Linoleic Acid Induces Mouse Embryonic Stem Cell Proliferation Via Ca^{2+} /PKC, PI3K/Akt, and MAPKs

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Key Words

Linoleic acid • Embryonic stem cell • Proliferation • PKC • PI3K/Akt • MAPKs

Abstract

Aims: This study investigated the effect of linoleic acid (LA) on cell proliferation and the related signaling cascade in mouse embryonic stem (ES) cells. **Materials & Methods:** To examine effects of LA, mouse ES cells (ES-E14TG2a) were used. Moreover, DNA synthesis, glucose production, protein and mRNA expressions were measured. **Results:** LA increased DNA synthesis in a concentration- ($\geq 10^{-9}$ M) and time- (≥ 24 h) dependent manner, as determined by [^3H] thymidine incorporation and increased cell number. LA increased intracellular Ca^{2+} levels via regulation of phospholipase C (PLC) and activated protein kinase C (PKC). LA activated phosphatidylinositol 3-kinase (PI3K)/Akt and p44/42 mitogen-activated protein kinases (MAPKs). U73122 (PLC inhibitor), staurosporine (PKC inhibitor), LY294002 (PI3K inhibitor), and Akt inhibitor blocked the phosphorylation of p44/42 MAPKs. In addition, LA stimulated gluconeogenesis through increase expression of glucose-6-phosphatase (G6Pase) and

phosphoenolpyruvate carboxykinase (PEPCK). LA-induced increases in the cell cycle regulatory proteins, cyclin D1, cyclin E, cyclin-dependent kinase (CDK) 2, and CDK 4, were blocked by U73122, staurosporine, LY294002, Akt inhibitor, PD98059, and metformin (gluconeogenesis inhibitor). **Conclusion:** LA stimulated cell proliferation via Ca^{2+} , PLC/PKC, PI3K/Akt, and p44/42 MAPKs signaling pathways in mouse ES cells.

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Introduction

Free fatty acids (FFAs) provide energy to the cell, contribute to cellular components, such as organelles and plasma membrane, and are also biologically active molecules that can regulate gene expression, enzyme activity, binding protein, and other cellular processes [1, 2]. FFAs are components in follicular fluid which influences the development of oocyte and embryo [3, 4]. In addition, a diet enriched in polyunsaturated fatty acids was found to enhance ovarian follicular development and accelerate early embryonic development in previous studies [5, 6]. FFAs are categorized by physical characteristics such as chain length and number and

configuration of double bonds including linoleic acid (LA, 18:2), α -linolenic acid (ALA, 18:3), oleic acid (OA, 18:1), and palmitoleic acid (PA, 16:1) [7, 8]. FFAs regulate gluconeogenesis via direct and indirect pathways. The indirect role of FFAs is through insulin secretion and action which relate to suppressing gluconeogenesis [9]. However, FFAs directly elevate gluconeogenesis independent of hormones in several ways [10]. FFAs also function directly as agonists at a number of G protein-coupled receptors (GPCRs) including GPR40, GPR41, and GPR43 [11, 12]. GPR40 is activated by both saturated and unsaturated medium- to long-chain fatty acids, such as LA and OA, and involves various signaling pathways [13-15].

Embryonic stem (ES) cells are pluripotent cell lines that are derived from an early mammalian embryo in the blastocyst-stage [16]. ES cells provide a new source for transplantation therapies and pharmaceutical testing, which have unique combination of long-term self-renewal properties and pluripotency [17]. In this respect, one of the first issues that need to be addressed is the necessity of expanding ES cells in vitro culture conditions. Numerous studies have been performed to test various supplements in support of ES cells maintenance such as human serum albumin, vitamin, antioxidants, trace mineral, lipids, and a specific list of cloned growth factors [17, 18]. It is important to study the effect of LA on ES cells because it induces cell growth in culture environment. Therefore, this study investigated the effects of LA on proliferation of mouse ES cells and its related signal pathways.

Materials and Methods

Materials

Mouse ES cells (ES-E14TG2a) were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum was purchased from Gibco (Rockville, MD, USA). Linoleic acid, fluorescence isothiocyanate (FITC)-conjugated goat anti-mouse IgM, FITC-conjugated goat anti-rabbit IgM, U73122, staurosporine, LY294002, Akt inhibitor, PD98059, metformin, and monoclonal anti- β -actin were obtained from Sigma-Aldrich (St. Louis, MO, USA). [3 H] Thymidine was purchased from PerkinElmer (Boston, MA, USA). Fluo-3/AM was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Anti-Oct4, pan-PKC, PKC α , PKC δ , PKC ζ , PEPCCK, cyclin D1, cyclin E, cyclin-dependent kinase (CDK) 2, and CDK 4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-Akt, Akt, phospho-p44/42, p44/42 antibodies, goat anti-rabbit IgG and goat-anti mouse IgG were purchased from New England Biolabs (West Grove,

PA, USA). All other reagents were purchased commercially and were of the highest purity available.

ES cell culture

Mouse ES cells were incubated in DMEM (Gibco-BRL, Gaithersburg, MD) supplemented with 3.7 g/L sodium bicarbonate, 1% penicillin and streptomycin, 1.7 mM L-glutamine, 0.1 mM β -mercaptoethanol, 5 ng/ml mouse leukemia inhibitory factor, and 15% fetal bovine serum (FBS) without a feeder layer for 5 days. The cells were grown on gelatinized 35-mm or 60-mm culture dishes in an incubator maintained at 37°C with an atmosphere containing 5% CO₂ in air. After 1-3 days, the cells were washed twice with phosphate-buffered saline (PBS) and maintained in serum-free DMEM including all supplements. The resulting cells were used for experiments after a 24 h incubation period.

Alkaline phosphatase staining

Mouse ES cells were washed twice with PBS and fixed with 4% formaldehyde (in PBS) for 15 min at room temperature. The cells were washed with PBS and incubated with alkaline phosphatase substrate solution (200 μ g/ml naphthol AS-MX phosphate, 2% N,N-dimethylformamide 0.1 M Tris [pH 8.2], and 1 mg/ml Fast Red TR salt [4-chloro-2-methyl-benzenediazonium salt; zinc chloride]) for 10 min at room temperature. The cells were washed with PBS and photographed.

Fluorescence activated cell sorter (FACS) analysis

The cells were incubated with LA (10⁻⁹ M) for 24 h and then cells were dissociated in trypsin/EDTA, pelleted by centrifugation, resuspended at approximately 10⁶ cells/ml in PBS containing 0.1% BSA. The cells were labeled with the mouse anti-SSEA 1 antibody (1:50, Santa Cruz, CA, USA) and then incubated with fluoresce in FITC-conjugated second antibodies (1:50, Santa Cruz, CA, USA). The cells were washed and resuspended in PBS and then read by flow cytometry (Beckman Coulter, CA, USA). Samples were analyzed using CXP software (Beckman Coulter, CA, USA).

Immunofluorescence staining with SSEA-1

Cells were fixed and treated with monoclonal antibody against mouse SSEA-1 (1:50 Santa Cruz, CA, USA) and then incubated for 30 min with FITC-conjugated second antibody (1:50). Fluorescence images were visualized with a fluorescence microscope (Fluoview 300, Olympus, Tokyo, Japan).

[3 H] Thymidine incorporation

The [3 H] thymidine incorporation experiments were carried out using the methodology reported by Chen et al. [19] and Zhang et al. [20], in which most ES cells are arrested in G0/G1 phase through serum deprivation. Furthermore, the synchronized ES cells successfully reenter a normal cell cycle after being resupplied with serum. In this study, the cells were cultured in one well until they reached 50% confluence. The cells were then washed twice with PBS and maintained in serum-free DMEM including all supplements. After 24 h incubation, the cells were washed twice with PBS and incubated

with fresh serum-free DMEM including all supplements and indicated agents. After the incubation period, 1 μ Ci of [methyl- 3 H] thymidine (specific activity: 74 GBq/mmol, 2.0 Ci/mmol; Amersham Biosciences, Buckinghamshire, UK) was added to the cultures and incubated for 1 h at 37°C. The cells were then washed twice with PBS, fixed in 10% trichloroacetic acid (TCA) at 23°C for 15 min, and washed twice with 5% TCA. The acid-insoluble material was dissolved in 0.2 N NaOH over a 12 h period at 23°C. Aliquots were removed, and the level of radioactivity was determined using a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA, USA). The control levels of [3 H] thymidine incorporation were determined under serum-free DMEM cell culture conditions. The values were converted from absolute counts to a percentage of the control in order to allow comparison between the experimental groups.

Cell counting

The cells were washed twice with PBS and trypsinized from the culture dishes. The cell suspension was mixed with 0.4% (w/v) trypan blue solution and the number of living cells was determined using a hemocytometer. Cells that failed to exclude the dye were considered non-viable.

Bromodeoxyuridine incorporation

The level of 5-bromo-2'-deoxyuridine (BrdU) (a thymidine analog) incorporation was measured in order to determine the level of DNA synthesis. ES cells grown on coverslips were serum-starved for 24 h prior to LA stimulation. The ES cells were then treated with LA for 24 h. Fifteen μ M BrdU was added during the last 16 h of incubation. After several washes with PBS, the cells were fixed with methanol [10% (vol/vol)] for 10 min at 4°C, followed by incubation in 1 N HCl for 30 min at room temperature. The cells were then washed and incubated with 0.1 M sodium tetraborate for 15 min and with Alexa Fluor 488-conjugated Mouse anti-BrdU Ab (1:200, Molecular Probes) in 2% BSA-PBS overnight at 4°C. After washing in PBS, coverslips were mounted onto glass slides with Dako Fluorescent mounting medium using gelvatol and examined under an optical microscope (Fluoview 300, Olympus, Tokyo, Japan).

For double-labeling experiments, the cells were fixed in acid alcohol and processed for Oct-4 and BrdU staining. The fixed cells were incubated successively with rabbit anti-Oct-4 and Alexa Fluor 555 anti-rabbit IgG (1:100, Molecular Probes) for 1 h at room temperature. This was followed by incubation in 1 N HCl, neutralization with 0.1 M sodium tetraborate, and incubation with Alexa Fluor 488-conjugated Mouse anti-BrdU Ab for 1 h at room temperature. After washing with PBS, the BrdU/Oct-4 stained cells were examined under confocal microscopy (Fluoview 300, Olympus, Tokyo, Japan).

Measurement of intracellular Ca^{2+} levels

The changes in intracellular Ca^{2+} levels were monitored using Fluo-3/AM dissolved in dimethyl sulfoxide. Mouse ES cells in 35-mm culture dishes were rinsed twice with Bath solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM HEPES, pH 7.4) and incubated in Bath

solution containing 3 μ M Fluo-3/AM with 5% CO_2 , 95% O_2 at 37°C for 40 min. The cells were then rinsed twice with Bath solution, mounted on a perfusion chamber, and scanned every second using confocal microscopy (x400) (Fluoview 300, Olympus, Tokyo, Japan). Fluorescence was excited at 488 nm, and the emitted light was read at 515 nm. All analyses of intracellular Ca^{2+} levels were processed at the single-cell level and the data are expressed as the relative fluorescence intensity.

RNA isolation and RT-PCR

Total RNA was obtained from mouse ES cells using STAT-60, a monophasic solution of phenol and guanidine isothiocyanate from Tel-Test, Inc. (Friendswood, TX, USA). Reverse transcription (RT) was carried out with 3 μ g RNA using a reverse transcription system kit (AccuPower RT PreMix, Bioneer, Daejeon, Korea) with oligo(dT) $_{18}$ primers. Five μ l of the RT products were then amplified using a polymerase chain reaction (PCR) kit (AccuPower PCR Premix) under the following conditions: denaturation at 94°C for 5 min and 30 cycles at 94°C for 45 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by 5 min of extension at 72°C. The primers used were: 5'-CGT GAG ACT TTG CAG CCT GA-3' (sense), 5'-GGC GAT GTA AGT GAT CTG CTG-3' (antisense) for Oct4 (519 base pair [bp]); 5'-TCT TAC ATC GCG CTC ATC AC-3' (sense), 5'-TCT TGA CGAAGC AGT CGT TG-3' (antisense) for FOXD3 (171 bp); 5'-GTG GAA ACT TTT GTC CGA GAC-3' (sense), 5'-TGG AGT GGG AGG AAG AGG TAA C-3' (antisense) for SOX2 (550 bp); 5'-GCT ATT CCT GGG GTG TGT GT-3' (sense), 5'-CCC TGT GAT GAG TCC CAA CT-3' (antisense) for GPR40 (434 bp); 5'-GTG AAT TAC CAA GAC TCC CAG-3' (sense), 5'-GCC CAT GGC ATG GCC AGA GGG-3' (antisense) for G6Pase (305 bp); and 5'-CTG GTT CCG GAA AGA CAA AA-3' (sense), 5'-GCT CGG AGC TCC CTC TCT AT-3' (antisense) for PEPCK (350 bp). PCR for β -actin was also carried out as a control for RNA quantity.

Real-time RT-PCR

Total RNA was extracted using STAT-60 (Tel-Test, Inc., Friendswood, TX, USA) from cells treated with each designated agent. Real-time quantification of RNA targets was then performed in the Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, NSW, Australia) using the QuantiTect SYBR Green RT-PCR kit (QIAGEN, CA, USA). The reaction mixture (20 μ l) contained 200 ng of total RNA, 0.5 μ M of each primer, appropriate amounts of enzymes, and fluorescent dyes as recommended by the supplier. The Rotor-Gene 2000 cyclor was programmed as follows: 30 min at 50°C for reverse transcription; 15 min at 95°C for DNA polymerase activation; 15 sec at 95°C for denaturation; and 45 cycles of 15 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C. Data was collected during the extension step (30 sec at 72°C). The PCR reaction was followed by a melting curve analysis to verify the specificity and the identity of the RT-PCR products; this analysis can distinguish the specific PCR products from the non-specific PCR product resulting from primer-dimer formation. The temperature of the PCR products was elevated from 65 to 99°C at a rate of 1°C/5 sec, and the resulting data were analyzed using the software provided by the manufacturer.

Fig. 1. Effect of LA on the characteristics of mouse ES cells. (A): Alkaline phosphatase enzyme activity was measured after 0 h, 6 h, 12 h, and 24 h of LA (10^{-9} M) treatment each, as described in Materials and Methods. (B, C): FOXD3 (171 bp), Oct-4 (519 bp), SOX-2 (550 bp), and β -actin (350 bp) mRNA expression in the presence or absence of LA was examined using RT-PCR and Real time RT-PCR. The examples are representative of three independent experiments. (D) Cells were washed with PBS, fixed, stained with SSEA-1, and analyzed by Flow cytometry and immunofluorescence to monitor the percentage of SSEA-1 positive cells in the presence or absence of LA were measured.

Small interfering RNA

The cells were grown in each dish until they reached 75% confluence. They were then transfected for 24 h with either a SMARTpool of the small interfering (si) RNAs specific to GPR40, PI3K, and p42 MAPKs (200 pmol/l) or a nontargeting siRNA (as negative control; 200 pmol/l; Dharmacon, Inc., Lafayette, CO, <http://www.dharmacon.com>) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Preparation of cytosolic and total membrane fractions

The medium was removed, and the cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation and resuspended in buffer A (137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.7 mM KCl, 1.5 mM KH_2PO_4 , 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 $\mu\text{g/ml}$ leupeptin, pH 7.5). The resuspended cells were lysed mechanically on ice by trituration using a 21.1-gauge needle. The lysates were first centrifuged at 1,000 $\times$ g for 10 min at 4°C. The supernatants were further centrifuged at 100,000 $\times$ g for 1 h at 4°C to prepare the cytosolic and total particulate fractions. The particulate fraction containing the membrane fraction was washed twice and resuspended in buffer A containing 1% Triton X-100. The protein level in each fraction was quantified using the Bradford method [21].

Western blot analysis

The cells were harvested, washed twice with PBS, and lysed with buffer (137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.7 mM KCl, 1.5 mM KH_2PO_4 , 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 $\mu\text{g/ml}$ leupeptin [pH 7.5]) for 30 min on ice. The lysates were centrifuged at 15,000 rpm for 10 min at 4°C, and the protein concentration was determined using the Bradford method [21]. Equal amounts of protein (40 μg) were resolved by 10% SDS-

polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were washed with TBST (10 mM Tris HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20), blocked with 5% skim milk for 1 h, and incubated with the appropriate primary antibodies at the dilutions recommended by the suppliers. The membranes were washed, and the primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG or goat-anti mouse IgG. The bands were then visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Measurement of glucose production

Glucose production in mouse ES cells was measured as previously described [10]. Briefly, cells were washed three times with warm phosphate-buffered saline to remove glucose, followed by treatment with 10^{-9} M LA for 30 min in glucose-free medium (Gibco 11966-025) containing gluconeogenic substrates (20 mM sodium lactate and 2 mM sodium pyruvate). Glucose concentration was determined with a glucose assay kit from Roche Applied Science (cat. No. 0716251) and normalized to the cellular protein concentration. Total glucose production was derived from both glycogenolysis and gluconeogenesis. Glucose production from glycogenolysis was measured in the absence of gluconeogenic substrates. The amount of glucose production by gluconeogenesis is defined as the difference between total glucose production and glycogenolysis.

Statistical analysis

The results are expressed as the mean $\pm$ standard error (S.E.). All experiments were analyzed by analysis of variance, and some experiments were examined by comparing the treatment mean with the control using the Bonferroni-Dunn test. The difference was considered statistically significant when $p < 0.05$.

Fig. 2. Effect of LA on cell proliferation. (A): Cells were incubated with 10^{-7} M LA, oleic acid, palmitic acid, or caproic acid for 24 h, then [3 H] thymidine incorporation was measured. (B): Mouse ES cells were incubated for 24 h with various LA concentrations (0 - 10^{-11} M) and were subsequently pulsed with 1μ Ci of [3 H] thymidine for 1 h prior to counting. (C): Mouse ES cells were incubated in the presence of LA (10^{-9} M) for varying periods of time (0 - 48 h) and pulsed with 1μ Ci of [3 H] thymidine for 1 h. (D): Mouse ES cells were treated with LA (10^{-9} M) for 24 h and counted using a hemocytometer. The values represent the mean $\pm$ S.E. of four independent experiments with triplicate dishes. * $p < 0.05$ versus Control, # $p < 0.05$ versus LA alone. (E): Mouse ES cells were incubated with LA (10^{-9} M) for 24 h and double-labeled with BrdU and Oct-4 antibodies. (F): The expression of GPR 40 mRNA and protein was detected by RT-PCR and Western blotting as described in Materials and Methods. The example shown is representative of three experiments.

Results

The effect of LA on mouse ES cell proliferation

The undifferentiated state of the mouse ES cells used in this experiment was confirmed by examining undifferentiated stem cell markers, such as FOXD3, Oct-4, SOX-2, and SSEA-1 gene expression as well as alkaline phosphatase activity. As shown in Fig. 1, mouse ES cells maintained an undifferentiated state under both control and LA treatment conditions.

To compare the effect of LA on DNA synthesis with that of other fatty acids, the cells were treated with 10^{-7} M OA (monounsaturated, long chain), LA (double-unsaturated, long chain) and palmitic acid (saturated, long chain), or caproic acid (saturated, short chain) for 24 h. [3 H] Thymidine incorporation was then measured. OA and LA increased [3 H] thymidine incorporation, whereas caproic acid failed to increase it. Moreover, palmitic acid slightly increased [3 H] thymidine incorporation (Fig. 2A). In order to determine the time- and dose-response effects of LA on [3 H] thymidine incorporation, the level of [3 H] thymidine incorporation in mouse ES cells was observed for varying time periods (0 - 48 h) and with various dosages of LA (0 - 10^{-11} M). As shown in Fig. 2B, when

mouse ES cells were treated with various LA doses for 24 h, a significant increase in [3 H] thymidine incorporation was observed in cells incubated with 10^{-9} M LA. [3 H] thymidine incorporation was stimulated in a time-dependent manner and the highest level of [3 H] thymidine incorporation was observed at 24 h after incubation with 10^{-9} M LA (Fig. 2C). Subsequently, there was a significant increase in the number of cells following 24 h incubation with 10^{-9} M LA (Fig. 2D). To validate these results, double labeling for Oct4 and BrdU was performed to determine whether LA exerts its growth-promoting effect on undifferentiated ES cells. The observed effects reflect the role of LA in undifferentiated ES cells, but not in spontaneously differentiated progeny (Fig. 2E). In addition, the mouse ES cells expressed GPR40 (Fig. 2F).

Involvement of Ca^{2+} /PKC in LA-induced cell proliferation

Intracellular Ca^{2+} mobility was measured in response to LA to determine whether ES cell proliferation involves LA-induced increases in intracellular Ca^{2+} levels. As shown in Fig. 3A, LA induced an increase in intracellular Ca^{2+} levels. In experiments to determine upstream signaling molecules, the LA-induced increase

Fig. 3. Effect of LA on PKC and intracellular Ca^{2+} levels. (A): Mouse ES cells were loaded with 2 μM fluo 3-AM in serum-free medium for 40 min and treated with LA (10^{-9} M). Mouse ES cells were pretreated with U73122 (10^{-6} M), EGTA (4×10^{-3} M), and BAPTA-AM (10^{-5} M) for 30 min prior to LA treatment and then Ca^{2+} influx was measured (a-d). The changes in intracellular Ca^{2+} levels were monitored by confocal microscopy, and the data are expressed as relative fluorescence intensity. The amplitude of LA-induced intracellular Ca^{2+} level transients in mouse ES cells, which were blocked by U73122, EGTA, and BAPTA-AM, was summarized (e). The values represent the mean $\pm$ S.E. of three independent experiments with triplicate dishes. * $p < 0.05$ versus Control, # $p < 0.05$ versus LA alone. (B): The pan-PKC protein and the PKC α , β , and ζ isoforms, which were present in either the cytosolic compartment or membrane compartment, were detected by Western blotting, as described in Materials and Methods. Each example shown is representative of three experiments. (C): After the transfection with either a SMARTpool of GPR40-specific siRNAs or a non-targeting control siRNA for 48 h, the pan-PKC protein in membrane compartment was detected. (D): Mouse ES cells were treated with U73122 (10^{-6} M) and staurosporine (10^{-6} M) for 30 min prior to LA (10^{-9} M) treatment for 24 h and subsequently pulsed with 1 μCi of [^3H] thymidine for 1 h prior to counting. The values represent the mean $\pm$ S.E. of three independent experiments with triplicate dishes. * $p < 0.05$ versus Control, # $p < 0.05$ versus LA alone.

of intracellular Ca^{2+} levels was significantly blocked by U73122 (PLC inhibitor, 10^{-6} M), EGTA (extracellular calcium chelator, 4×10^{-3} M), and BAPTA-AM (intracellular calcium chelator, 10^{-5} M). Western blotting analysis was performed to confirm the involvement of PKC in LA-induced cell proliferation. The translocation of PKC from the cytosol to the membrane compartment is believed to be the first step in PKC activation and was used to measure the level of PKC activation in response to LA in mouse ES cells. PKC was mainly present in the cytosolic fraction of untreated mouse ES cells. After 30 min treatment with LA, LA increased PKC redistribution from the cytosol to the membrane compartment. In addition, LA stimulated the

translocation of the PKC α , δ , and ζ isoforms to the membrane compartment (Fig. 3B). These results indicate that LA stimulates PKCs isotypes non-specifically. When cells were transfected with a pool of GPR40-specific siRNAs, LA-induced activation of PKC was blocked (Fig. 3C). As shown in Fig. 3D, pretreatment of mouse ES cells with U73122 and staurosporine (PKC inhibitor, 10^{-6} M) decreased the LA-induced increase in [^3H] thymidine incorporation.

Involvement of PI3K/MAPKs in LA-induced cell proliferation

LA induced the phosphorylation of Akt in a time-dependent manner, which was inhibited by GPR40-

Fig. 4. Effect of LA on the phosphorylation of Akt and MAPKs. Mouse ES cells were treated with LA (10^{-9} M) for various times (0-90 min), and the phosphorylation of Akt (A) and p44/42 MAPKs (C) was detected. (B): After the transfection with either a SMATpool of GPR40-specific siRNAs or non-targeting control siRNA for 48 h prior to LA treatment, Akt phosphorylation was detected by Western blotting as described in Materials and Methods. (C): Mouse ES cells were pretreated with LY294002 (10^{-6} M) and transfected with either a SMRTpool of PI3K-specific siRNAs or a non-targeting control siRNA for 48 h prior to LA treatment, and the phosphorylation of Akt was detected. (D), (E): Mouse ES cells were pretreated with either LY294002, Akt inhibitor (10^{-5} M), U73122 (10^{-6} M), or staurosporine (10^{-6} M) for 30 min prior to LA treatment, and the phosphorylation of p44/42 MAPKs was then detected. Each example shown is representative of three experiments. The lower panels (bars) denote the mean $\pm$ S.E. of three experiments for each condition determined from densitometry relative to total Akt or total p44/42 MAPKs, respectively. (F) Mouse ES cells were pretreated with LY294002, Akt inhibitor, and PD98059 (10^{-6} M) for 30 min prior to LA treatment for 24 h and then pulsed with 1 μ Ci of [3 H] thymidine for 1 h. The values represent mean $\pm$ S.E. of four independent experiments with triplicate dishes. * $p < 0.05$ versus Control, # $p < 0.05$ versus LA alone.

specific siRNAs (Fig. 4A and B). In addition, Akt activation was blocked by LY294002 (PI3K inhibitor, 10^{-6} M) and the PI3K-specific siRNAs (Fig. 4C). LA-induced phosphorylation of p44/42 MAPKs was also observed, which first appeared 10 min after stimulation with LA (Fig. 4D). LY294002 and Akt inhibitor (10^{-5} M) inhibited the LA-induced phosphorylation of p44/42 MAPKs (Fig. 4E). Moreover, U73122 and staurosporine decreased the level of LA-induced phosphorylation of p44/42 MAPKs (Fig. 4F). As shown in Fig. 4G, pretreatment of mouse ES cells with LY294002, Akt inhibitor, and PD98059 (MEK inhibitor, 10^{-5} M) decreased the LA-induced increase in [3 H] thymidine incorporation.

Effect of LA on gluconeogenesis and cell cycle regulatory proteins

LA increased G6Pase and PEPCK (gluconeogenic enzymes) mRNA expression level (Fig. 5A and B). LA also increased the expression of PEPCK protein which was inhibited by PD98059 and p42 MAPKs-specific siRNAs (Fig. 5C). These results indicate that LA can directly elevate levels of gluconeogenic gene transcripts in mouse ES cells. The effect of LA on glucose production was observed. As shown in Fig. 5D, glucose production was increased by treatment with LA and was blocked by PD98059. In addition, pretreatment of mouse ES cells with metformin (gluconeogenesis inhibitor, 10^{-3} M)

Fig. 5. Effect of LA on gluconeogenesis. (A), (B): Mouse ES cells were treated with LA for 30 min and the G6Pase and PEPCK mRNA levels were analyzed by RT-PCR or real-time RT-PCR. The values represent mean $\pm$ S.E. of four independent experiments with triplicate dishes. * $p < 0.05$ versus Control (C): Mouse ES cells were treated with LA (10^{-9} M) and PEPCK expression level was detected. In addition, mouse ES cells were pretreated with PD98059 (10^{-6} M) and transfected with either SMARTpool of p42 MAPKs-specific siRNAs or a non-targeting control siRNA for 48 h prior to LA treatment, and PEPCK expression was then detected by western blotting. (D): Mouse ES cells were incubated with LA and then glucose production was measured. Mouse ES cells were also pretreated with PD98059 (10^{-6} M) for 30 min prior to LA treatment for 60 min and then glucose production was measured as described in Materials and Methods. Each example shown is representative of three experiments. The lower panels (bars) denote the mean $\pm$ S.E. of three experiments for each condition determined from densitometry relative to β -actin. (E) Mouse ES cells were pretreated with metformin (10^{-3} M) for 30 min prior to LA treatment for 60 min and then pulsed with 1 μ Ci of [³H] thymidine. The values represent the mean $\pm$ S.E. of three independent experiments. * $p < 0.05$ versus Control, # $p < 0.05$ versus LA alone.

decreased the LA-induced increase in [³H] thymidine incorporation (Fig. 5E). The effect of LA on the expression of cell-cycle regulatory proteins was examined in order to confirm the effect of LA on cell proliferation. LA (10^{-9} M) increased the protein levels of cyclin D1, cyclin E, CDK2, and CDK4. In addition, U73122, staurosporine, LY294002, Akt inhibitor, PD98059, and metformin inhibited the LA-induced increases in the expression of

these proteins (Fig. 6A, B, and C).

Discussion

This study demonstrated that LA stimulated cell proliferation via Ca^{2+} , PLC/PKC, PI3K/Akt, and p44/42 MAPKs signaling pathways in mouse ES cells. Activated

Fig. 6. Effect of LA on cell cycle regulators. (A), (B), (C): Mouse ES cells were pretreated with U73122 (10^{-6} M), staurosporine (10^{-6} M), LY294002 (10^{-6} M), Akt inhibitor (10^{-5} M), PD98059 (10^{-6} M), and metformin (10^{-3} M) for 30 min prior to LA (10^{-9} M) treatment for 24 h. Each of the examples shown is representative of three independent experiments.

metabolites of FFAs play vital roles in many cellular processes such as cell proliferation. Biologically active metabolites of FFAs act as second messengers to promote progenitor cell proliferation and to induce proliferation of human cord blood hematopoietic stem cells [22]. On the other hand, FFAs could decrease the proliferation of neuroblastoma cell [23]. However, LA induced cell proliferation of mouse ES cells in the present study. This seeming discrepancy may be due to the different cell types and concentrations of LA used [24].

Unsaturated long-chain fatty acids, such as LA, preferentially activate GPR40 [25]. GPR40 couples mainly with a G-protein α -subunit of the Gq family. These G proteins play an essential role during embryonic development and also enhance mitogenesis and tumorigenicity [26, 27]. In the present study, LA increased intracellular Ca^{2+} levels, thereby activating PLC signaling cascades, as in previous studies [26, 28]. Furthermore, activation of the receptor resulted in elevation of intracellular Ca^{2+} levels, leading to activation of PKC and MAPKs and suggesting a possible role for GPR40. Consequently, these signaling molecules stimulate DNA synthesis and cell proliferation. Mouse ES cells were reported to express PKC α , β 1, γ , δ , and ζ , and the activation of these isoforms was implicated in mouse ES

cell proliferation [29]. In various cell types, the activation of PKC isoforms induced cell proliferation [30–32], consistent with our results that LA stimulated PKC α , δ , and ζ translocation. Akt phosphorylation could be triggered directly Ca^{2+} through Ca^{2+} /calmodulin-dependent protein kinase [33] as well as GPCR-induced growth factors acting through receptor tyrosine kinases [30, 34]. Akt is not only recognized as a downstream PI3K target that plays a key role in a variety of biological effects, cell survival, and the cell cycle, but is also important for the proliferation, survival, and maintenance of pluripotency in ES cells [35–37]. OA induced cell proliferation through the activation of PI3K/Akt pathways in various cells [38, 39]. Our results demonstrated that LA also activated PI3K/Akt pathways in mouse ES cells. Moreover, LA induced the activation of MAPKs pathways including p44/42 MAPKs, as previously reported [24, 40–42]. The p44/42 MAPKs lie downstream of PI3K in many cell systems and mitogenic signaling pathways and are involved in the signaling cascades that regulate several major cellular functions, including cell proliferation and differentiation [43, 44].

It is well-known that FFAs regulate gluconeogenesis via direct and indirect pathways. FFAs not only increase gluconeogenesis by serving as a source of substrates and

energy including acetyl-CoA, NADH, and ATP, but also regulate directly gluconeogenesis through transcription of key gluconeogenic gene such as G6Pase and PEPCK [10, 45-47]. In addition, previously, the Raf/MEK/ERK and p38 MAPK pathways were reported to be a redundant mechanism for the mediation of gluconeogenesis through the transcription of PEPCK and G6Pase genes [10, 48, 49]. Our results demonstrated that LA increased gluconeogenesis as well as the mRNA and protein expression of G6Pase and PEPCK via p44/42 MAPKs pathway. Fructose-1,6-bisphosphatase and PEPCK were found mainly in liver and kidney. However, the expression of these enzymes was also detected in other tissues such as prostate, ovary, testis, and heart [50]. Moreover, PEPCK was expressed by stimulation of insulin-like growth factor-binding protein receptor in embryoblast and trophoblast [51]. These evidences provide that ES cells may play an important role in the glucose homeostasis.

In the present study, LA induced the proliferation of mouse ES cells by enhancing the expression of cell cycle regulatory proteins, such as cyclin and CDK. Recent reports have demonstrated that the cyclin D-associated CDK activities are regulated by mitogenic signaling and are involved in the G1 to S phase transition in ES cells [52, 53]. Raf/MEK/ERK and PI3K/Akt signaling pathways can act synergistically to promote the G1-S phase cell cycle progression in both normal and cancer cells [54, 55]. In ES cells, the PI3K-dependent signaling pathways also regulate cyclin D1 expression [53]. On the other hand, Murata et al reported that eicosapentaenoic acid, a polyunsaturated fatty acids, induced gluconeogenesis through the PI3K/Akt pathway, but decreased MAPKs activity and cell proliferation [56]. Although there are a few previous reports about interaction between cell proliferation and gluconeogenesis, LA is sufficient to increase cyclin/CDK activities and cell proliferation related to the increase of gluconeogenesis. One element is the elevation of intracellular Ca^{2+} level. Elevation of intracellular Ca^{2+} levels is known to take part in cell proliferation and gluconeogenesis via activation of Ca^{2+} -dependent protein kinases [57, 58]. Peroxisome-proliferator activated receptors (PPARs) are transcription factors, including PPAR α , β/δ , and γ , and activated by fatty acids [59], which might involve the regulation of cells proliferation and gluconeogenesis [60, 61]. It may be also related to autocrine motility factor (AMF)/phosphoglucose isomerase (PGI), a cytosolic housekeeping enzyme that plays a key role in gluconeogenesis and is involved in cell proliferation

Fig. 7. The hypothesized model for the signal pathways involved in LA-induced stimulation of ES cell proliferation. LA activates GPR40, which stimulates PLC to generate IP_3 and DAG. In turn, IP_3 increases the concentration of intracellular Ca^{2+} . DAG activates PKC, which induces p44/42 MAPKs activation, continuously increasing cell cycle progression. In another pathway, LA also stimulates the phosphorylation of Akt and p44/42 MAPKs in mouse ES cells via PI3K leading to the increase of cell cycle progression. Moreover, the increase of G6Pase and PEPCK expression via the phosphorylation of p44/42 MAPKs stimulates the cell gluconeogenesis and proliferation. LA, linoleic acid; GPR40, G protein-coupled receptor 40; Gq, G-protein alpha (q) subunit; PLC, phospholipase C; IP_3 , 1,4,5-inositol-triphosphates; DAG, diacylglycerol; PKC, protein kinase C; MAPKs, mitogen-activated protein kinases; PI3K, phosphatidylinositol 3-kinase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; CDK, cyclin-dependent kinase. The solid line is the proposed pathways and the dashed line is suspected pathway.

together with up-regulation of cyclin/CDK activities [62-65]. These facts indicate that LA stimulated the proliferation and gluconeogenesis of mouse ES cells.

In conclusion, our study demonstrated that LA stimulated cell proliferation and gluconeogenesis via Ca^{2+} , PLC/PKC, PI3K/Akt, and p44/42 MAPKs signaling pathways in mouse ES cells (Fig. 7). These results indicate that LA provide a more efficient and safer large-scale culture system for pluripotent ES cells, and hence might contribute to the use of ES cells in therapeutic applications. We think that the discovery of the role played by LA in proliferation of ES cells, together with the other results shown in this study, represent a significant advance in our knowledge of how ES cells pluripotency is maintained by extra cellular factors and has application in the development of increasing chemically defined ES cells culture systems.

Acknowledgements

This research was supported by Grant SC 2270 from the Stem Cell Research Center of the 21st Century

Frontier Research Program and BK 21 project, funded by the Ministry of Education, Science, and Technology, Republic of Korea.

References

- Kotarsky K, Nilsson NE, Flodgren E, Owman C, Olde B: A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. *Biochem Biophys Res Commun* 2003;301:406-410.
- Sessler AM, Ntambi JM: Polyunsaturated fatty acid regulation of gene expression. *J Nutr* 1998;128:923-926.
- Iwata H, Inoue J, Kimura K, Kuge T, Kuwayama T, Monji Y: Comparison between the characteristics of follicular fluid and the developmental competence of bovine oocytes. *Anim Reprod Sci* 2006;91:215-223.
- Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A: Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* 2005;130:485-495.
- Thomas MG, Bao B, Williams GL: Dietary fats varying in their fatty acid composition differentially influence follicular growth in cows fed isoenergetic diets. *J Anim Sci* 1997;75:2512-2519.
- Thangavelu G, Colazo MG, Ambrose DJ, Oba M, Okine EK, Dyck MK: Diets enriched in unsaturated fatty acids enhance early embryonic development in lactating Holstein cows. *Theriogenology* 2007;68:949-957.
- Das UN: Biological significance of essential fatty acids. *J Assoc Physicians India* 2006;54:309-319.
- Mashek DG, Grummer RR: Effects of long chain fatty acids on lipid and glucose metabolism in monolayer cultures of bovine hepatocytes. *J Dairy Sci* 2003;86:2390-2396.
- Wyne KL: Free fatty acids and type 2 diabetes mellitus. *Am J Med* 2003;115 Suppl 8A:29S-36S.
- Collins QF, Xiong Y, Lupo EG, Jr., Liu HY, Cao W: p38 Mitogen-activated protein kinase mediates free fatty acid-induced gluconeogenesis in hepatocytes. *J Biol Chem* 2006;281:24336-24344.
- Milligan G, Stoddart LA, Brown AJ: G protein-coupled receptors for free fatty acids. *Cell Signal* 2006;18:1360-1365.
- Covington DK, Briscoe CA, Brown AJ, Jayawickreme CK: The G-protein-coupled receptor 40 family (GPR40-GPR43) and its role in nutrient sensing. *Biochem Soc Trans* 2006;34:770-773.
- Sawzdargo M, George SR, Nguyen T, Xu S, Kolakowski LF, O'Dowd BF: A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1. *Biochem Biophys Res Commun* 1997;239:543-547.
- Ma D, Lu L, Boneva NB, Warashina S, Kaplamadzhiev DB, Mori Y, Nakaya MA, Kikuchi M, Tonchev AB, Okano H, Yamashita T: Expression of free fatty acid receptor GPR40 in the neurogenic niche of adult monkey hippocampus. *Hippocampus* 2008;18:326-333.
- Brown AJ, Jupe S, Briscoe CP: A family of fatty acid binding receptors. *DNA Cell Biol* 2005;24:54-61.
- Guasch G, Fuchs E: Mice in the world of stem cell biology. *Nat Genet* 2005;37:1201-1206.
- Chase LG, Firpo MT: Development of serum-free culture systems for human embryonic stem cells. *Curr Opin Chem Biol* 2007;11:367-372.
- Gruen L, Grabel L: Concise review: scientific and ethical roadblocks to human embryonic stem cell therapy. *Stem Cells* 2006;24:2162-2169.
- Chen CH, Ho ML, Chang JK, Hung SH, Wang GJ: Green tea catechin enhances osteogenesis in a bone marrow mesenchymal stem cell line. *Osteoporos Int* 2005;16:2039-2045.
- Zhang E, Li X, Zhang S, Chen L, Zheng X: Cell cycle synchronization of embryonic stem cells: effect of serum deprivation on the differentiation of embryonic bodies in vitro. *Biochem Biophys Res Commun* 2005;333:1171-1177.
- Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-254.
- Chung JW, Kim GY, Mun YC, Ahn JY, Seong CM, Kim JH: Leukotriene B4 pathway regulates the fate of the hematopoietic stem cells. *Exp Mol Med* 2005;37:45-50.
- Zhu Y, Schwarz S, Ahlemeyer B, Grzeschik S, Klumpp S, Kriegstein J: Oleic acid causes apoptosis and dephosphorylates Bad. *Neurochem Int* 2005;46:127-135.
- Fang IM, Yang CH, Yang CM, Chen MS: Linoleic acid-induced expression of inducible nitric oxide synthase and cyclooxygenase II via p42/44 mitogen-activated protein kinase and nuclear factor- κ B pathway in retinal pigment epithelial cells. *Exp Eye Res* 2007;85:667-677.
- Tikhonova IG, Sum CS, Neumann S, Thomas CJ, Raaka BM, Costanzi S, Gershengorn MC: Bidirectional, Iterative Approach to the Structural Delineation of the Functional "Chemoprint" in GPR40 for Agonist Recognition. *J Med Chem* 2007;50:2981-2989.
- Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR, Jr., Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI: The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *J Biol Chem* 2003;278:11303-11311.
- Wettschureck N, Offermanns S: Mammalian G proteins and their cell type specific functions. *Physiol Rev* 2005;85:1159-1204.
- Lee DK, George SR, O'Dowd BF: Continued discovery of ligands for G protein-coupled receptors. *Life Sci* 2003;74:293-297.
- Heo JS, Han HJ: ATP stimulates mouse embryonic stem cell proliferation via protein kinase C, phosphatidylinositol 3-kinase/Akt, and mitogen-activated protein kinase signaling pathways. *Stem Cells* 2006;24:2637-2648.
- Han HJ, Han JY, Heo JS, Lee SH, Lee MY, Kim YH: ANG II-stimulated DNA synthesis is mediated by ANG II receptor-dependent Ca^{2+} /PKC as well as EGF receptor-dependent PI3K/Akt/mTOR/p70S6K1 signal pathways in mouse embryonic stem cells. *J Cell Physiol* 2007;211:618-629.
- Greco S, Muscella A, Elia MG, Salvatore P, Storelli C, Mazzotta A, Manca C, Marsigliante S: Angiotensin II activates extracellular signal regulated kinases via protein kinase C and epidermal growth factor receptor in breast cancer cells. *J Cell Physiol* 2003;196:370-377.

- 32 Zhao Y, Liu J, Li L, Liu L, Wu L: Role of Ras/PKC ζ /MEK/ERK1/2 signaling pathway in angiotensin II-induced vascular smooth muscle cell proliferation. *Regul Pept* 2005;128:43-50.
- 33 Yano S, Tokumitsu H, Soderling TR: Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature* 1998;396:584-587.
- 34 Rozengurt E: Mitogenic signaling pathways induced by G protein-coupled receptors. *J Cell Physiol* 2007;213:589-602.
- 35 Takahashi K, Murakami M, Yamanaka S: Role of the phosphoinositide 3-kinase pathway in mouse embryonic stem (ES) cells. *Biochem Soc Trans* 2005;33:1522-1525.
- 36 Dugourd C, Gervais M, Corvol P, Monnot C: Akt is a major downstream target of PI3-kinase involved in angiotensin II-induced proliferation. *Hypertension* 2003;41:882-890.
- 37 Stabile E, Zhou YF, Saji M, Castagna M, Shou M, Kinnaird TD, Baffour R, Ringel MD, Epstein SE, Fuchs S: Akt controls vascular smooth muscle cell proliferation in vitro and in vivo by delaying G1/S exit. *Circ Res* 2003;93:1059-1065.
- 38 Hardy S, Langelier Y, Prentki M: Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. *Cancer Res* 2000;60:6353-6358.
- 39 Yun MR, Lee JY, Park HS, Heo HJ, Park JY, Bae SS, Hong KW, Sung SM, Kim CD: Oleic acid enhances vascular smooth muscle cell proliferation via phosphatidylinositol 3-kinase/Akt signaling pathway. *Pharmacol Res* 2006;54:97-102.
- 40 Rao GN, Alexander RW, Runge MS: Linoleic acid and its metabolites, hydroperoxyoctadecadienoic acids, stimulate c-Fos, c-Jun, and c-Myc mRNA expression, mitogen-activated protein kinase activation, and growth in rat aortic smooth muscle cells. *J Clin Invest* 1995;96:842-847.
- 41 Katsuma S, Hatae N, Yano T, Ruike Y, Kimura M, Hirasawa A, Tsujimoto G: Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1. *J Biol Chem* 2005;280:19507-19515.
- 42 Hennig B, Lei W, Arzuaga X, Ghosh DD, Saraswathi V, Toborek M: Linoleic acid induces proinflammatory events in vascular endothelial cells via activation of PI3K/Akt and ERK1/2 signaling. *J Nutr Biochem* 2006;17:766-772.
- 43 Lopez-Illasaca M, Crespo P, Pellici PG, Gutkind JS, Wetzker R: Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase γ . *Science* 1997;275:394-397.
- 44 Hawes BE, Luttrell LM, van Biesen T, Lefkowitz RJ: Phosphatidylinositol 3-kinase is an early intermediate in the G $\beta\gamma$ -mediated mitogen-activated protein kinase signaling pathway. *J Biol Chem* 1996;271:12133-12136.
- 45 Lam TK, Carpentier A, Lewis GF, van de Werve G, Fantus IG, Giacca A: Mechanisms of the free fatty acid-induced increase in hepatic glucose production. *Am J Physiol Endocrinol Metab* 2003;284:E863-873.
- 46 Antras-Ferry J, Le Bigot G, Robin P, Robin D, Forest C: Stimulation of phosphoenolpyruvate carboxykinase gene expression by fatty acids. *Biochem Biophys Res Commun* 1994;203:385-391.
- 47 Bizeau ME, Hazel JR: Dietary fat type alters glucose metabolism in isolated rat hepatocytes. *J Nutr Biochem* 1999;10:709-715.
- 48 Lam TK, Poci A, Gutierrez-Juarez R, Obici S, Bryan J, Aguilar-Bryan L, Schwartz GJ, Rossetti L: Hypothalamic sensing of circulating fatty acids is required for glucose homeostasis. *Nat Med* 2005;11:320-327.
- 49 Barthel A, Schmoll D: Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol Endocrinol Metab* 2003;285:E685-692.
- 50 Yanez AJ, Nualart F, Droppelmann C, Bertinat R, Brito M, Concha, II, Slebe JC: Broad expression of fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase provide evidence for gluconeogenesis in human tissues other than liver and kidney. *J Cell Physiol* 2003;197:189-197.
- 51 Navarrete Santos A, Ramin N, Tonack S, Fischer B: Cell lineage-specific signaling of insulin and insulin-like growth factor I in rabbit blastocysts. *Endocrinology* 2008;149:515-524.
- 52 Han HJ, Heo JS, Lee YJ: Estradiol-17 β stimulates proliferation of mouse embryonic stem cells: involvement of MAPKs and CDKs as well as protooncogenes. *Am J Physiol Cell Physiol* 2006;290:C1067-1075.
- 53 Jirmanova L, Afanassieff M, Gobert-Gosse S, Markossian S, Savatier P: Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells. *Oncogene* 2002;21:5515-5528.
- 54 Shen F, Fan X, Liu B, Jia X, Du H, You B, Ye M, Huang C, Shi X: Overexpression of cyclin D1-CDK4 in silica-induced transformed cells is due to activation of ERKs, JNKs/AP-1 pathway. *Toxicol Lett* 2006;160:185-195.
- 55 Mirza AM, Gysin S, Malek N, Nakayama K, Roberts JM, McMahon M: Cooperative regulation of the cell division cycle by the protein kinases RAF and AKT. *Mol Cell Biol* 2004;24:10868-10881.
- 56 Murata M, Kaji H, Iida K, Okimura Y, Chihara K: Dual action of eicosapentaenoic acid in hepatoma cells: up-regulation of metabolic action of insulin and inhibition of cell proliferation. *J Biol Chem* 2001;276:31422-31428.
- 57 Roche E, Buteau J, Aniento I, Reig JA, Soria B, Prentki M: Palmitate and oleate induce the immediate-early response genes c-fos and nur-77 in the pancreatic β -cell line INS-1. *Diabetes* 1999;48:2007-2014.
- 58 Kraus-Friedmann N, Feng L: The role of intracellular Ca²⁺ in the regulation of gluconeogenesis. *Metabolism* 1996;45:389-403.
- 59 Grimaldi PA: Peroxisome proliferator-activated receptors as sensors of fatty acids and derivatives. *Cell Mol Life Sci* 2007;64:2459-2464.
- 60 Kim YH, Han HJ: High-glucose-induced prostaglandin E₂ and peroxisome proliferator-activated receptor δ promote mouse embryonic stem cell proliferation. *Stem Cells* 2008;26:745-755.
- 61 Xu J, Xiao G, Trujillo C, Chang V, Blanco L, Joseph SB, Bassilian S, Saad MF, Tontonoz P, Lee WN, Kurland IJ: Peroxisome proliferator-activated receptor α (PPAR α) influences substrate utilization for hepatic glucose production. *J Biol Chem* 2002;277:50237-50244.
- 62 Harrison RA: The detection of hexokinase, glucosephosphate isomerase and phosphoglucose activities in polyacrylamide gels after electrophoresis: a novel method using immobilized glucose 6-phosphate dehydrogenase. *Anal Biochem* 1974;61:500-507.
- 63 Silletti S, Raz A: Autocrine motility factor is a growth factor. *Biochem Biophys Res Commun* 1993;194:446-457.
- 64 Funasaka T, Hu H, Hogan V, Raz A: Down-regulation of phosphoglucose isomerase/autocrine motility factor expression sensitizes human fibrosarcoma cells to oxidative stress leading to cellular senescence. *J Biol Chem* 2007;282:36362-36369.
- 65 Tsutsumi S, Hogan V, Nabi IR, Raz A: Overexpression of the autocrine motility factor/phosphoglucose isomerase induces transformation and survival of NIH-3T3 fibroblasts. *Cancer Res* 2003;63:242-249.