</sup>. In breast cancer and to a varying degree in precancerous breast lesions this negative association is lost<sup>44</sup>. It has been speculated that this may represent an important early change in the genesis of breast cancer either reflecting a failure to downregulate ER as cells enter the cell cycle or a failure to suppress division of ER<sup>pos</sup> cancer cells<sup>44</sup>. Our data indeed are in favour of the latter possibility because ER<sup>pos</sup> HBECs do divide if TGFβ signalling is perturbed, a very likely scenario in cancer (for review see ref. 45). While in primary breast cancer a normal-like TGFβ signalling is still in operation to restrain growth, in metastatic breast cancer TGFβ signalling has shifted to that of an epithelial–mesenchymal transition response<sup>45</sup>.

**Figure 6 | ER<sup>pos</sup> cells in culture respond to oestrogen.** (a) Staining of PR (SAN27) in second-passage CD166<sup>high</sup> cells deprived of EGF and exposed to vehicle (EtOH) or oestrogen (E2) for 10 days. ER<sup>pos</sup> cells respond to oestrogen by increased expression of PR. (b) Quantification of cell number in four sets of pairwise triplicate cultures of EGF-deprived, oestrogen-stimulated CD166<sup>high</sup> low-passage cells grown for 4 days in second (P959) or third passage (P958), or 13 days in fourth passage (P959); medium-passage cells grown at high density before growth for 6 days (P958), 15 days (P957) and 14 days (P957 cultured without EGF since second passage) in fifth passage or cells with extended lifespan grown for 14 days in sixth passage; or high-passage cells (hTERT/shp16) in seventh passage grown for 13 days or in tenth to twelfth passage grown for 11, 15 and 11 days, respectively, in the presence of oestrogen (dark grey) or vehicle (light grey); or pairwise triplicate cultures of CD166<sup>high</sup> cells plus feeder grown for 8 days in the presence of oestrogen (dark grey) or vehicle (light grey). Second-passage cultures derived from two different biopsies (first and fourth set of bars) do not respond differently from third-passage cultures (second set of bars), irrespective of omission of EGF from TGFβR2i before co-culture (second set of bars). Omission of EGF throughout the entire experimental period reduced the total cell number, but did not augment the response to oestrogen (third set of bars). Error bars indicate s.d.'s. In all cases, oestrogen treatment significantly increases the growth of ER<sup>pos</sup> cells, independent of passage group (by analysis of variance (ANOVA), P < 0.05). The difference between experimental and control in each passage group is statistically significant by paired t-test (P values in each diagram). (c) Relative expression in triplicate of PGR and GREB1 from second (P957 and P959), third passages (P958), or fourteenth passage long-term (hTERT/shp16) cultured ER<sup>pos</sup> cells exposed to vehicle (EtOH, grey bars) or oestrogen (E2, black bars) for 4 (P958 and P959), 22 (P957) or 13 (hTERT/shp16) days, respectively. Transcription of downstream target genes of ER signalling, PGR and GREB1 is in all cases significantly upregulated by oestrogen as assessed by two-tailed t-test, P < 0.05. Error bars indicate s.d.’s.
Another implication of the TGFβR2i culture protocol is that it represents a much-in-demand cell-based assay for oestrogen action on normal cells. It is already well established that oestrogen is a mitogen for ER\textsuperscript{pos} breast cancer. However, its role in relation to ER\textsuperscript{pos} HBEcs has remained a mystery due to lack of ER\textsuperscript{pos} cell culture models, and because ectopic expression of ER in basal cell lines under standard culture conditions has provided the paradoxical result of growth inhibition\textsuperscript{11,12}. As a proof of principle, we here show that the TGFβR2i protocol serves as a physiologically relevant cell-based assay for oestrogen action, and moreover, in ER\textsuperscript{pos} cells, several oestrogen-responsive genes downstream of ER are upregulated. Thus, the TGFβR2i protocol represents a cell-based assay for gauging oestrogen action reminiscent of its action in vivo. Intriguingly, in the presence of fibroblasts, growth of ER\textsuperscript{pos} HBEcs is stimulated by oestrogen irrespective of the presence of EGF. This implies that stromal factors modulate ER activity. With the relevant representatives of ER\textsuperscript{pos} cells in hand, the complexity of this interaction can now be elucidated.

Our data further suggest that TGFβ signalling is key to the CD166\textsuperscript{high} phenotype, and the exact mechanisms by which TGFβR2i generate ER\textsuperscript{pos} cells clearly await further scrutiny. It cannot be excluded, however, that what we observe is part of a more general association between CD166 expression and TGFβ signalling. Thus, very recently others have found that TGFβ signalling is a main driver of the behaviour of CD166-expressing prostate cells.\textsuperscript{46}

The present protocol is the result of several years of experimentation including many reduction mammoplasties from different donors until the present conditions were established. We cannot exclude that further improvements are possible. The data illustrate, however, that apparent post-mitotic cells in vivo, given the right conditions may multiply considerably, yet still exhibiting a definitive lifespan, and the approach moreover serves as a platform for extending the lifespan of ER\textsuperscript{pos} cells into serial subcultures.

While we have reproducibly recovered and propagated ER\textsuperscript{pos} cells from all biopsies tested, we nevertheless wish to emphasize on a number of technicalities, which should be carefully observed when the protocol is applied. Biopsies inherently vary with respect to the frequency of ER\textsuperscript{pos} cells, which may eventually influence the definite number of ER\textsuperscript{pos} cells that can be derived from a given biopsy. Biopsies also vary with respect to growth potential, but in our hands they all perform well up to at least passage 4 (Table 2). It should be kept in mind, however, that the protocol releases only a minority of the total number of initially quiescent ER\textsuperscript{pos} cells, that is, up to 0.54% when plated at 4,000 cells per cm\textsuperscript{2} on sorting. Also, we strongly recommend that any attempts to validate Ks20.8 as well as co-expression of ER and PR cannot exclude that further improvements are possible. The data further suggest that TGFβ signalling is a main driver of the behaviour of CD166-expressing prostate cells.\textsuperscript{46}

Fluorescence-activated cell sorting. To reveal epithelial cell composition and to isolate single cells, organoids from 13 biopsies were trypsinized, filtered through a 100 \textmu m filter and resuspended in HEPES buffer supplemented with BSA (bovine fraction V; Sigma-Aldrich) and 2 mM EDTA (Merck), pH 7.5. The suspended cells were incubated for 45 min at 4 °C in the presence of conjugated monoclonal antibodies EpCAM/CD326-PerCP (clone Zyx20.4, 1:50, Cytokine Laboratories) to separate basal and luminal cells, and activated and P8C3.5 (clone 5F14, 1:20, Abcam) followed by 20 min incubation with secondary antibody Alexa Fluor 647 Goat Anti-IgG1 (1:500) and analysed by FACS. Overlay images were produced using Flowing Software 2.5.1 (University of Turku, Finland).

To establish fibroblast feeders, fourth-passage fibroblasts were incubated as described above with monoclonal antibodies CD26 (202–36, 1:200, Abcam) and conjugated CD117AF647 along with 67 kDa Laminin Receptor (MLuC5 1:50, Abcam) followed by secondary antibody AF488 (IgG1, 1:500) and analysed by FACS. Overlay images were produced using Flowing Software 2.5.1 (University of Turku, Finland).

Cell culture. On sorting, the primary cell populations were plated in Primaria T25 flasks (#3813, Becton Dickenson) in the presence of the FAD2‘ (DMEM, high glucose, no calcium, Life Technologies) 3:1 v/v with 2 mM glutamine, 0.5 mM hydrocortisone, 5 \mu g ml\textsuperscript{-1} insulin, 10 ng ml\textsuperscript{-1} cholera toxin (Sigma-Aldrich), 10 ng ml\textsuperscript{-1} epidermal growth factor (Peptrochem), 1.8 \times 10\textsuperscript{7} M adenine, 4.9 \times 10\textsuperscript{-7} M Y-27632 (Y0503, Sigma-Aldrich or 1683, Axon Medchem) and 5% fetal bovine serum (Sigma-Aldrich), modified from refs 26,27. On plating, which could take up to 2 days for the luminal ER\textsuperscript{pos} (CD166\textsuperscript{hi},CD117\textsuperscript{low}) cells and thus determined the time point for addition of TGFβR2i, a combination of the selective inhibitor of TGFβ type I receptor activin receptor-like kinase ALK5, RepSox was replaced by another TGFβ type I receptor activin receptor-like kinase ALK5 inhibitor, SB203580 (25 or 50 \mu M, R0158, Sigma-Aldrich) was added. To test the specificity of the effect of TGFβR2i, in some experiments SB203580 alone or the double concentration of SB203580 was used instead of TGFβR2i, or RepSox was replaced by another TGFβ type I receptor activin receptor-like kinase ALK5 inhibitor, SB203580 (50 \mu M, Tairos Bioscience). The vehicle, dimethyl sulfoxide (Sigma-Aldrich), was included in all experiments in appropriate concentrations for control cultures. Gentamicin (30 \mu g ml\textsuperscript{-1}, Biologica Industrias) was added to the cultures throughout the first week after sorting. Otherwise antibiotics were not included. To test the effect of ER\textsuperscript{pos} cells was restricted to the FAD2 medium, CD166\textsuperscript{hi},CD117\textsuperscript{low} cells sorted from one of the biopsies were plated at a density of 4,000 cells per cm\textsuperscript{2} on...
4,000 cells per cm² with or without TGFβ2 in other breast cells was further tested in MCF-10A, HMT-3522 (ref. 55), fibroblasts purified from normal breast tissue, CD117high cells purified from three different biopsies, as well as in basal cells isolated by FACS as described above and cultured on irradiated NIH-3T3 feeders before exposure to TGFβ2 to confirm the response of fibroblasts to TGFβ2 as previously followed by staining for Ks20.8 and ER. Fibroblasts were routinely grown to confluency on collagen-coated T25 flasks (Nunc, 8 µg collagen per cm², PureColl, CellSystems) in DMEM/F12, with 2 mM glutamine and 5% FBS before co-culture with CD166high/CD117low luminal cells. For phase contrast microscopy a Nikon Diaphot 300 microscope was used.

Cloning efficiency at low density in FAD2 was observed by plating the sorted basal cells, and the luminal populations CD166high/CD117low and CD166high/CD117low, at 400 cells per cm² for 14 days, followed by fixation in methanol for 5 min at −20 °C and counterstaining of nuclei with haematoxylin. To assess the Ks20.8 and ER expression of the sorted populations and to further assess whether starting the cultures at different densities would change the expression, cells were seeded at 3,000 cells per cm² and cultured for 9 days before immunocytochemical staining.

To quantify the frequency of Ks20.8high/ERpos colony-forming units CD166high/CD117low luminal cells from the different biopsies were plated at a density of 4,000 cells per cm² with or without TGFβ2 and grown for 13 days before staining for ER and Ks20.8 followed by quantification using an ocular grid. The number of stained colonies defined as presence of either marker in three areas of each culture relative to the initial number of seeded cells was calculated.

Low-density split cultures were initially seeded at 4,000 cells per cm² and grown for up to 15 days in primary culture in TGFβ2 or SB431542 alone. Next, the cultures were trypsinized and seeded at 4,000 cells per cm² in triplicate cultures, and subsequently passaged at the same density before the cultures reached confluency. Parallel cultures were stained to assess Ks20.8, ER and PR expression status. The number of cells was quantified manually using a counting chamber. Medium was changed at the same density that is, 8,000–20,000 cells per cm². For extended cultivation of low-density cultures sorted luminal Ks20.8high/ERpos (CD166high/CD117low) cells were first allowed to form colonies on irradiated NIH-3T3 feeders in modified basal medium (BBM) without HEPESTM (DMEM/F12 supplemented with glutamine, 100 µM ethanamine (Sigma, H0155), 1 µg/ml recombinant human insulin (Sigma, H0888), 9 µg/ml insulin (Sigma, I6634), 5 µg/ml transferrin (Sigma, T1147), 5.2 mM L-glutamine (sodium salt, BD Biosciences, 354201), 20 ng/ml basic fibroblast growth factor (Peprotech, 100-188) and 5nM amphotrelin (R&D Systems, 262-AR-100/CF) with the addition of Y-27632, adenine and the replication receptor B72 (20 µl/ml Life Technologies). TGFβ2 or with SB431542 alone. As an alternative approach to extend the lifespan of Ks20.8pos/ERpos cells, the method by Kiyono et al.27 was adapted by introducing human telomerase (hTERT) and shRNA p16 (shp16) to passage CD166high/CD117lowcells. The viral constructs, pLenti X2 Hyg/Int (IP2–16, AddGene #22264, a gift from Eric Campbell), and pBABE-neo-hTERT (AddGene #1774, a gift from Robert Weinberg) were prepared as follows: lentiviral particles containing the shp16 expressing construct pCMV–vesicular stomatitis virus glycoprotein as well as pol–vesicular stomatitis virus glycoprotein were generated by transient co-transfection of the vector construct into a pantropic retroviral packaging cell line GP2–293 (Clonetech), which stably expresses the retroviral packaging cell line GP2–293 (Clonetech), which stably expresses the calcium phosphate method with the vesicular stomatitis virus glycoprotein and the calcium phosphate method. The resulting virus particles were used to transduce CD166high/CD117low pLenti X2 Hygro/shp16 construct transduced cells with 100 µg/ml hygromycin (Sigma) for more than 2 weeks. The efficiency of the protocol was confirmed by hTERT/shp16 transducing second-passage CD166high/CD117low/Ks20.8pos/ERpos cells derived from a different biopsy.

To test the proliferative response to oestrogen in low-passage (p≤2) cultures, ERpos cells in TGFβ2 cultures were seeded with 4,000 cells per cm² in TGFβ2 with 25µM RepSox and without EGF, supplemented with oestrogen (10–8 M, β-oestradiol, E2758, Sigma–Aldrich) and cultured for up to 13 days with medium change every other day. A primary culture from another biopsy was cultured 27 days in the presence of oestrogen and without EGF before splitting. The cells were plated at 4,000 cells per cm², and the high-passage (p>27) cultures including two hTERT/shp16-transduced qRT–PCR cultures was passaged at 10,000–20,000 cells per cm² before seeding at 4,000 cells per cm², and the high-passage (p>13) cultures including two hTERT/shp16-transduced qRT–PCR cultures was passaged at 4,000 cells per cm² or split at up to 1:4 before the growth experiment with a seeding density of 6,000 cells per cm². The effect of an oestrogen antagonist (IC1182,780, Sigma–Aldrich), was tested in sixteenth passage, single-cell-cloned hTERT/shp16-transduced cells seeded at 4,000 cells per cm² in TGFβ2, exposed to vehicle or oestrogen (10–8 M) and increasing concentrations of IC1182,780 (10–10, 10–9 and 10–8 M). At day 6, cultures were trypsinized and quantified in a cell counter (Roche InnovaTec).

To assess whether the response of ERpos cells to oestrogen was modulated by the presence of fibroblasts, second- or third-passage ERpos cells, with or without fibroblasts purified from normal breast tissue, were seeded at 2,000 cells per cm² with or without TGFβ2 and grown for 5 days before staining for ER and Ks20.8. As an alternative approach to extend the lifespan of Ks20.8pos/ERpos cells, the method by Kiyono et al.27 was adapted by introducing human telomerase (hTERT) and shRNA p16 (shp16) to passage CD166high/CD117low cells. The viral constructs pLenti X2 Hyg/Int (IP2–16, AddGene #22264, a gift from Eric Campbell) and pBABE-neo-hTERT (AddGene #1774, a gift from Robert Weinberg) were prepared as follows: lentiviral particles containing the shp16 expressing construct pCMV–vesicular stomatitis virus glycoprotein were generated by transient co-transfection of the vector construct into a pantropic retroviral packaging cell line GP2–293 (Clonetech), which stably expresses the calcium phosphate method. The resulting virus particles were used to transduce CD166high/CD117low pLenti X2 Hygro/shp16 construct transduced cells with 100 µg/ml hygromycin (Sigma) for more than 2 weeks. The efficiency of the protocol was confirmed by hTERT/shp16 transducing second-passage CD166high/CD117low/Ks20.8pos/ERpos cells derived from a different biopsy.

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Immunohistochemistry and cytochemistry. Cryostat sections, smears of sorted cells and monolayer cultures were prepared and stained by immunoperoxidase or immunofluorescence, including negative controls without primary antibodies. Specifically, cellular smears for ER–PR staining were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) followed by fixation in ice-cold methanol:acetone (1:1) for 10 min at −20 °C. Smears were then washed twice for 3 min and blocked for 10 min in 0.3% saponin/10% goat serum in PBS. All subsequent washing and incubation steps were performed in 0.3% saponin/10% goat serum. To visualize hormone receptor expression smears were incubated with anti-ER (SP1, 1:10) and anti-PR (SP2, 1:10) for 2 h at room temperature followed by fixation with 30 min with Alexa Fluor 568 Goat anti-Rabbit IgG (1:500, Invitrogen). For quantification of ER–PR and Ks20.8 expression, smears were fixed as described above and subsequently incubated with anti-ER (SP1, 1:10), anti-PR (SP2, 1:10) and Ks20.8 (1:10) for 2 h at room temperature followed by incubation for 30 min with Alexa Fluor 568 Goat anti-Rabbit IgG (1:500, Invitrogen) and Alexa Fluor 488 Goat anti-Mouse IgG2a (1:500, Invitrogen). Slides were mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Life Technologies) and bright-field microscopes (Laborlux S or DM5500B, Leica), photographed using a laser-scanning microscope (LSM 510 or LSM700; Carl Zeiss MicroImaging, Inc.) and bright-field microscopes (Labophor S or DM5500B, Leica), respectively. For quantification of immunoperoxidase staining of ER, K8, K19 and p63, nuclei were counterstained with haematoxylin and counted in randomly selected fields using an ocular grid and given as the percentage of stained cells of a total of 1,000 cells evaluated with a ×20 objective in the confocal microscope.

Antibodies are listed in Table 1. Many antibodies stain independently of fixation procedure, but of note, to stain for ER and PR, cultures were rinsed in PBS, pH 7.4, or sections were air dried before fixation, for 5 min at room temperature, in 3.7% formaldehyde, two rinses in PBS, fixation in methanol:acetone (1:1) for 5 min at 20 °C, two rinses in PBS, permeabilization in 0.1% Triton X-100 in PBS, twice for 7 min, rinse in PBS and kept wet before application of UltraV Block (Thermo Scientific). ER and PR expression in cultures with long-term oestrogen exposure was assayed by immunoperoxidase for ER (SP1, prediluted) and PR (SAN27 or PgR636) by double-labeling immunofluorescence for ER (SP1, 1:10) and PR (PgR636, 1:10) with Alexa Fluor 488 Goat anti-Rabbit IgG (1:500) and Alexa Fluor 568 Goat anti-Mouse IgG1 (1:500) as secondary antibodies. To stain for Ks15, UltraV Block was substituted for 10% normal goat serum in PBS. Immunoreactivities and peroxidase staining were evaluated, quantified and photographed using a laser-scanning microscope (LSM 510 or LSM700; Carl Zeiss MicroImaging, Inc.) and bright-field microscopes (Labophor S or DM5500B, Leica), respectively. For quantification of immunoperoxidase staining of ER, K8, K19 and p63, nuclei were counterstained with haematoxylin and counted in randomly selected fields using an ocular grid and given as the percentage of stained cells of a total of 1,000 cells evaluated with a ×25 objective.

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