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Two novel type II receptors mediate BMP signalling and are required to establish left–right asymmetry in zebrafish

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Abstract

Ligands of the transforming growth factor β (TGFβ) superfamily, like Nodal and bone morphogenetic protein (BMP), are pivotal to establish left–right (LR) asymmetry in vertebrates. However, the receptors mediating this process are unknown. Here we identified two new type II receptors for BMPs in zebrafish termed bmpr2a and bmpr2b that induce a classical Smad1/5/8 response to BMP binding. Morpholino-mediated knockdown of bmpr2a and bmpr2b showed that they are required for the establishment of concomitant cardiac and visceral LR asymmetry. Expression of early laterality markers in morphants indicated that bmpr2a and bmpr2b act upstream of *pitx2* and the nodal-related *southpaw* (*spaw*), which are expressed asymmetrically in the lateral plate mesoderm (LPM), and subsequently regulate *lefty2* and *bmp4* in the left heart field. We demonstrated that bmpr2a is required for *lefty1* expression in the midline at early segmentation while bmpr2a/bmpr2b heteromers mediate left-sided *spaw* expression in the LPM. We propose a mechanism whereby this differential interpretation of BMP signalling through bmpr2a and bmpr2b is essential for the establishment of LR asymmetry in the zebrafish embryo.

Keywords: bmpr2a; bmpr2b; Left–right asymmetry; Cardiac laterality; Visceral laterality; Zebrafish

Introduction

During vertebrate development, establishment of proper left–right (LR) asymmetry is crucial for correct positioning and morphogenesis of the internal organs (Kishigami and Mishina, 2005; Levin, 2005). Nodal and Lefty, members of the transforming growth factor β (TGFβ) superfamily, are asymmetrically expressed in early vertebrate embryos and are known to play essential roles in the establishment and patterning of the left and right sides of the embryo (Kishigami and Mishina, 2005; Levin, 2005; Nakamura et al., 2006). Nodal, Lefty2 and Pitx2 form a highly conserved expression cassette generating robust left–right asymmetry in vertebrates (Hamada et al., 2002; Raya and Belmonte, 2006; Shen, 2007). In mouse, Nodal is expressed in the node and is required for its asymmetric expression in the left lateral plate mesoderm (LPM) (Shen, 2007 and references therein). This auto-induction mechanism leads to a rapid spread of Nodal expression throughout the left LPM and results in induction of *pitx2* and *lefty2* expression in the left LPM, and of *lefty1* in the axial midline. Lefty1 and Lefty2 antagonize Nodal activity, shaping a negative feedback loop that regulates the duration and extent of Nodal signalling in the left LPM (Raya and Belmonte, 2006). Lack of or bilateral expression of *nodal*, *lefty2* and *pitx2* in mice homozygous for a mutation in Smad5 suggests that upstream signalling by bone morphogenetic protein (BMP) is required to repress Nodal signalling in the right LPM, and that signalling by all of these

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ligands is intimately related (Chang et al., 2000). Analysis of chimeric mouse embryos with a high contribution of embryonic stem (ES) cells lacking the type I BMP receptor Activin receptor-Like Kinase 2 (ALK2), supported a repressive role for BMPs (Kishigami et al., 2004). In chick, Caronte activates Nodal by antagonizing BMP signalling in the left LPM (Rodriguez Esteban et al., 1999). By contrast, chimeric embryos containing ES cells with homozygous deletion of BMP4 (Fujiwara et al., 2002) suggested that BMP signalling coordinates LR asymmetry in mice by acting as a positive facilitator of Nodal expression in the left LPM. Moreover, transplantation of BMP2-expressing cells (Schlange et al., 2002) or BMP2-soaked beads (Piedra and Ros, 2002) into the left LPM of chick embryos induced *Nodal* expression, further supporting the role of BMP as activator of Nodal in the left LPM. What actually breaks symmetry is still debated but it seems likely that BMP signalling is involved.

In zebrafish, the first morphological evidence of a break in bilateral symmetry is the positioning of the heart to the left of the midline (Chen et al., 1997). The primitive heart tube is formed at the midline of the embryo and at 22 h post fertilization (hpf) the prospective atrial end moves abruptly to the left. This is known as cardiac jogging (Chen et al., 1997; Chin et al., 2000). After elongation and subsequent leftward jogging, the zebrafish heart changes from being linear into an S-shape (D-looping), whereby the future ventricle is ultimately placed anterior and to the right of the future atrium (Chen et al., 1997; Chin et al., 2000). The stronger left-sided (asymmetric) BMP4 expression in the heart predicts the (leftward) direction of cardiac jogging (Chen et al., 1997). Signalling by BMP4 regulates cardiac left–right asymmetry in frog and zebrafish (Branford et al., 2000; Chen et al., 1997; Schilling et al., 1999). Mutations in *chordin* (*dino*), *alk8* (*lost-a-fin*), *smad5* (*piggytail*) and *bmp7* (*snailhouse*) modify the asymmetric expression of BMP4 in the heart field and also result in defective heart jogging (Chen et al., 1997). Moreover, overexpression of BMP4 on the right, but not on the left, induces reversal of heart looping (Branford et al., 2000; Chen et al., 1997; Schilling et al., 1999), supporting the idea that enhanced one-sided BMP4 expression in the heart field determines its laterality.

Recently, it has been shown that temporally controlled overexpression of noggin3 at the tailbud stage in zebrafish induced bilateral expression of *southpaw* (*spaw*) in the LPM, whereas overexpression of bmp2b at the same stage resulted in no expression in either LPM (Chocron et al., 2007). This suggested that BMP signalling is necessary during early segmentation to repress *spaw* in the right LPM. Moreover, specific morpholino knockdown of bmp4, but not bmp7 or bmp2b in Kupfer's vesicle (KV) altered the expression of *spaw* in the LPM and *lefty1* in the notochord, identifying bmp4 in KV as the likeliest regulator of LR patterning in zebrafish (Chocron et al., 2007).

BMPs act through specific heteromeric type I/type II serine/threonine kinase receptor complexes. Three distinct type II receptors, BMPR2, Activin type IIA and IIB receptors (ActR2A and ActR2B) have been shown to bind BMPs and activate type I receptors within the complex (Liu et al., 1995; Rosenzweig et al., 1995; Yamashita et al., 1995). Type I receptors, termed ALKs, act downstream of serine/threonine kinase type II receptors and determine specificity within the receptor complex (Carcamo et al., 1994). The activated BMP type I receptors ALK1, ALK2, ALK3 and ALK6 phosphorylate downstream target Smads 1/5/8 (Heldin et al., 1997; Massague and Wotton, 2000) which then bind to Smad4, migrate to the nucleus and regulate transcription of target genes (Moustakas et al., 2001; ten Dijke et al., 2003).

Despite the evidence that the BMP pathway is involved in establishing LR asymmetry (reviewed in Kishigami and Mishina, 2005), the specific role of BMP type II receptors has not been elucidated. ActR2A and ActR2B mediate Activin and Nodal signalling and mutations in either of these genes cause laterality defects in mice (Chang et al., 2002; Hamada et al., 2002; Sakuma et al., 2002). Mice lacking BMPR2 however, arrest at the egg cylinder stage of development, prior to gastrulation (Beppu et al., 2000), precluding the analysis of its role in LR patterning. In zebrafish, orthologues of ActR2a and ActR2b have been described (Garg et al., 1999; Nagaso et al., 1999), although no evidence for disruption of LR asymmetry has been reported.

To investigate the role of BMP type II receptors in LR asymmetry in zebrafish, we probed the Ensembl Zebrafish database and found two candidates very similar to mouse and human BMPR2, termed bmpr2a and bmpr2b. Full length and dominant-negative bmpr2a and bmpr2b modified BMP signalling *in vivo*, demonstrating that they are *bona fide* BMP type II receptors. Injection of antisense morpholinos perturbed heart laterality concomitantly with laterality of the gut. Moreover, knockdown of bmpr2a or bmpr2b altered the asymmetric expression of *pitx2* and s*paw* in the LPM and *lefty2* and *bmp4* in the left heart field. Taken together, our experiments suggested that BMP signalling through bmpr2a and bmpr2b simultaneously regulates cardiac and visceral asymmetry upstream of Nodal signalling at early segmentation. Strikingly, we found that it is mainly bmpr2b that is required for *lefty1* expression in the (left) heart field at late segmentation whereas bmpr2a, but not bmpr2b, is pivotal for normal midline expression of *lefty1* at early segmentation. Finally, *in vitro* studies suggested that bmpr2a binds more efficiently to BMP2 and BMP4 than bmpr2b and that bmpr2b is more efficient in transducing Smad-dependent BMP signalling. This differential requirement suggests that bmpr2a and bmpr2b function non-redundantly and in concert to regulate complementary aspects of LR patterning by BMP in zebrafish. This represents a novel paradigm for signal output regulation of the BMP pathway in determining vertebrate LR asymmetry.

Materials and methods

Zebrafish maintenance and morphological scoring

Wild type zebrafish colonies were maintained using standard conditions for zebrafish husbandry, as described (Westerfield, 1995). Embryos were obtained by natural spawning of adult zebrafish and staged according to morphological criteria (Kimmel et al., 1995). The degree of dorsalization of injected embryos