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Competing interests statement

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Efp targets 14-3-3σ for proteolysis and promotes breast tumour growth

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Oestrogen exerts its influence on target organs through activating oestrogen receptors (ERs) and regulating downstream genes by means of their oestrogen-responsive elements. Efp, a target gene product of ERα[1–3], is a member of the RING-finger B-box coiled-coil (RBCC) motif family[4]. Efp is predominantly expressed in various female organs[2] as well as in breast cancers[5], and is thought to be essential for oestrogen-dependent cell proliferation and organ development—*Efp*-disrupted mice display underdeveloped uteri and reduced oestrogen responsiveness[6]. Here we show that Efp is a RING-finger-dependent ubiquitin ligase (E3) that targets proteolysis of 14-3-3σ, a negative cell cycle regulator that causes G2 arrest[7]. We demonstrate that tumour growth of breast cancer MCF7 cells implanted in female athymic mice is reduced by treatment with antisense Efp oligonucleotide. Efp-overexpressing MCF7 cells in ovariectomized athymic mice generate tumours in the absence of oestrogen. Loss of Efp function in mouse embryonic fibroblasts results in an accumulation of 14-3-3σ, which is responsible for reduced cell growth. These data provide an insight into the cell-cycle machinery and tumorigenesis of breast cancer by identifying 14-3-3σ as a target for proteolysis by Efp, leading to cell proliferation.

We have used MCF7 cells as a model system to explore the contribution of Efp to growth of breast tumours. MCF7 cells were implanted into intact female athymic mice followed by ovariectomy or administration of sense/antisense Efp oligonucleotides (Fig. 1a–d). Levels of Efp were markedly reduced in tumours obtained from ovariectomized or antisense Efp-treated mice compared with controls, suggesting that both treatments impaired Efp expression (Fig. 1a). Tumour volume did not increase in ovariectomized mice, consistent with the oestrogen-dependent nature of MCF7 cells (Fig. 1d). Tumour growth was observed in control mice (Fig. 1c, d); however, a dose-dependent inhibition of tumour growth was observed in mice treated with antisense Efp (Fig. 1b–d). We infer that tumour growth is modulated by Efp expression, implicating Efp as an oncogenic factor in breast cancers.

To test this hypothesis, we generated MCF7 cells that stably expressed Efp (Efp-MCF7 cells) and investigated whether Efp modulates progression of the cell cycle. Endogenous levels of $p21^{Cip1}$ and 14-3-3σ were reduced in Efp-MCF7 cells as compared with control MCF7 cells (vector-MCF7 cells) (Fig. 2a). These proteins are negative regulators of cell cycle progression and are important for G1 and G2 arrest after DNA damage[7,8], suggesting that Efp may regulate cell cycle checkpoints (Fig. 2a). This was confirmed by cell cycle analysis using flow cytometry, as a high percentage of Efp-MCF7 cells were in the proliferating stage, particularly in S phase, whereas vector-MCF7 cells were primarily in the G1 phase (Fig. 2b). To determine whether Efp overexpression influences tumour growth, we inoculated transfected MCF7 cells into intact female athymic mice. After two months, both vector-MCF7 and Efp-MCF7 cells showed tumour formation; however, the tumours were larger in Efp-MCF7 mice (data not shown). The role of Efp as a primary regulator of oestrogen-dependent signalling was verified by implanting transfected MCF7 cells into ovariectomized mice. Tumours did not grow in vector-MCF7 mice, whereas prominent tumour growth was observed in ovariectomized mice inoculated with Efp-MCF7 cells (Fig. 2c). To further confirm the role of Efp in tumour growth, transfected MCF7 cells were inoculated into intact athymic mice, followed by ovariectomy when the tumour volume reached 180 mm^3 (Fig. 2d). Tumour volume in vector-MCF7 animals did not increase further, whereas tumours in Efp-MCF7 animals continued to grow in the absence of gonadal oestrogens. This indicates that elevated levels of Efp promote cell growth, indicating that Efp might directly regulate the cell cycle machinery.

As a first step towards understanding the role of Efp in cell cycle progression, we used yeast two-hybrid screening to identify Efp-interacting proteins. Notably, a single interacting clone coding for 14-3-3σ was identified by screening 3×10^6 yeast clones transformed with a mouse embryo complementary DNA library. 14-3-3σ was originally identified as an epithelial-specific marker, HME1, which is downregulated in a few breast and colon cancer cell lines[7,9]. Although 14-3-3σ seems to be an important negative cell cycle

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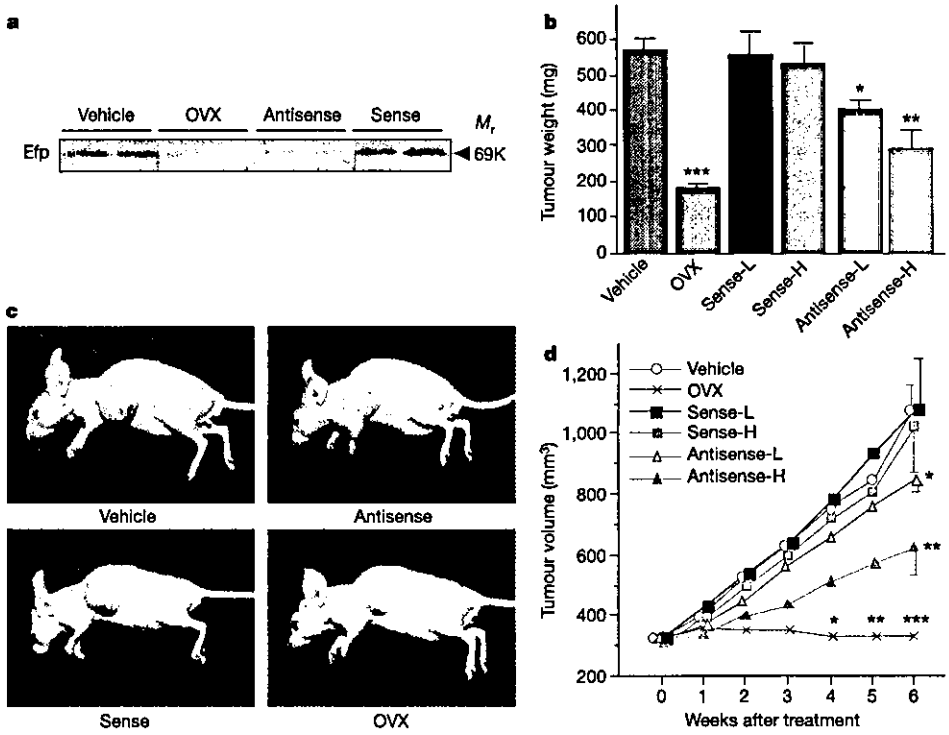

Figure 1 Inhibition of Efp expression suppresses tumour growth by MCF7 cells. Athymic female mice were inoculated with MCF7 cells then treated with an ovariectomy (OVX), or administration of vehicle (saline) or sense/antisense Efp oligonucleotides (sense-L and antisense-L, 20 μg per week; sense-H/sense and antisense-H/antisense, 100 μg per week). **a**, Efp expression in tumours obtained from mice inoculated with MCF7 cells. **b**, Weight of tumours caused by MCF7 cells in nude mice. **c**, Reduced tumour size in mice 6 weeks after inoculation with antisense Efp or with OVX. **d**, Reduced tumour volume in mice treated with antisense Efp or with OVX. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$; $n = 5$.

regulator, a regulatory mechanism for 14-3-3σ activity was previously unknown.

Next, we tested whether Efp can specifically interact with and regulate the activity of 14-3-3σ in mammalian cells. COS7 cells were transiently transfected with Efp and 14-3-3σ. Both proteins localized in the cytoplasm (Fig. 3a) and immunoprecipitation confirmed that Efp and 14-3-3σ formed a complex in the cells (Fig. 3b). In contrast, we did not detect interaction between Efp and other cell cycle checkpoints such as $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (data not shown). To define 14-3-3σ-binding domains in Efp, we constructed a series of Efp deletion mutants and analysed their binding to full-length 14-3-3σ by immunoprecipitation (Fig. 3c). Neither amino-terminal Efp (residues 1–122) containing the RING finger domain nor carboxy-terminal Efp (residues 440–630) containing the SPRY

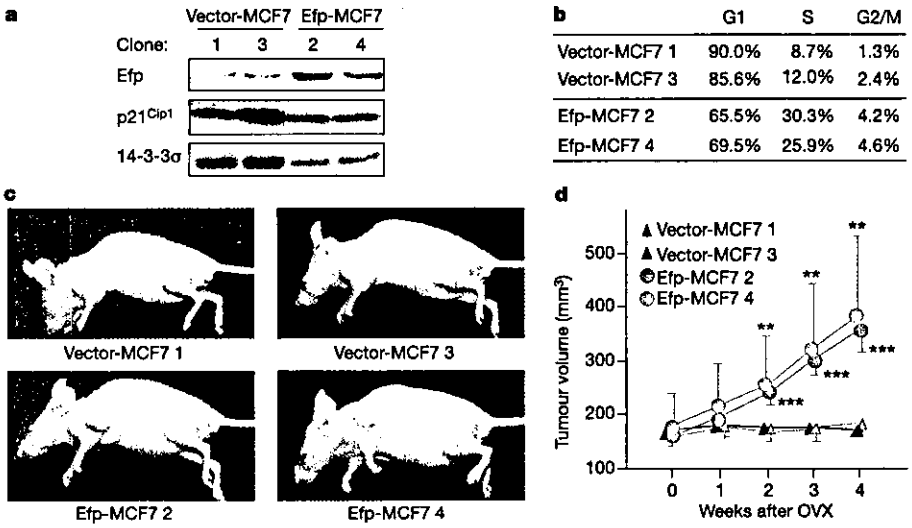

Figure 2 Efp promotes cell cycle progression and tumour growth by MCF7 cells in an oestrogen-independent manner. **a**, Efp overexpression downregulates levels of 14-3-3σ or $p21^{Cip1}$ in MCF7 cells. Efp-MCF7 cells stably express Efp. **b**, Efp overexpression increases the percentage of cells in the proliferation stage of the cell cycle. **c**, Hormone-independent tumour growth is observed in ovariectomized nude mice bearing Efp-MCF7 cells. Mice were inoculated with MCF7 cells and observed after 2 months. **d**, Tumour volume of mice bearing MCF7 transfectants after ovariectomy. Mice were ovariectomized at a tumour volume of 180 mm^3. Triple asterisk, $P < 0.001$.

domain could bind to 14-3-3σ. However, Efp (81–450) containing the B-box coiled-coil domain was able to interact with 14-3-3σ. The B-box coiled-coil domain often associates with a RING finger to form a larger conserved motif, RBCC[4], and it is this motif in Efp that specifically interacts with 14-3-3σ. It is notable that the RBCC motif family includes several oncogenic proteins such as PML[10] and TIF1 (ref. 11). We next investigated whether Efp overexpression affects the expression of 14-3-3σ. Co-transfection of Efp and 14-3-3σ into COS7 cells resulted in lower levels of 14-3-3σ protein compared with cells transfected with 14-3-3σ alone (Fig. 3d). Similar results were obtained in HEK293 cells (data not shown). To explore whether Efp overexpression can modulate 14-3-3σ turnover, we performed pulse and chase experiments in transfected COS7 cells (Fig. 3e). In the vector-transfected cells, ^{35}S-labelled 14-3-3σ immunoprecipitated by anti-haemagglutinin (HA) remains at more than 50% after 3 h, whereas the degradation rate of 14-3-3σ was explicitly enhanced in Efp-overexpressed cells (12.2% 14-3-3σ remaining after 3 h in Efp-overexpressed cells compared with 58.4% in control cells). The reduced levels of 14-3-3σ in MCF7 cells caused by adenoviral Efp transduction were recovered by MG132, a potent inhibitor of proteasome function[12] (Fig. 3f). The finding that MG132 increases the amount of 14-3-3σ binding to Efp was further confirmed by immunoprecipitation (Fig. 3g). These data suggest that Efp directly downregulates 14-3-3σ levels through a proteasome-dependent mechanism.

We next investigated how Efp downregulates expression of 14-3-3σ. One possible mechanism is that Efp functions as an E3 ubiquitin ligase similar to other RING finger proteins such as Rbx1 (refs

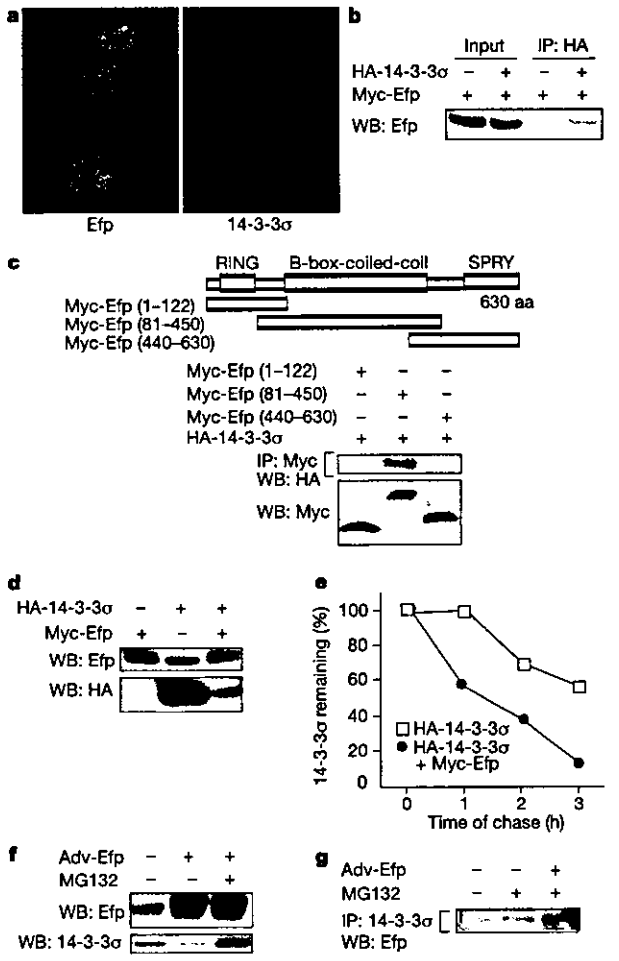

Figure 3 Efp conjugates to 14-3-3σ and promotes 14-3-3σ proteolysis in a proteasome-dependent manner. **a**, Immunoreactivities of Efp (fluorescein isothiocyanate (FITC), left panel) and 14-3-3σ (rhodamin, right panel) expressed in COS7 cells were co-localized in the cytoplasm. **b**, Interaction of Efp with 14-3-3σ. Lysates of COS7 cells transfected with indicated plasmids were analysed by immunoblotting using anti-Efp antibody (WB: Efp), combined without (input) or with (IP: HA) immunoprecipitation using anti-HA. **c**, Specific interaction of the B-box coiled-coil domain in Efp with 14-3-3σ. Lysates of transfected COS7 cells were immunoprecipitated with anti-Myc and analysed by immunoblotting using anti-HA (middle panel: IP: Myc, WB: HA). The amounts of Myc-Efp mutants (WB: Myc) are shown in the bottom panel. **d**, Efp overexpression downregulates exogenous 14-3-3σ. Lysates of transfected COS7 cells were analysed by anti-Efp (top panel) or anti-HA (bottom panel). **e**, 14-3-3σ degradation is accelerated by Efp expression. COS7 cells transiently transfected with HA-14-3-3σ and vector/Myc-Efp were pulse-labelled with [^{35}S]methionine for 30 min, and chased for the indicated time. **f**, Adenoviral Efp expression downregulates endogenous 14-3-3σ in a proteasome-dependent manner. MCF7 cells transduced with β-galactosidase (Adv-Efp −) or Efp (Adv-Efp +) adenoviruses were incubated with either vehicle (MG132 −) or MG132 (10 μM) for 8 h. Lysates were analysed by anti-Efp (top panel) or anti-14-3-3σ (bottom panel). **g**, MG132 enhanced *in vivo* interaction between adenovirally expressing Efp and endogenous 14-3-3σ. Lysates of MCF7 cells were immunoprecipitated with anti-14-3-3σ and analysed by anti-Efp.

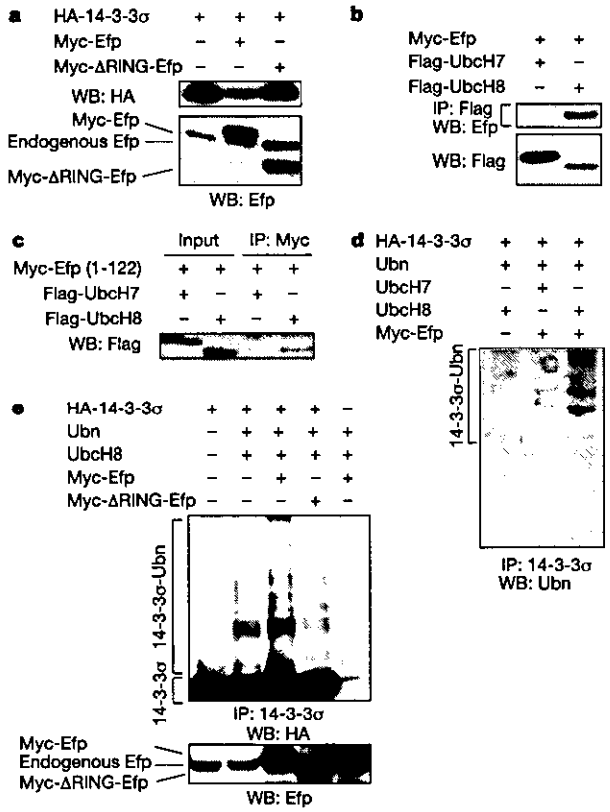

Figure 4 Efp is a RING-finger-dependent ubiquitin ligase that targets 14-3-3σ. **a**, 14-3-3σ downregulation is mediated through the RING finger in Efp. COS7 cells were co-transfected with HA-14-3-3σ and the indicated Myc-Efp mutants. Top panel, 14-3-3σ levels; bottom panel, expression of Efp with or without Myc-tagging. **b**, Association of Efp with UbcH8. COS7 cells were co-transfected with Myc-Efp and Flag-tagged UbcH7/UbcH8. **c**, The RING finger in Efp is required for interaction with UbcH8. COS7 cells were co-transfected with Myc-Efp (residues 1–122) and Flag-tagged UbcH7/UbcH8. **d**, Efp and UbcH8 promote 14-3-3σ ubiquitination. Anti-ubiquitin was used to detect ubiquitinated forms in 14-3-3σ immunoprecipitates obtained from transfected COS7 cells. **e**, The RING finger in Efp is required for 14-3-3σ ubiquitination. Anti-HA was used to detect HA-14-3-3σ and its ubiquitinated forms (Ubn) in 14-3-3σ precipitates obtained from transfected COS7 cells (top panel). The bottom panel shows expression of Efp with or without Myc-tagging.

13, 14), BRCA1 (ref. 15), Cbl (ref. 16), Mdm2 (ref. 17), parkin (ref. 18) and MID1 (ref. 19). The ubiquitination-dependent proteolysis is related to cell cycle progression and carcinogenesis[20–23]. We constructed a RING-finger-deleted Efp mutant that lacked the 31 N-terminal amino acids (ΔRING-Efp), and tested its function in COS7 cells. The ΔRING-Efp mutant failed to downregulate levels of 14-3-3σ, indicating that the RING finger in Efp is essential for regulation of 14-3-3σ (Fig. 4a). Furthermore, Efp preferentially bound to ubiquitin-conjugating enzyme UbcH8 rather than UbcH7 (Fig. 4b). This interaction is RING-finger-dependent because Efp (1–122) was immunoprecipitated with UbcH8 (Fig. 4c). We next examined whether Efp can ubiquitinate 14-3-3σ in a UbcH8-dependent manner. COS7 cells were transfected with Efp, 14-3-3σ, ubiquitin and UbcH8/UbcH7. Large amounts of ubiquitinated 14-3-3σ were observed in cells transfected with UbcH8 but not with UbcH7 (Fig. 4d). Cells transfected with the ΔRING-Efp mutant failed to ubiquitinate 14-3-3σ, confirming that ubiquitination requires the Efp RING finger (Fig. 4e). We conclude that Efp downregulates 14-3-3σ through ubiquitin-mediated degradation in which Efp functions as an E3 that selectively requires UbcH8.

We investigated further the effects of Efp abrogation on cell growth using mouse embryonic fibroblasts (MEFs) from $Efp^{+/+}$ and $Efp^{-/-}$ mice[6] as models. The growth of $Efp^{+/+}$ MEFs was normal, whereas that of $Efp^{-/-}$ MEFs was severely reduced (Fig. 5a). 14-3-3σ levels were markedly elevated in $Efp^{-/-}$ MEFs (Fig. 5b). The levels of Cdk2 and Cdc2 bound to $p21^{Cip1}$ or 14-3-3σ were elevated in $Efp^{-/-}$ MEFs (Fig. 5c), although the levels of Cdk2 and Cdc2 in both MEFs were not significantly different (Fig. 5c). This suggests that increased amounts of 14-3-3σ and $p21^{Cip1}$ in $Efp^{-/-}$ MEFs tend to entrap mitotic factors, leading to reduced growth. We examined the effects of 14-3-3σ antisense/sense oligonucleotides on MEF cell growth by collecting cells 24 or 48 h after initial incubation with oligonucleotides (Fig. 5d). The reduced cell growth in $Efp^{-/-}$ MEFs was rescued by the antisense oligonucletide in a dose-dependent manner, suggesting that 14-3-3σ is the principal checkpoint in $Efp^{-/-}$ MEFs. To explore whether the accumulation of 14-3-3σ in $Efp^{-/-}$ MEFs is due to stabilization of the protein rather than activation of transcription, we performed pulse and chase experiments (Fig. 5e). The half-life ($t_{1/2}$) of 14-3-3σ was 4 h in $Efp^{-/-}$ MEFs (95% confidence interval, 6.7–2.9 h), which was significantly longer than $t_{1/2}$ in $Efp^{+/+}$ MEFs (2.3 h, 95% confidence interval, 2.8–1.8 h, $P < 0.001$). The accumulation of 14-3-3σ in $Efp^{-/-}$ MEFs was reversed dose-dependently by adenoviral Efp transduction (Fig. 5f). The reduced levels of 14-3-3σ in $Efp^{-/-}$ MEFs adenovirally expressing Efp was rescued by MG132, which is consistent with the results indicating that Efp degrades 14-3-3σ through a ubiquitin-dependent pathway.

Our findings provide insight into the cell cycle machinery and growth of oestrogen-dependent tumours. In the classical scheme, DNA damage induces elevation of p53 levels and p53 promotes transcription of Cdk inhibitors, which recruit cyclin–Cdk complexes leading to cell cycle arrest and DNA repair[8,24–27]. Here we

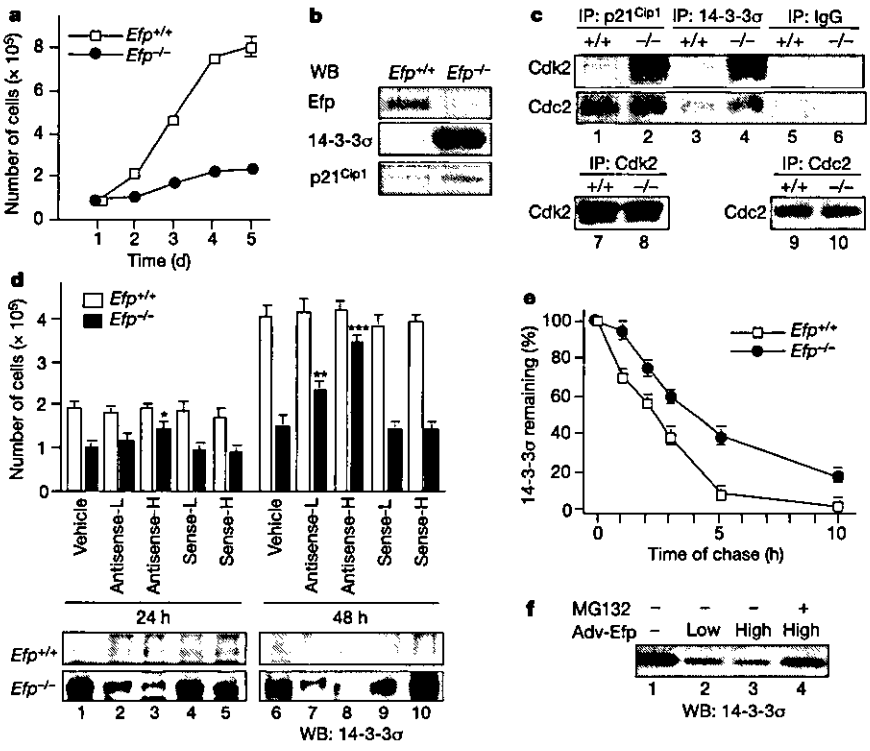

Figure 5 Loss of Efp function causes an accumulation of 14-3-3σ and inhibits cell proliferation. **a**, The growth of $Efp^{-/-}$ MEFs is reduced compared with $Efp^{+/+}$ MEFs. **b**, Accumulation of 14-3-3σ in $Efp^{-/-}$ MEFs. **c**, High amounts of Cdk2 (top panel) or Cdc2 (middle panel) associated with $p21^{Cip1}$ or 14-3-3σ are detected in $Efp^{-/-}$ MEFs. The amounts of precipitated Cdk2 and Cdc2 are shown in the bottom panel. **d**, Reduced growth in $Efp^{-/-}$ MEFs was rescued by antisense 14-3-3σ oligonucleotide. MEFs were incubated with antisense/sense oligonucleotides (antisense-L/sense-L, 100 nM; antisense-H/sense-H, 400 nM), or with vehicle. The graph shows the number of cells collected 24 or 48 h after initial oligonucleotide transfection. The bottom panel shows 14-3-3σ levels. Lanes 1–5 and 6–10 show lysates from indicated cells collected 24 and 48 h after initial transfection, respectively. Asterisk, $P < 0.05$ compared with vehicle at 24 h; double asterisk, $P < 0.01$ compared with vehicle at 48 h; triple asterisk, $P < 0.001$ compared with vehicle at 48 h. **e**, 14-3-3σ turnover is more stabilized in $Efp^{-/-}$ MEFs than in $Efp^{+/+}$ MEFs. Lysates were immunoprecipitated by anti-14-3-3σ. **f**, 14-3-3σ accumulation in $Efp^{-/-}$ MEFs is reduced by adenoviral Efp expression in a proteasome-dependent manner. MEFs adenovirally expressing β-galactosidase (lane 1) or Efp at a low dose (40 MOI) (lane 2) or at a high dose (100 MOI) (lanes 3 and 4) were subjected to immunoblotting using anti-14-3-3σ. Cells in lane 4 were treated with MG132 (10 μM).

demonstrate a mechanism in which the function of 14-3-3σ is modulated by Efp-mediated proteolysis. The degradation of 14-3-3σ is subsequently followed by dissociation of the protein from cyclin–Cdk complexes, leading to cell cycle progression and tumour growth. Indeed, it has been shown recently that downregulation of 14-3-3σ alone is sufficient to immortalize primary keratinocytes[28]. The present data suggest that Efp may be an essential oncogenic factor in breast cancers and perhaps other hormone-related malignancies. Our findings have important clinical relevance because targeting the Efp-mediated breakdown of 14-3-3σ could provide a new therapy to block breast tumour proliferation. □

Methods

MCF7 tumour growth in nude mice

MCF7 cells suspended in Matrigel (5×10^6 cells per 0.1 ml) were injected subcutaneously into female athymic mice (4-week-old BALB/c nu/nu). We calculated tumour volumes weekly by the tumour radii. When the tumour volume reached 300 mm^3, mice were ovariectomized or treated with Efp sense/antisense oligonucleotides (phosphorothioate-modified). Oligonucleotide sequences are: antisense, 5′-AGGGGGCACAGCTCTGCCAT-3′; sense, 5′-ATGGCAGAGCTGTGCCCCCT-3′. Six weeks later, animals were killed and tumours were analysed by immunoblotting using anti-Efp antibody[1]. In experiments for Efp-expressing MCF7 cells, cells were transfected with pEF[29] or pEF containing Efp. Two clones for each construct containing either pEF alone (vector-MCF7 number 1 and 3) or pEF-Efp (Efp-MCF7 number 2 and 4) were selected by G418. Each cell suspension (5×10^6 cells per 0.1 ml) was injected into five ovariectomized nude mice respectively. In other experiments, five intact female athymic mice injected with MCF7 cells were ovariectomized at a tumour volume of 180 mm^3.

Cell cycle analysis

Transfected MCF7 cells were incubated with phenol red free DMEM containing 0.2% charcoal-stripped fetal bovine serum (FBS) 24 h before analysis. Trypsinized cells were resuspended in hypotonic propidium iodide solution (50 $\mu g\,ml^{-1}$) containing 0.1% sodium citrate and 0.1% Triton X-100, and analysed on a FACScan flow cytometer (Becton Dickinson).

Yeast two-hybrid screen

Saccharomyces cerevisiae strain EGY48 was transformed with pEG202-NLS-human Efp, and further transformed with a mouse embryo cDNA library (OriGene). Selected positive clones according to the manufacturer's instructions were sequenced.

Immunoprecipitation assays

In experiments with COS7 cells, cells were lysed in Nonidet P-40 lysis buffer[30] 48 h after plasmid transfection. Lysates were immunoprecipitated with anti-Myc (Santa Cruz), anti-Flag (Sigma), or anti-14-3-3σ (Santa Cruz) antibodies. In experiments with MEFs, lysates were immunoprecipitated with anti-$p21^{Cip1}$ (Santa Cruz), anti-14-3-3σ, or goat immunoglobulin-γ (IgG), and immunoblotted by anti-Cdk2 (Santa Cruz) and anti-Cdc2 (Santa Cruz).

Pulse and chase experiments

MEFs were pulse-labelled with [^{35}S]methionine (200 $\mu Ci\,ml^{-1}$) in methionine-free medium containing 10% dialysed FBS for 2 h, washed twice with pre-warmed PBS, and chased by culturing in DMEM containing 10% FBS for 10 h. Lysates from pulse-labelled cells were immunoprecipitated with anti-14-3-3σ, resolved by SDS–polyacrylamide gel electrophoresis, and analysed with a PhosphorImager (Molecular Dynamics). In experiments with COS7 cells, cells were pulse-labelled for 30 min and chased for 3 h. Lysates were immunoprecipitated with anti-HA (Santa Cruz).

Generation of recombinant adenoviruses

Recombinant adenoviruses were generated by Adenovirus Expression Vector Kit (TaKaRa) and used at a multiplicity of infection (MOI) of 40–100 according to the manufacture's protocol.

In vivo ubiquitination assays

COS7 cells were transfected with plasmids including Myc-Efp, HA-14-3-3σ, ubiquitin, and Flag-tagged UbcH8 or UbcH7. Forty hours later, cells were incubated with MG132 (10 μM, Peptide Institute) for 8 h, then immunoprecipitated with anti-14-3-3σ. Precipitates were analysed by anti-ubiquitin (Santa Cruz) or anti-HA.

Preparation of *Efp*$^{+/+}$ and *Efp*$^{-/-}$ MEFs

We generated *Efp*$^{-/-}$ mice as described[6]. MEFs were obtained from wild-type or *Efp*$^{-/-}$ 13.5-day-old embryos and used at third to sixth generations. For growth rate analysis, cells were synchronized for 72 h in DMEM containing 0.1% FBS, then trypsinized and plated at a density of 1×10^5 cells per 35-mm dish in DMEM containing 10% FBS. In experiments for antisense oligonucleotide transfection, MEFs maintained for 24 h in DMEM containing 10% FBS were incubated with oligofectamine (Invitrogen) and either antisense/sense 14-3-3σ oligonucleotides (phosphorothioate-modified) or vehicle alone for 4 h according to the manufacture's protocol. At the end of incubation, 10% FBS was supplemented and maintained for the next 24 or 48 h. Antisense, 5′-ATCAGACTGGC TCTCTCCAT-3′; sense, 5′-ATGGAGAGAGCCAGTCTGAT-3′. In other experiments, cells were seeded and incubated with medium containing 10% FBS for 48 h, then lysed in Nonidet P-40 lysis buffer[30].

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Competing interests statement

The authors declare that they have no competing financial interests.

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Estrogen activates cyclin-dependent kinases 4 and 6 through induction of cyclin D in rat primary osteoblasts

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Abstract

Estrogen plays important roles in maintaining bone density and protecting against osteoporosis, but the underlying mechanisms of estrogen action via estrogen receptors (ERs) in bone remain to be clarified. In the present study, we isolated primary osteoblasts derived from transgenic rats harboring a dominant negative ER mutant, rat ERα (1–535) cDNA, and from their wild-type littermates. We observed that the rate of cell growth of osteoblasts from the transgenic rats was reduced compared to that of wild-type osteoblasts. Utilizing cDNA microarray analysis, we found that mRNA level of cyclin D2 was lower in the osteoblasts from the transgenic rats. D-type cyclins including cyclin D1, cyclin D2, and cyclin D3 are cell cycle regulators that promote progression through the early-to-mid G1 phase of the cell cycle. The protein levels of D-type cyclins including cyclin D2 and cyclin D3 but not cyclin D1 were elevated in wild-type osteoblasts with 17β-estradiol treatment, resulting in the activation of cyclin-dependent kinases 4 and 6 (Cdk4/6) activities and the promotion of cell growth. Moreover, an anti-estrogen ICI 182,780 abolished the induction of the expression of D-type cyclins by 17β-estradiol. Our findings indicate that estrogen and its receptors enhance Cdk4/6 activities through the induction of D-type cyclins, leading to the growth promotion of osteoblasts.

Keywords: Estrogen; Osteoblast; Cyclin D; Cdk; Estrogen receptor; Microarray

The bone homeostasis is based on the balanced actions of bone-forming osteoblasts and bone-resorbing osteoclasts. The imbalance of bone cell actions causes osteoporosis, which is characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture. Postmenopausal women are likely to be suffered from osteoporosis owing to estrogen deficiency [1,2], estrogen replacement therapy thus being a common treatment and prevention of postmenopausal osteoporosis [3–5].

Estrogen exerts various actions in its target cells including osteoblasts via two receptor proteins, estrogen receptor (ER)α and ERβ, which transactivate downstream estrogen-responsive genes [6–8]. Several lines of evidence have been accumulated regarding the stimulatory action of estrogen on bone formation and the inhibitory action of estrogen on bone resorption [9–11]; however, the precise role of estrogen in bone cell development and regulation still remains to be studied.

We have previously constructed a dominant negative ER (dnER) cDNA, namely a C-terminally truncated rat ERα cDNA fragment encoding amino acids 1–535, which inhibits the actions of not only ERα but also ERβ. We generated a transgenic rat line harboring the

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dnER cDNA. The impaired sensitivity to estrogen in the bones of the transgenic rats bearing the dnER was demonstrated by the fact that the administration of 17β-estradiol (E2) did not reverse the reduced bone mineral density (BMD) of the ovariectomized transgenic female rats, whereas E2 completely rescued the decrease of BMD of their wild-type littermates [12].

In the present study, using primary osteoblasts from the transgenic rats bearing the dnER and their wild-type littermates, we observed a reduced growth of osteoblasts from the transgenic rats compared to those from wild-type rats. Using cDNA microarray technology, we searched for genes that are possibly involved in the impaired growth of osteoblasts induced by the dnER transgene. We prepared cDNAs derived from two sources of mRNAs for hybridization: osteoblasts from the dnER transgenic rats and those from wild-type rats. Among genes that were differentially expressed, cyclin D2, one of the key regulators of the early-to-mid G1 phase of cell cycle, was downregulated in the osteoblasts from the transgenic rats. We also showed that E2 at a physiological concentration (10^{-8} M) induced the expressions of both cyclin D2 and cyclin D3, leading to the activation of cyclin-dependent kinases 4 and 6 (Cdk4/6) and the promotion of cell growth in osteoblasts. Our findings suggest a possible involvement of cyclin D and Cdk4/6 in the ER-mediated cell cycle control and growth regulation in osteoblasts.

Materials and methods

Cell culture. Rat primary osteoblasts were isolated from calvaria of 1-day-old neonatal rats by enzymatic digestion as described previously [13]. Calvaria were minced and incubated at 37 °C for 20 min with gentle shaking in magnesium-free phosphate-buffered saline containing 0.1% collagenase, 0.05% trypsin, and 4 mM Na_2 EDTA. The enzymatic digestion was repeated six times and cells were isolated at the last three digestions cultured separately in α-MEM containing 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin). Cells at the third passage were used for experiments.

Total RNA and mRNA isolation. Osteoblasts derived from wild-type rats and the dnER transgenic rats were maintained in 10-cm culture dishes with α-MEM containing 10% FBS until reaching confluency. Total RNAs were extracted from these cells using a ToTALLY RNA Kit (Ambion). Messenger RNA was extracted from 250 μg total RNA using an Oligotex-dT30 mRNA purification kit (Takara).

Genechip analysis. All procedures were performed according to the instructions from Affimetrix. For gene expression analysis, a double-stranded cDNA was prepared from 3 μg mRNA using SuperScript II Reverse Transcriptase (Invitrogen) and in vitro transcribed by Bio-Array RNA Transcript labeling Kit (Enzo Diagnostic). The cRNA products were purified and fragmented. Rat Genome U34A chips (Affimetrix) were hybridized with the fragmented cRNA samples for 16 h at 45 °C, washed, stained, and then scanned by a GeneArray Scanner (Affimetrix). Image analysis was performed using Microarray Suite 5.0 software. In this study, transcripts scored as increased or decreased more than 1.6-fold were considered as upregulated or downregulated, respectively.

Northern blot analysis. Northern blot analysis was performed using total RNA as described previously [14]. Rat cyclin D2 fragment containing full length of the open reading frame glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment [15] was labeled with [α-^{32}P]dCTP using BcaBEST Labeling kit (Takara) and used as probes. Autoradiography was carried out at −80 °C with intensifying screens for 24 h.

Cell proliferation analysis. Cell proliferation was evaluated by bromodeoxyuridine (BrdU) incorporation using BrdU Labeling and Detection Kit (Roche). Osteoblasts were plated into a 96-well microtiter plate at a density of 2×10^4 cells/well, washed twice by PBS after 24 h, and then incubated for 24 h in phenol red-free DMEM containing 2% charcoal dextran-treated FBS (CD-FBS). Cells were treated with E2, ICI 182,780, or both in phenol red-free DMEM containing 2% CD-FBS for another 24 h, including a 6-h pulse labeling with BrdU. Cells were subjected to immunostaining with anti-BrdU monoclonal antibody. The absorbance of the cells was determined using a microplate reader at 405 nm with a reference wavelength at 490 nm.

Western blot analysis. Cells were maintained in α-MEM containing 10% FBS and incubated in phenol red-free DMEM containing 0.5% CD-FBS for 24 h prior to experiments. Cells were treated with E2, ICI 182,780, or 0.1% ethanol for 16 h, rinsed twice with ice-cold PBS, and then lysed in Triton-X 100 buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton-X 100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 500 μM Na_3VO_4, 10 μg/ml aprotinin, 10 g/ml leupeptin, and 1 mM PMSF). Lysates were cleared by centrifugation at 15,000*g* for 30 min at 4 °C and subjected to immunoblotting. Antibodies against mouse cyclin D1, mouse cyclin D2, rat cyclin E, mouse Cdk4, and human Cdk6 were purchased from Santa Cruz and anti-mouse cyclin D3 antibody was from Upstate Biotech.

Kinase assays. Two hundred μg osteoblast lysates were immunoprecipitated with various antibodies using G-Sepharose beads (Amersham Pharmacia), washed four times with ice-cold Triton-X lysis buffer, washed twice with kinase buffer (80 mM sodium β-glycerophosphate, pH 7.4, 20 mM EGTA, 15 mM $Mg(OAc)_2$, and 1 mM dithiothreitol), and then mixed with 10 μl kinase buffer containing 50 μM ATP, 1.25 μCi [α-^{32}P]ATP, and 1 μg glutathione *S*-transferase-retinoblastoma protein (GST-Rb). The samples were incubated at 30 °C for 30 min and the reactions were terminated by addition of 30 μl Laemmli sample buffer. The samples were separated on 12.5% SDS–polyacrilamide gel and visualized by autoradiography.

Statistics. Differences between treatment group and the appropriate control were compared using Student's *t* tests. Data are expressed as means ± SD and $p < 0.05$ considered as statistically significant.

Results

The impaired cell growth of osteoblasts from the transgenic rats expressing dominant negative ER

To investigate the effects of the dnERagainst ER signaling pathways in the bone in detail, we prepared primary osteoblasts derived from the calvaria of the neonatal transgenic rats bearing the rat ERα (1–535) cDNA [12]. We confirmed the expression of the truncated ERα construct at mRNA as well as at protein levels in primary osteoblasts derived from the transgenic rats, as previously described [12]. We then compared the growth property of osteoblasts from the transgenic and wild-type rats. The cell growth of osteoblasts from the transgenic rats and control rats was assessed by BrdU incorporation assay. Osteoblasts from both groups were grown in α-MEM containing 10% FBS for 24 h and

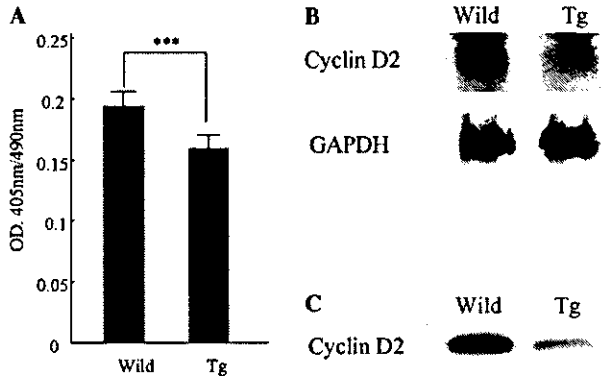

Fig. 1. Impaired cell growth and reduced expression of cyclin D2 mRNA and protein in osteoblasts derived from the dnER transgenic rats. (A) Reduced BrdU incorporation into osteoblasts from the dnER transgenic rats. Wild: osteoblasts from wild-type rats, Tg: osteoblasts from the dnER transgenic rats. Data are shown as means ± SD of six independent experiments. ***: $p < 0.001$ Wild versus Tg. (B) Reduced mRNA level of cyclin D2 in osteoblasts from the dnER transgenic rats. Twenty μg total RNA from osteoblasts was hybridized with the indicated probes. (C) Reduced protein level of cyclin D2 in osteoblasts from the dnER transgenic rats. Twenty μg cell extract from osteoblasts was analyzed by immunoblot.

incubated with BrdU for additional 6 h. As shown in Fig. 1A, BrdU incorporation was significantly reduced in the osteoblasts from the transgenic rats compared with the osteoblasts from wild-type rats ($p < 0.001$).

Identification of differentially expressed genes in osteoblasts from the transgenic rats

To identify which genes are differentially expressed in osteoblasts from the transgenic rats bearing the dnER compared to those from wild-type rats, cDNA microarray analysis was performed with mRNAs obtained from the two sources of confluent osteoblasts. Double-stranded cDNAs were prepared from mRNAs and labeled as described in Materials and methods, and then hybridized to Affymetrix Rat Genome U34A chips. Comparison of the gene expression profiles of the two osteoblast lines revealed 40 genes showing greater than 1.6-fold difference in transcript scores. In Table 1, we have shown 27 genes that were downregulated in the transgenic rat osteoblasts (Table 1A) and 13 genes that were upregulated in the transgenic rat osteoblasts (Table 1B). Among diffentially expressed genes that are related to cell cycle regulation, cyclin D2 gene, a member of D-type cyclins, was one of the downregulated genes in osteoblasts from the dnER transgenic rats. The mRNA level of cyclin D2 was decreased in osteoblasts from the transgenic rats compared to wild-type cells as confirmed by Northern blot analysis (Fig. 1B). The protein level of cyclin D2 was also decreased in the transgenic rat osteoblasts (Fig. 1C). These results showed that the inhibition of ER-mediated signaling by the dnER decreased the cyclin D2 expression at the transcriptional level.

Upregulations of D-type cyclins by estradiol

Since cyclin D2 expression was reduced in osteoblasts from the dnER transgenic rats, we next investigated whether E2 modulated expression of D-type cyclins in primary osteoblasts. D-type cyclins including cyclin D1, cyclin D2, and cyclin D3 are cell cycle regulators that are induced during the early-to-mid G1 phase [16–18]. The cell growth of primary osteoblasts from wild-type neonatal rats was analyzed by BrdU incorporation. E2 potentiated cell growth of osteoblasts in a dose-dependent manner with maximal effect at 10^{-8} M (Fig. 2A), consistent with previous reports [13–15]. We next examined the effect of E2 on the expressions of cyclins and cyclin-dependent kinases (Cdks) during the G1/S-phase transtition. Protein extracts were prepared from primary osteoblasts treated with E2 at 10^{-8} M and subjected to Western blot analysis (Fig. 2B). Cyclin D1 expression was not detected in osteoblasts treated with either vehicle or E2. Treatment with E2 increased the amounts of cyclin D2 and cyclin D3. To determine whether the action of E2 is mediated through ER, primary osteoblasts were treated with an ER antagonist ICI 182,780 (10^{-7} M) plus E2 (10^{-8} M). The amounts of cyclin D2 and cyclin D3 were reduced in cells treated with E2 plus ICI 182,780. The protein levels of cyclin E, Cdk4, and Cdk6 remained relatively constant either by E2 or by E2 plus ICI 182,780 treatment (Fig. 2B). These data suggest that cyclin D2 and cyclin D3 are E2-responsive cell cycle regulators in primary osteoblasts.

The expression pattern of cyclin D in primary osteoblasts was different from that in an osteoblast-like cell line derived from rat sarcoma, UMR106 cells. Cyclin D2 and cyclin D3 were co-expressed in UMR106 cells as well as in primary osteoblasts, whereas cyclin D1 was expressed in UMR106 cells but undetectable in primary osteoblasts (Fig. 2C).

Estrogen increases the amount of D-type cyclins bound to Cdk4 and Cdk6

D-type cyclins promote G1/S-phase transition by binding to Cdk4 or Cdk6 [16–18]. We next examined whether E2 treatment changed the amounts of cyclin D–Cdk4 and cyclin D–Cdk6 complexes. As expected, E2 treatment increased the amounts of cyclin D2–Cdk4/6 and cyclin D3–Cdk4/6 complexes in primary osteoblasts (Fig. 3A). Cyclin D1–Cdk4/6 complexes were not detected treated by either vehicle or E2 (data not shown).

Estrogen activates Cdk4 and Cdk6 kinase activities

Activation of cyclin D–Cdk4/6 complexes is an early-to-mid G1-phase event that elicits hyperphosphory-

Table 1
List of genes differentially expressed in osteoblasts derived from the transgenic rats bearing dominant negative ER (dnER)

Accession No.	Fold decrease	Descriptions
(A) Downregulated genes		
M36151	7.46	MHC class II A-β RT1.B-b-β gene, partial cds
M65251	6.50	Angiotensinogen gene-inducible enhancer-binding protein 1 mRNA, 3 end
L13406	4.29	Calcium/calmodulin-dependent protein kinase II δ subunit mRNA, partial cds
X05831	3.73	Fibronectin gene 5-end with prepro seq., I-1 and I-2 exons
AB012231	3.48	NFI-B2, partial cds
X76988	3.25	Gal-β-1,3-galNAc α-2,3-sialyltransferase
M28259	3.25	Fibronectin gene, exons 2b and 3a
Z29072	2.64	Mucin
M98327	2.46	Transfer RNA-Valine synthetase mRNA, partial cds
AB012235	2.30	NFI-X1, partial cds
X59601	2.30	Plectin
X56596	2.14	MHC class II antigen RT1.B-1 β-chain
AF034899	2.00	Olfactory receptor-like protein (SCR D-9) gene, complete cds
AF004811	2.00	Moesin mRNA
U23146	2.00	Mitogenic regulation SSeCKS (322) gene, complete cds
U20121	2.00	Type XI collagen α-1 chain (COL11A1) mRNA, partial cds
AB004277	2.00	Protocadherin 5, partial cds
U47312	1.87	R2 cerebellum DDRT-T-PCR
M61875	1.87	Glycoprotein CD44 (CD44) mRNA, complete cds
AB012234	1.87	NFI-X1, partial cds
D16308	1.87	Cyclin D2, complete cds
M15797	1.74	Entactin mRNA, 3 end
D10587	1.74	Eighty-five kDa sialoglycoprotein (LGP85), complete cds
L08505	1.74	Cytoplasmic dynein heavy chain (MAP 1C), mRNA, complete cds
U82612	1.74	Fibronectin (fn-1) gene, partial cds
AF081148	1.74	CL2AA mRNA, complete cds
X06564	1.62	One hundred and forty-kDa NCAM polypeptide
Accession. No.	Fold increase	Descriptions
(B) Upregulated genes		
M86912	3.48	Angiotensin receptor (AT1) gene, single exon
X62295	3.25	Vascular type-1 angiotensin II receptor
S49491	2.46	Proenkephalin [rats, heart, mRNA, 1250 nt]
M86912	2.46	Angiotensin receptor (AT1) gene, single exon
J04035	2.30	Tropoelastin mRNA, 3 end
M93257	2.30	Catechol-O-methyltransferase mRNA, 3-flank
M74054	2.00	Angiotensin II receptor mRNA, complete cds
U64030	2.00	dUTPase mRNA, complete cds
L07073	1.87	Clathrin-associated adaptor protein homolog (p47A) mRNA, complete cds
J04486	1.74	Insulin growth factor-binding protein mRNA, complete cds
A09811	1.74	BRL-3A-binding protein
U34843	1.62	Cell cycle progression-related D123 mRNA, complete cds
X13016	1.62	MRC OX-45 surface antigen

lation and inactivation of retinoblastoma protein (Rb) [10–12]. We next analyzed the Cdk4/6-dependent kinase activities in primary osteoblasts using GST-Rb as a substrate. Protein extracts were immunoprecipitated with anti-cyclin D2, cyclin D3, Cdk4 or Cdk6 antibody and assayed for Rb kinase activity (Fig. 3B). E2 treatment markedly enhanced all the Rb kinase activities. Cyclin D1-dependent Rb kinase activity was not detected in primary osteoblasts (data not shown). These results suggest that E2 promotes formation of cyclin D2–Cdk4/6 or cyclin D3–Cdk4/6 complexes and enhances Cdk4/6 kinase activities.

Discussion

In the present study, we demonstrated that the cell growth of osteoblasts from dnER transgenic rats was reduced compared to that of wild-type osteoblasts. Our present data and previous reports have shown that E2 stimulates osteoblast proliferation and anti-estrogens reduce the E2-mediated effect on the cellular proliferation [9–11].

Using cDNA microarray analysis, we identified cyclin D2, one of the D-type cyclins, as a potential target that promotes estrogen-ER-mediated osteoblast growth.

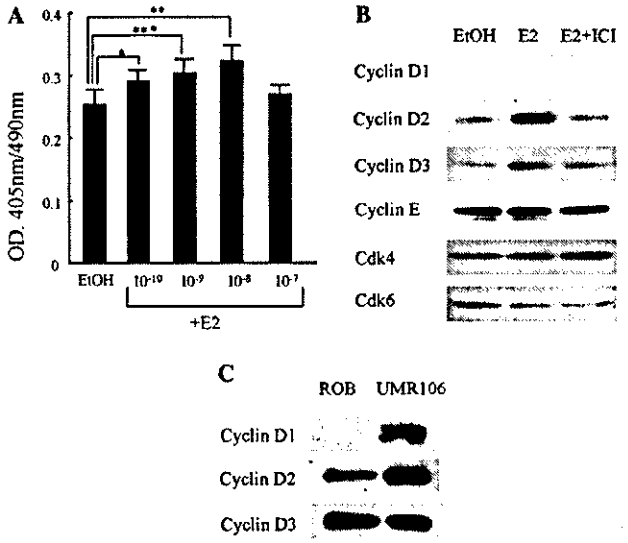

Fig. 2. Enhanced cell growth and induction of cyclin D2 expression in osteoblasts by E2 treatment. (A) BrdU incorporation into osteoblasts treated with either 0.1% ethanol (column 1, EtOH) or E2 (10^{-10}–10^{-7} M) (columns 2–5). Data are shown as means ± SD of six independent experiments. *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ E2 treatment versus EtOH treatment. (B) Protein levels of cyclin D2 and cyclin D3 in osteoblasts are elevated by E2 treatment and reduced by treatment with E2 plus ICI 182, 780. Cells were incubated with E2 (10^{-8} M), ICI 182, 780 (10^{-7} M), or 0.1% ethanol for 16 h. Twenty μg cell extract from osteoblasts was analyzed by immunoblot. (C) Expression of D-type cyclins proteins in E2 (10^{-8} M)-treated primary osteoblasts and rat osteoblast-like sarcoma cell line UMR106 cells.

Cyclin D is a cell cycle component that is induced during early-to-mid G1-phase in response to mitogen stimulation and plays a key role in the control of cellular proliferation. Cyclin D subsequently recruits Cdk4 and Cdk6 to form complexes that promote S-phase entry [16–18].

The present study showed that cyclin D2 and cyclin D3 but not cyclin D1 are predominantly expressed in primary osteoblasts. In contrast, cyclin D1 as well as cyclin D2/D3 could be detected in UMR106 osteosarcoma cell line. Previous literature reports that the tissue distribution and the regulatory mechanism of D cyclins seem to be subtype-specific. Cyclin D1 is predominantly expressed in epithelial tissues and it is a well-defined target of E2 action in ER-positive breast cancer cells, especially in MCF7 cells [20–25]. Cyclin D1 is overexpressed in nearly 50% of mammary carcinomas [26–28]. In contrast, cyclin D2 and cyclin D3 are predominantly expressed in hematopoietic tissues [19]. In breast cancer cells, cyclin D3 is expressed [29,30] but not cyclin D2 [31]. We also showed that Cdk4 and Cdk6 activities in primary osteoblasts were stimulated by estrogen-mediated induction of cyclin D2 and cyclin D3. The regulatory mechanism of cell cycle in primary osteoblasts is different from that in breast cancer cells, as estrogen stimulates Cdk4 activity but not Cdk6 activity through the induction of cyclin D1 expression [20–25]. Taken together, it is likely that each subtype of cyclin D may play a different functional role in various cell types and tissues by recruiting different Cdks and responding to subsequent signal transduction.

The protein levels of most cyclins (i.e., cyclin B1, cyclin A, and cyclin E) are regulated by degradation processes [32]. On the contrary, the levels of D-type cyclins are regulated by transcriptional induction, posttranscriptional mechanism, and translational mechanism [33–35]. Our results showed clearly that estrogen regulates cyclin D2 by transcriptional pathway. Consistent with our observation, a similar report has been made regarding the mRNA levels of cyclin D2 that were increased by follicle stimulating hormone (FSH) via its receptor-dependent pathway in ovarian granulosa cells [36]. E2 also induced cyclin D2 expression in granulosa cells [37]. These data suggest cyclin D2 might be one of the hormone-related genes also in other cell types.

It is not defined in the present study whether transcription of cyclin D2 and cyclin D3 genes is regulated directly through ERs. Reviewing nucleotide sequences of the promoter regions of rat cyclin D2 (Accession No. U87099) and cyclin D3 (U49935) genes up to 2-kb length from GenBank Data Bank, we did not find any typical estrogen-responsive elements (EREs) in those sequences. Several half-sites such as GGTCA or TGACC and imperfect EREs; however, were observed

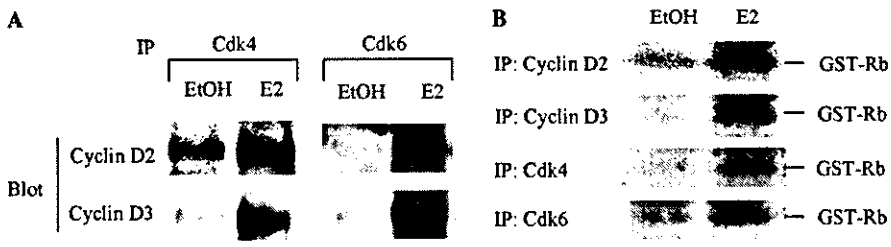

Fig. 3. Increase in assembly and activation of cyclin D–Cdk4/6 complexes by E2 treatment. (A) Amounts of cyclin D2–Cdk4/6 and cyclin D3–Cdk4/6 complexes in osteoblasts are increased by E2 treatment. Osteoblasts were incubated with either 0.1% ethanol (EtOH) or E2 (10^{-8} M) for 16 h. Four hundred μg cell extracts were immunoprecipitated with antibody against Cdk4 or Cdk6 and immunoblotted with anti-cyclin D2 or anti-cyclin D3 antibody. (B) Kinase activities of cyclin D–Cdk4/6 complexes in osteoblasts are enhanced by E2 treatment. In vitro kinase assay was performed in immunoprecipitates by antibody against cyclin D2, cyclin D3, Cdk4 or Cdk6 using GST-Rb as a substrate.

in those upstream regions. Previous literature has shown several lines of evidence that half-sites even without forming a complete palindromic ERE could relate to regulation of gene transcription [38–40]. We therefore consider a possibility that the transcription of cyclin D2 and cyclin D3 genes are regulated through direct interaction between half-sites and ERs.

Another possibility related to transcriptional regulation of cyclin D2 and cyclin D3 genes is the control by regulatory enhancer elements, which is a similar mechanism observed in the transcription of cyclin D1 genes. It has been reported that the promoter region of human cyclin D1 gene contains several enhancer elements including a cAMP response-like element and SP-1 binding sites that are known to be E2-responsive [41,42]. In the upstream region of cyclin D1 gene, ER seems to be involved in transcriptional regulation through the interaction with those enhancer elements. Regarding the promoter regions of rat cyclin D3 and cyclin D2 genes, both sequences seem to have putative cAMP response elements (CREs) and SP-1 binding sites ([43] and data from NCBI nucleotide). We do not know whether ER-mediated induction of cyclin D2 and cyclin D3 genes is regulated through those CREs or SP-1 binding sites. Future analysis will reveal which if any of these enhancer elements are involved or another mechanism is utilized in transcriptional regulation of cyclin D2 and cyclin D3 genes.

In summary, we have identified target genes of estrogen-ER-mediated proliferation in osteoblasts. These results suggest that the regulation of the expression of cyclin D2 and cyclin D3 may have important roles in the G1 progression of rat osteoblasts by estradiol through ERs. The observed increased expression of D-type cyclins and activity of Cdk4/6 by estrogen could be involved in the growth and the differentiation of osteoblasts in vivo. These results will enable us to identify the extracellular signals and intracellular pathways controlling cyclin D2/D3, Cdk4/6 activity, and cell cycle progression in future.

Acknowledgments

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Induction of Anti-Metallothionein Antibody and Mercury Treatment Decreases Bone Mineral Density in Mice

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Mercuric chloride ($HgCl_2$) is an industrial agent with toxic effects on the immune system, kidney, lung, and nervous tissue, but little is known about its effect on bone. Metallothionein (MT) is a cysteine-rich metal-binding protein that exerts cytoprotective effects against heavy metal toxins. It has been reported that the susceptibility of renal and pulmonary toxicity of mercury was markedly enhanced in MT-null mice compared to control mice. However, there is no report about the effects of anti-metallothionein (anti-MT) Ab induction on mercury toxicity. We investigated the effect of anti-MT Ab induction on mercury-induced bone injury. BALB/c mice were injected with MT (10 μg/mouse ic) five times to induce anti-MT Ab and then treated with $HgCl_2$ (1 mg/kg sc) three times per week for 3 weeks. MT immunization plus $HgCl_2$ treatment dramatically decreased bone mineral density (BMD), and the humoral bone formation indices, alkaline phosphatase (ALP) activity and osteocalcin. MT immunization or $HgCl_2$ treatment alone did not affect either BMD or serum ALP activity and osteocalcin levels. MT immunization impeded $HgCl_2$-induced increase of MT expression in the liver and led to an increase of mercury in serum and the liver but a decrease in the kidney. Furthermore, serum titers of IgE and IgG1 were significantly elevated in the MT-immunized plus $HgCl_2$ treatment group compared with those in the $HgCl_2$ treatment group. Similar results were also observed in splenic secretions of IL-4 and IL-10 based on anti-CD3 Ab stimulation. Taken together, our results indicate that anti-MT Ab induction causes mercury-induced bone injury in BALB/c mice and also enhances mercury-related immune disorders.

Key Words: alkaline phosphatase; autoimmunity; bone mineral density; cytokine; IgE; IgG subclass; mercuric chloride; metallothionein; anti-metallothionein Ab; osteocalcin.

Mercury is a well-known industrial agent and an environmental pollutant with toxic effects on the immune system, kidney, lung, and nervous tissues. The most conspicuous toxic effect of mercuric chloride ($HgCl_2$) is autoimmune disorders. In susceptible animals, $HgCl_2$ induces a systemic autoimmune disease characterized by a T cell-dependent polyclonal B cell activation. Increased serum levels of immunoglobulin G1 (IgG1)[1] and immunoglobulin E (IgE), production of anti-nucleolar autoantibodies (ANoA), and formation of immune complexes in the kidneys are also commonly affected. In *in vitro* experiments, bone marrow-derived cultured murine mast cells and mouse mast cells have been shown to release *N*-acetyl-β-D-hexosaminidase, interleukin-4 (IL-4), and tumor necrosis factor-α upon incubation with $HgCl_2$ (Dastych *et al.*, 1999). Little however is known about *in vivo* toxic effects of $HgCl_2$ on bone.

Metallothionein (MT) is a low-molecular-weight, cysteine-rich intracellular protein with a high affinity for metals such as mercury. It has an important cytoprotective effect on heavy metal toxicity. There are four isoforms of MT (MT I–IV). MT-I and MT-II are expressed in all tissues. Protection against metal toxicity has been attributed primarily to MT-I and MT-II. MT-III is localized mainly in the brain (Palmiter *et al.*, 1992) and plays a role in zinc homeostasis in neurons (Masters *et al.*, 1994b), whereas MT-IV is localized in stratified squamous epithelia (Quaife *et al.*, 1994). The function of MT-IV remains unclear.

Since MT gene knockout mice (MT-null mice) that do not express MT-I and MT-II genes have been established (Michalska and Choo, 1993; Masters *et al.*, 1994a), there have been many reports using MT-null mice to investigate the roles of MT in detoxification against heavy metals such as cadmium and mercury. The susceptibility of renal and pulmonary tissue to toxins of mercury is markedly enhanced in MT-null mice compared to control mice (Satoh *et al.*, 1997; Yoshida *et al.*, 1999). The cumulative median lethal dose of cadmium for wild-type mice is 6.9-fold higher than MT-null mice, suggesting MT plays a critical role in protecting against cadmium-induced lethality in rodents (Park *et al.*, 2001). There are no reports describing the effect of *in vivo* anti-MT Ab induction

[1] Abbreviations used: ALP, alkaline phosphatase; anti-MT Ab, anti-metallothionein antibody; ANoA, anti-nucleolar autoantibodies; BMC, bone mineral content; BMD, bone mineral density; DEXA, dual-energy X-ray absorptiometry; FBS, fetal bovine serum; FCA, Freund's complete adjuvant; FITC, fluorescein isothiocyanate; IgE, immunoglobulin E; IgG, immunoglobulin G; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; MT, metallothionein; PE, phycoerythrin; Th, T helper.

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