Maternal control of vertebrate dorsoventral axis formation and epiboly by the POU domain protein Spg/Pou2/Oct4

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Dorsoventral (DV) axis formation of the vertebrate embryo is controlled by the maternal genome and is subsequently refined zygotically. In the zygote, repression of ventralizing Bmp activity on the dorsal side through chordin and noggin is crucial for establishment of a dorsally located organizer. This interplay generates a zygotic Bmp activity gradient that defines distinct positional values along the DV axis. The maternal processes that control expression of the zygotic genes implicated in DV patterning are largely unknown. spiel-ohne-grenzen (spg/pou2) is a maternally and zygotically expressed zebrafish gene that encodes the POU domain transcription factor Pou2, an ortholog of mammalian Oct4/Pou5f1. We show that embryos that are genetically depleted of both maternal and zygotic pou2 function (MZspg) exhibit extreme DV patterning defects and, independently, a blastoderm-specific arrest of epiboly. Dorsal tissues expand to the ventral side at the expense of ventrolateral tissue in MZspg embryos. Dorsally expressed Bmp-antagonists, such as Chd and Nog1, and Gsc are ectopically activated at ventral levels in MZspg. Lack of ventral specification is apparent very early, suggesting that maternal processes are affected in MZspg. Indeed, maternal pou2 function is necessary to initiate zygotic expression of ventrally expressed genes such as bmp2b and bmp4, and for proper activation of bmp7, vox, vent and eve1. A constitutively active Alk8-TGFβ-receptor can ectopically induce bmp2b and bmp4 and rescues the dorsalization of MZspg. This indicates that pou2 acts upstream of Alk8, a maternally provided receptor implicated in the activation of zygotic bmp2b and bmp4 transcription. Consistent with this possibility, Bmp gene misexpression can rescue MZspg embryos, indicating that TGFβ-mediated signal transduction itself is intact in absence of Pou2. Inhibition of Fgf signaling, another pathway with early dorsalizing activity, can also restore and even ventralize MZspg embryos. The requirement for pou2 to initiate bmp2b expression can therefore be bypassed by releasing the repressive function of Fgf signaling upon bmp2b transcription. In transplantation experiments, we find that dorsalized cells from prospective ventrolateral regions of MZspg embryos are non cell-autonomously respecified to a ventral fate within wild-type host embryos. Analysis of pou2 mRNA injected MZspg embryos shows that pou2 is required on the ventral side of cleavage stage embryos. Based on the maternal requirement for pou2 in ventral specification, we propose that ventrally specified cells exhibit an active, pou2-dependent maternal induction step, rather than a default ventralizing program.

KEY WORDS: Zebrafish, Spg, Pou2, Oct4, Dorsoventral patterning, Bmp, Epiboly, Maternal effect, Axis formation

INTRODUCTION
During early development, three distinct asymmetries are established in bilaterian embryos, which are precursors to the dorsoventral (DV) and anteroposterior (AP) body axes. Axis formation depends largely on maternal and zygotic genes and involves a series of inductive cell interactions. In the fly, the frog and the zebrafish, an animal-vegetal (AV) polarity emerges upon localization of maternal components, including transcripts, proteins, cytoskeleton or mitochondria, to distinct cytoplasmic parts of the egg (Cheng and Bjerknes, 1989; Gard et al., 1997; Howley and Ho, 2000; Rand and Yisraeli, 2001; Volodina et al., 2003). Cells are endowed with different sets of these determinants and acquire their fates according to their position. AV-asymmetry probably prefigures DV polarity, as removal of vegetal yolk mass leads to lack of dorsal structures in the zebrafish (Mizuno et al., 1999; Ober and Schulte-Merker, 1999). The Drosophila AP axis is determined prior to DV patterning, both of which are executed by the activity of various maternal factors (reviewed by Roth, 2003). The chordate DV axis is inverted, and ventral specification involves signaling of Bmps, homologs of invertebrate Dpp (Arendt and Nübler-Jung, 1994; Holley and Ferguson, 1997). Other axis-defining pathways have not been conserved among vertebrates and invertebrates during evolution, and vertebrate DV specification by maternal factors is less well understood.

DV patterning in vertebrates integrates: (1) dorsalizing pathways involving dorsal stabilization of maternal β-catenin (reviewed by Wodarz and Nusse, 1998) (Schier, 2001); (2) ventralizing pathways depending on maternal and zygotic TGFβ signaling (Goutel et al., 2000; Bauer et al., 2001; Mintzer et al., 2001; Sidi et al., 2003) (reviewed by Hammerschmidt and Mullins, 2002) and zygotic Wnt signaling (Ramel and Lekven, 2004); and (3) noncanonical Wnt/Ca2+ signaling, which downregulates canonical Wnt signaling (reviewed in Pandur et al., 2002).

The zebrafish DV axis is determined prior to zygotic transcription, at the mid-blastula transition (MBT) (Kane and Kimmel, 1993; Stachel et al., 1993; Strähle and Jesuthasan, 1993). Maternal β-catenin is a key player to initiate dorsal identity (reviewed by Hibi et al., 2002). Specialized cells at the dorsal blastodermal margin, the prospective shield, become induced by the underlying yolk syncytial layer (YSL, the functional equivalent of the amphibian Nieuwkoop center) by maternal factors previously deposited into the egg (reviewed by Sakaguchi et al., 2002). The zebrafish shield is functionally equivalent to the Spemann-Mangold organizer (Shih...
A key activity of the shield is the transcriptional repression of ventralizing genes such as Bmp genes, vox and vent. This repression is mediated by boz (dharma – Zebrafish Information Network) (Yamanaka et al., 1998; Fekany et al., 1999; Koos and Ho, 1999; Kawahara et al., 2000a; Kawahara et al., 2000b; Leung et al., 2003a). During gastrulation, the coarse DV pattern is refined and involves formation of a Bmp gradient (Wilson et al., 1997; Dosch et al., 1997). This is achieved by dorsally emanating Bmp antagonists such as chordin and Noggin, which binds Bmps and prevents them from activating their receptors (reviewed by Schier, 2001; Hammerschmidt and Mullins, 2002).

The establishment of ventral identity is also controlled by maternal factors and involves the TGFβ factor Radar, which signals through the Alk8/Laf receptor to activate zygotic bmp2b and bmp4 expression (Goutel et al., 2000; Bauer et al., 2001; Mintzer et al., 2001; Payne et al., 2001; Kramer et al., 2002; Sidi et al., 2003). Zygotic bmp7 expression is probably activated by a different, yet unidentified, maternal pathway (Sidi et al., 2003). The early embryo is predominantly ventrally specified, except for a dorsal region devoid of Bmp gene expression, where the organizer is active. Later, during gastrulation, Bmps autoregulate via the Alk8 receptor (Bauer et al., 2001; Mintzer et al., 2001). Dorsal and ventral factors are crucial to keep the maternally established DV polarity equilibrated, as evidenced by zebrafish mutants lacking activity of Bmp7 (snh), Bmp2b (svr) or Smad5 (shn), which fail to develop proper ventral tissues because of dorsalization of the embryo, or by ventralized embryos mutated in chd (dino) or sizzled (ogon/mercedes) (reviewed by Hammerschmidt and Mullins, 2002).

The zebrafish genome is transcriptionally inactive during the first hours of embryogenesis, and zygotic transcription starts at MBT. Until this stage, development relies on maternal factors deposited in the oocyte (reviewed by Pelegri, 2003). pou2 encodes the POU domain transcription factor Spg/Pou2 (Belting et al., 2001; Burgess et al., 2002; Reim and Brand, 2002). pou2 mRNA is uniformly present during initiation of oogenesis and is subsequently confined to the animal oocyte region (Howley and Ho, 2000). After fertilization, pou2 transcripts are ubiquitously present in the embryo and overlap spatiotemporally with zygotically transcribed pou2 mRNA from late blastula until midgastrula stages (Takeda et al., 1994; Hauptmann and Gerster, 1995; Reim et al., 2004). pou2 plays a zygotic role in the establishment of the midbrain-hindbrain organizer (Schier et al., 1996; Belting et al., 2001; Burgess et al., 2002; Reim and Brand, 2002). An earlier pou2 function for endoderm initiation was detected through analysis of maternal-zygotic (MZ) spg mutant embryos (Reim et al., 2004; Lunde et al., 2004).

By analysis of MZ/Mspg embryos, we describe a novel maternal function of pou2 in establishing DV patterning and, independently, during epiboly. Our analysis indicates that maternal pou2 acts upstream of Alk8 signaling, and is required on the prospective ventral side to initiate and maintain zygotic expression of Bmp genes, vox, vent and eve1. Pou2 is therefore among the earliest maternal factors required for the initiation of ventral-specific fate. Based on the maternal requirement for pou2 in ventral specification, we propose that ventral specification employs an active, pou2-dependent maternal induction step, rather than a default ventralizing program.

RESULTS
Loss of pou2 function disturbs morphogenesis
To investigate an early developmental role for pou2 we examined the morphology of MZspg and Mspg mutant embryos devoid of both maternal and zygotic, or maternal pou2 function only, respectively, and find defects in axis patterning and epiboly (Fig. 1). MZspg embryos were indistinguishable from wild-type embryos during the early cleavage stage until period stage (Fig. 1A, A’). At dome stage, wild-type embryos undergo their first rearrangements, and during late blastula stages the blastoderm thins and spreads over the yolk cell (Fig. 1B). Doming continues into epiboly, a morphogenetic movement that drives the blastoderm, the enveloping layer (EVL) and the YSL vegetal-wards (Trinkaus, 1951) (reviewed by Kane and Adams, 2002). In MZspg embryos, doming is reduced (not shown), and the blastoderm does not flatten properly (Fig. 1B’). Normal gastrulation commences with the appearance of the circumferential marginal germling and the shield on the dorsal side, reflecting involution of cells around the embryonic margin (Fig. 1C,D). In MZspg, involution and shield formation starts on schedule; however, the blastoderm covers only 40%, rather than 50%, of the yolk (Fig. 1C’, D’). The width of the germling, including the shield, appears thicker when compared with the wild type (Fig. 1D’; arrowheads). During normal epiboly, the vegetal margin of the EVL and the YSL ‘lead the way’ (Fig. 1E,G), trailed by the edge of the blastoderm (Fig. 1E,G). In MZspg, epiboly of the blastoderm is delayed from the start (Fig. 1B’) and ceases at the beginning of gastrulation (Fig. 1E’-H’; red arrows indicate the blastoderm margin). By contrast,
epiboly of the underlying YSL continues normally in MZspg (Fig. 1E'-H'), as does the EVL (Fig. 1E'-H'; yellow arrows indicate the vegetal margin of the YSL and EVL; see Fig. S1L-R in the supplementary material). In the wild type, ventrolateral ectoderm and mesoderm converge to the dorsal side and extend along the AP axis during gastrulation (Fig. 1E-H). This process is disrupted in MZspg (Fig. 1E'-H'), where the prospective head region does not reach the animal pole on schedule but dives vegetally into the yolk (Fig. 1H'). Eventually MZspg mutants develop a dorsalized morphology (Fig. 1I-F').

**Disrupted blastoderm patterning in MZspg embryos**

We next investigated mesoderm and ectoderm patterning in MZspg embryos by in situ hybridization analysis. The notochord, the most dorsal mesodermal derivative, forms on schedule in MZspg embryos, though often bifurcated or split (Fig. 1I'), and expresses the notochord marker ntl (white arrowhead in Fig. 2D-H', white arrows in C,E). Split axes are also observed in epiboly mutants (Kane et al., 1996). Round cells line the notochord and the blastoderm margin (arrow in Fig. 1I'), that may have detached from the notochord, as judged by their expression of ntl and foxa1 (Fig. 2D, and not shown). MZspg embryos are severely shortened along their AP axis, as their notochord and ntl expression is shortened (Fig. 1I,I' and Fig. 2C-H'). After gastrulation, paraxial somites form dorsally in the wild type (Fig. 1I). Somites form on schedule in MZspg, but stretch to the ventral side (Fig. 2I'). Accordingly, myod expression is observed on the ventral side of MZspg embryos (Fig. 2A,B). pax2.1, a marker for the pronephric duct, a ventrolateral derivative, is missing in 19 out of 32 MZspg embryos (Fig. 2K,L; and data not shown). Endodermal differentiation also fails in MZspg, as described elsewhere (Reim et al., 2004; Lunde et al., 2004).

Dorsalization is also apparent in the neuroectoderm. krox20 is normally expressed in rhombomeres 3 and 5; rudimentary expression fuses ventrally in MZspg embryos (Fig. 2E,F, arrows). gbx2 marks the anterior hindbrain primordium (Fig. 2G, black arrow) and superimposes mesodermal gbx2 expression (white arrow). Neuroectodermal expression fails in MZspg (Reim and Brand, 2002) (Fig. 2H,H'), while mesodermal gbx2 expression...
fuses opposite of ntl expression in MZspg (Fig. 2H,H', white arrow; inset in H shows a ventro-animal view of an embryo expressing only gbx2). Dorsalization is also observed for brain-specific expression of pax2.1; however, expression is strongly reduced (arrowhead in Fig. 2I) as its activation requires zygotic pou2 (Reim and Brand, 2002). The forebrain expression of otx2 is strongly expanded in MZspg (Fig. 2O,P). Non-neural ectodermal expression of gata2, gata3, p63 and dlx3 is lacking in MZspg (Fig. 2Q-V; and data not shown). At late somitogenesis, MZspg embryos are severely malformed, and necrotic cells appear throughout the embryo (Fig. 1J-L), which eventually dies. In summary, MZspg embryos display a striking fusion of somites and expansion of anterior neuroectoderm at the expense of non-neural ectoderm reminiscent of dorsIALIZED phenotypes, as observed (for example) in Bmp-pathway mutant embryos or in embryos with excess Fgf signaling. In contrast to these embryos, MZspg embryos show ventrally fused expression of dorsal markers, which is not completely circumferential, and split notochords, which might be due to the epiboly defect.

To distinguish if MZspg embryos are morphologically abnormal, mostly owing to aberrant patterning, or if they also have a morphogenetic defect as well, we compared the movements of gastrulating wild-type and MZspg cells that were differentially labeled with rhodamine dextran or EGFP, respectively. A mixture of these cells was transplanted into the lateral germring of host embryos at the early shield stage (Fig. 2W). Transplanted MZspg cells survive and divide in wild-type
host embryos (Reim et al., 2004). When transplanted into MZspg embryos, wild-type and mutant cells behave the same: they do not converge but stay together in clusters (Fig. 2X), suggesting that cell movement is impaired in the mutant environment. Conversely, MZspg and wild-type cells within a wild-type host undergo normal convergence and extension movements (Fig. 2Y). These results suggest that abnormal morphogenesis of MZspg cells is strictly non cell-autonomous, and not due to a cell-intrinsic movement defect.

**pou2 is required to establish DV patterning**

Our above analysis suggests that abnormalities of MZspg embryos might stem from early defective Bmp-dependent DV patterning. Indeed, MZspg embryos show a failure of bmp2b and bmp4 initiation and a strong reduction of bmp7 expression from the beginning (Fig. 3A-C'). bmp7 is lost in MZspg embryos soon after its initiation. The homeobox genes vox and vent are ventrolaterally expressed targets of Wnt8 at late blastula stages, and of Bmp activity during gastrulation (Fig. 3D-E) (Ramel and Lekven, 2004; 2761).
Melby et al., 2000). Both repress transcription of dorsally expressed organizer genes such as boz, chd, gsc or axial (foxa2 – Zebrafish Information Network) in the wild-type blastula (Melby et al., 2000; Melby et al., 1999; Kawahara et al., 2000a; Kawahara et al., 2000b; Imai et al., 2001; Leung et al., 2003a). After normal initiation at sphere or dome stage, respectively, non-marginal expression of vox and vent are lost and marginal expression is reduced in MZspg from 40% E (epiboly) onwards (Fig. 3D, E). Importantly, MZspg embryos are not generally delayed, but initiate expression of unaffected markers on schedule with the wild type at all stages examined. This is substantiated by the proper timing of the initiation of morphological landmarks such as the shield or somites (Fig. 1C, C’, I’; Fig. 2A, B).

Genes required for dorsal identity, including the dorsal shield, are nog1, chd and gsc. Nog1 antagonizes Bmp signaling by binding and sequestering Bmp ligands (Zimmerman et al., 1996). nog1 expression initiates faintly at the dorsal side at oblong-sphere stage in the wild type (Fig. 3J) (Fürthauer et al., 1999). nog1 expression is stronger and expanded to the ventrolateral side in MZspg embryos shortly after its activation (Fig. 3J). This might explain the expansion of otx2 we observe in MZspg (Fig. 2N), as otx2 is a downstream target of nog in Xenopus (Gamse and Sive, 2001). Similarly, chd expression initiates between sphere and dome stage (Fig. 3K). Initiation of chd expression is normal in MZspg, although its ventral limitation is less sharp than in the wild type (Fig. 3K’). Between dome stage and 30% E chd strongly expands ventrolaterally in MZspg (Fig. 3L, M’). gsc expands similarly in MZspg after normal initiation at sphere–dome stage; however, ectopic expansion is restricted to the germling (Fig. 3N–O’). Expansion of nog1, chd and gsc lasts during blastula stages in MZspg. A further dorsaling component is Fgf8, which is expressed from blastula stages onwards. However, fgf8 expression and activity is normal at blastula and gastrula stages in MZspg embryos, as Fgf target genes such as spry4, emr and pea3 are normally expressed (see Fig. S1 in the supplementary material).

During gastrulation, DV patterning remains disrupted in MZspg embryos. After normal initiation, the ventral marker eve1 is strongly reduced in MZspg from 50% E onwards (Fig. 3F, F’; not shown). bmp2b, bmp4 and bmp7 expression remain lost within the blastoderm; however, bmp2b expression within the YSL is not affected in MZspg, similar to other dorsalized mutants (Fig. 3G–I’). Like gsc, bmp2b and bmp4 are also expressed in the wild-type shield during gastrulation, and are maintained in the shield and prechordal plate, respectively. bmp2b and bmp4 initiation within these domains is not affected in MZspg at shield stage, but expression is not maintained during gastrulation (Fig. 3G, H’; not shown). Other dorsal mesodermal genes expressed in the notochord (nog1 and ntl in the posterior axial mesoderm) or the prechordal plate/hatching gland [hgg1 (cstlb – Zebrafish Information Network) gsc or foxa2 in the anterior axial mesoderm] are ventrolaterally expanded in MZspg (not shown). The prechordal plate did not reach the wild-type level of the animal pole at tb stage (Fig. 1G, H’, and not shown). Despite the early and strong dorsализation, specific dorsal features remain intact in MZspg. For example, the shield (Fig. 1C–D’), or β-catenin-dependent expression of boz, hhhex and sqt was normal in MZspg (Fig. 3P–Q’; not shown).

With regard to DV patterning and epiboly, Mspg embryos look identical to MZspg embryos until beginning of gastrulation (not shown), but defects in epiboly always recovers until tb stage in Mspg embryos (Fig. 1E–H’). Expressivity and penetrance of dorsализation is highly variable in Mspg (Fig. 1M, N; see Fig. S1 in the supplementary material). ‘Strong’ Mspg mutants show severe reduction of non-neural ectoderm (not shown). A variable proportion of Mspg embryos even restores to the wild-type phenotype (not shown). Zspg embryos undergo normal epiboly and display no apparent DV patterning defect (Reim and Brand, 2002), suggesting that zygotic pou2 is not required to regulate DV specification and epiboly.

Establishment of axial asymmetry
Deposition of maternal mRNAs to the unfertilized egg is a common mechanism to propagate asymmetry to the embryo. In the zebrafish, different mRNA localization patterns reveal asymmetry established along the A/V axis during oogenesis, including pou2 (Howley and Ho, 2000). MZspg embryos lack pou2 transcripts (Reim et al., 2004), and might therefore derive from improperly polarized oocytes. However, we found that maternally deposited smad3a/3b/5, radar (gdf6a – Zebrafish Information Network) alk8, actRlb (acvr2b – Zebrafish Information Network), foxh1, zorba and brun (cugbp1 – Zebrafish Information Network) mRNAs are unaffected in spg– mutant oocytes and MZspg zygotes (not shown). One possibility is therefore that, rather than controlling asymmetric RNA localization itself, pou2 might control implementation of asymmetrically deposited information in the oocyte.

MZspg embryos can respond to Alk8- and Bmp-mediated signaling
In MZspg embryos, Bmp gene expression is either not initiated (bmp2b, bmp4) or is only partially initiated (bmp7), which prompted us to test if signaling pathway(s) leading to Bmp gene initiation are affected in MZspg embryos. Maternally expressed alk8/sg/ is the Type I TGFβ-receptor induced by the maternal ligand Radar and, presumably via its nuclear effectors Smad5 or Smad8, activates zygotic expression of bmp2b and bmp4, but not bmp7 (Hild et al., 1999; Bauer et al., 2001; Mintzer et al., 2001; Payne et al., 2001; Kramer et al., 2002; Sidi et al., 2003). Transcripts encoding components of this maternal signaling pathway, such as alk8, radar and smad5 are normal in MZspg embryos at early cleavage and blastula stages (not shown). To determine at which level pou2 might act during the establishment of DV patterning, we carried out epistasis experiments by injecting constitutively active (ca-) alk8 mRNA into MZspg zygotes. Wild-type embryos are ventralized upon ca-alk8 injection and can be distinguished from non-injected embryos by their spherical shape at the end of gastrulation (Fig. 4A, B) (Bauer et al., 2001). All injected MZspg embryos (n=45) are similarly ventralized to the wild type (Fig. 4B, B’). Expression analysis showed suppression of dorso-axial and neuroectodermal genes, or even ventralization, i.e. loss of somitic marker expression, depending on the amount of injected mRNA (Fig. 4D–F’).

Alk8 is later required for maintenance of Bmp2b/4/7 signal transduction (Bauer et al., 2001). ca-alk8-mediated reversion of dorsализation could reflect its function to maintain Bmp signaling during gastrulation, which would not allow a distinction from bypassing its maternal function in the initiation of Bmp gene expression. Therefore, we analyzed early activity of misexpressed ca-alk8. We observed an intensified bmp2b expression level in injected wild-type embryos at the sphere stage (Fig. 4J). MZspg embryos initiate bmp2b expression upon ca-alk8 misexpression at the oblong stage, and intensity of expression is comparable with the wild-type level (Fig. 4K, L). bmp4 is normally not activated prior to dome stage/30% E (Fig. 4L). ca-alk8 misexpression therefore elicits premature initiation of bmp4 in wild-type development.
embryos at sphere stage and MZspg embryos at oblong stage; however, in both cases the intensity of expression is comparable with the normal bmp4 initiation level (Fig. 4M,N, compare with Fig. 3C).

swr/bmp2b mutant analysis revealed that Bmp2b is required for self-maintenance of its expression during gastrulation (Kishimoto et al., 1997). Lack of pou2 prevents bmp2b initiation in MZspg. To test effects of exogenous Bmp, bmp2b mRNA was injected into MZspg embryos at the one-cell stage. MZspg embryos (50 of 50) responded to Bmp gene injection as observed for ca-alk8-injected embryos (Fig. 4C,C'). mRNA injection of bmp4 into MZspg rescues and ventralizes MZspg embryos in a similar fashion (Fig. 4G; compare with Fig. 4E-F'). Furthermore, pou2 mRNA injection cannot rescue Bmp gene mutants, and pou2 is expressed normally in snh/bmp7, sbn/smad5, swr/bmp2b mutant embryos at blastula and gastrula stages (not shown). Together, mutant in situ hybridization and epistasis analysis indicates that Alk8-mediated signal transduction is intact in pou2-deficient embryos. Therefore, maternal pou2 acts upstream of Bmp gene initiation and upstream of maternal Alk8 function.
In contrast to the rescue of DV patterning in MZspg embryos, ca-alk8/Bmp gene misexpression could not rescue endodermal sox17 expression (Fig. 4H; 40 out of 40 injected embryos), which is never expressed in MZspg (Reim et al., 2004). Bmp gene misexpression could not rescue the epiboly defect (Fig. 4C’); however, ca-alk8 misexpression led to a slight amelioration of epiboly in 50% of injected MZspg embryos (Fig. 4B’).

**Fgf-interference suppresses dorsalization of MZspg**

*pou2* is required in synergy with *fgf8* to activate hindbrain specific genes (Reim and Brand, 2002). With respect to DV patterning the relationship between *pou2* and *fgf8* appears rather antagonistic: *pou2* is required to activate Bmp genes (Fig. 3A-C’), while *fgf8* misexpression represses *bmp2b*, *bmp4* and *bmp7* (Fürthauer et al., 1997; Fürthauer et al., 2004). We therefore asked whether interference with Fgf signaling might cause a reverse effect in *pou2*-deficient embryos, by restoring Bmp gene expression. To analyze the effect of Fgf interference in MZspg embryos, mRNA encoding XFD, a dominant-negative FGFR (Amaya et al., 1991), was injected at the one-cell stage into MZspg. XFD injection caused different effects: *bmp2b* expression is restored and even expanded to the dorsal side at late blastula stages in MZspg embryos, as in injected wild-type embryos (Fig. 4O). By contrast, XFD injection had no effect on *bmp4* expression in the wild type (Fig. 4Q), and *bmp4* was not initiated in XFD-injected MZspg embryos (Fig. 4R). At the same time, ectopic and endogenous expression of *chd*, *gsc* and *nog1* was repressed in MZspg by XFD, as observed in the wild type (not shown) (Amaya et al., 1993). These experiments suggest that in the absence of *pou2* function, *bmp2b* expression, but not *bmp4*, can be restored by releasing Fgf-mediated transcriptional repression of *bmp2b*. This derepression can bypass the requirement for *pou2* to initiate *bmp2b* expression. Therefore, *bmp2b* is probably not directly activated by *pou2*.

**Dorsalized MZspg cells can be corrected by wild-type environment**

*pou2*-dependent control of proper Bmp gene initiation probably underlies the dorsalization of MZspg embryos. Therefore, it should be possible to rescue MZspg cells when transplanted into wild-type embryos. Rhodamine-dextrane labeled MZspg cells where transplanted into the ventral region of wild-type host embryos between 50% E and shield stage. The fate of donor cells was analyzed after 24 hpf. In the wild-type embryo, cells at ventral positions at late blastula stages give rise to ventral derivatives such as blood or the ventral tail fin (Fig. 5A,A’). Similarly, MZspg cells transplanted into wild-type embryos contribute to blood and ventral tail fin, but were not found in dorsal mesodermal tissues (Fig. 5B’-E’). This indicates an ability of strongly dorsalized MZspg cells to differentiate into ventral(-most) mesodermal derivatives in wild-type embryos. This finding illustrates that MZspg cells can show the maximal possible response to ventralizing signals such as Bmps from the wild-type environment.

**Spatial requirement for pou2 to establish ventral gene expression**

During early DV patterning stages, *pou2* transcripts are ubiquitously distributed in the blastula embryo, raising the question of where *pou2* is required. Theoretically, dorsalization in MZspg might be explained if Pou2 protein would be required for either: (1) induction of ventral-specifying genes ventrally; (2) repression of dorsal-specifying genes dorsally; or (3) for both. To analyze the spatial requirement for *pou2* function within the early embryo, we injected *pou2* mRNA into single marginal or non-marginal cells of the 16-32-cell stage of MZspg embryos, together with *egfp* mRNA. Co-injected embryos were fixed at shield initiation (Fig. 6A,B) or the beginning of gastrulation (Fig. 6C-N), analyzed for EGFP expression. Thereby, counted 16 injected MZspg embryos, ca *egfp* expression allowed for spatial allocation of *pou2*-injected cells, in relation to eventual *bmp4* expression domains. We find that *bmp4* expression can be restored only in MZspg embryos when ventral (Fig. 6E-F’,I-J) or ventrolateral blastomeres (Fig. 6A,B,C,D’,G-H) were injected with *pou2* mRNA (19 out of 26 injected MZspg embryos, the remaining embryos did not show *bmp4* expression). Restoration of *bmp4* expression appears cell autonomous in MZspg at the beginning of shield formation (Fig. 6A,B) and at the beginning of gastrulation (Fig. 6C-J). By contrast, *bmp4* expression was never observed in MZspg embryos injected with *pou2* mRNA into dorsal/dorsolateral or non-marginal blastomeres (Fig. 6K-N; 16 out of 16 injected MZspg embryos). Therefore, *pou2* is required from early cleavage stages in the prospective ventral blastomeres to initiate *bmp4*. 

![Fig. 5. The fate of dorsalized MZspg cells.](image_url)
**DISCUSSION**

**pou2 is required for the establishment of ventral positional information**

Although polarization is morphologically not apparent in the freshly laid zebrafish egg, maternal factors followed by differential zygotic gene expression impose DV asymmetry onto the embryo. Our analysis of MZspg and Mspg mutant embryos shows that the equilibrium of DV patterning is severely disrupted from the beginning in the absence of maternal *pou2*. As a consequence, cells in ventrolateral regions assume inappropriate dorsal fates soon after MBT (Fig. 3). Loss of *pou2* also interferes with maintenance of Bmp gene expression, as expression of these gene is also lacking at later stages (Fig. 3). This might be due to the failure of Bmp gene autoregulation. Together, this accounts for the later severity of the phenotype, i.e. ventral expansion of paraxial mesoderm and anterior neuroectoderm at the expense of non-neural ectoderm, and loss of ventral specification that is normally required for differentiation of blood and pronephros. The severity of the MZspg phenotype is comparable with the strongest class of dorsalized embryos (Mullins et al., 1996). A proportion of MZspg embryos has residual *gata1* and *pax2.1* expression within the prospective blood and pronephros, respectively. Residual expression of these genes was also observed in embryos devoid of Bmp signaling (Pyati et al., 2005). Unlike other dorsalized mutants, where somites and dorsal neuroectodermal markers extend around the circumference of the embryos, somites in MZspg embryos are displaced from the notochord, which is split. In this aspect, MZspg embryos resemble epiboly mutants (Kane et al., 1996), and altered epiboly may therefore explain the later split notochord and somite defect in MZspg embryos.

In DV patterning *pou2* might: (1) act as a repressor of dorsal gene expression; (2) activate ventral-specific genes, such as Bmp genes, required to restrict dorsalizing factors at blastula stages; or (iii) act both as repressor of dorsal and as an activator of ventral genes. At the time when dorsal markers start to expand to the ventral side (*nog1* at sphere-dome stage, followed by *chd* and *gsc* at dome stage-30% E), the transcriptional initiation of ventralizing genes such as *bmp2b/7/4* fails in MZspg embryos. Concomitant initiation of *eve1*, *vent* and *vox* expression is unaffected in MZspg embryos. From late blastula stages, however, maintenance expression of *vox* and *vent*,
as well as of eve1, fails in MZspg mutants, indicating that pou2 is required for activation and maintenance of ventral-specific genes. Normal expression of eve1, vox and vent is not sufficient to prevent nog1 expansion at blastula stages. nog1 overexpression is sufficient to cause ectopic germinating expression of gsc and eve1 reduction (Fürthauer et al., 1999). In accordance with this, uncontrolled nog1 expression in MZspg might cause ectopic chd and gsc expression. nog1 is unlikely to cause repression of bmp2 from their onset, as bmp2b/7 expression is already affected before nog1 becomes expanded in MZspg embryos. nog1 gain-of-function experiments would clarify whether nog1 would be sufficient to repress Bmp gene initiation.

In contrast to Bmp genes, induction of vox and vent is independent of zygotic gene expression but becomes partially wnt8 dependent during late blastula and Bmp gene-dependent during gastrula stages, respectively (Kawahara et al., 2000a; Kawahara et al., 2000b; Ramel and Lekven, 2004). The partial loss of vox/vent expression might cause ectopic expansion of chd and gsc in MZspg embryos at late blastula stages. This is similar to loss of both vox and vent function, which causes ectopic expansion of chd and gsc in MZspg embryos. Therefore, TGFβ signaling can function in Dorsal organizer function is normal in MZspg, including the organizer, is aberrant in the absence of pou2. However, pou2 is needed in ventral or ventrolateral blastomeres, respectively, from early cleavage stages onwards to restore bmp4 expression.

pou2 acts upstream to Alk8/Bmp-mediated signaling

Type I TGFβ receptors, including Alk8, mediate TGFβ signaling (reviewed by Hammerschmidt and Mullins, 2002). Alk8 transduces a maternal signal via the nuclear effector Smad5/8 that is required for initiation of bmp2b and bmp4, but is dispensable for bmp7 activation. The maternal signal for Alk8 might be Radar, a GDF-subgroup member of Bmps (Rissi et al., 1995; Sidi et al., 2003). Alk8, radar and smad5 are maternally and ubiquitously provided throughout early wild-type development (Mintzer et al., 2001; Goutel et al., 2000; Sidi et al., 2003; Dick et al., 1999; Hild et al., 1999). Smad5 is required to relay a maternal signal also to initiate bmp7 expression at blastula stages, and amorphic smad5 mutants exhibit strong maternal-zygotic effects, where embryos are earlier and more strongly dorsalized when compared with all thus far described DV patterning mutants, including compound bmp2b/7-mutant embryos (Hild et al., 1999; Schmid et al., 2000; Kramer et al., 2002). Later, during gastrulation, Smad5 also mediates autocrine/crossregulation of bmp2b/4/7 (Kishimoto et al., 1997; Schmid et al., 2000; Hild et al., 1999; Kramer et al., 2002). In case of bmp7 initiation, Alk3 and Alk6 are putative maternal TGFβ receptors; however, the ligand(s) of this signaling system are so far unknown (reviewed by Wilm and Solnica-Krezel, 2003).

Similar to the loss of smad5 and radar function, lack of ventral induction and dorsalization is very early manifested in MZspg embryos. Furthermore, radar-morphant embryos display ectopic expression of chd after normal initiation, which is probably due to reduced Bmp gene expression in these embryos (Sidi et al., 2003). As chd and gsc expression is normal in bmp2b mutants, Radar probably acts either via its zygotic target bmp4 or by other factor(s) to repress dorsal genes. Although maternal transcripts of alk8, smad5 and radar are present at normal levels in MZspg embryos, they are not sufficient to mediate proper Bmp gene activation. However, we did not analyze their translational or post-translational expression, which might be affected in MZspg embryos. Injection of an activated Alk8 receptor or bmp2b/4 is able to rescue and ventralize MZspg embryos. Therefore, TGFβ signaling can function in absence of Pou2, arguing that pou2 does not act downstream of Alk8 signaling to activate bmp2b/4. Instead, Pou2 probably acts upstream of the Alk8, Smad5 and, eventually, Bmp signaling, which would be in accordance with the strong dorsalization in MZspg embryos.

Ventral requirement for pou2 to initiate Bmp gene expression

pou2 transcripts are ubiquitously present in the early embryo; however, their uniform distribution does not reveal a distinct spatial requirement for pou2 in DV patterning. We analyzed the spatial requirement of pou2 by pou2 mRNA injection into MZspg embryos at early cleavage stages (Fig. 6). In the injection assay, we found that pou2 is needed in ventral or ventrolateral blastomeres, respectively, from early cleavage stages onwards to restore bmp4 expression.

Maternal pou2 antagonizes Fgf-mediated Bmp gene repression

Fgfs play an important role on the dorsal side, as dorsal XFD injections elicit more severe dorsalization than do ventral injections (Isaacs et al., 1994). In addition to organizer-derived secreted factors, Fgf signaling also modulates Bmp activity by transcriptional suppression (Fürthauer et al., 1997; Fürthauer et al., 2004). Antagonism between Egf/Fgf and Bmp signaling is thought to converge on Smad1, which is phosphorylated by MAPK, further preventing nuclear accumulation of Smad1 (Kretzschmar et al., 1997; Koshida et al., 2002; Pera et al., 2003). In turn, repression of Bmps at the protein level causes breakdown of Bmp gene-autoregulation in Xenopus and mouse (Ghosh-Choudhury et al., 2001; von Babnow and Cho, 2001). However, we did not observe overactivated or ectopic expression of Fgf genes or their downstream targets spry2/4 and pea3 in MZspg embryos at any stages, suggesting that the failure of Bmp gene activation does not involve ectopic Fgf signaling. In spite of normal dorsal Wnt signaling and elevated expression of organizer molecules such as Nog1 and Chd, blocking Fgf signaling was sufficient to repress dorsal development in MZspg embryos, which is reminiscent of interference with dorsal Wnt signaling in Xenopus (Gerhart et al., 1989). In particular, expression of bmp2b, but not bmp4, could be rescued in MZspg embryos. At the same time, chd, nog1 and gsc could be repressed. This suggests that dorsalizing pathways crossregulate each other, and removal of one dorsalizing component causes breakdown of the maintenance of other dorsalizing pathways. Moreover, in the absence of Fgf signaling, pou2 is dispensable for bmp2b expression at late blastula stages. By derepression, i.e. repression of an Fgf-dependent repressor, pou2 might therefore indirectly activate bmp2b expression at late blastula stages, which might already apply for bmp2b initiation. Fig. 7 summarizes the function of pou2 during establishment of DV patterning.

Dorsal organizer function is normal in MZspg

Cognate dorsalized Bmp gene mutants show normal gene expression within the dorsal organizer, suggesting that early maternal events in DV patterning and organizer establishment are normal in these mutants. By contrast, dorsal expression of gsc, nog1, chd, including the organizer, is aberrant in the absence of pou2. For example, gsc expansion in MZspg is similar as in LiCl-treated embryos and embryos with overactivated canonical Wnt
Maternal control of axis formation by Spg/Pou2/Oct4

Fig. 7. Spg/Pou2 is a maternally provided regulator of DV patterning. Schematic top view of blastula embryos. Arrows indicate a regulatory relationship that need not be direct. pou2 is required upstream of maternal TGFβ/Bmp signaling to activate ventral-specifying genes such as bmp2b, bmp4 and, partially, bmp7. pou2 is also required to maintain vox/vent and eve1 from late blastula stages onwards. In addition, pou2-dependent bmp2b expression involves repression of an Fgf-dependent repressor, either at the onset of bmp2b expression or at later blastula stages. In MZspg embryos, ventral-specifying genes completely or partially fail to be initiated, causing the embryo to be dorsalized. Specifically, ventral Bmp activity fails to establish, causing ectopic expression of dorsal organizer-derived genes nog1, chd and gsc.

Signaling, which provoke ectopic axes (Stachel et al., 1993). However, dorsalization in MZspg does not include ectopic organizer activity. boz is among the earliest genes initiated dorsally around MBT by maternal Wnt/nuclear β-catenin signaling and is normally required to initiate organizer-specific gene expression (Koos and Ho, 1999; Yamanaka et al., 1998; Feckany et al., 1999; Ryu et al., 2001; Leung et al., 2003a; Leung et al., 2003b). boz represses bmp2b and mutually represses vox/vent, thereby defining the extent of the initial dorsal organizer (Melby et al., 2000; Kawahara et al., 2000a; Kawahara et al., 2000b; Leung et al., 2003a). However, boz and another β-catenin-dependent gene, hhx (Ho et al., 1999), are not expanded, but are normally expressed in MZspg (Fig. 3). The reduction of vox and vent in MZspg embryos seems therefore not responsible for lateral expansion of boz, as previously indicated (Imai et al., 2001). Rather, ved is upregulated upon reduction of vox/vent, and able to repress boz (Gillardelli et al., 2004). Downstream of β-catenin, Nodal signaling is required for the function of the dorsal organizer, and the Nodal-related factor Sqt induces chd and nog (reviewed by Schier, 2001). However, expression of sqt and cyc, as well as of Nodal signaling is normal in MZspg embryos (Reim et al., 2004). Therefore, lack of pou2 does not cause ectopic function of the dorsal organizer. The broadened shield and various aberrant forms of axial tissue in MZspg embryos, including split and incomplete duplications, might be a consequence of reduced convergence combined with affected epiboly. Altogether, ventral expansion of nog, chd and gsc at late blastula stages are not sufficient to induce an ectopic organizer activity in MZspg embryos. Beside the organizer, gsc and chd are also expressed in non-axial mesendoderm during blastula stages, which might be predominantly affected in MZspg embryos. In addition, ectopic organizer activity might require deregulation of further organizer-specific genes like boz, sqt or cyc.

Aberrant migration of MZspg cells can be explained by disrupted DV patterning

Positional information controls both cell fates and movements. The Bmp gradient controls morphogenetic gastrulation movements of convergence-extension (CE) (reviewed by Myers et al., 2002). Highest Bmp activity at the ventral side specifies ventral regions and inhibits CE movements. Lowest Bmp activity at the dorsal side enforces extension, but reduces convergence movements, and intermediate Bmp activity at lateral regions of the embryo favor both convergence and extension. Therefore, Bmp activity tightly links DV patterning to CE movements. Wild-type and MZspg cells migrate in the same manner, which strictly depends on the genotype of the host embryo, but not of donor cells (Fig. 3). The cell non-autonomous convergence defect of MZspg cells is probably attributable to loss of Bmp gene function, which also leads to pronounced extension movements, most obvious at the dorsal axis of wild-type and dorsalized embryos (Fig. 3) (Myers et al., 2002). However, normal extension along the AP axis is probably obscured by stalled blastodermal epiboly in MZspg embryos.

pou2 is cell-autonomously dispensable at later stages of ventral differentiation

At the beginning of gastrulation, the position of a cell correlates to its prospective fate (Kimmel et al., 1990; Ho and Kimmel, 1993). We analyzed the fate of dorsalized MZspg cells from prospective ventral positions within wild-type embryos (Fig. 5). The MZspg/wild-type chimera analysis shows a dispensability of cell autonomous pou2 for late DV patterning. Therefore, a wild-type environment is able to bypass non-autonomously, by providing secreted Bnps, the early requirement of pou2 for Bmp gene activation. This result is reminiscent to smad5/sbn function: sbn mutant cells transplanted into wild-type embryos can give rise to ventral tissues, indicating that in a wild-type environment these cells can differentiate properly (Hild et al., 1999). Alternatively, pou2 expression in the wild-type environment could exert a cell non-autonomous function on transplanted MZspg cells, as pou2 is expressed in the tail region of wild-type embryos during somitogenesis (Hauptmann and Gerster, 1995). Similar to Smad5 function, ultimate specification of ventral fates is independent of cell autonomous pou2, in contrast to initiation of bmp2b/4 at blastula stages, which is pou2 and smad5-dependent.

Epiboly is differentially affected in MZspg embryos

Epiboly is a morphogenetic movement of the zebrafish embryo in which the blastoderm engulfs the spherical yolk (reviewed by Kane and Adams, 2002). Movement of the YSL occurs independently of blastoderm epiboly, and the EVL is linked to the YSL, whereby the yolk may act as a towing motor of epiboly (Trinkaus, 1951). This is in accordance with our findings, as EVL and YSL undergo normal epiboly in MZspg in spite of arrested blastoderm epiboly. It is therefore unlikely that the blastoderm is merely passively ‘stretched out’ between these two layers. The epiboly phenotype of MZspg is
reminiscent of epiboly mutants such as hab, ava, law and weg, which eventually arrest epiboly of deep blastodermal cells (Kane et al., 1996). These mutants are allelic series of E-cadherin/hab (Kane et al., 2005). The gap between the YSL/EVL and the blastoderm begins to widen at 80% E in hab, whereas in MZspg a much stronger delay between the leading EVL/YSL and the lagging deep blastodermal cells is obvious from shield stage onwards. In ava mutants, deep cells escape into the space between the YSL and the EVL. We similarly observe scattering of mesodermal MZspg cells (Fig. 1f, Fig. 2D,H) that might therefore be linked to the epiboly phenotype. This indicates that cell adhesion is impaired in MZspg and other epiboly deficient embryos; however, E-cadherin transcription is normal in MZspg during blastula and gastrula stages (not shown). The molecular basis of the affected epiboly in MZspg is currently unknown.

Maternal pou2 and MHB development

Previous analysis of Zspg embryos showed that zygotic pou2 is required to establish the MHB organizer (Schier et al., 1996; Belting et al., 2001; Burgess et al., 2002; Reim and Brand, 2002). The MHB appears slightly more impaired in MZspg when compared with strong Mspg or Zspg embryos (Fig. 2FJ, and not shown), although altered DV patterning and epiboly certainly contribute indirectly to the phenotype in MHB development, we cannot rule out that residual maternal Pou2 protein might also have a subtle function in MHB development.

Three independent maternal functions of pou2

MZspg embryos display failure of initial endoderm differentiation (Reim et al., 2004; Lunde et al., 2004), of initial DV patterning and epiboly. These components of the MZspg mutant phenotype probably reflect independent pou2 functions. (1) In MZspg embryos DV patterning is disturbed shortly after MBT, whereas endodermal development is only disrupted later, from the beginning of gastrulation onwards. (2) Embryos affected in Bmp signaling can differentiate into endoderm, and endoderm mutants do not fail in DV patterning. (3) DV patterning mutants, including strongly dorsIALIZED Mspg embryos, undergo normal epiboly, and epiboly-defective embryos have normal DV and endoderm differentiation (Kane et al., 1996; Bruce et al., 2005). (4) Mutants affected in endoderm formation, including strongly dorsIALIZED Mspg embryos, have no arrest in epiboly, and vice versa. (5) Rescue of dorsalization in MZspg restored DV patterning without any rescue of defective epiboly or endoderm. However, we cannot rule out a slight improvement of epiboly in ca-alk8 injected MZspg embryos, which might be Bmp gene-independent. (6) MZspg cells transplanted into wild-type embryos show similar morphogenetic movements as wild-type cells and adopt positions according to DV positional values of the wild-type host, suggesting that MZspg cells can overcome their epiboly and DV patterning defects. By contrast, in a similar transplantation experiment, endodermal differentiation remains defective (Reim et al., 2004). In summary, we suggest that the phenotype of MZspg mutant embryos reflects a mosaic of independent functions of pou2 in different processes, including patterning as well as morphogenesis during early development.

pou2 and Oct4 in early development

Oct4/Pou5f1 is a functional ortholog of pou2 in mammals (Burgess et al., 2002; Reim and Brand, 2002), raising the issue of whether mammalian Oct4 has a similar function as its zebrafish ortholog. Murine Oct4 is thought to be required for totipotency of stem cells, and in Oct4 mutant mice, extra-embryonic tissue forms at the expense of an embryo proper (Pesce and Schöler, 2000). This seems not to be the case in zebrafish, at least with respect to embryonic somatic and germ stem cells: embryonic tissue develops; primordial germ cells develop normally in MZspg embryos (G.R. and M.B., unpublished). Oct4 is also required for the transition of the primitive, premigratory into migratory hypoblast (Botquin et al., 1998) and functions in early embryonic differentiation of the primitive endoderm and the germ line (Niwa et al., 2000; Pesce and Schöler, 2000), reminiscent of the function of pou2 in controlling endodermal versus mesodermal fate choice (Lunde et al., 2004; Reim et al., 2004). A putative zygotic role in DV patterning in mammals might be obscured by the requirement for Oct4 before implantation. Recent observations in mammals suggest the presence of maternally derived asymmetries determining cleavage patterns (Zernicka-Goetz, 2004). Germline inactivation of Oct4 revealed its requirement for germ cell survival (Kehler et al., 2004), which so far precluded further analysis of Oct4 in early asymmetry of the mammalian egg. However, Xenopus XPou-25, which belongs to the same protein subclass as Pou2 and Oct4, can stimulate transcription of Xvent-2B and prevents differentiation of neuroectodermal and mesodermal tissues when misexpressed (Cao et al., 2004). Although the role of pou2 has been mainly studied by loss-of-function experiments, the Xenopus data are reminiscent of the role of pou2 in activation of ventral-specifying genes, neural patterning and differentiation into endoderm.

We thank members of the Brand laboratory for discussions; Günter Junghanns, Evelyn Lehmann and Marika Fischer for excellent fish care; Max Fürthauer, Matthias Hammerschmidt and our anonymous referees for comments on the manuscript; Erez Raz for providing the vasa-3UTR-gfp plasmid; and the zebrafish community for sharing many reagents. This work was supported by funds from the EU (ZI models), DFG SFB 655 and the Human Frontiers Science Program to M.B.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/14/2757/DC1

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