Original Article

# Divergent behavior of cyclin E and its low molecular weight isoforms to progesterone-induced growth inhibition in MCF-7 cells

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## Abstract

**Background:** Progesterone is a steroid hormone that modulates proliferation and differentiation in a cell phase and tissue-specific manner. Its function in breast cancer cells is of great significance since it can predict susceptibility of tumor cells to inhibitory effects of progesterone as adjuvant therapy.

**Materials and Methods:** Stable clones overexpressing cyclin E (EL) and its low molecular weight isoforms (LMW-Es) were generated and treated with various concentrations of progesterone. Cell proliferation was assessed 24 and 48 h after the treatment. Changes in progesterone receptor (PR) expression were measured by real-time polymerase chain reaction.

**Results:** Here we demonstrated that overexpression of EL and LMW-Es have divergent effects with regard to progesterone response. We found that progesterone could significantly decrease the growth rate of EL-expressing cells in the second cell cycle after treatment; however, progesterone was ineffective to arrest growth of LMW-Es expressing cells. PR expression level was at control level in EL-expressing cells but was downregulatedin LMW-Esexpressing clones.

**Conclusion:** These results were in line with progesterone response of studied cells. The drop in PR expression together with altered distribution of p21 and p27 can explain different effects of cyclin E isoforms expression on progesterone responsivity. These data bring cyclin E status of cancer cells as a marker for predicting the efficacy of progesterone treatment.

**Key Words:** Cyclin E, low molecular weight isoforms of cyclin E, progesterone, progesterone receptor

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**Received:** 22.07.2013 **Accepted:** 13.10.2013

| Access this article online | |
|---|---|
| Quick Response Code: | Website: www.advbiores.net |
| | DOI: 10.4103/2277-9175.148299 |

## INTRODUCTION

The steroid hormone progesterone is one of the principal regulators of human female reproductive organs, which also plays roles in non-reproductive tissues such as cardiovascular and central nervous system, underlining the omnipresent role of this hormone in physiology.[1,2] Progesterone mediates its effects through the progesterone receptor (PR),



**How to cite this article:** Montazeri H, Bouzari S, Azadmanesh K, Ostad SN, Ghahremani MH. Divergent behavior of cyclin E and its low molecular weight isoforms to progesterone-induced growth inhibition in MCF-7 cells. Adv Biomed Res 2015;4:16.

which is expressed at two isoforms PR-A and PR-B. These isoforms have identical ligand-binding (LBD) and DNA-binding domains (DBD) but the processing of the amino-terminal domain (NTD) in PR-A is different.[3,4] Contrary to proliferative effects of estrogen, progesterone can be either proliferative or differentiative in a tissue-specific manner.[5] As an example, progesterone collaborates with estrogen in proliferation of uterus stromal but inhibits estrogen-induced cell mitosis in the epithelium.[6] Use of progesterone in breast cancer is controversial since it does not exert the inhibitory as it shows in endometrial cancer; however, synthetic progestins have indomitable role in breast cancer treatment.[7,8] The course of action for the antitumor activity of progestins is yet to be identified but restraint of cell cycle within $G_1$ phase indicates that these compounds act by affecting expression of the genes involved in progression of cell cycle through $G_1$.[9] Investigations for identification of these target genes have mentioned the roles of cyclin-dependent kinases (CDKs) and cyclins including cyclin E.[10-14]

The role of cyclin E has been extensively studied in breast cancer.[15-17] The function of this molecule is to promote cell cycle from $G_1$ to S phase by coupling to its correspondent CDK, CDK2, and phosphorylating its target substrates.[18] Different mechanisms are known to be involved in cyclin E deregulation, such as gene amplification,[19] downregulation of hCDC4,[20] overexpression of miR-27a[21] and most importantly elastase-mediated cleavage of full length protein into low molecular weight isoforms (LMW-Es).[22,23] LMW-Es have higher affinity to CDK2 but are resistant to be inhibited by cyclin-dependent kinase inhibitors (CKIs), $p21^{cip1}$ and $p27^{kip1}$.[24,25] These hyperactive isoforms also exhibit higher kinase activity, comparing to full length protein, and thereby facilitating transition from $G_1$ to S phase.[23] Ultimately, LMW-Es generation results in genomic instability, tumorigenesis, and anti-hormonal therapy resistance.[24-26]

Emerging data suggest extensive crosstalk between progesterone signaling pathways and cell cycle regulators. Among them regulation of PR expression by cyclin D1[27]; Inhibition of cyclin D1, cyclin D3, and cyclin E[28]; and induction of CKIs, p21, p27, and $p18^{INK4c}$ by progesterone[29,30] can be mentioned. However, progesterone inhibitory effect in the presence of LMW-Es overexpression has not been studied yet. Because LMW-Es are refractory to inhibitory effects of CKIs,[24] we applied full length cyclin E and LMW-Es overexpressing models to interrogate whether progesterone response is different in these models.

## MATERIALS AND METHODS

### Cell culture and progesterone treatment

MCF-7 human breast cancer cell line purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (all from PAA, Austria) at 37°C with 5% $CO_2$. Generation of cell lines stably express cyclin E isoforms are described elsewhere.[31] For each isoform of cyclin E, two high expressing clones (HE 1 and HE 2) were selected and propagated for further investigations.

Three days before progesterone treatment, cells were switched to phenol red free RPMI 1640 medium containing 10% charcoal-treated fetal bovine serum (FBS). The day before treatment, cells were seeded in 96-well cell culture plates and on the day of the treatment the above medium was replaced witha medium containing 0.1and 1μM of water-soluble progesterone (Sigma, Germany). Proliferation of stable clones was measured 24 and 48 h after treatment by XTT cell viability dye (Roche, Germany) according to manufacturer's protocol. In each time point, the absorbance was measured at 500nm by Biotek micro plate reader.

### RNA extraction and transcript analysis

RNA extraction carried out using Tripure reagent (Roche, Germany) according to the manufacturer's specifications. The quality of RNA was checked by measuring A260/A280 absorbance ratio and by electrophoresis on 1% denaturing agarose gel. cDNA was prepared by reverse transcription of 1 μg of total RNA using 100 ng of random primer and 100 units of MuLV reverse transcriptase (Fermentas, Lithuania) under the conditions recommended by the supplier. Quantitative SYBR green polymerase chain reaction (PCR) reactions were performed in a total volume of 20 μL using Precision-R Master Mix (Primerdesign, UK) by StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, California, USA). All the reactions were performed in triplicate, and template volume was no more than one tenth of the totalreaction. Quantitative values defined as $C_t$ numbers and fold changes in gene expression were calculated by using the formula $2^{-\Delta\Delta Ct}$ and analyzed by REST software.[32] Results presented as N-fold change (decrease or increase) of target gene. Hypoxanthine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes. The primer sequences are as followings; for progesterone receptor: Forward 5'-GCAATGGAAGGGCAGCACAACT-3', reverse

5'-GCATCCAGTGCTCTCACAACTCTGAC-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5- ACACCCACTCCTCCACCTTG-3' and reverse 5'-TCCAC C ACCCTGTTGCTGTAG-3'; for hypoxanthine phosphoribosyltransferase (HPRT), forward 5- CTG G CGTCGTGATTAGTGATGATGA-3' and reverse 5'-TCGAGCAAGACGTTCAGTCCTGTC-3'.

## RESULTS

### Overexpression of full length cyclin E diminishes cell proliferation in the presence of progesterone

Because cyclin E-CDK2 complex are one of the targets of progesterone in growth arrest process,[28] we used stable clone overexpressing cyclin E isoforms to address the effect of cyclin E on the responsivity to progesterone. The stable clones expressing full length cyclin E (EL), Trunc1 (T1 encoding 44 and 45 kDa), middle Trunc ($T_{mid}$ encoding 40 kDa), and Trunc2 (T2 encoding 33 and 34 kDa) were used. For each isoforms, to limit clonal variations, we have used two high expresser clones (HE 1 and 2) for experiments. Our results revealed that progesterone treatment (at both 0.1 and 1 μM concentrations) had no significant effecton proliferation over a 24-h period for any of the clones (data not shown). However, at a concentration of 1 μM progesterone effectively inhibited proliferation in EL expressing clones. On the other hand, progesterone had no significant growth decrease in LMW-Es expressing cells [Figure 1]. In all the treatments, MCF-7 cell stably transfected with empty pcDNA 3.1 (MCF-7 3.1) was used as control cell line.

### Overexpression of LMW-Es but not full length cyclin E downregulates progesterone receptor levels

To gain a better understanding of cellular events leading to susceptibility of EL expressing cells to progesterone, we examined if expression of PR is affected by overexpression of cyclin E isoforms. The PR expression levels in stable transfectants were determined using real-time PCR and the results showed that only in LMW-Es overexpressing cells PR expression is downregulated [Table 1].

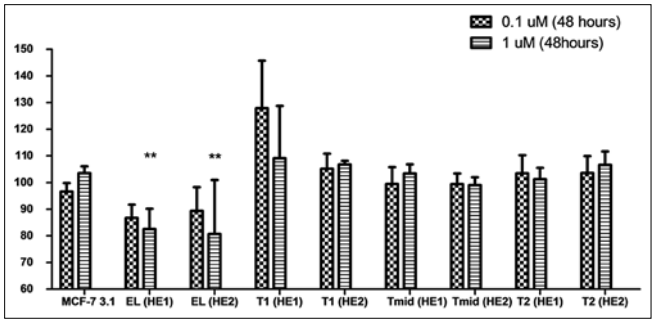


**Figure 1:** EL expressing cells respond distinctly to progesterone treatment. Cells were treated with 0.1 and 1 μM of progesterone and cell proliferation was determined by XTT assay at 48h after treatment (Mean ± SD, $n = 3$; Two-way ANOVA, Bonferroni post-test, $^{**}P < 0.01$ compared with MCF-7 3.1)

## DISCUSSION

In this study we investigatedthe effect of truncated isoforms of cylin E on the proliferation of MCF-7 cells affected by progesterone as a step toward unraveling the effects of progesterone on normal physiology as well as breast cancer. We showed that progesterone cannot inhibit cell proliferation of our target cells within 24 h after treatment, but obviously slowed down the growth rate of only EL expressing cells from the 48 h after treatment. Our results indicate that progesterone had no effect on the proliferation of MCF-7 cells. However, when cyclin E was overexpressed (EL clones), the proliferation was inhibited by progesterone. The clones expressing

**Table 1: Relative gene expression of PR in stable transfectants**

| Transcript | Type | Reaction efficiency | Expression | P (H1) | Result |
|---|---|---|---|---|---|
| EL (HE1) | | | | | |
| PR | TRT | 0.90 | 3.89 | 0.310 | NS |
| GAPDH | REF | 1.1 | 0.99 | | |
| HPRT | REF | 0.93 | 1.00 | | |
| EL (HE2) | | | | | |
| PR | TRT | 0.90 | 0.342 | 0.409 | NS |
| GAPDH | REF | 1.1 | 0.890 | | |
| HPRT | REF | 0.93 | 1.15 | | |
| T1 (HE1) | | | | | |
| PR | TRT | 0.90 | 0.021 | 0.039 | Down |
| GAPDH | REF | 1.1 | 1.05 | | |
| HPRT | REF | 0.93 | 0.952 | | |
| T1 (HE2) | | | | | |
| PR | TRT | 0.90 | 0.034 | 0.021 | Down |
| GAPDH | REF | 1.1 | 0.910 | | |
| HPRT | REF | 0.93 | 1.20 | | |
| T2 (HE1) | | | | | |
| PR | TRT | 0.90 | 0.135 | 0.041 | Down |
| GAPDH | REF | 1.1 | 0.980 | | |
| HPRT | REF | 0.93 | 1.02 | | |
| T2 (HE2) | | | | | |
| PR | TRT | 0.90 | 0.107 | 0.026 | Down |
| GAPDH | REF | 1.1 | 0.084 | | |
| HPRT | REF | 0.93 | 1.182 | | |
| $T_{mid}$ (HE1) | | | | | |
| PR | TRT | 0.90 | 0.042 | 0.012 | Down |
| GAPDH | REF | 1.1 | 0.934 | | |
| HPRT | REF | 0.93 | 1.07 | | |
| $T_{mid}$ (HE2) | | | | | |
| PR | TRT | 0.90 | 0.217 | 0.037 | Down |
| GAPDH | REF | 1.1 | 1.02 | | |
| HPRT | REF | 0.93 | 0.98 | | |

Relative gene expression measured as the fold change of the target gens with regard to housekeeping genes (GAPDH and HPRT) and compared with the gene expression in the control group using REST 2009 software. P(H1),the probability of alternate hypothesis that difference between sample and control groups is due only to chance. Data are representative of three independent experiments TRT: Target transcripts, PR: Progesterone receptor, REF: Reference transcripts, NS: Non significant (compared with MCF, 7 3.1), GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, HPRT: Hypoxanthine phosphoribosyltransferase

LMW-Es showed no significant changes in proliferation following progesterone treatments. As shown before progesterone markedly decreases cyclin E-CDK2 activity.[28] Based on these findings we expected to find our stable cell lines more responsive to progesterone inhibitory effect; however, this behavior was not seen in LMW-Es overexpressing cell lines.This phenomenon can be attributed in part by hyperactive nature of LMW-Es. Comparing to EL, these forms bind more effectively to CDK2 and are resistant to be inhibited by p21 and p27 despite efficient binding to these molecules.[24] However, p21 and p27 both show increased association with cyclin E-CDK2 complex following progesterone treatment.[28] Thus, p21 and p27 will be recruited to the complexes, which are refractory to their inhibition and as a result the cell cycle will be continued.

According to our experiments, there could be another explanation for different responses of EL and LMW-Es expressing cells. We found that PR expression was affected by LMW-Es expression and had been downregulated in LMW-Es overexpressing cell lines. The decrease in the level of PR was not associated with EL overexpression, indicating that EL and LMW-Es have different gene regulatory effects and regulation of PR expression lies downstream of LMW-Es levels. As progesterone mediates its effect through PR, lowerPR expression translates to lower activity of progesterone and consequently less effectiveness in controlling the proliferation of LMW-Es expressing cells.

Our results demonstrate regulatory effect of cyclin E expression on progesterone signaling pathways. These findings help us to have a better understanding of regulation of signaling pathways in which EL or LMW-Es are involved. Furthermore, EL and LMW-Es can be considered as markers of the efficacy of progesterone therapy in breast cancer.

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**Source of Support:** This work has been financially supported by a grant allocated for Hamed Montazeri's thesis from Pasteur Institute of Iran, **Conflict of Interest:** None declared.