

Original Article

ERK/CANP rapid signaling mediates 17 β -estradiol-induced proliferation of human breast cancer cell line MCF-7 cells

Guo-Sheng Wang¹, Yan-Gang Huang¹, Huan Li², Shi-Jie Bi¹, Jin-Long Zhao¹

¹The First Affiliated Hospital of Harbin Medical University, 23 Youzheng Str, Nangang District, Harbin 150001, Heilongjiang Province, P. R. China; ²School of Medicine, Shenzhen University, Nanhai Ave 3688, Shenzhen 518060, Guangdong Province, China

Received November 6, 2013; Accepted November 21, 2013; Epub January 15, 2014; Published January 30, 2014

Abstract: Objective: 17 β -estradiol (E2) exerts its functions through both genomic and non-genomic signaling pathways. Because E2 is important in breast cancer development, we investigated whether its actions in promoting breast cancer cell proliferation occur through the non-genomic signaling pathway via extracellular signal-regulated kinase 1/2 (ERK1/2)/calcium-activated neutral protease (CANP). Methods: MCF-7 breast cancer cells were treated with ERK1/2 inhibitor (PD98059) or CANP inhibitor (calpeptin) before exposure to 1×10^{-8} M E2. MTT colorimetry and flow cytometry were used to analyze effects on cell proliferation and cell cycle progression, respectively. Expression of phosphorylated-ERK (p-ERK), total ERK, and Capn4 proteins were assessed by Western blotting. Results: Cell proliferation increased in cells treated with E2 for 24 h ($P < 0.05$), and the proportion of cells in G₀/G₁ was decreased, accompanied by accelerated G₁/S. Calpeptin pre-treatment significantly inhibited the E₂-induced proliferation of MCF-7 cells ($P < 0.05$), while also ameliorating the effects of E2 on cell cycle progression. Further, expression of p-ERK was rapidly up-regulated (after 10 min) by E2 ($P < 0.05$), an effect that persisted 16 h after E2 exposure but which was significantly inhibited by PD98059 ($P < 0.05$). Conclusions: Finally, expression of Capn4 protein was rapidly up-regulated in E2-exposed cells ($P < 0.05$), but this change was significantly inhibited by PD98059 or calpeptin ($P < 0.05$) pre-treatment. Thus, the rapid, non-genomic ERK/CANP signaling pathway mediates E₂-induced proliferation of human breast cancer cells.

Keywords: Breast cancer cell, estradiol, cell proliferation, extracellular signal-regulated kinase

Introduction

17 β -estradiol (E2) participates in development of breast tissue and regulation of its physiological function. In addition to these normal functions, E2 can stimulate proliferation and infiltration of breast cancer cells, promoting progression of breast cancer [1]. These dual roles of E2 highlight the importance of proper regulation of its expression.

The biological effects of E2 can be mediated through two different signaling pathways. In its genomic (direct DNA-binding) signaling pathway, E2 and intracellular estrogen receptors (ER) form dimers before entering the cell nucleus [2-4]. These dimers then induce gene transcription and protein synthesis in target cells, thereby effecting a variety of cellular responses,

including proliferation, differentiation, and survival. All of these responses can be achieved within several hours. Using non-genomic signaling, mediated through protein-protein interactions rather than direct DNA binding, E2 can induce, among other responses, Ca²⁺ mobilization, which plays roles in cell motility, and phosphorylation-related activation of extracellular signal-regulated kinase 1/2 (ERK1/2), which is important for cell cycle regulation [5-9]. These effects can occur rapidly, within minutes. Such rapid, non-genomic signaling responses, combined with its ability to promote tumorigenesis, increase the importance of understanding E2 activity.

Within the context of E2 non-genomic signaling, one important downstream molecule is the intracellular calcium-activated neutral protease

(CANP). CANPs, or calpains, particularly CANP1 and CANP2, are widely expressed [10]. These proteins are dimer proteases activated by calcium; they share a common small subunit, CAPN4 (10). CANPs can be activated via phosphorylation and rapid activation of ERK by epidermal growth factor (EGF) [11], inducing cell migration. Further, disruptions in CANP expression and signaling are associated with various cancers; indeed, changes in expression and/or regulation of CANPs may promote tumorigenesis [12]. However, no studies have determined whether expression of and non-genomic signaling through E2 in breast cancer cells can activate CANP (via ERK) to stimulate cell proliferation and promote cancer progression.

To determine the effects of E2 overexpression on signaling through the ERK/CANP pathway, we exposed MCF-7 breast cancer cells to E2. Cells were analyzed for changes in proliferation, cell cycle, and expression of Capn4 and ERK1/2. Finally, signaling through this pathway was monitored by drug inhibition of ERK1/2 or CANP activity.

Materials and methods

Cell culture and proliferation assays

MCF-7 human breast cancer cells (Kunming Cell Bank, Chinese Academy of Sciences) were inoculated into a 96-well plate at cell density of 1×10^5 cells/mL, 100 μ L/well, and cultured at 37°C and 5% CO₂ in DMEM (Gibco) supplemented with 10% fetal bovine serum (TBD Co., Tianjin, China). After 24 h, cells were cultured with serum-free and phenol red-free DMEM (Gibco) medium for 24 h to deplete endogenous estrogen and synchronize cell cycle. Optimally effective concentration and time (10^{-8} M and 24 h) were selected according to preliminary test results. Six experimental groups were devised, as follows: 1) DMSO at isovolumetric concentration (control); 2) 10^{-8} M E2 (Sigma; dissolved in DMSO); 3) 10^{-8} M E2 + 10^{-7} M calpeptin (CANP inhibitor; Calbiochem, Germany); 4) 10^{-8} M E2 + 10^{-6} M calpeptin; 5) 10^{-8} M E2 + 10^{-5} M calpeptin; and 6) 10^{-8} M E2 + 10^{-6} M tamoxifen (TAM; Calbiochem; positive control). Each group included 10 parallel samples. Groups receiving E2 + calpeptin (varied concentrations) or E2 + TAM were pre-treated with calpeptin or TAM for 30 minutes before addition of E2, then cultured in complete medium

for 24 h. MTT (5 mg/mL; Amresco) solution was added, 20 μ L per well, and cells were continuously cultured for 4 h; subsequently, supernatant was discarded and DMSO was added to cultures, 150 μ L per well, with mixing for 10 minutes. Absorbance value (optical density, OD) for each well was measured by an ELISA plate reader at 492 nm wavelength. Cell proliferation rate was calculated using the following formula: Cell proliferation rate = (OD value for experimental group - OD value for blank group) / OD value in blank group \times 100%.

Flow cytometry

MCF-7 cells were inoculated in 25 mL bottles, 5×10^6 cells/bottle, cultured, and synchronized using above methods. Four experimental groups were included, as follows: 1) DMSO of isovolumetric concentration (control); 2) 10^{-8} M E2; 3) 10^{-8} M E2 + 10^{-5} M calpeptin; 4) 10^{-8} M E2 + 10^{-6} M TAM. Groups receiving E2 + calpeptin or E2 + TAM were pre-treated and cultured as above. Cells for each group were collected and centrifuged three times at 1000 rpm for 5 min. Supernatant was discarded, then 2 mL 70% ice-cold ethanol were added to cells with mixing. Cells were fixed overnight at 4°C. Following centrifugation, supernatant was discarded, and cells were washed twice with PBS. RNA enzyme was added to a final concentration of 50 g/L. The solution was incubated in a 37°C water bath for 30 min, placed in an ice bath to terminate effect of RNA enzymes, then centrifuged. To cell pellet was added propidium iodide (PI) at a final concentration of 50 mg/L; the solution was placed at 4°C for 15 min. Upward-flow cytometry (FACS Calibur, Becton-Dickinson) was used to determine cell cycle fractions. Trials were performed in triplicate.

Western blotting

MCF-7 cells treated with 10^{-8} M E2 were collected after 0, 10, or 30 min or 1, 2, 4, 8, or 16 h. Additionally, cells treated with 10^{-8} M E2, 10^{-8} M E2 + 20 μ M PD98059 (ERK inhibitor; Promega), and 10^{-8} M E2 + 10^{-5} M calpeptin were collected (pre-treated with PD98059 or calpeptin for 10 min, then treated with E2 for 30 min). Total protein was extracted after cell lysis, then loaded (30 μ g/well) onto 12% gels for SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane via electro-transfer. Membranes were incubated in

ERK/CANP in E₂-induced MCF-7 cells

Table 1. Proliferation rates of MCF-7 cells exposed to E2 alone or in after calpeptin

Treatment	n	Optical Density	Proliferation rate (%)
Control	10	0.202 ± 0.014	
E2	10	0.264 ± 0.012*	100.00 ± 0.00
E2 + 10 ⁻⁷ M calpeptin	10	0.221 ± 0.015*#	88.08 ± 6.29#
E2 + 10 ⁻⁶ M calpeptin	10	0.206 ± 0.011#▲	78.15 ± 6.40#▲
E2 + 10 ⁻⁵ M calpeptin	10	0.192 ± 0.014*#▲•	72.91 ± 6.73#▲•
E2 + TAM	10	0.183 ± 0.011*#▲•	71.24 ± 4.72#▲•
F		49.756	45.476
P		0.001	0.001

*P<0.05: vs. Control; #P<0.05 vs. E2 group; ▲P<0.05 vs. E2 + 10⁻⁷ M calpeptin group; •P<0.05 vs. E2 + 10⁻⁶ M calpeptin group.

Table 2. Cell cycle progression for MCF-7 cells treated with E2 alone or after calpeptin

Treatment	n	G ₀ /G ₁ stage (%)	S stage (%)	G ₁ /S stage (%)
Control	3	48.47 ± 0.70	35.55 ± 0.76	15.98 ± 0.06
E2	3	44.37 ± 0.86*	38.46 ± 1.06*	17.17 ± 0.21*
E2 + 10 ⁻⁵ M calpeptin	3	46.51 ± 1.03#	34.46 ± 0.74#	19.03 ± 0.42*
E2 + TAM	3	47.22 ± 1.09#	35.43 ± 0.72#	17.35 ± 0.46*#▲
F		10.211	12.967	43.863
P		0.004	0.002	0.001

*P<0.05: vs. Control; #P<0.05 vs. E2 group; ▲P<0.05 vs. E2 + 10⁻⁵ M calpeptin group.

5% powdered nonfat milk for 1 h at room temperature, then washed 3 times, 10 min each, with TBST. Membranes were then incubated with diluted anti-p-ERK or anti-ERK [rabbit anti-human polyclonal (Cell Signaling), 1:1000], anti-Capn4 [mouse anti-human monoclonal (Calbiochem), 1:2000], and anti-β-actin [mouse anti-human monoclonal (Wuhan Boster Biological Technology), 1:400] antibodies overnight at 4°C. After 3 10-min washes with TBST, corresponding secondary antibodies [horseradish peroxidase-labeled goat anti-mouse or goat anti-rabbit IgG (Wuhan Boster), 1:2000] were added for 1 h at room temperature. Staining was developed with enhanced ECL color system (Wuhan Boster). Target band intensities were analyzed using Quantity One System (BioRad); gray values were compared between target bands and β-actin band, with their ratios indicating relative expression levels of target bands. Experiment was performed in triplicate.

Statistical analysis

SPSS17.0 statistical software was used for data processing, and measurement data are

expressed as mean ± standard deviation ($\bar{x} \pm s$). Single-factor ANOVA and paired comparison (based on single-factor ANOVA) between groups (SNK) were used. Tests were two-sided, with α level of 0.05 and P<0.05 considered statistically significant.

Results

Calpeptin inhibited E₂-induced MCF-7 cell proliferation

Using MTT colorimetry to detect cell proliferation rates, we observed that treatment of MCF-7 breast cancer cells with 10⁻⁸ M E2 for 24 h increased cell proliferation (P<0.05) compared with the control group (Table 1). However, including calpeptin pre-treatment (10⁻⁷ to 10⁻⁵ M concentrations) to inhibit

CANP resulted in inhibition of the E₂-induced proliferation; the 10⁻⁵ M calpeptin pre-treatment produced the most dramatic effect on E₂-induced proliferation. Tamoxifen, an inhibitor of estrogen activity, was used as a positive control; tamoxifen treatment prior to 10⁻⁸ M E2 stimulation also resulted in reduced cell proliferation.

Calpeptin mitigated effects of E2 on MCF-7 cell cycle progression

Cell cycle progression was analyzed by flow cytometry after treatment of MCF-7 cells with 10⁻⁸ M E2 for 24 h. The proportion of E2-treated cells in G₀/G₁ was reduced compared with untreated cells, while the proportion in S phase was increased (Table 2). However, pre-treatment with 10⁻⁵ M calpeptin (only one concentration of calpeptin was used based on results in Table 1) or 10⁻⁶ M TAM (positive control) prior to E2 reduced the effects of E2 on cell cycle progression: the proportion of cells in G₀/G₁ increased, with a corresponding decrease at S phase; these proportions trended toward those of the control cells.

ERK/CANP in E₂-induced MCF-7 cells

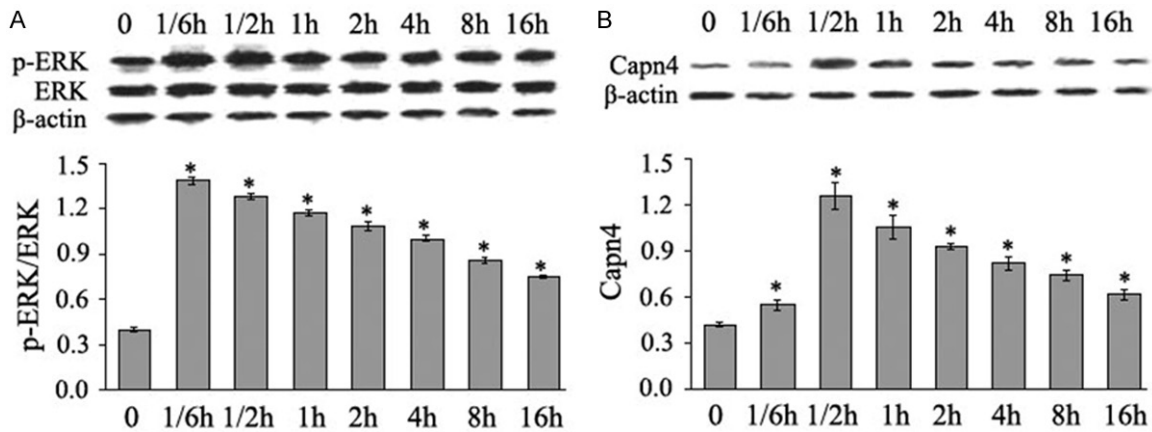


Figure 1. Expression of ERK, p-ERK, and Capn4 proteins in MCF-7 cells treated with E2 ($n=3$). A: *Top*, Bands detected by Western after indicated amount of time for which cells were exposed to E2. *Bottom*, Ratio of p-ERK to total ERK as compared by band intensity. B: *Top*, Bands detected by Western for Capn4 after indicated amount of time for which cells were exposed to E2. *Bottom*, Relative intensity of Capn4 expression. β -actin served as a control. * $P<0.05$: vs. Control group.

E2 rapidly induced p-ERK and Capn4 expression in MCF-7 cells

Western blots for expression of proteins in the non-genomic E2 signaling pathway revealed rapid changes in expression following E2 exposure. Following 10 min of treatment with 10^{-8} M E2, MCF-7 cells exhibited up-regulation of p-ERK expression ($P<0.05$). However, total ERK expression was not affected, resulting in an increased p-ERK/ERK ratio (**Figure 1A**); this effect was sustained for all E2 exposure times (up to 16 h). Similarly, 10 min treatment with E2 resulted in increased Capn4 expression ($P<0.05$; **Figure 1B**), and, although the highest Capn4 expression was observed after 30 min of E2 treatment, expression was increased for at least 16 h.

PD98059 and Calpeptin inhibited E₂-induced p-ERK and Capn4 protein expression in MCF-7 cells

To determine whether effects of E2 could be attenuated by altering the non-genomic E2 signaling pathway, Western blots were performed on MCF-7 cells pre-treated with inhibitors of CANP or ERK. Treatment of MCF-7 cells with 20 μ M PD98059, an inhibitor of ERK, or 10^{-5} M calpeptin, an inhibitor of CANP, prior to stimulation with E2 resulted in changes to the expression effects induced by E2 alone. PD98059 treatment significantly inhibited E₂-induced p-ERK expression ($P<0.05$), but had no effect on total ERK expression (**Figure 2A**). Pre-treatment with

either PD98059 or calpeptin reduced E₂-induced Capn4 protein expression ($P<0.05$, **Figure 2B**).

Discussion

E2 is critical to normal biological functions of estrogen, but has become increasingly important because of its roles in cancer biology. In particular, E2 is of interest because it exerts its biological effects through both genomic and non-genomic modes of action. These signaling pathways have distinct mechanisms, and, indeed, occur with different timing. Genomic signaling through E2 usually requires several hours or more to produce cellular responses, but non-genomic signaling can produce responses, including intracellular calcium mobilization and ERK 1/2 phosphorylation and activation [6, 7], within minutes. The rapid non-genomic signaling effects of E2 may not depend on estrogen receptor. For example, a recent report indicated that E2 can induce cell proliferation via angiotensin II receptors on the cell membrane [5].

Many effects of non-genomic signaling of E2 occur through the ERK pathway. For example, tamoxifen, which is used for endocrine therapy of breast cancer, can effectively block E2 genomic signaling, but also can induce non-genomic signaling through the ERK pathway [9]. ERK, an intracellular signaling molecule, can regulate cell proliferation through non-genomic signaling [13-15]. In addition, this protein can

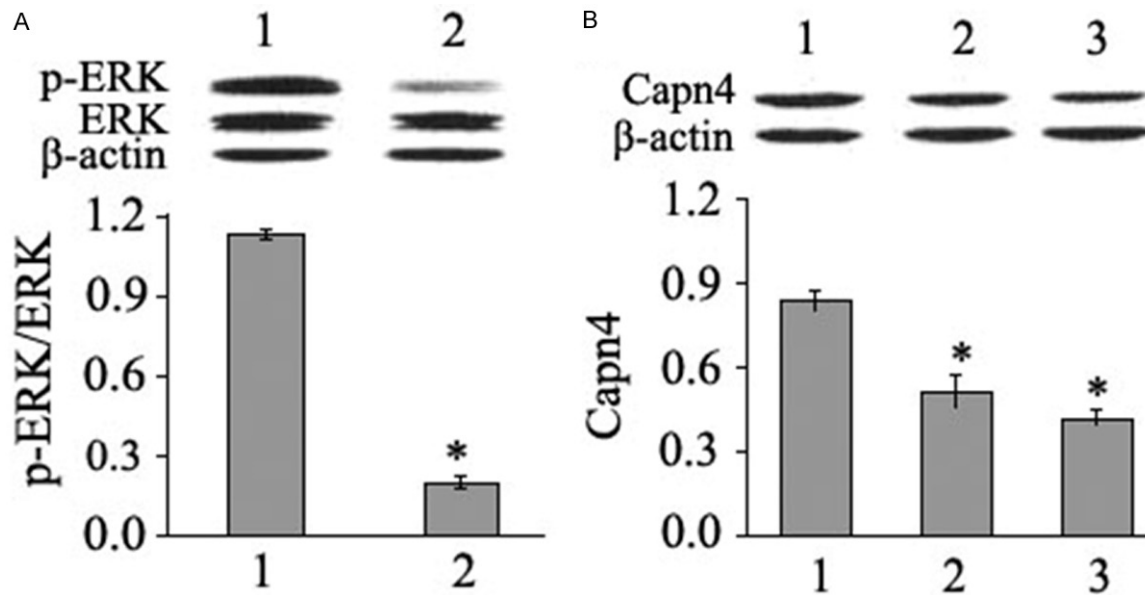


Figure 2. Inhibitory effect of PD98059 and calpeptin on expression of p-ERK and Capn4 in E₂-induced MCF-7 cells ($n=3$). A: Expression of p-ERK/ERK; B: Expression of Capn4. 1: 10⁻⁸ M E₂; 2: 10⁻⁸ M E₂ + 20 μmol/L PD98059; 3: 10⁻⁸ M E₂ + 10⁻⁵ M calpeptin. * $P<0.05$: vs. E₂ group.

induce rapid apoptosis of breast cancer cells through mediation of tamoxifen, an effect that is prevented by E₂ administration [6].

CANP is another apparent player in non-genomic E₂ signaling. CANP1 and CANP2 are widely expressed and exist as zymogens in the cytoplasm, regulating the biological activity of substrate proteins via limited cutting. *In vitro*, self proteolysis of Capn4 is the marker of CANP activation and positively correlates with the degree of CANP activation [16, 17]. Current evidence indicates that CANPs can promote apoptosis, angiogenesis, and cell proliferation [18-21] but also inhibit growth of high-density cell clones [22]. In rats, *Capn4* knockout does not affect cell growth and division, but impairs normal development of embryos [23]. Importantly, EGF can directly phosphorylate and activate CANP via ERK to induce cell migration, and in a calcium-independent manner [11]. Further, studies of brain cells indicate that E₂ can stimulate CANP activation through MAP kinases, which are related to ERK1/2 [24]. However, it remains unknown whether E₂, which is over-expressed in breast cancer, can activate CANP through ERK to stimulate cell proliferation.

In stimulating MCF-7 breast cancer cells with E₂ and investigating cell proliferation and cell cycle changes, we determined whether E₂

stimulation was occurring through the ERK/CANP non-genomic signaling pathway. Indeed, calpeptin inhibition of CANP prevented E₂-induced proliferation, indicating that CANP is involved in mediating this effect of E₂. Additionally, with only 30 min exposure to E₂, ERK phosphorylation was induced and Capn4 expression was increased, effects that were sustained over 16 h of treatment. We did not observe the previously-reported transient effect [9]. These effects, however, indicate that E₂ exposure triggers ERK and CANP signaling. E₂-induced phosphorylation and activation of ERK may stem from activation of EGF receptors in cell membrane by E₂ [15], which initiate Ras/Raf/MEK/ERK signaling in cells.

Inhibition of ERK phosphorylation reduced E₂ stimulation of Capn4 protein expression, indicating that E₂-induced ERK phosphorylation/activation is related to the rapid increase in expression of Capn4. Indeed, inhibition of CANP activity undermines E₂-induced up-regulation of Capn4 expression. Thus, E₂ induction of ERK phosphorylation/activation and up-regulation of Capn4 protein expression in breast cancer cells is characterized by rapid and persistent effects. The corresponding mechanism arise from the following two phenomena: 1) rapid up-regulation of Capn4 expression resulting from ERK phosphorylation/activation may

occur at the protein translation level, as part of the extra-nuclear effects of non-genomic signaling; and 2) when cytoplasmic ERK has been phosphorylated, it transfers into the nucleus to regulate gene transcription activity, thus inducing altering Capn4 expression during later stages.

In summary, E₂ can rapidly activate the non-genomic ERK/CANP signaling pathway to stimulate cell cycle progression and proliferation in breast cancer cells, resulting in increases in both Capn4 expression level and CANP activity. Both ERK and CANP inhibitors can block this effect, to delay cancer cell proliferation, thereby providing a theoretical basis for potential new clinical treatments for breast cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jin-Long Zhao or Guo-Sheng Wang, The First Affiliated Hospital of Harbin Medical University, 23 Youzheng Str, Nangang District, Harbin 150001, China. E-mail: jinlong-zhao123@126.com (JLZ); wgsydma@126.com (GSW)

References

- [1] Platet N, Cathiard AM, Gleizes M, Garcia M. Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion. *Crit Rev Oncol Hematol* 2004; 51: 55-67.
- [2] Marino M, Acconcia F, Ascenzi P. Estrogen receptor signaling: bases for drug actions. *Curr Drug Targets Immune Endocr Metabol Disord* 2005; 5: 305-314.
- [3] Cheskis BJ, Greger JG, Nagpal S, Freedman LP. Signaling by estrogens. *J Cell Physiol* 2007; 213: 610-617.
- [4] Acconcia F, Kumar R. Signaling regulation of genomic and nongenomic functions of estrogen receptors. *Cancer Lett* 2006; 238: 1-14.
- [5] Lim KT, Cosgrave N, Hill AD, Young LS. Nongenomic oestrogen signalling in oestrogen receptor negative breast cancer cells: a role for the angiotensin II receptor AT1. *Breast Cancer Res* 2006; 8: R33.
- [6] Zheng A, Kallio A, Härkönen P. Tamoxifen-induced rapid death of MCF-7 breast cancer cells is mediated via extracellularly signal-regulated kinase signaling and can be abrogated by estrogen. *Endocrinology* 2007; 148: 2764-2777.
- [7] Thomas W, Coen N, Faherty S, Flatharta CO, Harvery BJ. Estrogen induces phospholipase A2 activation through ERK1/2 to mobilize intracellular calcium in MCF-7 cells. *Steroids* 2006; 71: 256-265.
- [8] Walsh DE, Dockery P, Doolan CM. Estrogen receptor independent rapid non-genomic effects of environmental estrogens on [Ca²⁺]_i in human breast cancer cells. *Mol Cell Endocrinol* 2005; 230: 23-30.
- [9] Visram H, Greer PA. 17beta-estradiol and tamoxifen stimulate rapid and transient ERK activation in MCF-7 cells via distinct signaling mechanisms. *Cancer Biol Ther* 2006; 5: 1677-1682.
- [10] Ohshiro K, Schwartz AM, Levine PH, Kumar R. Alternate estrogen receptors promote invasion of inflammatory breast cancer cells via non-genomic signaling. *PLoS One* 2012; 7: e30725.
- [11] Glading A, Bodnar RJ, Reynolds IJ, Shiraha H, Satish L, Potter DA, Blair HC, Wells A. Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. *Mol Cell Biol* 2004; 24: 2499-2512.
- [12] Storr SJ, Carragher NO, Frame MC, Parr T, Martin SG. The calpain system and cancer. *Nat Rev Cancer* 2011; 11: 364-374.
- [13] Vijayanathan V, Venkiteswaran S, Nair SK, Verma A, Thomas TJ, Zhu BT, Thomas T. Physiologic levels of 2-methoxyestradiol interfere with nongenomic signaling of 17beta-estradiol in human breast cancer cells. *Clin Cancer Res* 2006; 12: 2038-2048.
- [14] Kleuser B, Malek D, Gust R, Pertz HH, Potteck H. 17 Beta-estradiol inhibits transforming growth factor-beta signaling and function in breast cancer cells via activation of extracellular signal-regulated kinase through the G protein-coupled receptor 30. *Mol Pharmacol* 2008; 74: 1533-1543.
- [15] Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* 2000; 14: 1649-1660.
- [16] Wang XD, Rosales JL, Magliocco A, Gnanakumar R, Lee KY. Cyclin E in breast tumors is cleaved into its low molecular weight forms by calpain. *Oncogene* 2003; 22: 769-774.
- [17] Daniel KG, Anderson JS, Zhong Q, Kazi A, Gupta P, Dou QP. Association of mitochondrial calpain activation with increased expression and autolysis of calpain small subunit in an early stage of apoptosis. *Int J Mol Med* 2003; 12: 247-252.
- [18] Lee MJ, Kee KH, Suh CH, Lim SC, Oh SH. Capsaicin-induced apoptosis is regulated by endo-

ERK/CANP in E₂-induced MCF-7 cells

- plasmic reticulum stress- and calpain-mediated mitochondrial cell death pathways. *Toxicology* 2009; 264: 205-214.
- [19] Su Y, Cui Z, Li Z, Block ER. Calpain-2 regulation of VEGF-mediated angiogenesis. *FASEB J* 2009; 20: 1443-1451.
- [20] Shimada M, Greer PA, McMahon AP, Bouxsein ML, Schipani E. In vivo targeted deletion of calpain small subunit, *Capn4*, in cells of the osteoblast lineage impairs cell proliferation, differentiation, and bone formation. *J Biol Chem* 2008; 283: 21002-21010.
- [21] Kashiwagi A, Schipani E, Fein MJ, Greer PA, Shimada M. Targeted deletion of *Capn4* in cells of the chondrocyte lineage impairs chondrocyte proliferation and differentiation. *Mol Cell Biol* 2010; 30: 2799-2810.
- [22] Xu Y, Mellgren RL. Calpain inhibition decreases the growth rate of mammalian cell colonies. *J Biol Chem* 2002; 277: 21474-21479.
- [23] Arthur JS, Elce JS, Hegadorn C, Williams K, Greer PA. Disruption of the murine calpain small subunit gene, *Capn4*: calpain is essential for embryonic development but not for cell growth and division. *Mol Cell Biol* 2000; 20: 4474-4481.
- [24] Zadran S, Qin Q, Bi X, Zadran H, Kim Y, Foy MR, Thompson Baudry M. 17-Beta-estradiol increases neuronal excitability through MAP kinase-induced calpain activation. *Proc Natl Acad Sci U S A* 2009; 106: 21936-21941.