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Estrogen receptor-negative/progesterone receptor-positive Evsa-T mammary tumor cells: a model for assessing the biological property of this peculiar phenotype of breast cancers

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Abstract

In 1986 we reported the appearance of a progestin binding protein in the human breast cancer cell line Evsa-T, originally described as lacking both estrogen and progesterone receptors (ER and PR). In this report we show that PR of this cell line displays a binding affinity for [^{3}H]ORG 2058 and a sucrose gradient sedimentation profile similar to those ascribed to PR from MCF-7 or T47D breast cancer cell lines. PR from Evsa-T cells is down-regulated by the progestin R-5020 as well as by the two antiprogestins, ZK 112.993 and ZK 98.299, but does not confer growth sensitivity to these compounds. ER remains undetectable by ligand binding assay, enzyme immunoassay and northern blotting. Our Evsa-T clone could be a valuable model for assessing the mechanisms leading the ER–/PR+ phenotype occurring occasionally in breast cancers and frequently in meningiomas. © 1997 Elsevier Science Ireland Ltd.

Keywords: Estrogen receptor; Progesterone receptor; Breast cancer; Cell lines

1. Introduction

Breast cancers that do not contain ER but contain PR when assessed by specific ligand binding may reflect a receptor profile in which ER is saturated or down-regulated by endogenous estrogens. While such a profile can be found in premenopausal women [1] numerous studies have identified a small percentage of ER–/PR+ lesions [2–4] without high levels of both circulating and intratumoral estrogen. The fact that such a peculiar ER–/PR+ phenotype is frequently seen in meningioma is additional evidence of the existence of an ER-independent PR expression [5]. The significance of the ER–/PR+ phenotype therefore merits investigation in regard to both biology and prognosis.

In 1986 we reported that steroid receptor negative Evsa-T cells cultured with fetal bovine sera (FBS) with a strong growth-promoting activity in MCF-7 cells acquired the capacity to incorporate the synthetic progestin [^{3}H]ORG 2058 without any evidence of the presence of ER [6]. Long term maintenance of these cells under such culture conditions stabilized this ER–/PR+ phenotype.

The finding that steroid hormone receptors are

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members of a large family of ligand inducible transcription factors [7,8] led us to confirm the true PR nature of this progestin binding protein as well as to clearly establish the absence of ER in Evsa-T cells under culture conditions. We investigated whether this protein displayed molecular properties ascribed only to PR including its ability to interact with anti-PR monoclonal antibodies, binding parameters for [^{3}H]ORG 2058 and sucrose gradient sedimentation characteristics. In parallel, we analyzed the possibility of an ER down-regulation process. These investigations are described by the data reported herein.

2. Materials and methods

2.1. Reagents, antibodies and culture materials

[^{3}H]E_2 (±100 Ci/mmol) and [^{3}H]ORG 2058 (±50 Ci/mmol) were purchased from Amersham (UK). Unlabeled estradiol (E_2), DNA-cellulose, RNase and bovine serum albumine (BSA) fraction V were obtained from Sigma (St. Louis, MD). Actinomycin D (AMD) and ATP were from Boehringer Mannheim (Germany). ZK 112.993 and ZK 98.299 were kindly provided by Dr M.R. Schneider (Schering, Berlin, Germany) and RU 58 668 was provided by Dr Van de Velde (Roussel Uclaf, Romanville, France). 4-Hydroxytamoxifen (OH-TAM) was a gift of Dr A. Wakeling (Zeneca, Macclesfield, UK). An *Eco*RI fragment (1300 bp) of pOR3 used as an ER mRNA probe was from the American Type Culture Collection (Rockville, MD). H-222 anti-ER monoclonal antibody was provided by Abbott Laboratories (North Chicago, IL). Earle's based minimal essential medium (MEM) with and without Phenol Red, FBS, L-glutamine, penicillin, streptomycin, gentamycin and TRIzol reagent were purchased from Life Technologies (GIBCO, Gent, Belgium) and culture materials were purchased from Falcon (Becton Dickinson, Gent, Belgium).

2.2. Culture conditions

Since their introduction to our laboratory, Evsa-T and MCF-7 cells have been maintained in monolayer culture at 37°C in MEM supplemented with 10% heat inactivated FBS and L-glutamine, penicillin, streptomycin and gentamicin at the usual concentrations. ER and PR assays as well as growth measurements were conducted on cells cultured in serum depleted of endogenous steroids by dextran-coated charcoal treatment (DCC) [9].

2.3. Receptor assays

Cells were detached from T-175 flasks with 1 mM EDTA in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} and harvested by 10 min centrifugation at $300 \times g$. Cells were washed twice with HBSS and once with 10 mM phosphate buffer (pH 7.4) containing 1.5 mM EDTA, 1 mM monothioglycerol and 10% glycerol before homogenization in this buffer by means of a teflon-glass homogenizer. Cytosolic ER and PR levels were assessed on 1 h $100\,000 \times g$ supernatant fraction of ultracentrifugation by multipoint DCC assays according to EORTC recommendations [10] using [^{3}H]E_2 and [^{3}H]ORG 2058 as labeling ligands. Receptor concentrations assessed by Scatchard plot analysis were expressed in fmol/mg protein, the latter being measured using the Bio-Rad reagent (Bio-Rad, Richemond, CA). Binding data were analyzed according to Scatchard with Ligand 4.5 program (P.J. Munson, NIH, Bethesda, MD 20892). Additional Abbott immunoassays (ER and PR-EIA) were carried out according to the manufacturer's instructions. The effect of poten-

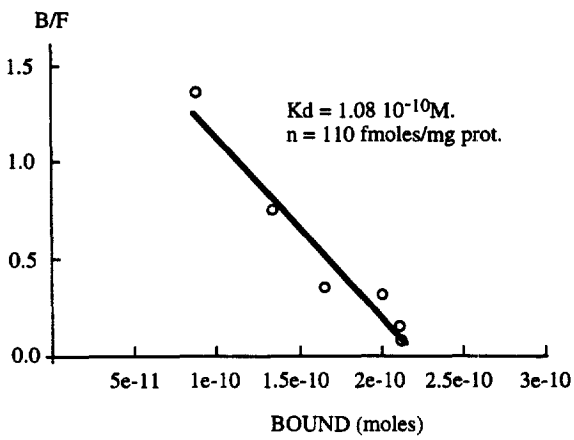

Fig. 1. Binding parameters of [^{3}H]ORG 2058 to a cytosol from Evsa-T cells. Data were analyzed by Scatchard plot analysis and curve fitting was done with a Ligand 4.5 program (figure shows a representative experiment). Concentrations are expressed as fmol/mg supernatant protein.

tial agonists or antagonists on the PR level was studied after 3 days of culture in their presence.

2.4. Sucrose gradient sedimentations

Cells were washed twice with HBSS and once with 10 mM Tris–HCl buffer (pH 8.5) with 1.5 mM EDTA (T10 E 1.5) before homogenization in this buffer by means of a teflon-glass homogenizer. Cytosol was obtained after 1 h ultracentrifugation at 100 000 × g. The supernatant was incubated for 1 h with 5 nM [^{3}H]ORG 2058 at 0–4°C. Following a DCC treatment to remove unbound ligand, 300 μl of the labeled cytosol was layered on the top of a linear 10–30% sucrose gradient in the presence or absence of KCl at 400 mM (10 mM Tris–HCl buffer (pH 8.5) with 1.5 mM EDTA; T10 E 1.5). After centrifugation (Beckman SW60 rotor at 50 000 rev./min for 16 h), gradients were divided into 100 μl fractions and their radioactivity measured by liquid scintillation. In the case of unlabeled cytosol, PR peaks were located within the gradient by measurement of the PR levels of each fraction by enzyme immunoassay (PR EIA from Abbott). BSA (4.4 S) was sedimented in parallel for assessing the sedimentation velocity of PR forms.

2.5. DNA cellulose adsorption

Aliquots of 300 μl of cytosol were labeled with 5 nM[^{3}H]ORG in the presence or in the absence of a 100-fold excess of unlabeled ligand. These preparations were then diluted with 100 μl of solution of ATP, KCl or RNase in 10 mM phosphate buffer (pH 7.4) containing 1.5 mM EDTA and 6 mM thioglycerol

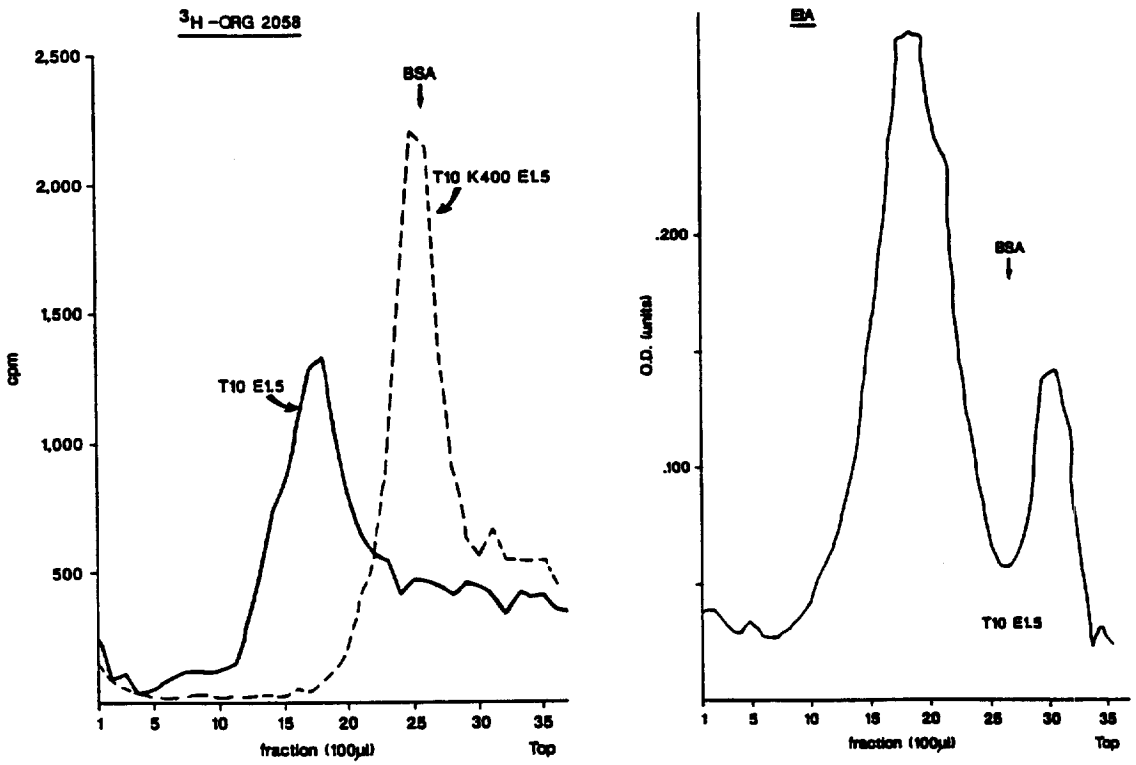

Fig. 2. (Left) Sucrose gradient sedimentation of [^{3}H]ORG 2058-labeled cytosol from Evsa-T cells. Cytosolic samples (100 μl) were sedimented either in the absence (T10 E1.5) or in the presence (T10 K400 E1.5) of 400 mM KCl. Addition of KCl to the sedimentation buffer produces a reduction of sedimentation velocity of the labeled receptor: control, ~8 S; high-salt, ~4 S. BSA refers to the sedimentation velocity of the bovine serum albumin standard (4.4 S). (Right) Sucrose gradient sedimentation of unlabeled cytosolic PR from Evsa-T cells. A cytosol sample (100 ml) was sedimented in a low-salt buffer (T10 E 1.5) and the fractions were analyzed by enzyme immunoassay (PR-EIA from Abbott) for the localization of PR in the gradient. The figures shows a high 8 S peak associated with a small 4 S peak.

(final concentrations: ATP, 40 mM; KCl, 300 mM; RNase, 1.2 mg/ml) to activate the receptor [11]. Control non-activated receptors were obtained by dilution of the sample either in the same buffer in the absence of ATP, KCl and RNase or in buffer containing 10 mM Na_2MO_4. After 1 h of incubation at 0–4°C, 500 μl of a suspension of DNA cellulose (40 μg DNA/tube) was added to these cytosol preparations and the pellets were washed three times with 1 ml of phosphate buffer. Pellets were finally extracted with 500 μl of ethanol and the radioactivity of the extracts was measured in an aliquot of 300 μl to evaluate the amount of PR bound to the matrix. DNA binding ability was assessed by comparison to the total PR content of the cytosol measured by ligand binding assay.

2.6. ER mRNA measurement

Total RNA was extracted with TRIzol reagent, dissolved in RNase-free water and quantified by spectrophotometry at 260–280 nM. Aliquots of 30 μg RNA/15 μl were electrophoresed through a 1% agarose formaldehyde gel, capillary transferred to a Hybond-N membrane (Amersham, UK), and treated according to the manufacturer's instructions. Blots were hybridized sequentially with a ^{32}P-labeled ER cDNA probe (10^9 cpm/mg cDNA, produced by random priming (Boehringer Mannheim, Germany)). Prehybridization (4 h) and hybridization (18 h) were performed at 42°C in 50% formamide, 5× SSPE (20× SSPE, 0.2 M phosphate buffer (pH 7.4), 2.98 M NaCl and 0.02 M EDTA), 0.1% polyvinylpyrrolidine, 0.1% Ficoll, 0.1% BSA, 0.1% SDS, 5% dextran sulfate and 100 mg/ml sheared DNA. The membranes were then washed with sodium citrate solutions (SSC) of increasing stringency, the last wash being performed in 0.3× SSC containing 0.1% SDS. Blots were visualized by exposure of the membranes for 1 day to Kodak XAR-5 film in an autoradiography cassette with an intensifying screen.

2.7. Growth experiments

The effect of hormones or antihormones on cell growth after 120 h of culture [12] was determined by measuring the DNA content of the cells by the diphenylamine method of Burton [13]. Briefly, cells maintained in monolayer culture were removed by trypsinization (trypsin 0.05%–EDTA 0.025%) and plated (±6 × 10^5 cells/well) in MEM supplemented with 10% DCC-treated FBS to produce a sub-culture at 37°C in a humidified 95% air, 5% CO_2 atmosphere. After 24 h, hormones or antihormones (from stock ethanolic solutions, diluted at the time of the experiment within the growth medium such that the final ethanol concentration was less than 0.1%; the same concentration of ethanol was added to the control cultures) were added to the medium and replaced 48 h later. Cells were harvested 72 h later and their growth was evaluated. All experiments were performed in quadruplicate.

3. Results

3.1. Main characteristics of PR from Evsa-T cells

Regular measurement of the binding properties for [^{3}H]ORG 2058 of cytosols from Evsa-T cells revealed a dissociation constant (K_d) of 1.2 ± 0.6 × 10^{-10} M; a value in the range ascribed to true PR (representative Scatchard plot in Fig. 1). The concentration of these binding sites fluctuates between 85 and 201 fmol/mg protein which remains relatively stable over time. This range of concentration is similar to that usually measured by us in MCF-7 cells but largely lower than that found in T47D cells.

Sucrose gradient sedimentation of [^{3}H]ORG 2058-labeled cytosol gave either a 4 S or 8 S peak whether or not the buffer gradient contained 400 mM KCl

Table 1
Binding ability to DNA cellulose of cytosolic PR from Evsa-T cells

Control	ATP (10 mM)	KCl (300 mM)	RNase[a] (1.22 mg/ml)
4.0 (1.2)[b]	29.0 (6.0)	15.4 (3.6)	
6.8 (5.6)[c]	31.0 (9.2)	20.7 (7.3)	13.1 (4.3)
Control MCF-7 cells			
10.4 (5.1)	40.9 (10.2)	19.6 (5.2)	16.0 (5.2)

[a]Only one experiment; RNase, 500 μg.
[b]Values in parentheses refer to cytosol fractions containing 10 mM sodium molybdate.
[c]Percentage of PgR concentration bound to DNA cellulose in two independent experiments.

(representative sedimentation profile of one out of two experiments in Fig. 2, left). Sedimentation of an unlabeled cytosol, in the absence of salt, gave an 8 S peak associated with a small 4 S peak when PR contents of the gradient fractions were assessed by Abbott's enzyme immunoassay (Fig. 2, right; sedimentation was solely run in low salt in view of the high cost of the experiment). This classical behavior of steroid hormone receptors confirmed the PR nature of the [^{3}H]ORG 2058 binding protein.

Activation of [^{3}H]ORG 2058-labeled PR by addition of ATP, KCl or RNase [11] increased its binding ability to DNA-cellulose in almost the same extent as was found with a control cytosol from MCF-7 cells (Table 1). Sodium molybdate, which is known to antagonize the activation potency of the receptor, strongly decreased its binding ability to the matrix.

Three day cultures of Evsa-T cells in the presence of increasing amounts of ORG 2058 (0.05, 0.5 and 1 nM) led to a progressive loss of their binding sites for the corresponding tritiated ligand indicating that their PR contents were subjected to down-regulation. Antiprogestins ZK 112.993 and ZK 98.299 also reduced PR although with a lower efficiency than ORG 2058. The extent of down-regulation was the same as that found with control MCF-7 cells (Table 2). Cells treated with either E_2 at 10 nM, OH-TAM at 1 μM or the pure antiestrogen RU 58 668 [14] at 1 μM failed to produce any modification on PR binding properties (K_d as well as contents of binding sites) (Table 3).

Table 2

Down-regulation of PR by ORG 2058 or antiprogestins ZK 112.993 and 98.299 in Evsa-T and MCF-7 cells

	MCF-7 (PR)		Evsa-T (PR)	
	K_d^a	n^b	K_d	n
Control	3.5	69	1.8	111
ORG 2058				
0.5×10^{-10} M	3.3	41	1.7	77
5×10^{-10} M	2.4	14	1.2	33
10^{-9} M	2.1	15	1.6	27

	MCF-7				Evsa-T	
	RO		PR		PR	
	K_d	n	K_d	n	K_d	n
Control	0.9	698	2.2	104	0.6	96
E_2 10^{-10} M	–	–	1.7	600	0.4	76
ZK 112993						
10^{-10} M	1.0	757	2.1	127	0.5	75
10^{-9} M	1.1	731	2.2	82	0.5	32
10^{-8} M	0.9	728	1.7	30	1.9	14
10^{-7} M	0.9	718	5.2	21	5.0	10
10^{-6} M	1.5	358	–	–	–	–
Control	1.2	633	1.6	106	1.1	114
E_2 10^{-10} M	–	–	1.6	385	1.3	110
ZK 98299						
10^{-10} M	0.9	652	1.3	94	1.1	131
10^{-9} M	0.8	673	1.1	93	0.9	111
10^{-8} M	0.9	747	2.1	64	1.2	93
10^{-7} M	1.2	698	1.3	64	2.3	68
10^{-6} M	4.0	453	4.1	34	12.4	55

Cells were incubated for 3 days with either ORG 2058, E_2 or an antiprogestin and their cytosolic binding properties for [^{3}H]ORG 2058 were subsequently measured. Control MCF-7 cells were submitted to the same treatment (in this case ER binding parameters were also measured).

[a] K_d, 10^{-10} M.

[b] n, fmol/mg protein.

Table 3
Effect of E2, OH-TAM and RU 58 668 on [^{3}H]ORG 2058 binding characteristics

	K_d (10^{-10} M)	n (fmol/mg protein)
Control	1.3	84
E_2 (10^{-10} M)	1.8	72
OH-TAM (10^{-6} M)	2.2	96
RU 58 668 (10^{-10} M)	1.5	89

Cells were incubated for 3 days with a given compound prior to ligand binding assay. Data were analyzed by Scatchard plot analysis and curve fitting was done in Ligand 4.5 program.

3.2. Absence of any detectable ER in Evsa-T cells

ER ligand binding and immunoassays performed over a period of 10 years (both in the cytosolic and nuclear fractions) have never suggested the presence of the receptor. Moreover, Northern blotting experiments performed twice (at a 2 year interval), failed to reveal any trace of ER mRNA while control MCF-7 cells displayed a characteristic 6.5 kb band (Fig. 3).

Dilution of cytosols from rat uterus or MCF-7 cells with a cytosol from Evsa-T cells (ratios 3:4, 2:4 and 1:4) failed to reveal the existence in the latter cells of any factor that may negatively interfere in ER assays; specific binding capacity for [^{3}H]E_2 was related to the dilution of the uterine or MCF-7 cells cytosol (data not shown).

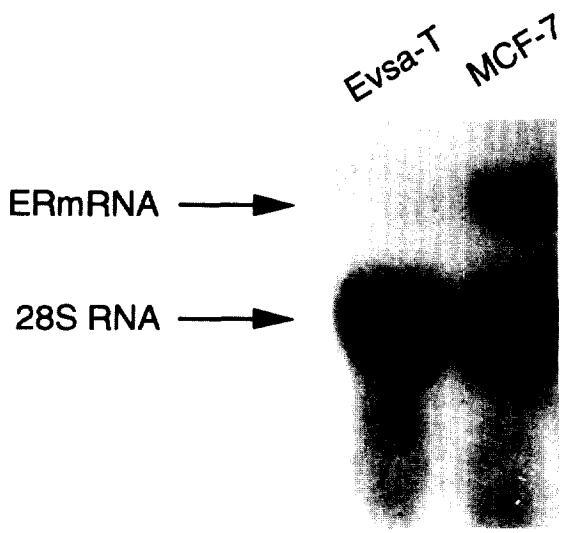

Fig. 3. Absence of ER mRNA in Evsa-T cells. Search for ER mRNA by Northern blot failed to reveal any positive signal in Evsa-T cells while it gave a classical positive spot of 6.5 kb in control MCF-7 cells.

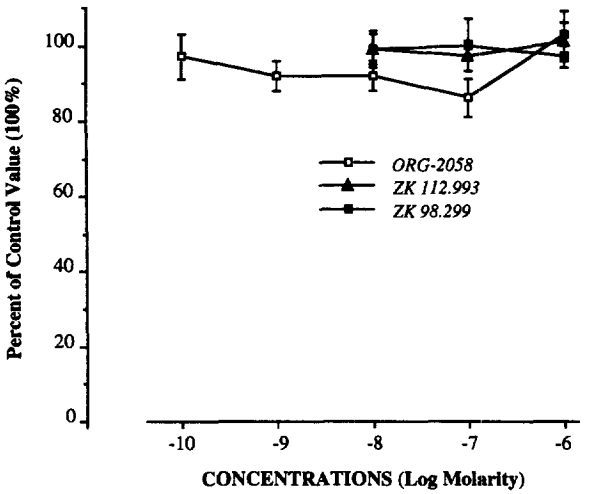

Fig. 4. Growth responses of Evsa-T cells to an increasing concentration of either ORG 2058, ZK 112.993 or ZK 98.299. Cells were harvested after 120 h of culture and their growth was evaluated by measuring their DNA content. Each value represents the mean ± SEM of two different experiments (each experiment in quadruplicate). The control value is taken as 100% (range of DNA: 20–25 μg/well in each experiment).

Estrogenic stimulation of ER-positive mammary tumor cells (e.g. MCF-7 cells) produces a rapid loss of both ER mRNA and peptide [15,16]. We therefore investigated the possibility of such a down-regulation process in Evsa-T cells induced by a potential endogenous estrogenic (or estrogenic-like) ligand. Cells were exposed to 1 μM AMD for 3 h since this intercalating agent was shown to block ER down-regulation (measures were carried out by [^{3}H]E_2 ligand binding assay) [16,17]. This treatment failed to produce any emergence of a specific [^{3}H]E_2 binding capacity. On the other hand, [^{3}H]ORG 2058 binding sites were not altered by this treatment, i.e. control, 140 fmol/mg protein versus 1 μM AMD, 135 fmol/mg protein. Hence, ER down-regulation produced by an endogenous ligand seems extremely unlikely.

3.3. Growth insensitivity of Evsa-T cells to hormones

Five-day cultures in the presence of either ORG 2058, ZK 112.993 or ZK 98.299 at doses producing PR loss (see above) did not modify the growth profile of the cells (Fig. 4). E_2 at 10 nM, OH-TAM at 1 μM

and RU 58 668 at 1 μM also failed to show any effect (control, 100%; E_2, 106 ± 6%; OH-TAM, 112 ± 4%; RU 58 668, 96 ± 5%; mean ± SD of three different experiments).

4. Discussion

Studies reported here clearly establish the presence of isolated PR in our Evsa-T cells, suggesting the selection of an ER–/PR+ clone. Although our Evsa-T cells may have some analogy with T47D cells, they differ from the latter by their lack of ER and their lower levels of PR. A subset of T47D cells have also retained PR expression despite the absence of ER [18]. Of note, the behavior of these cells was similar to our Evsa-T cells; neither their proliferation rate nor their PR contents were influenced by E_2. However, in contrast to our cells, this T47D variant line was derived from ER+/PR+ cells. In both cases the apparent constitutive expression of an estrogen-regulated gene may reflect the passage from an estrogen-regulated to a hormone insensitive status.

PR from our Evsa-T cells was activated under classical biochemical conditions (DNA cellulose adsorption) and down-regulated by the progestin R5020 as well as the two antiprogestins ZK 112.993 and ZK 98.299. Although the binding affinity for PR of the first of these compounds is only five-fold higher than the latter [19], ZK 112.993 was 100-fold more potent than ZK 98.299. Interestingly, these two antiprogestins failed to affect the growth of our cells while they were reported to inhibit the MCF-7 line [20]. As expected, no modulation of PR expression was observed under E_2, OH-TAM or RU 58 668 treatment. Hence, growth of Evsa-T cells appeared refractory to all tested hormonal treatments (progestins, antiprogestins, estrogens and antiestrogens) with PR down-regulation being the only response recorded.

Our Evsa-T cells failed to express significant levels of ER mRNA coding for the wild type (67 kDa) receptor. This lack of ER was not the result of a post-transcriptional dysfunction nor of an ER down-regulation process produced by a potential endogenous estrogenic production. We have not been able to exclude the presence of low amounts of an ER variant devoid of functional estrogen binding domain [21–23] nor very brief expression of wild type ER at a precise phase of the cellular cycle [24]. Concerning the first possibility, there is now considerable evidence for 'cross-talk' between growth factors and steroid hormone receptors; EGF and IGF-I have been shown to activate an ER devoid of ligand binding domain [25]. This receptor with functional A/B, C and D domains could not be measured either by ligand binding or by immunoassay.

The emergence of PR in a cell line originally described as ER– PR– is an intriguing occurrence. Although estrogens have long been recognized as the main regulator of PR expression in estrogen target tissues, IGF-I and EGF have also been reported to induce PR in breast cancer cells as well as in the uterus from the rat and the guinea pig (fetal cells) [26,27]. Activators of cAMP have also been reported to produce such induction [28]. One may therefore assume that long term maintenance of this cell line in sera containing appropriate growth factors for PR expression has led to the selection of the present phenotype. Supporting this concept is the observation that factors present in serum play a major role in PR induction mediated by both E_2 and growth factors; high serum concentrations (>1%) are required for an optimal E_2 effect and low concentrations are required for an optimal effect of IGF-I and cAMP [22].

Present Evsa-T cells may provide a valuable in vitro model for the assessment of ER-independent PR expression especially with regard to the investigation of PR modulation. Additionally, the similarity between our Evsa-T cells and the ER–/PR+ breast cancers or meningiomas [5] confers these cells with interest as regards the biology of these tumors.

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