

# Estrogen receptor- $\beta$ is expressed in stromal cells of fibroadenoma and phyllodes tumors of the breast

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**An estrogen dependency has been suggested for the growth of fibroadenomas: however, thus far, none of the steroid hormone receptors acting on breast tissues has been demonstrated in the stroma of breast fibroepithelial lesions. In this study, the expression of estrogen receptor (ER)- $\alpha$  and - $\beta$  was investigated by immunohistochemistry in 33 fibroadenomas and in 30 benign, three borderline and seven malignant phyllodes tumors, all with spindle cell growth and in one distant metastasis. In addition, the presence of ER- $\beta$  mRNA and its variants was evaluated by RT-PCR in microdissected stroma. The possible correlation between hormone receptor expression and differentiation processes of stromal cells was investigated by smooth muscle actin and calponin immunostaining. ER- $\beta$  was the only hormone receptor expressed by stroma of fibroadenomas and phyllodes tumors, both at protein and mRNA level. The highest percentage of ER- $\beta$  was observed in fibroadenomas with cellular stroma and in phyllodes tumors. In both lesions, ER- $\beta$ -positive stromal cells showed expression of smooth muscle actin and/or calponin, as demonstrated by double immunostaining. In addition, the mean age at diagnosis was significantly lower in patients with ER- $\beta$ -positive vs ER- $\beta$ -negative fibroadenomas. In contrast, in phyllodes tumors, ER- $\beta$  expression was higher in older patients. In conclusion, (i) only ER- $\beta$  is detected in the stroma of fibroadenomas and phyllodes tumors; (ii) its expression correlates with the expression of smooth muscle markers and suggests a role of ER- $\beta$  in myofibroblastic differentiation of stromal cells. These two results, together with the young age of patients carrying fibroadenomas with highly ER- $\beta$ -positive stroma cells, may further indicate a hormone-receptor mechanism involved in regulating the growth of fibroadenomas. Conversely, the older age of patients with ER- $\beta$ -rich phyllodes tumors suggests that mechanisms, probably independent from estrogen stimulation, act on the growth of these tumors.**

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Fibroadenoma and phyllodes tumor are biphasic lesions of the breast characterized by proliferation of both epithelial and stromal components of the terminal ductal unit. Proliferation of stromal cells is commonly considered the primary event, followed by secondary proliferation of epithelial cells. While studies on stromal cell clonality have shown

that malignant phyllodes tumors are neoplastic, most fibroadenomas are considered a result of hyperplastic processes involving the connective tissue of lobular units.<sup>1</sup> The natural history of fibroadenomas varies. They may grow, regress or remain unchanged, as the hormonal environment of the patient changes, but most stop growing after reaching 2–3 cm in diameter;<sup>2</sup> moreover, with aging, the stroma becomes less cellular and increases its hyalinization.<sup>3</sup> These data (occurrence in young women and sclerotic involution in the elderly) suggest a hormonal responsiveness of fibroadenoma. This hypothesis is supported by the recent finding of elevated plasma levels of estradiol in patients

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with fibroadenoma.<sup>4</sup> In the same patients, single-nucleotide polymorphisms of genes encoding for enzymes involved in estrogen metabolism have also been demonstrated.<sup>5</sup> In addition, tamoxifen treatment significantly reduces the proliferation activity in the stroma of fibroadenomas.<sup>6</sup> The classical mediator of the response to estradiol and tamoxifen is the estrogen receptor (ER)- $\alpha$ , but in fibroadenomas and phyllodes tumors, ER- $\alpha$  is mainly expressed by epithelial cells, while its expression by stromal cells is controversial.<sup>7–10</sup> A recent study demonstrated that only the ER- $\beta$  isoforms are found in stromal cells of the adult human mammary gland.<sup>11</sup> All these data taken together motivated us to investigate whether ER- $\beta$  could be expressed by the stromal cells in fibroadenomas and phyllodes tumors. In addition, following the recent observation of both the common smooth muscle cell differentiation in fibroepithelial lesions of the breast<sup>12</sup> and the high levels of ER- $\beta$  in muscle cells of other estrogen-dependent organs,<sup>13</sup> we decided to investigate the immunophenotype of the stromal cells using smooth muscle actin and calponin as markers.

## Materials and methods

### Case Series

We selected a series of 35 fibroadenomas and 30 benign, three borderline and seven malignant phyllodes tumors with fibrosarcomatous differentiation diagnosed in the four Pathology Departments. One of the cases gave rise to lung metastasis. All cases of fibroadenomas were reviewed and classified on the basis of stromal sclerosis and grade of cellularity. Sclerotic fibroadenomas were generally no more than 1 cm in diameter, and showed low cellularity with scattered thin spindle cells intermingled with bundles of sclerotic stroma. Cellular fibroadenomas were often larger than the sclerotic type (mean diameter 1.5 cm), their stroma was more myxoid and oedematous and was richly populated by cells with long extensions protruding from the large polygonal cytoplasm.

### Immunohistochemistry

All specimens were fixed in 4% formalin and embedded in paraffin. For immunohistochemistry, the endogenous peroxidase was inhibited by incubating specimens with 3% H<sub>2</sub>O<sub>2</sub> for 5 min prior to incubation with the primary monoclonal antibody. To study the hormone receptor status, sections were incubated with anti-ER- $\beta$  (clone 14C8, Abcam, Cambridge, UK) at a concentration of 5  $\mu$ g/ml overnight at 4°C in an humidified chamber, according to previously validated procedures.<sup>14</sup> The antibody reaction was revealed by EnVision + System (DakoCytomation, Glostrup, Denmark). Immunostaining was performed with the following antibodies: anti-

ER- $\alpha$  (clone 1D5, diluted 1:100, DakoCytomation), smooth muscle actin (clone 1A4, diluted 1:200; DakoCytomation) and calponin (clone CALP, diluted 1:100; BioGenex, San Ramon, CA, USA) mAbs using the automated immunostainer BenchMark XT (Ventana). Antigen retrieval was performed for all immunohistochemical reactions, with the exclusion of smooth muscle actin, by incubation in termostated bath in 1 mM citrate buffer, pH 6, for 40 min at 98°C. Primary antibodies were revealed by the universal iView-Dab detection kit (Ventana Medical System, Tucson, USA).

In two cases (one cellular fibroadenoma and one phyllodes tumor), two-step double immunostaining was performed to evaluate the coexpression of ER- $\beta$  with smooth muscle differentiation markers. In step one, anti-ER- $\beta$  nuclear binding was revealed with a 3,3'-diaminobenzidine solution (brown precipitate). The sections were then washed in phosphate-buffered saline (PBS), incubated for 30 min at 37°C in 0.01 M HCl and washed thoroughly with water (~2 h) to remove unbound antibody that could potentially interfere with the second part of the immunohistochemical reaction. In the second step, cytoplasmic binding of anti-smooth muscle actin mAb was visualized with a solution of 3,3'-diaminobenzidine and 0.3% NiCl<sub>2</sub> in 50 ml of PBS, pH 7.4 (black precipitate).

### Microdissection

To evaluate ER- $\beta$  mRNA expression, tissue samples of three cellular fibroadenomas, one malignant phyllodes tumor and one phyllodes tumor metastasis were frozen in liquid nitrogen and stored at -80°C until use. Two of these fibroadenomas and the phyllodes tumor with its metastasis were microdissected, in order to avoid epithelial contamination and to focus on mRNA of stromal tissue. ER- $\beta$  protein expression in stromal cells was first evaluated by immunohistochemistry. Sections were microdissected with a LaserScissors microdissector (Olympus/Cell Robotics Inc., Albuquerque, NM, USA). The microdissected stromal tissue was collected in microcentrifuge tubes and total RNA was extracted using Triagent solution (Genomed, Celbio, Pero, Italy) according to the manufacturer's instructions. RNA concentration was measured spectrophotometrically by determination at 260/280 nm, its purity and integrity was checked by electrophoresis on a 1.5% agarose gel.

### RNA Extraction and RT-PCR Analysis

Total RNA obtained from one not microdissected cellular fibroadenoma and from microdissected stromal tissues of the other specimens was extracted using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturers' instructions. Positive controls were MDA-MB231 breast cancer

**Table 1** Sequences of ER- $\beta$  and ER- $\alpha$  RT-PCR primers

Primer	Sequence	Amplification product (bp)
ER- $\beta$ 1-5 forward	5'-AGT ATG TAC CCT CTG GTC ACA GCG-3'	
ER- $\beta$ 1 reverse	5'-CCA AAT GAG GGA CCA CAC AGC AG-3'	408
ER- $\beta$ 2 reverse	5'-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3'	291
ER- $\beta$ 3 reverse	5'-GCA GTC AAG GTG TCG ACA AAG GCT GC-3'	400
ER $\beta$ 4 reverse	5'-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3'	608
ER $\beta$ 5 reverse	5'-CTT TAG GCC ACC GAG TTG ATT AGA G-3'	372
ER- $\alpha$ forward	5'-CAT AAC GAC TAT ATG TGT CC AGC C-3'	
ER- $\alpha$ reverse	5'-AAC CGA GAT GTA GCC AGC AGC-3'	659
$\beta$ 2-Actin forward	5'-CTC ACC CTG AAG TAC CCC ATC G-3'	
$\beta$ 2-Actin reverse	5'-CTT GCT GAT CCA CAT CTG CTG G-3'	885

cell line for ER- $\beta$  and MCF-7 breast cancer cell line for ER- $\alpha$ . Total RNA was reverse-transcribed at 42°C for 40 min using AMV reverse transcriptase (Finnzymes, Finland) and oligodT primer (Invitrogen, Groningen, The Netherlands). The PCR reaction system contained 5  $\mu$ l of 10  $\times$  PCR buffer, 10  $\mu$ l of RT product, 0.2 mM dNTP (Finnzymes, Espoo, Finland), 1.25 U *Taq* DNA polymerase (Finnzymes, Finland), 50 ng each of sense and antisense primers in a total volume of 50  $\mu$ l. ER- $\alpha$  and ER- $\beta$  primers are reported in Table 1.

Amplification was carried out as follows: for ER $\beta$  isoforms, 1  $\times$  (94°C, 1 min); 35  $\times$  (94°C, 30 s; 58°C, 30 s; 72°C, 30 s); and 1  $\times$  (72°C, 7 min); for ER- $\alpha$  and  $\beta$ -actin, 1  $\times$  (94°C, 1 min); 35  $\times$  (94°C, 30 s; 65°C, 30 s; 72°C, 30 s); and 1  $\times$  (72°C, 7 min); PCR products were electrophoresed on 2% agarose gel in the presence of ethidium bromide. Gels were photographed and analyzed with the PC Kodak 1D Image Analysis software (Eastman Kodak Company, Rochester, NY, USA).

### Statistical Analysis

Correlation between ER- $\beta$  expression and stromal cell percentage or smooth muscle differentiation was calculated with  $\chi^2$  test; correlation between age of patients and ER- $\beta$  expression was calculated with independent samples *t*-test. Statistical analysis was performed with SPSS for windows (V.9.0) software.

## Results

After revision, seven fibroadenomas were classified as low cellular 'sclerotic' and 26 as 'cellular' fibroadenomas.

### Immunohistochemistry

#### *Expression of hormone receptors*

ER- $\alpha$  were expressed in epithelial cells, but were not detectable in the stroma of fibroadenomas and phyllodes tumors, while ER- $\beta$  was expressed in both epithelial and myoepithelial cells and in a

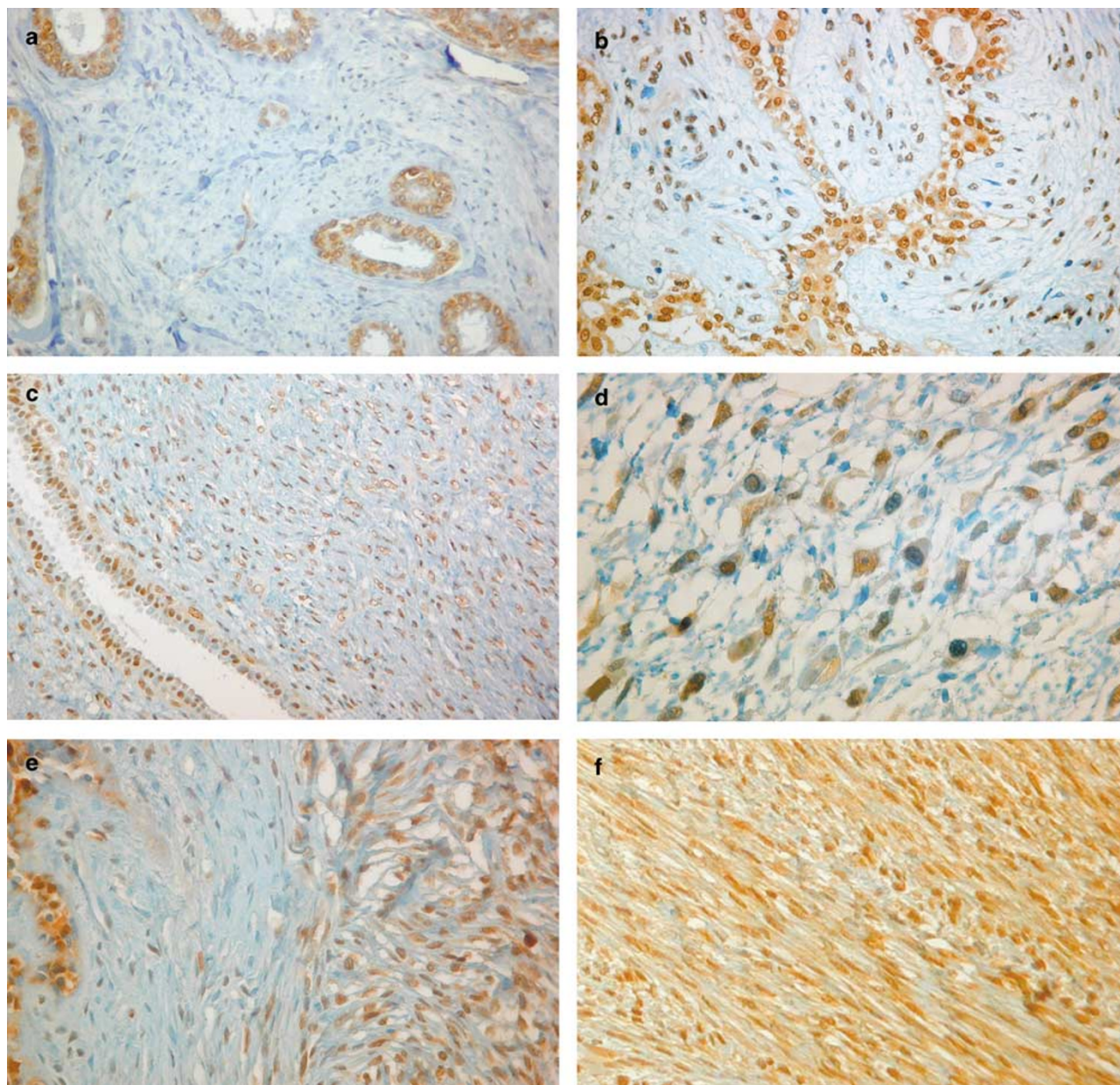
variable amount of nuclei of stromal cells and endothelial cells of small vessels. Three categories of ER- $\beta$  expression were considered on the basis of the percentage of nuclear staining of stromal cells: 0–5% (low), 6–30% (moderate) and 31–100% (high). In all seven sclerotic fibroadenomas ER- $\beta$  expression was absent or very low (Figures 1a and 2), whereas high and moderate levels of ER- $\beta$  were detected in 16 (62%) and six (23%) out of the 26 cellular fibroadenomas, respectively (Figures 1b and 2). In benign phyllodes tumors, the expression level of ER- $\beta$  was high in 14 cases (47%), moderate in 10 cases (33%) (Figure 1c) and low in six cases (20%). All borderline phyllodes tumors (Figure 1d) and 4/7 (57%) of the malignant phyllodes tumors together with the lung metastasis displayed high ER- $\beta$  expression (Figure 1e and f).

#### *Expression of smooth muscle actin and calponin*

In sclerotic fibroadenomas, the expression of smooth muscle actin and calponin was very low or undetectable; on the contrary, cellular fibroadenomas showed focal (six cases) (23%) or diffuse (20 cases) (77%) expression of at least one of the two markers. Moreover, smooth muscle differentiation significantly correlated with ER- $\beta$  expression (Figure 3) and the double immunostaining confirmed the coexpression of nuclear ER- $\beta$  and cytoplasmic smooth muscle actin in the same stromal cell (Figure 4a). In benign (21/30) phyllodes tumors, smooth muscle differentiation matched the nuclear expression of ER- $\beta$ , being prevalent in cases expressing high levels of the receptor (Figures 3 and 4b). Smooth muscle differentiation was observed in all borderline phyllodes tumors and in three out of four cases of malignant phyllodes tumors with high ER- $\beta$  level (Figures 3 and 4c). The metastasis of one of these cases showed diffuse expression of both smooth muscle differentiation markers and ER- $\beta$  (Figures 3 and 4d).

#### *ER- $\beta$ expression and patient age*

The correlation between percentage of ER- $\beta$  expression in fibroadenomas and phyllodes tumors and the age of patients is shown in Figure 5. In



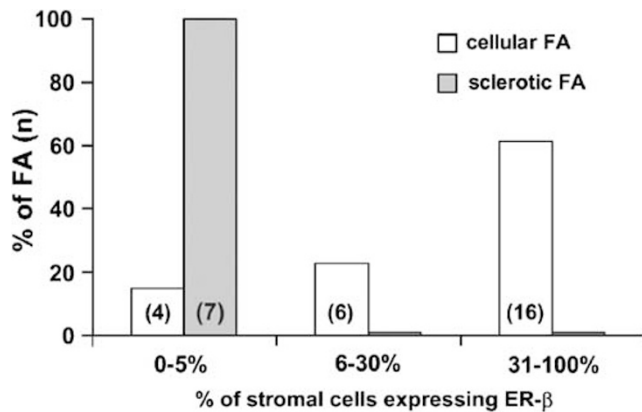
**Figure 1** ER- $\beta$  immunostaining in stromal cells. (a) Sclerotic fibroadenoma; intense nuclear staining is shown only by epithelial, myoepithelial and endothelial cells. (b) Cellular fibroadenoma, (c) benign phyllodes tumor, (d) borderline phyllodes tumor, (e) primary malignant phyllodes tumor and (f) its metastasis: in all these lesions, the stromal cells show positive nuclei.

fibroadenomas, the percentage of ER- $\beta$  expression was inversely correlated with the age of patients. In fact, the mean age of patients with cellular fibroadenomas with high or moderate levels of ER- $\beta$  expression was significantly lower (mean: 33.6 years, range: 16–46) than that of patients with sclerotic fibroadenomas (mean: 47.6 years, range: 40–67), or that of patients with cellular fibroadenomas expressing low level of ER- $\beta$  (mean: 50.5 years, range 49–57). In contrast, in benign phyllodes tumors, the high level of ER- $\beta$  expression correlated with an older age. All patients with malignant phyllodes tumor were in their sixth decade of life.

#### ER- $\beta$ and ER- $\alpha$ mRNA analysis

To confirm the specificity of the immunohistochemical results, we also demonstrated ER- $\beta$  gene expression by means of RT-PCR of mRNA extracted from microdissected and not microdissected stroma of fibroadenomas and phyllodes tumors. As shown in Figure 6, only the case of nonmicrodissected fibroadenomas expressed ER- $\alpha$  mRNA (lane 2), as a consequence of the presence of glandular epithelial cells, as confirmed by immunohistochemistry. Microdissected stromal compartment of the other cases did not show any evidence of ER- $\alpha$  mRNA.





**Figure 2** Relationship between stromal cellularity (empty bars)/sclerosis (solid bars) and stromal expression of ER- $\beta$  in fibroadenomas.  $\chi^2$  test: significant difference in low (0–5%) vs moderate (6–30%) ( $P=0.04$ ) ER- $\beta$  expression in sclerotic fibroadenomas and low (0–5%) vs high (31–100%) ( $P=0.001$ ) in cellular fibroadenomas.

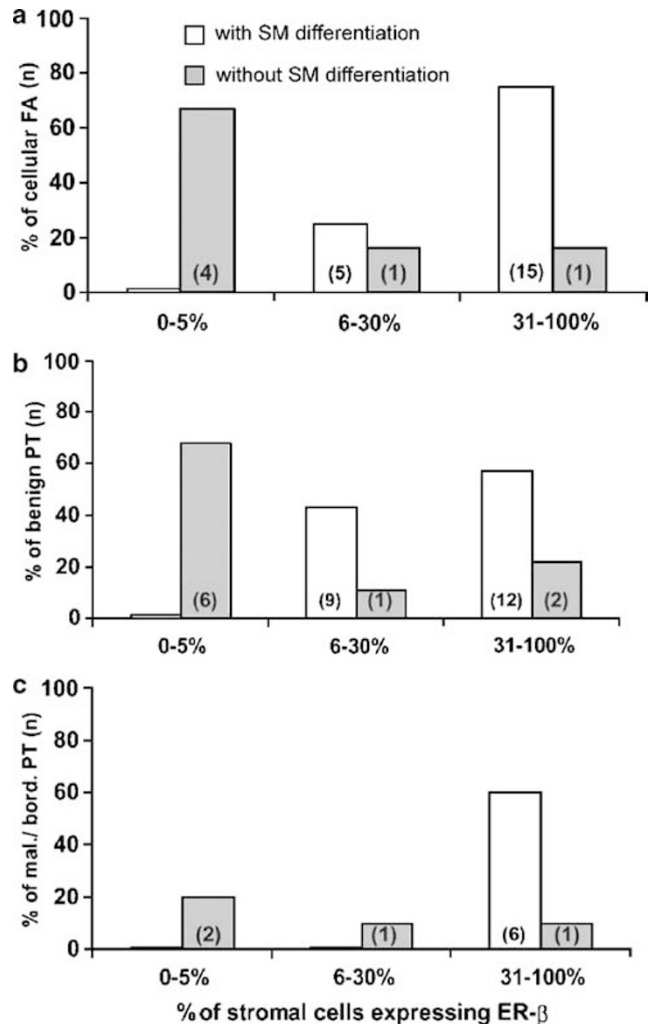
Conversely, ER- $\beta$ 1 (wild type) mRNA was detected in fibroadenomas (lanes 2, 3 and 4) and malignant primary and metastatic phyllodes tumors (lanes 5 and 6). The ER- $\beta$ 2, 3, 4 and 5 variants were not detected in any of the specimens examined.

## Discussion

In the present study, we demonstrate that ER- $\beta$  is expressed by stromal cells of fibroadenomas and phyllodes tumors both at the protein and the mRNA level, and that such expression is related to smooth muscle differentiation of stromal cells and to the age of patients.

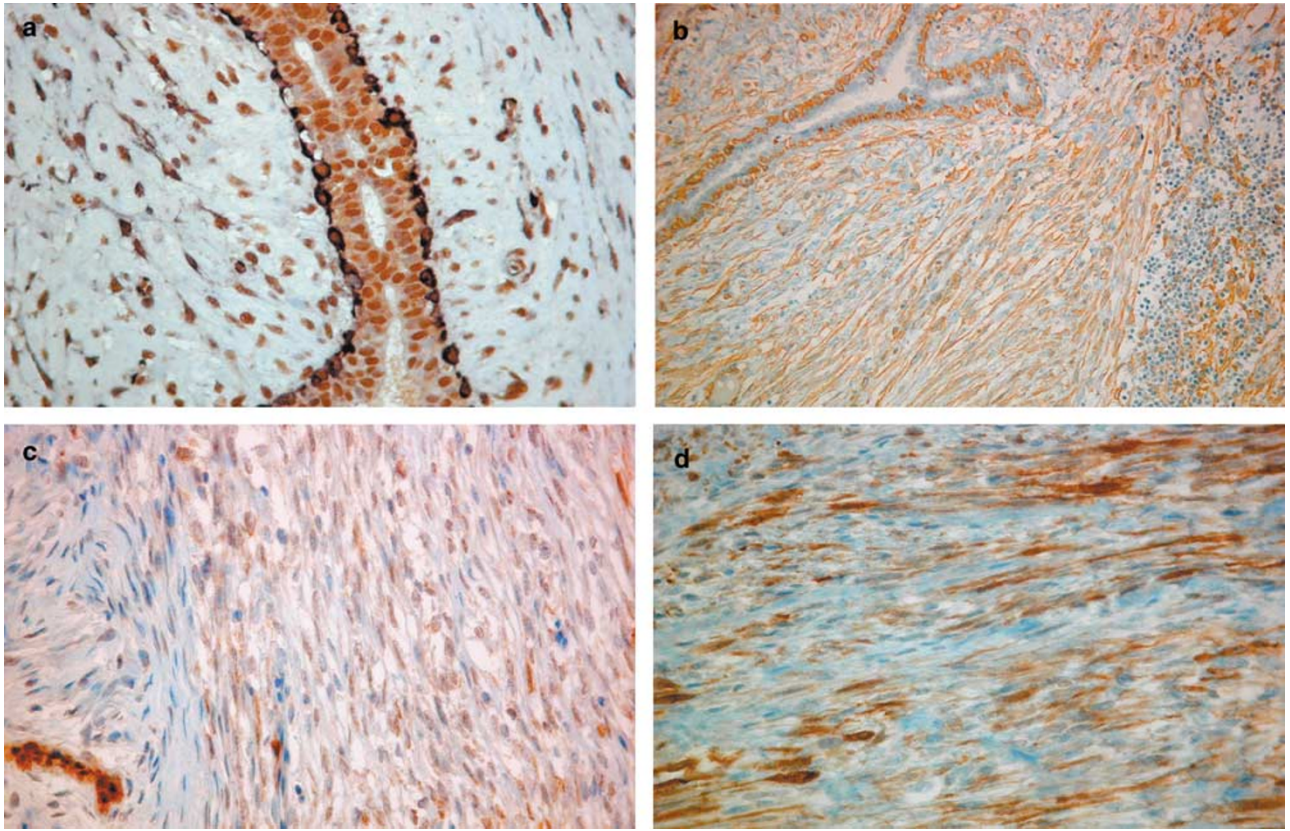
The stroma of the adult human and rodent mammary glands expresses ER- $\beta$ , but not ER- $\alpha$ .<sup>11,15,16</sup> The results of the present study confirm the absence of ER- $\alpha$  expression in stromal cells of fibroadenomas and phyllodes tumors and demonstrate the expression of the wild-type ER- $\beta$ , but not of the other ER- $\beta$  variants.

The role of ER- $\beta$  in the mammary gland epithelium has been widely studied, but, to our knowledge, no function for ER- $\beta$  in stroma has been reported. Our results suggest that, in addition to a well established antiproliferative effect on epithelial cells,<sup>17</sup> the expression of ER- $\beta$  in the breast is probably related to a prodifferentiative function on stromal cells. In fact, we here demonstrate that in fibroadenomas and phyllodes tumors, ER- $\beta$  expression goes in parallel with a smooth muscle differentiated phenotype of stromal cells, these cells being positive for ER- $\beta$ , smooth muscle actin and/or calponin by double immunostaining. ER- $\beta$  expression by mature smooth muscle cells of vessels<sup>18</sup> and uterus<sup>13,19</sup> may be crucial in the physiological response to ER-mediated transcriptional activity; our results suggest that ER- $\beta$  may also act on the

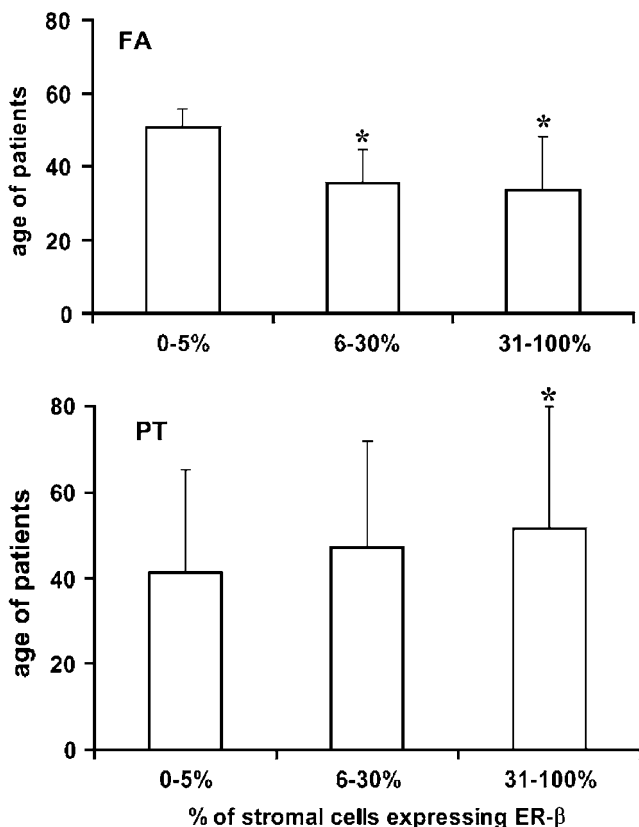


**Figure 3** Relationship between smooth muscle differentiation and ER- $\beta$  expression in cellular fibroadenomas (a), benign phyllodes tumors (b) borderline and malignant phyllodes tumors (c).  $\chi^2$  test: significant difference in low (0–5%) vs moderate (6–30%) ( $P=0.005$ ) ER- $\beta$  expression in non-smooth muscle-differentiated lesions and in low (0–5%) vs high (31–100%) ( $P=0.001$ ) ER- $\beta$  expression in smooth muscle-differentiated lesions.

differentiation process of fibroblasts towards a myofibroblastic phenotype. Previous works have shown the expression of smooth muscle actin by stromal cells of fibroepithelial lesions of the breast and a myofibroblastic phenotype of stromal cells of fibroadenomas and phyllodes tumors<sup>20,21</sup> has been confirmed by immunohistochemistry and electron microscopy.<sup>22,23</sup> In addition, similarly to our results, in the uterus ER- $\beta$  is detectable exclusively in myofibroblastic differentiated decidual cells of the stroma expressing desmin and actin.<sup>19,24</sup> Moreover, the possible hormone responsiveness of ER- $\beta$ -positive stromal cells is indirectly confirmed by clinical studies showing that an increased risk of fibroadenoma is associated with estrogen replacement therapy in postmenopausal women.<sup>25</sup> In the present study, the activation of a hormonal mechanism

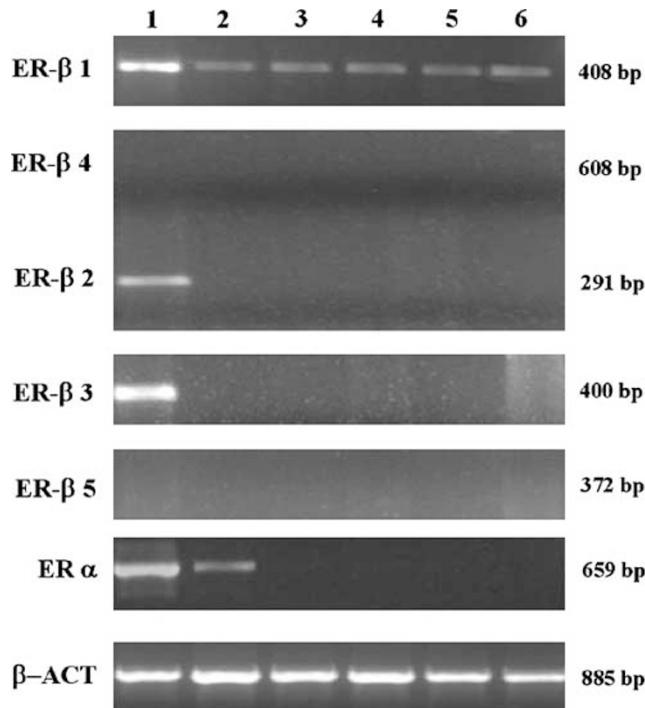


**Figure 4** Smooth muscle differentiation of stromal cells. (a) Double immunostaining of a cellular fibroadenoma; ER- $\beta$  (brown nuclei) and smooth muscle actin (black cytoplasm) are coexpressed by stromal cells. (b) Benign phyllodes tumor; smooth muscle actin immunostaining. (c) Primary malignant phyllodes tumor and (d) its metastasis; calponin immunostaining.



mediated by ER- $\beta$  may be suggested, since the expression of ER- $\beta$  is particularly high in cases of cellular fibroadenomas of young patients, in contrast to sclerotic fibroadenomas of elderly. However, the level of proliferative activity tested by Ki67 (data not shown) is very low in both type of fibroadenomas. Thus, the increase in size of fibroadenomas in young age and their progressive sclerotic involution is more probably an effect mediated by ER- $\beta$  on myofibroblastic differentiation and on the extracellular matrix remodelling. It is known that ER- $\beta$  is involved in elastin and collagen homeostasis either through transcriptional effects on their genes or through regulation of some of the proteases that are involved in the degradation of these proteins.<sup>18,26</sup> There is evidence from a variety of tissues that both male and female sex steroids regulate the extracellular matrix at different levels: specifically, 17- $\beta$  estradiol is known to reduce collagen biosynthesis.<sup>18,27</sup> In fact, estrogens protect against progression of glomerulosclerosis<sup>27</sup> and in menopause low

**Figure 5** Relationship between age and ER- $\beta$  expression in fibroadenomas and phyllodes tumors. Independent samples *t*-test: in fibroadenomas, ER- $\beta$  expression inversely correlates with patients age (\* $P < 0.04$ , low expression vs intermediate and high expression). In phyllodes tumor, ER- $\beta$  expression directly correlates with patient age (\* $P < 0.001$ , high vs low and moderate ER- $\beta$  expression).



**Figure 6** RT-PCR for ER- $\beta$  isoforms and ER- $\alpha$ . Lane 1: positive controls (MDA-MB231 cells for ER- $\beta$  isoforms and MCF-7 cells for ER- $\alpha$ ). Lane 2: non-microdissected fibroadenomas expressed ER- $\beta$ 1 and ER- $\alpha$  mRNA. Lanes 3 and 4, microdissected stroma of cellular fibroadenomas and lanes 5 and 6, primary and metastatic phyllodes tumors express only ER- $\beta$ 1; all the other ER- $\beta$  isoforms and ER- $\alpha$  are not detectable.

levels of estrogens increase the arterial stiffness.<sup>18</sup> Possibly, a similar mechanism may occur in fibroadenomas of elderly patients, where the low level of estrogen may increase collagen deposition and sclerosis process, inhibiting the matrix turnover mediated by stromal cells through ER- $\beta$  activation.

In contrast to these data, ER- $\beta$  expression was directly correlated to the age of patients in phyllodes tumors, suggesting an estrogen-independent tumor growth, possibly mediated by cytokines. It has been reported that ER- $\beta$  controls platelet-derived growth factor (PDGF),<sup>28</sup> a cytokine regulating the growth and the differentiation of fibroblasts into myofibroblasts.<sup>29</sup> Interestingly, PDGF is commonly expressed together with its receptors in the stroma of phyllodes tumor and rarely in fibroadenoma.<sup>30</sup> An ER- $\beta$ -PDGF pathway could therefore be involved in the smooth muscle differentiation in these lesions. Alternatively, recent findings indicate that cytosolic ER may modulate the activity of other transcriptional factors and in particular AP-1.<sup>31</sup> This complex is known to regulate fibroblast activation and proliferation<sup>32</sup> and myofibroblastic differentiation.<sup>33</sup>

In conclusion, the present work suggests that a heterogeneous fibroblast population with different lifespan is activated and recruited through ER- $\beta$  in the stroma of fibroadenomas and phyllodes tumors. In fibroadenomas, ER- $\beta$ -related myofibroblastic differentiation of fibroblasts may act on remodelling of

the matrix and inhibition of the sclerotic involution. On the other hand, the expression of ER- $\beta$  observed in phyllodes tumors leads to the assumption that in some cases the clonal proliferation of stromal cells described in these tumors<sup>1</sup> may be modulated through ER- $\beta$  activation, suggesting, thus, the potential use of ER- $\beta$ -selective agonists<sup>34</sup> as a new therapeutic option in malignant phyllodes tumors.

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