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Sox3 regulates both neural fate and differentiation in the zebrafish ectoderm

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ABSTRACT

Little is known of the first transcriptional events that regulate neural fate in response to extracellular signals such as Bmps and Fgfs. Sox3 is one of the earliest transcription factors to be expressed in the developing CNS and has been shown to be regulated by these signalling pathways. We have used both gain- and loss-of-function experiments in zebrafish to elucidate the role of Sox3 in determining neural fate. Ectopic Sox3 caused induction of neural tissue from a very early stage of cell specification in the ectoderm and this effect was maintained such that large domains of additional CNS were apparent, including almost complete duplications of the CNS. Knock-down of Sox3 using morpholinos resulted in a reduction in the size of the CNS, ears and eyes and subsequent inhibition of some aspects of neurogenesis. Our data also suggest that the pro-neural effects of Sox3 can compensate for inhibition of Fgf signalling in inducing neural tissue but it is not sufficient to maintain neural fate, suggesting the presence of Sox3-independent roles of Fgf at later stages.

Introduction

Neural development in vertebrates occurs in cells of the ectoderm in response to a range of intercellular signals, including the Bmps, Fgfs and Wnts (Stern, 2006; Wilson and Edlund, 2001). The balance of these signals results in cells adopting either a neural or non-neural fate. This response involves complex changes in gene expression mediated by altered activity of specific transcription factors. Despite many years of analysis, the relative roles of these different pathways is only now becoming clear and the link between the activation or repression of these pathways and specific transcription factor activities to elicit a neural response is poorly understood. This is reflected by the small number of transcription factors for which there is evidence of a direct role in mediating this response.

Sox3 is one of the earliest and most generally expressed transcription factors in neural development of vertebrates (Penzel et al., 1997; Rex et al., 1997; Wood and Episkopou, 1999; Zhang et al., 2004, 2003). Along with the other SoxB1 factors, Sox1 and Sox2, Sox3 has been implicated as a central player in the maintenance of the stem cell state of neural cells (Bylund et al., 2003; Graham et al., 2003). *Sox3* expression requires active Fgf signalling but it is generally repressed where Bmp signalling is active (Dee et al., 2007; Heeg-Truesdell and LaBonne, 2006; Kudoh et al., 2004; Rentzsch et al., 2004; Streit et al., 2000). However, *sox3* is also expressed prior to the partitioning of ectoderm into neural and non-neural domains (Dee et al., 2007; Rex et al., 1997). Despite its unique position as the earliest of the SoxB1 genes to be expressed, relatively little is known of the function and mechanism of Sox3 in neural development.

All three SoxB1 factors have also been shown to be able to impede the differentiation of neural stem cells in the spinal cord, while a construct that repressed target genes led to premature differentiation (Bylund et al., 2003; Graham et al., 2003). However, their role in the earlier fate choice of ectodermal cells to adopt a neural fate is less well understood. Loss of *Sox3* in mice appears to generate a relatively mild phenotype. Although initial studies in an inbred strain of mice showed loss of *Sox3* to be lethal as early as gastrulation, the genetic background proved to be critical, and later experiments using out-bred mice produced phenotypes varying from apparent wild-type to mild defects in brain and germ cell development (Rizzoti et al., 2004). In chick, although its expression is rapidly induced by the node or FGF signalling (Streit et al., 2000), the role of Sox3 in the central nervous system (CNS) has not been elucidated. In the peripheral nervous system (PNS), we have shown that Sox3 induces ectopic placode-like thickening (Abu-Elmagd et al., 2001) as has been described in Medaka (Koster et al., 2000). A role in even earlier events was demonstrated by Zhang et al. (2003, 2004) in which Sox3 appears to play a role in repressing mesendoderm formation. In *Drosophila* there are two SoxB genes, *SoxNeuro* and *Dichaete*, which have both been implicated in dorsoventral patterning of the neuroectoderm and subsequent neurogenesis (Girard et al., 2006; Overton et al., 2002; Zhao and Skeath, 2002). These data suggest that Sox3 is likely to play an early and central role in the choice of ectoderm to adopt a neural fate as well as regulating later neuronal differentiation.

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In this study we have analysed the role of Sox3 in neural fate in zebrafish using both gain-of-function and loss-of-function approaches. We show that ectopic expression of Sox3 leads ectoderm to acquire neural fate, with appearance of the earliest neural markers and expansion of the later neuroepithelium. Likewise, we observed loss of neural structures when Sox3 was knocked-down by morpholino injection. We also show that Sox3 can compensate for absence of Fgf signalling during the initiation of neural fate, but it is insufficient to compensate for later roles of Fgf signalling. In addition, we find that Sox3 is essential for subsequent neuronal differentiation, in both the CNS and PNS, and for normal development of the eye and ear.

Methods

Generation of constructs

A morpholino-sensitive *sox3*-GFP fusion construct (MS*sox3*-GFP) was produced to assess the efficacy of Sox3 morpholinos. The coding sequence of *sox3* and 189 bp of 5′ UTR, was amplified from zebrafish 70% epiboly stage cDNA by PCR using the Superscript III kit (Invitrogen) and the following primers: forward 5′-CCATCGATAGCTTAGCGCA-CAACTTT-3′ and reverse 5′-GCTCTAGAAATGTGGGTTAGGGGTAG-3′. The resulting product was ligated into the pCR-II vector (Invitrogen) and subcloned into the pCS2nlsGFP (gift from Dr. Marie-Anne J. O'Reilly) vector at the ClaI–XbaI site so that the final construct contains an in-frame fusion of *sox3* and GFP (with the nuclear localization signal and myc sequences of the parental vector removed).

Injection of RNA and morpholinos

Zebrafish embryos were collected and staged according to standard methods (Kimmel et al., 1995; Westerfield, 2000). Capped mRNAs for microinjection were produced from linearised cDNA template using mMessage-Machine kits (Ambion) according to the manufacturer's instructions.

Antisense morpholino oligonucleotides (MOs) designed to target the 5′ region of *sox3* were obtained from Genetools (Philomath, OR). The MO sequences were: *sox3*MO1 5′-GGTGCCAAGCACTCGAAAGAAAACG-3′, and *sox3*MO2 5′-CCATCATGTTATA-CATTCTTAAAAG-3′. Embryos were injected with a mixture of *sox3*MO1 (2.5 ng) and *sox3*MO2 (2.5 ng) in 0.5 nl volume at the 1–2 cell stage. The 5 bp mis-match (represented in lower case) control MO sequences were: *sox3*MO1-5 mm 5′-GGTcCCAAcCAgTCGAAAcAAAAgG-3′, and *sox3*MO2-5 mm 5′-CgATCATcTTATAgATTgT-TAAAtG-3′. Effective MO knock-down was tested by co-injecting with mRNA encoding MS*sox3*-GFP (125 pg).

Transplantations

Donor embryos were injected at the 1–2 cell stage with a mixture of *sox3* (50 pg) (including an HA tag) and GFP (50 pg) mRNA and cells removed at the oblong stage using a microelectrode connected to a 1 ml BD Plastipak syringe as previously described (Kane and Kishimoto, 2002). Donor cells were then introduced into the animal pole of similar stage wild-type host embryos, and the resulting embryos incubated in E2 buffer (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$) until time of collection. Following *in situ* hybridisation, transplanted cells were detected using rabbit polyclonal anti-HA ab9110 (1:2000; AbCam), anti-rabbit IgG HRP labelled secondary (1:2000; Vector labs) and visualized with DAB substrate (Vector labs).

TUNEL staining

Embryos were dechorionated at 24 hpf, fixed in 4% paraformaldehyde for 1 h at room temperature, dehydrated and stored at −20 °C over night. Following rehydration, embryos were treated with proteinase K (10 μg/ml in PBS) for 20 min at room temperature, and washed in PBS. TUNEL staining was then performed using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer's instructions. Images were captured using a Leica SP2 CLSM confocal microscope.

SU5402 treatments

SU5402 (Calbiochem) was dissolved in DMSO to give a 3.36 mM stock solution. This was used at a final concentration of 84 μM in fish water containing methyl blue. Embryos were treated at 28 °C, in the dark from the 512 cell stage until the required time of collection. Negative control embryos were treated with an equivalent dilution of DMSO.

Whole-mount in situ hybridisation and immunohistochemistry

Whole-mount *in situ* hybridisation was carried out as previously described (Jowett and Yan, 1996) using the following Digoxigenin (DIG)-labelled (Roche) riboprobes: *sox2* (Dee et al., 2007), *sox31* (Girard et al., 2001), *gata2* (Read et al., 1998), *ncad* (Lele et al., 2002), *pax2a* (Krauss et al., 1991), *pax8* (Pfeffer et al., 1998), *ngn1* (Sun et al., 2007), *phox2a* (Guo et al., 1999), *foxd3* (Stewart et al., 2006), *snai1b* (Stewart et al., 2006), *ntl* (Schulte-Merker et al., 1992), *shha* (Krauss et al., 1993), *emx1* (Morita et al., 1995), *krox20* (Sun et al., 2007), *otx2* (Mori et al., 1994), *hoxb1b* (EST obtained from RZPD: bq985541), *elavl3* (IMAGE: 7062042), *elavl4* (IMAGE: 6967800), *nefm* (IMAGE: 6968572). Alkaline phosphatase conjugated DIG antibody was detected using BM-purple (Roche). For the double *in situ* hybridisation, a fluorescein-labelled (Roche) *ntl* riboprobe was used and detected using alkaline phosphatase conjugated anti-fluorescein (Roche) and Fast Red (Sigma-Aldrich). To detect GFP, immunostaining was performed according to standard protocols using fluorescein conjugated anti-GFP (Ab6662, 1/1000; AbCam), and an alkaline phosphatase conjugated anti-fluorescein secondary antibody (Roche) visualized using Fast Red (Sigma-Aldrich). For sectioning after *in situ* hybridisation, embryos were dehydrated, transferred to ethanol and embedded in JB4 methacrylate (Agar Scientific) for microtome (Leica RM2265) sectioning. Sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). Sections were taken immediately caudal to the eye or posterior branchial arches (marked by *hoxb1b*) and the average cell count from 7 sections (10 μm thickness for brain or 12 μm for spinal cord) was taken.

Levels of Sox3 expression were assayed on frozen sections as follows. Embryos were soaked in 15% sucrose and embedded in 7.5% gelatin/15% sucrose. Blocks were frozen in isopentane and sectioned at 10 μm thickness onto Superfrost Plus (VWR international) slides. Antigen retrieval was carried out by fixing embryos in 4% paraformaldehyde then dehydrating in methanol overnight. Rehydrated embryos were treated with citrate buffer (10 mM citric acid, pH6) at 4 °C for 1 h, then transferred to citrate buffer at 100 °C

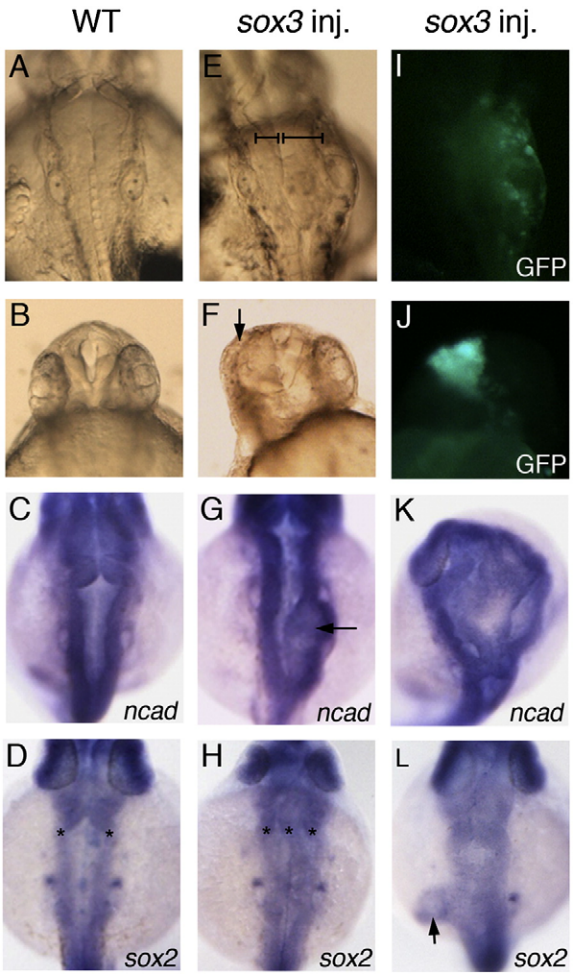

Fig. 1. Sox3 over-expression causes expansion of neural tissue. Wild-type control embryos (A–D) and embryos injected with *sox3* mRNA at 16–32 cell stage (E–L) are viewed dorsally aged 24 hpf. (E, I) Injection of *sox3* mRNA causes development of extra neural tissue visible in the live embryo (bars indicate unaffected and expanded sides of the CNS, E), and GFP fluorescence indicates this occurs in the region expressing exogenous *sox3* (I). (F, J) Live embryo showing over-expression of *sox3* causing loss of eye (arrow, F) when expressed in the rostral region of the CNS seen by GFP fluorescence (J). (C, D, G, H, K, L) *In situ* hybridisation for expression of neural markers *ncad* or *sox2* in wild-type embryos (C, D) and embryos injected with *sox3* mRNA (G, H, K, L) shows the development of ectopic tissue either within the brain (arrow, G; asterisks indicate multiple neural epithelia in panel H, 16/68 embryos), expansion of the brain (K, 2/21) or expansion of a local region of the trunk (arrow, L, 4/47).