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Oestrogen mediates the growth of human thyroid carcinoma cells via an oestrogen receptor – ERK pathway

Q. Zeng, G. G. Chen, A. C. Vlantis and C. A. van Hasselt

Department of Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong SAR, China

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Abstract. *Objectives*: Although thyroid cancer occurs much more frequently in females, the role of sex hormones in thyroid carcinogenesis is unknown. In this study, it has been investigated how 17β-oestradiol (E2) influenced proliferation and growth of thyroid cancer cells. *Materials and Methods*: Cell proliferation and its related molecules were examined in thyroid papillary carcinoma cells (KAT5), follicular thyroid carcinoma cells (FRO) and anaplastic carcinoma cells (ARO). Levels of oestrogen receptor (ER) α and β were regulated by their agonists (PPT and DPN), antagonists and siRNA. *Results*: E2 promoted cell proliferation. Such an effect was positively related to ERα but negatively to ERβ; PPT enhanced cell proliferation while DPN inhibited it. PPT increased Bcl-2 expression while DPN decreased it. DPN also elevated Bax expression. PPT elevated the level of phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2), suggesting a positive role of ERK1/2 in E2-induced cell proliferation. Knockdown of ERα significantly attenuated E2-mediated Bcl-2 and pERK1/2 expression. In contrast, knockdown of ERβ markedly enhanced them. *Conclusions*: Oestrogen stimulates proliferation of thyroid cancer cells, associated with increase in Bcl-2 and decrease in Bax levels in an ERK1/2-related pathway. Imbalance between ERα and ERβ may contribute to thyroid carcinogenesis.

INTRODUCTION

Thyroid cancer is the most common endocrine malignancy and accounts for the majority of endocrine cancer deaths each year (Correa & Chen 1995). Interestingly, incidence of thyroid cancer is roughly three times more frequent in females than males (Correa & Chen 1995; Rosenthal 1998), and this incidence decreases after menopause (Manole *et al*. 2001). The gender difference in development of thyroid cancer has been widely observed, suggesting that growth and progression of thyroid tumours may be influenced by sex hormones, particularly oestrogen (Ron *et al*. 1987; Hiasa *et al*. 1993; Segev *et al*. 2003).

In a previous study, we have demonstrated that both oestrogen receptors (ER) α and β existed in thyroid cancer cells (Lee *et al*. 2005). Levels of ER differ between normal and neoplastic thyroid cells, and between different types of neoplastic cell. There is evidence to

Correspondence: George G. Chen, Department of Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong SAR, China. Tel.: +852 26323934; Fax: +852 26450605; E-mail: gchen@cuhk.edu.hk

show that 17β-oestradiol (E2) stimulates growth of thyroid cells with functional ERs. ERs have traditionally been regarded as transcription factors that interact with the oestrogen-responsive element (Manole *et al*. 2001), leading to transcriptional activation of target genes. Studies have shown that E2 promotes cell proliferation and survival through a rapid non-genomic signalling pathway (Acconcia *et al*. 2005). Other studies have indicated that ERα is the primary endogenous mediator responsible for rapid action of E2, and is committed to cell proliferation and population growth (Clarke 2003). In contrast, ERβ promotes cell death through an ERβ-non-genomic pathway and thus may have a tumour suppressive function (Lazennec 2006).

Molecular pathways of ERα and ERβ have not been well defined, especially in thyroid carcinoma cells. Thus, this study has been undertaken to determine the molecular pathway by which E2 expression affects growth and proliferation of thyroid carcinoma cells.

MATERIALS AND METHODS

Cell culture and treatment

Human thyroid papillary carcinoma cells (KAT5), follicular thyroid carcinoma cells (FRO) and anaplastic carcinoma cells (ARO) were used for this study. Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal bovine serum (FBS). At 70–80% confluence, medium was changed to hormone-, growth factor- and phenol red-free RPMI 1640 medium supplemented with 0.5–1% charcoal-stripped FBS for 24 h before stimulation with either 10^{-8} M E2, 10^{-6} M PPT (a potent ERα-selective agonist) or 10^{-6} M DPN (a potent ERβ-selective agonist). Concentration of E2 at 10^{-8} M is within the serum level of E2 found in women in the follicular phase of their menstrual cycle (Mendelsohn & Karas 1999). MPP (a selective ERα antagonist) at 10^{-6} M, ICI182780 (a pure oestrogen antagonist) at 10^{-6} mol/L or PD098059 (a specific inhibitor of MAPK kinase) at 50×10^{-6} M were added to cells 1 h prior to the E2 treatment.

Assessment of cell proliferation and apoptosis

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to quantify cell proliferation based on cell viability and was performed as described elsewhere (Lee *et al*. 2005). Proliferation of cells was further confirmed using the bromodeoxyuridine (BrdU) method. BrdU, a thymidine analogue, replaces [^{3}H]thymidine becoming incorporated into S phase newly synthesized DNA strands of actively proliferating cells. The assay was performed using a BrdU cell proliferation assay kit (CHEMICON, Temecula, CA, USA).

Apoptosis was assessed by two methods, a DNA fragmentation assay, and annexin V/propidium iodide (PI) staining, DNA fragmentation being a typical feature of the apoptotic cell. Quantities of DNA fragments were measured using a DNA fragmentation ELISA kit (Roche, Mannheim, Germany). Phosphatidylserine externalization is a further feature of apoptosis, which can be analysed using flow cytometry with annexin V and PI staining.

Western blot analysis

Based on the result of cell proliferation assays, 10^{-8} M E2, 10^{-6} M PPT, 10^{-6} M DPN, 10^{-6} M MPP, 10^{-6} mol/L ICI182780 and 50×10^{-6} M PD098059 samples were chosen for Western blot analysis. Total protein was extracted from the cultured cells and was subjected to Western blotting (Lee *et al*. 2005). β-Tubulin or actin protein served as a control of equal loading.

RNA interference (siRNA)

siRNA was used to block ERα and ERβ. ERα siRNA, ERβ siRNA and the control vector were obtained from Dharmacon (Lafayette, CO, USA). Cells were seeded in six-well plates and were incubated in 2 mL normal growth medium containing serum, without antibiotics, overnight to allow cells to attach to the plates. The cells were then transfected with 50 nmol/L of siRNA using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Culture medium was replaced with growth medium containing serum 6 h after transfection, and cells were cultured at 37 °C in a CO_2 incubator for 24 h. After incubation, medium was changed to hormone-, growth factor- and phenol red-free RPMI 1640 medium supplemented with 0.5–1% charcoal-stripped FBS for 24 h before stimulation with 10^{-8} M E2 for 24 h. At the end of treatment, cells were harvested for Western blot analysis.

Statistical analysis

Data were presented as mean ± SD for at least three separate determinations for each group. Differences between groups were examined for statistical significance using Student's *t*-test or one-way ANOVA followed by Student's *t*-test. A *P*-value of < 0.05 was used to indicate statistically significant difference.

RESULTS

Effect of E2, PPT and DPN on cell viability

Our previous studies have shown that human thyroid cancer cells expressed both ERα and ERβ. Here, we have treated cells with E2 or PPT for different periods of time; their viability was examined by MTT assay and levels of cell proliferation by BrdU incorporation. E2, at concentrations of 10^{-7} M, 10^{-8} M, 10^{-9} M and 10^{-10} M gradually promoted cell proliferation for up to 48 h, as measured by MTT, in KAT5 cells (Fig. 1a). At 72 h, cell proliferation continued to increase only at an E2 concentration of 10^{-10} M, while cell proliferation at other concentrations of E2 dropped to levels similar to those at 12 h and 24 h. Similar to E2, the ERα agonist PPT stimulated cell proliferation (Fig. 1b). Maximal effect of PPT was achieved at 48 h after treatment. BrdU cell proliferation protocol confirmed the above data obtained by MTT assay (Fig. 1e–g). In the presence of ERα antagonist MPP or oestrogen antagonist ICI182780, E2 was unable to stimulate cell proliferation (Fig. 1c,g). Similar results were obtained in FRO and ARO cells (data not shown).

Quite interestingly, the effect of ERβ agonist DPN on cell proliferation was completed differently from that of the ERα agonist PPT. DPN inhibited rather than promoted cell proliferation (Fig. 1d) and maximal effect of inhibition was recorded at 48 h after treatment. To determine whether such inhibition was due to cell death by apoptosis, we measured DNA fragments of cells treated by DPN. We found that DPN significantly induced DNA fragmentation in a time-dependent manner (Fig. 2a). To confirm the occurrence of apoptosis, we used a further apoptosis assay, annexin V and PI staining, to assess apoptosis (Fig. 2b); the result (Fig. 2c) was in agreement with data obtained from the DNA fragmentation assay.

ERα and ERβ expression following E2, PPT and DPN exposure

Levels of ERα increased by E2 until 12 h, after which they began to decrease (Fig. 3). A significant increase in expression of ERα occurred at 12 h after treatment in KAT5 cells (Fig. 3a,b). It was found that induction of ERα by E2 was prevented by MPP, a special ERα inhibitor, and ICI182780, an ER inhibitor. PD098059, a specific inhibitor of MAPK kinase, had no influence

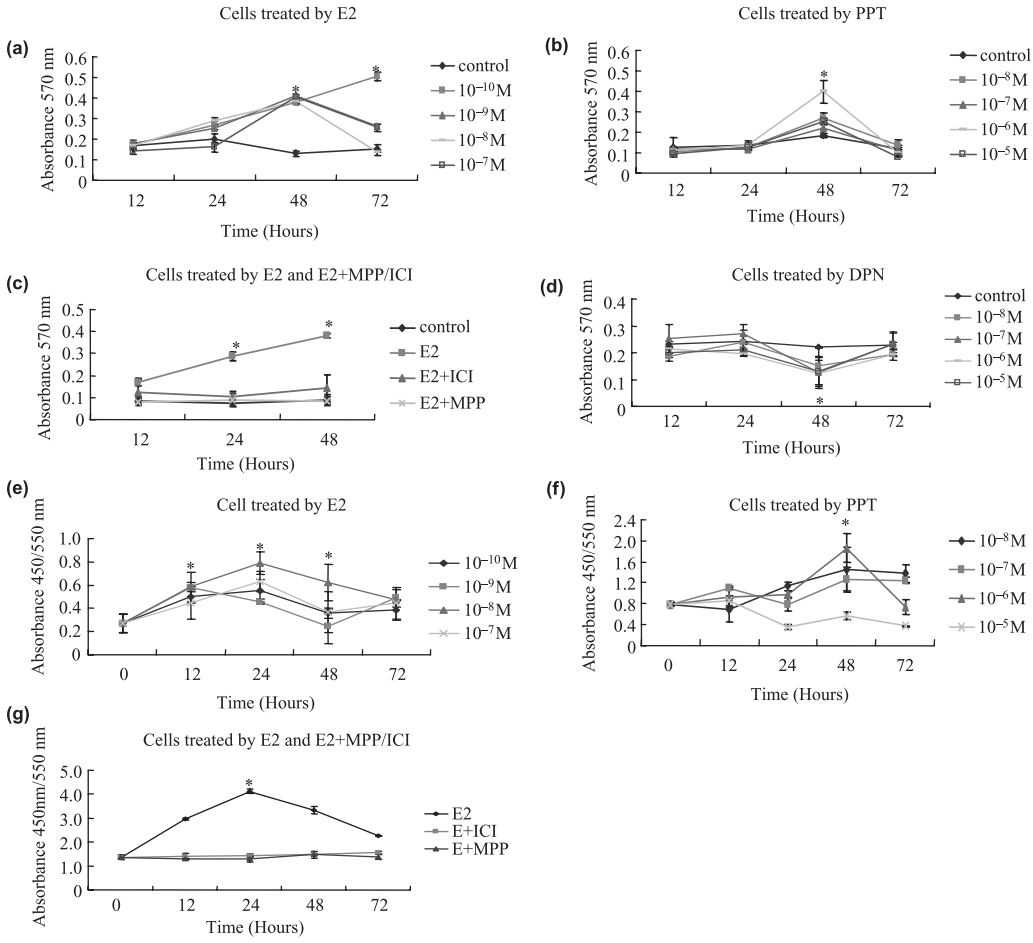


Figure 1. Cell proliferation. Thyroid cancer cells were treated with E2 (a), PPT (b), DPN (c) and E2 + ICI or E2 + MPP (d), and cell proliferation was measured by MTT assay. Compared to untreated samples, there was significant increase in cells at 48 h of treatment with 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M of E2 (all $*P < 0.05$) and the increase continued with 100 nM of E2 at 72 h ($*P < 0.05$) (a). The increase was inhibited by addition of MPP (10^{-6} M) and ICI (10^{-6} M) at 48 h after treatment (d). Cell proliferation was significantly elevated at 48 h after PPT (10^{-6} M) treatment ($*P < 0.05$) (b). An obvious decrease in cell proliferation occurred in cells treated with DPN at 48 h (c). Results of the MTT assay were confirmed by BrdU cell proliferation assay (e, f and g). Data are the mean ± SD of four separate experiments.

on ERα expression after E2 treatment. 10^{-6} M PPT alone significantly increased ERα expression. The promoting effect was maximal at 9 h and was maintained until at least 48 h after treatment. E2 also slightly stimulated expression of ERα in FRO and ARO cells after treatment for 12 h or longer (Fig. 3c). Although levels of ERβ in KAT5 cells were reduced after 6 h and 9 h treatment with E2, the change appeared not to be significant (Fig. 4a). However, the application of 10^{-6} M DPN significantly increased expression of ERβ after treatment for 12 h, and this elevated level was maintained until at least 48 h after treatment (Fig. 4b). In FRO cells, both E2 and DPN significantly enhanced levels of ERβ but they did not have any obvious effect on its expression in ARO cells (Fig. 4c).

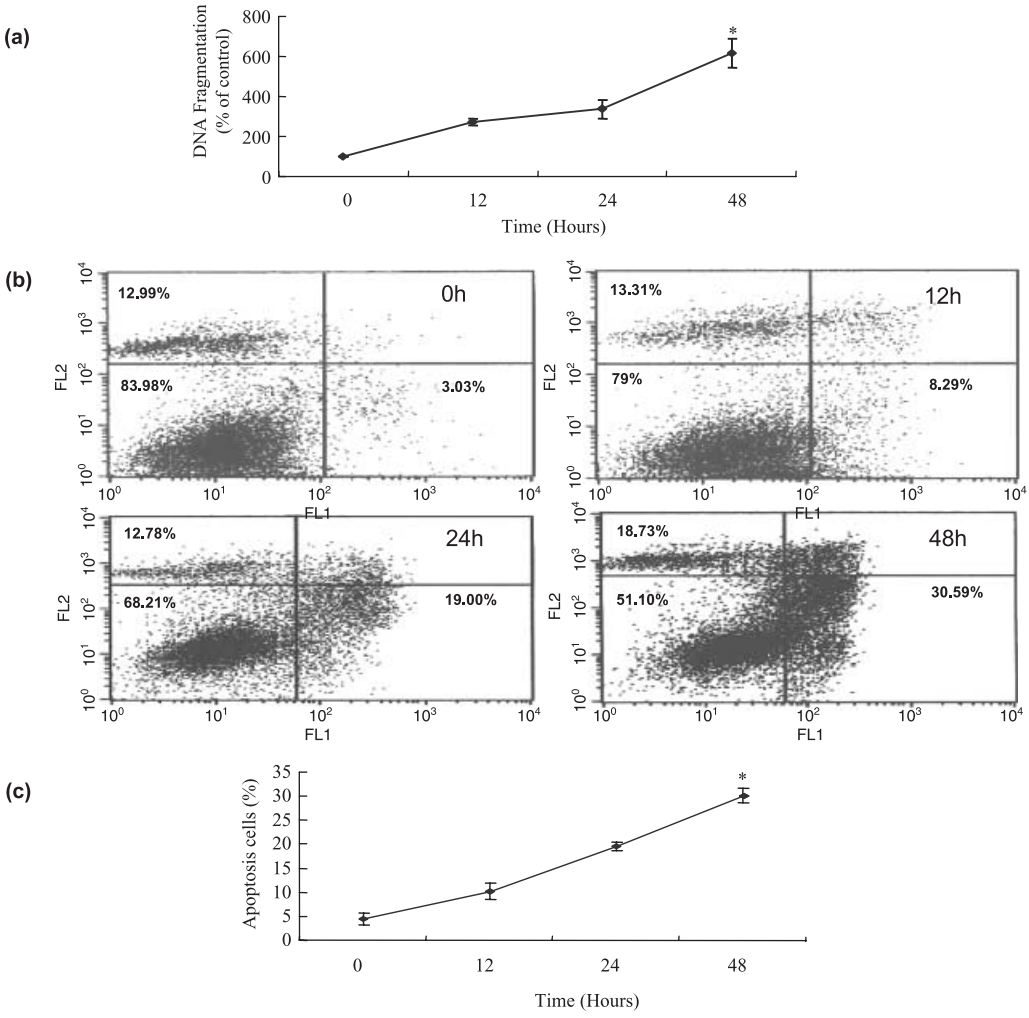


Figure 2. Measurement of apoptosis. After treatment with 10^{-6} M of DPN, DNA apoptotic bodies were detected using a DNA fragmentation ELISA kit (a). DNA fragmentation gradually increased and reached significance at 48 h after 10^{-6} DPN treatment, compared to untreated cells, $*P < 0.01$, $n = 3$. In addition to the DNA fragmentation assay, apoptotic cells were also detected using annexin V and propidium iodide staining (b). After 10^{-6} DPN treatment, viable, apoptotic and necrotic cells were determined. The percentage of apoptotic cells gradually increased and reached significance at 48 h after the treatment, compared to untreated cells, $*P < 0.01$, $n = 3$ (c). Results shown are representative of three independent experiments.

Bcl-2 and Bax expression following E2, PPT and DPN exposure

After E2 treatment, expression of Bcl-2 gradually increased and reached a peak at 12 h (Fig. 5). Effects of E2 on Bcl-2 could be offset by MPP, ICI182780 or PD098059. Similar to E2, PPT an ERα agonist, also increased expression of Bcl-2 in a time-dependant manner. However, unlike E2 and PPT, DPN, an ERβ agonist, inhibited expression of Bcl-2. The finding suggests that ERα and ERβ have opposing effects on the regulation of Bcl-2 in thyroid cancer cells. We also examined expression of Bax following E2, PPT and DPN exposure. E2 and PPT had no significant effect

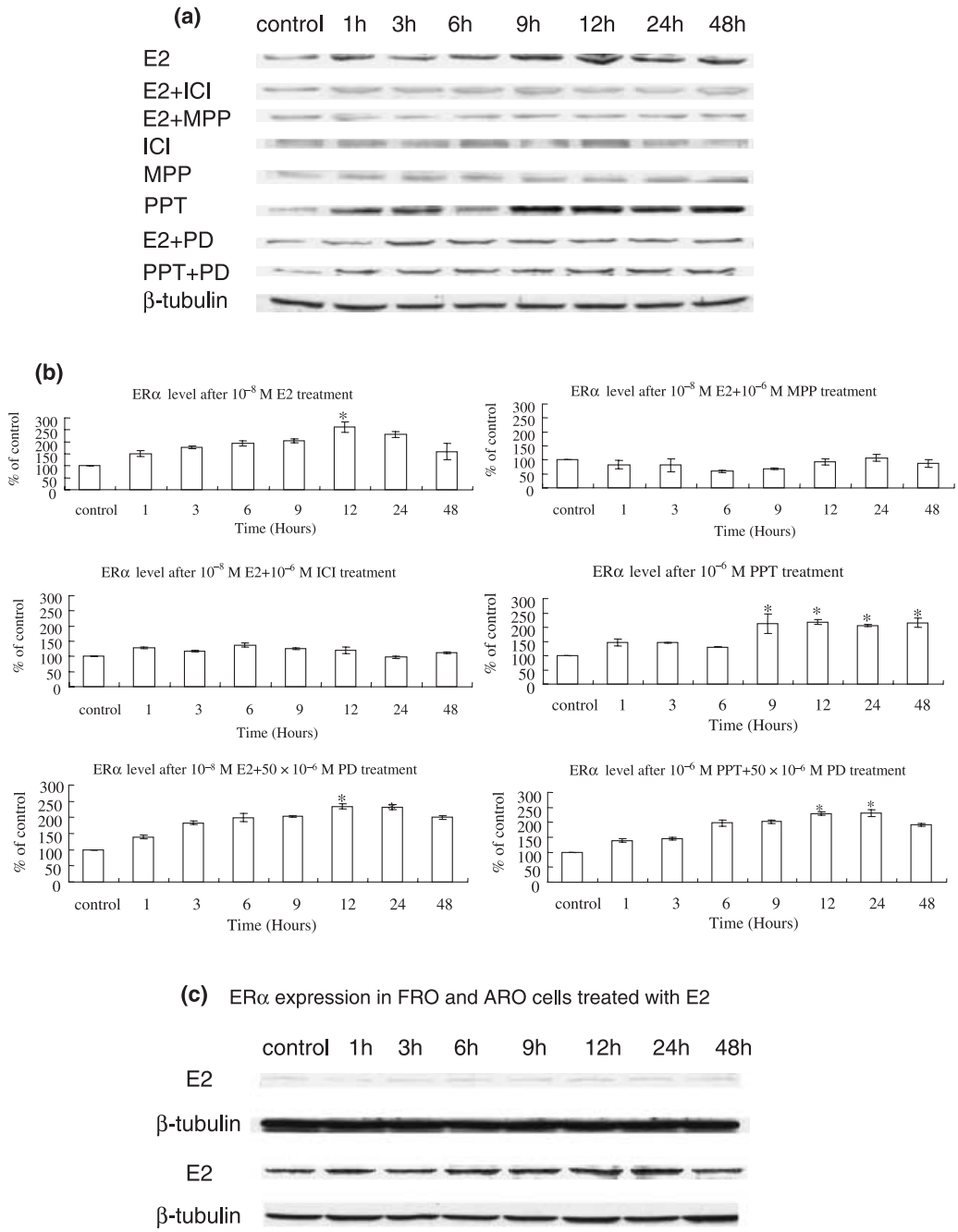


Figure 3. The expression of ERα protein in response to E2, 1PPT, E2 + MPP, 1E2 + ICI, E2 + PD and PPT + PD. Levels of ERα protein (67 kDa) in treated KAT5 cells were investigated by Western blot analysis and representative results of the independent experiments are shown (a). Densities of protein bands were determined and the average level is provided (b). A significant increase was observed in cells treated with E2 or E2 + PD from 3 h to 24 h and in cells treated with PPT from 9 h to 48 h or PPT + PD from 6 h to 24 h, compared to untreated cells, $*P < 0.01$, $n = 3$. E2 also slightly stimulated expression of ERα in FRO and ARO cells after treatment for 12 h or longer (c).

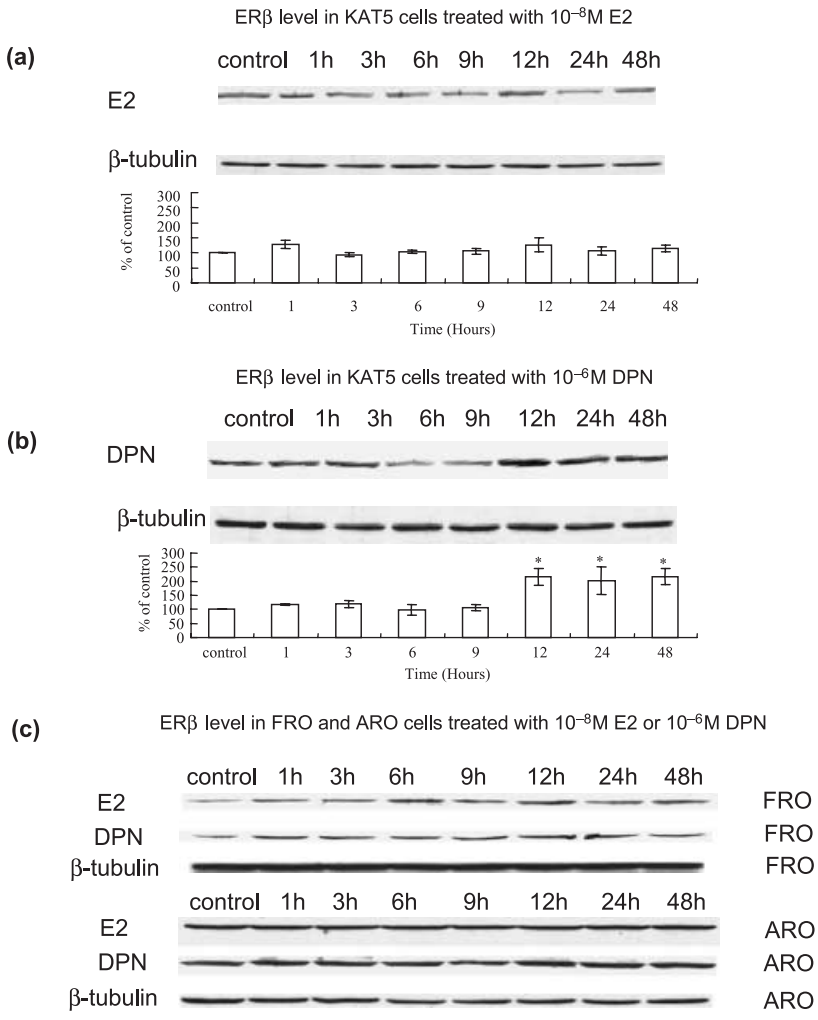


Figure 4. Expression of ERβ in response to E2 and DPN. Levels of ERβ protein (57 kDa) in KAT5 cells treated with E2 and DPN were determined by Western blot analysis. Representative results of the independent experiments are shown (a). Densities of protein bands were determined and the average level is provided (b). A significant increase in the level ERβ was observed in KAT5 cells treated with DPN from 12 h to 48 h, compared to untreated cells, $*P < 0.05$, $n = 3$. Both E2 and DPN significantly enhanced the level of ERβ in FRO cells but had no obvious effect on its expression in ARO cells (c).

on expression of Bax (Fig. 6). However, expression of Bax increased after DPN exposure, and was significant after 9 h of treatment.

Phosphorylation of ERK1/2 following E2, PPT and DPN exposure

Western blot analysis was used to determine expression of phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) in thyroid cancer cells treated with E2, PPT and DPN. This expression started to increase at 45 min after E2 treatment and reached a peak after 12 h in KAT5 cells (Fig. 7). The effect of E2 on expression of pERK1/2 was inhibited by the antagonists MPP and ICI182780. The effect of PPT on expression of pERK1/2 was similar to that of E2;

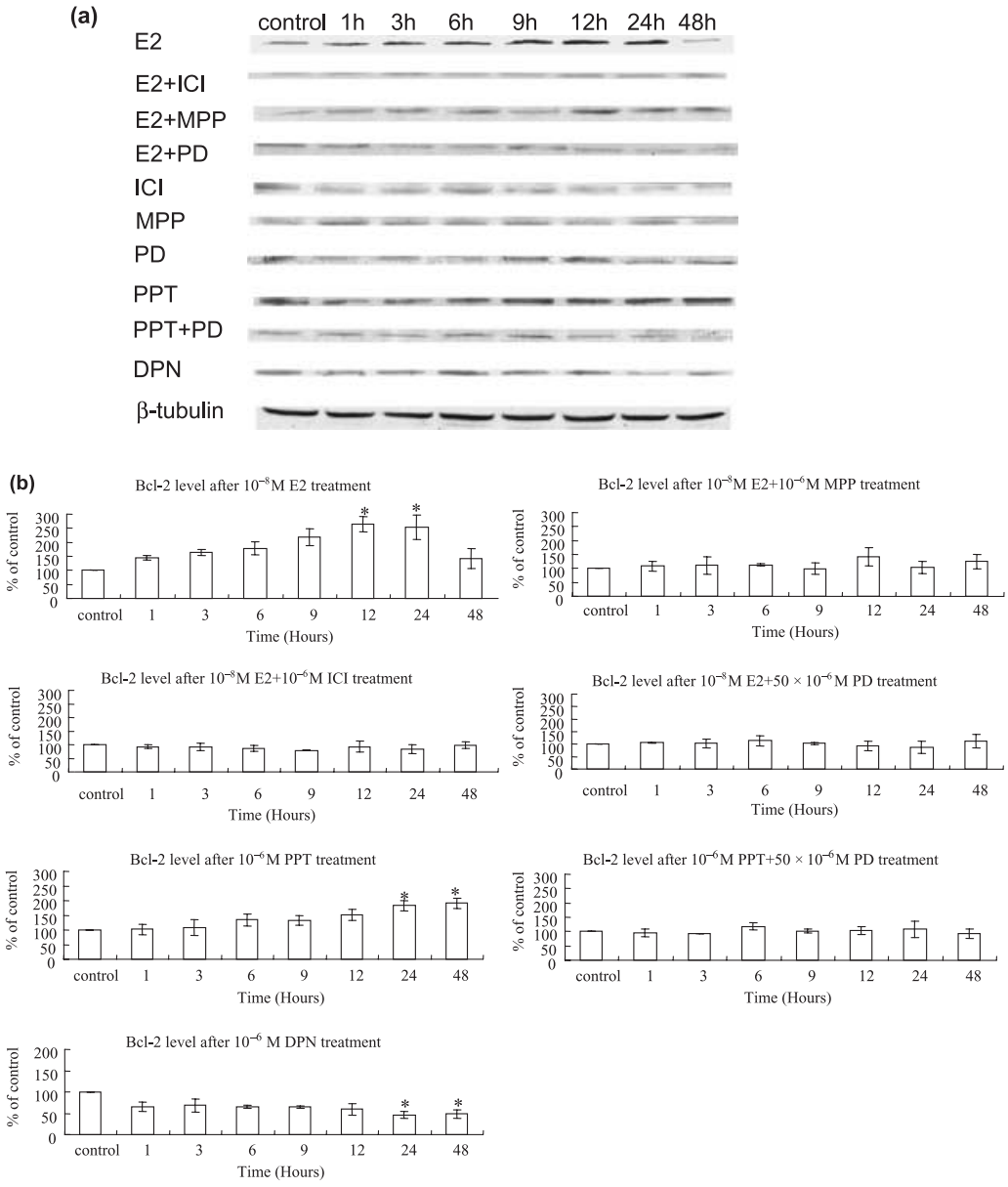


Figure 5. Expression of Bcl-2 in response to E2 and its related agents. Levels of Bcl-2 protein (28 kDa) in KAT5 cells treated with E2 and its related agents were investigated by Western blot analysis. Representative results of independent experiments are shown (a). Densities of protein bands were determined and the average level is provided (b). Compared to untreated cells, a significant increase was observed in cells treated with E2 from 9 h to 24 h, and with 10^{-6} M PPT from 12 h to 48 h (all $^*P < 0.05$, $n = 3$). In contrast, a significant decrease in the expression of Bcl-2 was observed in cells treated with 10^{-6} M DPN from 24 h to 48 h, $^*P < 0.05$, $n = 3$.

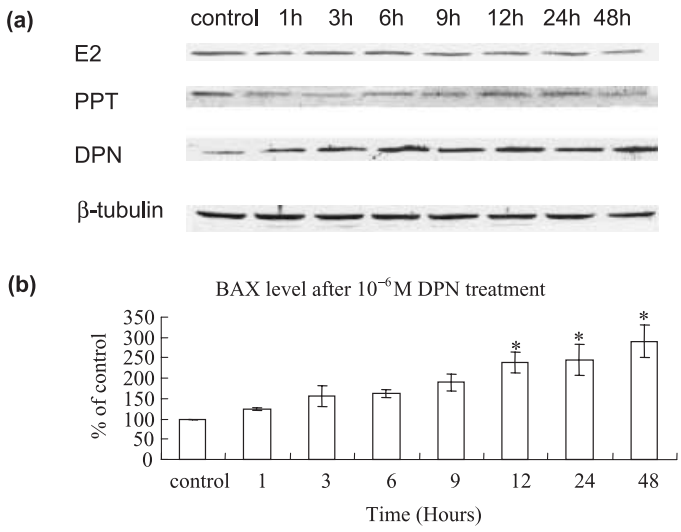


Figure 6. Expression of Bax in response to E2, PPT and DPN. Levels of Bax protein (23 kDa) in KAT5 cells treated with E2, PPT and DPN were investigated by Western blot analysis. Representative results of three independent experiments are shown (a). Densities of protein bands were determined and average level provided (b). Compared to untreated cells, a significant increase in Bax expression was observed in cells treated with DPN from 9 h to 24 h, $*P < 0.05$, $n = 3$.

however, it started earlier (15 min) and lasted longer (48 h) than E2. Maximal level of pERK1/2 also occurred much earlier in cells treated with PPT (3 h) than in those treated with E2 (12 h). DPN inhibited expression of pERK1/2 by 15 min after treatment and was maximal at 30 min after treatment; thereafter, expression returned to control levels. However, expression of total ERK1/2 was stable after treatment with E2 and its related reagents (Fig. 7c). In ARO cells, both E2 and DPN could enhance pERK1/2 at 5–35 min after treatment (Fig. 7d), and, thereafter, the level of pERK1/2 gradually returned to that close control. However, no similar effect was observed in FRO cells (Fig. 7d).

Bcl-2 and phosphorylation of ERK1/2 following E2, after ER-RNA interference

In order to determine whether ERs played a role in regulation of Bcl-2 and pERK1/2 expression, siRNA-directed against ERα or against ERβ was used to selectively block the ERs. siRNA was used at 50 nM, by which 80 ± 2.1% of ERα and 74 ± 3.4% of ERβ proteins were suppressed (Fig. 8a). At 24 h after E2 treatment, knockdown of ERα significantly attenuated E2-mediated Bcl-2 expression. In contrast, knockdown of ERβ markedly enhanced E2-induced Bcl-2 expression (Fig. 8b). Using the same strategy, similar results were obtained for pERK1/2 expression (Fig. 8b).

DISCUSSION

Both environmental and genetic factors, which are generally thought to predispose to neoplasia (such as radiation, nutrition and inheritance), have been particularly well studied and documented for thyroid cancer (Segev *et al*. 2003). However, there is increasing evidence to suggest that tumourigenesis in certain organs and tissues is influenced by endocrine function and hormones, particularly oestrogen (Ron *et al*. 1987; Manole *et al*. 2001). We have previously shown that

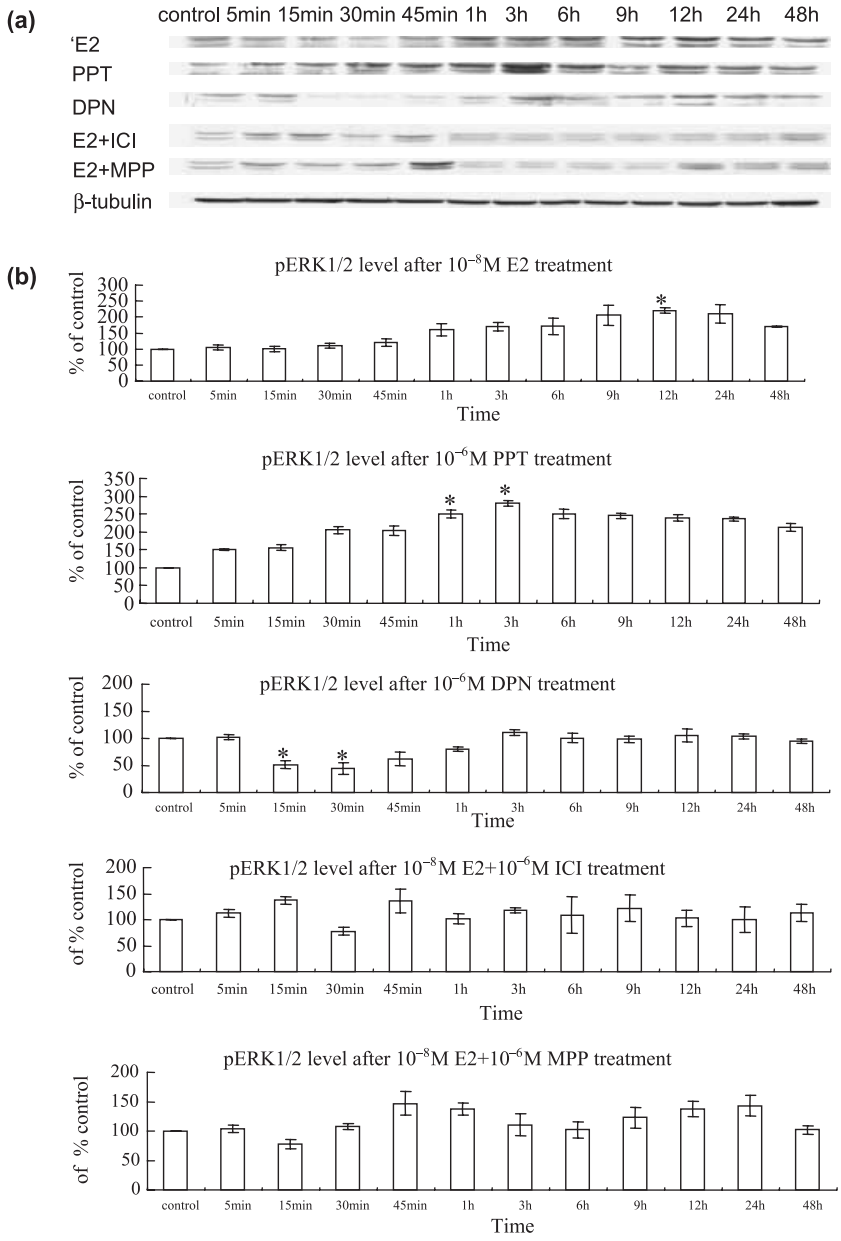
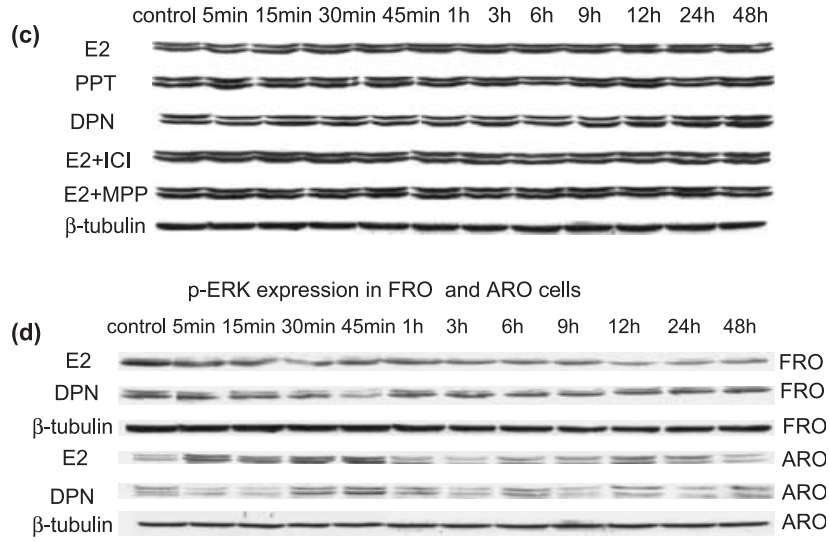


Figure 7. Expression of phosphorylated ERK1/2 in response to 10^{-8} M E2 and its related agents. Levels of pERK1/2 protein (42/44 kDa) in cells treated with E2 and its related agents were investigated by Western blot analysis. Representative results of independent experiments on KAT5 cells are shown (a). Densities of protein bands were determined and the average level provided (b). Compared to untreated cells, a significant increase of pERK1/2 was observed in cells treated with E2 at 12 h, and with PPT at 1 h and 3 h (all $*P < 0.01$, $n = 3$). However, a significant decrease was observed in cells treated with DPN at 15min and 30min ($*P < 0.01$, $n = 3$). Administration of either MPP or ICI prevented the effect of E2 on pERK1/2 protein expression. Besides, total ERK1/2 expression had no difference between E2 and its related agents in KAT5 cells (c). In ARO cells, both E2 and DPN could enhance pERK1/2 at 5–35 min after the treatment (d) and thereafter the level of pERK1/2 gradually returned to a level closed to control. No similar effect was observed in FRO cells (d).



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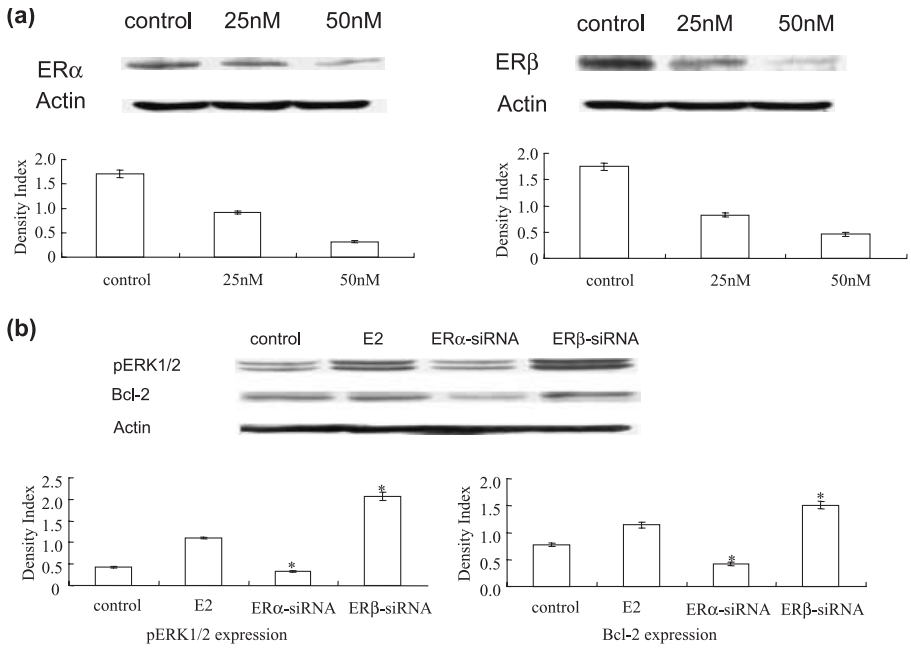

Figure 8. Block of ERα and ERβ by siRNAs. Following transfection of siRNA directed towards ERα and ERβ for 24 h, cells were treated with either E2 or vehicle alone for 24 h. Proteins were isolated from the cells and were subjected to Western blot analysis for both ERα and ERβ. Densities of protein bands were measured. Both ERα and ERβ could be significantly inhibited by 25 or 50 nM siRNA after 24 h treatment (a). Levels of Bcl-2 and pERK1/2 were measured after knockdown of ERα or ERβ (b). Data are the mean of three independent experiments,*$P < 0.05$, $n = 3$.

both ERα and ERβ are expressed in thyroid cancer cells and that E2 is able to promote their proliferation (Lee *et al*. 2005); here, we confirm that E2 promotes thyroid cancer cell proliferation. We have further demonstrated that the proliferative effect of E2 can be blocked, not only by E2 antagonist, but also by the ER antagonist, ICI182780, whose binding affinity with ERs is similar to that of E2 (Manole *et al*. 2001). This result suggests that ERs are necessary for the effect of E2. However, the result of ICI182780 application was unable to differentiate which ER subtype played the role in promotion of cell proliferation, as ICI182780 blocks both ERα and ERβ (Manole *et al*. 2001). Thus, we employed an ERα-selective antagonist, MPP (Sun *et al*. 2002), to specifically block ERα. The result demonstrated that cell proliferation induced by E2 could be effectively blocked to a similar level as that achieved by ICI182780 treatment, indicating that it is ERα rather than ERβ that plays this role in thyroid cancer cell proliferation induced by E2. In order to further clarify the roles of ERα and ERβ, we treated the cells with two ER-selective agonists, PPT (an ERα-selective agonist) and DPN (an ERβ-selective agonist). We observed that PPT promoted cell proliferation while DPN suppressed it. This result, for the first time, indicates that ERα and ERβ may have opposite functions in regulation of thyroid cancer cell proliferation, which mimics the findings obtained in mouse normal uteri, in which E2 and PPT markedly increased weight of the uterus while DPN reduced it (Frasor *et al*. 2003). The mechanism responsible for inhibitory effects of DPN and ERβ on cell proliferation appears to be associated with ability of DPN to induce apoptosis that we have determined by two different standard methods.

Findings that E2 stimulates cell proliferation and that ERα and ERβ have opposite functions in thyroid cancer cells have prompted us to assume that the expression of ERα and ERβ would be differentially regulated by E2. To test this hypothesis, expression of ERα and ERβ proteins was determined in our cells treated with E2, E2 agonists or E2 antagonists. We found that E2 treatment significantly increased expression of ERα but had no obvious effect on the level of ERβ except in FRO cells. Promotion of ERα but not of ERβ by E2 was consistent with the potential roles of ERα and ERβ, in which ERα enhanced while ERβ inhibited cell proliferation and growth. In work performed by Helguero *et al*. (2005), it was shown that E2 failed to stimulate proliferation of murine mammary epithelial cells in the presence of both ERα and ERβ. However, when expression of ERβ was blocked, E2 could significantly induce proliferation. They suggested that expression of ERβ may have a negative effect on expression of ERα in their cells. Our data suggest that a similar situation seems to apply, at least in thyroid papillary (KAT5) and anaplastic (ARO) cancer cells, in terms of influence of ERα and ERβ on cell proliferation, and levels of both receptors expressed. However, in human follicular thyroid cancer cells (FRO), E2 appears to promote both ERα and ERβ. This complicates understanding the mechanism responsible for a proliferation-promoting role of E2. Further experiments should explore expressional and functional balances of these two receptors in FRO cells to determine the roles of ERα and ERβ in them. Nevertheless, it is likely that different types of cell may respond differently to E2 to express ERα and ERβ, which may be physiologically and pathologically significant.

Having established the differential roles of ERα and ERβ in proliferation of the cells, we continued to investigate whether ERα and ERβ could influence molecules related to cell proliferation and apoptosis. In this regard, we chose to study Bcl-2 family members, as their levels are known to be altered in thyroid cancer (Kossmehl *et al*. 2003), and also are known to be regulated by oestrogen in some cell systems (Song & Santen 2003). We found that the anti-apoptotic protein Bcl-2 was up-regulated by E2 and PPT but down-regulated by DPN. We also observed that blocking of ERβ by its specific siRNA significantly increased E2-induced expression of Bcl-2 compared to the cells receiving the control vector. In contrast, the block of ERα by its specific siRNA decreased expression of Bcl-2. Thus, the result of siRNA experiments supports

the concept that E2 or ERα induces Bcl-2 while ERβ reduces it. It also suggests that increase in Bcl-2 level in thyroid cancer is likely to be a phenomenon resulting from interaction between ERα and ERβ. The roles of E2 and ERα in promotion of Bcl-2 were further supported by another experiment, in which up-regulatory effect of E2 on Bcl-2 was prevented by both the general ER antagonist, ICI182780, and the ERα-selective antagonist, MPP. In contrast to Bcl-2, the pro-apoptotic protein Bax was increased by DPN. However, E2 and PPT did not have any obvious effect on Bax expression. It is well known that apoptosis regulated by Bcl-2 family proteins is closely associated with mitochondria, and their aberrant expression usually results in change of cell function in mitochondria-mediated apoptotic pathways (Kossmehl *et al*. 2003). Therefore, E2, ERα and ERβ may regulate cell growth and death by participating in mitochondrial homeostasis.

Several investigators have demonstrated that functions of Bcl-2 family proteins are regulated by a number of protein kinases including ERK1/2 (Choi *et al*. 2003; Kurland *et al*. 2003; Trisciuoglio *et al*. 2005), but similar information in human thyroid cancer cells is lacking. Data obtained in this study indicate that E2 can induce pERK1/2, the finding of its up-regulation by E2 being reinforced by our protocol in which the ER antagonist, ICI182780, blocked this action. We further explored the possible role of ER subtypes in activation of ERK1/2 by E2. Results suggest that activation of ERK1/2 by E2 is mainly due to interaction between E2 and ERα. The ERα-selective antagonist, MPP, suppressed the effect of E2 on pERK1/2 and its inhibition was similar to that of the general ER antagonist, ICI182780. After application of ERβ siRNA, pERK1/2 was markedly up-regulated after E2 treatment. ERα siRNA had a similar effect to that of MPP on expression of pERK1/2 after E2 treatment. Thus, our findings indicate that ERK1/2 activation by E2 is positively associated with ERα in thyroid cancer cells. Although we did not directly investigate the consequence of ERK1/2 activation on cell proliferation, we showed that inhibition of ERK1/2 by PD098059 could significantly reduce the level of Bcl-2 induced by both E2 and the ERα agonist, PPT, and that increase in ERK1/2 occurred much earlier than change in levels of Bcl-2 treated with either. These results suggest that activation of ERK1/2 by either E2 or ERα is in favour of expression of anti-apoptotic Bcl-2 and that alteration of Bcl-2 is likely a downstream event in the E2/ERα-ERK1/2 pathway. These results are also consistent with our previous data that show that ERK1/2 activation protects thyroid cancer cells from apoptosis (Chen *et al*. 2004). To the best of our knowledge, activation of ERK1/2 by E2 or by ERα has not been reported previously in thyroid cancer cells. However, relationships between ERK1/2 and E2 or ER have been investigated in several other cell systems, including breast cancer cells, phaeochromocytoma cells, hippocampal neurones and male germ cells (Wu *et al*. 2005; Zivadinovic & Watson 2005; Alexaki *et al*. 2006; Vicini *et al*. 2006); our findings are consistent with results obtained in these. For example, loss of pERK1/2 leads to decrease in expression of Bcl-2, and activation of the ERK pathway results in induction of Bcl-2 in murine B-cell lymphoma cells (Kurland *et al*. 2003). Activation of ERK1/2 can also protect gastric epithelial cells from apoptosis by maintaining Bcl-2 expression (Choi *et al*. 2003). In neurones, increased calcium influx caused by E2 activates ERK, which subsequently enhances the level of Bcl-2 (Trisciuoglio *et al*. 2005). Collectively, results obtained from these studies are fully in agreement with our findings in human thyroid cancer cells, demonstrating that activation of ERK1/2 by either E2 or ERα promotes expression of anti-apoptotic Bcl-2 and thus provides a survival signal for the cells.

It is well known that E2 can function through either genomic or non-genomic signalling pathways. Our data suggest that action of E2 on activation of ERK1/2 is most likely *via* a non-genomic signalling pathway. Increased pERK1/2 occurs as early as 15 min after the treatment of cells with ERα-selective agonist, PPT, and an obvious elevation can be detected 1 h after the treatment. Similarly, ERβ-selective agonist, DPN, also acts within 15 min to down-regulate

pERK1/2. These results are in agreement with a recent study by Acconcia *et al*. (2005), which shows that E2 promotes HeLa cell survival through an ERα-non-genomic signalling pathway to activate ERK/MAPK, and induces cell death through an ERβ-non-genomic signalling pathway to inhibit ERK/MAPK.

This study has demonstrated a novel mechanism by which E2 contributes to proliferation and population growth of thyroid cancer cells. E2 treatment favours expression of ERα over ERβ, causing an imbalance between increased ERα and decreased ERβ in at least two types of thyroid cancer cell, thyroid papillary carcinoma cells (KAT5) and human anaplastic carcinoma cells (ARO). This imbalance may change the behaviour of the cells, providing them with the ability to proliferate and survive by enhancing ERK1/2 activity, and subsequently the ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax.

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