Developmental Biology 350 (2011) 484–495

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

FoxA transcription factors are essential for the development of dorsal axial structures

Sophie Dal-Pra, Christine Thisse, Bernard Thisse *

Department of Cell Biology, University of Virginia, Health Sciences Center, P.O. Box 800732, 1300 Jefferson Park Avenue, Charlottesville, VA 22908, USA

ARTICLE INFO

Article history:
Received for publication 13 August 2010
Revised 9 December 2010
Accepted 10 December 2010
Available online 21 December 2010

Keywords:
FoxA
Gastrula
Notochord
Floor plate
Hypochord

ABSTRACT

In vertebrates, embryonic structures present at the dorsal midline, prechordal plate, notochord, hypochord and floor plate share a common embryonic origin. In zebrafish, they derive from a pool of progenitors located within the embryonic shield at the onset of gastrulation. The molecular mechanisms responsible for the common development of these structures remain unknown. Based on their spatial and temporal expression, transcription factors of the Forkhead box A (FoxA) family appeared to be good candidates to play such a role. In agreement with this hypothesis, we found that simultaneous knockdown of FoxA2 and FoxA3 abolish the formation of all axial derivatives, while overexpression of these transcription factors strongly enlarges dorsal mesodermal territories. We establish that, in FoxA2–FoxA3 double morphants, precursors of axial tissues are correctly induced at early gastrula stage, but their dorsal midline identity is not maintained during development and we found that progenitors of these tissues are cell-autonomously re-specified to form muscle fibers as well as cells of the ventral neural tube. Our study provides the first example of a specific loss of all dorsal midline tissues and demonstrates that members of the FoxA family have redundant functions essential to maintain the axial identity of prechordal plate, notochord, floor plate and hypochord progenitors during gastrulation.

Published by Elsevier Inc.

Introduction

The vertebrate body plan is organized around a set of structures present at the dorsal midline, which includes the notochord, the organ that defines the phylum Chordata and contributes to the formation of the vertebral column. In zebrafish these structures also include the prechordal plate, which differentiates into hatching gland, the floor plate and the hypochord. The floor plate, located immediately above the notochord, corresponds to the ventral most part of the neural tube while the hypochord, located below the notochord, is a transient structure important for aorta development (Cleaver and Krieg, 1998). These axial tissues originate from a common pool of precursors located at the dorsal gastrula margin, within the embryonic shield, which is the equivalent of the Spemann organizer (Shih and Fraser, 1995; Melby et al., 1996; Latimer et al., 2002; Latimer and Appel, 2006). Consequently, surgical or genetic removal of the embryonic shield prevents the formation of prechordal plate, notochord, floor plate and hypochord (Shih and Fraser, 1996; Fekany et al., 1999; Saude et al., 2000). Similar observations of a common origin of these tissues have been reported in other vertebrates. In particular, cell-lineage analyses using quail-chick chimeras shows that the Hensen's node, the chick organizer, gives rise to notochord, floor plate and to dorsal endoderm (Catala et al., 1995, 1996; Charrier et al., 1999; Le Douarin et al., 1998).

At the molecular level, the dorsal axial territory is specified and patterned by Nodal activity (Saude et al., 2000; Gritsman et al., 2000; Thisse et al., 2000). Downstream of Nodal, two transcription factors expressed at the dorsal margin are important for axial structures formation: Notail (Ntl) the zebrafish homologue of Brachyury (Schulte-Merker et al., 1992, 1994) and Floating head (Flh), the zebrafish homologue of Not (Talbot et al., 1995). In *ntl* mutants, both the notochord and hypochord are missing and an excess of floor plate cells is generated (Schulte-Merker et al., 1994; Rissi et al., 1995; Halpern et al., 1997). *Flh* mutants display a complete lack of notochord, hypochord and part of the floor plate (Talbot et al., 1995; Halpern et al., 1995). Further analyses suggest that Flh is required in the chordamesoderm to maintain the identity of notochord progenitors by preventing acquisition of paraxial mesodermal fate (Halpern et al., 1995). Although Ntl and Flh are required for the formation of dorsal midline tissues, the hatching gland and part of the floor plate remain present in *ntl;flh* double mutant (Halpern et al., 1997).

To identify other transcription factors involved in the formation of axial structures, we searched for those expressed at early gastrula stages within the dorsal margin and the embryonic shield. Amongst them are members of Forkhead box A (FoxA) family. These proteins are characterized by the presence of a conserved Forkhead DNA binding domain (Weigel and Jackle, 1990). This domain displays a winged helix structure similar to the globular domain of histone H5,

* Corresponding author. Fax: +1 434 982 4081.
E-mail address: bernardthisse@virginia.edu (B. Thisse).

0012-1606/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.ydbio.2010.12.018

suggesting a role in chromatin remodeling (Clark et al., 1993). In zebrafish, the FoxA family is composed of four members (FoxA1, FoxA2, FoxA3 and FoxA). *FoxA2*, *foxA3* and *foxA* are co-expressed in the embryonic shield and in the chordamesoderm at early gastrula stage while the expression of *foxA1* starts later, after the end of gastrulation. Studies on orthologs of these genes have been reported in mouse, chicken and frog embryos (Odenthal and Nusslein-Volhard, 1998; Kaestner et al., 2000) and shown that they are also expressed in axial mesoderm (Ang et al., 1993; Bolce et al., 1993; Dirksen and Jamrich, 1992; Knochel et al., 1992; Lef et al., 1996; Monaghan et al., 1993; Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1993a, 1993b, 1995). Functional analyses of *foxA* genes in different species highlight their importance in the formation of axial structures. In Xenopus, gain of function of FoxA4a (Pintallavis) induces ectopic floor plate in the hindbrain (Ruiz i Altaba et al., 1993b), while overexpression of *foxA2* inhibits the formation of dorsal mesoderm (Suri et al., 2004). In mouse, targeted deletion of *foxa2* abolishes node formation, resulting in the lack of notochord and dorso-ventral patterning defects of the neural tube (Ang and Rossant, 1994; Weinstein et al., 1994). In zebrafish the phenotype of the *foxA2* mutant (*monorail*) is much weaker and is characterized by a failure of floor plate differentiation, while the rest of the axial structures remains unaffected (Norton, et al., 2005). No phenotype affecting the axial structures has been described for FoxA3 morphants.

Here we present evidences that FoxA1, FoxA2 and FoxA3 have redundant activities essential for the formation as well as differentiation of dorsal midline structures. The simultaneous loss of function of these three genes provides the first example of a phenotype restricted to the dorsal midline resulting in a complete absence of all axial structures in zebrafish embryos. We show that, in the absence of FoxA1, A2 and A3 activity, axial tissues are induced but are not maintained during gastrulation. Instead dorsal midline progenitors induced at early gastrula stage are cell-autonomously re-specified to paraxial mesoderm and ventral neurectoderm fates. This shows that FoxA proteins are required to maintain the axial identity of dorsal midline progenitors.

Results

FoxA2 and FoxA3 have redundant functions essential for the formation of all axial structures

To determine whether FoxA transcription factors are required for the formation of axial structures in zebrafish, we analyzed their function by using single or multiple morpholino knockdowns. Inactivation of FoxA2 leads to ventrally curved embryos that fail to differentiate the floor plate (Fig. 1B, G, Q, and V; 95%, $n=202$), a phenotype identical to *monorail*/*foxA2* mutant (Norton et al., 2005). Knockdown of FoxA3 results in an increased accumulation of its transcript in the embryonic axis as revealed by in situ hybridization (Fig. 2M and N). Therefore, the complete inactivation of FoxA3 requires the use of two morpholinos at high concentration. Under these conditions, knockdown of FoxA3 leads to an absence of hatching gland characterized by the lack of *hgg1* (a terminal differentiation marker of the hatching gland which encodes the Cathepsin L1b, one of the hatching enzymes) expression at 24 hpf (Fig. 2E; 100%, $n=311$). In these morphants, the prechordal plate markers *goosecoid* (*gsc*) and *noggin1* (*nog1*) are expressed normally at gastrula stage (Fig. 2F and G), but the expression of *hgg1* is not initiated (Fig. 2H). This shows that in FoxA3 morphants the anterior axial mesoderm, the prechordal plate, is properly induced but failed to differentiate into hatching gland. Looking for potential effects on the expression of other axial mesoderm markers, we found that FoxA3 morphants maintain expression of *ntl* in the prechordal plate during gastrulation, while transcript for this gene rapidly disappeared from this territory in wild-type (Fig. 2K and O). This may suggest that the lack of differentiation of prechordal plate into hatching gland may be due to the ectopic expression of *ntl* in this territory. However, injection of FoxA3 morpholinos in homozygous *ntl* mutants does not rescue the differentiation of the prechordal plate (Fig. 2P). Finally, in addition to the lack of hatching gland, a small fraction (8%, $n=311$) of FoxA3 morphants shows a locally disorganized notochord (data not shown).

While *foxa2* mutation has a dramatic effect on axial mesoderm development in mouse, the single inactivation of FoxA2 or FoxA3 in zebrafish only affects floor plate or hatching gland formation, respectively. Because these transcription factors are co-expressed at the dorsal midline they may be partially redundant for the formation of axial structures. We probed this hypothesis by performing combined knockdown of FoxA2 and FoxA3. For two-thirds of the embryos, the notochord is truncated caudally (Fig. 1D, I, and N; 62% $n=434$) and often presents local disorganizations, with notochordal cells invading the overlying neural tube (Fig. 1I and N; Fig. S1B and C). In addition, the remaining notochordal cells appear smaller than in wild-type (Fig. S1B and E). The last third of embryos displays a stronger phenotype with a complete deletion of notochord and fusion of somites underneath the neural tube (Fig. 1E, J, and O; 35%, $n=434$). In addition, double morphants have shorter and disorganized floor plate and hypochord (Fig. 1S and X) or do not show any floor plate or hypochordal cells (Fig. 1T and Y). When some floor plate cells are remaining in the embryo, instead of displaying their characteristic cuboidal shape, they appear elongated along the dorso-ventral axis of the neural tube (Fig. S1D–F).

In conclusion, the double inactivation of FoxA2 and FoxA3 strongly disrupts or even abolish the formation of all axial structures: hatching gland, notochord, floor plate and hypochord.

Because the penetrance of the strongest phenotype, characterized by a complete loss of midline structures in FoxA2–FoxA3 morphants, is rather low we hypothesized the existence of possible redundant activities carried by the other FoxA family members: FoxA1 and FoxA. While single knockdown of FoxA1 does not affect embryonic development (Fig. S2B and C), combined inactivation of FoxA1, FoxA2 and FoxA3 results in a complete lack of axial structures in almost all triple morphants (98% $n=55$; Fig. S2D, F, and I). This shows that FoxA1 contributes at least partially to the development of axial midline tissue. However, because its expression initiates much later than *foxA2* and *foxA3* (Fig. S2A, early somitogenesis), its function has not been analyzed further. Finally, morpholino knockdowns of FoxA did not reveal any role for this factor in the formation or differentiation of the axial structures (data not shown).

FoxA transcription factors are required for the maintenance of axial structures

The lack of dorsal midline structures in FoxA genes knockdown may be due either to an impairment of the induction of axial precursors at blastula and early gastrula stages, or to a defect in the maintenance and differentiation of these precursors. To distinguish between these two hypotheses, we analyzed the expression of axial and paraxial mesoderm markers during gastrulation. At late gastrula stage, *twist2* (a marker of chordamesoderm) and *myf5* (a marker of paraxial mesoderm) expression domains remained unaffected in FoxA2 morphants (Fig. 3B and G). In the absence of FoxA3, the chordamesoderm is slightly narrower while the paraxial mesoderm is weakly extended toward the midline (Fig. 3C and H). In double morphants, *twist2* expression is either strongly diminished or almost abolished (Fig. 3D and E), while *myf5* expression domain is dorsally expanded (Fig. 3I) and can even be fused along the axis (Fig. 3J; 6% $n=38$). Because the absence of axial midline could result from a failure in its induction, we looked at the presence of axial mesoderm precursors at the beginning of gastrulation using molecular markers specific of the dorsal margin at the shield stage: *flh* (Fig. 3K–N), *chordin* and *fadd* (data not shown). In the absence of FoxA2 and