A potential role of connexin 43 in epidermal growth factor-induced proliferation of mouse embryonic stem cells: Involvement of Ca^{2+} /PKC, p44/42 and p38 MAPKs pathways

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Abstract. *Objectives:* The gap junction protein, connexin (Cx), plays an important role in maintaining cellular homeostasis and cell proliferation by allowing communication between adjacent cells. Therefore, this study has examined the effect of epidermal growth factor (EGF) on Cx43 and its relationship to proliferation of mouse embryonic stem cells. *Materials and methods:* Expressions of Cx43, mitogen-activated protein kinases (MAPKs) and cell cycle regulatory proteins were assessed by Western blot analysis. Cell proliferation was assayed with [^3H]thymidine incorporation. Intercellular communication level was measured by a scrape loading/dye transfer method. *Results:* The results showed that EGF increased the level of Cx43 phosphorylation in a time- (≥ 5 min) and dose- (≥ 10 ng/mL) dependent manner. Indeed, EGF-induced increase in phospho-Cx43 level was significantly blocked by either AG 1478 or herbimycin A (tyrosine kinase inhibitors). EGF increased Ca^{2+} influx and protein kinase C (PKC) translocation from the cytosolic compartment to the membrane compartment. Moreover, pre-treatment with BAPTA-AM (an intracellular Ca^{2+} chelator), EGTA (an extracellular Ca^{2+} chelator), bisindolylmaleimide I or staurosporine (PKC inhibitors) inhibited the EGF-induced phosphorylation of Cx43. EGF induced phosphorylation of p38 and p44/42 MAPKs, and this was blocked by SB 203580 (a p38 MAPK inhibitor) and PD 98059 (a p44/42 MAPK inhibitor), respectively. EGF or 18α -glycyrrhetic acid (GA; a gap junction inhibitor) increased expression levels of the protooncogenes (*c-fos*, *c-jun* and *c-myc*), cell cycle regulatory proteins [cyclin D1, cyclin E, cyclin-dependent kinase 2 (CDK2), CDK4 and p-Rb], [^3H]thymidine incorporation and cell number, but decreased expression levels of the p21^{WAF1/Cip1} and p27^{Kip1}, CDK inhibitory proteins. Transfection of Cx43 siRNA also increased the level of [^3H]thymidine incorporation and cell number. EGF, 18α -GA or transfection of Cx43 siRNA increased 2-DG uptake and GLUT-1 protein expression. *Conclusions:* EGF-induced phosphorylation of Cx43, which was mediated by the Ca^{2+} /PKC, p44/42 and p38 MAPKs pathways, partially contributed to regulation of mouse embryonic stem cell proliferation.

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INTRODUCTION

Gap junctions are composed of transmembrane proteins called connexins; they play important regulatory roles in embryonic development, in cell population growth control, and in maintaining cellular homeostasis by allowing passage of small molecules between adjacent cells (Loewenstein 1979; Guthrie & Gilula 1989; De Maio *et al.* 2002). Earlier studies by Oyamada *et al.* (1996) showed that transcripts of connexin 43 (Cx43) were present in undifferentiated mouse ES cells (J1 cell line). Moreover, Nishi *et al.* (1991) detected Cx43 mRNA transcripts in a further mouse embryonic stem (ES) cell line (CCE), in undifferentiated F9 embryonic carcinoma cells, and in the inner cell mass of the mouse embryo. Other studies have also demonstrated the presence of Cx43 protein in undifferentiated P19 cells (Belliveau *et al.* 1997), NT2/D1 cells (Bani-Yaghoub *et al.* 1997), and pluripotent mouse and human ES cells, respectively. Recently, gap junction intercellular communication, deficient in mouse ES cells, induced loss of their pluripotent state (Todorova *et al.* 2008). However, the roles of gap junctions and connexins in ES cell proliferation are unclear and are still under investigation. In most cell types, changed gap junctional intercellular communication is involved in cell cycle progression, and gap junctional intercellular communication decreases in G₁, S and M phases (Ruch 1994). Several early reports demonstrated developmental abnormalities following severe restriction of embryonic gap junctions by injection of blocking antibodies (Lee *et al.* 1987) or connexin antisense RNA (Bevilacqua *et al.* 1989). 18 α -glycyrrhetic acid, *in vitro*, was also established in differentiation of human NT2/D1 cells (Bani-Yaghoub *et al.* 1999). However, physiological properties of ES cell gap junctions have not been characterized and there is no direct evidence to show that gap junctions have a role in growth factor-induced proliferation of ES cells.

Epidermal growth factor (EGF) is a powerful mitogen, which has an important role in the control of cell population growth, cell survival and mitogenesis, but not much is known concerning the effect of EGF on Cx43 expression in ES cells. The mitogenic effect of EGF has been shown to be related to activity of receptor tyrosine kinases, which induce other proteins' phosphorylation, and is associated with signal transduction from the plasma membrane to the nucleus (Maldonado *et al.* 1988). EGF induces serine phosphorylation on Cx43, mediated primarily by protein kinase A, protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and tyrosine kinase (Godwin *et al.* 1993; Kanemitsu & Lau 1993; Lampe & Lau 2004). It is generally believed that up-regulation of gap junctional intercellular communication is associated with inhibition of cell population growth, while down-regulation of gap junctional intercellular communication correlates with stimulation of its growth (Loewenstein & Rose 1992), although the molecular mechanisms remain largely unknown. Therefore, based on information from emerging literature showing that Cx43 has regulatory effects on proliferation in cell lines, we have hypothesized that Cx43 could be an important factor in EGF-induced ES cell proliferation. However, mechanisms relating the relationship between gap junctional communication and self-renewal of ES cells are still not fully understood.

This study used mouse ES cells, which have the ability to differentiate into cells of all the three germ layers, and have unlimited growth potential under certain conditions (Cogle *et al.* 2003). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with a leukaemia inhibitory factor (LIF) to maintain the undifferentiated state and to support derivation and expansion of ES cells (Evans & Kaufman 1981; Xie *et al.* 2002). These cells closely resemble their *in vivo* counterparts and provide a stable *in vitro* model of embryonic growth and development, as well as acting as a tool whereby a specific signalling system can be investigated (Heo *et al.* 2007; Lee *et al.* 2007, 2008). In this study, we tested the hypothesis that

increased Cx43 phosphorylation following stimulation of mouse ES cells with EGF would contribute to proliferation and its related signalling pathways.

MATERIALS AND METHODS

Materials

Mouse ES cells were obtained from the American Type Culture Collection (ES-E14TG2a). Foetal bovine serum was purchased from Biowhittaker (Walkersville, MD, USA). EGF, EGTA, AG 1478, herbimycin A, 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), PD 98059, SB 203580, staurosporine and Lucifer Yellow CH were acquired from Sigma Chemical Company (St. Louis, MO, USA). The Fluo-3/AM was obtained from Molecular Probes Inc. (Eugene, OR, USA). Bisindolylmaleimide I was supplied by Calbiochem (LaJolla, CA, USA). [^3H]thymidine and [^3H]-2-deoxyglucose were purchased from NEN (Boston, MA, USA), and anti-Pan PKC was obtained from Upstate Biotechnology (Charlottesville, VA, USA). Antibodies to phospho-Cx43, Ras, GLUT-1, cyclin D1, cyclin E, cyclin-dependent kinase 2 (CDK2), CDK4, p21^{Cip1}, p27^{Kip1}, p-Rb (Ser780) and β -actin were acquired from Santa Cruz Biotechnology (Delaware, CA, USA). Phospho-p44/42 MAPKs, p44/42 MAPKs, phospho-p38 MAPK and p38 MAPK antibodies were supplied by Cell Signalling Technology (Herts, UK), and goat anti-rabbit immunoglobulin G (IgG) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Liquiscint was obtained from National Diagnostics (Parsippany, NJ, USA). All other reagents were of the highest purity commercially available.

Embryonic stem cell culture

The mouse ES cells cultures were prepared using a modification of the method reported by Smith & Hooper (1987). They were cultured in 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 LIF, and 15% foetal bovine serum without a feeder layer, for 5 days in standard medium plus LIF. The cells were grown on either gelatinized 12-well plates or 60 mm culture dishes, in an incubator maintained at 37 °C with 5% CO₂. The medium was changed to serum-free DMEM with LIF for 24 h before the experiments.

Alkaline phosphatase staining

Cells were washed twice with phosphate-buffered saline (PBS) and were fixed for 15 min with 4% formaldehyde (in PBS) at room temperature. After washing with PBS, alkaline phosphatase substrate solution [200 $\mu\text{g/mL}$ naphthol AS-MX phosphate, 2% N,N-dimethylformamide, 0.1 M Tris (pH 8.2), and 1 mg/mL Fast Red TR salt (4-chloro-2-methylbenzenediazonium salt; zinc chloride)] for 10 min at room temperature. After washing with PBS, the cells were photographed.

RNA isolation and RT-PCR

Total RNA was extracted from the cells using STAT-60, which is a monophasic solution of phenol and guanidine isothiocyanate purchased from Tel-Test Inc. (Friendwood, TX, USA; <http://www.bioresearchonline.com>). Reverse transcription was carried out using 3 μL of RNA using a reverse transcription system kit (AccuPower® RT PreMix, Bioneer, Daejeon, South Korea; <http://www.bioneer.com>) with oligo(dT)18 primers. Five microlitres of RT products was then

amplified using a PCR kit (AccuPower® PCR PreMix, Bioneer) under the following conditions: denaturation at 94 °C for 5 min and 30 cycles at 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 5 min of extension at 72 °C. The primers used were 5'-CGTGAGACTTTGC AGCCTGA-3' (sense), 5'-GGCGATGTAAGTGATCTGCTG-3' (antisense) for Oct-4 (519 bp), 5'-GTGGAATTTGTCCGAGAC-3' (sense), 5'-TGGAGTGGGAGGAGGAGGTAAC-3' (antisense) for SOX-2 (550 bp), 5'-TCTTACATCGCGCTCATCAC-3' (sense), 5'-TCTTGACGAAGCAG TCGTTG-3' (antisense) for FOX-D3 (171 bp), 5'-CGTTGCAGACTGAGATTGCC-3' (sense), 5'-ACCGGACAGGTCCACATCTG-3' (antisense) for *c-fos* (356 bp), 5'-AACTCGGACCT-TCTCACGTCG-3' (sense), 5'-TGCTGAGGTTGGCGTAGACC-3' (antisense) for *c-jun* (355 bp), and 5'-TCCATTCCGAGGCCACAGCAAG-3' (sense), 5'-TCAGCTCGTTCCTCCTCTGACG-3' (antisense) for *c-myc* (266 bp). PCR of β -actin was also performed as control for the quantity of RNA. RT-PCR products were separated and visualized on 1.2% agarose gels. Data were then normalized to data obtained for β -actin.

Western blot analysis

Cell homogenates (20 μ g protein) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane. Blots were washed with TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], blocked with 5% skimmed milk for 1 h and incubated with appropriate primary antibodies at dilutions recommended by the supplier. Membranes were then washed, and primary antibody locations were detected using goat anti-rabbit IgG or goat anti-mouse IgG, conjugated to horseradish peroxidase. Bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Preparation of cytosolic and total membrane fractions

Preparation of cytosolic and total membrane fractions was performed using a slightly modified methodology of that reported by Mackman *et al.* (1991). DMEM of the mouse ES cells was exchanged at 12 h before the experiments. Medium was then removed and cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation, and re-suspended in a buffer A [137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 mg/mL leupeptin (pH 7.5)]. Re-suspended cells were then lysed mechanically on ice by trituration with a 21.1-G needle and lysates were initially centrifuged at 1000 *g* for 10 min at 4 °C. The supernatant was centrifuged at 100 000 *g* for 1 h at 4 °C to prepare cytosolic and total particulate fractions. The particulate fraction, which contained the membrane fraction, was washed twice and re-suspended in buffer A containing 1% (v/v) Triton X-100. Protein in each fraction was quantified using the Bradford procedure (1976).

Scrape loading/dye transfer assay

The gap junctional intercellular communication level was measured using a slightly modified scrape loading/dye transfer (SL/DT) method (El-Fouly *et al.* 1987; Zhang *et al.* 2006). The SL/DT assay was carried out using a non-cytotoxic dose of the samples, as determined by MTT assay. Cells on 35-mm dishes were rinsed twice with 2 mL of PBS, and 0.05% Lucifer Yellow was then applied to the centre of the dishes. Scrapes were performed in such a way to ensure that the scrape traversed a large group of confluent cells. Cells were incubated in the dye mix for precisely 1 min, rinsed quickly three times with PBS, and finally were examined by optical microscopy (fluoview 300, Olympus, Tokyo, Japan). Lucifer Yellow is a small molecule (443 Da) that can move freely through gap junctions from loaded cells to the neighbouring ones (Le & Musil 1998).

[³H]thymidine incorporation

[³H]thymidine incorporation experiment was performed using methodology described by Brett *et al.* (1993). Briefly, the mouse ES cells were serum-starved for 24 h before stimulating them with EGF. Immediately, before commencing the study, medium had been changed to DMEM in order to further supplement the EGF without serum. Cells were incubated in medium with or without EGF for 24 h, and then were pulsed with 1 μ Ci of [methyl-³H]thymidine for 1 h at 37 °C. The cells were washed twice with PBS, fixed in 10% trichloroacetic acid at 23 °C for 15 min, and then were washed twice with 5% trichloroacetic acid. Acid-insoluble material was dissolved in 2 N NaOH for 12 h at 23 °C. Aliquots were removed to determine the level of radioactivity using a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA, USA). All values are reported as mean [$\pm$ standard errors (SE)] of triplicate experiments. Values were converted from absolute counts to a percentage of the control in order to allow comparison between experiments.

Cell proliferation assay

In order to determine the number of cells, cultures were washed twice with PBS and then were trypsinized from the culture dishes. Cell suspensions were mixed with a 0.4% (w/v) trypan blue solution, and the number of live cells was determined using a haemocytometer. Cells failing to exclude the dye were considered non-viable.

2-Deoxyglucose uptake

2-Deoxyglucose (2-DG) uptake experiments were carried out using the methodology reported by Chirayath *et al.* (1998) and Lee *et al.* (2007); it was examined using the following procedure. Culture medium was removed by aspiration, and cells were gently washed twice with the uptake buffer [140 mM NaCl, 2 mM KCl, 1 mM KH₂PO₄, 10 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 5 mM L-alanine, 5 μ M indomethacin, and 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, (HEPES)/Tris, pH 7.4]. After the washing, cells were incubated in an uptake buffer containing 1 μ Ci/mL 2-DG at 37 °C for 30 min. At the end of the incubation period, cells were again washed three times with ice-cold uptake buffer, and were digested in 1 mL 0.1% sodium dodecyl sulfate. Intracellular 2-DG uptake was determined by removing 900 μ L of each sample and measuring the radioactivity using a liquid scintillation counter. The remainder of each sample was used to determine the protein level (Bradford 1976). Radioactivity of each sample was normalized to the protein level, and was corrected for zero-time uptake per milligram protein. All uptake measurements were performed in triplicate.

Cx43 small interfering ribonucleic acid transfection

The mouse ES cells were grown until they reached 50% confluence in each dish and then were transfected for 24 h with either ONTARGETplus SMARTpool small interfering RNAs (siRNAs) targeting Cx43 (200 pmol/L; cat. no. L-001042-00, Dharmacon Inc., Lafayette, CO, USA) or non-targeting siRNA (as negative control, 200 pmol/L; cat. no. D-001210-01-05, Dharmacon Inc.) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Measurement of intracellular Ca²⁺ concentration

The changes in [Ca²⁺]_i were monitored using Fluo-3/AM that had initially been dissolved in dimethyl sulfoxide and stored at -20 °C. Cells in the 35-mm culture dishes were rinsed twice with a bath solution [140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM glucose, 5.5 mM HEPES (pH 7.4)], were incubated in the bath solution containing 3 μ M Fluo-3/AM in an atmosphere containing 5% CO₂-95% O₂ at 37 °C for 40 min, rinsed again twice with bath

Figure 1. Characteristics of the undifferentiated state of mouse embryonic stem (ES) cells. (a) Alkaline phosphatase enzyme activity was measured as a representative marker of undifferentiated ES cells as described in the Materials and Methods section. The cells were treated with EGF (100 ng/mL), 18α-GA (10 μg/mL) or pre-treated with 18α-GA prior to EGF treatment for 24 h. (b) The cells were either cultured in EGF, 18α-GA or pretreated with 18α-GA before exposing them to EGF for 24 h. Oct-4, SOX2 and FOXD3 mRNA levels were determined by RT-PCR. The example shown is a representative of five independent experiments.

solution, mounted on to a perfusion chamber, and were scanned once a second using confocal microscopy (×400; fluoview 300, Olympus). Fluorescence was excited at 488 nm and emitted light was observed at 515 nm. All analyses of $[Ca^{2+}]_i$ were processed in a single cell, and the results are expressed as relative fluorescence intensity ($F/F_0\%$, arbitrary unit, F is the fluorescence captured at a particular time and F_0 is the initial fluorescence image).

Statistical analysis

Results are expressed as a mean ± SE. All the experiments were analysed by analysis of variance. In some experiments, this was followed by a comparison of the treatment means with the control using a Bonnferroni–Dunn test. A P value of < 0.05 was considered significant.

RESULTS

Effects of EGF on Cx43

To confirm the undifferentiated state of the mouse ES cells used in the experiments, we examined markers of undifferentiated stem cells, including alkaline phosphatase activity, and Oct-4 (POU domain transcription factor). As shown in Fig. 1a, the cells were either cultured in EGF, 18α-glycyrrhetic acid (GA), or were pre-treated with 18α-GA before exposing them to EGF for 24 h maintained for alkaline phosphatase enzyme activity. The cells were treated with EGF (100 ng/mL), 18α-GA (10 μg/mL), or were pre-treated with 18α-GA before the EGF treatment for 24 h and expressed equivalent levels of Oct-4, SOX-2 and FOXD3 mRNA as control cells (Fig. 1b). Therefore, the present results show that mouse ES cells maintained an undifferentiated state under our experimental conditions.

As shown in Fig. 2a, EGF (100 ng/mL) increased the level of Cx43 phosphorylation from 5 min, to reach a maximum at 60 min. When the cells were treated with different EGF

Figure 2. Effect of EGF on the Cx43 phosphorylation. (a) Cells were incubated in the presence of EGF (100 ng/mL) for various periods of time (0–360 min) under serum-free conditions. Phosphorylation of Cx43 was detected as described in the Materials and Methods section. (b) Cells were incubated with various concentrations of EGF (0–200 ng/mL) for 1 h and the level of Cx43 phosphorylation was examined by Western blotting. Cells were treated with AG 1478 (10^{-5} M) or herbimycin A (tyrosine kinase inhibitors, 10^{-6} M) for 30 min before the EGF treatment (100 ng/mL) for 1 h. Ras and phosphorylated Cx43 were detected by Western blotting. (c and d) The example shown is representative of four independent experiments. The lower part (a, b, c, d) depicting the bars denote the mean $\pm$ SE of four independent experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ versus control; ** $P < 0.05$ versus EGF alone.

concentrations (0–200 ng/mL) for 1 h, the level of Cx43 phosphorylation significantly increased from 10 ng/mL EGF (Fig. 2b). Involvement of EGF receptor tyrosine kinase activity in EGF-induced Cx43 phosphorylation was examined by pre-incubating the cells with AG 1478 (10^{-5} M) and herbimycin A (tyrosine kinase inhibitors, 10^{-6} M) for 30 min before EGF treatment. As shown in Fig. 2c,d, either AG 1478 or herbimycin A inhibited EGF-induced activation of Ras and Cx43 phosphorylation. Each inhibitor alone did not affect Cx43 phosphorylation (data not shown). An SL/DT assay was used to measure the gap junctional intercellular communication level. In the control, Lucifer Yellow diffused through the neighbouring cells, while 100 ng/mL EGF treatment for 1 h completely inhibited it. 18 α -GA (a gap junction channel blocker; 10 μ g/mL) also inhibited Lucifer Yellow transfer between the cells (Fig. 3).

Involvement of $[Ca^{2+}]_i$, PKC and MAPKs in EGF-induced phosphorylation of Cx43

Experiments were performed to determine whether Ca^{2+} , PKC and MAPK pathways were involved in the EGF-induced phosphorylation of Cx43. EGF stimulated Ca^{2+} influx (Fig. 4a) and PKC translocation from the cytosol to the membrane compartment (Fig. 4b). EGF-induced

Figure 3. Fluorescence photomicrographs of scrape/scratch experiments using Lucifer Yellow. (a) Mouse ES cells treated with EGF (100 ng/mL) or 18α-GA (10 μg/mL) and gap junctional intercellular communication analysis carried out using a SL/DT assay, as described in the Materials and Methods section. Scale bars, 20 μm (magnification, ×400). (b) Gap junction intercellular communication was monitored by counting cells stained with Lucifer Yellow CH dye. The example shown is a representative of nine independent experiments. * $P < 0.05$ versus control.

phosphorylation of Cx43 was blocked by either BAPTA-AM (an intracellular Ca^{2+} chelator, 10^{-5} M) or EGTA (an extracellular Ca^{2+} chelator, 4×10^{-3} M) (Fig. 4c) and bisindolymaleimide I or staurosporine (PKC blockers, 10^{-7} M) (Fig. 4d), respectively. In experiments aimed at determining involvement of MAPKs in EGF-induced phosphorylation of Cx43, phosphorylation of p38 MAPK was gradually increased in a time-dependent manner (Fig. 5a). In addition, the maximum level of p44/42 MAPK phosphorylation appeared 30 min after EGF treatment (Fig. 5b). PD 98059 (a p44/42 MAPK inhibitor, 10^{-5} M) or SB 203580 (a p38 MAPK inhibitor, 10^{-6} M) blocked EGF-induced phosphorylation of Cx43 (Fig. 5c,d). A EGF-induced increase in [^3H]thymidine incorporation was inhibited by these blockers (Fig. 5e). Each inhibitor alone did not affect Cx43 phosphorylation (data not shown). These results indicate the role of Ca^{2+} , PKC and MAPKs in EGF-induced phosphorylation of Cx43.

Relationship between Cx43 and cell cycle regulatory proteins

Experiments were carried out to examine the effect of EGF on protooncogene regulation. EGF increased levels of *c-jun*, *c-myc* and *c-fos* mRNA expression, and these changes were observed when the cells were treated with 18α-GA alone or EGF plus 18α-GA (Fig. 6). The cells were incubated with EGF (100 ng/mL) to determine whether EGF-induced phosphorylation of Cx43 is involved in an increase of mouse ES cell proliferation. In order to investigate the effect of 18α-GA on phosphorylation of Cx43, phospho-Cx43 was detected using Western blot analysis. 18α-GA did not induce phosphorylation of Cx43 (data not shown). As shown in Fig. 7a, EGF, 18α-GA, or a combined treatment, increased levels of cyclin D1, cyclin E, CDK2, CDK4 and p-Rb expression, while decreased levels of cyclin-dependent kinase inhibitory proteins, p21^{Cip1} and p27^{Kip1}. EGF, 18α-GA, or a combined treatment also increased levels of [^3H]thymidine incorporation (Fig. 7b) and cell number (Fig. 7c). In addition, cells were transfected with a pool of Cx43-specific siRNAs (200 pmol/L) or non-targeting siRNAs (200 pmol/L). In this experiment, disruption of Cx43 using Cx43 siRNA enhanced EGF-induced increase in [^3H]thymidine incorporation (Fig. 7d) and cell number (Fig. 7e). In a further study, EGF increased GLUT-1 protein expression level and 2-DG uptake (Fig. 8a,b). Moreover, transfection of Cx43 siRNA enhanced the EGF-induced increase in 2-DG uptake (Fig. 8c). This suggests that Cx43 is involved in EGF-induced cell proliferation and 2-DG uptake.

Figure 4. Effect of the $[Ca^{2+}]_i$ /PKC inhibitors on EGF-induced phosphorylation of Cx43. (a) Cells were loaded with 2 μ M Fluo-3/AM in serum-free medium for 40 min and treated with EGF (100 ng/mL), then EGF-induced Ca^{2+} influx was measured. Changes in $[Ca^{2+}]_i$ were monitored by confocal microscopy, and data are expressed as fluorescence intensity (F/F₀%, arbitrary unit). (b) The pan-PKC protein present in either the cytosolic compartment or the membrane compartment was detected by Western blotting. (c) Cells were pre-treated with BAPTA-AM (intracellular Ca^{2+} chelator, 10^{-5} M) or EGTA (extracellular Ca^{2+} chelator, 4×10^{-3} M) for 30 min before treatment with EGF for 1 h. Phosphorylation of Cx43 was then detected by Western blotting. (d) Cells were pre-treated with bisindolymaleimide I or staurosporine (PKC inhibitors, 10^{-7} M) for 30 min before treatment with EGF for 1 h. Phosphorylation of Cx43 was then detected by Western blotting. The example shown is representative of four independent experiments. The lower panels of (b), (c) and (d) depicting the bars denotes the mean $\pm$ SE of four experiments for each condition determined from densitometry relative to β -actin. * P < 0.05 versus control; ** P < 0.05 versus EGF alone.

DISCUSSION

The present results demonstrate that EGF-induced phosphorylation of Cx43, mediated by Ca^{2+} /PKC, p44/42 and p38 MAPKs pathways, partially contributed to regulation of mouse ES cell proliferation. Although a non-physiological dose of EGF was used here, previous studies have shown that in media enriched with EGF, IGF-I and IGF-II (1, 10, 100 and 1000 ng/mL),

Figure 5. Effect of the MAPK inhibitors on the EGF-induced phosphorylation of Cx43. Representative autoradiographs of the time course of p38 (a) and p44/42 MAPKs (b) phosphorylation by EGF. Cells were treated with EGF for different times (0–120 min). Then, they were treated with SB 203580 (10^{-6} M) (c) and PD 98059 (10^{-5} M) (d) for 30 min before the EGF treatment for 1 h. Phosphorylated Cx43 protein was detected by Western blotting, as described in the Materials and Methods section. The example shown is a representative of five independent experiments. The lower parts depicting the bars denotes means $\pm$ SE of five experiments for each condition determined from densitometry relative to total-p38 (a), total-p44/42 (b) and β -actin (c, d). (e) Cells were pre-treated with PD 98059 or SB 203580 for 30 min before the EGF treatment for 24 h, and then pulsed with 1 μ Ci of [^{3} H]thymidine for 1 h. The values are reported as the mean of five independent experiments with triplicate dishes. * P < 0.05 versus control; ** P < 0.05 versus EGF alone.

a significant increase in blastocyst cell proliferation rate, total blastocyst cell numbers and inner cell mass cell numbers were found when compared to controls (Glabowski *et al.* 2005). Moreover, in stem cell studies for examination of self-renewal or differentiation, diverse growth factors have been used as a high dose; neural stem cells were proliferated in EGF and FGF2 (from 0.6 to 80 ng/mL) and retained their self-renewing and multilineage potential (Tropepe *et al.* 1999; Pitman *et al.* 2004; Lange *et al.* 2005). In the present study, >100 ng/mL EGF significantly stimulated Cx43 phosphorylation compared to control levels, although 10 ng/mL EGF increased Cx43 phosphorylation slightly. Thus, we suggest that differences in effectiveness of various EGF concentrations may be due to unknown quality of EGF, difference of cell types, marker indices or experimental conditions (*in vitro* versus *in vivo*, serum versus serum-free media). Previous results demonstrated that the activated EGF receptor interacts with and phosphorylates several substrates with known biochemical functions, such as phospholipase C- γ , the Ras GTPase-activating protein and numerous protein kinases. In the present study, observation that PKC activation by EGF induces Cx43 phosphorylation suggests the possibility that EGF down-regulates

Figure 6. Effect of gap junction inhibitor on the EGF-induced stimulation of *c-fos*, *c-myc* and *c-jun* mRNA expression. Cells were either cultured in EGF, 18 α -GA, or were pre-treated with 18 α -GA before exposing them to EGF for 1 h. *c-fos*, *c-jun* and *c-myc* mRNA expression was then determined by RT-PCR. Bands represent 356 bp for *c-fos*, 355 bp for *c-jun*, 266 bp for *c-myc* and 350 bp for β -actin. Each example shown is representative of five independent experiments. The lower panel depicts the bars denote means $\pm$ SE of five independent experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ versus control.

gap junctional intercellular communication through PKC-dependent events, which might mediate the Ras effect. Indeed, the phorbol ester, TPA, is a classical inhibitor of cell communication in most cells examined (Ren *et al.* 1998). In parallel with our results, stimulation of Cx43 phosphorylation in T51B cells by EGF suggests the involvement of a protein kinase that transduces the signals from activated EGF receptors intracellularly (Kanemitsu & Lau 1993). PKC has been shown to activate a MAPK pathway, demonstrating cross-talk between it and PKC (Adams & Parker 1991; Schonwasser *et al.* 1998). Our results also demonstrated that Cx43 phosphorylation by EGF-induced PKC activation is mediated through MAPKs in mouse ES cells. This result is supported by the observation that several copies of the ERK1/2 consensus phosphorylation motif are present in the cytoplasmic C-terminal tail of Cx43 (Warn-Cramer *et al.* 1998; Camoron *et al.* 2003) and c-Jun N-terminal kinase and p38 MAPK directly inhibit gap junctional intercellular communication (Petrich *et al.* 2002; Polontchouk *et al.* 2002). To our knowledge, this is the first study that demonstrates involvement of MAPKs in regulation of Cx43 in mouse ES cells treated with EGF. Therefore, there is a possibility that Cx43 will join the growing number of MAPK substrates that include *c-myc* and *c-jun* as shown here. In the present study, treatment of 18 α -GA also increased mRNA levels of *c-myc*, *c-fos* and *c-jun*. 18 α -GA is a non-toxic gap junction inhibitor which reduces gap junction coupling; however, mechanisms of 18 α -GA are still unclear. A previous study has shown that 18 α -GA treatment induced not only Cx regulation but also endogenous expression of C/EBP (CAAT/enhancer-binding) α in skeletal muscle cells (Yamanouchi *et al.* 2007). C/EBP is a transcription factor, to which 3',5'-cyclic

Figure 7. Effect of the gap junction inhibitor on EGF-induced cell proliferation. (a) Cells were treated with EGF (100 ng/mL), 18α-GA (10 μg/mL), or pre-treated with 18α-GA prior to EGF treatment for 4 h, and then total lysates were subjected to SDS-PAGE and blotted with cyclin D1, cyclin E, CDK2, CDK4, p21, p27 and pRb antibodies. Each example shown is representative of four independent experiments. The cells were treated with EGF (100 ng/mL), 18α-GA (10 μg/mL) or pre-treated with 18α-GA prior to EGF treatment for 24 h, and then [³H]thymidine incorporation (b) and cell number counting (c) were carried out. Values are reported as the mean ± SE of four independent experiments with triplicate dishes. *P < 0.05 versus control; **P < 0.05 versus EGF alone. Cells were transfected with either a SMARTpool of Cx43 siRNAs (200 pmol/L) or a non-targeting control siRNA (200 pmol/L) using Lipofectamine 2000 and incubated for 24 h before exposing them to EGF for 24 h. Then, [³H]thymidine incorporation (d) and cell number counting (e) were carried out. Values are reported as a mean ± SE of four independent experiments. *P < 0.05 versus control; **P < 0.05 versus EGF alone.

adenosine monophosphate (cAMP) regulatory element can bind (Croniger *et al.* 1998). Consequently, cAMP-dependent pathways are known to activate various transcription factors, including *c-fos*, *c-myc* and *c-jun* (Ran *et al.* 1986; Murakami *et al.* 1991). Consistent with our results, association between Cx43 and several genes responsible for regulating the cell cycle are well documented (Tabernero *et al.* 2006).

A common early response of many cells to mitogens is rapid and transient inhibition of cell communication, suggesting that a cell may have to decrease its gap junction contact with its neighbours before it can undergo cell division (Madhukar *et al.* 1989; Moorby *et al.* 1995; Postma *et al.* 1998). Our scrape-loading study with Lucifer Yellow dye-coupling showed that the gap junction intercellular communication capacity was lower in EGF-treated mouse ES cells compare to controls. On the other hand, levels of cyclins D1 and E increased as a consequence

Figure 8. Effect of the gap junction inhibitor on EGF-induced stimulation of 2-DG uptake. (a) Cells were incubated with EGF or 18 α -GA for 24 h. Level of GLUT-1 protein expression was determined by Western blotting with plasma membrane protein as described in the Materials and Methods section. Bands represent 50–60 kDa of GLUT-1, and 41 kDa of β -actin, respectively. The example shown is representative of four independent experiments. The lower panel of (a) showing the bars denotes the mean $\pm$ SE of four independent experiments for each condition determined from densitometry relative to β -actin. (b) Cells were treated with EGF (100 ng/mL), 18 α -GA (10 μ g/mL) or pre-treated with 18 α -GA before the EGF treatment for 24 h. Level of 2-DG uptake was determined as described in the Materials and Methods section. (c) Cells were transfected with either a SMARTpool of Cx43 siRNAs (200 pmol/L) or a non-targeting control siRNA (200 pmol/L) using Lipofectamine 2000 and were incubated for 24 h before exposing them to EGF for 24 h and then 2-DG uptake examined. Values are reported as the mean $\pm$ SE of four independent experiments with triplicate dishes. * P < 0.05 versus control; ** P < 0.05 versus EGF alone.

of EGF-induced phosphorylation of Cx43. It could thus be proposed that these cyclins would be responsible for activating phosphorylation of p-Rb due to down-regulation of CDK inhibitors p21 and p27, and hence for the transition of the cells from the G_0/G_1 to S phase, observed after phosphorylation of Cx43. These results suggest that EGF-induced phosphorylation of Cx43 in ES cells can interrupt intercellular passage of cell regulatory molecules, which might be involved in mediating cell proliferation. Our results are consistent with previous data showing that thyroid hormone affects developmental expression of Cx43 (Stock *et al.* 1998; Gilleron *et al.* 2006) as well as Sertoli cell proliferation *in vivo* (Van Haaster *et al.* 1993) and *in vitro* (Cooke *et al.* 1994). Based on these results, we hypothesized that Cx43 actions on cell proliferation may have effects on cell cycle regulatory proteins. The present study has shown that 18 α -GA regulated cell cycle regulatory proteins. This mechanism can be associated with an increased level of the protooncogene *c-myc* by 18 α -GA. In agreement with this hypothesis, previous studies have shown that *c-myc* plays an important role in G_1/S transition as an upstream regulator of cell cycle regulatory proteins (Eilers *et al.* 1991). Alternatively, increased *c-myc* expression can inhibit the action of p21 and p27, major inhibitors of G_1 CDKs, and can relieve cyclin E/CDK2 inhibition to promote cell cycle progression (Prall *et al.* 1997). Moreover, as with the present results, it has been shown that inhibition of gap junctional intercellular communication can lead increased levels of p-Rb and D-type cyclins, which can respond to changes such as interruption of cell-to-cell communication (Matsushime *et al.* 1991; Tabernero *et al.* 2006). Even though the regulatory mechanism needs to be further verified, we suggest that inhibition of gap junctional intercellular communication by 18 α -GA is sufficiently effective to regulate cell cycle progression. Taken together, the results of our study provide evidence for involvement of

Figure 9. Hypothesized model for signal pathways involved through ES cell proliferation in EGF-induced phosphorylation of Cx43. EGF activates receptor tyrosine kinase, which stimulates Ca^{2+} , PLC and Ras. Then, PKC and MAPKs activate Cx43 phosphorylation. Finally, phosphorylation of Cx43 continuously induces an increase of protooncogene levels, cell cycle regulatory proteins and 2-DG uptake, partially contributing to regulation of ES cell proliferation. Cx43-p, Cx43 phosphorylation; EGFR, EGF receptor; TK, tyrosine kinase; PLC, phospholipase C; PKC, protein kinase C; CDK, cyclin-dependent kinase. The solid line is the proposed pathway and the dashed line is suspected pathway.

cyclins D1 and E as sensors of phosphorylation of Cx43 in mouse ES cells, and suggest that they may be mediators of the stimulatory effect of mouse ES cell proliferation observed after EGF treatment (Fig. 9). Furthermore, EGF-induced phosphorylation of Cx43 was involved in an increase of glucose uptake destined for an increase in glucose metabolism required for the higher rate of cell proliferation. Some studies have shown a parallel increase in expression of proliferation markers and GLUT1 (Ogawa *et al.* 1997; Furudoi *et al.* 2001). Moreover, expression of GLUT1 in fibroblasts is higher in proliferating cells when compared to contact-inhibited ones (Weber *et al.* 1984; Birnbaum *et al.* 1987). Thus, phosphorylation of Cx43 might contribute to maintaining mouse ES cells in a self-renewing state, in agreement with Todorova *et al.* (2008) who reported that gap junction intercellular communication is required to maintain ES cells in the proliferative state. Our data presented here provide the initial connection between Cx43 and cell cycle regulatory proteins plus a novel insight concerning how EGF signalling controls ES cell expansion and establishes the ES cell fate (Fig. 9). However, potential inter-relationships between Cx43 and cell cycle regulatory proteins still remain to be established in ES cells. In conclusion, EGF-induced phosphorylation of Cx43, which was mediated by the Ca^{2+} /PKC, p44/42 and p38 MAPKs pathways, partially contributed to regulation of mouse ES cell proliferation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of 18 α -GA on the Cx43 phosphorylation.

Figure S2. Effect of the EGF-induced stimulation of pan-PKC expression levels.

Figure S3. Effect of glucose uptake inhibitor on EGF-induced stimulation of [^{3} H]thymidine incorporation.

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