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The role of *Otx2* in organizing the anterior patterning in mouse

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ABSTRACT Understanding the molecular mechanism controlling induction and maintenance of signals required for specifying anterior territory (forebrain and midbrain) of the central nervous system is a major task of molecular embryology. The current view indicates that in mouse, early specification of the anterior patterning is established at the beginning of gastrulation by the anterior visceral endoderm, while maintenance and refinement of the early specification is under the control of epiblast-derived tissues corresponding to the axial mesendoderm and rostral neuroectoderm. In vertebrates a remarkable amount of data has been collected on the role of genes contributing to brain morphogenesis. Among these genes, the *orthodenticle* group is defined by the *Drosophila orthodenticle* and the vertebrate *Otx1* and *Otx2* genes, which contain a *bicoid*-like homeodomain. Mouse models and chimera experiments have provided strong evidence that *Otx2* plays an important role in the specification and maintenance of the rostral neuroectoderm destined to become forebrain and midbrain. In evolutionary terms, some of these findings lead us to hypothesize a fascinating and crucial contribution of the *Otx* genes to the genetic program underlying the establishment of the mammalian brain.

KEY WORDS: *Otx2, Otx1, gastrulation, AVE, head specification.*

Introduction

Fate and patterning of tissues depend on the activity of organizer cells emanating signals to a responding tissue which undergoes morphogenetic changes resulting in a specific differentiated fate (Spemann and Mangold, 1924; Waddington, 1932; Gurdon, 1987). Indeed, in amphibians, the dorsal lip of the blastopore induces a new, ectopic secondary axis when transplanted on the ventral side of a host embryo. Because of this ability, the dorsal lip of the blastopore has been called the organizer (Spemann and Mangold, 1924). Further experiments in amphibian and chick embryos have suggested that the age of the organizer tissue influences the extension of the induced neural plate as well as its regional identity. An organizer deriving from an early gastrula induces anterior as well as posterior neural tissue, whereas a late organizer induces only posterior tissue (Gallera, 1971; Nieuwkoop *et al.,* 1985; Storey *et al.,* 1992).

These results suggested the possibility that head and trunk inducing ability of the organizer might be associated to different cell populations coexisting in an early organizer. In mouse, at late streak stage the node is located at the rostral end of the primitive streak and is able to induce a secondary axis. Importantly, the secondary axis lacks any anterior neural tissues (Beddington, 1994) suggesting a functional parallelism with the amphibian late organizer. Inducing properties of an early mouse node have been tested in transplantation experiments, and also in this case it fails to induce anterior identity (Tam *et al.,* 1997), thus indicating that, unlike the amphibian organizer, the mouse node is unable to induce rostral neuroectoderm independently of its age.

Surprisingly, a number of evidences have highlighted a crucial role for the anterior region of the visceral endoderm (AVE) in specifying anterior neural patterning in mouse (reviewed in Beddington and Robertson, 1999). The visceral endoderm (VE) is an extraembryonic tissue surrounding the epiblast cells at the pre-early streak stage. Subsequently, the AVE cells move into the extraembryonic region and are replaced by the definitive endoderm that originates from the rostralmost portion of the primitive streak, the node. The definitive endoderm and the dorsal mesoderm constitute the so-called axial mesendoderm.

Expression analysis and cell lineage studies remarkably contributed to define the origin and the molecular identity of the AVE. The AVE precursor cells are located at the distal tip of the mouse pre-streak embryo and can be visualized by the expression of the *Hex* gene (Thomas *et al.,* 1998). This group of cells originates only anterior descendants giving rise to a first asymmetry along the

Abbreviations used in this paper: AVE, anterior visceral endoderm; *cer-l,* cerberus-like; CNS, central nervous system; d.p.c., days post coitum; *En,* engrailed; *gsc,* goosecoid; *otd,* Drosophila orthodenticle; RA, retinoic acid; VE, visceral endoderm.

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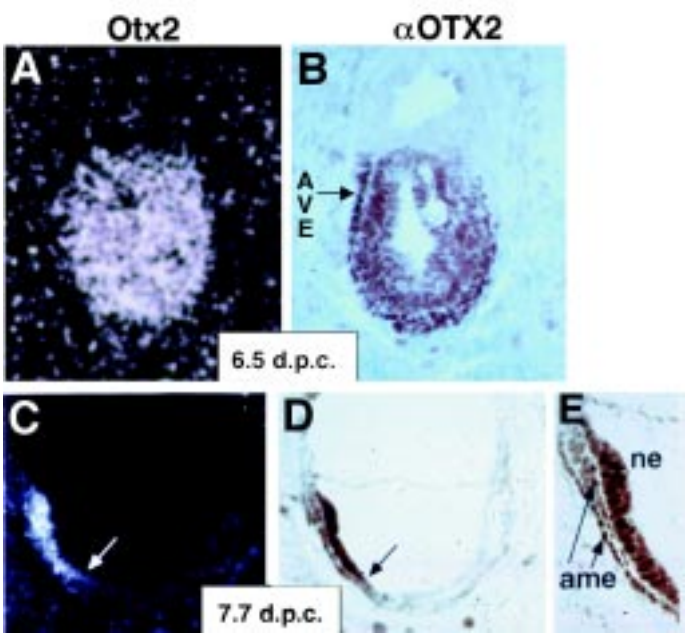

Fig. 1. *Otx2* mRNA and protein distribution in gastrulating mouse embryos. (A-E) *Otx2 is transcribed (A,C) and translated (B,D,E) in the AVE and epiblast cells at early-streak stage (6.5 d.p.c.) (A,B), and in the anterior neuroectoderm and axial mesendoderm at headfold stage (7.7 d.p.c.). Abbreviations: AVE, anterior visceral endoderm; ame, anterior mesendoderm; ne, neuroectoderm. The arrow in (C), (D) points to the Otx2 posterior border of expression.*

presumptive antero-posterior axis of the embryo. This asymmetry is reinforced by the expression of a number of genes such as *Hex, Hesx1, goosecoid (gsc), cerberus-like (cer-l), Lim1, Otx2* and *nodal* (Simeone *et al.,* 1993; Thomas and Beddington, 1996; Belo *et al.,* 1997; Varlet *et al.,* 1997; Dattani *et al.*, 1998; Thomas *et al.,* 1998).

Cell ablation and tissue recombination experiments have indicated the functional relevance of the AVE in inducing anterior patterning. Indeed, it has been shown that: i) the removal of a patch of AVE cells expressing the *Hesx1* gene prevents the subsequent expression of the gene in the rostral neuroectoderm which becomes reduced and abnormally patterned (Thomas and Beddington, 1996); ii) recombining chick epiblast with rabbit pre-streak AVE, the expression of forebrain markers is induced while chick hypoblast is unable to mediate the same response thus suggesting that chick hypoblast and murine AVE might not have shared the property of specifying anterior neural patterning (Knötgen *et al.,* 1999), and iii) chimera and transplantation experiments as well as the analysis of mouse mutants have indicated that a number of genes, including *Lim1, Otx2* and *nodal* are required in the AVE for proper specification of the anterior neuroectoderm (Acampora *et al.,* 1995, 1998b; Matsuo *et al.,* 1995; Shawlot and Behringer, 1995; Ang *et al.,* 1996; Varlet *et al.,* 1997; Rhinn *et al.,* 1998; Shawlot *et al.,* 1999). Therefore, altogether these findings support the notion that in mouse the AVE contains genetic information required to instruct the patterning of the rostral neuroectoderm.

Although the mouse node is unable to duplicate anterior structures in transplantation experiments, it gives rise to tissues such as the prechordal mesoderm, the definitive endoderm and the notochord that are similar to those originated by the amphibian organizer and expresses similar genes (Beddington, 1981; Lawson *et al.,* 1991). Moreover, there is clear evidence that the murine node and its derivatives are able to emanate neuralizing signals (Ruiz i Altaba, 1993, 1994; reviewed in Beddington and Robertson, 1999) and to induce the expression of the midbrain-hindbrain marker *engrailed* in the neuroectoderm (Rhinn *et al.,* 1998). Indeed, early patterning of the CNS primordium is also controlled by additional mechanisms involving vertical signals emitted from the axial mesendoderm underlying the neural plate and planar signals acting through the neuroectodermal plane (Doniach, 1993; Ruiz i Altaba, 1993, 1994). Furthermore, it has been shown that in *zebrafish* a small group of ectodermal cells located in the anteriormost head region is required for the patterning and survival of the anterior brain (Houart *et al.,* 1998; Ruiz i Altaba, 1998).

Therefore, these and other evidences (see below) indicate that while initiation of the anterior patterning is under the control of the AVE, epiblast-derived tissues are required to maintain and/or refine the rostral identity of the neural plate as well as to induce patterning of trunk and hindbrain. Among the genes required in the early specification of the anterior neural plate, the homeobox-containing gene *Otx2* plays a remarkable role in both induction and maintenance of the rostral neural patterning (Simeone, 1998; Acampora and Simeone, 1999). These and other potential roles are now subject of intense study that takes advantage from genetically modified mouse models and embryological approaches.

Specification of anterior patterning requires *Otx2* function in the AVE

The *orthodenticle* group is defined by the *Drosophila orthodenticle (otd)* and the vertebrate *Otx1* and *Otx2* genes (Finkelstein and Boncinelli, 1994; Chen *et al.,* 1997; Freud *et al.,* 1997). Murine OTX1 and OTX2 gene products share extensive sequence similarities even though in OTX1, downstream of the homeodomain, these regions of homology to OTX2 are separated by stretches of additional amino acids including repetitions of alanine and histidine residues (Simeone *et al.,* 1993). In mouse *Otx1* expression is first detected at the 2-5 somite stage throughout the fore- and midbrain neuroepithelium. *Otx2* is already transcribed before the onset of gastrulation in the epiblast and in the visceral endoderm (VE), and at the end of gastrulation in the axial mesendoderm and rostral neural plate (Simeone *et al.,* 1992, 1993; Ang *et al.,* 1994). During brain regionalization, *Otx1* and *Otx2* show largely overlapping expression domains with a posterior border coincident with the mesencephalic side of the isthmic constriction (Simeone *et al.,* 1992; Millet *et al.,* 1996). Therefore, during gastrulation *Otx2* is transcribed and translated in the cells that are believed to emanate signals in early specification and patterning of the neural plate (AVE and axial mesendoderm) as well as in those responding to these instructing signals (epiblast and anterior neuroectoderm) (reviewed in Simeone, 1998; Acampora and Simeone, 1999) (Fig. 1). The first indication that *Otx2* was responsive to inductive interactions came from explant-recombination experiments in gastrulating mouse embryos showing that a positive signal from anterior mesendoderm of headfold stage embryos is able to maintain *Otx2* expression in the anterior ectoderm of early streak embryos, and that a negative signal from the posterior mesendoderm, mimicked by exogenous retinoic acid (RA), represses *Otx2* expression in the anterior ectoderm of late streak embryos (Ang *et al.,* 1994). Similar interactions have been also demonstrated in *Xenopus* (Blitz and Cho, 1995).

The possibility that RA might contribute to the early distinction between fore-midbrain and hindbrain by controlling *Otx2* expression is supported by the finding that administration of exogenous RA at mid-late streak stage represses *Otx2* expression in both the axial mesendoderm and the posterior neural plate (Ang *et al.,* 1994; Simeone *et al.,* 1995; Avantaggiato *et al.,* 1996). This repression correlates with the appearance of microcephalic embryos that show early anteriorization of *Hoxb1* expression, hindbrain expansion (Sive and Cheng, 1991; Conlon and Rossant, 1992; Marshall *et al.,* 1992), loss of forebrain molecular and morphological landmarks, and gain of midbrain molecular markers in the rostralmost neuroectoderm (Simeone *et al.,* 1995; Avantaggiato *et al.,* 1996). Moreover, *Otx2* responsiveness to RA application is a common feature in different species including *Xenopus* and chick (Bally-Cuif *et al.,* 1995; Pannese *et al.,* 1995). Nevertheless, the question of whether endogenous RA plays a physiological role in rostral CNS demarcation by contributing to the establishment of the posterior border of *Otx2* expression, still remains open.

The evidence that *Otx2* may play a remarkable role in rostral CNS specification derives from *in vivo* genetic manipulation experiments performed in mouse and *Xenopus* which, to some extent, complement each other. In *Xenopus,* microinjection of synthetic *Otx2* RNA results in an abnormal reduction of the size of tail and trunk structures, and in the appearance of a second cement gland (Blitz and Cho, 1995; Pannese *et al.,* 1995). These phenotypes have been interpreted either with a possible *Otx2*-mediated interference with movements of extension and convergence during gastrulation (for trunk and tail reduction) and/or with an *Otx2*-requirement in the specification of anteriormost head structures (for the ectopic cement gland). Moreover, by using a dexamethasone-inducible OTX2 protein it has been shown that the *Xenopus Otx2* activity is regulated by regionally restricted factor(s), and that the cement gland-specific gene XCG may represent a direct target of the *Otx2* gene product (Gammill and Sive, 1997).

In mouse, *Otx2* null embryos die early in embryogenesis, lack the rostral neuroectoderm fated to become forebrain, midbrain and rostral hindbrain, and show heavy abnormalities in their body plan

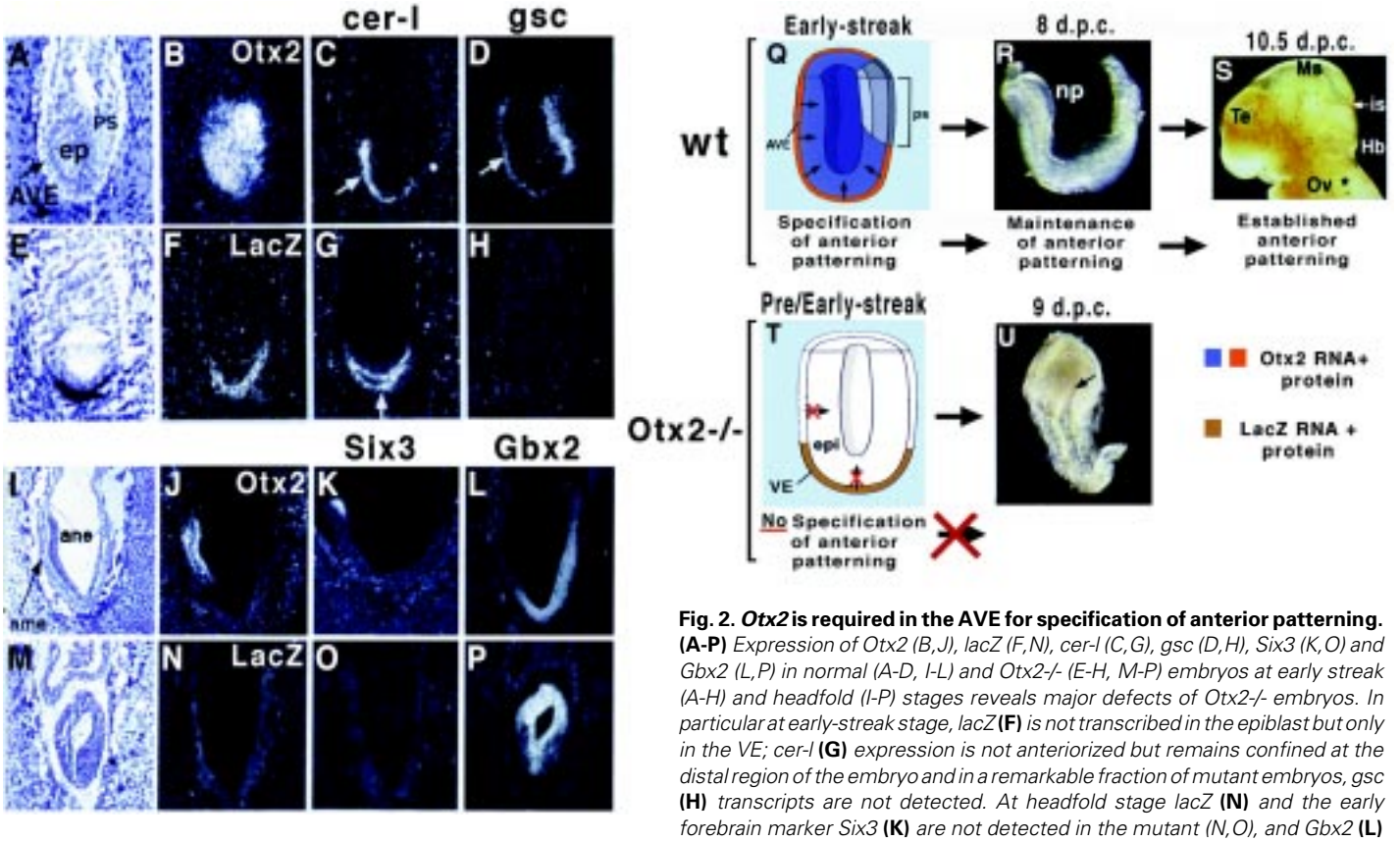

Fig. 2. *Otx2* is required in the AVE for specification of anterior patterning. **(A-P)** *Expression of Otx2 (B,J), lacZ (F,N), cer-l (C,G), gsc (D,H), Six3 (K,O) and Gbx2 (L,P) in normal (A-D, I-L) and Otx2-/- (E-H, M-P) embryos at early streak (A-H) and headfold (I-P) stages reveals major defects of Otx2-/- embryos. In particular at early-streak stage, lacZ* **(F)** *is not transcribed in the epiblast but only in the VE; cer-l* **(G)** *expression is not anteriorized but remains confined at the distal region of the embryo and in a remarkable fraction of mutant embryos, gsc* **(H)** *transcripts are not detected. At headfold stage lacZ* **(N)** *and the early forebrain marker Six3* **(K)** *are not detected in the mutant (N,O), and Gbx2* **(L)** *that is normally adjacent to the posterior border of Otx2* **(J)**, *is transcribed throughout all the presumptive neuroectoderm and proximal mesoderm* **(P)**. **(Q-U)** *Findings from Otx2 null embryos (T,U) suggest that in wild-type embryos (Q-S) at the early-streak stage Otx2 is required in the VE to mediate signal(s) (arrows in Q) that are directed to the epiblast and are required for specification of anterior patterning, while at late gastrula-headfold stage, Otx2 is required to maintain fore-midbrain regional identities (see also Fig. 3) even tough it cannot be assessed in which tissue (anterior mesendoderm or neural plate) it has this role. In null embryos (T-U) where Otx2 is replaced with the lacZ gene, the first impairment is seen at the pre-early streak stage* **(T)** *when lacZ transcription is lost in the epiblast and confined at the distal tip of the embryo. This observation indicates that at least one copy of the normal Otx2 allele is necessary in the VE for rescuing signal(s) necessary for its transcription in the epiblast, for specification of anterior patterning and proper gastrulation. These findings suggest that Otx2 is an important component of VE organizing properties. Abbreviations as in the previous Figure plus: ane, anterior neuroectoderm; epi, epiblast; ps, primitive streak; np, neural plate; Ov, otic vescicle; Te, telencephalon; Ms, mesencephalon; is, isthmus; Hb, hindbrain; VE, visceral endoderm. The arrow in (U) points to the rostral limit of the neuroectoderm in an Otx2-/- embryos.*

(Acampora *et al.,* 1995; Matsuo *et al.,* 1995; Ang *et al.,* 1996). Heterozygous *Otx2+/-* embryos in an appropriate genetic background show defects of the head such as serious brain abnormalities and craniofacial malformations, which are reminiscent of otocephalic phenotypes (Matsuo *et al.,* 1995). The analysis of *Otx2* null embryos revealed that at late streak stage, the rostral neuroectoderm is not specified and the primitive streak as well as the node and the axial mesendoderm are severely impaired. Therefore, one possibility is that the resulting headless phenotype might be due to the abnormal development of node-derived axial mesendoderm which lacks head organizer activity. A second possibility is that neural inducing properties of the AVE are severely impaired or abolished. In embryos replacing *Otx2* with the *lacZ* reporting gene, the first abnormality is already detected at the early streak stage (Acampora *et al.,* 1995). Indeed, at this stage, *lacZ* transcription and staining are abolished in the epiblast while they remain high in the VE of *Otx2-/-* embryos, the *goosecoid (gsc)* expression is undetectable or confined to the proximal region of the mutant embryos (Acampora *et al*., 1995) and the presumptive AVE does not anteriorize, thus remaining confined to the distal region of the embryo (Fig. 2). Therefore, these results indicate that headless phenotype and abnormal organization of the primitive streak may be determined very early at the pre-early-streak stages by an impairment of AVE properties.

Moreover, as revealed by the primitive streak early marker *Brackyury (T),* epiblast cells do not migrate posteriorly at the site of the primitive streak formation but remain for an abnormal longer period in the circumpherential ring close to embryonic-extraembryonic boundary. Only later and in a fraction of *Otx2-/-* embryos the presumptive AVE cells appear anteriorized. However, although abnormal, a primitive streak forms in all the *Otx2-/-* embryos (Acampora *et al.,* 1995, 1998b; Ang *et al.,* 1996). Indeed, mesodermal cells appear concentrated at the posterior third of the embryo and both the node and axial mesendoderm are heavily abnormal or absent as detected by histological and molecular analyses (Acampora *et al.,* 1995; Ang *et al.,* 1996).

These findings, therefore, favor a relation between primitive streak formation and anterior location of the AVE and suggest that *Otx2,* normally expressed in both epiblast cells and AVE, might be required in these two cell types in order to mediated proper positioning of the AVE and normal formation of the primitive streak. However, data deduced from the analysis of chimeric embryos and further mouse models indicate that early abnormalities in both AVE and primitive streak of *Otx2-/-* embryos should be ascribed to *Otx2* requirement in the VE (Acampora *et al.,* 1998b; Rhinn *et al.,* 1998; see below).

An intriguing feature of *Otx2-/-* embryos is that lack of anterior neuroectoderm correlates with failure of epiblast cells to express the *lacZ* reporter gene. Thus, since *Otx2* is already transcribed from the earliest stages (unfertilized egg in *Xenopus* and at least morula in mouse), this observation suggests that maintenance of *Otx2* transcription in the epiblast cells requires at least one normal allele expressed in the AVE while *Otx2* transcription in the latter is independent from the presence of a normal allele (Simeone, 1998; Acampora and Simeone, 1999). Moreover, these findings support the existence of *Otx2*-mediated signal(s) emitted from the AVE and directed to the epiblast cells (Fig. 2). Nevertheless, it is still unclear whether the same signal operates to mediate both specification of anterior patterning and *Otx2* transcription in epiblast cells or these two events are independently controlled.

The role of *Otx2* in induction and maintenance of rostral CNS

In order to understand the role of *Otx2* in the specification of the anterior patterning, it is of particular relevance to define its functional contribution to the different tissues where it is expressed during gastrulation. More direct evidence proving a role for *Otx2* in the AVE has been recently provided by generating murine chimeric embryos and new mouse models (Acampora *et al.,* 1998b; Rhinn *et al.,* 1998; Acampora *et al.,* unpublished results). Chimeric embryos containing *Otx2-/-* epiblast and wild-type VE rescue an early *Otx2-/-* neural plate but subsequently they fail to develop a brain, suggesting that *Otx2* is required in the AVE for induction of rostral neural plate and, subsequently, in the epiblast-derived tissues for specification of forebrain and midbrain regional identities. Conversely, when chimeric embryos consist of an *Otx2-/-* VE and an *Otx2+/+* epiblast, none of the phenotypic features of *Otx2-/-* embryos are rescued (Rhinn *et al.,* 1998). This latter result also argues, as previously suggested by *Otx2-/-* mice (Acampora *et al.,* 1995), that impaired axial mesendoderm of *Otx2-/-* embryos is a consequence of *Otx2* requirement at earlier stages in the VE.

Mice replacing *Otx2* with *Otx1* were originally generated in order to assess whether the two proteins shared functional equivalence or, alternatively, displayed unique properties specified by their limited amino acid divergence (Acampora and Simeone, 1999). Interestingly, homozygous mutant embryos replacing *Otx2* with the human *Otx1* (*hOtx1*) cDNA (*hOtx1*^{2}/*hOtx1*^{2}) recover anterior neural plate induction and normal gastrulation but show a headless phenotype from 9 days post coitum (d.p.c.) onwards. A combined analysis of both *hOtx1* RNA and protein distribution during early gastrulation has revealed that while the *hOtx1* mRNA is detected in the VE and epiblast, the hOTX1 protein is revealed only in VE (Fig. 3). Nevertheless, this VE-restricted translation of the *hOtx1* mRNA is sufficient to recover early anterior neural plate but fails to maintain fore-midbrain identities, and consequently *hOtx1*^{2}/*hOtx1*^{2} embryos display a headless phenotype with a normal body plan (Acampora *et al.,* 1998b). In fact at early-streak stage, *hOtx1*^{2}/*hOtx1*^{2} embryos recover anteriorization of the AVE, normal primitive streak and proper expression of *Lim1, cer-l, Hesx1, gsc* and *T*. At late gastrula stage, the anterior patterning of the rostral neural plate is correctly defined by the expression of fore-, mid- and hindbrain markers such as *Six3, Pax2, Gbx2* and *Hoxb1* and the axial-mesendoderm properly expresses *Lim1, Noggin, cer-l* and *Hesx1* thus, leading to argue that, as deduced in chimera study, *Otx2* is required in the AVE for initiating the specification of the anterior patterning and demarcation of forebrain and midbrain territories (Fig. 3 and compare to Fig. 2) (Acampora *et al*., 1998b).

Further analysis of *hOtx1*^{2}/*hOtx1*^{2} embryos reveals that at the early somite stage the forebrain markers *BF1* and the *hOtx1*^{2} mRNA are not detected in the rostral neuroectoderm where mid-hindbrain markers such as *Wnt1, En1, Fgf8, Gbx2* and *Pax2* appear to be co-expressed. Therefore, these findings lead to argue that the OTX2 gene product is required from the headfold stage onwards to maintain the anterior patterning previously established by the AVE. In this respect, the phenotype observed at the early somite stage appears to be the consequence of an antero-posterior repatterning involving the entire anterior neural plate (fore-midbrain) which, in the absence of any OTX gene product, adopts a more posterior fate (hindbrain) (Acampora *et al.,* 1998b; Acampora and Simeone, 1999). Therefore, the *hOtx1*^{2}/*hOtx1*^{2} mouse model allows to uncouple two distinct

phases both requiring *Otx2:* early induction of anterior neural patterning that is under the control of AVE and its subsequent maintenance that is likely mediated by epiblast-derived cells (the axial mesendoderm and neuroectoderm). However, since *Otx2* is normally transcribed and translated in both axial mesendoderm and rostral neuroectoderm, while *hOtx1*^{2} is transcribed but not translated neither in the axial mesendoderm nor in the rostral neuroectoderm, it cannot be deduced whether *Otx2* is required in one or both of these tissues to mediate maintenance properties of the anterior identity and whether *hOtx1* is functionally equivalent to *Otx2* also in these tissues. A cell-autonomous role of *Otx2* in the neuroectoderm is emerged by the analysis of chimeras with a moderate contribution of *Otx2-/-* cells (Rhinn *et al.,* 1999). From this analysis it has been shown that genes such as *Wnt1, Hesx1* and *R-cadherin* are selectively not expressed in *Otx2-/-* cells while other genes such as *En2* and *Six3* are uniformly expressed in neuroectoderm cells of both genotypes. Nevertheless, this finding does not help to understand whether *Otx2* plays a non-cell autonomous role in the axial mesendoderm. In this context, it is noteworthy that *Lim1* is correctly expressed in the anterior mesendoderm of *hOtx1*^{2}*/hOtx1*^{2} embryos and that *Lim1-/-* and *hOtx1*^{2}*/hOtx1*^{2} embryos show impressive phenotypic similarities. Recently, the stage and the cell type in which *Lim1* is required for head specification have been approached by chimeras and tissue recombination experiments (Shawlot *et al.,* 1999). These experiments have revealed that *Lim1* is required in the AVE for inducing anterior neural pattern and, subsequently, in the axial mesendoderm to refine and maintain the anterior identity. Therefore, since maintenance of anterior pattern requires *Otx2* in the axial mesendoderm or in the anterior neuroectoderm or in both tissues, it can be speculated that *Lim1* might contribute to mediating the release of axial-mesendoderm signal(s) instructing maintenance of anterior character and *Otx2* might confer to the neuroectoderm the competence in responding to this signal(s) emitted from the mesendoderm. This possibility is also compatible with a cell-autonomous role within the neuroectoderm and does not exclude an additional role also in the axial mesendoderm.

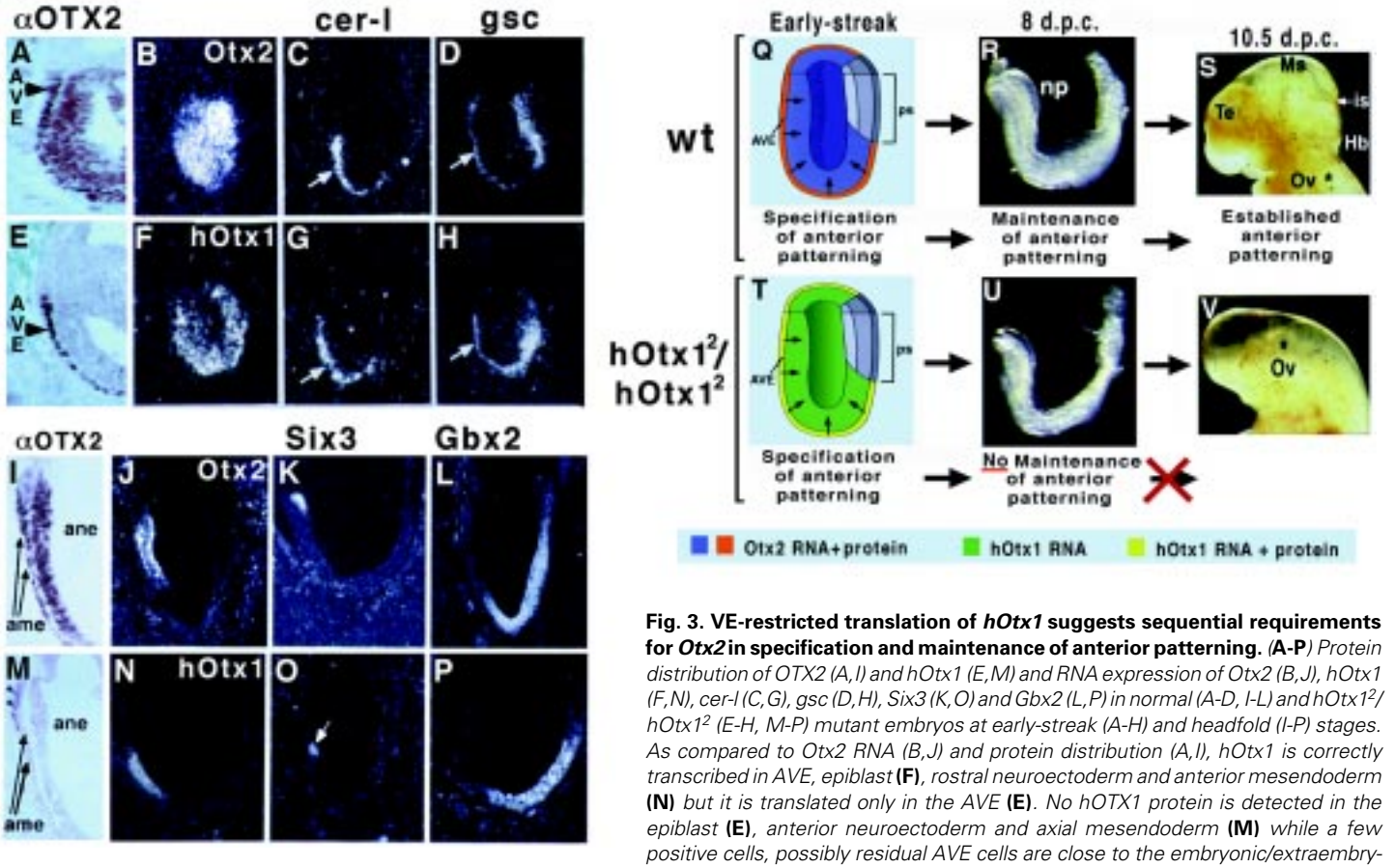

Fig. 3. VE-restricted translation of *hOtx1* suggests sequential requirements for *Otx2* in specification and maintenance of anterior patterning. (**A-P**) *Protein distribution of OTX2 (A,I) and hOtx1 (E,M) and RNA expression of Otx2 (B,J), hOtx1 (F,N), cer-l (C,G), gsc (D,H), Six3 (K,O) and Gbx2 (L,P) in normal (A-D, I-L) and hOtx1*^{2}*/ hOtx1*^{2} *(E-H, M-P) mutant embryos at early-streak (A-H) and headfold (I-P) stages. As compared to Otx2 RNA (B,J) and protein distribution (A,I), hOtx1 is correctly transcribed in AVE, epiblast* **(F)**, *rostral neuroectoderm and anterior mesendoderm* **(N)** *but it is translated only in the AVE* **(E)**. *No hOTX1 protein is detected in the epiblast* **(E)**, *anterior neuroectoderm and axial mesendoderm* **(M)** *while a few positive cells, possibly residual AVE cells are close to the embryonic/extraembryonic boundary (arrowheads in M).* **(Q-V)** *In wild-type embryos at early streak stage (6.5 d.p.c.) Otx2 mRNA and protein colocalize in VE and epiblast cells. At the same stage, in hOtx1*^{2}*/hOtx1*^{2} *embryos, hOtx1 transcripts are correctly detected in AVE and epiblast cells while, in contrast, the hOTX1 protein is restricted to the VE. Comparison of the two genotypes confirms that the OTX2 gene product is required in the AVE for specification of the early anterior neural plate and that hOTX1 and OTX2 proteins are functionally equivalent in the AVE. Moreover, the differential post-transcriptional control of hOtx1 transcripts makes it possible to distinguish between the specification and maintenance properties of Otx2. In fact, while the specification of the anterior neural plate and normal gastrulation are rescued by AVE-restricted hOTX1 protein, the maintenance of anterior patterning is not recovered for the absence of any OTX protein in the epiblast-derived cells (anterior neural plate and anterior mesendoderm). This finding leads to the attractive hypothesis that Otx2-mediated maintenance properties (Otx2 translation in epiblast cells) might have been acquired in early vertebrate evolution. This new function in epiblast cells might have represented a crucial molecular aspect in vertebrate head specification. Abbreviations as in previous Figures.*

A recent *in vitro* study supports the possibility of a direct protein-protein interaction between *Otx2* C-terminal and *Lim1* homeodomain (Nakano *et al.,* 2000). Furthermore, the *hOtx1*^{2} mouse model together with findings deduced from the analysis of mice lacking the *Cripto* gene (Ding *et al.,* 1998) may lead to some tentative considerations on the temporal stability of AVE signal(s). The *Cripto* gene encodes a membrane-associated protein containing epidermal growth factor-like motifs and is expressed shortly before the onset gastrulation in the proximal region of the epiblast where the primitive streak forms (Dono *et al.,* 1993; Ding *et al.,* 1998; Minchiotti *et al.,* 2000).

Cripto-/- embryos lack a primitive streak and do not anteriorize the AVE thus failing to convert the proximo-distal into antero-posterior axis (Ding *et al.,* 1998). Nevertheless, the AVE markers are correctly expressed but in a distal position and the epiblast does express rostral neuroectoderm markers such as *Otx2* and *En* until 8 - 8.5 d.p.c., thus indicating that in the absence of a node and derived tissues the epiblast acquires the identity of anterior neuroectoderm. Moreover, in *hOtx1*^{2}*/hOtx1*^{2} embryos, where the OTX1 protein is detected only in the AVE, anterior pattern is detected until 8 d.p.c. Similar results have been deduced from the analysis of chimeras composed of *Otx2-/-* epiblast and wild-type VE. Together these findings indicate that i) the AVE, even in an ectopic position may confer the anterior character to the surrounding epiblast cells; ii) in the absence of a node and axial mesendoderm, signal(s) emitted from the AVE may be efficient in initiating and maintaining the anterior specification until late streak-early somite stage and iii) *Otx2* codes for an important genetic component of the pathway leading the AVE to instruct the anterior patterning.

Otx genes and the evolution of brain complexity

In our opinion, one of the most interesting observations is the existence of a differential post-transcriptional control of the *hOtx1* mRNA between the VE and the epiblast cells. In heterozygous *hOtx1*^{2}*/Otx2* embryos the *Otx2* mRNA and protein colocalize while the *hOtx1* mRNA is translated only in the VE, thus suggesting that the *hOtx1* mRNA detected in epiblast cells is post-transcriptionally regulated by an *Otx2*-independent *cis*-acting control (Acampora *et al.,* 1998b).

In *Otx2+/-* embryos, the same *Otx2* region that is replaced by the *hOtx1* cDNA is substituted with the *lacZ* gene fused to the SV40 polyA site (Acampora *et al.,* 1995). In these embryos the *lacZ* mRNA is correctly detected in VE and epiblast while the staining is heavily reduced in the epiblast at early-mid streak stage (Acampora *et al.,* 1995). Furthermore, it is noteworthy that recently a new mouse model replacing the same *Otx2* region with the *Drosophila otd* cDNA generates a phenotype displaying impressive similarities with that of the *hOtx1*^{2}*/hOtx1*^{2} embryos. Also in this case, the *otd* mRNA is transcribed in epiblast and VE but the protein is detected only in the VE and the VE-restricted OTD protein is sufficient to rescue specification of anterior patterning and normal gastrulation but fails in maintenance of anterior patterning (Acampora *et al.,* unpublished results). Therefore, three different mouse models replacing the same *Otx2* genomic region with three different genes (*lacZ, hOtx1* and *otd*) converge towards the possibility that VE and epiblast cells are characterized by different post-transcriptional control properties.

Moreover, these mouse models also suggest that the *Otx2* replaced region, possibly the 3' UTR, might contain regulatory element(s) required for the *Otx2* translation in the epiblast cells. However, such molecular element(s) is actually unknown and *in vivo* experiments aimed at generating new mouse models specifically addressing this issue will certainly contribute towards unmasking eventual *Otx2* post-transcriptional control element(s) that at the moment can be only hypothesized. Nevertheless, since the *Otx2* locus is heavily engineered and does not contain introns, 3' UTR and part of the 5' UTR, it could be possible that the loss of mRNA translation in the epiblast might be also influenced by abnormal molecular event(s) affecting RNA stability, processing and transport of the chimeric knock-in transcripts. However, whatever the impairments are, since in the VE the mRNA is correctly translated, the absence of protein should be considered a peculiar event occurring in epiblast cells and their derivatives.

The finding that *Otx2* escapes this post-transcriptional control suggests that this is necessary for the maintenance of fore-midbrain territory specified by the VE (Acampora *et al.,* 1998b) and might have evolutionary implications. Indeed, the architectural components of the vertebrate brain (telencephalon, diencephalon and mesencephalon) are less clear in protochordates and a vertebrate-type brain, which is composed of a midbrain and six prosomers, firstly appears in Petromyzontoidea. It may be hypothesized that the specification of this prosomeric brain might have required the presence of a sufficient level of OTX2 protein in the early neuroectoderm cells and this event might have been acquired by modifying post-transcriptional control of *Otx2* transcripts rather that its coding sequence which would have retained common functional properties among the *otd*-related genes throughout evolution.

Comparative studies have demonstrated the existence of *otd*-related genes in all chordates (Simeone *et al.,* 1992; Li *et al.,* 1994; Bally-Cuif *et al.,* 1995; Mercier *et al.,* 1995; Pannese *et al.,* 1995) including urochordates (Wada *et al.,* 1996), cephalochordates (Williams and Holland, 1996), and agnates, where they are expressed in the rostralmost CNS independently of the complexity acquired by this area during evolution. In urochordates and cephalochordates, only one *Otx* gene has been identified so far that may be related to *Otx2* (Wada *et al.,* 1996; Williams and Holland, 1998). Indeed, in addition to similarities in amino acid sequence and expression, they are both expressed during gastrulation in endoderm cells which suggests that their oldest and primary role might be to mediate signals required to specify anterior neuroectoderm. Therefore, although this conservation in expression pattern and coding sequence might favor a remarkable role and functional equivalence of *otd/Otx* genes, it is unclear why the brain territory of protochordates has been so deeply and suddenly modified in a prosomeric territory that has been maintained in its basic topography until mammals (Rubenstein *et al.*, 1998).

A rather obvious answer to this question is that new genetic functions have been gained and recruited in developmental pathways. Indeed, it might be that conserved genes such as *Otx* genes acquired different roles even while retaining an evolutionary functional equivalence. Based on this hypothesis, it is expected that drastic evolutionary events should act on the regulatory control (transcription and translation) of *Otx*-related genes rather than on their coding sequences. Interestingly, the findings that: i) the *Drosophila otd* rescues in mouse most of the *Otx1-/-* impairments

(Acampora *et al.,* 1998a); ii) the human *Otx1* and *Otx2* rescue most of the *otd* defects in flies (Leuzinger *et al.,* 1998; Nagao *et al.,* 1998; Sharman and Brand, 1998); iii) *Otx1* rescues *Otx2* requirements in VE (Acampora *et al.,* 1998b); iv) *Otx2* rescues most of the *Otx1-/-* defects (Acampora *et al.,* 1999); v) the *Drosophila otd,* similarly to *Otx1,* rescues *Otx2* requirements in the VE (Acampora *et al.,* unpublished results), indicate that *Otx1, Otx2* and *otd* genes show a high degree of functional equivalence in the tissues and body regions where they are properly expressed. Therefore, these data support the notion that *otd/Otx* functions have been established in a common ancestor of fly and mouse and retained throughout evolution, while copy number and regulatory control of their expression have been modified and re-adapted by evolutionary events that have led to the specification of the vertebrate brain (Sharman and Brand, 1998; Simeone, 1998; Acampora and Simeone, 1999; Hirth and Reichert, 1999; Reichert and Simeone, 1999). Gene duplication and modification of the regulatory control of gene expression may greatly contribute to increase the complexity of the body plan and these events appear particularly relevant in the vertebrate evolution from protochordates (Garcia-Fernandez and Holland, 1994; Holland *et al.,* 1994; Williams and Holland, 1998). In fact, drastic evolutionary modifications in copy number and/or expression pattern might likely represent the most rapid and efficient mechanisms to confer morphological changes during embryonic development. Hence, gene duplication may allow the duplicated gene to acquire new specific function(s) either retaining or losing ancestral properties, and similarly, modification of the regulatory control of gene expression may establish new expression patterns which might alter preexisting cell-fates by generating new specialized cellular functions.

A likely consequence of both increased genomic complexity and modification of regulatory control of gene expression may result in an increase in the number of molecular interactions. Such increase in molecular interactions may contribute towards modifying relevant morphogenetic processes that in turn may confer a change in shape and size of the body plan as well as in the generation of cell-types with new developmental potentialities. On this basis, *Otx* gene duplication and subsequent or contemporary modification of regulatory control might have contributed to the evolution of the mammalian brain (e.g. by increasing the extent of neuroectodermal territory recruited to form the brain). This event might involve an improvement of proliferative activity of early neuronal progenitors (Acampora *et al.,* 1998a, 1999) and/or the positioning of the mes-met boundary (Acampora *et al.,* 1997, 1998b; Suda *et al.,* 1997). Additional property(ies) may be conferred to the duplicated gene by altering its coding sequence. Thus, the limited amino acid divergence between OTX1 and OTX2 might underlie modifications of their functional properties.

In this context, it is noteworthy that mice replacing *Otx1* with *Otx2* (Acampora *et al.*, 1999) rescued epilepsy, cortical impairments, eye and lachrymal/Harderian gland complex abnormalities, while they never recovered the lateral semicircular canal of the inner ear (Acampora *et al.,* 1996; Morsli *et al.*, 1999), strongly suggesting that, even though *Otx1* shares an extended functional equivalence to *Otx2* and *otd*, the ability to specify the lateral semicircular canal of the inner ear might represent a unique property of the *Otx1* coding sequence. Further mouse models will assess whether AVE-restricted equivalence of *Otx1* and *otd* to *Otx2* may be also extended to *Otx2* function in epiblast and derived tissues.

Conclusions

A remarkable amount of data indicates that, in mouse, early specification of the anterior patterning is under the control of AVE and that maintenance of this early specification may be contributed by both non-cell autonomous function(s) from the axial mesendoderm and cell autonomous function(s) of the anterior neuroectoderm. This review dealt with the role of *Otx2* during early events controlling specification and maintenance of anterior identity. Mouse models (*Otx2-/-; hOtx1^2/hOtx1^2; otd^2/otd^2)* and chimera experiments have largely contributed to defining differential roles of *Otx2* in the AVE and in epiblast-derived cells. These studies now indicate that *Otx2* is a major genetic requirement for early specification of the anterior identity. Future studies aimed at dissecting the genetic cascade, functional domains and regulatory control will certainly contribute to the understanding of the molecular basis governing development and evolution of the mammalian brain.

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References

ACAMPORA, D. and SIMEONE, A. (1999). Understanding the roles of *Otx1* and *Otx2* in controlling brain morphogenesis. *TINS* 22: 116-122.

ACAMPORA, D., AVANTAGGIATO, V., TUORTO, F. and SIMEONE, A. (1997). Genetic control of brain morphogenesis through *Otx* gene dosage requirement. *Development* 124: 3639-3650.

ACAMPORA, D., AVANTAGGIATO, V., TUORTO, F., BARONE, P., PERERA, M., CORTE, G. and SIMEONE, A. (1999). Differential transcriptional control as the major molecular event in generating *Otx1*-/- and *Otx2*-/- divergent phenotypes. *Development* 126: 1417-1426.

ACAMPORA, D., AVANTAGGIATO, V., TUORTO, F., BARONE, P., REICHERT, H., FINKELSTEIN, R. and SIMEONE, A. (1998a). Murine *Otx1* and *Drosophila otd* genes share conserved genetic functions required in invertebrate and vertebrate brain development. *Development* 125: 1691-1702.

ACAMPORA, D., AVANTAGGIATO, V., TUORTO, F., BRIATA, P., CORTE, G. and SIMEONE, A. (1998b). Visceral endoderm-restricted translation of *Otx1* mediates recovering of *Otx2* requirements for specification of anterior neural plate and proper gastrulation. *Development* 125: 5091-5104.

ACAMPORA, D., MAZAN, S., AVANTAGGIATO, V., BARONE, P., TUORTO, F., LALLEMAND, Y., BRÛLET, P. and SIMEONE, A. (1996). Epilepsy and brain abnormalities in mice lacking *Otx1* gene. *Nat. Genet.* 14: 218-222.

ACAMPORA, D., MAZAN, S., LALLEMAND, Y., AVANTAGGIATO, V., MAURY, M., SIMEONE, S. and BRÛLET, P. (1995). Forebrain and midbrain regions are deleted in *Otx2-/-* mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* 121: 3279-3290.

ANG, S.-L., CONLON, R.A., JIN, O. and ROSSANT, J., (1994). Positive and negative signals from mesoderm regulate the expression of mouse *Otx2* in ectoderm explants. *Development* 120: 2979-2989.

ANG, S.-L., JIN, O., RHINN, M., DAIGLE, N., STEVENSON, L. and ROSSANT, J. (1996). Targeted mouse *Otx2* mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* 122: 243-252.

AVANTAGGIATO, V., ACAMPORA, D., TUORTO, F. and SIMEONE, A. (1996). Retinoic acid induces stage-specific repatterning of the rostral central nervous system. *Dev. Biol.* 175: 347-357.

BALLY-CUIF, L., GULISANO, M., BROCCOLI, V. and BONCINELLI, E. (1995). c-*otx2* is expressed in two different phases of gastrulation and is sensitive to retinoic acid treatment in chick embryo. *Mech. Dev.* 49: 49-63.

BEDDINGTON, R.S.P. (1981). An autoradiographic analysis of the potency of embryonic ectoderm in the 8th day postimplantation mouse embryos. *J. Embryol. Exp. Morphol.* 64: 87-104.

BEDDINGTON, R.S.P. (1994). Induction of a second neural axis by the mouse node. *Development* 120: 613-620.

BEDDINGTON, R.S.P. and ROBERTSON, E.J. (1999). Axis development and early asymmetry in mammals. *Cell* 96: 195-209.

BELO, J.A., BOUWMEESTER, T., LEYNS, L., KERTESZ, N., GALLO, M., GOLLETTIE, M. and DE ROBERTIS, E.M. (1997) *Cerberus-like* is a secreted factor with neuralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev.* 68: 45-57.

BLITZ, I.L. and CHO, K.W.Y. (1995). Anterior neuroectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*. *Development* 121: 993-1004.

CHEN, S., WANG, Q.-L., NIE, Z., SUN, H., LENNON, G., COPELAND, N.G., GILBERT, D.J., JENKINS, N.A. and ZACK, D.J. (1997). *Crx*, a novel *Otx*-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. *Neuron* 19: 1017-1030.

CONLON, R.A. and ROSSANT, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine *Hox-2* genes in vivo. *Development* 116: 357-368.

DATTANI, M.T., MARTINEZ-BARBERA, J.-P., THOMAS, P.Q., BRICKMAN, J.M., GUPTA, R., MÅRTENSSON, I.-L., TORESSON, H., FOX, M., WALES, J.K.H., HINDMARSH, P.C., KRAUSS, S. BEDDINGTON, R.S.P. and ROBINSON, I.C.A.F. (1998). Mutations in the homeobox gene *HESX1/Hesx1* associated with septo-optic dysplasia in human and mouse. *Nat. Genet.* 19: 125-133.

DING, J., YANG, L., YAM, Y.-T., CHEN, A., DESAI, N., WYNSHAW-BORIS, A. and SHEN, M.M. (1998) *Cripto* is required for correct orientation of the anterior-posterior axis in the mouse embryo. *Nature* 395: 702-707.

DONIACH, T. (1993). Planar and vertical induction of antero-posterior pattern during the development of the amphibian central nervous system. *J. Neurobiol.* 24: 1256-1276.

DONO, R., SCALERA, L., PACIFICO, F., ACAMPORA, D., PERSICO, M.G. and SIMEONE, A. (1993). The murine cripto gene: expression during mesoderm induction and early heart morphogenesis. *Development* 118: 1157-1168.

FINKELSTEIN, R. and BONCINELLI, E. (1994). From fly head to mammalian forebrain: the story of *otd* and *Otx*. *Trends Genet.* 10: 310-315.

FREUD, C.L., GREGORY-EVANS, C.Y., KURUKAWA, T., PAPAIOANNOU, M., LOOSER, J., PLODER, L., BELLINGHAM, J., NG, D., HERBRICK, J.-A.S., DUNCAN, A., SCHERER, S.W., TSUI, L.-C., LOUTRADIS-ANAGNOSTOU, A., JACOBSON, S.G., CEPKO, C.L., BHATTACHARYA, S.S. and McINNES, R.R. (1997). Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (*CRX*) essential for maintenance of the photoreceptor. *Cell* 91: 543-553.

GALLERA, J. (1971). Primary induction in birds. *Adv. Morph.* 9: 149-180.

GAMMILL, L.S. and SIVE, H. (1997). Identification of *otx2* target genes and restrictions in ectodermal competence during *Xenopus* cement gland formation. *Development* 124: 471-481.

GARCIA-FERNANDEZ, J. and HOLLAND, P.W.H. (1994). Archetypal organization of the amphioux Hox gene cluster. *Nature* 370: 536-566.

GURDON, J.B. (1987). Embryonic induction - molecular prospects. *Development* 99: 285-306.

HIRTH, F. and REICHERT, H. (1999). Conserved genetic programs in insect and mammalian brain development. *BioEssays* 21: 677-684.

HOLLAND, P.W.H., GARCIA-FERNANDEZ, J., WILLIAMS, N.A. and SIDOW, A. (1994). Gene duplications and the origins of vertebrate development. *Dev. Suppl.* 125-133.

HOUART, C., WESTERFIELD, M. and WILSON, S.W. (1998). A small population of anterior cells patterns the forebrain during zebrafish gastrulation. *Nature* 391: 788-792.

KNÖTGEN, H., VIEBAHN, C. and KESSEL, M. (1999). Head induction in the chick by primitive endoderm of mammalian, but not avian origin. *Development* 126: 815-825.

LAWSON, K.A., MENESES, J.J. and PEDERSEN, R.A. (1991). Clonal analysis of the epiblast during germ layer formation in the mouse embryo. *Development* 113: 763-769.

LEUZINGER, S., HIRTH, F., GERLICH, D., ACAMPORA, D., SIMEONE, A., GEHRING, W., FINKELSTEIN, R., FURUKUBO-TOKUNAGA, K. and REICHERT. H. (1998). Equivalence of the fly *orthodenticle* gene and the human OTX genes in embryonic brain development of *Drosophila*. *Development* 125: 1703-1710.

LI, Y., ALLENDE, M.L., FINKELSTEIN, R. and WEINBERG, E.S. (1994). Expression of two zebrafish *orthodenticle*-related genes in the embryonic forebrain. *Mech. Dev.* 48: 229-244.

MARSHALL, H., NONCHEV, S., SHAM, M.H., MUCHAMORE, I., LUMSDEN, A. and KRUMLAUF, R. (1992). Retinoic acid alters hindbrain *Hox* code and induces transformation of rhombomeres 2/3 into 4/5 identity. *Nature* 360: 737-741.

MATSUO, I., KURATANI, S., KIMURA, C., TAKEDA, N. and AIZAWA, S. (1995). Mouse *Otx2* functions in the formation and patterning of rostral head. *Genes Dev.* 9: 2646-2658.

MERCIER, P., SIMEONE, A., COTELLI, F. and BONCINELLI, E. (1995). Expression patterns of two *Otx* genes suggest a role in specifying anterior body structures in zebrafish. *Int. J. Dev. Biol.* 39: 559-573.

MILLET, S., BLOCH-GALLEGO, E., SIMEONE, A. AND ALVARADO-MALLART, R.-M. (1996). Is the caudal limit of *Otx2* gene expression a marker of the midbrain/hindbrain boundary? A study using a chick-*Otx2* riboprobe and chick/quail homotopic grafts. *Development* 122: 3785-3797.

MINCHIOTTI, G., PARISI, S., LIGUORI, G., SIGNORE, M., LANIA, G., ADAMSON, E.D., LAGO, C.T. and PERSICO, M.G. (2000). Membrane-anchorage of Cripto protein by glycosylphosphatidylinositol and its distribution during early mouse development. *Mech. Dev.* 90: 133-142.

MORSLI, H., TUORTO, F., CHOO, D., POSTIGLIONE, M.P., SIMEONE, A. and WU, D.K. (1999). *Otx1* and *Otx2* activities are required for the normal development of the mouse inner ear. *Development* 126: 2335-2343.

NAGAO, T., LEUZINGER, S., ACAMPORA, D., SIMEONE, A., FINKELSTEIN, R., REICHERT, H. and FURUKUBO-TOKUNAGA, K. (1998). Developmental rescue of *Drosophila* cephalic defects by the human *Otx* genes. *Proc. Natl. Acad. Sci USA* 95: 3737-3742.

NAKANO, T., MURATA, T., MATSUO, I. and AIZAWA, S. (2000). OTX2 directly interacts with *Lim1* and *HNF-3β*. *Biochem. Bioph. Res. Comm.* 267: 64-70.

NIEUWKOOP, P.D., JOHNEN, A.G. and ALBERS, B. (1985). The Epigenetic Nature of Early Chordate Development. Inductive Interactions and Competence. Oxford University Press, Oxford.

PANNESE, M., POLO, C., ANDREAZZOLI, M., VIGNALI, R., KABLAR, B., BARSACCHI, G. and BONCINELLI, E. (1995). The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* 121: 707-720.

REICHERT, H. and SIMEONE, A. (1999). Conserved usage of gap and homeotic genes in patterning the CNS. *Curr. Opin. Neurobiol.* 9: 589-595.

RHINN, M., DIERICH, A., LE MEUR, M. and ANG, S.-L. (1999). Cell autonomous and non-cell autonomous functions of *Otx2* in patterning the rostral brain. *Development* 126: 4295-4304.

RHINN, M., DIERICH, A., SHAWLOT, W., BEHRINGER, R.R., LE MEUR, M. and ANG, S.-L. (1998). Sequential roles for *Otx2* in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* 125: 845-856.

RUBENSTEIN, J.L.R., SHIMAMURA, K., MARTINEZ, S. and PUELLES, L. (1998). Regionalization of the prosencephalic neural plate. *Annu. Rev. Neurosci.* 21: 445-477.

RUIZ I ALTABA, A. (1993). Induction and axial patterning of the neural plate: planar and vertical signals. *J. Neurobiology* 24: 1276-1304.

RUIZ I ALTABA, A. (1994). Pattern formation in the vertebrate neural plate. *Trends Neurosci.* 17: 233-243.

RUIZ I ALTABA, A. (1998). Deconstructing the organizers. *Nature* 391: 748-749.

SHARMAN, A.C. and BRAND, M. (1998). Evolution and homology of the neuron system: cross-phylum rescues of *otd/Otx* genes. *TIG* 14: 211-214.

SHAWLOT, W. and BEHRINGER, R.R. (1995). Requirement for *Lim-1* in head organizer function. *Nature* 374: 425-430.

SHAWLOT, W., WAKAMIYA, M., KWAN, M.K., KANIA, A., JESSELL, T.M. and BEHRINGER, R.R. (1999). *Lim1* is required in both primitive streak-derived tissues and visceral endoderm for head formation in the mouse. *Development* 126: 4925-4932.

SIMEONE, A. (1998). *Otx1* and *Otx2* in the development and evolution of the mammalian brain. *EMBO J.* 17: 6970-6978.

SIMEONE, A., ACAMPORA, D., GULISANO, M., STORNAIUOLO, A. and BONCINELLI, E. (1992). Nested expression domains of four homeobox genes in developing rostral brain. *Nature* 358: 687-690.

SIMEONE, A., ACAMPORA, D., MALLAMACI, A., STORNAIUOLO, A., D'APICE, M.R., NIGRO, V. and BONCINELLI, E. (1993). A vertebrate gene related to *orthodenticle* contains a homeodomain of the *bicoid* class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J.* 12: 2735-2747.

SIMEONE, A., AVANTAGGIATO, V., MORONI, M.C., MAVILIO, F., ARRA, C., COTELLI, F., NIGRO, V. AND ACAMPORA, D. (1995). Retinoic acid induces stage-specific antero-posterior transformation of rostral central nervous system. *Mech. Dev.* 51: 83-98.

SIVE, H. and CHENG, P. (1991). Retinoic acid perturbs the expression of *Xhox.lab* genes and alters mesodermal determination in *Xenopus laevis*. *Genes Dev.* 5: 1321-1332.

SPEMANN, H. and MANGOLD, H. (1924). Über induktion von Embryonanlagen durch Implantation artfremder Organisatoren. *Wilhelm Roux Arch. Entw. Mech. Organ.* 100: 599-638.

STOREY, K.G., CROSSLEY, J.M., DE ROBERTIS, E.M., NORRIS, W.E. and STERN, C.D. (1992). Neural induction and regionalisation in the chick embryo. *Development* 114: 729-741.

SUDA, Y., MATSUO, I. and AIZAWA, S. (1997). Cooperation between *Otx1* and *Otx2* genes in developmental patterning of rostral brain. *Mech. Dev.* 69: 125-141.

TAM, P.P.L., STEINER, K.A. and QUINLAN, G.A. (1997). Lineage and functional analyses of the mouse organizer. In *Cold Spring Harbor Symposia on Quantitative Biology LXII*, pp. 135-144, Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

THOMAS, P. and BEDDINGTON, R. (1996). Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. *Curr. Biol.* 6: 1487-1496.

THOMAS, P., BROWN, A. and BEDDINGTON, R.S.P. (1998). *Hex*: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* 125: 85-94.

VARLET, I., COLLIGNON, J. and ROBERTSON, E.J. (1997). *nodal* expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development* 124: 1033-1044.

WADA, S., KATSUYAMA, Y., SATO, Y., ITOH, C. and SAIGA, H. (1996). *Hroth*, an *orthodenticle*-related homeobox gene of the ascidian, *Halocynthia roretzi:* its expression and putative roles in axis formation during embryogenesis. *Mech. Dev.* 60: 59-71.

WADDINGTON, C.H. (1932). Experiments on the development of chick and duck embryos, cultivated *in vitro*. *Phil. Trans. Roy. Soc. Lond.* B 221: 179-230.

WILLIAMS, N.A. and HOLLAND, P.W.H. (1996). Old head on young shoulders. *Nature* 383: 490.

WILLIAMS, N.A. and HOLLAND, P.W.H. (1998). Gene and domain duplication in the chordate *Otx* family: insights from amphioxus *Otx*. *Mol. Biol. Evol.* 15: 600-607.