

# Endogenous aromatization of testosterone results in growth stimulation of the human MCF-7 breast cancer cell line

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## Abstract

Estrogens produced within breast tumors may play a pivotal role in growth stimulation of the breast cancer cells. However, it is elusive whether the epithelial breast cancer cells themselves synthesize estrogens, or whether the surrounding tumor stromal cells synthesize and supply the cancer cells with estrogen. The aromatase enzyme catalyzes the estrogen production, aromatizing circulating androgens into estrogens. The aim of this study was to investigate aromatase expression and function in a model system of human breast cancer, using the estrogen responsive human MCF-7 breast cancer cell line. Cells were cultured in a low estrogen milieu and treated with estrogens, aromatizable androgens or non-aromatizable androgens. Cell proliferation, expression of estrogen-regulated proteins and aromatase activity were investigated. The MCF-7 cell line was observed to express sufficient aromatase enzyme activity in order to aromatize the androgen testosterone, resulting in a significant cell growth stimulation. The testosterone-mediated growth effect was completely inhibited by the aromatase inhibitors letrozole and 4-hydroxy-androstenedione. Expression studies of estrogen-regulated proteins confirmed that testosterone was aromatized to estrogen in the MCF-7 cells. Thus, the results indicate that epithelial breast cancer cells possess the ability to aromatize circulating androgens to estrogens.

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## 1. Introduction

Estrogens are known to play a pivotal role in the development and promotion of human breast cancer. The highest frequency of breast cancer is found among postmenopausal women. These women have low levels of circulating estrogens, however, local synthesis of estrogens takes place in peripheral tissues, including the breast [1]. Estrogens are synthesized via aromatization of circulating C<sub>19</sub> androgens, a process catalyzed by the aromatase enzyme. For the last decades, antiestrogen therapy with tamoxifen has been the preferred treatment of estrogen responsive breast cancer. However, there is a shift towards treatment with aromatase inhibitors (AI), after third-generation inhibitors have shown superiority to tamoxifen [2–7]. However, as for other endocrine

treatments, many patients with advanced disease will develop resistance to treatment after a period with response [6]. The mechanisms responsible for development of acquired resistance are elusive and thus, studies are urgently required to fully understand the significance of local aromatase expression in breast cancer and the cellular and molecular consequences of AI treatment.

The aromatization of androgens has been observed to be more pronounced in malignant than normal breast tissues [8–10] and a locally increased estrogen production may stimulate proliferation of estrogen responsive breast cancer cells. It is still elusive which cell types that are responsible for intratumoral estrogen production, as aromatase protein has been immunohistochemically detected in both epithelial breast cancer cells and surrounding tumor stromal cells [11–15]. Further, *in vitro* cell culture studies have shown aromatase activity in both epithelial breast cancer cell lines [16–19] and breast-derived stromal fibroblasts from normal

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breast tissue and breast tumors [20–23]. The basal aromatase activity in both cell types is low, but the activity in fibroblasts can be highly stimulated with factors as IL-6, PGE<sub>2</sub>, TNF $\alpha$  and dexamethasone [20–23]. Only a moderate stimulation of aromatase enzyme activity has been reported in epithelial breast cancer cells [18,19]. Whether the modest activity observed in the cancer cells is sufficient to produce enough estrogen to cause intracellular biological effects remains controversial. Some studies have observed androgen-mediated cell growth stimulation via aromatization in epithelial breast cancer cells [17,24,25], and induction of the estrogen-regulated gene pS2 with testosterone [16], indicating that estrogen synthesis in the cancer cells is sufficient to induce biological effects. However, others have reported a growth-inhibitory effect of testosterone [26,27] and inhibition of E<sub>2</sub> upregulation of progesterone receptor (PR) protein expression [28], contradicting the biological significance of aromatization in epithelial breast cancer cells.

In order to further study the aromatase activity and function in human breast cancer cells, we have developed a model system with the estrogen responsive MCF-7 breast cancer cell line, grown in a low estrogen milieu. The focus of the present study was to investigate the biological relevance of endogenous aromatase activity in breast cancer cells. Further, it was examined whether androgens affected cancer cell growth by direct interaction with the estrogen receptor (ER) or the androgen receptor (AR). MCF-7 cells were treated with estrogens, aromatizable androgens or non-aromatizable androgen in a low estrogen milieu, alone or in combination with aromatase inhibitor (4-hydroxy-androstenedione or letrozole (Femara<sup>®</sup>)), the pure anti-estrogen ICI 182,780 (fulvestrant, Faslodex<sup>™</sup>) or the anti-androgen bicalutamide (Casodex<sup>®</sup>). Cell growth, estrogen receptor  $\alpha$  (ER $\alpha$ ) and PR protein expression and aromatase activity were examined.

## 2. Methods and materials

### 2.1. Hormones and inhibitors

Testosterone, androstenedione (Adione), estradiol (E<sub>2</sub>), estrone (E<sub>1</sub>) and 4-OH-androstenedione (4-OH-A) were purchased from Sigma–Aldrich, St. Louis, MO. Dihydrotestosterone (DHT) was purchased from Merck, Germany. Letrozole (Femara<sup>®</sup>) was a gift from Novartis Pharma AG, Basel, Switzerland. ICI 182,780 (fulvestrant, Faslodex<sup>™</sup>) and bicalutamide (Casodex<sup>®</sup>) were gifts from AstraZeneca, London, UK.

### 2.2. Cell culture

The MCF-7 cell line was obtained from the Human Cell Culture Bank, Mason Research Institute (Rockville, MD) and adapted to grow in a low serum concentration (1%) to obtain an estradiol concentration (approximately 1 pM) resembling postmenopausal concentrations of estra-

diol [29]. The AROM-1 cell line (MCF-7 cells stably transfected with the aromatase gene (CYP19)) was a gift from Dr. Mitch Dowsett's laboratory [30]. MCF-7 cells were maintained in FCS medium (DMEM/F12 medium (Gibco, Invitrogen, CA) without phenol red, 1% heat-inactivated fetal calf serum (FCS) (Life Technologies, Bethesda, MD), 6 ng/ml bovine insulin (Novo Nordic, Bagsværd, Denmark), 2.5 mM L-glutamine). AROM-1 was maintained in AROM-1 medium (RPMI medium (Gibco) with phenol red, 10% heat-inactivated FCS, 10  $\mu$ g/ml insulin, 2.5 mM L-glutamine, 600  $\mu$ g/ml G418). Cells were sub-cultivated by trypsinization once a week. In all experiments with MCF-7 cells, FCS was replaced with 5% newborn calf serum (NCS) (Life Technologies) and the medium was supplemented with  $2.5 \times 10^5$  U penicillin and 31.25  $\mu$ g/L streptomycin (NCS medium). NCS was used for experiments, as it contains low levels of growth factors and steroid hormones, facilitating detection of any stimulatory effects of supplemented hormones. Cells were incubated in a 5% CO<sub>2</sub> humidified incubator at 37 °C.

### 2.3. Cell proliferation assays

MCF-7 cells ( $2.5 \times 10^4$ ) were seeded into 24-well plates (2 cm<sup>2</sup> wells) and left to attach for 24 h in FCS medium, followed by 24 h starvation in NCS medium. The NCS medium was changed and supplemented with hormones and inhibitors (day 0) as indicated in the figures. Vehicle (ethanol) was added to the control culture. Culturing was sustained for 5 days, with replacement of medium, hormones and inhibitors on day 3. On day 5, the cells were rinsed in PBS and a crystal violet colorimetric assay, staining DNA, was used to obtain an indirect measure of the cell number [31]. The obtained optical density for each sample was expressed as a relative value in percent of the appropriate control.

### 2.4. Aromatase assay

MCF-7 cells and AROM-1 cells ( $2 \times 10^5$ ) were seeded in T25 flasks in triplicate. MCF-7 cells were cultured for 4 days in FCS medium, followed by 48 h culture in either FCS or NCS medium. AROM-1 cells were cultured for 6 days in AROM-1 medium. Medium was changed every second day. Prior to the assay, medium was removed and the cells were rinsed twice in serum-free DMEM/F12 medium. 2.5 ml of serum-free DMEM/F12 medium with 0.25  $\mu$ Ci 1 $\beta$ -<sup>3</sup>H-androstenedione (Perkin-Elmer Life Sciences Inc., Boston, MA) was added to each culture flask, as well as control flasks without cells for measurements of background radioactivity. Hundred nanomolar letrozole (MCF-7 cells) or 10  $\mu$ M letrozole (AROM-1 cells) was concurrently added to pre-selected flasks. Incubation was sustained for 24 h, the assay medium was collected and the cells were counted in a Bürker–Türk chamber. Aromatase activity was assayed by measuring the amount of <sup>3</sup>H present in the water phase after aromatization of 1 $\beta$ -<sup>3</sup>H-androstenedione according to a protocol developed by Dr. A. Purohit and Dr. M. Reed [32]. In brief, steroid was

removed from the medium by extraction with diethylether and dextran activated charcoal. Duplicate measures of  $^3\text{H}$  in the water phase of each sample were obtained with a liquid scintillation counter. The obtained background reading from the control flasks was subtracted from the sample values and the values were corrected for loss of  $^3\text{H}_2\text{O}$  during processing (approximately 20%, A. Purohit, personal communication). The aromatase activity was calculated as pmol androstenedione converted/ $10^6$  cells/24 h.

### 2.5. Western analysis

MCF-7 cells ( $3 \times 10^5$ ) were seeded in T25 flasks and cultured for 4 days in FCS medium, with medium change every second day. The cells were starved in NCS medium for 24 h and cultured in NCS medium with hormones  $\pm$  inhibitors for 24 h as indicated in the figure legends. Vehicle (ethanol) was added to the control culture. Cells were harvested by trypsinization, counted and divided into microtubes ( $5 \times 10^5$  cells/tube) and lysed with RIPA buffer (100 mM NaCl, 20 mM Tris base, 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% SDS, 1 mM EDTA, pH 8). Protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Munich, Germany). Ten micrograms of total protein per sample was separated on precast 4–12% Bis-Tris gels in MOPS buffer, using the X-Cell sureLock module (Invitrogen) and proteins were transferred using the X-Cell II blot module (Invitrogen) according to the manufacturer's instructions to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). Membranes were stained with Ponceau S to confirm equal loading and transfer of samples. Non-specific binding of antibodies was blocked by incubation of the membrane in 5% non-fat dry milk (1 h RT). Membranes were exposed for 1 h at RT to monoclonal rabbit estrogen receptor  $\alpha$  (ER $\alpha$  1:10,000), progesterone receptor (PR 1:2000) antibody (both LabVision, Fremont, CA) or monoclonal mouse cytokeratin-7 (K7 1:500,000) antibody. Specific binding was visualized by incubation for 1 h at RT with species-specific peroxidase-conjugated secondary antibodies (Dako Cytomation, Glostrup, Denmark) followed by visualization using the ECL<sup>PLUS</sup> kit (Amersham Pharmacia, Buckinghamshire, UK). Chemiluminescence was detected on Hyper Film<sup>TM</sup> (Amersham Pharmacia Biotech). Expression of K7 was used as loading control. The K7 antibody was kindly provided by Dr. Jiri Bartek (Danish Cancer Society, Copenhagen, Denmark).

### 2.6. Statistics

Levene's test was used to analyze for homogeneity of variance in all experiments and log transformation of data was performed in case of unequal variance. A linear model of analysis of variance, followed by two sided pair wise *t*-tests with Bonferroni's correction was used to detect differences between treatments. Results were considered significant when  $p < 0.05$ . Calculations were

performed using SAS, version 8.2 (SAS institute, Cary, NC).

## 3. Results

### 3.1. Growth of MCF-7 cells in FCS and NCS supplemented medium

The MCF-7 cells cultured in FCS supplemented medium had a high growth rate and adding estradiol ( $\text{E}_2$ , 100 pM) did not increase cell growth significantly (Fig. 1). Culturing cells in NCS supplemented medium significantly reduced the growth rate compared to cells cultured in FCS medium ( $p < 0.0001$ ). Addition of 100 pM  $\text{E}_2$  to the NCS medium significantly stimulated cell growth compared to the NCS control ( $p < 0.0001$ ), restoring growth to the level observed for the FCS culture. Thus, MCF-7 cells cultured in the NCS model system were dependent on  $\text{E}_2$  to retain their normal growth rate. The pure anti-estrogen ICI 182,780 (ICI, 10 nM) in a 100-fold excess concentration completely abolished the stimulatory effect of  $\text{E}_2$  ( $p < 0.0001$ ), bringing cell growth to the NCS culture level. No effect of the aromatase inhibitor letrozole (100 nM) was observed on  $\text{E}_2$  stimulated growth, indicating that letrozole did not have unspecific growth inhibitory effects on the cells in the applied concentration. Four independent quadruplicate experiments gave similar results.

### 3.2. Growth stimulation of MCF-7 cells with steroid hormones

Dose–response growth curves for MCF-7 cells were established with the estrogens  $\text{E}_2$  and estrone ( $\text{E}_1$ ) and for the androgens testosterone, androstenedione (Adione) and dihydrotestosterone (DHT). Fig. 2 shows a representative quadruplicate experiment for each hormonal treatment. All applied concentrations of  $\text{E}_2$  from 1 pM significantly stimulated cell growth ( $p < 0.0001$ ). A plateau with maximal stimulation approximately 10-fold above control level was reached at a concentration of 10 pM  $\text{E}_2$ .  $\text{E}_1$  significantly stimulated cell growth with concentrations from 10 pM ( $p < 0.0001$ ), with maximal growth stimulation obtained at a concentration of 100 nM. Thus,  $\text{E}_1$  was approximately 10 times less potent than  $\text{E}_2$ . All applied concentrations of testosterone significantly stimulated cell growth compared to the NCS control ( $p < 0.0001$ ). A plateau with maximal growth stimulation was reached with a concentration of 100 nM testosterone. DHT only stimulated cell growth significantly at concentrations from 100 nM ( $p < 0.0001$ ), and maximal effect was not established with the applied concentrations of DHT. Adione stimulated cell growth approximately 2.5-fold above the level of the NCS control culture at concentrations between 10–100 nM ( $p < 0.0001$ ). The effect of Adione leveled off at 1  $\mu\text{M}$ , which was repeatedly observed in the majority of performed experiments. Thus, all androgens tested were observed to stimulate MCF-7 cell growth, but with different

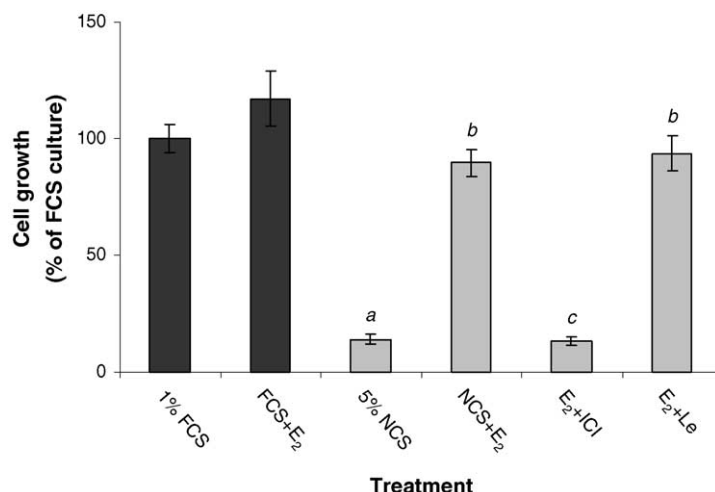


Fig. 1. Growth of MCF-7 cells in FCS and NCS supplemented medium. E<sub>2</sub>; estradiol, ICI; ICI 182,780, Le; letrozole. Black bars illustrate cells cultured in FCS medium  $\pm$  E<sub>2</sub> (100 pM). Gray bars illustrate cells cultured in NCS medium  $\pm$  E<sub>2</sub> (100 pM)  $\pm$  inhibitors (ICI 10 nM, Le 100 nM). Cells were cultured for 5 days with hormones and inhibitors and cell number was estimated by a colorimetric assay. Values are mean  $\pm$  S.D. from a representative quadruplicate experiment. Four independent quadruplicate experiments were performed. (a) Significantly different from the FCS culture, (b) significantly different from the NCS culture, and (c) significantly different from NCS + E<sub>2</sub> culture,  $p < 0.0001$ .

potency. Four independent quadruplicate experiments were performed for each steroid, and comparable dose–response curves were obtained, even though the fold stimulation varied between experiments. Concentrations of 100 pM E<sub>2</sub>, 100 nM testosterone and 100 nM DHT were selected for further experiments. Testosterone and DHT were selected in order to study effects of both an aromatizable androgen (testosterone) and a non-aromatizable androgen (DHT). The androgen concentration was based on the testosterone dose–response curve.

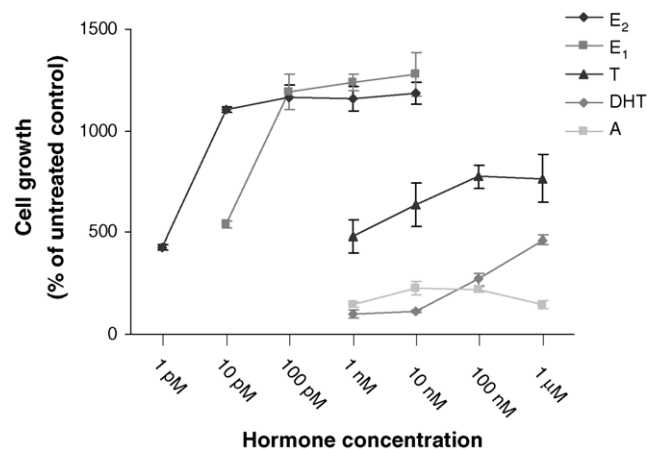


Fig. 2. Growth stimulation of MCF-7 cells with steroid hormones. E<sub>2</sub>, estradiol; E<sub>1</sub>, estrone; T, testosterone; A, androstenedione; DHT, dihydrotestosterone. Cells were cultured for 5 days in NCS medium supplemented with steroid hormones and cell number was estimated by a colorimetric assay. Values are mean  $\pm$  S.D. from a representative quadruplicate experiment for each steroid hormone. At least four independent quadruplicate experiments were performed for each steroid. Treatment with all steroid hormones, except 1 and 10 nM DHT, and 1 nM and 1  $\mu$ M A, resulted in a significantly higher MCF-7 cell growth compared to the NCS control culture ( $p < 0.0001$ ).

### 3.3. Inhibition of androgen-mediated MCF-7 cell growth

To elucidate the mechanism behind the androgen-mediated cell growth, the aromatase inhibitor letrozole, the anti-estrogen ICI and the anti-androgen bicalutamide were added to cells treated with the aromatizable androgen testosterone or the non-aromatizable androgen DHT (Fig. 3). Letrozole and ICI completely abolished the effect of testosterone ( $p < 0.0001$ ), whereas bicalutamide had no effect. Only ICI inhibited DHT-mediated cell growth ( $p < 0.0001$ ). Fig. 3

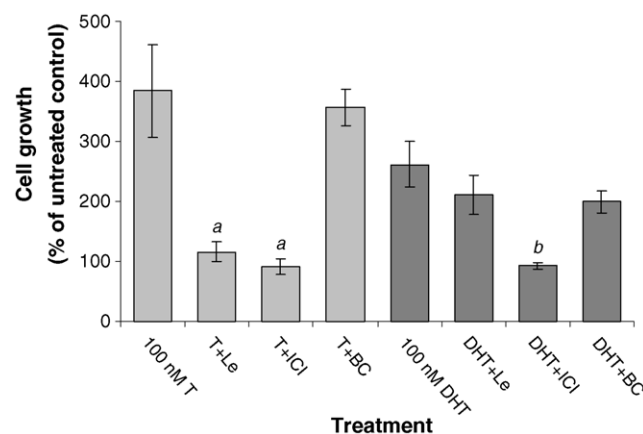


Fig. 3. Inhibition of androgen-mediated MCF-7 cell growth. T; testosterone, DHT; dihydrotestosterone, ICI; ICI 182,780, Le; letrozole, BC; bicalutamide. Cells were cultured for 5 days in NCS medium supplemented with 100 nM androgen  $\pm$  inhibitors (10 nM ICI, 100 nM Le, 100 nM BC). Cell number was estimated by a colorimetric assay. Values are mean  $\pm$  S.D. from a representative quadruplicate experiment. Four independent quadruplicate experiments were performed. (a) Significantly different from the T-treated culture and (b) significantly different from the DHT-treated culture,  $p < 0.0001$ .

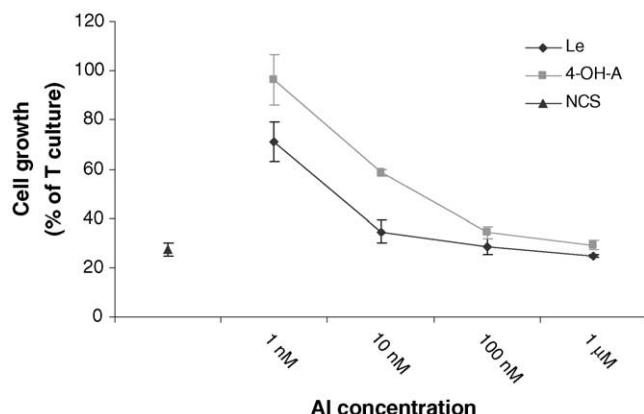


Fig. 4. Inhibition of testosterone-mediated MCF-7 growth with aromatase inhibitors. T; testosterone, Le; letrozole, 4-OH-A; 4-hydroxy-androstenedione, AI; aromatase inhibitor. Cells were cultured for 5 days in NCS medium with 100 nM testosterone plus increasing doses of aromatase inhibitor as indicated in the figure. Cell number was estimated by a colorimetric assay and expressed as percent of the testosterone-treated culture. Values are mean  $\pm$  S.D. from one quadruplicate representative experiment. At least four independent quadruplicate experiments were performed. All applied concentrations of the aromatase inhibitors, except for 1 nM 4-OH-A, resulted in inhibition of testosterone-mediated growth ( $p < 0.0001$ ).

shows a representative quadruplicate experiment. Four independent quadruplicate experiments gave comparable results. Thus, the estrogen receptor, but not the androgen receptor, appeared to be involved in testosterone and DHT-mediated growth. Aromatization of testosterone to estrogen was required for growth-stimulation with testosterone, whereas DHT may have interacted directly with the estrogen receptor.

### 3.4. Inhibition of aromatization of testosterone with aromatase inhibitors

Increasing concentrations of the non-steroidal aromatase inhibitor letrozole and the steroidal inhibitor 4-hydroxy-androstenedione (4-OH-A) were added to testosterone (100 nM) treated cultures of MCF-7 cells. Fig. 4 shows a representative quadruplicate experiment. All concentrations of letrozole, from 1 nM, reduced testosterone-mediated growth ( $p < 0.0001$ ), with complete inhibition down to NCS control level obtained at a concentration of 100 nM letrozole. 4-OH-A reduced testosterone-mediated growth at concentrations from 10 nM ( $p < 0.0001$ ), with complete inhibition to control level obtained at a concentration of 1  $\mu$ M 4-OH-A. Thus, both the steroidal and the non-steroidal aromatase inhibitor were able to completely abolish the growth-stimulatory effect of testosterone. Letrozole was approximately 10-fold more potent than 4-OH-A. Four independent experiments, each in quadruplicate, gave comparable results. Thus, testosterone-mediated growth stimulation appears to be dependent upon aromatization to estrogen.

### 3.5. Aromatase activity in MCF-7 and AROM-1 cells

The activity of the aromatase enzyme was measured using a tritium release assay [32], with  $1\beta$ - $^3$ H-androstenedione as the substrate in serum-free medium. A significant aromatase activity could be measured in MCF-7 cells (Fig. 5), and was significantly higher ( $p < 0.0001$ ) in cells primed in NCS supplemented medium (0.085 pmol androstenedione converted/ $10^6$  cells/24 h) compared to cells primed in FCS supplemented medium (0.011 pmol androstene-

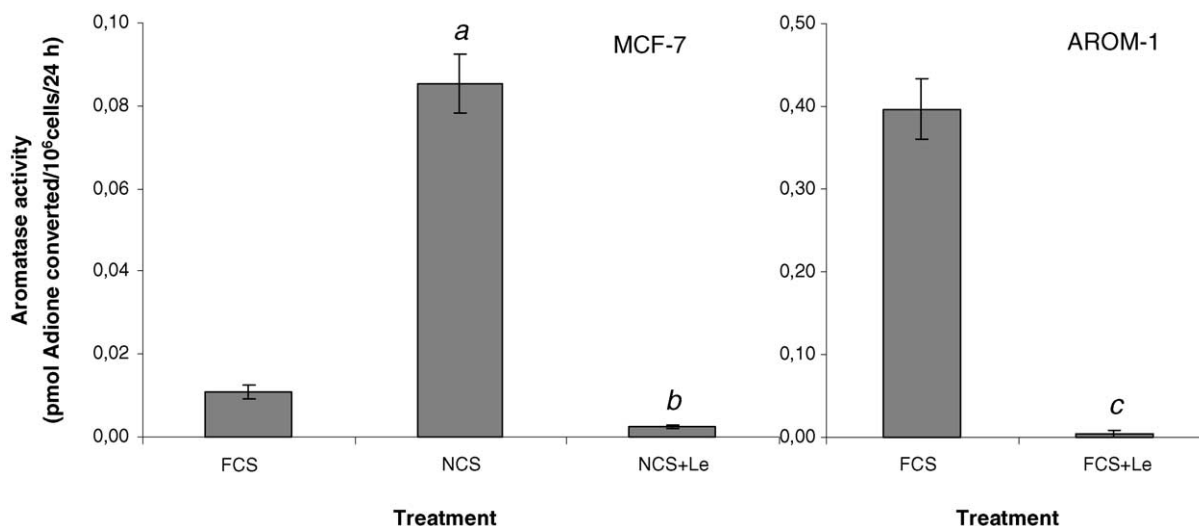


Fig. 5. Aromatase enzyme activity in MCF-7 and AROM-1 cells. Prior to the assay, MCF-7 cells were cultured for 4 days in FCS and 2 days in FCS or NCS medium. AROM-1 cells were cultured for 6 days in AROM-1 medium. Cells were incubated with 0.25  $\mu$ Ci  $1\beta$ - $^3$ H-androstenedione  $\pm$  letrozole (Le) in serum-free medium for 24 h. The letrozole concentration was 100 nM for MCF-7 cells and 10  $\mu$ M for AROM-1 cells. Medium was assayed for produced  $^3$ H $_2$ O and aromatase activity was calculated as pmol androstenedione (Adione) converted per  $10^6$  cells per 24 h. Values are mean  $\pm$  S.D. of a representative triplicate experiment out of four independent experiments. (a) Significantly different from the FCS-treated MCF-7 culture, (b) significantly different from the NCS-treated MCF-7 culture, and (c) significantly different from the FCS-treated AROM-1 culture,  $p < 0.0001$ .



dione converted/10<sup>6</sup> cells/24 h). Higher activity (0.4 pmol androstenedione converted/10<sup>6</sup> cells/24 h) was observed in the AROM-1 cells (MCF-7 cells stably transfected with the aromatase gene), which were used as a positive control in the assay. Thus, the amount of labeled substrate was not limiting for aromatization in the MCF-7 cells. Addition of 100 nM letrozole to MCF-7 cells cultured in NCS medium completely inhibited the aromatase activity ( $p < 0.0001$ ). Ten micromolar letrozole inhibited the aromatase enzyme in AROM-1 cells ( $p < 0.0001$ ). Experiments with 1,2 $\beta$ -<sup>3</sup>H-labeled testosterone as substrate were also performed, but the results were confounded by cell-independent release of <sup>3</sup>H from the substrate (data not shown).

### 3.6. PR and ER $\alpha$ protein expression in MCF-7 cells treated with estradiol or testosterone

The MCF-7 cell line is known to express PR and ER $\alpha$ , and only extremely low levels of the estrogen receptor  $\beta$  (ER $\beta$ ) [33]. PR is expressed in two forms, PR-A and PR-B. PR is an estrogen-regulated gene [34] and the expression is often used as a marker for the presence of functional ER $\alpha$ . ER $\alpha$  protein is significantly reduced after estrogen treatment of MCF-7 cells, both due to instability of the receptor in the presence of estrogen, and due to downregulation of the mRNA [35]. Ac-

cordingly, ER $\alpha$  and PR protein expression was examined in order to investigate whether testosterone stimulated MCF-7 cells via the ER $\alpha$ , both directly and/or after aromatization to estrogen. As shown in Fig. 6, ER $\alpha$  expression was downregulated in cells treated with E<sub>2</sub> and testosterone. PR expression was highly upregulated after exposure to E<sub>2</sub> and testosterone. Letrozole abolished the testosterone-mediated regulation of both ER $\alpha$  and PR, but not the regulation exerted by E<sub>2</sub>. ICI inhibited the effects of both E<sub>2</sub> and testosterone on PR expression, whereas the level of ER $\alpha$  remained low as ICI in itself downregulates ER $\alpha$  protein expression [35]. As letrozole completely inhibited the testosterone-mediated effect, aromatization of testosterone to estrogen was required for testosterone to affect the protein expression of these estrogen responsive genes. Further, regulation of ER $\alpha$  and PR expression was mediated via estrogen-interaction with ER $\alpha$  as ICI abolished the effect of aromatized testosterone.

## 4. Discussion

Locally produced estrogen is suggested to play a major role for proliferation of estrogen responsive breast cancer (recently reviewed in [36]). Intratumoral estrogens are produced from circulating androgens, catalyzed by the aromatase enzyme. Whether aromatase is localized in cancer cells, the surrounding stromal cells or both, has been debated since the first published immunohistochemical study of aromatase in breast carcinomas [37] and it still remains controversial [11–15]. The present study was undertaken in order to examine the presence of aromatase activity and its possible biological significance in human MCF-7 breast cancer cells.

A low but reproducible aromatase activity could be measured in the MCF-7 cells, which is in agreement with other reports [16–19]. The androgen testosterone was observed to stimulate MCF-7 cell growth significantly at concentrations as low as 1 nM, which is within the physiological range for this steroid hormone. The stimulation could be inhibited with aromatase inhibitors, indicating that aromatization of testosterone was responsible for the testosterone-mediated growth. Thus, in the present model system, the epithelial breast cancer cell line MCF-7 was observed to express sufficient aromatase activity in order to stimulate cell growth via aromatization of testosterone to E<sub>2</sub>. Expression of ER $\alpha$  and PR confirmed that testosterone was aromatized to estrogen, which then affected expression of these estrogen-regulated genes. Testosterone did not appear to interact directly with ER $\alpha$ . Further, the anti-androgen bicalutamide had no effect on T-stimulated growth, suggesting that the androgen receptor (AR) was not involved in the testosterone-mediated effects. Conflicting reports of testosterone-mediated effects on MCF-7 cells exist in the literature. Cell growth [17,24] and pS2 expression [16] have previously been observed to be stimulated as a consequence of aromatization of testosterone to estrogen. Further, testosterone has been reported to stimulate MCF-7 cell growth via direct interaction with ER [38]. Direct interac-

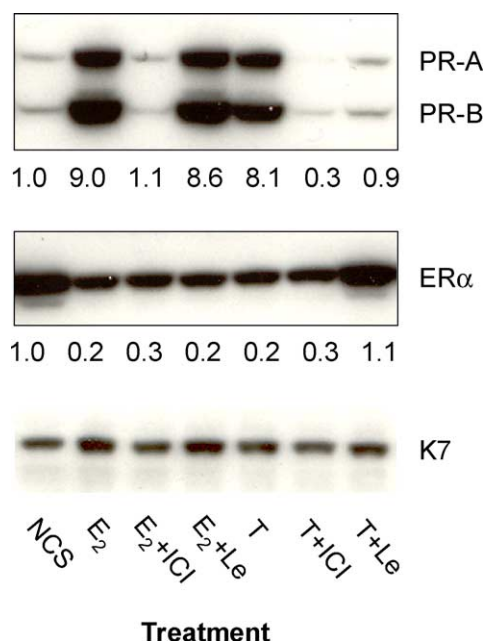


Fig. 6. PR and ER $\alpha$  protein expression in MCF-7 cells. PR-A and B; progesterone receptor A and B form, ER $\alpha$ ; estrogen receptor  $\alpha$ , E<sub>2</sub>; estradiol (100 pM), T; testosterone (100 nM), ICI; ICI 182,780 (10 nM), Le; letrozole (100 nM). Expression of cytokeratin 7 (K7) was used as loading control. Prior to treatment, cells were cultured for 4 days in FCS medium and starved for 2 days in NCS medium. Cells were treated with hormones  $\pm$  inhibitors for 24 h. Protein was extracted and ER $\alpha$  and PR expression measured by Western analysis. Numbers below lanes are protein expression levels normalized to the K7 expression and expressed as fold of the NCS culture. Three independent experiments gave comparable results.

tion of testosterone with ER has been reported at concentrations from 100 nM [39,40], which could not be confirmed in the present study, where testosterone only exerted effects after aromatization to estrogen. However, testosterone has also been observed to inhibit MCF-7 growth [26,27] and inhibit E<sub>2</sub>-mediated upregulation of PR protein expression [28], presumably via AR signaling [27,28].

Compared to testosterone, Adione only stimulated MCF-7 cell growth slightly, however, significantly. This was surprising as Adione based on circulating substrate availability, is believed to be the major substrate for the aromatase enzyme in peripheral tissues [41,42]. Yet, the concentration of Adione and the product from aromatization of Adione, E<sub>1</sub>, has been observed to be low in breast tumors, compared to the concentration of testosterone and E<sub>2</sub> [43]. This could indicate that the preferred substrate for the aromatase enzyme in breast cancer tissue is testosterone, produced in peripheral tissue from conversion of circulating Adione via type 5 17 $\beta$ -hydroxysteroid dehydrogenase (HSD) activity [1]. The low effect of Adione may thus indicate that the HSD activity is insufficient for the conversion of Adione to testosterone in the MCF-7 cells used in the present study. To our knowledge, the activity of type 5 HSD has not been studied in MCF-7 cells. The product from aromatization of Adione, E<sub>1</sub>, is a less potent estrogen than E<sub>2</sub>, and it could also be speculated that the amount of E<sub>1</sub> produced from aromatization of Adione was insufficient to stimulate MCF-7 growth, compared to the E<sub>2</sub> produced from testosterone. Yet, we found that E<sub>1</sub> stimulated MCF-7 cell growth at concentrations as low as 10 nM and the small growth stimulation obtained with Adione may thus not be ascribed to lack of E<sub>1</sub> potency. Other studies have also shown that aromatization of testosterone elicited estrogenic responses at concentrations where Adione was inactive [17,40], but also here was the reason unclear. The low aromatase activity obtained with 1 $\beta$ -<sup>3</sup>H-labeled androstenedione as substrate in the present study is in correspondence with the low growth stimulation obtained with androstenedione. It could be speculated that the aromatase activity would be higher, if labeled testosterone served as substrate. To investigate this, 1,2 $\beta$ -<sup>3</sup>H-labeled testosterone was used as substrate in the aromatase assay as 1 $\beta$ -<sup>3</sup>H-labeled testosterone is not commercially available. However, the results were confounded by cell-independent release of <sup>3</sup>H from the substrate, resulting in high background measures (data not shown). This may have been due to spontaneous release of <sup>3</sup>H from the 2 $\beta$ -position, which is not involved in aromatization of androgens to estrogens.

In addition to ER $\alpha$ , the MCF-7 cells were observed to express AR (data not shown). The non-aromatizable androgen DHT has a high affinity for the AR and it could be speculated that DHT mediated cell growth via AR. However, high concentrations, from 100 nM DHT, were needed to stimulate MCF-7 cell growth, which could indicate that the stimulation was not mediated by AR interaction. The inhibition study confirmed this, as the anti-androgen bicalutamide had no effect on DHT-mediated growth. On the

contrary, ICI completely blocked the effect, indicating that DHT may have mediated MCF-7 growth by direct ER $\alpha$  interaction. DHT has earlier been observed to bind to ER $\alpha$  in MCF-7 cells and elicit estrogenic actions, but only at pharmacological concentrations [38–40], which is in correspondence to our growth studies. Another study has reported that both physiological (1–10 nM) and pharmacological concentrations (100–1000 nM) of DHT stimulated proliferation after 48 h incubation, whereas longer time incubation with DHT inhibited proliferation [44]. In the present study, the effect of DHT was assessed after 5 days treatment and the inhibitory effect of DHT beyond 48 h incubation was not confirmed. In conclusion, only pharmacological concentrations of DHT had a stimulatory effect on MCF-7 cell growth, possibly mediated by direct interaction with ER $\alpha$ .

Whether androgens play a role in human breast cancer, besides being substrates for aromatization, is debated. Androgens have been suggested both directly to promote or to inhibit breast cancer cell growth, or to act differentially depending on the estrogen milieu, acting as inhibitors in a high estrogen milieu, but as promoters in a low estrogen milieu (reviewed in [45,46]). The hypothesis of androgens acting directly stimulatory in a low estrogen milieu could be of relevance for treatment benefit and disease progression in breast cancer patients deprived of estrogen via aromatase inhibitor treatment. However, the hypothesis was not supported by the present findings, as testosterone had no direct stimulatory effect but only affected the examined endpoints after aromatization, despite the low estrogen milieu. DHT may have stimulated MCF-7 cell growth via ER $\alpha$  interaction, however, only at pharmacological concentrations.

The reason for the conflicting results found in the literature regarding androgen-mediated growth effects on MCF-7 cells is unclear, but could be related to experimental design or differences between MCF-7 strains. Several comparative studies have shown that direct comparison between studies can be conflicting due to independent progression of MCF-7 strains [47–52]. Whether strain differences in regard of receptor content, E<sub>2</sub> responsiveness or aromatase enzyme expression may contribute to the differences can be speculated. One study has previously reported different aromatase activity in two MCF-7 strains [17]. Estrogen responsiveness is required for aromatized androgens to act as stimulators and may thus affect the observed effects of androgens. Our experience is that MCF-7 strains differ significantly with respect to estrogen responsiveness. In one study where testosterone inhibited MCF-7 cell growth, the cells appeared to be estrogen independent as the cells had a high growth rate in serum-free medium [26], possibly affecting the results. The MCF-7 cells used in the present study have been adapted to grow with a low serum concentration and may supposedly have become less dependent of, or hypersensitive to estrogens and growth factors. Hypersensitivity to E<sub>2</sub> could explain the growth stimulatory effects of testosterone if just a small amount was aromatized to E<sub>2</sub>. As seen in Fig. 2, our MCF-7 strain was indeed sensitive to E<sub>2</sub>, however, the sensitivity was

comparable to that of MCF-7 strains routinely maintained in 10% FCS (maximal growth stimulation at 100 pM  $E_2$ ) and not to that of hypersensitized MCF-7 cells (maximal growth stimulation at 100 fM  $E_2$ ), long-term deprived of estrogen (L-TED) [53,54]. Also, other studies have measured aromatase activity in MCF-7 cells only shortly deprived of estrogen and thus not hypersensitized [16,17]. Yet, the MCF-7 cells used in the present study may have acquired other changes from the long-term culture in a low estrogen milieu, low growth factor milieu (1% FCS), rendering this strain able to aromatize testosterone. Indeed, the aromatase activity was observed to be significantly higher in cells cultured in steroid poor NCS serum than FCS serum, which could indicate that growth in steroid poor serum further stimulated the aromatase activity in the cells. The concentration of estrogens in the NCS serum used in our laboratory has been routinely measured by specific radio-immune assay (RIA) and was comparable to the concentration in steroid-stripped FCS serum ( $E_2 < 40$  pM,  $E_2$ -sulphate  $< 200$  pM,  $E_1 = 100$  pM,  $E_1$ -sulphate = 400 pM). Also, MCF-7 cells adapted to grow on serum-free medium, yet retaining estrogen-responsiveness, have been observed to be growth-stimulated with androgens through conversion to estrogens [25]. It would be of great interest to repeat the experiments in different L-TED breast cancer cell lines, to see whether growth in a low estrogen milieu per se enables breast cancer cells to aromatize androgens. It is possible that a similar effect of estrogen deprivation in breast cancer patients may render the cancer cells able to produce endogenous estrogen.

In conclusion, a significant aromatase activity was detected in the MCF-7 cells. The activity was sufficient for the cells to aromatize testosterone to estrogen, resulting in significant cell growth stimulation at testosterone concentrations from 1 nM, as well as induction of estrogen responsive proteins. The results indicate that breast cancer cells, adapted to grow in a low estrogen milieu, possess the ability to synthesize estrogens from circulating androgens. Accordingly, estrogen may be an important intracrine factor in postmenopausal human breast cancer, where circulating estrogen levels are low.

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