L-Threonine Regulates G<sub>1</sub>/S Phase Transition of Mouse Embryonic Stem Cells via PI3K/Akt, MAPKs, and mTORC Pathways*<sup>S</sup>

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Although amino acids can function as signaling molecules in the regulation of many cellular processes, mechanisms surrounding L-threonine involvement in embryonic stem cell (ESC) functions have not been explored. Thus, we investigated the effect of L-threonine on regulation of mouse (m)ESC self-renewal and related signaling pathways. In L-threonine-depleted mESC culture media mRNA of self-renewal marker genes, [3H]thymidine incorporation, expression of c-Myc, Oct4, and cyclins protein was attenuated. In addition, resupplying L-threonine (500 μM) after depletion restores/maintains the mESC proliferation. Disruption of the lipid raft/caveolae microdomain through treatment with methyl-β-cyclodextrin or transfection with caveolin-1 specific small interfering RNA blocked L-threonine-induced proliferation of mESCs. Addition of L-threonine induced phosphorylation of Akt, ERK, p38, JNK/SAPK, and mTOR in a time-dependent manner. This activity was blocked by LY 294002 (PI3K inhibitor), wortmannin (PI3K inhibitor), mTOR in a time-dependent manner. This activity was blocked by LY 294002 (PI3K inhibitor), wortmannin (PI3K inhibitor), or an Akt inhibitor. L-threonine-induced activation of mTOR, p70S6K, and 4E-BP1 as well as cyclins and Oct4 were blocked by PD 98059 (ERK inhibitor), SB 203580 (p38 inhibitor) or SP 600125 (JNK inhibitor). Furthermore, L-threonine induced phosphorylation of raptor and rictor binding to mTOR was completely inhibited by 24 h treatment with rapamycin (mTOR inhibitor); however, a 10 min treatment with rapamycin only partially inhibited rictor phosphorylation. L-Threonine-induced translocation of rictor from the membrane to the cytosol/nuclear, which blocked by pretreatment with rapamycin. In addition, rapamycin blocked L-threonine-induced increases in mRNA expressions of trophoectoderm and mesoderm marker genes and mESC proliferation. In conclusion, L-threonine stimulated ESC G<sub>1</sub>/S transition through lipid raft/caveolae-dependent PI3K/Akt, MAPKs, mTOR, p70S6K, and 4E-BP1 signaling pathways.

A growing number of reports clearly demonstrate that amino acids are able to control many physiological functions, including regulation of cell signaling and gene expression, as well as transport and metabolism of amino acids themselves (1, 2). Although the molecular mechanisms involved in the control of gene expression by amino acid availability have been extensively studied in lower eukaryotes such as yeasts, the control of transcriptional events including signaling pathways, transcription factors, and their corresponding cis-acting DNA sequences is still unclear in stem cells. Moreover, it has been shown that the amino acid requirement is a developmentally regulated permissive event that occurs during a 4–8-h period at the early blastocyst stage in mice and that amino acids are involved in regulating early embryonic development (3). Therefore, it is now widely accepted that amino acids can stimulate signal transduction and function as signal molecules regulating many embryonic stem cell (ESC)<sup>2</sup> functions (4, 5). For instance, glutamine synthetase, which is the only enzyme which synthesizes glutamine de novo, is essential in early mouse embryogenesis (6, 7). In addition, L-proline induces formation of a distinct pluripotent cell population with primitive ectoderm characteristics in culture (8). Furthermore, interestingly, L-threonine-deprived culture media induces abnormal ESC colony growth and decreases alkaline phosphatase marker staining levels which present ESCs self-renewal (9). Nevertheless, amino acid-dependent regulation of ESC function has not been previously described and related signal pathways remain unclear.

Threonine, one of the essential amino acids, is an α-amino acid containing a polar hydroxyl group which enables participation in hydrogen bonding, an important factor in protein structure and amino acid transporters are associated with membrane rafts (10). It suggests that membrane raft/caveolae is important for the regulation of amino acid transporter functions. Amino acid and amino acid receptors have been shown to activate the mammalian target of rapamycin (mTOR) signaling pathway which exists in two distinct protein complexes, mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2). The mTORC1 complex mediates cell growth and protein synthesis (11). Unlike mTORC1, mTORC2 is thought to mediate cell proliferation and cell survival by direct phosphorylation of Akt at critical regulatory sites required for maximal Akt kinase activity (12). In addition, it is reported that activation of mTOR signaling is required for ESC proliferation (13). Cell cycle progression is tightly regulated by the

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<sup>2</sup>The abbreviations used are: ESC, embryonic stem cell; mTOR, mammalian target of rapamycin; mESC, mouse embryonic stem cell; CDK, cyclin-dependent kinase; mTORC1, mTOR complex1; mTORC2, mTOR complex2; MAPK, MAP kinase.
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coordinated action of cyclin and cyclin-dependent kinase (CDK) complexes (14). Furthermore, depletion of ractor and rictor increases cells in the G₁ phase while mTORC1 and mTORC2 inhibition elicits down-regulation of cyclin D1 and up-regulation of p27 levels (15). Moreover, mTOR (1L6-hydroxy-threonine) was purchased from Calbiochem (La Jolla, CA). All pharmacological inhibitors used in this study did not affect cell proliferation and signal pathway activation at treated concentrations. However, combination treatment of inhibitors for PI3K/Akt, MAPKs, and mTOR decreased [³H]thymidine incorporation (supplemental Figs. S1 and S2). [³H]thymidine was obtained from Dupont/NEN (Boston, MA). Phospho-ERK, phospho-JNK/SAPK, JNK/SAPK, phospho-p38, p38, phospho-Akt (Thr³⁰⁸ and Ser⁴⁷³), and Akt antibodies were purchased from New England Biolabs (Herts, UK). The Oct4, c-Myc, cyclin D1, and cyclin E antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The phospho-mTOR, phospho-p70S6K, and phospho-4E-BP1 antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA). The goat anti-rabbit IgG was supplied by Jackson ImmunoResearch (West Grove, PA). Liquiscint was obtained from National Diagnostics (Parsippany, NY). All other reagents were of the highest purity commercially available and were used as received.

ESC Culture—The mESCs were cultured in Dulbecco’s Modified Eagle’s Media (DMEM) (Invitrogen, Gaithersburg, MD) supplemented with 3.7 g/liter sodium bicarbonate, 1% penicillin, and streptomycin, 1.7 mM l-glutamine, 0.1 mM β-mercaptoethanol, 5 mg/ml mouse leukemia inhibitory factor (LIF), and 15% FBS, without a feeder layer. The cells were grown on gelatinized 12-well plates or 60-mm culture dishes in an incubator maintained at 37 °C in humidified atmosphere containing 5% CO₂. After 2 – 3 days of culture, cells were washed twice with phosphate-buffered saline (PBS) and maintained in serum- and l-threonine-free DMEM (WelGENE; Daegu, Korea), which contained all other supplements at the concentrations indicated above to synchronize ESCs. After a 24-h incubation period, the cells were washed twice with PBS and given fresh serum- and l-threonine-free media containing the designated agents for the time periods indicated. For the time course treatment, the cells were washed twice with PBS and given fresh serum- and l-threonine-free media, and then incubated for 24 h. After incubation, l-threonine was added to the medium in reverse order, and all samples were harvested at zero time point.

Alkaline Phosphatase Staining—Cells were washed twice with PBS and fixed with 4% formaldehyde for ~ 15 min at room temperature. After washing the cells with PBS, they were incubated with an alkaline phosphatase substrate solution (200 μg/ml naphthol AS-MX phosphate, 2% N,N-dimethylformamide, 0.1 M Tris, pH 8.2, and 1 mg/ml Fast Red TR salt) for 10 – 15 min at room temperature. After a wash with PBS, the cells were photographed.

Immunofluorescence Staining—Cells were fixed and labeled with rabbit anti-Oct4, or mouse anti-SSEA-1 (Santa Cruz Biotechnology) at a ratio of 1:50, followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM at 1:100. Alexa Fluor® 555 goat anti-rabbit IgG at 1:100, or labeled with FITC-conjugated mouse anti-BrdU each for 1 h at room temperature. Images were obtained using a Fluoview 300 fluorescence microscope (Olympus; Tokyo, Japan).

[³H]Thymidine Incorporation—The [³H]thymidine incorporation experiments were performed as previously described by Brett et al. (18). Briefly, mouse ESCs were synchronized in the G₀/G₁ phase by culture in serum- and l-threonine-free media for 24 h before stimulation with l-threonine. After the incubation period, 1 μCi of [methyl-³H]thymidine (specific activity: 74 GBq/mmol, 2.0 Ci/mmol; Amersham Biosciences; Buckinghamshire, UK) was added to the cultures for 1 hr at 37 °C. Cellular [³H]thymidine uptake was quantified by liquid scintillation counting of harvested cellular material. All values were,

Experimental Procedures

Materials—Mouse (m)ES cell lines ES-E14TG2a and ES-R1 were obtained from the American Type Culture Collection (Manassas, VA). In the present study, ES-E14TG2a cells were primarily used, and ES-R1 cells were used to examine whether the responses observed in ES-E14TG2a cells is depended on cell type. Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD). LY 294002, wortmannin, PD 98059, SB 203580, SP 600125, rapamycin, and monoclonal anti-β-actin were obtained from Sigma. Akt inhibitor (1L6-hydroxy-methyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-llycerocarbonate) was purchased from Calbiochem (La Jolla, CA). All pharmacological inhibitors used in this study did not affect cell proliferation and signal pathway activation at treated concentrations. However, combination treatment of inhibitors for PI3K/Akt, MAPKs, and mTOR decreased [³H]thymidine incorporation (supplemental Figs. S1 and S2). [³H]thymidine was obtained from Dupont/NEN (Boston, MA). Phospho-ERK,
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Western Blot Analysis—Cells were harvested, washed twice with PBS, and lysed in lysis buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mg/ml prolatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM sodium orthovanadate) for 30 min on ice. The lysates were cleared by centrifugation (30 min at 15,000 rpm, 4 °C), and the protein concentration was determined using the Bradford method (19). Equal amounts of protein (20 μg) were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were washed with TBST (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.01% Tween-20), blocked with TBST containing 5% skim milk for 1 h, and incubated with the appropriate primary antibodies at the dilutions recommended by the suppliers. The membranes were then washed and the primary antibodies detected with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. Immunoactive proteins were visualized by enhanced chemiluminescence (Amersham Biosciences; Buckinghamshire, UK).

Flow Cytometry for Proliferation Index—Cells were dissociated in trypsin/EDTA, pelleted by centrifugation, and resuspended at ~10^6 cells/ml in PBS containing 0.1% BSA. The cells were then fixed with 70% ice-cold ethanol for 30 min at 4 °C, followed by incubation in a freshly prepared nuclei staining buffer consisting of 250 μg/ml propidium iodide (PI) and 100 μg/ml RNase for 30 min at 37 °C. Cell cycle histograms were generated after analyzing the PI-stained cells by FACS (Beckman Coulter). The samples were analyzed using CXP software (Beckman Coulter) and the proliferation indices [(S + G2/M)/(G0/G1 + S + G2/M)] calculated.

Flow Cytometry for Oct4 Expression and BrdU Incorporation—BrdU-labeled cells were dissociated in trypsin/EDTA, pelleted by centrifugation, and resuspended at ~10^6 cells/ml in PBS containing 0.1% BSA. The cells were then fixed with 70% ice-cold ethanol for 30 min at 4 °C, followed by incubation with rabbit anti-Oct4 (Santa Cruz Biotechnology) at a ratio of 1:50, followed by Alexa Fluor® 555 goat anti-rabbit IgG at 1:100, or labeled with fluorescein isothiocyanate (FITC)-conjugated mouse anti-BrdU each for 1 h at room temperature.

MTT Cell Viability Assay—Cell viability was determined using the conversion of MTT to formazan via mitochondrial oxidation. mES cells were pretreated with indicated inhibitors prior to incubation with/without l-threonine for 24 h. MTT solution was then added to each well at a final concentration of 1 mg/ml per well and the plates were incubated at 37 °C for another 2 h. After incubation, 150 μl of DMSO was added to each well to dissolve the formazan formed and the absorbance was read at 570 nm using a spectrophotometer.

Statistical Analysis—Results are expressed as means ± S.E. All experiments were analyzed by ANOVA, and some experiments were examined by comparing the treatment means to the control using a Bonferroni-Dunn test. A p value of < 0.05 was considered significant.

RESULTS

Effect of l-Threonine on Transcriptional Regulation of Self-renewal—To determine whether mESCs maintain their pluripotency under long-term l-threonine depletion, we incubated the cells without l-threonine for various periods (0–4

converted from absolute counts to percentages of control and reported as means ± S.E. of triplicate experiments.

Cell Number Count—To determine total cell numbers, the cells were washed twice with PBS and trypsinized from the culture dishes. The cell suspension was mixed with a 0.4% (w/v) trypan blue solution, and the number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable.

RNA Isolation and Real-time Polymerase Chain Reaction (PCR)—Total RNA was extracted from cells treated with each of the designated agents using STAT-60, a monophasic solution of phenol and guanidine isothiocyanate (Tel-Test, Inc.; Friendswood, TX). Real-time quantification of RNA targets was performed in a Real-time thermal cycling system (Corbett Research; NSW, Australia) using QuantiTect SYBR Green RT-PCR Kits (Qiagen; Valencia, CA). The primers are comprised of the following 3 (sense) and 5 (antisense) pairs: 3'-GCUAUGGCAAGAUAUUCAUU and 5'-UGAAUAUUCUGCCAAUAGCUCU; 3'-GCACAUCUGGCGGUGUAUU and 5'-UACAAACCAGCCAGAGUGCUU; 3'-GCACAAUCGUGGACUGGCUAU and 5'-UGCCAGACGCAUUUCAGUGCUU; 3'-GCUCCGAAGAAGGUAUGGACUU and 5'-UGAAUAUCUUGCCAAUAGCUCU. The non-targeting siRNA was 3'-UAGGUAUAGCAUGGCUAUA-3'. After 24 h, transcription mixtures were replaced with serum- and l-threonine-free DMEM, and cells were maintained.

Immuno precipitation—The formation of mTORC1 and mTORC2 was analyzed by immunoprecipitation and Western blotting. Cells were lysed with lysis buffer (1% Triton X-100 in 50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 5 mM EDTA, 2 mM Na2VO4, 2.5 mM Na3PO4, 100 mM NaF, 200 mM microcystin lysine-arginine, and protease inhibitors). Cell lysates (300 μg) were mixed with 10 μg of mouse anti-mTOR antibody. The samples were incubated for 4 h, mixed with protein A/G PLUS-agarose immunoprecipitation reagent (Pierce) and then incubated for an additional 12 h. The beads were washed four times, and the bound proteins were released from the beads by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 5 min. Samples were analyzed by Western blotting with anti-phospho-mTOR, phospho-raptor, phospho-riotor, or mTOR antibodies.
days) and examined them for the expression of self-renewal and lineage specific differentiation markers. Depletion of L-threonine from the media decreased mRNA expression levels of Oct4, nanog, FOXD3, and Rex1, but increased Sox2 in a time-dependent manner. In addition, L-threonine depletion up-regulated trophoectoderm (Cdx2 and FGF4) and mesoderm (brachyury and MESP1) markers, but did not affect on endoderm and ectoderm markers expression, except Sox1 (Fig. 1A).

In agreement with mRNA expression results, we observed decreases in c-Myc and Oct4 protein expression levels (Fig. 1B). In addition, treatment with L-threonine (500 μM) recovered c-Myc, SSEA-1 (the stage-specific embryonic antigen-1 carbohydrate epitope), and Oct4 protein expression levels, which had been decreased by depletion of L-threonine for 1 day (Fig. 1C). L-Threonine-induced SSEA-1 expression was confirmed by immunofluorescence staining (Fig. 1D). To determine

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**FIGURE 1. Effect of L-threonine on transcriptional regulation of self-renewal of the mESC.** The mESCs were depleted of L-threonine for 4 days, and the total RNA was extracted as described under “Experimental Procedures.” The mRNA of the self-renewal marker genes (Oct4, Sox2, nanog, FOXD3, and Rex1), trophoectoderm marker genes (Cdx2, FGF4, and GATA3), endoderm marker genes (GATA4 and GATA6), mesoderm marker genes (brachyury and MESP1), and ectoderm marker genes (Sox1, NeuroD, and FGF5) were detected (A). Data are presented as means ± S.E. (standard error) of three different experiments, each from triplicate dishes. *, p < 0.05 versus control. Total cell lysates of mESCs (E14TG2a and R1) were subjected to depletion of L-threonine for 4 days and c-Myc and Oct4 expression were detected (B). The mESCs (E14TG2a and R1) were treated with 500 μM L-threonine after 24 h-incubation with L-threonine-free media for various times (0–48 h) and c-Myc, SSEA-1, and Oct4 expression was detected by Western blot analysis as described under “Experimental Procedures” (C). The lower parts depict the mean ± S.E. of three different experiments, each from triplicate dishes, *, p < 0.05 versus control. The cells were treated with L-threonine for 24 h, and then double labeled with SSEA-1 and PI (D). Scale bars represent 100 μm. The example shown is representative of four experiments. The mESCs were exposed to 500 μM L-threonine after 24-h incubation with L-threonine-free media for 24 h and double labeled with Oct4 and BrdU antibodies simultaneously (E). Scale bars represent 100 μm. The example shown is representative of four experiments. The mESCs were treated with 500 μM L-threonine for 24 h and dissociated in trypsin/EDTA. And then, double-labeled with Oct4 and BrdU antibodies and detected with flowcytometry (F). The percentage of cells described with the mean ± S.E. of three different experiments, each from triplicate dishes, *, p < 0.05 versus control. Alkaline phosphatase enzyme activity was assessed in mESCs treated with and without L-threonine (500 μM) for 24 h (G). Scale bars represent 100 μm. The example shown is representative of four experiments.
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Role of Lipid Raft/Caveolae on l-Threonine-induced ESC Proliferation—To examine whether the l-threonine effect operated through lipid raft of the plasma membrane, we incubated cells in l-threonine-depleted media with or without 0.01 μM methyl-β-cyclodextrin (MβCD) for 1 h or transfected them with cav-1 siRNA for 24 h, and then treat them with l-threonine (500 μM) for 24 h. Pretreatment with MβCD or cav-1 siRNA decreased the expression of l-threonine (500 μM)-induced cyclin D1, CDK4, cyclin E, and CDK2 protein expression levels (Fig. 3, A and B). In addition, disruption of lipid raft with MβCD and cav-1 knock-down decreased c-Myc and Oct4 protein expression levels (Fig. 3, C and D). Pretreatment with MβCD also decreased l-threonine-induced increases in [³H]thymidine incorporation (Fig. 3E). Consistent with these results, pretreatment with MβCD significantly decreased l-threonine-induced increases in the proliferation index and total cell numbers compared with the cells with l-threonine added (Fig. 3, F and G). These results suggest that the effects of l-threonine on mESC self-renewal are mediated by lipid raft/caveolae.

Involvement of PI3K/Akt and MAPKs Signaling—We treated cells incubated with threonine depletion media with 500 μM l-threonine for up to 120 min to detect Akt phosphorylation and examine whether the addition of l-threonine affects the PI3K/Akt signaling pathway to induce mESC proliferation. As shown in Fig. 4A, phosphorylation of Akt in phosphorylation sites Thr³⁰⁸ and Ser⁴⁷³ increased in a time-dependent manner and maximum phosphorylation was observed at 30–90 min after the addition of l-threonine. Pretreatment with MβCD and knock-down of cav-1 blocked l-threonine-induced Akt phosphorylation (Fig. 4, B and C). Furthermore, pretreatment with PI3K inhibitors (LY 294002, 10⁻⁶ M; or wortmannin, 10⁻⁷ M), or an Akt inhibitor (10⁻⁷ M) blocked the l-threonine-induced

whether threonine was involved in regulation of ESC self-renewal, we double-labeled cells for Oct4 expression and BrdU incorporation during threonine exposure. In these experiments, treatment with l-threonine maintained Oct4 expression and BrdU incorporation, which had decreased in threonine-depleted ESCs, reflecting the fact that threonine mediates G₁/S transition and maintenance of self-renewal in mESCs (Fig. 1, E and F). In addition, alkaline phosphatase stain levels were increased with the addition of l-threonine (Fig. 1G). Taken together, these results indicated that l-threonine affects the mESC self-renewal. Therefore, in the next series of experiments, we sought to identify the cell signaling pathways involved in regulation of mESCs with the addition of l-threonine.

Effect of l-Threonine on Cell Cycle Regulatory Protein Expression Levels and DNA Synthesis—To determine the effect of l-threonine on proliferation of mESCs, we incubated the cells with culture media without l-threonine and serum for 4 days. Depletion of l-threonine from the culture media decreased [³H]thymidine incorporation and expression of cyclin D1 and cyclin E in a time-dependent manner (Fig. 2, A and B). We next measured the effect of the addition of l-threonine on cell cycle regulatory protein expression levels and DNA synthesis. Addition of l-threonine after 24 h of deprivation increased expression of cyclins and [³H]thymidine incorporation in both time- and dose-dependent manners. Cyclin D1 and E expression levels were increased when incubated with 500 μM of l-threonine for 24 h (Fig. 2, C and D). Consistent with these results, we observed significant increases in [³H]thymidine incorporation after incubating cells with more than 100 μM l-threonine at 24 h (Fig. 2, E and F). These results suggest that l-threonine plays an important role in regulation of mESC G₁/S phase transition.
FIGURE 3. Relationship between lipid raft and L-threonine-induced mESC proliferation. Cells were preincubated with 0.01 μM MJCD for 1 h or transiently treated for 24 h with either a SMARTpool of cav-1 siRNA (200 pmol) or non-targeting control siRNA using Lipofectamine 2000 for 24 h prior to treatment with L-threonine (500 μM) for 24 h. After incubation, cyclin D1, cyclin E, c-Myc, and Oct4 expression were detected (A–D). The lower part depicts the mean ± S.E. of four different experiments, each from triplicate dishes. *, p < 0.05 versus control; **, p < 0.05 versus L-threonine alone. Cells (E14TG2a and R1) were pretreated with 0.01 μM MJCD for 1 h prior to being incubation with 500 μM L-threonine for 24 h. The mESCs were pulsed with [3H]thymidine for the last 1 h (E). Cells (E14TG2a and R1) were then washed with PBS, fixed, stained, and analyzed by flow cytometry (F). Gates were manually configured to determine the percentage of cells in S phase based on DNA content. The data were calculated using a proliferation index [(S/G2/M)/(G0/G1 + S + G2/M)] and reported as the mean ± S.E. of four different experiments, each conducted in triplicate. *, p < 0.05 versus control; **, p < 0.05 versus L-threonine alone. The mESCs were pretreated with MJCD prior to 24 h of incubation with L-threonine and the number of cells was counted using a hemocytometer (G). Values are means ± S.E. of three different experiments, each from triplicate dishes. *, p < 0.05 versus control. **, p < 0.05 versus L-threonine alone.

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recovery of cyclin D1, cyclin E, c-Myc, and Oct4 expression (Fig. 4, D and E). In accordance with these results, pretreatment with PI3K inhibitors and Akt inhibitor blocked L-threonine-induced increase in [3H]thymidine incorporation and total cell numbers (Fig. 4, F and G).

To examine the involvement of MAPKs signaling in L-threonine-induced mESC proliferation and maintenance of pluripotency, the cells were treated with L-threonine for various time points (0–120 min) and the phosphorylation levels of ERK, p38, and JNK/SAPK were measured. As shown in Fig. 5A, the maximum phosphorylation of ERK, p38, and JNK/SAPK were observed at 15 min, 60 min, and 15 min, respectively, after the addition of L-threonine. In addition, PI3K inhibitors (LY 294002 or wortmannin) or Akt inhibitor pretreatment blocked the L-threonine-induced phosphorylation of ERK, p38, and JNK/SAPK (Fig. 5B). These results suggest that ERK, p38, and JNK/SAPK act as downstream signal molecules of the L-threonine-induced activated PI3K/Akt signaling pathway. Moreover, pretreatment with PD 98059 (ERK inhibitor, 10−5 M), SB 203580 (p38 inhibitor, 10−6 M), or SP 600125 (JNK/SAPK inhibitor, 10−8 M) prior to the addition of 500 μM of L-threonine reduced the L-threonine-induced cyclin D1, cyclin E, c-Myc, and Oct4 expression (Fig. 5C and D). Furthermore, pretreatment with PD 98059, SB 203580, and SP 600125 blocked L-threonine-induced increase in [3H]thymidine incorporation and total cell numbers (Fig. 5E and F).

Relationship between mTORC1 and mTORC2 in Threonine-induced mESC Proliferation—We examined the phosphorylation of mTOR, raptor, 4E-BP1, p70S6K, and rictor to determine whether the mTORC1 and mTORC2 pathways participated in L-threonine induced mESC proliferation and maintenance of pluripotency. As shown in Fig. 6A, phosphorylation of mTOR, raptor, 4E-BP1, p70S6K, and rictor occurred in a time-dependent manner. To determine the relationship between L-threonine induced PI3K/Akt, MAPKs, and mTOR activation, we examined relationships between the pathways. Cells were pretreated with PI3K/Akt inhibitors or MAPKs inhibitors prior to the addition of 500 μM L-threonine, after which we looked for changes in mTOR, 4E-BP1, p70S6K, and rictor phosphorylation. Pretreatment with LY 294002, wortmannin, Akt inhibitor, PD 98059, SB 203580, or SP 600125 prior to 30 min L-threonine addition blocked L-threonine-induced phosphorylation of mTOR, 4E-BP1, and p70S6K (Fig. 6B, C and D). To examine whether L-threonine induced mTOR complex formation and activation, cells were pretreated with rapamycin for 10 min or 24 h prior to incubation with L-threonine, and phosphorylation of mTOR, raptor, and rictor analyzed by immunoprecipitation and Western blotting. As determined by immunoprecipitation experiments, raptor and rictor complexed with mTOR, and their phosphorylation levels were increased by threonine treatment. Furthermore, pretreatment with rapamycin for 24 h decreased the phosphorylation levels of mTOR, raptor, and rictor completely, while pretreatment with rapamycin for 10 min partially inhibited rictor phosphorylation (Fig. 6D). These results suggest that the treatment with threonine induced activation of mTORC1 and inactivation of mTORC2 through rictor phosphorylation. In order to examine mTORC1 and mTORC2 in response to L-threonine, immunofluorescence
staining of raptor and rictor was performed. Phospho-raptor, a critical component of mTORC1, localization was not affected by L-threonine treatment, but phospho-rictor, a critical component of mTORC2, was translocated from plasma membrane to cytosol/nuclear by treatment with L-threonine, which was blocked by rapamycin pretreatment (Fig. 6E). To examine the role of L-threonine-induced mTOR activity on maintenance of undifferentiated state and differentiation into specific lineage in mES cells, cells were pretreated with rapamycin prior to incubation in presence and absence of L-threonine. L-threonine

FIGURE 4. Involvement of PI3K/Akt activation on L-threonine-induced mESC proliferation. Cells were treated with 500 μM L-threonine after a 24 h of incubation with L-threonine-free media for different time periods (0–180 min). The phosphorylation of Akt Thr³⁰⁸ and Ser⁷²⁷ were detected as described under “Experimental Procedures” (A). Cells were transfected for 24 h with either a SMARTpool of cav-1 siRNA (200 pmol) or non-targeting control siRNA using Lipofectamine 2000 prior to treatment with L-threonine (500 μM) for 1 h (B). Cells were pretreated with 0.01 μM βCD for 1 h prior to the addition of L-threonine (500 μM) for 1 h (C). Cells (E14TG2a and R1) were pretreated with LY 294002 (PI3K inhibitor, 10⁻⁴ M), wortmannin (PI3K inhibitor, 10⁻⁵ M), or Akt inhibitor (10⁻⁵ M) for 30 min prior to being treated with L-threonine (500 μM) for 24 h. Total protein was extracted and blotted with cyclin D1, cyclin E, c-Myc, Oct4, or β-actin antibodies (D and E). The lower part depicted by bars denotes the mean ± S.E. of three different experiments for each condition determined from densitometry relative to β-actin. *, p < 0.05 versus control. **, p < 0.05 versus L-threonine alone. Cells (E14TG2a and R1) were pretreated with LY 294002, wortmannin, or Akt inhibitor for 30 min prior to being treated with L-threonine (500 μM) for 24 h. Cell were subsequently pulsed with 1 μCi of [³H]thymidine for 1 h (F). Data represent the mean ± S.E. of four independent experiments with triplicate dishes. *, p < 0.05 versus control. **, p < 0.05 versus L-threonine alone. The mESCs were pretreated with LY 294002, wortmannin, or Akt inhibitor prior to 24 h incubation with L-threonine and the number of cells was counted using a hemocytometer (G). Values are means ± S.E. of three different experiments, each from triplicate dishes. *, p < 0.05 versus control. **, p < 0.05 versus L-threonine alone.
altered undifferentiation markers (increase in Oct4 and nanog, decrease in Sox2), and decreased trophoectoderm and mesoderm markers, which was blocked by rapamycin treatment. In addition, there had no significant changes in markers expression in rapamycin alone compared with control (Fig. 6F). Furthermore, inhibition of mTOR signaling using rapamycin diminished l-threonine induced c-Myc, Oct4, cyclin D1, and cyclin E expression as well as [3H]thymidine incorporation (Fig.

FIGURE 5. Involvement of MAPKs activation on L-threonine-induced mESC proliferation. Cells were incubated with 500 μM L-threonine after 24 h of depletion of threonine for different time periods (0–180 min). Phosphorylation of ERK, p38, and JNK was detected as described under “Experimental Procedures” (A). The cells were pretreated with LY 294002, wortmannin, or Akt inhibitor for 30 min prior to being treated with L-threonine (500 μM) for 1 h, and then phosphorylation of ERK, p38, and JNK was detected (B). Cells (E14TG2a and R1) were pretreated with PD 98059 (ERK inhibitor, 10−8 M), SB 203580 (p38 inhibitor, 10−6 M), or SP 600125 (JNK inhibitor, 10−6 M) for 30 min prior to being treated with L-threonine (500 μM) for 24 h. Total protein was extracted and blotted with cyclin D1, cyclin E, c-Myc, Oct4, or β-actin antibodies (C and D). Each example shown is representative of three independent experiments. The lower part depicted by bars denotes the mean ± S.E. of four independent experiments with triplicate dishes. *, p < 0.05 versus control. **, p < 0.05 versus L-threonine alone. The mESCs were pretreated with PD 98059, SB 203580, or SP 600125 prior to 24 h incubation with or without 500 μM L-threonine. The number of cells was counted using a hemocytometer (E). Values are means ± S.E. of three different experiments, each from triplicate dishes. *, p < 0.05 versus control. **, p < 0.05 versus L-threonine alone.
To confirm the involvement of mTOR signaling on L-threonine-induced mESC proliferation, cells were pretreated with rapamycin prior to 24 h of incubation after the addition of L-threonine. And then, fluorescence-activated cell sorter (FACS) analysis and cell number count were performed. Pretreatment with rapamycin significantly blocked the L-threonine-induced increase in the proliferation index and total cell numbers (Fig. 6, J and K). Taken together, these results suggest that L-threonine-induced activation of mTOR signaling plays an important role in L-threonine-induced G1/S phase transition of mESC.

DISCUSSION

This study demonstrated that L-threonine stimulated ESC proliferation through lipid raft/caveolae-dependent PI3K/Akt, MAPKs, mTOR, p70S6K, and 4E-BP1 signaling pathways (Fig. 7). Recently, it was reported that specific amino acids could affect mESC properties. Deprivation of threonine deteriorates
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ESC colony growth and the self-renewal (9). In addition, l-proline can induce primitive ectoderm formation in embryos and embryoid bodies (8). These opposing results suggest that individual amino acids can differently regulate ESC proliferation and pluripotency according to the type of cell line and specific amino acid. In the present study, despite a relatively low plasma l-threonine concentration (77 μM), we chose to use 500 μM l-threonine, because it is close to the concentration in conventional mESC cells (798 μM) (20) which is known to exert obvious effects on mESC proliferation. l-threonine depletion significantly decreased ESC proliferation, suggesting that l-threonine, normally contained in culture media in vitro and in plasma in vivo, is closely associated with ESC proliferation. The addition of l-threonine restores and increases DNA synthesis and expression of cyclin D1 and cyclin E, which are rate-limiting activators of G1-to-S phase transition. Among the 20 amino acids, deprivation of l-threonine significantly decreased the number of normal ESC colonies and [3H]thymidine incorporation (9). In addition, l-threonine deprivation altered self-renewal markers and differentiation markers, suggesting that l-threonine plays an important role in maintenance of mESC. Indeed, these results are consistent with a previous report in which incubation of ESCs with culture media lacking threonine decreased alkaline phosphatase activity (9). Amino acid transporters are largely associated with cholesterol-rich lipid raft microdomains of the plasma membrane. This association with lipid rafts is important for transporter trafficking and function (21). Moreover, these have an important role in maintaining self-renewal of mESCs (10, 22). In this context, we investigated the involvement of lipid raft on threonine effects in mESC. In the present study, depletion of membrane cholesterol by MβCD or lipid raft/caveolae disruption using or cav-1 siRNA significantly inhibited l-threonine-induced ESCs proliferation. Although, in this study, we did not identify the specific threonine transporters, our results suggest that lipid raft/caveolae is involved in l-threonine-induced regulation of ESCs proliferation.

The mTOR is known as an evolutionarily conserved nutrient sensor that directs the cellular response to nutrient status, especially with regard to the availability of amino acids (23). The mTOR complex 1 is activated through a canonical signaling cascade triggered by the activation of class I PI3K/Akt (23) and resupplying amino acids can stimulate p70S6 kinase a downstream molecule of mTOR signaling (24–26). Indeed, these results are supported by previous studies that cellular amino acid deprivation reduces insulin-mediated phosphorylation of mTOR Ser2448, which is mediated by Akt (27). In addition, activation of the PI3K/Akt pathway is crucial for...
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inducing cyclin D1 (28). Activation of the PI3K/Akt pathway frequently elicits the activation of other intracellular signaling cascades such as MAPKs (29). In addition, mTOR signaling pathway and downstream molecules such as p70S6K and 4E-BP1 can be stimulated by PI3K/Akt and MAPKs pathways (30, 31). Translational control mediated by PI3K/Akt/mTOR is involved in regulation of mESC proliferation and lineage-restricted differentiation (32). Therefore, we examined the effects of t-threonine on the mTOR pathway through PI3K/Akt, ERK, p38, and JNK-dependent pathways. In the present study, we found that t-threonine stimulated MAPKs activation, which act as downstream molecules of threonine-induced PI3K/Akt activation in mESC. In addition, inhibition of MAPKs with pharmacologic antagonists reduced t-threonine-induced mESC proliferation.

Amino acids stimulate the phosphorylation of both raptor and rictor, critical components of mTORC1 and mTORC2, respectively (33, 34). Moreover, rictor is directly phosphorylated by S6K1 downstream of mTORC1 and this phosphorylation event exerts a negative regulatory effect on the mTORC2-dependent activation of Akt (Ser^{473}), due to the phosphorylation of the rictor subsequently binding with 14-3-3 and dampening mTORC2 ability to phosphorylate Akt (34). We found that threonine regulates ESC proliferation through fine-tuned regulation of mTORC1 and mTORC2 activation. The phosphorylation of raptor (Ser^{792}) and rictor (Thr^{1135}) was increased by treatment with threonine. In addition, pretreatment with rapamycin decreased the phosphorylation level of raptor or rictor assembled with mTOR. These effects of threonine and rapamycin on mTOR, raptor, or rictor phosphorylation are the same in total lysates, suggesting that the threonine-induced activation of the mTOR pathway works through regulation of phosphorylation levels of complex components rather than complex integrity. Furthermore, we observed that threonine is involved in the regulation of mTORC2 translocation. Threonine and rapamycin treatment did not affect localization of raptor, but pretreatment with rapamycin altered localization of rictor from membrane to cytosol/nuclear. It has been reported that long-term treatment with rapamycin triggers dephosphorylation of mTORC2 and cytoplasmic accumulation of rictor (35). Rictor phosphorylation is an mTORC1-dependent process and inhibition of rictor phosphorylation is not a secondary effect of disrupting the mTORC2 complex (34). Inhibition of mTOR activity using rapamycin blocked the t-threonine-induced maintenance of undifferentiation markers expression. In addition, addition of t-threonine inhibited the differentiation into trophoectoderm and mesoderm of mESC cultured with t-threonine depletion media, which were blocked by rapamycin. In consistent with these results, it has been reported that the increase in levels of Sox2 were led to rapid differentiation of ESC into neuroectoderm, mesoderm, and trophoectoderm, but did not alter the endoderm markers, which suggested that the Oct4 and Sox2 function as molecular rheostats in the control of the self-renewal and pluripotency of ESC (36). Moreover, Zhou et al. (37) reported that the mTOR activity is necessary to support long-term self-renewal and to suppress mesoderm and endoderm activities in hESC. These results suggest that t-threonine-induced mTOR activity play an important role on supporting self-renewal and suppression trophoectoderm and mesoderm activities in mESC. Furthermore, inhibition of mTOR pathways using pharmacological antagonists blocked t-threonine-induced mESC proliferation, suggesting that t-threonine-induced mTOR activation is critical to the regulation of mESC proliferation. Furthermore, t-threonine stimulated mTOR, which resulted in activation of p70S6K and 4E-BP1. Indeed in ESCs, t-proline induced phosphorylation of both 4E-BP1 and p70S6K, molecules implicated in the initiation of mRNA translation after mTOR activation (8). This activation of mTOR and its downstream molecules was blocked by PI3K inhibitors, Akt inhibitor, or MAPKs inhibitors. Taken together, our observations may support the possible role of threonine as a physiological regulator of the G_{1}-to-S phase transition of ES cells, which will provide valuable tools for modulating ES function and cell fate choice via the addition of small, nontoxic organic molecules. However, although the signal molecules suggested for amino acid regulation of ESC growth provide a rational framework for the available data, many aspects remain entirely speculative, and much additional work is necessary to identify the components and evaluate the relationships proposed. In conclusion, addition of t-threonine following a period of t-threonine deprivation stimulated ESC proliferation through lipid raft/caveolae-dependent PI3K/Akt, MAPKs, and mTORC signaling pathways.

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Threonine Regulates G_{1}/S Phase Transition of mESC

I-Threonine Regulates G₁/S Phase Transition of Mouse Embryonic Stem Cells via PI3K/Akt, MAPKs, and mTORC Pathways
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