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Pou5f1 contributes to dorsoventral patterning by positive regulation of *vox* and modulation of *fgf8a* expression

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ABSTRACT

Pou5f1/Oct-4 in mice is required for maintenance of embryonic pluripotent cell populations. Zebrafish *pou5f1* maternal–zygotic mutant embryos (*spiel ohne grenzen*; MZ*spg*) lack endoderm and have gastrulation and dorsoventral patterning defects. A contribution of Pou5f1 to the control of *bmp2b*, *bmp4* and *vox* expression has been suggested, however the mechanisms remained unclear and are investigated in detail here. Low-level overexpression of a Pou5f1-VP16 activator fusion protein can rescue dorsalization in MZ*spg* mutants, indicating that Pou5f1 acts as a transcriptional activator during dorsoventral patterning. Overexpression of larger quantities of Pou5f1-VP16 can ventralize wild-type embryos, while overexpression of a Pou5f1-En repressor fusion protein can dorsalize embryos. Lack of Pou5f1 causes a transient upregulation of *fgf8a* expression after mid-blastula transition, providing a mechanism for delayed activation of *bmp2b* in MZ*spg* embryos. Overexpression of the Pou5f1-En repressor induces *fgf8*, suggesting an indirect mechanism of Pou5f1 control of *fgf8a* expression. Transcription of *vox* is strongly activated by Pou5f1-VP16 even when translation of zygotically expressed transcripts is experimentally inhibited by cycloheximide. In contrast, *bmp2b* and *bmp4* are not activated under these conditions. We show that Pou5f1 binds to phylogenetically conserved Oct/Pou5f1 sites in the *vox* promoter, both in vivo (ChIP) and in vitro. Our data reveals a set of direct and indirect interactions of Pou5f1 with the BMP dorsoventral patterning network that serve to fine-tune dorsoventral patterning mechanisms and coordinate patterning with developmental timing.

Introduction

The transcription factor Pou5f1/Oct4 controls pluripotency of mouse embryonic inner cell mass cells (Nichols et al., 1998) and ES cell lines (Boiani and Scholer, 2005), and is an essential factor for somatic cell reprogramming (Takahashi and Yamanaka, 2006). *Pou5f1* gene homologues were identified in all vertebrate phyla starting from Gnathostomes: birds (Lavial et al., 2007), *Xenopus* (*XlPou91*, *XlPou25*, and *XlPou60*) (Hinkley et al., 1992), axolotl (Bachvarova et al., 2004), and zebrafish (Takeda et al., 1994). *Pou5f1* homologues show broad expression during pre-gastrulation and gastrulation stages (Bachvarova et al., 2004; Belting et al., 2001; Burgess et al., 2002; Downs, 2008; Lavial et al., 2007; Lunde et al., 2004; Morrison and Brickman, 2006), suggesting that their function is conserved at least in part during these stages. However, mammalian Pou5f1/Oct4 has evolved additional functions beyond Pou5f1/Pou2 (Niwa et al., 2008).

In mouse, loss of Oct4 in the embryo results in developmental arrest before the blastocyst stage (Nichols et al., 1998), precluding the analysis of Oct4 functions at later stages. Functional loss of Pou5f1 homologues in early development of lower vertebrates produces severe gastrulation and patterning abnormalities and results in embryonic lethality. Zebrafish *pou5f1* maternal–zygotic mutant embryos (*spiel ohne grenzen*; MZ*spg*) lack the endodermal germ layer, have gastrulation defects, and are dorsalized. Pou5f1 appears to have independent input into each of these developmental processes (Lachnit et al., 2008; Lunde et al., 2004; Reim et al., 2004; Reim and Brand, 2006). In *Xenopus*, simultaneous loss-of function for three co-orthologues of Pou5f1 (Xlpou25, Xlpou60 and Xlpou91) (Morrison and Brickman, 2006), or double knockdown of Oct25 and Oct60 (Cao et al., 2006), produces excess of endoderm, neuralization of the ectoderm and gastrulation defects. Although effects of Pou5f1

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loss on germ layer development differ between *Xenopus* and zebrafish, loss-of function phenotypes in both species can be rescued by overexpression of mouse Oct4 (Cao et al., 2006; Morrison and Brickman, 2006; Onichtchouk et al., 2010) arguing for conserved ancient roles of Pou5f1 proteins during gastrulation of vertebrates. A better understanding of interactions of Pou5f1 and its multiple downstream transcriptional targets with signaling pathways involved in patterning the embryo should shed light on mechanisms that coordinate control of pluripotency, cellular differentiation, and pattern formation. Further knowledge gained from studying *pou5f1* in different vertebrate organisms could aid efforts toward controlled ES cell differentiation.

Patterning of the vertebrate body axes relies on several signaling pathways that are active during early embryonic development and interact with each other to establish a genetic network, which specifies cell fates along the dorsoventral extent of the embryo. The best understood pathways in early embryonic patterning include the TGF-β (BMP, Nodal), WNT, and FGF pathways (for a review see Schier and Talbot, 2005).

Specification of dorsoventral cell fates is achieved by a BMP activity gradient along the dorsoventral axis (De Robertis et al., 2000). In zebrafish, as soon as the zygotic genome becomes activated, the expression of *bmp2b* and *bmp7* is initiated throughout the blastoderm. Within 30 min after beginning of sphere stage, *bmp2b* and *bmp7* transcripts become ventrally restricted due to transcriptional repression from the dorsal side, a process controlled by the transcriptional repressor Bozozok (Leung et al., 2003) and the FGF signaling pathway (Furthauer et al., 2004). The robustness of the molecular network generating the dorsoventral BMP activity gradient is ensured by cooperation of several partially redundant interdependent pathways. In zebrafish, dorsal inhibition of BMP signals during gastrulation is achieved by combined action of FGF signaling (Furthauer et al., 2004), and three dorsally expressed secreted molecules: Chordin (Schulte-Merker et al., 1997), Noggin 1 (Furthauer et al., 1999), and Follistatin-like 2 (Dal-Pra et al., 2006).

Experimental embryology demonstrated an essential role for Chordin in inhibiting ventralizing BMPs, thereby promoting the development of dorsal fates (Piccolo et al., 1996). While Chordin is the main player in establishing the proper level of BMP activity along the DV-axis, other factors, such as noggin and follistatin, play redundant roles (Dal-Pra et al., 2006). Anti-dorsalizing morphogenetic protein (ADMP) is a BMP receptor ligand that suppresses dorsal and anterior structures when overexpressed. In contrast to other BMP receptor ligands it is expressed dorsally, and maintained by Nodal signaling and indirect action of the transcription factor Bozozok (Lele et al., 2001). Results in zebrafish suggest that ADMP signaling causes the restriction of anterior and axial fates and cooperates with BMP signaling in establishing proper dorsoventral regionalization (Willot et al., 2002). A role of opposing ADMP and BMP signaling in providing self-regulating capacity of the dorsoventral gradient in the embryo was recently suggested (Ben-Zvi et al., 2008; Reversade and De Robertis, 2005).

WNT canonical signaling plays a dual role with respect to dorsoventral polarity in the zebrafish embryo: maternal WNT signaling induces the organizer before MBT, and zygotic WNT restricts organizer function during blastula and gastrula stages by maintaining the expression of the ventral homeobox transcriptional repressors Vox, Vent and Ved (Gilardelli et al., 2004; Kawahara et al., 2000a, 2000b; Melby et al., 2000; Shimizu et al., 2002). Expression of Vox, Vent and Ved is initiated by the maternally expressed transcription factor Runx2b (Flores et al., 2008) and maintained by WNT signaling at blastula until midgastrula stages (Ramel and Lekven, 2004; Varga et al., 2007). The positive cross-regulatory loop between Bmp pathway and Vox/Vent genes is first established at midgastrulation (Imai et al., 2001; Kawahara et al., 2000a, 2000b; Melby et al., 2000; Ramel and Lekven, 2004), while at earlier stages Vox, Vent and Ved are relatively independent of Bmp signaling.

Zebrafish Pou5f1 has been implicated in dorsoventral patterning based on the dorsalization of MZ*spg* mutants during gastrula stages, as judged from shifts in gene expression domains. It was previously shown to enhance transcription of *bmp2b* and *bmp4* genes upstream of the maternal Alk8 receptor, and to be involved in maintaining *vox* and *vent* expression (Reim and Brand, 2006). However, it remained unknown, whether the interactions between Pou5f1 and the components of dorsoventral patterning pathways are direct. Here, we combine analysis of MZ*spg* mutants by time-resolved transcriptional profiles for the genes involved in the BMP regulatory circuitry, and of overexpression of activator and repressor Pou5f1 fusion constructs. We employ assays with protein synthesis inhibition experiments to identify potentially direct interactions. Shortly after MBT in MZ*spg* embryos, we demonstrate a transient ectopic overactivation of expression of the dorsalizing genes *fgf8a*, *chd* and *noggin1*, and present evidence that *fgf8a* overactivation is caused by indirect action of Pou5f1. We show that Pou5f1 contributes to dorsoventral patterning by direct activation of the *vox* promoter, and that this is a phylogenetically conserved mechanism.

Materials and methods

Fish manipulation and care

Zebrafish embryos and larvae were raised at 28.5 °C in 0.3× Danieau's solution (Shih and Fraser, 1996). WT embryos of AB×TÜB strain crosses and MZ*spg* carrying the *m793* allele of the *spg* mutation were used (Belting et al., 2001). Developmental staging was performed according to Kimmel et al. (1995). Photographs of live embryos in 2% methylcellulose, 0.4% tricaine in 0.3× Danieau's solution, or of fixed embryos in 70–100% glycerol were taken with an Axiocam digital camera using a Zeiss Axiophot 2 or a LeicaMZ10 dissecting microscope.

Whole-mount in-situ hybridization and immunostaining

Whole-mount in situ hybridization was performed to visualize gene expression as described (Belting et al., 2001). The following probes were used: *bmp2b* (Kishimoto et al., 1997), *bmp4* (Martinez-Barbera et al., 1997), *chordin* (Miller-Bertoglio et al., 1997), *fgf8a* (previously called *fgf8*; Furthauer et al., 2004), *vox* and *vent* (Melby et al., 2000), and *ntl* (Schulte-Merker et al., 1994). Anti pSmad1/5/8 antibody against the phosphopeptide (NH2-CNPIS-S [PO3]-V-S [PO3]-COOH) was kindly provided by Dr. Ed Laufer and used in 1/500 dilution for anti-P-Smad immunostaining performed as in Stemple et al. (1996).

Plasmids and mRNA injection

For sense mRNA preparations: *Pou5f1-En* and *Pou5f1-VP16* fusion proteins have been described in Lunde et al. (2004). *caAlk3* (Nikaido et al., 1999), *fgf8a* (Furthauer et al., 2004) *Smad1* (Dick et al., 1999) and membrane-GFP from the RN3-EGFP-F plasmid (Weidinger et al., 2002) were also used. Dr. A. Tomilin kindly provided Mouse expression constructs CS2 + Oct4 and CS2 + mycOct4. To obtain the Vox-Luc reporter construct, 1 kb upstream of vox coding sequence (ZFISH7:13:52347190:52350403) was PCR amplified from zebrafish genomic DNA, using PCR primers with incorporated KpnI/BglII sites, cloned into PCRII-Topo vector, sequenced and subcloned into pGL4.26 (Promega) using KpnI/BglII sites. 3× FLAG-TAG sequence was amplified from the P3XFLAG-CMV-7-vector (Sigma-Aldrich, Catalog# E7408) and added 3′ to *pou5f1* by fusion PCR. The construct was subcloned into PCRII-Topo vector and sequenced. Finally the construct was EcoRI/XhoI cloned into the expression vector pCS2+. Primers used for cloning (5′ to 3′) were

Eco-Pou: GAATTCATGACGGAGAGAGCGCAGAG
FlagFuspou-rev: TCGCTGGTGAGATGACCCAC