Leftward Flow Determines Laterality in Conjoined Twins

Graphical Abstract

Highlights

- Conjoined *Xenopus* twins feature a fused and ciliated left-right organizer (LRO)
- Polarized and motile cilia produce a leftward flow on both sides of the fused LRO
- Flow in the right twin fails to repress *dand5* and to induce the Nodal cascade
- Laterality in conjoined twins is ruled by leftward flow as in singleton embryos

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In Brief
Organ asymmetry is normal in left and randomized in right conjoined twins. Tisler et al. report that cilia-driven leftward flow determines laterality in conjoined twins like in singletons. The right twin is randomized because flow is insufficient to repress the Nodal inhibitor *dand5* in the center domain between the twins.
Leftward Flow Determines Laterality in Conjoined Twins

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SUMMARY

Conjoined twins fused at the thorax display an enigmatic left-right defect: although left twins are normal, laterality is disturbed in one-half of right twins [1–3]. Molecularly, this randomization corresponds to a lack of asymmetric Nodal cascade induction in right twins [4]. We studied leftward flow [5, 6] at the left-right organizer (LRO) [7, 8] in thoracopagus twins in Xenopus, which displayed a duplicated, fused, and ciliated LRO. Cilia were motile and produced a leftward flow from the right LRO margin of the right to the left margin of the left twin. Motility was required for correct laterality in left twins, as knockdown of dynein motor dnah9 prevented Nodal cascade induction. Nodal was rescued by parallel knockdown of the inhibitor dand5 [9, 10] on the left side of the left twin. Lack of Nodal induction in the right twin, despite the presence of flow, was due to insufficient suppression of dand5. Knockdown of dand5 at the center of the fused LRO resulted in asymmetric Nodal cascade induction in the right twin as well. Manipulation of leftward flow and dand5 in a targeted and sided manner induced the Nodal cascade in a predictable manner, in the left twin, the right one, both, or neither. Laterality in conjoined twins thus was determined by cilia-driven leftward fluid flow like in single embryos, which solves a century-old riddle, as the phenomenon was already studied by some of the founders of experimental embryology, including Darenste [11], Fol and Warwinsky [12], and Spemann and Falkenberg [13] (reviewed in [14]).

RESULTS AND DISCUSSION

Conjoined Xenopus Twins Display a Duplicated and Fused Left-Right Organizer

To systematically investigate left-right (LR) axis specification in conjoined twins, we analyzed several salient benchmarks during Xenopus development [6]. Twins were induced in a side-directed manner, by injecting synthetic β-catenin (βcat) mRNA into the left or right ventral blastomere at the four-cell stage [4] (Figure S1A).

The Xenopus left-right organizer (LRO) is represented by the gastrocoel roof plate (GRP), a transient ciliated epithelium that develops from the superficial mesoderm (SM) while this patch of epithelial cells involves during gastrulation [15] (Figure 1A). A characteristic SM marker gene is foxj1, a key transcription factor of motile ciliogenesis [16] (Figure 1B). Figure 1C shows that foxj1 was expressed in the SM of the primary as well as the induced secondary axis, and that the two SM tissues were clearly separated. GRP cells are flanked by sox17α-positive endodermal cells (Figure 1D) [17]. Analysis of sox17α expression in dorsal explants prepared from neurula stage induced twins (stage 17) revealed a U-shaped region devoid of sox17α mRNA (Figure 1E), suggestive of a fused GRP. GRP cells express the axonemal dynein motor gene dnah9 [18] (Figure 1F). Figure 1G shows dnah9 expression in a pattern complementary to sox17α, confirming the fused nature of the common GRP. Previous analyses of conjoined twins induced in the frog Xenopus revealed nodal1 expression in the left lateral plate mesoderm (LPM) of left twins at late neurula and early tadpole stages, whereas mRNA was consistently absent from the right LPM [4, 19]. We reproduced and extended these data to include the two other genes of the Nodal cascade, lefty2 and pitx2c (Figures S1B–S1D), which together direct the morphogenesis and placement of asymmetric organs (heart, lung, and gastro-intestinal tract; GIT). Expression of all three genes was restricted to the left LPM of the left twin, irrespective of the side of twin induction (Figure S1H). Assessment of organ asymmetry was restricted to the heart of stage 45 larvae, because the GIT was generally common to both twins and looping was abnormal in most cases (Figures S1E–S1G). The heart, as reported in experimental twins and human patients [4, 13, 20], was randomized in the right twin, whereas the left displayed situs solitus (Figures S1E–S1H; Movie S1). Identical results of marker gene expression and heart situs were obtained when twinning was induced by injection of siamois1 or wnt8a mRNA (Figures S1I and S1J).

Motile and Polarized Cilia at the Fused LRO Produce a Leftward Flow of Extracellular Fluids

Vertebrates break symmetry during neurulation: polarized monocilia rotate to produce a leftward flow of extracellular fluids at the LRO [21]. Cilia were present on all cells of the fused GRP region, as visualized by scanning electron microscopy (SEM) of dorsal explants (Figures 1H–1K; Figure S2), suggesting that the U-shaped area represented an incomplete fusion of the GRPs of the two twins. Central GRP cilia are motile and polarized,
whereas the flanking lateral domains harbor predominantly non-polarized and immotile cilia [22], with the latter cilia presumably sensing flow directionality [5, 6, 23, 24]. When cilia localization was analyzed in SEM close-ups, polarized cilia were found in central regions on both sides of the U (Figures S2C and S2D). In contrast, central cilia and cells devoid of cilia were seen at the fused center of the twinned GRP (Figure S1E), as well as in both lateral domains bordering the endodermal cells (not shown). Primary and induced sides of the GRP did not differ with respect to cilia polarization, length of cilia, and ciliation rate, whereas the fused central domain revealed fewer ciliated cells (Figures 1I–1K). Identical results on GRP ciliation were obtained when SEM photographs of siamois1-induced conjoined twins were analyzed (not shown). The presence of polarized cilia suggested directed fluid flow on the left and right sides of the fused GRP. To assess flow directly, we prepared and cultured dorsal explants of conjoined twins in the presence of fluorescent microbeads [22]. Beads were transported with identical, i.e., leftward, directionality and similar velocity on both sides (Figures 1L and 1M; Movie S2). Primary and induced parts of the fused GRP thus were indistinguishable with respect to ciliation, cilia polarization, and leftward flow (Figure 1N).

Cilia Motility and Leftward Flow Are Required for Normal Laterality Development in Left Conjoined Twins

To abrogate cilia motility and directly test their role in LR specification in twins, we used previously characterized antisense morpholino oligonucleotides (MOs) against the axonemal dynein gene dnah9 [18]. Control MO (CoMO) or dnah9MO was injected into the endogenous GRP lineage, and secondary axes were induced by $\beta$cat on the left or right side (Figure 2A). CoMO did not interfere with the induction of nodal1 in the left LPM of the left twin (Figures 2B and 2E). Knockdown of cilia motility interfered with nodal1 induction when the endogenous, dnah9MO-injected twin was on the left side (Figures 2C and 2E), whereas no effect was seen when the induced twin, i.e., the one that did not receive dnah9MO, was on the left (Figures 2D and 2E). Flow analysis of morphants demonstrated that beads at the
GRP of flow-compromised left twins moved only slowly and without directionality, whereas flow was normal in right twins. A representative specimen is shown in Movie S3. These data demonstrated that motile cilia were required for LR axis determination in conjoined twins but acted exclusively on the left side of the fused GRP.

Cells flanking the LRO co-express nodal1 and its inhibitor dand5 [6, 9, 10]. Flow downregulates dand5, releases Nodal
repression, and—following transfer to the left LPM—induces the Nodal signaling cascade [6, 10]. Therefore, dand5 was analyzed next in order to assess the impact of flow. Three expression domains of nodal1/dand5 were observed in twinned GRPs (right, center, and left; Figures 2F–2L), indicating a fusion of the right lateral domain of the left twin to the left lateral domain of the right one. Specimens in about 75% of cases displayed a complete fusion (Figures 2F–2L), whereas the anterior-most aspect of the fused central domain was bifurcated to various degrees in one-quarter of twinned GRPs (Figure 2J). Importantly, nodal1 expression did not differ during and after flow (Figures 2F and 2G). In contrast, the left dand5 domain of the left twin was clearly reduced at post-flow stages (Figures 2H and 2L). The left domain of the right twin was partially reduced as well, which was best seen in specimens with anterior bifurcations of the fused central domain, in agreement with the observed flow in both twins (Figures 2J–2L). This partially reduced signal, however, was still consistently stronger than the one on the left side of the left twin (Figures 2K and 2L). This result is in perfect agreement with the observed situs solitus in left twins (repression of dand5 followed by left-asymmetric induction of the Nodal cascade) and the randomization on the right side (absence of Nodal cascade gene expression).

The lack of Nodal cascade induction in the LPM tissue between the twins, anterior to the bifurcation of the axes, however, was puzzling, posing the question of whether this tissue was competent to be induced. If it were competent, the unchanged patterns at flow and post-flow stages would indicate that flow was insufficient to repress dand5 expression. If laterality in conjoined twins was determined by cilia-driven leftward flow, which repressed dand5 at the left GRP margin of the left twin as in single embryos (Figure 4F), the Nodal cascade was not induced in the right twin, because flow—although present—was insufficient to reduce dand5 expression in the fused central domain to a level that abolishes Nodal repression (Figure 4F). Although our data on laterality determination in conjoined twins clearly demonstrated that cilia and flow were required for Nodal cascade induction, they do not exclude the formal possibility that earlier LR asymmetries exist that developmentally precede cilia-based LR patterning [25, 26]. Together with previous analyses of leftward flow in singletons [10, 22, 27–30], these experiments unequivocally demonstrate, however, that potential early asymmetries, which certainly were present before leftward flow evolved [8, 31], have been superseded by cilia-based symmetry breakage.

Other vertebrate model organisms have not been systematically investigated for situs defects in conjoined twins, short of...
a means to induce twinning during embryogenesis. We have identified a single embryonic day 8.5 (E8.5) conjoined mouse twin that expressed Pitx2 selectively in the left LPM of the left twin (Figure S3), as in our βcat-induced twins in Xenopus (Figures S1D and S1H). Only a certain type of human conjoined twins, which together account for some 70% of cases [1], displays situs randomization in the right twin, namely twins in which the thorax is fused (dicephalic, thoracopagus). Twins joined at the head or pelvis, however, develop situs solitus in both twins [1, 14, 32]. Our experimental manipulations in Xenopus only allow for the generation of thoracopagus twins, which—like in humans—show strict randomization of the right twin. Vertebrate LROs are only transiently present in the developing embryo [8]; they generally form and function between the 6- and 20-somite stage, i.e., at anterior-posterior positions corresponding to the upper chest region during later development and adulthood. This notion supports the view that twins joined at the head or pelvis region each retain separate LROs and thus develop without LR defects. How the differential specification of laterality in conjoined thoracopagus twins is determined has remained enigmatic. It has been argued that twinning is induced too early to be influenced by cilia and leftward flow, and that induced twins must pick up laterality information from a primary organizer in the endogenous twin [33–35]. The unclear mechanism of differential situs determination in conjoined twins has remained a last serious objection against the general acceptance of cilia-driven symmetry breakage in fish, amphibians, and mammals. The predictable Nodal cascade induction in conjoined twins by manipulation of just flow itself and the flow target gene dand5 demonstrates that cilia-driven leftward flow represents the decisive mechanism for symmetry breakage in conjoined twins, as in single embryos.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.12.049.
AUTHOR CONTRIBUTIONS

M.T., T.T., A.S., and M.B. designed experiments. M.T., T.T., and I.S. performed experiments. M.T. and M.B. wrote the manuscript.

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