Atypical PKC, regulated by Rho GTPases and Mek/Erk, phosphorylates Ezrin during eight-cell embryo compaction

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Phosphorylation of Ezrin T567 plays an important role in eight-cell embryo compaction. Yet, it is not clear how Ezrin phosphorylation is regulated during embryo compaction. Here, we demonstrated that inhibition of Mek/Erk or protein kinase C (PKC) signaling reduced the phosphorylation level of Ezrin T567 in eight-cell compacted embryos. Interestingly, the Rho GTPase inhibitor C3-transferase caused basolateral enrichment of atypical PKC (aPKC), as well as basolateral shift of phosphorylated Ezrin, suggesting aPKC may be a key regulator of Ezrin phosphorylation. Moreover, inhibition of PKC, but not Mek/Erk or Rho GTPases, affected the maintenance of Ezrin phosphorylation in compacted embryos. We further identified that aPKC is indeed required for Ezrin phosphorylation in eight-cell embryos. Taken together, Rho GTPases facilitate the apical distribution of aPKC and Ezrin. Subsequently, aPKC and Mek/Erk work together to promote Ezrin phosphorylation at the apical region, which in turn mediates the apical enrichment of filamentous actin, stabilizing the polarized apical region and allowing embryo compaction. Our data also suggested that aPKC might be the Ezrin kinase during eight-cell embryo compaction.

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Introduction

During mouse preimplantation embryogenesis, the first cell differentiation event leads to the segregation of the inner cell mass (ICM) and the trophectoderm (TE), which initiates after the compaction of blastomeres at the late eight-cell stage. Upon compaction, blastomeres become polarized (Ducibella and Anderson, 1975; Johnson and Ziomek, 1981; Lehtonen, 1980; Ziomek and Johnson, 1980). A polarized blastomere can give rise to two polarized outside cells through symmetric division, or one apolar inside cell and one polarized outside cell through asymmetric division. Subsequently, inside cells and outside cells develop into the ICM and the TE, respectively (Chen et al., 2010; Cockburn and Rossant, 2010; Johnson, 2009; Johnson and McConnell, 2004; Zernicka-Goetz et al., 2009). Cell polarization appears to be a critical factor for the cell fate choice. Down-regulation of polarity molecules, such as PAR3 and the atypical protein kinase C (aPKC), promotes inside localization of blastomeres and the ICM fate (Plusa et al., 2005).

Many proteins have been shown to participate in eight-cell blastomere polarization, including apically distributed JAM1, the polarity protein PAR3 and PAR6, aPKC, Erk2, Ezrin and filamentous actin (F-actin), as well as basolaterally localized PAR1 and E-cadherin (Louvet et al., 1996; Lu et al., 2008; Pauken and Capco, 2000; Thomas et al., 2004; Vestweber et al., 1987; Vinot et al., 2005). Ezrin is a member of the ERM (Ezrin, Radixin, Moesin) protein family, which serves as cross-linkers between F-actin and the plasma membrane (Chen et al., 1995; Kondo et al., 1997; Lamb et al., 1997; Martin et al., 1995; Takeuchi et al., 1994). The cross-linker function of Ezrin requires the phosphorylation of T567. In resting cells, Ezrin is in a dormant state, where the F-actin binding site is masked through the interaction between the N-terminal ERM homology (FERM) domain and the C-terminal tail domain. Phosphorylation of Ezrin T567 disrupts the interaction between the FERM and tail domains, exposing the F-actin binding site (Bretscher et al., 2002; Matsui et al., 1998; Shaw et al., 1998; Simons et al., 1998). Ezrin is expressed throughout preimplantation embryo development. It is distributed evenly around the cell cortex from the zygote to the early eight-cell stage, and becomes restricted to the apical region after compaction (Louvet et al., 1996). It has been suggested that blastomere
polarization and compaction of eight-cell embryos rely on protein post-translational modifications, but not protein synthesis (Bloom and McConnell, 1990; Kidder and McClachlin, 1985; Levy et al., 1986). Consistently, phosphorylation of Ezrin T567 plays an important role in the compaction and the polarization of eight-cell embryos (Dard et al., 2004). The mutation of T567 into an aspartate (T567D), mimicking a phosphorylated residue, renders Ezrin to localize all around the cell cortex, instead of polarized apical distribution in eight-cell embryos. Consequently, the compaction of eight-cell embryos and the blastocoeil formation in blastocysts are compromised by the Ezrin-T567D mutation. Oppositely, replacing T567 by an alanine (T567A), which prevents phosphorylation at this residue, does not affect compaction at the eight-cell stage. However, developmental defect is observed at the 16-cell stage and later, due to the aberrant redistribution of the Ezrin-T567A mutant to the basolateral cortex and reduced surface of adherens junctions (Dard et al., 2004).

Moreover, some signaling molecules, such as PKC, Mek/Erk and Rho family GTPases, are also involved in blastomere polarization and compaction of the eight-cell embryo (Clayton et al., 1999; Lu et al., 2008; Maekawa et al., 2007; Winkel et al., 1990). Both PKC, and Erk2 show apical distribution in compacted eight-cell embryos (Lu et al., 2008; Faulken and Capco, 2000). Moreover, activation of PKC by phorbol ester leads to premature compaction at the four-cell stage, while inhibition of PKC with sphingosine blocks induced premature compaction as well as normal compaction of eight-cell embryos (Winkel et al., 1990). Mek/Erk signaling has been suggested to activate Cdx2 and to suppress Nanog, thereby regulating the differentiation of the ICM and the TE (Lu et al., 2008). Yet, the functional significance of the apical distribution of Erk2 in late eight-cell embryos remains unclear. Is it the cause or the consequence of blastomere polarization? Although there is no evidence for asymmetric distribution of Rho GTPases in eight-cell embryos, it has been demonstrated that Rho GTPases are required for blastomere polarization and eight-cell embryo compaction (Clayton et al., 1999). Even though many molecules involved in eight-cell blastomere polarization have been identified, the genetic and biochemical interactions of these molecules remain elusive. Here, we demonstrated that Mek/Erk, PKC and Rho GTPases regulate Ezrin T567 phosphorylation through different mechanisms. Rho GTPases regulate the distribution of aPKC, Ezrin and phosphorylated Ezrin (p-Ezrin). Both aPKC and Mek/Erk signaling are required for Ezrin T567 phosphorylation during embryo compaction. aPKC appears to be the Ezrin kinase in eight-cell embryos. Phosphorylation of Ezrin T567 in turn mediates the formation of F-actin at the apical surface, and promotes embryo compaction. Thus, our studies reveal a pivot role of Ezrin in eight-cell blastomere polarization and embryo compaction, as well as the regulation of Ezrin phosphorylation by multiple signaling pathways.

Materials and methods

Cell culture

V6.5 and iRasES Embryonic stem (ES) cells were cultured in Dulbecco’s modified Eagle’s medium (high glucose DMEM, GIBCO), supplemented with 15% fetal bovine serum (FBS, Hyclone), 2 mM l-glutamine, 5000 U/ml penicillin and streptomycin, 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), and 1000 U/ml LIF (ESGRO, Chemicon). To induce Ras expression, iRasES cells were plated on gelatin-coated tissue culture dish in mouse ES medium supplemented with 1 µg/ml doxycycline. ES-derived trophectoderm stem (ES-TS) cells (Lu et al., 2008) and trophectoderm stem (TS) cells were cultured in TS culturing medium containing RPMI 1640, 20% FBS (Hyclone), Fgf4 (25 ng/ml), heparin (1 ng/ml), 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM l-glutamine, penicillin and streptomycin (Invitrogen).

Phosphoproteomic analysis with mass spectrometry

Ras expression was induced in iRasES cells with 1 µg/ml doxycycline (Dox). Cells were harvested at indicated time points, and phosphoproteins were purified with Phosphoprotein Purification Kit (Qiagen). LC–MS/MS analysis was performed with the ThermoElectron Finnigan LTQ by the mass spec core facility in the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai.

Embryo culture

Female ICR mice (4–6 weeks) were induced to superovulate by intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin (PMSG, Calbiochem) and 48 h later 5 IU human chorionic gonadotropin (hCG, Sigma). Then females were paired with ICR males overnight and checked for vaginal plugs the following morning. Two-cell embryos were flushed from oviducts at 42–48 h post-hCG and cultured in groups of 20–30 in a 50 µl droplet of potassium simplex optimization medium (KSO) with amino acids (Millipore) covered by mineral oil (Sigma, for embryo culture) in a 37 °C incubator with 6.5% CO2. Inhibitors were added at appropriate stages at the following concentrations: PD98059 (Calbiochem), 20 µM; PD0325901 (Sigma), 1 µM; C3-transferase (Cytoskeleton), 1 µg/ml; H-1112 (Calbiochem), 1 µM; cytchalasin D (Calbiochem), 0.5 µg/ml; ω-Sphingosine (Sigma), 2.5 µM; Ro-31-8220 (Calbiochem), 5 µM; Gö6976 (Calbiochem), 1 µM; PKCζ pseudosubstrate inhibitor (Myristoylated-SIYRRGARRW/KL-0H, Calbiochem), 10 µM. All experiments were performed with groups of more than 10 embryos and repeated three times.

Immunofluorescence

Embryos at desired stages were fixed in 4% paraformaldehyde for 20 min, and then permeabilized with 0.2% Triton X-100 for 30 min. After being blocked with 5% goat serum for 2 h, embryos were incubated with primary antibodies for 4–6 h at room temperature or overnight at 4 °C. Then embryos were washed and incubated with secondary antibodies and/or rhodamine-phalloidin (Molecular Probe). We used the following primary antibodies: anti-Ezrin (BD Transduction Laboratories), phospho-Ezrin (Thr567)/Radixin (Thr564/Moesin (Thr558) antibody (Cell Signaling Technology), anti-ε-Cadherin (BD Transduction Laboratories), anti-aPKC (Santa Cruz). We used Alexa Fluor 488 anti-mouse, Alexa Fluor 488 anti-rabbit and Alexa Fluor 594 anti-rabbit as secondary antibodies (Molecular Probe), and Hoechst 33342 (Sigma) for nuclei staining. Epifluorescent images were taken with Olympus IX81 microscope. Confocal images were captured using Leica TCS SP5 confocal microscope.

Western blot

Cells were lysed, and total protein concentration was measured using BCA Protein Assay Kit (Beyotime) to ensure equal loading in western blot analysis. The samples were resolved by SDS-PAGE followed by transferring onto a PVDF membrane (Millipore). Membranes were probed with anti-phospho-ERM antibody (Cell Signaling Technology), anti-Ezrin (Sigma), anti-Ras (Upstate), and anti-Actin (Abcam). Bound primary antibodies were recognized by HRP-linked secondary antibodies (GE Healthcare). HRP activity was detected by ECL Plus (Beyotime) and Kodak light film. For protein lysates from embryos, pools of
embryos were collected in the sample buffer, lysed and boiled for 10 min, followed by SDS-PAGE.

Real-time RT-PCR

Total RNA was extracted from pools of embryos using RNeasy Micro Kit (Qiagen) with an on-column DNA digestion using RNase-Free DNase Set (Qiagen). cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instruction. Transcript levels were determined by real-time PCR using SYBR Green Real-time PCR Master Mix (TOYOBO) in BioRad iQ5 system. Running conditions were as follows: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s, and then a melting curve of the amplified DNA was acquired. Quantification of target genes was normalized with β-Actin. Primer sequences were as follows: Ezrin forward GAAAGGAAACAGACCTTGGCCTGG, Ezrin reverse GCCTTCTTGTCGATGGGCTTAATG; β-Actin forward CAGAAGGAGATTACTGCTCTGGCT, β-Actin reverse TACTCCTGTGCTTGCTGATCCACATC; PKCε forward ATCCTGACTAGGCTTAAGGTCCTC; PKCε reverse CGCTGCATATGAAACATGAGGTCC.

Transfection of TS cells

Short interfering RNAs (siRNA) were synthesized by GenePharma Shanghai Corp. siRNA was transfected into TS cells at 70% confluence with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. 24 h later, cells were harvested. RNA was purified and subjected to real-time RT-PCR analysis. The siRNA sequences were as follows: PKCε-1 GGGAGAGCAAGTGCCATT; PKCε-2 CGCGTTCTCTGTTGTAAA; PKCε-3 CAGAGGGCATAGTGATTCT; PKCε-2 CGTGCAAGACAGAACATATCAGATCAGATGACTAT.

Two-cell embryo injection

For embryo injection, 2-cell embryos were collected from superovulated F1 females mated with F1 males (both C57BL/6xDBA). 10 μM control or PKC siRNAs, together with 0.03 μg/μl H2B-Cherry mRNA, were injected into one blastomere of late 2-cell stage mouse embryos. Microinjection was performed in M2 medium on a Leica DMI3000B inverted microscope equipped with Leica micromanipulator and Eppendorf FemtoJet microinjector as described elsewhere (Na et al., 2007). After injection, embryos were cultured until the 8-cell stage in KSOM medium in incubator at 37 °C supplied with 5% CO₂.

Results

Ezrin becomes phosphorylated upon Ras induction in ES cells

It has been demonstrated that ectopic expression of an activated form of human H-Ras (Hras1Q61L) in mouse embryonic stem (ES) cells activates the Mek/Erk signaling pathway, which in turn activates the key trophectodermal transcription factor Cdx2, and suppresses the pluripotency gene Nanog, resulting in differentiation toward trophectoderm (Lu et al., 2008). We utilized this technique to study the phosphorylation of Ezrin T567 in response to Ras induction. The results revealed that Ezrin becomes phosphorylated upon Ras induction in ES cells, which is consistent with previous studies. This finding suggests that Ezrin plays an important role in the regulation of trophectodermal differentiation.

Fig. 1. Phosphorylation of Ezrin T567 was associated with trophectodermal differentiation. (A) In iRasES cells, Ras induction increased the phosphorylation of Ezrin T567 (p-Ezrin), while the expression of Ezrin remained steady. (B) Immunoblot analysis revealed that the expression levels of Ezrin were similar in V6.5 ES cells, ES–TS and TS cells. However, the levels of p-Ezrin in ES–TS and TS cells were significantly higher than that in ES cells and (C) Confocal images of mouse embryos from the two-cell to the blastocyst stage. Phosphorylated Ezrin became detectable in compacted eight-cell embryos, and was distributed in the apical region. The apical enrichment of p-Ezrin was maintained throughout preimplantation embryogenesis. Hoechst 33342 staining marked the nucleus. Bar, 20 μm.
in vitro differentiation system to study how Mek/Erk signaling regulates the transcription of Nanog and Cdx2, as well as other potential functions of Mek/Erk signaling in trophectodermal differentiation. Mek/Erk signaling is transduced through a cascade of kinases. Thus, we set out to identify proteins being phosphorylated after Ras induction. Inducible Ras ES (iRasES) cells were harvested at 0, 12, and 24 h after Ras induction, and phosphoproteins were purified and subjected to mass-spectrometry analysis. Proteins detected at 12 and/or 24 h, but not at 0 h, were listed in supplemental Table S1.

Among these candidates, Ezrin was of particular interest for us. Ezrin is one of the molecules involved in eight-cell blastomere polarization. It is distributed evenly around the cell cortex before the early eight-cell embryo stage, and becomes enriched at the apical region after compaction (Louvet et al., 1996). Moreover, phosphorylation of Ezrin at T567 has been shown to be critical for eight-cell embryo compaction (Dard et al., 2004). We first confirmed our mass-spectrometry result by western blot. Ras induction did not change Ezrin expression level significantly, while phosphorylated Ezrin was increased after Ras induction (Fig. 1A). We further examined the expression of Ezrin and p-Ezrin in ES cells, a trophectoderm stem cell line derived from iRasES cells (ES–TS) and embryo-derived trophectoderm stem (TS) cells. More p-Ezrin was detected in ES–TS and TS cells than in ES cells, whereas Ezrin expression levels in all three types of cells were similar (Fig. 1B). It implied that Ezrin phosphorylation might play a role in the segregation of the TE and the ICM lineages.

Inhibition of Mek/Erk signaling blocks Ezrin phosphorylation in late eight-cell embryos

To study the role of Ezrin phosphorylation in early embryogenesis, we determined the expression dynamics of Ezrin and the level of p-Ezrin during early embryo development. Consistent with previous report, Ezrin expression was detected throughout preimplantation embryo development. Ezrin was located at the cell cortex before compaction, and became apically distributed in compacted embryos (Louvet et al., 1996). In contrast, p-Ezrin was not detected before the early eight-cell embryo stage. Clear apical p-Ezrin staining appeared in compacted eight-cell embryos, and was maintained in morula and blastocysts (Fig. 1C).

We have shown that Ezrin became phosphorylated upon Ras induction in ES cells. Our previous study has identified Mek/Erk signaling as a major downstream mediator of Ras during iRasES cell
differentiation into the trophectodermal lineage (Lu et al., 2008). We then asked whether Mek/Erk signaling regulates Ezrin phosphorylation in developing embryos. Two-cell embryos were treated with the Mek inhibitor PD98059 for 24 h, and the expression of p-Ezrin in the resulting late eight-cell embryos was examined by immunostaining. Inhibition of Mek/Erk signaling did not impair embryo compaction (Fig. S1). However, p-Ezrin level decreased significantly in PD98059 treated embryos, whereas the expression level and the apical distribution of Ezrin were not affected by PD98059 (Fig. 2A and B). Immunoblot assay further confirmed that PD98059 treatment blocked the phosphorylation of Ezrin (Fig. 2C). Due to the low affinity of Ezrin antibody, Ezrin expression in eight-cell embryos was undetectable by western blot. Instead, Ezrin mRNA levels in PD98059 treated and control embryos were measured by reverse transcription followed by real-time PCR. Ezrin mRNA expression was not affected by PD98059 treatment (Fig. 2D). To ensure that the reduced p-Ezrin level is due to inhibition of Mek/Erk signaling, but not non-specific effect of PD98059, we treated two-cell embryos with another Mek inhibitor PD0325901. Consistently, 24-h treatment with PD0325901 suppressed the phosphorylation of Ezrin in compacted eight-cell embryos (Fig. S2). These data suggested that the Mek/Erk signaling pathway is involved in phosphorylating Ezrin during embryo compaction.

We further analyzed whether inhibition of Mek/Erk signaling affects other polarized molecules in compacted embryos. The basolateral distribution of E-cadherin and the apical localization of aPKC and F-actin were not changed by PD98059 treatment (Fig. 2E–G). However, the F-actin staining became attenuated in PD98059 treated embryos, consistent with the fact that Ezrin phosphorylation is required for the interaction between Ezrin and F-actin (Fig. 2E). To distinguish whether apical enrichment of F-actin is up-stream or down-stream of Ezrin phosphorylation, a well-known microfilament-disrupting agent cytochalasin D (CD) was used to treat four-cell embryos. Shown in Fig. S2, CD treatment prevented embryo compaction. However, the overall level of p-Ezrin in eight-cell embryos was not reduced by CD treatment. These data suggested that Ezrin phosphorylation is up-stream of apical F-actin enrichment, and down-stream of Ezrin and aPKC polarization during embryo compaction.

Rho GTPases regulate the distribution of Ezrin, p-Ezrin and aPKC

Rho-associated kinases, as well as PKCα, PKCβ, and PKCγ, have been shown to phosphorylate the conserved threonine in the ERM proteins in vitro and in cultured cells (Chuan et al., 2006; Matsui et al., 1998; Ng et al., 2001; Oshiro et al., 1998; Pietromonaco et al., 1998; Shaw et al., 1998; Simons et al., 1998; Wald et al., 2008). Coincidently, both Rho GTPases and PKC are involved in eight-cell embryo compaction (Clayton et al., 1999; Winkel et al., 1990). Thus, it raised the possibility that Rho GTPases or PKC might be the Ezrin kinase in eight-cell embryos. To test whether Rho GTPases regulate the phosphorylation of Ezrin T567 during embryo compaction, four-cell embryos were treated with a Rho inhibitor C3-transferase for 12 h. The resulting eight-cell embryos were immunostained for Ezrin and p-Ezrin, F-actin, aPKC and E-cadherin. Hoechst 33342 staining marked the nucleus. Confocal images were shown. 

![Embryo compaction and blastomere polarization](image)

Fig. 3. Embryo compaction and blastomere polarization were compromised by inhibition of Rho GTPases. Four-cell embryos were cultured in medium with or without 1 μg/ml C3-transferase for 12 h. The resulting eight-cell embryos were immunostained for Ezrin and p-Ezrin, F-actin, aPKC and E-cadherin. Hoechst 33342 staining marked the nucleus. Confocal images were shown. (A) The apical localization of Ezrin and p-Ezrin was impaired by C3-transferase. Moreover, the overall level of p-Ezrin was reduced in C3-transferase treated embryos. The bright green circles outside eight-cell embryos were background signals of Zona pellucida. The apical Ezrin and p-Ezrin in control embryos were marked with arrows, while the basolateral Ezrin and p-Ezrin were highlighted with triangles. (B) F-actin colocalized with p-Ezrin in the basolateral cortex of C3-transferase treated embryos. (C) C3-transferase treatment led to basolateral distribution of aPKC, which overlapped with F-actin and (D) C3-transferase did not interfere the basolateral localization of E-cadherin. Bar, 20 μm.
12 h, and the resulting eight-cell embryos were subjected to immunofluorescence analysis. Consistent with previous report (Clayton et al., 1999), 80% (69 out of 86) of embryos treated with C3-transferase failed to compact (Fig. S1). Moreover, inhibition of Rho GTPases reduced the overall level of p-Ezrin, and disrupted the apical restriction of Ezrin and p-Ezrin. Ezrin and p-Ezrin were also detected in the basolateral cortex in C3-transferase treated embryos (Fig. 3A). In addition, F-actin and aPKC co-localized with p-Ezrin at the basolateral cortex (Fig. 3B and C). These data implicated that Rho GTPases regulate the spatial distribution of the Ezrin kinase in eight-cell embryos. Inhibition of Rho GTPases changes the spatial pattern of p-Ezrin, subsequently the distribution of F-actin. Co-localization of aPKC and p-Ezrin at the basolateral region implied that aPKC might be a candidate for Ezrin kinase or an associated factor of Ezrin kinase. Treatment of four-cell embryos with another highly specific and potent Rho kinase inhibitor (H-1152) recaptured the phenotypes caused by C3-transferase, including reduced p-Ezrin level and aberrant basolateral distribution of p-Ezrin in eight-cell embryos (Fig. S2). These data further confirmed that Rho GTPases are involved in regulating the spatial distribution and the activity of the Ezrin kinase in eight-cell embryos.

Inhibition of Rho GTPases affects not only Ezrin phosphorylation, but also the microfilament cytoskeleton and the formation of the apical region. Therefore, it is possible that the effect of Rho GTPase inhibition on Ezrin phosphorylation is due to the disruption of the microfilament cytoskeleton. However, disrupting microfilament formation by CCD did not reduce the overall level of p-Ezrin or affect the apically enriched p-Ezrin in eight-cell embryos, even though the embryo compaction was blocked (Fig. S2). Although aberrantly basolaterally distributed p-Ezrin was also observed in CCD treated embryos, incomplete blastomere polarization and the failure of embryo compaction might be responsible for the appearance of p-Ezrin at the basolateral region. These data suggested that the regulatory effect of Rho GTPases on Ezrin phosphorylation is not due to the disruption of the microfilament cytoskeleton.

PKC inhibition by Ro-31-8220 prevents Ezrin phosphorylation, but does not alter the distribution of p-Ezrin

Next, we studied the potential role of PKC signaling in Ezrin phosphorylation during embryo compaction. The PKC inhibitor Ro-31-8220 was used to treat embryos from the two-cell stage. In contrast to Rho kinase inhibition, inhibition of PKC by Ro-31-8220 affected neither eight-cell embryo compaction nor the distribution of Ezrin and p-Ezrin (Figs. 4A, B, and S1). Instead, the overall level of p-Ezrin was reduced by Ro-31-8220 treatment (Fig. 4A–C). Increasing level of E-cadherin at the apical region in eight-cell embryos (Fig. 4E), implying that PKC signaling might be involved in the exclusion of E-cadherin from the apical domain. F-actin appeared not to be well organized in Ro-31-8220 treated embryos (Fig. 4A, C). This might be caused by non-specific effect of Ro-31-8220.

PKC activity is required for p-Ezrin maintenance in compacted embryos

We have shown that Mek/Erk, Rho GTPases, and PKC regulate Ezrin phosphorylation during eight-cell embryo development, and that inhibition of Mek/Erk or PKC reduces the level of p-Ezrin in
compact eight-cell embryos. Yet, it is still unclear which kinase in these two pathways phosphorylates Ezrin. We reasoned that the Ezrin kinase should contribute to the establishment of Ezrin phosphorylation during embryo compaction, as well as the maintenance of p-Ezrin in compact embryos. Thus, we asked which pathway is responsible for the maintenance of p-Ezrin after embryo compaction. Compacted eight-cell embryos, in which Ezrin has been phosphorylated, were treated with Mek inhibitors PD98059 and PD0325901, Rho kinase inhibitors C3-transferase and H-1152, and PKC inhibitors sphingosine and Ro-31-8220, for 3 h. Immunofluorescence assay revealed that inhibition of Mek/Erk and Rho GTPases did not affect the level of p-Ezrin in compacted embryos (Fig. 5). However, inhibition of PKC by sphingosine or Ro-31-8220 suppressed the phosphorylation of Ezrin (Fig. 5). These data implied that PKC, but not Mek/Erk and Rho GTPases, is likely to be the Ezrin kinase in compacted eight-cell embryos.

**aPKC Is required for the phosphorylation of Ezrin in eight-cell embryos**

To further narrow down which subtype of PKC regulates Ezrin phosphorylation, PKC inhibitors with subtype specificities were used to treat two-cell embryos. Gö 6976, an inhibitor for PKCa and PKCB, did not affect Ezrin phosphorylation in eight-cell embryos (Fig. 6A). In contrast, a selective PKCd pseudosubstrate inhibitor (Myristoylated-SIYRGARRWRKL-OH, PSI) prevented Ezrin phosphorylation in eight-cell embryos. The treatment with PSI caused extensive embryo death, and only 6 out of 30 embryos (20%) developed to the eight-cell embryo stage. Nevertheless, these six surviving embryos all displayed similar phenotypes to embryos treated with Ro-31-8220 (Fig. 6B). The level of p-Ezrin was reduced, but the apical distribution of p-Ezrin was not disturbed. These data not only confirmed that PKC regulates Ezrin phosphorylation in eight-cell embryos, but also implied that the aPKC subtypes might be responsible for Ezrin phosphorylation in eight-cell embryos.

To avoid the issue of non-specific effect associated with inhibitors, siRNAs were applied to prove the regulation of Ezrin phosphorylation by aPKC. The knockdown efficiencies of siRNAs targeting PKCa or PKCb were first tested in TS cells. As shown in supplemental Fig. S4, two PKCa siRNAs downregulated PKCa efficiently, whereas only siRNA c-2 knocked down PKCb to 50%. Combination of siRNA a-1, a-2 and c-2 allowed simultaneous downregulation of PKCa and PKCb. These siRNAs, together with H2B-RFP mRNA, were injected into one blastomere of the two-cell embryo. Injection of control, PKCa or PKCb siRNA individually did not affect the level of p-Ezrin in progenies of the injected cell. Only when both PKCa and PKCb were down-regulated, Ezrin phosphorylation was reduced in the eight-cell blastomeres derived from the injected cell (10 out of 40 injected embryos) (Figs. 6C and S5). These data suggested that both subtypes of aPKC phosphorylate Ezrin.

**Discussion**

It has been demonstrated that spatial and temporal regulation of Ezrin T567 phosphorylation is critical for embryo compaction (Dard et al., 2004). Our data further support the importance of Ezrin T567 phosphorylation in embryo compaction. When embryos were treated with C3-transferase, Ezrin and p-Ezrin are located to the basolateral region, and embryos fail to compact. This phenotype is similar to the defect caused by Ezrin T567D mutation, mimicking a phosphorylated state. Ezrin T567D mutant localizes all around the cell cortex in eight-cell embryos, leading to embryo compaction failure (Dard et al., 2004). Taken together, abnormal p-Ezrin at the basolateral region, which might prevent Ezrin from moving to the apical domain, initiates F-actin formation aberrantly at the basolateral region, interferes with tight junction formation, and blocks embryo compaction. In contrast, Ezrin T567A mutation does not impair embryo compaction. Rather, Ezrin T567A mutant is almost cytoplasmic until the eight-cell stage, and accumulates at the basolateral area in 16-cell embryos, thus preventing further development of embryos (Dard et al., 2004). These data suggest that unphosphorylated Ezrin does not block embryo compaction. However, Ezrin phosphorylation is essential for anchoring Ezrin at the apical region,
stabilizing the polarized apical domain and further embryo development. Consistently, PD98059 and Ro-31-8220 do not affect embryo development, even though both of them reduce the p-Ezrin level at the apical region of eight-cell embryos. We did not examine whether PD98059 and Ro-31-8220 treatment could affect embryo development at later stages, because these inhibitors might impair embryo development through other mechanisms in addition to Ezrin phosphorylation. It has been demonstrated that embryos treated with PD98059 from the eight-cell stage show a marked developmental delay in blastocoel formation. This defect might be attributed to the reduced expression of a key trophectodermal transcription factor Cdx2 (Lu et al., 2008). Decreased Ezrin phosphorylation might also contribute to the developmental delay.

Despite the important role of Ezrin phosphorylation at T567 in embryo compaction (Dard et al., 2004), it is not clear which kinase is responsible for Ezrin phosphorylation during embryo compaction. Our data suggest that aPKC might phosphorylate Ezrin in eight-cell embryos. First, it has been demonstrated that Rho-associated kinases and various subtypes of PKC phosphorylate the conserved threonine in the ERM proteins in vitro and in cultured cells (Chuan et al., 2006; Matsui et al., 1998; Ng et al., 2001; Oshiro et al., 1998; Pietromonaco et al., 1998; Shaw et al., 1998; Simons et al., 1998; Wald et al., 2008). In C3-transferase treated embryos, co-localization of aPKC, p-Ezrin and F-actin at the basolateral cortex implicates that aPKC might be the Ezrin kinase or a factor associated with the Ezrin kinase. Second, inhibition of PKC by Ro-31-8220 or PSI, but not by the PKCα/β inhibitor Gö 6976, reduces the overall level of p-Ezrin in eight-cell embryos. Moreover, simultaneous downregulation of PKCa and PKCc by siRNA injection reduces the level of p-Ezrin in eight-cell blastomere progenies of the injected cell. In addition, depletion of PKCa does not affect embryo compaction and blastomere polarization at the eight-cell stage, but impaired the stabilization of cell polarity at the 16-cell stage (Dard et al., 2009). Consistently, Ezrin is detected in the basolateral area in PKCa depleted 16-cell embryos, implying that Ezrin is not efficiently phosphorylated in these embryos (Dard et al., 2009). Third, we showed that PKC, but not Rho GTPases or Mek/Erk, is required for maintaining the level of p-Ezrin in compacted embryos. All these data support that aPKC is the Ezrin kinase in eight-cell embryos.

In addition to aPKC, we also demonstrated that other signaling pathways, including Rho GTPases and Mek/Erk, regulate Ezrin phosphorylation in eight-cell embryos. During normal embryo development, p-Ezrin appears in the apical region of compacted eight-cell embryos, and persists throughout the rest of preimplantation embryogenesis. Inhibition of Rho GTPases changes the distribution of Ezrin, p-Ezrin and aPKC, from the apical surface to
the basolateral region. Aberrantly localized p-Ezrin leads to F-actin formation at the basolateral region, which might interfere with the formation of tight junctions at the basolateral region, hence preventing embryo compaction. These data suggest that Rho GTPases facilitate polarized distribution of Ezrin and aPKC during embryo compaction. More investigations are required to understand how Rho GTPases regulate Ezrin and aPKC polarization. Our data also implied that Mek/Erk signaling is involved in Ezrin phosphorylation, as inhibition of Mek/Erk prevents the phosphorylation of Ezrin T567 in eight-cell embryos. However, inhibition of Mek/Erk does not interfere with the polarization of Ezrin and aPKC. Therefore, Mek/Erk appears to act at a step between aPKC/Ezrin polarization and Ezrin phosphorylation. Given that Mek/Erk activity is not required for the maintenance of p-Ezrin in compacted embryos, Mek/Erk might regulate the activity of the Ezrin kinase aPKC in compacting embryos. Once aPKC is activated in compacted embryos, Mek/Erk becomes dispensable for Ezrin phosphorylation. Alternatively, Mek/Erk might facilitate the interaction between aPKC and Ezrin to promote Ezrin phosphorylation by aPKC. How Mek/Erk regulates Ezrin phosphorylation catalyzed by aPKC during compaction remains to be elucidated.

Taken together, we propose a working model for these events during embryo compaction (Fig. 6D). During eight-cell embryo compaction, activation of Rho GTPases may facilitate the apical region. The involvement of multiple signaling pathways in regulating Ezrin phosphorylation allows better temporal and spatial control of the Ezrin kinase activity. Coincidently, during normal embryo development, molecules mediating PKC and Mek/Erk signaling, such as PKCζ and Erk2, localize to the apical region of eight-cell embryos, restricting the Ezrin kinase activity to the apical region (Lu et al., 2008; Pauken and Capco, 2000). Thus, Ezrin is only phosphorylated at the apical cortex. Phosphorylated Ezrin mediates the apical enrichment of F-actin, stabilizing the polarized apical region and securing normal embryo development.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.01.002.

References


