available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/modo

Selective inactivation of Otx2 mRNA isoforms reveals isoform-specific requirement for visceral endoderm anteriorization and head morphogenesis and highlights cell diversity in the visceral endoderm

Dario Acampora[a,c], *Luca Giovanni Di Giovannantonio*[a], *Michela Di Salvio*[a], *Pietro Mancuso*[a], *Antonio Simeone*[a,b,c,*]

[a]*CEINGE Biotecnologie Avanzate, Via Comunale Margherita 482, 80145 Naples, Italy*
[b]*SEMM European School of Molecular Medicine – Naples Site, Italy*
[c]*Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, Via P. Castellino 111, 80131 Naples, Italy*

ARTICLE INFO

Article history:
Received 19 June 2009
Accepted 7 July 2009
Available online 15 July 2009

Keywords:
Otx2
Visceral endoderm
Head morphogenesis
A–P axis conversion
Gastrulation
Forebrain
Midbrain
Dkk1
Lefty1

ABSTRACT

Genetic and embryological experiments demonstrated that the visceral endoderm (VE) is essential for positioning the primitive streak at one pole of the embryo and head morphogenesis through antagonism of the Wnt and Nodal signaling pathways. The transcription factor Otx2 is required for VE anteriorization and specification of rostral neuroectoderm at least in part by controlling the expression of *Dkk1* and *Lefty1*. Here, we investigated the relevance of the *Otx2* transcriptional control in these processes. Otx2 protein is encoded by different mRNAs variants, which, on the basis of their transcription start site, may be distinguished in distal and proximal. Distal isoforms are prevalently expressed in the epiblast and neuroectoderm, while proximal isoforms prevalently in the VE. Selective inactivation of *Otx2* variants reveals that distal isoforms are not required for gastrulation, but essential for maintenance of forebrain and midbrain identities; conversely, proximal isoforms control VE anteriorization and, indirectly, primitive streak positioning through the activation of *Dkk1* and *Lefty1*. Moreover, in these mutants the expression of proximal isoforms is not affected by the lack of distal mRNAs and vice versa. Taken together these findings indicate that proximal and distal isoforms, whose expression is independently regulated in the VE and epiblast-derived neuroectoderm, functionally cooperate to provide these tissues with the sufficient level of Otx2 necessary to promote a normal development. Furthermore, we discovered that in the VE the expression of *Otx2* isoforms is tightly controlled at single cell level, and we hypothesize that this molecular diversity may potentially confer specific functional properties to different subsets of VE cells.

1. Introduction

Before the onset of gastrulation, the mouse embryo is composed by two germ layers, the epiblast (Ep) and the visceral endoderm (VE). A large body of evidence indicates that the VE in mouse and the hypoblast and endoblast in chick play a relevant role in embryonic axis formation, positioning of the primitive streak and initial specification of

* Corresponding author. Address: CEINGE Biotecnologie Avanzate, Via Comunale Margherita 482, 80145 Naples, Italy. Tel.: +39 081 3737868; fax: +39 081 3737808.
E-mail address: simeone@ceinge.unina.it (A. Simeone).
0925-4773/$ - see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mod.2009.07.003

anterior neural structures (Beddington and Robertson, 1999; Stern, 2001, 2004; Foley et al., 2000; Tam and Gad, 2004; Srinivas, 2006). In mouse VE cells are established before gastrulation and, although it has for long time believed that they contribute exclusively to extraembryonic structures, very recently it has been shown that a relevant portion of them persists in the embryo proper and contributes to the gut (Kwon et al., 2008; Stern, 2008). At the two germ layers stage the main embryonic axis is oriented proximo-distally and, just prior the primitive streak forms, the proximal–distal (Pr–D) axis is permanently converted in the anterior–posterior (A–P) axis. Axis conversion involves the anterior displacement of the distal VE (DVE) into the anterior VE (AVE) and the posterior positioning of primitive streak (Beddington and Robertson, 1999; Srinivas et al., 2004; Tam and Gad, 2004; Foley et al., 2000; Stern, 2001, 2004; Rivera-Pérez et al., 2003; Rodriguez et al., 2005). Molecular and embryological evidence suggests that the VE plays a relevant role in this process by inhibiting Nodal and Wnt signaling through the production of antagonizing molecules such as Lefty1, Cer-l and Dkk1 (Bertocchini and Stern, 2002; Perea-Gomez et al., 2002; Kimura-Yoshida et al., 2005; Yamamoto et al., 2004; Glinka et al., 1998). Besides this role in A–P axis conversion, embryological evidence suggests that the AVE is also required for specification of anterior neural structures (Tam and Steiner, 1999; Beddington and Robertson, 1999; Stern, 2001). In this context several transcription and signaling molecules such as Otx2, Dkk1, Lim1, Foxa2 and Nodal are required in VE cells for proper development of rostral neuroectoderm (Rhinn et al., 1998; Acampora et al., 1998; Mukhopadhyay et al., 2001; Lewis et al., 2008; Dufort et al., 1998; Filosa et al., 1997; Shawlot et al., 1999; Varlet et al., 1997). *Otx2* null mutants exhibit headless phenotype and severe impairment of gastrulation (Acampora et al., 1995; Simeone, 2004; Matsuo et al., 1995; Ang et al., 1996). In particular, *Otx2*$^{-/-}$ embryos fail to convert the DVE into the AVE and to confine the primitive streak at the posterior pole of the embryo (Acampora et al., 1998; Rhinn et al., 1998; Kimura et al., 2000; Perea-Gomez et al., 2001). Embryological and genetic studies showed that Otx2 may be required in the VE for its anterior displacement by activating the expression of *Lefty1* and *Dkk1* (Perea-Gomez et al., 2001; Kimura et al., 2000; Zakin et al., 2000). Remarkably, *Otx2*-driven expression of *Dkk1* in the VE and Ep of *Otx2* null embryos is sufficient to rescue axis conversion by promoting AVE formation (Kimura-Yoshida et al., 2005). In the neuroectoderm, from mid-late-headfold stage *Otx2* is necessary to maintain forebrain and midbrain identity by antagonizing *Gbx2* and positioning the midbrain-hindbrain boundary (MHB) (Acampora et al., 1998; Martinez-Barbera et al., 2001). Therefore, the complexity of *Otx2* functions led us to investigate how the regulatory control of its expression is functionally involved in these processes and whether the different *Otx2* functions are encoded by specific mRNA isoforms. Previous studies showed the existence of at least three different *Otx2* mRNAs differing only in the 5′ untranslated region (UTR) (Courtois et al., 2003; Fossat et al., 2005). Here, we first extended these studies and then analyzed in vivo the functional requirement of the *Otx2* mRNAs. Selective inactivation of the distal isoforms, expressed prevalently in the Ep, and proximal isoforms, encoding for most of the Otx2 protein in the VE, revealed that distal isoforms are not required for gastrulation and axis conversion, but are crucial for maintenance of forebrain and midbrain regional identities; conversely, proximal isoforms are important for axis conversion, but much less for maintenance of forebrain and midbrain territories. Furthermore, we also discovered within the VE a marked cell diversity in the *Otx2* transcriptional control determining at single cell level the expression of proximal and/or distal variants, which suggests a high degree of complexity of this tissue.

2. Results

2.1. *Otx2* transcriptional organization and distribution of Otx2 mRNA isoforms

Previous studies (Fossat et al., 2005; Courtois et al., 2003) have already shown that the Otx2 protein is encoded by at least three different mRNA isoforms. In order to extend these findings, we performed a detailed and exhaustive analysis of the *Otx2* transcriptional organization. Four major *Otx2* isoforms were identified. Three of them, named *A*, *B* and *D* corresponded to those previously reported and a fourth isoform, named *C*, represented a novel *Otx2* mRNA (Fig. 1A and B). Isoforms *A* and *B* exhibited different 5′ exon and a common splice site (SS) at −119 bp upstream of the *Otx2* methionine (met) and were referred to as distal transcripts; isoforms *C* and *D* were colinear with the *Otx2* genomic region upstream of the *Otx2* met and were referred to as proximal transcripts (Fig. 1A and B). The transcription start site (TSS) for *A*, *B* and *D* mRNAs was determined at position −5015, −2599 and −208 from the *Otx2* met (Fig. 1A) (Courtois et al., 2003); while for isoform *C*, we identified a major TSS at position −687 (Fig. 1A). Then, we investigated the relative distribution and abundance of *A*–*D* variants at E6.5, E7.5 and E10.5. In this context since the *D* mRNA was included in the *C* isoform, a *D* specific primer could not be designed, and, therefore, the level of the *D* mRNA could only be indirectly deduced in RT-PCR experiments as the difference between *C* + *D* and *C* alone. At E6.5 the *A* and *D* isoforms were the most represented, while between E7.5 and E10.5, the most abundant mRNA was the isoform *A* (Fig. 1C and D). Then, the distribution of the *A*–*D* mRNAs was analyzed on dissected VE and Ep (Fig. 1E). In the Ep the *Otx2* mRNA was primarily contributed by isoform *A*, while in the VE by isoform *D* (Fig. 1E). Ep contamination in the VE and vice versa was negligible as revealed by the expression of *Oct4* and *Cer-l* (Fig. 1E). These findings were supported by in situ hybridization experiments showing that at E6.5 isoform *A* was the most abundant in the Ep (Supplementary Fig. 1B) and isoforms *C* + *D* the most expressed in the VE (Supplementary Fig. 1E). At E10.5 isoform *A* was the prevalent *Otx2* transcript (Supplementary Fig. 1G–K). Next, we studied at E6.5 and E10.5 whether the *Otx2* expression was also controlled at the translational level. According to the length of β-actin (350 amino acids) and Otx2 (289 amino acids) an efficiently translated mRNA should be respectively concentrated in the first two and in the second and third fractions (Acampora et al., 2001). We found that isoforms *A* and *D* were efficiently translated at both stages (Fig. 1F and I); isoform *B* was distributed in the first five fractions, which was indicative