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Development of the vertebral morphogenetic field in the mouse: Interactions between Crossveinless-2 and Twisted Gastrulation

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ABSTRACT

Crossveinless-2 (Cv2), Twisted Gastrulation (Tsg) and Chordin (Chd) are components of an extracellular biochemical pathway that regulates Bone Morphogenetic Protein (BMP) activity during dorso-ventral patterning of *Drosophila* and *Xenopus* embryos, the formation of the fly wing, and mouse skeletogenesis. Because the nature of their genetic interactions remained untested in the mouse, we generated a null allele for *Cv2* which was crossed to *Tsg* and *Chd* mutants to obtain *Cv2*; *Tsg* and *Cv2*; *Chd* compound mutants. We found that *Cv2* is essential for skeletogenesis as its mutation caused the loss of multiple bone structures and posterior homeotic transformation of the last thoracic vertebra. During early vertebral development, Smad1 phosphorylation in the intervertebral region was decreased in the *Cv2* mutant, even though CV2 protein is normally located in the future vertebral bodies. Because *Cv2* mutation affects BMP signaling at a distance, this suggested that CV2 is involved in the localization of the BMP morphogenetic signal. *Cv2* and *Chd* mutations did not interact significantly. However, mutation of Tsg was epistatic to all CV2 phenotypes. We propose a model in which CV2 and Tsg participate in the generation of a BMP signaling morphogenetic field during vertebral formation in which CV2 serves to concentrate diffusible Tsg/BMP4 complexes in the vertebral body cartilage.

Introduction

Pattern formation in the developing embryo is controlled by gradients of morphogens in which localization, local concentration, and binding to receptors must be precisely regulated. Extracellular protein–protein interactions ultimately determine how much signal is sensed by a cell at a specific position and its behavioral response within a morphogenetic field. The Bone Morphogenetic Protein (BMP) family of morphogens is involved in many developmental processes ranging from dorso-ventral patterning (De Robertis and Kuroda, 2004; Little and Mullins, 2006) to organogenesis and skeletal development (Hogan, 1996; Massague and Chen, 2000; Zhao et al., 2002). Central to the establishment of BMP morphogenetic gradients are the secreted BMP-binding proteins Chordin (Chd), Chordin-like-1 (Chdl-1), Chordin-like-2 (Chdl-2), Noggin, Twisted Gastrulation (Tsg) and Crossveinless-2 (CV2). Chd, Tsg and CV2 have been reported to function both as pro-and anti-BMPs depending on the model system studied (Little and Mullins, 2006; Oelgeschläger et al., 2000; Scott et al., 2001; Eldar et al., 2002; Nosaka et al., 2003; Coles et al., 2004; Petryk et al., 2004; Zakin and De Robertis, 2004; Ikeya et al., 2006; O'Connor et al., 2006; Zhang et al., 2007). Chdl-1 (Coffinier et al., 2001; Nakayama et al., 2001) and Chdl-2 (Nakayama et al., 2004; Zhang et al., 2007) are molecules that function as BMP antagonists in combination with Tsg, behaving similarly to Chd. The Chordin system is regulated by the Tolloid family of metalloproteinases that inactivate Chd by proteolytic cleavage and release previously inactive BMPs, which are then able to signal (Piccolo et al., 1997; Larrain et al., 2001).

Cv2 was first identified in *Drosophila* as a gene required for the formation of the wing crossveins, structures that require high BMP signaling (Conley et al., 2000; O'Connor et al., 2006; Blair, 2007). Mouse CV2 contains 5 cysteine-rich (CR) domains (also found in Chd, which function as BMP-binding modules) (De Robertis and Kuroda, 2004), a partial Von Willebrand Factor-D domain (vWFD), a trypsin inhibitor-like (TIL) domain involved in protein–protein interactions and/or oligomerization (Coffinier et al., 2001), and a heparin/extracellular matrix binding site within the vWFD domain that is thought to limit CV2 diffusion (Rentzsch et al., 2006; Serpe et al., 2008). The amino terminal domain of CV2 binds BMP, blocking the sites that are recognized by BMP receptors type I and II, thus preventing signaling (Zhang et al., 2008). One difference with Chd is that the *Cv2* gene product is constitutively secreted as two disulfide-linked fragments generated by an auto-catalytic cleavage occurring in the secretory pathway (Binnerts et al., 2004; Serpe et al., 2008; Ambrosio et al., 2008). Unlike Chd, vertebrate CV2 is completely resistant to digestion by Tolloid metalloproteinases (Ambrosio et al., 2008).

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Both pro- and anti-BMP effects of CV2 have been reported depending on the experimental situation. Anti-BMP effects were described *in vitro* during the differentiation of endothelial cells (Moser et al., 2003), osteoblasts and chondrocytes (Binnerts et al., 2004). However, the co-transfection of *Cv2*, *Smad1* and *Bmp4* in Cos7 cells revealed a pro-BMP activity (Kamimura et al., 2004). The electroporation of *Cv2* in the neural tube of chick embryos produced pro-BMP phenotypes (Coles et al., 2004). In the mouse, *Cv2* has been reported to function as a pro-*Bmp4* in developing embryos (Ikeya et al., 2006). Likewise, pro-BMP effects were described in zebrafish embryos in morpholino knock-down experiments (Rentzsch et al., 2006; Moser et al., 2007). However, potent anti-BMP effects for CV2 were demonstrated in biochemical studies and in *Xenopus* and zebrafish overexpression experiments (Coles et al., 2004; Rentzsch et al., 2006; Zhang et al., 2007). In *Xenopus*, depletion of CV2 with morpholinos increased BMP signaling and strongly synergized with the depletion of the BMP antagonist *Chd*, indicating that the overall function of CV2 in early development is to inhibit BMP signaling (Ambrosio et al., 2008). Like Chd, CV2 binds directly to Tsg protein, which greatly increases its BMP inhibitory activity (Ambrosio et al., 2008).

In the fly wing, CV2 functions as a co-factor for Dpp/BMP that may concentrate the activity of this ligand in the crossvein. First, Dpp/Gbb BMP ligands form a diffusible complex with Sog/Chd and the dTsg-2/Cv-1 protein (Vilmos et al., 2005; Shimmi et al., 2005), and are subsequently released at the crossvein for signaling after cleavage of Sog by the Tolloid-related metalloproteinase Tlr (Ralston and Blair, 2005; Blair, 2007). Recent work by Serpe et al. (2008) has shown that CV2 also acts as a co-receptor that binds to Thickveins (Tkv, a Type I BMP receptor), facilitating BMP signaling at intermediate CV2 concentrations and inhibiting signaling at high CV2 concentrations. In the *Drosophila* wing, the CV2 secreted protein binds to the glypican Dally and stays locally, not signaling beyond one or two cell diameters from its site of synthesis (Serpe et al., 2008).

In the *Xenopus* gastrula, CV2 is expressed in the ventral (high-BMP) center. Biochemical studies have shown that mouse CV2 binds with high affinity (1 nM) to Chd protein, and even more strongly to Chd/BMP complexes or to Chd fragments resulting from cleavage of Chd by tolloids (Ambrosio et al., 2008). This led to the proposal that the pro-BMP effects of CV2 are caused by directing the flow of Tsg/Chd/BMP complexes towards more ventral regions in which CV2 is tethered to the cell surface (Ambrosio et al., 2008; Bier, 2008). The flow of BMP4 from dorsal toward ventral regions has been observed directly in the *Xenopus* gastrula, and shown to require endogenous Chd (Ben-Zvi et al., 2008). We now propose that a similar flow of Tsg/Chd/BMP4 toward prevertebral cartilage regions expressing CV2 protein at their surface might explain the formation of a BMP morphogenetic gradient during mammalian vertebral development.

In the present study we investigated the role of *Cv2* during mouse embryogenesis and its interactions with *Chd* and *Tsg* in controlling BMP signaling using mouse loss-of-function mutations. To analyze *Cv2* function *in vivo*, we inactivated the *Cv2* gene, characterized the mutant phenotype, and crossed heterozygous mice with the *Chd* (Bachiller et al., 2003) and *Tsg* (Zakin and De Robertis, 2004) mutant strains to generate compound *Cv2*; *Chd* and *Cv2*; *Tsg* mutants. We found that $Cv2^{-/-}$ pups died at birth (in agreement with Ikeya et al., 2006), of respiratory failure and displayed multiple skeletal abnormalities. Little or no interaction between *Cv2* and *Chd* was observed. Surprisingly, the $Cv2^{-/-}$ lethality and skeletal phenotype were completely suppressed in $Cv2^{-/-}$; $Tsg^{-/-}$ double mutants. This rescue indicated that *Tsg* is epistatic over *Cv2*. The results also indicate that *Tsg*, *Cv2*, and possibly other BMP- and Tsg-binding proteins (such as Chdl-1, Chdl-2 and Chd), interact during skeletal development. We propose that the function of CV2 is to generate and maintain a BMP activity gradient in the developing vertebral morphogenetic field.

Materials and methods

Generation of Cv2 mutant mice

Genomic DNA for *Cv2* recombination arms was isolated from a 129/SVJ mouse BAC library (Incyte Genomics) and subcloned into the pGN vector (Supplementary Fig. 1A) (Le Mouellic et al., 1990). The plasmid was electroporated into 129/SVJ ES cells and stable transfection was achieved after G418 selection. Clones were screened for homologous recombination by PCR using the following primers, CV2-5′2 (5′TCC ACC TTC TCA TTC ACA AC3′) and CV2-3′2 (5′CGG GCC TCT TCG CTA TTA CG3′) which yielded a diagnostic band of 2530 bp and homologous recombination was confirmed by Southern Blot (Supplementary Fig. 1B). The *Cv2* mutant strain was backcrossed into the hybrid strain B6SJLF1/J (Jackson Laboratories). The *Tsg* and *Chd* mutant strains were previously described (Bachiller et al., 2003; Zakin and De Robertis, 2004) and maintained in the same genetic background. The *Cv2*, *Tsg* and *Chd* mutant mouse strains used in this work are publicly available from Jackson Laboratories through an agreement made possible by the Howard Hughes Medical Institute (http://jaxmice.jax.org, stock numbers are 007552 for *Chd*, 007553 for *Tsg* and 007554 for *Cv2*).

Genotyping and RT-PCR

Genotyping of the *Cv2* mutant strain was performed by PCR (Supplementary Fig. 1A) using the following primers: pGN1 (5′ACC CTC TGT GTC CTC CTG TTA A3′), Cv2down (5′AGT CTC CTC CTA TGT TTC TTG C3′) and Cv2up (5′TCT CTT TGG TGA TGC TAT TGT T3′). Tsg22 (5′ AGC CTG AAT GTT TGA ATG TTT A3′), Tsg23 (5′CTT GAA TCC TTA CCT GAA TGA G 3′) and LacZ3 (5′TCT GCC AGT TTG AGG GGA CGA C3′) were used to genotype the *Tsg* mutant strain as previously described (Zakin and De Robertis, 2004) and Neo2 (5′GTT CCA CAT ACA CTT CAT TCT CAG3′), Null Low (5′GGT AGG AGA CAG AGA AGC GTA AAC T3′) and Null Up2 (5′GAG TTA GGA GGT GGA GCT CTA CAC T3′) for the *Chd* mutant strain (Bachiller et al., 2000).

For measuring transcripts by RT-PCR, the primers were RTCv2up (5′CTC CTT CCT GAC AGG GTC TG3′) and RTCv2down (5′GGG TAC AAC CTT TGC ATC GT3′) for *Cv2*, RTLacZdown (5′TTG AAA ATG GTC TGC TGC TG3′) and RTlacZup (5′TAT TGG CTT CAT CCA CCA CA3′) for *LacZ*, and HPRTforward (5′CAC AGG ACT AGA ACA CCT GC3′) and HPRTreverse (5′GCT GGT GAA AAG GAC CTC T3′) for HPRT. They yielded bands of 295 bp, 234 bp and 249 bp respectively.

In situ hybridization, histology, immunohistochemistry and skeletal preparations

Procedures for *in situ* hybridization on whole-mount and cryostat sections were as described (http://www.hhmi.ucla.edu/derobertis/) using the following probes: *Cv2* (Coffinier et al., 2002), *Tsg* (Zakin and De Robertis, 2004), *Chd* (Bachiller et al., 2000), *Chdl-1* (previously designated as *Neuralin 1*, Coffinier et al., 2001), *Chdl-2* (Genbank accession number AF 338222), and *Bmp1*/*Tll1* (Scott et al., 1999). Alcian Blue/Alizarin Red skeletal preparations, β-galactosidase, Hematoxylin and Eosin (H&E) and Mallory's tetrachrome stainings were as described (Zakin and De Robertis, 2004). For antibody staining, embryos were fixed in 4% paraformaldehyde in PBS, dehydrated, embedded in paraffin, serially sectioned at 7 μm and sections processed as described (http://www.hhmi.ucla.edu/derobertis/). Rabbit anti-pSmad1 (Cell Signaling #9511L) and anti-CV2 (R and D AF2299) antibodies were used at a 1:100 dilution.

Preparation of mouse embryonic fibroblasts (MEFs)

MEFs were prepared as previously described (Hogan et al., 1994), with the following modifications. 12.5 days post coïtum (d.p.c.)