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Research Article

Interaction of galectin-1 with caveolae induces mouse embryonic stem cell proliferation through the Src, ERas, Akt and mTOR signaling pathways

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Abstract. Galectins have the potential to provide a promising alternative for unveiling the complexity of embryonic stem (ES) cell self-renewal, although the mechanism by which galectins maintain ES cell self-renewal has yet to be identified. Galectin-1 increased [^{3}H]-thymidine incorporation as well as cyclin expression and decreased p27^{kip1} expression. Src and caveolin-1 phosphorylation was increased by galectin-1, and phospho-caveolin-1 was inhibited by PP2. In addition, inhibition of caveolin-1 by small interfering RNA and methyl-β-cyclodextrin (Mβ-CD) decreased galectin-1-induced cyclin expression and [^{3}H]-thymidine incorporation. Galectin-1 caused Akt and mTOR phosphorylation, which is involved in cyclin expression. Galectin-1-induced phospho-Akt and -mTOR was inhibited by PP2, ERas siRNA, caveolin-1 siRNA and Mβ-CD. Furthermore, mTOR phosphorylation was decreased by LY294002 and Akt inhibitor. Galectin-1-induced increase in cyclin expression and decrease in p27^{kip1} was blocked by Akt inhibitor and rapamycin. In conclusion, galectin-1 increased DNA synthesis in mouse ES cells via Src, caveolin-1 Akt, and mTOR signaling pathways.

Keywords. ES cell, proliferation, galectin-1, Src, caveolin-1.

Introduction

A major issue that needs to be addressed is the need to expand embryonic stem (ES) cells in *in vitro* culture conditions to improve the potential of ES cells for regenerative medicine. However, although significant advances are being made in this field, available media for ES cell culture are unsatisfactory due either to their undefined composition or, when composition is known, to their suboptimal performance. Galectins are a family of carbohydrate-binding proteins with an affinity for β-galactoside-containing glycoconjugates. To date, 15 members have been identified [1, 2], of which all can be classified according to their carbohydrate recognition domains [3]. Galectin-1 is a lectin that binds preferentially to the lactosamine structure in glycans [4 – 6]. Galectin-1 is expressed by various stem cells, including embryonic, hematopoietic and keratinocyte stem cells [7 – 11]. Until now, there has been little research into the function of lectins in ES cells. Recently, Yanagisawa et al, [12] reported that galectin-1 is expressed on adult neural stem cells and promotes their proliferation through its carbohydrate-

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binding ability. Galectins also play important functional roles in determining cell fate such as self-renewal, proliferation and differentiation. Recent studies clearly implicate the functional relevance of glycoconjugates in ES cells in mediating signal transduction, cell-cell interaction and adhesion [13]. This evidence raises the possibility that galectins play functional roles in ES cells. Therefore, galectins may provide a promising alternative for unveiling the complexity of ES cell self-renewal, although the mechanism has yet to be identified in detail.

In various cell systems, galectins interact with caveolin-1 [14, 15]. Caveolins are functionally and structurally highly conserved and initiate formation of caveolae from raft-derived components. Caveolae are specialized lipid raft microdomains that form 50- to 100-nm-sized flask-shaped invaginations within the plasma membrane. Caveolae have been suggested to function in signaling events through compartmentalization-signaling molecules that interact with caveolin proteins. Thus, in addition to the structural role of caveolins in the formation of caveolae, there is considerable evidence for their involvement in the modulation of cell signaling. They are found in various cell types and are involved in various functions, including signal transduction [16–19]. The recent identification of various signaling molecules and their interaction with caveolin suggests that caveolin may participate in transmembrane signaling. Indeed, many of the signaling molecules that interact with caveolin-1 mediate mitogenic signals [20, 21]. However, the cell-cycle control of ES cells remains poorly understood. Mouse ES cells, derived from the ICM of mouse blastocysts, have been established as permanent lines and are characterized by their capacity for self-renewal *in vivo* and *in vitro*. ES cells can differentiate into all three germ layers and have unlimited growth potential under certain conditions [22]. In this study, ES cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with leukemia inhibitory factor (LIF) to maintain their undifferentiated state and to support the derivation and expansion of ES cells [23, 24]. These cells closely resemble their *in vivo* counterparts and provide a stable *in vitro* model of embryonic growth and development. In addition, they provide a tool whereby specific signaling systems can be investigated [25–27]. In this study, we examined the effect of galectin-1 on ES cell proliferation and analyzed the underlying mechanisms for its growth regulatory activities in mouse ES cells.

Materials and methods

Materials

Mouse ES cells (ES-E14TG2a) were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum was purchased from Biowhittaker (Walkersville, MD). Recombinant mouse galectin-1 was obtained from R&D Systems (Minneapolis, MN, USA). Methyl-β-cyclodextrin, β-lactose, sucrose, LY 294002, Akt inhibitor, rapamycin, fluorescence isothiocyanate (FITC)-conjugated goat-anti rabbit IgM and β-actin were acquired from Sigma Chemical Company (St. Louis, MO, USA). [^{3}H]-Thymidine was purchased from NEN (Boston, MA, USA). Anti-phospho-Src, phospho-caveolin-1 and caveolin-1 antibodies were acquired from Santa Cruz Biotechnology (Delaware, CA, USA). Phospho-Akt 308, phospho-Akt 473, phospho-mTOR, phospho-p70S6K and phospho-4E-BP1 antibodies were supplied by New England Biolabs (Herts, UK). Goat anti-rabbit IgG was purchased from Jackson Immunoresearch (West Grove, PA, USA). All other reagents were purchased commercially and were of the highest purity available.

ES cell culture

The mouse ES cells were cultured for five days in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 3.7 g/l of sodium bicarbonate, 1% penicillin and streptomycin, 1.7 mM L-glutamine, 0.1 mM β-mercaptoethanol, 5 ng/ml mouse leukemia inhibitory factor (LIF) and 15% fetal bovine serum (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 an atmosphere containing 5% CO_2. The medium was exchanged with serum-free DMEM containing LIF for 24 h before the experiments.

Caveolin-1 siRNA transfection

The cells were grown to 75% confluence in each dish and transfected for 48 h with either a SMARTpool of small interfering RNAs (siRNAs) specific to caveolin-1 and ERas (200 pmol/l) or non-targeting siRNA (as negative control; 200 pmol/l; Dharmacon, Inc., Lafayette, CO, USA) using Dharmafect (Dharmacon, Inc., Lafayette, CO, USA) according to the manufacturer's instructions.

[^{3}H]-Thymidine incorporation

The [^{3}H]-thymidine incorporation experiment was performed using the methodology described by Brette et al. [28]. The cells were incubated in medium with or without galectin-1, and the cells were then pulsed with

1 μCi of [methyl-^{3}H]-thymidine for 1 h at 37°C. The ES cells were washed twice with phosphate-buffered saline (PBS), fixed in 10% trichloroacetic acid (TCA) at 23°C for 15 min, and then washed twice with 5% TCA. The acid-insoluble material was dissolved in 2 N NaOH for 12 h at 23°C. Aliquots were removed in order to determine the level of radioactivity using a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA, USA). All values are reported as mean (± S.E.M) of triplicate experiments.

Cell-proliferation assay

In order to determine the number of cells, 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.

Detergent-free purification of caveolin-rich membrane fraction

Caveolin-enriched membrane fractions were prepared as described previously [29]. Cells 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; Branson Sonicator 250, Branson Ultrasonic Corp., Danbury, CT, USA). 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 containing 250 mM sodium carbonate) and centrifuged at 40,000 rpm for 20 h in an SW 41 rotor (Beckman Instruments). Twelve 1-ml fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Immunofluorescence staining

Cells were fixed and permeabilized for 10 min with 0.1% (v/v) Triton X-100 and washed. In order to decrease non-specific binding of the antibody, the cells were preincubated with 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) in PBS for 20 min. The cells were then incubated for 60 min with primary antibody in a solution containing 1% (v/v) BSA in PBS. After three washes with PBS and incubation with 1% (v/v) BSA for 5 min, the cells were incubated for 60 min with FITC-conjugated secondary antibody in PBS with 1% (v/v) BSA. After washing, the samples were mounted on slides and visualized using a confocal microscope (Fluoview 300, Olympus) with a 400× objective.

Western blot analysis

The cell homogenates (30 μg protein) were separated by 10% 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] and blocked with 5% skimmed milk for 1 h. The blots were then incubated with the appropriate primary antibody at the dilution recommended by the supplier. The membrane was washed, and primary antibodies were detected with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. The bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK).

Statistical analysis

The results are reported as the mean ± standard error (S.E.M.). All experiments were analyzed by analysis of variance (ANOVA). In some experiments, this analysis was followed by a comparison of the treatment means with the control using the Bonferroni-Dunn test. A P value <0.05 was considered significant.

Results

Effect of galectin-1 on DNA synthesis and cyclin expression

The time course of galectin-1 effects was examined to determine the effective treatment time. As shown in Figure 1A, incubating mouse ES cells with galectin-1 significantly increased [^{3}H]-thymidine incorporation from 8 h. The dose-response effect of galectin-1 on [^{3}H]-thymidine incorporation was then examined. Mouse ES cells were incubated with various galectin-1 concentrations (0–100 ng/ml) for 24 h. Galectin-1 significantly increased [^{3}H]-thymidine incorporation at concentrations of $\geq$0.1 ng/ml. The maximum increase was observed at 100 ng/ml galectin-1 (Fig. 1B). In an experiment examining cyclin expression levels, galectin-1 also significantly increased cyclin D1 and cyclin E expression in a time-dependent manner. In addition, galectin-1 decreased the cyclin-dependent protein kinase (CDK) inhibitor protein, $p27^{kip1}$ (Fig. 1C). In further experiments to examine the effect of galectin-1 on cell proliferation, a significant increase in the number of cells was observed (Fig. 1D). In order to determine the involvement of carbohydrate-binding activity of galectin-1, the cells were treated with galectin-1 either in the presence or absence of lactose (10 mM) or sucrose (10 mM; used as an osmolarity control) for 24 h. In the experiment,

galectin-1-induced increase in [^{3}H]-thymidine incorporation was attenuated by lactose (Fig. 1E). In addition, the effect of intrinsic galectin-1 on ES cell proliferation was examined. As shown in Figure 1F and G, we observed that galectin-1 was expressed in mouse ES cells and was not detected in culture media, and [^{3}H]-thymidine incorporation was not decreased by lactose treatment under control conditions.

Involvement of Src and caveolin-1 in galectin-1-induced increase of DNA synthesis

In the present study, galectin-1 increased the levels of Src and caveolin-1 phosphorylation (Fig. 2A). In addition, we investigated whether galectin-1-induced tyrosine phosphorylation of caveolin-1 was associated with Src in mouse ES cells. As shown in Figure 2B, inhibition of Src by PP2 (Src inhibitor, 5×10^{-6} M) abrogated galectin-1-induced caveolin-1 phosphorylation, suggesting that Src contributes to caveolin-1-related signaling. In addition, these results establish galectin-1 as an inducer of caveolin-1 phosphorylation in mouse ES cells. Caveolin-1 directly binds to cholesterol, the most important cofactor in caveolae morphogenesis. In order to confirm the structural importance of membrane rafts in caveolin-1-dependent signaling, we first examined the effect of methyl-β-cyclodextrin (Mβ-CD) on caveolae structure. In the discontinuous sucrose density gradient fractionation, we confirmed that the caveolin-enriched membrane fraction (Fig. 2C, fraction 5) was decreased after treatment with 10 mM Mβ-CD for 1 h (Fig. 2C). In addition, organization of caveolin-1 was assessed by confocal microscopy. As shown in Figure 2D, when cholesterol was depleted from the membrane by means of Mβ-CD, caveolae organization was disrupted. We next examined whether Src and caveolin-1 were involved in galectin-1-induced cell cycle regulatory protein expression and DNA synthesis. Figure 3A shows that PP2 attenuated galectin-1-induced cell cycle regulatory protein (cyclin D1, cyclin E and $p27^{kip1}$) expression. In order to confirm the effect of caveolin-1 siRNA in caveolin-1 expression, we treated caveolin-1 siRNA under normal ES cell culture conditions. As shown in Figure 3C, caveolin-1 siRNA significantly downregulated the caveolin-1 expression level in a dose-dependent manner. In addition, Mβ-CD and caveolin-1 siRNA inhibited galectin-1-induced cell cycle regulatory protein expression (Fig. 3C, D). This result suggests that not only caveolin-1 expression but also the structural function of caveolae is needed for galectin-1-induced cell cycle progression. Furthermore, Figure 3E shows that the galectin-1-induced increase in [^{3}H]-thymidine incorporation was blocked by PP2, Mβ-CD and caveolin-1 siRNA, which was consistent with the results shown in Figure 3A–D.

Involvement of Src, caveolin-1 and ERas in the galectin-1-induced Akt phosphorylation

We next defined the roles of Src and caveolin-1 in the activation of immediate downstream targets in galectin-1 signaling. Galectin-1 induced the phosphorylation of Akt in a time-dependent manner (0–240 min) as early as 10 min after stimulation with galectin-1 (Fig. 4A). In order to determine if activation of Src was involved in galectin-1-induced Akt phosphorylation, mouse ES cells were treated with PP2 for 30 min prior to galectin-1 treatment. Figure 4B shows that PP2 decreased galectin-1-induced phosphorylation of Akt. To further verify the role of caveolin-1 in galectin-1-induced Akt phosphorylation, the cells were either pretreated with Mβ-CD or caveolin-1 siRNA for 30 min prior to galectin-1 treatment. As shown in Figure 4C and D, phosphorylation of Akt by galectin-1 was inhibited by Mβ-CD and caveolin-1 siRNA. These data show that normal protein expression of caveolin-1 and an intact caveolar structure are necessary for Akt phosphorylation. In addition, we carried out the experiments to determine the involvement of ERas in galectin-1-induced Akt phosphorylation. In the experiment, Akt phosphorylation was attenuated by ERas siRNA in a dose-dependent manner, and glaectin-1-induced increase in Akt phosphorylation was also inhibited by ERas siRNA (Fig. 4E, F).

Involvement of Src and caveolin-1 in galectin-1-induced mTOR signaling

The level of mTOR/p70S6K/4E-BP1 phosphorylation was examined. As shown in Figure 5A, phosphorylation of mammalian target of rapamycin (mTOR), p70S6K and 4E-binding protein 1 (4E-BP1) was induced by galectin-1 in a time-dependent manner (0–240 min). In addition, phosphorylation of mTOR was attenuated by pretreatment with LY 294002 or Akt inhibitor (Fig. 5B). This suggests that the phosphoinositide 3-kinase (PI3K)/Akt signaling pathways are involved in mTOR phosphorylation. Therefore, we next investigated whether Src and caveolin-1 are involved in galectin-1-induced phosphorylation of mTOR in mouse ES cells. As shown in Figure 5C, galectin-1-induced phosphorylation of mTOR was inhibited by pretreatment with PP2. In addition, treatment with Mβ-CD and caveolin-1 siRNA blocked galectin-1-induced mTOR activation, confirming that caveolin-1 is required for mTOR-mediated signaling (Fig. 5D, E).

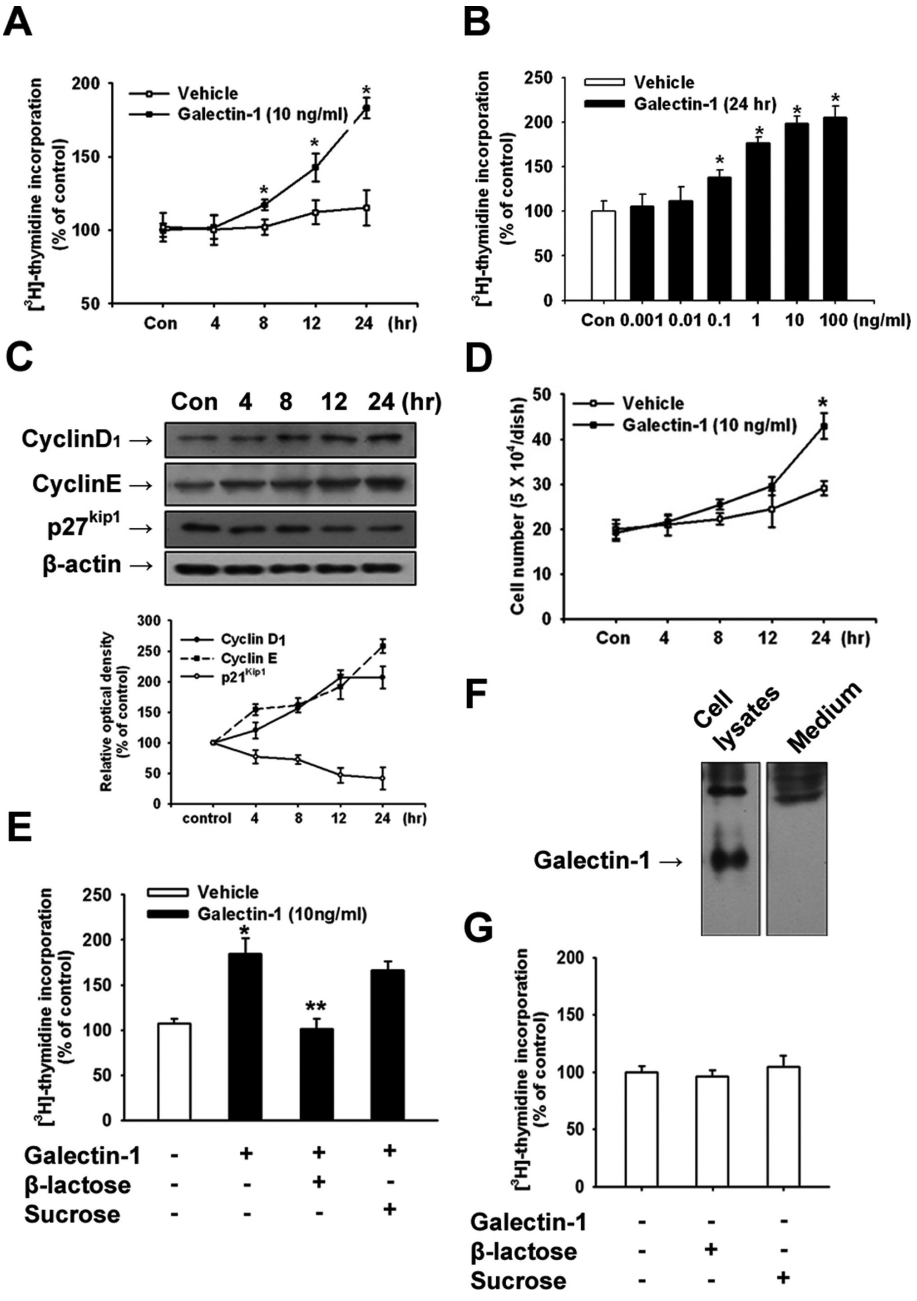

Figure 1. Effect of galectin-1 on [^{3}H]-thymidine incorporation and cell cycle regulatory protein expression. Time (*A*) and dose (*B*) response of galectin-1 effect on [^{3}H]-thymidine incorporation. Mouse ES cells were treated with galectin-1 (10 ng/ml) for various times (0–24 h) or with different doses of galectin-1 (0–100 ng/ml) for 24 h and then pulsed with 1 μCi of [^{3}H]-thymidine for 1 h prior to counting. (*C*) The time course of cyclin D1, cyclin E and p27^{kip1} protein expression was analyzed by Western blot analysis as described in 'Materials and methods'. The cells were treated or not treated with galectin-1 (10 ng/ml) for various times (0–24 h). Each example shown is representative of three experiments. The graphs denote the mean ± S.E.M. of three experiments for each condition determined from densitometry relative to β-actin. *, $P<0.05$ vs. control. (*D*) The cells were treated with or without galectin-1 (10 ng/ml) for various times (0–24 h) and the number of cells was counted using a hemocytometer. (*E*) The cells were treated with galectin-1 either in the presence or absence of lactose (10 mM) or sucrose (10 mM; used as an osmolarity control) for 24 h and pulsed with 1 μCi of [^{3}H]-thymidine for 1 h prior to counting. (*F*) The cells were cultured in serum-free medium for 24 h, and then galectin-1 was detected from total cell lysates or ES cell culture medium by Western blot analysis as described in 'Materials and methods'. Each example shown is representative of three experiments. (*G*) The cells were cultured in the absence or presence of lactose or sucrose for 24 h and then pulsed with 1 μCi of [^{3}H]-thymidine for 1 h prior to counting. [^{3}H]-thymidine incorporation data are reported as the mean ± S.E.M. of four independent experiments, each conducted in triplicate. *, $P<0.05$ vs. vehicle; **, $P<0.05$ vs. galectin-1 alone.

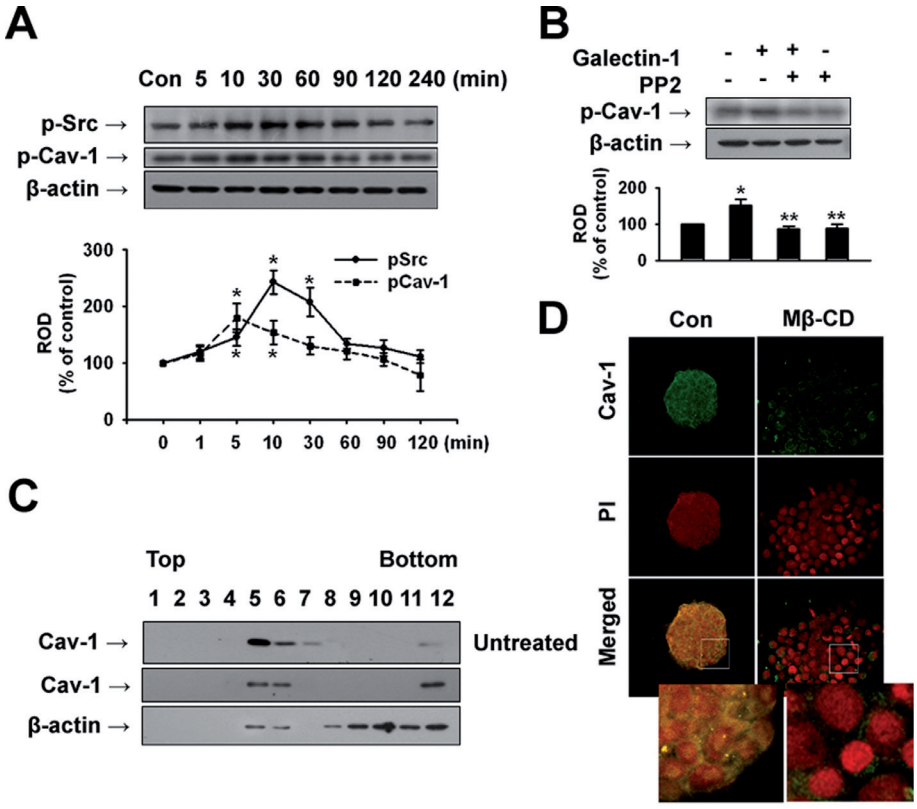

Figure 2. Involvement of Src and caveolin-1 in effect of galectin-1. (*A*) Mouse ES cells were treated with galectin-1 (10 ng/ml) for various times (0–240 min), and the phosphorylation of Src and caveolin-1 was detected by Western blot analysis. (*B*) The cells were pretreated with PP2 (Src inhibitor, 5×10^{-6} M) for 30 min before the galectin-1 treatment, and the phosphorylation of Src and caveolin-1 was detected. Each example shown is representative of three independent experiments. The graphs denote the mean ± S.E.M. of three experiments for each condition determined from densitometry relative to β-actin. *, $P<0.05$ vs. control. (*C*) Lysates of untreated (control) or 10 mM methyl-β-cyclodextrin (Mβ-CD)-treated cells were subjected to discontinuous sucrose density gradient fractionation. Each fraction was assessed by Western blot analysis. (*D*) Control and 10 mM Mβ-CD-treated cells were immunostained for caveolin-1 (green), and each nucleus was stained with propidium iodide (red). Each example shown is representative of three experiments.

Involvement of Akt and mTOR in galectin-1-induced cell cycle progression

To determine whether the Akt and mTOR pathways are involved in galectin-1-induced DNA synthesis, we examined the expression levels of cell cycle regulatory proteins. As shown in Figure 6A, the galectin-1-induced increase in cyclin D1 and cyclin E and the decrease in $p27^{kip1}$ were blocked by Akt inhibitor or rapamycin (mTOR inhibitor, 10^{-5} M). We further investigated whether Akt inhibitor or rapamycin similarly modulated the cell cycle profile. Galectin-1 significantly increased the percentage of the cell population in the S phase as compared to control. However, pretreatment with Akt inhibitor or rapamycin decreased the accumulation in the S phase induced by galectin-1 (Fig. 6B, C).

Discussion

In the present study, galectin-1 induced DNA synthesis and expression of cell cycle regulatory proteins in mouse ES cells and lactose [30] significantly down-regulated extracellular galectin-1-induced increases in [^{3}H]-thymidine incorporation. In addition, galectin-1 in the ES cell culture media was not detected, although ES cells express galectin-1 endogenously. Therefore, our results suggest that galectin-1 stimulates ES cell proliferation via a carbohydrate-dependent mechanism and secreted intrinsic galectin-1 is not enough or too low level to exhibit its role in ES cell growth under our experimental conditions.

In this study, caveolin-1 was phosphorylated in response to galectin-1 treatment. These results demonstrated that galectin-1-mediated engagement of caveolin-1 plays a critical role in mouse ES cell proliferation. The present study has extended our understanding of the biological function of galectin-1 in mouse embryonic stem (ES) cell growth. To our

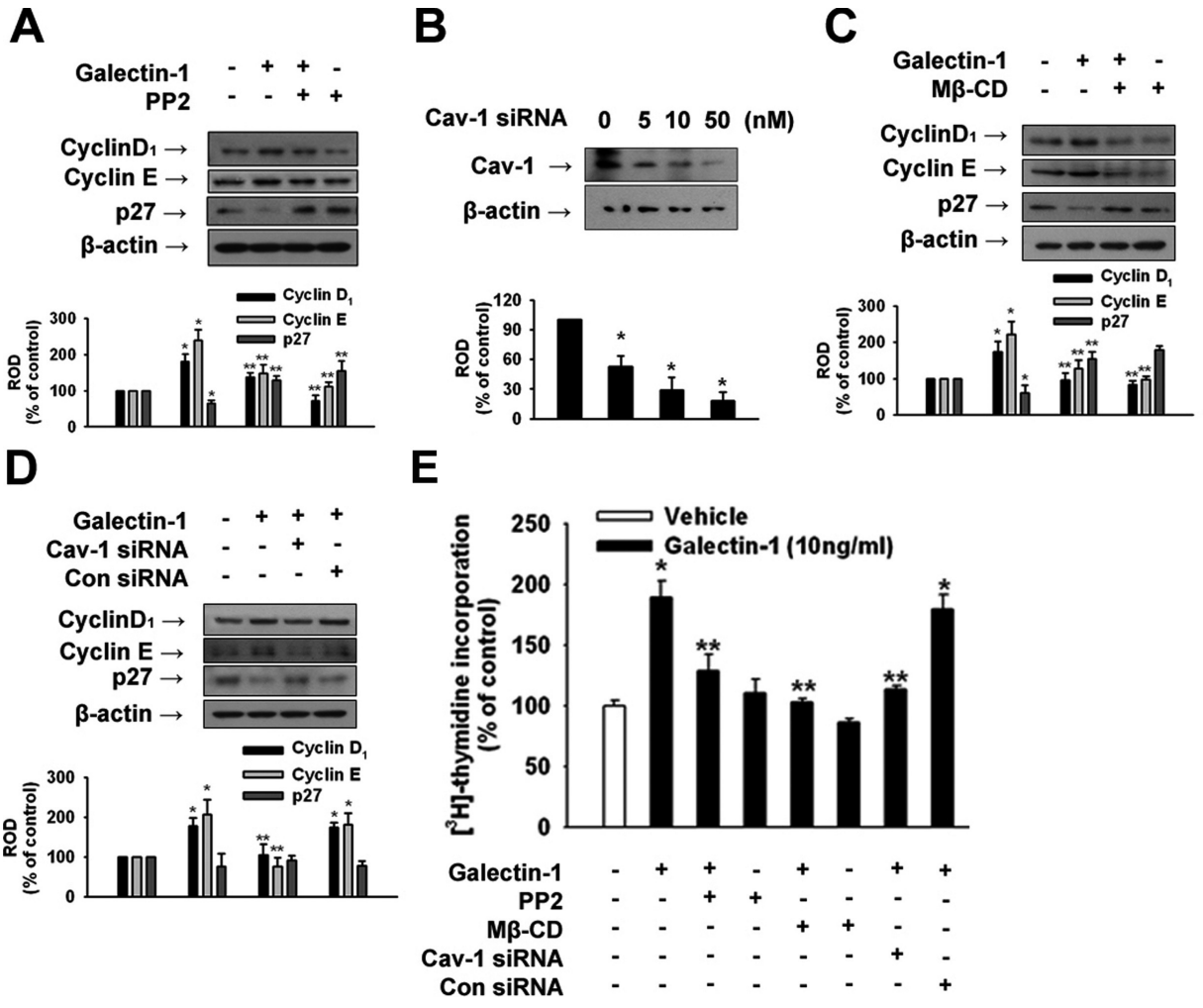

Figure 3. Involvement of Src and caveolin-1 in galectin-1-induced cell cycle regulatory protein expression and DNA synthesis. (*A*) Mouse ES cells were pretreated with PP2 for 30 min prior to galectin-1 (10 ng/ml) treatment for 24 h, and the expression of cyclin D1, cyclin E and $p27^{kip1}$ was detected by Western blot analysis. (*B*) The cells were transfected for 36 h with different doses of SMARTpool of caveolin-1 siRNA (0–50 nM), and caveolin-1 expression was detected by Western blot analysis. (*C*) The cells were pretreated with 10 mM Mβ-CD for 1 h prior to galectin-1 treatment for 24 h, and the expression of cyclin D1, cyclin E and $p27^{kip1}$ was detected by Western blot analysis. (*D*) The cells were transfected for 36 h with either a SMARTpool of caveolin-1 siRNAs or non-targeting control siRNA prior to galectin-1 treatment for 24 h, and the expression of cyclin D1, cyclin E and $p27^{kip1}$ was detected by Western blot analysis. Each example shown is representative of three independent experiments. The graphs denote the mean ± S.E.M. of three experiments for each condition determined from densitometry relative to β-actin. (*E*) The cells were pretreated with PP2 for 30 min or 10 mM Mβ-CD for 1 h, and transfected for 36 h with either a SMARTpool of caveolin-1 siRNAs or a non-targeting control siRNA for 24 h before galectin-1 treatment, and then pulsed with 1 μCi of [^{3}H]-thymidine for 1 h prior to counting. The data are reported as the mean ± S.E.M. of three independent experiments, each carried out in triplicate. *, $P<0.05$ vs. control; **, $P<0.05$ vs. galectin-1 alone.

knowledge, this effect of galectin-1, through its functional interaction with caveolin-1, has not previously been reported in mouse ES cells. Caveolin-1 directly binds to cholesterol, the most important cofactor in caveolae morphogenesis [31]. Conversely, the importance of membrane rafts in cell signaling can be inferred from the blocking effects of cholesterol depletion. In this study, caveolin-1 was phosphorylated by galectin-1 treatment. In addition, galectin-1-induced increases in cyclin D1 and cyclin E expression and [^{3}H]-thymidine incorporation were decreased by caveolin-1 siRNA and by disruption of caveolae-like structures by methyl-β-cyclodextrin (Mβ-CD). These results suggest that caveolin-1 protein expression as well as structurally functional caveolae is required for signal transduction in galectin-1-induced ES cell proliferation. Thus, we hypothesized that phosphorylation of caveolin-1 may be an intermediate step in the initiation of downstream signaling cascades.

In recent years, caveolin-1 has emerged as an important regulatory molecule in signal transduction. Some controversy regarding the effect of caveolin has arisen from the multiple and sometimes opposite roles played by caveolin and caveolae. Caveolin-1 has

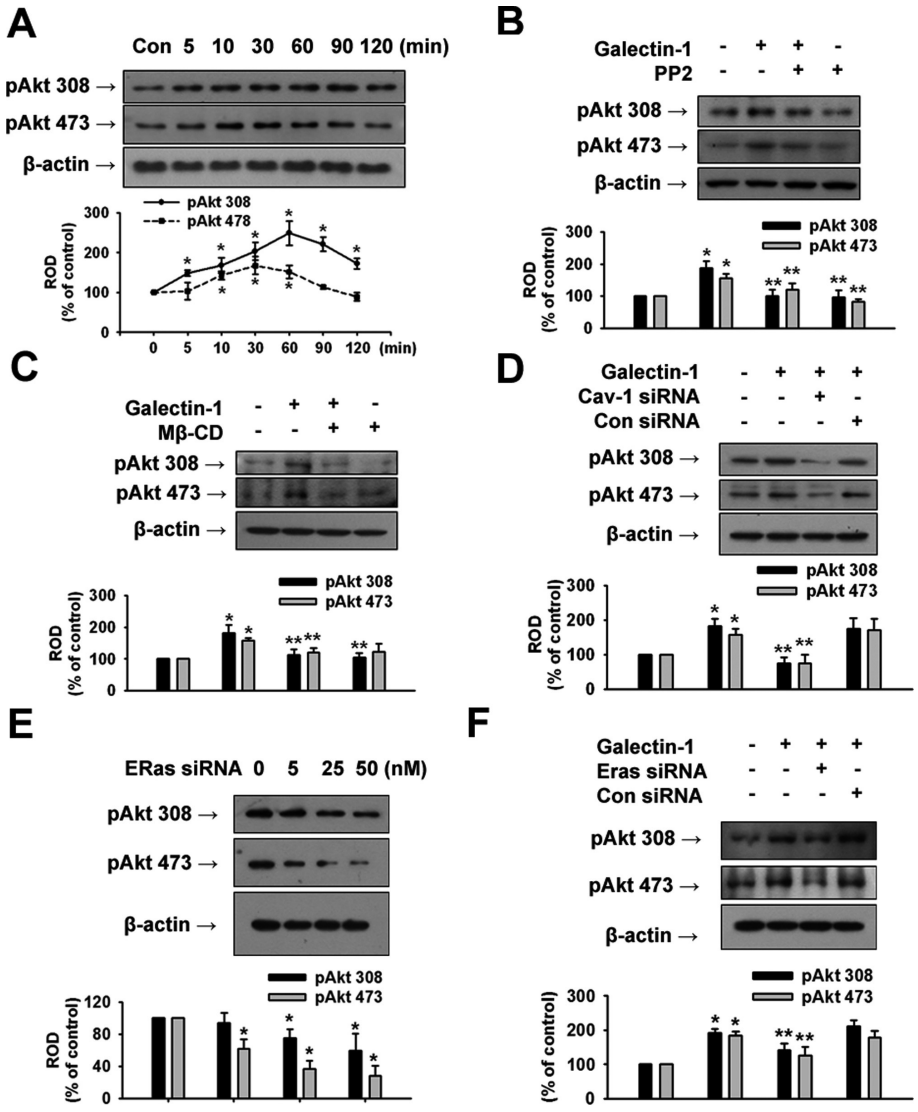

Figure 4. Involvement of Src and caveolin-1 in galectin-1-induced Akt phosphorylation. (*A*) Mouse ES cells were treated with galectin-1 (10 ng/ml) for various times (0–240 min), and the phosphorylation of Akt (Ser^{473}, Thr^{308}) was detected by Western blot analysis. (*B*) The cells were pretreated with PP2 (Src inhibitor, 5×10^{-6} M) for 30 min prior to galectin-1 treatment for 24 h, and the phosphorylation of Akt was detected by Western blot analysis. (*C*) The cells were pretreated with 10 mM Mβ-CD for 1 h before galectin-1 treatment, and the phosphorylation of Akt was detected by Western blot analysis. (*D*) The cells were transfected for 36 h with either a SMARTpool of caveolin-1 siRNAs or a non-targeting control siRNA prior to galectin-1 treatment for 36 h, and the phosphorylation of Akt was detected by Western blot analysis. (*E*) The cells were transfected for 36 h with different doses of SMARTpool of ERas siRNA (0–50 nM), and the phosphorylation of Akt was detected by Western blot analysis. (*F*) The cells were transfected for 36 h with either a SMARTpool of ERas siRNAs or a non-targeting control siRNA prior to galectin-1 treatment for 36 h, and the phosphorylation of Akt was detected by Western blot analysis. Each example shown is representative of three independent experiments. The graphs denote the mean ± S.E.M. of three experiments for each condition determined from densitometry relative to β-actin. *, $P<0.05$ vs. control; **, $P<0.05$ vs. galectin-1 alone.

previously been shown to be phosphorylated in response to growth factors such as EGF and insulin [32, 33]. However, caveolin-1 phosphorylation seems to be cell-type specific and stimulus specific [34, 35]. The functional consequences of caveolin tyrosine phosphorylation are not yet fully understood. It has been postulated that tyrosine phosphorylation of caveolin-1 at residue 14 could confer subsequent growth-stimulatory or oncogenic activity [33]. In the present study, galectin-1-induced tyrosine phosphorylation of caveolin-1 was sensitive to the c-Src inhibitor PP2. In addition, pretreatment with PP2 attenuated galectin-1-induced increases in cell cycle regulatory protein expression and [^{3}H]-thymidine incorporation. A previous report suggested that constitutive phosphorylation of caveolin on tyrosine 14 is induced by activated Src [36]. c-Src is localized on the plasma membrane and is often associated with lipid rafts [37–39] and cell proliferation [40]. Indeed, Ret-mediated mitogenesis requires Src kinase activity in

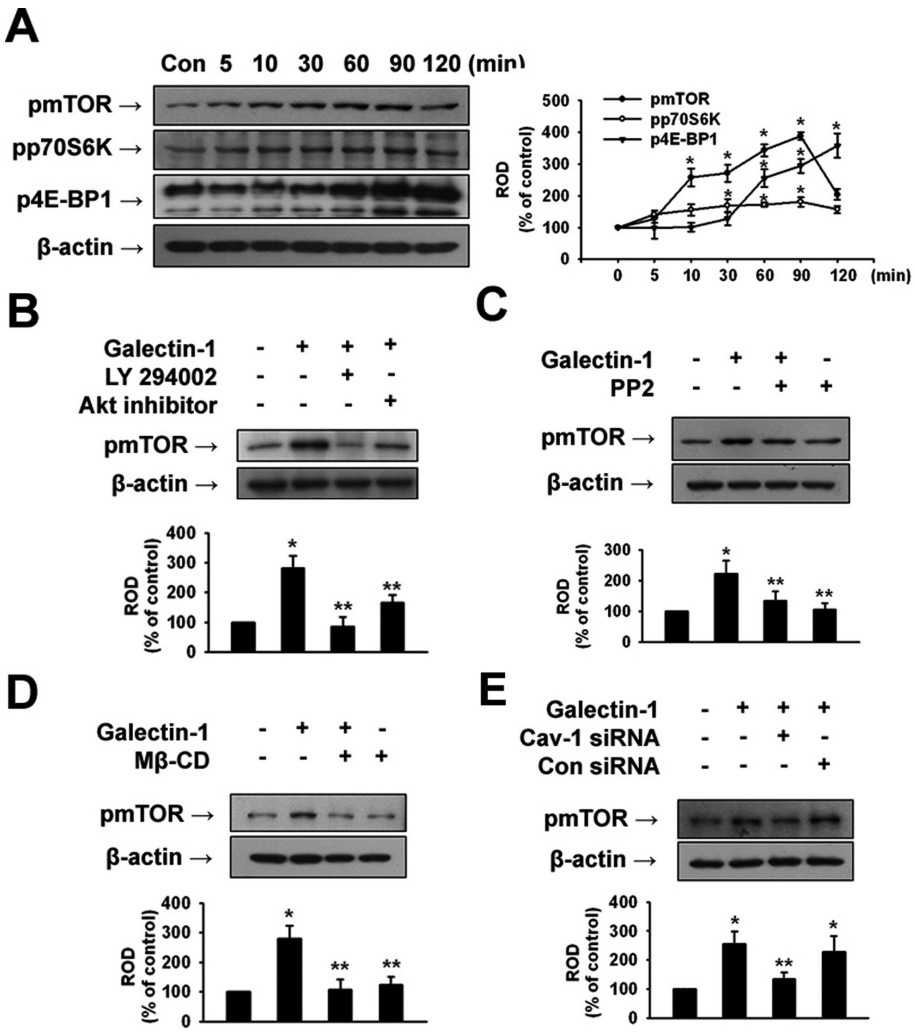

Figure 5. Involvement of Src and caveolin-1 in galectin-1-induced mTOR signaling. (*A*) Mouse ES cells were treated with galectin-1 (10 ng/ml) for various times (0–240 min), and the phosphorylation of mTOR, p70S6K and 4E-BP1 was detected by Western blot analysis. (*B*) Cells were pretreated with LY 294002 (PI3K inhibitor, 10^{-6} M) or Akt inhibitor (10^{-5} M) for 30 min before being incubated with galectin-1, and the phosphorylation of mTOR was detected by Western blot analysis. (*C*) Cells were pretreated with PP2 (Src inhibitor, 5×10^{-6} M) for 30 min prior to galectin-1 treatment for 24 h, and phosphorylation of mTOR was detected by Western blot analysis. (*D*) The cells were pretreated with Mβ-CD for 1 h prior to galectin-1 treatment, and phosphorylation of Akt was detected by Western blot analysis. (*E*) The cells were transfected for 36 h with either a SMARTpool of caveolin-1 siRNA or non-targeting control siRNA before galectin-1 treatment for 24 h, and phosphorylation of mTOR was detected by Western blot analysis. Each example shown is representative of three independent experiments. The graphs denote the mean ± S.E.M. of three experiments for each condition determined from densitometry relative to β-actin. *, $P<0.05$ vs. control; **, $P<0.05$ vs. galectin-1 alone.

other cell types. In addition, Src is important for embryonic stem cell self-renewal [41]. These observations suggest that galectin-1-induced phosphorylation of caveolin-1 is caused by c-Src and that activated Src plays an important role in galectin-1-induced ES cell proliferation.

In this study, galectin-1 increased the phosphorylation of Akt. Activated Akt is important in cell proliferation and survival because it phosphorylates various substrates. Previous reports have suggested that the Akt signaling pathway is downstream of caveolin-1 [20, 42, 43]. Indeed, we observed that depletion of cholesterol by Mβ-CD not only decreased phospho-caveolin-1 levels but also downregulated phosphorylation of Akt. Knockdown of caveolin-1 with siRNA also decreased Akt phosphorylation levels. Several studies have reported that caveolin-1 is a potent activator of the phosphoinositide 3-kinase (PI3K)/Akt pathway [20, 43]. Therefore, these results establish a molecular link between caveolin-1 and Akt activation. Previous studies reported that HRas activated by extracellular stimuli interacts with intracellular galectin-1 [44, 45] and HRas directly interacts with the catalytic subunit of PI3K in the activation PI3K/Akt pathway [46–48]. Therefore, we examined whether ERas is involved in glaectin-1-induced Akt activation. Our results show that ERas (HRas2) siRNA strongly downregulated Akt phosphorylation levels in a dose-dependent manner. In addition, we observed that ERas siRNA blocked galectin-1-induced Akt phosphorylation. Therefore, we suggest that extracellular galectin-1 has an effect on intracellular galectin-1 activation, which then activates Ras and thus Akt signaling.

mTOR is a strong candidate as a downstream target of PI3K signaling in ES cells [50]. Many growth factors, including insulin, activate PI3K, which phosphorylates phospatidylinositol-4,5-bisphosphate [PtdIns-(4,5)P_2] at the 3′ position to generate PtdIns(3,4,5)P_3 and activate Akt [49]. In accordance with these reports, Akt inhibitor blocked the increase in mammalian target of rapamycin (mTOR) phosphorylation. Furthermore, in this study, Mβ-CD and caveolin-1

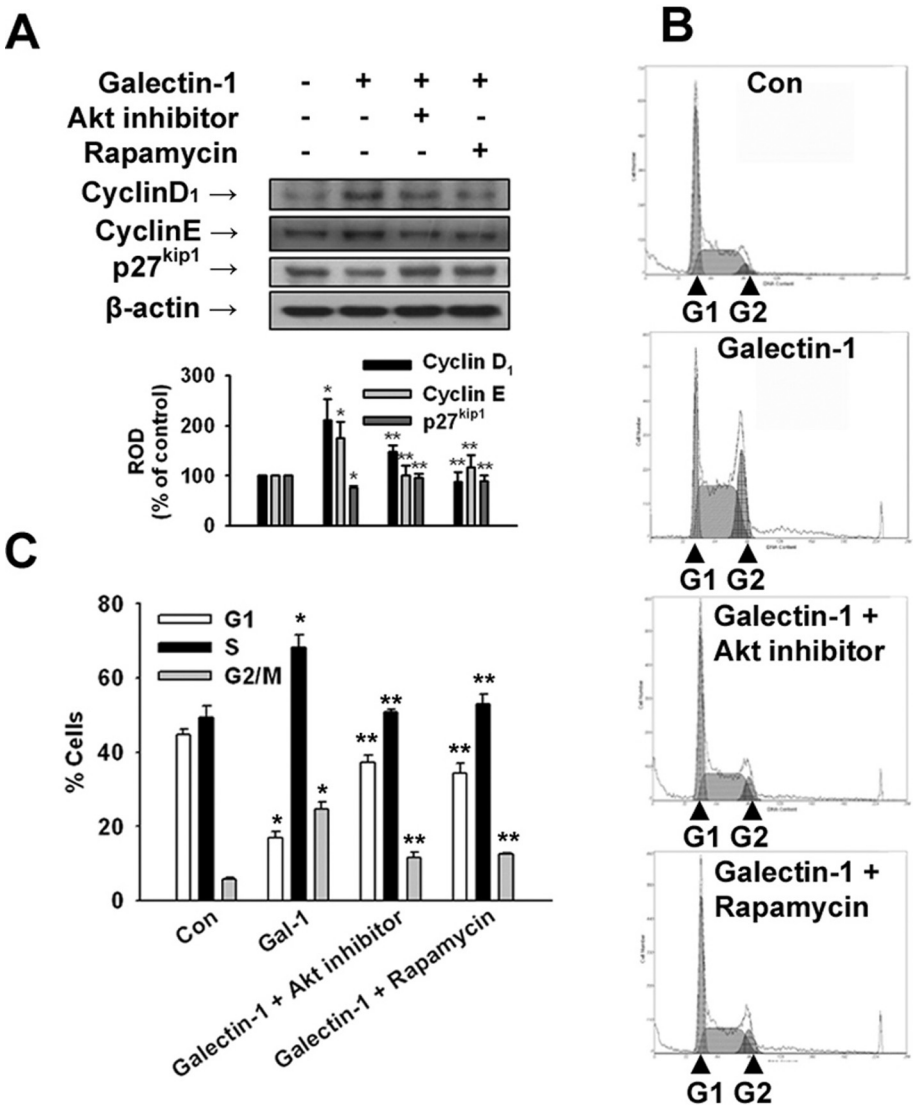

Figure 6. Involvement of Akt and mTOR in galectin-1-induced cell cycle progression. (*A*) Mouse ES cells were pretreated with Akt inhibitor (10^{-5} M) and rapamycin (mTOR inhibitor, 10^{-9} M) prior to galectin-1 (10ng/ml) treatment for 24 h. The levels of cyclin D1, cyclin E and $p27^{kip1}$ were assessed by Western blot analysis. Each of the examples shown is representative of three independent experiments. The graphs denote the mean ± S.E.M. of three experiments for each condition determined from densitometry relative to β-actin. (*B, C*) Representative FACS data and corresponding histograms for mouse ES cells that were pretreated with Akt inhibitor and rapamycin before galectin-1 treatment for 24 h. The cells were washed with PBS, fixed, stained and analyzed by flow cytometry. The gates were configured manually to determine the percentage of cells in S phase based on the DNA content. *, $P<0.05$ vs. control; **, $P<0.05$ vs. galectin-1 alone.

siRNA inhibited galectin-1-induced mTOR phosphorylation. This strongly suggests that PI3K signaling is involved in regulating the phosphorylation of mTOR and that both signaling molecules are regulated by caveolin-1 in mouse ES cells. In the present study, we observed that Akt inhibitor and rapamycin attenuated galectin-1-induced cell cycle progression. PI3K/Akt signaling are known to be highly active in ES cells and involved in cell proliferation, survival and maintenance of pluripotency in ES cells [48, 50, 51]. Indeed, Jirmanova et al. [52] demonstrated the role of PI3K in the cell-cycle control of ES cells. They showed that inhibition of PI3K markedly increased the population of ES cells in the G0/G1 phase. PI3K-dependent signaling is also known to regulate the level of $p27^{kip1}$, a major inhibitor of G1 CDKs [53, 54]. PI3K activates mTOR, which in turn upregulates cyclin D1 translation via phosphorylation of the ribosomal S6 protein [55, 56]. In addition, a previous study showed that mTOR is essential for mouse ES cell proliferation [57]. Thus, Akt and mTOR signaling pathways respond to mitogenic stimuli to promote cell cycle progression.

Taken together, galectin-1-induced activation of caveolin-1 regulates Akt and mTOR signaling pathways, which correlate with ES cell proliferation. In conclusion, galectin-1-induced cell cycle progression is mediated by activation of Src, which induces caveolin-1 phosphorylation and Ras, subsequently inducing Akt activation, which correlates with mTOR phosphorylation (Fig. 7). We think that the discovery of the role played by galectin-1 in stimulating ES cell self-renewal, together with the other results shown in this study, represents a significant advance in our knowledge of how ES cell pluripotency is maintained by extracellular factors and has application in the development of ES cell culture systems aiming for cell

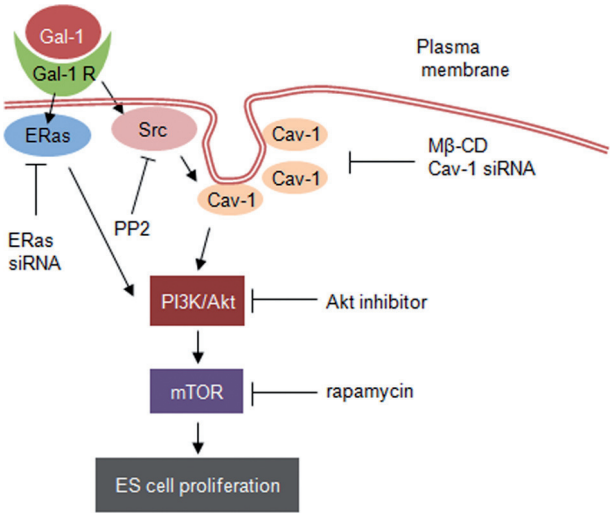

Figure 7. The hypothetical model of the signaling pathways involved in galectin-1- induced cell proliferation. Galectin-1 activates caveolin-1, which is induced by activated Src. In addition, galectin-1-activated caveolin-1 stimulates Akt to induce mTOR activation. In turn, activated mTOR regulates cell proliferation. Gal-1, galectin-1; Gal-1 R, galectin-1 receptor; cav-1, caveolin-1; mTOR, mammalian target of rapamycin. The solid line is the proposed pathway.

replacement therapy alone or in combination with other small molecules.

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