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Hormonal Regulation of Endocrine-resistant Breast Cancer

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ACADEMIC DISSERTATION
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ABSTRACT

Breast cancer is the most common type of cancer among women in western world. In Finnish women, approximately 4000 – 5000 new breast cancers are diagnosed every year. Approximately 70 percent of breast cancers express estrogen receptor (ER). These tumors use estrogen as their main growth stimulus and they are considered to be hormone-dependent. Endocrine therapy targeting ER is the most effective treatment of hormone-dependent breast cancer therapy both in the adjuvant and metastatic setting.

In premenopausal women, the main source of estrogen is the ovaries, while in postmenopausal women, the enzyme aromatase converts estrogen from androgens in peripheral tissues such as adipose, skin or bone. In hormone-dependent breast cancer, the suppression of tumor growth can be achieved by reducing estrogen levels with chemical ovarian ablation (occasionally with surgery or radiation) in premenopausal women, or with aromatase inhibitors in postmenopausal women. Also, the interaction of estrogen and ER can be blocked by using selective ER modulators (SERMs) or by downregulating the expression of ER with selective ER downregulators (SERDs) both in premenopausal and postmenopausal women.

Endocrine therapy is the most effective treatment for the hormone receptor-positive breast cancer. Still, not all patients receiving endocrine therapy respond to treatment (*de novo* resistance), and others will eventually relapse despite an initial response (acquired resistance). Knowledge of resistance mechanisms originates mainly from tumor biopsies of tamoxifen-resistant breast cancer, and from *in vitro* cell culture models of antiestrogen resistance. Several different mechanisms have been hypothesized to be involved in *de novo* or acquired resistance to endocrine therapy. These mechanisms include the activation of growth factor receptors and their downstream signaling pathways, which lead to an increased estrogen-sensitivity of ER or ligand-independent activation of ER. Such mechanisms may lead breast cancer cells from an estrogen-dependent phenotype which responds to endocrine therapy, to a non-responding estrogen-independent phenotype.

This study was undertaken in order to examine the alterations in gene expression when hormone-dependent breast cancer cells were grown over a long-term period without estrogen, or additionally in the presence of the SERM toremifene, eventually leading to estrogen-independent (which mimics resistance to aromatase inhibitor) and toremifene-resistant phenotypes. Our results show that observed alterations in gene expression after long-term culture of the cells were already present as soon as the cells started proliferating after a period of quiescence. This indicates that pre-existing resistant subpopulations were selected, without involving any changes in individual cells. The overexpression of the fos-like antigen 1 (FOSL1) was shown to be critical for the growth of toremifene-resistant cells. We also observed changes in the regulation of genes by toremifene, which took place later in the cell culture process, indicating that the resistant cells acquired a toremifene-stimulated phenotype. This may not have happened in the early selection process.

There has been renewed interest in alternative hormonal treatments both in early disease and in the advanced setting when conventional ER-targeting therapies fail. We assessed the effect of different nuclear receptor ligands on the growth of hormone-sensitive breast cancer cells and endocrine-resistant sublines. In our study, physiological concentrations of vitamin A (retinol) inhibited the growth of cell lines, especially in the presence of estrogen. This is interesting since low-dose estrogen therapy is a promising new approach in treating estrogen-independent and antiestrogen-resistant hormone receptor-positive breast cancer. Another intriguing finding was that medroxyprogesterone acetate dose-dependently and at low pharmacological concentrations inhibited the growth of estrogen-independent cells, and that the presence of estrogen abrogated the effect. These findings warrant further study on the effects of combined low-dose estrogen and retinoids, and aromatase inhibitor and synthetic progestins on acquired aromatase inhibitor-resistant breast cancer.

TIIVISTELMÄ

Rintasyöpä on naisten yleisin syöpä. Vuosittain suomessa todetaan 4000–5000 uutta rintasyöpätapausta. Rintasyövistä noin 70 % on hormonireseptoripositiivisia eli niissä ilmenee estrogeenireseptori. Estrogeeni, joka vaikuttaa estrogeenireseptorin kautta, on hormonireseptoripositiivisen, eli hormoniherkän rintasyövän tärkein tunnettu kasvutekijä. Hormoniherkissä syövissä kaikki hormonaaliset hoidot tähtäävätkin estrogeenin kasvuvaikutuksen eliminoimiseen.

Premenopausaalisilla naisilla estrogeenia tuottavat munasarjat, kun taas postmenopausaalisilla naisilla estrogeenia tuotetaan androgeeneista perifeerisissä kudoksissa (esim. rasvakudos) aromataasientsyymin toimesta. Hormoniherkän rintasyövän hoidossa premenopausaalisilla potilailla munasarjojen estrogeenituotanto lopetetaan kemiallisesti (tai sädehoidolla tai munasarjojen kirurgisella poistolla). Postmenopausaalisilla potilailla hoitona voidaan käyttää aromataasi entsyymin estäjiä (aromataasinestäjät). Näiden lisäksi estrogeenin sitoutuminen reseptoriinsa voidaan estää antiestrogeeneillä kuten tamoksifeeni, toremifeeni tai fulvestrantti. Antiestrogeenit soveltuvat sekä pre- että postmenopausaalisille potilaille.

Hormonaaliset hoidot ovat tehokkaita hoitomuotoja sekä liitännäishoitoina annettuna leikkauksen jälkeen että levinneen (metastaattisen) rintasyövän hoidossa. Kuitenkin osalla hormonireseptoripositiivisista potilaista ei todeta vastetta hoidolle eli syöpäkasvain on resistenssi hormonaalisille hoidoille (*de novo* resistenssi), ja osalla potilaista rintasyöpä uusiutuu sellaisena että se ei enää reagoi annettuun hormonaaliseen hoitoon (hankittu resistenssi). Sekä *de novo* että hankitun resistenssin syntymekanismeista on saatu tietoa laboratoriossa tehdyistä soluviljelykokeista (*in vitro* tutkimus) sekä tutkimalla kasvaimista otettuja kudosnäytteitä (*in vivo* tutkimus). Resistenssien mahdollisia syitä ovat mm. kasvutekijäreseptorien sekä näihin liittyvien solun signaalireittien yliaktivaatiot. Nämä tapahtumat aiheuttavat syöpäkasvaimen estrogeenireseptorin yliherkistymistä estrogeenille tai reseptorin aktivoitumista ilman estrogeenia, ja saattavat lopulta johtaa syöpään joka kasvaa estrogeenista riippumatta.

Tutkimuksessamme tutkimme soluviljelymallien avulla geenien ilmenemisen muutoksia prosessissa, jossa hormoniherkästä rintasyövästä kehittyy resistenssi antiestrogeenihoidolle (toremifeeni), ja kun hormoniherkkä rintasyöpä alkaa kasvaa ilman estrogeenia. Jälkimmäinen tilanne mallintaa resistenssiä aromataasinestäjälle. Tutkimuksemme osoittaa että pitkäaikaisen kasvatuksen (1 vuosi) jälkeen havaitut muutokset geenien ilmenemisessä voidaan nähdä soluissa jo heti kun ne alkavat kasvamaan ilman estrogeenia tai antiestrogeenista huolimatta, noin 2-3 kuukauden jälkeen. Tuloksemme tukevat näkemystä että osalla soluista on kyky kasvaa muuttuneissa olosuhteissa ja ne valikoituvat (selektio) kasvamaan tässä prosessissa. Osoitimme myös että fos-like antigen 1 – geenin yli-ilmentyminen on tärkeä ominaisuus toremifeeni-resistenttien solujen kasvulle. Lisäksi osoitimme että selektiota ei tapahdu vain resistenssin syntymisen alkuvaiheessa, sillä toremifeeni vaikutti joidenkin geenien säätelyyn vasta pidemmän altistuksen, noin 12 kuukauden jälkeen.

Estrogeenin kasvuvaikutuksen eliminoimiseen tähtäävät hoitomuodot tehoavat hormoniherkkään rintasyöpään vaihtelevasti ja hoidoille resistenttien syöpien ilmeneminen on yleistä. Tämän takia myös muiden hormonaalisten hoitomuotojen tutkiminen resistenteillä syöpäsoluilla on tärkeää. Tutkimuksessamme fysiologisena konsentraationa A-vitamiini hidasti resistenttien solujen kasvua, etenkin annettuna yhdessä estrogeenin kanssa. Havaitsimme myös että synteettinen progestiini, medroxyprogesteroni asetaatti (MPA) jo matalina farmakologisina konsentraatioina esti estrogeenista riippumattomien solujen kasvua, mutta estrogeeni poisti tämän vaikutuksen.

CONTENTS

ABBREVIATIONS

AF	activation function
AIB1	amplified in breast cancer-1
AKT	v-akt murine thymoma viral oncogene homolog
AP-1	activator protein-1
AR	androgen receptor
cDNA	complementary deoxyribonucleic acid
CYP2D6	cytochrome P450, family 2, subfamily D, polypeptide 6
CX	cycloheximide
DBD	DNA-binding domain
DCC-FBS	dextran charcoal-treated foetal bovine serum
DEX	dexamethasone
DHT	dihydrotestosterone
DLG5	discs, large (Drosophila) homolog 5
DMEM/F-12	Dulbecco´s Modified Eagle´s Medium/Nutrient Mixture F-12 Ham
DNA	deoxyribonucleic acid
DPN	diarylpropionitrile
EGFR	epidermal growth factor receptor
EI	estrogen-independent
ERBB2	v-erb-b2 erythroblastic leukemia viraloncogene homolog 2, neuro/glioblastoma -derived oncogene homolog (avian)
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
ERR	estrogen related receptor
FKBP54	FK506 binding protein 5
FOSL1	fos-like antigen 1
GDF15	growth differentiation factor 15
GR	glucocorticoid receptor
HER2	human epidermal growth factor receptor 2
HRE	hormone response element
HRP	horse radish peroxidase
Hsp	heat shock protein

IGF1R	insulin-like growth factor 1 receptor
INSR	insulin receptor
L1CAM	L1 cell adhesion molecule
LBD	Ligand-binding domain
LE	long-term estrogen-treated
LTED	long-term estrogen-deprived
MAPK	mitogen-activated protein kinase
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin (serine/threonine kinase)
MYBL2	v-myb myeloblastosis viral oncogene homolog (avian)-like 2
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NCOA	nuclear receptor coactivator
NCOR	nuclear receptor corepressor
NF-κB	nuclear factor κB
NPY1R	neuropeptide Y receptor Y1
NTD	N-terminal domain
p70S6K	ribosomal protein S6 kinase, 70kDa, polypeptide 1
PAGE	polyacrylamide gel
Pak1	p21 protein (Cdc42/Rac)-activated kinase 1
PDGF/Abl	platelet-derived growth factor/c-abl oncogene 1, non-receptor tyrosine kinase
PgR	progesterone receptor
PI3K	phosphoinositide-3-kinase
pMCF-7	parental MCF-7
PTP4A1	protein tyrosine phosphatase type IVA member 1
RPLP0	ribosomal phosphoprotein, large subunit P0
RT-PCR	reverse transcriptase polymerase chain reaction
S100P	S 100 calcium binding protein P
SDS	sodium dodecyl sulphate
ser	serine
SERD	selective estrogen receptor downregulator
SERM	selective estrogen receptor modulator

siRNA	small interfering ribonucleic acid
SMRT	silencing mediator for retinoid and thyroid hormone receptors
SP-1	specificity protein 1
SRC-1	steroid receptor coactivator-1
TBS	tris-buffered saline
TFF1	trefoil factor 1
TIMP1	TIMP metallopeptidase inhibitor 1
TR	toremifene-resistant
ZFP36L1	zinc finger protein 36 C3H type-like 1

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I Pennanen PT, Sarvilinna NS, Purmonen SR, Ylikomi TJ. Changes in protein tyrosine phosphatase type IVA member 1 and zinc finger protein 36 C3H type-like 1 expression demonstrate altered estrogen and progestin effect in medroxyprogesterone acetate-resistant and estrogen-independent breast cancer cell models. Steroids. 2009 Apr-May;74(4-5):404-9.

II Pennanen PT, Sarvilinna NS, Ylikomi TJ. Gene expression changes during the development of estrogen-independent and antiestrogen-resistant growth in breast cancer cell culture models. Anticancer Drugs. 2009 Jan;20(1):51-8. Erratum in: Anticancer Drugs. 2009 Nov;20(10):956.

III Pennanen PT, Sarvilinna NS, Toimela T, Ylikomi TJ. Inhibition of FOSL1 overexpression in antiestrogen-resistant MCF-7 cells decreases cell growth and increases vacuolization and cell death. Steroids. 2011 Sep-Oct;76(10-11):1063-8.

IV Pennanen PT, Eronen H, Sarvilinna NS, Ylikomi TJ. The effect of nuclear receptor ligands on the growth of antiestrogen-resistant and estrogen-independent breast cancer cells. Submitted.

INTRODUCTION

Breast cancer is the most common type of cancer among women in the western world. Approximately 70 percent of breast cancers express ER. These tumors use estrogen as their main growth stimulus and they are considered to be hormone-dependent. Thus, endocrine therapy targeting ER is the most effective treatment of hormone-dependent breast cancer both in the adjuvant and metastatic settings (Conzen 2008).

In premenopausal women, the main source of estrogen is the ovaries, while in postmenopausal women estrogen is converted from androgens by the enzyme aromatase in peripheral tissues such as adipose, skin or bone. In hormone-dependent breast cancer, the suppression of tumor growth can be obtained by reducing estrogen levels with chemical ovarian ablation (occasionally with surgery or radiation) in premenopausal women, or with aromatase inhibitors in postmenopausal women. Also, the interaction of estrogen and ER can be blocked by using selective ER modulators (SERMs) or by downregulating the expression of ER with selective estrogen receptor downregulators (SERDs), both in premenopausal and postmenopausal women (Normanno et al. 2005).

Aromatase inhibitors (such as exemestane, anastrozole and letrozole) inhibit the aromatase enzyme by binding to its substrate binding site, thus blocking the conversion of adrenal androgens to estrogens. In this way, they lower circulating estrogen levels (Chumsri et al. 2011). SERMs (such as tamoxifen, toremifene and raloxifene) are synthetic molecules which compete with estrogen to bind to ER, and effectively block the potential for estrogen stimulation. However, SERMs exhibit mixed tissue-dependent ER-antagonist/agonist activity, which means that the SERM-bound receptor can activate or inhibit transcription (Jordan and O'Malley 2007). SERDs, for example fulvestrant (ICI 182780), SR 16234 and ZK 191703, are synthetic steroidal antiestrogens which bind to ER, but unlike SERMs they completely block transcription and increase receptor degradation, resulting in ER downregulation (Howell et al. 2000).

Endocrine therapy is the most effective treatment for hormone receptor-positive breast cancer. However, not all patients receiving endocrine therapy respond to treatment (*de novo* resistance), and others can eventually relapse despite an initial response (acquired resistance). *De novo* resistance means that the ER-positive tumor, against prediction, does not respond to endocrine therapy. In the case of acquired resistance, breast tumors that initially respond to endocrine treatment develop resistance over time. Knowledge of the mechanisms of resistance originates mainly from tumor biopsies of tamoxifen-resistant breast cancer, and especially from *in vitro* cell culture models of estrogen independency and antiestrogen resistance (Musgrove and Sutherland 2009).

In the *in vitro* studies, the most often used cell line is the ERα-positive MCF-7 breast cancer cell line. The proliferation of MCF-7 cells is highly dependent on estrogen and it expresses functional ER and PgR, which makes it a good model for *in vitro* studies of endocrine resistance (Levenson and Jordan 1997; Wong and Chen 2012). Several different mechanisms have been hypothesized to be involved in *de novo* or acquired resistance to endocrine therapy. These mechanisms include changes in the expression levels of ER and its cofactors and progesterone receptor, and the activation of growth factor receptors and their downstream signaling pathways, which lead to an increased estrogen-sensitivity of ER or the ligand-independent activation of ER. Such mechanisms may lead breast cancer cells from an estrogen-dependent phenotype which responds to endocrine therapy, to a non-responding estrogen-independent phenotype (Jordan and O'Malley 2007; Riggins et al. 2007).

LITERATURE REVIEW

1. Steroid receptors

Steroid hormones, including estrogens, progestins, androgens, glucocorticoids and mineralocorticoids, are small lipophilic molecules which regulate a wide array of cellular and physiological functions such as proliferation, differentiation, survival, organ development and metabolism. Most of the cellular actions of these molecules are mediated through binding to their nuclear receptors. Steroid hormone receptors include estrogen related receptors (ERRα, ERRβ and ERRγ), estrogen receptors (ERα and ERβ), progesterone receptors (PgR-A and PgR-B), androgen receptor (AR), glucocorticoid receptors (GRα and GRβ) and mineralocorticoid receptor (MR). All steroid receptors are implicated in breast cancer, but the role of ERs and PgRs is pivotal, especially in the management of hormone-dependent breast cancer. In addition to steroid hormone receptors, the nuclear receptor superfamily includes receptors for thyroid hormones, active forms of vitamin A (retinoids) and vitamin D3 (calcitriol), as well as different orphan receptors which lack a defined endogenous ligand, and many other nuclear receptors comprising altogether 48 members in humans (Conzen 2008; McEwan 2009).

1.1 Structure of steroid receptors

Like all other nuclear receptors, steroid receptors comprise five regions or domains: 1) a variable N-terminal domain (NTD), 2) a conserved DNA-binding domain (DBD), 3) a hinge D region, 4) a conserved ligand-binding domain (LBD) and 5) in some nuclear receptors, a C-terminal region of unknown function. Nuclear receptors are transcription factors also containing regions which required for transcriptional activation. The NTD of many receptors contains an autonomous transcriptional activation function (AF-1), that contributes to constitutive ligand-independent transcriptional activation by the receptor. A second transcriptional activation

domain (AF-2) is located in the C-terminus of LBD. Unlike the AF-1 domain, the activity of the AF-2 domain is strictly ligand-dependent (Huang et al. 2010).

1.2 Mechanism of action of steroid receptors

Upon ligand-binding, the receptors dimerize and bind to specific DNA sequences in target genes known as hormone response elements (HREs) (Brosens et al. 2004). When bound to HREs, the liganded receptor recruits coregulators (coactivators), such as steroid receptor coactivator-1 (SRC-1) (Onate et al. 1995) and amplified in breast cancer 1 (AIB1) (Anzick et al. 1997)), and the transcriptional machinery necessary to activate transcription. This is called the classical genomic pathway of activating transcription (A in Fig. 1.). Unliganded or antagonist-bound steroid receptors interact with corepressors (such as nuclear receptor corepressor (NCOR) (Chen and Evans 1995) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) (Horlein et al. 1995), shutting off gene transcription (Jackson et al. 1997; Lavinsky et al. 1998).

The ligand-bound steroid receptor complexes can also regulate gene expression by co-activating other transcription factors. For example, ER can interact with activator protein 1 (AP-1) (Bjornstrom and Sjoberg 2004; Philips et al. 1993), or specificity protein 1 (SP-1) (Saville et al. 2000) through a process called transcription factor cross-talk. This is called the nonclassical genomic pathway (B in Fig. 1.). The transcriptional activity of steroid hormones is also regulated by ligand-independent mechanisms. Activated growth factor receptors such as epidermal growth factor receptor (EGFR) and their downstream signaling pathways, can activate steroid receptors by phosphorylation. This is referred to as the ligand-independent genomic pathway (Kato et al. 1995; Tremblay et al. 1999) (C in Fig. 1.). In addition to the genomic mechanisms of steroid receptors, some receptors (such as ER) can regulate cellular functions through nongenomic mechanisms, where the binding of ER to DNA is not needed. In this case steroid hormones can interact with their steroid receptors at the plasma membrane and activate various protein kinases and their downstream signaling cascades (Losel and Wehling 2003) (D in Fig. 1.).

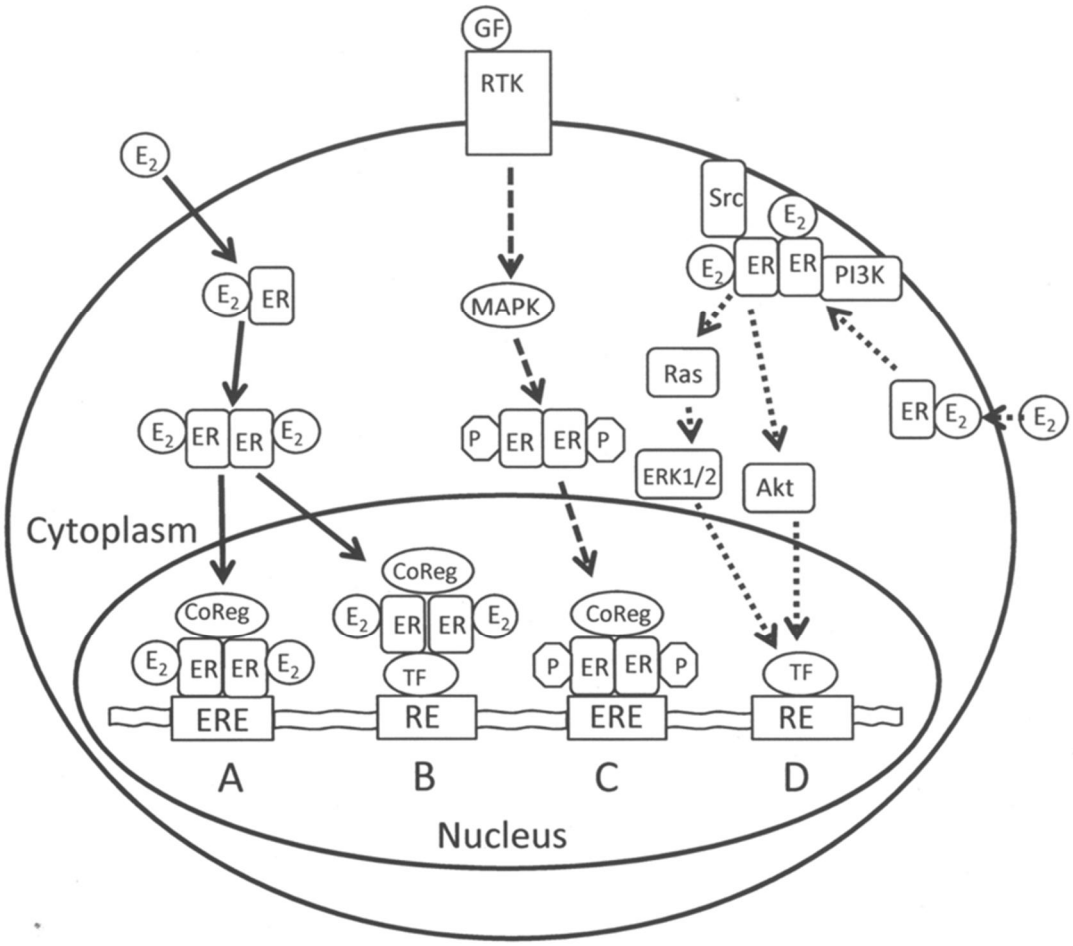

Figure 1. Estrogen receptor (ER) signaling pathways. Pathway A is called the classical genomic pathway. Pathway B is the noncalssical genomic pathway, which involves transcription factor (TF) cross-talk. Pathway C is estrogen-independent and activates ER through phosphorylation induced by the growth factor (GF) -activated receptor tyrosine kinases (RTK). Pathway D is the nongenomic pathway, which involves ERs located close to the plasma membrane. Through the recruitment of protein kinases (Src and PI3K), ligand-bound ER activates signaling cascades (Ras, Erk1/2, Akt) at the plasma membrane. ERE, estrogen response element. RE, response elements to other transcription factors. P, phosphorylation. CoReg, coregulators. E_2, estrogen. MAPK, mitogen-activated protein kinase. Modified from (Le Romancer et al. 2011).

2. Hormone-dependent breast cancer and its therapy

The steroid hormones estrogen and progesterone play a critical role in normal mammary gland development. Estrogen is the primary driver of ductal elongation and the growth of the lobuloalveolar system during mammary gland development in puberty. Progesterone is required for the formation of lobuloalveolar structures in the mammary gland during pregnancy (Topper and Freeman 1980). In the human breast, ERα (ER) and PgRs are expressed only in a minority of luminal epithelial cells (Anderson and Clarke 2004), and the normal mammary epithelial cells that express these receptors are typically non-proliferating (Clarke et al. 1997; Russo et al. 1999). However, in breast cancers ER and PgR are highly expressed and have an important role in the treatment of hormone-dependent breast cancer.

About 70 to 75 percent of breast cancers are ER-positive. The presence of ER is a very strong predictive factor for a response to endocrine therapies, although approximately 40 to 50 percent of women with ER-positive breast cancer do not benefit from endocrine therapy. This means that some ER-positive tumors are intrinsically (*de novo*) resistant to endocrine therapy. Approximately 50 percent of breast tumors express PgR. ER regulates the expression of PgR, hence, the presence of PgR usually indicates that the estrogen-ER pathway is intact and functional. It is thought that the presence of both ER and PgR is a stronger marker for the benefit of endocrine therapy than ER alone. In contrast, only a small minority of ER/PgR-negative patients respond to endocrine therapy (Conzen 2008; Harvey et al. 1999; Le Romancer et al. 2011). Presently, AR, GRs and MR have little role in the management of hormone-dependent breast cancer (Conzen 2008). Taken together, ER is the most important biomarker in breast cancer. A majority of invasive breast tumors express ER, and these tumors use the steroid hormone estrogen as their main growth stimulus. These findings provide a strong rationale for therapies that disrupt the actions of estrogen and ER.

Endocrine therapies for breast cancer target steroid hormone receptors. Approaches in endocrine therapy include antiestrogens, antiprogestins and analogues of luteinizing hormone-releasing hormone (Klijn et al. 1996). Based on its demonstrated efficacy, endocrine therapy targeting ER has become the most important component of hormone-dependent breast cancer therapy, both in the adjuvant and metastatic settings. By using endocrine therapy targeting ER, the suppression of tumor growth can be obtained by: 1) reducing estrogen levels with surgical, radiation, or chemical ovarian ablation in premenopausal women, or with aromatase inhibitors in postmenopausal women, 2) blocking the interaction between estrogen and ER with selective ER modulators (SERMs), and 3) downregulating the expression of ER with selective ER downregulators (SERDs) (Colozza et al. 2007).

2.1 Mechanism of action of SERMs, SERDs and aromatase inhibitors

Selective estrogen receptor modulators (SERMs) are synthetic molecules which can be divided into tamoxifen-like (for example toremifene, droloxifene and idoxifene) and fixed ring compounds (for example raloxifene, arzoxifene, EM-800). SERMs compete with estrogen for the binding to ERα LBD and effectively block the potential for estrogen stimulation. However, all SERMs exhibit mixed tissue-dependent ER-antagonist/agonist activity. The outcome of SERM binding on transcription is determined by several factors: 1) ER isoform (ERα or ERβ), 2) The structure of the SERM which determines the conformation of the ER-SERM complex and thus, which coregulators are recruited (coactivators or corepressors), 3) Tissue dependency: The relative and absolute levels of transcriptional coregulators differ between tissues, altering the effect of SERMs, 4) post-translational modifications like phosphorylation and methylation of the ER, and/or the coregulators with which they interact, can also impact the transcriptional activity of the ER-SERM complex (Jordan and O'Malley 2007).

Selective estrogen receptor downregulators (SERDs), for example fulvestrant (ICI 182780), SR 16234 and ZK 191703, are steroidal antiestrogens. SERDs have no

known estrogen agonist activity, and because of this they are also termed pure antiestrogens. SERDs bind to ER, but unlike SERMs, they impair receptor dimerization, disrupt nuclear localization and increase receptor turnover by destabilizing it, resulting in ER downregulation. As a consequence of ER downregulation, ER-mediated transcription is completely attenuated (Howell et al. 2000).

Aromatase enzyme catalyzes the rate-limiting and the final step of estrogen synthesis. Aromatase expression has been found in many tissues including ovaries, adipose, bone, brain and skin (Chumsri et al. 2011; Simpson et al. 1994). Some breast tumors are also characterized by an overexpression and increased activation of aromatase (Bulun et al. 1993; Esteban et al. 1992; Harada 1997; Miller and O'Neill 1987). Aromatase inhibitors inhibit the aromatase enzyme by binding to its substrate binding site, thus blocking the conversion of adrenal androgens to estrogens. In this way they lower circulating estrogen levels. Currently approved aromatase inhibitors in clinical use are the steroidal exemestane and non-steroidal anastrozole and letrozole. Steroidal aromatase inhibitors irreversibly inactivate aromatase, while non-steroidal agents reversibly bind to aromatase (Chumsri et al. 2011).

2.2 Endocrine therapy for hormone-dependent breast cancer

2.2.1 Premenopausal patients

Selecting the optimal endocrine therapy for hormone-dependent breast cancer depends on the menopausal status of the patient. In premenopausal women, the ovaries are the major source of estrogen production, and a SERM (generally tamoxifen) is the main treatment option. With local and local-regional (non-metastatic) operable breast cancer, tamoxifen is offered as an adjuvant therapy after surgery. Five years of adjuvant tamoxifen safely reduces 15-year risks of breast cancer recurrence and death, whether or not chemotherapy is also given (Early Breast Cancer Trialists' Collaborative et al. 2011). For premenopausal patients with

advanced metastatic breast cancer, the treatment of choice is a SERM. After relapse, the recurrent disease may be treated with a SERD. Aromatase inhibitors are contraindicated in women who are premenopausal (Cruz Jurado et al. 2011).

The role of ovarian ablation/suppression in the management of premenopausal women with early breast cancer has been investigated in several randomized trials. Notably, the efficacy of adjuvant ovarian ablation appears to be similar to that of adjuvant tamoxifen. There seems to be no added benefit for combining ovarian ablation with tamoxifen. However, this treatment option is used in selected cases, mainly in young patients (<40 years) with a high risk of recurrence (Emens and Davidson 2009).

2.2.2 Postmenopausal patients

In postmenopausal women, peripheral aromatization is the primary source of estrogen. In postmenopausal early breast cancer, the treatment of choice for endocrine therapy is an aromatase inhibitor. In the adjuvant setting, several studies have demonstrated that aromatase inhibitors are tolerated well and offer improved disease-free survival compared to tamoxifen (Arimidex et al. 2008; Coates et al. 2007; Coombes et al. 2007) or a placebo (Goss et al. 2003). An aromatase inhibitor can be offered as a primary therapy, or sequentially with tamoxifen, or as an extended therapy after tamoxifen. It is recommended that therapy with an aromatase inhibitor should not extend beyond five years in either the primary or extended adjuvant settings. In the sequential setting, it is recommended that patients receive an aromatase inhibitor after two or three years of tamoxifen (or converse) for a total of five years of adjuvant endocrine therapy (Hurvitz and Pietras 2008).

For postmenopausal women, the benefits of aromatase inhibitors over tamoxifen have also been demonstrated in the metastatic setting (Bonneterre et al. 2000; Mouridsen et al. 2003; Nabholtz et al. 2000; Paridaens et al. 2003). In a recurrent disease, endocrine therapy often includes SERMs, SERDs and aromatase inhibitors in sequence. At the moment, there is no clearly recommended sequencing of these

agents, but studies are undergoing (Burstein et al. 2010; Emens and Davidson 2009; Hurvitz and Pietras 2008).

3. Endocrine-resistant breast cancer and its therapy

3.1 *In vivo* mechanisms of endocrine resistance

There are two types of resistance to endocrine therapies. A proportion (around 30 percent) of ER-positive tumors are *de novo* resistant to endocrine therapy, which means that the tumor, against prediction, does not respond to therapy (Riggins et al. 2007). The other type of resistance is acquired endocrine resistance. In this case, the breast tumors that initially respond to endocrine treatment develop resistance over time. Almost all *in vivo* data of the possible molecular mechanisms of *de novo* or acquired resistance comes from tumor biopsies of tamoxifen-resistant breast cancer. Similar *in vivo* data of the mechanisms underlying resistance to aromatase inhibitors or SERDs are rare. For the most part, the molecular determinants of *de novo* and acquired endocrine resistance are the same and include changes in the functional activity of ER, changes in the expression levels of ER and its cofactors and PgR, and the activation of various growth factor receptors and their downstream signaling pathways (Jordan and O'Malley 2007; Musgrove and Sutherland 2009; Riggins et al. 2007).

3.1.1 *De novo* tamoxifen resistance

One potential cause of the *de novo* resistance to tamoxifen in ER-positive breast cancer involves the metabolism of tamoxifen. Patients who have defective variants (polymorphism) of cytochrome P450, family 2, subfamily D, polypeptide 6 (CYP2D6) enzyme are unable to transfer tamoxifen to its more active metabolite endoxifen and have a higher risk of recurrence (Beverage et al. 2007; Schroth et al. 2007). It is also likely that impaired expression of some coregulator genes like SRC-1 (known as nuclear receptor coactivator 1, NCOA1) (Berns et al. 1998), AIB1 (known as nuclear receptor coactivator 3, NCOA3) (Osborne et al. 2003) and

nuclear receptor corepressor 1 (NCOR1) (Girault et al. 2003), may be involved in tamoxifen resistance. One mechanism by which ER regulates gene expression is through protein-protein interactions with other transcription factors like activator protein-1 (AP-1) or the nuclear factor κB (NF-κB). Elevated AP-1 or NF-κB transcriptional activity is associated with the *de novo* tamoxifen resistance of human breast tumors (Schiff et al. 2000; Zhou et al. 2007).

Data from breast cancer biopsies indicate that altered expression and/or modification of several growth factor receptors and downstream signaling pathways correlates with de novo tamoxifen resistance. High expression and/or activation of EGFR, the insulin-like growth factor 1 receptor (IGF1R) and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma-derived oncogene homolog (avian) (ERBB2, also known as HER2) can confer resistance by the activation of phosphoinositide-3-kinase (PI3K), p38 (mitogen-activated protein kinase 4, MAPK14), p21 protein (Cdc42/Rac)-activated kinase 1 (Pak1), PI3K/v-akt murine thymoma viral oncogene homolog 1 (AKT) pathways, and the cell proliferation pathway mediated by ERK1/2 (mitogen-activated protein kinase 3/mitogen-activated protein kinase 1, MAPK3/1) (Arpino et al. 2004; Dowsett et al. 2005; Gee et al. 2005; Holm et al. 2006; Kirkegaard et al. 2005). Intrinsic resistance to tamoxifen can also be found in a subset of ER-positive breast tumors that are PgR-negative, ERBB2-positive and have an increased expression of coactivator NCOA3 (Osborne et al. 2003; Shou et al. 2004).

3.1.2 Acquired tamoxifen resistance

Acquired resistance occurs in a significant number of patients in the adjuvant setting and is inevitable in metastatic breast cancer. Acquired resistance develops for all three classes of endocrine treatment compounds; SERMs, SERDs and aromatase inhibitors, but the *in vivo* data of acquired resistance comes solely from tamoxifen studies. *In vivo* studies have implicated the loss of ER expression or ER mutations as potential mechanisms of acquired resistance. However, the loss of ER expression has been demonstrated in only 17-28 percent of patients during the acquisition of tamoxifen resistance (Gutierrez et al. 2005; Johnston et al. 1995), and less than one

percent of ER-positive tumors have ER mutations. Elevated AP-1 transcriptional activity is also associated with acquired tamoxifen resistance in human breast tumors (Johnston et al. 1999). Activated growth factor pathways are involved in the development of resistant tumors. EGFR/ERBB2 signaling is modestly increased in acquired tamoxifen resistance (Gee et al. 2005). Similarly, an increased expression of ERBB2 (emergence of ERBB2 positivity), together with an enhanced expression of phosphorylated MAPK14, was observed in tumors with acquired tamoxifen resistance (Dowsett et al. 2005; Gutierrez et al. 2005).

3.2 Therapy for endocrine-resistant breast cancer

The availability of ER-positive breast cancer cells and the development of cell culture models of endocrine resistance have been essential in the development of treatments for endocrine-resistant breast cancer. The interaction of ER and growth factor receptor pathways is currently a major focus of research and treatment of these cancers. There are on-going preclinical and clinical trials investigating the inhibition of growth factor receptors ERBB2, IGF-1R and EGFR, and their downstream signaling pathways MAPK and PI3K/AKT/mTOR (Giuliano et al. 2011).

In ER-positive/ERBB2 endocrine-resistant breast cancer, the addition of the anti-ERBB2 monoclonal antibody trastuzumab to anastrozole versus anastrozole alone (Kaufman et al. 2009), or combined letrozole plus tyrosine kinase inhibitor labatinib, versus letrozole alone (Schwartzberg et al. 2010), have shown a significantly improved progression-free survival in the combination arm. There are on-going trials investigating the EGFR pathway after acquired resistance. These studies compare antihormone treatments such as tamoxifen or AIs, with and without EGFR inhibitors gefinitib and erlotinib (Cristofanilli et al. 2010; Osborne et al. 2011). Also, targeting the AKT/mTOR pathway with combined AI and the mTOR inhibitor everolimus or temsirolimus has shown significant benefit in comparison to AI alone (LoRusso 2013).

4. MCF-7 cell culture models of estrogen deprivation and acquired resistance to endocrine therapy

The lack of tumor tissue for detailed studies of acquired resistance to endocrine therapy is a major hindrance for *in vivo* studies. It is difficult to obtain paired tumor tissues for biomarker studies immediately before treatment and again at the time that resistance develops. This type of study will be crucial to elicit which pathways become activated during the development of endocrine resistance. At the moment, studies of acquired resistance to SERMs and aromatase inhibitors emerge from breast cancer cell culture models. In these models, cells that are estrogen-independent and/or resistant to different forms of endocrine therapies have been established from hormone-dependent parental cells by long-term culture (Dowsett et al. 2005; Musgrove and Sutherland 2009). In these studies, the most often used cell line is the ER-positive MCF-7 breast cancer cell line. The proliferation of MCF-7 cells is highly dependent on estrogen and it expresses functional ER and PgR, which makes it a good model for *in vitro* studies of endocrine resistance. Other ER-positive cell lines, T47D, ZR75-1, MDA-361 and HCC-1428 have also been used to study ER-mediated response and endocrine resistance (Levenson and Jordan 1997; Miller et al. 2010; Wong and Chen 2012).

4.1 Cell models for acquired resistance to aromatase inhibitors

The mechanism of acquired resistance to aromatase inhibitors (estrogen deprivation) has been studied primarily with MCF-7 breast cancer cells. Cell culture models have been designed to mimic surgical oophorectomy in premenopausal women and the use of aromatase inhibitors in postmenopausal women with advanced breast cancer. Surgical oophorectomy lowers estrogen levels of approximately 200 pg/ml to 10

pg/ml (20-fold). Secondary therapy with aromatase inhibitors lower estrogen levels further tenfold to 1-2 pg/ml (Santen et al. 1990). In the most common model, MCF-7 cells have been grown in long-term estrogen deprivation (LTED) *in vitro*. In these conditions, after several weeks of reduced growth or quiescence, the LTED cells start to grow and reach a growth rate similar to the estrogen-treated original cells. This means that the proliferation of LTED cells has become estrogen-independent. However, the cells may not be fully estrogen-independent, as it has been shown that the LTED cells can become hypersensitive to estrogen possibly due to residual estrogen within the growth medium. Thus, in the clinical setting, tumor cells may become growth-stimulated by the low estrogen levels induced by LTED, which could mean that functional ER signaling remains in these cells. Estrogen hypersensitivity is characterized by the ability of LTED cells to respond to levels of estrogen at concentrations 2-3 log lower than what is required to stimulate wild-type cells (Chan et al. 2002; Masamura et al. 1995; Yue et al. 2002).

4.1.1 The role of estrogen receptor in LTED

Estrogen receptor has a pivotal role in the estrogen-mediated growth of ER-positive breast cancer cells. Because of this, it has been interesting to study possible changes in the expression level and activity of ER in the process of LTED. The *in vitro* studies have shown that both the mRNA and protein levels of ER are frequently increased (around two- to tenfold) in MCF-7 cells (Brunner et al. 1993; Chan et al. 2002; Clarke et al. 1989; Jeng et al. 1998; Katzenellenbogen et al. 1987; Martin et al. 2003; Martin et al. 2005b; Welshons and Jordan 1987) or ZR-75-1 and T-47D (Daly and Darbre 1990) cells that have adapted to grow without estrogen. Some of these studies have also assessed the expression of PgR. The protein expression of PgR was found to be low in LTED cells, but inducible by estrogen, implying that the ER is still functional in these cells (Brunner et al. 1993; Clarke et al. 1989; Jeng et al. 1998; Katzenellenbogen et al. 1987; Welshons and Jordan 1987).

The functionality of ER in LTED MCF-7 cells has also been studied with ER transcriptional reporter assays. These studies have reported substantially increased basal ER transcriptional activity in LTED cells in comparison to wild-type cells

(Jeng et al. 1998; Jeng et al. 2000; Martin et al. 2005b; Martin et al. 2011; Miller et al. 2010). One way to activate ER involves phosphorylation of ER at residue ser118. This is the major site of estrogen-induced phosphorylation of ER, and studies have reported hyperphosphorylation of ER ser118 in LTED cells (Chan et al. 2002; Martin et al. 2003; Martin et al. 2005a; Martin et al. 2011). Altogether, these studies indicate that the expression of ER and its basal activity are induced in LTED cells, possibly to circumvent low estrogen conditions.

4.1.2 The role of growth factor receptors and signaling pathways in LTED

It is evident that various growth factor signaling kinases can cross-talk with nuclear ER by phosphorylating its key regulatory sites like Ser118 and Ser167. It is conceivable that this ER phosphorylation could enhance ER transcriptional activity in a ligand-independent manner and lead to estrogen-independent growth (Martin et al. 2003). Several studies have attempted to assess signaling pathways that could possibly be involved in estrogen hypersensitivity or the estrogen-independent growth of LTED MCF-7 cells. It has been shown that growth factor receptors like ERBB2, EGFR, insulin receptor (INSR) and IGF1R and their downstream signaling pathways MAPK and PI3K/AKT have been implicated in resistance to estrogen deprivation.

Elevated levels of activated MAPK have been detected in MCF-7 LTED cells (Jeng et al. 2000; Martin et al. 2003; Martin et al. 2011; Yue et al. 2002), and increased expression and phosphorylation of ERBB2 was observed together with elevated MAPK activity. This implies that enhanced ERBB2 could result in elevated MAPK activity (Martin et al. 2003). However, it was also shown that the suppression of MAPK was unable to block the elevated basal activity of ER (Jeng et al. 2000) or ER ser118 phosphorylation (Martin et al. 2003), indicating that MAPK activation is not an exclusive mechanism for estrogen hypersensitivity, but that other signal pathways might also be involved.

In addition to the MAPK pathway, estrogen independency is also characterized by increased PI3K activity in LTED MCF-7 cells (Martin et al. 2003; Miller et al.

2010; Sanchez et al. 2011; Yue et al. 2003). In these studies, increased phosphorylation of PI3K substrate AKT and mammalian target of rapamycin (serine/threonine kinase) (mTOR) substrate p70S6K (ribosomal protein S6 kinase, 70kDa, polypeptide 1, RPS6KB1) has been detected in LTED MCF-7 cells, indicating a hyperactivation of the PI3K/AKT/mTOR pathway (Miller et al. 2010; Sanchez et al. 2011; Yue et al. 2003). In two recent studies, it was shown that INSR and IGF1R are activated (phosphorylated) in MCF-7 LTED cells (Miller et al. 2010), and that INSR and IGF1R are required for estrogen-independent cell growth (Fox et al. 2011). Furthermore, an increased expression of IGF1R has been shown in MCF-7 LTED cells (Martin et al. 2005b). These studies together suggest that acquired hormone-independent growth could be associated with hyperactivation of the IGF1R/INSR/PI3K/mTOR pathway, in addition to MAPK. In fact, it has been shown that the dual blockage of both MAPK and PI3K completely reversed estrogen hypersensitivity in LTED cells, which further implicates the importance of both of these signaling pathways in LTED (Yue et al. 2003).

4.1.3 Microarray studies of LTED in MCF-7 cells

The gene-expression changes implicated in LTED have been studied with microarrays in MCF-7 cells, providing various gene expression signatures. In the study by Sadler et al. (2009) the progression of LTED was monitored over the time period of a year. Alterations in gene expression were greatest in the early weeks, but were still observed at the last time points up to 55 weeks. Forty eight percent of the genes that were estrogen-regulated in the parental cells retained this responsiveness during LTED (Sadler et al. 2009). Similarly, in another study, wild-type MCF-7 and LTED cells responded to estrogen with a similar degree of certain genes, and with divergent responses of other genes (Santen et al. 2005). In a recent study (Weigel et al. 2012), temporal changes in gene expression were assessed during the adaptation of MCF-7 cells to LTED. The platelet-derived growth factor/c-abl oncogene 1, non-receptor tyrosine kinase (PDGF/Abl) pathway was significantly elevated as early as one week post-estrogen-deprivation. It was concluded that the PDGF/Abl pathway could be a novel therapeutic target for overcoming endocrine resistance. The expression profiles of LTED cells have also revealed a signature of v-myc

myelocytomatosis viral oncogene homolog (avian) (MYC) activation. It was concluded that MYC signaling may substitute for estrogen-induced ER signaling in a subset of breast cancers (Miller et al. 2011).

4.1.4 Aromatase-transfected MCF-7 cell models of acquired aromatase inhibitor resistance

Breast cancer is also characterized by an overexpression and increased activity of aromatase enzyme (Bulun et al. 1993; Esteban et al. 1992; Harada 1997; Miller and O'Neill 1987). MCF-7 cells express aromatase at very low levels. To engineer an aromatase-positive breast cancer cell model, an aromatase overexpressing (stably) MCF-7 cell line was created (MCF-7aro) (Zhou et al. 1990). MCF-7aro cells were subsequently cultured long-term in the presence of testosterone (as a substrate for aromatase) and tamoxifen to get acquired tamoxifen-resistant cells, and without hormones to get acquired aromatase inhibitor-resistant cells (LTEDaro). Alternatively, aromatase inhibitor-resistant cells were also obtained by the long-term culture of MCF-7aro cells in the presence of testosterone and an aromatase inhibitor (anastrozole, letrozole or exemestane) (Masri et al. 2008). Microarray analysis has been performed with these cell lines. Results showed that gene signatures unique to aromatase inhibitor resistance are clearly different from LTEDaro and tamoxifen-resistant gene signatures. An interesting finding was that LTEDaro, and letrozole- and anastrozole-resistant cells contained a constitutively active ER that does not require estrogen for activation. This ligand-independent activation of ER was not observed in MCF-7aro, and tamoxifen- or exemestane-resistant cells (Masri et al. 2008).

Two additional aromatase inhibitor-resistant cell models have been described from MCF-7aro cells by other groups. The UMB-1Ca cell line is an LTED model for acquired aromatase inhibitor resistance. MCF-7aro cells were cultured long-term in an estrogen-free medium until cells acquired the ability to grow in the absence of estrogen. UMB-1Ca cells were characterized by increased protein levels of EGFR2, ER and increased levels of phosphorylated AKT (Sabnis et al. 2005). A long-term letrozole-treated cell line, modeling acquired letrozole resistance (LTLT-Ca), was also generated from the MCF-7aro cells. The MCF-7aro cells were injected into

ovariectomized mice, which were then treated with letrozole for 56 weeks. Subsequently, cells were then extracted from this tumor xenograft and cultured *in vitro*. This LTLT-Ca cell line was characterized by an increased protein expression of growth factor receptor pathways, in particular ERBB2 and MAPK (Jelovac et al. 2005).

4.2 Cell culture models for acquired tamoxifen resistance

Similar to the LTED studies, the development of acquired antiestrogen resistance has been studied primarily with the MCF-7 breast cancer cell line. The resistant cell lines have been established by long-term culture in the presence of SERM and in the absence of estrogen. The SERM used in these studies has been almost solely tamoxifen. Culturing the estrogen-dependent cells in estrogen-deprived conditions and with tamoxifen mimics the tamoxifen treatment of the hormone-responsive breast cancer in the clinic: The patients are postmenopausal, which means that the resistance to tamoxifen has been developed in estrogen-deprived conditions. The molecules and pathways implicated in antiestrogen (tamoxifen) resistance *in vitro* have been extensively reviewed by other authors (Musgrove and Sutherland 2009; Riggins et al. 2007; Ring and Dowsett 2004).

4.2.1 The role of estrogen receptor in acquired tamoxifen resistance

Several studies have shown that ER expression is equal or lower in tamoxifen-resistant MCF-7 cells when compared to wild-type cells (Badia et al. 2000; Brunner et al. 1993; Herman and Katzenellenbogen 1996; Knowlden et al. 2003; Lykkesfeldt et al. 1994; Treeck et al. 2004; Vendrell et al. 2005). It has been shown in transcriptional reporter assays that ER is functional in tamoxifen-resistant cells although its transcriptional activity can be altered or suppressed in these cells. An example of this phenomenon is changes in the regulation of the known estrogen-induced genes PgR and pS2 (trefoil factor 1, TFF1). The loss of PgR induction by estrogen is frequently observed, but at the same time the estrogen induction of pS2

is retained in these cells (Badia et al. 2000; Herman and Katzenellenbogen 1996; Vendrell et al. 2005; Zhou et al. 2012).

4.2.2 The role of growth factor receptors and signaling pathways in acquired tamoxifen resistance

Studies on the expression and activation of growth factor receptors and their downstream signaling pathways have yielded variable results in MCF-7 cells with acquired tamoxifen resistance. Several studies have reported that the long-term treatment of MCF-7 cells with tamoxifen does not change the mRNA or protein expression of ERBB2 in these cells (de Cremoux et al. 2003; Fan et al. 2007; Larsen et al. 1999; Treeck et al. 2004; Vendrell et al. 2005). Similarly, no change in the expression of EGFR was observed in tamoxifen-resistant cells (de Cremoux et al. 2003; Fan et al. 2007; Larsen et al. 1999; Treeck et al. 2004). On the other hand, increased expression and especially phosphorylation (activation) of EGFR and ERBB2 has been observed in some studies (de Cremoux et al. 2003; Fan et al. 2006; Knowlden et al. 2003; Vendrell et al. 2005). Similar to this, MAPK or PI3K/AKT activation has not been observed in one study (Fan et al. 2007), but in other studies an increased activation of PI3K/AKT (Jordan et al. 2004) and MAPK (Knowlden et al. 2003; Vendrell et al. 2005) has been detected in the acquired tamoxifen resistance of MCF-7 cells. It has also been observed that tamoxifen-resistant cells are more sensitive (growth inhibited) to EGFR- and ERBB2-specific inhibitors than parental control cells (Fan et al. 2006; Fan et al. 2007; Knowlden et al. 2003). It has been proposed that long-term tamoxifen exposure causes the translocation of ER to the cytoplasm, and that ER-EGFR interaction is enhanced without increases in the expression of these receptors. This nongenomic function of ER via co-operation with EGFR could be one of the mechanisms responsible for acquired tamoxifen resistance (Fan et al. 2007; Yue et al. 2007). Taken together, these studies show that enhanced signaling via EGFR/ERBB2 growth factor receptors and their downstream signaling pathways MAPK and PI3K/AKT may play an important role in tamoxifen resistance.

Studies concerning the role of IGF1R signaling in tamoxifen resistance have also been variable. It has been shown that tamoxifen-resistant MCF-7 cells have lower

levels of IGF1R than their wild-type MCF-7 counterparts (Brockdorff et al. 2003; Fagan et al. 2012; Knowlden et al. 2005; McCotter et al. 1996), but the level of phosphorylated IGF1R is equivalent to wild-type and resistant cells (Knowlden et al. 2005). It has also been shown that the selective IGF1R inhibitor AG1024 and the anti-IGF1R monoclonal antibody alphaIR-3 both block the growth of tamoxifen-resistant MCF-7 cell variants (Jones et al. 2004; Parisot et al. 1999). On the other hand, in a recent study the IGF1R antibody dalotuzumab inhibited the growth of parental cells but not the growth of tamoxifen-resistant cells (Fagan et al. 2012). Nevertheless, it has been postulated that the role of IGF1R signaling in the acquired tamoxifen resistance of MCF-7 cells could be mediated in part through crosstalk with EGFR or could involve the scaffolding molecule insulin receptor substrate 1 (Cesarone et al. 2006; Knowlden et al. 2005).

4.2.3 Microarray studies of acquired tamoxifen resistance in MCF-7 cells

Microarrays have also been used to assess the acquisition of tamoxifen resistance in MCF-7 cells. In these studies, acquired tamoxifen resistance is associated with the downregulation of pro-apoptotic genes and the upregulation of genes coding for apoptosis inhibitors (Scott et al. 2007; Treeck et al. 2004). There is no change in the expression of ER, but changes in ER transcriptional coregulators have been observed (Scott et al. 2007; Vendrell et al. 2005). The estrogen regulation of genes has also been compared between wild-type MCF-7 cells and their tamoxifen-resistant subline. An ER-positive phenotype was maintained in tamoxifen-resistant cells, but they had altered the estrogen regulation of a cohort of genes that were estrogen-regulated in wild-type MCF-7 cells. In some of these genes, altered promoter DNA methylation profiles were observed, which could explain both the upregulation (promoter hypomethylation) and downregulation (promoter hypermethylation) of genes (Fan et al. 2006).

5. MCF-7 cell models of *de novo* estrogen independency and tamoxifen resistance

Most ER+ breast cancer patients respond well to ER-targeted therapies. However, approximately 30 percent of ER-positive breast cancers do not respond to tamoxifen therapy and are considered to be *de novo* resistant to tamoxifen (Riggins et al. 2007). In clinical tumor samples, ERBB2 overexpression and increased ERBB2/EGFR or IGF1R signaling has been shown to positively correlate with tamoxifen resistance (Arpino et al. 2004; Dowsett et al. 2005; Gee et al. 2005). Also, high AKT activation is a marker of possible *de novo* resistance to tamoxifen (Kirkegaard et al. 2005). MCF-7 cells have been transfected to overexpress these growth factor receptors in order to mimic the *in vivo* intrinsic resistance to endocrine therapy. Transfection of MCF-7 cells with ERBB2 has been shown to promote estrogen independence and tamoxifen resistance. ERBB2 transfection was shown to cause ligand-independent activation (phosphorylation) of ER and subsequent induction of estrogen regulated PgR protein (Pietras et al. 1995), and the hyperactivation of MAPK (Kurokawa et al. 2000). The transfection of MCF-7 cells with EGFR (Miller et al. 1994) or IGF1R (Guvakova and Surmacz 1997) has been shown to reduce estrogen dependency of the cells. In one study, transfection of MCF-7 cells with ERBB2 or EGFR caused the hyperactivation of MAPK which induced a loss of ER expression (Oh et al. 2001). MCF-7 cells have also been transfected to overexpress constantly activated AKT. It induced ER transcriptional reporter activity without estrogen (Kurokawa and Arteaga 2003), or activated ER in the absence of estrogen by phosphorylating ER ser-167 (Campbell et al. 2001). Furthermore, in these studies AKT overexpression conferred tamoxifen resistance of MCF-7 cells.

Aims of the study

The aims of the present study were:

1. To find genes which are estrogen- and progestin-regulated, and to assess the role of these genes in acquired estrogen-independent growth and the acquired progestin-resistant growth of breast cancer cells (I)
2. To find genes which are implicated in the development of acquired estrogen independency and acquired antiestrogen resistance in breast cancer cells (II)
3. To ascertain whether the overexpression of fos-like antigen-1 (FOSL1) could account for the acquired antiestrogen-resistant growth of breast cancer cells (III)
4. To study the effect of different steroid receptor ligands on the growth of estrogen-independent and antiestrogen-resistant breast cancer cells (IV)

6. Materials and methods

6.1 Cell culture (I-IV)

MCF-7 cells and the MCF-7-derived sublines were routinely cultured in 75 cm^2 flasks at 37 degrees Celsius in a humidified atmosphere of five percent CO2/95 percent air. The cells were maintained in phenol red-free Dulbecco´s Modified Eagle´s Medium/Nutrient Mixture F-12 Ham (DMEM/F-12; Sigma, St. Louis, MO, USA), supplemented with five percent dextran charcoal-treated foetal bovine serum (DCC-FBS; Gibco/BRL, Invitrogen Life Technologies, Gaithesburg, MD, USA), penicillin (100 IU/ml)/streptomycin (100µg/ml) (Gibco) and insulin (10 ng/ml) (Gibco). The cell culture media with appropriate hormones were changed every two or three days.

The establishment of medroxyprogesterone acetate-resistant (MR), long-term estrogen-treated (LE), estrogen-independent (EI) and estrogen-independent and toremifene-resistant (TR) sublines has been described previously (Sarvilinna et al. 2006). Briefly, estrogen and antiestrogen-sensitive, estrogen- and progesterone receptor-positive MCF-7 human breast cancer cells were used as parent cells to generate these sublines. MR cells received 1 nM estrogen (Sigma) + 100 nM medroxyprogesterone acetate (MPA) (Sigma), LE cells received vehicle (96 percent ethanol) + 1 nM estrogen, TR cells received 1 µM toremifene citrate (provided by Orion Pharma (Turku, Finland)) and EI cells received vehicle only continuously for nine months.

In the first study (I), steroid hormones (diluted in absolute ethanol) were used at the following concentrations: 1 nM E_2, 10 nM MPA, 10 nM R5020 (Schering

Aktiengesellschaft, Berlin, Germany), 10 nM dihydrotestosterone, 10 nM progesterone (Merck, Darmstadt, Germany), and 10 nM dexamethasone (Sigma). Cycloheximide (CX) (Sigma) was used at a 10 µg/ml concentration (diluted in absolute ethanol). In the cDNA microarray experiments, MCF-7 cells that were routinely grown in the presence of E_2, were compared with cells grown without E_2 for 72 hours to reveal E_2-regulated genes. The cells that were grown in the presence of E_2 were treated with MPA for six or 24 hours or absolute ethanol (vehicle) only to reveal MPA-regulated genes. For the real-time RT-PCR experiments, E_2-deprived (for 72 hours) MCF-7 cells were subsequently treated with E_2 for 72 hours. The cells that were routinely grown in the presence of E_2 were treated with MPA, progesterone, R5020, dexamethazone, dihydrotestosterone or vehicle for indicated time. In the CX experiment, cells that were grown in the presence of E_2 were treated with CX for one hour before addition of MPA or vehicle for six hours.

In the second study (II), for the cDNA microarray and RT-PCR experiments, LE, TR and EI cells were plated on the flasks and grown with appropriate hormones for one passage. After reseeding, the LE and TR cells were grown without hormones, and EI cells without vehicle, for six days in order to eliminate any residual hormonal effects on the gene expression. For the RT-PCR experiments, pMCF-7, LE, TR and EI cells were also grown for one week with the appropriate hormones. In the third study (III), the cells were treated as indicated in the RNA interference and automated pattern analysis by machine vision system -sections below.

In the fourth study (IV), the following hormones (concentrations) were used in the cell growth assay: TAM (Orion Pharma) (0.1 µM and 1 µM), MPA (0.1 nM, 1nM and 10 nM), retinol (Sigma) (0.1 µM, 1 µM and 10 µM), calcitriol (Leo Pharmaceuticals, Denmark) (1 nM, 10 nM and 100 nM) and DPN (Orion Pharma) (1 nM, 10 nM and 100 nM). The treatments were done both in the presence and absence of 1 nM E_2.

6.2 Cell growth assay (I-IV)

The cells were seeded to 96-well plates 1000 cells/well and allowed to attach for 24 hours. The cells were subsequently treated with the indicated drugs for six (IV) or seven (I-III) days. Growth medium and drugs were renewed every other day. After six or seven days treatment the cells were fixed, stained and the relative number of cells was assessed with a modification of the crystal violet staining method by Kueng et al (Kueng et al. 1989). Briefly, cells were fixed with one percent glutaraldehyde and stained by an addition of 0.1 percent solution of crystal violet on the plates. The bound dye was dissolved to 100 µl of 10 percent acetic acid on the plates. Absorbances from each well were measured at 590 nm wavelength using a Victor 1420 Multilabel Counter (Wallac, Turku, Finland).

6.3 cDNA microarray (I, II)

In the first study (I), cDNA was synthesized and 33P dCTP labelled (10 mCi/ml with a specific activity of 3000 Ci/mmol) (Amersham Pharmacia Biotech, Uppsala, Sweden) using 1 µg total RNA from the MCF-7 cells treated with the appropriate hormones. The integrity of the RNA samples was assessed by electrophoresis on a denaturing one percent agarose gel, and the concentration and purity of the RNA was determined using spectrophotometer (GeneQuant II, Pharmacia Biotech Ltd., England). Release I of the Human GeneFilters (GF200 I, Research Genetics, Huntsville, AL, USA) was used for differential expression screening. After hybridisation (for 16 hours) the filter was exposed to a high resolution screen (Storage Phosphor Screen, Molecular Dynamics, CA, USA) for 20 hours. The screen was analysed with a phosphor imager (PhosphorImager SI, Molecular Dynamics) at a resolution of 100 µm using Image Quant software (Molecular Dynamics). The image from the phosphor imager was further analysed using Pathways software (Research Genetics). The experiment was strictly performed according to standardized protocols from the manufacturer (Research genetics).

In the second study (II), an Atlas Human Cancer 1.2 Array (Clontech, CA, USA) containing 1176 cancer-related genes was used to study the basic gene expression

differences between LE, TR and EI cells. The integrity of the RNA samples was assessed by electrophoresis on a denaturing one percent agarose gel, and the concentration and purity of the RNA was determined using a spectrophotometer (GeneQuant II, Pharmacia Biotech Ltd.). The microarray experiments were done according to the manufacturer's protocol. Briefly, 50 µg of total RNA from each sample was reverse-transcribed to [α-33P]dATP –labeled cDNA with the Atlas Pure Total RNA Labeling System. The radiolabelled cDNAs were hybridized to the array membranes overnight, and a phosphorimaging screen (Storage Phosphor Screen, Molecular Dynamics) was exposed with the membranes overnight. The phosphorimaging screen was scanned with a Storm Phosphorimager (Molecular Dynamics) at a resolution of 100 µm. Imagequant files from these scans were analyzed using ArrayVision software (Imaging Research, St. Catharines, Ontario, Canada). A template, which contains the spot layout of the array, was overlaid on the phosphorimage, and the pixel intensity of each spot on the array was determined. Spot intensities were background subtracted and globally normalized.

6.4 Real-time RT-PCR (I, II, III)

In the first study (I), one-step real-time RT-PCR was performed with a LightCycler instrument (Roche, Basel, Switzerland) in a total volume of 20 µl containing 100 ng of total RNA, 3.25 mM Mn(oAc)2 and 0.5 µM of each primer. The LightCycler RNA Master SYBR Green I kit (Roche) was used. Reverse transcription was performed for 20 minutes at 61 degrees Celsius and denaturation for two minutes at 95 degrees Celsius. Forty-two PCR cycles were run. Each PCR-cycle included denaturation at 95 degrees Celsius, five seconds of primer annealing at 55 degrees Celsius and extension (extension time dependent on the length of the product) at 72 degrees Celsius. After amplification, the specificity of the PCR product was verified by melting curve analysis. Analysis of the LightCycler data was performed employing LightCycler analysis software (Roche).

In the second (II) and third (III) studies, a two-step real-time RT-PCR was performed. The RNA samples were reverse-transcribed to cDNA with a High Capacity Archive Kit (Applied Biosystems, CA, USA) following the manufacturer's

instructions. Real-time RT-PCR was done with a SYBR Green PCR Master Mix Kit in an ABI PRISM 7000 Detection System according to the manufacturer's instructions (Applied Biosystems). The following PCR conditions were used: denaturation at 95 degrees Celsius for 10 minutes followed by 40-50 cycles at 95 degrees Celsius for 15 seconds (denaturation) and 60 degrees Celsius for one minute (elongation). The data was analyzed by ABI PRISM 7000 SDS Software (Applied Biosystems).

The final real-time RT-PCR results, expressed as N-fold relative differences (ratio) in gene expression between the studied samples and the control (or 'calibrator') sample, were calculated according to the following equation (Pfaffl 2001): Ratio = $((E_{target})^{\Delta CP\ target\ (control-sample)})/((E_{ref})^{\Delta CP\ ref\ (control-sample)})$. E_{target} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCP_{target} is the CP (crossing point) deviation of control – sample (subtraction) of the target gene transcript; ΔCP_{ref} is the CP deviation of control – sample of the reference gene (RPLP0; ribosomal protein, large, P0) transcript. Real-time PCR efficiencies (E) were calculated according to $E = 10^{[-1/slope]}$.

6.5 RNA interference (III)

The small interfering RNA method was used to knock down FOSL1 mRNA in TR cells. SiRNA SMARTpool® reagents were from Dharmacon (Lafayette, CO, USA), including FOSL1 siRNA, Non-Targeting siRNA, 5X siRNA buffer and DharmaFECT®1 Transfection Reagent. The TR cells were seeded to 25 cm^2 flasks 1 x 10^5 cells/flask and allowed to attach for 24 hours. For transfection, the cells were then subjected to 50 nM target siRNA (FOSL1), 50 nM Non-target siRNA (siRNA control), transfection reagent (Mock transfection without siRNA), or medium only for 24 hours according to the manufacturer's instructions. After transfection, the cells were cultured in a normal growth medium for 24-72 hours (for subsequent RT-PCR analysis) or 72 hours (for subsequent Western blot analysis) and then subjected to Trizol (GIBCO) reagent for RNA extraction or M-PER® (PIERCE) reagent for protein extraction. For the cell growth studies, the cells were seeded to 96-well

plates 1 x 10^3 cells/well and allowed to attach for 24 hours. Next, the cells were transfected for 24 hours with the indicated siRNA concentrations. The transfection medium was then replaced by a normal growth medium in the presence or absence of 1 µM toremifene. The medium was renewed every second day. The cells were fixed, stained and the relative number of cells was counted every 24 hours using the crystal violet staining method as described by Kueng et al. (Kueng et al. 1989). Absorbance was measured at 590 nm wavelength using a Victor 1420 Multilabel counter (Wallac Inc., Turku, Finland).

6.6 SDS-PAGE and Western blot (III)

Cells were subjected to M-PER® (PIERCE, Rockford, IL, USA) reagent modified with protease inhibitors (Complete Mini Protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Indianapolis, IN, USA)) for protein extraction according to the manufacturer's instructions. Total protein concentrations were measured using a BCA Protein Assay Kit (PIERCE) according to the manufacturer's instructions. One hundred µg of total protein was mixed with two percent of sodium dodecyl sulfate (SDS) buffer, boiled for five minutes and analyzed by electrophoresis in 12 percent polyacrylamide gel (PAGE). Proteins separated by PAGE were transferred (two hours) to the nitrocellulose membrane (0.45µm pore, Schleicher and Schuell, Germany) at room temperature (RT) using a transfer buffer containing 25mM Tris, 192mM glycine and 20 percent methanol, pH 8.3. Membranes were incubated for one hour at RT in a Tris buffer containing salt and Tween (TBST) (50mM Tris-HCL, 150mM NaCL, 0,05 percent Tween 20, pH 8.0) and five percent non-fat dry milk powder to saturate the non-specific protein binding sites. Membranes were then incubated with FOSL1 primary antibody (Fra-1, C-12) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:10000 in five percent BSA-TBST overnight at four degrees Celsius with mild agitation. The membranes were washed three times for 10 minutes with TBST and incubated for one hour with horse radish peroxidase (HRP) -conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology Inc.) in 1:4000 dilution in five percent non-fat milk-TBST with mild agitation at RT. The membranes were washed three times for 10 minutes with TBST and subjected to enhanced chemiluminescence reagents (ECL,

UK) according to the manufacturer's instructions and exposed to X-ray film. 3611-RF nuclear extract (Santa Cruz Biotechnology Inc.) was used as a positive control for FOSL1 primary antibody, and anti-beta-actin (AC-15) (Sigma) was used as a loading control.

6.7 Automated pattern analysis by machine vision system (III)

The automated cell culturing and pattern analysis platform (Cell-IQ, Chip-Man Technologies, Tampere, Finland) was used to measure cell growth and to analyze changes in cell morphology in the siRNA experiment. The Cell-IQ system consists of a special cell culture incubator with an inbuilt microscope and camera system. The system and its applications have been described previously (Toimela et al. 2008). In the siRNA experiment, the cells were seeded to 24 well plates 4 x 103 cells/well and allowed to attach for 24 hours. For transfection, the cells were then subjected to 10 nM target siRNA (FOSL1), 10 nM Non-target siRNA (siRNA control), transfection reagent (Mock transfection without siRNA), or a medium only for 24 hours. The transfection medium was then replaced by normal growth medium (1000 µl/well). The 24-well plate was then placed in the incubator and the automated monitoring and image capturing were continued for seven days. The growth medium was not changed during this time. The experiment was carried out in the absence of toremifene. There were four parallel wells for each treatment, and three parallel images were taken at random spots from every well, every 15 minutes. The resultant captured images were analyzed with the Cell-IQ software. In this experiment, the analyzer was taught to detect stable, dead and vacuolated cells.

6.8 Statistical analysis

All quantitative data are expressed as geometric mean ± geometric standard error. Comparisons were made by the Mann-Whitney U-test and $p<0.05$ was considered as a statistically significant difference.

7. Results

7.1 Estrogen- and progestin-regulated genes in MCF-7 cells

The cDNA microarray experiment revealed four genes that were previously reported (by other authors) to be estrogen- and/or progestin regulated in human breast cancer cells. Those genes included S 100 calcium binding protein P (S100P), FK506 binding protein 5 (FKBP54), neuropeptide Y receptor Y1 (NPY1R) and discs, large (Drosophila) homolog 5 (DLG5). The regulation pattern of these genes in our study was consistent with the other studies. In addition to these, we found two novel medroxyprogesterone acetate (MPA) and/or estrogen-regulated genes in MCF-7 cells: zinc finger protein 36, C3H type-like 1 (ZFP36L1) and protein tyrosine phosphatase type IVA, member 1 (PTP4A1). The expression of ZFP36L1 mRNA was upregulated by MPA, whereas the expression of PTP4A1 was upregulated by estrogen and downregulated by MPA. MPA regulation of these genes was dependent on estrogen: When the cells were grown without estrogen, the genes were no longer regulated by MPA.

The induction of ZFP36L1 expression by MPA was already evident after two hours. This indicated that ZFP36L1 could be a direct target of MPA. To study if the MPA regulation of ZFP36L1 and PTP4A1 was a direct effect that does not require *de novo* protein synthesis, MCF-7 cells were treated simultaneously with the protein synthesis inhibitor cycloheximide (CX) and MPA. CX was not able to block the upregulation of ZFP36L1 mRNA by MPA, suggesting that ZFP36L1 was indeed a direct target of MPA. On the contrary, the treatment caused an induction of PTP4A1 expression, indicating that PTP4A1 was not a direct target of MPA action.

In addition to MPA, we studied the effect of progesterone, progesterone agonist (R5020), androgen (dihydrotestosterone, DHT) and glucocorticoid (dexamethasone, DEX) on the regulation of ZFP36L1 and PTP4A1 expression in MCF-7 cells grown in the presence of estrogen. At a 10 nM concentration, all hormones regulated the expression of the genes in a similar way. MPA had the most potent effect, statistically significant regulation was also observed by DEX and R5020.

The estrogen and MPA regulation of the genes was also studied in estrogen-independent (EI), MPA-resistant (MR) and long-term estrogen-treated (LE) MCF-7 sublines. In these cell lines the regulation of ZFP36L1 was not altered, with the exception of LE cells where ZFP36L1 was no longer regulated by combined estrogen and MPA. However, PTP4A1 was downregulated by estrogen in EI cells and upregulated by combined estrogen and MPA in MR cells. This regulation profile of PTP4A1 in endocrine-resistant EI and MR cells was clearly opposite to that observed in MCF-7 or LE cells.

7.2 Gene expression changes in estrogen-independent and antiestrogen-resistant MCF-7 cells

Parental MCF-7 (pMCF-7) cells were grown for nine months in different hormonal environments in order to establish long-term estrogen-treated (LE), estrogen-independent (EI) and estrogen-independent and toremifene-resistant (TR) sublines. The gene expression profiles of pMCF-7, LE, EI and TR cells were compared with cDNA microarray and RT-PCR to find out which genes are implicated in estrogen independency and/or antiestrogen resistance. We observed an altered expression of fos-like antigen-1 (FOSL1), TIMP metallopeptidase inhibitor 1 (TIMP1), L1 cell adhesion molecule (L1CAM) and growth differentiation factor 15 (GDF15) in these cells. The expression of FOSL1 was induced in TR cells. The expression of TIMP1 was downregulated in LE and EI cells, but in TR cells its expression was maintained at the same level as in pMCF-7 cells. The expression of L1CAM was induced in EI cells. L1CAM and GDF15 proved to be downregulated by toremifene in TR cells.

7.2.1 Gene expression changes during the acquisition of estrogen-independent growth and antiestrogen resistance

As discussed above, pMCF-7 cells were grown for nine months in different hormonal environments to establish LE, EI and TR sublines. In order to assess temporal changes in gene expression during the long term culture, we determined the expression levels of FOSL1, TIMP1, L1CAM and GDF15 at four time points during the nine month growth period: pMCF-7 (first time point), two passages from the middle, and from the last passage (late time point) of the sublines. It was shown that the changes in FOSL1, TIMP1 in TR cells and L1CAM expression in EI cells happened early and remained constant during the long-term culture. There were also transient changes in gene expression as GDF15 was upregulated in EI cells during the middle passages, but its expression fell down to the level of pMCF-7 cells in the last passage of EI cells.

7.2.2 Switch of toremifene-activity from estrogen antagonist to agonist in toremifene-resistant cells

The cDNA microarray and subsequent real-time RT-PCR experiments showed that mRNA expression of L1CAM was downregulated in pMCF-7 or LE cells by estrogen, but not by toremifene. Similar to this, L1CAM was not regulated by toremifene in the middle passages of TR cells. Surprisingly, L1CAM became toremifene-downregulated in the last passage of TR cells, as it was estrogen-downregulated in pMCF-7 and LE cells.

7.3 Inhibition of FOSL1 expression in antiestrogen-resistant MCF-7 cells

TR cells overexpress FOSL1 mRNA and protein when compared to EI, LE or pMCF-7 cells. The inhibition of FOSL1 mRNA expression by small interfering RNA (siRNA) downregulated FOSL1 mRNA and protein in TR cells to the level observed in the other sublines. This downregulation drastically inhibited the growth of TR cells and restored the growth-inhibitory effect of toremifene in these cells. After siRNA treatment, an automated pattern analysis machine vision system was

used to observe changes in the morphology of the cells. A growth-inhibition and eventual death of FOSL1-siRNA-treated TR cells was preceded by an increase of cytosolic vesicles, which is characteristic to autophagic cell death.

7.4 The effect of different nuclear receptor ligands on the growth of MCF-7 cells and estrogen-independent and antiestrogen-resistant sublines

We wanted to study how tamoxifen, MPA, 1,25-dihydroxyvitamin D3 (calcitriol), retinol and estrogen receptor-β-selective ligand diarylpropionitrile (DPN) affected the proliferation of TR, EI and pMCF-7 cells. The nuclear receptor ligands were used in concentrations which are close to the observed physiological concentrations of the natural nuclear receptor ligands, or pharmacological concentrations of the synthetic ligands. Treatments were carried out both in the presence and absence of 1 nM estrogen.

ERβ agonist DPN stimulated the growth of pMCF-7, TR and EI cells at the highest 100 nM concentration. The level of growth stimulation by 100 nM DPN was similar to that of 1 nM estrogen in all sublines.

Tamoxifen inhibited the growth of original pMCF-7 cells in the presence of E_2. The growth of EI cells was also inhibited by TAM, but in this case the inhibition was diminished in the presence of E_2. TR cells were highly resistant to TAM. MPA alone inhibited the growth of EI cells, whereas TR cells were resistant to it. In the presence of E_2, the growth inhibitory effect was lost in EI cells.

Calcitriol inhibited the growth of all cell lines at pharmacological 100 nM concentration. In the presence of E_2, the same pattern of inhibition was observed but it did not reach statistical significance in EI and TR cells. Retinol alone inhibited the growth of EI and TR cells, but not pMCF-7 cells. Furthermore, 1 µM retinol stimulated the growth of E_2-deprived pMCF-7 cells. When retinol was administered together with E_2, all cell lines were growth-inhibited, and the inhibition was markedly stronger in comparison retinol alone.

8. Discussion

8.1 Estrogen- and progestin-regulated genes in MCF-7 breast cancer cells and in the endocrine-resistant sublines

cDNA microarrays were applied to identify genes that have altered hormonal regulation or are differentially expressed in parental pMCF-7 cells and in the endocrine-resistant sublines. The cDNA microarrays used in these studies (I, II) were nylon-based filters which allowed us to study a limited number of genes (1000 – 3000 genes). The approach was to find possible novel hormone-regulated genes or genes that might contribute to endocrine resistance, and verify their expressions by another method that measures RNA expression, namely real-time RT-PCR.

In the first study (I) we discovered two novel estrogen and/or progestin (MPA)-regulated genes in MCF-7 breast cancer cells: zinc finger protein 36, C3H type-like 1 (ZFP36L1) and protein tyrosine phosphatase type IVA, member 1 (PTP4A1). The microarray analysis also revealed four genes, which have been previously shown to be estrogen- and/or progestin-regulated in breast cancer cells. These genes included S 100 calcium binding protein P (S100P) (Jacobsen et al. 2002), FK506 binding protein 5 (FKBP54) and neuropeptide Y receptor Y1 (NPY1R) (Wan and Nordeen 2002), and discs, large (drosophila) homolog 5 (DLG5) (Purmonen et al. 2002). The estrogen and progestin-regulation pattern of these genes in our microarray analysis was consistent with the observed regulation in previous studies.

ZFP36L1 is a transcription factor and member of the 12-O-tetradecanoylphorbol-13-acetate (TPA)-inducible sequence 11 (TIS11) family of early response genes (Carrick and Blackshear 2007). Similarly, in our study the expression of ZFP36L1 was induced early (at two hour time-point) by MPA, and it proved to be a direct target of the hormone. There is only one study where ZFP36L1 has been linked to

breast cancer. In this study, where invasive ductal breast carcinomas were grouped into lymph node-negative and positive groups, ZFP36L1 was found to be overexpressed in lymph node-positive tumors (Abba et al. 2007). In our study, ZFP36L1 was downregulated in the estrogen-independent (EI) cells when compared to the long-term estrogen-treated (LE) control cells, whereas in MPA-resistant (MR) cells its expression level was similar to LE cells. The potential role of ZFP36L1 in breast cancer remains unknown. PTP4A1 overexpression has been observed in the transformed phenotype of cells and it might be involved in tumorigenesis (Cates et al. 1996; Diamond et al. 1994; Wang et al. 2002). However, PTP4A1 transcripts were significantly decreased in malignant breast tumors when compared with their paired normal tissues (Dumaual et al. 2012). We observed that PTP4A1 expression was decreased in resistant EI and MR cells when compared to LE cells. This may suggest that resistant EI and MR cells represent less differentiated cancer cells and higher grade tumors.

8.2 Alterations in gene expression in the development of estrogen-independent and antiestrogen-resistant breast cancer

In the second study (II) we assessed the gene expression changes that may contribute to the development of estrogen-independent growth, acquired resistance to antiestrogen treatment and ultimately the development of possible antiestrogen-stimulated growth of breast cancer cells. We showed that the expression of five genes was altered in EI and estrogen-independent and antiestrogen-resistant (TR) cells when compared to estrogen-dependent LE cells. These genes included v-myb myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2), growth differentiation factor 15 (GDF15), fos-like antigen-1 (FOSL1), L1 cell adhesion molecule (L1CAM) and TIMP metallopeptidase inhibitor 1 (TIMP1), all of which have been reported to have a role in breast cancer.

MYBL2 is a transcription factor that belongs to proliferation-associated genes. This group of genes is characterized by genes whose increased expression is correlated with increased proliferation rates of breast cancer cells (Whitfield et al. 2006). In

our study, the expression of MYBL2 was high in estrogen-dependent parental MCF-7 (pMCF-7) and LE cells in the presence of estrogen. In the absence of estrogen (non-proliferating cells), the expression of MYBL2 was diminished in these cells. These results further highlight the role of MYBL2 as a proliferation marker. Transcription factor FOSL1 dimerizes with proteins of JUN family, forming the transcription factor complex AP-1 (Young and Colburn 2006). Crosstalk with other transcription factors like AP-1 is one mechanism by how activated ER can regulate transcription (Dahlman-Wright et al. 2012). A high expression of FOSL1 has been detected in breast epithelioid carcinoma cells (Kustikova et al. 1998; Zajchowski et al. 2001) and in ER-negative breast cancer cell lines (Milde-Langosch et al. 2004; Zajchowski et al. 2001). TIMP1 inhibits the action of metalloproteinases. It has been suggested that TIMP1 expression is induced by FOSL1 in breast cancer cells (Belguise et al. 2005). In our study, the expression of FOSL1 and TIMP1 was induced in TR cells. L1CAM has been shown to be involved in epithelial-mesenchymal transition-like events in MCF-7 cells, where its overexpression increases motility and promotes the scattering of epithelial cells from compact colonies (Shtutman et al. 2006). The expression of L1CAM was induced in EI cells in our study. Finally, it has been shown that the expression of GDF15 is induced by PI3K/AKT in MCF-7 cells conferring antiestrogen resistance (Campbell et al. 2001; Wollmann et al. 2005). In agreement with this, GDF15 expression was induced in the toremifene-resistant TR cells. Altogether, the results of our study further confirm that the expression of these genes could be altered in endocrine-resistant breast cancer.

8.2.1 Evidence of a selection process during the development of resistant cell lines

An altered expression of FOSL1 and TIMP1 was observed in the TR cells, whereas L1CAM expression was upregulated in EI cells. The induction of these genes was evident already in the early passage number of TR or EI cells. When TR and EI were established, there was at first a proliferative quiescence which lasted for approximately two to three months. After this period, the cells reached a constant proliferation rate that was prominent for the remaining time of the establishment process of these cells (Sarvilinna et al. 2006). In the present study, the cells of early

passage number represented the cells that had reached a constant proliferation rate soon after the quiescence. As discussed above, the expression changes were already present in these cells. This could indicate that there was a process during the quiescence period, when those cells that possessed the phenotype required for survival in the new hormonal environment were selected to grow. There are studies which support the conclusion that the MCF-7 cell line is heterogeneous and that selection conditions allow the growth of pre-existing phenotypes (Coser et al. 2009; Leung et al. 2010). In the study by Coser et al. this was proven in a system where MCF-7 cells were treated with tamoxifen for 21 days. The monoclonal sublines that survived were compared with the control sublines that were unselected by tamoxifen. Tamoxifen-selected sublines shared the same genomic DNA aberrations, which were distinct from the control sublines. These genomic DNA aberrations cannot have happened during the 21 days of tamoxifen treatment. So, there must have been a selection of pre-existing tamoxifen-resistant subpopulations, without involving changes in individual cells (Coser et al. 2009).

In addition to the selection of pre-existing clones with different DNA aberrations, epigenetic changes could be involved later in the long-term culture. After a favorable epigenetic event, the selection process follows. Epigenetic regulation involves three mutually interacting events: DNA methylation, histone modifications and chromatin remodeling. DNA methylation has been studied in the tamoxifen resistance of MCF-7 cells (Badia et al. 2007). For example, some proto-oncogenes that are implicated in drug resistance to endocrine therapy, have been found to be upregulated in acquired tamoxifen-resistant MCF-7 breast cancer cells through the hypomethylation of their promoters (Fan et al. 2006). Similarly, DNA hypermethylation and histone acetylation can play a role in acquired tamoxifen resistance in MCF-7 cells. In these studies, many estrogen-regulated genes were not active anymore in tamoxifen-resistant cells, possibly due to hypermethylation and histone acetylation (Fan et al. 2006; Shen et al. 2011).

We have previously shown that the growth of the antiestrogen-resistant TR cells is stimulated by toremifene (Sarvilinna et al. 2006). The results of the present study also suggest that toremifene might function as an estrogen agonist in TR cells. It has been shown previously, that long-term tamoxifen-treated MCF-7 cells exhibit

growth stimulation by this antiestrogen (Herman and Katzenellenbogen 1996; Wiseman et al. 1993). Supporting this hypothesis in our study was the expression pattern of L1CAM and GDF15. Both of these estrogen-regulated genes became, in a similar way, toremifene-regulated, but only in the last passage of TR cells, that is, late in the adaptation process. This could indicate that after prolonged treatment with antiestrogen, the antiestrogen-resistant cells may eventually generate an estrogen-agonistic response to antiestrogen, probably acquiring an additional growth advantage. Thus, epigenetic changes late in the adaptation process cannot be ruled out.

8.3 The role of FOSL1 overexpression in antiestrogen resistant cells

Studies have demonstrated that a high expression of the AP-1 component FOSL1 has a crucial role in malignant transformation, increased motility and invasiveness of breast cancer cell lines (Baan et al. 2010; Belguise et al. 2005; Kustikova et al. 1998; Tkach et al. 2003; Zajchowski et al. 2001). High FOSL1 expression has been shown to correlate with the absence of ER expression and with estrogen-independent growth in breast cancer cell lines (Bamberger et al. 1999; Belguise et al. 2005; Nakajima et al. 2007; Philips et al. 1998). Unlike in these *in vitro* studies, the negative correlation of ER and FOSL1 has not been clearly demonstrated in the *in vivo* studies investigating breast carcinomas ranging from benign disease to aggressive breast carcinomas. There are *in vivo* studies which show that FOSL1 expression correlate negatively with the ER status in breast cancer tissues (Bamberger et al. 1999; Nakajima et al. 2007). Yet, in some studies such correlation has not been demonstrated (Chiappetta et al. 2007; Song et al. 2006). It has been shown that high AP-1 binding activity could be the result of a high FOSL1 expression in breast cancer cells (Philips et al. 1998), and that enhanced AP-1 activity may bypass hormone dependence and/or associate with an agonistic tamoxifen response in ER-positive tamoxifen-resistant breast cancers (Dumont et al. 1996; Johnston et al. 1999; Riggins et al. 2008; Schiff et al. 2000; Zhou et al. 2007). Furthermore, it has been shown that the inhibition of AP-1 activity can restore the

tamoxifen-sensitivity of resistant breast cancer cells (Riggins et al. 2008; Zhou et al. 2007).

In our study (III), we used the MCF-7-derived estrogen-independent and antiestrogen-resistant subline (TR cells) which overexpress FOSL1 (Pennanen et al. 2009). In the cell growth studies, the inhibition of cell growth by partial FOSL1-knockdown was drastic in these cells. Furthermore, toremifene-sensitivity (inhibiting cell growth) was restored in TR cells by the inhibition of FOSL1 expression. These results support the idea that targeting FOSL1 or AP-1 could be an efficient therapy for the endocrine-resistant, both ER-negative and ER-positive, breast cancers that overexpress FOSL1.

8.4 The effect of different nuclear ligands on the growth of the sublines

In the fourth study (IV), we assessed the effect of different nuclear receptor ligands (DPN, tamoxifen, calcitriol, MPA and retinol) on the growth of pMCF-7 cells and the endocrine-resistant EI and TR sublines. DPN is an ERβ selective (agonist) ligand. In normal breast cells, ERβ seems to act like an antagonist of ERα activity, inhibiting the ability of estrogen to stimulate proliferation (Pettersson et al. 2000; Strom et al. 2004; Treeck et al. 2010). ERβ may also have a protective role in breast tumors by inhibiting proliferation and invasion (Behrens et al. 2007; Lazennec et al. 2001; Williams et al. 2008). We have previously shown that pMCF-7, EI and TR cell lines express ERβ, and its expression is induced by estrogen in these cells (Sarvilinna et al. 2006). In the fourth study (IV), our results show that the highest concentration (100 nM) of DPN stimulates the growth of pMCF-7, EI and TR cell lines similarly to 1 nM E_2. It could be concluded that DPN alone may not be suitable to treat endocrine-resistant breast cancer. Cross-resistance of tamoxifen-resistant cells with toremifene has been previously described in several studies (Nolan et al. 1998; O'Regan et al. 1998; Osborne et al. 1994; Vogel et al. 1993), but the tamoxifen resistance of toremifene-resistant cells has not been reported earlier. Thus, a new observation is that TR cells are cross-resistant to tamoxifen.

Previous studies have reported that calcitriol and its analogues inhibit the growth of breast cancer cells irrespective of the presence of ER (Abe et al. 1991; Chouvet et al. 1986; Love-Schimenti et al. 1996). The time- and dose-dependent growth inhibition by high doses of calcitriol in original MCF-7 cells has been established in previous studies (Meyers et al. 2001; Vink-van Wijngaarden et al. 1994). It has also been shown that calcitriol inhibits growth and induces apoptosis in antiestrogen-resistant variants of ER-positive breast cancer cells (Nolan et al. 1998). In our study, pharmacological doses of calcitriol inhibited the growth of pMCF-7, EI and TR cells, both in the presence and absence of estrogen. Our results are in line with observations that the sensitivity to calcitriol is not reduced during the progression to estrogen-independence and antiestrogen resistance (Nolan et al. 1998).

Several reports have demonstrated that physiological and pharmacological concentrations of retinol, or its active metabolite all-trans retinoic acid (ATRA), inhibit the growth of MCF-7 cells (Butler and Fontana 1992; Lacroix and Lippman 1980; Prakash et al. 2001; Stephen and Darbre 2000). There are only a few *in vitro* studies on the effect of retinoids on the growth of endocrine-resistant breast cancer cells. Butler et al. (1992) showed that TAM-resistant subclone of MCF-7 cells was resistant to 1 µM ATRA. In the study of Stephen et al. (2000), estrogen-independent MCF-7 cells remained growth-inhibited by 1 µM ATRA. In our study, physiological concentrations of retinol inhibited the growth of all cell lines, especially in the presence of E_2. This is interesting since low-dose estrogen therapy could be a new approach to treat estrogen-independent and antiestrogen-resistant hormone receptor-positive breast cancer (Ellis et al. 2009). In clinical trials, it has been shown that retinoids such as all-trans retinoic acid or 13-cis retinoic acid do not have significant activity in patients with hormone-refractory metastatic breast cancer (Cassidy et al. 1982; Sutton et al. 1997). This lack of congruence with our study could be due to possible low estrogen levels in these patients. The combined effect of retinoids and low-dose estrogen should be taken into account in future studies concerning endocrine-resistant breast cancer.

MPA is a synthetic progestin which has been used in hormonal therapy for the treatment of metastatic breast cancer (Parazzini et al. 1993). Several studies have also shown that MPA inhibits the estrogen-stimulated growth of MCF-7 cells

(Lippert et al. 2001; Schoonen et al. 1995; Seeger et al. 2003a; Seeger et al. 2003b). The use of MPA declined when tamoxifen and aromatase inhibitors became standard care for endocrine responsive breast cancer, but there has been renewed interest in alternative hormonal treatments such as MPA, for use both in early disease and in the advanced setting when conventional therapies fail (Focan et al. 2004; Otani et al. 2004; Zaucha et al. 2004). We showed that pMCF-7 cells were only slightly growth inhibited by MPA, and that TR cells were resistant to it. This was observed both in the presence and absence of estrogen. However, the growth of EI cells was dose-dependently inhibited by MPA at low pharmacological concentrations, and estrogen totally blocked the inhibitory effect. Thus, it could be hypothesized that if aromatase inhibitor is the first line treatment, then it would be beneficial to maintain an aromatase inhibitor if MPA is the second line treatment. This is because withdrawing AI-treatment would allow the production of E_2, which in the EI cells blocked the growth inhibitory effect of MPA.

SUMMARY AND CONCLUSIONS

The ER- and PgR-positive MCF-7 breast cancer cell line has been widely used in studies concerning resistance to aromatase inhibitors (estrogen independency) and to antiestrogens *in vitro* and in xenografts. Both *de novo* and acquired resistance cell models have been created. Studies have focused on changes in the regulation and activation of ER and its coregulators. Also, the expression and activation of growth factor receptors and their downstream signaling pathways MAPK and PI3K/AKT have been under thorough investigation in hormone-sensitive cells and their resistant variants. The appearance of gene expression microarrays has vastly broadened our knowledge of gene expression changes in the development of estrogen independency and antiestrogen resistance. On the other hand, microarrays have shown the great differences between various breast cancer cell lines, and even between the same cell lines obtained from different laboratories.

The *in vitro* results originating from hormone-sensitive and -resistant variants of the MCF-7 cell line underline the importance of ER function and the activation of growth factor receptor pathways. When long-term estrogen-deprived or tamoxifen-treated MCF-7 cells escape from estrogen-dependency, ER has become sensitive to very low levels of estrogen or is activated without ligand in these cells. This is a consequence of highly activated growth factor receptor pathways which are able to phosphorylate and activate ER. Furthermore, the growth of these cells is still inhibited by SERD fulvestrant, demonstrating the role of ER in the proliferation of these cells. It has been shown that long-term treatment of these cells with fulvestrant might lead to a loss of ER expression and the development of a fully estrogen-independent phenotype.

The results of our gene expression studies show that when hormone-dependent breast cancer cells are treated with antiestrogen or subjected to estrogen deprivation, the cells that possess a resistant phenotype are selected to grow. The cells reached a constant proliferation rate after two to three months of quiescence. The gene expression changes observed at this point were also present after 12 months culture of the cells. An exception was the genes that were regulated by toremifene. This regulation was observed only after 12 months culture of the cells. In this case, we

cannot exclude changes in individual cells, which could have taken place later in the long-term culture of the cells. One of the genes whose expression was induced early in TR cells was the fos-like antigen 1 (FOSL1). Our studies show that induced expression of FOSL1 was critical for the growth of TR cells. Inhibiting the expression of FOSL1 could be a potent strategy for treating breast cancers that overexpress FOSL1.

Physiological concentrations of vitamin A (retinol) inhibited the growth of the cell lines, especially in the presence of estrogen. This is interesting since low-dose estrogen therapy is a promising new approach in treating estrogen-independent and antiestrogen-resistant hormone receptor-positive breast cancer. An interesting finding was that medroxyprogesterone acetate dose-dependently and at low pharmacological concentrations inhibited the growth of estrogen-independent cells, and that the presence of estrogen abrogated the effect. These findings warrant further studies on the effects of combined low-dose estrogen and retinoids, and aromatase inhibitor and synthetic progestins on acquired aromatase inhibitor-resistant breast cancer.

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Changes in protein tyrosine phosphatase type IVA member 1 and zinc finger protein 36 C3H type-like 1 expression demonstrate altered estrogen and progestin effect in medroxyprogesterone acetate-resistant and estrogen-independent breast cancer cell models

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ABSTRACT

Estrogen stimulates proliferation in hormone-responsive breast cancer cells. Progestins inhibit the estrogen-mediated growth in these cells and are used in the treatment of mammary carcinomas. We applied cDNA microarray and real-time RT-PCR methods to reveal 17β-estradiol- and medroxyprogesterone acetate (MPA)-regulated genes in MCF-7 breast cancer cells. We identified six genes, two of which were novel MPA and/or 17β-estradiol-regulated genes: protein tyrosine phosphatase type IVA, member 1 (PTP4A1) and zinc finger protein 36, C3H type-like 1 (ZFP36L1). PTP4A1 expression was upregulated by 17β-estradiol and this was opposed by MPA treatment of the cells. ZFP36L1 proved to be a direct target of MPA. Since MPA has also been shown to bind to glucocorticoid- and androgen receptors, we studied the regulation of the genes with progesterone, synthetic progestin R5020, dexamethasone and dihydrotestosterone. We also assessed the expression and hormonal regulation of PTP4A1 and ZFP36L1 mRNA in MCF-7-derived MPA-resistant and estrogen-independent sublines. The regulation of PTP4A1 expression upon 17β-estradiol and combined 17β-estradiol and MPA treatment was completely reversed in the estrogen-independent and MPA-resistant sublines, respectively. This study suggests an important role for PTP4A1 in cell growth, and shows that MPA may potentiate the effect of 17β-estradiol in the MPA-resistant breast cancer cells.

1. Introduction

Estrogen and progesterone, acting via their specific nuclear receptors, are essential for the development of mammary gland. Estrogen is essential in normal development as well as in the induction and progression of mammary carcinoma [1]. Estrogen has been shown to increase the proliferation of normal breast epithelium both *in vivo* and *in vitro*. In the *in vivo* studies estrogen increases the number of cycling epithelial cells [2], and in the *in vitro* studies it consistently increases proliferation of normal breast epithelium [3–6]. Estrogen is also known to stimulate the growth of hormone-responsive breast cancer cell lines like T-47D, ZR75-1 and MCF-7 cells, but the degree of estrogen sensitivity is considerably influenced by cell culture conditions [7]. In the *in vitro* studies, where MCF-7 cells are deprived of estrogens, the short-term absence of estrogen inhibits the proliferation of these breast cancer cells. In these short-term estrogen-withdrawn cells, addition of estrogen to the cell culture medium again stimulated cell proliferation markedly [8,9]. In the *in vivo* studies, MCF-7 cells transplanted in athymic or ovariectomized nude mice produced progressively growing tumors only when supplemented with exogenous estrogen and these tumors regressed rapidly following estrogen ablation [10–13].

The major developmental role of progesterone in the normal breast is to stimulate the formation of lobulo-alveolar structures during pregnancy [14]. The effects of progesterone on the proliferation of normal mammary gland epithelium have been variable *in vivo* and *in vitro*, showing that progestins can be either stimulatory or inhibitory, or without an effect [2–6]. In breast cancer cell lines progesterone and synthetic progestins have an inhibitory effect on proliferation and they stimulate differentiation. Many of the growth inhibitory effects of progestins like progesterone,

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medroxyprogesterone acetate (MPA), R5020 and norethisterone (NET) are thought to be due to their ability to oppose the proliferative effect of estrogen [15–23]. Altogether, the mechanism of estrogen-stimulated growth and the opposing effect of progestins in breast cancer cells are not well defined.

In this study, the cDNA microarray method was applied to discover 17β-estradiol (E_2) and progestin (MPA)-regulated genes in MCF-7 breast cancer cells. MPA is a synthetic progestin, which is used as a second line hormonal therapy for the treatment of metastatic breast cancer. MPA also inhibits the E_2-stimulated growth of MCF-7 cells [17,18,20,21]. Based on our cDNA microarray results two novel E_2- and/or MPA-regulated genes, protein tyrosine phosphatase type IVA, member 1 (PTP4A1), and zinc finger protein 36, C3H type-like 1 (ZFP36L1), were selected for verification and further studies by real-time RT-PCR. In addition to the progestagenic effect, MPA has been shown to bind to androgen [24–26] and glucocorticoid [27–29] receptors, and it has been suspected that MPA may exert its influence in part through these steroid receptors. Therefore we also studied the time-dependent mRNA regulation of PTP4A1 and ZFP36L1 genes with progesterone, synthetic progestin R5020, dexamethasone (glucocorticoid) and dihydrotestosterone (androgen). Finally, the expression of the genes was assessed in E_2-independent and in MPA-resistant cell lines which have been established by long-term culture of MCF-7 cells in the absence of E_2, or in the presence of 1 nM E_2 and 100 nM MPA, respectively [30].

2. Experimental

2.1. Cell culture and RNA extraction

MCF-7 cells were routinely grown in Phenol-red free Dulbecco's Modified Eagles Medium (DMEM) (Sigma, St. Louis, MO, USA) with 5% Dextran-Coated Charcoal-Treated Fetal Calf Serum (DCC-FCS), 2% penicillin/streptomycin (Gibco/BRL, Invitrogen Life Technologies, Gaithesburg, MD, USA), 10 ng/ml insulin (Gibco) and 1 nM E_2 (Sigma) at 37 °C and 5% CO_2. Cell culture media were changed every 2–3 days. Steroid hormones (diluted in absolute ethanol) were used at following concentrations: 1 nM E_2, 10 nM MPA (Sigma), 10 nM R5020 (Schering Aktiengesellschaft, Berlin, Germany), 10 nM dihydrotestosterone, 10 nM progesterone (Merck, Darmstadt, Germany), and 10 nM dexamethasone (Sigma). Cycloheximide (CX) (Sigma) was used at 10 μg/ml concentration (diluted in absolute ethanol).

In the cDNA microarray experiments, MCF-7 cells that were grown routinely in the presence of E_2, were compared with cells grown without E_2 for 72 h to reveal E_2-regulated genes. The cells that were grown in the presence of E_2 were treated with MPA for 6 or 24 h or absolute ethanol (vehicle) only to reveal MPA-regulated genes. For the real-time RT-PCR experiments E_2-deprived (72 h) MCF-7 cells were subsequently treated with E_2 for 72 h. The cells that were grown routinely in the presence of E_2 were treated with MPA, progesterone, R5020, dexamethazone, dihydrotestosterone or vehicle for indicated time. In the CX experiment, cells that were grown in the presence of E_2 were treated with CX for 1 h before addition of MPA or vehicle for 6 h. The establishment of MPA-resistant (MR), E_2-independent (EI) and long-term E_2-treated (LE) MCF-7 sublines has been described previously [30]. In the present study, EI, MR and LE cells were grown in the presence of 1 nM E_2 or 10 nM MPA or vehicle for 6 days. The cells were also grown with 1 nM E_2 for 4 days and subsequently treated with 10 nM MPA for 48 h. In all of the experiments, the cells were grown so that in the last time point of the time course the cells were in ~80% confluence. Cell culture procedures were repeated 2–4 times for real-time RT-PCR experiments. Total RNA was extracted using TRIzol Reagent (Gibco/BRL) according to manufacturer's protocol.

2.2. Cell growth experiments

MCF-7 cells were seeded to 96-well plates 1000 cells/well without E_2 or in the presence of 1 nM E_2. Cells were allowed to attach for 24 h. Cells were grown without E_2, with 1 nM E_2 or with 1 nM E_2 and 10 nM MPA. The media and hormones were renewed every second day. The cells were fixed, stained and the relative number of cells was counted every 24 h by Crystal Violet method as described by Kueng et al. [31]. Absorbance was measured at 590 nm wavelength using a Victor 1420 Multilabel counter (Wallac Inc., Turku, Finland).

2.3. cDNA microarray

cDNA was synthesized and 33P dCTP labelled (10 mCi/ml with a specific activity of 3000 Ci/mmol) (Amersham Pharmacia Biotech, Uppsala, Sweden) using 1 μg total RNA. Release I of the Human GeneFilters (GF200 I, Research Genetics, Huntsville, AL, USA) was used for differential expression screening. After hybridisation (16 h) the filter was exposed to a high resolution screen (Storage Phosphor Screen, Molecular Dynamics, CA, USA) for 20 h. The screen was analyzed with phosphor imager (PhosphorImager SI, Molecular Dynamics) and Image Quant software (Molecular Dynamics). The image from phosphor imager was further analyzed using Pathways software (Research Genetics). The experiment was performed strictly according to standardized protocols from the manufacturer (Research genetics). Human Cot-1 DNA, 5× First Strand Buffer, DTT, and Superscript II Reverse Transcriptase were purchased from Invitrogen Life Technologies. Ultrapure dNTP Kit was purchased from Amersham Pharmacia. Bio-Spin 6 Chromatography Column was from Bio-Rad (Hercules, CA, USA).

2.4. Real-time RT-PCR

One-step real-time RT-PCR was performed with a LightCycler instrument (Roche, Basel, Switzerland) in a total volume of 20 μl containing 100 ng of total RNA, 3.25 mM Mn(oAc)2 and 0.5 μM each primer. LightCycler RNA Master SYBR Green I kit (Roche) was used. Reverse transcription was performed for 20 min at 61 °C and denaturation for 2 min at 95 °C. 42 PCR cycles were run. Each PCR-cycle included denaturation at 95 °C, 5 s of primer annealing at 55 °C and extension (extension time dependent on the length of the product) at 72 °C. After amplification a melting curve was obtained by heating at 20 °C/s to 95 °C, cooling at 20 °C/s to 65 °C and heating at 0.1 °C/s to 95 °C with fluorescence data collection at 0.1 °C intervals. Quantitative analysis of the LightCycler data was performed employing LightCycler analysis software (Roche). Gene expression studies which involved the MCF-7-derived sublines were done with the SYBR Green PCR Master Mix Kit in ABI PRISM 7000 Detection System according to the manufacturer's instructions (Applied Biosystems, CA, USA). The RNA samples were reverse transcribed to cDNA with High Capacity Archive Kit (Applied Biosystems) following the instructions of the manufacturer. The following PCR conditions were used: denaturation at 95 °C for 10 min followed by 40–50 cycles at 95 °C for 15 s (denaturation) and 60 °C for 1 min (elongation). The data were analyzed by ABI PRISM 7000 SDS Software (Applied Biosystems). In both RT-PCR systems the final results, expressed as *N*-fold relative differences (ratio) in gene expression between samples, were calculated according to the following equation [32]:

$$\text{ratio} = ((E_{\text{target}})^{\Delta\text{CP target (control-sample)}})/((E_{\text{ref}})^{\Delta\text{CP ref (control-sample)}}).$$

E_{target} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCP target (control-sample) is the CP (crossing point) deviation of control-sample (subtraction) of the target gene transcript; ΔCP ref (control-sample) is the CP deviation of control-sample

of reference gene transcript. Real-time PCR efficiencies (E) were calculated, according to $E = 10^{[-1/\text{slope}]}$. Geometric average and geometric standard error were calculated from the repeated experiments. The experiments were repeated two to four times. Statistical significance of differences was analyzed using the non-parametric Mann–Whitney U-test. The following primer pairs (Amersham Pharmacia Biotech, Uppsala, Sweden) were used in the LightCycler quantitative real-time RT-PCR: PTP4A1-forward, 5′-CTG GTT GTT GTA TTG CTG TTC-3′ and PTP4A1-reverse, 5′-CAG TTG TTT CTA TGA CCG TTG-3′. ZFP36L1-forward, 5′-ATG TAA ACA GAA CTG GCA CAC-3′ and ZFP36L1-reverse, 5′-CTT ACT GGC AAA GTC AAA CC-3′. RPLP0-forward, 5′-GGC GAC CTG GAA GTC CAA CT-3′ and RPLP0-reverse, 5′-CCA TCA GCA CCA CAG CCT TC-3′. For ABI PRISM the primer pairs (TAG, Copenhagen A/S, Denmark) were: PTP4A1-forward, 5′-TTC ATA AGA CAA AAG CGG CGT G-3′ and PTP4A1-reverse, 5′-CCG TTG GAA TCT TTG AAA CGC A-3′. ZFP36L1-forward, 5′-AGG ATG ACC ACC ACC CTC GTG TCT-3′ and ZFP36L1-reverse, 5′-CCC CCT GCA CTG GGA GCA CTA-3′. RPLP0-forward, 5′-AAT CTC CAG GGG CAC CAT T-3′ and RPLP0-reverse, 5′-CGC TGG CTC CCA CTT TGG T-3′.

3. Results

3.1. E_2- and MPA-regulated genes in cDNA microarray

The MCF-7 cells proliferated rapidly in the presence of 1 nM E_2 when compared to the E_2-deprived cells. MPA inhibited the growth of E_2-treated MCF-7 cells by 15 ± 7% (data not shown). The Research Genetics GF200 microarray filter was applied to identify genes that could be implicated in the growth regulation of MCF-7 cells by these hormones. The genes that were regulated less than 2.0-fold, or had expression level close to background level in the microarray filter, were excluded from further studies. The genes that have been previously reported to be E_2- and/or progestin-regulated in human breast cancer cells, were also excluded from further studies. Those genes included S 100 calcium binding protein P (S100P) [33], FK506 binding protein 5 (FKBP54), neuropeptide Y receptor Y1 (NPY1R) [34] and discs, large (Drosophila) homolog 5 (DLG5) [35]. The regulation pattern of these genes in our cDNA microarray analysis was consistent with the studies mentioned above.

Protein tyrosine phosphatase type IVA, member 1 (PTP4A1) and zinc finger protein 36, C3H type-like 1 (ZFP36L1) were found to be novel E_2- and/or progestin-regulated genes. E_2 had no effect on the expression of ZFP36L1 whereas PTP4A1 expression was upregulated by E_2. In the E_2-treated MCF-7 cells, MPA upregulated ZFP36L1 expression at the 6 h time point. In these cells, PTP4A1 was downregulated by MPA in the 6 and 24 h time points (Table 1). PTP4A1 and ZFP36L1 genes were selected for verification and further studies by real-time RT-PCR in MCF-7 cells.

Table 1
Genes regulated in the cDNA microarray by 17β-estradiol (E_2) and medroxyprogesterone acetate (MPA) in MCF-7 breast cancer cells.

Gene name	Accession no.	MPA 6 h	MPA 24 h	E_2
S100P*	X65614	–	7.5 ↑	–
FKBP54*	U42031	–	5.2 ↑	–
DLG5*	AB011155	–	3.3 ↑	–
ZFP36L1	X79067	4.5 ↑	–	–
NPY1R*	M84755	2.6 ↑	2.3 ↓	2.3 ↑
PTP4A1	U48296	4.2 ↓	2.3 ↓	3.3 ↓

MCF-7 cells grown in the presence of E_2 were treated with MPA for 6 h (MPA 6 h) and 24 h (MPA 24 h). E_2-deprived MCF-7 cells were also compared to the cells that were grown in the presence of E_2 (E_2). The numbers indicate the fold of regulation of the particular gene. An arrow pointing up (↑) shows upregulation, and an arrow pointing down (↓) shows downregulation of gene expression. Line (–) indicates that the gene was not regulated. Genes that have been previously described in the literature as E_2- and/or progestin-regulated are shown (*).

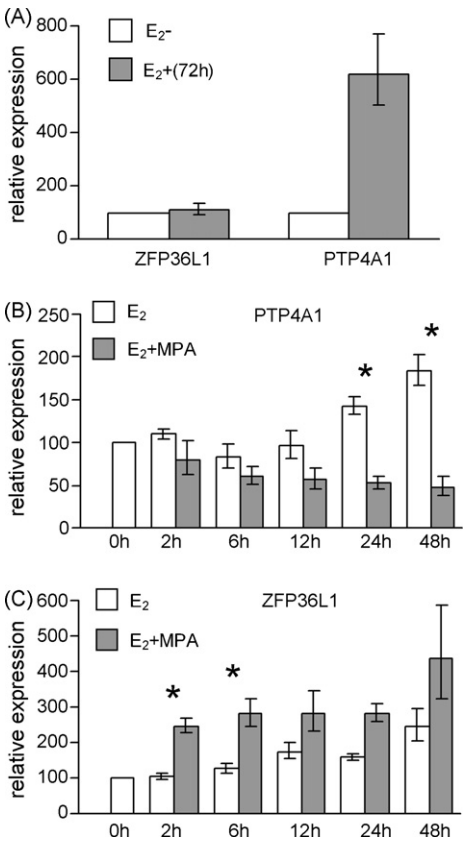

Fig. 1. Real-time RT-PCR results of 17β-estradiol (E_2) and medroxyprogesterone acetate (MPA) regulation of PTP4A1 and ZFP36L1 mRNA in MCF-7 breast cancer cells. Regulation of PTP4A1 and ZFP36L1 mRNA by E_2 was studied by comparing E_2-deprived (E_2-) cells with cells that were first E_2-deprived and then subjected to E_2 for 72 h (E_2+ (72 h)) (A). E_2- cells were given the arbitrary value 100 and the expression levels of E_2+ (72 h) cells were calculated relative to the E_2- cells. The results are average of two independent experiments (geometric mean ± geometric standard error). Regulation of PTP4A1 (B) and ZFP36L1 (C) by MPA was studied for indicated time. The 0 h time point (0 h) was given the arbitrary value 100. The expression levels of the other time points are calculated relative to the 0 h time point. The results are average of four independent experiments (geometric mean ± geometric standard error). *$P < 0.05$ in the Mann–Whitney U-test.

3.2. Regulation of PTP4A1 and ZFP36L1 gene expression by steroid hormones

To verify the E_2 regulation of ZFP36L1 and PTP4A1 genes, MCF-7 cells that were E_2-deprived for 7 days were compared to the cells that were first E_2-deprived for 7 days and then subjected to E_2 for 72 h. Consistent with the cDNA microarray result, no E_2 regulation of ZFP36L1 gene was detected, and PTP4A1 was markedly upregulated in the cells that received E_2 for 72 h (Fig. 1A). Next, the regulation of the genes by MPA was studied in the cells grown with E_2. Significant downregulation of PTP4A1 by MPA could be observed at 24 and 48 h time point (Fig. 1B). The mRNA level of ZFP36L1 increased more than two-fold at the 2 and 6 h time points in the MPA-treated cells (Fig. 1C). This early induction of ZFP36L1 mRNA indicated that it could be a direct target of MPA.

Next, MCF-7 cells were treated simultaneously with the protein synthesis inhibitor cycloheximide (CX) and MPA to determine if the hormone regulation of ZFP36L1 and PTP4A1 is a direct effect that does not require *de novo* protein synthesis. The change in the expression level of the genes was measured at 6 h time point. CX was not able to block the upregulation of ZFP36L1 mRNA by MPA, suggesting that ZFP36L1 is indeed a direct target of MPA action (Fig. 2A). The treatment of MCF-7 cells with CX alone or CX with MPA caused an induction of PTP4A1 gene. The increase in PTP4A1 mRNA in the

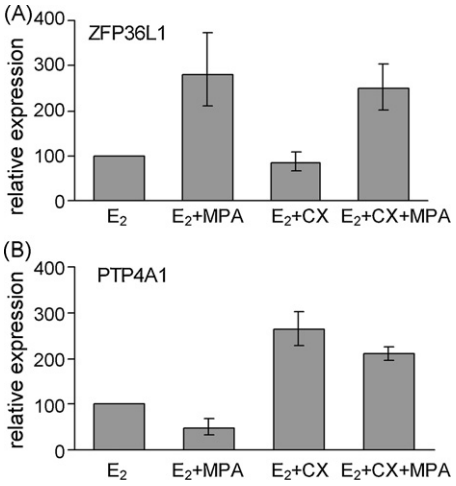

Fig. 2. Real-time RT-PCR results of ZFP36L1 (A) and PTP4A1 (B) gene expression in the presence of 17β-estradiol (E_2), E_2 and cycloheximide (E_2 + CX), E_2 and medroxyprogesterone acetate (E_2 + MPA) and E_2 and CX and MPA (E_2 + CX + MPA) in MCF-7 breast cancer cells. mRNA expression levels were measured after 6 h treatment with MPA and/or CX. The control sample (E_2) was given the arbitrary value 100. The expression levels of the other samples are calculated relative to the control sample. The results are average of two independent experiments (geometric mean ± geometric standard error).

presence of CX could be due to the inhibition of synthesis of specific inhibitor protein (Fig. 2B).

In addition to MPA, we studied the effect of progesterone, progesterone agonist R5020, androgen (dihydrotestosterone, DHT) and glucocorticoid (dexamethasone, DEX) on the regulation of ZFP36L1 and PTP4A1 expression in MCF-7 cells grown in the presence of E_2. ZFP36L1 was significantly upregulated by DEX and R5020 (Fig. 3A), whereas PTP4A1 was significantly regulated by DEX (Fig. 3B). Overall, MPA was the most potent regulator of gene expression of the two genes. The potency was in order: MPA > DEX > R5020 > progesterone > DHT. The steroid hormone regulation of PTP4A1 and ZFP36L1 expression was also studied in the E_2-deprived MCF-7 cells. The cells were grown 72 h without E_2 and then subjected to 10 nM MPA, progesterone, R5020, DEX, DHT or vehicle alone for 6 and 48 h. All these hormones failed to regulate the PTP4A1 and ZFP36L1 expression in the E_2-deprived cells (data not shown).

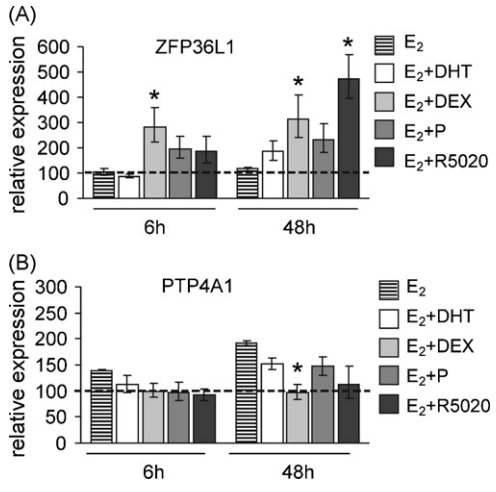

Fig. 3. Real-time RT-PCR results of ZFP36L1 (A) and PTP4A1 (B) regulation by progesterone (P), dihydrotestosterone (DHT), R5020, dexamethasone (DEX) or 17β-estradiol only (E_2) in MCF-7 cells. Cells were grown in the presence of 17β-estradiol (E_2) and treated with the hormones for indicated time. 0 h time point is given the arbitrary value 100 (broken line). The expression levels of the other time points are calculated relative to the 0 h time point. The results are average of three independent experiments (geometric mean ± geometric standard error). $^*P < 0.05$ in the Mann–Whitney *U*-test.

3.3. Altered expression of PTP4A1 and ZFP36L1 in MPA-resistant and E_2-independent cell lines

The expression of PTP4A1 and ZFP36L1 was assessed in MCF-7-derived long-term estrogen-treated (LE), MPA-resistant (MR) and estrogen-independent (EI) cell lines. LE cells were included in the comparison as the duration of subculture for LE cells was equal to EI and MR cells (9 months). ZFP36L1 mRNA was reduced to a low level in EI cells, whereas the expression of PTP4A1 was low in both EI and MR cells when compared to LE cells (Fig. 4A). The response of the genes to E_2 and MPA was also studied in EI, MR and LE cells. In EI cells PTP4A1 expression was downregulated by E_2, and in MR cells PTP4A1 mRNA was more than 8-fold upregulated by MPA in the presence of E_2 (Fig. 4B). This regulation profile was clearly opposite to that in MCF-7 cells (Fig. 1A and C) and LE cells. ZFP36L1 was more than 4-fold upregulated by MPA in the presence of E_2 in both EI and

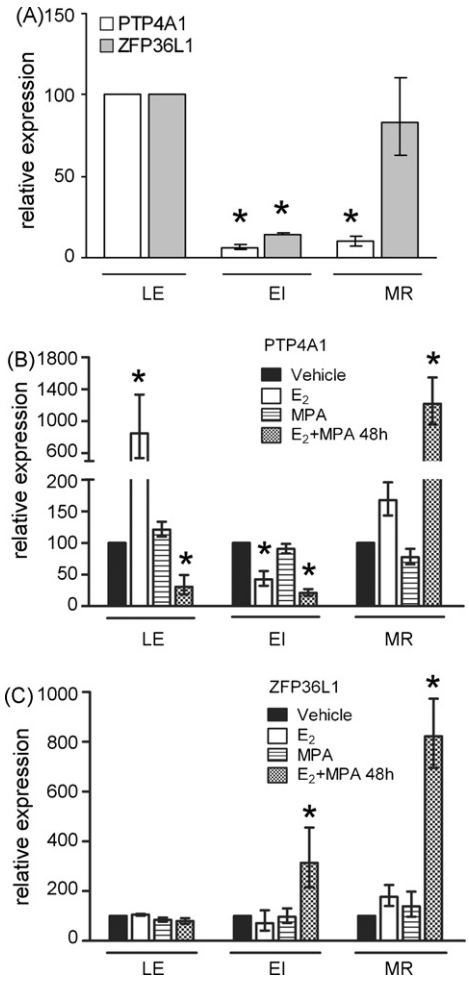

Fig. 4. PTP4A1 and ZFP36L1 mRNA levels were assessed in long-term estrogen-treated (LE), estrogen-independent (EI) and medroxyprogesterone acetate (MPA)-resistant (MR) cells (A). Expression levels in LE cells were given the arbitrary value 100. Expression levels in EI and MR cells were calculated relative to LE cells. Hormonal regulation of PTP4A1 (B) and ZFP36L1 (C) was studied in LE, EI and MR cells. Cells were grown without hormones, in the presence of 17β-estradiol (E_2) or MPA, or in the presence of 17β-estradiol and treated by MPA for 48 h (E_2 + MPA 48 h). Expression levels were calculated relative to control (vehicle) sample, which was given the arbitrary value 100. The results are average of three independent experiments (geometric mean ± geometric standard error). $^*P < 0.05$ in the Mann–Whitney *U*-test.

MR cells (Fig. 4C). The regulation profile of ZFP36L1 in EI and MR cells was similar to MCF-7 cells (Fig. 1B and C). On the contrary, ZFP36L1 was not upregulated by combined E_2 and MPA in LE cells as it was in MCF-7 cells.

4. Discussion

Gene expression microarray and real-time RT-PCR methods were used to identify E_2- and MPA-regulated genes in MCF-7 breast cancer cells. Six MPA and/or E_2-regulated genes were found (Table 1), and the regulation of two MPA and/or E_2-regulated genes, PTP4A1 and ZFP36L1, was verified and studied further by real-time RT-PCR. To our knowledge, this is the first time that the progestin regulation of ZFP36L1 or E_2 and progestin regulation of PTP4A1 is described. The expression of ZFP36L1 and PTP4A1 was also regulated by R5020 and DEX similarly to MPA. This finding is in agreement with the observations where MPA was shown to have glucocorticoid-like effects in addition to progestin effects. ZFP36L1 is a transcription factor and member of the tristetraprolin (TTP) family of tandem zinc finger proteins [36]. In a recent study, breast-invasive ductal carcinomas were grouped into lymph node negative and lymph node positive groups, and ZFP36L1 was found to be overexpressed in lymph node positive tumors [37], suggesting that ZFP36L1 is implicated in breast cancer. PTP4A1 overexpression has been implicated in transformed phenotype of cells and it may be involved in tumorigenesis [38–40]. The expression of PTP4A1 has been studied in a number of cancer cell lines, and high levels of its mRNA were found in a variety of tumor cell lines of different tissue origin compared to untransformed control cell lines [41].

In this study, the effect of E_2 or MPA on ZFP36L1 expression did not correlate with the effect of these hormones on cell growth. On the contrary, the expression of PTP4A1 was induced in highly proliferating E_2-treated cells, but downregulated in MPA-treated or E_2-deprived cells, indicating that the expression of PTP4A1 is dependent on proliferation. This is further supported by the observation that PTP4A1 regulation by E_2 was reversed in EI cells that no longer require E_2 for proliferation. Similarly, the regulation of PTP4A1 by E_2 and MPA together was reversed in MR cells which are adapted to grow rapidly in the presence of combined E_2 and MPA. We have previously shown that MR cells exhibit lowered growth response to E_2 compared to parental MCF-7 and LE cells [30]. This could also be seen in a lower induction of PTP4A1 mRNA by E_2 in MR cells.

Loss of PTP4A1 and ZFP36L1 regulation by DEX, DHT and all progestins was observed in the E_2-deprived MCF-7 cells (data not shown). The same effect is shown in EI, MR and LE cells where MPA alone was not sufficient to regulate the expression of either gene (Fig. 4B and C). Several studies have shown that progesterone receptor protein (PRα and PRβ) is decreased even below detection by Western blot already after 48 h or 7 days E_2 deprivation of MCF-7 cells [42,43]. PR mRNA is also markedly downregulated in EI cells and E_2-deprived MR cells [30]. This marked downregulation of PR could be one reason for the inability of the progestins to regulate ZFP36L1 and PTP4A1 gene expression in E_2-deprived cells. On the other hand, the observed loss of DEX regulation cannot be explained by decreased glucocorticoid receptor (GR) abundance, since previous studies have shown that GR mRNA is abundant in E_2-deprived MCF-7 cells [44,45]. Thus, the reason for the loss of DEX regulation of these genes in E_2-deprived MCF-7 cells is not known.

MPA is at present used in hormonal therapy for the treatment of metastatic breast cancer, and in hormone replacement therapy (HRT) in the treatment of perimenopausal symptoms. Some clinical studies suggest that progestin presence in HRT increases breast cancer incidence [46]. We have showed that long-term E_2- and MPA-treated MR cells have probably acquired additional growth advantage from MPA, which is implicated in the reversed PTP4A1 mRNA regulation by combined E_2 and MPA. This is particularly intriguing, since MPA potentiates the effect of E_2 on the PTP4A1 expression in these cells. On the other hand, our results also indicate that if MPA was the progestin used in HRT, it may not be a progestagenic mechanism alone, which is causing the increase in the breast cancer incidence in HRT users.

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Gene expression changes during the development of estrogen-independent and antiestrogen-resistant growth in breast cancer cell culture models

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We have established estrogen-independent and antiestrogen-resistant cell lines from hormone-dependent MCF-7 breast cancer cells by long-term culture in the absence of estrogen, or in the presence of antiestrogen toremifene, respectively. By using a cDNA microarray we compared gene expression profiles among estrogen-independent, antiestrogen-resistant and long-term estrogen-treated MCF-7 cells. We also determined how the expression of the differentially expressed genes has developed during the long-term culture of the cell lines. Of the screened 1176 cancer-related genes, FOSL1, TIMP1, L1CAM, GDF15, and MYBL2 were found to be differentially expressed between the cell lines. A change in FOSL1 and TIMP1 expression could be attributed to the development of antiestrogen resistance, whereas induced L1CAM expression was implicated in the development of estrogen-independent growth of the cells. Estrogen regulated genes GDF15 and L1CAM became regulated by toremifene in the later passage number of toremifene-resistant cells, which might be an indication of the developed estrogen-agonistic activity of toremifene in these cells. Our findings suggest a pattern where the hormone-responsive cancer cells, which survive E_2 deprivation and/or antiestrogen treatment, first acquire necessary changes in gene expression for transition to maximal growth in the new hormonal environment. Then, after prolonged treatment with antiestrogen, the antiestrogen-resistant cells may eventually generate an E_2-agonistic response to antiestrogen, probably acquiring additional growth advantage. ***Anti-Cancer Drugs*** **20:51–58 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The ability to reduce breast tumor growth through the administration of antiestrogens has played an important role in the endocrine therapy of hormone-dependent breast cancer (reviewed in Refs [1,2]). Although the initial response rates to antiestrogen are high among ER-positive tumors, the majority of the patients that respond will eventually develop antiestrogen resistance. A number of mechanisms leading to the development antiestrogen resistance *in vivo* have been proposed including estrogen receptor (ER) mutations (reviewed in Ref. [3]), alterations in the expression levels of ERα, ERβ and progesterone receptor, and their coactivators and corepressors [4–11]. Growing evidence *in vivo* suggests that high expression and/or activation of epidermal growth factor receptor and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma-derived oncogene homolog (avian) (ERBB2) can confer resistance by the activation of downstream signaling pathways. Among these activated pathways are the cell survival pathway mediated by PI3 kinase and AKT/PKB, and the cell proliferation pathway mediated by the mitogen-activated protein kinase ERK1/2 [11–13]. The involvement of growth factor signaling in antiestrogen resistance is also supported by in-vitro studies with breast cancer cell lines [14–17].

The in-vitro studies elucidating mechanisms of antiestrogen-resistant growth emerge from breast cancer cell culture models, in which cells that are resistant to different antiestrogens have been established from hormone-dependent parental cells by long-term culture. Most of these cell lines have been obtained by in-vitro selection of the MCF-7 breast cancer cells by tamoxifen [15,18–22], but cell lines that are resistant to other antiestrogens or pure antiestrogens have also been established [23–28]. Another feature of hormone-dependent breast cancers is the development of estrogen-independent (EI) growth of cancer cells in the absence of estrogen. Several groups have used in-vitro MCF-7 breast cancer model systems to characterize the adaptive changes occurring in the development of EI phenotype [29–33].

We have earlier established three MCF-7 cell line-derived sublines by long-term (9 months) culture in the absence

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of estrogen (E_2), or in the absence of E_2 and presence of antiestrogen toremifene (TOR). These sublines serve as in-vitro models of EI or EI and toremifene-resistant (TR) breast cancer, respectively. The third, long-term estrogen-treated cell line (LE) was established by 9 months culture of MCF-7 cells in the presence of E_2 [28]. In this study, we compared gene expression profiles among TR, EI, and LE cells. Of the 1176 cancer-related genes screened by cDNA microarray we found five differentially expressed genes. In addition to these three cell lines, the original (pMCF-7) cells were included in the subsequent verification of the microarray result and further studies by real-time RT-PCR. We have frozen earlier passage numbers of the studied cells during the long-term culture. This enabled us to determine how the expression of the differentially expressed genes has developed as the cells have adapted to grow in these different hormonal environments.

Materials and methods

Hormones and reagents

17β-estradiol (E_2) and insulin were purchased from Sigma (St Louis, MO, USA). The antiestrogen toremifene citrate was kindly provided by Orion Pharma (Turku, Finland). Stock solutions were prepared by dissolving the hormones in 96% ethanol, stored at −20°C and added to culture media to yield ethanol concentration not exceeding 0.1%. The antibiotics, penicillin and streptomycin, were obtained from GIBCO (Invitrogen Corporation, Paisley, UK).

Cell growth experiments

The establishment of LE, TR and EI MCF-7 sublines has been described earlier [28]. Briefly, estrogen and antiestrogen-sensitive, estrogen- and progesterone receptor-positive MCF-7 human breast cancer cells were used as parent cells to generate these sublines. LE cells have received vehicle (96% ethanol) + 1 nmol/l E_2, TR cells 1 μmol/l TOR and EI cells vehicle only continuously for 9 months. In this study, parent MCF-7 cells (pMCF-7) and the MCF-7-derived sublines with different passage numbers were routinely cultured in 75 cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2: 95% air in phenol red-free Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12; Sigma) supplemented with 5% dextran charcoal-treated fetal bovine serum (DCC-FBS; GIBCO), penicillin (100 IU/ml), streptomycin (100 μg/ml) and insulin (10 ng/ml). The media with appropriate hormones were changed every 2 or 3 days. All disposable cell culture materials were purchased from Nunc (Apogent Technologies Inc., Portsmouth, NH, USA).

In the cDNA microarray experiments, LE, TR and EI cells were plated on the flasks and grown with appropriate hormones for one passage. After reseeding, the LE and TR cells were grown without hormones, and EI cells without vehicle, for 6 days to eliminate any residual hormonal effects on the gene expression, and then subjected to TRIzol reagent (Gibco, Invitrogen, CA, USA) for RNA extraction according to the manufacturer's instructions. In the real-time RT-PCR experiments, pMCF-7, LE, TR and EI cells were grown as explained above in the cDNA microarray experiment (i.e., hormonal deprivation for 6 days) before RNA extraction. Parallel to this, pMCF-7, LE, TR and EI cells were grown for 1 week with appropriate hormones and then subjected to RNA extraction to obtain RNA samples from the cells that have been grown routinely in normal growth conditions (i.e., no hormonal deprivation).

cDNA microarray

Atlas Human Cancer 1.2 Array (Clontech, CA, USA) containing 1176 cancer-related genes was used to study the basic gene expression differences among LE, TR, and EI cells. The integrity of the RNA samples was assessed by electrophoresis on a denaturing 1% agarose gel, and the concentration and purity of the RNA was determined using a spectrophotometer (GeneQuant II, Pharmacia Biotech Ltd, UK). The microarray experiments were performed according to the manufacturer's protocol. Briefly, 50 μg of total RNA from each sample was reverse transcribed to [α-^{33}P]dATP-labelled cDNA with the Atlas Pure Total RNA Labeling System. The radiolabelled cDNAs were hybridized to the array membranes overnight, and the phosphorimaging screen was exposed with the membranes overnight. The phosphorimaging screen was scanned with a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, California, USA) at a resolution of 100 μm. Imagequant files from scans were analyzed using ArrayVision software (Imaging Research, St Catharines, Ontario, Canada). A template, which contains the spot layout of the array, was overlaid on the phosphorimage, and the pixel intensity of each spot on the array was determined. Spot intensities were background subtracted and globally normalized.

Real-time RT-PCR

The RNA samples were reverse transcribed to cDNA with High Capacity Archive Kit (Applied Biosystems, California, USA) following the instructions of the manufacturer. Real-time RT-PCR was done with the SYBR Green PCR Master Mix Kit in an ABI PRISM 7000 Detection System according to the manufacturer's instructions (Applied Biosystems). The following PCR conditions were used: denaturation at 95°C for 10 min followed by 40–50 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (elongation). The data were analyzed by ABI PRISM 7000 SDS Software (Applied Biosystems). The final results, expressed as *N*-fold relative differences (ratio) in gene expression between the studied samples and the control (i.e., calibrator) sample, were calculated according to the following equation [34]: $\text{Ratio} = [(E_{\text{target}})^{\Delta CP\ \text{target}\ (\text{control}-\text{sample})}]/[(E_{\text{ref}})^{\Delta \text{CP}\ \text{ref}\ (\text{control}-\text{sample})}]$. E_{target} is the real-time PCR

efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCP_{target} is the *CP* (crossing point) deviation of control–sample (subtraction) of the target gene transcript; ΔCP_{ref} is the *CP* deviation of control–sample of reference gene (RPLP0; ribosomal protein, large, P0) transcript. Real-time PCR efficiencies (*E*) were calculated, according to $E = 10^{(-1/slope)}$. The following primers (TAG, Copenhagen, Denmark) were used: MYBL*2* forward primer (f) 5′-GCCG AGATCGCCAAGATG-3′ and MYBL*2* reverse primer (r) 5′-TGATGGTAGAGTTCCAGTGATTCTTC-3′. FOSL1 (f) 5′-GGAGGAAGGAACTGACCGACTT-3′ and FOSL1 (r) 5′-TGCAGCCCAGATTTCTCATCT-3′. GDF15 (f) 5′-TGCCCGCCAGCTACAATC-3, and GDF15 (r) 5′-TCTTTGGCTAACAAGTCATCATAGGT-3′. L1CAM (f) 5′-CCACAGATGACATCAGCCTCAA-3′ and L1CAM (r) 5′-GGTCACACCCAGCTCTTCCTT-3′. TIMP1 (f) 5′-GATACTTCCACAGGTCCCACAAC-3′ and TIMP1 (r) 5′-GCAAGAGTCCATCCTGCAGTTT-3′. RPLP0 (f) 5′-AATCTCCAGGGGCACCATT-3′ and RPLP0 (r) 5′-CG CTGGCTCCCACTTTGGT-3′. The primers were designed using Primer Express software for ABI PRISM 7000 detection system (Applied Biosystems).

Statistical analysis

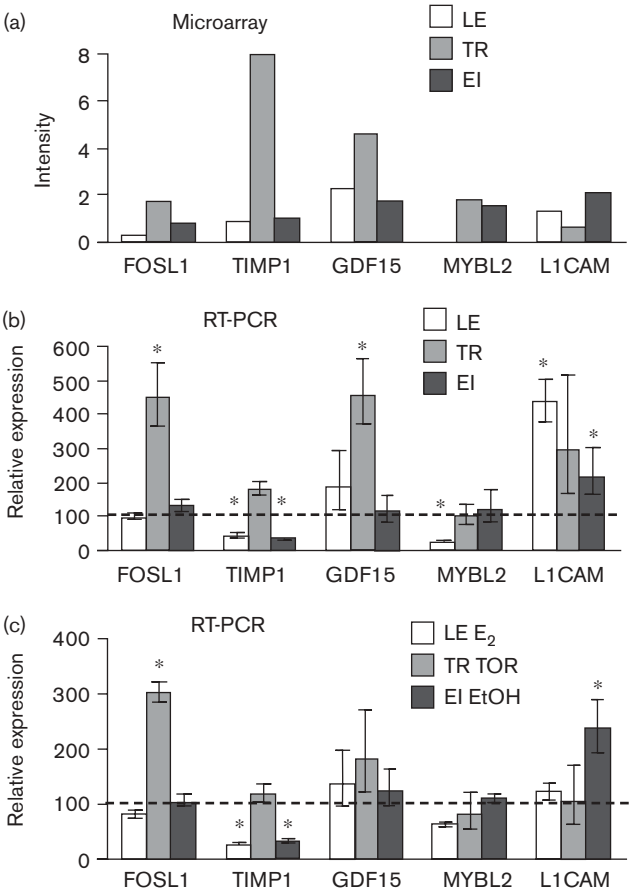
All experiments were repeated three times. The quantitative data are expressed as geometric mean ± geometric standard error. Comparisons were made by the Mann–Whitney *U* test and *P* value of less than 0.05 was considered as statistically significant difference.

Results

Differentially expressed genes in LE, TR and EI cells

The cDNA microarray method was used to reveal differences in basal gene expression between long-term E_2-treated (LE) cells, toremifene-resistant (TR) and estrogen-independent (EI) cells (Fig. 1a). To find differences in basal gene expression, LE and TR cells were deprived of hormone for 6 days (E_2 and TOR-deprived, respectively). Five genes (FOSL1, TIMP1, L1CAM, GDF15, and MYBL*2*) were found to be differentially expressed among the three sublines. FOSL1, TIMP1, and GDF15 were upregulated in TR cells whereas L1CAM was downregulated in these cells. MYBL*2* proved to be downregulated in LE cells. The cDNA microarray results were verified by real-time RT-PCR, both in the hormone-deprived cells (Fig. 1b) and in the cells that were grown with their appropriate hormones (LE cells with E_2 and TR cells with TOR) (Fig. 1c). In the real-time RT-PCR experiments the expression level of the genes in LE, TR and EI cells was calculated relative to their expression level in original pMCF-7 cells that were grown in the presence of estrogen. By comparing the gene expressions between the hormone-deprived cells (Fig. 1b) and the cells grown with the appropriate hormones (Fig. 1c), it was evident that the expression of FOSL1 and TIMP1 was not changed. However, the expression of GDF15 was downregulated by TOR in TR cells. Similarly, the expression of L1CAM was downregulated by TOR in TR cells, and it was also downregulated by E_2 in LE cells. MYBL*2* was upregulated by E_2 in LE cells.

Fig. 1



Relative expression of five differentially expressed genes in the long-term estrogen-treated (LE), toremifene-resistant (TR), and estrogen-independent (EI) cells. Basal expression differences studied by cDNA microarray (a), verification of the cDNA microarray result by real-time RT-PCR (b), and expression in cells grown with the appropriate hormones studied by real-time RT-PCR (c), are shown. The cDNA microarray results represent intensity values obtained from the microarray filter. Intensity value 0 is equivalent to the level of background intensity in the filter. In the real-time RT-PCR experiments, the expression of each gene was calculated relative to its expression in original (pMCF-7) cells. The expression of each gene in pMCF-7 cells was given the arbitrary value 100 (broken line). pMCF-7 cells were grown in the presence of estrogen. The values represent the geometric average and geometric standard error (*$P<0.05$). E_2, estradiol; EtOH, ethanol; TOR, toremifene.

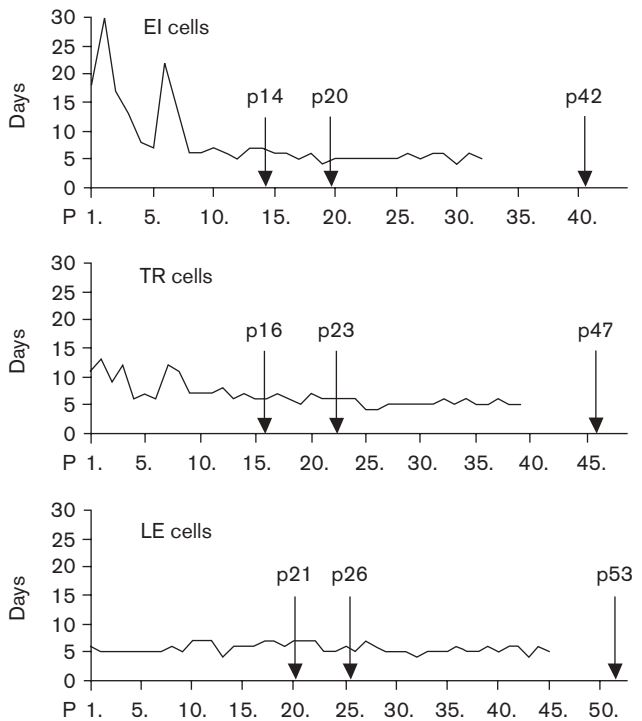
Gene expression changes during the acquisition of estrogen-independent growth and antiestrogen resistance

Several different passage numbers of the sublines were used in this study. The time to reach confluence, after constant number of cells seeded to flasks, was measured during the 9-month culture period to follow the growth

rate of the sublines. The cell passages used in this study, and their growth rates, are shown in Fig. 2. The growth rate study did not include the last passages of the cell lines. However, we observed no changes in the growth rate of these cells when compared with the cells representing earlier passages, and therefore it is apparent that all the passages used in the study represented proliferating cells that have similar growth rates.

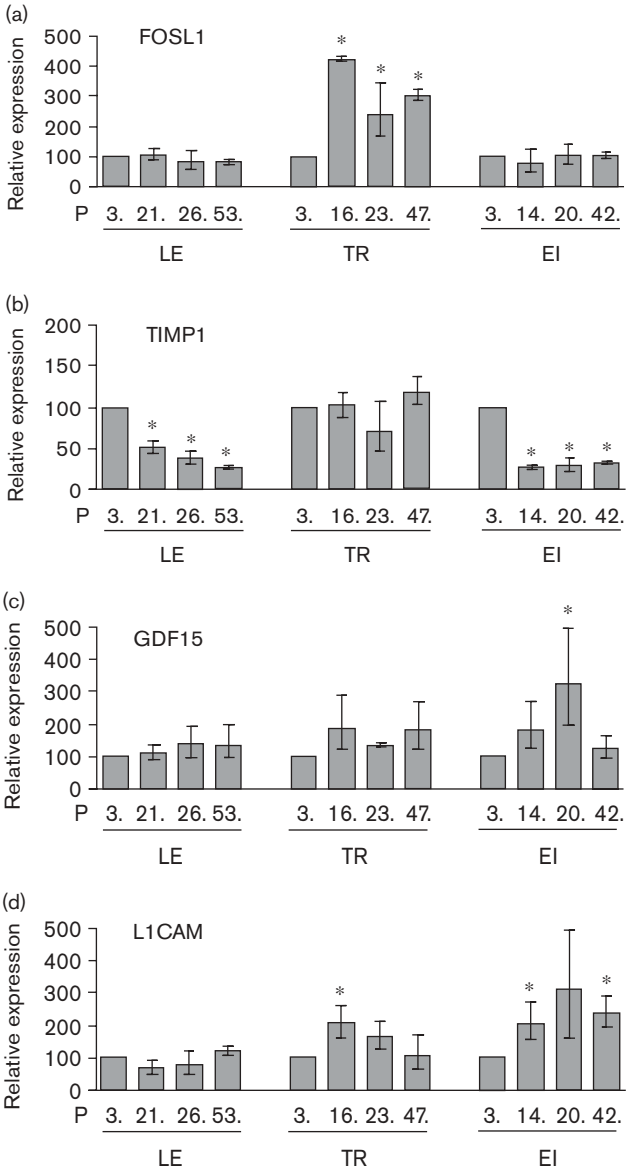
We wanted to determine how the changes in the expression pattern of the five genes have developed as the cells have adapted to grow in different hormonal environments during the long-term culture. The expression levels of the genes were studied in LE, TR, and EI cells grown with the appropriate hormones. Four time points from each subline were studied including original pMCF-7 cells, two passages from the middle of the subculture period, and passages that represented the sublines after 9 months culture. FOSL1 expression level remained unchanged in LE and EI cells, but its induction was evident already in passage number 16 (p16) in TR cells (Fig. 3a). TIMP1 mRNA levels decreased in LE and EI cells as the passage number increased, but in TR cells its mRNA level remained at the same level as in pMCF-7 cells (Fig. 3b). The expression of GDF15 remained modestly induced in the last passage of TR cells, but not in the last passage of EI cells (nonsignificantly) (Fig. 3c). On the contrary, L1CAM expression remained upregulated in the last passage of EI cells, but in TR cells its expression fell back to the level observed in pMCF-7 cells

Fig. 2



Growth rate of the estrogen-independent (EI), toremifene-resistant (TR) and long-term estrogen-treated (LE) cell lines during the 9-month cell culture period. The *y*-axis shows the time (days) to reach confluence after a constant number of cells were reseeded to flasks. The *x*-axis shows the growing passage (P) numbers of the cell lines. The passage numbers that were used in this study are shown for each cell line (arrows).

Fig. 3



The expressions of FOSL1 (a), TIMP1 (b), GDF15 (c), and L1CAM (d) were studied by real-time RT-PCR in long-term estrogen-treated (LE), toremifene-resistant (TR), and estrogen-independent (EI) cells representing different passage numbers. The cell lines were grown with the appropriate hormones. The growing passage (P) numbers are shown (*x*-axis), starting from the original (pMCF-7) cells representing passage number 3. The expressions of the genes in different cell lines were calculated relative to the expression of the gene in pMCF-7 cells, which were given the arbitrary value 100. The values represent the geometric average ± geometric standard error (*$P < 0.05$).

(Fig. 3d). No changes in MYBL*2* gene expression were seen during the long-term culture in LE, TR, or EI cells (data not shown).

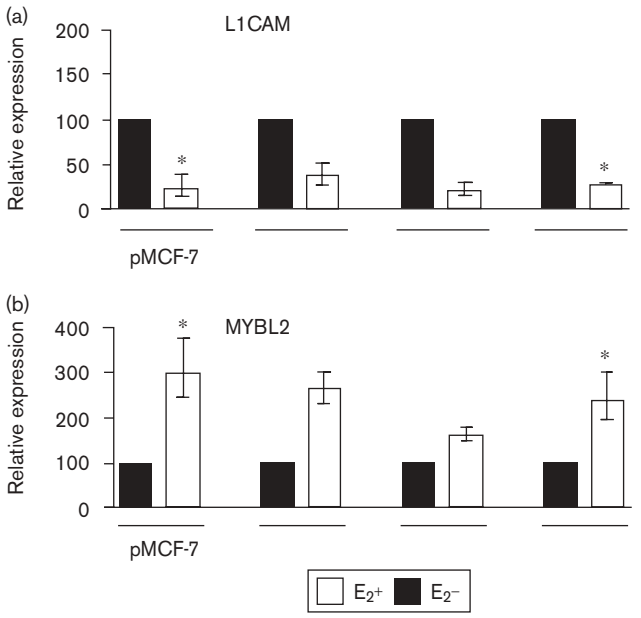
Estrogen-regulated genes

The cDNA microarray and real-time RT-PCR results indicated that L1CAM and MYBL*2* were regulated by E_2 in LE cells, whereas GDF15, FOSL1, and TIMP1 expression was not altered by the hormone. We assessed the E_2 regulation of L1CAM and MYBL*2* in the pMCF-7 cells and in three passages of LE cells (p21, p26, and p53). L1CAM and MYBL*2* proved to be E_2-regulated in the original pMCF-7 cells and in the long-term cultured LE cells representing passage numbers 21, 26, and 53 (Fig. 4a, b). It was also evident that the E_2 regulation of these genes remained relatively similar throughout the subculture period.

Toremifene-regulated genes

The cDNA microarray and real-time RT-PCR results indicated that GDF15 and L1CAM were regulated by TOR in TR cells. We wanted to study whether this TOR regulation existed already in the original pMCF-7 cells, or had evolved during the long-term culture. Therefore we assessed the TOR regulation of these genes in pMCF-7 and three passages of LE cells (p21, p26 and p53), and in three passages of TR cells (p16, p23 and p47). The expression of both GDF15 and L1CAM was regulated in TR cells by TOR in the last passage of the cells only (Fig. 5b, d GDF15 text is missing from Fig. 5b. Similarly L1CAM text is missing from Fig. 5c, d. GDF15 text is correctly in Fig. 5a). GDF15 was modestly regulated by TOR in pMCF-7 cells and all passages of LE cells (Fig. 5a), whereas the expression of L1CAM was mostly unaltered by TOR in these cells (Fig. 5c). Interestingly, TOR regulation of L1CAM in TR cells was essentially similar to its regulation by E_2 in pMCF-7 and LE cells (compare with Fig. 4a).

Fig. 4



Estrogen (E_2) regulation of L1CAM (a) and MYBL2 (b) expression in original (pMCF-7) cells and in long-term estrogen-treated (LE) cells representing different passage numbers. The expressions were studied by real-time RT-PCR in the cells that were grown routinely with E_2 (E_2+), and in the cells that were E_2-deprived for 6 days (E_2-). The expressions of the genes in (E_2+) – samples were calculated relative to the (E_2-) – samples, which were given the arbitrary value 100. The values represent the geometric average ± geometric standard error (*$P<0.05$).

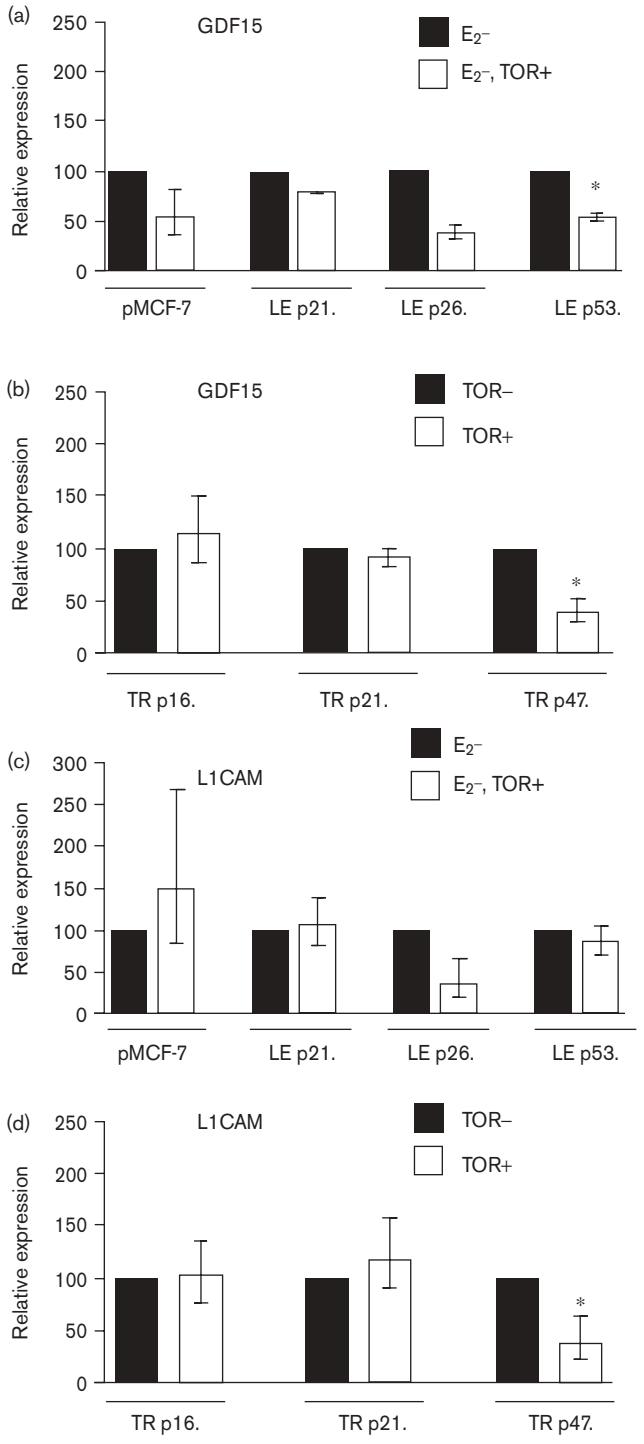
Discussion

Cell culture models to study EI growth and antiestrogen resistance exist, but they are relatively few in number, and almost all are based on the MCF-7 human breast cancer cell line [35]. Large-scale gene expression studies from these cell lines are limited, and comparing the results from these studies is complex as the cell sublines often show different characteristics that originate from variations in cell culture conditions and hormone treatments; for example, the different antiestrogens used [18,36–38]. Two different MCF-7 cell-derived models of estrogen independence and antiestrogen resistance have been compared by De Cremoux *et al.* [39]. The conclusion of the study was that the two cell model systems displayed some similar but also markedly different gene expression characteristics, implying that more gene expression studies of antiestrogen-resistant cell lines are needed for elucidating the mechanisms involved in the development of acquired resistance to antiestrogens.

We have previously established MCF-7 human breast cancer cell variants that serve as in-vitro models of E_2-independent growth (EI cells), and both E_2-independent and antiestrogen-resistant (toremifene) growth (TR cells) [28]. These cell culture models facilitated our study of the gene expression changes that may contribute to the development of E_2-independent growth, acquired resistance to antiestrogen treatment and ultimately development of possible antiestrogen-stimulated growth of breast cancer cells. Of the 1176 genes that were screened, we found five genes (FOSL1, TIMP1, L1CAM, GDF15, and MYBL*2*) that were differentially expressed between the cell lines and differentially regulated by E_2 and/or TOR.

Altered expression of FOSL1 and TIMP1 was observed in the development of E_2-independent and antiestrogen-resistant TR cells whereas L1CAM upregulation was characteristic of EI cells. The induction of FOSL1 and TIMP1 genes was evident already in the early passage number of TR cells, and E_2 or TOR treatment of TR cells

Fig. 5



Toremifene (TOR) regulation of GDF15 and L1CAM expression was studied by real-time RT-PCR in original (pMCF-7), long-term estrogen treated (LE) and toremifene-resistant (TR) cells that represented different passage numbers. E_2-deprived (E_2-) pMCF-7 and LE cells, and TOR-deprived (TOR-) TR cells were treated with 1 μmol/l TOR. GDF15 regulation by TOR is shown in pMCF-7 and LE cells (a) and in TR cells (b). Similarly, L1CAM regulation by TOR is shown in pMCF-7 and LE cells (c) and in TR cells (d). The values represent the geometric average ± geometric standard error (*$P < 0.05$).

had no effect on the expression of these genes. FOSL1 is a transcription factor implicated in multiple human cancers including breast cancer. Elevated FOSL1 expression has been detected in breast epithelioid carcinoma cells [40,41], and a higher expression of FOSL1 is seen, particularly in ER-negative breast cancer cell lines [42,43]. It could be hypothesized that a state that resembles ER negativity, and thus facilitates FOSL1 upregulation, is achieved in our ER-expressing TR cells by the presence of antiestrogen in addition to E_2 deprivation in the culture medium. Long-term E_2 deprivation alone (EI cells) apparently did not cause FOSL1 or TIMP1 induction. TIMP1 expression was downregulated in LE and EI cells as the passage number increased, but maintained in TR cells at the level of original (pMCF-7) cells. Belguise *et al.* [43] have shown a correlation with FOSL1 expression and the expression of some genes implicated in malignant progression and these genes included TIMP1. The authors suggested that FOSL1 upregulated TIMP1 expression in breast cancer cells, though modestly. This regulation could also explain higher TIMP1 expression in our TR cells.

We have previously shown that the growth of the antiestrogen-resistant TR cells is stimulated by TOR [28]. The results of this study also suggested that TOR might function as an E_2 agonist in TR cells. Supporting this hypothesis was the expression pattern of L1CAM and GDF15 in the sublines. L1CAM was found to be E_2-regulated gene in E_2-dependent LE and original pMCF-7 cells, and it became similarly regulated by TOR in the late passage of TR cells. GDF15 seemed to be regulated by TOR in pMCF-7 and LE cells; however, in TR cells the regulation of GDF15 was lost in the passages representing the mid-phase of the long-term culture of TR cells, being retained again in the last passage of the cells. Taken together, E_2 agonism of TOR has probably developed late during the establishment of antiestrogen-resistant phenotype as the E_2 agonism was not observed in the earlier passage numbers of the TR cells. GDF15, a divergent member of transforming growth factor-β superfamily, and the cell adhesion molecule, L1CAM, are both implicated in breast cancer. GDF15 is upregulated by v-akt murine thymoma viral oncogene homolog/protein kinase β (AKT/PKB) in MCF-7 cells conferring antiestrogen resistance [44,45]. L1CAM has been shown to play a role in epithelial–mesenchymal transition-like events in MCF-7 cells in which its overexpression increases motility of the cells and promotes the scattering of epithelial cells from compact colonies [46].

These results show that the gene expression changes that accompany EI growth are different from those observed in the development of antiestrogen-resistant growth. Our findings also suggest a pattern in which the hormone-responsive cancer cells that survive from the E_2 deprivation and/or antiestrogen treatment of several weeks or

months, first acquire necessary changes in gene expression for transition to maximal growth in the new hormonal environment. Then, after prolonged treatment with antiestrogen, the antiestrogen-resistant cells may eventually generate E_2-agonistic response to antiestrogen, probably acquiring additional growth advantage.

Acknowledgements

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Erratum

Anti-Cancer Drugs 2009, **20**:956

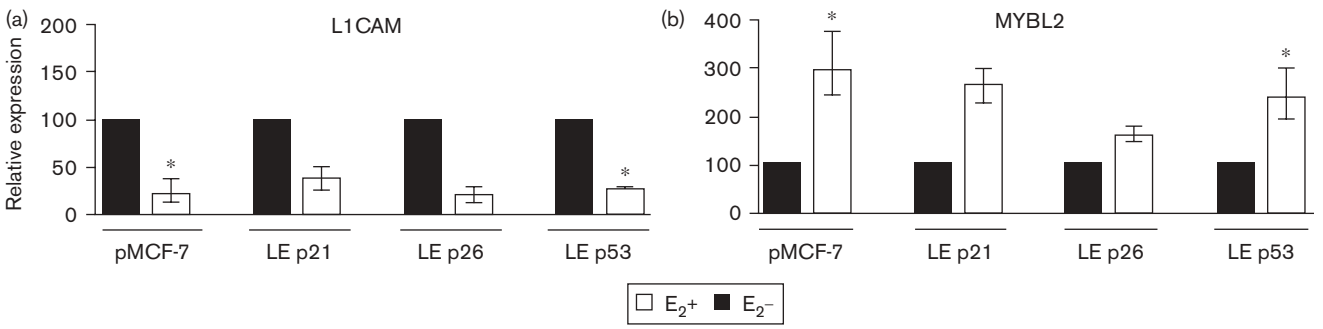
Typographical errors were published in the article 'Gene expression changes during the development of estrogen-independent and anti-estrogen-resistant growth in breast cancer cell culture models' by Pasi T. Pennanen, Nanna S. Sarvilinna and Timo J. Ylikomi, which appeared on pages 51–58 of Anti-Cancer Drugs, Volume 20, Issue 01.

A correct version of figure 4 has been published; the on-line version has now been amended and the correct figure appears below.

Toremifene-regulated genes

The following text was printed incorrectly, '(Fig. 5b, d GDF15 text is missing from Fig. 5b. Similarly L1CAM text is missing from Fig. 5c, d GDF15 text is correctly in Fig. 5a).'

The paragraph should correctly read, 'The cDNA microarray and real-time RT-PCR results indicated that GDF15 and L1CAM were regulated by TOR in TR cells. We wanted to study whether this TOR regulation existed already in the original pMCF-7 cells, or was it evolved during the long-term culture. Therefore we assessed the TOR regulation of these genes in pMCF-7 and three passages of LE cells (p21, p26, and p53), and in three passages of TR cells (p16, p23, and p47). The expression of both GDF15 and L1CAM was regulated in TR cells by TOR in the last passage of the cells only (Fig. 5b, d). GDF15 was modestly regulated by TOR in pMCF-7 cells and all passages of LE cells (Fig. 5a), whereas the expression of L1CAM was mostly unaltered by TOR in these cells (Fig. 5c). Interestingly, TOR regulation of L1CAM in TR cells was essentially similar to its regulation by E2 in pMCF-7 and LE cells (compare with Fig. 4a).



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Inhibition of FOSL1 overexpression in antiestrogen-resistant MCF-7 cells decreases cell growth and increases vacuolization and cell death

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ABSTRACT

Elevated activator protein-1 (AP-1) activity in breast cancer cells has been linked to Tamoxifen (TAM) resistance. Fos-like antigen-1 (FOSL1) is a member of the AP-1 transcription factor and is overexpressed in a variety of human cancers including breast tumors. We have previously established an estrogen-independent and antiestrogen Toremifene (TOR)-resistant subline of MCF-7 breast cancer cells. In these cells, the expression of FOSL1 is upregulated when compared to the parental cells. In the present study, partial inhibition of FOSL1 expression in these cells by small interfering RNA resulted in a marked decrease of cell growth. The inhibition of cell growth paralleled with changes in cell morphology such as increased formation of vacuoles followed by an increase in the number of dead cells. The inhibition of FOSL1 expression in these cells also restored sensitivity to TOR. Our results suggest that chemotherapy targeting overexpression of FOSL1 could be a potent strategy for treating endocrine resistant breast cancers.

1. Introduction

Elevated activator protein-1 (AP-1) DNA binding activity has been linked to Tamoxifen (TAM)-resistant breast cancer. AP-1 transcription factor functions as a dimer mainly consisting of proteins encoded by JUN (C-JUN, JUNB, JUND) and FOS (C-FOS, FOSL1, FOSL2, FOSB) protein families. In the AP-1 driven mechanism, estrogen (or antiestrogen) binds to estrogen receptor (ER), which in turn binds to dimerized AP-1. This complex can modulate gene expression at the promoters that contain AP-1 sites. It has been suggested that the enhanced AP-1 activity may bypass hormone dependence and/or associate with an agonistic TAM response in ER-positive TAM-resistant breast cancers [1–5].

The role of the AP-1 component Fos-like antigen-1 (FOSL1, also known as FRA-1) has been studied extensively in breast cancer. Several studies have demonstrated a crucial role for high FOSL1 expression in transformation, increased motility and invasiveness of breast cancer cell lines [6–12]. FOSL1 expression also correlated with ER-negativity of breast cancer cells *in vitro*. FOSL1 mRNA and protein levels were found to be high in ER-negative breast cancer cell lines, whereas its expression was low or undetectable in breast cancer cell lines that were ER-positive [10,13–16]. In contrast with the *in vitro* data, the negative correlation of ER and FOSL1 has not been clearly demonstrated in the *in vivo* studies investigating breast carcinomas ranging from benign disease to aggressive breast carcinomas. However, the conclusion of these *in vivo* studies was that FOSL1 overexpression is associated with aggressive and highly malignant breast carcinomas [14,15,17,18].

We have previously established an MCF-7-derived cell line which is estrogen-independent and antiestrogen toremifene (TOR)-resistant (TR cells). These cells are ER-positive, are growth-stimulated by estrogen and stably overexpress FOSL1 mRNA [19,20]. This cell line provided us a model where FOSL1 overexpression is not negatively correlated with ER expression. This expression pattern has not been shown previously *in vitro* in breast cancer cell lines, but it exists *in vivo* as discussed above. In the present study, we assessed the effect of the inhibition of FOSL1 mRNA on the growth of TR cells, both in the presence and absence of TOR. An automated pattern analysis machine vision system was applied to evaluate morphological changes induced by FOSL1 inhibition in these cells.

2. Experimental

2.1. Hormones and cell culture reagents

17β-Estradiol (E_2), insulin and phenol red-free DMEM/F-12 (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham)

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were purchased from Sigma (St. Louis, MO, USA). The anti-estrogen toremifene citrate (TOR) was kindly provided by Orion Pharma (Turku, Finland). The antibiotics penicillin and streptomycin, and DCC-FBS (dextran charcoal-treated foetal bovine serum) were obtained from GIBCO (Invitrogen, CA, USA). All disposable cell culture materials were purchased from Nunc (Apogent Technologies Inc., Portsmouth, NH, USA).

2.2. Cell culture

The establishment of long-term estrogen-treated (LE), estrogen-independent (EI) and estrogen-independent toremifene-resistant (TR) MCF-7 sublines has been described previously [19]. Briefly, E_2- and antiestrogen-sensitive, ER- and PgR-positive MCF-7 human breast cancer cells representing passage number 11 were used as parent cells to generate these sublines. LE cells have received vehicle (96% ethanol) and 1 nM E_2, TR cells 1 μM TOR and EI cells vehicle only continuously for 9 months. In the present study, parental MCF-7 (pMCF-7), LE, EI and TR cells were routinely cultured in 75 cm^2 flasks at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in phenol red-free DMEM/F-12 supplemented with 5% DCC-FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml) and insulin (10 ng/ml). The medium with appropriate hormones was changed every 2 or 3 days.

2.3. RNA interference

SiRNA SMARTpool® reagents were from Dharmacon (Lafayette, CO, USA), including FOSL1 siRNA, Non-Targeting siRNA, 5× siRNA buffer and DharmaFECT®1 Transfection Reagent. For RNA and protein studies, the cells were seeded to 25 cm^2 flasks 1×10^5 cells/flask and allowed to attach for 24 h. For transfection, the cells were then subjected to 50 nM target siRNA (FOSL1), 50 nM non-target siRNA (siRNA control), transfection reagent (Mock transfection without siRNA), or medium only for 24 h according to the manufacturer's instructions. siRNA concentrations higher than 50 nM proved to be too toxic for the cells to survive the 72 h experiment. After transfection, the cells were cultured in normal growth medium for 24–72 h (RT-PCR) or 72 h (Western Blot) and then subjected to Trizol (GIBCO) reagent for RNA extraction or M-PER® (PIERCE) reagent for protein extraction. For the cell growth studies, the cells were seeded to 96-well plates 1×10^3 cells/well and allowed to attach for 24 h. Next, the cells were transfected for 24 h with the indicated siRNA concentrations. The transfection medium was then replaced by normal growth medium in the presence or absence of 1 μM TOR. Medium was renewed every second day. Here, lower siRNA concentrations were used (5–20 nM), for the 50 nM siRNA concentrations proved to be too toxic for the cells to survive the 7-days cell growth experiment. The cells were fixed, stained and the relative number of cells was counted every 24 h using crystal violet staining method as described by Kueng et al. [21]. Absorbance was measured at 590 nm wavelength using a Victor 1420 Multilabel counter (Wallac Inc., Turku, Finland).

2.4. Automated pattern analysis by machine vision system

The automated cell culturing and pattern analysis platform (Cell-IQ, Chip-Man Technologies, Tampere, Finland) was used to measure cell growth and to analyze changes in cell morphology in the siRNA experiment. The Cell-IQ system consists of a special cell culture incubator with an inbuilt microscope and camera system. The system and its applications have been described previously [22]. In the siRNA experiment, the cells were seeded to 24 well plates 4×10^3 cells/well and allowed to attach for 24 h. For transfection, the cells were then subjected to 10 nM target siRNA (FOSL1), 10 nM non-target siRNA (siRNA control), transfection reagent (Mock transfection without siRNA), or medium only for 24 h. The transfection medium was then replaced by normal growth medium (1000 μl/well). The 24-well plate was then placed in the incubator and the automated monitoring and image capturing were continued for 7 days. Growth medium was not changed during this time. The experiment was carried out in the absence of TOR. There were 4 parallel wells for each treatment, and 3 parallel images were taken at random spots from every well in every 15 min. The resultant captured images were analyzed with the Cell-IQ software. In this experiment, the analyzer was taught to detect stable, dead and vacuolized cells.

2.5. SDS–PAGE and Western Blot

Cells were subjected to M-PER® (PIERCE, Rockford, IL, USA) reagent modified with protease inhibitors (Complete Mini Protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Indianapolis, IN, USA)) for protein extraction according to manufacturer's instructions. Total protein concentrations were measured using BCA Protein Assay Kit (PIERCE) according to manufacturer's instructions. 100 μg of total protein was mixed with 2% sodium dodecyl sulfate (SDS) buffer, boiled for 5 min and analyzed by electrophoresis in 12% polyacrylamide gel (PAGE). Proteins separated by PAGE were transferred (2 h) to the nitrocellulose membrane (0.45 μm pore, Schleicher and Schuell, Germany) at room temperature (RT) using transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3. Membranes were incubated for 1 h at RT in Tris buffer containing salt and Tween (TBST) (50 mM Tris–HCl, 150 mM NaCl, 0,05% Tween 20, pH 8.0) and 5% non-fat dry milk powder to saturate the non-specific protein binding sites. Membranes were then incubated with FOSL1 primary antibody (Fra-1, C-12) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:10,000 in 5% BSA-TBST overnight at 4 °C with mild agitation. The membranes were washed 3 times for 10 min with TBST and incubated for 1 h with horse radish peroxidase (HRP) – conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology Inc.) in 1:4000 dilution in 5% non-fat milk-TBST with mild agitation at RT. The membranes were washed 3 times for 10 min with TBST and subjected to enhanced chemiluminescence reagents (ECL, UK) according to manufacturer's instructions and exposed to X-ray film. 3611-RF nuclear extract (Santa Cruz Biotechnology Inc.) was used as positive control for FOSL1 primary antibody, and anti-beta-actin (AC-15) (Sigma) was used as loading control.

2.6. Real-time RT-PCR

The RNA samples were reverse transcribed to cDNA with High Capacity Archive Kit (Applied Biosystems, CA, USA) following the instructions of the manufacturer. The real-time RT-PCR was done with SYBR Green PCR Master Mix Kit (Applied Biosystems) in ABI PRISM 7000 Detection System (Applied Biosystems) according to the manufacturer's instructions. The data were analyzed by ABI PRISM 7000 SDS Software (Applied Biosystems). The final results, expressed as *N*-fold relative differences (ratio) in gene expression between the studied samples and the control (i.e. calibrator) sample, were calculated according to the following equation [23]: $\text{ratio} = ((E_{\text{target}})^{\Delta \text{CP target (control}-\text{sample)}})/((E_{\text{ref}})^{\Delta \text{CP ref (control}-\text{sample)}})$. E_{target} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; $\Delta CP_{\text{target}}$ is the CP (crossing point) deviation of control – sample (subtraction) of the target gene transcript; ΔCP_{ref} is the CP deviation of control – sample of reference gene (RPLP0; ribosomal protein, large, P0) transcript. Real-time PCR efficiencies (E) were calculated, according to $E = 10^{[-1/\text{slope}]}$. The following primers (TAG, Copenhagen, Denmark) were used: FOSL1 (f) 5′-GGA GGA AGG AAC TGA CCG ACT T-3′ and FOSL1 (r) 5′-TGC AGC CCA GAT TTC TCA TCT-3′.

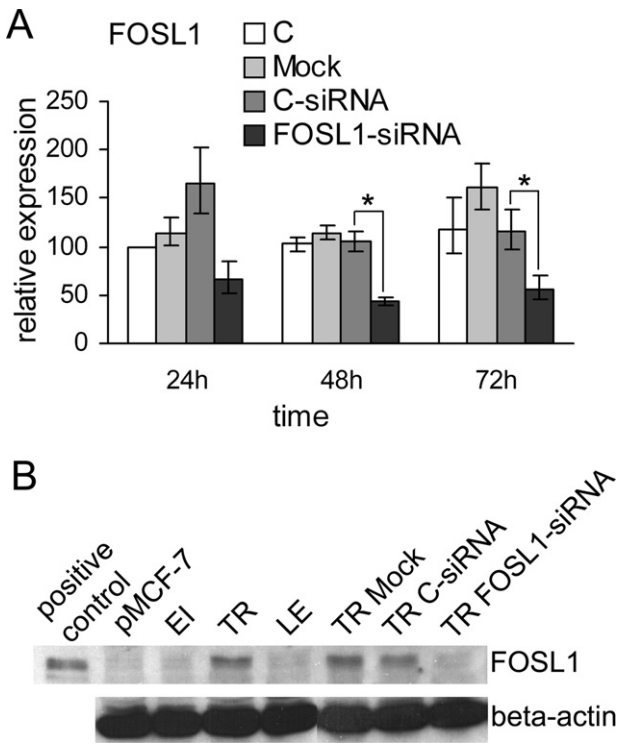

Fig. 1. The effect of small interfering RNA (siRNA) transfections on the expression level of FOSL1 mRNA and protein in antiestrogen-resistant cells. For the mRNA studies (A), cells were grown in the presence of normal medium (C) and treated with transfection reagent (Mock), control siRNA (C-siRNA) or FOSL1 siRNA (FOSL1-siRNA). Expressions were calculated relative to the 24 h control sample, which was given the arbitrary number 100. Values represent the average and standard deviation of three independent experiments (*$P < 0.05$). FOSL1 protein levels (B) were analyzed by Western Blot in original MCF-7 (pMCF-7), estrogen independent (EI), long-term estrogen-treated (LE), antiestrogen-resistant (TR) cells, and in 3611-RF nuclear extract (positive control). TR cells were incubated with transfection reagent only (TR mock), control siRNA (TR C-siRNA) and FOSL1 siRNA (TR FOSL1-siRNA). Western Blots represent the 72 h time point. Beta-actin was used as a loading control.

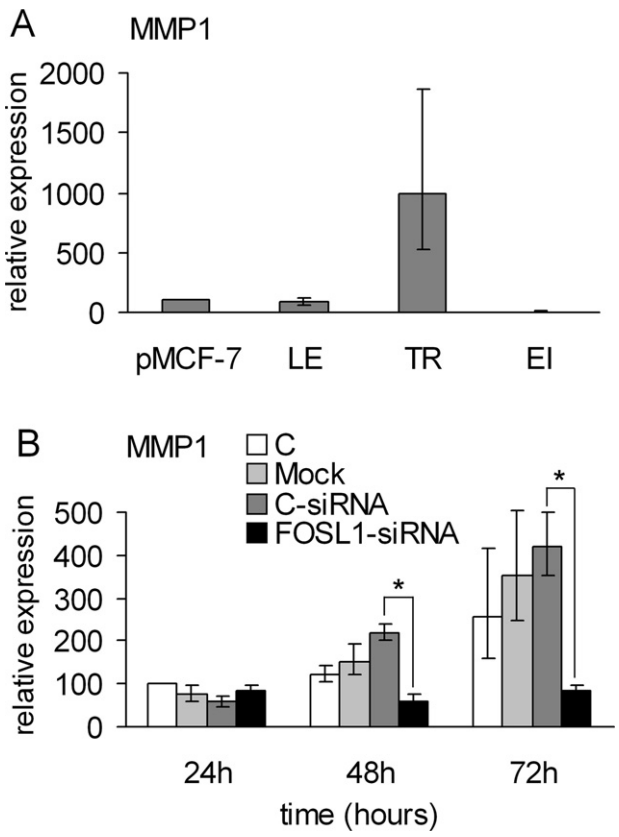

Fig. 2. The mRNA expression of MMP1. (A) The expression of MMP1 was measured in parental MCF-7 cells (pMCF-7), long-term estrogen-treated (LE), antiestrogen-resistant (TR) and estrogen-independent (EI) cells. Expressions were calculated relative to the pMCF-7 sample which was given the arbitrary value 100. (B) The effect of small interfering RNA (siRNA) transfections on the expression of MMP1 gene in TR cells. The cells were grown in the presence of normal medium (C) and treated with transfection reagent (Mock), control siRNA (C-siRNA) or FOSL1 siRNA (FOSL1-siRNA). Expressions were calculated relative to the 24 h control sample, which was given the arbitrary number 100. The values represent the geometric average and geometric standard error of four independent experiments (*$P < 0.05$).

MMP1 (f) 5′-TTC ACC AAG GTC TCT GAG GGT CA-3′ and MMP1 (r) 5′-GCA AGA TTT CCT CCA GGT CCA-3′. RPLP0 (f) 5′-AAT CTC CAG GGG CAC CAT T-3′ and RPLP0 (r) 5′-CGC TGG CTC CA CTT TGG T-3′. The primers were designed using Primer Express software for ABI PRISM 7000 detection system (Applied Biosystems).

3. Results

3.1. FOSL1 was upregulated in toremifene-resistant cells

The level of FOSL1 mRNA is upregulated (3-fold) in toremifene-resistant (TR) cells when compared to estrogen-independent (EI), long-term estrogen treated (LE) and parental pMCF-7 sublines [20]. The upregulation of FOSL1 in TR cells was also evident at the protein level (Fig. 1B). To investigate the role of FOSL1 in TR cells, we inhibited its expression in these cells by siRNA method. FOSL1-siRNA treatment of TR cells down-regulated FOSL1 mRNA and protein close to the level observed in the other sublines, thus the siRNA-mediated knock-down of FOSL1 was partial in TR cells (Fig. 1A and B).

Matrix metallopeptidase 1 (MMP1) expression has been shown to be induced in a system where MCF-7 cells were stably transfected to overexpress FOSL1 [10,24]. In the present study, we measured the mRNA expression of MMP1 in the subclones, and in the siRNA transfected TR cells. MMP1 mRNA was indeed highly induced in the FOSL1 overexpressing TR cells when compared to pMCF-7, EI and LE cells (Fig. 2A). FOSL1 downregulation by siRNA resulted in a decrease of MMP1 mRNA expression in TR cells showing that the partial inhibition of FOSL1 was effective (Fig. 2B).

3.2. FOSL1 inhibition suppressed cell growth

We then investigated the effect of FOSL1 inhibition on the growth of TR cells by crystal violet staining method. Inhibition of FOSL1 decreased the growth rate of TR cells drastically, and the effect was dose-dependent. Similar effect, but considerably weaker, could be seen in the control siRNA-transfected cells. Transfection reagent alone (Mock) also produced a slight decrease in cell growth (Fig. 3A–C). We have previously shown that TOR stimulates the growth of TR cells [19]. Similarly, in the present study TOR increased the growth of untreated and Mock-treated cells, whereas the stimulatory effect was not observed in 10 nM and 20 nM control siRNA-transfected cells. In the cells transfected with 10 nM and 20 nM FOSL1 siRNA, the effect of TOR was reversed from growth stimulation to growth inhibition measured after seven days of growth. However, this finding was not statistically significant when compared to 10 nM and 20 nM control siRNA treated cells. As seen with control siRNA, the siRNA treatment itself reduces the viability of the cells and renders them more sensitive to TOR. Nevertheless, the growth of FOSL1-siRNA-treated cells was inhibited by TOR more efficiently than the growth of control-siRNA-treated cells (Fig. 3D and E).

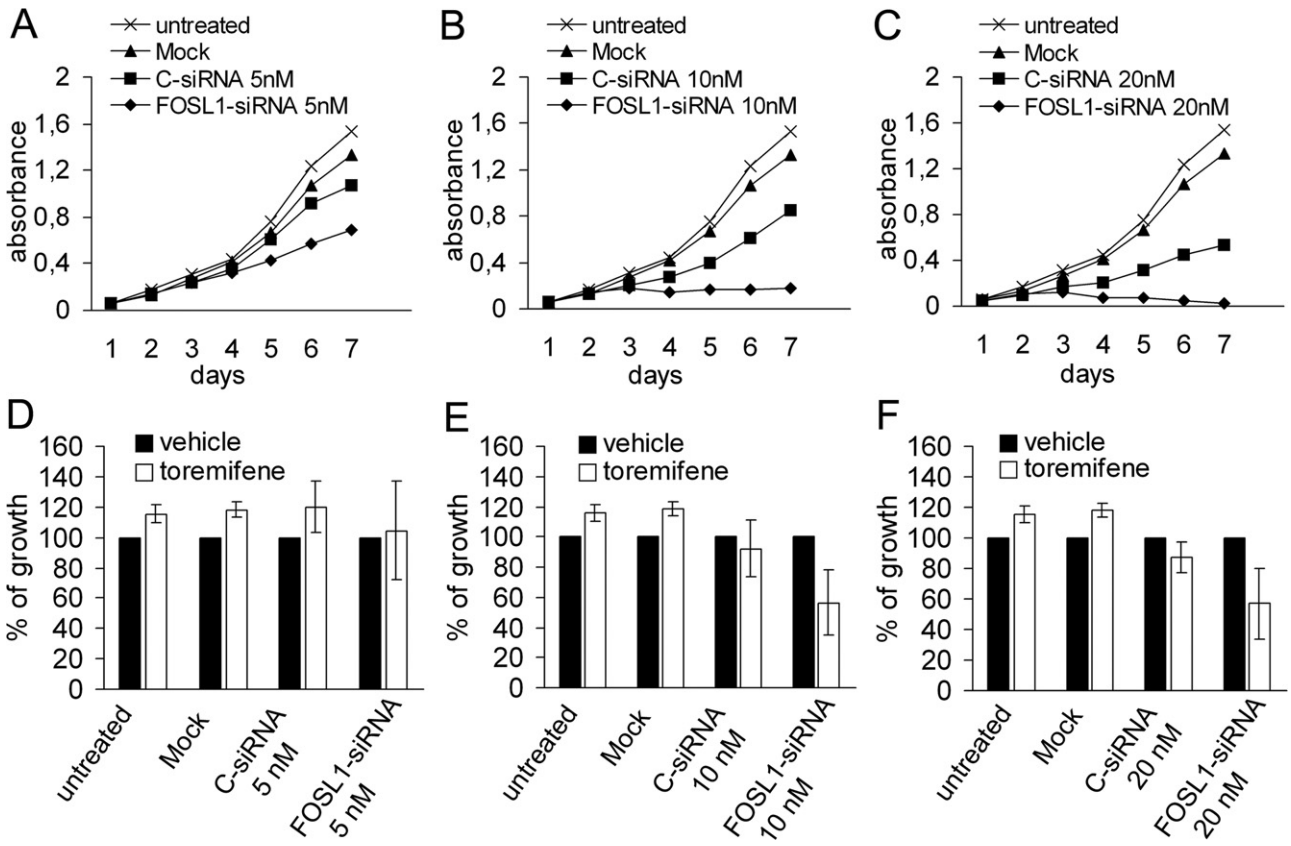

Fig. 3. The growth of antiestrogen-resistant cells. (A–C) The growth curves of untreated cells (untreated) and cells treated with transfection reagent (Mock), 5–20 nM control siRNA (C-siRNA) and 5–20 nM FOSL1 siRNA (FOSL1-siRNA) are shown. The cells were grown in the absence of toremifene. The values represent the average of four independent experiments. (D–F) The effect of toremifene on the growth of antiestrogen-resistant cells treated with Mock, 5–20 nM C-siRNA, 5–20 nM FOSL1-siRNA and untreated cells. Percent (%) of change in cell growth caused by 1 μM toremifene with different treatments is shown. The values represent the average and standard deviation of three independent experiments.

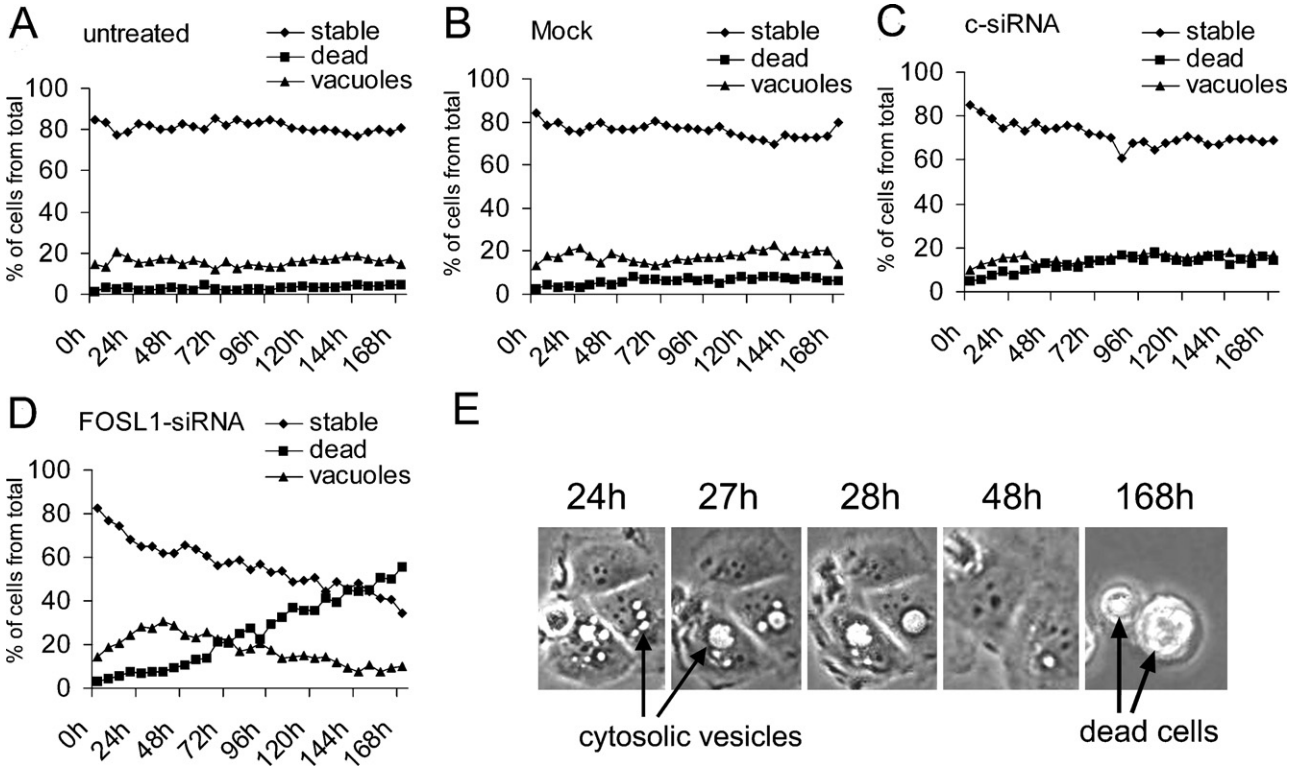

Fig. 4. Pattern recognition by automated machine vision system. (A–D) Stable, dead and vacuolized (cytosolic vesicles) cells counted by the automated machine vision system in different treatments. (E) Cytosolic vesicles containing cells and dead cells (arrows) shown in different time points (24–168 h) from the start of the analysis. Untreated cells and cells treated with transfection reagent (Mock), 10 nM control siRNA (c-siRNA) and 10 nM FOSL1 siRNA (FOSL1-siRNA) are shown.

3.3. Distinct cellular morphological changes caused by FOSL1 inhibition

Changes in cell growth and morphology were assessed by automated pattern analysis machine vision system. 10 nM siRNA concentrations were used in this experiment. The effect of siRNAs on cell growth measured by machine vision was similar to the crystal violet staining method. The analyzer software was taught to detect stable (living), dead and vacuolized cells. The FOSL1-siRNA-treated group contained higher amount of vacuolized cells during the first 48 h, and there was a subsequent rise in the number of dead cells and a decline in the number of stable cells towards the end of the experiment. These features were not observed in other treatment groups (Fig. 4A–D). Fig. 4E shows the formation of several smaller vacuoles which then merge to form larger vacuoles in FOSL1-siRNA-treated cells. This is followed by a decrease in the amount of vacuoles and an increase in the amount of dead cells.

4. Discussion

High FOSL1 expression has been shown to correlate with the absence of ER expression and with estrogen-independent growth in breast cancer cell lines [10,13–15]. Similarly, in the *in vivo* studies by Bamberger et al. [15] and Nakajima et al. [14] FOSL1 expression correlated negatively with the ER status in breast cancer tissues [14,15]. Yet, this correlation might not be dominant *in vivo*, since some recent studies demonstrated that high FOSL1 expression did not correlate with ER-negativity in breast cancers [17,18]. In the present study, we used an MCF-7-derived estrogen-independent and antiestrogen-resistant subline (TR cells), which is an example of the *in vivo* existing ER-positive phenotype since the cells express functional ER [19] and overexpress FOSL1 [20].

In the present study, the expression of FOSL1 was decreased in TR cells by siRNA method. It is noteworthy, that the decrease was partial i.e., the mRNA and protein level of FOSL1 decreased to a level observed in the other sublines. In the cell growth studies, the inhibition of cell growth by FOSL1 siRNA was drastic in these cells. This indicates that FOSL1 overexpression is essential for the growth of TR cells. Another observation from the cell growth studies was that the effect of TOR was reversed from growth stimulation to growth inhibition upon FOSL1-siRNA treatment.

The effect of FOSL1 inhibition was analyzed further by automated pattern analysis machine vision system. The analysis revealed an increased formation of vacuoles during the first 48 h and eventually increased cell death in the FOSL1-treated cells. The observed vacuoles could be autophagic vesicles and related to autophagic cell death. Autophagic cell death is characterized by the appearance of cytoplasmic vesicles engulfing bulk cytoplasm or cytoplasmic organelles such as mitochondria and endoplasmic reticulum [25].

The mRNA expression of MMP1 was highly induced in TR cells when compared to the other sublines, and down-regulated by FOSL1 siRNA treatment. MMP1 has an AP-1 site in its promoter and it is a known AP-1 [26] and FOSL1-upregulated [10,24] gene in breast cancer cells. This could mean that TR cells possess increased AP-1 binding activity due to induced FOSL1 expression. It has been shown, that high AP-1 binding activity could be a result of high FOSL1 expression in breast cancer cells [13]. Furthermore, it has been shown that the inhibition of AP-1 activity can restore TAM sensitivity of resistant breast cancer cells [2,3]. Similarly, TOR sensitivity was restored in TR cells by inhibition of FOSL1 expression. Taken together, our study supports the idea that targeting FOSL1 or AP-1 could be a highly sensitive therapy for the endocrine-resistant, both ER-negative and ER-positive, breast cancers that overexpress FOSL1.

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The effect of nuclear receptor ligands on the growth of antiestrogen-resistant and estrogen-independent breast cancer cells

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Abstract

The aim of this study was to determine how physiological or pharmacological concentrations of tamoxifen (TAM), medroxyprogesterone acetate (MPA), 1,25-dihydroxyvitamin D_3 (calcitriol), retinol and estrogen receptor-β-selective ligand diarylpropionitrile (DPN) affect the cell growth of parental MCF-7 breast cancer cells (pMCF-7) and their toremifene-resistant (TR) and estrogen-independent (EI) sublines. Treatments were carried out both in the presence and absence of 17β-estradiol (E_2). ERβ-selective ligand DPN stimulated the growth of the cell lines similarly to E_2, but for that DPN was needed at 100-fold higher concentration than E_2. This suggests that DPN alone may not be suitable for treatment of hormone-resistant breast cancer. A novel finding was also that TR cells were highly cross-resistant to (TAM). Calcitriol inhibited the growth of all cell lines and E_2 had no marked effect on that, but the growth inhibitory effect of retinol was clearly increased in the presence of E_2 in all cell lines. An intriguing observation was that MPA dose-dependently inhibited the growth of EI cells, and that the presence of E_2 abrogated the inhibitory effect. These findings warrant further studies on the effects of combined low-dose estrogen and retinoids in breast cancer, and combined aromatase inhibitor (AI) and synthetic progestins on AI-resistant breast cancer.

Key words: breast cancer, estrogen-independent, antiestrogen resistance, vitamin D, progestin, retinoid

Introduction

The *in vivo* data of the molecular mechanisms of acquired endocrine resistance in breast cancer originates largely from tumor biopsies. It has been shown that the molecular determinants of acquired endocrine resistance include changes in the functional activity of ERα, changes in the expression levels of ERα and its cofactors and progesterone receptor (PgR), and activation of various growth factor receptors and their downstream signaling pathways [1-3]. However, lack of tumor tissue for detailed studies of acquired resistance to endocrine therapy is a major hindrance of *in vivo* studies. It is difficult to obtain paired tumor tissues for biomarker studies immediately before treatment and again at the time that resistance develops. At the moment, a substantial amount of studies concerning acquired resistance to antiestrogens and aromatase inhibitors (AI) emerge from breast cancer cell culture models. In these models, cells that are estrogen-independent and/or resistant to different forms of endocrine therapies have been established from hormone-dependent parental cells by long-term culture [4-6].

We have previously established MCF-7-derived cell lines by long-term culture in the absence of estrogen (17β-estradiol, E_2), or in the absence of E_2 and presence of antiestrogen toremifene (TOR) [7]. Majority of the studies with endocrine-resistant cell lines have focused on ER function or on the role of growth factor receptors and their down-stream signaling pathways [8-13]. Less attention has been drawn to the effects of different nuclear receptor ligands on the growth of the resistant cell lines.

The aim of the present study was to study the effects of different nuclear receptor ligands on the growth of estrogen-independent (EI), TOR-resistant (TR) and parental MCF-7 cells (pMCF-7). The cells were treated with medroxyprogesterone acetate (MPA), 1α,25$(OH)_2D_3$ (calcitriol), tamoxifen (TAM), retinol and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) with and without 17β-estradiol (E_2). In the present study, the nuclear receptor ligands were used in concentrations which are close to the observed physiological concentrations of the natural nuclear receptor ligands, or pharmacological concentrations of the synthetic ligands (Table 1).

Experimental

Hormones and Antihormones

17β-Estradiol (E_2), 6α-methyl-17α-hydroxy-progesterone acetate (MPA), retinol and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antiestrogens toremifene (TOR) and tamoxifen (TAM) were provided by Orion Pharma (Turku, Finland). 1α,25$(OH)_2D_3$ (Vitamin D_3) was obtained from Leo Pharmaceuticals (Denmark). 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) was a gift from Orion Pharma. Stock solutions of hormones and antiestrogens were diluted in 96 % ethanol, stored at –20 °C and added to the treatment medium. All controls received ethanol (vehicle) at a concentration equal to that of the hormone-treated cells. The concentration of ethanol did not exceed 0.1 %.

Cell Lines

The establishment of TOR-resistant (TR) and estrogen-independent (EI) cell lines has been described previously. Briefly, MCF-7 breast cancer cells were used as parent cells. The TR cells were established by culturing the MCF-7 cells in the presence of 1μM TOR (without E_2) for 9 months. EI cells were established by culturing the MCF-7 cells in the absence of E_2 for 9 months, and they model both postmenopausal and AI-treated breast cancer. The original MCF-7 cells were used both as control cells and as a model of premenopausal breast cancer. The cell lines have similar basal expression (i.e. expression without hormones) of ERα and ERβ mRNA, and slight differences in the expression of PgR mRNA. The expression of PgR mRNA is highly induced by E_2 in these cell lines. As PgR is a known E_2-regulated gene, this would indicate that ERα is still functional in these cells [7]. The expression profiles of VDR, RAR or GPR30 have not been studied in these cell lines

Cell Culture

The cells were routinely cultured in T-75 flasks at 37 °C under an atmosphere of 5 % CO_2. The medium used was phenol red-free DMEM/F12 (Sigma-Aldrich) supplemented with 5 % dextran charcoal-stripped (steroid-depleted) fetal bovine serum (GIBCO, Invitrogen Corporation, UK), penicillin (100 IU/ml), streptomycin (100μg/ml) and insulin (10ng/ml). All disposable cell culture materials were purchased from Nalge Nunc International (NY, USA).

Cell Growth Assay

The cells were grown without hormones for 6 days, and then seeded on 96-well plates (750 cells /well). The cells were allowed to attach for 2 days (without hormones) and subsequently treated with the indicated hormones for six days. Growth medium and hormones were renewed every other day. After six days treatment the cells were fixed, stained and the relative number of cells was assessed with the crystal violet staining as described Kueng et al. [14]. Briefly, cells were fixed with 1 % glutaraldehyde and stained by addition of 0.1 % solution of crystal violet on the 96-well plates. The bound dye was solubilized to 100 μl of 10 % acetic acid. The absorbance was measured using a Victor 1420 Multilabel counter (Wallac Inc., Turku, Finland) at 590nm wavelength. Each experiment was repeated three times. The following hormones (concentrations) were used: TAM (0.1 μM and 1 μM), MPA (0.1 nM, 1nM and 10 nM), retinol (0.1 μM, 1 μM and 10 μM), calcitriol (1 nM, 10 nM and 100 nM) and DPN (1 nM, 10 nM and 100 nM). The treatments were done both in the presence and absence of 1 nM E_2.

Statistical Analysis

All experiments were repeated three times. The quantitative data are expressed as geometric mean ± geometric standard error. Significance was assessed by using Student's paired t-test. P value of less than 0.05 was considered as statistically significant difference.

Results

First, we studied the effect of the ERβ-selective ligand DPN on the growth of pMCF-7, EI and TR cells. The cells were treated with three concentrations of DPN. DPN stimulated the growth of estrogen-dependent pMCF-7 cells at the highest 100 nM concentration. The level of growth stimulation by 100 nM DPN was similar to that of 1 nM E_2 in pMCF-7 cells (Fig. 1A-B). When DPN was added together with 1 nM E_2, no additive stimulatory effect on growth was observed, but a lower concentration of DPN (10 nM) slightly inhibited the stimulatory effect of E_2 on pMCF-7 (Fig. 1C).

TAM inhibited the growth of original pMCF-7 cells in the presence of E_2. The growth of EI cells was also inhibited by TAM, but in this case the inhibition was diminished in the presence of E_2. As expected, TR cells were highly resistant to TAM (Fig. 2A-B). MPA alone inhibited the growth of EI cells, whereas TR cells were resistant to it. In the presence of E_2, the growth inhibitory effect was lost in EI cells (Fig. 2C-D). We also wanted to see if DPN could restore sensitivity to TAM in TR cells, or potentiate the effect of 10 nM MPA in EI cells. DPN was used in 1 nM concentration because of the observed growth stimulation of higher concentrations of DPN, which could be caused by its known binding to ERα [15]. Nevertheless, 1 nM DPN did not change the effect of TAM on TR cells, and it did not enhance or block the effect of MPA on EI cells (data not shown).

Calcitriol inhibited the growth of all cell lines at pharmacological 100 nM concentration. In the presence of E_2, the same pattern of inhibition was observed but it did not reach statistical significance in EI and TR cells (Fig. 3A-B). Retinol alone inhibited the growth of EI and TR cells, but not pMCF-7 cells, and furthermore, 1 µM retinol stimulated the growth of E_2-deprived pMCF-7 cells. When retinol was administered together with E_2, all cell lines were growth-inhibited, and the inhibition was markedly stronger compared to retinol alone (Fig. 3C-D).

Discussion

There is evidence that ERβ may act as a tumor suppressor in breast cancer [16-18], but studies on the effect of selective ERβ ligands on endocrine-resistant breast cancer are lacking. Nair et al. (2011) showed that letrozole-resistant LTLT cells and its parental MCF-7aro (aromatase-transfected) cells were growth inhibited by DPN *in vitro*, and that ERβ expression was required for this effect. However, in their LTLT mouse xenografts, combined DPN/letrozole was needed to suppress tumor growth, and DPN alone stimulated tumor growth [19]. Our cell lines express ERβ and its expression is induced by E_2 in these cells [7]. In the present study, DPN alone (similarly to 1 nM E_2) did not inhibit the growth of resistant cell lines, and it stimulated the growth of pMCF-7 cells. It could be concluded that DPN may not be suitable to treat hormone-dependent breast cancer.

In the present study, TR cells were highly resistant to TAM both in the presence and absence of E_2. This has not been reported in earlier studies, although cross-resistance of TAM-resistant cells with TOR has been previously described in several studies [20-22]. We have previously shown that TOR inhibits the growth of EI cells [7]. The growth of these cells was also inhibited by TAM, but the inhibitory effect was diminished in the presence of E_2.

Original pMCF-7 cells were only slightly growth-inhibited by MPA in the presence of E_2, and TR cells were largely resistant to it, both in the presence and absence of E_2. However, the growth of EI cells was dose-dependently inhibited by MPA, and E_2 totally blocked the inhibitory effect. EI cells model AI-treated breast cancer. It could be hypothesized that if AI is the first line treatment, then AI-treatment would be maintained if MPA was used as the second line treatment after relapse. This is because withdrawing AI-treatment would allow production of E2, which in our EI-cells blocked the growth inhibitory effect of MPA.

Previous studies have shown that calcitriol and its analogues inhibit the growth of breast cancer cells irrespective of the presence of ER [23-25]. Calcitriol also inhibits the growth of antiestrogen-resistant derivatives of ER-positive breast cancer cells [26]. In our study, calcitriol inhibited the growth of all cell lines and E_2 had no marked effect on that. We did not observe any increased sensitivity of resistant cells towards treatment with calcitriol.

Several reports have demonstrated that physiological and pharmacological concentrations of retinol, or its active metabolite all-trans retinoic acid (ATRA), inhibit the growth of MCF-7 cells [27-30]. There are only few *in vitro* studies on the effect of retinoids on the growth of endocrine-resistant breast cancer cells. Butler et al. showed that TAM-resistant subclone of MCF-7 cells was resistant to 1 µM ATRA [27]. In the study of Stephen et al., estrogen-independent MCF-7 cells remained growth inhibited by 1 µM ATRA [30]. In our study, retinol inhibited the growth of all cell lines except E_2-deprived pMCF-7 cells. A new finding was that the effect of retinol was potentiated in the presence of E_2 in all cell lines.

Taken together, the most interesting findings of the present study were the combined effects of E_2 and retinol, and the observation that MPA inhibited the growth of EI cells, but the inhibition was diminished in the presence of E_2. In our study, physiological concentrations of retinol inhibited the growth of all cell lines, especially in the presence of E_2. This is interesting since low-dose estrogen therapy is a new promising approach to treat estrogen-independent and antiestrogen-resistant hormone receptor-positive breast cancer [31]. In clinical trials, it has been shown that retinoids like all-trans retinoic acid or 13-cis retinoic acid do not have significant activity in patients with hormone-refractory metastatic breast cancer [32, 33]. This lack of congruence with our study could be due to possible low estrogen levels in these patients. An intriguing finding was that MPA dose-dependently and at low pharmacological concentrations inhibited the growth of estrogen-independent cells, and that the presence of E_2 abrogated the effect. These findings warrant further studies on the effects of combined low-dose estrogen and retinoids in breast cancer, and AIs and synthetic progestins on acquired AI-resistant breast cancer.

Acknowledgements

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Figure legends

Fig. 1. The effect of 17β-estradiol (E_2) (A) and different concentrations of 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) on the growth of original MCF-7 (pMCF-7), estrogen-independent (EI) and toremifene-resistant (TR) cells in the absence (B) and presence (C) of 1 nanomolar (nM) E_2. Growth of the treated cells was calculated as percent (%) of growth compared to vehicle (ethanol) or E_2 –treated cells, which were given the value 100 %. The values represent the geometric average and geometric standard error of three independent experiments (*$P < 0.05$).

Fig. 2. The effect of different concentrations of tamoxifen (TAM) and medroxyprogesterone acetate (MPA)on the growth of original MCF-7 (pMCF-7), estrogen-independent (EI) and toremifene-resistant (TR) cells in the absence (A and C) and presence (B and D) of 1 nanomolar (nM) 17β-estradiol E_2. Growth of the treated cells was calculated as percent (%) of growth compared to vehicle (ethanol) or E_2 –treated cells, which were given the value 100 %. The values represent the geometric average and geometric standard error of three independent experiments (*$P < 0.05$).

Fig. 3. The effect of different concentrations of calcitriol and retinol on the growth of original MCF-7 (pMCF-7), estrogen-independent (EI) and toremifene-resistant (TR) cells in the absence (A and C) and presence (B and D) of 1 nanomolar (nM) 17β-estradiol E_2. Growth of the treated cells was calculated as percent (%) of growth compared to vehicle (ethanol) or E_2 –treated cells, which were given the value 100 %. The values represent the geometric average and geometric standard error of three independent experiments (*$P < 0.05$).

Table 1.Physiological and pharmacological concentrations of nuclear receptor ligands

Nuclear receptor ligands	Physiological/pharmacological concentrations (references)	concentrations used in the study
Estradiol	< 2 nM* [33, 34]	1 nM
Tamoxifen	0.2 – 0.8 µM** [35, 36]	0.1 – 1 µM
DPN	-	1 – 100 nM
MPA	1 nM – 9.3 µM** [37]	0.1 – 10 nM
Calcitriol	0.05 – 0.15 nM* [38]	1 – 100 nM
Retinol	1.1 – 2.8 µM* [39]	0.1 – 10 µM

-, no data; *, physiological concentration; **, pharmacological concentration; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; MPA, medroxyprogesterone acetate.

Fig. 1.

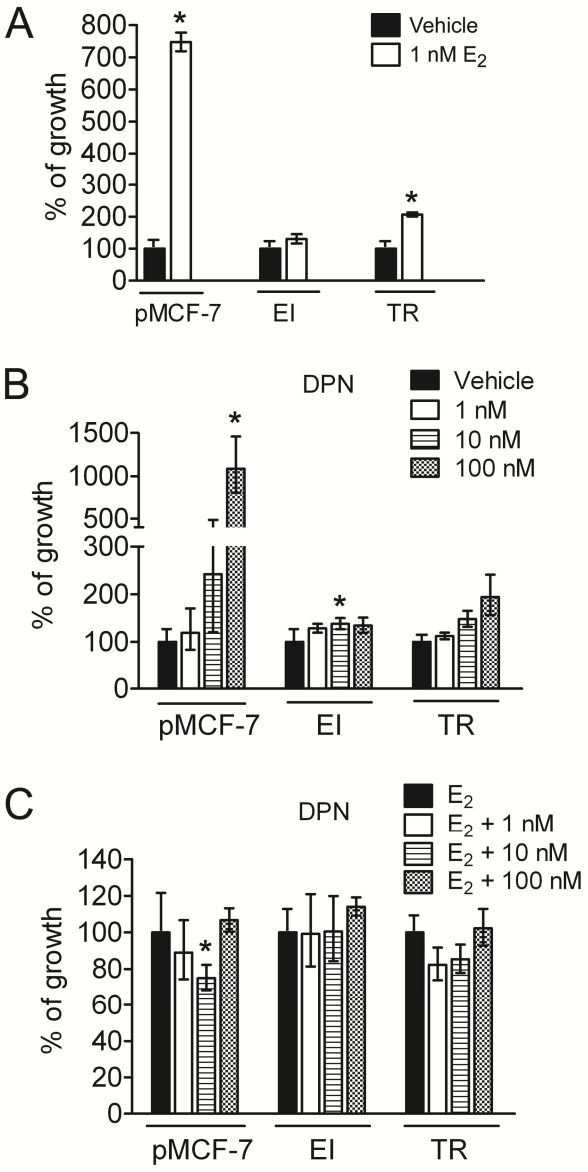

A
800
700
600
500
400
300
200
100
0
% of growth
*
*
Vehicle
1 nM E2
pMCF-7
EI
TR
B
DPN
Vehicle
1 nM
10 nM
100 nM
1500
1000
500
300
200
100
0
% of growth
*
*
pMCF-7
EI
TR
C
DPN
E2
E2 + 1 nM
E2 + 10 nM
E2 + 100 nM
140
120
100
80
60
40
20
0
% of growth
*
pMCF-7
EI
TR

Fig. 2.

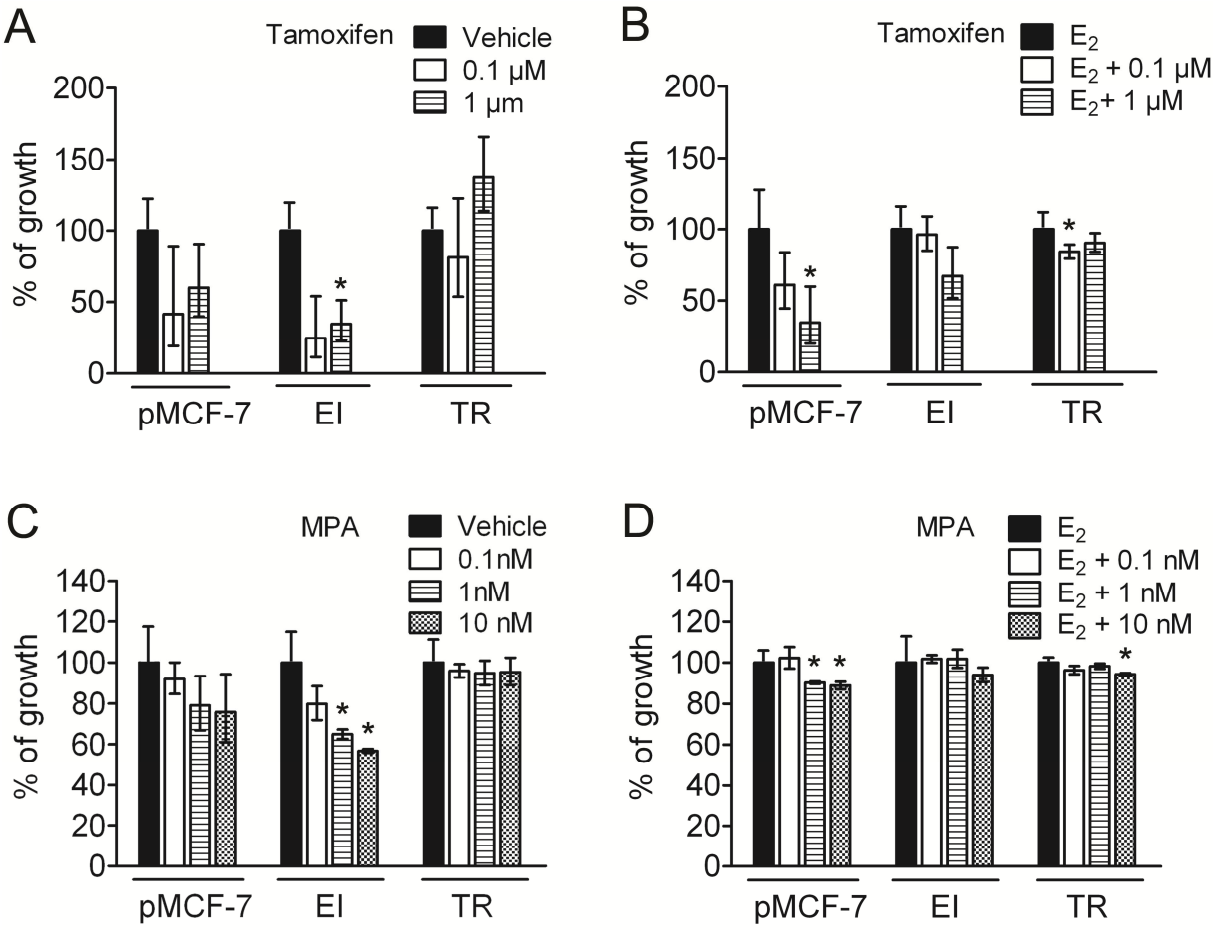
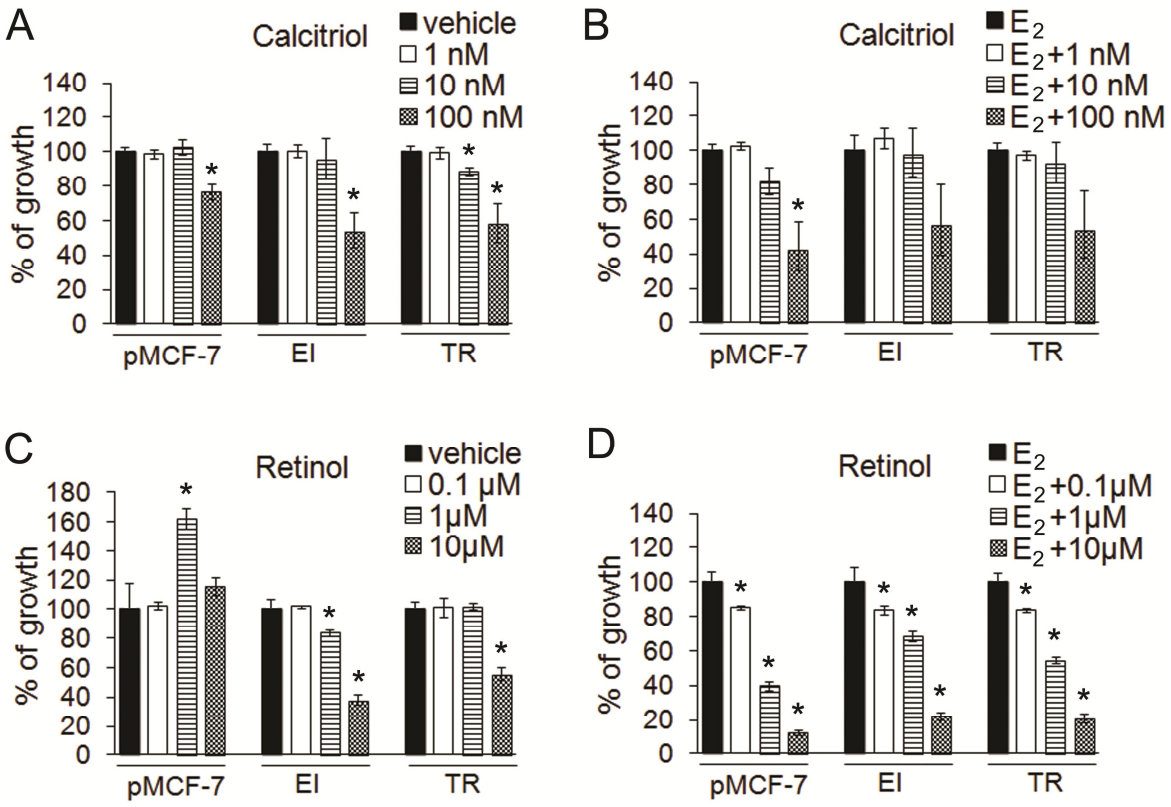

A
Tamoxifen
Vehicle
0.1 µM
1 µm
% of growth
200
150
100
50
0
pMCF-7
EI
TR
B
Tamoxifen
E2
E2 + 0.1 µM
E2+ 1 µM
% of growth
pMCF-7
EI
TR
C
MPA
Vehicle
0.1nM
1nM
10 nM
% of growth
140
120
100
80
60
40
20
0
pMCF-7
EI
TR
D
MPA
E2
E2 + 0.1 nM
E2 + 1 nM
E2 + 10 nM
% of growth
pMCF-7
EI
TR

Fig. 3.


A
Calcitriol
vehicle
1 nM
10 nM
100 nM
% of growth
pMCF-7
EI
TR
B
Calcitriol
E2
E2+1 nM
E2+10 nM
E2+100 nM
C
Retinol
vehicle
0.1 μM
1μM
10μM
D
Retinol
E2
E2+0.1μM
E2+1μM
E2+10μM