Caveolin-1 plays important role in EGF-induced migration and proliferation of mouse embryonic stem cells: involvement of PI3K/Akt and ERK

Jae Hong Park and Ho Jae Han

Department of Veterinary Physiology, Biotherapy Human Resources Center (BK21), College of Veterinary Medicine, Chonnam National University, Gwangju, Korea

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Park JH, Han HJ. Caveolin-1 plays important role in EGF-induced migration and proliferation of mouse embryonic stem cells: involvement of PI3K/Akt and ERK. Am J Physiol Cell Physiol 297: C935–C944, 2009. First published July 22, 2009; doi:10.1152/ajpcell.00121.2009.—The involvement of caveolin-1 in the regulation of embryonic stem (ES) cell growth by epidermal growth factor (EGF) is by no means clear cut. Thus we examined the relationship between EGF and caveolin-1 in mouse ES cell migration and proliferation. The results revealed that EGF increased Src, caveolin-1, focal adhesion kinase (FAK), Akt, and extracellular signal-regulated kinase-1/2 (ERK) phosphorylation levels. Especially, phosphorylation of caveolin-1 is attenuated by AG1478, herbinycin A (tyrosine kinase inhibitors), and pyrazolopyrimidine 2 (PP2, Src inhibitor) and EGF-induced ERK activation was blocked by PP2, methyl-β-cyclodextrin (MβCD), caveolin-1 small interfering RNA (siRNA), LY-294002 [phosphoinositol-3 kinase inhibitor (PI3K)], and Akt inhibitor. In addition, EGF promoted the cell migration, which was attenuated by PP2, caveolin-1 siRNA, FAK siRNA, LY-294002, Akt inhibitor, and PD-98059. EGF also increased matrix metalloproteinase (MMP-2) expression levels and EGF-induced MMP2 expression was inhibited by caveolin-1 siRNA, FAK siRNA, LY-294002, Akt inhibitor, and PD-98059. Furthermore, EGF-induced increase of cell cycle proteins expression level and [3H]thymidine incorporation was blocked by MMP inhibitor. EGF also significantly increases [3H]thymidine incorporation and cell number, which were significantly blocked by AG 1478, PP2, MβCD, caveolin-1 siRNA, FAK siRNA, LY-294002, and PD-98059 (ERK inhibitor). EGF-induced increase of protooncogenes (c-fos, c-myc, and c-Jun) and cell cycle regulatory proteins (cyclin D1, CDK4, cyclin E, and CDK2) expression levels were also attenuated by caveolin-1 siRNA and FAK siRNA. In conclusion, these results demonstrated that EGF-induced DNA synthesis and cell migration are mediated by caveolin-1, which is activated by Src, FAK, PI3K/Akt, ERK, and MMP-2 signals in mouse ES cells.

mouse embryonic stem cells; epidermal growth factor; caveolin-1; phosphoinositol-3-kinase/Akt; mitogen-activated protein kinases; cell proliferation

THE ROLE OF LIPID RAFT and caveolin in signaling by several growth factor receptors has been studied but none more so than the epidermal growth factor receptor (EGFR). We will therefore be paying particular attention to this receptor. The EGFR signaling pathway is an important mediator of cell proliferation, maintenance, and survival (6, 18, 34). Caveolin-1 is not anymore the only protein known to be required for caveola formation, and functions for caveolin-1 outside of caveolae are being unveiled. As more in-depth knowledge about caveolin functions is generated, new questions arise that will need to be answered. Under normal conditions, caveolin-1 has been shown to be phosphorylated in response to EGF (17, 19, 23). However, caveolin-1 phosphorylation seems to be cell type- and stimulus-specific because it was not observed in adrenal cortex endothelial cells after VEGF stimulation (7), even though this phosphorylation was similary demonstrated in NIH 3T3 cells (32). Activation of the pathway in caveolin-1-positive cells resulted in activation of the phosphoinositol-3 kinase (PI3K)/Akt pathway. This led to cell cycle progression G1 and entry into the S phase. The extracellular signal-regulated kinase-1/2 (ERK1/2) pathway is another example of caveolin-1-dependent regulation of signaling and is associated with the promotion of cell survival (21, 27). Caveolin-1 was also recently implicated in the regulation of cell migration (10, 11, 15). From these reports, we hypothesize that caveolin-1 phosphorylation in embryonic stem (ES) cells may affect divergent actions on cell migration, proliferation, and survival. However, the precise role of caveolae in EGF receptor-mediated signal transduction is still unclear, and the physiological significance of EGF-mediated caveolin-1 phosphorylation in ES cells has not been determined.

ES cells hold a great deal of promise for the modeling of early development and also may evidence therapeutic potential. These cells are defined as cells with self-renewal capacity and the ability to generate multiple differentiated cell types (29, 36). Recently, approaches in the field of stem cell biology have emphasized the potential of signaling molecule phenotypic screens, not only for the identification of small molecules that can alter stem cell fate and define the relevant corresponding molecular pathways, but also in terms of their general biological implications in the field of stem cell research. In this study, we investigated the role of caveolin-1 phosphorylation on EGF signaling in ES cell migration and proliferation and its related signal pathways. Therefore, our results will provide the novel insights for the study of ES cell self-renewal mechanisms.

MATERIALS AND METHODS

Materials. Mouse ES cells were obtained from the American Type Culture Collection (ES-E14T2a, www.atcc.org). Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD). EGF, PD-98059, and methyl-β-cyclodextrin (MβCD) were purchased from Sigma (St. Louis, MO). Pyrazolopyrimidine 2 (PP2), matrix metalloproteinase (MMP) inhibitor, and the Akt inhibitor were purchased from Calbiochem-Novabiochem (La Jolla, CA). LY-294002 was purchased from Biomol (Butler Pike, PA), and [3H]thymidine was purchased from NEN (Boston, MA). Antibodies against phospho-caveolin-1, caveolin-1, cyclin D1, cyclin E, cyclin-dependent kinase (CDK)2, CDK4, phospho-Akt (Thr308, Ser473), Akt, phospho-EGFR, EGFR, E-cadherin, fibronectin, and β-actin were all purchased from Santa Cruz Biotechnology (Delaware, CA). The phospho-Src, phospho-ERK, phospho-Fak, integrinβ1, and total ERK antibodies were purchased from NEN (Boston, MA). Antibodies against phospho-caveolin-1, caveolin-1, cyclin D1, cyclin E, cyclin-dependent kinase (CDK)2, CDK4, phospho-Akt (Thr308, Ser473), Akt, phospho-EGFR, EGFR, E-cadherin, fibronectin, and β-actin were all purchased from Santa Cruz Biotechnology (Delaware, CA). The phospho-Src, phospho-ERK, phospho-Fak, integrinβ1, and total ERK antibodies were purchased from NEN (Boston, MA).

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supplied by Cell Signaling Technology (Herts, UK). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG were purchased from Jackson Immunoresearch (West Grove, PA). Liquiscint was obtained from National Diagnostics (Parsippany, NJ). All other reagents were of the highest commercially available purity.

Mouse ES cells were maintained in standard medium [Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO-BRL; Gaithersburg, MD) supplemented with 3.7 g/l of sodium bicarbonate, 1% penicillin-streptomycin, 1.7 mM L-glutamine, 0.1 mM β-mercaptoethanol, 5 ng/ml mouse LIF, and 15% fetal bovine serum]. Cells were cultured without a feeder layer for 5 days on either gelatinized 12-well plates or 60-mm culture dishes in an incubator maintained at 37°C with 5% CO2. Media were changed to serum-free standard media for 24 h before experimentation.

Western blot analysis and immunoprecipitation. Proteins (20 μg) from cell homogenates were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Each membrane was washed with Tris-buffered saline Tween-20 [TBST: 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween-20], blocked with 5% skim milk for 1 h, and incubated with appropriate primary antibodies at dilutions recommended by the supplier. Membranes were washed in TBST and probed with an appropriate HRP-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse IgG). For immunoprecipitation, lysates were incubated with appropriate antibodies and protein A-Sepharose beads with gentle shaking overnight. Samples were washed three times with lysis buffer and analyzed by SDS-PAGE. Bands were visualized with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech; Buckinghamshire, UK).

Isolation of caveolin-rich membrane fraction. Caveolin-enriched membrane fractions were prepared as described previously (30). Four confluent 100-mm dishes were washed twice with ice-cold PBS and scraped into 2 ml 500 mM sodium carbonate (pH 11.0), transferred to a plastic tube, and homogenized with a sonicator (three 20-s bursts). The homogenate was then adjusted to 45% sucrose by the addition of 2 ml 90% sucrose prepared in MES-buffered solution [MBS: 25 mM MES (pH 6.5), 0.15 M NaCl] and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed above (4 ml 5% sucrose, 4 ml 35% sucrose, both in MBS

Table 1. Primers used for PCR

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ES cell culture. Mouse ES cells were maintained in standard medium [Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO-BRL; Gaithersburg, MD) supplemented with 3.7 g/l of sodium bicarbonate, 1% penicillin-streptomycin, 1.7 mM L-glutamine, 0.1 mM β-mercaptoethanol, 5 ng/ml mouse LIF, and 15% fetal bovine serum]. Cells were cultured without a feeder layer for 5 days on either gelatinized 12-well plates or 60-mm culture dishes in an incubator maintained at 37°C with 5% CO2. Media were changed to serum-free standard media for 24 h before experimentation.

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Supp Fig. 1. Characteristics of undifferentiated mouse embryonic stem (ES) cells. EGF did not influence Oct4, SOX2, and FOXD3 mRNA levels (A) and Oct4 and Nanog protein expression levels (B). Bands correspond to Oct4 (50 – 60 kDa), Nanog (35 kDa), and β-actin (41 kDa). The mRNA levels were determined by real-time RT-PCR, and protein levels were determined by Western blot analysis. Examples represented 5 independent experiments.
containing 250 mM sodium carbonate) and centrifuged at 40,000 rpm for 20 h in an SW 41 rotor (Beckman Instruments, Fullerton, CA). Twelve 1-ml fractions were collected from the top and analyzed by SDS-PAGE.

**Immunofluorescence.** The cells were fixed and permeabilized in 0.1% Triton X-100 and washed. To decrease nonspecific binding of the antibody, the cells were preincubated with 1% bovine serum albumin (BSA) (Sigma) in PBS for 20 min. The cells were then incubated for 60 min with primary antibody in a solution containing 1% BSA in PBS. After three washes with PBS and incubation with 1% BSA for 5 min, the cells were incubated for 60 min with FITC-conjugated secondary antibody in PBS with 1% BSA. The fluorescent images were visualized with a Fluoview 300 fluorescence microscope (Olympus, Tokyo, Japan).

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**Fig. 2.** Effects of EGF on caveolin-1 phosphorylation. (A, C) Mouse ES cells were treated with EGF for various time periods (0–120 min) under serum-free conditions. Src, caveolin-1, and focal adhesion kinase (FAK) phosphorylation were detected by Western blot analysis. B: cells were treated with AG-1478 (EGFR-specific inhibitor, 10^{-5} M) or herbimycin A (tyrosine kinase inhibitor, 10^{-6} M) for 30 min before EGF treatment (100 ng/ml) for 15 min. Phosphorylated Src was detected by Western blot analysis. D: cells were pretreated with AG-1478 or herbimycin A for 30 min before EGF treatment for 15 min. (E, F) Cells were pretreated with pyrazolopyrimidine-2 (PP2, Src inhibitor, 10^{-6} M) for 30 min or MβCD for 1 h before EGF treatment. Phosphorylated caveolin-1 and FAK levels were detected by Western blot analysis. G: caveolin-enriched membrane fraction was immunoprecipitated with an anti-caveolin-1 antibody and blotted with an antibody directed against phospho-EGFR, Src, and FAK, respectively. The example blot was representative of 4 independent experiments. The values represented the mean values of 4 independent experiments conducted in triplicate. *P < 0.05 vs. control. **P < 0.05 vs. EGF alone.
Determination of [3H]thymidine incorporation and cell numbers. The [3H]thymidine incorporation experiments were performed as previously described (2). Briefly, ES cells were arrested in the G0/G1 phase by serum deprivation for 24 h (37). Serum-free standard medium supplemented with EGF were added to cells. Cells were pulsed with 1 μCi [methyl-3H]thymidine for 1 h at 37°C after 24 h incubation, 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 materials were dissolved in 2 N NaOH at 23°C for 12 h, and the levels of radioactivity were measured with a liquid scintillation counter (LS 6500, Beckman Instruments). Absolute counts were converted to a percentage of control (untreated) counts to facilitate comparisons between conditions. All results were presented as means ± SE values of triplicate experiments. On the other hand, trypsinized cell suspensions were mixed with 0.4% (wt/vol) trypan blue solution, and live cell counts were determined using a hemocytometer. Cells with dye uptake were considered nonviable.

RNA isolation and RT-PCR. Total RNA was extracted from mouse ES cells using STAT-60 (a monophasic solution of phenol and guanidine isothiocyanate, Tel-Test, Friendwood, TX, http://www.bioresearchonline.com). Reverse transcription was conducted with a reverse transcription system kit (AccuPower RT PreMix; Bioneer; Daejeon, Korea, http://www.bioneer.com), extracted RNA (3 μl), and oligo(dT)18 primers. RT products (5 μl) were amplified using a polymerase chain reaction (PCR) kit (AccuPower PCR PreMix, Bioneer) under the following conditions: denaturation at 94°C for 5 min, 30 amplification cycles (94°C for 45 s, 55°C for 30 s, and 72°C for 30 s), and extension for 5 min at 72°C. Amplification of the Oct4, FOXD3, SOX2, c-fos, c-jun, and c-myc cDNAs were performed in mouse ES cells using the primers described in Table 1, and β-actin PCR was performed as a control for RNA quantity.

siRNA preparation and treatment. Mouse ES cells were grown to 50% confluence and transfected for 24 h with an small interfering RNA (siRNA) construct targeting caveolin-1 [3′-GCUAUUGGCA-
GAUAUUCAUU (sense) and 5'-UGAUAAUUCUGCAAGCUU (antisense); 3'-GCACACUGGCUUGUAGCUU (sense) and 5'-GAUCAGUACCUUGGCUU (antisense); 3'-GUCCAUAAUCCCAGUGCUU (sense) and 5'-GAUCACAGAAGAGCUU (antisense); 3'-GUCCAUCUUCAGCUGUU (sense) and 5'-UUCCAAACAACCACUUC (antisense); 3'-GGGCAUCAUUCAGAAGAUA (sense) and 5'-UCGGAGUCCACGUAUUUGCUU (antisense); 3'-GUCCAUAAUCCCAGUGCUU (sense) and 5'-GAUCAGUACCUUGGCUU (antisense); 3'-UCGGAGUCCACGUAUUUGCUU (antisense); 3'-GUCCAUAAUCCCAGUGCUU (sense) and 5'-GAUCAGUACCUUGGCUU (antisense); 3'-GUCCAUAAUCCCAGUGCUU (sense) and 5'-GAUCAGUACCUUGGCUU (antisense). (ONTARGETplus SMARTpool; 200 pmol/l, catalog no. L-058415-00; Dharmacon; Lafayette, CO), focal adhesion kinase (FAK) [3'-GAAGUUGGUGGUUUUGAA (sense) and 5'-UUCCAAACAACCACUUC (antisense); 3'-GGGCAUCAUUCAGAAGAUA (sense) and 5'-UAUCUCGUGUGCCAGAGCUU (antisense); 3'-GUCCACUGAAGACCCAGAGCUU (sense) and 5'-UGAUAAUUCUGCAAGCUU (antisense); 3'-GUCCAUAAUCCCAGUGCUU (sense) and 5'-GAUCAGUACCUUGGCUU (antisense)]. (ONTARGETplus SMARTpool; 200 pmol/l, catalog no. L-058415-00; Dharmacon) using LipofectAMINE 2000 (Invitrogen; Carlsbad, CA) according to the manufacturer’s instructions.

Cell migration assays. The Oris Cell Migration and Calcein AM were from Platypus Technologies (Madison, WI) and Invitrogen. Mouse ES cells were seeded at 100 μl per well and incubated for 12 h to permit cell adhesion. After cells had reached 70% confluence in the
dishes, inserts were carefully removed and wells were gently washed with culture media. The cells were then incubated with EGF and fresh media. Migrations were observed microscopically for varying incubation times. Cell populations in end-point assays were stained with 5/1000000 Calcein AM for 30 min. Migrated cells were quantified through measurement of fluorescence signals using the microplate reader at 485 nm excitation and 515 nm emission.

Fluorescence-activated cell sorter analysis. Mouse ES cells were serum-starved for 24 h before EGF stimulation, as previously described. Cells were dissociated in trypsin/EDTA and pelleted by centrifugation. Cells were resuspended at 10^6 cells/ml in PBS containing 0.1% BSA. Cells were fixed in 70% ice-cold ethanol and incubated with freshly prepared nuclei staining buffer (250 μg/ml propidium iodide and 100 μg/ml RNase) for 30 min at 37°C. Cell cycle histograms were generated by analyzing propidium iodide-stained cells using fluorescence-activated cell sorter (FACS, Beckman Coulter). At least 10^4 events were recorded for each sample. Samples were analyzed using CXP software (Beckman Coulter).

Statistical analysis. All results were expressed as means ± SE values. All experiments were analyzed by ANOVA. Several experiments were also evaluated by comparing the treatment means to the control using a Bonferroni-Dunn test. Statistical significance was defined at P values <0.05.

RESULTS

Effect of EGF on caveolin-1 phosphorylation. We examined the markers of pluripotent ES cells to confirm the undifferentiated state of mouse ES cells used in the present experiments. Oct4, SOX-2, and FOXD3 mRNA levels (Fig. 1A) and Oct4 and Nanog protein expression levels (Fig. 1B) were not influenced by EGF treatment. These results suggested that mouse ES cells maintained pluripotent state under the experimental conditions in this study. In experiments to determine the structural importance of caveolae in caveolin-1-dependent sig-

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

![Graph F](image6)

Fig. 5. Effects of MMP inhibitors on EGF-induced stimulation of cell proliferation. A: MMP-1, -2, -3, and -9 and β-actin mRNA expression levels were determined by real-time RT-PCR using RNA from cells cultured with or without EGF for 24 h. MMP-2 and -9 protein expression levels were detected in ES cell treated with EGF in various time points. B: cells were transfected for 24 h with caveolin-1/FAK siRNAs or control siRNAs before 24 h EGF treatment. Total protein was extracted and blotted with anti-MMP-2 or MMP-9 antibodies. C: cells were pretreated with LY-294002, Akt inhibitor, or PD-98059 for 30 min before 24 h EGF treatment. MMP-2 expression levels were detected by Western blot analysis. D: mouse ES cells were pretreated with MMP inhibitors for 30 min before 12 h EGF treatment. Total protein was extracted and blotted with the cyclin D1, cyclin E, CDK 2, and CDK 4 antibodies. E: cells were treated with EGF and MMP inhibitors or were pretreated with MMP inhibitors and EGF treatment for 24 h. Cells were pulsed with [3H]thymidine (1 μCi) for 1 h. F: cell cycles were analyzed by flow cytometry. Gates were manually configured to determine the percentage of cells in G1, S, and G2 phases based on DNA content. Values represented means ± SE values of 5 independent experiments. *P < 0.05 vs. control. **P < 0.05 vs. EGF alone.
naling, EGFR was almost exclusively found in caveolae, and the EGFR protein levels in caveolae were increased at 12 h after EGF treatment (Fig. 1C, fraction 5). Furthermore, caveolin-enriched membrane fraction was decreased after treatment with methyl-β-cyclodextrin (MβCD; lipid raft disruptor, 10^{-4} M) for 30 min (Fig. 1D). Indeed, the immunostaining of caveolin-1 reveals that caveolin-1 was significantly reduced from the membrane after treatment of MβCD (Fig. 1E).

EGF treatment was sufficient to increase Src phosphorylation and maximum Src values were achieved 2 min after EGF treatment (Fig. 2A), which was ameliorated by inhibition of EGF receptor with AG-1478 and herbimycin A (tyrosine kinase inhibitor) (Fig. 2B). Similarly, caveolin-1 and FAK phosphorylation reached maximum levels at 15 min after EGF treatment (Fig. 2C), which was blocked by EGFR inhibition (Fig. 2D). In addition, EGF-induced caveolin-1 and FAK phosphorylation were attenuated by MβCD (Fig. 2E) or PP2 (Src inhibitor) (Fig. 2F). These results suggest that EGF-induced activation of Src and structural function of caveolae are required for caveolin-1 and FAK phosphorylation. Furthermore, we found that phosphorylated EGFR, Src, and FAK coimmunoprecipitated with Cav-1 in the presence EGF (Fig. 2G).

Relationship between caveolin and Akt or ERK phosphorylation. EGF treatment enhanced Akt phosphorylation on Thr308 and Ser473 and ERK phosphorylation (Fig. 3, A and 6). Effects of caveolin-1 siRNA on EGF-induced cell proliferation. Mouse ES cells were pretreated with AG-1478, PP2, or MβCD (lipid-raft disruptor, 10^{-4} M) for 30 min before 24 h EGF treatment. [3H]thymidine incorporation (A) and cell number counting (B) were measured. C: cells were transfected for 24 h with caveolin-1/FAK siRNAs or control siRNAs before 1 h EGF treatment. Gene expression levels of c-fos, c-jun, and c-myc were analyzed by RT-PCR. Amplified gene products were produced for c-fos (356 bp), c-jun (355 bp), c-myc (266 bp), and β-actin (350 bp). Each example represented 4 independent experiments. D: cells were transfected for 24 h with caveolin-1/FAK siRNAs or control siRNAs before 12 h EGF treatment. Expression levels of the cell cycle regulator proteins were detected by Western blot analysis. Each blot was representative of 4 independent experiments. E: cells were transfected for 24 h with caveolin-1/FAK siRNAs or control siRNAs before 24 h EGF treatment. Cells were pulsed with [3H]thymidine (1 μCi) for 1 h. F: cells were counted using trypan blue staining. The values represented the mean values of 4 independent experiments conducted with triplicate dishes. G: cells were washed with phosphate-buffered saline, fixed, stained, and analyzed by flow cytometry. Gates were manually configured to determine the percentage of cells in the G1, S, and G2 phases based on DNA content. H: cells were pretreated with LY-294002, Akt inhibitor, or PD-98059 for 30 min before 24 h EGF treatment. [3H]thymidine incorporation was then measured. The values represented the mean values of 4 independent experiments conducted with triplicate dishes. *P < 0.05 vs. control. **P < 0.05 vs. EGF alone.
Involvement of caveolin-1 in EGF-induced ES cell migration and MMP-2 expression. Cell migration was significantly stimulated in response to EGF in a time-dependent manner, which was decreased by PP2, LY-294002, and PD-98059 (ERK inhibitor) (Fig. 4, A–C). In addition, caveolin-1, FAK siRNA, and MMP inhibitor significantly blocked EGF-induced increases in cell migration (Fig. 4, D–G). Next, in experiments to examine involvement of integrin-β1 and focal adhesion complexes, we observed that EGF enhanced integrin-β1 and fibronectin protein expression, whereas decreased E-cadherin expression (Fig. 4H). Furthermore, EGF increased MMP-2 mRNA and protein expression levels (Fig. 5A), which was significantly attenuated by caveolin-1 and FAK siRNA. However, EGF-induced MMP-9 expression was not attenuated by caveolin-1 siRNA or FAK siRNA (Fig. 5B). On the other hand, LY-294002, Akt inhibitor, or PD-98059 decreased EGF-induced MMP-2 expression (Fig. 5C). In further experiment, we observed that MMP-2 inhibitor blocked EGF-induced increase of cell cycle regulatory protein expressions (Fig. 5D). Consistent with these results, EGF-induced increase of [3H]thymidine incorporation and the percentage of S phase cells were inhibited by MMP inhibitor (Fig. 5, E and F). These results suggested that caveolin-1 may act as a positive regulator of MMP-2 activity, which is important in EGF-induced stimulation of cell cycle progression.

Involvement of caveolin-1 in EGF-induced ES cell proliferation. We examined the effects of caveolin-1 on EGF proliferative effects since EGF induced DNA synthesis and caveolin-1 activation in mouse ES cells. Pretreatment of AG 1478, PP2, and MβCD prevented EGF-induced increase in [3H]thymidine incorporation and cell number (Fig. 6, A and B). Next, we transfected mouse ES cells with caveolin-1/FAK-specific siRNAs (200 pmol/l) to examine the relationship between caveolin-1 and expression of the proto-oncogenes or cell cycle regulatory proteins. EGF treatment significantly stimulated c-fos, c-myc, and c-jun mRNA expression levels, which were inhibited by caveolin-1/FAK siRNA pretreatment (Fig. 6C). These results suggested that EGF stimulated the proliferation of mouse ES cells through caveolin-1 and FAK-dependent proto-oncogene transcription. We examined the effects of EGF on the expression of cell cycle regulatory proteins, cyclin D1, cyclin E, CDK4, and CDK2 (all of which are possible essential factors in G1/S progression) to confirm the EGF effects on ES cell proliferation. EGF treatment yielded significant increases in cyclin D1, cyclin E, CDK2, and CDK4 levels. Importantly, all effects were inhibited by pretreatment with caveolin-1/FAK siRNA (Fig. 6D). These results suggested that caveolin-1 mediated EGF-induced cell proliferation occurred through modulation of cell cycle regulatory protein levels. Downregulation of caveolin-1/FAK by siRNA also inhibited the EGF-induced increases in [3H]thymidine incorporation and cell number (Fig. 6, E and F). We performed cell cycle analysis using FACS to elucidate the nature of this growth phenotype. Cell transfection with caveolin-1/FAK-specific siRNA before EGF treatment significantly decreased the percentage of S phase cells and increased the percentage of G0/G1 cells compared with control samples (Fig. 6G). Finally, pretreatment with PI3K/Akt and ERK inhibitors blocked the EGF-induced increase in [3H]thymidine incorporation (Fig. 6H).

FIGURE 7. The hypothesized model for the caveolin-1 signal pathways involved in EGF-induced ES cell proliferation. EGF-activated receptor tyrosine kinase (TK), which stimulates Src kinase. Src activated caveolin-1, which stimulated FAK activation. These effects were partially mediated by the activation of PI3K/Akt. Finally, these molecules may induce ERK1/2, which increases cell cycle regulatory proteins and MMP-2 expression.

DISCUSSION

In the present study, EGF increased caveolin-1 phosphorylation, which affected EGF-mediated PI3K/Akt and MAPK as well as subsequent ES cell migration and proliferation. Our results show that caveolin-1 may play multiple roles in the regulation of EGF activity and of proliferation of ES cells. This interrelationship between caveolin-1 and EGFR is further complicated by recent evidence suggesting that caveolae contained an abundance of EGFR and interacts with caveolin-1 through a binding sequence located in its intracellular kinase domain (9, 16, 22). We found that the EGF was highly enriched in caveolae and EGF promotes the interaction between phosphorylated EGFR and caveolin-1. From various previous reports, there is complexity and versatility on the role of caveolin-1 in the EGF-induced signaling pathway. They showed that caveolin-1 play an activatory or inhibitory role (5, 12, 28). This suggests that other factors are involved in EGF-induced signaling or some of which are cell-type specific. Thus the recognition of caveolin-1 as both a negative and positive regulator of proliferation through its interaction with both upstream and downstream components of the EGF-induced signaling cascade may provide interesting new tools for the study of the role of this protein in self-renewal of ES cells. We also demonstrated that phosphorylation of caveolin-1 by EGF resulted in activation of the PI3K/Akt and ERK1/2 pathways. Our data suggested that caveolin-1 served as a positive regulator of cellular proliferation because EGF-mediated ES cell proliferation was significantly inhibited by caveolin-1 siRNA.
pretreatment before EGF treatment. However, it is not yet possible to say what are the exact roles of caveolins and further work is required to give a better understanding of the situation.

Caveolin-1 has been shown to interact with B1-integrins in a complex that regulated adhesion and signaling through FAK and the Src family kinases (24). FAK is a nonreceptor protein tyrosine kinase that localizes to focal contact sites and has been linked to the generation of cell survival, cell cycle progression, and cell migration signals (13, 20). It was reported that the stimulated phosphorylation of FAK at Tyr-397 creates an SH2 binding motif (26) that is required for the FAK-mediated promotion of cell migration (8, 33). In addition, caveolin-1 depletion was shown to result in the loss of focal adhesion sites, FAK phosphorylation, and cell adhesion (1, 35). Our results showed that EGFRs are in caveolae and EGF promotes activation and formation of focal adhesion complex with caveolin-1, which contribute to cell migration and proliferation. Furthermore, we observed that MMP-2 expression increased migration mobile abilities under EGF-mediated cellular proliferative stimulation. Therefore, this study suggest that caveolin-1 had a critical role in mediating EGF effects on the control of MMP-2 expression. Indeed, MMP-2 and its activator MT1-MMP are also targeted to caveolin-1-rich microdomains on endothelial cells and thereby restrict matrix proteolysis to a limited microenvironment at the cell surface (25). We also assessed the role of caveolin-1 in EGF-induced proliferation of mouse ES cells. Despite the controversy, it is generally accepted that cavolin-1 functions in cell growth was heavily dependent on cellular-specific contexts. Our results demonstrated that inhibition of caveolin-1 expression with siRNA abolished the EGF-induced upregulation of several proto-oncogenes (c-myc, c-fos, c-jun) and cell cycle regulatory proteins (cyclin D1-CDK 4 and cyclin E-CDK 2). Previously it was reported that EGF stimulate c-fos through ERK or PI3K-dependent pathways (3). Additionally, many of the gene targets downstream of EGF signaling (cyclin D1, c-myc, c-fos, and cyclin E) were targets of ERK signaling and part of the ERK-dependent induction of mitosis (4, 14, 31). We demonstrated that EGF induced ES cell proliferation via ERK and PI3K/Akt signals dependent on caveolin-1. These results suggested that caveolin-1 had an important role in ES cell maintenance and self-renewal and provided novel insights concerning the possible mechanisms for the caveolin-1 role in the modulation of EGFR-receptor-associated signaling (Fig. 7). This is an important step in the expansion of stem cells in vitro. Further studies evaluating the relationship between EGFR and caveolin will probably see more reports of caveolins functions, which will enhance our knowledge of caveolin physiological roles and reinforce the potential of caveolin in self-renewal of ES cells. In conclusion, our findings suggested that EGF stimulated cellular proliferation and motility by caveolin-1 activation. Caveolin-1 signaling to the PI3K/Akt-ERK pathway was required for ES cell proliferation and partially necessary for migration.

GRANTS

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