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The involvement of Eph–Ephrin signaling in tissue separation and convergence during *Xenopus* gastrulation movements

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ABSTRACT

In *Xenopus* gastrulation, the involuting mesodermal and non-involuting ectodermal cells remain separated from each other, undergoing convergent extension. Here, we show that Eph–ephrin signaling is crucial for the tissue separation and convergence during gastrulation. The loss of EphA4 function results in aberrant gastrulation movements, which are due to selective inhibition of tissue constriction and separation. At the cellular levels, knockdown of EphA4 impairs polarization and migratory activity of gastrulating cells but not specification of their fates. Importantly, rescue experiments demonstrate that EphA4 controls tissue separation via RhoA GTPase in parallel to Fz7 and PAPC signaling. In addition, we show that EphA4 and its putative ligand, ephrin-A1 are expressed in a complementary manner in the involuting mesodermal and non-involuting ectodermal layers of early gastrulae, respectively. Depletion of ephrin-A1 also abrogates tissue separation behaviors. Therefore, these results suggest that Eph receptor and its ephrin ligand might mediate repulsive interaction for tissue separation and convergence during early *Xenopus* gastrulation movements.

Introduction

The morphogenetic movements of gastrulation are important for the establishment of three germ layers and basic body plan during early embryogenesis. In *Xenopus* embryos, gastrulation begins with the invagination of the bottle cells, followed by the coordinated involution of the mesoderm and epiboly of the ectoderm (Solnica-Krezel, 2005). The endoderm moves toward the dorsal ectoderm via a process called vegetal rotation. Vegetal rotation plays a significant role in directing involution (Winklbauer and Schurfeld, 1999). The involuting mesoderm undergoes convergent extension (CE) movements, which establish the anterior–posterior axis of the embryo. At the onset of CE movements, dorsal mesodermal cells are polarized along the mediolateral axis and adopt a bipolar shape. Then, the narrowing and elongation of the tissue is achieved by coordination of the convergence along the mediolateral axis and extension along the anterior–posterior axis (Keller et al., 2000). Concomitantly with CE movements, the involuting mesoderm and endoderm (mesendoderm) become separated from the non-involuting neuroectoderm by a process called tissue separation (Wacker et al., 2000). As a result, the cleft of Brachet is formed between the mesendoderm and the ectoderm. The anterior domain of Brachet's cleft is generated by vegetal rotation and the posterior cleft is developed when the mesoderm invaginates through the blastopore lip (Winklbauer and Schurfeld, 1999). Fibronectin deposition as a stable interface occurs between migrating mesodermal cells and their substrate layer, blastocoel roof (BCR) for normal gastrulation (Winklbauer, 1998; Winklbauer and Keller, 1996; Winklbauer et al., 1996).

Several proteins including the Wnt/planar cell polarity (PCP) pathway components have been shown to regulate CE movements and/or tissue separation of gastrulation (Wang and Steinbeisser, 2009). In particular, Frizzled-7 (Fz7) and paraxial protocadherin (PAPC) were implicated in both processes. Fz7 and PAPC control tissue separation behaviors in a non-redundant manner through PKCα and Rho GTPase, respectively (Medina et al., 2004; Winklbauer et al., 2001). In addition, Fz7 and PAPC were shown to regulate CE movement (Unterseher et al., 2004). Recently, a novel FGF target gene named ankyrin repeats domain protein 5 (xANR5) was also involved in cell protrusion formation and tissue separation (Chung et al., 2007). However, more novel factors need to be identified to elucidate the precise molecular mechanisms of tissue separation. Moreover, how CE movements and tissue separation are coordinated at the same time by the same molecules such as Fz7 and PAPC remains unclear.

Eph–ephrin signaling is critical for various cellular events including cell migration, boundary formation, and cell shape control. Eph receptors, the member of receptor tyrosine kinases (RTKs), are divided into two subclasses (A- and B-class) based on their affinities for ephrin ligands and on the amino acid sequence similarities. Ephrins are also

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divided into A-class, which is linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, and B-class, members of which have a transmembrane domain and a cytoplasmic tail. In general, A-class Eph receptors typically bind to A-class ephrin ligands, and B-class Ephs interact with B-class ephrins. However, some of the receptors such as EphA4 can bind to both classes of ephrins (Pasquale, 2005). The interactions of Ephs with ephrins are restricted to the sites of cell–cell contact, and bidirectional signals can be introduced by the contact-dependent communication between cells since both Ephs and ephrins are attached to plasma membrane (Egea and Klein, 2007; Pasquale, 2008). A well-known function of Eph forward signaling is the retraction of the cell periphery following contact with the ephrin-expressing cells. This repulsive mechanism of Eph-ephrin signaling is particularly important for the growth cone retraction in axon guidance and cell sorting in embryonic patterning (Holder and Klein, 1999; Kullander and Klein, 2002; Poliakov et al., 2004).

In *Xenopus leavis*, five members of Ephs (XEphA2, A4, B1, B3, and B4) and four members of ephrins (Xephrin-A1, -A3, -B1, and -B3) have been identified. Among them, *Xenopus* EphA4 (XEphA4) has critical roles in the boundary formation of hindbrain (Xu et al., 1995) and the migration of branchial neural crest cells (Smith et al., 1997). Overexpression of mouse EphA4 was shown to induce ectopic posterior protruding structure which is dependent on FGF signaling (Park et al., 2004). In addition, studies with chimeric EGFR/XEphA4 (EPP) receptor showed that the catalytic activity of XEphA4 is related to cell adhesion and polarity (Bisson et al., 2007; Winning et al., 1996, 2001). Although XEphA4 shows restricted expression in the involuting mesoderm at the gastrula stages (Winning and Sargent, 1994), which undergoes extensive morphogenetic processes, its roles at this time has not been investigated.

Our initial hypothesis for this work was that the tissue separation between the involuting mesoderm and non-involuting ectoderm at gastrulation might be achieved by repulsive interactions of these two germ layers. Thus, we investigated the roles in tissue separation of Eph–ephrin signaling, which is the most typical repulsive signaling pathway. In this study, we found that XEphA4 and its putative ligand, ephrin-A1 are expressed in a mutually exclusive manner in the involuting mesoderm and non-involuting ectoderm of early gastrulae, respectively. Blockade of XEphA4 or Xephrin-A1 function interfered with the tissue separation of those two layers but not their specification. XEphA4 controls tissue separation via RhoA GTPase in parallel to Fz7 and PAPC signaling pathways. Furthermore, we observed that EphA4 coordinates cell polarity of the involuting mesoderm in the mediolateral direction to control selectively the convergence in CE movements. Thus, these results suggest that the Eph–ephrin signaling plays critical roles in tissue separation behaviors and convergence during gastrulation.

Materials and methods

Embryo, in situ hybridization and RT-PCR

In vitro fertilization and embryo culture were performed as described in (Sive et al., 2000). The developmental stages of embryos were determined according to Nieuwkoop and Faber (1994). Whole mount *in situ* hybridization and quantitative RT-PCR were carried out as described previously (Park et al., 2005, 2007). For the anti-sense RNA probes, XEphA4ΔC, Xephrin-A1, *Chordin* (Sasai et al., 1994), *Goosecoid* (Cho et al., 1991) and *Xbra* (Smith et al., 1991) constructs were used. The sequences of PCR primers used here are as follows: *Chordin*, 5′-AACTGCCAGGACTGGATGGT-3′ (forward) and 5′-GGCAG GATTTAGAGTTGCTTC-3′ (reverse); *Goosecoid*, 5′-ACAACTGGAAG CACTGGA-3′ (forward) and 5′-TCTTATTCCAGAGGAACC-3′ (reverse); *Xbra*, 5′-GGATCGTTATCACCTCTG-3′ (forward) and 5′-GTGTAGTCTG TAGCAGCA-3′ (reverse); ODC, 5′-GTCAATGATGGAGTGTAT GGATC-3′ (forward) and 5′-TCCATTCCGCTCTCCTGAGCAC-3′ (reverse).

Morpholino oligonucleotides, DNA constructs and RNA synthesis

Anti-sense morpholino oligos (MO) were obtained from Gene Tools. The sequences of MOs are as follows: XEphA4 MO, 5′-ATAGGCATCTCT TAATCCACCTCCG-3′; and Xephrin-A1 MO, 5′-CCGCC GCTCTGTA CAACTCCATCAT-3′. Control MO is a standard morpholino oligo from Gene Tools whose sequence is 5′-CCTCTTACCTCAGTTACAATTTATA-3′. XEphA4-HA and Xephrin-A1-HA constructs were generated by inserting the complete coding regions of *Xenopus* EphA4 and ephrin-A1 into pCST-HA vector, respectively. We produced 5′UTR-XEphA4-HA by subcloning the PCR product encompassing its coding region and MO target site (5′ UTR) into pCST-HA plasmid. The rescue construct, sm-XEphrin-A1-HA, was generated by introducing point mutations into eight base pairs of its MO target sequence through site-directed mutagenesis (Fisher and Pei, 1997). The mutated MO target sequence is 5′-ATGATGGAacTtTAtcGcG CaGCaG-3′ (the substituted base pairs are indicated by lower-case letters). Dominant negative XEphA4 (XEphA4ΔC) construct (comprising amino acids 1–611) was obtained by removing its intracellular region containing the kinase domain. The PCR product encoding XEphA4ΔC was cloned into *EcoR* I/*Xba* I site of pCS2+ plasmid. The following constructs were described previously: XPAPC and DN-XPAPC (Kim et al., 1998); XFz7 and XFz7ΔC (Medina et al., 2000); Dsh-Myc, DshΔDIX, DshΔPDZ and DshΔDEP (Miller et al., 1999); Myc-RhoA, DN-RhoA and CA-RhoA (Wunnenberg-Stapleton et al., 1999); and PKCα (Otte and Moon, 1992). Capped synthetic mRNAs for microinjections were synthesized using mMessage mMachine Kit (Ambion).

Scanning electron microscopy (SEM)

For SEM analysis, the embryos were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4 °C overnight. Embryos were bisected sagittally through the dorsal midline with vibratome blade and then coated with 1% osmium tetroxide pH 7.4 for 1 h at room temperature in a dark condition. Samples were washed three times in 0.1 M cacodylate buffer, pH 7.4, serially dehydrated with 25% to 100% ethanol and dried. Completely dried samples were mounted onto metal stub with double sided carbon tape and coated with gold in a thin layer on the sample using an automated sputter coater.

In vitro tissue separation assay

In vitro tissue separation assay was performed as described previously (Wacker et al., 2000). Cell aggregates from the DMZ explants were placed on the BCR whose rolling up was prevented by applying a strip of BSA-saturated (5%, 30 min) coverslip resting on silicone grease. After 1 h, we examined whether the cell aggregates were present on the surface of BCR or have sunk into the BCR.

Elongation, cell polarity, and migration in Keller explants

Embryos were injected with the indicated reagents with or without membrane-targeted Venus mRNA in the marginal region of two dorsal blastomeres at 4-cell stage. Keller explants were dissected at stage 10.5 and for elongation assay, cultured in 1×MR (0.1 M NaCl, 1.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES-NaOH, pH 7.4) containing bovine serum albumin (10 mg/ml) and gentamicin (50 mg/ml) until stage 19. To control the size of each of Keller explants, they were made by cutting out an approximately same-sized rectangle of tissue reaching from animal pole to dorsal blastopore lip and 60–90° wide around the equator. The long protrusion formed in each of the explants represents the involuting marginal zone (IMZ), which undergoes extensive convergence. As defined in Fig. 4B, the initial length (Li), total length (Lt), total width (w) and width of the involuting marginal zone (IMZ) (c) were measured to determine the elongation (Lt/Li) and constriction (w/c) of explants (Unterseher et al., 2004). For cell polarity assay, Keller explants were placed on the fibronectin-coated (20 ug/ml) coverslip and

cultured in 1×MR until stage 13. Alternatively, those explants were dissociated in Ca^{2+}/Mg^{2+}-free MBS and cultured on the fibronectin-coated slide glass for 6 h to observe formation of protrusions of dorsal mesodermal cells. The elongation and orientation of the long axis of each cell were analyzed under a confocal laser scanning fluorescence microscope (Zeiss LSM 510) as described by Wallingford et al. (2000). For cell migration assay, anterior Keller explants were cultured overnight at 4 °C on the fibronectin-coated culture dishes containing 1×MR. Cell migration was quantified by measuring the farthest distance travelled by cell and calculating the average distances (Winklbauer, 1990).

Immunocytochemistry

The subcellular localization of protein was monitored as described by Park et al. (2005). Animal caps and DMZ tissues were dissected at stage 10.5 from the injected embryos and then fixed in 4% paraformaldehyde. Tissues were incubated in PBSTB (PBS, 0.1% Triton X-100, and 2% BSA) to block nonspecific antibody binding, followed by a standard immunostaining procedure. Image analysis was performed using a confocal laser scanning fluorescence microscope (Zeiss LSM510). The following abtibodies are used: rabbit anti-c-myc polyclonal antibody (Santa Cruz Biotechnology, SC-789) and goat anti-rabbit IgG conjugated with Alexa Fluor 488 antibody (Invitrogen, A11034).

RhoA activity assay and Western blot analysis

To measure the activity of RhoA GTPase, DMZ tissues were isolated at stage 10.5 from the injected embryos, cultured *in vitro* to stage 12, and then extracted in lysis buffer (50 mM Tris pH 7.2, 1% Triton X-100, 500 mM NaCl, 10 mM $MgCl_2$ and a cocktail of proteinase inhibitors). Subsequently, the GST-RDB binding assay was performed as described by Ren et al. (1999). For Western blot analysis, tissue samples were homogenized in Triton X-100 lysis buffer (20 mM Tris–HCl pH 8.0, 1% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM Sodium orthovanadate, 50 mM NaF, and a cocktail of proteinase inhibitors). Western blot was performed according to standard protocol as previously described (Park et al., 2007). Protein detection was performed using Fujifilm Image Reader LAS-3000 System and software (FujiFilm). The following antibodies are used: mouse anti-HA monoclonal (SC7392), mouse anti-c-Myc monoclonal (SC-40), rabbit anti-β-catenin polyclonal (SC-7199) and rabbit anti-actin polyclonal (SC1616-R) antibodies (Santa Cruz Biotechnology).

Results

Loss of XEphA4 function inhibits gastrulation movements

To examine the involvement of Eph–ephrin signaling in the morphogenetic movements at *Xenopus* gastrulation, we employed EphA4, which was expressed only in the involuting mesoderm of early gastrulae (Winning and Sargent, 1994). We first generated the loss-of-function phenotypes of XEphA4 using its anti-sense morpholino oligo (MO) and a dominant negative construct (XEphA4ΔC), which lacks its cytoplasmic region including the kinase domain (Park et al., 2004). Our designed XEphA4 MO, but not control MO, could inhibit specifically the translation of XEphA4 mRNA with its target sequence

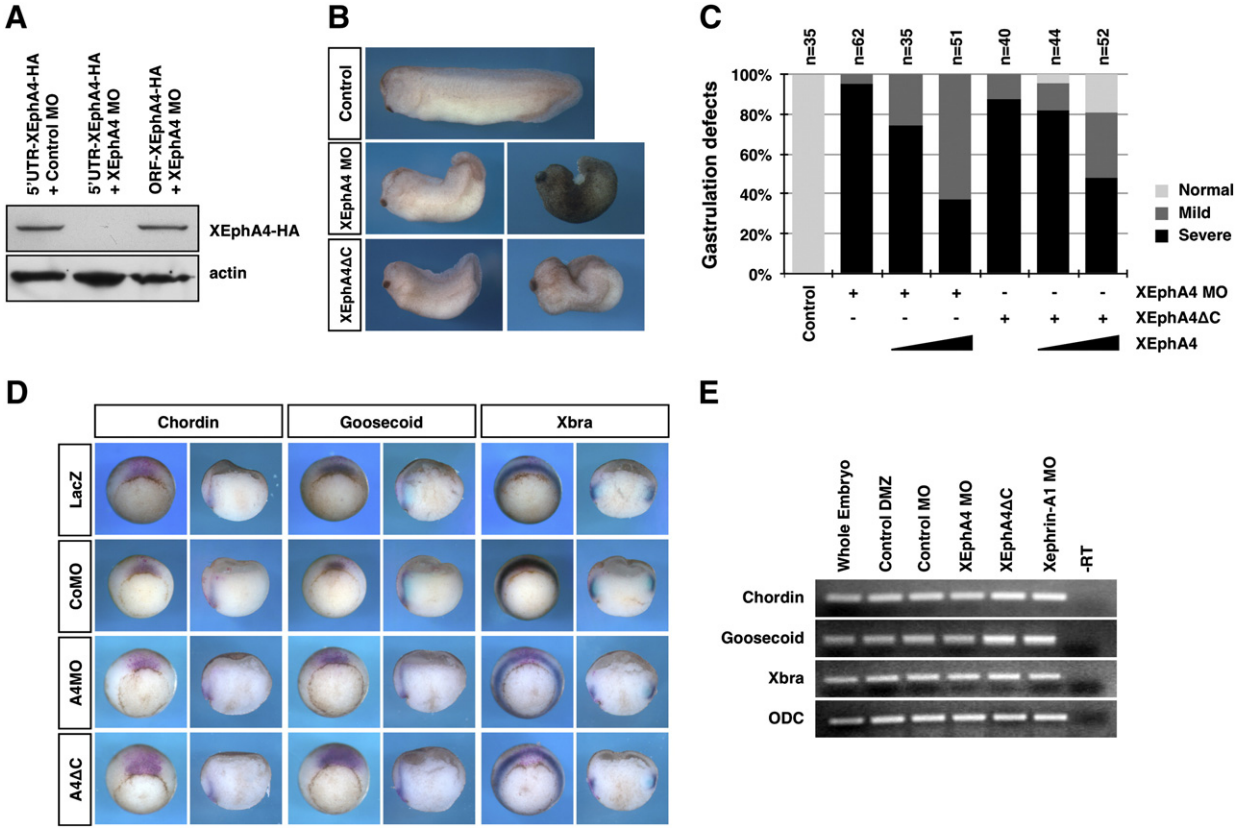

Fig. 1. Blockade of XEphA4 activity causes defective gastrulation movements. (A) Western blotting analysis showing the efficacy and specificity of XEphA4 MO. Actin serves as a loading control. (B) Gastrulation-defective phenotypes of the loss of XEphA4 function. (C) Quantification of the injection phenotypes in (B). Four-cell stage embryos were injected dorsally with XEphA4 MO (80 ng) or XEphA4ΔC RNA (3 ng) with or without MO-resistant XEphA4 mRNA (2–4 ng) and cultured to the tadpole stages. The mild phenotypes indicate bending with or without shortened anterior–posterior axis and severe ones kinked axis with open blastopore and neural tube. n, the total number of injected embryos. (D, E) No effects of the loss of XEphA4 function on the expression of mesodermal marker genes. (D) *In situ* hybridization against mesodermal markers. Four-cell stage embryos were injected dorsally with LacZ RNA (250 pg) as a lineage tracer with or without the indicated MOs and RNA, fixed at stage 10.5, stained with Red-Gal. Right panels for each marker show the embryos sectioned after *in situ* hybridization. In left panels, dorsal is up. (E) Quantitative RT-PCR analysis of mesodermal markers in dorsal marginal zone (DMZ) explants. DMZ tissues were dissected from the injected embryos at stage 10.5. ODC is a loading control. -RT, a control in the absence of reverse transcriptase.

(5′UTR-XEphA4). In contrast, XEphA4 mRNA lacking the MO target site (ORF-XEphA4) was not sensitive to the MO (Fig. 1A). Notably, dorsal injection of XEphA4 MO or XEphA4ΔC RNA produced gastrulation defects including a dorsally kinked and shortened body axis as well as defective blastopore and neural tube closures (Fig. 1B, C). These phenotypes could be rescued by coexpression of MO-resistant XEphA4 mRNA in a dose-dependent manner (Fig. 1C), indicating the specificity of the MO effects.

To see if these phenotypes result from a defect in mesodermal differentiation, we further analyzed the expression of molecular markers in embryos devoid of EphA4 activity. As shown in Fig. 1D, *in situ* hybridization revealed that the expression of mesodermal markers such as *Chordin*, *Goosecoid*, and *Xbra* was not affected by XEphA4 MO or XEphA4ΔC RNA as observed in LacZ or control MO-injected embryos. Consistently, this was confirmed by the quantitative RT-PCR analysis (Fig. 1E). Thus, these results suggest that the gastrulation defects caused by the loss of EphA4 activity are due to abnormal morphogenetic movements rather than defective mesodermal patterning.

Tissue separation depends on XEphA4 function

We also found that the loss of XEphA4 activity could interfere with tissue separation between the involuting mesoderm and non-involuting ectoderm of early gastrulae, which corroborates our initial hypothesis on the role of repulsive signaling for this process. Scanning electron microscope (SEM) analysis revealed that in the embryos injected dorsally with control MO, the involuting mesoderm remained separated from the non-involuting ectoderm on the dorsal side and the posterior Brachet's cleft was clearly visible (Fig. 2A, B). In contrast, dorsal injection of XEphA4 MO or XEphA4ΔC RNA strongly perturbed the tissue separation, thereby reducing or abrogating the formation of posterior Brachet's cleft on the dorsal side without effects on ventral cleft formation, which serves an internal control (Fig. 2C, D). The defects in posterior Brachet's cleft formation caused by XEphA4 MO could be partially recovered by coinjection of XEphA4 RNA (Fig. 2I).

The role of EphA4 in tissue separation was also confirmed by *in vitro* explant assay described in Fig. 2E. When cell aggregates from dorsal mesoderm are placed on the inner layer of blastocoel roof (BCR), they remain on the surface and do not integrate into the BCR tissues because of their separation behavior (Wacker et al., 2000). Notably, DMZ cell aggregates from the control MO-injected embryos remained separated from the BCR tissue (Fig. 2F), whereas when XEphA4 function was inhibited by XEphA4 MO or XEphA4ΔC RNA, the cell aggregates lost their separation ability, sank and mixed with the BCR tissues (Fig. 2G, H).

Since XEphA4 is expressed in all the marginal zones from dorsal to ventral during gastrulation (Winning and Sargent, 1994), we also analyzed the effects of XEphA4 on the formation of Brachet's cleft in the lateral and ventral marginal zones (LMZ and VMZ). When XEphA4 MO or XEphA4ΔC RNA was injected in those regions of embryos, the formation of the posterior Brachet's cleft was severely reduced as in the DMZ (Fig. S1A, B). Overall, these results suggest that XEphA4 has a critical role in the tissue separation between the involuting mesoderm and non-involuting ectoderm of early gastrulae.

XEphA4 controls tissue separation in parallel to XFz7 and XPAPC

The demonstrated role of EphA4 in tissue separation suggests the possibility that it might be functionally correlated with Fz7 and PAPC which are previously characterized regulators of this process (Medina et al., 2004; Winklbauer et al., 2001). To test this assumption, we first examined the ability of Fz7 or PAPC to rescue the defective tissue separation in the XEphA4-depleted embryos. As shown above,

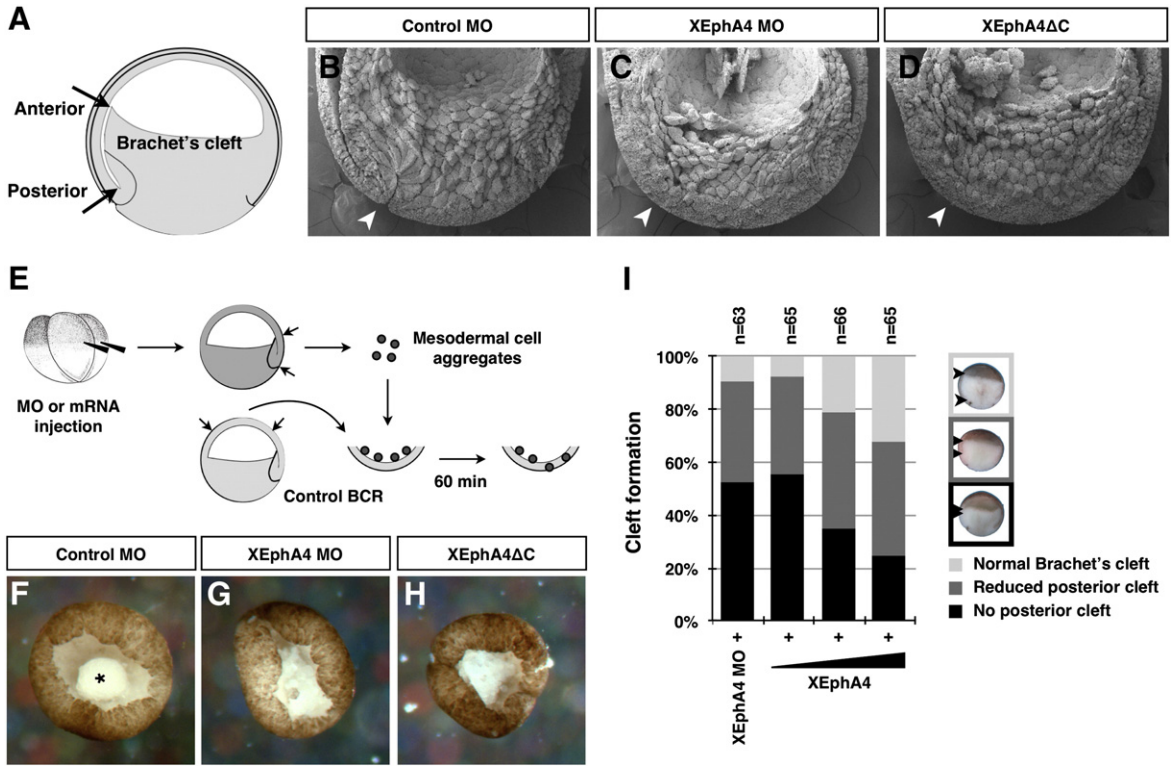

Fig. 2. Inhibition of XEphA4 activity abrogates tissue separation between the involuting mesoderm and non-involuting ectoderm. (A) Scheme showing the formation of Brachet's cleft at stage 10.5. Arrows indicate the anterior and posterior ends of the Brachet's cleft. (B–D) Scanning electron micrographs (SEM) of the Brachet's cleft in embryos injected with control MO (B), XEphA4 MO (C) or XEphA4ΔC RNA (D). Arrowheads mark presumptive dorsal blastopore. (E) Schematic diagram of *in vitro* tissue separation assay (see Materials and methods for details). (F–H) Mesodermal cell aggregates from the control MO-injected embryos were separated from the BCR, but not those from the XEphA4 MO or XEphA4ΔC RNA-injected embryos. Asterisk indicates the mesodermal cell aggregates. (I) Rescue by coinjection of XEphA4 RNA (1–4 ng) of defective tissue separation caused by XEphA4 MO. n, the total number of injected embryos.

knockdown of XEphA4 impaired the formation of the posterior Brachet's cleft (Fig. 3A, lane 1). This inhibitory effect could not be restored effectively by the coexpression of XFz7 or XPAPC RNA (Fig. 3A; lanes 2–7), which is sufficient on its own to elicit tissue separation. Furthermore, coexpression of XEphA4 could not also recover the disruptive tissue separation generated by a dominant negative XPAPC or XFz7ΔC (Fig. S2). Thus, these results indicate their non-redundant functions in tissue separation. In line with this, the suboptimal doses of dominant negative XEphA4, XFz7 or XPAPC RNA alone were not able to disrupt strongly the formation of the posterior Brachet's cleft (Fig. 3A; lanes 8–10), whereas when combined, they inhibited synergistically the tissue separation behavior (Fig. 3A; lanes 11–12). Together, these data imply that XEphA4 regulates the tissue separation in parallel to XFz7 and XPAPC signaling pathways.

XEphA4 signals through RhoA to regulate tissue separation

To identify downstream effectors of XEphA4 to regulate tissue separation, we performed the rescue experiments with candidate components. RhoA has been shown to mediate Eph receptor signaling in a variety of physiological and pathological processes (Pasquale, 2008). Moreover, it mediates PAPC signaling to establish the Brachet's cleft during gastrulation (Medina et al., 2004). As shown previously, the injection of dominant negative RhoA (DN-RhoA) RNA inhibited the tissue separation in a dose-dependent fashion (Fig. 3B; lanes 1–3; Medina et al., 2004). Importantly, a constitutively active form of RhoA (CA-RhoA) could rescue efficiently the impaired tissue separation in the XEphA4-depleted embryos (Fig. 3B; lanes 4–7), suggesting that RhoA acts downstream of XEphA4 in control of tissue separation behaviors. Consistent with this, the gain- and loss-of XEphA4 function could increase and decrease RhoA activity in the involuting mesodermal tissues, respectively (Fig. 3C). In contrast, PKCα, a mediator of Fz7 signaling for tissue separation (Winklbauer et al., 2001), could not restore the defective tissue separation caused by XEphA4 MO (Fig. 3B, lanes 8–11), thus confirming the non-redundant functions of XEphA4 and Fz7 signaling. Considering that Dishevelled (Dsh), a Wnt/Fz pathway component, specifically interacts with the B-class of Eph receptors, ephrin ligands to mediate Eph–ephrin induced cell repulsion (Tanaka et al., 2003) and furthermore, the B-class of Eph/ephrin signaling regulates gastrulation movements in zebrafish (Chan et al., 2001; Kida et al., 2007), we also tested whether Dsh might function as a downstream effector of XEphA4. However, it could not rescue the inhibition of tissue separation by XEphA4 MO (Fig. S3A; lanes 1–5). Even, injection of truncated Dsh mutant RNAs including DshΔDIX, DshΔPDZ and DshΔDEP had no effect on tissue separation (Fig. S3A; lanes 6–8). Moreover, overexpression and inhibition of XEphA4 signaling did not affect the phosphorylation and subcellular localization of Dsh (Fig. S3B, C). Taking together, we argue that

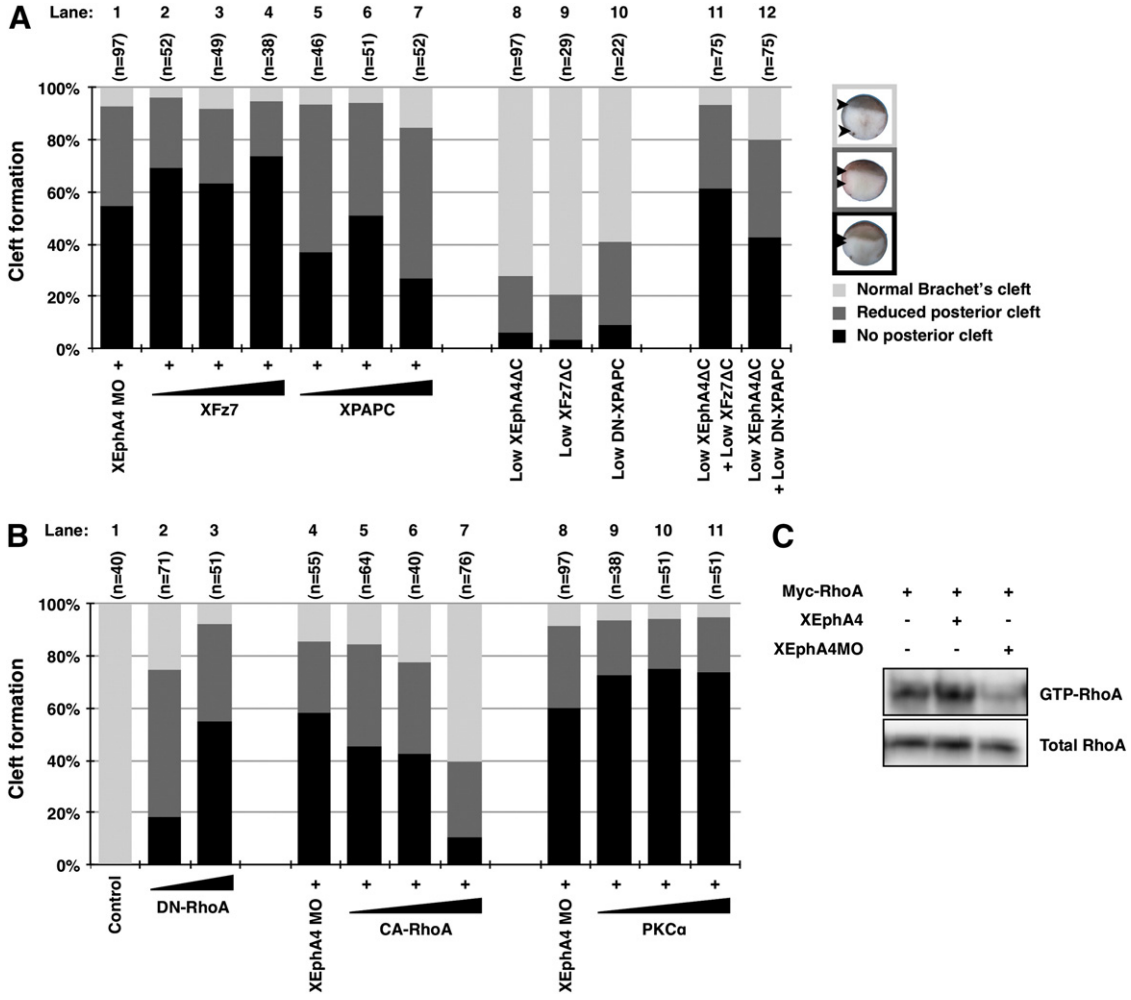

Fig. 3. XEphA4 regulates tissue separation via RhoA in parallel to Fz7 and PAPC. (A) Compilation of the experiments demonstrating the non-redundant functions of XEphA4, XFz7, and XPAPC in tissue separation. (B) Rescue by RhoA GTPase of the defective Brachet's cleft in the XEphA4-depleted embryos. (A, B) Four-cell stage embryos were injected with LacZ RNA as a lineage tracer along with the indicated combination of XEphA4 MO (80 ng), XFz7 (1–4 ng), XPAPC (1–4 ng), XEphA4ΔC (2 ng), XFz7ΔC (2 ng), DN-XPAPC (2 ng), DN-RhoA (1–2 ng), CA-RhoA (5–25 pg), PKCα (0.5–2 ng) RNAs, fixed at stage 11, stained with Red-Gal, bisected sagittally through the dorsal midline and scored. n, the total number of injected embryos. (C) DMZ explants from the embryos injected with RhoA-myc RNA (250 pg) with or without XEphA4 mRNA (2 ng) or XEphA4 MO (80 ng) were subjected to RhoA activity assay.

XEphA4 regulates via RhoA tissue separation independently of the Fz7/PKC/Dsh signaling pathway.

Loss of XEphA4 function impairs tissue convergence, cell polarity and migratory activity

Besides tissue separation, XEphA4 was also found to affect convergence in convergent extension (CE) movements of mesodermal tissues. As demonstrated by the Keller explant elongation assay, the dorsal marginal zone (DMZ) explants from XEphA4 MO or XEphA4ΔC RNA-injected embryos became normally elongated compared to control MO-injected tissues (Fig. 4A, C, D) whereas their constriction was significantly perturbed (Fig. 4A, E), suggesting that the inhibition of XEphA4 function causes the failure of cells to converge properly. In contrast, the control MO-injected DMZ tissues exhibited normal narrowing and elongation as a result of CE (Fig. 4A). Sagittal section of the DMZ explants also revealed increased thickness in the involuting marginal zone of the XEphA4 MO-injected tissues (Fig. S4A-C). However, the overall number of cells per section was not changed by depletion of XEphA4 (Fig. S4D), suggesting that the increase in thickness of explants is not due to enhanced cell proliferation. Importantly, the defective convergence of DMZ tissues caused by XEphA4 MO could be rescued by coexpression of the constitutively active form of RhoA (Fig. 4A, E), which has been shown to regulate CE movements downstream of PAPC and Wnt/Fz7 signaling (Unterseher et al., 2004). Thus, these results indicate that the XEphA4-dependent signaling regulates via RhoA tissue convergence as well as separation behaviors like PAPC and Wnt/Fz7 signaling.

Since CE movements involve polarization and protrusive activity of gastrulating cells (Wallingford et al., 2002), we next investigated the effects of the loss of XEphA4 function on these cellular events. First, a membrane-targeted Venus (mVenus) RNA was coinjected with control MO or XEphA4 MO to see the cell polarization of the involuting mesodermal cells. While the DMZ cells from control MO-injected embryos showed the typical bipolar morphology (Fig. 5A), the majority of the cells in the XEphA4 MO-injected tissues lost this normal cell shape (Fig. 5B), suggesting the inhibitory effects of XEphA4 depletion on cell polarization. We further quantified the effects of XEphA4 MO on cell polarity by determining the elongation and orientation of the long axis of each cell in the involuting mesodermal tissues (Wallingford et al., 2000). As shown in Fig. 5C–E, while the majority of DMZ cells from control MO-injected embryos elongated toward the mediolateral axis, XEphA4 MO-injected DMZ cells showed random angular distribution and no elongation, which results from the loss of cell polarity.

We next checked whether knockdown of XEphA4 could affect cell migration by measuring the migratory activity of mesodermal cells on fibronectin-coated slide glass. Notably, DMZ cells from the control MO-injected embryos spread out from the planted site *in vitro* (Fig. 5F, I) as they migrate toward anterior axis *in vivo*. In contrast, injection of XEphA4 MO or XEphA4ΔC RNA perturbed significantly this migratory activity of mesodermal cells (Fig. 5G-I).

We further examined the polarity and protrusion formation of the individual cells, which are responsible for the migratory activity of mesodermal cells. Control cells from the control MO-injected tissues displayed polarized shape and active protrusions (Fig. 5J), whereas mesodermal cells lacking XEphA4 activity lost these characteristics (Fig. 5K, L). Together, these data suggest that the inhibition of tissue constriction by loss of EphA4 function might result from the impairment in cell polarization and migration.

Xephrin-A1 is a putative ligand for XEphA4 in control of tissue separation

We assumed that if XEphA4, which is localized to the involuting mesoderm, mediates repulsive interaction for tissue separation, its ligand would be expressed in a complementary region of early gastrulae, the ectoderm. Given this, *Xenopus* ephrin-A1 (Xephrin-A1) is a potential candidate because it is expressed specifically in the ectodermal region of embryo and physically interacts with XEphA4 (Weinstein et al., 1996). We confirmed that they are expressed in the mutually exclusive

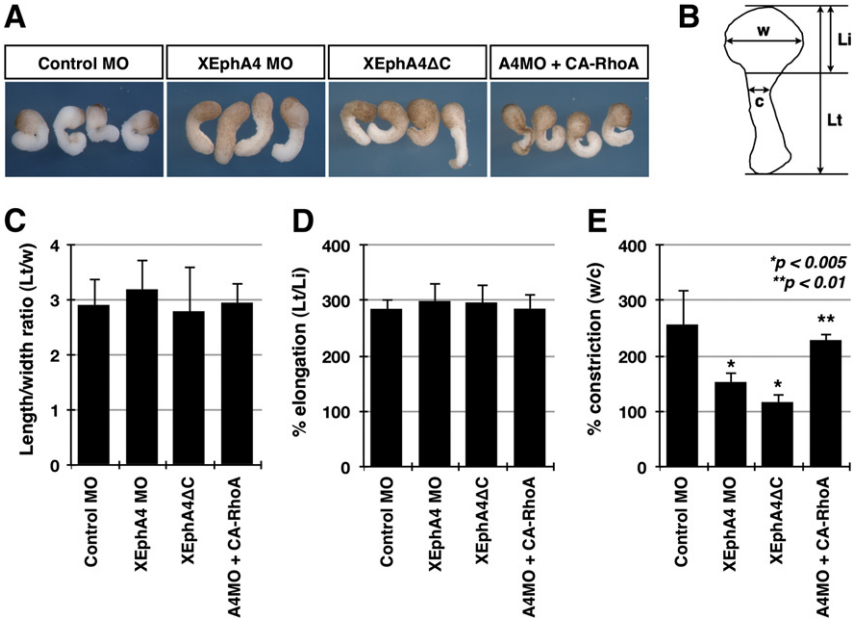

Fig. 4. XEphA4 is required for tissue constriction in CE movements. (A) *In vitro* elongation assay showing that XEphA4 MO and XEphA4ΔC RNA perturb the constriction of DMZ tissues with no effect on their elongation . Of note, CA-RhoA rescued the defective constriction of DMZ tissues caused by XEphA4 MO. (B) Definition of the initial length (Li), total length (Lt), width (w) and constriction (c) of explants. (C–E) Summaries of the length/width ratio (C), elongation (D) and constriction (E) of the DMZ explants shown in (A). The total number (n) of the explants measured for each injection: control MO, n = 27; XEphA4 MO, n = 29; XEphA4ΔC, n = 25; XEphA4 MO + CA-RhoA, n = 15. Error bars denote standard deviation. Asterisks indicate *p-value* compared to the control MO-injected (*) or XEphA4 MO-injected (**) explants.

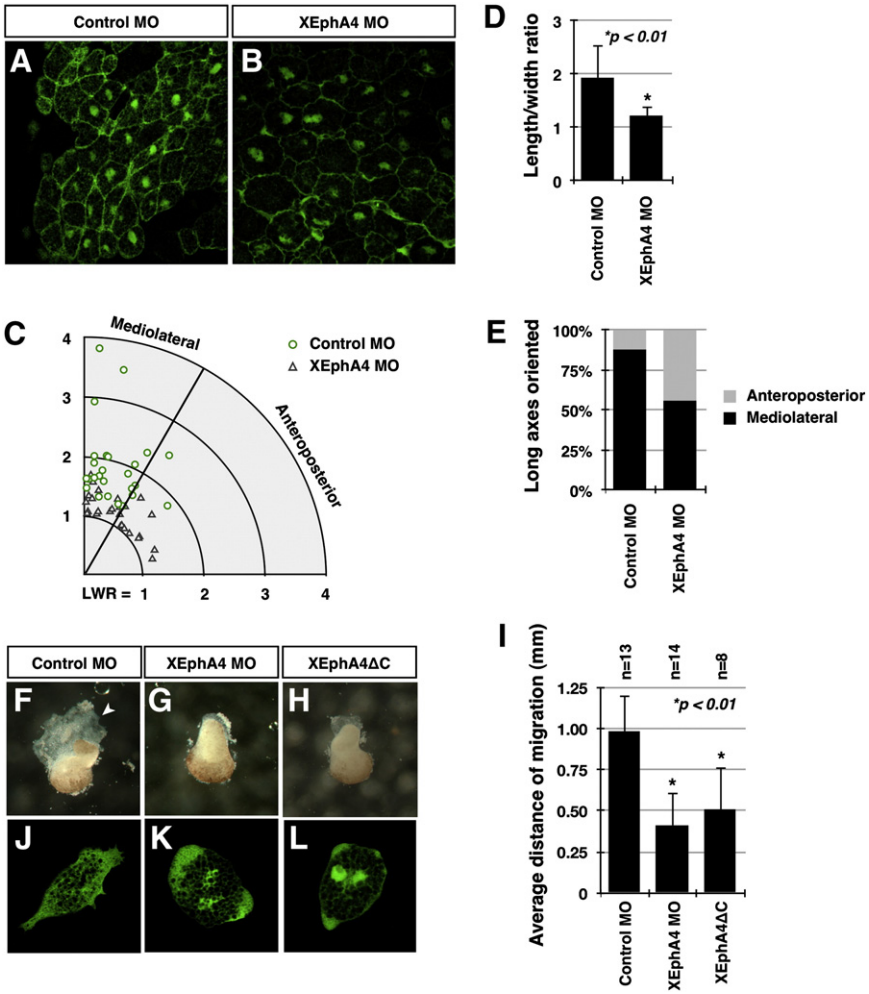

Fig. 5. XEphA4 controls cell polarity and migration. (A, B) Depletion of XEphA4 disrupts cell polarization, leading to the failure of cells to align and elongate. Four-cell stage embryos were injected dorsally with membrane-targeted Venus (mVenus) mRNA (500 pg) with control MO or XEphA4 MO, and DMZ tissues were dissected at stage 10.5, placed on the cover slip coated with fibronectin, cultured to stage 13 and observed with confocal microscope. (C, D) Length/width ratio (LWR) and LWR versus angle of major axis for each of the cells scored in (A, B). (E) Orientation of long axes for each of the cells in (C, D). (F–H) Loss of XEphA4 activity interferes with cell migration. Anterior DMZ tissues were excised at stage 10.5 from the embryos injected with the indicated MO or RNA and cultured on the dishes coated with fibronectin overnight at 4 °C. An arrowhead points to the migrating cells. (I) Measurement of the distances moved by cells shown in (F–H). (J–L) Loss of XEphA4 function impairs the polarity and protrusive activity of mesodermal cells. DMZ tissues from the embryos injected as described in (A, B) were dissociated in Ca^{2+}/Mg^{2+}-free MBS, and the dissociated cells were plated on a fibronetin-coated slide glass and cultured for 6 h. n, the number of explants measured. Error bars denote standard deviation. Asterisks indicate *p-value* compared to the control MO-injected explants.

domains of early gastrulae, the involuting mesoderm and non-involuting neuroectoderm (Fig. 6A). Thus, we further investigated the effects of the loss of Xephrin-A1 function on tissue separation behaviors. Our designed Xephrin-A1 MO could inhibit the production of protein from Xephrin-A1 with the MO target sequence but not from a mutated Xephrin-A1 (sm-Xephrin-A1) which has silent mutations at eight base pairs in the MO target site (Fig. 6B). Like XEphA4 MO, dorsal injection of Xephrin-A1 MO caused aberrant gastrulation movements in a dose-dependent manner (Fig. 6C, D). Furthermore, bisection of Xephrin-A1-depleted gastrulae revealed the severe defects in Brachet's cleft formation, which could be effectively rescued by sm-Xephrin-A1 (Fig. 7A, B). The role of Xephrin-A1 in tissue separation was also confirmed by *in vitro* assay depicted in Fig. 7C. Notably, mesodermal cell aggregates remained separated from the BCR tissues from control MO-injected embryos (Fig. 7D), whereas upon being apposed to the BCR tissues from Xephrin-A1 MO-injected embryos, they sank and fused with the BCR explants (Fig. 7E). In contrast, knockdown of Xephrin-A1 had no effects on the expression of mesodermal marker genes as analyzed by *in situ* hybridization and RT-PCR (Figs. 7A, 1E). Taking together, we conclude that the repulsive signals mediated by XEphA4 and Xephrin-A1 might be critical for normal tissue separation between the involuting mesoderm and non-involuting ectoderm.

Discussion

During *Xenopus* gastrulation, the endodermal and mesodermal cells which are brought into the interior of embryos come into close contact with ectodermal cells, but they do not mix with and remain separated from each other (Wacker et al., 2000). We initially hypothesized that the mechanism separating these tissues might involve repulsive interaction mediated by the Eph–ephrin signaling. Supporting this assumption, knockdown of a specific Eph receptor, EphA4 and its putative ligand, ephrin-A1 could impair the separation of the involuting mesoderm and non-involuting ectoderm as assayed *in vivo* and *in vitro*. Furthermore, they are expressed in a complementary manner in the involuting mesoderm and non-involuting ectoderm of early embryo, respectively, suggesting their positions to cause those tissues to repel each other. Eph receptors and ephrins expressed in opposing cells interact in trans and activate bidirectional signaling whereas when coexpressed in the same cells, they interact in cis and interfere with the trans interaction and/or signaling (Arvanitis and Davy, 2008). In light of this, the partial rescue by EphA4 of its MO phenotypes could be due to ectopic expression of injected EphA4 in the overlying ectoderm, which might impede the bidirectional interaction. Notably, while *Xenopus* ephrin-A1 is maternally expressed in the ectodermal layer (Weinstein et al., 1996), EphA4

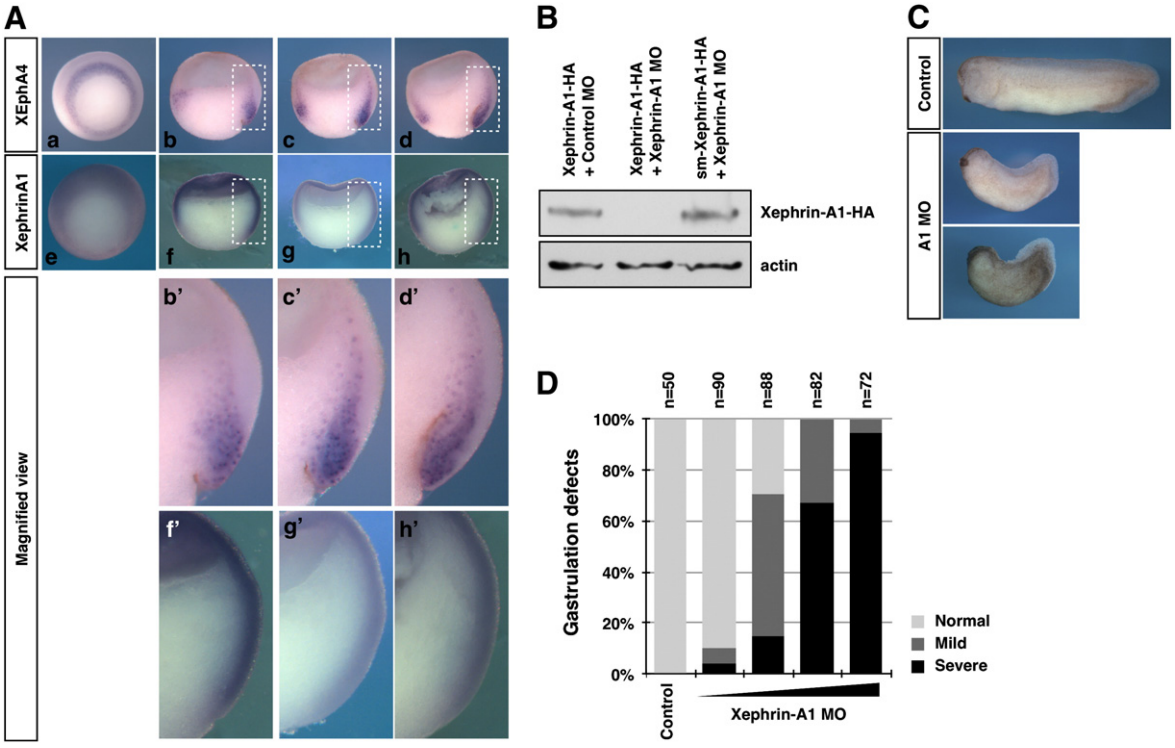

Fig. 6. Complementary expression pattern of XEphA4 and Xephrin-A1 during gastrulation. (A) Comparison of the spatial expression patterns of XEphA4 and Xephrin-A1 in early gastrulae. XEphA4 (a–d) and Xephrin-A1 (f–i) are expressed in the involuting mesoderm and entire ectoderm, respectively. (a, e) Vegetal view of stage 10 embryos. (b–d, f–h) Sagittal sections of stage 10 (b, f), stage 10.5 (c, g), and stage 11 (g, h) embryos. (b'–d', f'–h') Enlarged views of the expression of XEphA4 and Xephrin-A1 in the rectangular areas in (b–d) and (f–h). (B) Efficacy and specificity of Xephrin-A1 MO. (C) Phenotype of embryos injected dorsally with Xephrin-A1 MO (80 ng). (D) Summary of the effects of Xephrin-A1 MO on gastrulation movements. n, the total number of injected embryos.

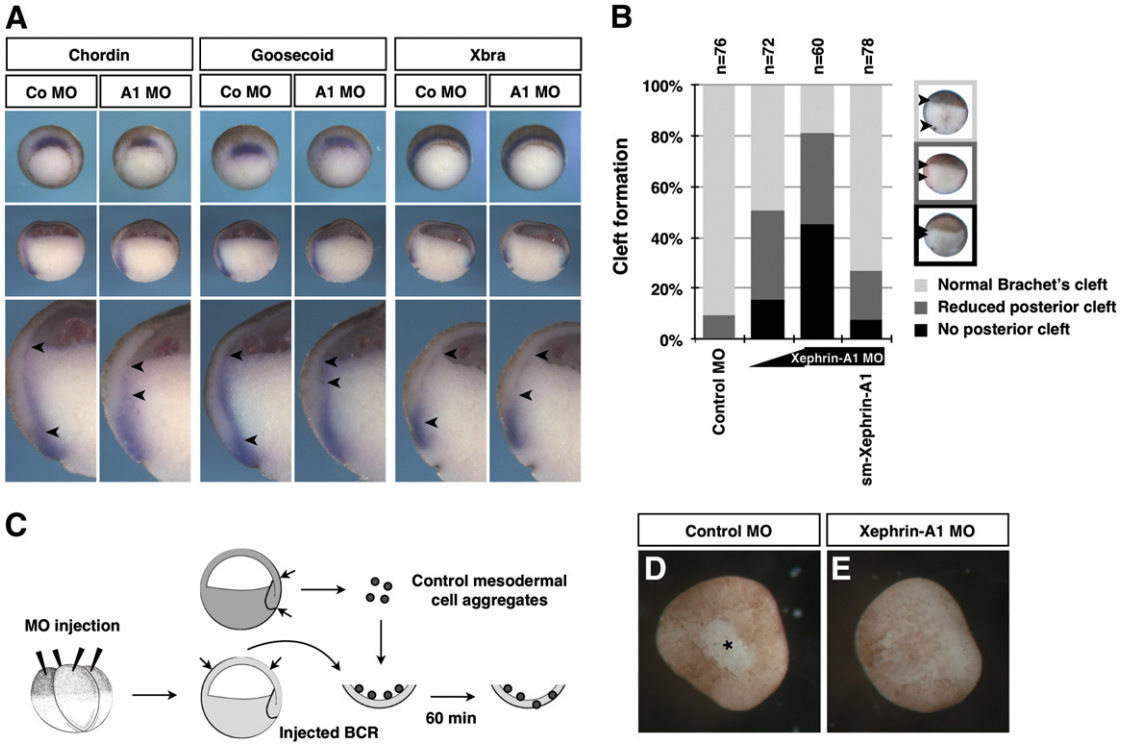

Fig. 7. Loss of Xephrin-A1 function inhibits tissue separation. (A) Knockdown of Xephrin-A1 abrogates the formation of Brachet's cleft but not the expressions of mesodermal markers, *Chordin*, *Goosecoid*, and *Xbra*. Arrowheads indicate the anterior and posterior ends of Brachet's cleft. (B) Summary of Brachet's cleft formation in the Xephrin-A1-depleted embryos. n, the total number of injected embryos. (C) Schematic diagram of *in vitro* tissue separation assay. (D, E) Mesodermal cell aggregates were separated from the BCR of control MO-injected embryos but fused into Xephrin-A1 MO-injected BCR. Asterisk indicates the mesodermal cell aggregates.

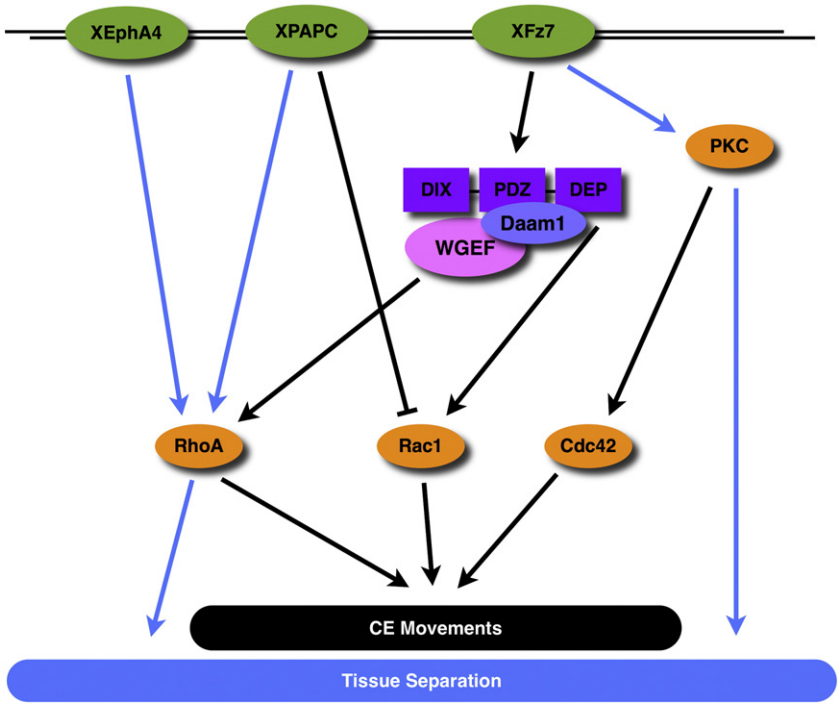

Fig. 8. Model for tissue separation. XEphA4 activates RhoA, XPAPC modulates RhoA and Rac1 activity, and XFz7 signals through PKCα, RhoA, Rac1, and Cdc42. The balanced combination of RhoA and PKCα activity regulated by XEphA4, XPAPC or XFz7 is essential for tissue separation behaviors in gastrulation movements. Blue arrows indicate signals for tissue separation and black ones for CE movements.

is zygotically induced in the marginal regions of embryo from the onset of mesoderm involution onward (Winning and Sargent, 1994), indicating that the Eph–ephrin signaling occurs in the right place at the right time to regulate the tissue separation. To convert the initial adhesive contact into repulsion, the Eph–ephrin signaling system employs the proteolytic cleavage of the extracellular domain of the ephrin ligand from its membrane tether, which is mediated by the metalloprotease such as A-Disintegrin-And-Metalloprotease (ADAM) 10 (Hattori et al., 2000; Janes et al., 2005). When we injected a dominant negative mutant of ADAM 10 in the marginal zone of embryo, the separation of the involuting mesoderm and non-involuting ectoderm was strongly impeded (Fig. S5). This observation substantiates the possibility that the repulsive contact controlled by the Eph–ephrin signaling is responsible for the tissue separation behavior.

The Eph–ephrin complex produces bidirectional signals which affect both Eph-expressing and ephrin-expressing cells (Pasquale, 2008). Given this, the XEphA4 forward and Xephrin-A1 reverse signaling would modulate the adhesive properties of mesodermal and ectodermal cells, respectively, for tissue separation. Thus, it is noteworthy that the inhibition of the EphA4 forward signaling in dorsal mesoderm by using its kinase-dead mutant reduced strongly the Brachet's cleft formation (data not shown). This EphA4 forward signaling appears to be mediated by RhoA as the defective tissue separation caused by XEphA4 MO was rescued by RhoA. Although PAPC and Fz7 signaling also involve RhoA activation and are able to induce tissue separation on their own, they could not restore efficiently the impaired Brachet's cleft formation in the XEphA4-depleted embryos. It seems therefore likely that the Eph–ephrin signaling regulates more than RhoA-mediated cytoskeletal reorganization and the resulting changes in cell adhesion. In *Xenopus* gastrulae, fibronectin (FN) fibril assembly occurs on the surface of the blastocoel roof (BCR) (Winklbauer, 1998). The interaction of integrin and the FN fibril appears critical for not only migration of the involuting mesodermal cells, but separation of the mesodermal and ectodermal germ layers. Study on the somitic boundary formation in zebrafish demonstrates that the Eph–ephrin signaling controls integrin clustering and FN assembly (Julich et al., 2009). Thus, it is tempting to speculate that the XEphA4 forward and/or Xephrin-A1 reverse signaling could also affect integrin signaling and FN assembly on the BCR to segregate the involuting mesoderm and non-involuting ectoderm and to modulate migration of mesodermal cells during early gastrulation.

Apart from its role in tissue separation, the Eph–ephrin signaling seems involved in tissue convergence during gastrulation movements. We showed that the inhibition of the XEphA4 activity could interfere with constriction, but not elongation, of the dorsal marginal zone tissues *in vitro*. While convergence and extension have been considered inseparable during CE movements in *Xenopus*, there are some pieces of evidence supporting their uncoupling. For example, depletion of PAPC or Fz7 inhibits selectively constriction with no effect on extension in Keller explants (Unterseher et al., 2004). In addition, the RhoA-dependent kinase (ROK) has been shown to influence only convergence in zebrafish (Marlow et al., 2002). Given these findings with the activity of XEphA4, the separation of constriction and extension movements seems general, which needs to be further investigated. This selective abrogation of tissue constriction might result from the defective polarization and protrusion of gastrulating cells as observed in the PAPC-depleted embryos (Unterseher et al., 2004). Tissue convergence occurs in both the involuting mesoderm and neuroectoderm of gastrulae. Given the bidirectional signaling of the Eph–ephrin complex, the EphA4 forward and ephrin-A1 reverse signaling which are generated by their reciprocal interaction are likely to control tissue convergence in the mesoderm and ectoderm, respectively. It is also possible that they would function independently of each other. Fz7 and PAPC signaling regulate tissue constriction in a non-redundant manner (Unterseher et al., 2004). Besides, the non-canonical Wnt signaling as well as FGF and integrin signaling is implicated in tissue convergence and extension in gastrulation movements (Hammerschmidt and Wedlich, 2008; Wang and Steinbeisser, 2009). Importantly, the Eph receptors and/or ephrin ligands have been shown to communicate with other cell surface signaling pathways including FGF, integrin and Ryk (Arvanitis and Davy, 2008). Thus, EphA4 might regulate tissue

convergence through crosstalks with these signaling pathways without binding to ephrin-A1 ligand. The interplay between Eph–ephrin signaling and other signaling pathways appears to converge at the level of cytoplasmic kinases such as MAPK and/or small GTPases including RhoA. Therefore, the combined and balanced activities of these downstream effectors might be critical for normal tissue convergence and separation (Fig. 8).

In conclusion, we have demonstrated that the Eph–ephrin signaling is essential for tissue convergence and separation during gastrulation movements. The underlying mechanism for this involves control of cell polarity, adhesion and protrusive activity. Further studies are warranted to investigate how the convergent extension and separation of tissues are coordinated simultaneously by the same signaling components and whether the crosstalks between Eph–ephrin signaling and other cell surface signaling pathways are relevant to tissue separation and convergence in gastrulation.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.12.012.

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