Cell cycle arrest in node cells governs ciliogenesis at the node to break left-right symmetry

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SUMMARY
Cilia at the node generate a leftward fluid flow that breaks left-right symmetry. However, the molecular mechanisms that regulate ciliogenesis at the node are largely unknown. Here, we show that the epiblast-specific deletion of the gene encoding the BMP type 1 receptor (Acvr1) compromised development of nodal cilia, which results in defects in leftward fluid flow and, thus, abnormalities in left-right patterning. Acvr1 deficiency in mouse embryonic fibroblasts (MEFs) resulted in severe defects in their quiescence-induced primary cilia. Although the induction of quiescence in wild-type MEFs leads to an increase in the level of the cyclin-dependent kinase inhibitor p27Kip1 and to rapid p27Kip1 phosphorylation on Ser10, MEFs deficient in Acvr1 show a reduction in both p27Kip1 protein levels and in p27Kip1 Ser10 phosphorylation. The observed defects in cilium development were rescued by the introduction of p27Kip1 into Acvr1-deficient MEFs, implying that BMP signaling positively controls p27Kip1 stability in the G0 phase via p27Kip1 Ser10 phosphorylation, which is a prerequisite for induction of primary cilia. Importantly, in control embryos, p27Kip1 protein is clearly present and strongly phosphorylated on Ser10 in cells on the quiescent ventral surface of the node. By contrast, the corresponding cells in the node of Acvr1 mutant embryos were proliferative and showed a dramatic attenuation in both p27Kip1 protein levels and phosphorylation on Ser10. Our data suggest that cell quiescence controlled by BMP signaling via ACVR1 is required for transient formation of nodal cilia, and provide insight into the fundamental question of how the node represents the mechanistic ‘node’ that regulates the development of left-right symmetry in vertebrates.

KEY WORDS: BMP signaling, Cell cycle, Cilia, Left-right determination, Node

INTRODUCTION
The node in mammals is structurally and functionally homologous to Kupffer’s vesicle in zebrafish, Spemann’s organizer in amphibia and Hensen’s node in chick (Hamada et al., 2002; Hirokawa et al., 2006; Levin, 2005; Sulik et al., 1994; Tabin, 2006). This transient embryonic structure has the ability to induce the axial specification during establishment of the embryonic body plan (Tam and Behringer, 1997). In the mouse embryo, the fully formed node is located at the anterior end of the primitive streak (Lee and Anderson, 2008). It has dorsal and ventral surfaces, and contains 200 to 300 ciliated cells (Hamada et al., 2002; Sulik et al., 1994). Recent studies have shown that nodal cilia are functional and that their rotational movement generates a leftward fluid flow (nodal flow), breaking the left-right symmetry in mouse embryos (Becker-Heck et al., 2011; Blum et al., 2007; Hirokawa et al., 2006; Nonaka et al., 1998). Despite the importance of cilia at the node, very little is known about the fundamental mechanisms that regulate ciliogenesis at the node in vivo.

Bone morphogenetic proteins (BMPs) play an essential role in establishment of the left-right patterning in vertebrates (Fujiwara et al., 2002; Furtado et al., 2008; Mine et al., 2008; Ramsdell and Yost, 1999; Yokouchi et al., 1999). We have previously reported that one of the BMP type 1 receptors, Acvr1, is required for the left-right patterning in the mouse (Kishigami et al., 2004). However, molecular details underlying the role of BMP signaling in this process are still unclear. In this paper, we took advantage of a conditional gene knockout approach to assess the requirement of BMP signaling during establishment of left-right asymmetry. We found that BMP signaling through a type 1 receptor (ACVR1) is important for formation of nodal cilia. BMP signaling positively controls the phosphorylation of a cyclin-dependent kinase inhibitor p27Kip1 (Cdkn1b – Mouse Genome Informatics), which maintains cells in quiescence, a crucial process for the induction of primary cilia. Notably, both p27Kip1 and phospho-p27Kip1 are produced exclusively at the node under the tight regulation of BMP signaling when embryos start to break left-right symmetry. Our results suggest that BMP signaling is essential for inducing cell cycle arrest at the node and for triggering development of nodal cilia.

MATERIALS AND METHODS

Animal and genotyping analysis
Generation of Acvr1 heterozygous mice, Acvr1 floxed mice, Mox2-Cre mice and ROSA26 reporter mice have been reported previously (Kaartinen and Nagy, 2001; Komatsu et al., 2007; Mishina et al., 1999; Soriano, 1999; Tallquist and Soriano, 2000). All mouse experiments were performed in accordance with University of Michigan’s guidelines covering the humane care and use of animals in research.

In situ hybridization and immunohistochemistry
In situ hybridization was performed using standard procedures (Komatsu et al., 2007). Immunohistochemistry was performed as described previously (Bellomo et al., 1996). Antibodies used in this study were against: acetylated tubulin (Sigma, T6793), γ-tubulin (Sigma, T5326), γ-tubulin (Sigma, T6557), Ki-67 (Dako, M7249), p27 (BD Transduction Laboratories, 610241), p27 phospho Ser10 (Abcam, ab62364), p21 (BD Transduction Laboratories, 556431), p57 (Santa Cruz, sc-1039), retinoblastoma (BD Pharmingen, 554136), cyclin D1 (Santa Cruz, sc-8396), phospho SMAD1/5/8 (Cell Signaling, 9511) and β-Actin (Sigma, A5441).
Scanning electron microscopy
Embryos were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde. Following postfixation in 1% buffered osmium tetroxide, the samples were dehydrated with ethanol and critical point-dried using carbon dioxide as the transition solvent. Embryos were mounted on aluminum stubs with silver paste and sputter-coated with a 20 nm thickness of gold:palladium alloy 60:40. Samples were examined with a JEOL scanning electron microscope using an accelerating voltage of 20 kV. Cilia length was measured by ImageJ software.

Observation of the nodal flow
Nodal flow observation was performed as described previously (Nonaka et al., 1998). Beads movements were observed with an Olympus BX-51 microscope by Olympus PlanApo 60x/1.40 objective lens. Images were captured with a CCD camera (KP-M1U, Hitachi Denshi), converted (ADV-C-300, Canopus Co.) and recorded. Embryos were genotyped by PCR after nodal flow observation.

Generation of mouse embryonic fibroblast (MEF) and adenovirus transduction
Acvr1<sup>fl/+</sup> carried with ROSA26 reporter MEF were established from E12.5 embryos. MEF were grown in DMEM with 10% fetal bovine serum (Hyclone). Adenovirus (Ad)-lacZ, Ad-GFP, Ad-Cre (Vector Development Laboratory, Baylor College of Medicine) and Ad-p2<sup>Cre</sup>i (Vector Biolabs) were used. After transduction of Ad-lacZ or Ad-Cre, MEF were stained with fluorescein di-β-galactoside (Invitrogen) and sorted by flow cytometry.

Immunoblot analysis
MEF extracts were prepared using RIPA buffer. Cell lysates were applied for SDS-PAGE and transferred to Amersham Hybond-P membrane. Signal was detected using ECL western blotting reagents (GE Healthcare).

RESULTS AND DISCUSSION
We disrupted Acvr1 in an epiblast-specific manner by using the Mox2-Cre driver line (Kaartinen and Nagy, 2001; Komatsu et al., 2007; Mishina et al., 1999; Tallquist and Soriano, 2000) (see Fig. S1 in the supplementary material) (Acvr1 cKO hereafter). Unlike conventional Acvr1 knockout embryos (Mishina et al., 1999), Acvr1 cKO embryos developed beyond gastrulation and died around embryonic day 11.5 (E11.5). Acvr1 cKO embryos displayed altered head structures, and abnormal embryonic turning, heart looping and limb bud development (Fig. 1A; see Fig. S2 in the supplementary material); these features are frequently observed in mice displaying defects in left-right patterning. Therefore, we first examined expression of the left side-specific genes Nodal (Collignon et al., 1996; Lowe et al., 1996), Lefty2 (Menio et al., 1998) and Pitx2 (Yoshioka et al., 1998) (Fig. 1B). In Acvr1 cKO embryos, expression of Nodal, Lefty2 and Pitx2 in the lateral plate mesoderm was either left-sided (=40%) or bilateral (=60%). We also analyzed the midline marker Lefty1, which is required for attenuation of the left side-specific genes on the right side of the embryo (Menio et al., 1998). Although the floor plate markers Shh and Foxa2 were expressed normally (Echelard et al., 1993; Sasaki and Hogan, 1993), Lefty1 expression was downregulated in Acvr1 cKO embryos (Fig. 1B). These results confirm that Acvr1 is required for correct left-right patterning during early mouse development.

To assess whether Acvr1 has a primary role in the establishment of left-right asymmetry, we investigated structural features of the node at E7.75 to E8.0 using scanning electron microscopy. When viewed from the ventral side, wild-type embryos showed a cup-shaped node with evenly distributed ciliated cells; the Acvr1 cKO embryos displayed morphological changes at the node that were characterized by a flat shape, the presence of atypical bulging cells (Fig. 2A) and notably shortened cilia (Fig. 2B). These data indicate that although the node was formed in the mutant embryos, the formation of nodal cilia was compromised. Because the shortened cilia may lead to abnormal nodal flow (Beckers et al., 2007), we examined whether nodal flow was altered in Acvr1 cKO embryos. The majority of Acvr1 cKO embryos (13/16) showed random and Brownian motion movement of the beads, which required more time to reach to the left edge of the node compared with that of wild-type embryos (Fig. 2C; see Movies 1 and 2 in the supplementary material). These results indicate that BMP signaling through ACVR1 is required for appropriate formation of nodal cilia.

Fig. 1. BMP signaling through ACVR1 regulates left-right patterning. (A) Whole-mount view of wild-type and Acvr1 cKO embryos at E10.5. Acvr1 cKO embryos showed abnormalities in head (white asterisks, top panel), embryonic turning (middle panel) and cardiac looping (bottom panel). (B) Expression patterns of Nodal, Lefty2, Pitx2, Shh, Foxa2 and Lefty1 are shown. Ventral view for Nodal. Frontal view for Lefty2, Pitx2, Foxa2 and Lefty1. Lateral view for Shh. Red arrows show aberrant right-sided expression of Nodal, Lefty2 and Pitx2 (second panel from the top), and absent midline expression of Lefty1 (bottom panel) in Acvr1 cKO mutants. A, anterior; P, posterior; L, left; R, right.
Although the molecular mechanisms that initiate formation of transient nodal cilia are largely unknown, it has been reported that node cells are quiescent in mouse embryos at E7.75-E8.2 (Bellomo et al., 1996), the crucial period when the formation of nodal cilia is initiated. In addition, it has been shown that cells need to be quiescent in order to develop primary cilia in tissue culture (Tucker et al., 1979). Together with our previous discovery that Acvr1 is expressed in ventral surface of the node (Kishigami et al., 2004), these observations prompted us to test the hypothesis that the primary role of BMP signaling through ACVR1 is to induce cellular quiescence, thus allowing node cells to develop cilia. To address the role of ACVR1 in regulation of cell cycle and cilia development, we generated Acvr1-deficient mouse embryonic fibroblasts (MEFs) (see Fig. S3 in the supplementary material). When cultured to the high cell density, 60% of control MEFs (Ad-lacZ), but only about 20% of Acvr1-deficient MEFs (Ad-Cre), developed primary cilia (Fig. 3A,B). We also analyzed the cells for the presence of Ki-67, which marks actively dividing cells (Starborg et al., 1996). Fewer than 20% of control MEFs, but still more than 50% of Acvr1-deficient MEFs stained positive for Ki-67 (Fig. 3A,B). Both control and Acvr1-deficient MEF cells grew normally (see Fig. S4 in the supplementary material). These results reveal that BMP signaling through ACVR1 is required for induction of the quiescence, a prerequisite for developing primary cilia (Tucker et al., 1979). Cell cycle arrest in MEFs can be induced by the Cip/Kip family of cyclin-dependent kinase inhibitors, including p27Kip1 (Toyoshima and Hunter, 1994). It also has been suggested that phosphorylation of p27Kip1 on Ser10 is required to stabilize p27Kip1 protein during the quiescent phase (Kotake et al., 2005). Thus, we investigated p27Kip1 protein levels and levels of p27Kip1 phosphorylation on Ser10 in control and Acvr1-deficient MEFs. During primary cilia induction, both p27Kip1 protein levels and phosphorylation on Ser10 were increased in control MEFs (Fig. 3C). However, in Acvr1-deficient MEFs, the level of p27Kip1 protein was decreased (Fig. 3C; see Fig. S5 in the supplementary material) and p27Kip1 phosphorylation on Ser10 was attenuated (Fig. 3C). The retinoblastoma (Rb) and cyclin D1 protein levels were comparable in control and Acvr1-deficient MEFs (Fig. 3C), showing initial cell cycle progression occurred normally in both MEFs. In addition, we observed that formation of primary cilia was restored by introduction of p27Kip1 using adenoviral gene transfer (Ad-p27) into Acvr1-deficient MEFs (Fig. 3D,E). These data suggest that BMP signaling through ACVR1 positively regulates the stabilization of p27Kip1 to keep MEFs in the quiescent phase to allow formation of primary cilia.

Next, we analyzed expression of p27Kip1 at the node of mouse embryos. Importantly, all the cells on ventral surface of the node stained positive for p27Kip1 and for p27Kip1 phosphorylation on Ser10 in control and Acvr1-deficient MEFs. During primary cilia induction, both p27Kip1 protein levels and phosphorylation on Ser10 were increased in control MEFs (Fig. 3C). However, in Acvr1-deficient MEFs, the level of p27Kip1 protein was decreased (Fig. 3C; see Fig. S5 in the supplementary material) and p27Kip1 phosphorylation on Ser10 was attenuated (Fig. 3C). The retinoblastoma (Rb) and cyclin D1 protein levels were comparable in control and Acvr1-deficient MEFs (Fig. 3C), showing initial cell cycle progression occurred normally in both MEFs. In addition, we observed that formation of primary cilia was restored by introduction of p27Kip1 using adenoviral gene transfer (Ad-p27) into Acvr1-deficient MEFs (Fig. 3D,E). These data suggest that BMP signaling through ACVR1 positively regulates the stabilization of p27Kip1 to keep MEFs in the quiescent phase to allow formation of primary cilia.

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proliferative, as demonstrated by positive Ki-67 staining (Fig. 4A). A role in the regulation of quiescence at the node seems to be specific for p27Kip1, as the other members of the Cip/Kip family of Cdk inhibitors (p21Cip1 and p57Kip2) were not produced at the node (see Fig. S7 in the supplementary material). These data demonstrate that BMP signaling via ACVR1 is required at the node for the induction of quiescence by stabilizing the p27Kip1 protein, which is prerequisite for development of the nodal cilia. As a result, the nodal flow is generated that, in turn, triggers the breaking of left-right symmetry (Fig. 4B).

Primary cilia are regarded as postmitotic structures of quiescent cells (Santos and Reiter, 2008). Ciliary dynamics thus appears to be precisely coordinated with a cell cycle progression, but the molecular cues that trigger ciliogenesis at the node are poorly understood. Our results demonstrate that BMP signaling through ACVR1 controls the stability of p27Kip1 via p27Kip1 phosphorylation on Ser10 to induce the cell quiescence that leads to the formation of nodal cilia. Although mutant mice lacking either p27Kip1 or p27Kip1 phospho-Ser10 are viable (Chu et al., 2008), the situs of the visceral organs and ciliogenesis, including nodal cilia, in these mutants have not been examined. Importantly, several mouse models of polycystic kidney disease generated by mutating ciliogenic genes show downregulation of p27Kip1 (Alcalay et al., 2008; Cadieux et al., 2008). Thus, it will be interesting to determine whether dysregulation of cell cycle via p27Kip1 causes primary ciliary dyskinesia and situs inversus.

Breaking of left-right symmetry is dependent on nodal cilia-generated leftward fluid flow at the node (Hamada et al., 2002; Hirokawa et al., 2006; Nonaka et al., 1998). But why nodal cilia are formed exclusively at the node during a limited time window...
Fig. 4. Cell cycle arrest regulated by BMP signaling is crucial for the transient nodal cilia formation. (A) Levels of both p27\(^{\text{Kip1}}\) and p27\(^{\text{Kip1}}\) phosphorylation on Ser\(^{10}\) (P-p27\(^{\text{Ser10}}\)) (green staining) were significantly lower on the ventral surface of the node in Acvr1 cKO embryos at E7.75. In wild-type embryos, cells on ventral surface of the node were quiescent (no detectable Ki-67 staining), whereas corresponding cells in Acvr1 cKO embryos were proliferative (stained positive for Ki-67). A, anterior; P, posterior. (B) A mechanism by which BMP signaling through ACVR1 regulates development of nodal cilia in mouse embryo. The presence of ACVR1 leads to a stabilization of p27\(^{\text{Kip1}}\) via phosphorylation on Ser\(^{10}\), thus inducing quiescence. Subsequently, nodal cilia develop and nodal flow is generated, which triggers the establishment of left-right symmetry. If Acvr1 is deleted, phosphorylation on Ser\(^{10}\) is attenuated, leading to a destabilization of p27\(^{\text{Kip1}}\) at the node. As a result, nodal cilia are not developed appropriately, which leads to a failure of nodal flow and to a failure to break left-right symmetry.
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