

ROCK-I regulates closure of the eyelids and ventral body wall by inducing assembly of actomyosin bundles

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Rho-associated kinase (ROCK) I mediates signaling from Rho to the actin cytoskeleton. To investigate the *in vivo* functions of ROCK-I, we generated ROCK-I-deficient mice. Loss of ROCK-I resulted in failure of eyelid closure and closure of the ventral body wall, which gave rise to the eyes open at birth and omphalocele phenotypes in neonates. Most ROCK-I^{-/-} mice died soon after birth as a result of cannibalization of the omphalocele by the mother. Actin cables that encircle the eye in

the epithelial cells of the eyelid were disorganized and accumulation of filamentous actin at the umbilical ring was impaired, with loss of phosphorylation of the myosin regulatory light chain (MLC) at both sites, in ROCK-I^{-/-} embryos. Stress fiber formation and MLC phosphorylation induced by EGF were also attenuated in primary keratinocytes from ROCK-I^{-/-} mice. These results suggest that ROCK-I regulates closure of the eyelids and ventral body wall through organization of actomyosin bundles.

Introduction

The small GTPase Rho contributes to cellular functions such as cell motility, adhesion, and cytokinesis through reorganization of the actin cytoskeleton. Rho is activated by extracellular signals such as lysophosphatidic acid as well as during progression of the cell cycle. Rho activation results in assembly of contractile actin–myosin bundles, which include stress fibers in interphase cells and the contractile ring in dividing cells (Narumiya, 1996; Etienne-Manneville and Hall, 2002). The actions of Rho are mediated by downstream Rho effectors, a variety of which have been identified on the basis of their selective interaction with the active form of Rho. One such effector is Rho-associated kinase (ROCK), which includes ROCK-I (also known as ROK β) and ROCK-II (also known as Rho-kinase or ROK α ; Riento and Ridley, 2003). ROCK mediates Rho signaling and reorganizes the actin cytoskeleton through phosphorylation of several substrates that contribute to the assembly of actin filaments and contractility. For example, ROCK phosphorylates both the

myosin regulatory light chain (MLC) and myosin phosphatase, thereby regulating the level of MLC phosphorylation both directly and indirectly (Amano et al., 2000). Phosphorylation of MLC induces smooth muscle contraction as well as the formation of both stress fibers and focal adhesions. ROCK also phosphorylates and activates LIM-kinase, which in turn phosphorylates the cofilin/actin-depolymerizing factor complex and thereby inhibits its ability to mediate depolymerization and severing of actin filaments (Maekawa et al., 1999).

Elucidation of the roles of ROCK in the intact mammalian body has been facilitated by the introduction of a specific ROCK inhibitor, Y-27632 (Uehata et al., 1997). Studies with this inhibitor have revealed that ROCK regulates various physiological and pathological processes including smooth muscle contraction associated with hypertension and asthma, and cell migration associated with inflammation and tumor metastasis. However, relatively little is known of the role of ROCK in development. In addition, given that Y-27632 inhibits the activity of both ROCK-I and ROCK-II, studies with this inhibitor provide limited information on the specific functions of each ROCK isoform. To explore such specific functions of ROCK-I and ROCK-II, we have generated mice deficient in these enzymes individually. We previously described the generation and phenotype of ROCK-II knockout mice (Thumkeo et al., 2003). Most

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Abbreviations used in this paper: α -SMA, α -smooth muscle actin; dpc, d postcoitum; EGFR, EGF receptor; EOB, eyes open at birth; JNK, c-Jun NH₂-terminal kinase; MLC, myosin regulatory light chain; ROCK, Rho-associated kinase.

ROCK-II-deficient embryos manifest extensive thrombus formation in the placenta, resulting in placental dysfunction, intrauterine growth retardation, and fetal death. These animals thus revealed an important role for ROCK-II in placental homeostasis during the perinatal period.

Closure and subsequent reopening of the eyelids are common to all mammals during development. In mice, eyelid closure occurs between 15.5 and 16.5 d postcoitum (dpc), when the outermost epithelial layer of the eyelid rim extends toward the center to cover the entire ocular surface and fuses with the opposite eyelid epithelium. The eyelids are tightly closed at birth and remain so until 12 d after birth, with complete separation occurring 1 or 2 d later (Findlater et al., 1993; Kaufman, 2002). Mutations of several genes cause the eyes open at birth (EOB) phenotype. Implication of EGF receptor (EGFR) signaling in the eyelid closure and its similarity to dorsal closure in *Drosophila* are discussed (Martin and Parkhurst, 2004; Xia and Karin, 2004).

Another example of tissue closure during mammalian development is closure of the ventral body wall. At midgestation, visceral organs rapidly expand in volume and exceed the space of the peritoneal cavity, resulting in protrusion of the midgut loop from this cavity through the umbilical ring. This "physiological umbilical hernia" is first apparent at 10.5 dpc in mice. At 15.5 dpc, the midgut loop begins to return to the peritoneal cavity, and by 16.5 dpc the physiological umbilical hernia has disappeared with closure of the umbilical ring and resorption of the space formerly occupied by the gut within the umbilical cord (Kaufman, 2002). Failure of umbilical ring closure results in omphalocele, in which organs such as the liver and gut protrude from the peritoneal cavity.

We now show that ROCK-I knockout mice exhibit both EOB and omphalocele phenotypes as a result of disorganization of actomyosin cables in the eyelid epithelium and defective actin assembly in the umbilical ring.

Results

Targeted disruption of the mouse ROCK-I gene

We disrupted the ROCK-I gene by replacing exons 3 and 4, which encode part of the kinase domain, with genes for β -galactosidase and neomycin resistance (Fig. 1 A). Chimeric mice were generated, and males were mated with C57BL/6N females to produce heterozygous ROCK-I^{+/-} mice, which were then intercrossed. The genotypes of offspring were determined by Southern blot analysis and PCR with DNA obtained from the tail (Fig. 1, B and C). Loss of ROCK-I protein was confirmed by immunoblot analysis (Fig. 1 D). A 160-kD protein corresponding to ROCK-I was detected in the adult brain of wild-type mice but not in that of ROCK-I^{-/-} mice. In contrast, the abundance of ROCK-II did not differ between the two genotypes, indicating that the expression of ROCK-II did not increase to compensate for the loss of ROCK-I. We made use of the *lacZ* reporter gene inserted in-frame with the initiator methionine codon of ROCK-I in the targeting construct to examine the pattern of ROCK-I gene expression by whole-mount staining of ROCK-I^{-/-} embryos for β -galactosidase activity with

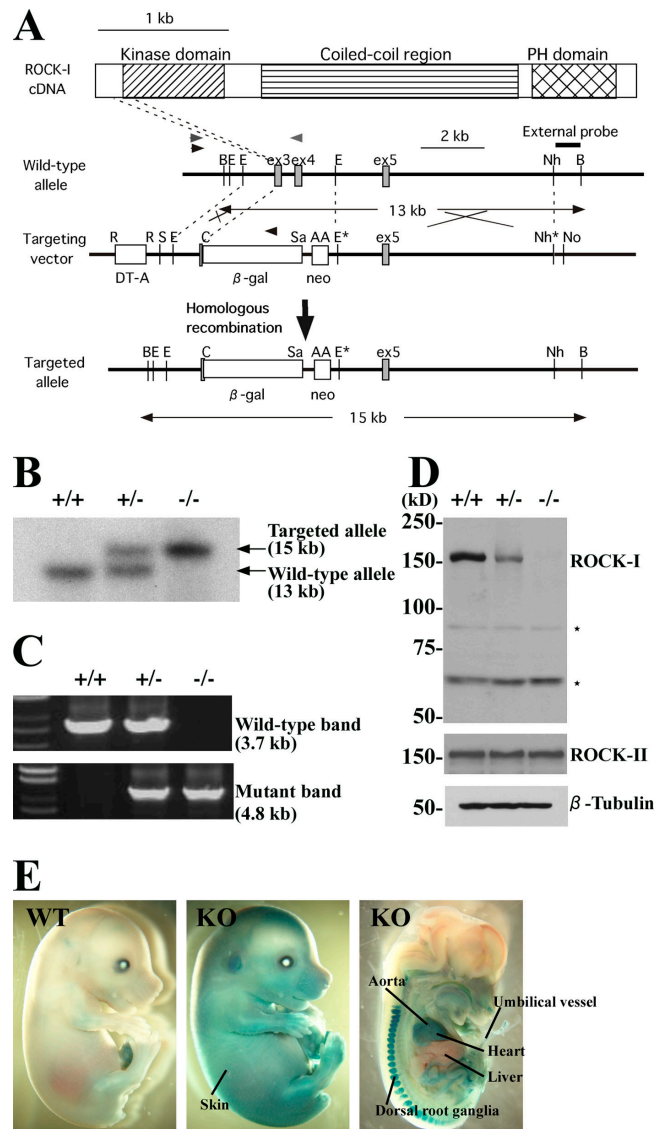


Figure 1. Generation of ROCK-I-deficient mice. (A) Schematic representations of the domain structure of mouse ROCK-I cDNA, the wild-type ROCK-I allele, the targeting vector, and the targeted allele. Positions of β -galactosidase (β -gal), neomycin resistance (neo), and diphtheria toxin A (DT-A) genes; of restriction sites for BglII (B), EcoRV (E), NheI (Nh), RsrII (R), SmaI (S), ClaI (C), SalI (Sa), Ascl (A), and NotI (No); and of exons 3 (ex3), 4, and 5 are shown. The restriction sites indicated by asterisks are lost due to blunt-end ligation. Positions of primers for PCR analysis are indicated by arrowheads. The external probe is a unique 3' genomic probe that distinguishes the 13-kb wild-type BglII fragment from the 15-kb BglII fragment generated by the targeted allele. (B) Southern blot analysis of genomic DNA obtained from mouse tail. The genotypes of the wild-type, heterozygous, and homozygous knockout mice are shown as +/+, +/-, and -/-, respectively. (C) Genotyping by PCR analysis of genomic DNA from mouse tail. The leftmost lane contains molecular size standards. (D) Immunoblot analysis of whole-brain lysates of adult ROCK-I^{+/+}, ROCK-I^{+/-}, and ROCK-I^{-/-} mice with antibodies specific for either ROCK-I, ROCK-II, or β -tubulin. Positions of molecular mass markers are shown on the left. Asterisks indicate nonspecific bands. (E) X-Gal staining of wild-type (WT) and ROCK-I^{-/-} (KO) embryos at 15.5 dpc (left and middle) or 13.5 dpc (right, sagittal section). X-Gal staining was detected in the skin, heart, aorta, umbilical blood vessels, and dorsal root ganglia of ROCK-I^{-/-} embryos.

the substrate X-Gal. Such staining was detected in many locations throughout the embryo, including the skin, heart, aorta, umbilical blood vessels, and dorsal root ganglia (Fig. 1 E).

Table I. Genotypes of offspring obtained by crossing of ROCK-1^{+/-} mice

Stage	Total no. of offspring	No. of mice of each genotype		
		+/+	+/-	-/-
14.5 dpc	40	12	20	8
15.5 dpc	145	40	73	32
16.5 dpc	56	14	28	14
18.5 dpc	344	86	168	90
4 wk	316	101	203	12

EOB and omphalocele phenotypes of ROCK-1-deficient mice

Analysis of genotype distribution in offspring from heterozygote crosses revealed that the homozygous mutant mice were present in the expected Mendelian ratio during embryonic development at all stages including 18.5 dpc. However, the ROCK-1^{-/-} mice were greatly underrepresented among littersmates at 4 wk old (Table I).

Examination of embryos at 18.5 dpc revealed EOB and omphalocele phenotypes that were fully penetrant in ROCK-1^{-/-} mice (Fig. 2 A). At this stage of development, wild-type mice manifest fused eyelids and resolution of the physiological umbilical hernia. There was no substantial difference between wild-type and ROCK-1^{+/-} littermates in this regard (unpublished data). Different degrees of fusion impairment were

apparent in the eyelids of ROCK-1^{-/-} mice; 50% (17/34) of such embryos had fully open eyelids, whereas the eyelids of the remaining 50% (17/34) of embryos were almost fully fused but manifested a hole with a diameter of <1 mm (Fig. 2 B). Some of the surviving ROCK-1^{-/-} mice gradually developed apparent proliferative inflammation of the eyelid that almost covered the entire eye; this phenotype was observed in 50% (6/12) of ROCK-1^{-/-} mice at 5–6 mo old (Fig. 2 C). This eye lesion may result from secondary effects due to the open state of the eye in neonates.

Omphalocele was apparent also in varying degrees and was classified into three types (Fig. 2 A): severe omphalocele, including the liver and small intestine (9/34, 26.5%); moderate omphalocele, with only the small intestine protruding (13/34, 38.2%); and mild omphalocele, in which the intestine protrudes upon crying (12/34, 35.3%). There was no correlation between the extent of EOB and that of omphalocele. The presence of an omphalocele does not affect survival in utero, and we found that the loss of ROCK-1^{-/-} mice occurred immediately after birth. The umbilicus was not closed and intraperitoneal bleeding was evident in the dead ROCK-1^{-/-} neonates (Fig. 2 D). We further noted that parts of several visceral organs, notably the liver and intestine, had been gnawed at in mutant neonates (Fig. 2, E and F). Because we confirmed that visceral organs were fully intact in 18.5 dpc mutant (not depicted), these results indicate that the mother had cannibalized the omphalocele together with the protruded organs in the process of clearing

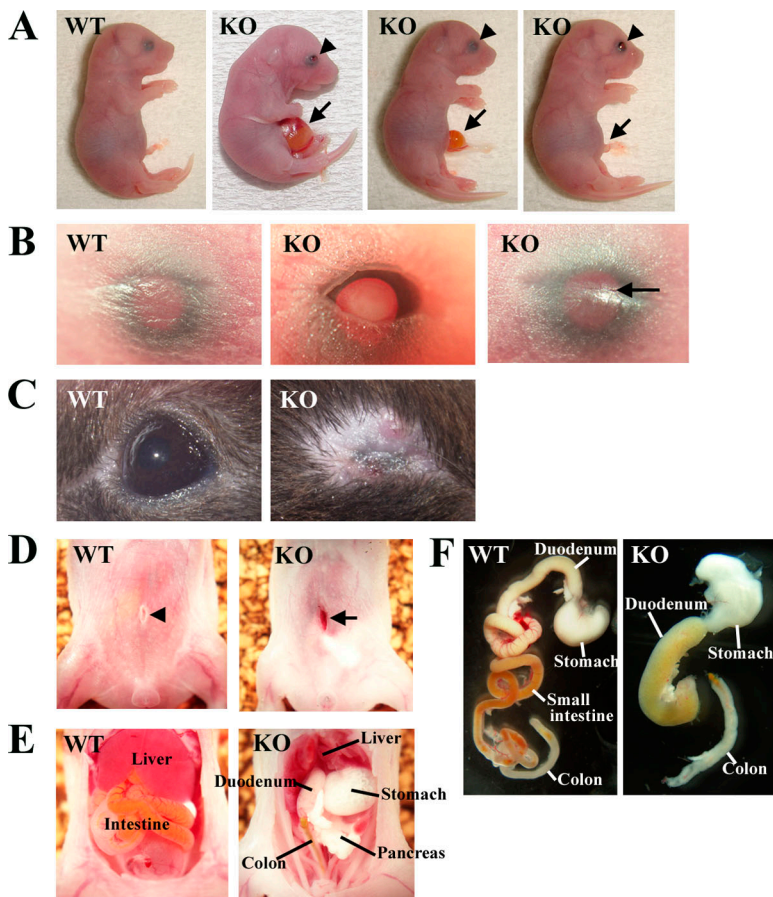
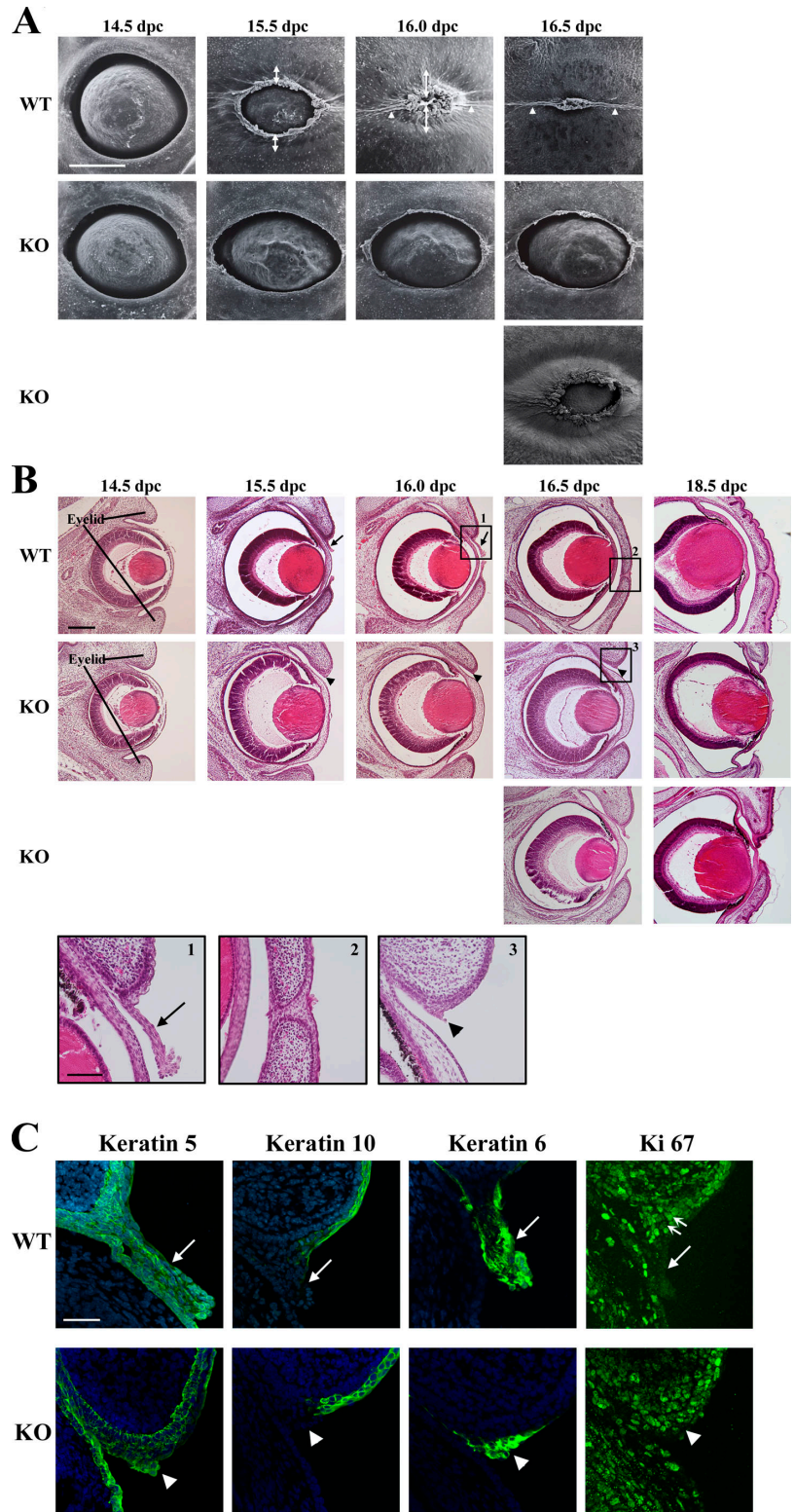


Figure 2. EOB and omphalocele phenotypes of ROCK-1-deficient mice. (A) Wild-type and ROCK-1^{-/-} embryos at 18.5 dpc. Arrowheads and arrows indicate EOB and omphalocele phenotypes, respectively. Severe, moderate, and mild forms of omphalocele in the mutant embryos are shown from left to right. (B) Eyes of wild-type and ROCK-1^{-/-} mice at 18.5 dpc. The eyes of the ROCK-1^{-/-} embryos are either fully open (middle) or partially open (right; arrow indicates a small hole). (C) Eyes of adult wild-type and ROCK-1^{-/-} mice. (D) Umbilical region of wild-type and ROCK-1^{-/-} neonates. The umbilical ring in the wild-type neonate is closed (arrowhead), whereas that in the ROCK-1^{-/-} neonate remains open (arrow). (E) Visceral organs in the abdominal cavity of the neonates shown in D. The ventral body wall was removed to render the visceral organs visible. Portions of the liver and intestine are absent in the ROCK-1^{-/-} neonate. (F) Gastrointestinal tract from the stomach to the colon of the neonates shown in D and E. A part of the small intestine is absent in the ROCK-1^{-/-} mouse.

Figure 3. Impaired eyelid closure in *ROCK-1*^{-/-} embryos. (A) Scanning electron micrographs of the eyes of wild-type (top) and *ROCK-1*^{-/-} (middle and bottom) embryos from 14.5 to 16.5 dpc. Bidirectional arrows indicate extension of the eyelid rim. Arrowheads indicate eyelid fusion in the wild-type embryos. Bar, 500 μ m. (B) Hematoxylin-eosin staining of transverse eye sections from wild-type and *ROCK-1*^{-/-} embryos from 14.5 to 18.5 dpc. Arrows indicate the eyelid epithelial sheet extending from the rim of the eyelid in the wild-type embryos. Eyelid epithelial extension was impaired in mutant embryos, with arrowheads indicating the cell mass at the expected site of sheet formation. Bar, 200 μ m. Boxed regions 1–3 are also shown enlarged. Bar, 20 μ m. (C) Immunofluorescence staining (green) for keratins 5, 10, and 6 as well as for Ki67 in frozen sections of the eyelids of wild-type or *ROCK-1*^{-/-} embryos at 16.0 dpc. Nuclei are stained blue. Large arrows indicate the eyelid epithelial sheet; arrowheads indicate the epithelial cell mass. The two small arrows indicate the base of the eyelid epithelial sheet. Bar, 50 μ m.



the placenta and umbilicus, causing the death of these neonates. Most *ROCK-1*^{-/-} neonates therefore do not survive, leaving only a small proportion to grow to adulthood.

Most of the surviving *ROCK-1*^{-/-} mice, both male and female, subsequently developed normally, with the exception of the eye lesions described above, and the adult mutant animals

were fertile and apparently healthy. Two adult *ROCK-1*^{-/-} mice were killed and tissue sections were stained with hematoxylin-eosin. No obvious abnormality was observed in the brain, spinal cord, heart, aorta, lung, trachea, kidney, or bladder; in endocrine organs such as the thyroid, pituitary, and adrenal glands; in hematopoietic or lymphoid organs such as bone mar-

row, spleen, thymus, and lymph nodes; in the gastrointestinal tract from the tongue to the rectum; in reproductive organs including testis, epididymis, seminal vesicle, prostate gland, ovary, and uterus; or in the epidermis and dermis of the skin (unpublished data). In addition, no marked difference in systolic blood pressure, as measured by a noninvasive tail-cuff method, was found between wild-type and ROCK-I^{-/-} mice (138 ± 10 and 134 ± 6 mmHg, respectively, *n* = 5).

Impaired extension of the eyelid epithelial sheet in ROCK-I^{-/-} embryos

To elucidate the molecular mechanism responsible for the failure of eyelid closure in ROCK-I^{-/-} embryos, we first examined wild-type and homozygous mutant embryos at various stages by scanning electron microscopy (Fig. 3 A). Typically, at 14.5 dpc, wild-type and ROCK-I^{-/-} embryos manifested open eyes of similar shape and with no distinct morphological difference. Differences between the genotypes were evident at 15.5 and 16.0 dpc, however. In wild-type embryos, the rims of the top and bottom eyelids extended toward each other and fused from both nasal and lateral margins. In contrast, the eyelids of many ROCK-I^{-/-} embryos failed to extend during this period, leaving the ocular surface exposed. Whereas the eyelids of wild-type embryos were completely fused at the midline by 16.5 dpc, two different degrees of failure of eyelid closure (fully open eyes or partially fused eyelids) were apparent in the eyes of ROCK-I^{-/-} mice at this time.

We next compared eyelid closure in wild-type and ROCK-I^{-/-} embryos by histological analysis (Fig. 3 B). Similar to the skin in other regions of the body, the eyelid consists of epithelium and dermis. In wild-type embryos, the eyelid epithelium comprises a single layer of basal cells at 14.5 dpc and becomes multilayered as a result of proliferation and differentiation of the basal cells between 15.5 and 16.0 dpc. Around this time, the eyelid epithelial sheet extends from both top and bottom eyelid tips to cover the ocular surface. The two sheets meet at the center and eventually fuse each other at 16.5 dpc. The eyelid morphology of ROCK-I^{-/-} embryos appeared identical to that of their wild-type littermates at 14.5 dpc. The eyelid epithelium also became multilayered in the mutant mice between 15.5 and 16.0 dpc. However, the ROCK-I^{-/-} embryos failed to extend the eyelid epithelial sheet. At 16.5 dpc, the ROCK-I^{-/-} embryos had only partially extended the eyelid epithelial sheets or had not done so at all. Consequently, the eye of ROCK-I^{-/-} embryos was open by varying degrees at 18.5 dpc. These results suggest that the EOB phenotype of ROCK-I^{-/-} mice is caused by impaired or delayed extension of the eyelid epithelial sheet.

To characterize the cells present in the eyelid epithelial sheet, we performed immunofluorescence staining for keratins 5, 10, and 6, which are markers of epithelial cell differentiation (Fig. 3 C; Fuchs, 1996; Mazzalupo and Coulombe, 2001). The eyelid epithelial sheet of wild-type embryos was positive for keratin 5 and negative for keratin 10, indicative of its basal cell composition. The sheet was also positive for keratin 6. A similar staining pattern was apparent for the cell mass at the expected site of sheet formation in ROCK-I^{-/-} mice. In addition, the base of the eyelid epithelial sheet in wild-type embryos and

that of the cell mass in ROCK-I^{-/-} embryos were rich in cells positive for Ki67, a marker of cell proliferation. These results indicate the presence of a population of normally differentiated cells in the eyelid of ROCK-I^{-/-} mice.

Impaired formation of actomyosin cables in the eyelid epithelial cells of ROCK-I^{-/-} embryos

Given that ROCK induces myosin-mediated cross-linking of actin filaments and consequently makes actin bundles such as stress fibers in cultured cells, we investigated whether loss of ROCK-I might impair formation of the actin architecture required for extension of the eyelid epithelial sheet and thereby cause the EOB phenotype. Whole-mount phalloidin staining of the eyelids of wild-type embryos at 15.5 and 16.0 dpc revealed the presence of thick actin cables that coursed continuously from cell to cell at the leading edge of the eyelid epithelial sheet and encompassed the circumference of the eye like a ring (Fig. 4, A–D). Pronounced phalloidin staining was also apparent in individual cells of the eyelid around the eye circumference in ROCK-I^{-/-} embryos, but most of these actin filaments were not organized into cables that extended beyond each cell (Fig. 4, A and E–G). Phalloidin staining of transverse frozen sections from wild-type mice revealed that the actin cables were localized in a few layers of cells in the eyelid epithelial sheet (Fig. 4 H); they were first apparent in the eyelid tips at 15.5 dpc and had increased in number by 16.0 dpc. In contrast, the assembly of such structures was minimal in most ROCK-I^{-/-} embryos at 15.5 or 16.0 dpc, although a few actin bundles were detected in some of the mutant embryos at 16.0 dpc (Fig. 4 H).

Given that ROCK regulates the phosphorylation of MLC both directly and indirectly *in vitro* (Amano et al., 2000), we next examined MLC phosphorylation in the eyelid epithelial sheet by staining sections of the eyelid at 16.0 dpc with antibodies specific for phosphorylated MLC (Matsumura et al., 1998). Myosin itself was uniformly stained throughout the eyelid epithelium with no difference between wild-type and ROCK-I^{-/-} embryos (unpublished data). However, whereas staining for phosphorylated MLC was pronounced and associated with actin cables in the eyelid epithelial sheet of wild-type embryos, it was markedly reduced in ROCK-I^{-/-} embryos (Fig. 4 I). We also prepared rabbit polyclonal antibodies to mouse ROCK-I in order to examine ROCK-I distribution during eyelid closure. Immunofluorescence staining with these antibodies revealed the presence of ROCK-I in the eyelid epithelial sheet and skin of wild-type embryos but not in ROCK-I^{-/-} embryos (Fig. 4 J). A prominent ROCK-I signal was apparent in the eyelid epithelial sheet. However, staining was not specifically increased in the cells containing the actomyosin cables but rather was ubiquitous in the eyelid epithelial cells. These results suggest that local ROCK-I activation, rather than the expression level of ROCK-I, regulates the assembly of actomyosin cables during eyelid closure. Together, these observations indicate that phosphorylation of MLC occurs in a ROCK-I-dependent manner in the eyelid epithelial sheet and that this phosphorylation is required for extension of the eyelid epithelial sheet and eyelid closure.

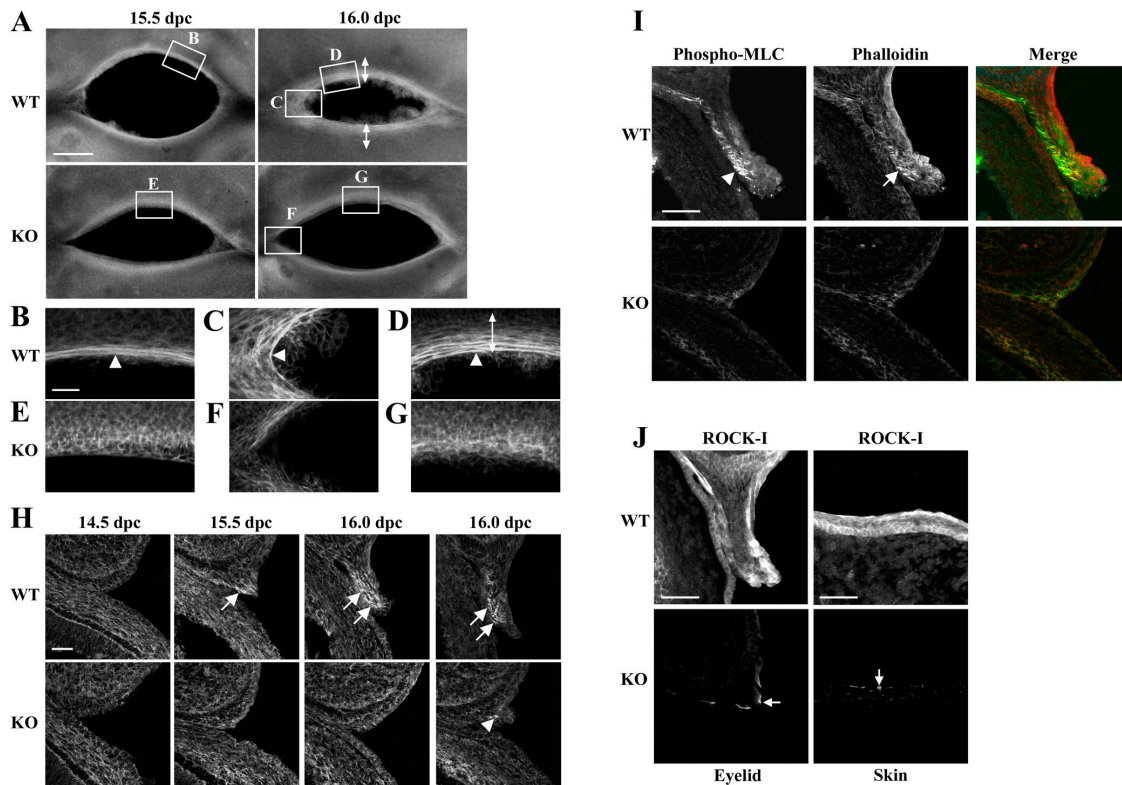


Figure 4. ROCK-I-dependent formation of actomyosin cables in the eyelid epithelial sheet. (A) Whole-mount phalloidin staining of the eyelids of wild-type (top) and *ROCK-I*^{-/-} (bottom) embryos at 15.5 and 16.0 dpc. Bidirectional arrows indicate extension of the eyelid epithelial sheet. Bar, 500 μ m. (B–G) Higher magnification views of the boxed regions in A. Arrowheads indicate actin cables in the eyelid epithelial sheet of wild-type embryos. Bidirectional arrows indicate extension of the eyelid epithelial sheet. *ROCK-I*^{-/-} embryos failed to assemble continuous actin cables. Bar, 50 μ m. (H) Phalloidin staining of transverse sections of the eyelid rim of wild-type (top) and *ROCK-I*^{-/-} (bottom) embryos from 14.5 to 16.0 dpc. A substantial number of actin cables was apparent in a few layers of the eyelid epithelial sheet of wild-type embryos (arrows). Little filamentous-actin accumulation was detected in mutant embryos, with the exception of a few actin bundles in some embryos (arrowhead). Bar, 50 μ m. (I) Immunofluorescence staining for phosphorylated MLC (green) and phalloidin staining (red) in sections of the eyelid of wild-type (top) and *ROCK-I*^{-/-} (bottom) embryos at 16.0 dpc. Phosphorylated MLC was enriched in the eyelid epithelial sheet of wild-type embryos (arrowhead), where it was colocalized with actin cables (arrow). Little phosphorylated MLC staining was detected in *ROCK-I*^{-/-} embryos. Bar, 50 μ m. (J) Immunofluorescence staining for ROCK-I in sections of the eyelid (left) and skin (right) of wild-type (top) and *ROCK-I*^{-/-} (bottom) embryos at 16.0 dpc. Nonspecific signals are indicated by arrows. Bars, 50 μ m.

ROCK-I mediates MLC phosphorylation induced by EGF in primary keratinocytes

Studies with several lines of mutant mice have implicated EGF signaling in eyelid closure (Miettinen et al., 1995; Sibilias and Wagner, 1995; Threadgill et al., 1995). Therefore, we next examined the role of ROCK-I in EGF signaling in primary keratinocytes. Primary keratinocytes were isolated from wild-type and *ROCK-I*^{-/-} embryos at 18.5 dpc and their identity was confirmed by staining for keratin 5; the presence or absence of ROCK-I, respectively, was also confirmed by immunoblot and immunofluorescence analyses (unpublished data). Measurement of the incorporation of BrdU revealed that the proliferation capacity of the *ROCK-I*^{-/-} keratinocytes did not differ from that of the wild-type cells (Fig. 5 A), consistent with our Ki67 immunostaining data (Fig. 3 C).

Phalloidin staining showed the presence of actin filaments around the circumference of serum-deprived wild-type keratinocytes (Fig. 5 B); a few short actin bundles were also detected inside the cells. Serum-deprived *ROCK-I*^{-/-} keratinocytes also contained circumferential actin filaments and short actin bundles. Stimulation of wild-type cells with EGF (10 ng/ml) resulted

in the formation of thick actin bundles that resembled stress fibers and spanned the cell length. These actin bundles were observed in 33 and 56.4% of wild-type cells at 30 and 120 min, respectively (Fig. 5, B and C). Similar structures were also induced by EGF in *ROCK-I*^{-/-} cells, but they were thinner and fewer in number. In addition, only 11 and 20% of *ROCK-I*^{-/-} cells contained the actin bundles at 30 and 120 min, respectively. These results suggest that ROCK-I plays an important role in the EGF-induced formation of stress fibers in keratinocytes. The EGF-induced formation of actin bundles in both wild-type and *ROCK-I*^{-/-} cells was prevented by the ROCK inhibitor Y-27632 (Fig. 5 B), suggesting that ROCK-II also contributes to this process.

Given that ROCK acts downstream of Rho, we next examined the possible effect of EGF stimulation on Rho activity in primary keratinocytes with the use of a pull-down assay for the GTP-bound (active) form of Rho. EGF induced a small but reproducible increase in the amount of the GTP-bound form of Rho in both wild-type and *ROCK-I*^{-/-} keratinocytes (Fig. 5 D). Together, these results thus indicate that the Rho–ROCK signaling pathway is activated by EGF in primary keratinocytes. To elucidate the signaling events downstream of ROCK-I in

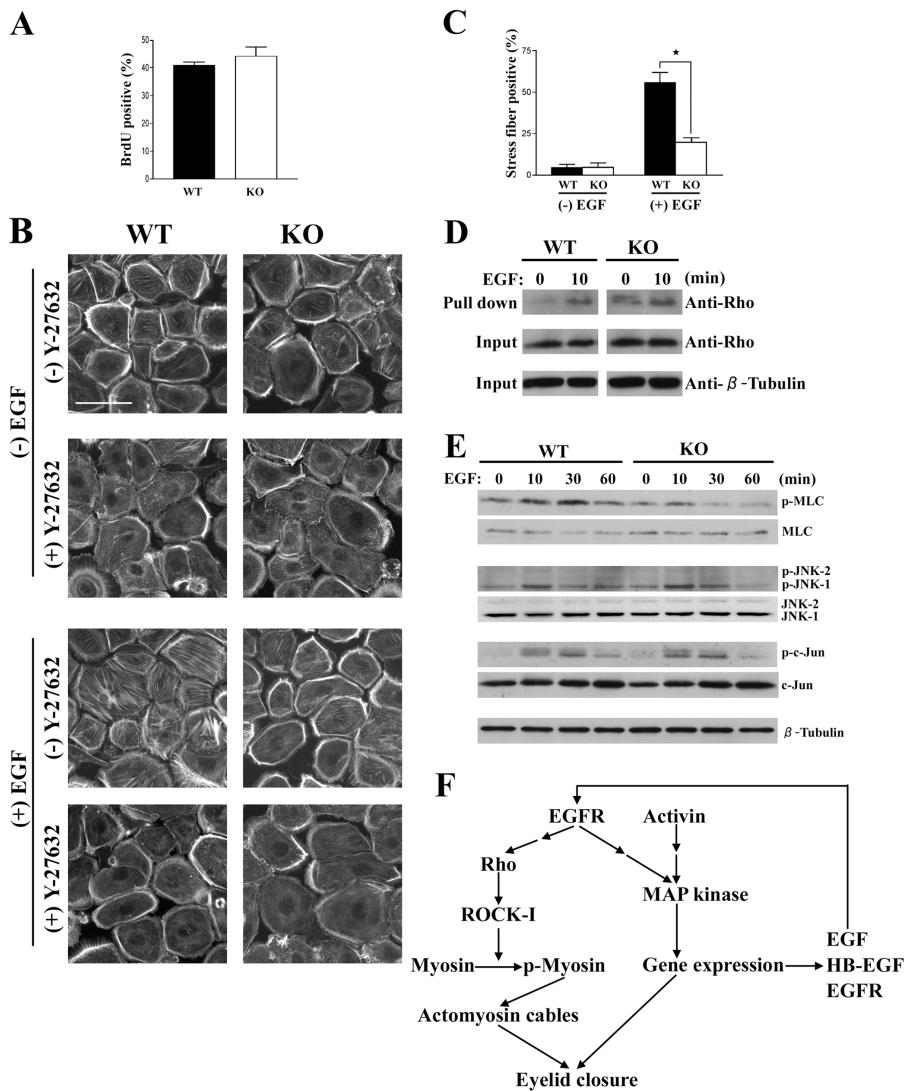


Figure 5. Impairment of EGF-induced formation of actin stress fibers in primary keratinocytes derived from ROCK-1^{-/-} embryos. (A) Cell proliferation. Primary keratinocytes derived from wild-type or ROCK-1^{-/-} embryos were cultured in the presence of EGF (10 ng/ml) and evaluated for cell proliferation by measurement of incorporation of BrdU. Data are expressed as the percentage of cells that were BrdU positive and are means \pm SEM of values from five independent experiments. (B) Phalloidin staining of keratinocytes. Wild-type and ROCK-1^{-/-} keratinocytes were maintained in serum-free medium for 24 h, cultured with or without EGF (10 ng/ml) for 2 h in the absence or presence of 10 μ M Y-27632, and then subjected to staining with phalloidin. Bar, 50 μ m. (C) Quantitative analysis of EGF-induced stress fiber formation. Cells incubated with or without EGF for 2 h and stained with phalloidin as in B were analyzed for determination of the percentage of cells with stress fibers. A total of 100 cells were examined for each condition. Data are means \pm SEM of values from five independent experiments. *, $P < 0.05$. (D) EGF-induced Rho activation. Wild-type and ROCK-1^{-/-} keratinocytes were cultured in serum-free medium for 24 h, stimulated with EGF (10 ng/ml) for 0 or 10 min, and then subjected to a pull-down assay for the GTP-bound (active) form of Rho. Rho-GTP precipitated from cell lysates was detected by immunoblot analysis with antibodies to Rho (top), and cell lysates (input) were similarly analyzed for the total amounts of Rho (middle) and β -tubulin (bottom). (E) EGF-induced MLC, JNK, and c-Jun phosphorylation. Wild-type and ROCK-1^{-/-} keratinocytes were maintained in serum-free medium for 24 h before stimulation with EGF (10 ng/ml) for the indicated times. Cell lysates were then subjected to immunoblot analysis with antibodies to MLC, to phosphorylated (p-) MLC, to JNK, to phosphorylated JNK, to c-Jun, to phosphorylated c-Jun, and to β -tubulin (control). (F) Proposed role for ROCK-1 in mouse eyelid closure. A Rho-ROCK-1-myosin cascade triggered by activation of the EGFR is required for the assembly of the purse stringlike actin cables that contribute to extension of the eyelid epithelial sheet. An autocrine-paracrine pathway mediated by EGFR or activin results in activation of a MAPK cascade and consequent transcription of EGF, heparin-binding EGF (HB-EGF), and EGFR genes.

triggered by activation of the EGFR is required for the assembly of the purse stringlike actin cables that contribute to extension of the eyelid epithelial sheet. An autocrine-paracrine pathway mediated by EGFR or activin results in activation of a MAPK cascade and consequent transcription of EGF, heparin-binding EGF (HB-EGF), and EGFR genes.

EGF signaling in these cells, we examined MLC phosphorylation and the activation of c-Jun NH₂-terminal kinase (JNK) and c-Jun. The basal level of MLC phosphorylation did not differ between wild-type and ROCK-1^{-/-} keratinocytes (Fig. 5 E). EGF induced a rapid and transient increase in the extent of MLC phosphorylation in wild-type keratinocytes; this effect was detectable at 10 and 30 min but no longer at 60 min. EGF-induced phosphorylation of MLC was also apparent in ROCK-1^{-/-} keratinocytes at 10 min, but its extent was markedly reduced compared with that observed in wild-type cells. Moreover, MLC phosphorylation in the mutant cells had returned to the basal level by 30 min. In contrast, EGF induced the phosphorylation of JNK, as well as that of its substrate c-Jun, to similar extents in wild-type and ROCK-1^{-/-} keratinocytes (Fig. 5 E). This latter finding was supported by immunofluorescence staining that revealed similar levels of c-Jun and phosphorylated c-Jun in the eyelid epithelial cells of both wild-type and ROCK-1^{-/-} littermates at 16.0 dpc (unpublished data).

Impaired umbilical ring closure in ROCK-1^{-/-} mice

The other major phenotype of ROCK-1^{-/-} mice was omphalocele, herniation of the gut and liver through the umbilical ring in neonates (Fig. 6 A). The umbilical ring was open in wild-type embryos at 15.5 dpc, and the midgut loop protruded through the umbilical ring from the abdominal cavity (Fig. 6 B). The sac of this physiological hernia consisted of an internal lining of peritoneum and an external covering of amnion. ROCK-1^{-/-} mice also showed the physiological umbilical hernia at this stage. By 16.5 dpc, the umbilical ring had closed and the gut had returned to the abdominal cavity in wild-type embryos. In contrast, the umbilical hernia persisted in ROCK-1^{-/-} embryos at this time, resulting in varying degrees of omphalocele in neonates. A ridge-like structure that appeared to constrict the ventral abdominal wall at the site of entry of the umbilical cord was evident in wild-type embryos but not in ROCK-1^{-/-} embryos (Fig. 6 A). Histological analysis revealed that this ridge consisted of

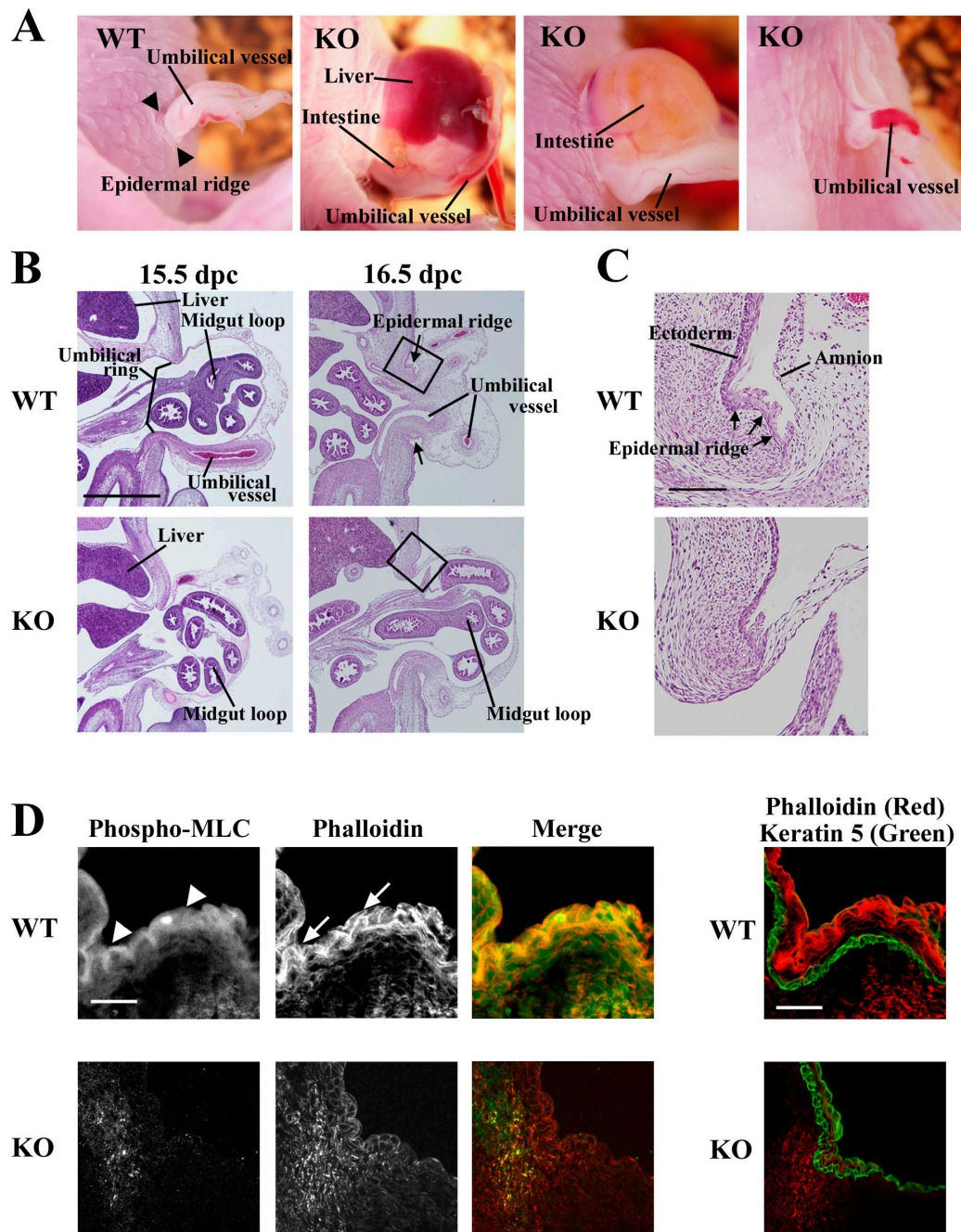


Figure 6. **Impaired closure of the umbilical ring in *ROCK-1^{-/-}* mice.** (A) Omphalocele in *ROCK-1^{-/-}* embryos. Wild-type and *ROCK-1^{-/-}* embryos at 18.5 dpc are shown. Severe, moderate, and mild forms of omphalocele are apparent in the mutant mice shown in the center left, center right, and right panels, respectively. Arrowheads indicate the epidermal ridge in the wild-type embryo. (B) Hematoxylin-eosin staining of sagittal sections through the umbilical region of wild-type (top) and *ROCK-1^{-/-}* (bottom) embryos at 15.5 (left) and 16.5 (right) dpc. Boxes indicate the region of the ectodermal–amnion transition. Arrows indicate the epidermal ridge. Bar, 1 mm. (C) Higher magnification images of the boxed regions in B. Arrows indicate the epidermal ridge in the wild-type embryo. Bar, 200 μ m. (D) Staining of the epidermal ridge. Sections of the epidermal ridge of wild-type (top) and *ROCK-1^{-/-}* (bottom) embryos at 16.5 dpc were stained with an antibody to phosphorylated MLC (green, arrowheads) and phalloidin (red, arrows) in the left panels, and with phalloidin (red) and an antibody to keratin 5 (green) in the right panel. Bars, 50 μ m.

the epidermis and was apparent at the transition between the embryo ectoderm and the amnion of wild-type mice at 16.5 dpc (Fig. 6, B and C). To characterize the function of the epidermal ridge, we stained it for F-actin, phosphorylated MLC, and keratin 5 (Fig. 6 D). Intense phalloidin staining and phosphorylated MLC was detected in the epithelial layer covering the ridge of wild-type embryos. Both of these signals were absent in the ep-

ithelium of *ROCK-1^{-/-}* embryos. These results indicate that specific actin bundles are localized in the epithelial cells of the umbilical ring. Together, these results suggest that the impaired closure of the umbilical ring in *ROCK-1^{-/-}* embryos is due to failure of myosin phosphorylation and actin assembly in the epithelium at the site of formation of the epidermal ridge at the ectoderm–amnion transition.

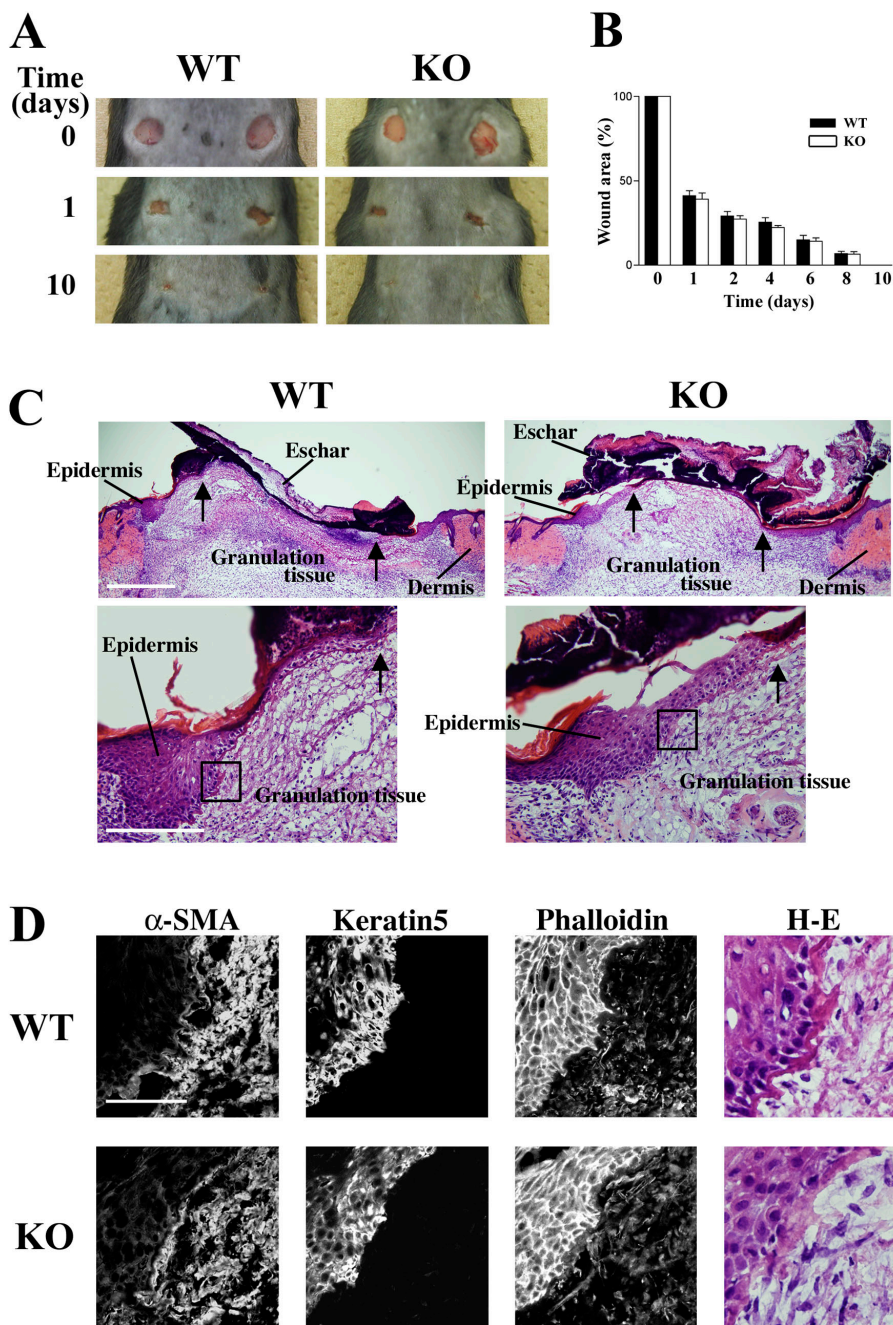


Figure 7. Wound healing in adult wild-type and ROCK-I^{-/-} mice. (A) Adult wild-type or ROCK-I^{-/-} mice were shaved on their backs and a 6-mm-diam cylinder of skin (full thickness) was punched out bilaterally. Healing of the wounds was monitored for 10 d. (B) Wound area was expressed as a percentage of the initial value. Quantitative data are means \pm SEM ($n = 8$ wounds for each group). (C) Hematoxylin-eosin staining of the transverse sections of the wound on day 5 from wild-type (left) and ROCK-I^{-/-} mice (right). Arrows indicate the foremost tips of the migrating epithelial sheet. Areas of the migrating epithelial sheet are enlarged in the bottom panels. Bars: (top) 500 μ m; (bottom) 200 μ m. (D) Staining of the wound edge. Serial sections of the wound from wild-type (top) and ROCK-I^{-/-} (bottom) mice were stained with antibodies to α -SMA, keratin 5, phalloidin, and hematoxylin-eosin (H-E). The fields of view shown correspond to the regions boxed in C. Bar, 50 μ m.

Wound healing in adult ROCK-I^{-/-} mice

Finally, we examined the effect of ROCK-I deficiency on wound healing in adult mice. We punched out a full-thickness piece of skin and monitored closure of the excision wound in both wild-type and ROCK-I^{-/-} animals. No apparent repair defect was detected in ROCK-I^{-/-} mice, with wound healing after a time course similar to that observed in wild-type controls (Fig. 7, A and B). Histological examination of the wound on day 5 revealed that the epidermal edges of the wound migrate forward to cover the denuded wound surface in ROCK-I^{-/-} mice as in wild-type controls (Fig. 7 C, arrows). In both groups, myofibroblasts expressing α -smooth muscle actin (α -SMA) were detected underneath the migrating epidermis in granulation tissue (Fig. 7 D). These myofibroblasts were simi-

larly stained with phalloidin in the two groups. These results suggest that ROCK-I is not essential for migration of keratinocytes and appearance of myofibroblasts in adult wound healing process.

Discussion

We have disrupted the gene for ROCK-I, one of the two isoforms of ROCK, by homologous recombination in mice in order to elucidate the functions of this protein in vivo. Mice homozygous for the disrupted ROCK-I allele manifested the EOB and omphalocele phenotypes. Analysis of the molecular mechanisms responsible for these abnormalities has revealed an evolutionarily conserved role of ROCK-I in animal development.

ROCK-I-dependent assembly of actomyosin cables: conservation in mammalian eyelid closure and in *Drosophila* dorsal closure

Monitoring of the morphological changes associated with eyelid closure revealed that the extension of the eyelid epithelial sheet that occurs in wild-type embryos was markedly impaired in ROCK-I^{-/-} mice. Staining with phalloidin and antibodies to phosphorylated MLC revealed the presence of thick actomyosin cables that course around the circumference of the eye and join neighboring cells together in the eyelid epithelial sheet of wild-type mice. This actomyosin structure was specifically lost in the eyelids of ROCK-I^{-/-} mice. Given the functions of ROCK in actomyosin dynamics, these findings indicate that ROCK-I is activated in cells of the eyelid epithelial sheet, catalyzes MLC phosphorylation, and thereby triggers the cross-linking of actin filaments and the assembly of actomyosin bundles, the latter of which then contract and provide the driving force necessary for eyelid closure. The loss of such ROCK-I function is thus the likely cause of the EOB phenotype in ROCK-I^{-/-} mice.

This process that we propose for eyelid closure is similar to that which underlies dorsal closure of the fly epidermis (Jacinto et al., 2002). In both instances, the epithelium extends as a sheet and actomyosin cables are present in these sheets. On the basis of the contribution of a MAPK cascade (see below) to both processes, Xia and Karin (2004) suggested the operation of an evolutionarily conserved mechanism both in dorsal closure in *Drosophila* and in eyelid closure in mammals. Consistent with this proposal and with our present findings, genetic analysis in *Drosophila* has implicated the Rho pathway in dorsal closure (Harden et al., 1999; Magie et al., 1999). Moreover, disruption of the *Drosophila* gene for the myosin binding subunit of myosin phosphatase resulted in impairment of dorsal closure (Mizuno et al., 2002). Given that the myosin binding subunit of myosin phosphatase is the major substrate of ROCK in its indirect regulation of phosphorylated MLC, this finding suggests the participation of ROCK in dorsal closure. Laser-ablation experiments have shown that the actin cables generate the contractile force responsible for movement of the epithelial sheet during dorsal closure, functioning in a manner analogous to a purse string (Kiehart et al., 2000; Hutson et al., 2003). Therefore, we propose that actomyosin cable in eyelid epithelial sheet contracts like a purse string, and thus drive the eyelid epithelial sheet movement forward as seen in dorsal closure. In addition to dorsal closure, purse stringlike actin assembly occurs at the margin of wounds in chick embryos, and experiments with C3 exoenzyme have indicated that Rho mediates this assembly (Brock et al., 1996; Wood et al., 2002). Therefore, it is likely that Rho-ROCK signaling also contributes to wound closure in chick embryos. Thus, ROCK-mediated assembly and contraction of actomyosin cables appears to be an evolutionarily conserved mechanism for tissue closure.

In contrast to the findings in chick embryos, we found that ROCK-I deficiency did not affect wound closure in adult mice. Wound healing in adult skin is a complex process. After wounding, a fibrin clot and then granulation tissue form in the wound,

after which a sheet of keratinocytes migrates from the wound edges to repair the epidermal layer (Martin, 1997). This migration of the epithelium occurs by integrin-dependent extension of lamellipodia over the ECM. Failure of ROCK-I deficiency to block this process suggests that ROCK-I is not required for cell migration over ECM in adult skin. Consistent with this conclusion, an in vitro assay of wound healing with a monolayer of cultured keratinocytes revealed that keratinocytes derived from wild-type or ROCK-I^{-/-} mice migrated in response to EGF stimulation in similar manners (unpublished data).

Role of ROCK-I in EGF signaling during eyelid closure

EGFR-deficient mice (Miettinen et al., 1995; Sibilio and Wagner, 1995; Threadgill et al., 1995) exhibit an EOB phenotype similar to that of ROCK-I^{-/-} mice. In addition, mice deficient in TGF- α (Luetteke et al., 1993; Mann et al., 1993), an EGFR ligand, and mice deficient in Gab1 (Itoh et al., 2000), an adaptor molecule of the EGFR, also manifest the open-eye phenotype, suggesting that EGFR signaling is critical for eyelid closure. The EGFR is activated in the same region of the eyelid epithelial sheet (Li et al., 2003) as that found to contain actomyosin cables in the present study. Given that Rho is activated by EGFR signaling in cultured cells (Ridley and Hall, 1992), Rho-ROCK signaling likely functions downstream of the EGFR in eyelid closure. We have now shown that EGF activates Rho and induces both phosphorylation of MLC and formation of stress fibers in a ROCK-I-dependent manner in primary mouse keratinocytes, supporting the notion that ROCK-I functions in induction of formation of the actomyosin cables in the eyelid epithelial sheet.

We also examined whether the Rho-ROCK pathway might play a role in EGFR signaling other than that which leads to the formation of actomyosin cables. Knockout mouse studies have implicated a MAPK cascade in EGF signaling during eyelid closure. For example, MEKK1-deficient mice exhibit the EOB phenotype (Zhang et al., 2003). JNK1^{+/-}/JNK2^{-/-} mice (Weston et al., 2004) as well as mice with keratinocyte-specific knockout of c-Jun (Li et al., 2003; Zenz et al., 2003) are also defective in eyelid closure. In all of these mutant mice, the EOB phenotype is associated with reduced expression of the EGFR ligands EGF or heparin-binding EGF, indicating the presence of a positive feedback loop. The recent observation that RhoA stimulates the expression of c-Jun by activating JNK (Marinissen et al., 2004) led us to examine the possible role of ROCK-I in this signaling cascade. However, JNK activation and phosphorylation of c-Jun were not altered in ROCK-I^{-/-} keratinocytes, excluding a role for ROCK-I in this MAPK cascade. On the basis of these various observations, we propose the model shown in Fig. 5 F for the role of ROCK-I in the EGFR signaling associated with eyelid closure.

Umbilical ring closure is mediated by ROCK-I-dependent actin assembly

A reduced level of MLC phosphorylation and consequent failure of actomyosin assembly in the epithelium may also explain the omphalocele phenotype of ROCK-I^{-/-} neonates. Accumu-

lation of filamentous-actin and MLC phosphorylation were observed in the epithelial cells at the ectoderm–amion transition that formed a ridge-shaped structure encircling the umbilical ring in wild-type mouse embryos. Neither such filamentous-actin accumulation nor MLC phosphorylation was detected and the epidermal ridge was not formed in ROCK-I^{-/-} embryos. In contrast, although omphalocele can be caused by defects or malformation of the ventral body wall itself (Eggenschwiler et al., 1997; Zhang et al., 1997; Brewer and Williams, 2004), this structure was intact in ROCK-I^{-/-} embryos. These results indicate that ROCK-I^{-/-} embryos are defective in the final step of umbilical ring closure, which we propose is executed by ROCK-I-mediated actomyosin contraction of the epithelium in the epidermal ridge. In contrast to the EOB phenotype, omphalocele is not observed in mice defective in EGFR signaling. Moreover, the extents of the EOB phenotype and of omphalocele were not correlated in ROCK-I^{-/-} embryos, suggesting that the upstream signaling in umbilical closure differs from that in eyelid closure. The omphalocele phenotype of the ROCK-I^{-/-} mice is reminiscent of that of human neonatal omphalocele, for which failure of umbilical ring closure is proposed as a major cause. The incidence of human omphalocele has been estimated as 1 in 4,000–7,000 live births (Weber et al., 2002). Characterization of the detailed mechanism of the failure of umbilical ring closure in ROCK-I^{-/-} mice may thus provide insight into the pathophysiology of omphalocele and its treatment in humans.

Further considerations

Given the importance of ROCK in Rho signaling, the phenotype of ROCK-I^{-/-} mice described in the present study as well as that of ROCK-II^{-/-} mice we described previously (Thumkeo et al., 2003) might be considered milder than expected. Both types of knockout mice manifest perinatal problems, but those that survive develop in an apparently normal manner to adulthood. In addition, in the present study, some ROCK-I^{-/-} embryos exhibited partial extension of the eyelid epithelial sheet and associated actin bundling. EGF induced a low level of stress fiber formation in ROCK-I^{-/-} keratinocytes, and this effect was abolished by Y-27632, suggesting a contribution, albeit little, by ROCK-II in the same process. Consistently, we recently found that a substantial proportion of ROCK-II^{-/-} embryos also exhibited the EOB phenotype and omphalocele (unpublished data). These results suggest that ROCK-I and ROCK-II function in a redundant manner, and indicate a possibility that each is able to compensate functionally for the loss of the other in most systems without up-regulation except the tissues such as the placenta (Thumkeo et al., 2003) and the eyelid and umbilical ring (this study). The generation of double knockout mice deficient in both ROCK-I and ROCK-II should therefore provide further insight into the role of the ROCK family of kinases in development.

Materials and methods

Generation of ROCK-I-deficient mice

Several clones containing various portions of the ROCK-I gene were isolated from a 129/SvJ lambda FIXII genomic library (Stratagene). A 1.1-kb

fragment containing the first 3 bp and 5' flanking region of exon 3 was generated by PCR with a 3.8-kb genomic DNA fragment containing exons 3 and 4 as the template and with a forward primer containing a SmaI site and a reverse primer containing a ClaI site. The amplified fragment was subcloned as a short arm into pBluescript SK+ (Stratagene). The plasmid DNA was cut with ClaI and Sall and fused with a ClaI–Sall fragment containing the β -galactosidase gene. The multicloning site of pKOScrambler915 (Stratagene) was modified to contain the restriction enzyme sites RsrII–EcoRI–XhoI–Ascl–SacII–ClaI–SmaI–BglII–AvrII–Sall–NotI, yielding mpKO915. The latter plasmid was cut with Ascl, and the Ascl fragment of the phosphoglycerate kinase promoter-neomycin resistance (neo) cassette was introduced at this site. The diphtheria toxin A gene was then inserted at the RsrII site of the mpKO915 plasmid, after which the SmaI–Sall fragment containing the short arm of the ROCK-I gene and the β -galactosidase gene from pBluescript SK+ was subcloned into the EcoRI–XhoI site. Finally, a 7.8-kb EcoRV–NheI fragment of genomic DNA of ROCK-I was inserted as the long arm into the SmaI site of the vector to generate the targeting construct (Fig. 1 A). The targeting vector was linearized with NotI and introduced into embryonic stem cells (line RW4; Genome System) by electroporation. G418-resistant clones were isolated, and homologous recombination was verified in three clones by PCR-based genotyping. Cells of each of the three clones were injected into blastocysts, and the chimeric male offspring obtained were mated with C57BL/6N females. ROCK-I^{+/-} heterozygous mice were then intercrossed to produce ROCK-I^{-/-} homozygotes. Mice studied in the present work had been backcrossed to the C57BL/6N strain for more than five generations. The morning of the day on which a vaginal plug was detected was designated as 0.5 dpc. All animal experiments were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine and were performed according to the guidelines for the protection of experimental animals of Kyoto University, Kyoto, Japan.

Genotyping

Genotyping was performed by Southern blot or PCR analysis with genomic DNA isolated from embryonic stem cells, embryonic visceral yolk sacs, or the tail of mice. For Southern analysis, 10 μ g of genomic DNA were digested with BglII for 12 h at 37°C, and the resulting fragments were fractionated by electrophoresis on a 0.7% agarose gel, transferred to a Hybond-N⁺ nylon membrane (Amersham Biosciences), and subjected to hybridization overnight at 42°C in hybridization buffer (Ultrasensitive Hybridization buffer; Ambion) with the external probe (Fig. 1 A) that had been randomly labeled with α -[³²P]dCTP (1 \times 10⁶ to 2 \times 10⁶ cpm/ml) with the use of Ready-To-Go DNA Labeling Beads (Amersham Biosciences). For PCR analysis, the primers were 5'-CACTACAATCAAG-TAAGCTTCATCC-3' (forward primer for the wild-type and targeted allele), 5'-GCTGAGCAGCTTCATAGCATAAAC-3' (reverse primer for the wild-type allele), and 5'-GACCAGACCGTTCATACAGAACTGG-3' (reverse primer for the targeted allele).

Production of antibodies to mouse ROCK-I

A GST fusion protein of fragment 1b of mouse ROCK-I (amino acids 400–662) was produced in *Escherichia coli* BL21 cells, and purified with the use of glutathione-Sepharose 4B beads (Amersham Biosciences). GST was cleaved, and ROCK-I (1b) was used as the antigen and injected into rabbits. Specific antibodies to mouse ROCK-I were then affinity purified from serum with the use of ROCK-I (1b) immobilized on CNBr beads (Amersham Biosciences).

Immunoblot analysis

The entire brain of adult mice or confluent monolayers of primary keratinocytes were homogenized, and used for immunoblotting as described previously (Thumkeo et al., 2003) with antibodies to either ROCK-I, ROCK-II (H-85; Santa Cruz Biotechnology, Inc.), β -tubulin (TUB 2.1; Sigma-Aldrich), phosphorylated MLC (Matsumura et al., 1998), MLC (A-10; Santa Cruz Biotechnology, Inc.), phosphorylated JNK (Promega), JNK (BD Biosciences), phosphorylated c-Jun (Cell Signaling Technology), or c-Jun (H-79; Santa Cruz Biotechnology, Inc.).

Whole-mount staining for β -galactosidase

Embryos isolated at 13.5 or 15.5 dpc were processed for whole-mount β -galactosidase staining as described previously (Thumkeo et al., 2003).

Histological and immunofluorescence analyses

Samples were fixed in 10% formaldehyde, dehydrated with a graded series of ethanol solutions, and embedded in paraffin. 8- μ m sections were prepared, subjected to removal of paraffin by immersion in xylene, rehy-

drated, and stained with hematoxylin-eosin. The samples were embedded in Tissue Tek OCT compound (Sakura) and frozen on dry ice. Cryostat sections (12 μm) were prepared and subjected to indirect immunofluorescence analysis with antibodies to either phosphorylated MLC (Matsumura et al., 1998), myosin (BT-561; Biomedical Technologies), keratin 5 or keratin 10 (BabCo), keratin 6 (McGowan and Coulombe, 1998), Ki67 (Novocastra), or α -SMA (1A4; Sigma-Aldrich). Antibody to keratin 6 was a gift from B.A. Coulombe (Johns Hopkins University of Medicine, Baltimore, MD). Immunocomplexes were detected with Alexa Fluor 488-conjugated donkey antibodies to rabbit or mouse IgG (Molecular Probes). TOPRO-3 iodide and phalloidin labeled with either Oregon green or Texas red (Molecular Probes) were used for staining of nuclei and filamentous actin, respectively. Primary keratinocytes were fixed with 4% PFA for 15 min at RT, permeabilized for 5 min with PBS containing 0.1% Triton X-100, and stained for filamentous actin with Oregon green phalloidin. Specimens were examined with the LSM 510 confocal microscope, at RT, with both 40 \times (Plan-Neofluor; NA 1.3) and 63 \times (Plan-Apochromat; NA 1.4) objective lens. Images were acquired with LSM 510 AIM software (Carl Zeiss Microimaging, Inc.). All pseudo-color representations were assembled using Adobe Photoshop version 6.0 for illustrative purpose only.

Scanning electron microscopy

The head of embryos at 14.5–16.5 dpc was fixed in 0.1 M sodium phosphate buffer, pH 7.4, containing 1% glutaraldehyde and 4% formaldehyde, washed with 0.1 M sodium phosphate buffer containing 0.1 M sucrose, and dehydrated with a graded series of ethanol solutions. Specimens were then dried and sputter coated according to standard procedures before examination with an S4700 scanning electron microscope (Hitachi).

Whole-mount phalloidin staining

Eyelids isolated together with the skin around the eye from embryos at 15.5 or 16.0 dpc were fixed in 4% PFA for 30 min, washed with PBS, incubated for 1 h with 200 μl of Oregon green phalloidin diluted 1:50 in PBS containing 0.2% Triton X-100, and washed five times with the PBS Triton X-100 solution. Specimens were examined under a fluorescence microscope (model BX50; Olympus) equipped with a charge-coupled device camera (model DP-70; Olympus) as well as with the LSM 510 confocal imaging system (Carl Zeiss Microimaging, Inc.).

Primary culture of keratinocytes

Primary culture of mouse keratinocytes was performed as described previously (Sano et al., 1999). For pull-down assay, keratinocytes were lysed in pull-down lysis buffer (Upstate Biotechnology), and the lysate was subjected to the pull-down assay using a GST fusion protein containing the Rho binding domain of Rhotekin (Upstate Biotechnology). For BrdU incorporation, keratinocytes were cultured for 24 h with EGF (10 ng/ml), after which BrdU (Roche) was added to the culture medium at 10 μM . After further incubation for 1 h, the cells were fixed and stained with antibodies to BrdU (BMC 9318; Roche).

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