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Effect of Leukotriene D_4 on Mouse Embryonic Stem Cell Migration and Proliferation: Involvement of PI3K/Akt As Well As GSK-3β/β-Catenin Signaling Pathways

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ABSTRACT

The actual leukotriene D_4 (LTD_4) signaling pathways that regulate cell proliferation have not been elucidated thoroughly although fatty acid and its metabolites play a key role in regulations of embryonic functions. Thus, this study investigated the response of mouse embryonic stem (ES) cells exposed to LTD_4 and elucidated the signaling pathways as well. LTD_4 increased DNA synthesis in concentration-dependent ($\geq 10^{-7}$ M) and time-dependent ($\geq$12 h) manners, as determined by [^{3}H] thymidine incorporation and increased cell number. LTD_4 induced the phosphorylation of signal transducer and activator of transcription-3 (STAT3) and the increase of intracellular Ca^{2+} levels via cysteinyl leukotriene (CysLT) 1 and 2 receptors. LTD_4 increased Akt activation and calcineurin expression, which were blocked by STAT3 inhibitor and calcium chelators. LTD_4-induced glycogen synthase kinase (GSK)-3β phosphorylation was decreased by LY294002, Akt inhibitor, and cyclosporine A. LTD_4 inhibited the phosphorylation of β-catenin. In addition, LTD_4-stimulated migration through increased activation of focal adhesion kinase (FAK) and paxillin which were blocked by Akt inhibitor and cyclosporine A. LTD_4-induced increases in protooncogene and cell cycle regulatory proteins were blocked by cyclosporine A, FAK siRNA, and β-catenin siRNA. In conclusion, LTD_4-stimulated mouse ES cell proliferation and migration via STAT3, phosphoinositide 3-kinases (PI3K)/Akt, Ca^{2+}-calcineurin, and GSK-3β/β-catenin pathway. J. Cell. Biochem. 111: 686–698, 2010.

KEY WORDS: EMBRYONIC STEM CELL; LEUKOTRIENE; MIGRATION; PROLIFERATION

The primary concern of stem cell augmentation from any source is getting true undifferentiated embryonic stem (ES) cells to increase in numbers in culture. Regulatory mechanisms which are involved by lipid metabolites modulate the growth and microenvironmental influences and act as a store house of factors to selectively affect the extent to which stem cells engage in proliferation, migration, and differentiation [Guasch and Fuchs, 2005; Chase and Firpo, 2007]. Of particular interest is the use of fatty acid metabolites including prostacyclin, prostaglandin, thromboxane, and leukotrienes (LT). Cysteinyl leukotrienes (CysLTs), such as LTC_4, LTD_4, and LTE_4, are derived from the ubiquitous membrane component arachidonic acid and are multipotential lipid mediators [Capra, 2004; Akino et al., 2006]. They play a key role in paracrine or autocrine regulations of embryonic and fetal functions, which are regulated by 5-lipoxygenase [Schafer et al., 1996; Sato et al., 2008]. However, there is limited knowledge concerning a potential role for LT and their cognate receptors in modulating ES cell proliferation. LTD_4 is the most potent of the CysLTs and its effects are primarily mediated through at least two distinguishable G protein-coupled receptors, CysLT1 receptor (CysLT1R) and CysLT2R [Lewis et al., 1990; Evans, 2003; Paruchuri and Sjolander, 2003]. CysLTRs is expressed and localized in lung, prostate, placenta, brain, and other tissues [Sarau et al., 1999]. Previous reports have shown that LTD_4 induces a proliferative cell survival response and migration via separate signaling pathways in various cell lines [Saegusa et al., 2001; Braccioni et al., 2002; McMahon et al., 2002; Paruchuri et al., 2002; Paruchuri and Sjolander, 2003]. It was also reported that LTD_4 stimulated the migration but not proliferation of endothelial cells

Min Hee Kim and Yu Jin Lee contributed equally to this work.

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mediated by the CysLT1R via the extracellular signal-regulated kinase (ERK) 1/2 pathway [Yuan et al., 2009]. In accordance with a role for LTD_4 in cell survival, it causes an up-regulation of factors such as COX-2, β-catenin, and cell survival protein Bcl-2 [Ohd et al., 2000]. However, the actual LTD_4 signaling pathways that regulate thoroughly cell proliferation and migration have not been elucidated.

ES cells are pluripotent cell lines that are derived from the inner cell mass of the mammalian embryo in the blastocyst stage [Guasch and Fuchs, 2005]. The distinctive features of ES cells contain the ability to maintain and expand the cells in culture for an extended time while maintaining their normal karyotype and pluripotent nature [Semb, 2005]. Because of these features, ES cells provide a new source for transplantation therapies and pharmaceutical testing [Chase and Firpo, 2007]. In this regard, the possible effect of LTD_4 on ES cells is worthy of study, since LTD_4 induces cell growth and mobility in a culture environment. However, very little is known about CysLT-induced effects and signaling in ES cells. To answer to this question, in the present study we examined the response of mouse ES cells exposed to LTD_4 and elucidated the signaling pathways.

MATERIALS AND METHODS

MATERIALS

Mouse ES cells (ES-E14TG2a) were obtained from the American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) and DMEM were purchased from Gibco (Rockville, MD). Fluorescence isothiocyanate (FITC)-conjugated rabbit anti-goat IgG, LY294002, and monoclonal anti-β-actin were obtained from Sigma–Aldrich (St. Louis, MO). STAT3 inhibitor, Akt inhibitor, and cyclosporine A were acquired from Calbiochem (La Jolla, CA). LTD_4, MK-571, and Bay-u9773 were from Cayman Chemical (Ann Arbor, MI). [Methyl-^{3}H] thymidine was purchased from Amersham Biosciences (Buckinghamshire, UK). Fluo-3/AM was obtained from Molecular Probes, Inc. (Eugene, OR). Oct4, calcineurin, phospho-paxillin, paxillin, phospho-FAK, FAK, fibronectin, phospho-glycogen synthase kinase (GSK)-3β, GSK-3β, phospho-β-catenin, β-catenin, cyclin D1, cyclin E, cyclin-dependent kinase (CDK) 2, and CDK4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Akt, Akt, goat anti-rabbit IgG, and goat-anti mouse IgG were purchased from New England Biolabs (West Grove, PA). All other reagents were purchased commercially and were of the highest purity available.

ES CELL CULTURE

Mouse ES cells were incubated in Dulbecco's modified Eagle's medium (DMEM) 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% FBS without a feeder layer for 5 days. The cells were passaged with 0.05% trypsin/EDTA onto gelatinized 12-well plates or onto 60 mm-diameter culture dishes without a feeder layer and maintained at 37°C in an atmosphere containing 5% CO_2 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. After a 24 h incubation period, the cells were washed twice with PBS and incubated with fresh serum-free DMEM including all supplements and the designated agents for the indicated period before the experiments.

ALKALINE PHOSPHATASE STAINING

Approximately 70% confluent mouse ES cells were washed twice with PBS and fixed with 4% formaldehyde (in PBS) for approximately 15 min at room temperature. The cells were washed with PBS and incubated using an alkaline phosphatase substrate solution (200 μg/ml naphthol AS-MX phosphate [3-hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate], 2% *N*,*N*-dimethyl-formamide, 0.1 M Tris [pH 8.2], and 1 mg/ml Fast Red TR salt [4-chloro-2-methylbenzenediazonium salt; zinc chloride]) for 10 min at room temperature. After being washed with PBS, the cells were photographed.

[^{3}H] THYMIDINE INCORPORATION

[^{3}H] Thymidine incorporation experiments were carried out as previously described under this condition, most of the serum-deprived ES cells arrest in G_0/G_1 phase [Chen et al., 2005; Zhang et al., 2005]. 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 μCi of [methyl-^{3}H] thymidine (specific activity: 74 GBq/mmol, 2.0 Ci/mmol) 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 LS 6500 liquid scintillation counter (Beckman Instruments, Fullerton, CA). 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 to allow comparison between the experimental groups.

FLOW CYTOMETRY ANALYSIS

The cells were incubated with or without LTD_4 for 12 h, and the cells were then dissociated in trypsin/EDTA, pelleted by centrifugation, washed in PBS, and fixed at 4°C in 70% ethanol for 12 h. The cells were then resuspended in PBS containing 0.1% bovine serum albumin (BSA). When required, a propidium iodide (PI) solution (500 μg/ml PI in 3.8×10^{-2} M sodium citrate, pH 7.0) and boiled RNase (10 mg/ml prepared in 10 mM Tris–HCl, pH 7.5) were added to the cells in the dark at room temperature, and the cells were incubated 37°C for 30 min. The sample was read by flow cytometry and analyzed using CXP software (Beckman Coulter, Fullerton, CA).

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 incorporation of 5-bromo-2′-deoxyuridine (BrdU; a thymidine analog) was measured to determine the level of DNA synthesis. ES cells grown on coverslips were serum-starved for 12 h prior to LTD_4 stimulation. The ES cells were then treated with LTD_4 for 24 h. BrdU (15 μM) was added during the last 16 h of incubation. After several washes with PBS, the cells were fixed with methanol [10% (v/v)] 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 antibody (diluted 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 the double-labeling experiments, the cells were fixed in acidified alcohol and processed for Oct-4 staining, which was followed by BrdU staining. The fixed cells were incubated with the rabbit anti-Oct-4 antibody (1:100; Santa Cruz Biotechnology) for 1 h at room temperature 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 and neutralization with 0.1 M sodium tetraborate and then with Alexa Fluor 488-conjugated mouse anti-BrdU mAb for 1 h at room temperature. After being washed with PBS, the BrdU/Oct-4-stained cells were examined by confocal microscopy (Fluoview 300; Olympus).

IMMUNOFLUORESCENCE STAINING WITH CYSLT1R OR CYSLT2R

Cells were fixed with 3.5% paraformaldehyde in PBS and permeabilized for 10 min with 0.1% (v/v) Triton X-100 and washed three times with PBS, for 10 min each wash. Cells were pre-incubated with 10% bovine serum albumin (BSA; Sigma–Aldrich) in PBS for 20 min to decrease non-specific antibody binding. Cells were then incubated for 60 min with primary antibody in a solution containing 1% (v/v) BSA in PBS, and washed with PBS as above. Cells were incubated with 1% (v/v) BSA for 5 min and then incubated for 60 min with FITC-conjugated secondary antibody in PBS containing 1% (v/v) BSA, and washed with PBS as above. Samples were mounted on slides and visualized with a FluoView 300 confocal microscope (Olympus).

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 μM Fluo-3/AM with 5% CO_2 and 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 (400×; Fluoview 300, Olympus). 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 REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was obtained from mouse ES cells using STAT-60, a monophasic solution of phenol and guanidine isothiocyanate (Tel-Test, Friendswood, TX). Purified total RNA was subjected DNase I digestion, followed by column purification using the DNA Free RNA Kit (Zymo Research, Orange, CA). RT was carried out with 3 μg RNA using a reverse transcription system kit (AccuPower RT PreMix, Bioneer, Daejeon, Korea) with oligo$(dT)_{18}$ primers. Five microliter 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 s, 55°C for 30 s, and 72°C for 30 s, followed by 5 min of extension at 72°C. The primers used were: 5′-CAACGAACTATCCACCTTCACC-3′ (sense), 5′-AGCCTTCTCCTAAAGTTTCCAC-3′ (antisense) for mCysLT1R (162 bp); 5′-GTCCACGTGCTGCTCATAGG-3′ (sense), 5′-ATTGGCTGCAGCCATGGTC-3′ (antisense) for mCysLT2R (180 bp); 5′-CGTTGCAGACTGAGATTGCC-3′ (sense), 5′-ACCGGACAGGTCC-ACATCTG-3′ (antisense) for c-fos (356 bp); 5′-AACTCGGACCTTCT-CACGTCG-3′ (sense), 5′-TGCTGAGGTTGGCGTAGACC-3′ (antisense) for c-jun (355 bp); 5′-TCCATTCCGAGGCCACAGCAAG-3′ (sense), 5′-TCAGCTCGATTCCTCCTCTGACG-3′ (antisense) for c-myc (266 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) 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, New South Wales, Australia) using the QuantiTect SYBR Green RT-PCR kit (QIAGEN, Valencia, CA). The reaction mixture (20 μl) contained 200 ng of dyes as recommended by the supplier. The Rotor-Gene 2000 cycler was programmed as follows: 30 min at 50°C for reverse transcription; 15 min at 95°C for DNA polymerase activation; 15 s at 95°C for denaturation; and 45 cycles of 15 s at 94°C, 30 s at 55°C, 30 s at 72°C. Data were collected during the extension step (30 s at 72°C). PCR 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 s, and the resulting data were analyzed using the software provided by the manufacturer.

LIVE CELL IMAGING MICROSCOPY

Cells were placed in temperature/CO_2 control chambers (Tokai) attached to an Olympus IX81-ZDC zero-drift microscope. Images continued to be collected for 0–24 h at 5-min intervals, using a Cascade 512B camera (Roper Scientific) operated by the multi-dimensional acquisition package of MetaMorph v. 7.01 software (Molecular Devices).

CELL MIGRATION ASSAYS

The Oirs™ Cell Migration and Calcein AM were from Platypus Technologies (Madison, WI) and Invitrogen (Carlsbad, CA) for each. Mouse ES cells were seeded at 100 μl/well and incubated in chamber for 12 h to permit cell adhesion. After the cells were grown to 70% confluence in the dishes, the insert were carefully removed and wells were gently washed with culture medium. Then, cells were incubated with new fresh medium and treatment LTD_4. For varying incubation times, we microscopically observed migration. The cell populations in the endpoint assay were stained with 0.5 mM Calcein AM for 30 min. The migrated cells were quantified by measuring the fluorescence signal using a microplate reader at excitation and emission wavelengths of 485 and 515 nm, respectively, and examined under an optical microscope (Fluoview 300; Olympus).

SMALL INTERFERING RNA (siRNA)

Cells were grown in each dish until they reached 75% confluence. They were then transfected for 24 h with either a SMARTpool of the siRNAs specific to FAK and β-catenin (200 pmol/L) or a nontargeting siRNA (as negative control; 200 pmol/L; Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

PREPARATION OF CYTOSOLIC AND MEMBRANE FRACTION

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 phenylmethanesulphonylfluoride (PMSF), 10 μg/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*g* for 10 min at 4°C. The supernatants were further centrifuged at 100,000*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 as previously described [Bradford, 1976].

NUCLEAR AND NON-NUCLEAR PROTEIN FRACTIONATION

Cells were lysed in hypotonic buffer (20 mM Hepes pH 7, 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP-40, 1 mM sodium orthovanadate) containing a cocktail of protease inhibitors. Lysates were homogenized gently (30 strikes in a syringe with a 19-gauge needle, and 30 strikes with a 25-gauge -needle, and centrifuged at 1,500*g* for 5 min at 4°C. The supernatants, containing the nonnuclear fraction, were cleared by centrifugation at 12,000*g* for 5 min and conserved at −70°C. The pellets, containing the nuclear fraction, were washed three times in hypotonic buffer. After homogenation in hypertonic buffer (hypotonic buffer containing 0.5 M NaCl and a cocktail of protease inhibitors), they were centrifuged at 12,000*g* for 10 min, and the supernatants were conserved at −70°C.

WESTERN BLOT ANALYSIS

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 μg/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 as previously described [Bradford, 1976]. Equal amounts of protein (40 μg) were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) 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, Piscataway, NJ).

STATISTICAL ANALYSIS

The results are expressed as the mean ± standard error (SE). 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 $P < 0.05$.

RESULTS

EFFECT OF LTD_4 ON ES CELL PROLIFERATION

The undifferentiated state of the mouse ES cells used in this experiment was confirmed by examining the undifferentiated stem cells markers, which included the FOXD3, Oct-4, and SOX-2 gene expression levels as well as the alkaline phosphatase activity. As shown in Figure 1A,B, the mouse ES cells maintained an undifferentiated state with or without LTD_4 treatment.

To determine the time–response and dose–response effects of LTD_4 on cell proliferation in mouse ES cells, the level of [^{3}H] thymidine incorporation was observed for varying time periods (0–48 h) and with various dosages of LTD_4 (0–10^{-5} M). As shown in Figure 2A, when mouse ES cells were treated with various LTD_4 doses for 12 h, a significant increase in [^{3}H] thymidine incorporation was observed in cells incubated with 10^{-7} M LTD_4. [^{3}H] thymidine

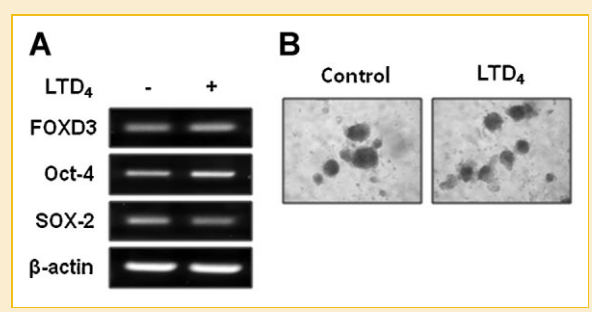

Fig. 1. Effect of LTD_4 on the characterization of mouse ES cells. A: FOXD 3 (171 bp), Oct4 (519 bp), SOX 2 (550 bp), and β-actin (350 bp) mRNA expression levels was examined after LTD_4 (10^{-7} M) treatment for 12 h using RT-PCR. B: The alkaline phosphatase enzyme activity was measured in the cells after 12 h of LTD_4 treatment, as described in Materials and Methods Section. The examples are representative of three independent experiments.

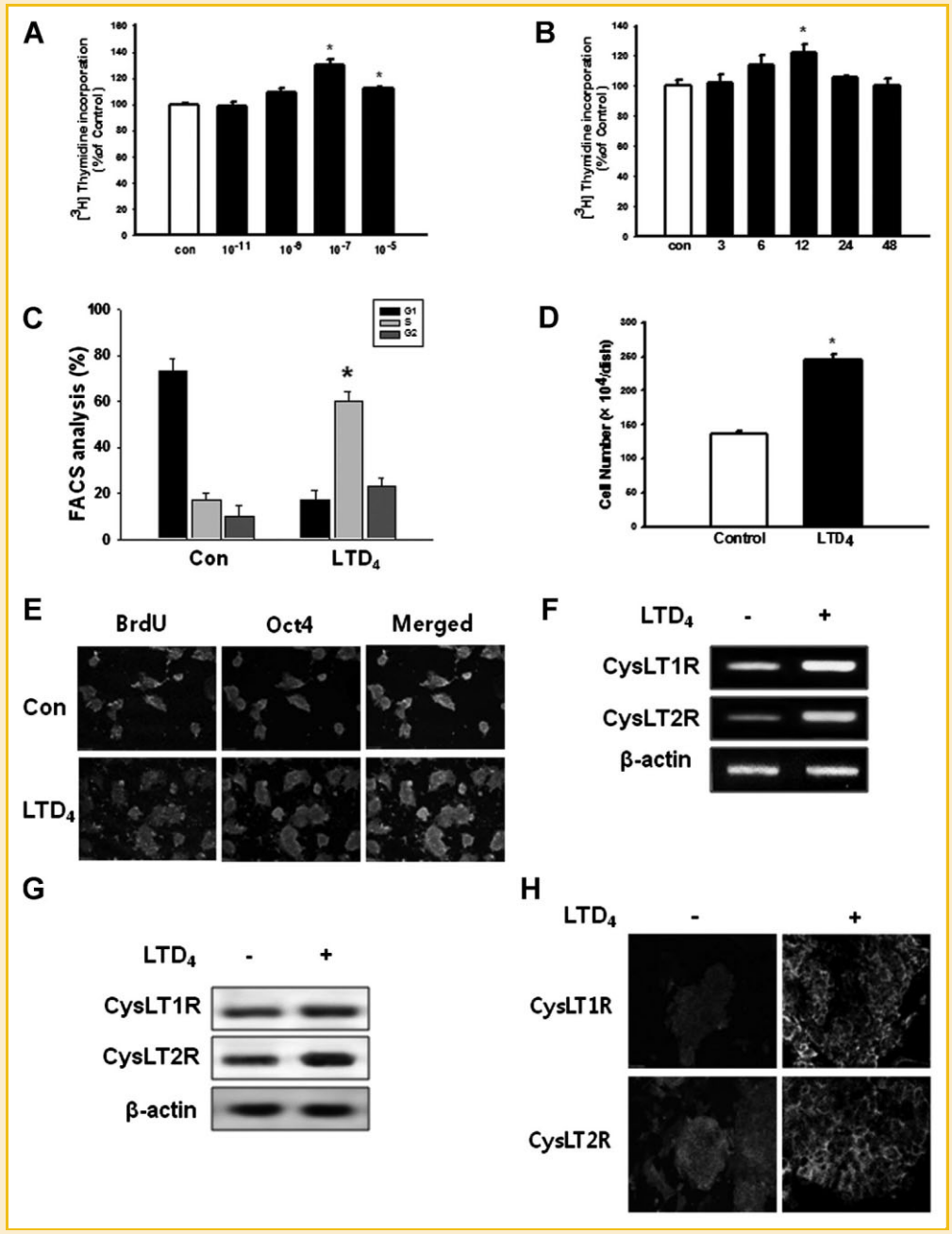

Fig. 2. Effect of LTD_4 on cell proliferation. A: Mouse ES cells were incubated for 12 h with various LTD_4 concentrations (0–10^{-5} M) and were subsequently pulsed with 1 μCi of [^{3}H] thymidine for 1 h prior to counting. B: The cells were incubated in the presence of LTD_4 (10^{-7} M) for varying periods of time (0–48 h) and pulsed with 1 μCi of [^{3}H] thymidine for 1 h. C: Cell cycles were also analyzed by flow cytometry to confirm the LTD_4-induced proliferation. The gates were configured manually to determine the percentage of cells in G1, S, and G2 phases based on DNA content. D: The cells were treated with LTD_4 (10^{-7} M) for 12 h and counted using a hemocytometer. E: The cells were incubated with LTD_4 for 12 h and double-labeled with BrdU and Oct-4 antibodies. Expression of CysLT1R and CysLT2R mRNA was detected by RT-PCR (F), Western blotting (G), and immunofluorescence staining (H) as described in Materials and Methods Section. The example shown is representative of three experiments. The values represent the mean ± SE of four independent experiments with triplicate dishes. $^*P < 0.05$ versus control.

incorporation was stimulated in a time-dependent manner and the highest level of [^{3}H] thymidine incorporation was observed at 12 h after incubation with 10^{-7} M LTD_4 (Fig. 2B).

As shown in Figure 2C, treatment with LTD_4 significantly increased the percentage of S and G2 phase cells (Control: about 27%; LTD_4: about 83%) and decreased the percentage of G_0/G_1 phase cells (Control: about 73%; LTD_4: about 17%) as compared with control, consistent with the observed increase in the level of [^{3}H] thymidine incorporation. Subsequently, there was a significant increase in the number of cells following 12 h incubation with 10^{-7} M LTD_4 (Fig. 2D). To validate these results, double labeling for Oct4 and BrdU was performed to determine whether

LTD_4 exerted its growth-promoting effect on undifferentiated ES cells (Fig. 2E). The observed effects reflected the role of LTD_4 in undifferentiated ES cells, but not in spontaneously differentiated progeny. In addition, mouse ES cells expressed CysLT1R and CysLT2R mRNA and protein (Fig. 2F–H).

INVOLVEMENT OF STAT3, Ca^{2+}, Akt, AND CALCINEURIN

To confirm the LTD_4 induced the phosphorylation of STAT3 via CysLTRs as well as the relation of STAT3 and Akt, Western blotting was carried out. LTD_4 induced the phosphorylation of STAT3 in a time-dependent manner (Fig. 3A). STAT3 phosphorylation was blocked by MK-571 (CysLT1R antagonist, 10^{-6} M) and Bay-u9773 (CysLT2R antagonist, 10^{-6} M; Fig. 3B). Moreover, LTD_4 induced the phosphorylation of Akt in a time-dependent manner (Fig. 3C). The phosphorylation of Akt was blocked by STAT3 inhibitor (10^{-6} M) and LY294002 (PI3K inhibitor, 10^{-6} M; Fig. 3D,E). These results indicated that STAT3 phosphorylation via CysLTRs regulated the activation of Akt.

Intracellular Ca^{2+} mobility was measured in response to LTD_4 to determine whether LTD_4 through CysLT1R and CysLT2R could induce increases in intracellular Ca^{2+} levels. As shown in Fig. 4A–C, LTD_4 induced an increase in intracellular Ca^{2+} levels that was significantly blocked by MK-571 and Bay-u9773. In addition, Akt activation was inhibited by EGTA (extracellular calcium chelator, 4×10^{-3} M) and BAPTA-AM (intracellular calcium chelator, 10^{-5} M; Fig. 4D). LTD_4-induced increase of calcineurin expression was also observed, which first appeared after 1 h stimulation with LTD_4 (Fig. 4E). Akt inhibitor (10^{-5} M) inhibited the LTD_4-induced

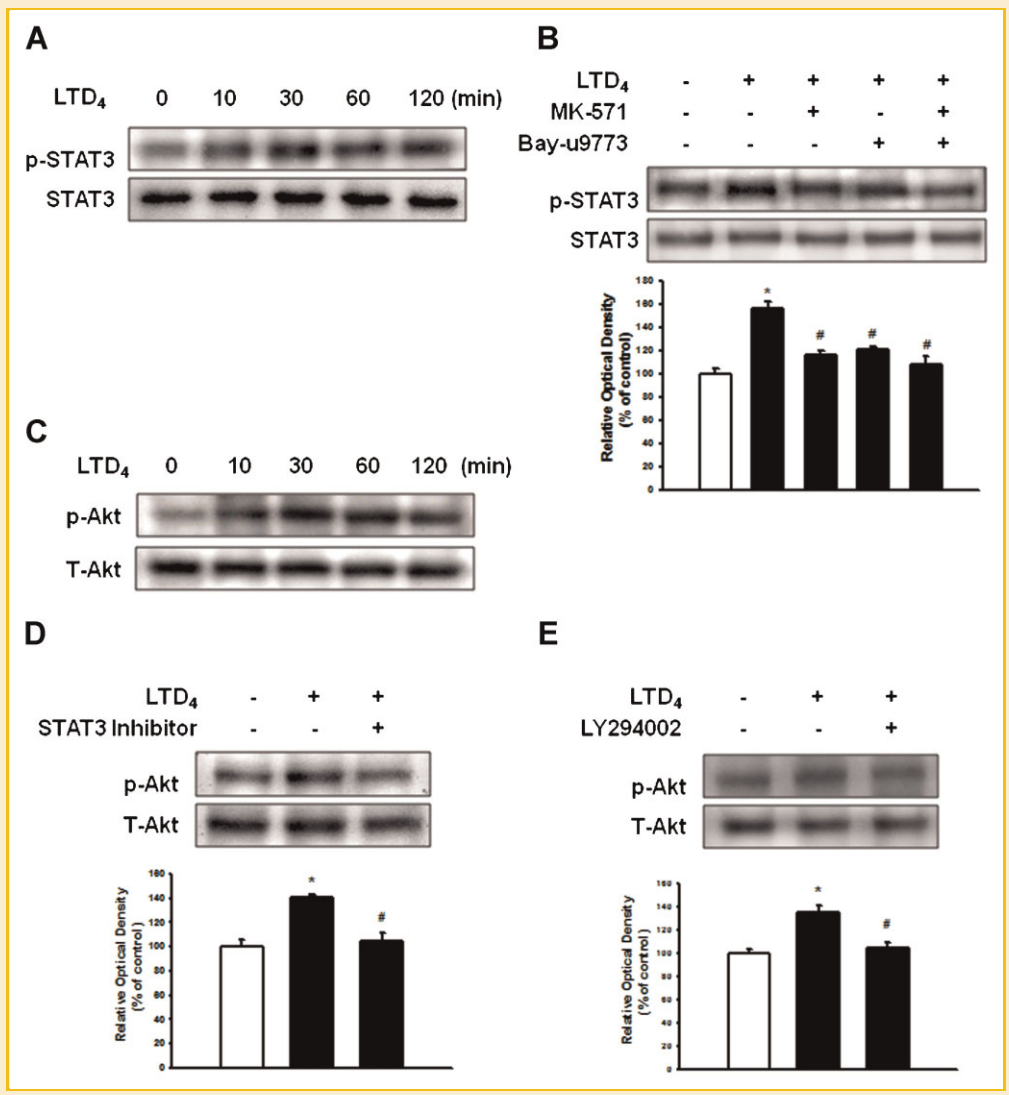

Fig. 3. Effect of LTD_4 on the phosphorylation of STAT3 and Akt. A: Mouse ES cells were incubated with LTD_4 (10^{-7} M) for 0–120 min and the total protein was extracted and blotted with antibodies against phospho-STAT3 and total STAT3. B: The cells were pretreated with either MK-571 (CysLT1R antagonist, 10^{-6} M) or Bay-u9773 (CysLT2R antagonist, 10^{-6} M) for 30 min prior to LTD_4 treatment, and the phosphorylation of STAT3 was then detected. C: The cells were treated with 10^{-7} M LTD_4 for 0–120 min, and the phosphorylation of Akt was detected. The cells were pretreated with STAT3 inhibitor (10^{-6} M) (D), LY294002 (PI3K inhibitor, 10^{-6} M) (E). Each example shown is representative of three experiments. The lower panels (bars) denote the mean ± SE of three experiments for each condition determined from densitometry relative to total STAT3 and Akt, respectively. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus LTD_4 alone.

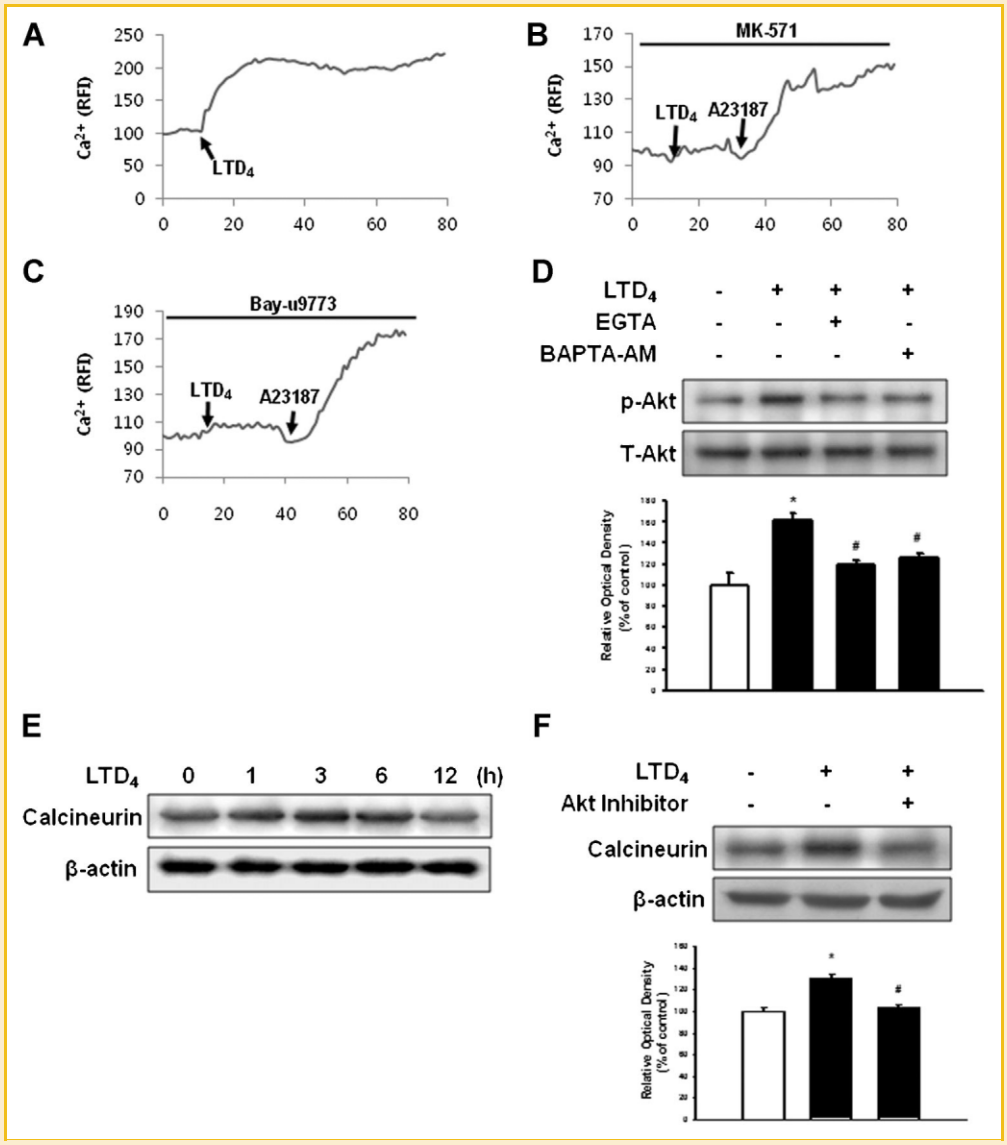

Fig. 4. Effect of LTD_4 on intracellular Ca^{2+} levels and calcineurin expression. Mouse ES cells were loaded with 2 μM Fluo 3-AM in serum-free medium for 40 min and treated with LTD_4 (10^{-7} M) (A). The cells were pretreated with MK-571 (B) and Bay-u9773 (C) for 30 min prior to LTD_4 treatment and then Ca^{2+} influx was measured. The changes in intracellular Ca^{2+} levels were monitored by confocal microscopy, and the data are expressed as relative fluorescence intensity. D: The cells were pretreated with EGTA (extracellular calcium chelator, 4×10^{-3} M), and BAPTA-AM (intracellular calcium chelator, 10^{-5} M) for 30 min prior to LTD_4 treatment, and the phosphorylation of Akt was detected. E: The cells were incubated with LTD_4 incubation for 0–12 h and the total protein was then extracted and blotted with antibody against calcineurin. F: The cells were pretreated with Akt inhibitor (10^{-5} M) for 30 min prior to LTD_4 treatment and the expression of calcineurin was then detected. Each example shown is representative of three experiments. The lower panels (bars) denote the mean ± SE of three experiments for each condition determined from densitometry relative to total Akt or β-actin, respectively. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus LTD_4 alone.

activation of calcineurin (Fig. 4F). These indicated that LTD_4 increased Ca^{2+} influx via CysLTRs, which stimulated the expression of calcineurin via Akt activation.

INVOLVEMENT OF GSK-3β/β-CATENIN AND NFATc1

We next sought to determine the effect of LTD_4 on the expression of GSK-3β and β-catenin. LTD_4-induced phosphorylation of GSK-3β was also observed, which first appeared after 30 min stimulation with LTD_4 (Fig. 5A). LY294002, Akt inhibitor, and cyclosporin A (calcineurin inhibitor, 10^{-5} M) inhibited the LTD_4-induced phosphorylation of GSK-3β (Fig. 5B,C). LTD_4 increased the expression of β-catenin in a time-dependent manner (Fig. 5D). In addition, the phosphorylation of β-catenin was blocked by LTD_4 and LiCl (GSK-3β inhibitor, 10^{-6} M; Fig. 5E). The translocation of β-catenin from the cytosol to the nucleus compartment is believed to be essential for β-catenin activation, and so was used to measure the level of β-catenin activation in response to LTD_4 in mouse ES cells. After 3 h treatment with LTD_4, β-catenin redistribution from the cytosol to the membrane compartment was increased (Fig. 5F). Moreover, LTD_4 increased mRNA expression of NFATc1, which was

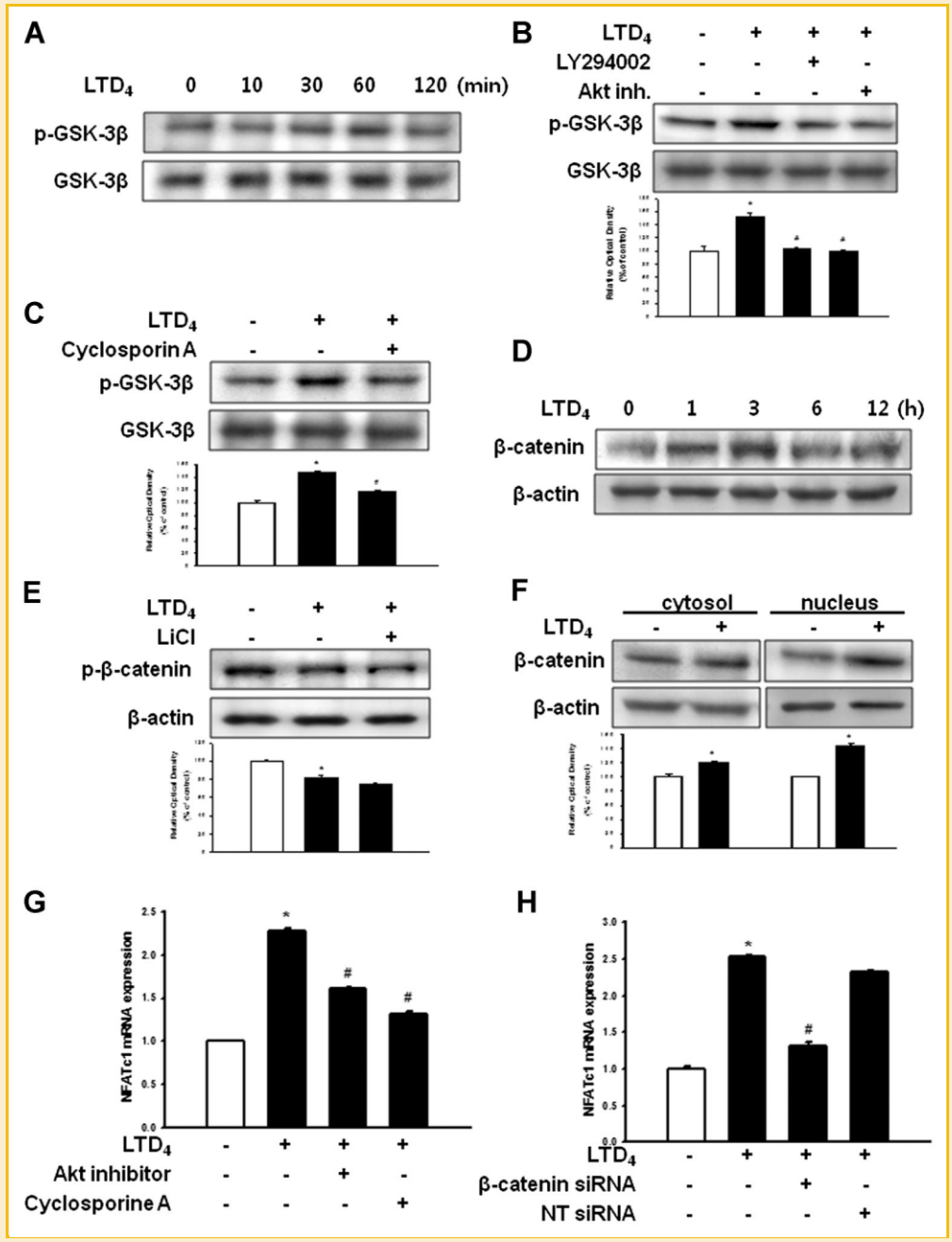

Fig. 5. Effect of LTD_4 on the regulation of GSK-3β, β-catenin, and NFATc1. A: Mouse ES cells were incubated with LTD_4 incubation for 0–120 min, and the total protein was extracted and blotted with antibody against phospho-GSK3β. The cells were pretreated with LY294002, Akt inhibitor (B), and cyclosporin A (calcineurin inhibitor, 10^{-5} M) (C) for 30 min prior to LTD_4 treatment, and the phosphorylation of GSK-3β was detected. D: The cells were treated with LTD_4 (10^{-7} M) for various time (0–12 h) and the expression of β-catenin was detected. E: The cells were pretreated with LiCl (GSK-3β inhibitor, 10^{-6} M) for 30 min prior to LTD_4 treatment and the phosphorylation of β-catenin was detected. F: Expression of β-catenin, which was present in either the cytosolic compartment or nucleus compartment, was detected by Western blotting. G: The cells were pretreated with Akt inhibitor and cyclosporin A for 30 min prior to LTD_4 treatment and mRNA expression of NFATc1 was detected. H: After transfection with either a SMARTpool of β-catenin-specific siRNAs or non-targeting control siRNA for 48 h prior to LTD_4 treatment, NFATc1 expression was detected by real-time RT-PCR. Each example shown is representative of three experiments. The lower panels (bars) denote the mean ± SE of three experiments for each condition determined from densitometry relative to total GSK-3β or β-actin, respectively. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus LTD_4 alone.

blocked by Akt inhibitor, cyclosporine A, and β-catenin siRNA (Fig. 5G,H). These results indicated that LTD_4 induced the GSK-3β inhibition and the β-catenin activation via Akt signaling as well as NFATc1 expression via the Akt, calcineurin, and β-catenin pathway.

EFFECT OF LTD_4 ON MIGRATION AND CELL CYCLE REGULATORY PROTEINS

LTD_4 induced the phosphorylation of FAK and paxillin in a time-dependent manner, which first appeared after 30 min stimulation

with LTD_4 (Fig. 6A). Akt inhibitor and cyclosporin A decreased the LTD_4-induced phosphorylation of FAK and paxillin (Fig. 6B). In addition, we conducted live cell imaging analysis to examine that LTD_4 has a pro-migratory effect on mouse ES cells. As shown in Figure 6C, ES cell proliferation and migration were markedly occurred from 12 to 24 h after LTD_4 treatment compared to 0 h. Moreover, LTD_4-indued increase of cell migration was inhibited by FAK siRNA (Fig. 6D). These results indicate that LTD_4 can elevate cell migration by regulating of focal adhesion-associated proteins such as FAK and paxillin in mouse ES cells. The effect of LTD_4 on the expression of protooncogene and cell-cycle regulatory proteins was examined to confirm the effect of LTD_4 on cell proliferation. LTD_4

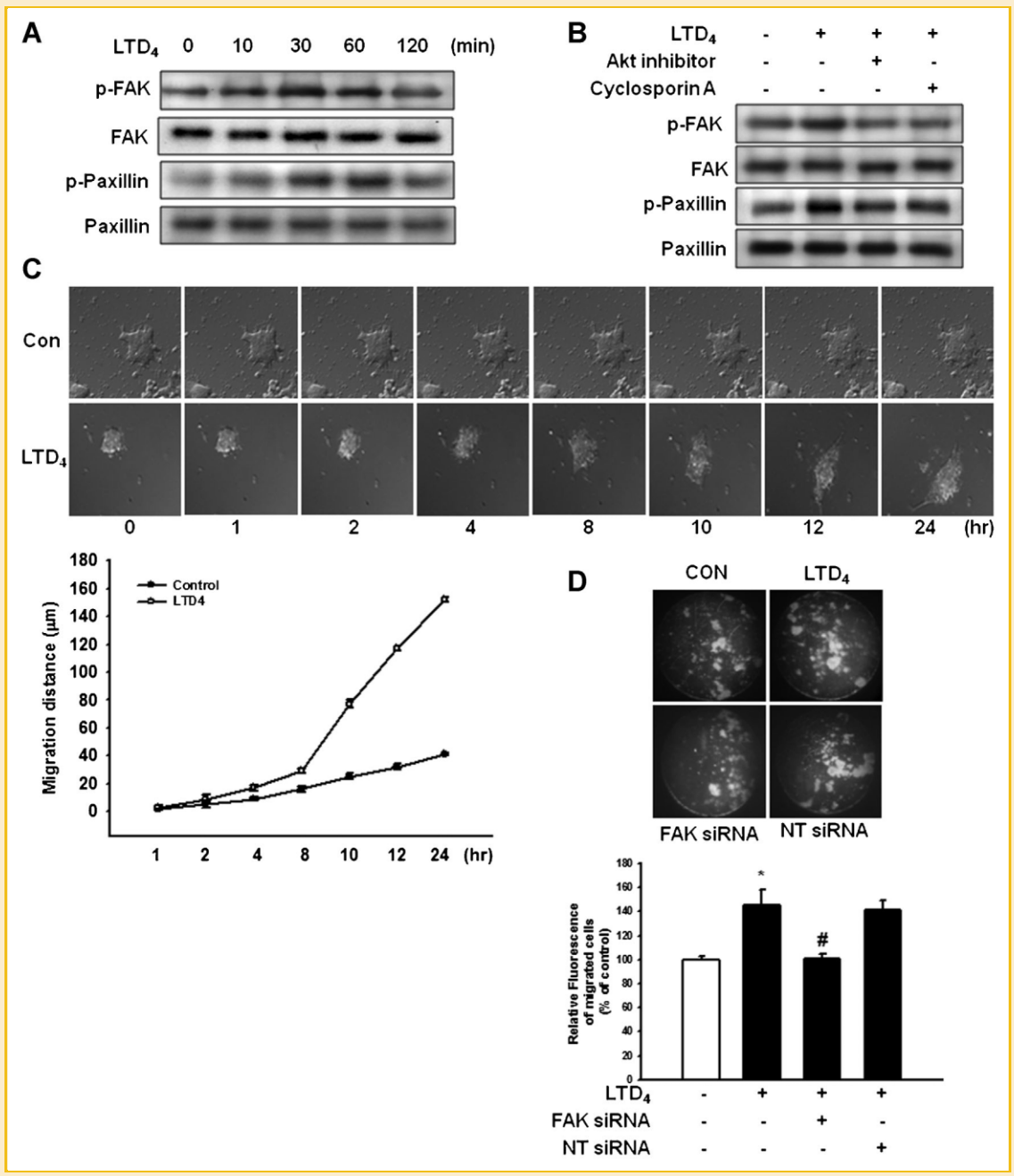

Fig. 6. Effect of LTD_4 on cell migration. A: Mouse ES cells were treated with LTD_4 (10^{-7} M) for various time (0–120 min) and the phosphorylation of FAK and paxillin was detected. B: The cells were pretreated with Akt inhibitor and cyclosporin A for 30 min prior to LTD_4 treatment and the phosphorylation of FAK and paxillin was detected. C: A typical time-lapse imaging experiment shows directed migration of mouse ES cells with treatment of LTD_4. The line graph denotes the distance of cell migration with treatment of LTD_4 analyzed by the multidimensional acquisition package of MetaMorph v. 7.01 software (Molecular Devices). D: To measure LTD_4-induced cell migration, calcein AM-induced fluorescence in the analytic zone was quantified by using a plate reader and observed under an optical microscope as described in Materials and Methods Section. Each example shown is representative of three experiments. The lower panels (bars) denote the mean ± SE of three experiments for each condition determined from densitometry relative to total FAK, paxillin, or β-actin, respectively. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus LTD_4 alone.

increased the protooncogene expression including c-fos, c-jun, and c-myc, which were decreased by β-catenin siRNA (Fig. 7A). Moreover, LTD_4 promoted the protein expression levels of cyclin D1, cyclin E, CDK2, and CDK4. These increases were inhibited by cyclosporin A and FAK siRNA (Fig. 7B,C). As shown in Figure 7D,E, pretreatment of mouse ES cells with cyclosporin A as well as FAK and β-catenin siRNA decreased the LTD_4-induced increase in [^{3}H] thymidine incorporation. These results indicated that LTD_4 increased the cell proliferation via β-catenin, calcineurin, and FAK pathway.

DISCUSSION

In the present study, we have demonstrated that LTD_4 stimulates mouse ES cell migration and proliferation through STAT3, PI3K/Akt, Ca^{2+}-calcineurin, and GSK-3β/β-catenin pathways via CysLTRs. We provide several lines of evidence that support our hypothesis that the interaction of LTD_4 with its plasma membrane receptors can influence ES cells cycle and proliferation. These findings, taken collectively, highlight the role of the CysLTRs as a

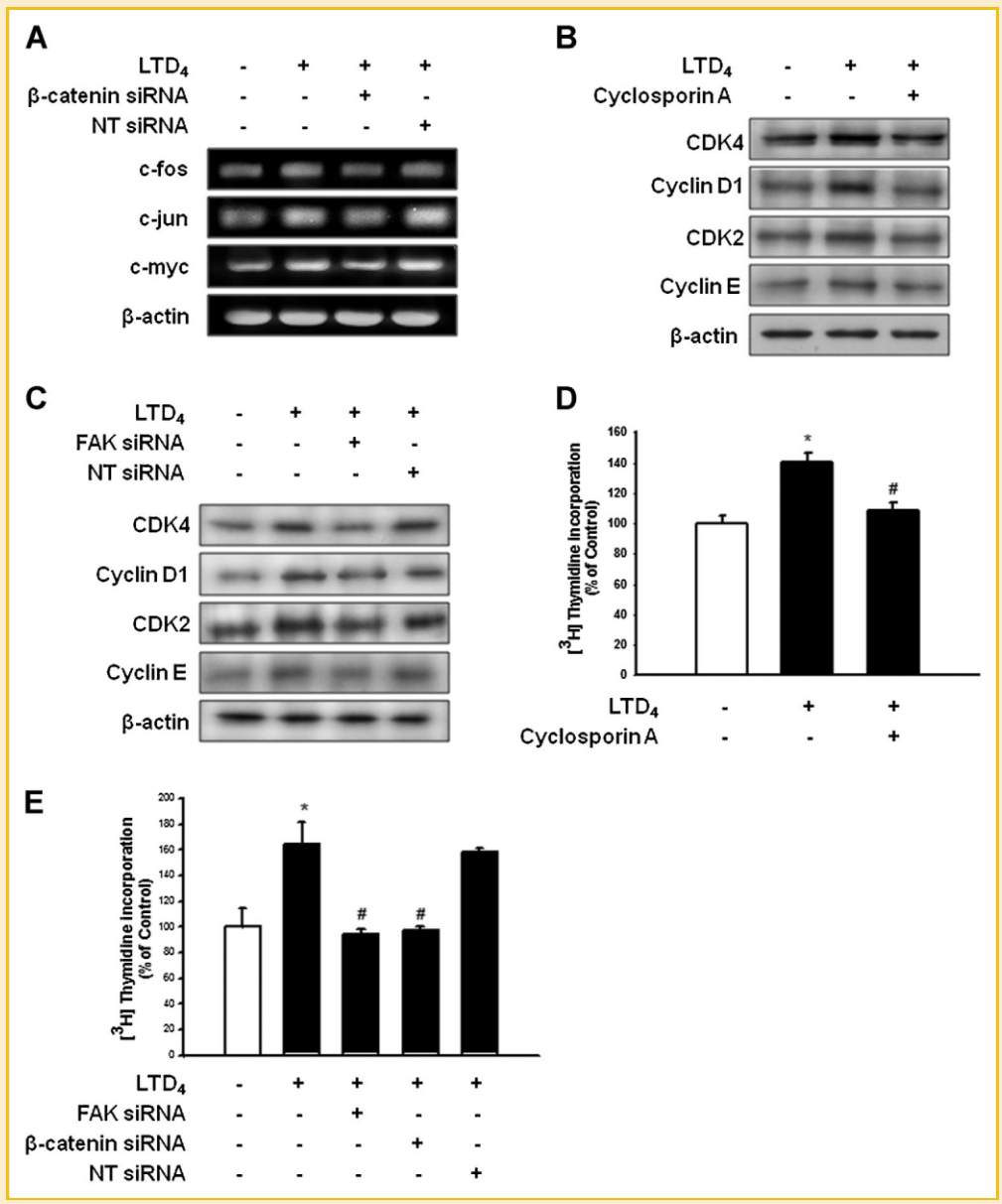

Fig. 7. Effect of LTD_4 on proto-oncogene and cell cycle regulatory proteins. A: After transfection with either a SMARTpool of β-catenin-specific siRNAs or non-targeting control siRNA for 48 h prior to LTD_4 treatment, expression of c-fos, c-jun, and c-myc were detected by RT-PCR. B: The cells were pretreated with cyclosporine A for 30 min prior to LTD_4 treatment and the expression of CDK2/4 and cyclinD1/E was detected. C: Expressions of CDK2/4 and cyclinD1/E were detected by Western blotting after the transfection with either a SMARTpool of FAK-specific siRNAs or non-targeting control siRNA for 48 h prior to LTD_4 treatment. The cells were treated with LTD_4 (10^{-7} M) and and pulsed with 1 μCi of [^{3}H] thymidine for 1 h. In addition, mouse ES cells were pretreated with cyclosporine A (D) and transfected with either SMARTpool of FAK and β-catenin-specific siRNAs or a non-targeting control siRNA for 48 h prior to LTD_4 treatment (E), and [^{3}H] thymidine incorporation were then measured. Each example shown is representative of three experiments. The values represent the mean ± SE of four independent experiments with triplicate dishes. $^*P < 0.05$ versus control, $^\#P < 0.05$ versus LTD_4 alone.

direct and potent regulator of ES cell proliferation, providing new and novel insights into the direct regulatory effects of the CysLTs on other biological processes which may involve ES cell proliferation. To our knowledge, this is the first study analyzing the responses associated with CysLT1 signaling in ES cells. In our study, when LTD_4 of 10^{-7} M concentration showing clear the growth promoting effect of LTD_4 on various cells types [Vannella et al., 2007; Bosse et al., 2008; Profita et al., 2008] was added exogenously in absence of FBS, DNA synthesis and cell proliferation increased in ES cells cultures. Hence, we show for the first time that ES cells may be expanded in the presence of LTD_4 in culture media. It was reported that endogenous urinary LTD_4 concentration in normal human was <0.005 nM [Mayatepek et al., 2005]. Moreover, dose-dependent LTD_4 stimulation of oocyte CysLTRs was observed with a half-maximal effective concentration of 0.4 nM and a threshold response of about 10^{-11} M [Lynch et al., 1999]. These great contrasts between the in vivo and in vitro conditions might be the reasons that the effect of LTD_4 in vivo may be indirect and mediated by a paracrine loop involving the secretion of cell mitogens [Bosse et al., 2008].

LTD_4-induced activation of STAT3 state through CysLTRs contributed to maintaining the pluripotent state of mouse ES cells and regulation of the downstream molecules for cell fate including proliferation and migration. Additionally, LTD_4-induced elevation of intracellular free Ca^{2+} concentration, which was prevented by antagonist of CysLTRs and induced Akt pathway. Our previous study also demonstrated that linoleic acid-induced proliferation maintains the pluripotent status of mouse ES cells through intracellular Ca^{2+} influx and the Akt pathway [Kim et al., 2009]. It has been reported that these PI3K/Akt cascades activate two potentially independent pathways; one regulated by Ras signaling and the other regulated by the STAT3-containing complex [Hsu et al., 2004; Lee et al., 2007; Lu et al., 2008]. These results imply that the LTD_4-induced PI3K/Akt pathway might play a critical role in ES cells via STAT3 activation as well as via an increase of intracellular Ca^{2+} concentration. In addition, the inhibition of GSK3, a negative regulator of the Wnt/β-catenin pathway, significantly promotes self-renewal of ES cells by leading to the activation of β-catenin [Sato et al., 2004; Marikawa, 2006]. In the present study, LTD_4-induced GSK-3β phosphorylation mediated the increase of β-catenin level via the activation of PI3K/Akt and calcineurin. These observations are consistent with previous reports concerning LTD_4 promotion of antiapoptosis or oncogenic target genes such as c-myc and cyclin D1, through PI3K-mediated increased β-catenin level [Mezhybovska et al., 2005, 2006]. In addition, GSK-3β acts a negative regulator of nuclear factor of activated T-cells (NFAT) by inducing phosphorylation and export from the nucleus. Inactivation of GSK-3β by phosphorylation on Ser9 by Akt is essential to insure NFAT-dependent transcription [Lipskaia et al., 2003]. It is well-known that transcriptional activity of NFATs is not only dependent on dephosphorylation by calcineurin in response to increased intracellular calcium, but also regulates gene expression during embryonic development [Schulz and Yutzey, 2004; Buchholz and Ellenrieder, 2007]. In our results, LTD_4 particularly increased expression of NFATc1, whereas other NFATs did not display significant changes of expression (data not shown). NFATc1 has been implicated in the migration and proliferation of vascular smooth muscle cell and targeted disruption of NFATc1 results in embryonic lethality [Chow et al., 2008; Nguyen and Di Giovanni, 2008].

In the present study, activation of FAK and/or paxillin was decreased by PI3K/Akt and calcineurin/NFAT inhibitors, which regulate assembly of focal adhesions in migrating cells and participate in transduction pathway informing the nucleus for contact with matrix. Moreover, LTD_4 increased the expression of CDKs/cyclins and protooncogene including c-jun, c-myc, and c-fos, which have already been investigated as key molecules in the proliferation of mouse ES cells [Han et al., 2007; Kim and Han, 2008]. Previous studies reported LTB_4-induced cell proliferation in hematopoietic and neural stem cells, and LTD_4-induced cell migration in fibroblasts [Chung et al., 2005; Kato et al., 2005; Wada et al., 2006]. In addition, proliferation is generally known to be decreased in migrating cell front, allowing theses cells to maintain their cell-to-cell communication and form a continuous moving front. Nevertheless, the area in some tissues or cells can support without the limitation of additional new cells and promote the proliferation in a region behind the moving cell front [Zelenka and Arpitha, 2008]. FAK, as an intracellular non-receptor tyrosine kinase and a major mediator of signal transduction by integrins, has been linked to the generation of cell survival, cell cycle progression, and cell migration signals [Hanks et al., 2003; Liu et al., 2007; Luo and Guan, 2010]. Moreover, it was previously reported that integrin-dependent cell migratory signals play an essential role in promotion of cell growth in a variety of cell types including mouse ES cells [Fashena and Thomas, 2000; Mitra and Schlaepfer, 2006; Hayashi et al., 2007; Park and Han, 2009; Kim et al., 2010]. In the present study, CDKs/cyclins expressions were decreased by FAK inhibition. Recent studies reported that the expression of cyclin D3 is increased by FAK overexpression as well as that the formation of focal adhesion promotes the proliferation by the mediation of fibronectin [Sonoda et al., 2008; Tanaka et al., 2009]. The important issue raised by our study is that none of the described cell lines reproduces LTD_4 signaling events found in ES cells. Collectively, these data show a very complex picture of LTD_4 signaling pathway that is dependent on cell type, model used, and phenotype analyzed. Thus, the results regarding LTD_4 signaling obtained from specific cells should be verified in ES cells before further conclusions can be made. Therefore, additional work is required to address the relevance of these possibilities in self-renewal of ES cells. In conclusion, our results demonstrate that LTD_4 promotes mouse embryonic stem cell proliferation and migration through STAT3, PI3K/Akt, Ca^{2+}-calcineurin, and GSK-3β/β-catenin pathways via CysLTRs.

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