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FoxA3 and goosecoid promote anterior neural fate through inhibition of Wnt8a activity before the onset of gastrulation

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Abstract

Formation of the nervous system initially requires the acquisition of neural identity, which is achieved through the inhibition of epidermalizing factors. A regional patterning then takes place within the neural plate through the activity of caudalizing factors. These two processes are tightly regulated early in development by the dorsal organizer. Here, we show that, in zebrafish embryos, two transcription factors, FoxA3 and Goosecoid, coexpressed at the dorsal blastula margin, are required for the definition of anterior neural fate. Their inactivation results in deletions of anterior head structures associated with an increase of Wnt8 activity at the dorsal blastula margin. These phenotypes can be fully rescued by overexpression of Wnt inhibitors or by inactivation of *wnt8a*. Altogether, *foxA3* and *goosecoid* cooperate to promote formation of anterior neural tissue by protecting, as early as blastula stage, presumptive anterior neural cells from an irreversible caudalization by the posteriorizing factor Wnt8a.

Keywords: Goosecoid; *foxA3*; *foxA2*; *dkk1*; *frzb*; *Wnt8a*; Nervous system patterning; Posteriorization; Antero-posterior axis; Zebrafish; Embryo; Blastula

Introduction

The neuroectoderm of the vertebrate gastrula was proposed by Nieuwkoop to be regionalized into forebrain, midbrain, hindbrain and spinal cord by a two-step process. In the activation step, the Spemann gastrula organizer induces neuroectoderm with anterior character, followed with posteriorization by a transforming signal, thereby generating a complete anteroposterior (AP) succession of neural fates (Nieuwkoop et al., 1952). More recently, simultaneous inhibition of BMP and Wnt signaling was shown to induce head formation in frog embryos (Glinka et al., 1997, 1998; Piccolo et al., 1999). Consistent with this, multiple Wnt, Nodal and BMP inhibitors, including Noggin, Chordin, Follistatin, Frzb, Cerberus, Antivin and Dickkopf are expressed in discrete, overlapping domains of the organizer during gastrulation (De Robertis et al., 2000). However, although most of these secreted factors bind to and antagonize the activity of Wnt and/or BMP proteins produced by non-organizer cells, these factors do not repress *wnt* or *bmp* transcription, and are therefore not sufficient to exclude *wnt* and *bmp* expressions from the organizer. As ectopic activation of the zygotic Wnt or BMP pathways inhibits organizer function, a second class of inhibitors may exist which represses the transcription of *wnt* and *bmp* genes in the Spemann organizer. One candidate for such an organizer-specific transcriptional repressor is the homeobox gene *goosecoid* (*gsc*).

Gsc is found across animal phyla, from hydra to human (Blum et al., 1994; Broun et al., 1999; De Robertis et al., 1994; Lemaire and Kodjabachian, 1996). During gastrulation of the vertebrate embryo, *gsc* is expressed in organizer cells—the Spemann organizer in *Xenopus*, the embryonic shield in zebrafish, the node in mouse and chick—and the conservation of Gsc structure and expression suggests an important function in early development (Blum et al., 1992; Blumberg et al., 1991; Izpisua-Belmonte et al., 1993; Schulte-Merker et al., 1994). In *Xenopus*, *gsc* expression peaks at the early gastrula stage in the dorsal mesendoderm that constitutes the Spemann organizer, and injection of ventral blastomeres with *gsc* mRNA induces the formation of a secondary body axis (Cho et al., 1991; Steinbeisser et al., 1993), suggesting that it may be an essential component of the gastrula organizer. Consistent with this idea, overexpression of *gsc* inhibits the expression of *Xwnt8* and

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bmp4 (Christian and Moon, 1993; Fainsod et al., 1994; Steinbeisser et al., 1995), which are expressed in non-dorsal mesoderm, and antagonize organizer function and axis formation (Christian and Moon, 1993; Dale et al., 1992; Fredieu et al., 1997; Hemmati-Brivanlou and Thomsen, 1995; Hoppler and Moon, 1998; Hoppler et al., 1996; Jones et al., 1992, 1996; Tian et al., 1999).

However, additional functional studies in the mouse reveal that knock-out of *gsc* leads to normal embryos and the *gsc*-null mice are born alive. They die soon after birth, however, with craniofacial defects, suggesting that *gsc* is not essential for organizer activity but that it is required later during embryogenesis for craniofacial and rib development (Belo et al., 1998; Rivera-Pérez et al., 1995; Yamada et al., 1995, 1997; Zhu et al., 1998). Given that organizer expression of *gsc* is absolutely conserved in vertebrates, the lack of an early phenotype suggests that functionally redundant genes are expressed in the gastrula embryo which compensate for the loss of *gsc* function.

To investigate this hypothesis, we searched for potential candidates by the use of a large-scale in situ screen we are currently performing in the aim of identifying the expression pattern of the whole zebrafish genome. Among our collection of 14,000 different genes analyzed, only two of them, *gsc* and *foxA3*, shared the same expression pattern at early developmental stages, in both the dorsal marginal blastoderm and in the dorsal marginal part of the yolk syncitial layer. FoxA3 belongs to the Hepatocyte Nuclear Factor 3 (HNF3 or FoxA) family, known to play a crucial role in the regulation of metabolism and in the differentiation of tissues such as pancreas and liver (Kaestner, 2000). Nevertheless, no information was available about its possible function during early developmental stages except a partial description of its expression pattern at early stages (Odenthal and Nüsslein-Volhard, 1998). We therefore investigated its potential role during early development by inhibiting its activity using the morpholino knock-down technology. We also reexamined in zebrafish the function of *gsc* using the same strategy and tested by double knock-down the potential redundant function of these genes during early developmental stages. In this report, we show that *foxA3* and *gsc* cooperate to promote formation of anterior neural tissue by protecting presumptive anterior neural cells from the caudalizing activity of Wnt8a. We further demonstrate that rostral presumptive neural tissues need to be protected from Wnt8a stimulation very early in development, as early as blastula stage, to prevent their irreversible caudalization by this posteriorizing factor.

Materials and methods

Whole-mount in situ hybridization

All whole-mount in situ hybridizations were performed as described previously (Thisse et al., 2004a). *foxA3*, *frzb*, *dkk1*, *foxi1*, *pax2.1*, *hoxa1*, *otx5* and *pax6* were isolated in the course of a large-scale in situ hybridization screen (http://zfin.org) and antisense RNA made through *Not*I linearization and T7 transcription. The other clones used in this study have been previously described: *gsc* (Thisse et al., 1994), *emx1* (Morita et al., 1995), *eng2* (Ekker et al., 1992) and *wnt8a* (Kelly et al., 1995).

DNA, mRNA and morpholino injections

The *wnt8a* (a gift of R. Moon) and *dkk1* ORFs were subcloned into *Bam*HI/*Xho*I sites of pCS2+. The *gsc* and *frzb* ORFs were subcloned into the *Eco*RI/*Xho*I sites of pCS2+. Plasmids were then linearized with *Not*I and sense RNA transcribed with SP6 RNA polymerase using the mMessage mMachine Kit (Ambion).

Morpholinos (Gene Tools) were resuspended in Danieau 1×, stored at −20°C as a 4 mM stock solution and diluted before use to the appropriate concentration. The sequences of the morpholinos used are:

Mo-gsc: CAAGCGAAAAGATGTGTGAGATTTG
Mo-gsc(D): AGTAAAAAAATACCTGTAGGAATAC
Mo-gsc(A): GCGCTGTCATAACCTGAAAATAAGA
Mo-foxA3: CTCGTAAGAAACGGGATAGTGACTG
Mo-dkk1: GAGAGCATGGCGATGTGCATCATGT
Mo-wnt8a: ACGCAAAAATCTGGCAAGGGTTCAT
Mo-foxA2: CCTCCATTTTGACAGCACCGAGCAT

For mRNA and morpholino injections, embryos were dechorionated using Pronase E and injected with either RNA or morpholinos diluted in 0.2% Phenol Red and 0.1 M KCl, using an Eppendorf 5426 microinjector.

Cells transplantation

Transplantation experiments were performed by suction as described (Saùde et al., 2000). Donor embryos were injected at the 1–2 cell stage with dkk1 plus GFP RNA (100 and 50 pg, respectively) or GFP RNA alone (50 pg) as a control. Host embryos were injected at the 1–2 cell

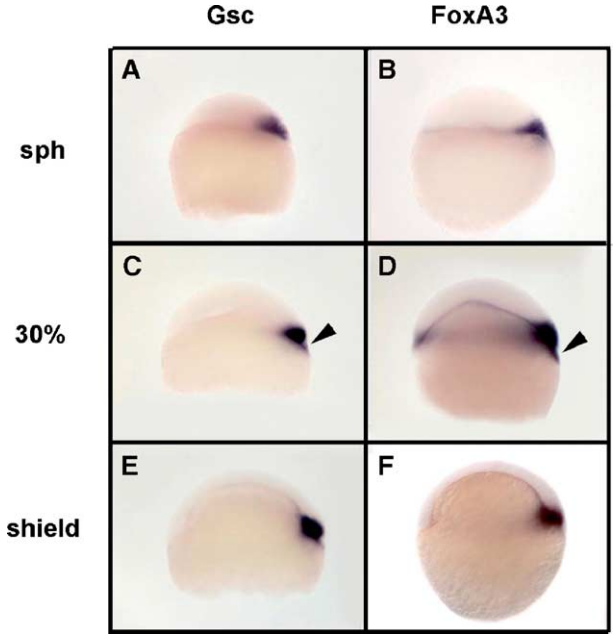

Fig. 1. Expression of gsc and foxA3 during early developmental stages. (A, B) At sphere stage, *gsc* and *foxA3* are expressed at the dorsal margin. (C, D) At blastula stage (30% epiboly), *gsc* and *foxA3* transcripts colocalize at the dorsal margin with strong expression in blastodermal cells and YSL (arrowhead). (E, F) At the onset of gastrulation, *gsc* and *foxA3* are expressed in the central part of the embryonic shield. Transcripts of both genes are no longer observed in the YSL. Embryos are in lateral view, dorsal towards the right.