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The mouse *Ovol2* gene is required for cranial neural tube development

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Abstract

The *Ovo* gene family encodes a group of evolutionarily conserved transcription factors and includes members that reside downstream of key developmental signaling pathways such as Wg/Wnt and BMP/TGF-β. In the current study, we explore the function of *Ovol2*, one of three *Ovo* paralogues in mice. We report that *Ovol2* is expressed during early–mid embryogenesis, particularly in the inner cell mass at E3.5, in epiblast at E6.5, and at later stages in ectodermally derived tissues such as the rostral surface (epidermal) ectoderm. Embryos in which *Ovol2* is ablated exhibit lethality by E10.5, prior to which they display severe defects including an open cranial neural tube. The neural defects are associated with improper *Shh* expression in the underlying rostral axial mesoderm and localized changes of neural marker expression along the dorsoventral axis, as well as with expanded cranial neural tissue and reduced cranial surface ectoderm culminating in a lateral shift of the neuroectoderm/surface ectoderm border. We propose that these defects reflect the involvement of *Ovol2* in independent processes such as regionalized gene expression and neural/non-neural ectodermal patterning. Additionally, we present evidence that *Ovol2* is required for efficient migration and survival of neural crest cells that arise at the neuroectoderm/surface ectoderm border, but not for their initial formation. Collectively, our studies indicate that *Ovol2* is a key regulator of neural development and reveal a previously unexplored role for *Ovo* genes in mammalian embryogenesis.

Keywords: *Ovo*; *Ovol2*; Neural tube; Brain; *Shh* signaling; Neuroectoderm; Surface ectoderm (epidermal ectoderm); Neural crest

Introduction

The embryonic brain develops from a region of specialized dorsal ectoderm known as the neural plate, within which the specification of the anterior neuroectoderm is initiated by the anterior visceral endoderm and is subsequently maintained by signals from the underlying anterior axial mesendoderm derived from the node (Brennan et al., 2001; Wilson and Houart, 2004). Ectoderm also gives rise to the surface ectoderm, precursor of the epidermis and its appendages. Studies in *Xenopus*, zebrafish, and chick suggest that the induction of neural plate and patterning of neural and non-neural ectoderm are governed by a complex interplay between BMP, Wnt, and FGF signaling pathways (Altmann and Brivanlou, 2001; Bally-Cuif and Hammerschmidt, 2003). Specifically, a BMP signaling gradient, set forth by BMPs and their antagonists, is proposed to differentially pattern the ectoderm; inhibition of BMP activity induces a neural fate, high BMP activity specifies an epidermal fate, and intermediate BMP activity induces the formation of neural crest cells at the neural/non-neural border (Hemmati-Brivanlou and Melton, 1997; Marchant et al., 1998; Nguyen et al., 1998; Wilson et al., 1997). Additionally, FGF signaling promotes a neural fate, whereas active Wnt signaling inhibits the ectoderm's response to FGF signaling, thereby permitting epidermal specification (Wilson et al., 2001; Wittler and Kessel, 2004). Also implicated in this process, largely by studies in *Xenopus*, are transcription factors that either promote (e.g., the Zic family members) or inhibit (e.g., Msx1, Dlx3) neural plate differentiation (Feledy et al., 1999; Kuo et al., 1998; Luo et al., 2001; Mizuseki et al., 1998; Nakata et al., 1997; Suzuki et al., 1997). To date, little is known about the genetic pathways underlying neural/epidermal patterning in mammals, as studies have been hampered by the in utero development of embryos and by functional redundancy between multiple members of particular gene families.

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A number of important signaling centers are formed to elaborate anterior–posterior (A–P) and dorsal–ventral (D–V) patterning of the prospective brain (reviewed in Joyner, 2002; Rubenstein and Beachy, 1998). The rostral domain of axial mesendoderm including the prechordal plate specifies the ventral aspect of the cranial neuroectoderm, while the surface ectoderm adjacent to the neural plate emanates BMP signals that specify a dorsal neural fate. The balance between these opposing activities determines the final D–V specification of the cranial neural tube (Joyner, 2002). Shh is an important ventralizing signal from the prechordal plate and the notochord. It induces the expression of itself and of *Ptch1*, encoding an Shh receptor and inhibitor, in the ventral neuroectoderm, as well as the expression of ventral forebrain markers such as the transcription factor Nkx2.1 (Briscoe and Ericson, 2001; Dale et al., 1997; Goodrich et al., 1997; Jessell, 2000; Shimamura and Rubenstein, 1997). In the dorsal neuroectoderm, transcription factors Pax3 and Msx1/2 are expressed in response to dorsalizing BMP signals and are required for dorsal neural differentiation (Goulding et al., 1993; Liem et al., 1995). Understanding the function of known and yet uncharacterized transcription factors and how they interact with the morphogenetic signaling events is key to understanding the genetic networks that underlie the formation of complex tissues such as the brain.

Cranial neurulation is an integral component of brain morphogenesis. This process requires morphogen-induced cell shape changes and movements, a delicate balance between proliferation and apoptosis in the neuroepithelium, expansion of the cranial mesoderm, and efficient migration of neural crest cells away from the neural plate (Copp et al., 2003). Disruption of any of these cellular and morphological events leads to exencephaly, ultimately resulting in defective brain formation. The medial expansion of the surface ectoderm provides a major driving force for folding and subsequent closure of the neural plate (Smith and Schoenwolf, 1997). Genetic studies in mice have elucidated a critical involvement of a number of genes in cranial neurulation (Copp et al., 2003; Smith and Schoenwolf, 1997), including *twist*, which encodes a basic helix–loop–helix protein expressed in head mesenchyme, and the transcription factor AP-2, which is expressed in cranial surface ectoderm. Thus, factors present outside of the neuroepithelium can also affect the morphogenesis of the cranial neural tube.

The *Ovo* gene family encodes evolutionarily conserved zinc-finger transcription factors whose function in embryogenesis is under-explored. The founding member of this family, *Drosophila ovo*, is required for epidermal denticle formation and oogenesis and has been shown to be genetically downstream of canonical Wg/Wnt signaling (Mevel-Ninio et al., 1995; Oliver et al., 1994; Payre et al., 1999). *lin-48*, the *ovo* homologue found in *C. elegans*, is required for cell fate specification during hindgut development (Chamberlin et al., 1999). There are three *ovo* homologues in mammals, designated *Ovol1*, *Ovol2*, and *Ovol3*. In mice, *Ovol1* is required for hair follicle differentiation, where it is regulated by nuclear effectors of Wnt signaling, and also for kidney and male germ cell development (Dai et al., 1998; Li et al., 2002b, 2005). Recently, human *OVOL1* has been identified as a gene that is responsive to TGF-β1/BMP7 treatment via a Smad4-dependent pathway (Kowanetz et al., 2004). That members of the *ovo* gene family act downstream of signaling pathways required for diverse processes during both early and late stages of embryonic development raises the possibility that *ovo* genes might be important for embryogenesis.

Previous studies revealed *Ovol2* expression in brain, testis, and epithelial tissues such as skin and intestine of adult mice (Li et al., 2002a). In this study, we show that it is also expressed during early–mid embryogenesis, particularly in the epiblast and its ectodermal derivatives in the developing head. Using a gene targeting approach, we ablated *Ovol2* expression and observed that *Ovol2* is required for the development of the embryonic brain. We further demonstrate that *Ovol2* is required for maintaining the proper expression of signaling molecules such as *Shh* and *Wnt1* along the cranial D–V axis, for positioning the neuroectoderm/surface ectoderm border in the head region, for maintenance of migrating neural crest cells, and for closure of the cranial neural tube.

Materials and methods

Generation of Ovol2 mutant mice

A 120 kb BAC clone containing the *Ovol2* locus was identified by screening a 129/Sv genomic library (Incyte Genomics) and was used to generate a targeting vector designed to delete exons 1a, 1b, and 2 (E1A, E1B, E2) that contain the start codon of the Ovol2 open reading frame (Fig. 1A; Li et al., 2002a). A 2 kb *Bam*HI fragment corresponding to the genomic region upstream of E1A was cloned as the 5′ arm into the pPGKneobpA-lox2PGKDTA vector at the *Hin*dII site by blunt-end ligation. Primers corresponding to sequences in E2 and E3 were used to amplify a 14 kb genomic sequence which was subsequently cloned into the TOPO-TA vector (Invitrogen). A 6.8 kb *Pst*I fragment was released from the resulting plasmid and blunt-end ligated into the *Not*I site of the targeting vector as the 3′ arm. A 1 kb *Sma*I–*Afl*II fragment encoding the EGFP protein (from pEGFP-N1, Clontech) was inserted downstream of the 5′arm at the *Nhe*I site by blunt-end ligation.

The targeting vector was linearized with *Sac*II and electroporated into E14 embryonic stem (ES) cells. After selection in the presence of G418, clones were screened by PCR and Southern hybridization (Figs. 1B–D), two of which were injected into blastocysts. The resulting chimeras were mated with wild-type C57BL/6J females to produce F1 progeny of a C57BL6/J×129Ola (B6×129) mixed genetic background, which were further intercrossed to produce homozygous mutant F2 progeny for study. Subsequent breeding of F1 heterozygous males with wild-type CD1 outbred females generated heterozygous offspring enriched in a CD1 background that were also used to produce homozygous mutant mice for study. Mutant phenotypes in mice derived from both ES clones were identical.

PCR genotyping, Southern blot, and RT-PCR analysis

Genomic DNA or lysate isolated from ES cells, tail clippings, or yolk sacs was used for PCR genotyping using the following primer sequences (designated as arrows in Fig. 1A): PCRi: 5′-TGCTTCGTGGGTTGGCCTGAGAAC-3′ and 5′-CAGATGTCCATCGGTGCCTTGGGC-3′; PCRii: 5′-GTTTCGCTTGGTG-GTCGAATGGGCAG-3′ and 5′-CACCACAGAGGCTGGGAGTGACATC-3′; PCRiii: 5′-CGCTCCTTCTTTCTAGCAAGTCTCCCG-3′ and 5′-AAG-TCGTGCTGCTTCATGTG-3′; PCRiv: 5′-GCTGACCCTGAAGTTCATCT-GACCA-3′ and 5′-CGCTTCTCGTTGGGGTCTTTGCTCA-3′. Southern blot analysis was performed essentially as described (Sambrook and Russell, 2001) using the probe indicated in Fig. 1A. RT-PCR was performed using RNA isolated from either single embryos (Superscript One-Step RT-PCR with Platinum *Taq* kit, Invitrogen) or pooled embryos (Superscript III Reverse Transcriptase, Invitrogen)