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Phenotypic Analyses of Mouse Embryos With Ubiquitous Expression of Oct4: Effects on Mid–Hindbrain Patterning and Gene Expression

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***Oct4* is a transcription factor that has been associated with pluripotency and fate determination in the initial cell lineages of mammals. On the other hand, *Pou2*, the ortholog of *Oct4* in zebrafish, serves additional later functions during brain development acting as a differentiation switch. In mice, *Oct4* is expressed throughout the neural plate of embryos until embryonic day (E) 8.0. In this study, we produced transgenic mouse embryos that ubiquitously express *Oct4* and analyzed the consequences during development. We show that, at E8.0, a higher dosage of *Oct4* in the neuroectoderm is sufficient to transiently alter mid–hindbrain patterning and produced a strong up-regulation of *Pax2*, indicating that *Oct4* can regulate this gene in vivo. After E9.5, ectopic *Oct4* in this region produced cell death and affected the development of the forebrain, suggesting that, at these later stages, *Oct4* down-regulation is necessary for normal development to proceed. The phenotype of the transgenic embryos was also accompanied with an increase of *Fgf8* expression in several of its endogenous domains, suggesting the possibility that Oct4 can participate in the regulation of expression of this ligand. Our observations support the hypothesis that *Oct4*, like zebrafish *Pou2*, has a conserved function during early brain patterning in mouse. *Developmental Dynamics 232:180–190, 2005.* © 2004 Wiley-Liss, Inc.**

Key words: Oct4; MHB; Fgf8; Pax2; organizer; brain; mouse

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INTRODUCTION

Oct4 is a remarkable member of the POU transcription factor family. It was first identified in embryonic stem (ES) and embryonic carcinoma (EC) cell lines as well as in pluripotent cells of the preimplantation embryo (Schöler et al., 1990b; Rosner et al., 1990; Okamoto et al., 1990). Its unique expression pattern suggested an involvement of this factor in the establishment or preservation of cell totipotency. In the mouse, zygotic *Oct4* gene expression begins before the eight-cell stage in all blastomeres of the morula; later, it becomes restricted to the inner cell mass (ICM) of the blastocyst. During implantation *Oct4* expression is transiently up-regulated and subsequently shutdown in those cells of the ICM that give rise to the primitive endoderm. By E9.0, expression is still observed in the neural groove, posterior neuropore, the hindgut area where the primordial germ cells (PGCs) are located, the paraxial mesoderm, and the unsegmented presomitic mesoderm.

The expression of this factor becomes down-regulated from anterior to posterior as development progresses. After E9.5, the only cells that express high levels of the *Oct4* gene are the PGCs

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(Yeom et al., 1996). *Oct4* expression is shutdown during sexual differentiation of PGCs, but it is again up-regulated in the mature oocyte (Pesce et al., 1998).

Gene inactivation of *Oct4* is embryonic lethal at the time of implantation due to a failure to specify the ICM (Nichols et al., 1998). In vitro culture of *Oct4*−/− embryos resulted in the differentiation of all blastomeres into trophectoderm, indicating that down-regulation of *Oct4* also might be crucial for trophectoderm differentiation. Evidence obtained from studies with mouse embryonic cell lines such as ES, EC, and embryonic germ (EG) cells confirmed the importance of *Oct4* in the maintenance of pluripotency and supported a participation of this protein in the control of cell fate. In one of these studies (Niwa et al., 2000), by decreasing *Oct4* levels in ES cells, it was possible to establish that the minimal amount required for the preservation of cell totipotency was 50% of the regular endogenous *Oct4* level. Below that, cells differentiated into trophectoderm. On the other hand, up-regulation of *Oct4* above 150% of the regular level resulted in differentiation into primitive endoderm and mesoderm. Moreover, it was shown recently that sustained up-regulation of *Oct4* in serum-free leukemia inhibitory factor (LIF)-deficient medium promotes ES differentiation into neuroectoderm cells and subsequent neuronal differentiation (Shimozaki et al., 2003). From these observations, it was concluded that *Oct4* could be acting as a trigger for the specification of different cell lineages; therefore, changes in its expression levels during development could lead to cell fate alterations in vivo.

Oct4 ortholog genes were initially identified in several mammalian species. The original thought that *Oct4* homologs were absent in the genomes of nonmammalian species led to the concept that it was a specific mammalian gene. In this regard, the identification of the *Pou2* gene as an ortholog of *Oct4* in zebrafish was a significant finding (Burgess et al., 2002). The *Pou2* gene was identified as the disrupted gene in *spiel-ohne-grenzen* (*spg*) mutant zebrafish. Analysis of the *spg* phenotype revealed that this gene has an early function during the proliferation of blastomeres similar to the one of mouse *Oct4* during the formation of the ICM. In addition, the *Pou2* gene was shown to have a specific function during midbrain and hindbrain formation (Burgess et al., 2002; Reim and Brand, 2002).

The anterior/posterior patterning of the vertebrate midbrain and cerebellum is regulated by a signaling center termed the mid–hindbrain boundary (MHB), which is localized in the junction between these two brain territories. The MHB produces secreted proteins with signaling activity and transcription factors participating in a complicated molecular network that underlies the establishment, refinement, and function of the organizer.

Among the secreted factors, Fgf8 and Wnt1 are key components. Other critical genes expressed in this region are the transcriptional factors *Otx2*, *Gbx2*, *Pax2*, and *En1/2* (reviewed by Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001). The initial positioning of the MHB depends on the junction between the posterior expression domain of *Otx2* and the anterior expression domain of *Gbx2* (Simeone et al., 1992). Subsequently, *Pax2* is activated around the *Otx2-Gbx2* interface followed by *En1/2*, *Wnt1*, and *Fgf8* expression (Davis and Joyner, 1988; Rowitch and McMahon, 1995; Crossley and Martin, 1995).

Although a participation of *Oct4* during mouse brain morphogenesis has not been documented, different observations suggest that *Oct4* could be an important player in the MHB establishment in mice. First, *Oct4* is expressed in the early neural plate (Fig. 1E; Rosner et al., 1990; Schöler et al., 1990a; Burgess et al., 2002). Second, murine *Oct4* rescues the brain phenotype of zebrafish *spg* mutants. Third, in *spg* mutants, *Pax2.1* expression is almost eliminated. In the mouse, the regulatory region of *Pax2* contains an enhancer region with recognition sequences for POU domain proteins. This enhancer is active during the early expression of *Pax2* in the MHB, suggesting that the initial activation of this gene depends on a POU transcription factor (Pfeffer et al., 2002).

The studies of a possible involvement of *Oct4* in developmental processes, other than the maintenance of totipotency, are precluded in the knockout mouse; therefore, alternative approaches to alter *Oct4* levels in vivo are required. In this work, we produced mouse lines ubiquitously expressing *Oct4* in the postimplantation embryo. In one of these lines, in which the levels of *Oct4* expression allowed for the survival of embryos up to midgestation, we studied the effects of this gene during brain formation. We report that overexpression of *Oct4* in the neuroectoderm leads to misregulation of some MHB markers. Remarkably a strong up-regulation of initial *Pax2* expression provides new evidence indicating that *Oct4* regulates this gene in vivo. In addition, we found that *Oct4* had a strong specific influence on *Fgf8* expression in most of its endogenous domains, including regions outside the brain. Our study uncovered previously undescribed functions for *Oct4* in mouse development.

RESULTS

Ubiquitous Activation of *Oct4* Expression by Cre-Mediated Recombination

To explore the in vivo dosage effects of the *Oct4* gene in the embryo, we aimed to produce transgenic mice in which *Oct4* was expressed at high levels in the whole animal from the zygote stage onward. To obtain these embryos, a Cre-*loxP* approach was required, given that an early phenotype could be expected. For this purpose, the tissue nonspecific alkaline phosphatase $(TNAP)^{Cre}$ and Oct4-*loxP* transgenic lines were used. The $TNAP^{Cre}$ is a reported line that targets PGCs for restricted genomic alterations (Lomelí et al., 2000); and the Oct4-*loxP* lines were generated for the present work as described in the methods. The mating of these lines produced offspring that included mice overexpressing *Oct4* in primordial germ cells and gametes. Targeted mice called $TNAP^{Cre}$/Oct4-*loxP* were selected and used in subsequent crosses with wild-type CD1 as shown in Figure 1A. The $TNAP^{Cre}$/Oct4-*loxP* modified gametes, which did not present an apparent phenotype, allowed the generation of mouse embryos with ectopic expression of *Oct4* in the zygote and thereafter in every cell of the embryo.

Four different Oct4-*loxP* lines derived from two alternative constructs were available (see Experimental Procedures section for details). Before mating with the TNAP^{Cre} mouse, these lines were analyzed by β-galactosidase activity to determine the transgene expression onset and localization. This analysis showed that, in all four lines, transgenic expression was present at the blastocyst stage. At later stages (E9.0) the *lacZ* activity was still detected in all the lines; however, the intensity was higher for the Oct4-*loxP7* and Oct4-*loxPF12* lines. In the former one, the reporter gene activity was followed up to day 14 and was found to be ubiquitous all over this period. This line was the one used for most of the analyses of the present work.

For a preliminary determination of the viability of transgenic embryos, progenies from different developmental stages derived from crosses of the TNAP^{Cre}/Oct4-*loxP7* with wild-type females, were genotyped by PCR. Embryos with activated Oct4-*loxP* alleles were selected and referred to hereafter as tgO7; nonexcised littermates were identified and used as controls. In agreement with the previously determined excision efficiency for the TNAP^{Cre} mouse (Lomelí et al., 2000), an average of 32% of the progeny expressed exogenous *Oct4*. Based on the report by Niwa et al. (2000), in which a higher dosage of *Oct4* in ES cells resulted in differentiation into primitive mesendodermal cells, early lethality of tgO7 embryos was a possible outcome. Surprisingly, tgO7 embryos were found in expected ratios until E9.5, indicating that they were able to survive beyond the blastocyst stage.

To assess *Oct4* expression in tgO7 embryos, the presence of the *Oct4* transcript was detected by ISH. As shown in Figure 1B, at midgestation, a strong transcriptional expression of *Oct4* was found in tgO7 embryos. Quantitative immunoblot analyses was also performed to compare the Oct4 protein endogenous expression in ES cells, with the transgenic expression in whole E9.5 and dissected brains of E12.5 tgO7 embryos (Fig. 1C). This experiment revealed that the amount of Oct4 in E9.5 embryos was 50% of that observed in ES cells. This quantity represented the minimal amount of protein per cell in the transgenic embryos, because it is likely that Oct4 expression is silenced in some cell types. A quantification of Oct4 in transgenic blastocysts by immunoblot was not possible; however, immunofluorescence studies showed that Oct4 protein was not significantly overexpressed in the tgO7 blastocysts compared with the wild-type ones (not shown). This finding can explain the lack of phenotype in tgO7 blastocysts. Of interest, transgenic blastocysts derived from Oct4-*loxPF12* mice presented a significantly higher level of Oct4 expression. A phenotypic analysis in such embryos is currently under way.

Embryos Ubiquitously Expressing *Oct4* Presented Several Morphological Abnormalities and Died Around E13.5

Resorption of some tgO7 embryos was first observed at E9.5. This phenomenon increased in the following days up to E13.5, after which, live embryos with excised transgenic alleles were not longer detected. Histological analysis of E12.5 tgO7 mutants suggested that lethality was due to a cardiac failure as this tissue was severely congested and embryos were highly edematous.

Significant defects were detected in tgO7 embryos beyond E9.5, including abnormalities in the head, trunk, tail, and limbs (Fig. 1D). The head was misshapen and visibly smaller. The first branchial arch seemed hypoplastic, and the gap between the maxillary and mandibular components was wider in tgO7 embryos. At E12.5, craniofacial malformations were evident, nasal and maxillary processes were reduced, and the lower jaw was poorly developed. The tail was engrossed and dorsally kinked. Tail abnormalities were consistent with an accumulation or defective migration of mesodermic cells from the tail bud. The neural tube presented a defective closure, which was noticed in the tail or as an irregular midline in the trunk region. In later stages (E13.5), abnormalities were expressed in the trunk where Alcian blue staining revealed alterations in vertebrate formation. Often an extra rib was present. Both the tail and vertebrate malformations would fit with defective somitogenesis. This possibility is presently being investigated. Limb defects were present with different degrees of severity. They included failure of skeletal development affecting mainly digits and zeugopod elements.

Mutant embryos derived from the other Oct4-*loxP* transgenic lines (Oct4-*loxPF12*, *-G9*, and *-20*) were also evaluated. In general, they all presented the axial phenotypes, both in the tail and vertebrate regions. Head defects were also visible in the tgOF12 embryos (derived from Oct4/*loxPF12*). These embryos were being lost from the progeny at earlier stages (E8). In contrast, embryos derived from the Oct4/*loxP20* and Oct4/*loxPG9* lines (which showed a lower *lacZ* staining) presented milder phenotypes, like the absence of craniofacial malformations and neural tube defective closure and survived to birth, after which, they were not followed. Head defects in these embryos were not measured but were not obvious. The limb phenotype only was observed in the tgO7 embryos.

Influence of *Oct4* Dosage on the Expression of Brain Molecular Markers During Mid–Hindbrain Regional Determination

Oct4 expression in the neuroectoderm of wild-type mice is significant during the regional determination of the brain (Fig. 1E; Rosner et al., 1990; Schöler et al., 1990a; Burgess et al., 2002). Additionally, there is evidence demonstrating that an *Oct4* ortholog in zebrafish is implicated in the MHB development, and recently, an enhancer that can bind *Oct1* and *Oct4* proteins has been identified in the *Pax2* regulatory region. These findings together suggested the possibility that *Oct4* could act as a regulator of gene expression in the mouse MHB organizer. If this were the case, an increased dosage of *Oct4* in the neuroectoderm during MHB development, would result in the altered expression of genes involved in brain patterning, suggesting the possible participation of *Oct4* in this process. For this rea-

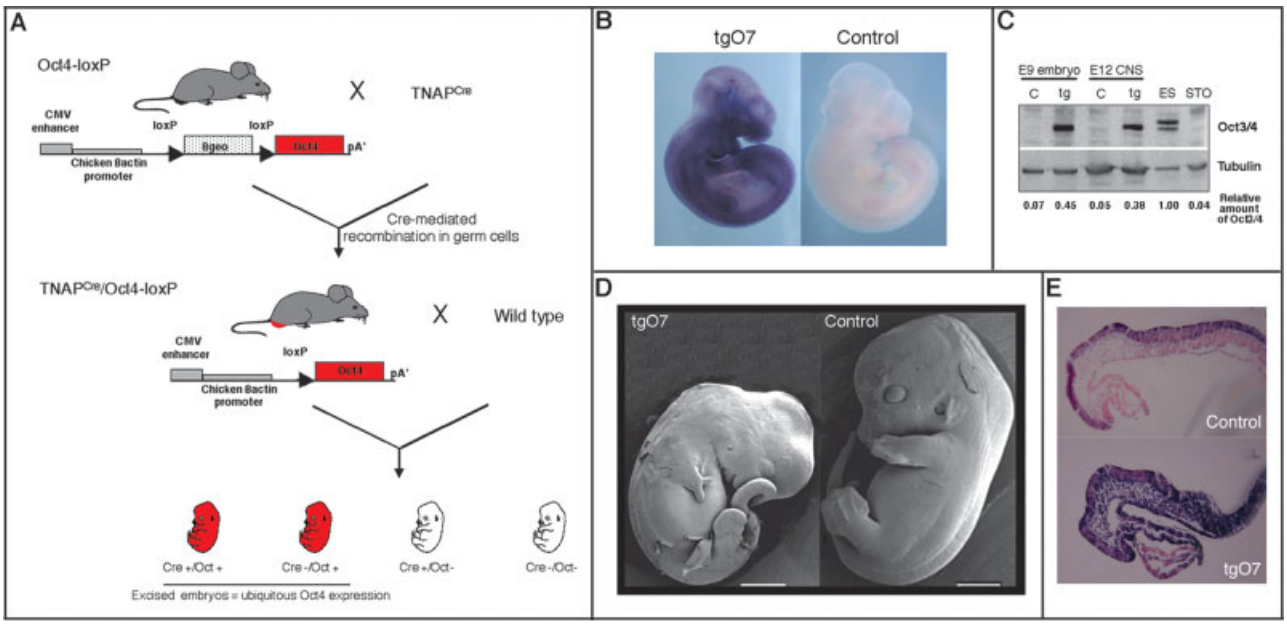

Fig. 1. Embryos with Oct4 ubiquitous expression. **A:** Schematic representation of the transgenic alleles carried by the Oct4-*loxP* and the TNAP^{Cre}/Oct4-*loxP* lines. In the Oct4-*loxP* line, expression of the β*geo* reporter is found in the entire mouse. In TNAP^{Cre}/Oct4-*loxP* mice, after Cre recombination the β*geo* reporter is removed and *Oct4* expressed in the germ line. TNAP^{Cre}/Oct4-*loxP* males are mated with CD1 females. The modified gametes of the TNAP^{Cre}/Oct4-*loxP* allow the generation of embryos with general expression of *Oct4*. **B:** Ubiquitous *Oct4* expression was detected by in situ hybridization in embryonic day (E) 10.5 in tgO7 embryos (left). **C:** Oct3/4 was detected in protein extracts of tgO7 E9 whole embryos and tgO7 E12.5 central nervous system (CNS) but not in equivalent extracts of control littermates. β-Tubulin was used as a standard for protein loading. Relative amounts of Oct3/4 were obtained with respect to endogenous expression of Oct3/4 in ES cells. STO cells were used as negative control. **D:** Scanning electron micrograph of E12.5 tgO7 (left) and control (right) embryos of the same litter. **E:** Sagittal sections of an in situ hybridization showing *Oct4* expression in the neuroectoderm of E8.5 control and tgO7 embryos. Scale bar = 1 mm in D.

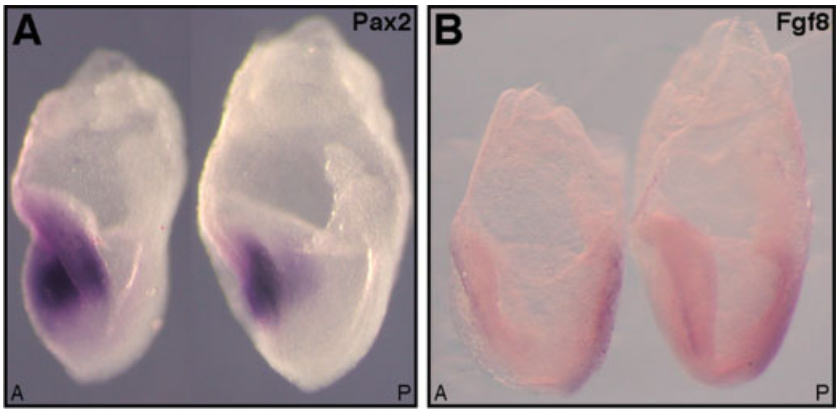

Fig. 2. *Pax2* is up-regulated in embryonic day (E) 7.5 tgO7 embryos. **A,B:** Lateral view of whole-mount in situ hybridization of E7.5 tgO7 (left) and control (right) embryos with *Pax2* (A) and *Fgf8* (B) probes. *Pax2* expression is expanded to anterior regions (A), and no difference was found in *Fgf8* expression at this stage (B). A, anterior; P, posterior.

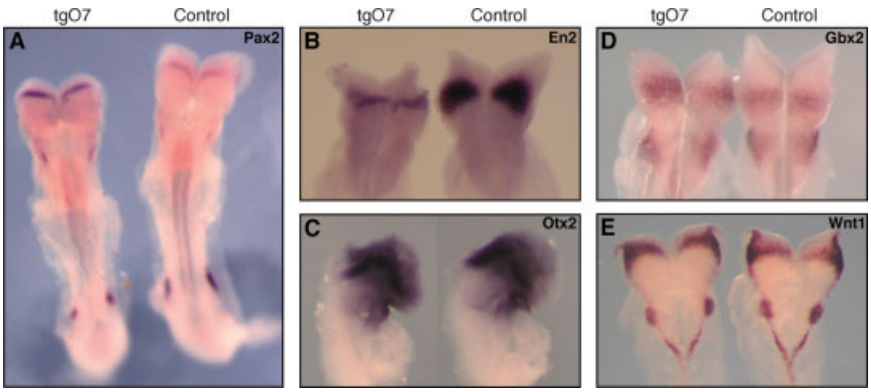

Fig. 3. Expression profile of mid–hindbrain boundary (MHB) genes in E8.5 tgO7 embryos. A–E: Whole-mount in situ hybridization of embryonic day (E) 8 embryos: dorsal views (A,B and D,E) and lateral view (C). **A–D:** In tgO7 embryos (left), *Pax2* expression is up-regulated (A) and *En2* expression is down-regulated (B); in contrast, expression of *Otx2* (C) and *Gbx2* (D) seems unaffected. **E:** *Wnt1* expression is slightly decreased in the ventral region.

son, we did whole-mount in situ hybridization in tgO7 embryos to follow the expression of several genes that are known to participate in the network of transcription and secreted factors that control the function of the MHB organizer.

At E7.5, *Pax2* expression is initiated in cells surrounding the Otx2/Gbx2 boundary (Rowitch and McMahon, 1995). In tgO7 embryos, *Pax2* was found up-regulated toward its anterior limit (Fig. 2A). At this stage, we have examined other genes like *Fgf8* (Fig. 2B), *Otx2*, and *Gbx2* (data not shown), but none of them presented an altered expression pattern.

At E8.5, *Pax2* increase in the MHB was maintained (Fig. 3A). This up-regulation was also observed in the branchial arches but not in the pronephros, another region of *Pax2* endogenous expression at this stage. *Engrailed 2* (*En2*), a gene that is expressed in a broad region covering the mes/met domain at this time (Davis and Joyner, 1988), was down-regulated in tgO7 embryos (Fig. 3B). *Otx2*, *Gbx2*, *Grg4*, and *Wnt1* expression were also investigated. *Otx2* and *Gbx2*, which are expressed in the midbrain and hindbrain boundaries, respectively (Ang et al., 1994; Bouillet et al., 1995), did not show alterations (Fig. 3C,D); *Wnt1*, whose expression is restricted to the *Otx2*-positive side (Bally-Cuif et al., 1995), showed a subtle and transient decrease in the ventral region (Fig. 3E). *Grg4* showed an unaltered expression pattern (not shown).

By E9.5, *Pax2* recovered its wild-type expression level (Fig. 4A). Similarly, *En2* expression level was recovered, but its caudal border presented an abnormal concavity (arrow in Fig. 4B). Although the amount of expression of *Otx2* and *Gbx2* did not show alterations in the tgO7 embryos, both genes presented an irregular but complementary shape in their expression borders at E9.5 (Fig. 4C,D). By this stage, *Wnt1* also showed a normal expression pattern (not shown). *Pax2* expression was also examined in tgOF12 embryos at E7.5 and E8.5. This experiment confirmed the up-regulation, which was restricted as well to the MHB domain (not shown).

Sox2, a gene encoding for a transcription factor that interacts with Oct4 is normally expressed in the presumptive neuroectoderm during gastrulation and in the neural tube at early somitogenesis. There is evidence suggesting that *Oct4* and *Sox2* coregulate each other (Avilion et al., 2003). Therefore, we examined the *Sox2* expression in tgO7 mutants but found no difference with respect to control embryos. Similarly, *Fgf4*, a recognized target for Oct4 (Yuan et al., 1995), did not present any change of expression at E8.5. Finally, we analyzed the expression of *Shh*, a gene that presents a longitudinal pattern along the ventral midline of the neuroectoderm and in the underlying mesoderm. At E10.5, the expression pattern of *Shh* seemed normal. Together, our results indicate that *Oct4* overexpression produced effects on specific genes and at specific regions. This observation underlies the importance of particular cofactors for the function of Oct4 in different developmental processes.

Oct4 Transgenic Expression Affected the Regulation of *Fgf8* Endogenous Expression

Fgf8 acts as a potent organizing molecule and is required during gastrulation, somitogenesis, limb morphogenesis, regionalization of the neural plate, telencephalon patterning, and formation of the heart field, among others (Meyers et al., 1998; Sun et al., 1999; Moon and Capecchi, 2000; Reifers et al., 2000; Dubrulle et al., 2001). Functional studies of the *spg/Pou2* mutant in zebrafish revealed phenotypic similarities with *acerebellar/Fgf8* (*ace*), suggesting that these two genes could act in the same or synergistic pathways (Reim and Brand, 2002). Specifically for zebrafish, Reim and Brand (2002) demonstrated that Pou2 is a factor that mediates competence to respond to Fgf8 in the neuroectoderm. To determine whether *Oct4* transgenic expression affected the regulation of *Fgf8* expression in the tgO7 mouse, we determined the expression pattern for this gene in transgenic embryos.

At E7.5, *Fgf8* expression in the primitive streak appeared unaffected (Fig. 2B). However, at E8.5, *Fgf8* signal was augmented in the branchial arches and presomitic mesoderm, and few ectopic-positive cells were found in the lateral mesoderm (Fig. 5A). In the MHB and anterior neural ridge (ANR), 30% of the embryos presented a broader and less-confined domain of expression. At E9.5, *Fgf8* expression adjacent to the zona limitans intrathalamica (ZLI) was completely abrogated; but in the MHB, expression was no longer affected (Fig. 5B). Other regions outside the brain such as the tail bud and branchial arches presented up-regulation at this stage (Fig. 5B). At E10.5, *Fgf8* was up-regulated in the tail bud, the maxillary and mandibular components of the first branchial arch, and in the nasal pits. In the limbs, *Fgf8* expression was either completely lost or expressed in patches. This lack of expression correlated with an increased limb phenotype (Fig. 5C).

Fgf8 expression was also analyzed in tgOF12 embryos at E8.5, E9.5, and E10.5. At E9.5 and E10.5, the results were the same as in the tgO7 embryos, in the zona limitans and tail bud. In the branchial arches, expression was increased but only in the maxillary region (not shown). At E8.5, *Fgf8* expression in tgOF12 embryos was not yet affected.

Changes of *Fgf8* expression in the presomitic mesoderm and MHB (where *Oct4* is normally expressed at E8.5) are likely due to a higher dosage of this protein. On the other hand, the effects in the branchial arches, the zona limitans, and the limbs are due to an ectopic *Oct4* expression. However, a remarkable observation is that, regardless of whether *Oct4* is overexpressed or ectopic, the observed effects on *Fgf8* expression are not ectopic, but mostly restricted to its endogenous domains.

Oct4 Gain-of-Function in the Neuroectoderm After E9.0 Leads to a Size Reduction in the Forebrain and Midbrain Regions

At E9, *Oct4* is not longer expressed in the neural tube. Therefore, a phenotypic effect after this stage could not be associated to an endogenous function. However, because *Oct4* has been recognized as a cell fate determinant, whose concentration is relevant at specific differentiation steps,

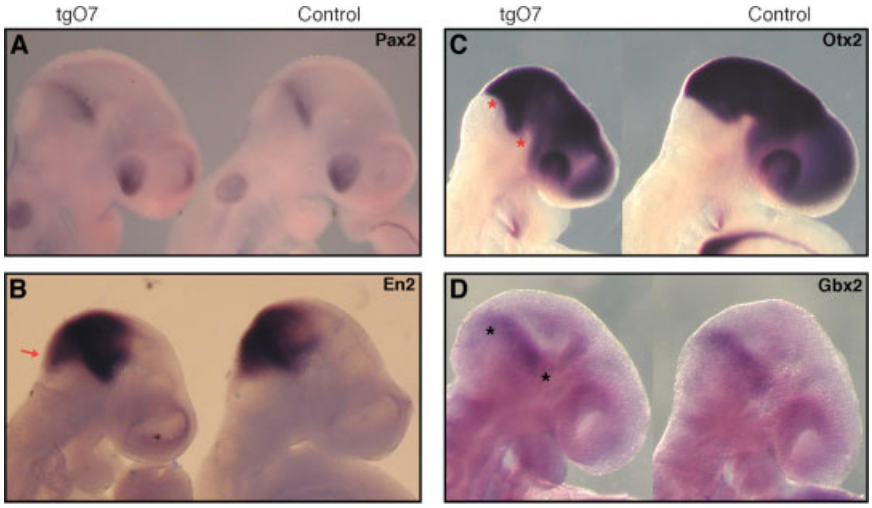

Fig. 4. Gene expression in the mid–hindbrain boundary (MHB) of embryonic day (E) 9 embryos. **A–D:** Whole-mount in situ hybridization in control (right) and transgenic (left) E9 embryos for *Pax2* (A), *En2* (B),*Otx2* (C), and *Gbx2* (D) genes. *Pax2* (A) and *En2* (B) recovered their wild-type expression level in the MHB. B–D: *En2* (arrow in B) and *Otx2* (asterisks in C) show an irregular shape in its caudal border, and *Gbx2* shows an irregular shape in its rostral border (asterisks in D).

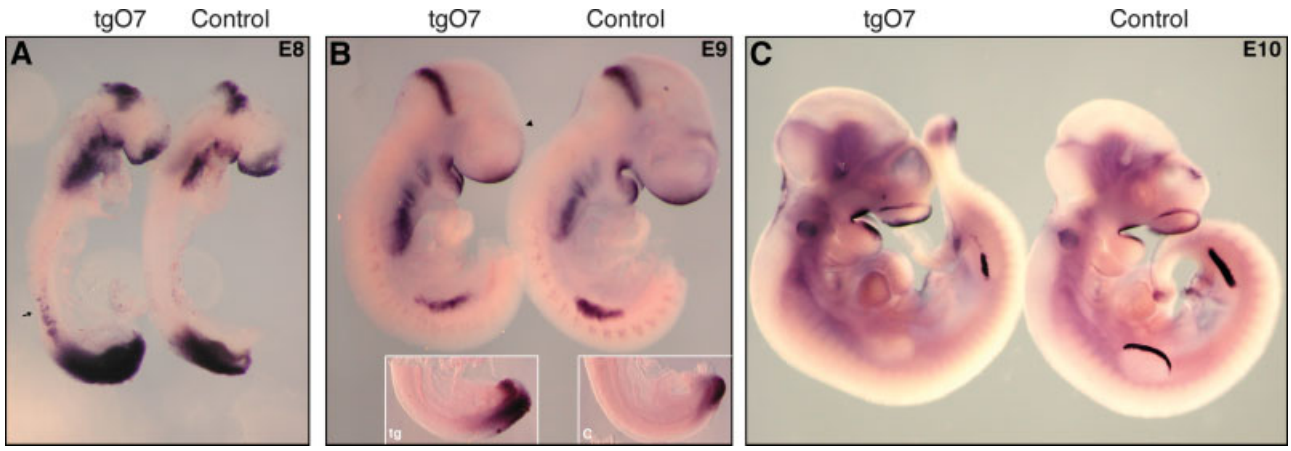

Fig. 5. *Fgf8* expression is altered in tgO7 embryos. **A:** *Fgf8* expression in embryonic day (E) 8 embryos. Up-regulation is found in the branchial arches and presomitic mesoderm and for several embryos (30%), in the mid–hindbrain boundary (MHB) and the anterior neural ridge (ANR). Ectopic *Fgf8*-positive cells are present in the lateral mesoderm (arrow). **B:** At E9.5, *Fgf8* recovered its wild-type expression at the MHB but is still up-regulated in the branchial arches and the tail bud (inset panels in B). In all of the tgO7 embryos, *Fgf8* expression is abolished at the zona limitans (arrowhead). **C:** At E10.5, *Fgf8* is up-regulated in the tail, the maxillary and mandibular component of the first branchial arch and the nasal pits. In the limbs, *Fgf8* expression was either completely lost or expressed in patches.

we considered it interesting to explore whether ectopic *Oct4* could produce further alterations in brain development. As the main morphological distinction between control and tgO7 embryos was the reduced head size (Fig. 6A), we carried out a comparative analysis of the brain size in different areas of tgO7 and wild-type heads at the 24-somite stage. The brain was subdivided into four regions, and the length of each was measured along the anteroposterior axis in four control and four tgO7 embryos. One region, localized in the presumptive telencephalon (Tel), spanned from the optic level of the ANR to the diencephalic constriction; a second region corresponded to the presumptive diencephalon (Die); another region included the midbrain (MB); and the fourth region corresponded to the anterior hindbrain (aHB), spanning from the isthmus to the otic vesicle (Fig. 6B). Tel, Die, and MB regions were 22 to 25% reduced in tgO7 embryos ($P < 0.05$), whereas no significant difference was found in the aHB (Fig. 6C).

To determine whether the size reduction of the brain was accompanied of an abnormal morphology, we performed a histological analysis of E12.5 tgO7 brains. Gross brain morphology was not altered, and the neuroepithelium presented a normal organization (not shown).

Ectopic Expression of *Oct4* Increased Cell Death in the Brain of tgO7 Embryos

The reduced size of the tgO7 developing brain could be reflecting either a decrease in proliferation or an increase in cell death of neuroepithelial cells. To examine the first possibility, we performed an analysis of BrdU incorporation in E9.5 embryos. There was not a significant difference in BrdU incorporation between tgO7 and wild-type littermates.

To address whether cell death contributed to the reduction in size of tgO7 brains, we performed the TUNEL assay on serial sections of E9.5 embryos. At the 21- to 22-somite stage, tgO7 brain

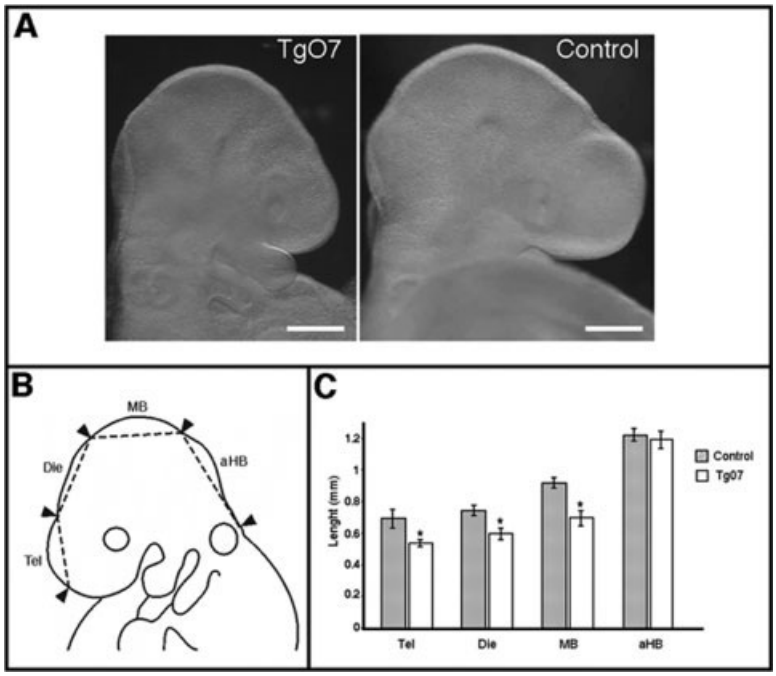

Fig. 6. Size reduction of anterior brain regions in tgO7 embryos. **A:** At embryonic day (E) 9.5, heads of tgO7 embryos (left) are smaller than those of control littermates (right). **B:** Schematic representation of brain regions measured. **C:** Lengths of brain regions from 24-somite stage tgO7 and control embryos (n = 4, for each) were plotted. According to the Student's *t*-test, size reduction was significant in the telencephalon (Tel), diencephalon (Die) and midbrain (MB). Asterisks indicate $P < 0.05$. In contrast, the anterior hindbrain (aHB) did not present a significant difference. Scale bar = 500 μm in A.

sections taken at a similar rostrocaudal level showed an increase in terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL)-positive cells in the neuroepithelium (Fig. 7A). For a quantitative study of cell death, the neuroepithelium was divided into an anterior region (midbrain and forebrain) and a posterior region (anterior hindbrain), and TUNEL-positive cells were counted in each part. We found that cell death in the anterior neuroepithelium of tgO7 embryos increased nearly four times in comparison to control brains (Fig. 7B). By contrast, cell death in the posterior neuroepithelium of tgO7 mutants, did not show a difference with respect to control embryos. Dead cells increased mainly in regions of normal cell death, like the optic vesicles and the dorsal diencephalon (Fig. 7A). We also found ectopic cell death in the mesencephalic region (not shown). These findings indicate that cell death is a likely cause for the size reduction in the anterior part of transgenic brains. Despite the increased cell death that we observed in the forebrain region, the molecular identity of organizing structures such as the ANR and the ZLI seemed to be conserved, as evidenced by the expression patterns of *Fgf8* and *Shh*, respectively.

DISCUSSION

In this work, we have studied the phenotype of mice, ubiquitously expressing the transcriptional factor Oct4 throughout development. This strategy was used in an attempt to explore the effects of increasing the levels of *Oct4* at early embryogenesis. A general evaluation of the amount of protein present in embryos of the tgO7 line at different developmental stages indicated that the Oct4 protein increased gradually, not being significantly augmented in the preimplantation period. The first time in development when we detected a phenotype was at E7.5 where we found evidence indicating that *Pax2* was upregulated. Detectable morphological abnormalities were seen only at E9.5 in which the amount of Oct4 protein had accumulated to a level that was 50% that of ES cells.

The relative low increase of the Oct4 protein achieved in the tgO7 line might be due to a deficient translation of the mRNA. This possibility is suggested because the tgOF12 line containing a different *Oct4* transgenic construct, presented a faster accumulation of the protein at the blastocyst stage (according to immunostaining studies), although similar levels of mRNA were detected by ISH in both lines at midgestation. Nonetheless, the generalized expression of *Oct4* in the tgO7 line resulted in severe morphogenetic abnormalities.

Although a complex phenotype was found at mid-gestation, a distinction can be made between *Oct4* ectopic and overexpression effects. Of interest, the two major phenotypic alterations that were present in the tgO7 embryos, i.e., anterior neural plate patterning and paraxial–tail bud defects, could be associated to regions where *Oct4* is endogenously expressed. The axial defects were not addressed in the current study; therefore, it is not clear when they are initiated. Nevertheless, either they started at gastrulation or at somitogenesis and became visible when cells of the presomitic mesoderm and somites still express *Oct4* (Yeom et al., 1996). Early brain patterning defects, which are mainly focused on in this study are also apparent at a time when endogenous *Oct4* is still expressed in the neuroectoderm (E7.5–E8; Fig. 1E; Rosner et al., 1990; Schöler et al., 1990a; Burgess et al., 2002). Among the ectopic *Oct4* effects, one that was studied was the forebrain and midbrain size reduction, which is detected at E9.5 and is likely due to increased cell death. Defects in the limbs found in tgO7 embryos, also could not be associated to *Oct4* endogenous expression, because *Oct4* is never present in this region. Considering the previous description, the phenotype of tgO7 embryos is more specific than what could have been predicted for a general regulator of totipotency.

Another interesting observation about the phenotype is related to the finding that *Oct4* had a strong specific influence on *Fgf8* expression. In this regard, it is remarkable that *Fgf8* has significant functions on mid–hind-

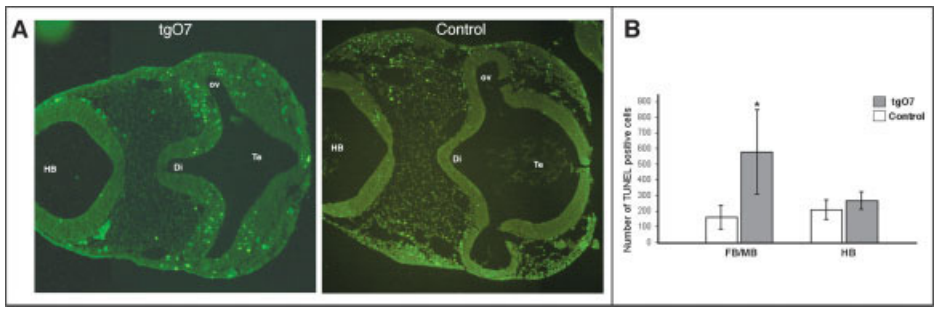

Fig. 7. Oct4 ectopic expression induces apoptosis in anterior brain regions. **A:** Brain sections of 21- to 22-somite stage embryos assayed for terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL). The cell death in the neuroepithelium of tgO7 embryos (left) is increased in comparison to control littermates (right). **B:** Consistent with the size reduction of anterior brain regions (forebrain and midbrain, FB/MB) in tgO7 embryos, neuroectoderm of these regions had a significantly increased number of TUNEL-positive cells The asterisk indicates $P < 0.05$, in comparison to control littermates ($n = 4$, for each). By contrast, hindbrain (HB) did not present any difference in TUNEL-positive cells. Di, diencephalon; Te, telencephalon; ov, optic vesicle. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

brain determination, migration of mesodermic cells at gastrulation, axis segmentation, and limb formation, all regions where *Fgf8* expression is affected. Therefore, the tgO7 phenotype is consistent with *Fgf8* misexpressions.

Oct4 Gain-of-Function in the Neuroectoderm Altered Gene Expression

Recent studies in zebrafish indicated that the *Pou2* gene, encoding a POU domain transcription factor orthologous to Oct4, was required during the establishment of the mid–hindbrain boundary and in hindbrain development. These data suggested the possibility that *Oct4* had a function in brain formation in mammals. If this were true, a gain-of-function situation could reveal possible target genes for Oct4 in the brain. As a first approximation, we analyzed the expression of different genes that are key players in the establishment of the MHB organizer. We found that an increased amount of *Oct4* in the anterior neural plate, strongly up-regulated *Pax2* gene expression in tgO7 and tgOF12 embryos at E7–E8. This finding is significant because it has been demonstrated that, in mouse, an enhancer that binds *Oct1* and *Oct4* in vitro controls the early expression of *Pax2* in the anterior neural plate. This enhancer has been shown to be an essential control element for the early expression of *Pax2* (Pfeffer et al., 2002). Therefore, our results combined with these previous data, strongly argues that *Oct4* plays an important role during mid–hindbrain patterning in the mouse, regulating the timing and spatial expression of *Pax2*. Similar observations had been described for zebrafish, where *Pou2*, the ortholog of *Oct4*, was shown to activate *Pax2.1* expression. Although, we cannot exclude the possibility that *Oct1* also participates in such regulation, it is worth mentioning that the inactivation of this gene does not result in brain patterning defects (Wang et al., 2004). Because Oct4−/− embryos are peri-implantation lethal, the functional essentiality of Oct4 for the initiation of *Pax2* expression can only be demonstrated with a conditional knock out of this gene in the anterior neural plate.

Other POU-factors like the class III Brn proteins (Brn-1, Brn-2, Brn-4, and Oct6) and the class IV Brn-3 are widely expressed in the developing nervous system and during neural tube formation (He et al., 1989). However, although patterns of expression in the embryonic brain have been established for most of these proteins, none of such studies has addressed the specific expression in the neural plate at E7.5 (He et al., 1989; Eng et al., 2001; Heydemann et al., 2001; Sim et al., 2002). For this reason, to consider other candidates besides *Oct4* as possible *Pax2* regulators, it would be important to precisely identify which other POU factors are present in the anterior neural plate when *Pax2* expression is initiated. On the other hand, in vivo deletion studies of all the above-mentioned POU factors, only lead to abnormalities in limited brain regions at later stages of development, indicating that, if they were involved in *Pax2* regulation, none of them is essential for such role (Philips and Luisi, 2000).

In the tgO7 line, *Pax2* up-regulation was strictly limited to its endogenous expression domain in the neural tube. The stimulation of *Pax2* gene expression was also confined in time to early somite embryos. This specificity suggests that a cofactor is restricting the domain of action of Oct4 and that it is acting as a heterodimeric complex. If this were the case, our results indicate that Oct4 is able to form a functional complex with the specific partner involved in *Pax2* activation. The only known protein whose expression overlaps with *Oct4* in this *Pax2* domain of expression that can interact with POU transcription factors is Sox2; however, no binding sites for HMG proteins are present in the *Pax2* regulatory region. The possibility also remains that *Oct4* could be acting as a homodimer or monomer on the *Pax2* enhancer sequence. This situation would only be compatible with our data if a general repressor of *Pax2* expression were active all over the embryo with the exception of the anterior neural plate.

En2, whose expression turns on a few hours after *Pax2* initiates expression, was also transiently altered in tgO7 embryos. However, this gene was down-regulated in E8.5 embryos but recovered its normal expression level by E9.5. Because the *En2* regulatory region does not contain recognition sequences for POU proteins, we believe that *En2* is down-regulated by Pax proteins. The *En2* gene contains

regulatory regions that bind Pax2/5/8, and it has been shown that Pax proteins are required for the positive regulation of *En2* expression in the MHB region (Song and Joyner, 2000). However, higher concentrations of Pax protein could lead to a decreased transcriptional activity (Chi and Epstein, 2002).

Except for the effect on *En2* and *Fgf8*, no other gross alterations of the expression were found in the MHB of tgO7 embryos. This lack of effect on other genes might be due to the transient character of both *Pax2* and *En2* regulation. In turn, for both genes it is established that activation and maintenance of expression, is controlled by separate enhancers (Song and Joyner, 2000; Pfeffer et al., 2002), all of which is in congruence with our data. Similarly, the histological analyses of tg07 brains indicated that no significant morphological changes in midbrain and cerebellar structures were present at E12.5. It is possible that transient morphological defects appear in earlier stages coincident with the early alterations of expression. Such defects could partially explain both the change of shape in the patterns of expression of *Otx2* and *Gbx2* at E9.5, and the detected midbrain size reduction. These abnormalities would be reverted as gene expression becomes normal, indicating a high level of plasticity in brain formation at this stage of development.

Oct4 Had an Influence on *Fgf8* Expression

An interesting question that our work raises is why *Fgf8* expression is affected by Oct4 in the two lines of transgenic embryos tested, and whether this influence is meaningful in vivo. The specificity of this phenomenon is supported by the finding that several other genes whose expression was tested in tgO7 embryos, did not show variations at midgestation, including demonstrated targets of Oct4, like *Fgf4* and *Sox2*.

Our study does not demonstrate an effect on the regulation of *Fgf8* gene expression. In fact, in the *Fgf8* regulatory region, no binding sites for POU transcription factors have been identified. Then a direct binding of Oct4 to such regions seems unlikely. Other mechanisms for the regulation of *Fgf8* expression by Oct4 are possible. An indirect one is that Oct4 induces the expression of one or various transcriptional regulators of *Fgf8*. A second alternative is that *Oct4* could function as a coactivator (or corepressor) by interacting with a factor that directly binds to the *Fgf8* regulatory region. In this situation, Oct4 would not be required to bind *Fgf8* regulatory sequences. For example, Oct4 is known to form a transcriptional complex with the Ets type transcription factor Ets2. This transcription factor complex functions to repress *tau interferon* genes in the absence of DNA binding of Oct4 (Ezashi et al., 2001). An appealing speculation is that an unknown factor could assemble with Oct4 to regulate *Fgf8* expression.

Instead of regulating its expression, *Oct4* could be increasing Fgf8-positive cell populations. This effect could be due to an enhanced Fgf8 signaling that could initially lead to alterations on proliferation or cell survival. This kind of synergistic action has been demonstrated during early neural development in zebrafish for these two factors. In this instance, it was found that *Pou2* makes cells competent to Fgf8 signaling (Reim and Brand, 2002). In our tgO7 embryos, increased *Fgf8* expression in the tail bud is accompanied with growth of the corresponding cell population.

Forebrain and Midbrain Size Reductions Are Likely Due to Cell Death

A visible consequence of the transient deregulated expression of brain markers in our tgO7 mice was the 20 to 25% size reduction in forebrain and midbrain at E9.5 and abnormal craniofacial and branchial arch morphogenesis. A TUNEL assay indicated that cell death in these parts of the brain was also significantly increased. In a close examination of sections from control and tgO7 embryos, we noticed that cell death in the forebrain was augmented mainly in regions where apoptosis normally occurs. Regions of normal cell death that showed an increase were localized in the optic vesicles, and within the telencephalon. The progenitor cells of much of the telencephalic and optic domains are contained in the ANR. Furthermore, Fgf8 has been implicated in mediating some of its functions. In 30% of E8.5 tgO7 embryos, *Fgf8* expression was increased in the ANR (Fig. 5A). On the other hand, apoptosis was also increased in the dorsal diencephalon, where the domain of dying cells was expanded away from the midline. In the tgO7 E9.5 embryos, we found a striking shutdown of *Fgf8* in the vicinity of the ZLI. This band is adjacent to the diencephalic domain where cell death is increased. Therefore, areas where apoptosis is affected seem to overlap with, or be close to domains where *Fgf8* expression is altered, suggesting that cell death increases could be associated to these variations.

Recently, it was shown that *Fgf8* regulates a cell survival pathway in the telencephalon (Storm et al., 2003). The effects of varying the level of *Fgf8* expression on cell death in the forebrain, were determined. It was found that either eliminating or increasing *Fgf8* leads to decreased cell survival. The molecular pathway that has been identified on this regulation of cell survival involves the activation of *Foxg1* by Fgf8 (Storm et al., 2003). The cell death phenotype that we observe is in agreement with this report.

In contrast with the situation in the forebrain, cell death in the midbrain seemed to be ectopic and not easily associated to changes of *Fgf8* expression in the MHB. Instead, cell death in the metencephalon could be related to the loss of *En1/2* expression, because increased cell death has been reported in the prospective midbrain of *En1*−/− embryos (Chi et al., 2003).

Concluding Remarks

The ectopic expression of *Oct4* in midgestation embryos had important effects on several phenotypic aspects. Of interest, these effects were more specific than predicted. A molecular explanation for this observation could be that regulation by Oct4 requires the assembly of very specific complexes on DNA enhancer sequences. For this reason, only very specific partnerships would become functional during development.

Our work provides evidence for additional biological properties besides the ones associated to conservation of pluripotency and the fate of initial cell

lineages in mammals. Furthermore, our data allow us to conclude that Oct4 can affect the activation of *Pax2* during early brain development and confirms the participation of Fgf8 signaling during brain cell death. Our results also suggest the possibility that Oct4 participates either in the regulation of *Fgf8* expression or on the modulation of Fgf8 signaling activity. Conditional *Oct4* mouse mutants will be required to further explore these suggestions.

EXPERIMENTAL PROCEDURES

Mice

Oct4 ubiquitous expression was produced by using the Cre/*loxP* site-specific recombination system. The TNAP^{Cre} line previously described in Lomelí et al. (2000) provides Cre expression under the control of the TNAP gene promoter. To create the Oct4-*loxP* lines, we used the pCALL vector (Lobe et al., 1999), which contains the lacZ/neomycin fusion (β*geo*) reporter gene flanked by *loxP* sites. After the second *loxP* site, *Xho*I and *Bgl*II restriction sites allowed the insertion of the *Oct4* cDNA sequence. Gene expression in this vector is driven by the CMV enhancer/chicken β-actin hybrid gene promoter (pCAGG), which has been shown to provide a consistent and strong ubiquitous expression in transgenic embryos (Lobe et al., 1999). For the Oct4-*loxP7* and Oct4-*loxP20* lines, the *Oct4* coding sequence was inserted in *Bgl*II. As this construct accidentally carried a strong ATG preceding the *Bgl*II site, we predicted an effect on the efficiency of translation of *Oct4* from its own ATG initiation codon. For this reason, a second construct was generated in which the *Oct4* cDNA was inserted into the *Xho*I site. This construct was used for the generation of the Oct4-*loxPG9* and Oct4-*loxPF12* lines. All Oct4-*loxP* lines express *lacZ* before Cre-mediated recombination.

We mated TNAP^{Cre} males with Oct4-*loxP* females to produce double-transgenic TNAP^{Cre}/Oct4-*loxP* mice in which Cre excision removed β*geo* allowing the expression of *Oct4* in gametes (Fig. 1A). Males were genotyped by PCR, and those carrying the two transgenic alleles, TNAP^{Cre} and Oct4-*loxP*, were used for a subsequent cross with a wild-type CD1 female. This cross produced a proportion of embryos with ubiquitous expression of *Oct4* (Fig. 1A). Mating was assumed to have occurred at midnight; E0.5 was the day on which vaginal plug was found.

Genotyping

To establish the genotype, ear punches (adults) or yolk sacs (embryos) were analyzed by PCR (lysis buffer 50 mM KCl, 10 mM Tris pH 8.3, 2 mM $MgCl_2$, 0.01% gelatin, 0.45% NP-40, 0.45% Tween 20, 100 μg/ml of proteinase K). Primers for the TNAP^{Cre} line (forward, CACGTCGATGGCCGCTCTA; reverse, TAAGGGCCAGCTCATTCCTCC) generated a 262-base pair (bp) fragment; primers for the Oct4-loxP line (forward, CCTACAGCAGATCACTCA; reverse, CAGAAGTCAGATGCTCAAG) generated a 518-bp fragment. To identify excised embryos, the pair of primers (forward, GCTCTAGAGCCTCTGCTAAC; reverse, AGCCTGGTCCGATTCCAG) generated a 372-bp fragment only after Cre excision removed β*geo*.

Scanning Electron Microscopy

Embryos were fixed in Karnovsky's fixative (3% glutaraldehyde, 0.1 M sodium cacodylate) overnight at 4°C, post-fixed with 1% OsO_4, and ethanol-dehydrated; then they were dried by the critical point method, sputtered-coated with gold, and examined with scanning electron microscope (JEOL mod. 5410 LV).

Western Blot

Cells were lysed in lysis buffer (25 mM Hepes pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 1 mM phenylmethyl sulfonyl fluoride, 10 mM BGP, 10 mM NAF, 200 μM $NaVO_4$, 1 μg/ml leupeptin, 10 μg/ml aprotinin). Tissues were lysed with ice-cold RIPA buffer plus 1× complete protease inhibitor cocktail (Roche). A total of 40 μg of protein were loaded to sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. We used antibodies directed against Oct3/4 (BD Transduction Laboratories) and β-tubulin (Santa Cruz). Relative levels of Oct3/4 protein were determined by normalization of densitometric values of Oct3/4 corrected for the amount of β-tubulin.

Whole-Mount RNA In Situ Hybridization

Mouse embryos were fixed and processed for in situ hybridization as described previously (Hogan et al., 1994). Antisense RNA probes were synthesized by using digoxigenin RNA labeling mix (Roche).

TUNEL Assay

Wild-type and mutant embryos were dissected at E9 and fixed with 4% paraformaldehyde in PBS. Eight-micrometer-thick paraffin transverse sections were processed for TUNEL assay by using the In Situ Cell Death Detection Kit (Roche). TUNEL-positive cells were counted in the neuroepithelium of 21- to 22-somite stage tgO7 ($n = 4$) and control ($n = 4$) embryos. Differences were analyzed by Student's *t*-test to determine the level of significance.

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