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XEphA4 is a cellular receptor that functions to regulate cell and tissue interactions in amphibian embryos via a repulsive mechanism that involves actin cytoskeleton reorganization. Ectopic EphA4 signaling in Xenopus embryos results in a loss of cell-adhesion and rounded cell morphology, and this phenotype is consistent with EphA4 signaling in cultured A6 cells. How EphA4 achieves its effects on the actin cytoskeleton at the molecular level is largely unknown. One known step in the pathway is that EphA4 causes inhibition of the small GTPase RhoA. RhoA has many downstream effectors that cause cytoskeletal reorganization; the most recognized of these are the ROCK proteins (Rho-associated kinases). ROCK exists in two isoforms, ROCKI and ROCKII. We hypothesize that ROCK inhibition is one step in EphA4 signaling. To test our hypothesis we used ROCK inhibitors and mutants. ROCK inhibitors resulted in loss of cell-adhesion, but not change in cell shape in *Xenopus* embryo, and rounded morphology in A6 culture. Dominant-negative ROCKII did not result in a phenotype similar to EphA4 and constitutively-active ROCKII did not rescue *Xenopus* embryos from the EphA4 phenotype. Expression of dominant-negative ROCKI in A6 culture produces a phenotype similar to EphA4 and more importantly expression of constitutively-active ROCKI in Xenopus embryos resulted in partial rescue of the embryos from the effects of EphA4 signaling. These data support a role for ROCKI but not ROCKII in EphA4 signaling. However, the lack of a change in cell shape in *Xenopus* embryos suggests RhoA has multiple downstream effectors involved in EphA4 signaling.

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The Involvement of Rho-Associated Kinases (ROCKs) in EphA4 Signaling in *Xenopus laevis*

By

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Abstract

XEphA4 is a cellular receptor that functions to regulate cell and tissue interactions in amphibian embryos via a repulsive mechanism that involves actin cytoskeleton reorganization. Ectopic EphA4 signaling in *Xenopus* embryos results in a loss of cell-adhesion and rounded cell morphology, and this phenotype is consistent with EphA4 signaling in cultured A6 cells. How EphA4 achieves its effects on the actin cytoskeleton at the molecular level is largely unknown. One known step in the pathway is that EphA4 causes inhibition of the small GTPase RhoA. RhoA has many downstream effectors that cause cytoskeletal reorganization; the most recognized of these are the ROCK proteins (Rho-associated kinases). ROCK exists in two isoforms, ROCKI and ROCKII. We hypothesize that ROCK inhibition is one step in EphA4 signaling. To test our hypothesis we used ROCK inhibitors and mutants. ROCK inhibitors resulted in loss of cell-adhesion, but not change in cell shape in *Xenopus* embryo, and rounded morphology in A6 culture. Dominant-negative ROCKII did not result in a phenotype similar to EphA4 and constitutively-active ROCKII did not rescue *Xenopus* embryos from the EphA4 phenotype. Expression of dominant-negative ROCKI in A6 culture produces a phenotype similar to EphA4 and more importantly expression of constitutively-active ROCKI in *Xenopus* embryos resulted in partial rescue of the embryos from the effects of EphA4 signaling. These data support a role for ROCKI but not ROCKII in EphA4 signaling. However, the lack of a change in cell shape in *Xenopus* embryos suggests RhoA has multiple downstream effectors involved in EphA4 signaling.

Introduction

For proper animal development some cells must migrate to specific target locations within the embryo. This means a cell must travel along a path that causes it reach its target site. How a cell finds its way to that target site and then recognizes its destination once there is due to the regulation of cell adhesion (Steinberg, 1996; Tepass *et al.*, 2002).

Eph receptors, the largest family of receptor tyrosine kinases (RTKs), and their membrane-bound ligands, the ephrins, are key regulators of cell migration, adhesion and

targeting in a broad range of tissues (for reviews, see Cowan and Henkemeyer, 2002; Kullander and Klein, 2002; Tepas *et al.*, 2002; Murai and Pasquale, 2003; Pasquale, 2005). The Eph-ephrin interaction seems to control cell migration by regulating cell adhesion. Our focus is on the Eph receptor EphA4. EphA4 is expressed in a specific subset of tissues in the early embryo including involuting mesoderm during gastrulation, forebrain beginning at neurula stage, rhombomeres r3 and r5 of the hindbrain, cranial neural crest of visceral arch 3 and transiently in dorsal otic vesicle and developing pronephros (Winning and Sargent, 1994). The effects of EphA4 have been investigated in *Xenopus laevis* embryos using a chimeric form of the receptor, known as EPP, which consists of an extracellular (ligand binding) domain of EGFR (epidermal growth factor receptor) fused to the transmembrane and intracellular (catalytic) domains of EphA4. This chimeric receptor is therefore activated by the ligands of EGFR, EGF (epidermal growth factor) and TGF-α (transforming growth factor alpha), while the cellular effects are mediated by EphA4 catalytic activity (Winning *et al.*, 1996). A chimeric receptor was chosen in order to prevent the potential for promiscuity in ligand-receptor interactions; this approach has been successful in the analysis of other RTKs, including the Eph-class gene Elk (Lhotak and Pawson, 1993). Ectopic expression and activation of EPP in early *Xenopus* embryos has been found to result in loss of cell adhesion in the affected cells (Winning *et al.*, 1996). In addition to loss of cell adhesion, it has also been demonstrated that activation of EphA4 signaling results in a change in cell shape to a rounded morphology, reduction of microvilli on the apical surfaces of superficial cells, a loss of apical/basolateral polarity, and that a preferential separation of adherens junctions initiates the loss of cell adhesion (Winning *et al.*, 2001). The observed effects of EphA4 signaling therefore suggest the actin cytoskeleton as the target of the receptor.

The mechanism in which EphA4 achieves its effects on the actin cytoskeleton is largely unknown. The Src-like tyrosine kinase p59fyn has been found to bind to phosphotyrosine in the juxtamembrane region of EphA4 (Ellis *et al.*, 1996) and activation of the kinase mimics EphA4 signaling (Winning, unpublished results). Furthermore, inhibition of the small GTPase RhoA, in an ephexin-independent pathway, is a downstream effect of EphA4 signaling (Winning *et al.*, 2002). Recently, it has been shown that xPAK1 is recruited to EphA4 by Nckβ (Grb4) and activation of xPAK1 sequesters Cdc42 leading to the down-regulation of RhoA (Bisson *et al.*, 2007).

Rho cycles between an active (GTP-bound) and inactive (GDP-bound) state that is regulated by guanine nucleotide exchange factors (GEFs), which activate Rho, and GTPase-

activating proteins (GAPs), which cause Rho to become inactive (reviewed in Schmitz *et al.*, 2000). The GTP-bound, active state, of Rho provides the ability for the selective interaction with downstream targets, called effector proteins. One of these effector proteins, ROCK, is well known for its interaction with Rho.

ROCK proteins are important regulators of cell growth, migration, and apoptosis via control of the actin cytoskeleton assembly. They regulate cell contraction through serine-threonine phosphorylation of adductin, ezrin-radixin-muesin (ERM) proteins, LIM kinase, Myosin light-chain phosphatase (MLCP), and Na/H exchanger (NHE)1 (Denker *et al*., 2000; Riento and Ridley, 2003). ROCK proteins consist of an N-terminal kinase domain followed by a central putative coiled-coil region, a pleckstrin homology domain, and a cysteine-rich domain at the C terminus (Leung *et al.*, 1996; Matsui *et al.*, 1996; Ishizaki *et al.*, 1996). The Rho-binding domain (RBD) within the predicted amphipathic α-helical coiled-coil is responsible for the recognition and binding of the active Rho proteins (Fujisawa *et al.*, 1996). Two ROCK isoforms have so far been identified, ROCKI/ROK(beta)/p160ROCK and ROCKII/ROK(alpha)/Rho kinase, that share 65% overall identity and 95% homology (Leung *et al.*, 1996; Matsui *et al.*, 1996; Fujisawa *et al.*, 1996; Amano *et al.*, 1996).

Due to the involvement of ROCK proteins in organization of the actin cytoskeleton as well as its well-characterized interaction with Rho, we hypothesize that EphA4 achieves its cellular effects by inhibiting ROCK.

In order to test this hypothesis we used inhibitors of ROCK as well as dominant-negative mutant forms of ROCK to see if nonfunctional ROCK results in a phenotype similar to EPP in both *Xenopus* embryos and A6 (renal epithelial) cultured cells. In *Xenopus* embryos we inhibited ROCK using Y-27632, a selective inhibitor of the ROCK family of kinases (Uehata *et al*., 1997). Using a dominant-negative form of ROCKII we tested to see if a nonfunctional ROCKII alone would cause EPP phenotype. In addition, we treated A6 cells with multiple selective ROCK inhibitors: Y-27632, Hydroxyfasudil, and Rho-kinase Inhibitor I (Uehata *et al*., 1997; Shimokawa *et al*., 1999; Ikenoya *et al*., 2002 respectively), as well as a dominant-negative version of each ROCK isoform.

In addition, rescue experiments were performed using each isoform to see if constitutively-active ROCK would save *Xenopus* embryos from the EPP phenotype.

Our results suggest that inhibition of ROCKI, but not ROCKII, is part of the EphA4 signaling pathway.

Methods

Clones and in vitro transcription

Construction of the chimeric receptor clone EPP is described elsewhere (Winning *et al*., 1996). Synthesis of EPP and TGF-α RNA for injection into embryos is described elsewhere (Winning *et al*., 2002). The constitutively active and dominant negative ROCKII mutants, CAT and RB/PH respectively, were provided by Kozo Kaibuchi (Nagoya University, Japan) in a pEF-Bos-myc plasmid. The ROCKII CAT was subcloned into pSP64 and RB/PH into a TOPO vector using an Invitrogen TOPO Cloning kit. The CAT was linearized using *NotI* and followed by transcription using an Ambion mMessage mMachine SP6 transcription kit. RB/PH RNA was produced by linearizing the plasmid using *KpnI* and transcription was done using an Ambion mMessage mMachine T7 transcription kit. Both RNAs underwent polyA tailing using an Ambion Poly(A) Tailing kit. Purification of the mRNA was done previous to polyA tailing via Lithium Chloride precipitation and the final RNA solution was filtered through a Duropore 0.22 μm filter (Duropore). Purified mRNAs were quantified by UV spectroscopy and electrophoresis. The dominant-negative and constitutively-active ROCKI clones (in pCAG-my plasmids), KD-IA and Δ3 respectively, were provided by Shuh Narumiya (Kyoto University, Japan).

Microinjection

Adult *Xenopus laevis* were purchased from Xenopus I (Ann Arbor, MI) or Nasco (Ft. Atkinson, WI). Eggs from these frogs were obtained and fertilized as outlined in Jonas *et al*. (1989) and were staged according to Nieuwkoop and Faber (1994). Fertilized eggs, at the one-cell stage, were injected with RNA in the animal hemisphere using a Nanoject microinjector. Detailed injection methods have been described elsewhere (Jonas *et al*., 1989). The RNAs injected consisted of 500 pg of EPP plus 250 pg TGF-α, 1ng, 3.75 ng or 7.5 ng ROCKII RB/PH, and 500 pg ROCKII CAT in a volume of 10 nl.

For Y-27632 (purchased from Calbiochem) inhibitor studies, fertilized eggs were injected with 250 pg or 1 ng of the drug in a volume of 10 nl.

The ROCKI rescue experiment used active ROCKI protein (purchased from Upstate Biotechnology) instead of mRNA. Active protein was diluted with ddH20 to a concentration of 50 or 100 pg in a volume of 10 nl.

Cell transfection

Drug inhibitors used were Y-27632, Hydroxyfausfadil and Rho-Kinase Inhibitor I (Calbiochem).

Xenopus renal epithelial A6 cells were plated on 15mm diameter Thermanox® coverslips, for SEM studies, in Leibovit's L-15 (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin. For light microscopy, the cells were grown in 12 well dishes. After culture for 1 day (confluence of 50-60%), cells were transfected with plasmid DNA (pCAG-myc ROCKI KD-IA or pEF-Bos-myc RB/PH) or drug inhibitor using LipofectAMINE Plus transfection reagents (Gibco). Transfections of 4ug, 6ug, and 10ug were done of all inhibitors and DNA samples. Previous to transfection cell media was replaced with serum/antibiotic free L-15. Three hours after incubation with the lipofectamine-DNA/inhibitor coprecipitates, medium was added containing 10% FBS.

Scanning Electron Microscopy

Embryos at mid-blastula stage were fixed overnight at 4^{o}C in 2.5% glutaraldehyde in 1x PBS. After rinsing three times in 1 x PBS, fixed embryos were bisected with a scalpel blade, and examined under a dissecting microscope for morphology and presence or absence of cellular dissociation. Embryos were then prepared for analysis by scanning electron microscopy as described in Winning *et al*. (2001).

A6 cell culture were fixed at confluence overnight at 4^{o}C in 2.5% glutaraldehyde in 1x PBS. After washing three times in 1x PBS the cells were post-fixed in 1% (V/V) osmium tetroxide for one hour and then washed twice in distilled water, immersed in 1% (v/v) tannic acid for one hour and slowly dehydrated through a graded series of ethanol to two changes of 100%. Following ethanol dehydration, the embryos were dried using hexamethyldisalazane (Nation, 1983). The coverslips were then affixed to stubs with double-stick, carbon-permeated tape and stabilized with colloidal graphite. Samples were then off-gassed in a vacuum overnight, and sputter-coated with gold.

Results

Inhibition of the ROCK Family of Kinases by Y-27632 Causes Loss of Cell-Cell Adhesion, but No Change in Cell Shape in Xenopus Embryos

Our hypothesis states that activation of EphA4 results in the inhibition of ROCK. Therefore, we predict that inhibition of ROCK without the presence of activated EphA4 signaling should result in phenotypic effects similar to those previously seen during EPP activation; namely a loss of cell-cell adhesion (Winning *et al*., 1996) and a change in cell shape (Winning *et al*., 2001).

Y-27632 is a selective inhibitor of the ROCK family of kinases (Uehata *et al*., 1997). Y-27632 competes for the ATP-binding site, and thereby inhibits both kinase isoforms with equal potency (Ishizaki *et al*., 2000). Initial characterization revealed that Y-27632 inhibits the ROCK family of kinases 100 times more potently than other kinases, including protein kinase C, cAMP-dependent kinase and myosin light chain kinase (Uehata *et al*., 1997).

We used Y-27632 to test whether inhibition of ROCK resulted in phenotypic effects similar to those of EPP activation. In order to test this, we injected embryos with Y-27632 and looked at the effects on the blastula-stage embryo.

Compared to water-injected embryos (Fig 1A), embryos injected with Y-27632 (Fig 1B) at the one-cell stage exhibited a loss of cell adhesion that was similar to that seen in the EPP phenotype (Fig 1C) where the blastocoel was improperly formed. In this experiment, embryos were bisected at blastula stage, assessed under a dissecting microscope, and scored in one of three categories: 'Normal Blastocoel' indicates no cellular dissociation and therefore an intact blastocoel; 'No Blastocoel' indicates massive loss of cell adhesion resulting in an indistinguishable blastocoel; and 'Abnormal Blastocoel' indicates embryos where some cellular dissociation has occurred, but the blastocoel is still somewhat intact. The results of the scoring are shown in Table 1.

Table 1 Y-27632 causes loss of cell-adhesion in *Xenopus* blastulas

	Injection			
	Uninjected	Water	Y-27632 (250 pg)	Y-27632 (1000 pg)
Normal Blastocoel	100% (119)	95.8% (69)	11.6% (13)	0% (0)
Abnormal Blastocoel	0% (0)	4.2% (3)	77.7% (87)	49.5% (45)
No Blastocoel	0% (0)	0% (0)	10.7% (12)	50.5% (46)

Embryos were injected at one-cell stage with Y-27632 at concentration listed. After embryos reached blastula stage, embryos were bisected and assessed for blastocoel morphology and cellular dissociation. 'Normal Blastocoel' indicates no cellular dissociation; 'Abnormal Blastocoel' indicates some cellular dissociation, but a blastocoel is still distinguishable; 'No Blastocoel' indicates cellular dissociation resulting in no blastocoel formation. Percentages indicate the fraction of embryos expressing each phenotype and parentheses are the number of embryos exhibiting each phenotype.

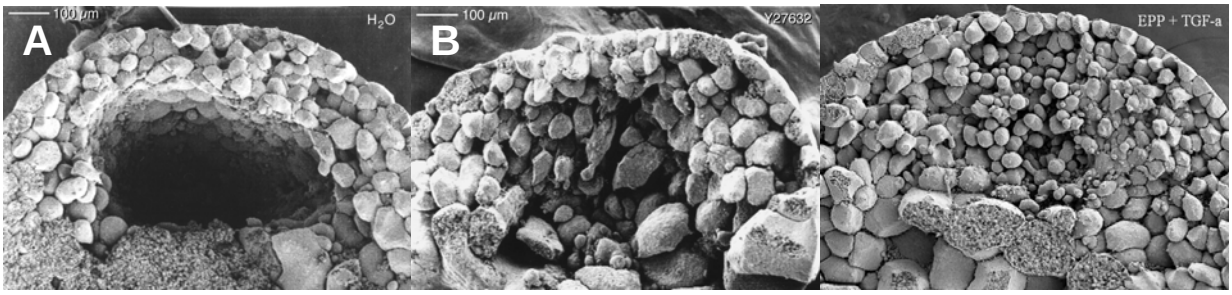

Fig. 1 Inhibition of ROCK family of kinases using Y-27632 results in loss of cell adhesion. Embryos were injected with water **(A)** or 1000pg Y-27632 **(B)**. The EPP phenotype **(C)** (separate experiment) can be compared with the inhibition of ROCK.

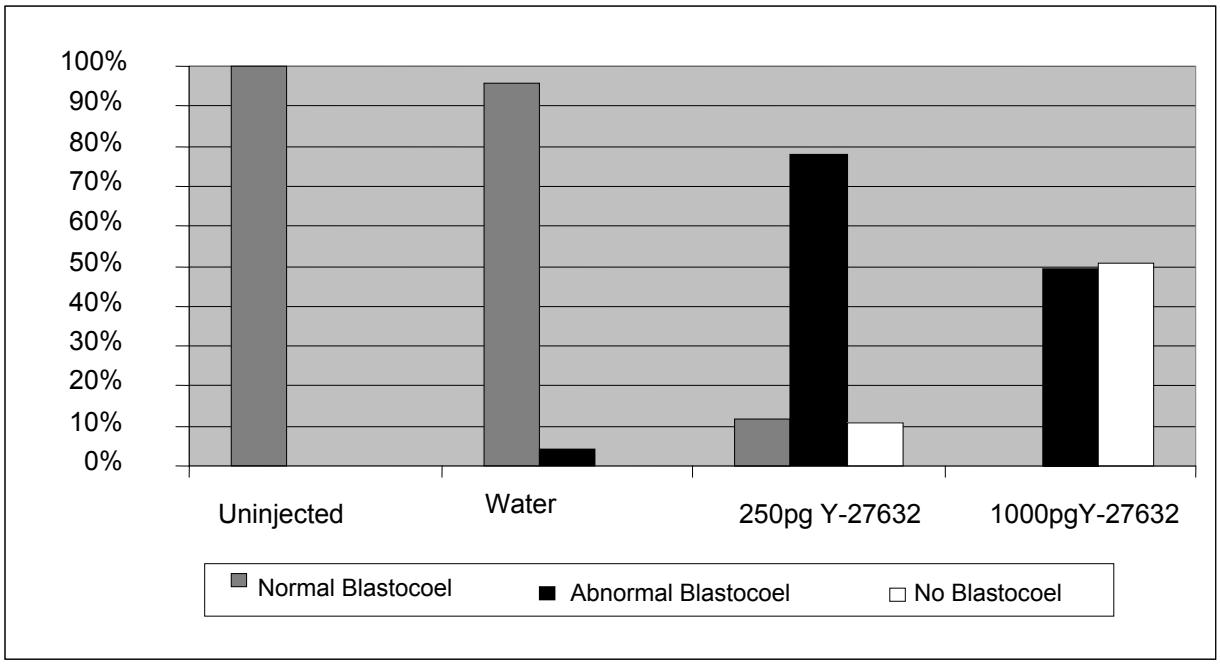

Fig. 2 Graphical representation of Table 1 data. The bars represent the percentage of embryos with each phenotype. Phenotypes indicated by the color of the bar; 'Normal Blastocoel' is gray, 'Abnormal Blastocoel' is black and 'No Blastocoel' is White.

Although a loss of cell adhesion was observed in the experimental embryos, consistent with the phenotype observed upon EPP activation, no noticeable change in cell shape occurred. Using scanning electron microscopy (SEM) we were able to easily view cell morphology. Figure 3 shows a scanning electron micrograph of embryos that were water injected (A), expressed activated EPP (B), or were injected with Y-27632 (C). The water-injected embryos served as a control, with their cells exhibiting the normal angular appearance. The cells that were affected by EPP activation changed their morphology to a rounded appearance. No noticeable change in cell morphology is observed in the Y-27632 affected cells.

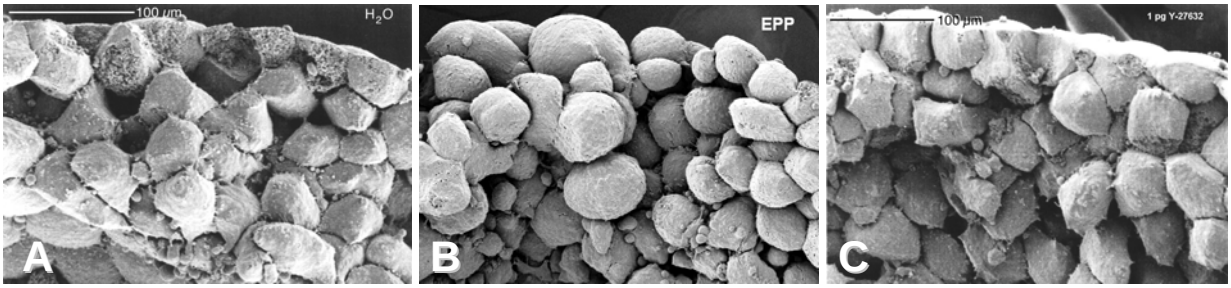

Fig. 3 Inhibition of ROCK family of kinases does not cause a change in cell shape. Embryos were injected with water **(A)**, or EPP and TGF-α **(B)**, or 1 ng Y-27632 **(C)**. At blastula stage, embryos were bisected and viewed under SEM.

Transfection of A6 Cells with ROCK Inhibitors Causes Rounded Cell Bodies with Neurite Extentions

When EPP is activated in A6 cultured cells the flat cells round and detach from each other and the substrate (Winning unpublished results). We looked at the effects of three different ROCK inhibitors on A6 cell culture to see if ROCK inhibition causes similar effects as EPP activation: Y-27632, which has previously been described; Hydroxyfasudil, an active metabolite of fasudil (Satoh *et al*., 2001), which is a specific ROCK kinase inhibitor with an inhibitory effect on ROCK 100 times greater than on PKC and 1,000 times greater than on MLCK (Shimokawa e*t al*., 1999); and Rho-Kinase Inhibitor I, an isoquinolinesulfonamide derivative which is a strong inhibitor of ROCK and a poor inhibitor of other serine/threonine kinases (Ikenoya *et al*., 2002). Cells were lipofected with the different inhibitors, prepared for SEM, and observed.

Inhibition of ROCK by different ROCK inhibitors causes a change in cell morphology from the flat normal cells. Hydroxyfasudil and Rho-Kinase Inhibitor I caused more of the affected cells to be spherical whereas Y-27632 resulted in cells that were long and puckered. Fig 4 shows the results of the different inhibitors.

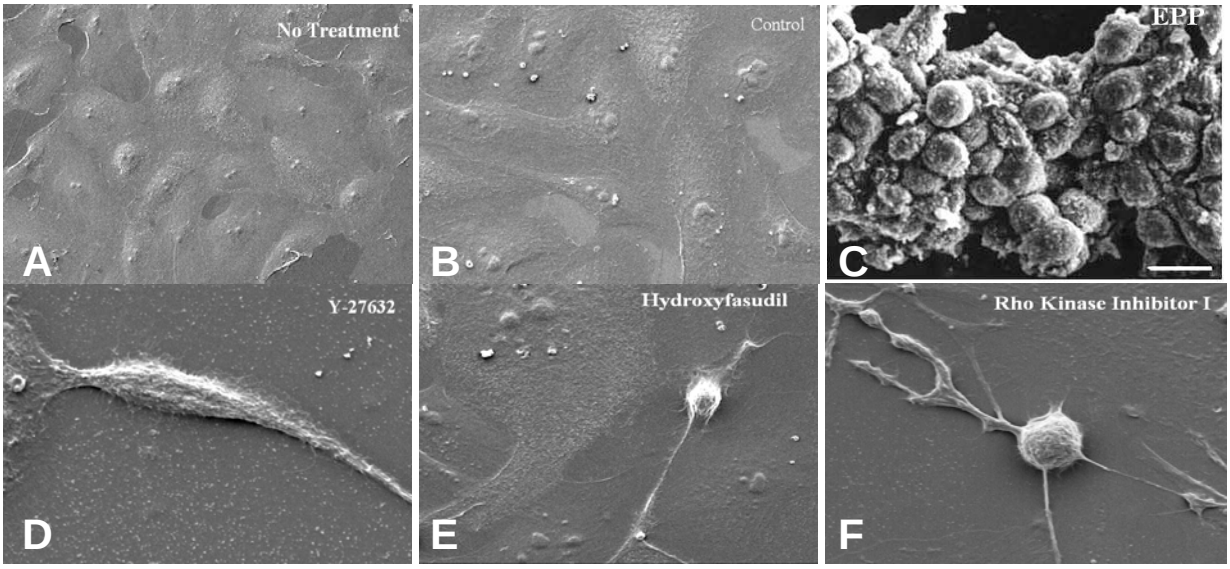

Fig. 4 Inhibition of ROCK in A6 cell culture causes change in cell morphology. Cells were not treated **(A)**, treated with Lipofection reagents with no DNA **(B)**, 10 µg Y-27632 **(D)**, 10 µg Hydroxyfasudil **(E)**, or 10 µg Rho Kinase Inhibitor I **(F)**. The picture of cells with activated EPP shown in **(C)** is from Winning unpublished results.

Dominant-Negative ROCKII Does Not Cause a Loss of Cell Adhesion in Xenopus Embryos or Change in Cell Morphology of Cultured A6 Cells

As previously mentioned, ROCK exists in two isoforms (ROCKI and ROCKII). The ROCK inhibitors tested above inhibit both isoforms of ROCK. We used mutant forms of both isoforms in order to determine the role of each in the loss of cell adhesion witnessed in the *Xenopus* embryo experiment and the change of cell morphology in the A6 cell culture experiment.

The dominant-negative version of ROCKII (referred to as RB/PH). This protein has point mutations in the Rho-binding (RB) and PH domains, preventing Rho binding activity and abolishing ROCKII activity (Amano *et al*., 1997). The internal morphology of the *Xenopus* embryos that had RB/PH expressed in them is shown in Table 2. A graph of the table is shown in Figure 5. A6 cell culture was also lipofected with RB/PH; these cells showed no visible change in their morphology (Figure 6).

Table 2 ROCKII RB/PH does not cause loss of cell-adhesion in *Xenopus* blastulas

	Injection				
	Water	1ng RB/PH	3.75ng RB/PH	7.5ng RB/PH	EPP+TGF- α
Normal Blastocoel	91.5% (74)	80.2% (73)	86.0% (80)	82.2% (65)	44.1% (30)
Abnormal Blastocoel	2.5% (2)	11.0% (10)	3.2% (3)	8.9% (7)	14.7% (10)
No Blastocoel	6.2% (5)	8.8% (8)	10.8% (10)	8.9% (7)	41.2% (28)

Embryos were injected at one-cell stage with the mRNA listed. Embryos were then assessed at blastula stage for blastocoel morphology and cellular dissociation by phenotypes described in Table 1. Percentages indicate the fraction of embryos exhibiting each of these phenotypes and parentheses are the numbers of embryos exhibiting each phenotype.

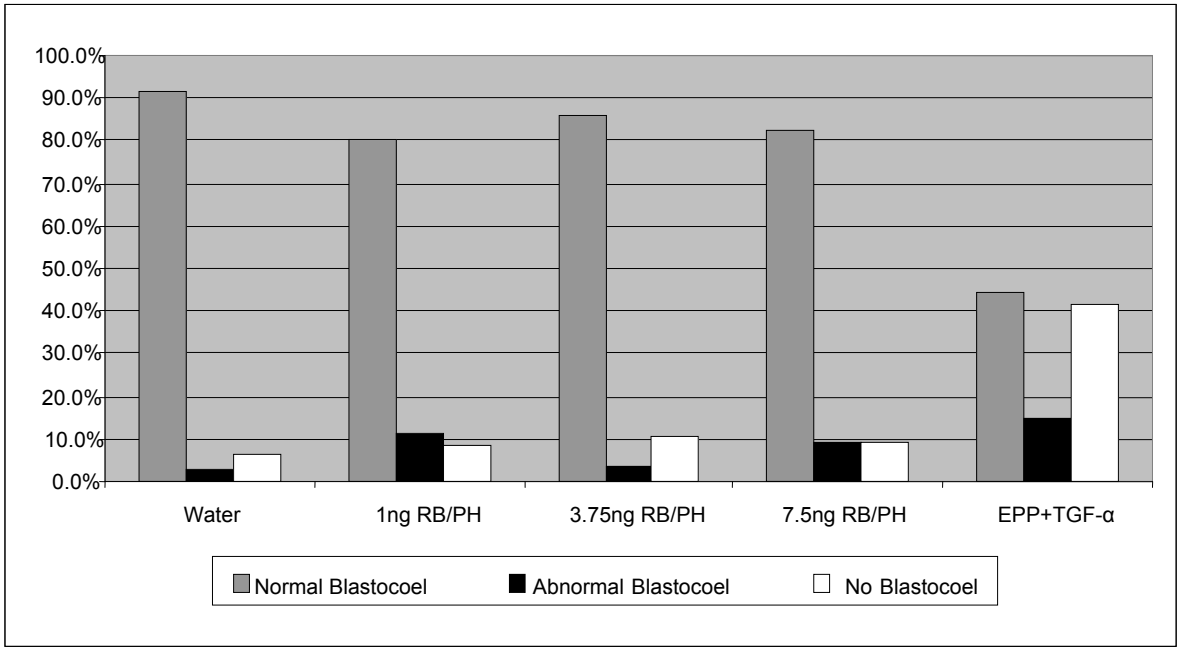

Fig. 5 Graphical representation of Table 2. The bars represent the percentage of embryos with each phenotype. Phenotypes indicated by the color of the bar; 'Normal Blastocoel' is gray, 'Abnormal Blastocoel' is black and 'No Blastocoel' is White.

Dominant-Negative ROCKI Causes a Change in Cell Morphology in Cultured A6 Cells

The dominant-negative version of ROCKI is called KDIA. KDIA is a full length protein which is made dominant-negative by point mutations both in the kinase and the Rho-binding domains (Ishizaki *et al*., 1997). Lipofection of A6 cells with KDIA caused cell rounding with filopodial extension, similar to the morphology of cells treated with Hydroxyfasudil and Rho-Kinase Inhibitor I. The cells were photographed under the phase-contrast microscope (Figure 6C) and a more detailed look was done using SEM (Figure 7).

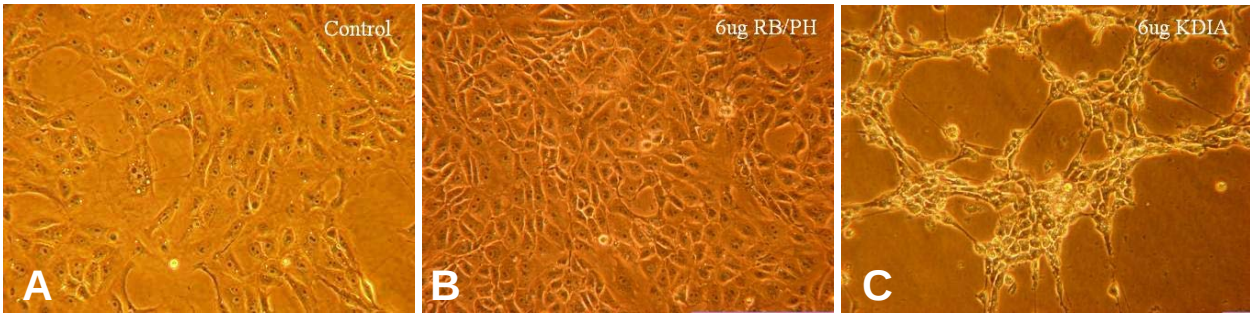

Fig. 6 Dominant-negative ROCKII does not cause a change in cell morphology while dominant-negative ROCK1 does. Cells were transfected with lipofection reagents but no DNA for a control **(A)**, 6 µg RB/PH **(B)**, or 6 µg KDIA **(C)**. Cells were photographed under a phase-contrast microscope.

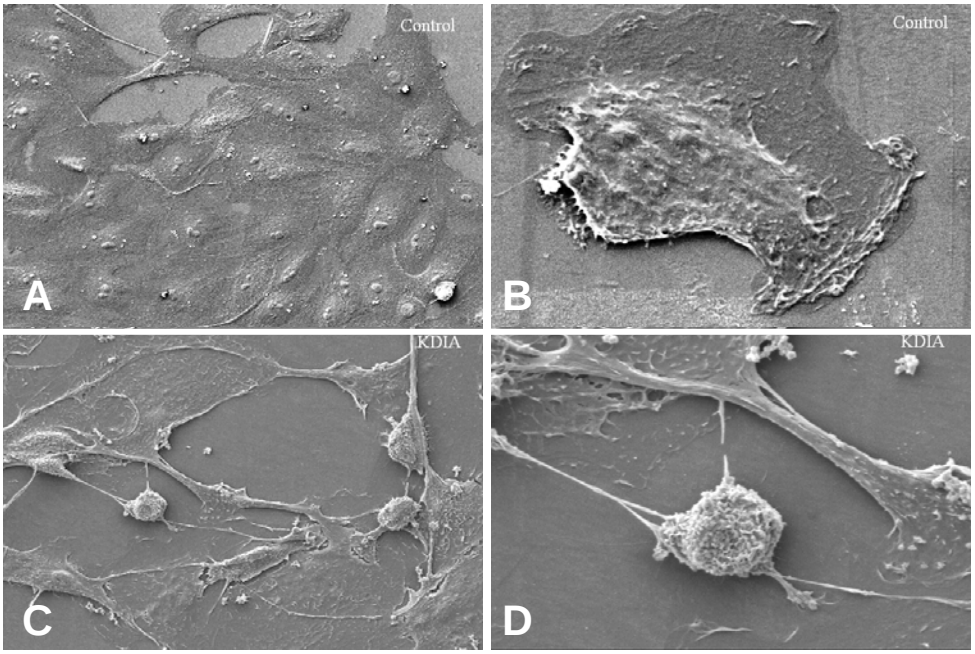

Fig. 7 Comparison of control A6 cells and those lipofected with KDIA shows KDIA causes cell rounding. The top pictures **(A, B)** show control cells and the bottom pictures **(C, D)** show cells lipofected with 6µg KDIA.

Constitutively-active ROCKI Shows Partial Rescue of EPP Phenotype While Constitutively-active ROCKII Does Not Rescue Embryos from EPP Phenotype

Because our hypothesis states that EphA4 activation causes ROCK inhibition, expressing constitutively-active ROCK along with EPP activation should result in rescue of embryos from the EPP phenotype.

In order to test this for ROCKII, mRNA encoding constitutively-active ROCKII was co-injected with mRNA for EPP and TGF-α into *Xenopus* embryos at the one-cell stage. The constitutively-active form of ROCKII is known as CAT. This mutant is created by deleting the

COOH-terminal portion of ROCKII, which results in the NH_2-terminal portion containing the catalytic domain becoming constitutively active (Amano *et al*., 1997). The injected embryos were scored for their blastocoel at blastula stage. Results of the scoring are shown in Table 3 and a graph of the data is shown in Figure 8. These results show little difference in blastocoel morphology between embryos with activated EPP and embryos which have CAT expressed in addition to activated EPP. As expected, injection of CAT alone has little effect on embryos as compared to water-injected controls

Table 3 ROCKII CAT does not cause rescue of EphA4 phenotype

	Injection			
	Water	EPP + TGF-α	EPP+TGF-α + 500 pg CAT	500 pg CAT
Normal Blastocoel	95.4% (62)	9.4% (6)	14.5% (11)	71.5% (40)
Abnormal Blastocoel	3.1% (2)	14.1% (9)	22.4% (17)	12.5% (7)
No Blastocoel	1.5% (1)	76.5% (49)	63.1% (48)	16.1% (9)

Embryos were injected at the one-cell stage with the mRNA combinations listed. Assessment of embryos was done by evaluating blastocoel morphology and cell dissociation at blastula stage. Phenotypes listed are described in Table 1. Percentages indicate the fraction of embryos exhibiting each phenotype while parentheses indicate the number of embryos exhibiting each phenotype.

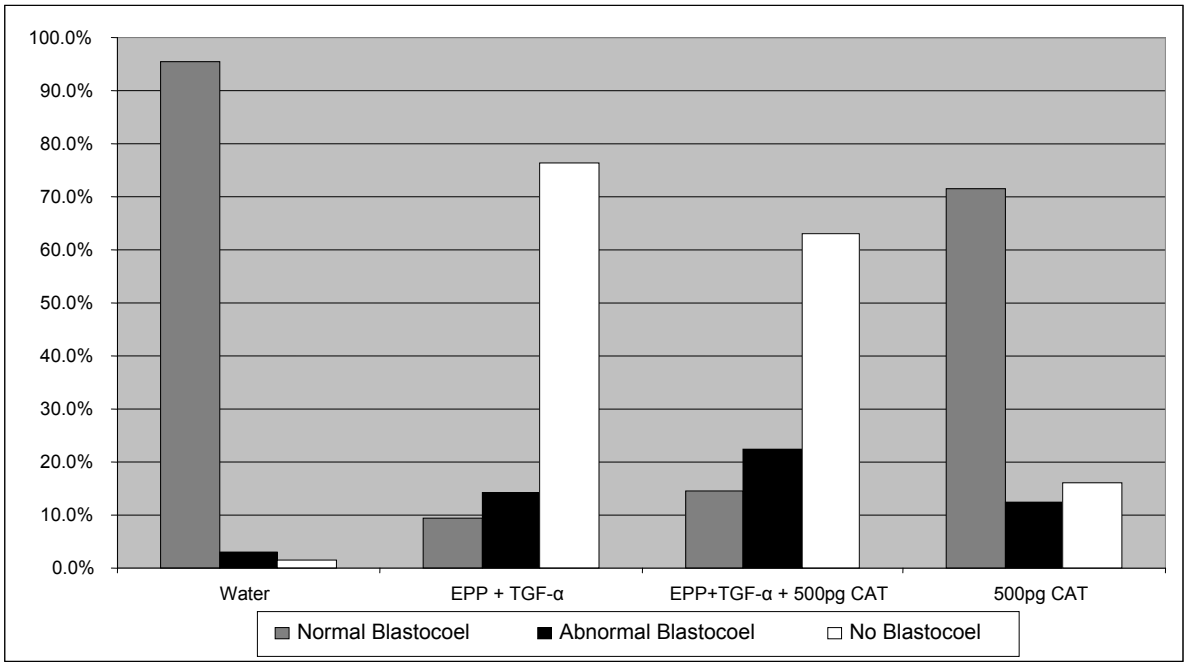

Fig. 8 Graphical representation of Table 3. The bars represent the percentage of embryos with each phenotype. Phenotypes indicated by the color of the bar; 'Normal Blastocoel' is gray, 'Abnormal Blastocoel' is black and 'No Blastocoel' is White.

For the ROCKI rescue experiment, active ROCKI protein was co-injected with mRNA for EPP and TGF-α into *Xenopus* embryos at the one-cell stage. The embryos were scored based on their blastocoel at blastula stage. Results of the scoring are shown in Table 4 and a graph of the data is shown in Figure 9. These results show that co-injection of active ROCKI protein in addition to EPP activation results in a detectible reduction in the amount of embryos with the lack of a blastocoel.

Table 4 Active ROCKI causes limited rescue of EphA4 phenotype in *Xenopus* blastulas

	Injection				
	Water	EPP+TGF-α	EPP+TGF-α + 50pg ROCKI	EPP+TGF-α + 100pg ROCKI	100pg ROCKI
Normal Blastocoel	60.0% (21)	0.0% (0)	13.6% (6)	13.6% (6)	65.1% (28)
Abnormal Blastocoel	14.3% (5)	2.9% (1)	22.7% (11)	25.0% (11)	18.6% (8)
No Blastocoel	25.7% (9)	97.1% (34)	63.6% (28)	61.4% (27)	16.3% (7)

Embryos were injected at the one-cell stage with the mRNA and protein combinations listed. Assessment of embryos was done by evaluating blastocoel morphology and cell dissociation at blastula stage. Phenotypes listed are described in Table 1. Percentages indicate the fraction of embryos exhibiting each phenotype while parentheses indicate the number of embryos exhibiting each phenotype.

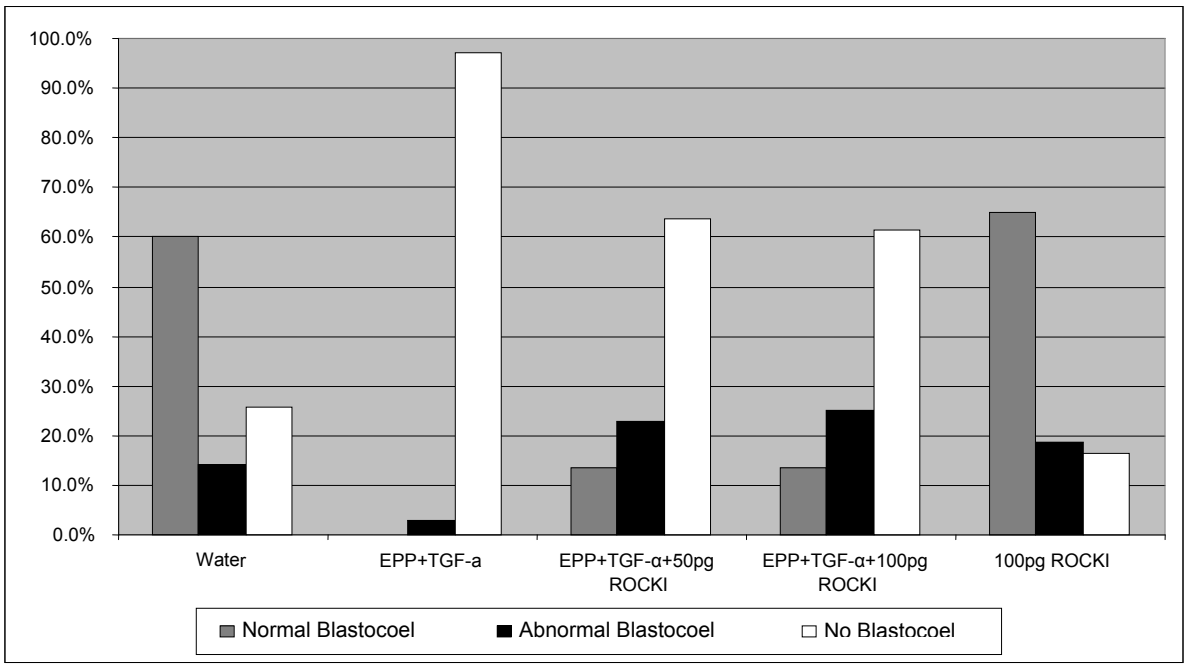

Fig. 9 Graphical representation of Table 4. The bars represent the percentage of embryos with each phenotype. Phenotypes indicated by the color of the bar; 'Normal Blastocoel' is gray, 'Abnormal Blastocoel' is black and 'No Blastocoel' is White.

Discussion

Our results have shown that ROCK inhibition by treatment with ROCK inhibitors produces a phenotype similar to that of EPP activation; in *Xenopus* embryos it causes a loss of cell-cell adhesion and in cultured A6 cells it produces a change in cell morphology. Mutants were used to differentiate the roles of each ROCK isoform. Inhibition of ROCKII through the expression of a dominant-negative form did not cause a loss of cell-cell adhesion in *Xenopus* embryos or a change in cell morphology in cultured A6 cells. Furthermore, constitutively-active ROCKII failed to rescue embryos with activated EPP from the EPP phenotype. In contrast, expression of dominant-negative ROCKI in cultured A6 cells caused a rounded cell morphology and co-expression of active ROCKI protein with activated EPP in *Xenopus* embryos resulted in partial rescue of the EPP phenotype. The results of our experiments provide evidence that ROCKI, but not ROCKII, is inhibited in EphA4 signaling.

In order to further solidify the involvement of ROCKI in EphA4 signaling, more research must be done. First, a study in *Xenopus* embryos using a dominant-negative version of ROCKI was never performed. This experiment should be completed in order to assess whether dominant-negative ROCKI causes loss of cell adhesion resulting in the lack of a blastocoel, similar to the EPP phenotype. In addition, the ROCKI rescue, which took place in *Xenopus* embryos, should be modified to include co-injection of constitutively-active ROCKI mRNA in addition to the mRNA used for EPP activation, as opposed to active ROCKI protein. More protein needs to be injected into the one-cell embryo than mRNA to get the same effect, yet injecting too much protein results in death of the embryos. As a result, mRNA injection is more desirable, because less mRNA can be injected and higher expression achieved, which may possibly result in a larger proportion of rescued embryos. In addition, to see if the rescue experiment can be mimicked in cultured A6 cells, a lipofection should be performed that includes DNA encoding active EPP as well as constitutively-active ROCKI.

ROCK has many downstream effectors (Listed in Loirand *et al*., 2006). Two prominent effectors are Myosin Light Chain (MLC) phosphatase and LIM kinase (LIMK) (Kimora *et al*., 1996; Maekawa *et al*., 1999). Phosphorylation of MLC and myosin regulatory proteins results in increased stress fiber formation (Chrzanowska-Wodnicka and Burridge, 1996). LIMK phosphorylation by ROCK causes LIM to inactivate cofilin (Maekawa *et al*., 1999). Cofilin promotes actin depolarization, and cofilin inactivation promotes actin stability (Moon and Drubin, 1995). It has been shown in Madin-Darby canine kidney (MDCK) cells that using a

MLC kinase inhibitor and dominant-active mutant of cofilin inhibits the formation of stress fibers and focal adhesions (Takaish *et al*., 2000). These results suggest that ROCK inhibition results in the inhibition of stress fiber formation, through the effectors MLC and LIMK.

It has also been shown in MDCK cells that treatment with C3 transferase induces the disappearance of stress fibers and focal adhesions, followed by disruption of cell-cell adhesions and cell-rounding (Kotani *et al*., 1997; Takaishi *et al*., 1997). C3 transferase is an inhibitor of RhoA, B, and C (Aktories and Hall, 1989; Aktories *et al*., 2000). Using C3 in *Xenopus* embryos also causes the loss of cell-cell adhesion and cell rounding (Winning *et al*., 2002). Additionally, when dominant-negative ROCKI is expressed in MDCK cells, a similar phenotype is observed. This is achieved by ROCK inhibiting stress fiber and focal adhesion formation or the localization of ERM proteins at the peripheral bundles (Nakano *et al*., 1999). Furthermore, dominant-negative ROCKI induced cell rounding and disruption of E-cadherin-based cell-cell adhesion (Nakano *et al*., 1999). However, although the dominant-negative ROCKI induced phenotype is similar to that seen during Rho inhibition; the results are not the same. We had also seen this in the comparison of ROCK inhibition to the active EPP phenotype.

Inhibition of ROCK does not result in the complete EphA4 phenotype. In *Xenopus* embryos ROCK inhibition using the selective inhibitor Y-27632 results in the loss of cell-cell adhesion and therefore the lack of blastocoel, consistent with EPP activation, yet it does not result in a rounded cellular morphology which is also associated with EPP activation. Therefore, the change in cell shape must be due to some other downstream effect of EphA4 activation.

Inhibition of RhoA causes change in cell shape in the form of cellular rounding in *Xenopus* embryos (Winning *et al*., 2002). Because ROCK does not show a change in cell shape when inhibited, RhoA must have at least one other downstream effector, which causes change in cell shape. RhoA has multiple downstream effectors, including PKN, Citron kinase, PI3 kinase, Dia (1 and 2 in mammals), and PI-4-P5 kinase among others (reviewed in Bishop and Hall, 2000).

Other than ROCK proteins, Dia is required for Rho-induced assembly of stress fibers and focal adhesions (Watanabe *et al*., 1997). The catalytic domain of ROCK alone does not induce organization of stress fibers correctly; however, it has been reported that when combined with an active version of Dia, stress fibers are induced (Watanabe *et al*., 1999; Nakano *et al*., 1999; Wasserman, 1998). Dia (1 and 2 in mammals) is a member of the formin-homolgy (FH) family of proteins. Dia proteins contain two FH domains (Wasserman, 1998). The FH1 sequence which

contains multiple proline-rich motifs allows binding to the G-actin-binding protein, profilin (Watanabe *et al*., 1999), an interaction that allows Dia to contribute to actin polymerization and F-actin organization into stress fibers (Wasserman *et al*., 1998). It has been shown that the morphology of stress fibers in MDCK cells coexpressing active mutants of ROCKI and mDia1 is not identical to the V14RhoA-induced (constitutively-active RhoA) morphology, however, it is more similar than the active ROCKI alone-induced morphology (Nakano *et al*., 1999).

Phosphatidylinositol-4-phosphate 5-kinase, PI-4-P5K, may also provide a link between Rho and the stimulation of new actin polymerization and of focal adhesion assembly. Overexpression of PI-4-P5K to produce PIP_2 induces actin polymerization in COS-7 cells (Desrivieres *et al*., 1998). Furthermore, PIP_2 binds capping proteins, such as gelsolin, inducing their release from actin-filament barbed ends. This provides a mechanism where PIP_2 could increase actin polymerization (Janmey *et al*., 1987). A physical interaction between Rho and PI-4-P5K has been detected in Swiss-373-cell lysates; however the interaction may not be direct (Ren *et al*., 1996).

Because the kinase domains of both ROCKI and ROCKII isoforms are nearly identical, it has been assumed that both isoforms share the same substrates. Because data support a role for ROCKI in EphA4 signaling, but not ROCKII, it is possible that ROCKI and ROCKII have different downstream targets. The observation that only ROCKI and not ROCKII binds to and phosphorylates RhoE is the first evidence that ROCKI and ROCKII have different targets (Riento *et al*., 2005). Furthermore, ROCKI and ROCKII regulate different aspects of myosin II activity where ROCKI is important in stress fiber formation and ROCKII acts in regulating the microfilament bundle and focal adhesion site (Yoneda *et al*., 2005). The differences in substrate specificity may be, in part, due to the N-terminal regions upstream of the kinase domains, which can be involved in the interaction with the substrates (Riento *et al*., 2003).

In conclusion, our data suggest a role for ROCKI in EphA4 signaling, but not ROCKII. ROCK proteins have many downstream targets, but those most likely in the reorganization of the actin cytoskeleton due to EphA4 signaling include MLC, LIMK, and ERM. ROCK inhibition does not completely reproduce the phenotypes of EPP activation or RhoA inhibition, because it does not cause change in cell shape in *Xenopus* embryos. This suggests another downstream effector of RhoA to be involved; and the literature suggests Dia1 and PI-4-P5K as probable candidates.

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