Histol Histopathol (2009) 24: 1381-1390

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

MUC-1$^-$/ESA$^+$ progenitor cells in normal, benign and malignant human breast epithelial cells

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Summary. The existence of mammary epithelial stem/progenitor cells has been demonstrated in MUC-1$^-$/ESA$^+$ subpopulations of breast epithelial cells. However, knowledge about the expression and localization in benign and malignant breast lesions is unknown. Using a double-staining immunohistochemistry method, we investigated MUC-1$^-$/ESA$^+$ cells in 10 normal breast tissues, 49 cases with fibrocystic disease, 40 fibroadenomas, 36 invasive ductal carcinomas and the breast cancer cell lines MCF-7 and MDA-MB-468. In normal breast tissues MUC-1$^-$/ESA$^+$ cells were mainly found in the suprabasal layer, but under the apical surface of the duct/alveolus. In the hyperplastic areas of fibrocystic disease, the number of this subpopulation of cells was higher than that in hypoplastic areas and in fibroadenomas. In invasive ductal carcinoma, the MMUC-1$^-$/ESA$^+$ cells were heterogeneously present in different carcinoma nests. In the MCF-7 cell line most cells were MUC-1$^-$/ESA$^+$, and in the MDA-MB-468 cell line MUC-1$^-$/ESA$^+$ cells and MUC-1$^-$/ESA$^+$ cells were almost equal. Our results show that the MUC-1$^-$/ESA$^+$ subpopulation increases in fibrocystic disease within the hyperplastic areas, and varies in benign and malignant breast tumours, indicating that breast carcinogenesis may develop from malignant changes of normal MUC-1$^-$/ESA$^+$ cells.

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Key words: Human mammary gland, Stem/progenitor cell, Immunohistochemistry

Introduction

The terminal duct/lobular unit is the basic structure in the breast, and it consists of a branching ductal-alveolar system lined by an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells. Luminal and myoepithelial cells can be identified by their expression of various surface and cytoskeletal proteins. Luminal cells are characterised by their expression of epithelial membrane antigen (EMA) (O'Hare et al., 1991), epithelial specific antigen (ESA) (Stingl et al., 1998) and cytokeratins (K) 7, 8, 18 and 19 (Taylor-Papadimitriou et al., 1989). Myoepithelial cells express α-smooth muscle actin (α-SMA), α6 integrin, vimentin (Guelstein et al., 1988) and K5 and K14 (Jarasch et al., 1988).

Evidence is accumulating for the existence of mammary stem cells that can form all epithelial lineages. Three types of human breast epithelial progenitors have been identified through marker expression in in vitro cultures (Stingl et al., 1998). Bipotent progenitor cells with a capability of generating colonies with a central core of cells expressing the luminal marker ESA, surrounded by cells expressing the myoepithelial marker K14, have also been identified (Stingl et al., 2001). Increasing evidence supports the hypothesis that myoepithelial cells are derived from luminal precursors. Gudjonsson and coauthors (Gudjonsson et al., 2002) reported cell markers associated with breast stem cell properties. In their experiment, luminal cells from primary cultures were separated immunomagnetically by sialomucin (MUC-1, identical to EMA) and ESA into

subsets MUC-1$^-$/ESA$^+$ and MUC-1$^+$/ESA$^+$. Two cell lines were further established by transduction of the E6/E7 genes from human papilloma virus type 16. In clonal cultures, the MUC-1$^+$/ESA$^+$ epithelial cell line revealed luminal epithelial cells in its differentiation repertoire, while the MUC-1$^-$/ESA$^+$ epithelial cell line was able to generate both MUC-1$^-$/ESA$^+$ and MUC-1$^-$/ESA$^+$ epithelial cells, in addition to Thy-1$^+$/α-smooth muscle actin$^+$ (ASMA$^+$) myoepithelial cells. Within a reconstituted basement membrane, the MUC-1$^+$/ESA$^+$ epithelial cell line formed acinus-like spheres, but the MUC-1$^-$/ESA$^+$ epithelial cell line formed, in contrast, elaborate branching structures resembling uncultured terminal duct lobular units both by morphology and marker expression, indicating that MUC-1$^-$/ESA$^+$ epithelial cells within the luminal epithelial lineage may function as stem/precursor cells of terminal lobular units in the human breast. In other independent experiments on human breast (Alvi et al., 2003; Clayton et al., 2004), similar results were reported. Further studies indicate that MUC-1$^-$/ESA$^+$ is not the sole phenotype progenitor cell, and CD49f^{high}/EpCAM^{low} and ESA$^+$/EpCAM^{low} are also indicated to be markers for primitive cell types (Stingl et al., 2006a,b; Stingl and Caldas, 2007).

Knowledge about the localization of stem/progenitor cells in breast, especially in different breast lesions, is still not fully elucidated. Our hypothesis is that if the MUC-1$^-$/ESA$^+$ cells are representatives of normal progenitor cells, and these cells may also be identified in fibrocystic disease with hypoplastic areas, hyperplastic areas, adenomas, and even invasive malignant lesions, this will allow us search for evidence of malignant transformation of normal stem cells. To verify this, we performed double-staining immunohistochemistry (DIHC) of ESA and MUC-1 in normal breast tissue, benign breast lesions, breast carcinomas and breast cancer cell lines (MCF-7 and MDA-MB-468). We found that in normal breast tissues, MUC-1$^-$/ESA$^+$ cells were mainly present in the suprabasal layer, under the apical surface of the duct or alveolus wall. In hyperplastic areas of fibrocystic disease, the number of these subpopulation cells was higher than that in hypoplastic areas and in fibroadenoma. In invasive ductal carcinoma, the MUC-1$^-$/ESA$^+$ cells heterogeneously appeared in different carcinoma nests. In the MCF-7 cell line, however, most cells were MUC-1$^-$/ESA$^+$, while in MDA-MB-468 cell line MUC-1$^-$/ESA$^+$ cells and MUC-1$^+$/ESA$^-$ cells were almost equal. Our results indicate that carcinogenesis of breast may be derived, at least partly, from a malignant change of normal MUC-1$^-$/ESA$^+$ cells.

Material and methods

Materials

Our study comprised 125 breast lesions and 10 normal breast tissues. Surgically removed samples from patients with breast fibroadenoma (40 cases), breast fibrocystic disease (49 cases) and invasive ductal carcinoma (IDC) (36 cases) were included in this study. All the patients were diagnosed at The Department of Pathology, The First Affiliated Hospital of Zhengzhou University, Henan, China, between 2006 and 2007. The cases were re-examined independently by two surgical pathologists. No chemotherapy or radiotherapy was given prior to surgery. Normal breast samples (n=10) were obtained from patients undergoing reduction mammoplasty for cosmetic reasons, with no previous history of breast disease.

Cell culture

MCF-7 and MDA-MB-468 breast cancer cell lines were maintained in our lab. The cells were cultured in DMEM, supplemented with 10% FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin (GIBCO).

Cell block preparation

Cells from the cell lines were removed from the culture dish with trypsin and EDTA (Sigma), washed and centrifuged at 2000 rpm for 10 minutes. After removal of the supernatant, 3 drops of plasma and 2 drops of thrombin were added to the sedimentation before carefully mixing by tube rotation. One minute later, the mixture was coagulated. Then 4% buffered formalin was added. The coagulated mass was then placed in linen paper for further conventional paraffin block making process. Four μm sections made from these blocks were used for immunohistochemistry.

Double Staining Immunohistochemistry (DIHC)

4 μm thick sections were cut from the formalin-fixed and paraffin embedded tissue or cell blocks. The sections were dewaxed with xylene and gradually hydrated. The following primary antibodies were used in this study: primary mouse anti-human MUC monoclonal antibody, (IgG2a/κ, clone E29, Maixin-bio, China); primary mouse anti-human ESA monoclonal antibody, (lgG1, clone Vu-1D9, Maixin-bio, China). Double staining was performed by a Strept-Avidin-Biotin (DouSP^{TM} IHC double stain kit, Maixin-bio, China) method according to the supplier's protocol. All incubations were carried out at room temperature for 1 h. The MUC-1 antibody was firstly visualized with BCIP/NBT/Alkaline Phosphatase, and the positive signal was purple or dark on the cell membrane. The ESA immunoreaction was demonstrated with H_2O_2/AEC/Streptavidin Peroxidase, and the positive signal was carmine red on the cell membrane or cytoplasm. Afterwards, the slides were briefly counterstained with hematoxylin. To detect myoepithelial cells, two myoepithelial markers, smooth muscle actin (SMA, IgG2a/κ, clone 1A4, Maixin-bio, China) and P63 protein (IgG2a/κ, clone 4A4, Maixin-bio, China), were examined respectively using UltraSensitive S-P kit (Maixin-bio, China). Antigen retrieval was done in a pressure cooker containing 0.1

mol/L sodium citrate for 15 min, and the sections were incubated overnight at 4°C with the primary antibodies. The peroxidase reactions were performed using 0.5 mg/mL 3,3'-diaminobenzidine for 5 min and the positive signal was brown. As a negative control, corresponding non-immune IgG1 or IgG2 from the same company was used at the same concentration. Known positive tumours for the above antibodies were used as positive control for this study. All controls provided satisfactory results.

DIHC evaluation

In normal breast tissue and fibroadenomas, three high power (HP) visual fields of typical area were firstly located in every slide and then MUC-1$^-$/ESA$^+$ cells were counted. The mean of the numbers of MUC-1$^-$/ESA$^+$ cells per HP in three regions was calculated for each case. For fibrocystic disease we observed more variation with areas full of ducts and lobular structures and other areas with dominance of fibrosis. To deal with this variation, we divided all the cases into two histological types: hyperplastic areas (the lobules were more than 3 or alveoli more than 30 in low visual field) and hypoplastic areas (the lobules were less than 2, and alveoli less than 15 in low visual field). The densities of MUC-1$^-$/ESA$^+$ cells in 60 hyperplastic areas and 60 hypoplastic areas were then calculated as described above, respectively. In invasive ductal carcinoma (IDC), three high power visual fields of the most cellular areas were firstly located in every slide and then MUC-1$^-$/ESA$^+$ cells were counted and the mean of the counting was obtained as described above.

Statistical analysis

Student's t-test was used to discover the difference in densities of MUC-1$^-$/ESA$^+$ cells. 2-tailed P of less than 0.05 was considered significant.

Results

Normal breast tissues

MUC-1 immunoreactivity was only found on the apical surface of luminal epithelium, and ESA positive signals were also shown on the suprabasal cells. Therefore, the first layer to lumen was ESA$^+$/MUC1$^+$. MUC-1$^-$/ESA$^+$ cells were observed in the middle layer of ducts/alveoli wall, or in small cell groups showing no lumen structure (Fig. 1). The density of the MUC-1$^-$/ESA$^+$ cells in normal breast tissue was 0.5~6/HP, mean of 1.1±0.208/HP. The basal cells were constantly negative for these two factors (Fig. 1a). Further analyses verified the myoepithelial features. The basal cells showed cytoplasmic staining for SMA, and nuclear immunoreactivity for p63 protein (Fig. 2). Occasionally, a few myoepithelial cells could be observed admixed in the small MUC$^-$/ESA$^+$cell groups (Fig. 1b).

Breast fibroadenoma

The duct/alveoli with threadlike lumen were compressed by increased fibrous connective tissue. The inner layer of the epithelium was MUC-1$^+$/ESA$^+$, and the outer layer was MUC-1$^-$/ESA$^-$. Occasionally MUC-

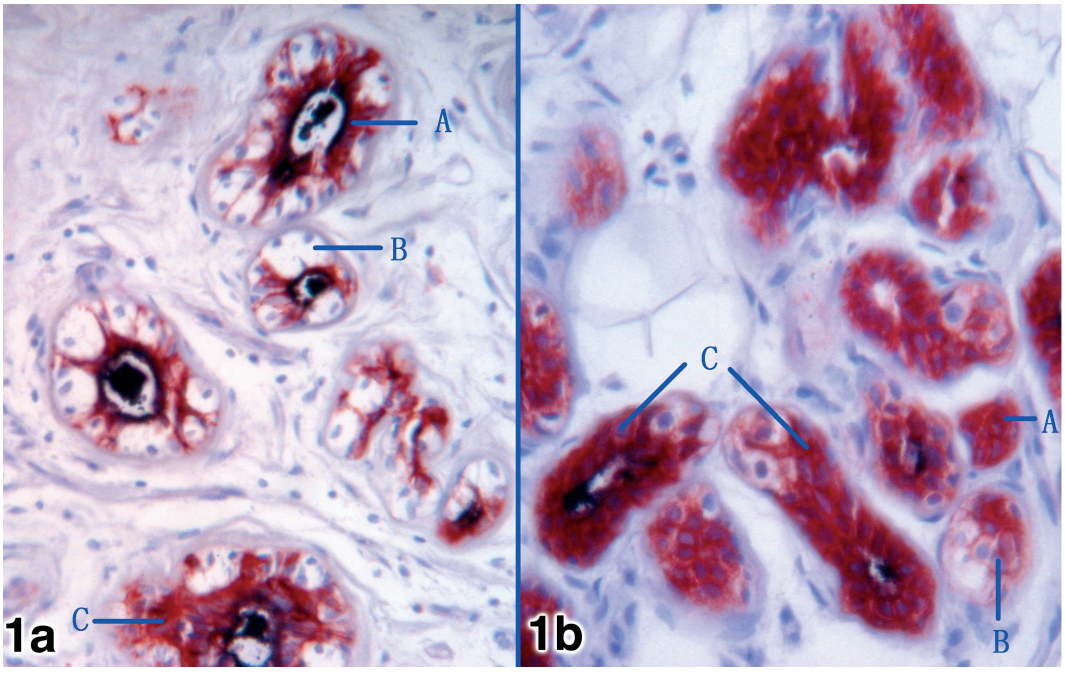

Fig. 1. Double staining for MUC-1 and ESA in normal breast tissue. MUC-1: black. ESA: carmine. **a.** MUC-1$^+$/ESA$^+$ cells (apical layer) (A); MUC-1$^-$/ESA$^-$ cells (myoepithelial cells) (B) and MUC-1$^-$/ESA$^+$ cells (C). **b.** A group of MUC-1$^-$/ESA$^+$ cells (A); a group of MUC-1$^-$/ESA$^+$ cells with MUC-1$^-$/ESA$^-$ cells (B); two groups with mixed ESA$^+$/MUC-1$^+$ cells, MUC-1$^-$/ESA$^+$ cells and MUC-1$^-$/ESA$^-$ cells (C). x 40 objective.

1⁻/ESA cells could be observed between the two layers (Fig. 3). The density of the MUC-1⁻/ESA⁺ cells in fibroadenoma was 0.3~5/HP, mean of 2.1±0.216/HP, significantly higher than that in normal breast tissue ($p<0.05$), (Table 1).

Fibrocystic disease

We selected 60 hyperplastic areas and 60 hypoplastic areas in 49 cases with fibrocystic disease of the breast. In hyperplastic areas crowded with ducts and alveoli with small and irregular lumens, we observed three types of epithelia: an inner single layer of MUC-1⁺/ESA⁺ cells, a single layer of MUC-1⁻/ESA⁻ cells outside, and multiple layers with MUC-1⁻/ESA⁺ in between (Fig. 4). The densities of MUC-1⁻/ESA⁺ cells in lobule hyperplastic sections were 2~50/HP(mean was 14.3±1.121/HP), significantly higher than that in normal breast tissue and fibroadenomas ($p<0.05$). In hypoplastic areas, ducts/alveoli were occasionally found in the fibrotic tissues. The MUC-1⁻/ESA⁺ cells were difficult to find in the epithelium of ducts/alveoli, which comprised of mainly a single layer of MUC-1⁺/ESA⁺ cells accompanied occasionally by MUC-1⁻/ESA⁻ cells (Fig. 5). The MUC-1⁻/ESA⁺ cells in the hypoplastic areas were 0~1/HP (mean 0.3±0.001/HP), significantly less than in the hyperplastic sections and normal breast tissue ($p<0.05$), (Table 1).

Table 1. Density of MUC-1⁻/ESA⁺ cells in breast lesions.

Lesion	n	Density (x±s) (Mean cell number/HP)	*p*
Normal breast tissue	10	1.1±0.208	<0.05[1]
fibroadenoma	40	2.1±0.216	<0.05[2]
Fibrocystic disease with hyperplasia	60	14.3±1.121	<0.05[3]
Fibrocystic disease with hypoplasia	60	0.3±0.001	<0.05[4]

[1]: between fibroadenoma and normal tissue; [2]: between fibroadenoma and hyperplasia; [3]: between hyperplasia and normal tissue; [4]: between hypoplasia and normal tissue.

Invasive ductal carcinoma

MUC-1⁻/ESA⁺ cells could be seen in invasive ductal

Table 2. Densities (cell number/HP) of different cell types in breast IDC.

	MUC-1⁻/ESA⁺	MUC-1⁺/ESA⁺	MUC-1⁺/ESA⁻	MUC-1⁻/ESA⁻
Median	80	3.5	1	1
Min	0	0	0	0
Max	148.3	96	41	40

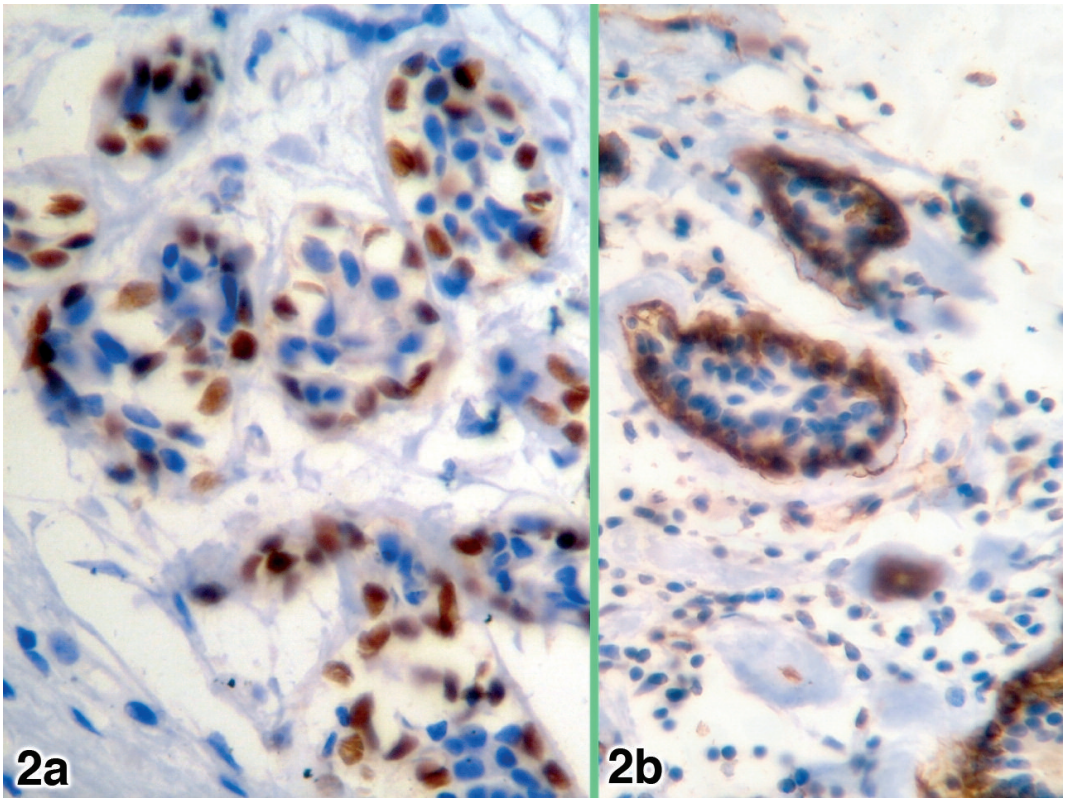

Fig. 2. IHC for p63 protein **(a)** and SMA **(b)**. Positive signals were yellow. Basal cells showing nuclear staining for p63 protein **(a)**; basal cells with cytoplasmic staining for SMA **(b)**, indicating that the basal cells are myoepithelial. x 40 objective.

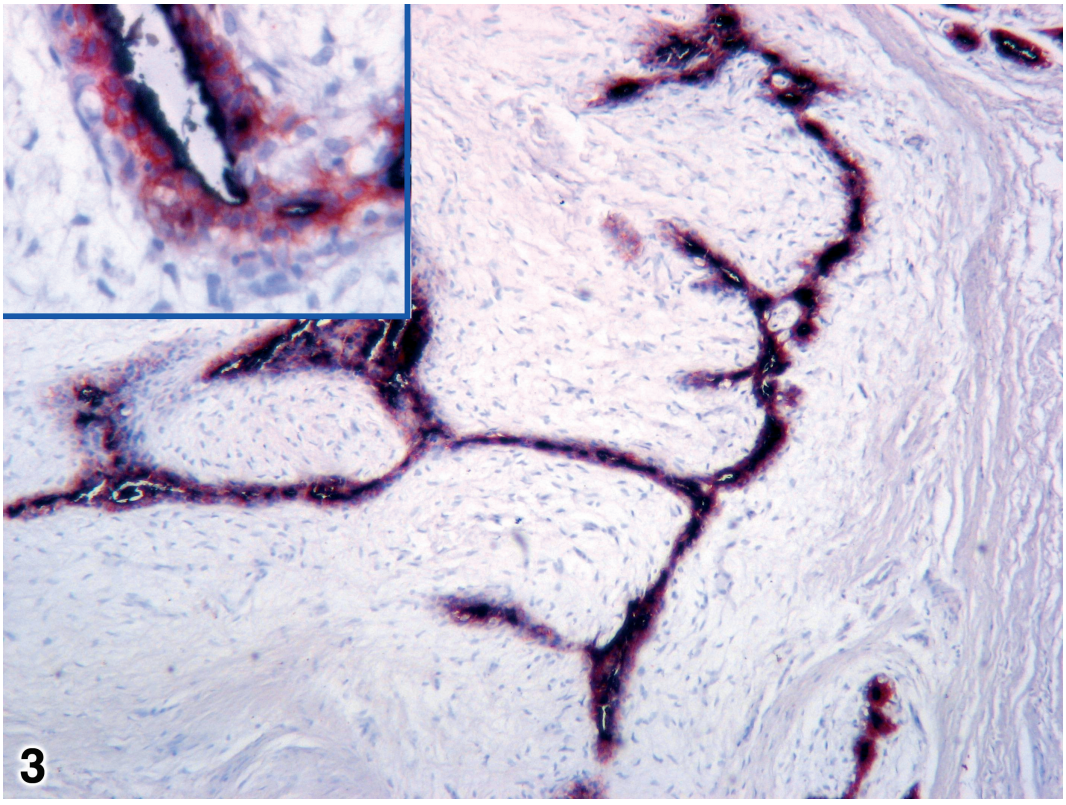

Fig. 3. Double staining for MUC-1 (black) and ESA (carmine) in breast fibroadenoma. A few MUC-1⁻/ESA⁺ cells are shown. x10 objective; inset, x 40 objective.

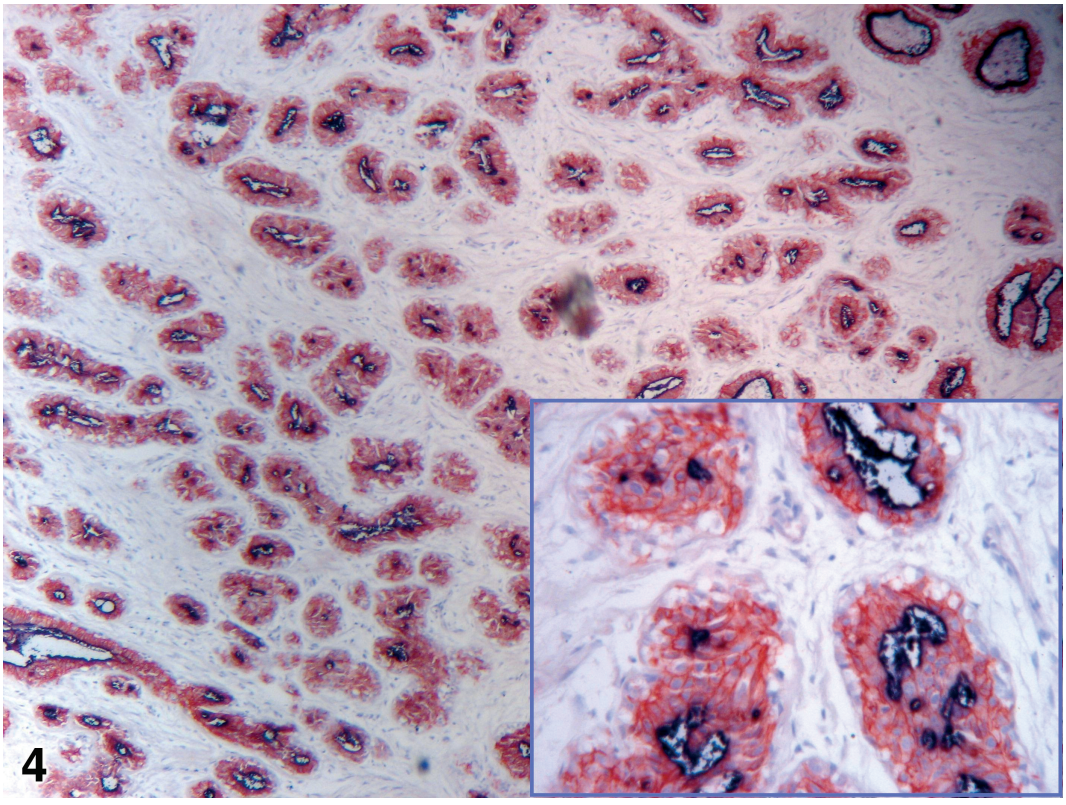

Fig. 4. Double staining for MUC-1 (black), ESA (carmine) in fibrocystic disease with hyperplasia showing that proliferating cells are MUC-1⁻/ESA⁺. x10 objective; inset, x 40 objective.

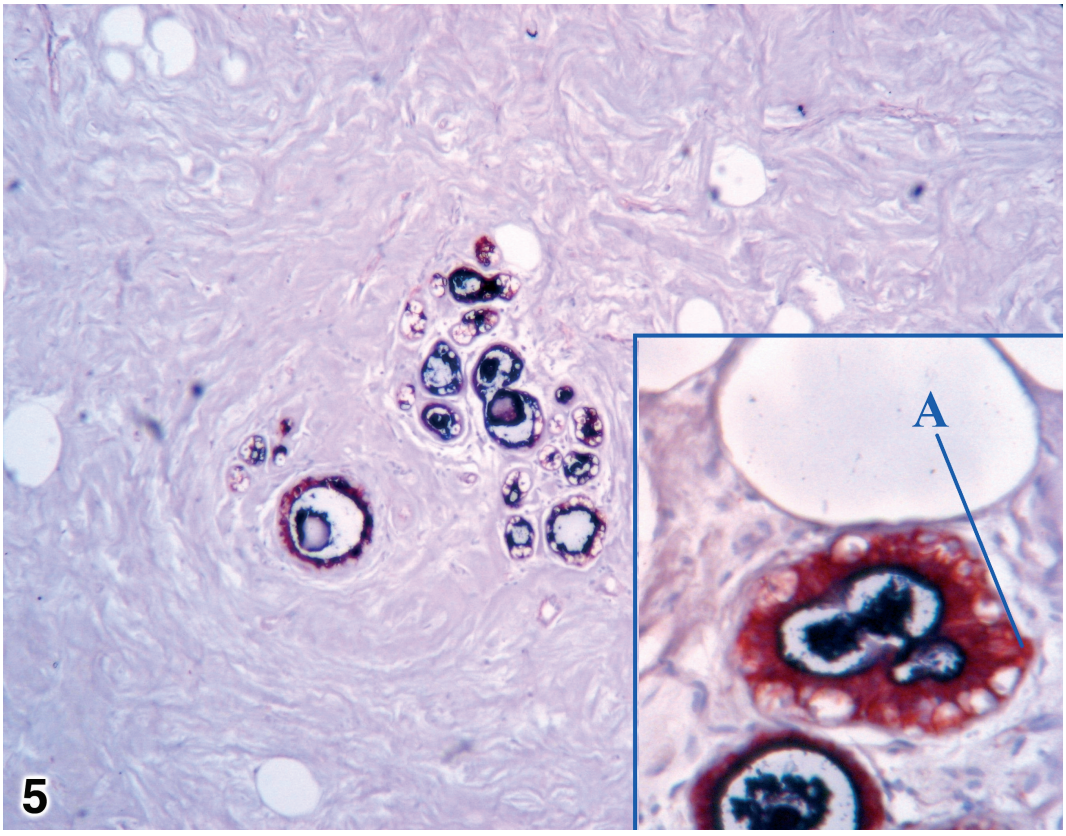

Fig. 5. Double staining for MUC-1 (black), ESA (carmine) in fibrocystic disease with hypoplasia. ?10 objective, the inset is ?40 objective. A: MUC-1⁻/ESA⁺ cell. x10 objective; inset, x 40 objective.

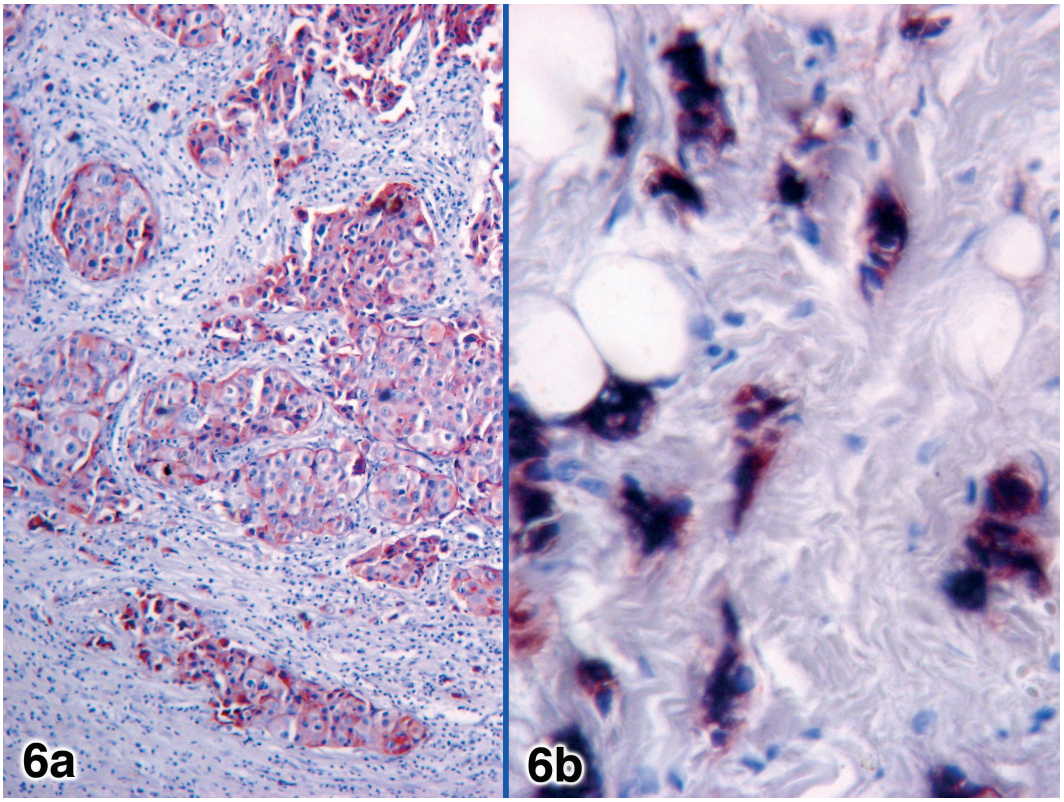

Fig. 6. Double staining for MUC-1 (black), ESA (carmine) in IDC. **a.** The cancer nests composed of MUC-1⁻/ ESA⁺ cells. **b.** Cancer nests with MUC-1⁺ / ESA⁺ cells. x 10 objective.

carcinoma with irregular expression patterns. In high grade carcinomas, the separate nests consisted of a pure cell type, either MUC-1$^-$/ESA$^+$, or MUC-1$^+$/ESA$^+$ (Fig. 6). In low grade carcinomas, the nests were composed of a mixture of cells. MUC-1$^-$/ESA$^+$, MUC-1$^+$/ESA$^+$ could all be seen in the same nests (Fig. 7). Furthermore, blurry ducts or alveoli could be found in well-differentiated nests, with MUC-1$^+$ cells in the apical surface (facing lumen) and ESA$^+$ cells in the outside layers, similar to the findings in benign lesions (Fig. 7). Due to the great difference in quantity in IDCs, we take the median as the mean value of each cell type rather than arithmetic mean (Table 2).

Cell lines

In the MCF-7 cell line, most cells were MUC-1$^-$/ESA$^+$ (Fig. 8). However, in the MDA-MB-468 cell line, MUC-1$^-$/ESA$^+$ cells and MUC-1$^+$/ESA$^+$ cells were almost equally present (Fig. 9).

Discussion

The existence of normal mammary stem cells was established as early as in 1959, when DeOme and colleagues observed that epithelium isolated from several different regions of the mammary gland was able to generate fully functional mammary outgrowths (Deome et al., 1959). Recently, it has been established that the entire mouse mammary gland can develop from a single stem cell (Shackleton et al., 2006, Stingl et al., 2006a,b) . However reports about localization and number of stem/progenitor cells are still not very clear. The present study was aimed at disclosing the alteration of the MUC-1$^-$/ESA$^+$ cells, so-called breast stem/progenitor cells, in different breast lesions.

We observed that the MUC-1$^-$/ESA$^+$ cells located mostly in the suprabasal, but beneath the apical surface in ducts and alveoli in normal breast tissues and benign breast lesions. The same findings were seen in well differentiated IDC tumours with rather distinct glandular structures. However, the density of this cell type varied considerably in the different lesions. In fibrocystic disease with lobular hyperplasia numerous MUC-1$^-$/ESA$^+$ cells were present, appearing in the ducts or alveolar walls or even as a pure cell group without other cell phenotypes. On the other hand, in lobular hypoplastic areas very few of such cells were present, and were actually difficult to observe. In the same areas other cell phenotypes were present, such as MUC-1$^-$/ESA$^-$ cells (myoepithelium) and groups of MUC-1$^-$/ESA$^+$ cells with MUC-1-/ESA- cells (myoepithelium) and MUC-1$^+$/ESA$^+$ cells (apical cells). In our series we did not observe any isolated pure clone of MUC-1$^-$/ESA$^-$ cells, which was not mixed with other cell types. Our findings may support the suggestion that during the

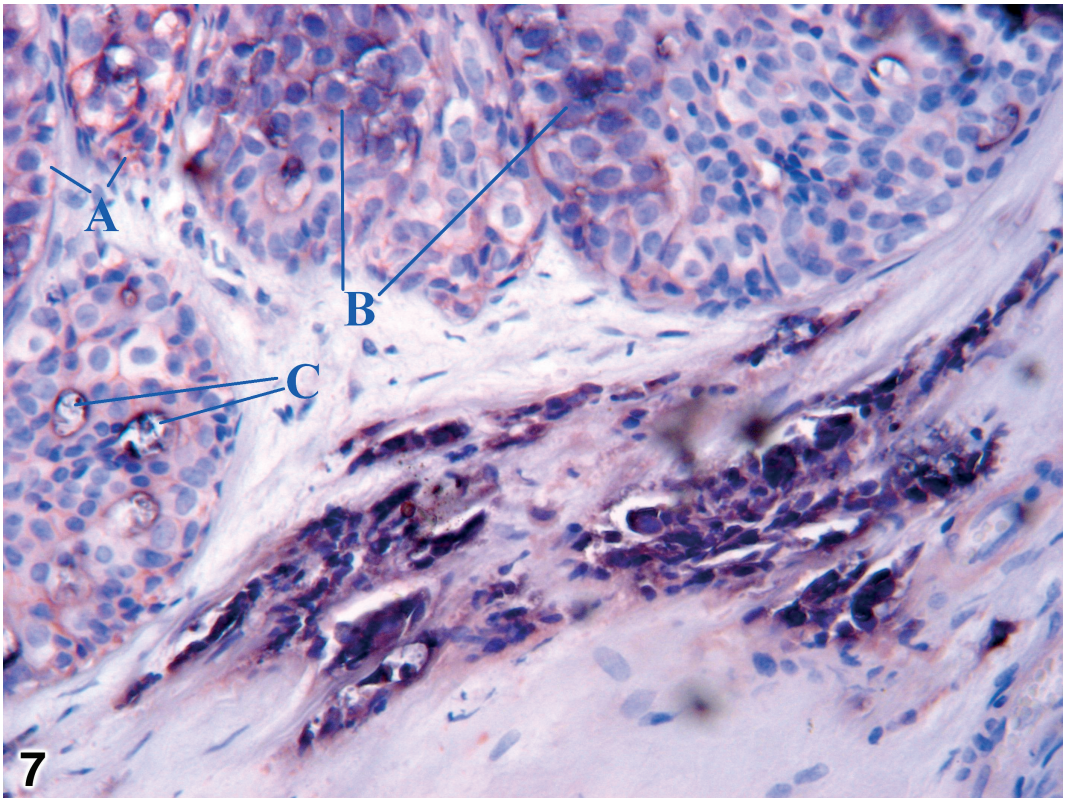

Fig. 7. Double staining for MUC-1 (black), ESA (carmine) in IDC. Top: Well differentiated cancer nests composed of cells with variable cell phenotypes. A: cells with MUC-1$^-$/ESA$^+$; B: cells with MUC-1$^+$/ESA$^+$; C: blurry duct lumina could be seen, with MUC-1 cells in the apical surface (facing lumen) and ESA cells in the outside layers. Tumour cells in the rather poorly differentiated area (bottom) showing pure cell phenotype of MUC-1$^-$/ESA$^+$. x 10 objective.

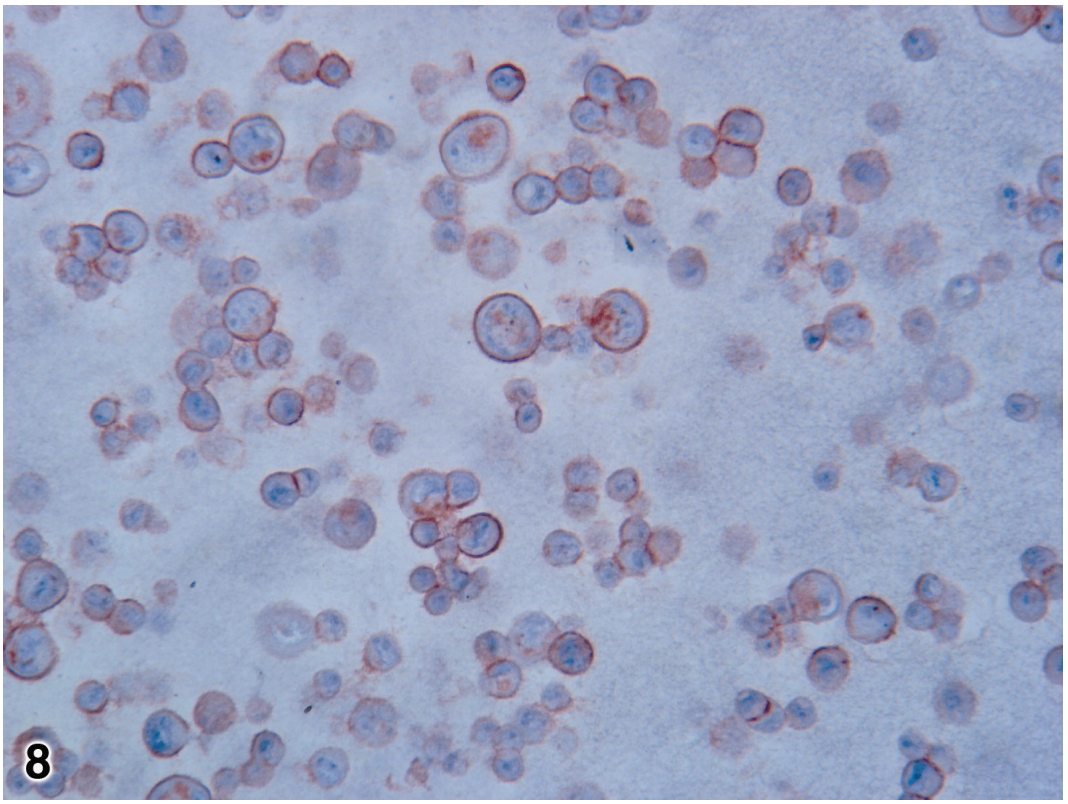

Fig. 8. Double staining for MUC-1 (black), ESA (carmine) in MCF-7 cell line. Most cells are MUC-1$^{-/}$ESA^{+}. x 40 objective.

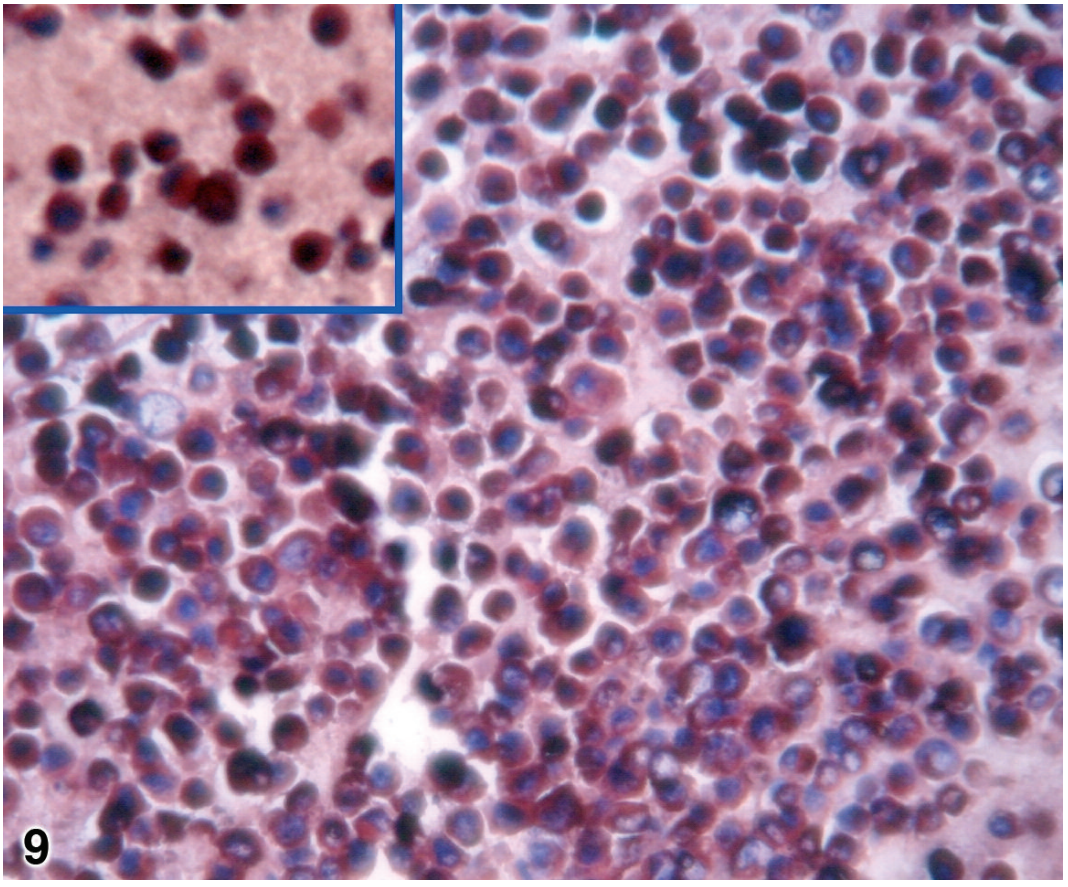

Fig. 9. Double staining for MUC-1(black), ESA (carmine) in MDA-MB-468 cell line. MUC-1^{-}/ESA^{+} cells and MUC-1^{+}/ESA^{+} cells are mixed in this cell line. The inset shows MUC-1^{+}/ESA^{+} cells. x 40 objective.

menstrual cycle the following is happening: the limited number of MUC-1$^-$/ESA$^+$ cells in the ducts and alveoli are generally inactive in function; upon growth stimulation during the luteal phase, these cells proliferate, duplicate and partly differentiate to other cell phenotypes, including myoepithelial cells, and in this way provide an accumulation of MUC-1$^-$/ESA$^+$ cells in fibrocystic disease. After luteal phase, the MUC-1$^-$/ESA$^+$ cells stop proliferating, resulting in a reduced number of cells. Under conditions with hormone disorders, the extent and rhythm of the MUC-1$^-$/ESA$^+$ cells proliferation were disturbed, leading to the fibrocystic disease with hyperplastic areas and / or hypoplastic areas. This would also be valuable for malignant breast lesions, since our results show a variable number of MUC-1$^-$/ESA$^+$ cells in malignant neoplastic lesions.

One question may be raised about whether the MUC-1$^-$/ESA$^+$ cell proliferation is a risk factor for breast cancer development. In the present study, the proportion of MUC-1$^-$/ESA$^+$ cells in the lobular hyperplastic areas is 14.3%, much higher than that in lobular hypoplasia and fibroadenoma (0.3% and 2.1%). Lobular hyperplasis has been proved to be a risk factor (Inai et al., 1992) for breast cancer development and could be explained with the increasing number of MUC-1$^-$/ESA$^+$ cells.

Besides the stem/progenitor property of the MUC-1$^-$/ESA$^+$ cells in the human breast, other cell types have also been proved to be breast epithelial cell progenitors. In Villadsen et al's experiment (Villadsen et al., 2007), cells from ducts and lobules collected under the microscope were functionally characterized by colony formation on cell culture, mammosphere formation in suspension culture, and morphogenesis in laminin-rich extracellular matrix gels. Staining for the lineage markers keratins K14 and K19 further revealed K19$^+$/K14$^+$ multipotent cells in the stem cell zone, and three lineage-restricted cell types (K19$^+$/K14$^-$, K19?/K14?, K19?/K14+) outside this zone. Compared to the other stem-like marker MUC-1$^-$/ESA$^+$ cells, which were also found by their group previously, they propose that there was a stem cell hierarchy, and that K14$^+$/K19$^+$ cells most certainly were precursors to MUC$^-$/ESA$^+$ cells.

Increasing evidence supports the notion that cancer stem/progenitor cells are the driving force for tumours and such cells exist in all types of malignant tissues as a small fraction of the tumour cells (Al-Hajj et al., 2003; Bapat, 2007; Song and Miele, 2007; Tang et al., 2007; Tysnes and Bjerkvig, 2007; Zheng et al., 2007). Furthermore, increasing evidence is appearing that cancer stem cells may develop from normal stem or progenitor cells in an organ (Bapat, 2007; Polyak, 2007;Tysnes and Bjerkvig, 2007). Neither MUC-1$^-$/ESA$^+$ cells nor K19$^+$/K14$^+$ cells have been fully explored for other stem cell surface markers. In our present study, we show that the densities of MUC-1$^-$/ESA$^+$ cells differ greatly in invasive ductal carcinomas (from 0-148.3). Due to progenitor properties in normal breast epithelium, MUC-1$^-$/ESA$^+$ cells may be related to progenitor tumour cells in these invasive carcinomas as well. Five molecular subtypes (Perou et al., 2000; Hu et al., 2006, Sorlie et al., 2006) of breast cancer have been revealed: basal-like, luminal A, luminal B, HER2$^+$/ER$^-$, and normal breast-like, which are conserved across ethnic groups and are already evident at the ductal carcinoma in situ stage (Yu et al., 2004). Further studies are merited as to how MUC-1$^-$/ESA$^+$ cells progress in breast carcinoma in relation to the molecular classification proposed.

We have found that MUC-1$^-$/ESA$^+$ phenotypic cells vary in breast cancer cell lines as well. In the breast cancer cell line MCF-7, most cells showed MUC-1$^-$/ESA$^+$. The majority of the cells within the MCF-7 cell line exhibit luminal cell characteristics (Charafe-Jauffret et al., 2006; Elstrodt et al., 2006), indicating the breast tumor with luminal cells feature may manifest MUC-1$^-$/ESA$^+$ phenotypic expression, most probably tumour progenitor cells as well. However, we still do not know whether the MUC-1$^-$/ESA$^+$ tumour cell population is a direct link to its normal counter part. MDA-MB-468 was derived from basal type cells (Charafe-Jauffret et al., 2006, Elstrodt et al., 2006). The significant variation of MUC-1$^-$/ESA$^+$ cells present in these breast cancer cell lines may indicate that tumours derived from different subtype cells in the same histological tumour type may have different tumour stem /progenitor cell phenotypes.

In summary, we have demonstrated that the normal breast progenitor subpopulation MUC-1$^-$/ESA$^+$ increases during the process of hyperplasia, and this subpopulation varies greatly in benign and malignant breast lesions. That more MUC-1$^-$/ESA$^+$ cells are present in the MCF-7 cell line compared to MDA-MB-468 cell lines, may suggest that cell lines derived from different subtype tumours may harvest different stem/progenitor markers.

Acknowledgements. This work is supported by the Norwegian Cancer Society.

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Accepted May 13, 2009