Biochemical and Biophysical Research Communications 450 (2014) 659–665

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pick1 modulates ephrinB1-induced junctional disassembly through an association with ephrinB1

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ARTICLE INFO

Article history:
Received 26 May 2014
Available online 14 June 2014

Keywords:
Ephrinb1
Pick1
Cell–cell junctions
Metastatic diseases
Xenopus

ABSTRACT

Members of the Eph family have been implicated in the formation of cell–cell boundaries, cell movement, and positioning during development in the context of cancer progression. De-regulation of this signaling system is linked to the promotion of more aggressive and metastatic tumor phenotypes in a large variety of human cancers, including breast, lung, and prostate cancer, melanoma, and leukemia. Thus, it is interesting to consider the case of cancer progression where de-regulation of the Eph/ephrin signaling system results in invasion and metastasis. Here, we present evidence that Pick1, one of the essential components of the adherens junction, recovers ephrinB1-induced cell–cell de-adhesion. Loss of Pick1 leads to dissociation of epithelial cells via disruption of the adherens junction, a phenotype similar to ephrinB1 overexpression. In addition, overexpressed ephrinB1-induced disruption of the adherens junction is rescued via binding to Pick1. These data indicate that Pick1 is involved in regulating the cell–cell junction in epithelial cells, and this may influence therapeutic strategy decisions with regards to cell adhesion molecules in metastatic disease.

1. Introduction

Cell–cell adhesion is essential to maintain intercellular connections between epithelial cells. It is a critical, initial step required for morphogenetic movement during normal development as well as in oncogenesis and metastasis of cancer cells. Cell–cell junctions, including tight junctions (TJs), adherens junctions (AJs), desmosomes, and gap junctions are fundamental, ultra-physical structures involved in the maintenance of complex cellular organization in all epithelial tissues [1]. Since cell–cell contacts allow the epithelium to function as a coordinator between adjacent tissues, they must be tightly regulated during development of tissue architecture of an organism. The TJs are crucial to the paracellular characterization of the epithelial cell barrier. The AJs need to be competent to establish appropriate tissue morphogenesis in epithelial tissues. E-cadherin is a vital transmembrane protein in the AJs, whose inactivation is attributed to cancer progression. E-cadherin interactions in the cell–cell junction regulate several biological process, including growth, migration, differentiation, and fundamental localization of junctional complexes. In addition to their structural function, cell–cell adhesion plays important roles in various cell signaling pathways such as Eph/ephrin and Wnt [2,3].

The Eph/ephrin signaling pathway is associated with the regulation of several morphogenetic processes such as axon outgrowth, neural crest and retinal progenitor cell migration, hindbrain segmentation, skeletal patterning, and angiogenesis [4,5]. The Ephs/ephrins are categorized based on their sequence similarity and preferential binding to a subset of ligands that are tethered to the cell surface by either a glycosyl phosphatidyl inositol-linkage (ephrinA) or a transmembrane domain (ephrinB). Eph/ephrin signaling is bidirectional and plays a key role in the regulation of cell–cell adhesion via apical junctional complexes [6]. Studies from several laboratories, including our own, have revealed a new paradigm for the signal transduction cascade initiated by ligand/

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http://dx.doi.org/10.1016/j.bbrc.2014.06.027
0006-291X/

receptor interactions, where a transmembrane Eph ligand (ephrinB) not only activates a cognate Eph RTK residing on a neighboring cell, but also transduces a signal through its own intracellular domain that affects cell adhesion and movement [7,8].

Protein interacting with C kinase 1 (Pick1) is a peripheral membrane protein that binds to lipid molecules and participates in subcellular localization of a variety of membrane proteins [9]. Pick1 interacts directly with junctional adhesion molecules (JAMs), and has been implicated in the regulation of both TJs and AJs in epithelial cells [10]. Pick1 was shown to interact with ephrinB1 in the yeast two-hybrid-system, and to co-cluster with ephrinB1 and EphB receptors at synapses in the mouse brain [11]. Pick1 has been reported to bind to F-actin and the Arp2/3 complex, and thereby inhibit Arp2/3-mediated actin polymerization [12]. Pick1 has also been shown to regulate trafficking and function of some membrane proteins, such as AMPA receptor and ephrinB1 [9,13]. Pick1 binds to PKC, which has been reported to participate in TJ disassembly, whereas PKC and aPKC are involved in TJ formation in epithelial cells [14]. These associations with proteins that affect TJs, such as the actin cytoskeleton, JAM, and PKC, make Pick1 a promising candidate for interactions with ephrinB1.

In this study, we used an antisense morpholino oligonucleotide (MO) approach to determine whether Pick1 is necessary for TJ/AJ formation and stability in the *Xenopus* ectoderm. We demonstrated the interaction between ephrinB1 and Pick1 in *Xenopus* oocytes. We found that once tyrosine residues in the cytoplasmic domain of ephrinB1 are phosphorylated, this interaction is severely reduced. Moreover, we found that Pick1 morphants showed significant reduction in phosphorylated E-cadherin expression in AJs. In addition, overexpressed ephrinB1-induced disruption of the adherens junction is rescued via binding to Pick1. These data indicate that Pick1 is involved in the regulation of cell–cell junctions in epithelial cells, which may influence therapeutic strategy decisions with regards to cell adhesion molecules in metastatic disease.

2. Materials and methods

2.1. Plasmids and reagents

cDNA clones encoding full-length ephrinB1 and Pick1 were obtained from the American Type Culture Collection (ATCC). Tagged proteins ephrinB1 (HA, Flag, Pyo) and Pick1 (HA, Flag) were synthesized by polymerase chain reaction (PCR), and sub-cloned into expression vectors pCS2+ and pCS107, respectively. FGFR1 KE (FGFR1 K562E) and FGFR1 KD (FGFR1 C289R/K420A) constructs in pCS2+ were used [15].

2.2. mRNA synthesis and Xenopus embryo microinjection

For injections, capped mRNAs were synthesized using the SP6 mMessage mMachine kit (Ambion; Austin, TX, USA). For HA-tagged pCS2+ ephrinB1, Flag was linearized with KpnI, whereas for HA- and Flag-tagged pCS107/Pick1 were cut with KpnI and ApaI, respectively. Flag-tagged pCS107/4MT Pick1 was linearized by ApaI. FGFR1 KE (FGFR1 K562E) and FGFR1 KD (FGFR1 C289R/K420A) protein constructs in pCS2+ were linearized by NotI and BamHI, respectively. The ephrinB1MO was 25-nucleotides long with the base composition 5′-GGAGCCCTTCCATCCGCACAGGTGG-3′. Pick1MO had the following sequence: 5′-ATCAGAAAACATCTTAGAAACGGCC-3′ (Gene Tools; Philomath, OR, USA). mRNA and MOs were microinjected into both blastomeres of 2-cell stage embryos.

2.3. Whole-mount in situ hybridization

The embryos were fixed in MEMFA [4% paraformaldehyde, 0.1 M MOPS (pH 7.4), 1 mM $MgSO_4$, 2 mM EGTA] overnight at 4 °C and then hydrated before storing in 100% methanol at −20 °C. For making the anti-sense DIG-labeled probe, Pick1 DNA templates were linearized using ClaI and generated using T7 RNA polymerase (Ambion; Austin, TX, USA). EphrinB1 anti-sense probe was also transcribed with T7 RNA polymerase on the BamHI-linearized plasmid. Probes were attached to alkaline phosphatase-labeled anti-digoxigenin antibody and BM purple dye. Whole-mount *in situ* hybridization was performed for stage 22 and stage 32 embryos using these RNA probes.

2.4. Co-immunoprecipitation and Western blot analysis

Lysates were incubated with 2 μg antibodies against HA and Flag (Santa Cruz Biotechnology; Santa Cruz, CA, USA) in the same buffer as that used in the western blot for 1 h at 4 °C. Samples were immunoprecipitated with 15 μL protein-A/G agarose (Santa Cruz Biotechnology; Santa Cruz, CA, USA) overnight at 4 °C. The agarose beads were washed three times with a low salt concentration lysis buffer. Immunoblots were performed using anti-Flag-HRP conjugated (Sigma; St. Louis, MO, USA), anti-HA-HRP conjugated (Roche; Indianapolis, IN, USA), anti-phosphotyrosine-HRP conjugated (Upstate Biotechnology; Billerica, MA, USA), and anti-phosphotyrosine (Cell Signaling Technology; Danvers, MA, USA) antibodies.

2.5. Cryosections

Xenopus embryos injected with mRNA or mMO were embedded in 15% cold water fish gelatin containing 15% sucrose for 24 h at room temperature. Embedded embryos were transferred to a mold and rapidly frozen on dry ice for a few seconds. The frozen samples were longitudinally sectioned with 10 μm thickness from animal pole to vegetal pole, with a thermo microtome cryostat HM 525 (Thermo Scientific; Kalamazoo, MI, USA). Post sectioning, samples were mounted on a glass slide and stored at −80 °C.

2.6. Immunofluorescence

Slides were incubated with blocking buffer (PBS containing 1% bovine serum albumin, 5% HTLS) after fan-drying within a fume hood and double- or triple-stained with primary antibodies [polyclonal anti-P-E-cadherin (EPITOMICS; Burlingame, CA, USA, 1/400) and monoclonal anti-HA or/and anti-Flag antibodies (Applied Biological Materials; Richmond, BC, Canada, 1/400, respectively)] to identify *Xenopus* embryo cells expressing ephrinB1 and Pick1. The cells were visualized using Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG, and Alexa Fluor 350 goat anti-mouse IgG secondary antibodies (Molecular Probes; Invitrogen; Grand Island, NY, USA; 1/400).

3. Results

3.1. The expression pattern of Pick1 partially overlapped with that of ephrinB1

An interaction between ephrinB1 and the scaffold protein Pick1 has been previously reported [16]. We have also previously identified Pick1 as an interacting protein in the yeast two-hybrid screening system using the intracellular domain of ephrinB1.

We verified the spatio-temporal expression patterns of ephrinB1 and Pick1 by whole-mount *in situ* hybridization. Expression of Pick1 partially overlapped with that of ephrinB1 along the anterior border of the eye-field, forebrain, and pronephros at the tailbud stage (Supplementary Fig. 1), while Pick1 was more broadly expressed in the early stage (Supplementary Fig. 1; black arrow).

This expression pattern suggests that ephrinB1 and Pick1 may have the opportunity to interact during development.

3.2. Pick1 interacts with ephrinB1

To verify the results of the yeast two-hybrid screen, co-IP analysis was performed with exogenously expressed proteins in *Xenopus* oocytes. RNA encoding full-length *Xenopus* Pick1 with a C-terminal HA epitope tag (Pick1-HA) was co-injected with RNA encoding *Xenopus* ephrinB1 containing a C-terminal Flag epitope tag (ephrinB1-Flag) in *Xenopus* oocytes. Lysates were prepared and co-IP/western blot analyses were performed (Fig. 1A). Both ephrinB1 and Pick1 were exogenously expressed in embryos, and results of co-immunoprecipitation (Co-IP) assays confirmed the interaction between the two proteins. When Pick1-HA was immunoprecipitated with HA-conjugated agarose beads, ephrinB1-Flag was detected in the immune complexes. This result suggests that Pick1 interacts with ephrinB1, and thereby may be involved in ephrinB1 signaling.

3.3. Tyrosine phosphorylation in the intracellular domain of ephrinB1 disrupts the interaction with Pick1

EphrinB1 can be phosphorylated in response to binding to a cognate Eph receptor [17,18] or the TJ-associated protein claudin [19], or in response to fibroblast growth factor receptor (FGFR) activation [20]. We tested whether tyrosine phosphorylation of ephrinB1 affected the association between ephrinB1 and Pick1. A constitutively active form of the FGFR1 (FGFR1-KE) or a kinase dead form (FGFR1-KD) was co-expressed with ephrinB1-Pyo and Pick1-HA in *Xenopus* oocytes. Co-expression of FGFR1-KD had no effect on the binding between Pick1-HA and ephrinB1-Pyo, and no detectable tyrosine phosphorylation of ephrinB1 was observed (Fig. 1B). Of note, co-expression of FGFR1-KE resulted in a high level of ephrinB1 phosphorylation and concomitant loss of co-IP between ephrinB1-Pyo and Pick1-HA (Fig. 1B). Co-IP analysis using mutants of ephrinB1 harboring single Phe substitutions for Tyr at either tyrosine 324, 325, or both, showed no reduction of ephrinB1/Pick1 binding as compared to wild type ephrinB1 in the presence of FGFRKE (Fig. 1C). Together, these data indicate that phosphorylation of tyrosines 324 and 325 in ephrinB1 prevents or disrupts an interaction between ephrinB1 and Pick1, and may represent a critical step in the regulation of ephrinB1/Pick1 signaling.

3.4. Loss of Pick1 leads to epithelial cell dissociation

To investigate the functions of Pick1 in development, we designed anti-sense MO to block endogenous Pick1 translation, and injected these into both blastomeres at the 2-cell stage of

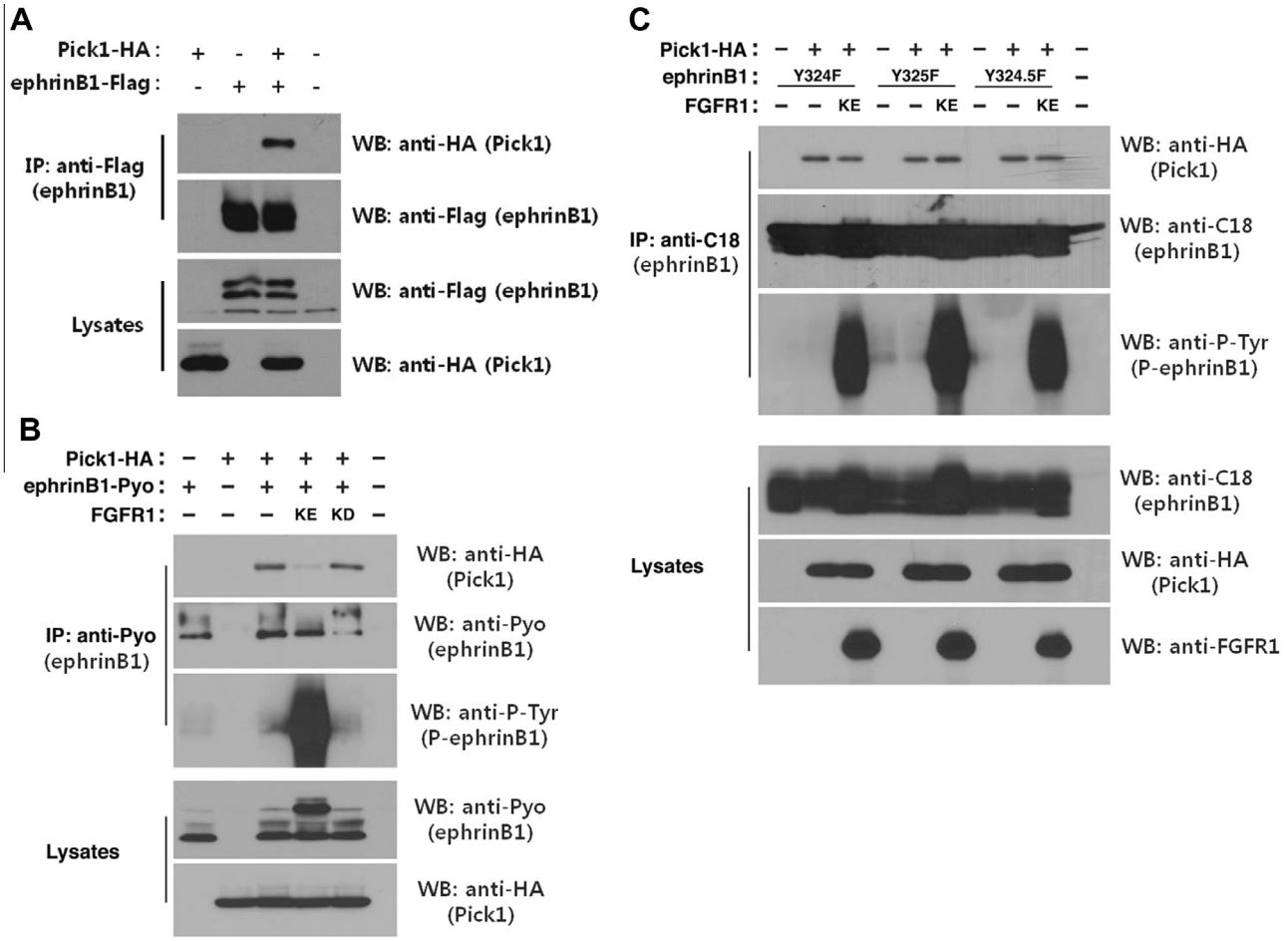

Fig. 1. Interaction between Pick1 and ephrinB1. (A) Full-length Pick1 fused to a C-terminal HA epitope tag (10 ng Pick1-HA) and full-length *Xenopus* ephrinB1 harboring a Flag epitope tag at the C-terminus (10 ng ephrinB1-Flag) were co-expressed in *Xenopus* oocytes for Co-IP/western blot analysis. Oocyte lysates were immunoprecipitated with Flag antibody and western blots were performed with the indicated antibodies. (B) *Xenopus* oocytes were each injected with 10 ng RNAs as indicated and lysates were subjected to Co-IP/western blot analysis. Co-expression with constitutively active FGFR1 K562E (KE) resulted in loss of binding between Pick1 and ephrinB1. However, co-expression with kinase dead FGFR1 C289R/K420A (KD) had no effect on the Pick1 and ephrinB1 interaction. (C) *Xenopus* oocytes were each injected with 10 ng RNAs as indicated and lysates were subjected to Co-IP/western blot analysis. Even co-expression of mutant ephrinB1 on PBM (Y324F, Y325F, Y324.5F) with constitutively active FGFR1 KE resulted in no effect on the Pick1 and ephrinB1 interaction.

embryos. Loss of Pick1 (Pick1MO-injected) in embryos showed that the phenotypes, including bent and shorter body axis, inhibition of eye development, and reduced brain size, were similar to those observed with loss of ephrinB1 (Fig. 2A; Data not shown). Moreover, loss of Pick1 in embryos showed cell dissociation in the ventral epithelium in a dose-dependent manner (Fig. 2A). This inhibitory effect could be mitigated by a moderate level of *Pick1* RNA that was resistant to MO (4MT Pick1). Co-injection of Pick1MO and 4MT Pick1 into the embryos significantly restored the phenotypic defects as compared to Pick1MO alone (Fig. 2B). In particular, the percentage of ventral lesion and bent axis of Pick1MO/4MT Pick1-injected embryos decreased by over 80% in Pick1MO (Fig. 2C), while over-expression of Pick1 showed minor defects such as a slightly bent axis (Fig. 2C). The expression of 4MT Pick1 was identified by western-blot (Fig. 2D), suggesting that the 4MT Pick1 was adequately expressed and was functional. These data suggest that Pick1 may be directly involved in formation of cell junctions in epithelial cells through the ephrinB1 interaction.

3.5. Loss of Pick1 leads to disruption of AJs

Having established that an interaction between ephrinB1 and Pick1 exists (Supplementary Fig. 1) and Pick1 may be critical for AJ maintenance (Fig. 2), we performed loss-of-function studies in *Xenopus* embryos using MO to check the specific markers for

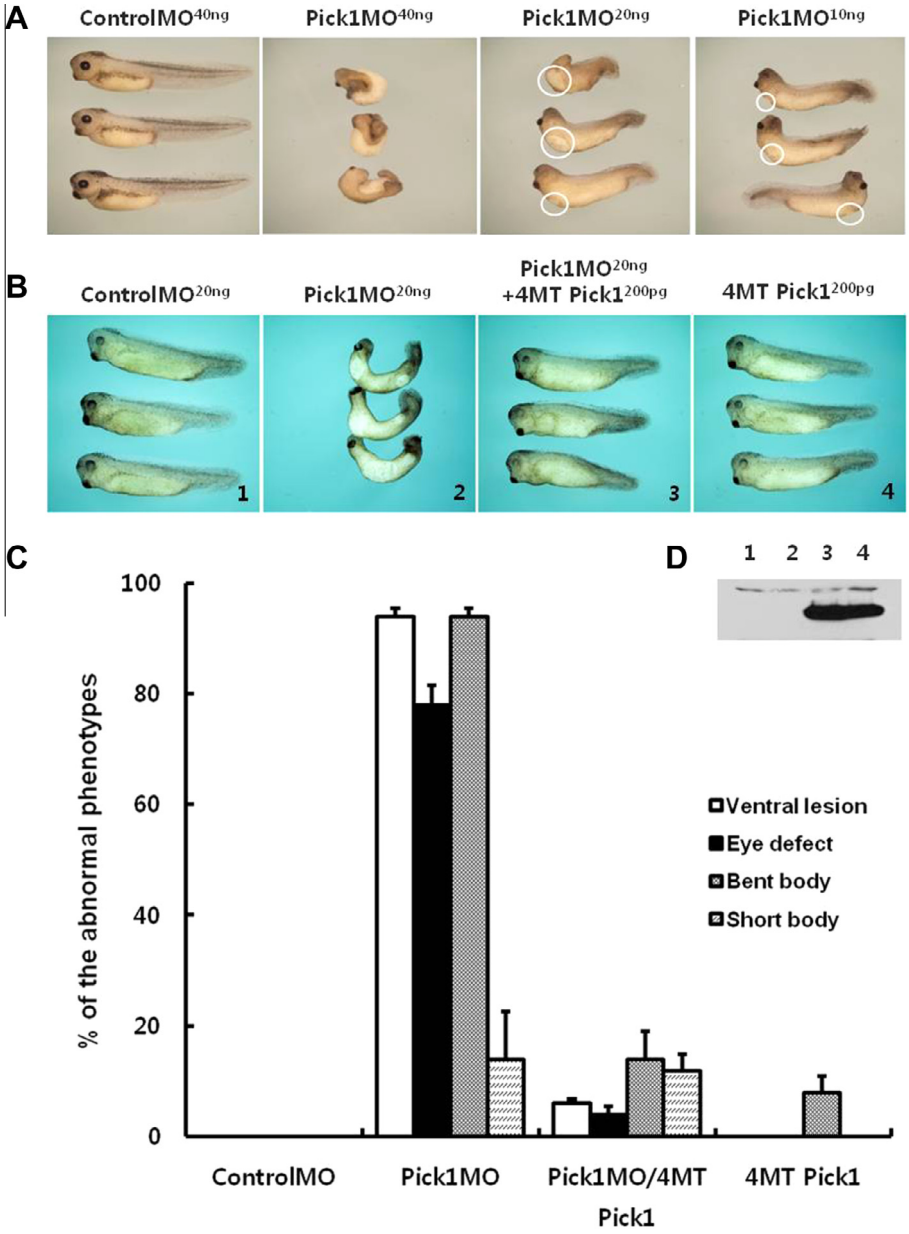

Fig. 2. Pick1 knock-down causes defects in ventral trunk formation and epidermal cell dissociation. (A) Embryos injected with Pick1MO (10 ng, 20 ng, 40 ng) into both blastomeres at the 2-cell stage. Pick1MO embryos showed bent and shorter body axis, inhibition of eye development, and reduced brain size. The ventral cell dissociation is shown (white circle). (B) Abnormal phenotypes including the ventral lesion caused by the loss of Pick1 significantly rescued by co-injection with 200 pg of 4MT Pick1 mRNA. (C) The histogram denotes the percentage of embryos with defects as described in (B). Note: Pick1 MO-injected embryos showed significant developmental defected embryos (ventral lesion, 94.0 ± 1.41%; eye defect, 78.0 ± 3.54%; bent body, 94.0 ± 1.41%; short body, 14.0 ± 8.49%; $n = 60$). Co-injection of Pick1MO and 4MT Pick1 into the embryos significantly restored the phenotypic defects as compared to Pick1MO alone (ventral lesion, 6.0 ± 0.71%; eye defect, 4.0 ± 1.41%; bent body, 14.0 ± 4.95%; short body, 12.0 ± 2.83%; $n = 55$), while over-expression of Pick1 showed minor defects such as a slightly bent axis (bent body, 8.0 ± 2.83%; $n = 55$). (D) 4MT Pick1-injected embryos were tested for expression. Western blotting was performed using an anti-Flag antibody.

cell–cell junctions. Since Pick1MO induced cell dissociation in the ventral ectoderm, we injected Pick1MO into both the blastomeres of the 2-cell stage embryos to test whether loss of Pick1 affects AJ formation in the early embryonic ectoderm (Fig. 3A). We found that MO blocked expression of endogenous Pick1 (Fig. 3B) and caused a redistribution of the AJ-associated protein, phosphorylated E-cadherin, while that of control (MO-injected) embryos was intact (Fig. 3C and D). In addition, 4MT Pick1 RNA, which is resistant to Pick1MO, was capable of recovering Pick1 expression, resulting in the restoration of proper localization of phosphorylated E-cadherin, while Pick1 RNA alone showed no significant changes in localization of phosphorylated E-cadherin (Fig. 3E and F). Previous studies have shown that Pick1 is localized at basolateral membranes of epithelial cells with polarized epithelial cells [10,21]. These results demonstrate that Pick1 is one of the critical components for maintaining appropriate localization of AJ-associated proteins since blocking the expression of Pick1 redistributed the AJ-associated proteins.

3.6. Pick1 rescues ephrinB1-induced disruption of AJs

We previously reported that over-expression of ephrinB1 induces cell–cell dissociation in the embryonic ectoderm [22]. Furthermore, we found evidence that Pick1 is one of the key regulators in the assembly of AJs in cell–cell junctions. From these findings, we hypothesized that Pick1 may be a key regulator of ephrinB1 signaling in cell adhesion. The role of Pick1 as a mediator of ephrinB1 function was evaluated using knock-in experiments with *Xenopus* embryo ectoderm. Pick1 and ephrinB1 RNA co-injected embryos showed significantly reduced cell–cell dissociation in comparison to ephrinB1 alone (Supplementary Fig. 2).

On the basis of previous results, we investigated the functional relationship between Pick1 and ephrinB1 *in vivo*. We co-injected *Pick1* and *ephrinB1* RNAs and performed immunofluorescence to visualize AJs in epithelial cells. ControlMO and Pick1 over-expressed embryos had well-established AJs at the cell–cell boundaries. Although this experiment was performed with a high dose of Pick1 RNA as compared to previous experiments (Figs. 2 and 3), we obtained similar results (Fig. 4). It has been reported previously that over-expressed ephrinB1 is localized within E-cadherin-based AJs at the cell–cell boundary and caused disruption of the formation of cell–cell junctions (Fig. 4C) [22,23], while over-expressed Pick1 showed no significant changes in localization of phosphorylated E-cadherin (Fig. 4E). Interestingly, co-injection of *Pick1* RNA significantly rescued the over-expressed ephrinB1-induced AJ-defects at cell–cell boundaries (Fig. 4D). These data suggest that

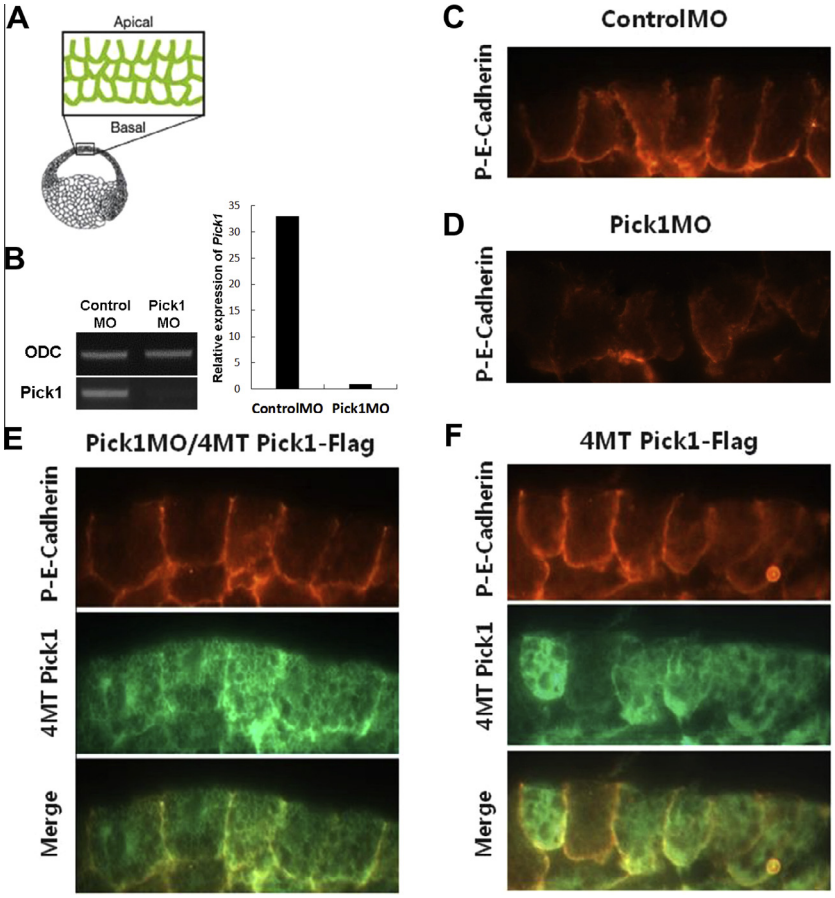

Fig. 3. Pick1 knock-down causes the disruption of AJs. Embryos were injected with or without 20 ng control MO, 20 ng Pick1 MO, and 200 pg 4MT Pick1 RNA in both the blastomeres of 2-cell stage embryos and those collected at stage 10.5. Immunostaining was performed with phospho-E-cadherin and Flag antibodies on cryosections. (A) Experimental scheme. Schematic showing the region of embryo isolated and examined by immunofluorescence microscopy. Adapted from Lee HS et al. [22]. (B) RT-PCR showed that *Pick1* expression was significantly down-regulated, while *ephrinB1* was not changed in Pick1MO-injected embryos. The histogram shows the relative intensity of *Pick1* mRNA expression. (C) ControlMO-injected embryos showed stable localization of phosphorylated-E-cadherin along cell–cell boundaries in cell junctions. (D) Localization of phosphorylated-E-cadherin was greatly diminished in AJs of Pick1MO-injected embryos. (E) The redistribution of phosphorylated-E-cadherin by Pick1MO was significantly rescued by appropriate amounts of 4MT Pick1 RNA. (F) The localization of Pick1 partially overlapped with that of phosphorylated-E-cadherin, indicating that Pick1 can be present in AJs of epithelial cells and over-expressed Pick1 does not affect cell junction formations. Embryos were injected with 20 ng controlMO, 20 ng Pick1MO, and 200 pg 4MT Pick1 mRNA in both the blastomeres of 2-cell stage embryos. 1st row (Red: phosphorylated-E-cadherin), 2nd row (green: Pick1), 3rd row (merged). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Pick1 may act as a downstream mediator of ephrinB1 signaling or Pick1 may interact with over-expressed ephrinB1, leading to the disruption of TJs and redistribution of over-expressed ephrinB1 from the cell junction.

4. Discussion

Cell movements induce the disruption of cell–cell adhesion and the loss of contact inhibition, eventually leading to unregulated cell proliferation, tissue invasion, and finally metastasis to other organs.

Pick1 is a scaffold protein that links various components of the AJ complex in epithelial cells and interacts with various transmembrane components of both AJs and TJs [21]. Pick1 was first demonstrated as a protein containing a PDZ domain, interacting with the PDZ binding motif of PKCα [24,25]. Pick1 is also found in the cytoplasm of cancer cells and may play an important role in cancer [26].

EphrinB1, an important juxtacrine factor in inducing cell movement and de-adhesion, can be regulated by Pick1, which in turn is one of the key proteins shown to be involved in maintenance of AJs and TJs in the *Xenopus* ectoderm. Since EphB/ephrinB interactions are directly related to cell–cell contact events, presumably, ephrinBs must be located at the sites of contact in receptor-expressing cells [27]. Evidence for a causal relationship between over-expression of ephrinB1 in *Xenopus* embryos and dissociation of the blastomeres in ectodermal tissue has been previously reported [27]. It is not likely that this effect is merely a result of the adhesive properties of the interaction between Eph receptor and ephrin since adhesion was shown to be disrupted by over-expressing an ephrinB1 ligand lacking the receptor-binding domain [27]. A particularly important aspect of ephrinB reverse signaling that is beginning to emerge is its role in cell–cell junctions, and we have focused our efforts on the mechanistic aspects of this function of ephrinB1.

Here, we proposed a model for ephrinB1 regulation of cell–cell dissociation using *Xenopus* stem-like cells. Our results confirmed that Pick1 is an important adhesion protein (Figs. 2 and 3), and we have demonstrated that ephrinB1-induced cell–cell dissociation was repaired by Pick1, which eventually mediated the formation of AJs and TJs (Fig. 4). These data strongly suggest that novel molecular mechanisms exist to regulate ephrinB1-modulated cell–cell de-adhesion.

Further investigation of the connection between Cdc42, Par-6, and Pick1 could provide new means to understand the regulation of cell junction formation. Identifying these mechanisms would help to elucidate several other physiological and pathological functions of ephrinB1 signaling, such as morphogenesis, differentiation, proliferation, and migration.

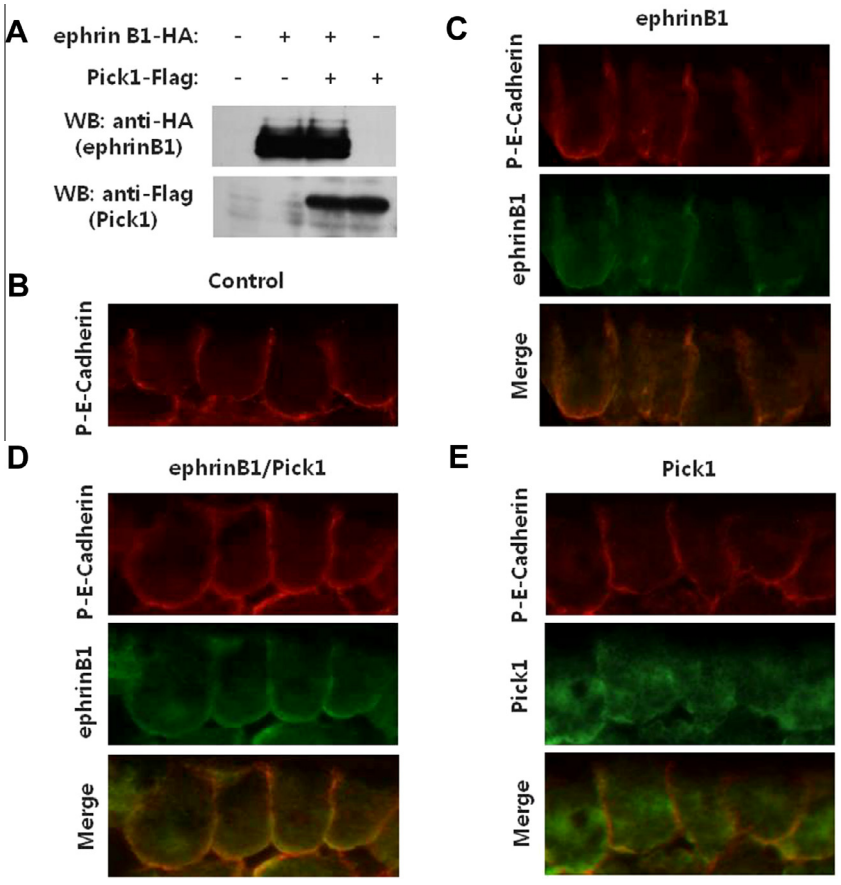

Fig. 4. Pick1 repairs ephrinB1-induced disruption of AJs. Embryos were injected with or without 400 pg ephrinB1 and 500 pg Pick1 RNAs in both the blastomeres of 2-cell stage embryos and those collected at stage 10.5. Immunostaining with phospho-E-cadherin, Flag, and HA antibodies was performed on cryosections. (A) Lysates from embryos were tested for the expression of ephrinB1 and Pick1. Western blot was performed using anti-HA and anti-Flag antibodies. (B) Control embryos showed stable localization of phosphorylated-E-cadherin along cell–cell boundaries in cell junctions. (C) The over-expressed ephrinB1 embryo with disrupted cell–cell junctions. (D) The redistribution of phosphorylated-E-cadherin by ephrinB1 over-expression was significantly repaired by appropriate amounts of Pick1 RNA. (E) The over-expressed Pick1 did not affect cell junction formations. Red: Phosphorylated-E-cadherin, Green: ephrinB1, Blue: Pick1, Last row (merged). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (NRF-2012R1A1A2042334), and by a Grant from the Next-Generation BioGreen 21 Program (PJ009564), Rural Development Administration, Republic of Korea.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.027.

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