学位論文

Doctor's Thesis

論文名： **Studies on a role of *Otx2* gene in forebrain formation**

（前脳形成におけるOtx2遺伝子の役割）

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Summary

Recent analyses employing chimeric and transgenic rescue experiments have suggested that mouse paired-like homeodomain transcription factor *Otx2*, a homologue of *Drosophila orthodenticle* gene, is required in the neuroectoderm for development of the forebrain region. In order to overcome the disadvantages of conventional genetic studies and elucidate the precise role of *Otx2* in forebrain development, we attempted to generate an allelic series of *Otx2* mutations by Flp- and Cre-mediated recombination for the production of conditional knock-out mice. Unexpectedly, the *neo*-cassette insertion at the first intron created a hypomorphic *Otx2* allele: consequently, the phenotype of compound mutant embryos carrying both a hypomorphic and a null allele ($Otx2^{frt\text{-}neo/-}$) was analyzed. $Otx2^{frt\text{-}neo/-}$ mutant mice died at birth displaying rostral head malformations. Molecular marker analysis showed that the anterior visceral endoderm (AVE), the anterior definitive endoderm (ADE) and the axial mesendoderm developed normally in $Otx2^{frt\text{-}neo/-}$ mutant embryos, demonstrating $Otx2^{frt\text{-}neo/-}$ mutants appeared to undergo antero-posterior (A-P) axis generation; also indicating the anterior neuroectoderm was induced appropriately. However, these mutants subsequently failed to specify correctly the forebrain region. As the rostral margin of the neural plate, termed the anterior neural ridge (ANR), plays essential roles with respect to neural plate specification (Shimamura and Rubenstein, 1997; Houart et al., 1998), we examined expression of molecular markers for ANR and neural plate; moreover, neural plate explant studies were performed. Analyses revealed that telencephalic gene expression did not occur in mutant embryos due to defects of the neural plate; however, the mutant ANR bore normal induction activity on gene expression. These results further suggest that *Otx2* dosage may be crucial in the neural plate with respect to response to inductive signals primarily from ANR for forebrain specification.

List of publications

1. **E Tian, Chiharu Kimura, Naoki Takeda, Shinichi Aizawa and Isao Matsuo.** (2002). *Otx2* is required to respond to signals from anterior neural ridge for forebrain specification. *Developmental Biology* **242**, 204-223.

2. **Chiharu Kimura, Kazuya Yoshinaga, E Tian, Misao Suzuki, Shinichi Aizawa and Isao Matsuo.** (2000). Visceral endoderm mediates forebrain development by suppressing posteriorizing signals. *Developmental Biology* **225**, 304-321.

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Abbreviations

ADE;	anterior definitive endoderm
ANR;	anterior neural ridge
A-P;	antero-posterior
AVE;	anterior visceral endoderm
AxM;	axial mesendoderm
BMPs;	bone morphogenetic proteins
CNS;	central nervous system
D-V;	dorso-ventral
En;	*Engrailed*
ES;	embryonic stem
FGFs;	fibroblast growth factors
Gsc;	*goosecoid*
is;	isthmic constriction
mes;	mesencephalon
met;	metencephalon
neo;	neomycin
neo-cassette;	*PGKneopA*-cassette
P-D;	proximal-distal
PME;	prechordal mesendoderm
pros;	prosencephalon
RT-PCR;	reverse transcription PCR
rh;	rhombencephalon
Sonic hedgehog;	*Shh*

1. Introduction

Despite the sophisticated architecture of the vertebrate adult brain, its primordium is a simple planar sheet of neuroepithelium produced during gastrulation, named as neural plate (Rubenstein et al., 1998). The neurulation converts the neural plate into the neural tube to establish central nervous system (CNS) along antero-posterior (A-P) and dorso-ventral (D-V) axes. As development proceeds, enlarged anterior neural tube generates the approximate boundaries between the primordia of the forebrian, midbrain and hindbrain, and additional transverse constrictions continue to subdivide the brain region. The prosencephalon is the most anterior portion of CNS. During development, it gives rise to a variety of tissues including the eyes, cerebral cortex, basal ganglia, hypothalamus and thalamus that are comprise of forebrain, which is among the most complex biological structures and has higher cognitive functions. Recent advances have been made in the understanding of the molecular and cellular mechanisms involved in the formation and patterning of the vertebrate forebrain.

(1) The formation of vertebrate embryonic axis

1) The amphibian Spemann organizer and its molecular nature

Among the vertebrates, the mechanism of embryonic axis formation is best understood in amphibian. Spemann and Mangold found that the dorsal blastopore lip of an early newt gastrula could induce an entire ectopic axis on the ventral part of host embryo (Spemann and Mangold, 1924). This induced axis, with structures head to tail, derived not from the cells of the graft but from the ventral ectoderm of the host. They demonstrated that the interaction between dorsal ectoderm and involuting dorsal mesoderm was essential for CNS initiation at gastrulation. Their conclusion have been confirmed in *Xenopus* by modern lineage tracers (Gimlich and Cooke, 1983). This suggests that in normal development the mesoderm of the organizer region is an initial source of axial induction (Nieuwkoop et al., 1985; Harland, 2000). Recent evidence

from amphibians suggested that several molecules existed in the *Xenopus* organizer are encoded by *Noggin* (Smith and Harland, 1992), *Chordin* (Sasai et al., 1994), *Follistatin* (Hemmati-Brivanlou et al., 1994), *Xnr3* (Smith et al., 1995) and *Cerberus* (Bouwmeester et al., 1996). All of these organizer molecules are able to block BMPs signaling (Piccolo et al., 1996, 1999; Zimmerman et al., 1996; Fainsod et al., 1997; Hansen et al., 1997). Releasing BMP inhibitory signals is sufficient to cause neural development in the ectoderm during axis formation. The ectoderm cells assumed a neural fate when inhibitory signaling (BMPs) was interrupted, furthermore, the organizer molecules might locally antagonize these epidermalizing signals, allowing dorsal ectoderm to follow its "default" neural fate (Fig. 1A; Wilson and Hemmati-Brivanlou, 1997; Streit and Stern, 1999).

2) The node of the mouse as a gastrula organizer

At the late-streak stage in mouse embryo, the special node structure forms at the anterior end of the primitive streak as the streak reaches to the distal end of the embryo (Fig. 1C). The node of mouse embryo has been shown to be the initial source of the progenitors for the notochord, the floor plate and the gut endoderm (Tam and Beddington, 1987, 1992). Dr. Beddington first demonstrated that the mouse node was functionally equivalent to an amphibian organizer since it could induce a heterotopic axis during gastrulation (Beddington, 1994). Extirpation of mouse node resulted in the defects of the ventral neural tube and lateral body axis, but A-P axis developed normally (Davidson et al., 1999). The lack of the anterior development in the induced axis implies that the specific head organizing activity may be absent from the node. The study employed dye labeling, *lacZ* and *GFP* tracing showed that the node is the part of cell population presented in the mouse gastrula organizer (Kinder et al., 2001). In contrast, the transplantations of rabbit and mouse nodes to chick caused the formation of an entire patterned ectopic axis (Knoetgen et al., 2000). This phenomenon may attribute to the different competence of the responding epithelium during axis induction by node tissue. Knock-out analyses in mouse homologus of *Xenopus* Spemann organizer genes showed no obvious effect on the early axis formation. The *Follistatin*

mutant mice had no early phenotype but died perinatally (Matzuk et al., 1995). The *Noggin* mutants underwent normal gastrulation and CNS development (McMahon et al., 1998). But *Chordin* and *Noggin* double homozygous mutant mice displayed severe prosencephalon defects (Bachiller et al., 2000). Also, candidate genes *Brachyury/T*, *Nodal* and *Cripto* are the earliest markers of primitive streak, knock-out them resulted in failure of gastrulation (Herrmann, 1991; Conlon et al., 1994; Ding et al., 1998). And *Foxa2* and *Wnt3* genes are known as importance for the node formation and the posterior structures development (Ang et al., 1994; Liu et al., 1999a). It is conceivable that mouse gastrula organizer may have more complicated mechanisms and evolved functions than the *Xenopus* Spemann organizer during neural axis generation.

3) The mouse anterior visceral endoderm (AVE) and anterior patterning

The mouse-to-mouse grafting experiment by node tissue exhibited the ectopic neural axis lacking the anterior structure, suggesting the node acts as a trunk organizer (Fig. 1B; Beddington and Robsertson, 1999). The mouse visceral endoderm (VE) is an extraembryonic cell layer around the epiblast that appears prior to gastrulation, it is a derivative of the primitive endoderm and its main role has been considered to entail nutrient uptake and transport (Bielinska et al., 1999). Recent studies have suggested that the anterior visceral endoderm (AVE) may play quite important role for the brain development (Fig. 1B). An increasing number of genes have been identified that are specifically expressed in the AVE before or during gastrulation, including *Otx2* (Acampora et al., 1995), *Lim1* (Shawlot et al., 1999), *Hesx1/Rpx* (Thomas and Beddington, 1996), *Hex* (Thomas et al., 1998), *Nodal* (Varlet et al., 1997), *Foxa2* (Dufort et al., 1998), *Cer-1* (Belo et al., 1997), *mdkk-1* (Pearce et al., 1999), *Gsc* (Belo et al., 1997) and *Lefty1* (Meno et al., 1999). Surgical ablation of the AVE results in the failure of *Hesx1* expression in the prospective forebrain region (Thomas and Beddington, 1996). Knock-out mutation of *Otx2*, *Lim1*, *Hesx1* and *Hex* genes revealed that they are crucial for forebrain formation (Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995; Ang et al., 1996; Dattani et al., 1998; Martinez-Barbera et al., 2000a). And the gene inactivation studies have shown that molecules (*Gsc*, *Foxa2*,

Lim1) coordinate and interact in anterior development (Filosa et al., 1997; Perea-Gomez et al., 1999; Jin et al., 2001). Chimeric analyses on *nodal*, *Foxa2*, *Otx2*, *Lim1* and *Hesx1Rpx* mutant mice have also indicated that the visceral endoderm is essential for the forebrain development (Varlet et al., 1997; Dufort et al., 1998; Rhinn et al., 1998; Shawlot et al., 1999; Martinez-Barbera et al., 2000b). The genetic deletion of node tissue in *Foxa2* homozygous mutant mice showed that the forebrain markers were still expressed, probably due to that the AVE tissue functions normally. (Klingensmith et al., 1999). Thus, the mouse AVE is proposed to be a head organizer (Beddington and Robsertson, 1999; Stern, 2001). One report from Dr. Tam's group indicated that the whole neural axis requires the synergistic interaction of the AVE, the node precursor cells and the anterior epiblast in the early mouse gastrula (Tam and Steiner, 1999). It is possible that mice have split the early amphibian organizer into AVE and the node, whereas AVE involved in anterior patterning and the node in posterior patterning.

(2) The molecular mechanism of the CNS subdivision

The discovery of the amphibian Spemann organizer, and the determination of the node of mouse gastrula and the mouse AVE have provided a promising model of axis induction on the initial formation of the CNS. The next step in CNS development mainly includes A-P and D-V patterning.

1) The molecular mechanism of A-P patterning in vertebrates

The A-P subdivision of the future CNS begins early, at least by the open neural plate stage (Saha and Grainger, 1992), it leads to the generation of distinct transverse domains at different axial positions in the CNS. Experiments suggested that vertical signals from underlying axial mesendoderm and planar signals from the organizer contribute to the specification of A-P regional differences (Ruiz i Altaba, 1994). The works in amphibian embryos demonstrated that signals from the organizer not only established the neural plate but also imposed rudimentary A-P patterning, in which distinct organizer signals directly induce neural tissue of a different A-P type (Hamburger, 1988). The evidences have shown that neural development is initiated by organizer molecules and then later

signals transform the posterior specification to the induced neural tissues (Nieuwkoop, 1952a and b). Nieuwkoop and collaborators postulated a two-step model which divided CNS development into the initial step of forebrain induction and the second step of caudalization (Neiuwkoop and Nigtevecht, 1954). According to a wealth of data, this model has been modified, which is including the steps of activation, stabilization and transformation (Fig. 2; Stern, 2001). The early markers (*Otx2*, *Lim1*, etc.) expressed in the AVE and the node precursor cells might be an initial activation of the forebrain development. In chick, the hypoblast transiently induced the expression of *Otx2* and *Sox3*, suggesting this activation is not sufficient for forebrain specification (Foley et al., 2000). The graft experiments have proposed that the node produced axial mesoendoderm provides signals for stabilization step in development of forebrain (Ang and Rossant, 1993; Foley et al., 1997; Camus et al., 2000). During the transformation step of posterior CNS development, fibroblast growth factors (FGFs) have been proposed as a mediator (Cox and Hemmati-Brivanlou, 1995). The mouse *Fgf8* is required for various stages of development including gastrulation, forebrain formation and mid/hindbrain boundary establishment (Heikinheimo et al., 1994; Crossley et al., 1995), and the conditional knock-out mice of *Fgf8* displayed different defects in brain and posterior development (Meyers et al., 1998). Another posteriorization factor is retinoic acid (RA). In *Xenopus*, RA can transform prospective anterior CNS into posterior CNS (Ruiz i Altaba and Jessell, 1991; Sharpe, 1991). However, the spatial and temporal distribution of RA and the *in vivo* roles for RA remain unclear. The studies in mouse have suggested that *Nodal*, *Cripto* and *Wnt3* are essential factors for posterior structure development during transformation step (Conlon et al., 1994; Varlet et al., 1997; Ding et al., 1998; Liu et al., 1999; Kimura et al., 2001).

2) The molecular mechanism of D-V patterning in vertebrates

The famous graft experiment by Spemann and Mangold showed that the secondary neural tube induced by the grafted dorsal lip has a clear D-V polarity (Spemann and Mangold, 1924), indicating the organizer not only induced A-P patterning but also D-V axis. Which factors initiate early D-V patterning in the neural plate? In *Xenopus*, when

animal caps were treated by *Noggin*, both dorsal and ventral CNS markers are induced in different areas of the explants, suggesting that neural tissue induced in the explant is already patterned along D-V polarity (Knecht et al., 1995). Furthermore, the antagonists of organizer neural inducers, BMPs, seem to play a role in the D-V patterning of the CNS (Liem et al., 1995), but the D-V character of neural tissue produced by BMPs inhibition alone is not clear. Since BMPs family has opposite activities to the organizer factors in neural induction and CNS patterning, an intriguing possibility is that BMPs involved neural induction and the CNS D-V patterning may share similar signaling mechanisms (Sasai and De Robertis, 1997). The Winged-Helix transcription factor *Foxa2* (previous name is *HNF3β*) and the secreted factor *Sonic hedgehog* (*Shh*), which are expressed in the floor plate and the notochord, are excellent candidates for determination of CNS D-V patterning (Dirksen and Jamrich, 1992; Echelard et al., 1993). In *Xenopus*, *Shh* can not induce neural tissue from the ectoderm cells, this is different from organizer molecules *Noggin* and *Chordin*, but can change the D-V pattern of preexisting neural tissue (Ekker et al., 1995). Misexpression of *Foxa2* in the dorsal neural tube results in the ectopic expression of floor plate markers in mouse and *Xenopus* (Sasaki and Hogan, 1994; Ruiz i Altaba et al., 1993). In mice, *Foxa2* is required for *Shh* expression in the notochord and floor plate (Ang and Rossant, 1994). *Foxa2* induces *Shh* expression in the neural tube and *Shh* can in turn induce the expression of *Foxa2* (Echelard et al., 1993). Several additional classes of transcription factors are also expressed in the early neural plate and important for D-V patterning: the *Pax* family (Gruss and Walther, 1992), the *Lim* family (Tsuchida et al., 1994), the *Nkx* class (Price, 1993), the *Gli* family (Hui et al., 1994), the POU-domain factor *xlpou2* (a frog homologue of mouse *Brn-4*; Witta et al., 1995) and Sry-related HMG factor *Sox* (Grosschedl et al., 1994). These factors are good candidates for effector genes acting closely downstream of the neural inducing signaling pathway.

3) The role of axial mesendoderm (AxM) in the CNS subdivision

The descendants of the node of mouse gastrula form the axial mesendoderm (AxM) structures (Fig. 1C; Beddington and Robertson, 1999). The AxM subdivides into

prechordal plate, head process, notochord and the definitive endoderm. The prechordal plate underlies the forebrain, and the notochord underlies the ventral midline of the midbrain, hindbrain and spinal cord. The definitive endoderm contributes to gut tissues. *in vitro* experiments have shown that the AxM retains much of the neural inducing ability of early organizer cells (Kinder et al., 2001), suggesting that AxM could represent a later source of neural inducing signals for CNS subdivision. Removal of the AxM tissue resulted in a truncation of the head accompanied by the loss of forebrain markers (Camus et al., 2000). In the co-culture experiments, the anterior mesendoderm, which including the AxM, has ability to induce and maintain the neuroectoderm markers *En* and *Otx2* expression in epiblast explants (Ang and Rossant, 1993; Ang et al., 1994). By the late allantoic bud substage of neural plate stages in mouse, the AxM also subdivides the CNS along ventral midline. In the most rostral region of AxM, *Gsc* and *mdkk-1* are expressed in the prechordal plate underlying the prospective forebrain (Belo et al., 1998; Pearce et al., 1999). And *Otx2*, *Lim1*, *Foxa2* are expressed in the AxM region underlying the prospective midbrain (*Otx2* and *Lim1*) or hindbrain (Acampora et al., 1995; Matsuo et al., 1995; Shawlot et al., 1999; Filosa et al., 1997). Chimeric experiments and germ layer recombination assays have provided genetic evidence that *Lim1* is required in anterior mesendoderm for forebrain formation (Shawlot et al., 1999). Overexpression of zebrafish homologue *dkk1* promoted anterior neuroectoderm development and the formation of AxM (Hashimoto et al., 2000). The *mdkk-1* null mutants lacked head structures and analysis of chimeric embryos implicated the requirement of *mdkk-1* in the anterior axial mesendoderm for brain induction (Mukhopadhyay et al., 2001). At more posterior portion, the notochord that derives from the node expresses *Brachyury/T*, *Foxa2*, *Shh*, *Nodal*, *Cripto* and *Noggin* (Wilkinson et al., 1990; Chiang et al., 1996; Filosa et al., 1997; Varlet et al., 1997; Ding et al., 1998; McMahon et al., 1998). Several genes, *Hesx1/Rpx*, *Hex*, *Cer-1* and *Otx2* (Thomas and Beddington, 1996; Belo et al., 1997; Pearce et al., 1999; Kimura et al., 2000), are also expressed in the anterior definitive endoderm (ADE). Knock-out mice of *Hesx1/Rpx* and *Hex* showed a significant reduction of forebrain region (Dattani et al.,

1998; Martinez-Barbera et al., 2000a). Chimeric analyses of *Hex*, *Hesx1/Rpx* and *Smad2* demonstrated that their functions are required for specification of the definitive endoderm lineage for forebrain development (Martinez-Barbera et al., 2000a and b; Tremblay et al., 2000). But the precise roles of ADE in CNS development have not yet been determined.

Fig. 1 The models of CNS formation in vertebrates.

(A) The default model of early *Xenopus* gastrula. At early gastrula in *Xenopus*, ectoderm cells have an autonomous tendency to differentiate into neural tissue, and this tendency is inhibited by BMPs signaling (blue arrows). The endogenous BMPs antagonists *Noggin*, *Chordin*, *Follistatin*, *Xnr3* and *Cerberus* are expressed in the organizer. All of them are able to block BMPs activities for axis induction (red lines). An. animal pole; Veg, vegetal pole; D, dorsal; V, ventral; The Spemann organizer is shown in orange. (B) At pre-gastrula stage in mouse, the genes expressed in the anterior visceral endoderm (AVE), such as *Otx2*, *Lim1*, *Hesx1*, *Hex*, *Nodal*, *Foxa2*, *Cer-l*, *mdkk-1*, *Gsc* and *Lefty1*, are crucial for forebrain induction and development (Acampora et al., 1995; Shawlot et al., 1999; Thomas and Beddington, 1996; Thomas et al., 1998; Varlet et al., 1997; Dufort et al., 1998; Belo et al., 1997; Pearce et al., 1999; Belo et al., 1997; Meno et al., 1999). (C) At late gastrula in mouse, the genes *Noggin*, *Chordin*, *Follistatin*, *T*, *Nodal*, *Cripto*, *Foxa2*, *Lim1* and *Wnt3* are expressed in primitive streak and/or node, which are required for formation of posterior structures (Herrmann, 1991; Ang et al., 1994; Conlon et al., 1994; Matzuk et al., 1995; Ding et al., 1998; McMahon et al., 1998; Liu et al., 1999; Shawlot et al., 1999; Bachiller et al., 2000). Genes expressed in the axial mesoendoderm (AxM) and the anterior definitive endoderm, like *Otx2*, *Lim1*, *Foxa2*, *Shh*, *Gsc*, *Cer-l*, *mdkk-1* and *Smad2*, are essential for CNS

subdivision (Belo et al., 1998; Pearce et al., 1999; Acampora et al., 1995; Matsuo et al., 1995; Chiang et al., 1996; Filosa et al., 1997; Belo et al., 1998; Pearce et al., 1999; Shawlot et al., 1999; Tremblay et al., 2000). The mouse gastrula appears to have distinct node trunk organizer and AVE head organizer. The orientation of embryo is anterior to the left and posterior to the right.

Fig. 2 The modified Nieuwkoop's model of CNS development.

There are three steps in modified Nieuwkoop's model. The first step occurs before gastrulation, in which the AVE, the hypoblast and/or together with node precursors (e.g. posterior epiblast) activate a labile, pre-neural and prosencephalon state. In the second step, neural fate should be retained, cells must also receive stabilizing signals from the node and/or its descendants like axial mesendoderm. The third step is to transform the node caudalized signals to specify midbrain, hindbrain and spinal cord progressively. (modified from Stern, 2001)

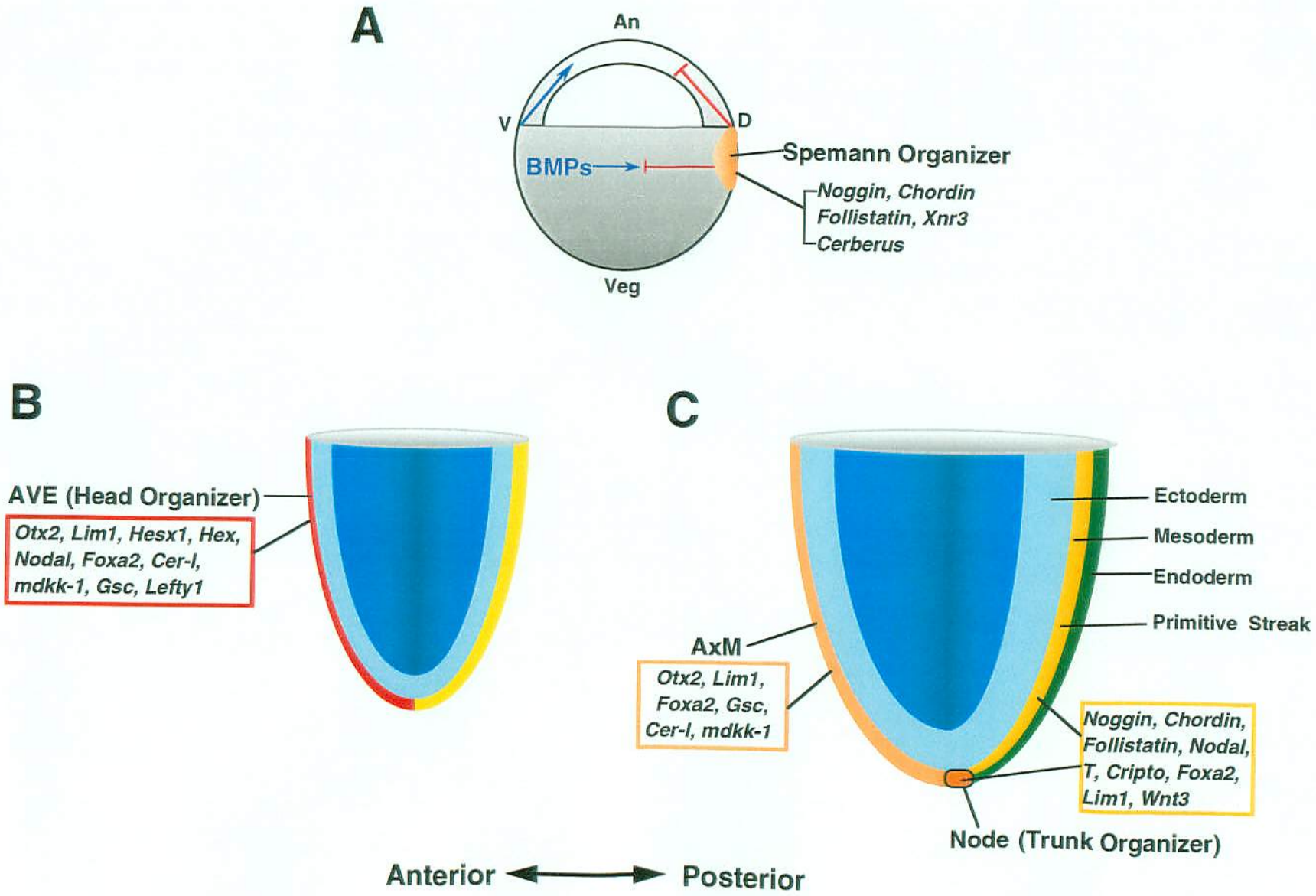

A
An
V
D
BMPs
Spemann Organizer
Noggin, Chordin
Follistatin, Xnr3
Cerberus
Veg
B
AVE (Head Organizer)
Otx2, Lim1, Hesx1, Hex,
Nodal, Foxa2, Cer-l,
mdkk-1, Gsc, Lefty1
C
Ectoderm
Mesoderm
Endoderm
Primitive Streak
AxM
Otx2, Lim1,
Foxa2, Gsc,
Cer-l, mdkk-1
Noggin, Chordin,
Follistatin, Nodal,
T, Cripto, Foxa2,
Lim1, Wnt3
Node (Trunk Organizer)
Anterior
Posterior

Fig. 1

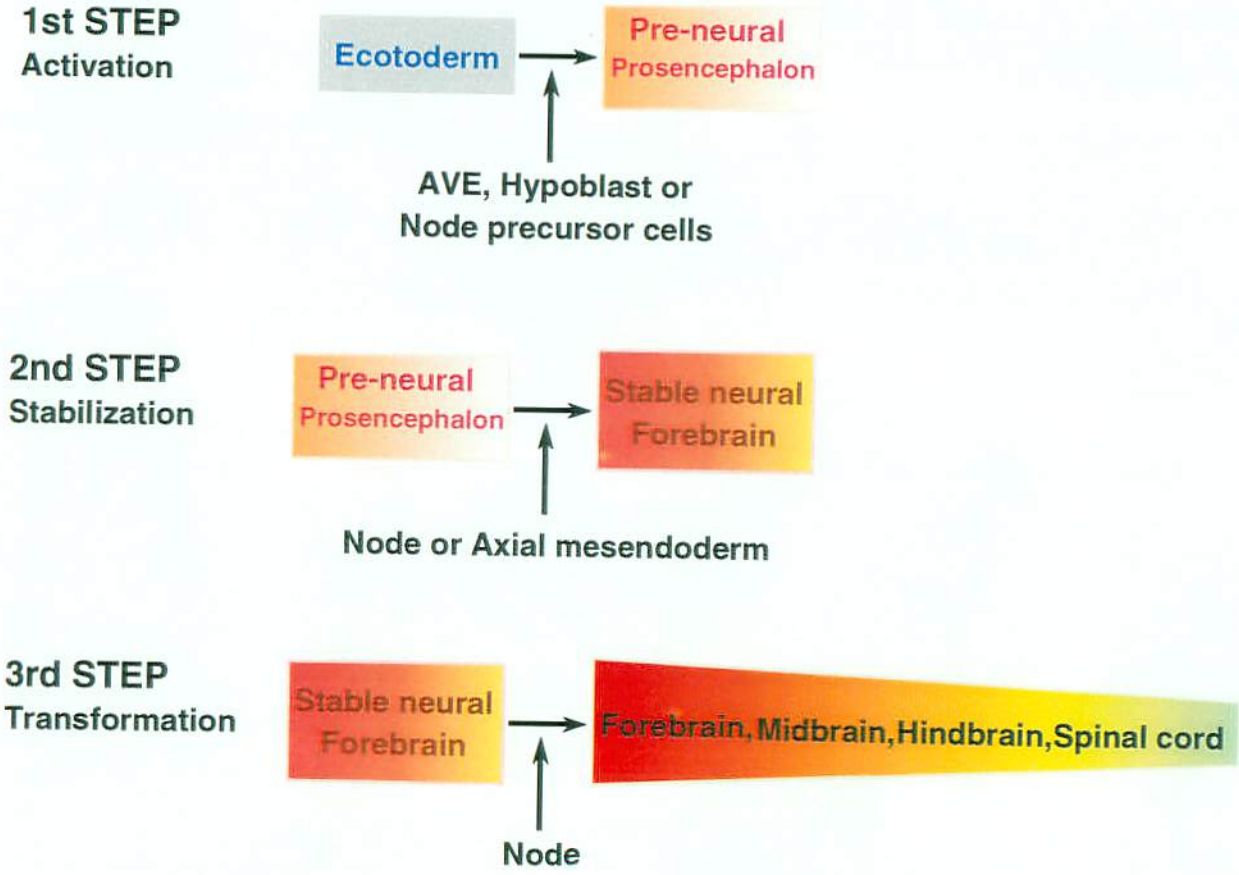

1st STEP
Activation
Ecotoderm
Pre-neural
Prosencephalon
AVE, Hypoblast or
Node precursor cells
2nd STEP
Stabilization
Pre-neural
Prosencephalon
Stable neural
Forebrain
Node or Axial mesendoderm
3rd STEP
Transformation
Stable neural
Forebrain
Forebrain, Midbrain, Hindbrain, Spinal cord
Node

Fig. 2

(3) Embryonic forebrain regionalization

Gene expression patterns provide insights into the onset, location and developmental consequences of inductive processes that produce regionalization in the developing CNS. During the past ten years, scientific researchers have identified many regulatory genes in the embryonic neural tube patterning which are providing insights into brain organization (Rubenstein et al., 1998). These genes have restricted expression patterns in the neural plate, and related to the longitudinal and/or transverse dimensions of the CNS.

1) The longitudinal regionalization of forebrain during CNS development

Longitudinal patterns extend along part or entire of A-P axis and mark the prospective regions of the basal, floor and alar plates (Shimamura et al., 1995). The genes expressed in longitudinal patterns are along the medial aspect of the neural plate. *Shh* is expressed along the entire A-P extent of the axial mesendoderm (the prechordal plate, head process and notochord). *Shh* induces the expression of some genes (e.g., *Foxa2, Nkx2.1*). For instance, different genes of *Nkx* family are expressed at different axial position. *Shh* regulates *Nkx2.1* expression only in the anterior neural plate whereas *Nkx6.1* expression is induced at the posterior neural plate, suggesting the neural plate is patterned along the A-P axis and leads to distinct competence to response to inductive signals produced by the axial mesendoderm (Fig. 3A; Ericson et al., 1995; Shimamura and Rubenstein, 1997). Both gain-of-function and loss-of-function experiments indicated medial signaling is regulated by *Shh* (Echelard et al., 1993; Hynes et al., 1995; Chiang et al., 1996). Experimental analyses have suggested that lateral signaling is regulated by members of the TGFβ superfamily, like BMPs mediate dorsalizing signal from the epidermal ectoderm for neural plate regionalization (Fig. 3A; Liem et al., 1995). The detail roles of BMPs in mouse have not yet been determined.

2) The transverse regionalization of forebrain during CNS development

In transverse regions of the anterior neural plate, the expression boundaries of some genes mark molecular discontinuities along the A-P axis (Rubenstein et al., 1998). Gain-

and loss-of-function studies have identified a number of homeobox genes including *Emx1* and *Emx2* (Yoshida et al., 1997), *Hesx1/Rpx* (Dattani et al., 1998), *Six3* (Kobayashi et al., 1998) etc. that may have roles in regulation of the early regional subdivision of the prospective forebrain into telencephalic, optic and diencephalic territories. There is a major transverse boundary in the neuroepithelium at the transition between the prechordal plate and notochord. This boundary is respected by the posterior limit of *Nkx2.1* expression in the medial neural plate and by posterior limit of *Emx2* expression in the lateral neural plate. The more anterior transverse boundary is also apparent for *Otx1* and *Emx2* expression (Shimamura et al., 1997). *Pax6* is expressed in most of the lateral prosencephalic neural plate. The posterior boundary of *Pax6* in the alar plate abuts the mesencephalon (Stoykova and Gruss, 1994). In addition, several genes that are implicated in midbrain and cerebellum development, like *Wnt1*, *En1* and *Pax2*, have anterior expression limits that appear near the posterior boundary of prospective forebrain at early stages of development (Rowitch and McMahon, 1995). After the generation of basic A-P and D-V organization of CNS, A-P patterning generates transverse domains with differential competence within the neural plate while D-V patterning creates longitudinally aligned areas. The combination of A-P and D-V patterning then generates a grid-like organization of distinct histogenic forebrain region (Fig. 3). Later events appear to be other mechanisms that specify additional levels of complexity in the anterior neural plate. Some of local patterning centers arise in regulation these processes. These organizers are likely to be at boundary zones. These boundaries may be at the transition between neural and non-neural tissues, or at the position of histogenic region, such as the ANR and isthmic constriction (Fig. 3B).

3) The role of the anterior neural ridge (ANR) in forebrain regionalization

Ablation, transplantation and explant studies have suggested that the ANR, cells at the rostral margin of the neural plate, play an important role in the telencephalic development (Shimamura and Rubenstein, 1997; Houart et al., 1998). The ANR is a morphologically defined structure at the boundary of the anterior neural plate and the non-neural ectoderm (Couly and Le Douarin, 1988). The fate mapping study using two

fluorescent labels in *Xenopus* provided a model of ANR folding and the cell movements for differential proliferation patterns which contribute to brain morphogenesis (Eagleson et al., 1995). The ANR gives rise to the olfactory and hypophyseal placodes as well as to forebrain tissues (Papalopulu, 1995; Baker and Bronner-Fraser, 2001). In zebrafish, the telencephalic gene expression (e.g., *dlx2*, *Emx1*) is reduced or disappeared when cells at the margin of the prospective neural plate (row-1 cells) are excised, and these rostral cells can induce ectopic telencephalic gene expression when transplanted to more posterior region of the neural plate (Houart et al., 1998). Studies in mice by explant experiments showed that the telencephalic marker *BF-1* expression is lost when the ANR is ablated, and FGF8 protein can restore *BF-1* expression in ANR-denuded neural plate explants, supporting the notion that one or more signals from the margin of the neural plate promote telencephalic development (Shimamura and Rubenstein, 1997). *Fgf8* starts its expression in the ANR around 4 somites stage in mice, and later at the midline of the telencephalon while *BF-1* expression in neuroectoderm is detectable after 7 somites stage. This temporal relationship indicates one possible mechanism that the onset of *BF-1* expression in the neuroectoderm is induced by *Fgf8* signal produced from the ANR. The inhibitors of *Fgf* function reduce *BF-1* expression in neural plate (Ye et al., 1998). However, because telencephalon is still formed in *Fgf8*-deficient mice and fish, *Fgf8* may not the primary inducer of the telencephalon or cooperate with other *Fgf* factors (Meyers et al., 1998; Shanmugalingam et al., 2000; Shinya et al., 2001). Thus the ANR may be a localized signaling center that utilizes FGF8, and probably other molecules, for mediating regional specification and growth of the anterolateral neural plate (Fig. 3B).

Fig. 3 Model of neural plate patterning between 4-8 somites stages in mouse.

Schemes represent dorsal-ventral (D-V) patterning (A) and local signaling centers (B) for forebrain development. (A) Ventral (medial, M) patterning signals, such as *Shh* (green arrows), emanating from the axial mesendoderm consists of prechordal plate (pp) and notochord (nc), which is under the prosencephalon (pros), the mesen- (mes) and metencephalon (met), respectively. Dorsal (lateral, L) patterning signals, like *Bmps* (blue arrows) are proposed to emanate from the non-neural ectoderm (ect). Studies have indicated *Shh* signaling (green arrows) can induce *Nkx2.1* expression in the anterior prosencephalic neural plate (represented by yellow area) (Ericson et al., 1995; Shimamura and Rubenstein, 1997). The ventral signals are thought to induce the basal plate (bp), whereas the lateral signals pattern the alar plate (ap). (B) Localized *Fgf8* signals (orange arrows) emanating from the anterior neural ridge (ANR) and the isthmus (Is), the boundary between the mesencephalon and metencephalon. There is evidence that signals from the ANR, which include *Fgf8*, regulate expression of the *BF-1* gene that is required for telencephalic development. FGF8 protein applied to the prosencephalic neural plate induces *BF-1* (represented by blue area), whereas applied to the mesencephalic neural plate and the anterior rhombencephalon induces *En2* expression, demonstrating distinct domains of competence. (modified from Rubenstein and Beachy, 1998)

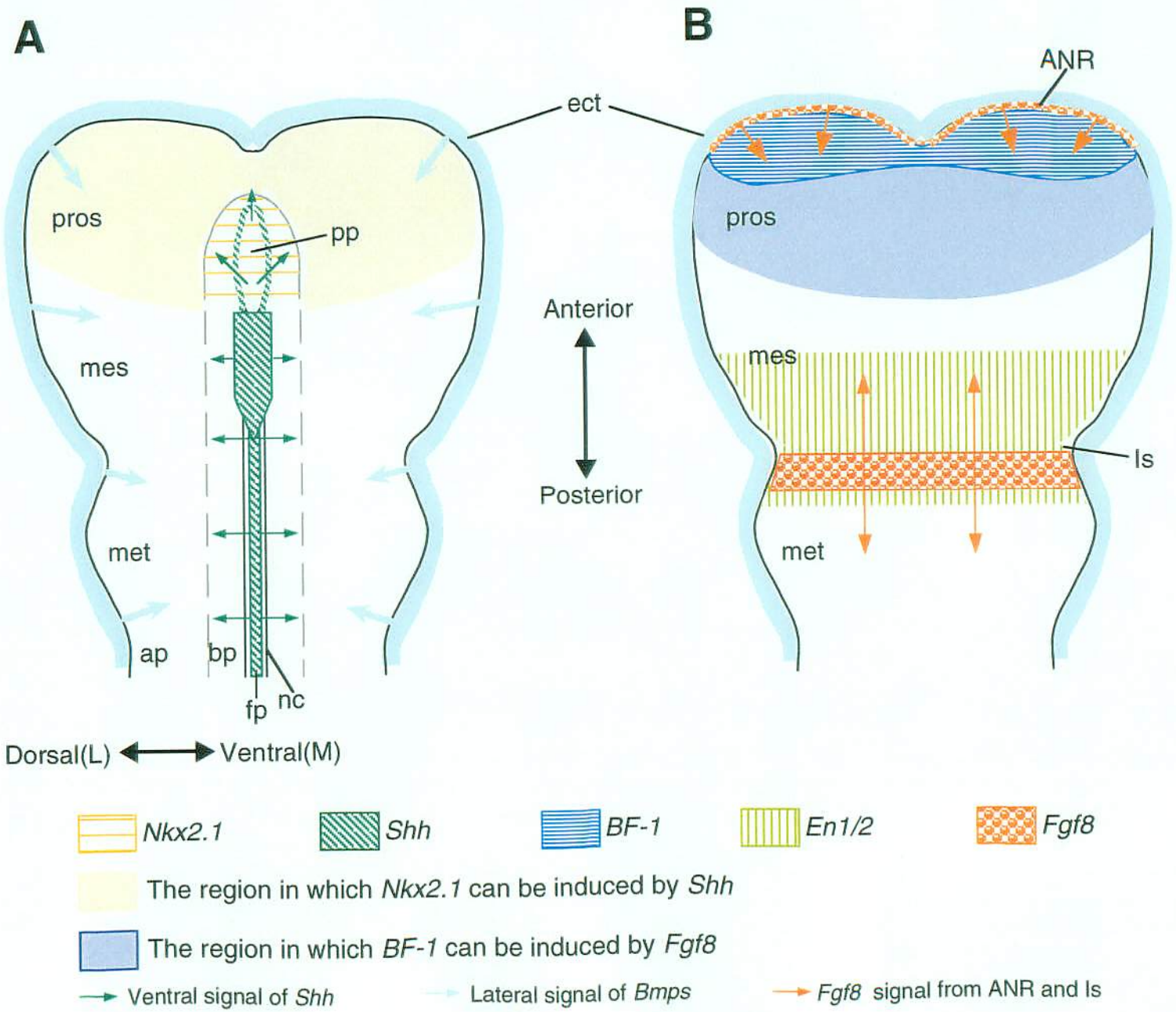

A
B
ect
ANR
pros
pp
mes
met
ap
bp
fp
nc
Anterior
Posterior
pros
mes
ls
met
Dorsal(L)
Ventral(M)
Nkx2.1
Shh
BF-1
En1/2
Fgf8
The region in which Nkx2.1 can be induced by Shh
The region in which BF-1 can be induced by Fgf8
Ventral signal of Shh
Lateral signal of Bmps
Fgf8 signal from ANR and ls

Fig. 3

(4) Tissue-specific conditional gene targeting by Cre/*loxP* and Flp/*frt* recombinant systems

1) The conventional gene targeting and its limitation

Gene targeting, defined as the introduction of site-specific modifications into the genome by homologous recombination, has revolutionized the field of mouse genetics and allowed the analysis of various aspects of gene function *in vivo* (Muller, 1999). The conventional genetic study is that insertion of neomycin resistance gene (*neo*), which is under the control of a strong promoter, into the coding region of a particular targeted gene. The analysis of gene function at later developmental stages may be precluded when null allelles cause early embryonic lethality. The genes related to brain development sometimes show the important roles in early neural plate formation and prosencephalon specification, such as *Otx2*, *Lim1*, *Fgf8*, etc. *Otx2* knock-out mice died before 9.5 dpc (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996) and this prevented further analyses of detail *Otx2* function in later brain development.

2) Conditional knock-out and application of recombinase systems

More recently, methods have been developed for controlling gene targeting in a time- and tissue-specific manner. These so called conditional gene targeting approaches are extremely useful in cases where complete gene inactivation leads to a lethal or otherwise adverse phenotype that prevents further analysis. Tissue-specific gene inactivation may be obtained by first generating transgenic mice that express Cre or Flp recombinase under the control of a tissue-specific promoter. Then these mice may be mated to target mice that contain *loxP* or *frt* sites flanking the interested genomic region (Fig. 4). The Cre/*loxP* system is the first well-developed recombination system. The Cre recombinase from P1 bacteriophage belongs to the integrase family of site-specific recombinases (Hamilton and Abremski, 1984). It is a 38 kDa protein that efficiently catalyzes recombination between two of its consensus 34 bp DNA recognition *loxP* sites, which is consist of a core spacer sequence of 8 bp and two 13 bp palindromic flanking sequences. By applying the Cre recombinase system, we can excise or invert *loxP*-flanked DNA fragments of generate intermolecular recombination between different

DNA molecules, or introduce specific point mutations to large site-specific chromosomal aberrations. Furthermore, using the recombinase activity as a genetic activation or inactivation switch, conditional transgenesis or conditional knock-out became available (Nagy, 2000). Another useful site-specific recombinase is Flp that is from the yeast *Saccharomyces cerevisiae*, but its efficiency is relatively low in mammalian cells (Dymecki, 1996). A themostable improved Flpe has been reported recently that mediates high recombination efficiencies *in vivo* similar with those of Cre (Buchholz et al., 1998). Although use of the Flp/*frt* system is unlikely to be as widespread as that of the Cre/*loxP* system, it continues to be developed as a genetic tool and will be useful as a supplementary system. In most cases, *loxP* or *frt* sites are placed in introns, sometimes are inserted in 5' - or 3' -flanking regions without compromising gene expression (Lewandoski, 2001). The conditional alleles should have wild-type activity before recombination. The ability to inactivate an endogenous gene in the mouse in a temporally and spatially controlled manner is not only useful for circumventing early lethal phenotypes but also allows biological questions to be addressed with exquisite accuracy. By crossing a mouse line with a conditional allele flanked by *loxP* sites to an effector mouse line that expresses *cre* in a tissue-specific manner, offsprings are produced in which the conditional allele is inactivated only in those tissues or cells that express *cre*. After the first publication on tissue-specific knock-out (Gu et al., 1994), the use of this strategy has steadily increased (Trumpp et al., 1999; Utomo et al., 1999; Ashery-Padan et al., 2000; Lewandoski et al., 2000; Sund et al., 2001).

3) The generation of hypomorphic allele and its application

It has been determined that cryptic splice sites in *neo* interfere with normal splicing and therefore reduce wild-type mRNA levels (Meyers et al., 1998; Nagy et al., 1998). Besides *neo* can be removed by Cre-mediated recombination event *in vitro* or *in vivo*. The insertion of *neo* can generate a hypomorphic allele, an allele that results in a reduction of wild-type levels of gene product or activity, often causing a less severe phenotype than a null allele, retaining it can often be advantageous. If combine such an

allele with the null or conditional alleles, it can be very informative. Martin's group reported the allelogenic strategy recently (Meyers et al., 1998). A hypomorphic allele was generated as part of an allelic series from one mouse line by floxing an essential region of the *Fgf8* gene, and then flanking the *neo* cassette by *frt* sites in order to remove it by Flp-mediated recombination. Three mouse lines can be generated using this methodology. The original line (*neo* insertion) expresses a hypomorphic allele. Second is one line expresses a floxed allele, which is generated by mating the original line to a *flp*-expression mouse. Third is one line carries a null allele, generated by breeding the original line to a *cre*-expressing mouse. Such an allelic series at the *Fgf8* locus has been instrumental in studying the role of *Fgf8* in gastrulation (Sun et al., 1999), left-right axis determination (Meyers and Martin, 1999), branchial arch development (Trumpp et al., 1999) and limb development (Lewandoski et al., 2000). Using this approach, an allelic series has been generated at the *Fgf* receptor 1 locus, including hypomorphic and semi-dominant alleles (Partanen et al., 1998). Thus, this approach requires the production of only one mouse line and therefore no more laborious than a traditional knock-out technique. It should provide a widely applicable method for obtaining the means to perform an extensive genetic analysis of the genes of interest.

Fig. 4 Controlling gene expression by Cre/*loxP* and Flp/*frt* recombinases.

(A) Sequences of *loxP* and *frt* sites. (B) The principle of Cre- or Flp-mediated recombination. (A) The 34-bp *loxP* and *frt* sites each consist of two 13-bp inverse repeats (black) that flank an 8-bp core sequence (red in *loxP* and blue in *frt*). The core sequence confers directionality to these sites (black arrows under the sequences). (B) Dimers of Cre or Flp (pink) catalyse in cis the conservative recombination between two directly repeated *loxP* or *frt* sites (orange arrowheads), resulting in the formation of a synaptic structure, the excision of region "b" and the juxtaposition of regions "a" and "c". If region "b" is an essential region of a gene, then the recombination event results in gene inactivation. Recombination can also activate gene expression. For example,

A

loxP

ATAACTTCGTATAATGTATGCTATACGAAGTTAT

frt

CTTCAAGGATAAGAGATCTTTGTATAGGAACTTC

B

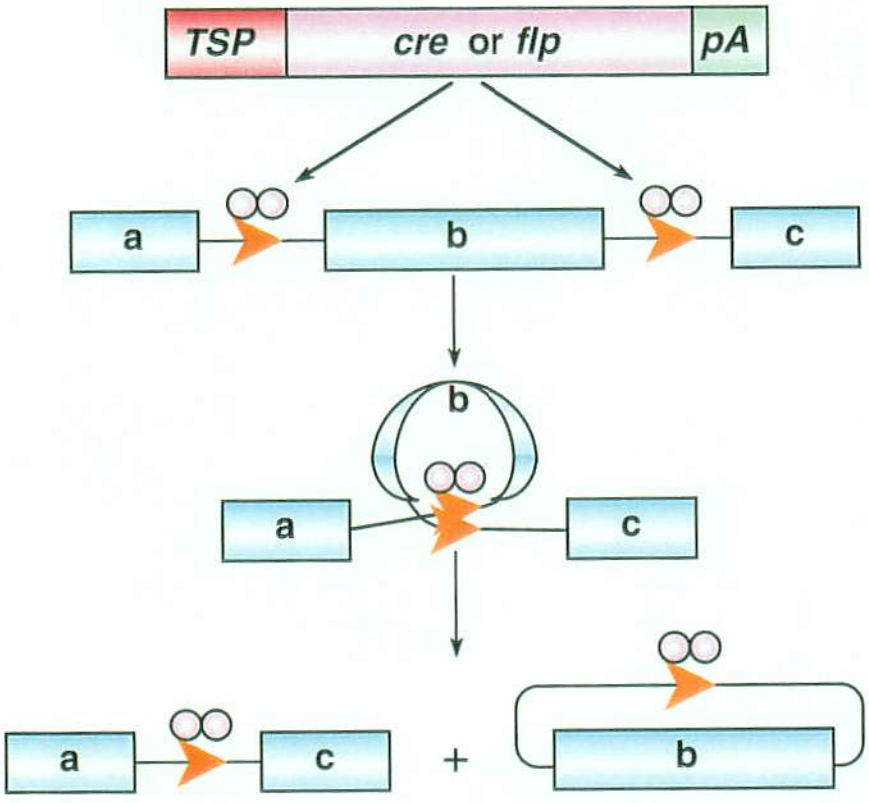


Fig. 4

transcription from a promoter in region "a" could fail to reach protein-coding sequences in region "c" if polyadenylation sites exist in region "b"; excising region "b" would therefore activate transcription of region "c". Abbreviation: pA, polyadenylation site; TSP, tissue-specific promoter. (modified from Lewandoski, 2001)

(5) The purpose of current research on the role of *Otx2* gene for forebrain development

The murine paired-like homeobox gene *Otx2* is a homologue of *Drosophila* head gap gene *orthodenticle* (Simeone et al., 1992). *Otx2* is thought to play key roles on brain development at various developmental stages (Simeone et al., 1992, 1993; Frantz et al., 1994). Several reports have been shown *Otx2* is essential in forebrain development, the entire forebrain and midbrain fail to develop in *Otx2* homozygous mutant embryos (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Chimeric and genetic studies have indicated that *Otx2* is required at an earlier stage prior to and during gastrulation for the formation of AVE (Rhinn et al., 1998; Kimura et al., 2000). Nevertheless, analysis of chimeric embryos, *Otx1* knock-in mutation and transgenic rescue experiments have suggested that *Otx2* is also required in the neuroectoderm for forebrain development (Acampora et al., 1998; Rhinn et al., 1998, 1999; Suda et al., 1999; Kimura et al., 2000). Additionally, *Otx2* functions in the formation of mesencephalic territory by determining the position of the isthmic organizing center cooperating with *Otx1* (Suda et al., 1996, 1997; Acampora et al., 1997). Furthermore, *Otx2* is essential for diencephalon development in cooperation with *Emx2* (Suda et al., 2001). However, the molecular mechanism by which *Otx2* functions in forebrain development, also including the issues discussed in previous sections, remains unclear.

Since *Otx2* homozygous null mutants caused early embryonic lethality before 9.5 dpc (Matsuo et al., 1995), standard genetic approaches are not suitable for further functional analysis of *Otx2* at the later developmental stages. In order to elucidate the

precise role of *Otx2* in forebrain specification at the neural plate stage, we designed a strategy for tissue-specific conditional knock-out of *Otx2* gene based on Cre/*loxP* and Flp/*frt* recombination systems. We have attempted to produce an allelic series of *Otx2* mutations by this methodology. In the course of the generation of various alleles, we found that the *neo*-cassette insertion creates a hypomorphic *Otx2* allele. The phenotype of the mutant embryo carrying both a hypomorphic allele and a null allele was analyzed. This investigation revealed that mutant embryos failed to form the forebrain region correctly. Analyses employing molecular markers and neural plate explant studies have indicated that *Otx2* may be required to respond to signals from the ANR with respect to forebrain specification. This study brings the new insights on the *Otx2* functions of the mechanisms of forebrain development.

2. Materials and Methods

(1) Construction of the *Otx2* targeting vector containing *loxP* and *frt* sites

To generate an *Otx2* allele that can be modified by both Cre- and Flp-mediated recombination, the targeting vector was effected via initially subcloning a 3-kb *Eco*RI-*Sph*I fragment of mouse *Otx2 3'* homologous region into modified pBluescriptII with a DT-A fragment (Yagi et al., 1993). Subsequently, the *PGKneopA*-cassette (*neo*-cassette), flanked by *frt* sites and including a *loxP* site inserted in 5' fashion relative to the *frt* site, was inserted into 5' of the *Otx2 3'* region adjacent to the *Nhe*I and *Eco*RI sites. Following *neo*-cassette insertion, a 2.5-kb *Bam*HI-*Nhe*I fragment, containing *Otx2* promoter and the first coding exon, was inserted 5' of the *neo*-cassette. Finally, a *Sma*I-*BamH*I 5.2-kb 5' *Otx2* region possessing a *loxP* site at the 3' end was inserted 5' homologous of the *Otx2* promoter. Standard techniques were used for all constructs during molecular cloning (Sambrook et al., 1989). Products and reagents from Amersham Pharmacia Biotech, Boehringer Mannheim, GIBCO BRL, New England Biolabs, TaKaRa and TOYOBO company were used. The two *frt* sequences were generated by annealing the two synthetic oligo DNA strands at 70°C, 10 minutes (5'-CCGGATCCGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGG AACTTCACTGCAGGG-3' and 5'-CCCTGCAGTGAAGTTCCTATACTTTCTAG AGAATAGGAACTTCGGAATAGGAACTTCGGATCCGG), and by PCR amplification (primers: 5'-GCCAAGCTTGAAGTTCCTATTCCGAAGTTC-3' and 5'-CCGGTACCGAATTCTGAAGTTCCTATACTTTCTAG-3'), respecitively. The two *loxP* sequences were obtained from pBS246 plasmid (GIBCO BRL) by PCR amplification (primers: 5'-CGGGATCCTGATATCGGCTGGACGTAAACTCCTC TT-3' and 5'-AAAGATCTCGATCATATTCAATAACCCTT-3', and 5'-AGAGATC TAATAACTTCGTATAGCATA-3' and 5'-CGGGATCCTTAATATAACTTCGTA TA-3', respectively). The PCR program included 25 cycles of: 94°C, 45 seconds; 37°C, 25 seconds; 72°C, 2 minutes. PCR fragments were recovered from 9% polyacrylamide

gels for cloning and checked sequences by ABI PRISM™ 310 genetic analyzer (PE Biosystems) according to the manufacturer's instructions.

(2) Generation and genotyping of mice carrying the different *Otx2* alleles

The targeting vector was linearized with *Sal*I and introduced into TT2 embryonic stem (ES) cells via electroporation as described before (Yagi et al., 1993), by Gene Pulser™ (BIO-RAD, Model No. 1652075) under the condition of 0.8 KV, 3 μF, 0.1 second. G418-resistant ES clones were identified by Long PCR by primers 5'-GCTTGAAGTTCCTATTCCGAAGTTC-3' (Forward, neomycin) and 5'-ACTGATTGAGATGGCTGGTAACAGC-3' (Reverse, *Otx2* 3rd exon). The PCR program as follow: 1 cycle of 94°C, 1 minute; 45 cycles of 96°C, 30 seconds and 68°C, 6 minutes; 1 cycle of 72°C, 15 minutes. And confirmed by Southern blot analysis. Four kinds of probes were employed for Southern blot. *Hin*dIII-digested genomic DNAs were hybridized with Probe A (*Sph*I-*Hin*dIII- digested 230-bp fragment of the *Otx2* third coding exon). *Xho*I-digested genomic DNAs were hybridized with Probe B (*Bam*HI-*Pst*I-digested 650-bp fragment of the *neo* gene). *Bam*HI-digested genomic DNAs were hybridized with Probe C (*Eco*RI-*Eco*RV-digested 600-bp fragment of the *Otx2* 5' region). *Nhe*I-digested genomic DNAs were hybridized with Probe D (*Eco*RI-*Kpn*I-digested 750-bp fragment of the *Otx2* first intron) (Figs. 5A and 5B).

Two independent homologous recombinant clones were injected into morula-stage embryos to generate chimera mice. Male chimeras were mated with CBA or C57BL/6 females in order to produce heterozygous mutants ($Otx2^{frt\text{-}neo/+}$). These $Otx2^{frt\text{-}neo/+}$ mice were crossed with *Flpe* transgenic mice carrying the *CAG-Flpe* transgene (provided by Dr. F. Stewart) in order to delete the *neo*-cassette, resulting in the production of $Otx2^{flox/+}$ heterozygous mice (Fig. 5A). The $Otx2^{flox/+}$ mice can be inverted into $Otx2^{\Delta pro\text{-}ex1/+}$ heterozygous mice, which are characterized by deletion of the *Otx2* promoter and first coding exon, through mating with *Cre* transgenic mice (Fig. 5A; Lewandoski et al., 1997). The $Otx2^{frt\text{-}neo/+}$ mice can also be changed to $Otx2^{null\text{-}neo/+}$ heterozygous mice by Cre-mediated recombination (Fig. 5A). These mutant phenotypes were analyzed

mainly in the CBA genetic background. Genotypes of mutant mice were performed routinely by PCR of genomic DNA extracted from adult mice tail or embryonic yolk sacs (Fig. 5C). The PCR primers were used as follow:

F1: 5'-GTATTTTCCTTGCTACCAAACTGCCGAGTG-3' (*Otx2* 1st intron)

N1: 5'-TCGTGCTTTACGGTATCGCCGCTCCCGATT-3' (*neomycin*)

R1: 5'-CTGGAGGGAAGCCACACCTCTAAGGATTAA-3' (*Otx2* 1st intron)

P1: 5'-TAGAGGGTATTTAAATAAGGAACGACTGGG-3' (*Otx2* 5' region)

P2: 5'-GTCCTTCTAAGAGAACTGGATTGTTAACGA-3' (*Otx2* 5' region)

T1: 5'-CCTATACTTTCTAGAGAATAGGAACTTCGG-3' (*frt*)

GP1: 5'-CGACGTTCTGGAAGCTCTGTTTGCCAAGAC-3' (*Otx2* 2nd coding exon)

OX2A: 5'-GCACCCTGGATTCTGGCAAGTTGATTTTCA-3' (*Otx2* 2nd coding exon)

The PCR program for F1/N1/R1 (*frt-neo*), F1/R1 (*flox*), P1/R1 (*Δpro-ex1*), P1/T1 (*null-neo*) and OX2A/N1/GP1 (null) primers have 35 cycles of 94°C, 45 seconds; 63°C, 25 seconds and 72°C, 2 minutes. For P1/P2 (1st *loxP* site) primers had 35 cycles of 94°C, 45 seconds; 50°C, 25 seconds and 72°C, 2 minutes. PCR products were visualized on 1% agarose gels or 9% polyacrylamide gels depend on fragment size.

(3) Generation of transgenic mice

The plasmids were linearized by restriction enzyme, and recovered by 1% LMP-agarose gels (GIBCO BRL). Then treated by GELase™ agarose gel-digesting preparation (EPICENTRE TECHNOLOGIES), further purified by ELUTIP-d column (Schleicher & Schuell) according to protocol. DNAs were diluted to 1ng/μl by injection buffer (0.5mM Tris-HCl, 0.1mM EDTA. pH7.4) and sterilized by 0.22μm filter (MilliPore). Transgenic mice were generated by microinjection of DNA into fertilized eggs as described (Hogan et al., 1994). Genotypes of offsprings were carried out by PCR of genomic DNA extracted from adult mice tail or embryonic yolk sacs. The PCR primers were used as follow:

Flp F1: 5'-CCACCTAAGGTCCTGGTTCGTCAGTTTGTG-3'

Flp R1: 5'-ATACAAGTGGATCGATCCTACCCCTTGCGC-3'

Cre401: 5'-CTCTAGCGTTCGAACGCACTGATTTCGACC-3'

Cre850: 5'-GGTATCTCTGACCAGAGTCATCCTTAGCGC-3'

The PCR program of both Flp and Cre primers contain 35 cycles of 94°C, 45 seconds; 58°C, 25 seconds and 72°C, 2 minutes. PCR amplified 300bp *Flp* fragment and 450bp *Cre* fragment were checked by 1% agarose gels.

(4) Reverse transcription (RT)-PCR analysis

RNA was isolated from 6.5, 7.5 or 8.5 dpc embryos and total RNA was prepared by TRIZOL reagent (GIBCO BRL). First strand cDNA synthesis was performed with oligo-dT primers and Superscript kit (GIBCO BRL). The cDNA was utilized as substrate for PCR by standard protocols. The PCR primers are used as follow:

E1: 5'-TATGGACTTGCTGCATCCCTCCGTGGGCTA-3' (*Otx2* 1st coding exon)

E3: 5'-TGGCAGGCCTCACTTTGTTCTGACCTCCAT-3' (*Otx2* 3rd coding exon)

N0: 5'-TTGACAAAAAGAACCGGGCGCCCCTGCGCT-3' (*neomycin*)

N1: 5'-TCGTGCTTTACGGTATCGCCGCTCCCGATT-3' (*neomycin*)

N2: 5'-AATCGGGAGCGGCGATACCGTAAAGCACGA-3' (*neomycin*)

The PCR program included 35 cycles of 94°C, 45 seconds; 63°C, 25 seconds and 72°C, 2 minutes. PCR products were visualized on 3% agarose-1000 gels (GIBCO BRL).

(5) Histological analysis

Embryos were fixed in Bouin's fixative, dehydrated, embedded in paraplast (OXFORD) and sectioned at 8 μm by rotary microtome HM355. Sections were then dewaxed, rehydrated and stained with Hematoxylin-Eosin solution (Wako). Whole mount in situ hybridization specimens were treated by Histoclear II agent (National Diagnostics). Slices were visualized under Nikon ECLIPSE E600 light microscope and photographed by LEITZ DMD microscope (Leica) using FUJI ISO100 color film.

(6) Skeletal analysis

Skeletal preparation and staining were conducted as described previously (Kelly et al., 1983; Matsuo et al., 1995). 18.5dpc mice head region were placed in 70% alcohol to remove skin and eyes, then fixed in formalin-acetic acid-70% alcohol (1:1:8) about one hour. Cartilage staining was performed for 36~48 hours by 0.03% alcian blue 8GX (Wako) solution and bone was stained about 24 hours by 0.02% alizarin red S (Merck). Specimens were stored in 80% glycerol and photographed by WILD HEERBRUGG microscope (Leica) using FUJI ISO100 color film.

(7) Whole mount RNA in situ hybridization

Embryos were dissected in PBS and fixed in 4% paraformaldehyde (PFA) /PBS (Nacalai Tesque) at 4°C, followed by gradual dehydration in methanol/PBS (Wako) containing 0.1% Tween-20 (PBT). Specimens were stored in 100% methanol at -20°C. Whole mount in situ hybridization was performed by digoxigenin (DIG)-labeled riboprobes (Boehringer Mannheim) as described (Wilkinson, 1993). Two-color whole-mount in situ hybridization was carried out as described (Hauptmann and Gerster, 1994; Stern, 1998). Fluorescein-labeled probe (Boehringer Mannheim) was visualized with BM Purple (Roche), and DIG-labeled probe was visualized with INT/BCIP (Roche). The following probes were employed: *Otx1* and *Otx2* (Matsuo et al., 1995), *En2* (Davis and Joyner, 1988), *Fgf8* (Crossley and Martin, 1995), *Gbx2* (Bolfone et al., 1993), *BF-1* (Tao and Lai, 1992; new name as *Foxg1*), *Emx2* (Yoshida et al., 1997), *Six3* (Oliver et al., 1995), *Pax6* (Stoykova and Gruss, 1994), *Nkx2.1* (Kimura et al., 1996), *mdkk-1* (Glinka et al., 1998), *Cer-1* (Belo et al., 1997), *Foxa2* (Sasaki and Hogan, 1993), *goosecoid* (Blum et al., 1992), *Shh* (Echeland et al., 1993), *Foxd4* (Kaestner et al., 1995; previously referred as *fkh-2*), *Cripto* (Ding et al., 1998) and *Brachyury/T* (Herrmann, 1991). Pictures were taken by LEITZ DMD (Leica) or WILD HEERBRUGG (Leica) or LEICA MZ FLIII microscope using FUJI ISO100 or 400 color film.

(8) Explant culture and FGF8b-bead implantation

Mice were mated and fertilization was assumed to occur at midpoint of the dark cycle. 0-5 somites embryos were collected in chilled Hank's solution. The cephalic region was isolated from the embryos and digested with 2.5-3.5% pancreatin (Sigma) and 0.5-0.7% trypsin (wako) in $MgCl_2$- and $CaCl_2$-depleted Hank's solution depending on embryonic stages. Germ layer separation was achieved with tungsten needles (Shimamura and Rubenstein, 1997).

The neural plate from 0-2 somites wild type embryos was isolated exclusively (Fig. 11, Exp. 1; Shimamura and Rubenstein, 1997). The anterior axial mesendoderm, which consists of the prechordal plate and anterior portion of the head process from wild type or mutant embryos at the 0-2 somites stage, was transplanted to the wild type neural plate, respectively (Fig. 11, Exps. 2 and 3). These explants were cultured on a nuclepore filter (Whatman #110614) floating in DMEM (GIBCO BRL) supplemented with 20% fetal bovine serum (Cell Culture Technologies, LOT#20120642), 1X non-essential amino acids (GIBCO BRL) and 0.1XPenicillin-Streptomycin (GIBCO BRL) in a CO_2 incubator at 37°C for 24 hours (Shimamura and Rubenstein, 1997; Kimura et al., 2000).

Heparin acrylic beads (Sigma) were rinsed in PBS several times and approximately 50 beads were soaked in 5 μl 0.2 mg/ml FGF8b recombinant protein solution (R&D) overnight at 4°C (Liu et al., 1999). The beads were rinsed in PBS prior to use. Control beads were soaked in 50 mg/ml BSA-PBS in an identical manner. The left side of the ANR was removed from the wild type neural plate (Fig. 14, Exp. 1). The ANR from wild type or mutant embryos at the 3-5 somites stage was transplanted to the wild type neural plate in the region where the left side of the ANR was ablated (Fig. 14, Exp. 2). On the other hand, wild type ANR (3-5 somites stage) transplanted to entire mutant neural plate was also did (Fig. 14, Exp. 3). The intact mutant neural plate was isolated exclusively (Fig. 14, Exp. 4). A single heparin acrylic bead soaked in BSA or FGF8b was implanted to the left side of the wild type neural plate where ANR had been excised unilaterally (Fig. 14, Exp. 5) or to the intact mutant neural plate (Fig. 14, Exp. 6). These explants were cultured as same conditions as above.

At the end of culture, the membranes were transferred into fresh 4% PFA/PBS to let the explants float off the membrane. Then the explants were fixed in 4% PFA/PBS at 4°C, dehydrated through 25%, 50% and 75% methanol/PBT and stored in methanol at -20°C for in situ hybridization.

(9) Germ-layer explant recombination assay

The assay was carried out as previous reports (Ang and Rossant, 1993; Kimura et al., 2000). The 6.5 dpc GFP embryos at pre- to early streak stages, the 7.5 dpc embryos at headfold stages were collected. The extraembryonic regions of 7.5 dpc headfold stage embryos were divided for PCR genotyping to decide mutant embryos. The stages of embryos were staged according to morphological landmarks (Downs and Davies, 1993). The embryos were treated in 0.25% pancreatin and 0.5% trypsin in Hank's solution at room temperature for 5-10 minutes (pre- to early stages) or 15-20 minutes (headfold stages). Then by using tungsten needles, 6.5 dpc epiblast was separated from visceral endoderm, and 7.5 dpc anterior definitive endoderm (ADE) with cranial mesoderm was isolated from ectoderm. In the recombination experiments, a single epiblast piece was reaggregated with ADE and cultured as described above. After 24 hours culture, explants were fixed in 4% PFA/PBS and observed under LEICA MZ FLIII fluorescence microscope and took digital pictures by HAMAMATSU color chilled 3CCD camera controller. Then did dehydration and stored at -20°C for in situ hybridization.

3. Results

(1) Generation of an allelic series of *Otx2* mutants controlling by Flp- or Cre-mediated recombination

To generate a series of *Otx2* mutant alleles, a targeting vector was designed in which the *Otx2* promoter and first coding exon region were flanked with two *loxP* sites; additionally, a *PGKneopA* (*neo*-) cassette flanked by two *frt* sites was inserted into the *Otx2* first intron (Fig. 5A). We planned to excise the *neo*-cassette in the first intron by Flp-mediated recombination and the first coding exon by Cre-mediated recombination; the latter should eliminate *Otx2* function as this exon encodes the translational start site. Following electroporation in TT2 ES cells, eight of 182 G418-resistant ES clones were isolated as homologous recombinants by PCR and finally confirmed by Southern blot (Fig. 5B; data not shown). Chimeric mice and their mutant offspring were generated from two independent recombinant ES clones (E90 and E92). No phenotypic difference was evident between these two mutant lines (see below). Heterozygous offspring of chimera bearing the $Otx2^{frt\text{-}neo}$ allele was viable and fertile; offspring exhibited no noticeable phenotype in both CBA and C57BL/6 genetic background (Figs. 7A and 7C; data not shown). However, $Otx2^{frt\text{-}neo/frt\text{-}neo}$ homozygous mutants died at birth or by weaning, and displayed slight brain abnormalities (Figs. 7A and 7D, 7F and 7G; Table 1; data not shown).

$Otx2^{frt\text{-}neo/+}$ mice were mated to *CAG-Flpe* transgenic animals, which ubiquitously express Flpe, a variant *Flp* recombinase possessing enhanced thermostability, in order to remove the *neo*-cassette (Buchholz et al., 1998). The mutant progeny carrying the transgene effectively inherited the recombined allele ($Otx2^{flox}$ allele) (Figs. 5B and 5C). Subsequently, $Otx2^{\Delta pro\text{-}ex1/+}$ heterozygous mutants were obtained by mating $Otx2^{flox/+}$ mice with *β-actin-cre* mice, which ubiquitously express Cre under the human *β-actin* promoter (Lewandoski et al., 1997) (Figs. 5B and 5C). Another heterozygous mutant

mice $Otx2^{null\text{-}neo/+}$ were produced by crossing $Otx2^{frt\text{-}neo/+}$ mice with *cre* transgenic mice (Figs. 5B and 5C). $Otx2^{flox/+}$, $Otx2^{flox/flox}$, $Otx2^{\Delta pro\text{-}ex1/+}$ and $Otx2^{null\text{-}neo/+}$ mutant mice thus produced were fertile and showed no noticeable phenotype in the CBA genetic background. In order to reduce *Otx2* dosage more, $Otx2^{frt\text{-}neo/+}$ mice were then crossed with $Otx2^{+/-}$ mutant mice, which carry a null allele (Matsuo et al., 1995), resulting in offspring containing both a *frt-neo* and a null allele. Unexpectedly, all the $Otx2^{frt\text{-}neo/-}$ mutant fetuses exhibited dramatic head malformations such as excencephaly and acephaly at 18.5 dpc (Fig. 7E, data not shown). The compound $Otx2^{frt\text{-}neo/\Delta pro\text{-}ex1}$ mutant embryos showed similar phenotypes as the cases of $Otx2^{frt\text{-}neo/-}$ mutants (Table 1, data not shown). In contrast, $Otx2^{flox/-}$ fetuses were viable and demonstrated no apparent abnormalities, suggesting that $Otx2^{flox}$ allele functions as wild type *Otx2* allele. All phenotypes of different *Otx2* allelic mutants were summarized in Table 1.

Fig. 5 Generation of an allelic series of *Otx2* mutations.

(A) Strategy for production of an allelic series of mutations at the *Otx2* locus. Schematic representation of the *Otx2* wild type allele (a), the targeting vector (b), the $Otx2^{frt\text{-}neo}$ allele (c), the $Otx2^{null\text{-}neo}$ allele (d), the $Otx2^{flox}$ allele (e) and the $Otx2^{\Delta pro\text{-}ex1}$ allele (f), respectively. The $Otx2^{frt\text{-}neo}$ allele is obtained by the standard method of homologous recombination (c). Mice heterozygous for $Otx2^{frt\text{-}neo}$ allele are crossed with *Cre* transgenic mice to generate mice carrying the $Otx2^{null\text{-}neo}$ allele which the *Otx2* promoter and first coding exon are excised but *neo*-cassette is remained (d). $Otx2^{frt\text{-}neo/+}$ mice can also be crossed with *Flpe* transgenic mice to generate animals bearing the $Otx2^{flox}$ allele (e), in which the *neo*-cassette is excised. Heterozygotes for the $Otx2^{flox}$ allele are further crossed with *Cre* transgenic mice to generate mice carrying the $Otx2^{\Delta pro\text{-}ex1}$allele (f), in which the *Otx2* promoter and first coding exon are deleted. Horizontal thick lines represent *Otx2* genomic DNA, whereas the thin line represents pBluescript

sequences. Filled boxes represent *Otx2* coding exons. Red arrowheads and blue arrowheads denote *loxP* and *frt* sites, respectively. Yellow box and *Neo*^{r}: neomycin-resistant gene with the PGK promoter and polyadenylation signal. DT-A: diphtheria toxin A fragment gene with MC1 promoter. Restriction enzyme sites: B, *Bam*HI; H, *Hind*III; E, *Eco*RI; N, *Nhe*I; NI, *Not*I; S, *Sph*I; SI, *Sal*I; Sm, *Sma*I; X, *Xho*I. Probes A, B, C and D are used for Southern blot identification. P1, P2, T1, F1, N1 and R1 show primers utilized for the identification of the different *Otx2* alleles by PCR analysis. (B) Identification and characterization of mutant allele by Southern blot analysis. (a) Genomic DNAs from wild type and mutant mice were digested with *Hind*III and hybridized with probe A to examine the correct 3' recombination. The 5.6- and 3-kb bands represent wild type and targeted alleles, respectively. (b) Digested with *Xho*I and hybridized with probe B for the *neo*-cassette identification. The 8-kb band represents targeted allele. (c) Digested with *Bam*HI and hybridized with probe C for the correct 5' recombination and the presence of the first *loxP* site. Recombinants generate a 7-kb band in addition to the 20-kb band of a wild type allele. (d) Digested with *Nhe*I and hybridized with probe D for identification of $Otx2^{frt\text{-}neo}$ (4-kb), $Otx2^{flox}$ (2-kb) and $Otx2^{\Delta pro\text{-}ex1}$ (5-kb) alleles, as well as 8-kb band of wild type allele. (C) PCR analysis of genotyping for different alleles. (a) PCR amplification employing a combination of primers F1, N1 and R1 generates a 550- and 360-bp product for the $Otx2^{frt\text{-}neo}$ and wild type allele, respectively. (b) Primers P1 and P2 amplified a 220- and 160-bp product indicating the presence of the *loxP* site located 5' upstream in the mutant allele and the absence of the *loxP* site in the wild type allele, respectively. (c) In the $Otx2^{flox}$ allele, a 560-bp PCR product is amplified by primers F1 and R1. (d) Primers P1 and R1 are used to detect the $Otx2^{\Delta pro\text{-}ex1}$ allele (300-bp). (e) Primers P1 and T1 produced a 180-bp fragment represents the $Otx2^{null\text{-}neo/+}$ allele.

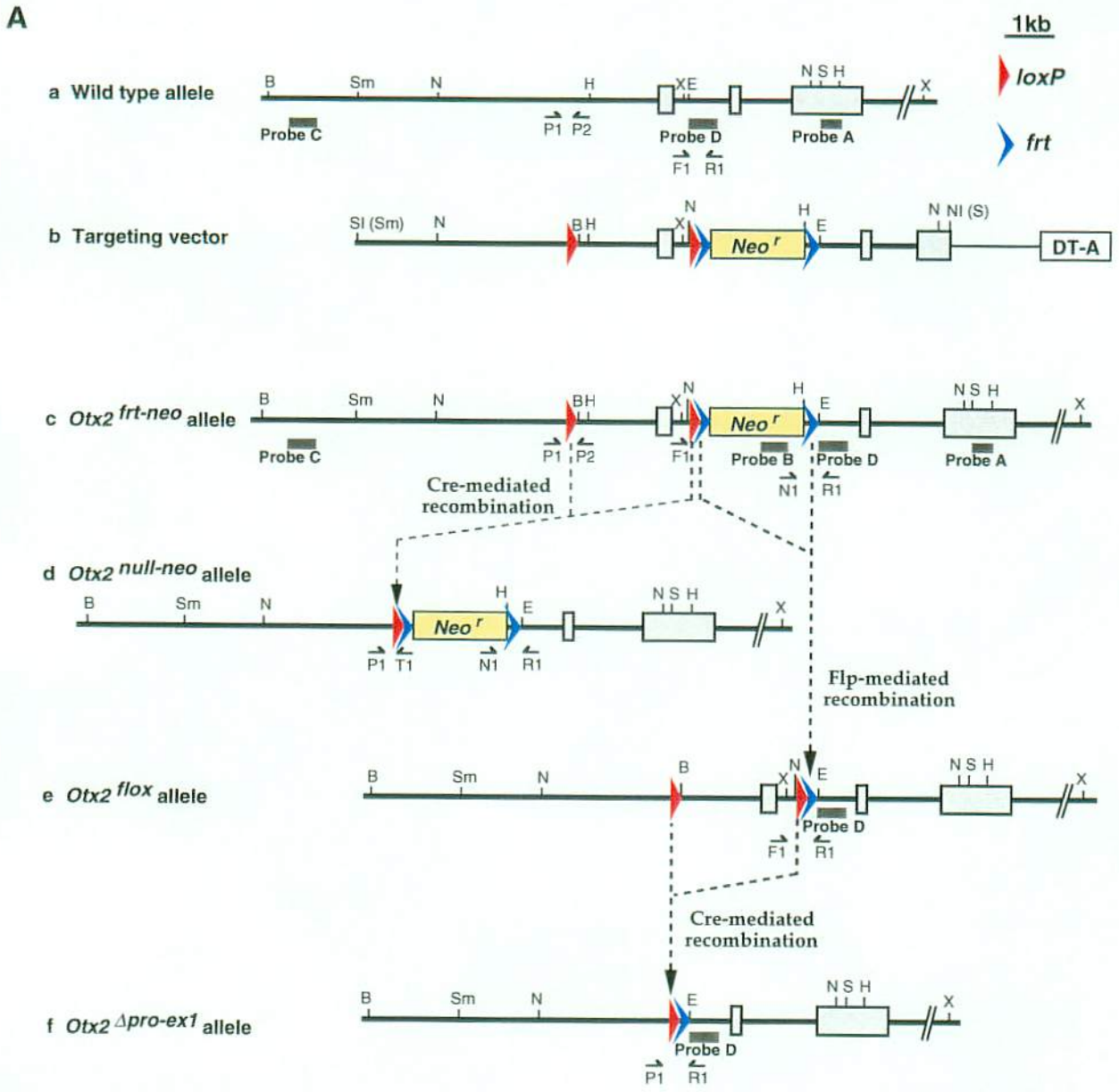
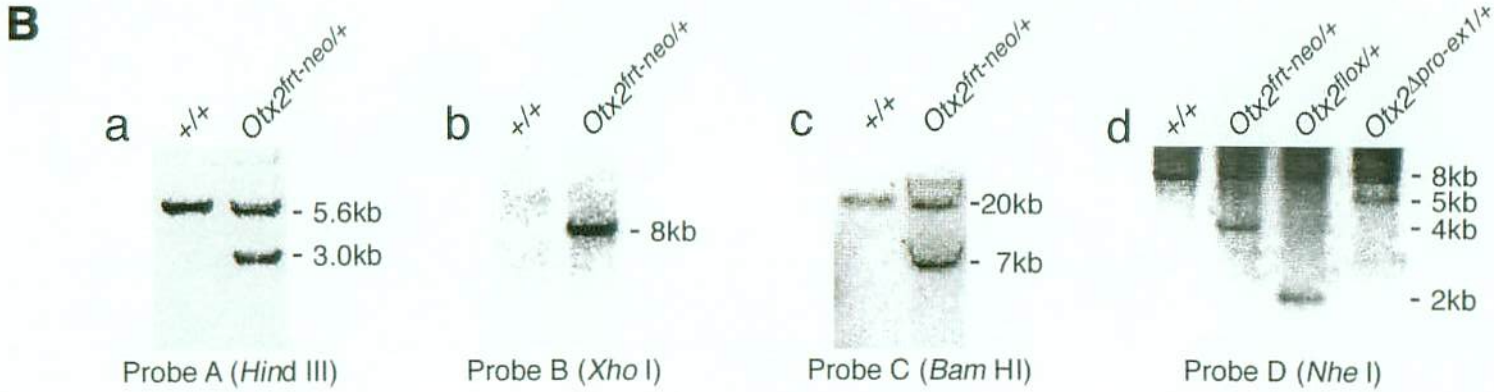

A
1kb
loxP
frt
a Wild type allele
B Sm N H XE N S H X
Probe C
P1 P2
Probe D
F1 R1
Probe A
b Targeting vector
Sl (Sm) N BH X N Neo r H E N Nl (S)
DT-A
c Otx2 frt-neo allele
B Sm N BH X N Neo r H E N S H X
Probe C
P1 P2
F1
Probe B
N1
Probe D
R1
Probe A
Cre-mediated recombination
d Otx2 null-neo allele
B Sm N H Neo r E N S H X
P1 T1
N1
R1
Flp-mediated recombination
e Otx2 flox allele
B Sm N B X N E N S H X
Probe D
F1
R1
Cre-mediated recombination
f Otx2 Δpro-ex1 allele
B Sm N E N S H X
Probe D
P1
R1

Fig. 5-1


B
a
+/+
Otx2frt-neo/+
- 5.6kb
- 3.0kb
Probe A (Hind III)
b
+/+
Otx2frt-neo/+
- 8kb
Probe B (Xho I)
c
+/+
Otx2frt-neo/+
-20kb
- 7kb
Probe C (Bam HI)
d
+/+
Otx2frt-neo/+
Otx2flox/+
Otx2Δpro-ex1/+
- 8kb
- 5kb
- 4kb
- 2kb
Probe D (Nhe I)

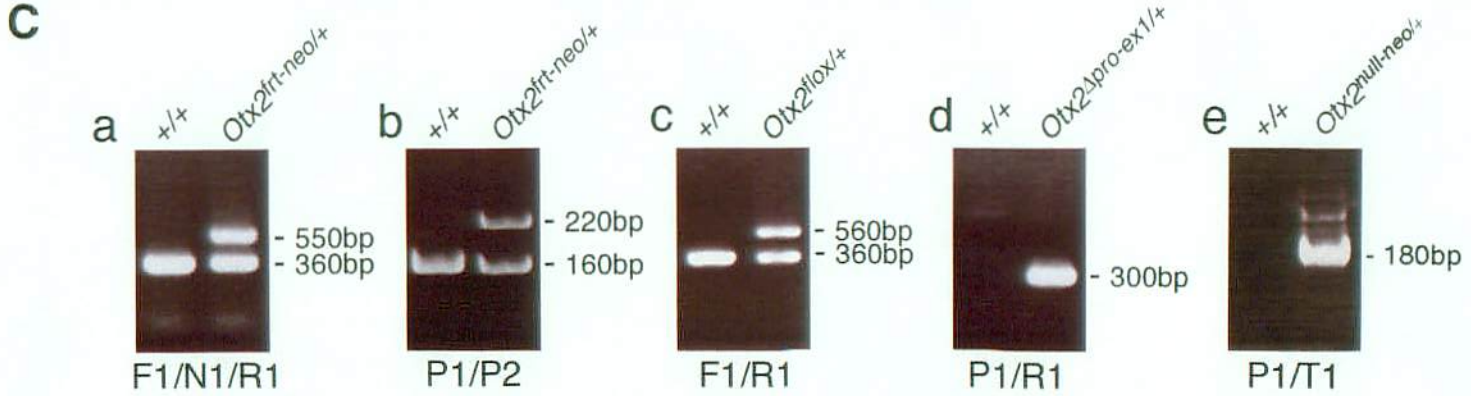

C
a
+/+
Otx2frt-neo/+
- 550bp
- 360bp
F1/N1/R1
b
+/+
Otx2frt-neo/+
- 220bp
- 160bp
P1/P2
c
+/+
Otx2flox/+
- 560bp
- 360bp
F1/R1
d
+/+
Otx2Δpro-ex1/+
- 300bp
P1/R1
e
+/+
Otx2null-neo/+
- 180bp
P1/T1

Fig. 5-2

Table 1 Phenotypes of different *Otx2* mutant allelic combinations

Genotypes	Stage analysed	No. examined	Phenotypes
Otx2 frt-neo/+	Postnatal	68	Normal
Otx2 frt-neo/frt-neo	6.5 dpc ~ postnatal	25	Death at birth or by weaning, dwarf
Otx2 frt-neo/- [*1]	6.5 ~ 18.5 dpc	347	• Normal gastrulation • Open brain (exencephaly) • Truncation of rostral brain • Abnormal craniofacial development
Otx2 flox/+	Postnatal	41	Normal; fertile
Otx2 flox/- [*2]	10.5 dpc ~ postnatal	10	Normal
Otx2 flox/flox	Postnatal	73	Normal; fertile
Otx2 Δpro-ex1/+	Postnatal	36	Normal; fertile
Otx2 null-neo/+	Postnatal	13	Normal; fertile
Otx2 frt-neo/Δpro-ex1 [*3]	9.0 ~ 18.5 dpc	19	Similar with *Otx2 frt-neo/-*

1. *Otx2*$^{frt\text{-}neo/-}$ mutants were generated by crossing *Otx2*$^{frt\text{-}neo/+}$ mice with *Otx2*$^{+/-}$ mutant mice, which carry a null allele (Matsuo et al., 1995). In this study, *Otx2*$^{frt\text{-}neo/-}$ mutants were examined at various stages: 6.5 dpc (17), 7.5 ~ 7.8 dpc (46), 8.5 dpc (224), 9.5 ~ 10.5 dpc (44), 12.5 ~ 15.5 dpc (9) and 18.5 dpc (7). The numbers in the bracket represent the number of *Otx2*$^{frt\text{-}neo/-}$ embryos analysed.

2. The *Otx2*$^{flox/-}$ fetuses and mice were obtained by mating *Otx2*$^{flox/+}$ mice with *Otx2*$^{+/-}$ mutant mice.

3. *Otx2*$^{frt\text{-}neo/\Delta pro\text{-}ex1}$ mutants were generated by mating *Otx2 frt-neo/+* mice with *Otx2*$^{\Delta pro\text{-}ex1/+}$ heterozygous mice, which displayed similar abnormal brain phenotypes with *Otx2*$^{frt\text{-}neo/-}$ mutants.

(2) *Neo*-cassette insertion creates a hypomorphic *Otx2* allele

Neo-cassette was supplied as a selectable marker to isolate homologous recombinant clones; however, it has been reported to contain potential cryptic splice sites that may interfere with gene expression (Carmeliet et al., 1996; Meyers et al., 1998; Nagy et al., 1998). To examine whether the presence of the *neo*-cassette in the intron affected *Otx2* gene expression by aberrant splicing, reverse transcription (RT)-PCR analysis was performed at several developmental stages (6.5, 7.5 and 8.5 dpc) using primers from *Otx2* and *neo* sequences (Fig. 6A). Indeed, the *neo*-cassette included both a cryptic splice acceptor and a splice donor (Fig. 6). Aberrant splicing already happened at pre- or early-streak stages, PT-PCR showed same patterns at three stages (Fig. 6A, data not shown). Sequence analysis of RT-PCR products indicated that the splice acceptor is located 5 bp upstream of the *neo* translation start site, whereas the splice donor is located 15 bp downstream of the *neo* TGA stop codon (Fig. 6B). In addition, two types of *neo* fusion transcripts were produced: one contained the *Otx2* second exon, which encodes homeodomain, whereas the second lacked this exon as a consequence of aberrant splicing (Fig. 6B). Therefore, $Otx2^{frt\text{-}neo/frt\text{-}neo}$ homozygous embryos exhibited three kinds of transcripts, wild type normal transcripts and the two types of *Otx2-neo* fusion mRNA transcripts (Fig. 6). Nucleotide sequences of two aberrant transcripts predicted that neither produced normal Otx2 protein; transcripts contained only 33 amino acids in the N-terminal peptides of Otx2 in fusion with entire neo protein products (Fig. 6B). Furthermore semi-quantitative PCR analysis indicated that the expression level of wild type mRNA transcripts was significantly reduced in $Otx2^{frt\text{-}neo/frt\text{-}neo}$ mutant embryos (Fig. 6A). Analysis of *Otx2* expression by in situ hybridization using the third exon as a probe demonstrated that *neo*-cassette insertion did not direct ectopic *Otx2* mRNA expression in $Otx2^{frt\text{-}neo/-}$ mutant embryos (Fig. 6C).

Defects in $Otx2^{frt\text{-}neo/-}$ mutants were more severe than those in $Otx2^{frt\text{-}neo/frt\text{-}neo}$ and $Otx2^{+/-}$ mutants; however, defects were milder than those observed in $Otx2^{-/-}$ mutants (Fig. 7, and data not shown). This finding demonstrated that the *frt-neo* allele was not functioning normally. Analysis of mRNA expression and phenotype, taken together,

indicated that *Otx2*$^{frt\text{-}neo}$ is a hypomorphic allele and that the defects in mutant mice carrying the *Otx2 frt-neo* allele are due to a reduction in the amount of functional *Otx2* gene expression brought about by the presence of the *neo*-cassette in the first intron of the *Otx2* gene. As described below, detailed analysis of the defects caused by the hypomorphic allele has provided new insight into *Otx2* function regarding the mechanism of forebrain development.

Fig. 6 The *Otx2*$^{frt\text{-}neo}$ allele produces aberrant *Otx2* transcripts by the *neo*-cassette insertion.

(A) (a) RT-PCR analysis with primers E1 and N2 utilizing cDNAs from wild type (left) and *Otx2*$^{frt\text{-}neo/frt\text{-}neo}$ homozygous mutant embryo (right) at 8.5 dpc. The 800-bp products in the mutant indicates that the *neo*-cassette contains a splice acceptor. (b) RT-PCR analysis with primers N1 and E3 indicates the presence of two types of aberrant mRNA transcripts (see below). (c) RT-PCR analysis with primers E1, N0 and E3. The 272-bp products correspond to wild type mRNA transcripts, whereas the 200-bp products correspond to *Otx2* and *neo* hybrid mRNA, respectively. In the mutant embryo, the level of wild type mRNA expression is downregulated. (B) (a) Schematic diagram of the *Otx2*$^{frt\text{-}neo}$ allele. Shaded boxes represent *Otx2* coding exons, an open box represents *neo*-cassette. Filled and opened arrowheads denote *loxP* and *frt* sites, respectively. The arrows indicate primers used in RT-PCR analysis. (b, c) Schematic diagrams representing exon structures of mRNAs produced from this allele. The exon structures were determined by sequence analysis of RT-PCR products. Asterisks indicate the stop codon of *neo*. Aberrant *Otx2* transcripts were also evident in *Otx2*$^{frt\text{-}neo/frt\text{-}neo}$ mutant embryos at 6.5 and 7.5 dpc stages (data not shown). (C) Whole mount in situ hybridization of *Otx2* expression in wild type (a) and *Otx2*$^{frt\text{-}neo/-}$ mutant embryos (b) at 8.5 dpc.

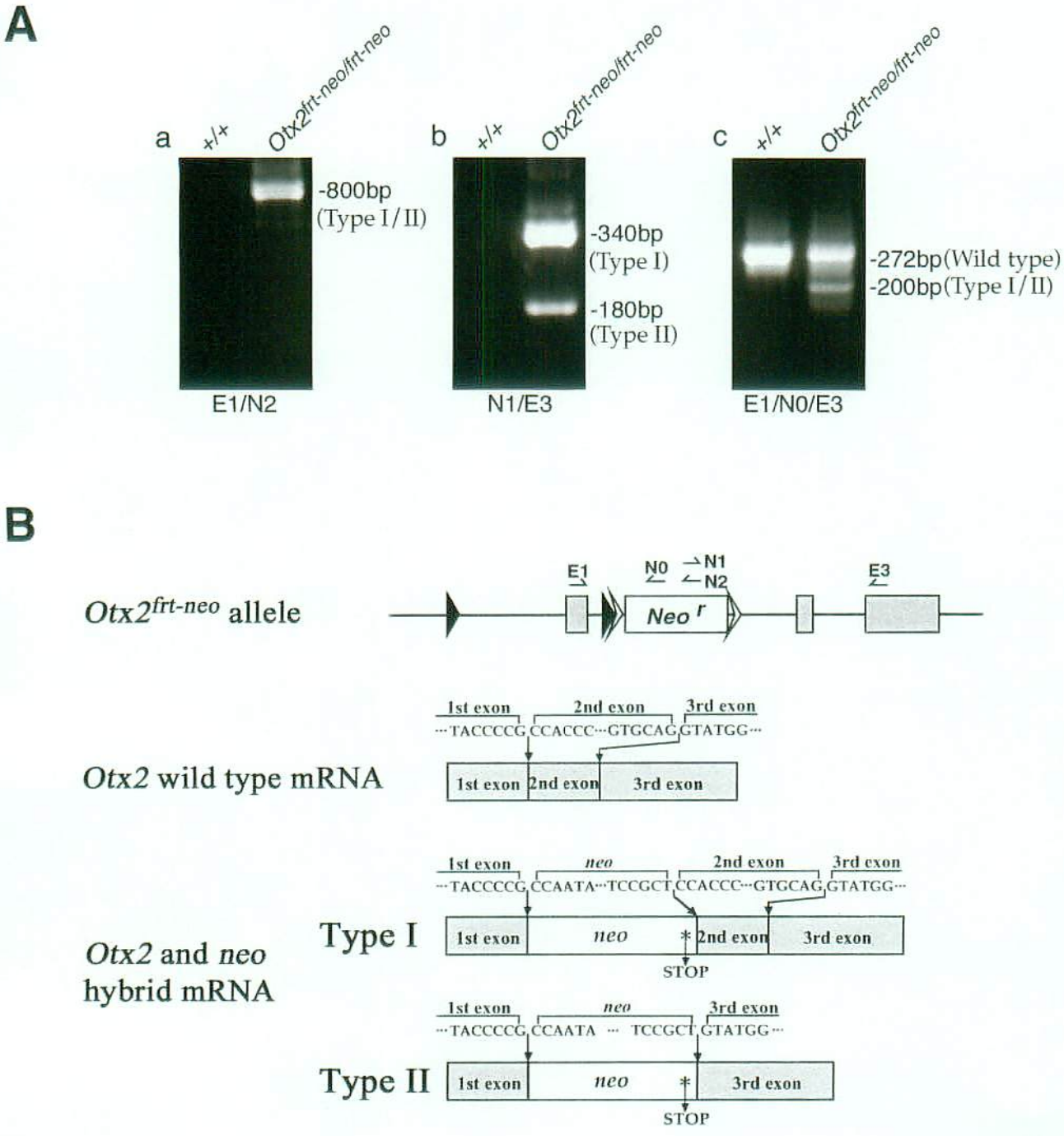

A
a
+/+
Otx2frt-neo/frt-neo
-800bp
(Type I/II)
E1/N2
b
+/+
Otx2frt-neo/frt-neo
-340bp
(Type I)
-180bp
(Type II)
N1/E3
c
+/+
Otx2frt-neo/frt-neo
-272bp(Wild type)
-200bp(Type I/II)
E1/N0/E3
B
Otx2frt-neo allele
E1
N0
N1
N2
Neo r
E3
Otx2 wild type mRNA
1st exon
2nd exon
3rd exon
···TACCCCG CCACCC···GTGCAG GTATGG···
Otx2 and neo hybrid mRNA
Type I
neo
STOP
Type II
C
a
+/+
b
Otx2frt-neo/-

Fig. 6

(3) Forebrain development was arrested in *Otx2*$^{frt\text{-}neo/-}$ hypomorphic mutant embryos

Previously, we reported that mice heterozygous for the *Otx2* mutation display a craniofacial malformation which is mainly characterized as the loss of the lower jaw depending on the genetic background of the C57BL/6 strain (Matsuo et al., 1995). The majority of skull elements at the level of premandibular and distal portions of the mandibular regions were lost or severely affected by this mutation (Matsuo et al., 1995). However, 18.5 dpc *Otx2*$^{frt\text{-}neo/-}$ mutants developed upper and lower jaws appropriately (Figs. 6I and 6K, 6J and 6L), but the sagittal sections at 15.5 dpc showed lacking fore- and midbrain and craniofacial structures in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos (Figs. 7F and 7H). Skeletal analysis also indicated that most of the skull elements in premandibular and distal components of the mandibular regions were present and developed although the morphology of these structures was distorted (Figs. 7I-L). These structures are derived from mesencephalic neural crest cells (Couly et al., 1993); therefore, the neural crest at the level of mesencephalon appeared to be formed appropriately in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos.

18.5 dpc *Otx2*$^{frt\text{-}neo/-}$ mutant mice did not develop rostral brain and eyes (Fig. 7E). We then examined earlier defects in the mutant brain. At 10.5 dpc, the rostral aspect of the brain appeared to be truncated with the failure in neural tube closure (Figs. 7M and 7N, data not shown). Histological examination indicated the complete absence of the structures of the telencephalic vesicles and diencephalon (Figs. 7O and 7P). Additionally, the mesencephalon and rostral portion of metencephalic regions, including the isthmus, also appeared to be malformed in the mutant embryos (Figs. 7O and 7P). These morphological data indicate that the majority of the rostral brain failed to develop normally in *Otx2*$^{frt\text{-}neo/-}$ embryos at 10.5 dpc.

Fig. 7 Morphological features of *Otx2*$^{frt\text{-}neo/-}$ mutant embryos.

(A-D) Lateral view of wild type (A), *Otx2*$^{+/-}$ (B), *Otx2*$^{frt\text{-}neo/+}$ (C), *Otx2*$^{frt\text{-}neo/frt\text{-}neo}$ (D) and *Otx2*$^{frt\text{-}neo/-}$ (E) embryos at 18.5 dpc on the CBA genetic background, respectively. *Otx2*$^{+/-}$ and *Otx2*$^{frt\text{-}neo/+}$ show no noticeable abnormalities in the CBA background (B, C). *Otx2*$^{frt\text{-}neo/frt\text{-}neo}$ mutants seems normal at outlook, but died at birth or within weaning (D). *Otx2*$^{frt\text{-}neo/-}$ mutants fail to develop dorsal and rostral regions of the head; however, they do undergo appropriate formation of ventral portions of the cranial region (E). (F-G) Sagittal section at 15.5 dpc in wild type (F), *Otx2*$^{frt\text{-}neo/frt\text{-}neo}$ (G) and *Otx2*$^{frt\text{-}neo/-}$ mutant embryos (H). The midbrain region is slightly deformed in *Otx2*$^{frt\text{-}neo/frt\text{-}neo}$ mutant embryos (G), but no rostral brain and craniofacial structures existed in *Otx2*$^{frt\text{-}neo/-}$ mutants (H). Skull morphologies of wild type (I, K) and *Otx2*$^{frt\text{-}neo/-}$ mutant embryos (J, L) in the CBA background at 18.5 dpc. Ventral views of the whole mount skull without mandibles (I, J) and separated mandibles (K, L) are shown. Most of the skull elements are formed, although slightly distorted, in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos. Lateral view (M, N) and sagittal sections (O, P) of wild type (M, O) and *Otx2*$^{frt\text{-}neo/-}$ (N, P) embryos at 10.5 dpc. The telencephalon and diencephalon are not formed in mutant embryos (N, P). The isthmic constriction (arrows in M, O) is not clearly observed in mutants (N, P). The first branchial arch (ba1) develops normally in the mutant embryo (M, N). Abbreviations: as, alisphenoid; bs, basisphenoid; bo, basioccipital; cb, cerebellum; d, diencephalon; dt, dorsal thalamus; eo, exoccipital; m, mesencephalon; mt, metencephalon; mx, maxillar; ob, olfactory bulb; p, pons; pl, palatine; pm, premaxillar; pt, pretectum; sp, spinal cord; sy, symphysis; t, telencephalon; tm, tegmentum; vt, ventral thalamus.

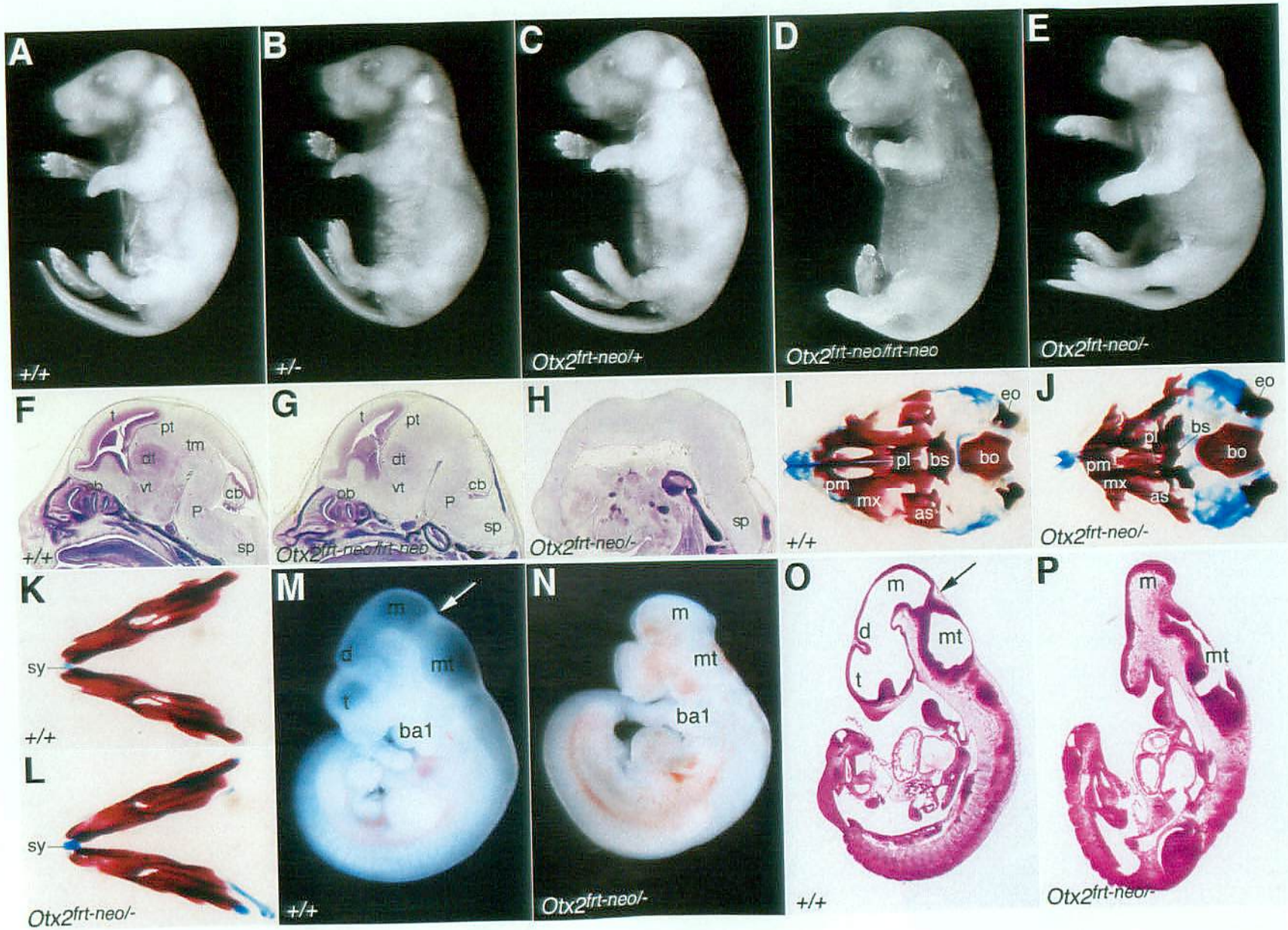

A
+/+
B
+/-
C
Otx2frt-neo/+
D
Otx2frt-neo/frt-neo
E
Otx2frt-neo/-
F
t
pt
tm
dt
vt
cb
P
sp
+/+
G
t
pt
dt
vt
cb
P
sp
Otx2frt-neo/frt-neo
H
sp
Otx2frt-neo/-
I
eo
pl
bs
bo
pm
mx
as
+/+
J
eo
pl
bs
bo
pm
mx
as
Otx2frt-neo/-
K
sy
+/+
L
sy
Otx2frt-neo/-
M
m
d
mt
t
ba1
+/+
N
m
mt
ba1
Otx2frt-neo/-
O
m
d
mt
t
+/+
P
m
mt
Otx2frt-neo/-

Fig. 7

(4) Neuroectodermal marker analysis at 9.5 dpc in *Otx2*$^{frt\text{-}neo/-}$ hypomorphic mutant embryos

Since the development of rostral brain in *Otx2*$^{frt\text{-}neo/-}$ mutant was already defected during mid- to late-gestation, expression of neuroectoderm markers by whole mount in situ hybridization at 9.5 dpc was subsequently analyzed in order to define rostral brain abnormalities in *Otx2*$^{frt\text{-}neo/-}$ embryos (Fig. 8). Expression of molecular markers for forebrain and isthmus were absent or severely affected in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos.

Normally, *BF-1*, a winged-helix transcription factor, which is expressed in most of the telencephalic region and important for forebrain development (Fig. 8A; Tao and Lai, 1992; Xuan et al., 1995); however, its expression in this region was not detected in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos (Fig. 8B). *Emx2*, a homeobox transcription factor, is also expressed in dorsal telencephalon and diencephalon of wild type 9.5 dpc embryos (Fig. 8C). *Emx2* expression was absent in *Otx2*$^{frt\text{-}neo/-}$ hypomorphic mutants (Fig. 8D). At this stage, *Six3* is expressed in anterior forebrain region and eyes in wild type (Fig. 8E; Oliver et al., 1995); however, its expression was undetectable in mutant embryos (Fig. 8F). Normally, *Nkx2.1*, a homeodomain gene, is expressed in ventral forebrain (Fig. 8G; Kimura et al., 1996); *Nkx2.1* expression was not observed in mutant embryos (Fig. 8H). At 9.5 dpc, the secreted factor *Fgf8* is expressed in the commissural plate of telencephalon, the dorsal diencephalon and the isthmic constriction in wild type (Fig. 7I; Heikinheimo et al., 1994; Ohuchi et al., 1994). In *Otx2*$^{frt\text{-}neo/-}$ mutant embryos, *Fgf8* expression in these regions was not found; rather, *Fgf8* was expressed throughout the entire rostral region including non-neural ectoderm (Fig. 8J). The homeobox gene *Engrailed-2* (*En2*) expression, which covers the midbrain and anterior hindbrain at 9.5 dpc in wild type (Fig. 7K; Davis et al., 1988), expressed normally but underwent an anterior shift in *Otx2*$^{frt\text{-}neo/-}$ hypomorphic embryos (Fig. 8L). Thus, the expression of anterior neuroectodermal markers are missed or severe reduced in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos at 9.5 dps.

Fig. 8 Molecular analysis of anterior neural markers at 9.5 dpc.

BF-1 (A, B), *Emx2* (C, D), *Six3* (E, F), *Nkx2.1* (G, H), *Fgf8* (I, J) and *En2* (K, L) expression by whole mount in situ hybridization in wild type (A, C, E, G. I, K) and $Otx2^{frt\text{-}neo/-}$ mutant embryos (B, D, F, H, J, L) at 9.5 dpc. *BF-1* expression occurs in the telencephalic region of the wild type (A); however, *BF-1* expression is not detected in this region (B). Normally, *Emx2* and *Six3* are expressed in the dorsal forebrain and most anterior forebrain, respectively (C, E); however, expression of these genes is not detected in mutant embryos (D, F). *Nkx2.1* expression is found in the ventral forebrain of the wild type (arrow in G); however, it is not observed in the mutant embryo (H). *Fgf8* expression, seen in the telencephalic commissural plate and isthmic constriction in the wild type (arrows in I), is present throughout the entire rostral region in mutant embryos (arrowheads in J). The expression domain of *En2*, which covers the mesencephalon and anterior metencephalon in the wild type (arrows in K), is shifted anteriorly in mutant embryos (arrows in L). Arrowheads indicate the presence of an *En2*- negative domain in the most rostral portion of the mutant neuroectoderm (L).

Fig. 8

(5) Anterior neuroectodermal defects in 8.5 dpc *Otx2*$^{frt\text{-}neo/-}$ hypomorphic mutant embryos

To determine the initial defects in brain formation, expression of neuroectoderm markers at 8.5 dpc were further analyzed (Fig. 9). We found that prosencephalon was not specified correctly in *Otx2*$^{frt\text{-}neo/-}$ embryos.

At 8.5 dpc, *Emx2* expression, a marker for the prospective telencephalon and dorsal diencephalon, was not detected in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos (Figs. 9A and 9B). Expression of *Six3*, the earliest anterior ectoderm marker, was observed; however, expression was significantly reduced in mutant embryos (Figs. 9C and 9D). At this stage, the paired domain containing transcription factor *Pax6* is evident in the wild-type forebrain (Fig. 9E; Stoykova and Gruss 1994; Shimamura et al., 1997), was absent in mutant embryos (Fig. 9F). *Nkx2.1* expression was observed in neither mutant embryos at this stage (data not shown). *Otx1*, which is expressed in the prospective forebrain and midbrain in wild type (Fig. 9G; Simeone et al., 1993), was shifted to the anterior side in mutant embryos (Fig. 9H). *En2* expression, a marker for mesencephalon and anterior metencephalon (Fig. 9I), also underwent a shift to the anterior side in mutant embryos (Fig. 9J). Similarly, the domain of homeodomian gene *Gbx2* expression, a marker for anterior metencephalon (Fig. 9K; Bouillet et al., 1995), was shifted anteriorly in mutant embryos (Fig. 9L). It is noteworthy that an *En2*-negative region was present in the most anterior region of the mutant neuroectoderm (Fig. 9J), suggesting the occurrence of anterior structures rostral to mesencephalon in mutant embryos.

Analysis of these aforementioned markers suggests that in *Otx2*$^{frt\text{-}neo/-}$ hypomorphic mutants, the prospective forebrain region marked by *Six3* and *Otx1* expression is formed; however, the forebrain is not specified correctly as judged by the markers for the prospective telencephalon and diencephalon (see Discussion).

Fig. 9 Molecular analysis of neural markers at 8.5 dpc.

Emx2 (A, B), *Six3* (C, D), *Pax6* (E, F), *Otx1* (G, H), *En2* (I, J), *Gbx2* (K, L) and *Shh* (M, N) expression by whole mount in situ hybridization in wild type (A, C, E, G, I, K, M) and $Otx2^{frt\text{-}neo/-}$ mutant (B, D, F, H, J, L, N) embryos at 8.5 dpc. *Emx2* expression is detected in the prospective dorsal forebrain of the wild type embryo (A); however, it is not observed in the mutant embryo (B). Although *Six3* expression, a marker for the most anterior neuroectoderm (C), is present in mutant embryos, the expression domain is significantly reduced (D). *Pax6* expression, a marker for the prospective diencephalon (E), is not evident in the mutant (F). *Otx1* expression, a marker for prospective forebrain and midbrain (G), also occurs in the mutant embryo (H). The *En2* positive region is shifted to the anterior side (I, J); however, the *En2*-negative domain is still present in the anterior neuroectoderm of the mutant embryo (J, arrowheads). *Gbx2* expression domain, a marker for anterior metencephalon (K), is expanded and shifted anteriorly in the mutant embryo (L). *Shh* expression, a marker for the ventral midline of neural tube and axial mesendoderm (M), is unchanged in the $Otx2^{frt\text{-}neo/-}$ mutant embryo (N).

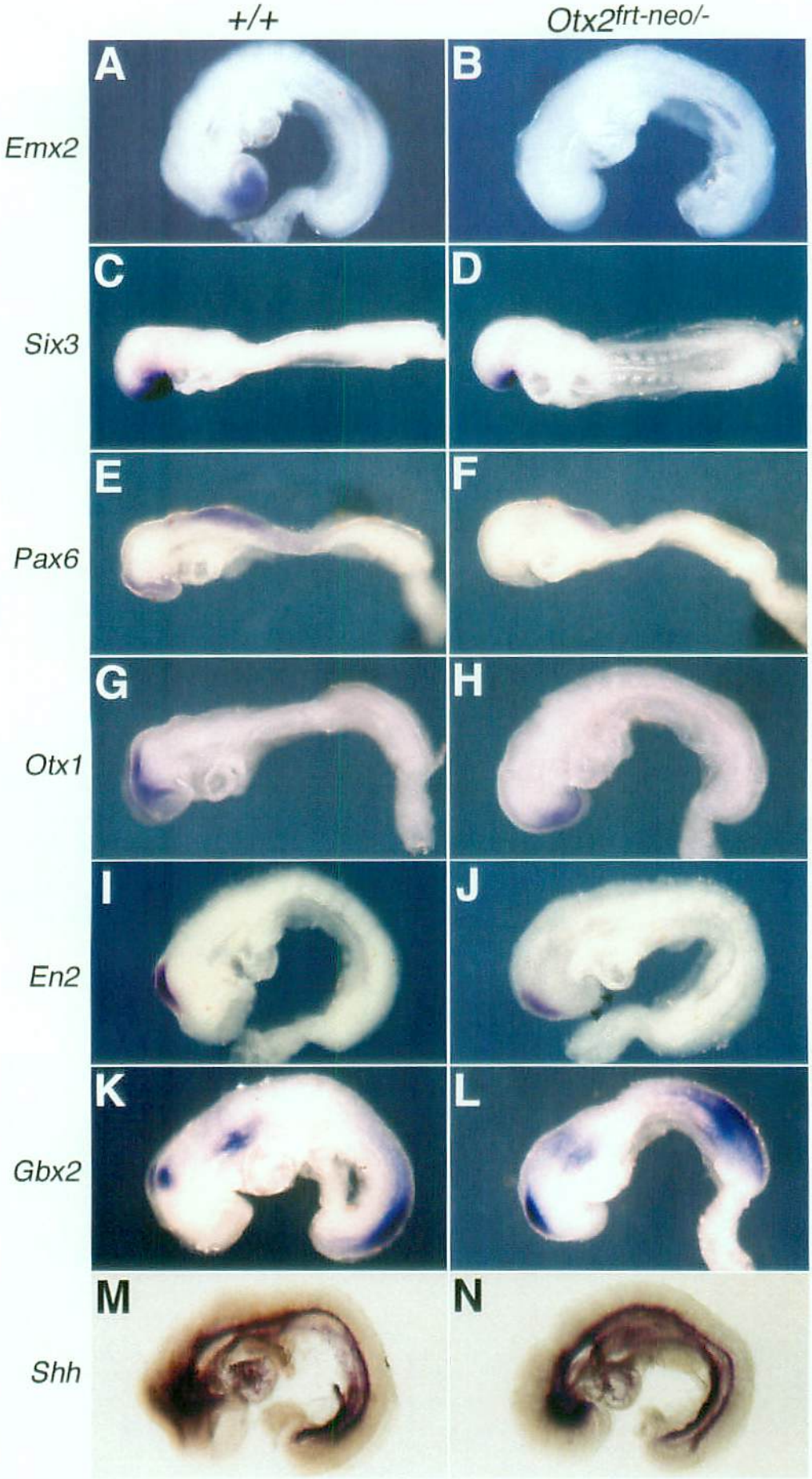

+/+
Otx2frt-neo/-
A
B
Emx2
C
D
Six3
E
F
Pax6
G
H
Otx1
I
J
En2
K
L
Gbx2
M
N
Shh

Fig. 9

(6) *Otx2*$^{frt\text{-}neo/-}$ hypomorphic mutants form AVE and ADE normally

Induction of the anterior neuroectoderm has been proposed to result from signals emanating from AVE prior to and in ADE and/or axial mesendoderm after gastrulation (Ang and Rossant, 1993; Beddington and Robertston, 1999; Shawlot et al., 1999; Camus et al., 2000; Kimura et al, 2000; Wilson and Rubenstein 2000; Kiecker and Niehrs, 2001). To examine the possibility that the failure in forebrain specification is a consequence of defects in these tissues, expression of molecular markers was analyzed. We then found that marker expression was unchanged in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos (Fig. 10). In *Otx2*$^{-/-}$ mutant embryos, expression of AVE markers such as *Cer-l* and *Lim*1 occurred; however, expression remained in the distal visceral endoderm even at 6.5 dpc; that is, expression does not shift to the anterior side (Acampora et al., 1998; Kimura et al., 2000). In contrast, *mdkk-1* expression (Pearce et al., 1999) is specifically lost in *Otx2*$^{-/-}$ mutant visceral endoderm (Zakin et al., 2000; Kimura et al., 2001; Perea-Gomez et al., 2001). In the epiblast of *Otx2*$^{-/-}$ mutants, the posterior markers, *T* and *Cripto* (Herrmann, 1991; Ding et al., 1998), were expanded in the proximal ectoderm (Kimura et al., 2000). Thus, the generation of the A-P axis fails in *Otx2*$^{-/-}$ mutant embryos. In *Otx2*$^{frt\text{-}neo/-}$ mutant embryos, however, expression of both *Cer-l* and *mdkk-1* was detected in the AVE (Figs. 10A-D). Moreover, *Cripto* and *T* expressions were observed normally in the posterior epiblast in *Otx2*$^{frt\text{-}neo/-}$ mutants (Figs. 10E-H).

At the late-streak stage, expression of the winged-helix hepatic nuclear factor *Foxa*2 (previously named as *HNF-3β*), a marker for head process, node and notochord (Fig. 10I; Sasaki and Hogan, 1993), was unaffected in mutant embryos (Fig. 10J). The homeobox gene *goosecoid* (*Gsc*) is expressed in prechordal plate at 7.8 dpc (Fig. 10K; Blum et al., 1992), was evident in mutant embryos (Fig. 10L). At this stage, *mdkk-1*, a marker for prechordal plate and the lateral region of ADE (Fig. 10M; Kiecker and Niehrs 2001), and *Cer-l*, a marker for ADE (Fig. 10O; Belo et al., 1997), were also present in mutant embryos, which is the normal occurrence (Figs. 10N and 10P). In addition, expression of *Six3* and *Foxd4*, the earliest anterior neuroectoderm markers (Oliver et al.,

1995; Kaestner et al., 1995), were also unaffected in mutant embryos at 7.8 dpc (Figs. 10Q-T), supporting that the prospective forebrain region is once formed appropriately in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos. These results suggest that *Otx2*$^{frt\text{-}neo/-}$ hypomorphic mutant embryos can establish the A-P axis and form ADE and anterior axial mesendoderm tissues normally.

Fig. 10 Normal development of AVE and ADE in *Otx2*$^{frt\text{-}neo/-}$ mutant embryos.
Wild type (A, C, E, G, I, K, M, O, Q, S) and *Otx2*$^{frt\text{-}neo/-}$ mutant embryos (B, D, F, H, J, L, N, P, R, T). 6.5 dpc (A-H), 7.5 dpc (O, P) and 7.8 dpc (I-N, Q-T). The direction of embryo is anterior to the left and posterior to the right (except I-L, which are frontal views). *Cer-1* and *mdkk-1* expression in the mutant AVE at 6.5 dpc are not distinguishable from the wild type embryo (A-D). Expression of posterior markers, *Cripto* and *T*, are present in the posterior ectoderm or primitive streak (p) in both wild type and mutant embryos (E-H). *Foxa2* expression is evident in the head process (hp) and node (n) appropriately in mutant embryos at 7.8 dpc (I, J). *Gsc* is expressed in prechordal plate (pp) normally in the mutant embryos at 7.8 dpc (K, L). Expression of *mdkk-1* and *Cer-1* in the prechordal plate and ADE, respectively, is not affected in *Otx2*$^{frt\text{-}neo/-}$ mutants at 7.5-7.8 dpc (M-P). Expression of *Six3* (Q) and *Foxd4* (S) in the anterior neuroectoderm (ANE) is unchanged in the mutant embryos at 7.8 dpc (R, T). Abbreviations: ADE, anterior definitive endoderm; AVE, anterior visceral endoderm.

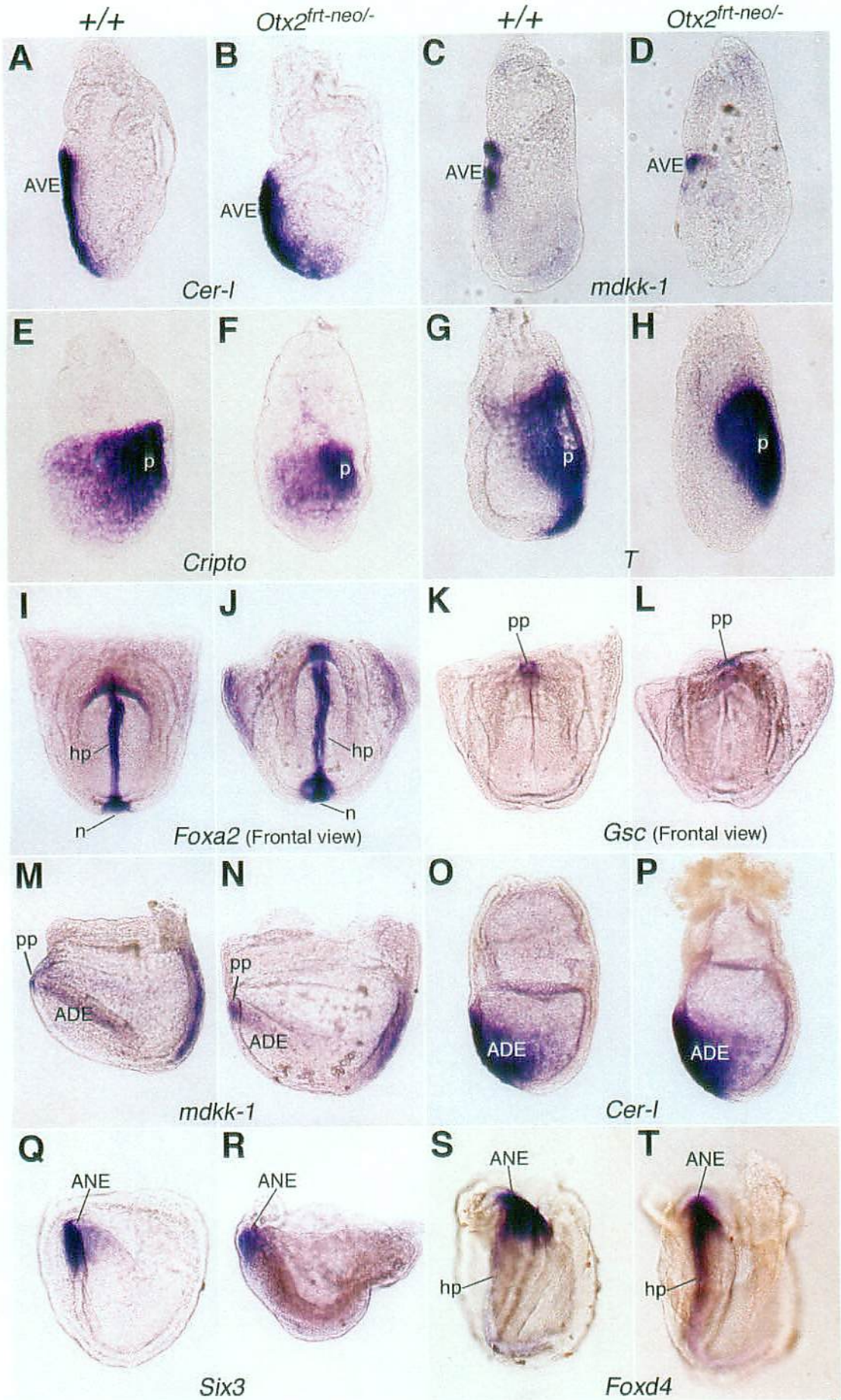

+/+
Otx2frt-neo/-
+/+
Otx2frt-neo/-
A
B
C
D
AVE
AVE
AVE
AVE
Cer-l
mdkk-1
E
F
G
H
p
p
p
p
Cripto
T
I
J
K
L
hp
hp
n
n
pp
pp
Foxa2 (Frontal view)
Gsc (Frontal view)
M
N
O
P
pp
pp
ADE
ADE
ADE
ADE
mdkk-1
Cer-l
Q
R
S
T
ANE
ANE
ANE
ANE
hp
hp
Six3
Foxd4

Fig. 10

(7) The anterior axial mesendoderm and ADE of *Otx2*$^{frt\text{-}neo/-}$ hypomorphic mutants functions normally

Notably, the signals of *Shh* from anterior axial mesendoderm has been shown to induce and maintain *Nkx2.1* expression in the neuroectoderm (Ericson et al., 1995; Shimamura and Rubenstein, 1997; Pabst et al., 2000). Indeed, *Nkx2.1* expression was not evident in *Otx2*$^{frt\text{-}neo/-}$ hypomorphic mutant embryos at 8.5 and 9.5 dpc stages (Fig. 8F and data not shown). But the expression of *Shh* was normal in 8.5 dpc mutant embryos. Thus, in order to investigate whether the inductive signals produced in the mutant anterior axial mesendoderm are defective, neural plate explant analysis was performed (Fig. 11). As reported previously, the anterior axial mesendoderm fragment, which consists of the prechordal plate and the anterior portion of the head process from wild type embryos at 0-2 somites stage, could induce *Nkx2.1* expression in neural plate explants from wild type embryos at the identical stage, followed by culture for 24 hours (Figs. 11B and 11C; Shimamura and Rubenstein, 1997). Unexpectedly, the anterior axial mesendoderm from mutant embryos was also able to induce *Nkx2.1* expression transplanted on wild type neural plate explants (Fig. 11D; n=5). This result indicates that the mutant anterior axial mesendoderm is capable of normal induction of *Nkx2.1* expression. Furthermore, these findings suggest that failure in the forebrain specification of mutant embryos does not result from defects of the axial mesendoderm.

Moreover, additional explant analysis was conducted in order to establish whether mutant anterior definitive endoderm (ADE) is able to induce and/or maintain *Otx2* expression transplanted to the wild type epiblast (Fig. 12; Ang *et al.*, 1994). The ADE from mutant embryos could induce and/or maintain *Otx2* expression in the wild type epiblast (Fig. 12E-F; n=3). These explant studies suggest that failure in forebrain specification of *Otx2*$^{frt\text{-}neo/-}$ mutant embryos is not a consequence of defects in either the ADE or the anterior axial mesendoderm; rather, failure is due to later and/or other developmental events.

Fig. 11 The anterior axial mesendoderm of *Otx2*$^{frt\text{-}neo/-}$ embryos functions normally in neural plate explant assays.

(A) Schematic representation of the neural plate and anterior axial mesendoderm of wild type and *Otx2*$^{frt\text{-}neo/-}$ mutant embryos at the 0-2 somites stage, respectively, and the explant experiments (Exps. 1-4). (B-E) Whole mount in situ hybridization analysis of *Nkx2.1* expression following 24-hour culture of neural plates. In neural plate explant obtained from wild type embryos exclusively, *Nkx2.1* expression is not evident (B; n=6). The anterior axial mesendoderm from wild type (C; n=8) or mutant embryos (D) can induce *Nkx2.1* expression in the wild type neural plate.

Fig. 12 The ADE of *Otx2*$^{frt\text{-}neo/-}$ mutant embryos has inductive activity in germ-layer explant recombinant co-culture.

(A, C, E) Dark-field views under the fluorescence microscope after 24-hour culture of recombinant explants. (B, D, F) Whole mount in situ hybridization analysis of *Otx2* expression after fluorescence observation. (A) Epiblast from pre- to early streak GFP-positive embryos only, no *Otx2* expression can be detected in the epiblast explants (B; n=7). (C) Epiblast from pre- to early streak GFP-positive embryos are combined with the ADE from headfold stage wild type embryos, *Otx2* expression is induced or maintained in the GFP-positive epiblast and mesendoderm (D; n=12). (E) Same source of epiblast are cultured with the ADE from headfold stage *Otx2*$^{frt\text{-}neo/-}$ mutant embryos, induction of *Otx2* expression is detected in the GFP-positive epiblast and mesendoderm (F).

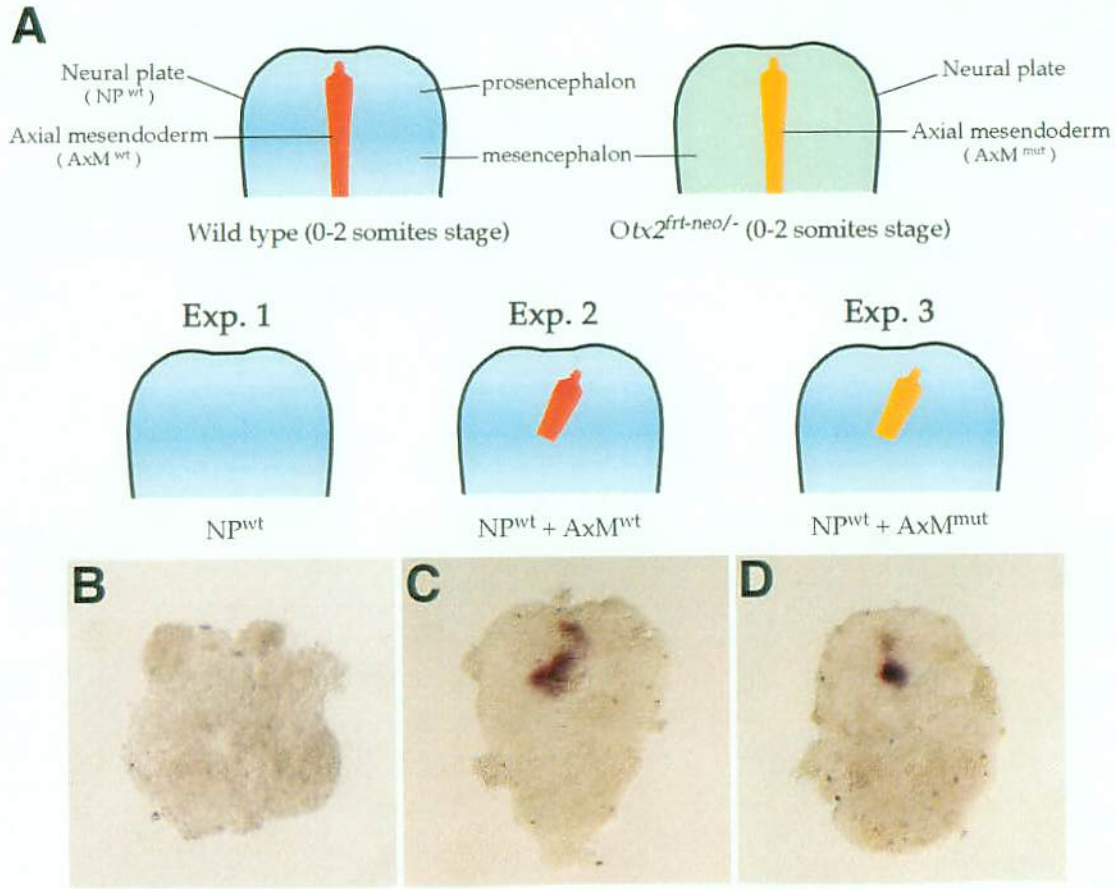

A
Neural plate
(NP wt)
Axial mesendoderm
(AxM wt)
prosencephalon
mesencephalon
Wild type (0-2 somites stage)
Neural plate
Axial mesendoderm
(AxM mut)
Otx2frt-neo/- (0-2 somites stage)
Exp. 1
Exp. 2
Exp. 3
NPwt
NPwt + AxMwt
NPwt + AxMmut
B
C
D

Fig. 11

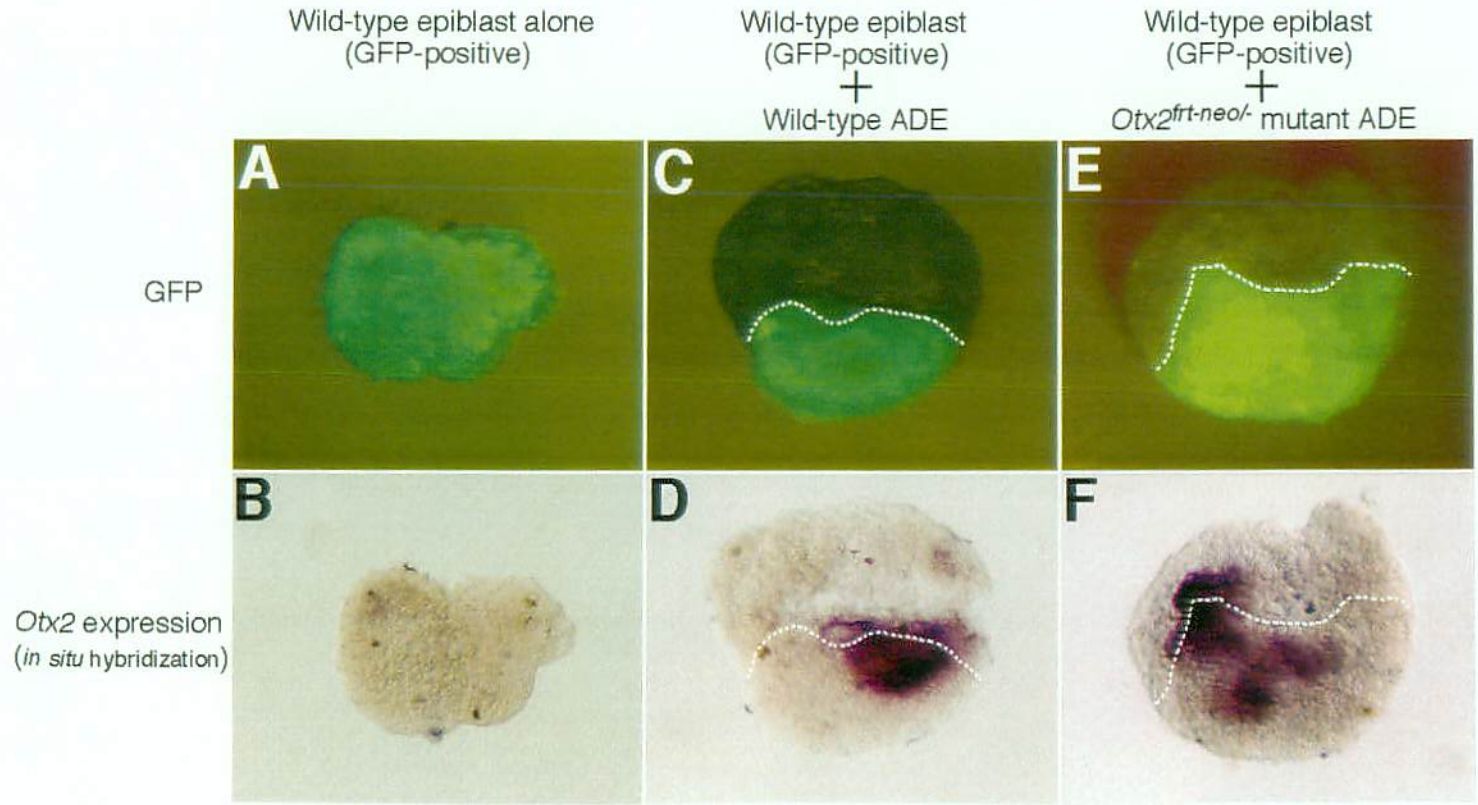

Wild-type epiblast alone
(GFP-positive)
Wild-type epiblast
(GFP-positive)
+
Wild-type ADE
Wild-type epiblast
(GFP-positive)
+
Otx2frt-neo/- mutant ADE
GFP
Otx2 expression
(in situ hybridization)
A
B
C
D
E
F

Fig. 12

(8) Gene expression is not initiated in the neuroectoderm of mutant embryos

Several reports have indicated that ANR functions as a local signaling center essential for forebrain specification (Shimamura and Rubenstein, 1997; Houart et al., 1998). *Fgf8* is expressed in the ANR and later in the commissural plate of telencephalon. Indeed, *Fgf8* has been proposed to play an important role in forebrain specification, in particular, to induce and/or maintain *BF-1* expression (Shimamura and Rubenstein, 1997).

In order to investigate the possibility that mutant defects are due to the failure in forebrain specification by ANR, expression of markers for the ANR and prosencephalic neural plate was analyzed at early somites stages. We found that *BF-1* and *Fgf8* expression of the non-neural ectoderm occurred properly; however, expression of *BF-1* and *Fgf8* was absent in the neural plate of $Otx2^{frt\text{-}neo/-}$ mutants (Fig. 13). Normally, *BF-1* expression is initially found in non-neural ectoderm adjacent to the anterior edge of the neural plate around the 2 to 3 somites stage (data not shown). Subsequently, *BF-1* expression begins in the rostral margin of the ectoderm after the 5 somites stage in wild type (Fig. 13A and Shimamura and Rubenstein, 1997). In mutant embryos, *BF-1* expression was detected in the ANR at this stage, as was the case with wild type (Fig. 13B). At the ensuing 8 somites stage, neuroectodermal expression was induced in wild type embryos (Fig. 13C); however, induction was not evident in $Otx2^{frt\text{-}neo/-}$ mutant embryos (Fig. 13D). Even at the 12 somites stage, *BF-1* expression remained undetectable in the neuroectoderm of mutant embryos (Figs. 13E and 13F). Similarly, *Emx2* expression is initiated in the laterocaudal neural plate at the 3 somites stage (Shimamura et al., 1995; data not shown). However, no *Emx2* expression was detected in mutant embryos at the 8 somites stages (Figs. 13G and 13H).

Normally, *Fgf8* expression commences in the non-neural ectoderm around the 4 somites stage and in neuroectoderm following the 6 somites stage (Shimamura and Rubenstein, 1997; data not shown). In mutant embryos at the 8 somites stage, however, *Fgf8* expression was not observed in the neural plate; on the other hand,

normal *Fgf8* expression was displayed in non-neural ectoderm (Figs. 13I-L). At the subsequent 12 somites stage, *Fgf8* expression in the prospective commissural plate remained undetectable in mutant embryos (Figs. 13M and 13N). Furthermore, in double-staining whole mount in situ hybridization at 9.5 dpc, *BF-1* is expressed in most of the telencephalic region as well as in otic vesicle, olfactory placode, pharyngeal pouches and foregut (Tao and Lai, 1992; Fig. 13O). In $Otx2^{frt\text{-}neo/-}$ mutant embryos, only the telencephalic *BF-1* expression was not found at all, although *Fgf8* was expressed throughout the entire rostral region (Fig. 13P). Thus, in mutant embryos, expression of telencephalic genes *BF-1* and *Fgf8* was not initiated in neuroectoderm, although their expression began normally in non-neural ectoderm.

Fig. 13 Defects of neural plate in the $Otx2^{frt\text{-}neo/-}$ hypomorphic mutant embryos.
BF-1 (A-F), *Emx2* (G, H) and *Fgf8* (I-N) expression by whole mount in situ hybridization at the 5 somites (A, B), 8 somites (C, D, G-L), 12 somites (E, F, M, N) and double-staining whole mount in situ hybridization at 9.5 dpc (O, P) stages in wild type (A, C, E, G, I, K, M, O) and $Otx2^{frt\text{-}neo/-}$ mutant embryos (B, D, F, H, J, L, N, P). (K, L) displays sagittal sections of embryos shown in (I, J), respectively. All whole mount embryos are lateral views. At the 5 somites stage, *BF-1* expression occurs in the non-neural ectoderm (arrowhead) of the wild type embryo but not in the neuroectoderm (A). *BF-1* expression is not changed in the non-neural ectoderm of the mutant embryo at the 5 somites stage (B, arrowhead). *BF-1* expression is found in the neuroectoderm (arrow) and non-neural ectoderm (arrowhead) in the wild type embryo at the 8 somites stage (C). In the mutant embryo, *BF-1* expression is detected solely in the non-neural

ectoderm (D, arrowhead). At the 12 somites stage, *BF-1* expression is evident throughout the prospective telencephalic region of wild type (E, arrow). At this stage, *BF-1* expression is not induced in the neural ectoderm; however, it remains apparent in the non-neural ectoderm of mutant embryos (F, arrowhead). At the 8 somites stage, *Emx2* expression is present in the neuroectoderm in the wild type (G). However, no *Emx2* expression occurs in the mutant embryo at this stage (H). At the 8 somites stage, *Fgf8* expression is observed in the neuroectoderm (arrow) and non-neural ectoderm (arrowhead) of the wild type embryo (I and K); in contrast, *Fgf8* expression occurs in the non-neural ectoderm (arrowheads) but not in the neuroectoderm in mutant embryos (J and L). At the 12 somites stage, *Fgf8* expression is present in the prospective telencephalic commissural plate in the wild type embryo (M, arrow). In the mutant embryo, expression of *Fgf8* is not initiated in the neuroectoderm; however, it remains evident in the non-neural ectoderm (N, arrowhead). At 9.5 dpc, both *BF-1* (purple) and *Fgf8* (red) expression are found in the telencephalic region of the wild type (O). *Fgf8* expression is seen throughout the entire rostral region of the mutant embryo but *BF-1* expression is not detected in this region (P, arrowheads). Abbreviations: be, branchial ectoderm; fg, foregut; Is, isthmic constriction.

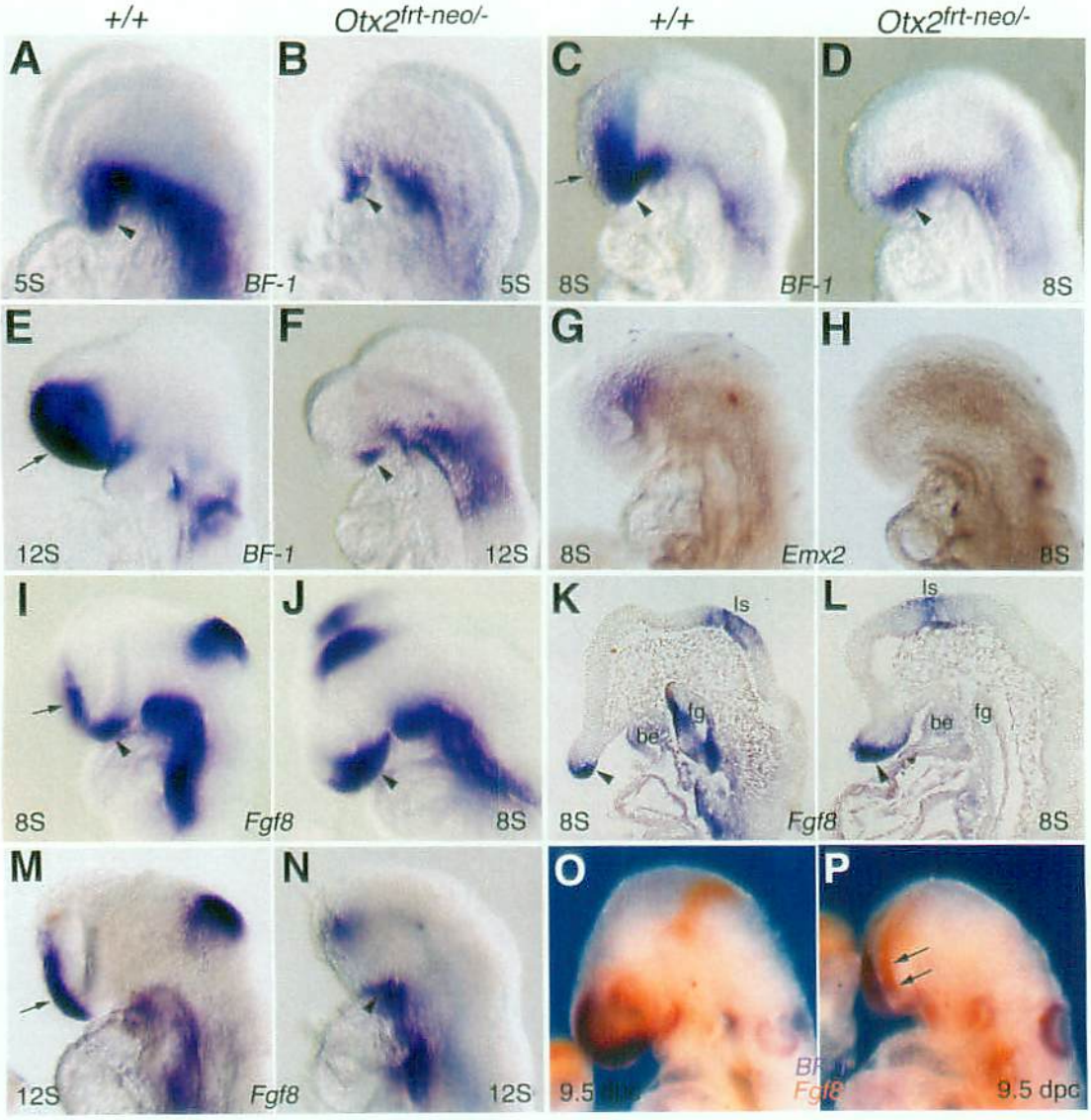

+/+
Otx2frt-neo/-
+/+
Otx2frt-neo/-
A
B
C
D
5S
BF-1
5S
8S
BF-1
8S
E
F
G
H
12S
BF-1
12S
8S
Emx2
8S
I
J
K
L
ls
fg
be
ls
be
fg
8S
Fgf8
8S
8S
Fgf8
8S
M
N
O
P
12S
Fgf8
12S
9.5 dpc

Fig. 13

(9) *Otx2*$^{frt\text{-}neo/-}$ mutant neural plate cannot respond to the *Fgf8* signal from the ANR

To investigate the additional possibility that mutant ANR retains the ability to induce telencephalic markers while the mutant neural plate loses competence to respond to the inductive signals from the ANR, neural plate explant assays were conducted (Fig. 14; Shimamura and Rubenstein, 1997). Wild type anterior neural plate explants lose *BF-1* expression upon excision of the ANR at the 3 to 5 somites stages followed by culture for 24 hours (Fig. 14B, n=9; Shimamura and Rubenstein, 1997). Initially, we examined whether the ANR fragment from mutant embryos (ANR^{mut}) can restore *BF-1* expression in wild type neural plate explants in a manner similar to the ANR fragment from wild type (ANR^{WT}) (Fig. 14C; n=10). Indeed, the ANR^{mut} was able to induce *BF-1* expression transplanted on the wild type explants (Fig. 14D; n=14). In order to investigate the competence of the mutant neural plate, the ANR^{WT} was transplanted on the mutant neural plate (Figs. 14E and 14F). The ANR^{WT}, however, was unable to induce *BF-1* expression (Fig. 14F; n=7). Further, a FGF8b-bead was transplanted on the mutant neural plate; a FGF8b-bead is demonstrated to be sufficient for *BF-1* induction in the neural plate (Figs. 14G and 14H; n=14; Shimamura and Rubenstein, 1997). A FGF8b-bead, however, could not induce *BF-1* expression in the mutant neural plate (Fig. 14I; n=11). Therefore, failure in forebrain specification of mutant embryos results from the defects of the neural plate, but not of the ANR. This finding suggests that *Otx2* may be required in the neural plate for proper response to signals produced in the ANR.

Fig. 14 The mutant neural plate fails to respond to the signals from the ANR.

(A) Schematic representation of neural plate of wild type and *Otx2*$^{frt\text{-}neo/-}$ mutant embryos at the 3 to 5 somites stage and explant experiments (Exps. 1-6). (B-I) Whole mount in situ hybridization analysis of *BF-1* expression following 24-hour culture of neural plates. In wild type explants lacking left side ANR, *BF-1* expression is completely lost on the left side (B). The ANR fragment from wild type embryos can induce *BF-1* expression on the transplanted side where ANR is excised (C, arrowhead). The ANR from *Otx2*$^{frt\text{-}neo/-}$ mutants restores *BF-1* expression in the neural plate (D, arrowhead). No *BF-1* expression was detected in *Otx2*$^{frt\text{-}neo/-}$ mutant explants alone (E; n=6). In *Otx2*$^{frt\text{-}neo/-}$ mutant neural plate explant. the ANR from wild type cannot induce *BF-1* expression (F). Heparin-acrylic beads soaked with BSA (G) or FGF8b (H) implanted to the left side of the neural plate explant in the area of ANR ablation. A BSA-bead cannot induce *BF-1* expression in the neural plate (G; n=14); in contrast, an FGF8b-bead can induce *BF-1* expression (H). *BF-1* expression is not induced by a FGF8b-bead transplanted on the mutant neural plate (I).

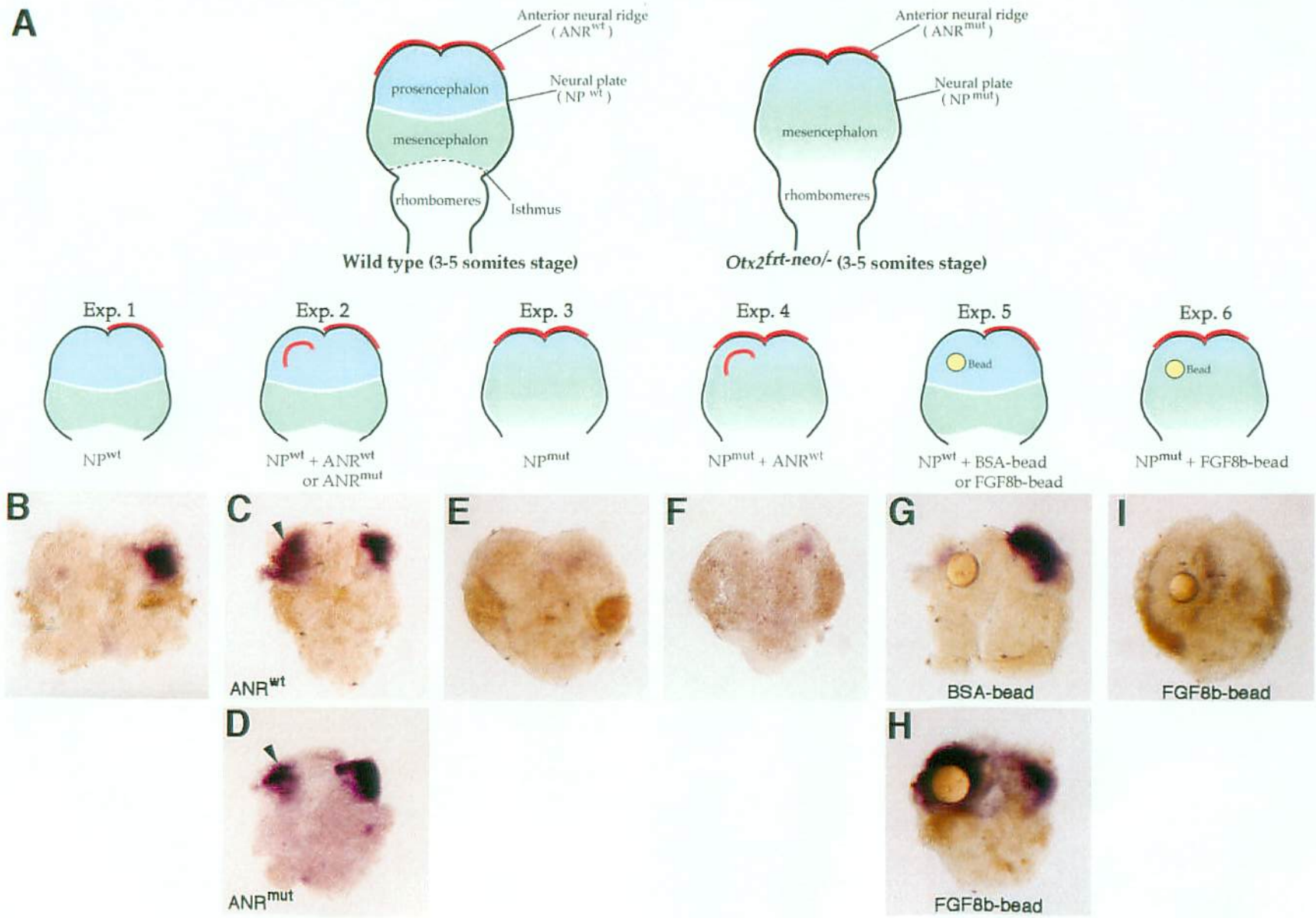

A
Anterior neural ridge (ANR wt)
Neural plate (NP wt)
prosencephalon
mesencephalon
rhombomeres
Isthmus
Wild type (3-5 somites stage)
Anterior neural ridge (ANR mut)
Neural plate (NP mut)
mesencephalon
rhombomeres
Otx2frt-neo/- (3-5 somites stage)
Exp. 1
NPwt
Exp. 2
NPwt + ANRwt or ANRmut
Exp. 3
NPmut
Exp. 4
NPmut + ANRwt
Exp. 5
Bead
NPwt + BSA-bead or FGF8b-bead
Exp. 6
Bead
NPmut + FGF8b-bead
B
C
ANRwt
D
ANRmut
E
F
G
BSA-bead
H
FGF8b-bead
I
FGF8b-bead

Fig. 14

4. Discussion

(1) Generation of *Otx2* hypomorphic mutation by *neo* insertion

A series of mutant *Otx2* alleles were generated through a single gene targeting, which contained *neo* cassette insertion into the *Otx2* intron and the recognition sequences of two different DNA recombinases. The targeted *Otx2* allele can be converted to a null allele or reverted to an allele useful for Cre-mediated tissue-specific gene inactivation experiments simply by crossing the targeted mouse line to *cre* or *flpe* transgenic mice. Intriguingly, the $Otx2^{frt\text{-}neo}$ allele was a hypomorph, and the $Otx2^{frt\text{-}neo/-}$ hypomorphic mutant embryos displayed a unique phenotype, which permitted normal A-P axis generation and gastrulation. However, the mutants failed to specify the forebrain region. These results provide new insights into the roles of *Otx2* with respect to forebrain specification.

RT-PCR analysis demonstrated that normal *Otx2* mRNA expression of the hypomorphic allele was significantly reduced as this cassette contains cryptic mRNA splice sites and directs two kind of aberrant splicing patterns (Fig. 6). These abnormal mRNA splice occurred before gastrulation, it is possible that the transcriptional onset time of wild type *Otx2* and aberrant splicing may be at same point. Our data and previous studies showed same location of splice acceptor and donor in the *neo* sequence (Fig. 6; Meyers et al., 1998: Nagy et al., 1998)

Predicted protein products consist of only 33 amino acids of N-terminal residues of Otx2 fused with an entire neo protein resulting from the aberrant splicing. As $Otx2^{frt\text{-}neo/+}$ hypomorphic mutant mice develop normally, it is unlikely that *neo*-fused products exert dominant negative effects. It seems the Otx2 protein level is not so critical for gastrulation but is essential for neuroectoderm development. Thus, the forebrain defects observed in the $Otx2^{frt\text{-}neo/-}$ mutant embryos may be due to the subcritical thresholds of *Otx2* expression in the anterior neuroectoderm. Recently, *neo* cassette insertion into the intron has been shown to create a hypomorphic mutation by targeting of other genes (Meyers et al., 1998; Nagy et al., 1998; Gage et al., 1999; Lowe et al., 2001). Early

embryonic lethality resulting from complete loss-of-function mutations renders milder alleles valuable in the elucidation of roles at later developmental stages; consequently, this approach may provide an applicable method for extensive genetic analysis of genes of interest.

(2) *Otx2* functions in the neuroectoderm to induce forebrain gene expression

Our detailed analysis of $Otx2^{frt\text{-}neo/-}$ hypomorphic mutant embryos provides evidence that *Otx2* is required in the neural plate to induce telencephalic gene expression for forebrain specification. Previous studies have suggested that *Otx2* expression in AVE can direct the induction of anterior neuroectoderm but is not sufficient to form the forebrain region correctly (Acampora et al., 1998; Suda et al., 1999; Kimura et al., 2000). Moreover, it was suggested that *Otx2* expression in the neuroectoderm is essential for forebrain development, based on investigations involving mouse chimeras containing $Otx2^{-/-}$ and wild type cells (Rhinn et al., 1998; 1999). Consistent with the aforementioned reports, it appears that the anterior neural plate marked by *Six3*, *Foxd4* and *Otx1* expression can be formed once in $Otx2^{frt\text{-}neo/-}$ hypomorphic mutant embryos normally (Figs. 9 and 10). Notably, *Six3* demarcates the most anterior neural plate (Oliver et al., 1995). Overexpression of *Six3* leads to enlarged forebrain, whereas the dominant activator form of *Six3* results in the reduction of forebrain in zebrafish (Kobayashi et al., 1998; 2001). This observation suggests that *Six3* may define the forebrain territory.

Recently, we found the *cis*-regulatory region of *Otx2*, which governs transgene expression in AVE and ADE but not in anterior neuroectoderm within endogenous *Otx2* expression (Kimura et al., 2000). Transgenic rescue studies with this *cis*-regulatory region have demonstrated that *Otx2* expression in the anterior neural plate is not required for induction of *Six3* expression (Kimura et al., 2000). Furthermore, the fact that $Otx2^{-/-}$ cells express *Six3* based on chimeric studies (Rhinn et al., 1999), in concert with the aforementioned findings regarding *Otx2*, support the possibility that *Otx2* expression in neural plate may not function in an essential capacity in formation of the

prospective forebrain territory. Consistently, marker expression of AVE, primitive streak, ADE and anterior axial mesendoderm, which have been proposed to play important roles in forebrain development in mouse (Beddington and Robertson, 1999; Shawlot et al., 1999; Camus et al., 2000), was not affected during gastrulation in *Otx2*$^{frt\text{-}neo/\text{-}}$ mutant embryos (Figs. 9M and 9N, 10, and 11). Indeed, the ADE from *Otx2*$^{frt\text{-}neo/\text{-}}$ mutant embryos was able to induce or maintain *Otx2* expression transplanted on the epiblast from wild type embryos at early streak stage (Fig. 12). Moreover, the mutant anterior axial mesendoderm could induce *Nkx2.1* expression transplanted on wild type neural plate explants, suggesting that the mutant anterior axial mesendoderm functions normally (Fig. 11). These data indicate that AVE, ADE and anterior axial mesendoderm form appropriately in mutant embryos; furthermore, these results support the transient formation of the prospective prosencephalic neural plate in mutant embryos (summarized in Fig. 15A).

In *Otx2*$^{frt\text{-}neo/\text{-}}$ mutant embryos, however, subsequent forebrain region marked by *BF-1*, *Emx2*, *Nkx2.1* and *Pax6* expression was not formed correctly (Figs. 8, 9 and 13). Notably, loss of function analysis revealed that *BF-1* is essential in the development of the cerebral hemispheres (Xuan et al., 1995). *Emx2* functions in the formation of dorsal telencephalon and diencephalon (Yoshida et al., 1997; Suda et al., 2001). Small eye mutation analyses have demonstrated that *Pax6* is required for the development and regionalization of diencephalon (Stoykova et al., 1996; Warren and Price, 1997; Grindley et al., 1997). Furthermore, overexpression experiments suggest that *Pax6* also defines the dien-mesencephalic boundary via repression of *En1* and *Pax2* (Matsunaga et al., 2000). *Nkx2.1* is essential to ventral forebrain development (Kimura et al., 1996; Sussel et al., 1999). Therefore, *Otx2* may be involved in forebrain development through direct or indirect transactivation of these forebrain genes crucial for subsequent forebrain specification. On the other hand, expression of the mesencephalic marker, *En2*, was not altered; however, the expression domain underwent an anterior shift in mutant embryos (Figs. 8 and 9). This result is supported by the previous report that *Otx2* expression in the neural plate may not be required for the initiation, but rather the maintenance, of

En2 expression indirectly (Rhinn et al., 1999). It is noteworthy that *En2* was not expressed in the most anterior portion of mutant neuroectoderm (Figs. 8L and 9J), suggesting the presence of the prospective prosencephalic neural plate in mutant embryos. In conjunction, these observations suggest that A-P axis development and induction of the prospective prosencephalic neural plate appear to have been undergone normally once; however, at subsequent stages, the forebrain region failed to be specified in $Otx2^{frt\text{-}neo/-}$ mutant embryos (summarized in Fig. 15B).

(3) *Otx2* may define the competence of prosencephalic neural plate to inductive signals from ANR

In this study, expression of forebrain markers such as *BF-1* and *Emx2* was not induced in $Otx2^{frt\text{-}neo/-}$ mutant embryos from its initial manifestation (Fig. 13). Recently, ANR in mouse or the first row of cells in zebrafish has been proposed to function as a local signaling center necessary for forebrain specification (Shimamura and Rubenstein, 1997; Houart et al., 1998). Removal of the ANR results in the loss of expression of the telencephalic gene, *BF-1*; additional ANR can induce ectopic *BF-1* expression in mouse neural plate explants (Shimamura and Rubenstein, 1997). To assess the cause of the $Otx2^{frt\text{-}neo/-}$ mutant defects, molecular markers were examined for ANR and prosencephalic neural plate during early somites stage in mutant embryos. At this juncture, molecular defects of $Otx2^{frt\text{-}neo/-}$ mutant embryos beginning in the anterior neural plate were detected by the 8 somites stage at the time of forebrain regionalization; expression of *BF-1*, *Fgf8* and *Emx2* was not induced in the neuroectoderm of mutant embryos, whereas expression of *Fgf8* and *BF-1* was not affected in non-neural ectoderm (Fig. 13). It is open to question as to whether ANR or the neural plate is defective for the inductive interaction between the ANR and the neural plate in mutant embryos.

In order to address this issue, neural plate explants studies were performed. These experiments demonstrated that mutant ANR was able to induce *BF-1* expression when transplanted on the wild-type neural plate; in contrast, neither wild type ANR nor a FGF8b-bead, which mimics the properties of the ANR, induce *BF-1* expression in

mutant neural plate (Fig. 14). This evidence suggests that mutant embryos can form ANR bearing normal induction activity; however, mutants are unable to respond to signals of induction produced in the ANR. Concomitantly, *Otx2* is expressed in the anterior neuroectoderm but not in the ANR of wild type embryos at the 5 somites stage (data not shown). Thus, it is likely that due to the incompetence of mutant neural plate, expression of *BF-1* and *Emx2* genes, which are crucial for telencephalon development (Xuan et al., 1995; Yoshida et al., 1997), cannot be initiated in $Otx2^{frt\text{-}neo/-}$ mutant embryos. Consequently, mutant neural plate might result in the loss of the entire forebrain region at later stages. More interestingly, this study suggests that *Otx2* may be involved downstream of the signals from the ANR, e.g., *Fgf* signaling, to mediate the induction of telencephalic genes for forebrain regionalization. *Otx2* is a transcription activating factor (Simeone et al., 1993); therefore, it is likely that *Otx2* transactivates telencephalic genes as a target directly or indirectly in response to *Fgf* signals. It is notable that $Otx2^{-/-}$ cells in the forebrain region subsequently underwent apoptosis in chimeric embryos (Rhinn et al., 1999). Apoptosis in these cells may be induced as a result of the incompetence of mutant neural plate with respect to growth or differentiation signals (e.g., *Fgfs* produced by the ANR for forebrain specification). Additional molecular analysis is required in order to determine which point(s) of the pathway is/are regulated by *Otx2* in the prosencephalic neural plate.

Recently available evidence suggests the existence of differential competence within the neuroectoderm to inductive signals produced by ANR or isthmic organizer (Shimamura and Rubenstein, 1997; Rubenstein et al., 1998; Rubenstein and Beachy, 1998). For example, *Fgf8* induces *BF-1* expression in the prospective telencephalic neural plate, whereas in more caudal regions of the neural plate, *Fgf8* induces *En2* or *Gbx2* expression (Shimamura and Rubenstein, 1997; Rubenstein et al., 1998). Thus, such distinct neuroectodermal gene expression at differing positions along the A-P axis within the neural plate is due to intrinsic differences in competence to the signals. $Otx2^{frt\text{-}neo/-}$ mutant embryos does not express *BF-1*; however, expression of *En2* and *Gbx2* is normal (Figs. 8, 9 and 13). Indeed, FGF8b-beads induced neither *BF-1* nor *En-2*

expression in the prospective prosencephalic region of mutant neural plates (Fig. 14, and data not shown), implying that the competence of prosencephalic neural plate may not be transformed to that of the mesencephalic neural plate; rather, prosencephalic competence may be lost exclusively in this *Otx2*$^{frt\text{-}neo/-}$ mutant embryo. The study in chick embryos by electroporation has shown that *Six3* can regulate *BF-1* and *Nkx2.1* expression responding to *Fgf8* and *Shh* signals at the different portions of anterior neural plate, respectively, whereas the other homeodomain transcription factor *Irx3* regulates *En2* and *Nkx6.1* expression in the posterior region. And the mutual repression of *Six3* and *Irx3* demonstrated the regional distinct competence during neural plate specification (Kobayashi et al. in press). In our work, *Six3* expression initiated normally in the anterior neuroectoderm (Fig. 10), however, *BF-1* expression was not found in the forebrain region of *Otx2*$^{frt\text{-}neo/-}$ mutant embryos (Figs 8 and 13). One possibility is that *Six3* expression is not sufficient to induce *BF-1* in these mutants. Nevertheless, given the arguments stated above, we believe that *Otx2* most likely involves the competence of the prosencephalic neural plate with regard to signals produced mainly in the ANR (summarized in Figs. 15B and 15C).

Additional transplant and genetic studies are necessary in order to identify the components involved in the differential competence of the anterior neural plate. Recent report in zebrafish has shown that development of subpallial telencephalon needs the cooperation between *Fgf8* and *Fgf3*, and also the Fgf/Ras/MAPK signaling cascade (Shinya et al., 2001). It is important to investigate other molecular activators for forebrain formation and to understand *Otx2* involves what kind of signaling pathways during forebrain specification.

(4) *Otx2* is involved in critical processes for forebrain development

In mouse, the A-P axis is initially generated in a proximal-distal (P-D) direction around 5.5 dpc; subsequently, a process of axis rotation occurs prior to primitive streak formation (Beddington and Robertson, 1999). As a result of this process, the expression of genes that mark the distal visceral endoderm shifts to the anterior visceral

endoderm, whereas markers expressed in the proximal epiblast shift to the posterior aspect where the primitive streak is to be formed (Beddington and Robertson, 1999). It has been suggested that *Otx2* possesses a crucial role in A-P axis formation in P-D orientation from its initial manifestation in cooperation with *Cripto* in epiblast (Kimura et al., 2001) and the subsequent autonomous anterior movement of distal visceral endoderm cells (Kimura et al., 2000). The AVE thus formed does not induce anterior neuroectoderm directly; rather, AVE mediates forebrain development by repressing posteriorizing signals (Tam and Steiner, 1999; Klingensmith et al., 1999; Kimura et al., 2000; Stern, 2001). Moreover, *Otx2* is also essential to this suppression (Kimura et al., 2000).

At subsequent mid-to-late-streak stages, ADE derived from the primitive streak also plays an important role in the correct patterning of neuroectoderm (Ang and Rossant, 1993; Ang et al., 1994). Requirement of ADE in forebrain development is also suggested by germ-layer explant studies, surgical removal experiments and chimeric analysis of *Lim1* mutant embryos (Ang et al. 1994; Shawlot et al., 1999; Camus et al., 2000). Thus, ADE may function in the establishment or maintenance of anterior territory that is initiated by AVE (Shawlot et al., 1999; Kimura et al., 2000). However, the precise role of *Otx2* expression in ADE remains to be determined.

Within the anterior neuroectoderm, the neural plate is transversely subdivided into forebrain, midbrain and hindbrain regions, each with characteristic morphological and molecular properties. Two local signaling centers, ANR and mid/hindbrain junction, termed the isthmus, play crucial roles in the refinement of the identity within neuroectoderm along the A-P axis (Joyner, 1996; Shimamura and Rubenstein, 1997; Rubenstein et al., 1998). *Otx2* interacts in a repressive manner with *Fgf8* and *Gbx2* for the correct positioning of the isthmic organizing center (Acampora et al., 1997; Suda et al., 1997; Liu et al., 1999; Martinez et al., 1999; Joyner et al., 2000; Garda et al., 2001; Liu and Joyner, 2001). In the present investigation, we found that *Otx2* is also essential for the longitudinal subdivision of the forebrain by defining the competence of the neural plate to signals from the ANR (summarized in Figs. 15B and 15C).

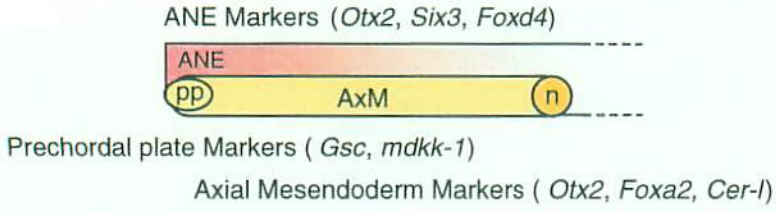
Further elucidation of *Otx2* function in processes of brain specification and regionalization will be resolved through conditional knock-out experiments. These studies can be performed via the mating of mice carrying the $Otx2^{flox}$ allele, which we have generated, with mice that express Cre under the control of appropriate tissue-specific regulatory regions.

Fig. 15 Schematic representation of the requirement of *Otx2* gene in forebrain development.

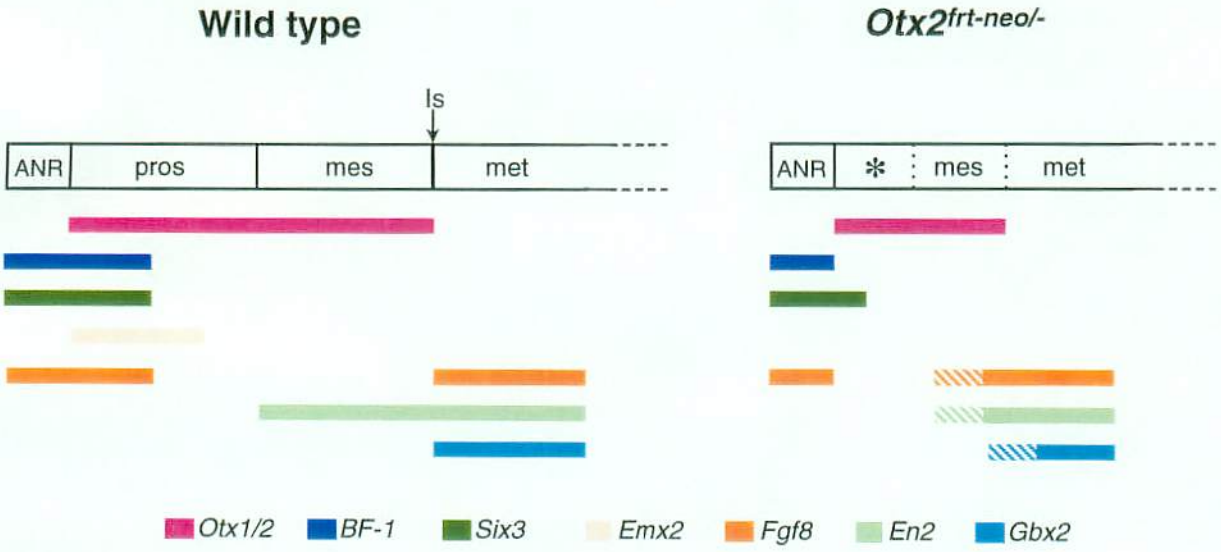
(A) At the stages of 7.5~7.8 dpc during mid- to late gastrulation, $Otx2^{frt\text{-}neo/-}$ mutants undergo normal A-P axis generation and induce anterior neuroectoderm (ANE) once at its onset. The ANE markers (*Otx2, Six3, Foxd4*), the axial mesendoderm markers (*Otx2, Foxa2, Cer-1*) and prechordal plate markers (*Gsc, mdkk-1*) are expressed normally in the mutant embryos as wild type embryos. (B) During 5~8 somites stages, the brain areas acquire a specific regional identity in parallel with a progressive restriction of molecular markers. In $Otx2^{frt\text{-}neo/-}$ mutants, the expression of *Otx2* and *Otx1* in the prosencephalon (pros) and mesencephalon (mes) regions were reduced due to the neural plate defect. The telencephalic gene *BF-1* could not be induced in the prosencephalon since the mutant neural plate lost competence to response to inductive signals from ANR (e.g. *Fgf8*). The expression of early ANE marker *Six3* was decreased, and the *Emx2* expression was lost in the prosencephalon. The mesen- metencephalon (mes-met) markers, *Fgf8, En2* and *Gbx2* are normally expressed in mutant embryos but shifted to more anterior region (shadow bars). The anterior *En2*- and *Gbx2*-negative region was existed in the mutant embryos (indicated by asterisk). (C) *Otx2* is required in the competence of the anterior neural plate. During 5~8 somites stages, local signals arising from the ANR (e.g. *Fgf8*, red arrows) induce *BF-1* expression in the prosencephalic region. *Otx2* is required to respond to *Fgf8* inductive signal for the telencephalic *BF-1* expression. At the posterior limit of mesencephalon, *Otx2* and *Gbx2* set up the mid-

hindbrain boundary corresponding to isthmus (Is) (Broccoli et al., 1999; Simeone, 2000). The signals from axial mesendoderm (e.g. *Shh*) are essential for ventral patterning of the anterior neural plate (Shimamura et al., 1995; Shimamura and Rubenstein, 1997; Rubenstein et al., 1998). Other abbreviations are same as above.

A 7.5~7.8 dpc Wild type & *Otx2*$^{frt\text{-}neo/-}$



B 8.5 dpc (5~8 somites)



C

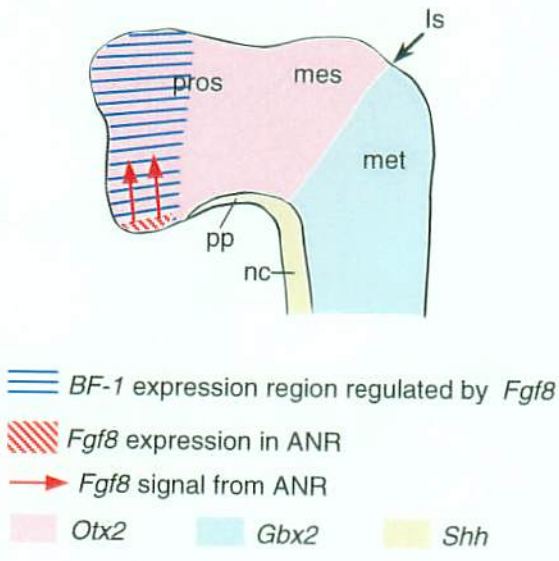


Fig. 15

5. Conclusions

Among a series of *Otx2* mutant alleles generated by a single gene targeting based on application of Cre/*loxP* and Flp/*frt* recombinant systems, we obtained a informative hypomorphic allele, *frt-neo*, which had *neo*-cassette insertion into *Otx2* first intron. The compound mutant containing this allele and a null allele ($Otx2^{frt\text{-}neo/-}$) provided new insight into *Otx2* function regarding the mechanism of forebrain development. Molecular marker analyses demonstrated that $Otx2^{frt\text{-}neo/-}$ mutants produced normal A-P axis and prospective prosencephalon was induced appropriately at its onset. But these mutants subsequently failed to specify correctly the forebrain region and displayed rostral brain defects. Further analyses together with explant culture assays revealed that telencephalic gene expression did not occur in mutant embryos due to loss competence in the neural plate; however, the mutant ANR bore normal induction activity on gene expression. Our results suggest that *Otx2* dosage may be essential for the longitudinal subdivision of the forebrain development by defining the competence of the neural plate to response signals from the ANR. Future investigation will be focused on detail mechanisms of *Otx2* in forebrain patterning by application of floxed mice models, which were created by this work.

6. REFERENCES

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A. and Brulet, P. (1995). Forebrain and midbrain regions are deleted in *Otx2-/-* mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121**, 3279-3290.

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., Briata, P., Corte, G. and Simeone, A. (1998). Visceral endoderm-restricted translation of *Otx1* mediates recovery of *Otx2* requirements for specification of anterior neural plate and normal gastrulation. *Development* **125**, 5091-5104.

Ang, S-L. and Rossant, J. (1993). Anterior mesendoderm induces mouse *Engrailed* genes in explant cultures. *Development* **118**, 139-149.

Ang, S-L. and Rossant, J. (1994). *HNF-3β* is essential for node and notochord formation in mouse development. *Cell* **78**, 561-574.

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). A 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.

Ashery-Padan, R., Marquardt, T., Zhou, X. and Gruss, P. (2000). Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev.* **14**, 2701-2711.

Bachiller, D., Klingensmith, J., Kemp, C., Belo, J. A., Anderson, R. M., May, S. R., McMahon, J. A., McMahon, A. P., Harland, R. M., Rossant, J. and De Robertis, E. M. (2000). The organizer factors Chordin and Noggin are required for mouse forebrain development. *Nature* **403**, 658-661.

Baker, C. V., Bronner-Fraser, M. (2001). Vertebrate cranial placodes I. Embryonic induction. *Dev. Biol.* **232**, 1-61.

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

Beddington, R. S. P. & Robertson, E. J. (1999). Axis development and early asymmetry in mammals. *Cell* **96**, 195-209.

Belo, J. A., Bouwmeester, T., Leyns, L., Ketrtesz, N., Gallo, M., Follettie, M., and De Robertis, E. M. (1997). *Ceberus-like* is a secreted factor with neuralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev.* **68**, 45-57.

Bielinska, M., Narita, N. and Wilson, D. B. (1999). Distinct roles for visceral endoderm during embryonic mouse development. *Int. J. Dev. Biol.* **43**, 183-205.

Blum, M., Gaunt, S. J., Cho, K. W., Steinbeisser, H., Blumberg, B., Bittner, D. and De Robertis, E.M. (1992). Gastrulation in the mouse: the role of the homeobox gene goosecoid. *Cell* **69**, 1097-1106.

Bouillet, P., Chazaud, C., Oulad-Abdelghani, M., Dolle, P. and Chambon, P. (1995). Sequence and expression pattern of the Stra7 (Gbx-2) homeobox-containing gene induced by retinoic acid in P19 embryonal carcinoma cells. *Dev. Dyn.* **204**, 372-382.

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B. and De Robertis, E. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Broccoli, V., Boncinelli, E. and Wurst, W. (1999). The caudal limit of *Otx2* expression positions the isthmic organizer. *Nature* **401**, 164-168.

Buchholz, F., Angrand, P-O. and Stewart, A. F. (1998). Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nat. Biotech.* **16**, 657-662.

Bulfone, A., Puelles, L., Porteus, M. H., Frohman, M. A., Martin, G. R., Rubenstein, J. L. (1993). Spatially restricted expression of *Dlx-1*, *Dlx-2* (*Tes-1*), *Gbx-2*, and *Wnt-3* in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J. Neurosci.* **13**, 3155-3172.

Camus, A., Davidson, B. P., Billiards, S., Khoo, P-L., Rivera-Perez, J. A., Wakamiya, M., Behringer, R. R. and Tam, P. P. L. (2000). The morphogenetic role of midline mesendoderm and ectoderm in the development of the forebrain and the midbrain of the mouse embryo. *Development* **127**, 1799-1813.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Monns, L., Collen, D., Risau, W. and Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B. and Robertson, E. J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* **120**, 1919-1928.

Couly, G. and Le Douarin, N. M. (1988). The fate map of the cephalic neural primordium at the presomitic to the 3-somite stage in the avian embryo. *Development* **103**, Suppl:101-113.

Couly, G. F., Coltey, P. M. and Le Douarin, N. M. (1993). The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. *Development* **117**, 409-429.

Cox, W. G. and Hemmati-Brivanlou, A. (1995). Caudalization of neural fate by tissue recombination and bFGF. *Development* **121**, 4349-4358.

Crossley, P.H., and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in developing embryo. *Development* **121**, 439-451.

Dattani, M. T., Martinez-Barbera, J. P., Thomas, P. Q., Brickman, J. M., Gupta, R., Martensson, I. L., Toresson, H., Fox, M., Wales, J. K., Hindmarsh, P. C., Krauss, S., Beddington, R. S. P. and Robinson, I. C. (1998). Mutations in the homeobox gene *HESX1/Hesx1* associated with septo-optic dysplasia in human and mouse. *Nat. Genet.* **19**, 125-133.

Davidson, B. P., Kinder, S. J., Steiner, K., Schoenwolf, G. C. and Tam, P. P. (1999). Impact of node ablation on the morphogenesis of the body axis and the lateral asymmetry of the mouse embryo during early organogenesis. *Dev. Biol.* **211**, 11-26.

Davis, C. A. and Joyner, A. L. (1988). Expression pattern of the homeobox containing genes *En-1* and *En-2* and the proto-oncogene *int-1* diverge during mouse development. *Genes Dev.* **2**, 1736-1744.

Ding, J., Yang, L., Yan, 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.

Dirksen, M. L. and Jamrich, M. (1992). A novel, activin-inducible, blastopore lip specific gene of *Xenopus laevis* contains a fork-head DNA binding domain. *Genes Dev.* 6, 599-608.

Downs, K. M. and Davies, T. (1993). Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118**, 1255-1266.

Dufort, D., Schwartz, L., Harpal, K. and Rossant, J. (1998). The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. *Development* **125**, 3015-3025.

Dymecki, S. M. (1996). Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **93**, 6191-6196.

Eagleson, G., Ferreiro, B. and Harris, W. A. (1995). Fate of the anterior neural ridge and the mophogenesis of the Xenopus forebrain. *J. Neurobiol.* **28**,146-158.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Ekker, S. C., McGrew, L. L., Lai, C. J., Lee, J. J., Von Kessler, D. P., Moon, R. T. and Beachy, P. A. (1995). Distinct expression and shared activities of members of the hedgehog gene family of *Xenopus laevis*. *Development* **121**, 2337-2347.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. *Cell* **81**, 747-756.

Fainsod, A., Deissler, K., Yelin, R., Marom, K., Epstein, M., Pillemer, G., Steinbeisser, H. and Blum, M. (1997). The dorsalizing and neural inducing gene *follistatin* is an antagonist of BMP-4. *Mech. Dev.* **63**, 39-50.

Filosa, S., Rivera-Perez, J. A., Gomez, A. P., Gansmuller, A., Sasaki, H., Behringer, R. R. and Ang, S. L. (1997). Goosecoid and *HNF-3β* genetically interact to regulate neural tube patterning during mouse embryogenesis. *Development* **124**, 2843-2854.

Foley, A. C., Storey, K. G. and Stern, C. D. (1997). The prechordal region lacks neural inducing ability, but can confer anterior character to more posterior neuroepithelium. *Development* **124**, 2983-2996.

Foley, A. C., Skromne, I. and Stern, C. D. (2000). Reconciling different models of forebrain induction and patterning: a dual role for the hypoblast. *Development* **127**, 3839-3854.

Frantz, G. D., Weimann, J. M., Levin, M. E. and McConnell, S. K. (1994). Otx1 and Otx2 define layers and regions in developing cerebral cortex and cerebellum. *J. Neurosci.* **14**, 5725-5740.

Gage, P. J., Suh, H. and Camper, S., A. (1999). Dosage requirement of Pitx2 for development of multiple organs. *Development* **126**, 4643-4651.

Garda, A-L., Echevarria, D. and Martinez, S. (2001). Neuroepithelial co-expression of *Gbx2* and *Otx2* precedes *Fgf8* expression in the isthmic organizer. *Mech. Dev.* **101**, 111-118.

Gimlich, R. L. and Cooke, J. (1983). Cell lineage and the induction of second nervous system in amphibian development. *Nature* **306**, 471-473.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.

Grindley, J. C., Hargett, L. K., Hill, R. E., Ross, A., and Hogan, B. L. (1997) Disruption of *PAX6* function in mice homozygous for the *Pax6Sey-1Neu* mutation produces abnormalities in the early development and regionalization of the diencephalon. *Mech. Dev.* **64**, 111-126.

Grosschedl, R., Giese, K. and Pagel, J. (1994). HMG domain proteins: Architectural elements in the assembly of nucleoprotein structures. *Trends Genet.* **10**, 94-99.

Gruss, P. and Walther, C. (1992). Pax in development. *Cell* **69**, 719-722.

Gu, H., Marth, J. D., Orban, P. C., Mossmann, H. and Rajewsky, K. (1994). Deletion of a DNA polymerase β- gene segment in T cells using cell type-specific gene targeting. *Science* **265**, 103-106.

Hamburger, V. (1988). The Heritage of Experimental Embryology: Hans Spemann and the Organizer (New York: Oxford University Press).

Hamilton, D. L. and Abremski, K. (1994). Site-specific recombination by the bacteriophage P1 lox-Cre system. Cre-mediated synapsis of two lox sites. *J. Mol. Biol.* **178**, 481-486.

Hansen, C. S., Marion, C. D., Steele, K., George, S. and Smith, W. C. (1997). Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3. *Development* **124**, 483-492.

Harland, R. (2000). Neural induction. *Curr. Opin. Genet. Dev.* **10**, 357-362.

Hashimoto, H., Itoh, M., Yamanaka, Y., Yamashita, S., Shimizu, T., Solnica-Krezel, L., Hibi, M. and Hirano, T. (2000). Zebrafish Dkk1 functions in forebrain specification and axial mesendoderm formation. *Dev. Biol.* **217**, 138-152.

Hauptmann, G. and Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and Drosophila embryos. *Trends Genet.* **10**, 266.

Heikinheimo, M., Lawshe, A., Shackleford, G. M., Wilson, D. B. and MacArthur, C. A. (1994). *Fgf-8* expression in the post-gastrulation mouse suggests roles in the development of the face, limbs, and central nervous system. *Mech. Dev.* **48**, 129-138.

Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemman organizer and displays direct neuralizing activity. *Cell* **77**, 283-295.

Herrmann, B. G. (1991). Expression pattern of *brachyury* gene in whole-mount Twis/Twis mutant embryos. *Development* **113**, 913-917.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). "Manipulating the Mouse Embryo; A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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.

Hui, C. C., Slusarski, D., Platt, K. A., Holmgren, R. and Joyner, A. L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol.* **162**, 402-413.

Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P. A. and Rosenthal, A. (1995). Induction of midbrain dopaminergic neurons by Sonic hedgehog. *Neuron* **15**, 35-44.

***Jin*, O., Harpal, K., Ang, S-L. and Rossant, J.** (2001). *Otx2* and *HNF3β* genetically interact in anterior patterning. *Int. J. Dev. Biol.* **45**, 357-365.

Joyner, A. L. (1996). *Engrailed*, *Wnt* and *Pax* genes regulate midbrain-hindbrain development. *Trends Genet.* **12**, 15-20.

Joyner, A. L., Liu, A. and Millet, S. (2000). *Otx2*, *Gbx2* and *Fgf8* interact to position and maintain a mid-hindbrain organizer. *Curr. Opin. Cell Biol.* **12**, 736-741.

Kaestner, K. H., Monaghan, A. P., Kern, H., Ang, S-L.,Weitz, S., Lichter, P. and Schutz, G. (1995). The mouse *fkh-2* gene. *J. Biol. Chem.* **270**, 30029-30035.

Kelly, W. L., and Bryden, M. M. (1983). A modified differential stain for cartilage and bone in whole mount preparations of mammalian fetuses and small vertebrates. *Stain Technology* **58**, 131-134.

Kiecker, C. and Niehrs, C. (2001). The role of prechordal mesendoderm in neural patterning. *Curr. Opin. Neurobiol.* **11**, 27-33.

Kimura, S., Hara, Y., Fernandez-Salguero, P., Fox, C. H., Ward, J. M. and Gonzalez, F. J. (1996). The *T/ebp* null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituiary. *Genes Dev.* **10**, 60-69.

Kimura, C., Yoshinaga, K., Tian, E., Suzuki, M., Aizawa, S. and Matsuo, I. (2000). Visceral endoderm mediates forebrain develpoment by suppressing posteriorizing signals. *Dev. Biol.* **225**, 304-321.

Kimura, C., Shen, M. M., Takeda, N., Aizawa, S. and Matsuo, I. (2001). Complementary functions of *Otx2* and *Cripto* in initial patterning of mouse epiblast. *Dev. Biol.* **235**, 12-32.

Kinder, S. J. Tsang, T. E. Wakamiya, M., Sasaki, H., Behringer, R. R. Nagy, A. and Tam, P. P. L. (2001). The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. *Development* **128**, 3623-3634.

Klingensmith, J., Ang, S-L., Bachiller, D. and Rossant, J. (1999). Neural induction and patterning in the mouse in the absence of the node and its derivatives. *Dev. Biol.* **216**, 535-549.

Knecht, A. K., Good, P. J., Dawid, I. B. and Harland, R. M. (1995). Dorsal-ventral patterning and differentiation of noggin-induced neural tissues in the absence of mesoderm. *Development* **121**, 1927-1936.

Knoetgen, H., Teichmann, U., Wittler, L., Viebahn, C. and Kessel, M. (2000). Anterior neural induction by nodes from rabbits and mice. *Dev. Biol.* **225**, 370-380.

Kobayashi, D., Kobayashi, M., Ogura, T., Nakafuku, M. and Shimamura, K. Early subdivision in the neural plate define distinct competence for inductive signals. *Development* in press.

Kobayashi, M., Toyama, R., Takeda, H., Dawid, I. B., and Kawakami, K. (1998). Overexpression of the forebrain-specific homeobox gene *six3* induces rostral forebrain enlargement in zebrafish. *Development* **125**, 2973-2982.

Kobayashi, M., Nishikawa, K., Suzuki, T., and Yamamoto, M. (2001). The homeobox protein *six3* interacts with the groucho corepressor and acts as a transcriptional repressor in eye and forebrain formation. *Dev. Biol.* **232**, 315-326.

Lewandoski, M., Meyers, E. N. and Martin, G. R. (1997). Analysis of *Fgf8* gene function in vertebrate development. *Cold Spring Harbor Symp. Quant. Biol.* 159-167.

Lewandoski, M., Sun, X. and Martin, G. R. (2000). Fgf8 signalling from the AER is essential for normal limb development. *Nature Genet.* **26**, 460-463.

Lewandoski, M. (2001). Conditional control of gene expression in the mouse. *Genetics* **2**, 743-755.

Liem, K. F., Jr., Tremmi, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of the neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Liu, A., Losos, K. and Joyner, A. L. (1999). FGF8 can active *Gbx2* and transform regions of the rostral mouse brain into a hindbrain fate. *Development* **126**, 4827-4838.

Liu, A. and Joyner, A. L. (2001). EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region. *Development* **128**, 181-191.

Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R. and Bradley, A. (1999a). Requirement for Wnt3 in vertebrate axis formation. *Nat Genet.* **22**, 361-365.

Lowe, L. A., Yamada, S, and Kuehn, M. R. (2001). Genetic dissection of *nodal* function in patterning the mouse embryo. *Development* **128**, 1831-1843.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Martinez-Barbera, J. P., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D. and Beddington, R. S. P. (2000a). The homeobox gene *Hex* is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127**, 2433-2445.

Martinez-Barbera, J. P., Rodriguez, T. A. and Beddington, R. S. P. (2000b). The homeobox gene *Hesx1* is required in the anterior neural ectoderm for normal forebrain formation. *Dev. Biol.* **223**, 422-430.

Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. and Martin, G. R. (1999). FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar develpopment via a repressive effect on *Otx2* expression. *Development* **126**, 1189-1200.

Matsunaga, E., Araki, I., and Nakamura, H. (2000). *Pax6* defines the di-mesencephalic boundary by repressing *En1* and *Pax2*. *Development* **127**, 2357-2365.

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.

Matzuk, M. M., Lu, N., Vogel, H., Sellheyer, K., Roop, D. R. and Bradley, A. (1995). Mutiple defects and perinatal death in mice deficient in follistatin. *Nature* **374**, 360-363.

McMahon, J. A., Takada, S., Zimmerman, L. B., Fan, C. M., Harland, R. M. and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **12**, 1438-1452.

Meno, C., Gritsman, K., Ohishi, S., Ohfuji, Y., Heckscher, E., Mochida, K., Shimono, A., Kondoh, H., Talbot, W., Robertson, E. J., Schier, A. F. and Hamada, H. (1999). Mouse *lefty2* and zebrafish *Activin* are feedback inhibitors of *Nodal* signaling during vertebrate gastrulation. *Mol. Cell* **4**, 287-298.

Meyers, E. N., Lewandoski, M. and Martin, G. R. (1998). An *Fgf8* mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat. Genet.* **18**, 136-141.

Meyers, E. N. and Martin, G. R. (1999). Differences in left-right axis pathways in mouse and chick: functions of FGF8 and SHH. *Science* **285**, 403-406.

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D. W., Glinka, A., Grinberg, A., Huang, S-P., Niehrs, C., Belmonte, J. C. I. and Westphal, H. (2001). *Dickkopf1* is required for embryonic head induction and limb morphogenesis in the mouse. *Dev. Cell* **1**, 423-34.

Muller, U. (1999). Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. *Mech. Dev.* **82**, 3-21.

Nagy, A., Moens, C., Ivanyi, E., Pawling, J., Gertsenstein, M., Hadjantonakis, A-K., Pirity, M. and Rossant, J. (1998). Dissecting the role of *N-myc* in development using a single targeting vector to generate a series of alleles. *Curr. Biol.* **8**, 661-664.

Nagy, A. (2000). Cre recombinase: The universal reagent for genome tailoring. *Genesis* **26**, 99-109.

Nieuwkoop, P. D. (1952a). Activation and organization of the central nervous system in amphibians. Part I, Induction and activation. *J. Exp. Zool.* **120**, 1-31.

Nieuwkoop, P. D. (1952b). Activation and organization of the central nervous system in amphibians. Part II, Differentiation and organization. *J. Exp. Zool.* **120**, 33-81.

Nieuwkoop, P. D. and Nigtevecht, G. V. (1954). Neural activation and transformation in explants of competent ectoderm under the influence of fragments of anterior notochord in Urodeles. *J. Embryol. Exp. Morph.* **2**, 175-193.

Nieuwkoop, P. D., Johnen, A. G. and Albers, B. (1985). The Epigenetic Nature of Early Chordate Development: Induction and Competence (Cambridge: Cambridge University Press).

Ohuchi, H., Yoshioka, H., Tanaka, A., Kawakami, Y., Nohno, T. and Noji, S. (1994). Involvment of androgen-induced growth factor (FGF-8) gene in mouse embryogenesis and morphogenesis. *Biochem. Biophys. Res. Comm.* **204**, 882-888.

Oliver, G., Mailhos, A., Wehr, R., Copeland, N. G., Jenkins, N. A. and Gruss, P. (1995). *Six3*, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development* **121**, 4045-4055.

Pabst, O., Herbrand, H., Takuma, N., Arnold, H. H. (2000) NKX2 gene expression in neuroectoderm but not in mesendodermally derived structures depends on sonic hedgehog in mouse embryos. *Dev. Genes. Evol.* **210**, 47-50.

Papalopulu, N. (1995). Regionalization of the forebrain from neural plate to neural tube. *Perspect Dev. Neurobiol.* **3**, 39-52.

Partanen, J., Schwartz, L. and Rossant, J. (1998). Opposite phenotypes of hypomorphic and Y766 phosphorylation sites mutations reveal a function for Fgfr1 in anteroposterior patterning of mouse embryos. *Genes Dev.* **12**, 2332-2344.

Pearce, J. J. H., Penny, G. and Rossant, J. (1999). A mouse cerberus/Dan-related gene family. *Dev. Biol.* **209**, 98-110.

Perea-Gomez, A., Shawlot, W., Sasaki, H., Behringer, R. R. and Ang, S-L. (1999). *HNF3β* and *Lim1* interact in the visceral endoderm to regulate primitive streak formation and anterior-posterior polarity in the mouse embryo. *Development* **126**, 4499-4511.

Perea-Gomez, A., Lawson, K. A., Rhinn, M., Zakin, L., Brulet, P, Mazan, S. and Ang, S-L. (2001). *Otx2* is required for visceral endoderm movement and for the restriction of posterior signals in the epiblast of the mouse embryo. *Development* **128**, 753-765.

Piccolo, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP4. *Cell* **86**, 589-598.

Piccolo, S., Agius, E., Leyns, L., Bhattacharya, S., Grunz, H., Bouwmeister, T. and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-710.

Price, M. (1993). Members of the Dlx- and Nkx2-gene families are regionally expressed in the developing forebrain. *J. Neurobiol.* **24**, 1385-1399.

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.

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.

Rowitch, D. H. and McMahon, A. P. (1995). Pax-2 expression in the murine neural plate precedes and encompasses the expression domains of Wnt-1 and En-1. *Mech. Dev.* **52**, 3-8.

Rubenstein, J. L. R., Martinez, S., Shimamura, K. and Puelles, L. (1994). The embryonic vertebrate forebrain: the prosomeric model. *Science* **266**, 578-580.

Rubenstein, J. L. R., Shimamura, K., Martinez, S. and Puelles, L. (1998). Regionalization of the prosencephalic neural plate. *Annu. Rev. Neurosci.* **21**, 445-477.

Rubenstein, J. L. R. and Beachy, P. A. (1998). Patterning of the embryonic forebrain. *Curr. Opin. Neurobiol.* **8**, 18-26.

Ruiz i Altaba, A., and Jessell, T. M. (1991). Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos. *Development* **112**, 945-958.

Ruiz i Altaba, A., Prezioso, V. R., Darnell, J. E. and Jessell, T. M. (1993). Sequential expression of *HNF-3β* and *HNF-3α* by embryonic organizing centers: the dorsal lip/node, notochord and floor plate. *Mech. Dev.* **44**, 91-108.

Ruiz i Altaba, A. (1994). Pattern formation in the vertebrate neural plate. *Tends Neurosci.* **17**, 233-243.

Saha, M. S. and Grainger, R. M. (1992). A labile period in the determination of the anterior-posterior axis during early neural development in *Xenopus*. *Neuron* **8**, 1003-1014.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Sasaki, H. and Hogan, B. (1994). *HNF-3β* as a regulator of floor plate development. *Cell* **76**, 103-115.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.

Sasai, Y. and De Robertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. *Dev. Biol.* **182**, 5-20.

Shanmugalingam, S., Houart, C., Picker, A., Reifers, F., Macdonald, R., Barth, A., Griffin, K., Brand, M. and Wilson, S. W. (2000). Ace/Fgf8 is required for forebrain commissure formation and patterning of the telencephalon. *Development* **127**, 2549-2561.

Sharpe, C. R. (1991). Retinoic acid can mimic endogenous signals involved in transformation of the *Xenopus* nervous system. *Neuron* **7**, 239-247.

Shawlot, W. and Behringer, R. R. (1995). Requirement for *Lim1* in head-organizer function. *Nature* **374**, 425-430.

Shawlot, W., Wakamiya, M., Kwan, K. M., 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.

Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L. R. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-3933.

Shimamura, K. and Rubenstein, J. L. R. (1997). Inductive interactions direct early regionalization of the mouse forebrain. *Development* **124**, 2709-2718.

Shimamura, K., Martinez, S., Puelles, L. and Rubenstein J. L. R. (1997). Patterns of gene expression in the neural plate and neural tube subdivide the embryonic forebrain into transverse and longitudinal domains. *Dev. Neurosci.* **19**, 88-96.

Shinya, M., Koshida, S., Sawada, A., Kuroiwa, A. and Takeda, H. (2001). Fgf signalling through MAPK cascade is required for development of the subpallial telencephalon in zebrafish embryos. *Development* **128**, 4153-4164.

Simeone A, Acampora D, Gulisano M, Stornaiuolo A, Boncinelli E. (1992). Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358**, 687-690.

Simeone, A., Acampora, D., Mallamci, 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 neuroectderm of the gastrulating mouse embryo. *EMBO J.* **12**, 2735-2747.

Simeone, A. (2000). Positioning the isthmic organizer where *Otx2* and *Gbx2* meet. *Trends Genet.* **16**, 237-240.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* **70**, 829-840.

Smith, W. C., Mckendry, R., Ribisi, S.J. and Harland, R. M. (1995). A nodal-related gene defines a physical and functional domain within the Spemann organizer. *Cell* **82**, 37-46.

Spemann, H. and Mangold, H. (1924). Uber Induktion von Embryonanlagen durch Implantation artfremder Organisatoren. *Arch. mikr. Anat. EntwMech.* **100**, 599-638.

Stern, C. D. (1998). *Current Topics in Developmental Biology*. **36**, pp. 223-243, Oxford, UK: Academic Press.

Stern, C. D. (2001). Initial patterning of the central nervous system: How many organizers? *Nat. Rev. Neurosci.* **2**, 92-97.

Stoykova, A. and Gruss, P. (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J. Neurosci.* **14**, 1395-1412.

Stoykova, A., Fritsch, R., Walther, C., Gruss, P. (1996). Forebrain patterning defects in *Small eye* mutant mice. *Development* **122**, 3453-3465.

Streit, A. and Stern, C. D. (1999). Neural induction. A bird's eye view. *Trends. Genet.* **15**, 20-24.

Suda, Y., Matsuo, I., Kuratani, S. and Aizawa, S. (1996). *Otx1* function overlaps with *Otx2* in development of mouse forebrain and midbrain. *Genes to Cells* **1**, 1031-1044.

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.

Suda, Y., Nakabayashi, J., Matsuo, I. and Aizawa, S. (1999). Functional equivalency between *Otx2* and *Otx1* in development of the rostral head. *Development* **126**, 743-757.

Suda, Y., Hossain, Z. M., Kobayashi, C., Hatano, O., Yoshida, M., Matsuo, I. and Aizawa, S. (2001). *Emx2* directs the development of diencephalon in cooperation with *Otx2*. *Development* **128**, 2433-2450.

Sun, X., Meyers, E. N., Lewandoski, M. and Martin, G. R. (1999). Targeted distruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev.* **13**, 1834-1846.

Sund, N. J., Vatamaniuk, M. Z., Casey, M., Ang, S-L., Magnuson, M. A., Stoffers, D. A., Matschinsky, F. M. and Kaestner, K. H. (2001). Tissue-specific deletion of *Foxa2* in pancreatic beta cells results in hyperinsulinemic hypoglycemia. *Genes Dev.* **15**, 1706-1715.

Sussel, L., Marin, O., Kimura, S., and Rubenstein, J. L. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126**, 3359-3370.

Tao, W. and Lai, E. (1992). Telencephalon-restricted expression of *BF-1*, a new member of the *HNF-3/folk head* gene family in the developing rat brain. *Neuron* **8**, 957-966.

Tam, P. P. and Beddington, R. S. P. (1987). The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* **99**, 109-126.

Tam, P. P. and Beddington, R. S. P. (1992). Establishment and organization of germ layers in the gastrulating mouse embryo. *Ciba Found Symp.* **165**, 27-49.

Tam, P. P. and Steiner, K. A. (1999). Anterior patterning by synergistic activity of the early gastrula organizer and the anterior germ layer tissue of the mouse embryo. *Development* **126**, 5171-5179.

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

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

Tremblay, K. D., Hoodless, P. A., Bikoff, E. K. and Robertson, E. J. (2000). Formation of the definitive endoderm in mouse is a Smad2-dependent process. *Development* **127**, 3079-3090.

Trumpp, A., Depew, M. J., Rubenstein, J. L. R., Bishop, J. M. and Martin, G. R. (1999). Cre-mediated gene inactivation demonstratesthat FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev.* **13**, 3136-3148.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.

Utomo, A. R. H., Nikitin, A. Y. and Lee, W. H. (1999). Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat. Biotechnol.* **17**, 1091-1096.

Varlet, L., 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.

Warren, N. and Price, D. J. (1997). Roles of *Pax-6* in murine diencephalic development. *Development* **124**, 1573-1582.

Wilkinson, D. G., Bailes, J. A. and McMahon, A. P. (1987). Expression of the proto-oncogene *int-1* is restricted to specific neural cells in the developing mouse embryos. *Cell* **50**, 79-88.

Wilkinson, D. G. (1993). *In situ* hybridization. In *Essential Developmental Biology: A practical Approach* (ed. Stern, C. D. and Holland, P.W.H.), pp. 257-274. Oxford, UK: IRL Press.

Wilson, P. A. and Hemmati-Brivanlou, A. (1997). Vertebrate neural induction: inducers, inhibitors, and a new synthesis. *Neuron* **18**, 699-710.

Wilson, S. W. and Rubenstein, J. L. R. (2000). Induction and dorsoventral patterning of the telencephalon. *Neuron* **28**, 641-651.

Witta, S. E., Agarwai, V. R. and Sato, S. M. (1995). Xlpou2, a noggin-inducible gene, has direct neuralizing activity. *Development* **121**, 721-730.

Xuan, S., Baptista, C. A., Balas, G., Tao, W., Soares, V. C., Lai, E. (1995). Winged helix transcription factor *BF-1* is essential for the development of the cerebral hemispheres. *Neuron* **14**, 1141-1152.

Yagi, T., Nada, S., Watanabe, N., Tamemoto, H., Kohmura, N., Ikawa, Y. and Aizawa, S. (1993). A novel negative selection for homologous recombinants using diphtheria toxin A fragment gene. *Aanal. Biochem.* **214**, 77-86.

Ye, W., Shimamura, K., Rubenstein, J. L. R., Hynes, M. A. and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-766.

Yoshida, M., Suda, Y., Matsuo, I., Miyamoto, N., Takeda, N., Kuratani, S. and Aizawa, S. (1997). *Emx1* and *Emx2* functions in development of dorsal telencephalon. *Development* **124**, 101-111.

Zakin, L., Reversade, B., Virlon, B., Rusniok, C., Glaser, P., Elalouf, J. M. and Brulet, P. (2000). Gene expression profiles in normal and *Otx2-/-* early gastrulating mouse embryos. *Proc. Natl. Acad. Sci. USA.* **97**, 14388-14393.

Zimmerman, L. B., De Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.