

Rho kinases play an obligatory role in vertebrate embryonic organogenesis

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SUMMARY

Rho-associated kinases (Rho kinases), which are downstream effectors of RhoA GTPase, regulate diverse cellular functions including actin cytoskeletal organization. We have demonstrated that Rho kinases also direct the early stages of chick and mouse embryonic morphogenesis. We observed that Rho kinase transcripts were enriched in cardiac mesoderm, lateral plate mesoderm and the neural plate. Treatment of neurulating embryos with Y27632, a specific inhibitor of Rho kinases, blocked migration and fusion of the bilateral heart primordia, formation of the brain and neural tube, caudalward movement of Hensen's node, and establishment of normal left-right asymmetry. Moreover, Y27632 induced precocious expression of

cardiac α -actin, an early marker of cardiomyocyte differentiation, coincident with the upregulated expression of serum response factor and GATA4. In addition, specific antisense oligonucleotides significantly diminished Rho kinase mRNA levels and replicated many of the teratologies induced by Y27632. Thus, our study reveals new biological functions for Rho kinases in regulating major morphogenetic events during early chick and mouse development.

Key words: Rho kinase, Y27632, Cardia bifida, Cardiomyocyte differentiation, Chick

INTRODUCTION

RhoA GTPase and other Rho family proteins are regulators of actin cytoskeletal architecture and components of signal transduction cascades (reviewed by Hall, 1994; Van Aelst and D'Souza-Schorey, 1997). Genetic analysis of RhoA in *Drosophila* indicated its obligatory role during early developmental processes including gastrulation, head involution, dorsal closure, segmentation and the generation of tissue polarity (Barrett et al., 1997; Magie et al., 1999; Strutt et al., 1997). RhoA is also important for vertebrate morphogenesis with respect to head formation in *Xenopus* embryos (Wunnenberg-Stapleton et al., 1999). These observations suggest that RhoA plays an important role in regulating embryonic morphogenetic events through its effects on actin cytoskeleton reorganization which controls cell movement and differentiation.

The mammalian Rho-associated kinase family (hereafter referred to as Rho kinases) is comprised of Rho kinase/ROK α /ROCK2 and p160ROCK/ROCK1/ROK β (Ishizaki et al., 1996; Matsui et al., 1996). These serine/threonine protein kinases, identified as direct effectors of RhoA, are implicated in the regulation of cytoskeletal

organization (Amano et al., 1997; Leung et al., 1996). They regulate myosin light chain (MLC) phosphorylation directly by phosphorylating MLC and by inactivating myosin phosphatase (Kimura et al., 1996). Phosphorylation of MLC induces smooth muscle contraction, formation of stress fibers and focal adhesions. Rho kinases also phosphorylate LIM kinase (Maekawa et al., 1999), ezrin/radixin/moesin proteins and adducin (Fukata et al., 1999), thereby regulating actin dynamics, cell adhesion, membrane ruffling and cell motility. Although there is considerable evidence that Rho kinases mediate many in vivo functions of RhoA, their roles in regulating embryonic morphogenesis remain unknown.

We asked whether Rho kinases play a morphogenetic role during the elaboration of embryonic tissues. A pyridine derivative, Y27632, was discovered to specifically inhibit the activity of Rho kinases (Uehata et al., 1997). Subsequently, this compound has been widely used to evaluate the roles of Rho kinases in a variety of systems (Sawada et al., 2000; Itoh et al., 1999; Kuwahara et al., 1999). Our studies demonstrate that Y27632 is a potent teratogen that blocked the migration of precardiac mesoderm and cardiac tube fusion in cultured chick and mouse embryos. Rho kinase inhibition by this compound also blocked brain and somite formation, and induced laterality

defects. Treatment of chick embryos with Rho kinase antisense oligonucleotides also caused severe defects in head formation and cardiac tube fusion/looping. Last, our evidence indicates that Rho kinases may repress cardiac cell differentiation as their inhibition resulted in precocious activation of the cardiac α -actin gene expression.

MATERIALS AND METHODS

Isolation of chick p160ROCK and ROK α cDNAs

Avian p160ROCK and ROK α cDNAs were isolated from a lambda ZAP chicken embryonic heart library (stage 17-18) (Stratagene, La Jolla, CA), using a 1.5 kb cDNA fragment corresponding to the 5'-coding region of human p160ROCK. Nine clones were isolated for p160ROCK, which represented an overlapping sequence of 2598 bp containing the kinase domain and a partial coiled coil domain (Accession Number AF347075). Three clones were isolated for ROK α , corresponding to an overlapping sequence of 2138 bp containing partial kinase domain and partial coiled coil domain (Accession No: AF347076). The nucleotide sequence homologies of the partial chick p160ROCK and ROK α cDNAs to the human homologous regions were 82% and 81%, respectively.

In vitro culture and drug treatment of chick and mouse embryos

Embryos were removed from fertilized White Leghorn eggs (A&M University, College Station, TX) incubated at 37°C and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos developed between stages 4 and 8 were placed inside of Watman paper rings and laid ventral side-up on albumin-agar plates (Sundin and Eichele, 1992). Y27632 compound diluted to 200 μ M in Yolk-Tyrode's solution was added (20 μ l) to the surface of the embryos. Embryos were then incubated in humidified 37°C incubator for 4-20 hours to reach developmental stages 8-12 and processed for whole-mount in situ hybridization as described below. Mouse embryos (C57BL/6) were cultured in the roller system, as described (Sturm and Tam, 1993). Embryos of 7.5 days post-coitum (E7.5) or E8.0 (five per 60 ml bottle) were cultured at 38°C for 48 hours in 5 ml of rat serum with or without Y27632 (10 μ M) to reach E8.5-9.0 or E9.0-9.5, respectively. Because chick embryos were cultured on a semi-solid support and both the paper ring and the agar absorbed the compound, a higher concentration (200 μ M) was required to induce developmental defects. However, mouse embryos were cultured in liquid medium; thus, a lower concentration of Y27632 (10 μ M) was sufficient to produce similar defects.

Whole-mount in situ hybridization of chick and mouse embryos

Whole-mount in situ hybridization of chick embryos was carried out as described (Yamada et al., 1999). To examine the expression pattern of each Rho kinase isoform, three different antisense probes were synthesized from chick partial cDNAs and showed similar expression patterns. No signal was observed with sense probes (data not shown). One antisense probe was generated for each of the following genes: cardiac α -actin (Hayward and Schwartz, 1986), GATA4 (Laverriere et al., 1994), GATA5 (Laverriere et al., 1994), serum response factor (SRF; Croissant et al., 1996), Nkx2.5 (Schultheiss et al., 1995), Irx4 (Bao et al., 1999), AMHC1 (Yutzey et al., 1994), Sonic hedgehog (Shh) and Nodal (Levin et al., 1995). After color development, 10 μ M sections were cut from paraffin-embedded whole mounts of cardiac α -actin.

Whole-mount in situ hybridization of mouse embryos was carried out as described (Yamada et al., 1999). Partial mouse p160ROCK and ROK α cDNA clones were obtained from ATCC (Rockville, Maryland) and sequences were confirmed by DNA sequencing. The cDNA of the mouse p160ROCK clone covers nucleotides 1244-1985

(Accession Number, U58512) and that of ROK α covers nucleotides 1458-3170 (Accession Number, U58513). Both antisense and sense probes were synthesized from these partial cDNAs, and no signal was observed with sense probes (data not shown). The Nkx2.5 antisense probe was generated from a cDNA containing full-length coding region (Lints et al., 1993).

Immunohistochemistry analysis

Mouse embryos were equilibrated in 20% sucrose in PBS, embedded in OCT compound (Tissue-Tek), frozen in dry ice and sectioned at 10 μ m on a cryostat. Sections were fixed in 4% paraformaldehyde, incubated with anti- α -actinin (Sigma) at 1:500 dilution and then with biotin labeled anti-mouse IgG (Sigma) at 1:200 dilution. The secondary antibody was visualized with Vectastain ABC kit (Vector Laboratories).

Antisense oligonucleotide treatment

Two antisense oligonucleotides for Rho kinases and two randomized control oligonucleotides were used. Their sequences are: p160-AS (20-mers), 5'-ATTCCAAAGCTGCTTGCAGC-3'; ROK α -AS (23-mers), 5'-TTCATGCAAGTGCCAAATCTGC-3'; control oligo 1 (20-mers), 5'-GGTGGTCCGTACATAAGGTA-3'; control oligo 2 (23-mers), 5'-AAGTACGTTACGGTTTTAGACG-3'. Control oligo 1 and oligo 2 have the same GC content as that of p160-AS and ROK α -AS, respectively. For each oligonucleotide, the first three and the last three bases were phosphorothioate modified. All oligonucleotides were applied as droplets of 20 μ l at 20-40 μ M concentration on top of the cultured chick embryos of stages 4-6. DMSO (1%) was added to the oligonucleotide solution to improve oligonucleotide penetration into cells. General toxicity effects were not apparent with oligonucleotide concentrations of 10 to 40 μ M. Embryos were collected 4 hours later for RT-PCR analysis or were incubated for 20 hours (stage 12) for morphological analysis by whole-mount in situ hybridization with cardiac α -actin probe.

RT-PCR analysis

RNA was harvested from the treated or control embryos at different time points as indicated using TRIZOL (Gibco-BRL, Gaithersburg, MD). Each RNA sample was prepared from a pool of three identically treated embryos. First strand cDNA synthesis was carried out with the SuperScript Preamplification System (Gibco-BRL) in a volume of 50 μ l using 2.5 μ g of RNA and 125 ng of Oligo (dT)₁₂₋₁₈. PCR reaction was then carried out with 2 μ l of first strand cDNA and one set of specific primers. For each primer set, two or three cycle numbers were tested to be certain that PCR product accumulated within a linear range. PCR reactions including trace amount of α -³²P[dCTP] were separated by 6% polyacrylamide gel electrophoresis and quantitated by PhosphorImage analysis. GAPDH (10 to 15 cycles) was amplified as a control marker with primers as described (Barron et al., 2000). Other genes were amplified each with a specific primer set: chick GATA4 (20 to 25 cycles), Nkx2.5 (15 to 20 cycles) and SRF (20 to 22 cycles; Barron et al., 2000); chick cardiac α -actin (20 to 25 cycles), forward 5'-TAAGCACTTTTCCCTACATT-3', reverse 5'-AAGCA-ATAAATATTAGAAGC-3'; chick p160ROCK (23 to 25 cycles), forward 5'-GATTTTGCCTCCTCTATTGAC-3', reverse 5'-GCTGC-AAGCAGCTTTGGAATC-3'; chick ROK α (23 to 25 cycles), forward 5'-CAATGGAACGTGCAAGTTGTT-3', reverse 5'-CAGGAGAAT-AATCATCTTTCAG-3'. Expression of each gene was then normalized to GAPDH.

RESULTS

Embryonic expression of Rho Kinases in regions of early organogenesis

We asked if Rho kinases were expressed during early

embryogenesis, particularly focusing on early cardiac organogenesis. In vertebrates, the cardiac mesoderm involutes early during gastrulation and becomes specified when it reaches its position bilaterally in the anterior lateral plate mesoderm (Lyons, 1996). These paired cardiac fields (or cardiac crescent) form at the definitive streak stage (stage 4 for chick embryos, E7.0 for mouse embryos). As neurulation proceeds, precardiac mesoderm cells migrate towards the midline and fuse to form the definitive heart tube (stage 9 for chick embryos, E8.0 for mouse embryos).

In situ hybridization analysis in chick using ROK α and p160ROCK probes showed that transcripts for both Rho kinase isoforms displayed a diffuse expression pattern before the appearance of the first somite (stage 6; Fig. 1A,E). They then became enriched in the head fold at the one-somite stage (stage 7) and in the developing neural system at four-somite stage (stage 8; Fig. 1B,C,F,G). p160ROCK was also enriched in somites and Hensen's node, the organizer region involved in gastrulation and laterality (Fig. 1B,C). Both ROK α and p160ROCK were expressed in the neural ectoderm, and p160ROCK was also enriched in the lateral mesoderm, notochord and Hensen's node (Fig. 1D,H).

Analysis of mouse embryos at E7.5 showed the accumulation of p160ROCK transcripts in the cardiac crescent (Fig. 1I), which became restricted to the linear and looping heart at later stages (Fig. 1K). Transverse and sagittal sections revealed p160ROCK transcripts enriched throughout the myocardium and endocardium (Fig. 1J-L). In contrast, ROK α was ubiquitously expressed in the early embryos (E7.5), becoming more enriched in the head at later stages (Fig. 1M,N). These observations indicate that Rho kinases are expressed in both chick and mouse embryos, in regions involved with early organogenesis.

Rho kinase inhibitor, Y27632, induced cardia bifida and severe neural defects in cultured chick and mouse embryos

To investigate the role of Rho kinases in early development, we treated cultured chick embryos with the selective Rho kinase inhibitor, Y27632. Strikingly, approximately 90% of the embryos first exposed to Y27632 between stages 4-6 formed two laterally positioned beating hearts (cardia bifida; Fig. 2B; Table 1). In the most severe cases, the heart tubes were well separated and located anteriorly in the embryos. In other cases, partial migration of the heart promordia was observed but the heart tubes did not fuse in the midline (see Fig. 4D). In addition, there was a stage-dependency of initial exposure to Y27632, as only 19% of embryos treated from stages 7-8 displayed cardia bifida and about 27% had fused heart tubes though with abnormal looping (Table 1). These results suggest that Rho kinases regulate the migration of the cardiac precursors to the ventral midline. In the most severe cases, neurulation was disrupted, resulting in either a poorly formed brain, or its complete absence, and the failure to form a closed neural tube (Fig. 2D). Somitogenesis was also disrupted as somites failed to condense, and in some cases, anterior somites appeared to be fractured into minisomites (see Fig. 5C). Thus, the cardiac, neural and somite developmental defects induced by Y27632 correlate with the expression pattern of ROK α and p160ROCK in early chick embryos.

Similarly, dramatic developmental defects were observed in

Table 1. Y-27632, a Rho kinase inhibitor, elicited specific avian cardiac teratologies*

Kinase inhibitors‡	Percentage of embryos with each phenotype			
	Cardia bifida§	Abnormal heart¶	Dead	Normal
Y27632 (200 μ M)				
HH 4-6 ($n=26$)	88.5	0	0	11.5
HH 7-8 ($n=48$)	18.8	27.1	0	54.1
Y27632 (100 μ M)				
HH 4-6 ($n=36$)	16.7	30.5	0	52.8
H7 (200 μ M)				
HH 4-6 ($n=17$)	0	11.8	0	88.2
H9 (200 μ M)				
HH 4-6 ($n=19$)	10.5	21.1	21.1	47.3
ML-9 (1 mM)				
HH 4-6 ($n=9$)	0	11.1	11.1	77.8
Calyculin A (50 nM)				
HH 4-6 ($n=12$)	8.3	16.7	75.0	0
HH 7-8 ($n=9$)	0	33.3	22.2	44.5
Control				
HH 4-6 ($n=27$)	0	11.1	3.7	85.2
HH 7-8 ($n=35$)	0	11.4	0	88.6

*Chick embryos were cultured on albumin-agar plates from Hamburger and Hamilton (HH) stages 4-6 or 7-8 with or without different kinase inhibitors, as indicated. The embryos continued to develop under this condition until stage 12 for cardiac morphological analysis.

‡H7 and H9 are PKA and PKC inhibitors, ML-9 is a MLC kinase inhibitor, and calyculin A is a phosphatase inhibitor. Several doses below the highest molar concentration that the embryos would tolerate have been tested for H7, H9, ML-9 and calyculin A, and no significant number of embryos with cardia bifida was observed for all the doses tested. Only one dose is reported here.

§Cardia bifida: embryos have two beating heart tubes that were not fused into a single heart tube.

¶Abnormal heart: this includes reversed and/or abnormal looping.

cultured mouse embryos from E7.5 or E8.0 treated with Y27632 (Fig. 2F,H,J). Embryos cultured without Y27632 ($n=12$) developed a normally looped heart with proper chamber formation as in vivo (Fig. 2E,G,I). Cardia bifida was observed in 83% ($n=12$) of mouse embryos treated from E7.5, while mouse embryos treated from E8.0 displayed a linear beating heart tube across the body, and chamber formation was not grossly evident (100%, $n=10$). In addition, the normal embryonic process of turning was blocked, and the linear heart tube was located anteriorly to the head, which was also severely affected.

We then asked if other protein kinase inhibitors would elicit embryonic defects similar to those caused by Y27632. The affinity of Y27632 for Rho kinases is about 200 times greater than that for PKA and PKC (Uehata et al., 1997). We observed that neither H7 nor H9, protein kinase inhibitors with greater affinities for PKA and PKC than for Rho kinases, efficiently caused cardia bifida (Table 1). As Rho kinases regulate the phosphorylation state of MLC, we also asked if MLC phosphorylation was a target primarily responsible for the morphogenetic defects caused by Rho kinase inhibition. ML-9, a MLC kinase inhibitor, and calyculin A, a MLC phosphatase inhibitor, have previously been shown to affect smooth muscle cell contraction (Uehata et al., 1997); however, neither drug efficiently induced cardia bifida in chick embryos (Table 1). Thus, the cardiac teratology induced by Y27632 was neither primarily due to inhibition of MLC phosphorylation nor to the nonspecific inhibition of other protein kinases.

Y27632 induced midline structural and left-right asymmetry defects

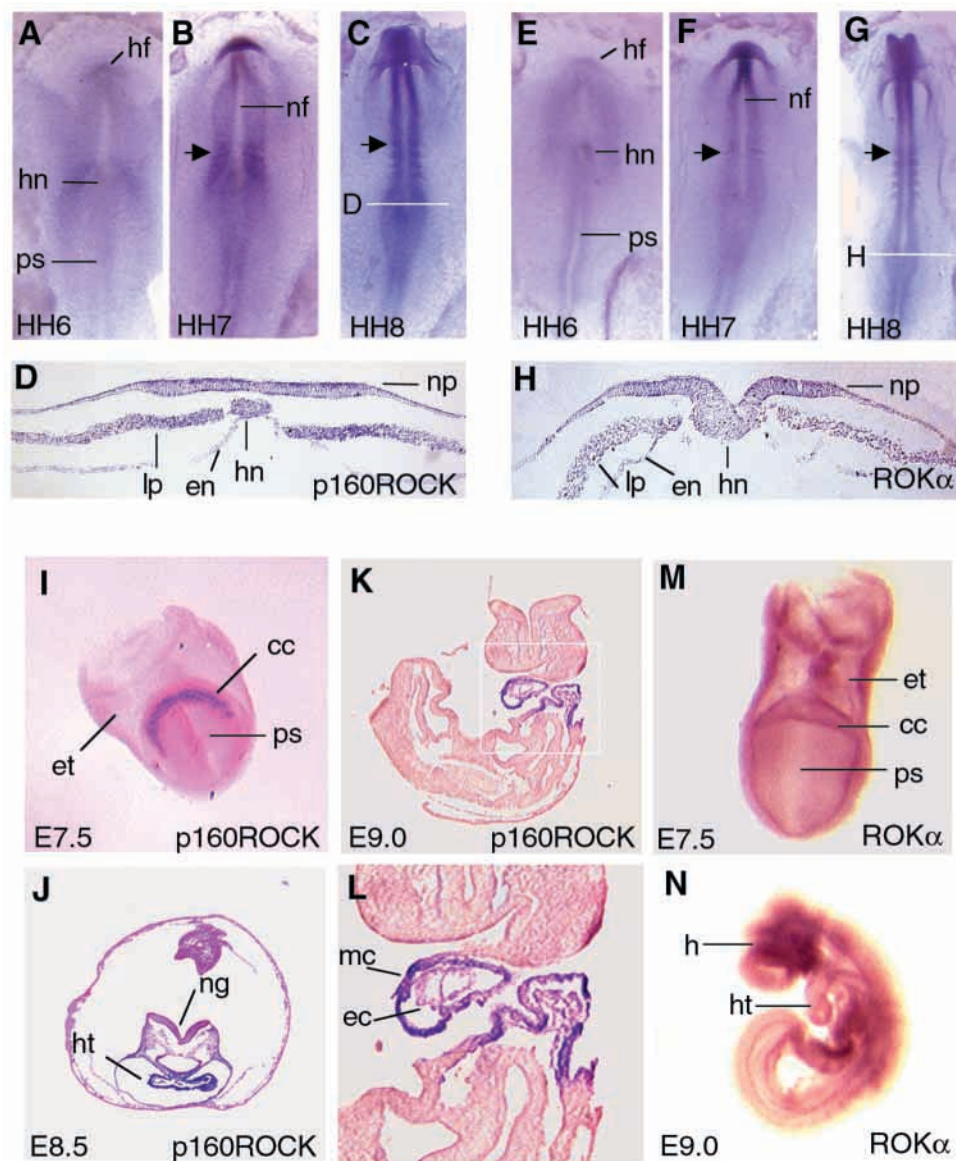
In normal embryos, Hensen's node regresses, driven by its caudal region (Charrier, et al., 1999), and leaves in its wake the notochord. The regression begins at stage 6 and reaches the end of the primitive streak by stage 10. Y27632 treatment beginning between stages 4-6 halted regression of Hensen's node. Thus, the node remained anteriorly located; consequently, the posterior half of the embryo lacked Hensen's node-derived structures including the notochord (Fig. 3A). Both Hensen's node and the midline structure are crucial for the development of the left-right axis. An asymmetric signaling cascade is initiated from Hensen's node during gastrulation, and the midline structure is thought to serve as a barrier maintaining the integrity of the left-right signaling cascade (Burdine and Schier, 2000). Nodal is asymmetrically expressed within the left lateral plate at stage 7, and this asymmetry remains until stage 11 in control embryos. Interestingly, in embryos treated with Y27632 from stages 4-6, Nodal was bilaterally expressed (Fig. 3B),

indicating a central role for Rho kinases in left-right axis formation.

Chick embryos treated with Y27632 expressed GATA5, a molecular marker of both mesoderm and endoderm differentiation

Accumulating evidence indicates that endoderm influences the process of heart development and cardia bifida is associated in some cases with a defective endoderm (Narita et al., 1997; Alexander et al., 1999; Reiter et al., 1999). To determine whether Y27632 induced endoderm developmental defects, we examined the expression pattern of GATA5. GATA5 has been shown to be abundant in both the precardiac mesoderm and the underlying endoderm, which migrates ventrally with the precardiac mesoderm to generate the ventral floor of the foregut upon fusion of the precardiac mesoderm (Fig. 4A,C,E; Laverriere et al., 1994). We observed that GATA5 was expressed in the endocardium, the myocardium and the underlying endoderm of drug treated embryos (Fig. 4B,D,F), indicating that endoderm is apparently specified correctly.

Fig. 1. Transcripts of Rho kinases are enriched in regions of early organogenesis. (A-H) Whole-mount in situ hybridization analysis of p160ROCK (A-C) and ROK α (E-G) expression in chick embryos at Hamburger and Hamilton (HH) stages 6 to 8 (indicated in the bottom left-hand corner of each photograph). Ventral views of stage 6 (A,E), stage 7 (B,F) and stage 8 (C,G) chick embryos. The position of the first pair of somites is indicated by the arrows. (D,H) Transverse sections of stage 8 embryo at the levels indicated in C and G. (I-N) Whole-mount in situ hybridization analysis of p160ROCK (I-L) and ROK α (M,N) expression in mouse embryos at E7.5 to E9.0. (I,M) Ventral views of E7.5 embryos. (J) Transverse section of an E8.5 embryo. (K) Sagittal section of an E9.0 embryo. (L) Enlargement of the box areas in K. (N) Lateral view of an E9.0 embryo. The following embryonic tissues are marked: cc, cardiac crescent; ec, endocardium; en, endoderm; et, extra-embryonic tissue; h, head; hf, head fold; hn, Hensen's node; ht, heart tube; lp, lateral plate mesoderm; mc, myocardium; nf, neural fold; ng, neural groove; np, neural plate; ps, primitive streak.



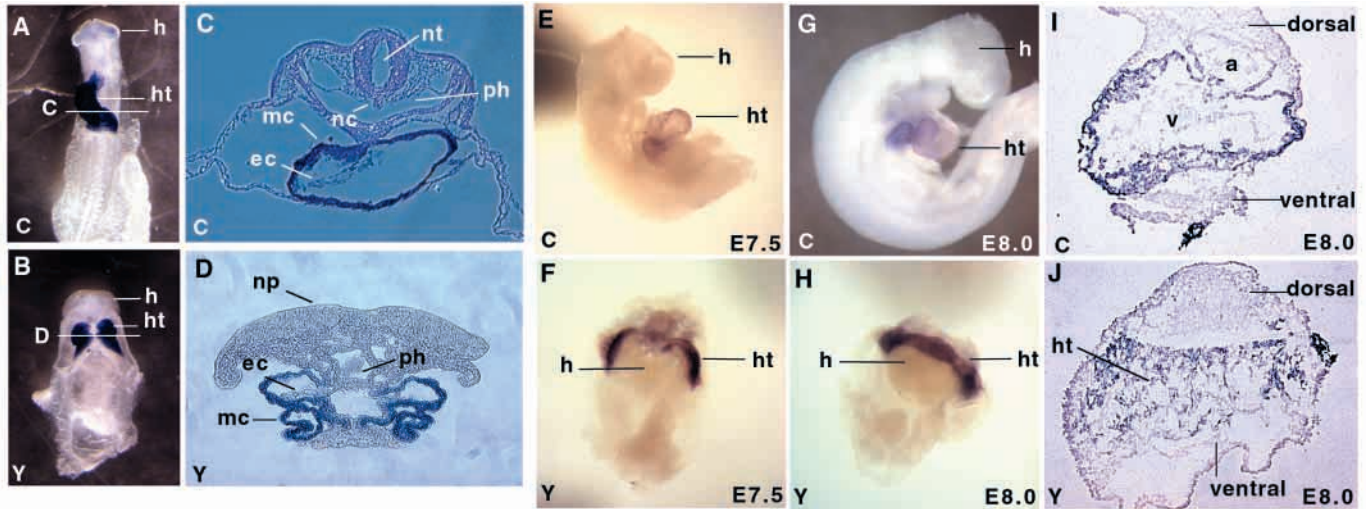


Fig. 2. Y27632, a specific Rho kinase inhibitor, caused severe cardiac and neural developmental defects. (A,B) Ventral views of chick embryos cultured with or without Y27632 (indicated in the bottom left-hand corner of each photograph) from stages 4–6. Whole-mount in situ hybridization was performed with cardiac α -actin. (C,D) Transverse sections at levels indicated in A,B. (E–H) Mouse embryos cultured with (F,H) or without (E,G) Y27632 from E7.5 (E,F) or E8.0 (G,H). Embryos are shown in lateral (E,G) or dorsal view (F,H). Whole-mount in situ hybridization was performed with Nkx2.5. In the drug-treated mouse embryos from stage E8.0 (H), the head formation and the turning process were disrupted so that the head was under the heart tube. (I,J) Immunostaining for α -actinin of transverse sections of mouse embryos cultured with (J) or without (I) Y27632. No lumen was formed in the heart of drug-treated embryos. The following embryonic tissues are marked as a, atrial; ec, endocardium; h, head; ht, heart tube; mc, myocardium; nc, notochord; np, neural plate; nt, neural tube; ph, pharynx; v, ventricle.

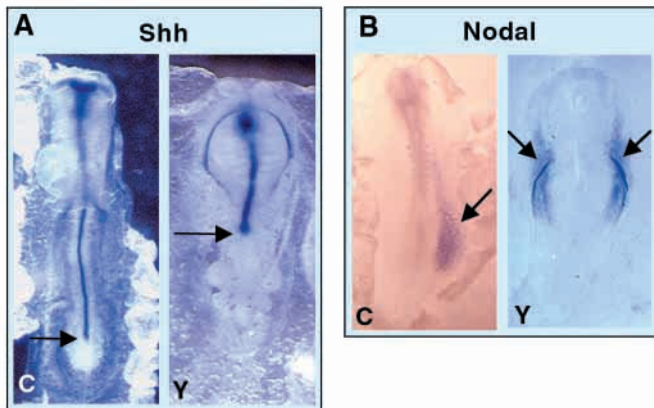


Fig. 3. Y27632 prevented the regression of Hensen's node and induced a laterality defect. Chick embryos of stage 5 were treated with or without Y27632 (indicated in the bottom left-hand corner of each photograph) and continued to grow to stage 12 at evaluation for Shh expression (A) or to stage 9 for Nodal expression (B) by whole-mount in situ hybridization. Shh was expressed in the notochord anterior to Hensen's node (indicated by arrows), thus showing the position of Hensen's node. Nodal expression (indicated by arrow) was detected on the left-hand side of the lateral plate in the control embryo, but on both sides of the drug-treated embryo. All embryos are shown in ventral view.

However, endoderm morphogenesis was affected as the formation of the ventral floor of the foregut was disrupted (Fig. 4F), perhaps owing to defects in the migration.

Y27632 upregulated cardiac gene activity and expanded the paired heart fields

In addition to their midline migration, specified cells in the

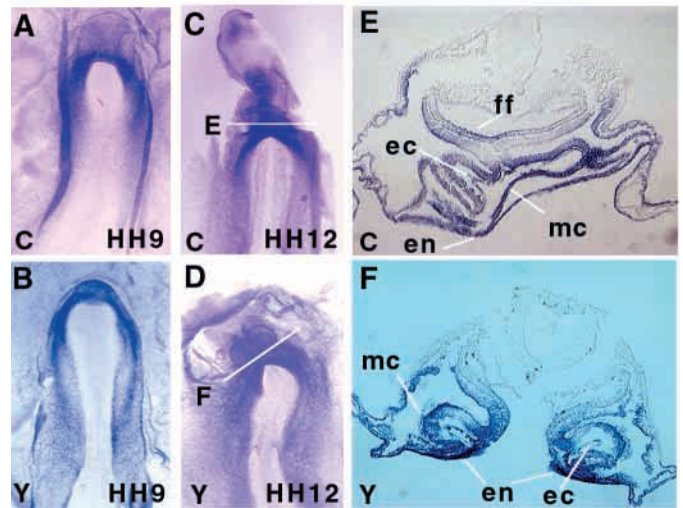
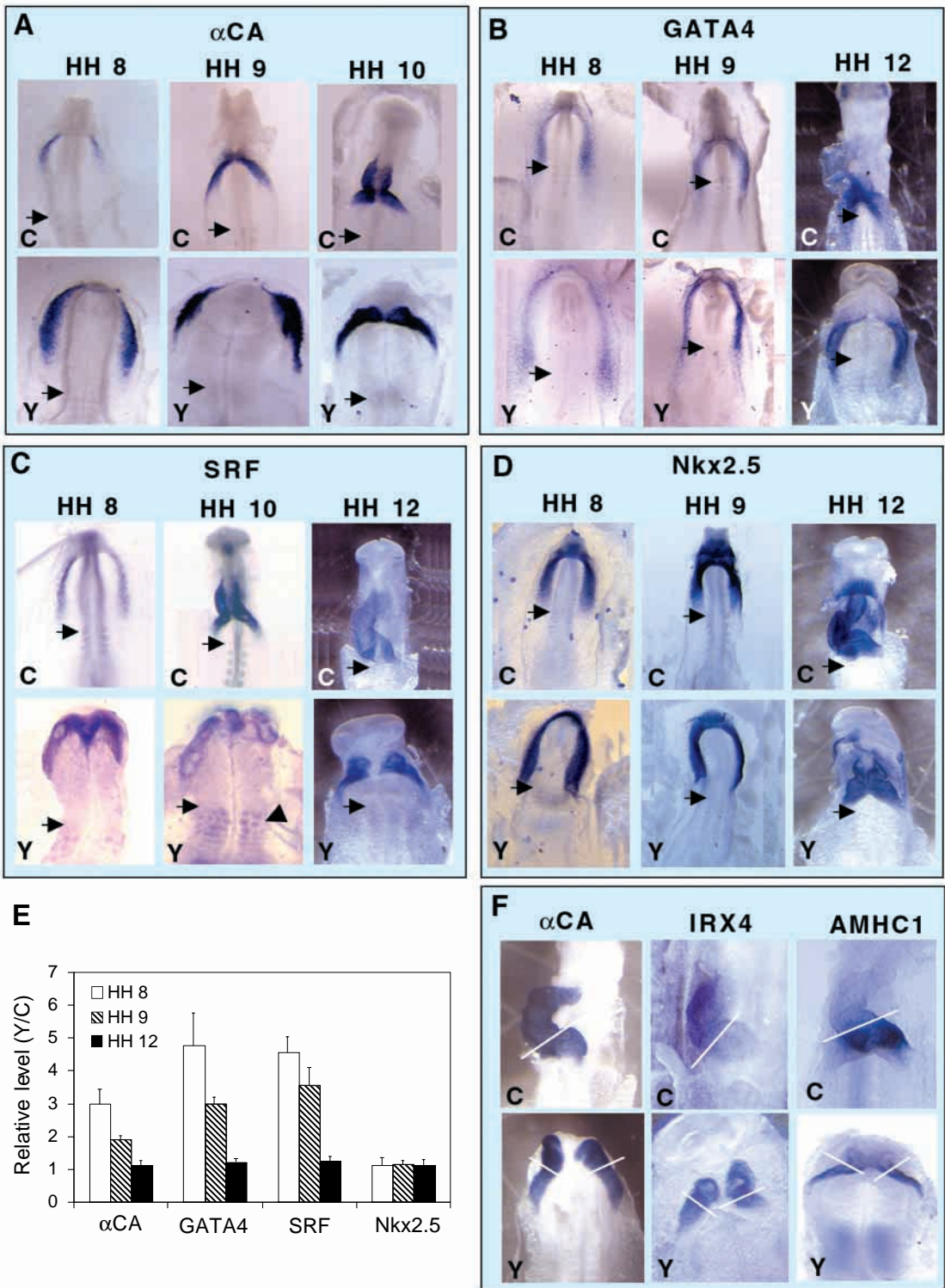


Fig. 4. Y27632 did not inhibit expression of GATA5, an endoderm differentiation marker. (A–D) Whole-mount in situ hybridization of GATA5 expression in chick embryos that were treated with or without Y27632 (indicated as Y or C, respectively, in the bottom left-hand corner of each photograph) from stages 5–6 and allowed to develop to stages 9–12 as indicated. (E,F) Transverse sections at levels indicated in C,D. GATA5 expression was abundant in cardiac mesoderm, the underlying endoderm and gut endoderm. The following embryonic tissues are marked: ec, endocardium; en, endoderm; mc, myocardium; ff, ventral floor of the foregut.

paired cardiac fields undergo terminal differentiation. In chick embryos, borders of the heart fields can be determined by expression of Nkx2.5 and GATA4 at stage 5 (Chen and Fishman, 2000), while markers of terminal differentiation

Fig. 5. Y27632 induced precocious expression of cardiac α -actin, expanded the paired heart fields at early stages and partially retained cardiac anteroposterior polarity. (A-D) Whole-mount in situ hybridization analysis of cardiac α -actin (A), GATA4 (B), SRF (C) and Nkx2.5 (D) expression in chick embryos which were treated with (Y) or without Y27632 (C in corner) from stages 5-6 and continued to grow to stages 8-12 as indicated. The position of the first pair of somites in each embryo is indicated by an arrow. Abnormal somite formation in drug-treated embryos is indicated by an arrowhead in C. (E) The timecourse of expression of cardiac α -actin, GATA4, SRF or Nkx2.5 was analyzed by semi-quantitative RT-PCR with the pool of three embryos for each sample. For each gene, two or three cycle numbers were tested to be certain that PCR product accumulates within a linear range. Their expression was normalized to GAPDH and is shown for Y27632-treated embryos relative to control embryos. Data represent averages of three to five PCR reactions + s.e.m. (vertical bars). (F) Whole-mount in situ hybridization analysis of Irx4 and AMHC1 compared with that of cardiac α -actin. A bar separates the ventricular and atrial regions in the control embryos. A bar separates the anterior and posterior regions of bilateral heart tubes in the embryos treated with Y27632. All embryos are shown in ventral view.



such as cardiac α -actin become detectable at stage 8. To analyze myocardial differentiation in Y27632 treated embryos, we examined the expression of cardiac α -actin, GATA4, SRF and Nkx2.5. In stage 8 embryos, cardiac α -actin expression was significantly upregulated (about threefold) by Y27632, indicating precocious activation of the cardiac α -actin gene, coincident with the posterior expansion of GATA4

expression (Fig. 5A,B). As SRF and GATA-4 transcripts in stage 8 embryos were also increased four- to fivefold by Y27632 (Fig. 5E), they may be partly responsible for precociously activating SRF-dependent cardiac α -actin expression in the expanded heart fields. By stage 12, no significant difference in the expression levels of these cardiac genes was observed between Y27632 treated and control

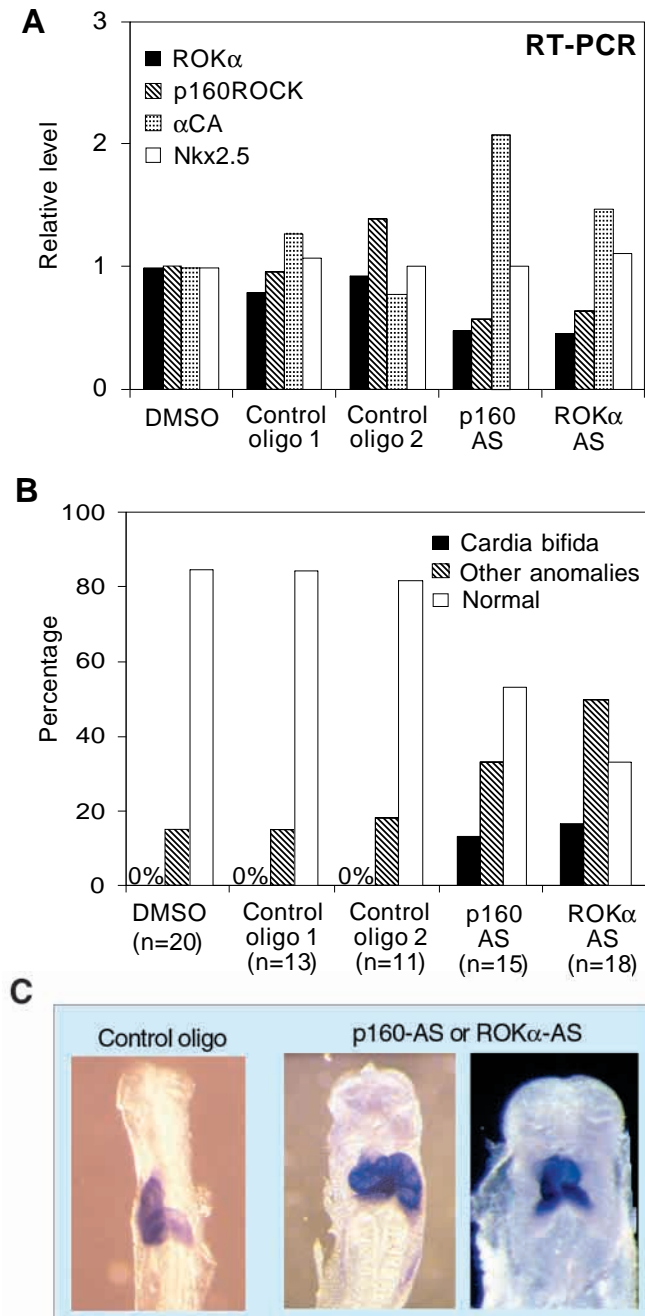


Fig. 6. Rho kinase antisense oligonucleotides reduced Rho kinase transcripts, reproducing Y27632-induced embryonic defects. (A) Semi-quantitative RT-PCR analysis of chick embryos after treatment with p160ROCK antisense oligonucleotide (p160-AS) or ROKα antisense oligonucleotide (ROKα-AS) or randomized control oligonucleotides (control oligo 1 and control oligo 2) from stage 5 for 4 hours. Three identically treated embryos were pooled. For each gene, two or three cycle numbers were tested to be certain that PCR product accumulates within a linear range. Their expression was normalized to GAPDH as described in Materials and Methods and is shown for oligonucleotide-treated embryos relative to control embryos treated with DMSO. (B) Incidence of heart anomalies induced by antisense oligonucleotide treatment. In addition to cardia bifida, other heart anomalies include reversed and/or abnormal looping and poor contractility. (C) Ventral view of chick embryos treated with control or antisense oligonucleotides and the deformities produced. Whole-mount in situ hybridization was performed with cardiac α-actin.

in control embryos (Fig. 5F). In Y27632-treated chick embryos, the expression of *Irx4* was more enriched in the anterior regions but was expanded to the posterior regions of the heart tubes, whereas *AMHC1* was more restricted to the posterior regions of each heart tube (Fig. 5F). In addition, *GATA4* transcripts were also restricted to the posterior region of the heart tubes in the drug-treated embryos (Fig. 5B). During stages 5-7, the cardiogenic mesoderm cells in the paired heart fields undergo a directed craniomedial migration and meet in the midline over the developing head fold to form a cardiogenic crescent. The posterior expansion of *Irx4* expression was probably due to defects in craniomedial migration of the anterior precardiac cells. The restricted posterior expression patterns of *AMHC1* and *GATA4* in Y27632-treated embryos suggested that cardiomyogenic lineage diversification was not affected.

Application of antisense oligonucleotides reproduced Y27632 induced embryonic defects

We used a complementary approach to analyze the function of Rho kinases in embryogenesis through their mRNA depletion using antisense oligonucleotides. Two antisense oligonucleotides (p160-AS and ROKα-AS) were specifically designed each to target its respective Rho kinase isoform. They both decreased the mRNA level of their targeted isoform up to 50%, while two random control oligonucleotides had no significant effect relative to non-oligo controls (Fig. 6A). While ROKα-AS has some homology (two mismatches) to the p160ROCK sequence, p160-AS has no homology to the ROKα sequence. However, both p160-AS and ROKα-AS reduced the mRNA level of their non-targeted cognate isoform, suggesting a potential positive feedback mechanism for transcriptional regulation of Rho kinases. Thus, the expression of one isoform seems to maintain the expression of the other isoform and vice-versa. Interestingly, cardiac α-actin expression was upregulated, whereas *Nkx2.5* expression remained unchanged (Fig. 6A). This is consistent with precocious activation of the cardiac α-actin gene in Y27632-treated chick embryos. Morphological defects like those associated with the drug inhibition of Rho kinases were also observed in chick embryos treated with antisense oligonucleotides (i.e. cardia bifida, abnormal looping of the heart tube, abnormal head and somite formation; Fig. 6B,C). The incidence of abnormal embryos as well as the severity of the defects (in cardia bifida, the

embryos as the developing hearts reached terminal differentiation (Fig. 5E). Thus, Rho kinases regulate the appropriate onset of myocardial differentiation.

Anteroposterior polarity of the heart is partially conserved in Y27632-treated chick embryos

During early cardiogenesis, the anterior region of the paired heart fields becomes the developing ventricles whereas the posterior region becomes the developing atria upon fusion into a definitive heart tube. To analyze whether this anteroposterior pattern was affected in the unfused heart tubes of Y27632-treated chick embryos, we examined the expression of a ventricle-specific marker, *Irx4*, and an atrial-specific marker, *AMHC1*. *Irx4* and *AMHC1* expression patterns do not overlap

independently beating heart tubes were very close) was comparable with that induced by Y27632 at a lower concentration (Table 1). This may be due to the partial depletion of Rho kinase mRNA and the pre-existing Rho kinase protein, which may persist.

DISCUSSION

This study demonstrates that Rho kinases play an essential role in major morphogenetic events, establishing them as a novel class of signaling molecules in vertebrate embryogenesis. We have shown that the normal migration of the precardiac mesoderm and the caudalward movement of Hensen's node require Rho kinases. Indeed, they may regulate the intrinsic migratory properties of these two unrelated tissues. Mechanisms through which Rho kinases regulate cell migration have been extensively studied in cell culture systems (Fukata et al., 1999). The best characterized of the mechanisms is regulation of the phosphorylation state of MLC by Rho kinases (Kimura et al., 1996). In this study, however, the defective cardiac morphogenesis induced by the Rho kinase inhibitor did not result primarily from inhibition of MLC phosphorylation (Table 1). Alternatively, Rho kinases have been shown to regulate cell migration via phosphorylation of adducin, which mediates Rho kinase-dependent membrane ruffling and cell migration (Fukata et al., 1999). Another potential mediator of Rho kinases in regulating embryonic morphogenesis is LIM kinase, which is highly enriched in the developing neural system and cardiac mesoderm during early mouse development (Cheng and Robertson, 1995). Rho kinases activate LIM kinase (Maekawa et al., 1999), which in turn phosphorylates and inactivates the actin-depolymerizing protein cofilin, thereby regulating actin dynamics (Arber et al., 1998). We are now investigating role of adducin and LIM kinase in early embryonic morphogenesis.

Previous studies have shown that ectopic addition of retinoic acid (Sundin and Eichele, 1992; Osmond et al., 1991) or depletion of GATA4 or GATA5 (Jiang et al., 1998; Molkenstein et al., 1997; Kuo et al., 1997; Reiter et al., 1999) disrupted precardiac mesoderm migration resulting in cardia bifida. Could these factors mediate the morphogenetic actions of Rho kinases in embryogenesis? As Hensen's node regressed normally in chick embryos treated with retinoic acid (Sundin and Eichele, 1992; Osmond et al., 1991), it is likely that different mechanisms mediate the effects of Rho kinases versus retinoic acid on embryogenesis. Moreover, GATA4 (Fig. 5B) was expressed in Y27632-treated embryos at higher levels than in control embryos, suggesting that the effects of Rho kinases are not mediated by a deficit of this transcription factor. Studies in chick, mouse and zebrafish embryos have indicated that cardia bifida may be secondary to endoderm defects (Narita et al., 1997; Alexander et al., 1999; Reiter et al., 1999). GATA5, a mesoderm and endoderm differentiation marker, was expressed in both endoderm and mesoderm of drug-treated embryos (Fig. 4), indicating that cardia bifida did not result from a lack of endoderm differentiation. However, the possibility that the underlying endoderm is unable to provide a permissive environment for the migration of precardiac cells in drug-treated embryos can not be excluded. However, a recent study in zebrafish has shown that cardia bifida was induced by

mutation of the sphingosine-1-phosphate (S1P) receptor (Kupperman et al., 2000), a G-protein-coupled receptor that activates several downstream signaling molecules including RhoA. As Rho kinases function downstream of RhoA, effects of S1P receptor-mediated signaling on precardiac cell migration may be mediated in part by Rho kinases.

Our study also shows that Rho kinases are involved in preventing cardiomyocyte differentiation. Precocious activation of cardiac α -actin observed in Rho kinase inhibitor and antisense oligonucleotide-treated embryos suggests that Rho kinases may be required to prevent terminal myocyte differentiation before fusion of the paired heart tubes occurs. During early embryogenesis, signals inhibiting cardiac differentiation are thought to originate from within the precardiac mesoderm and also from dorsal/medial structures, including the neural plate, neural fold and notochord (Chen and Fishman, 2000). As neural development is also severely affected by the inhibition of Rho kinases, inhibitory signals from neural tissues could also be attenuated and in part cause precocious cardiomyocyte differentiation. It is worth noting that Rho kinase inhibitor-induced precocious activation of cardiac α -actin may be partly responsible for abnormal migration of the precardiac mesoderm. Previous studies on the actin family show that the striated cardiac and skeletal α -actins compose contractile sarcomeres and smooth muscle α and γ -actins form smooth muscle myofibrils, whereas the ubiquitous β - and γ -cytoplasmic actins are involved in cell motility (Vandekerckhove and Weber, 1978). The functional roles of these actin isoforms are not equivalent (Schildmeyer et al., 2000). Thus, precocious activation of cardiac α -actin might promote formation of sarcomeric structure that could impair myocyte motility.

The mechanism by which Rho kinases suppress cardiac α -actin (and perhaps other cardiac specific) gene expression in precardiac cells may play a central role in heart development. Rho kinase signaling may be mediated indirectly through the actin cytoskeleton, which has been shown to mediate Rho kinase-dependent gene expression (Hahn et al., 2000; Iwamoto et al., 2000). Rho kinases may also regulate cardiac α -actin gene activity directly through transcription factors like SRF. We have observed that SRF is an *in vitro* and *in vivo* substrate for Rho kinases and that phosphorylation of SRF blocks its DNA-binding activity, thus repressing SRF-dependent cardiac α -actin promoter activation (D. Iyer, L. W., N. S. Belaguli, T. Otani, A. Balasubramanyam and R. J. S., unpublished). We have previously reported that SRF cooperates with GATA4 to synergistically activate numerous muscle-specific or non-muscle promoters containing serum response elements including cardiac α -actin and SRF itself (Belaguli et al., 2000). Interestingly, precocious activation of the cardiac α -actin gene in Y27632-treated embryos is associated with up-regulation of GATA4 and SRF expression, suggesting that increased expression of SRF and GATA4 may be responsible in part for activation of cardiac α -actin gene transcription.

In summary, we have demonstrated that Rho kinases regulate various vertebrate embryonic morphogenetic events, including cell migration, cell differentiation and axis formation. These observations reveal new biological functions for Rho kinases in early embryogenesis, particularly cardiac organogenesis. In addition, the present study indicates that Y27632, a potential therapeutic drug for the treatment of hypertension, vascular proliferative disorders and cancer

(Uehata et al., 1997; Sawada et al., 2000; Itoh et al., 1999; Kuwahara et al., 1999), is also a powerful teratogenic agent. Information regarding the teratogenic effects of this compound will be useful in designing clinical trials of this and other Rho kinase inhibitors.

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