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Lack of the murine homeobox gene Hesx1 leads to a posterior transformation of the anterior forebrain

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The homeobox gene Hesx1 is an essential repressor that is required within the anterior neural plate for normal forebrain development in mouse and humans. Combining genetic cell labelling and marker analyses, we demonstrate that the absence of Hesx1 leads to a posterior transformation of the anterior forebrain (AFB) during mouse development. Our data suggest that the mechanism underlying this transformation is the ectopic activation of Wnt/β-catenin signalling within the Hesx1 expression domain in the AFB. When ectopically expressed in the developing mouse embryo, Hesx1 alone cannot alter the normal fate of posterior neural tissue. However, conditional expression of Hesx1 within the AFB can rescue the forebrain defects observed in the Hesx1 mutants. The results presented here provide new insights into the function of Hesx1 in forebrain formation.

KEY WORDS: Mouse, Hesx1, Anterior forebrain, Wnt, β-catenin, Cre, Rosa26

INTRODUCTION

The homeobox gene Hesx1 is a member of the paired-like family of homeobox proteins and functions as a transcriptional repressor (Thomas et al., 1995; Hermesz et al., 1996; Dasen et al., 2001). Hesx1 and its orthologues in other vertebrates (Anf genes) are expressed in the rostral region of the embryo during gastrulation and neurulation in all species analysed (Hermesz et al., 1996; Thomas and Beddington, 1996; Kazansky et al., 1997; Knoetgen et al., 1999). In mouse, Hesx1 expression is very dynamic, and Hesx1 transcripts are detected in anterior regions of the visceral endoderm, definitive endoderm and neural ectoderm during early development. Later, Hesx1 expression is restricted to the Rathke’s pouch.

Hesx1-deficient embryos show a reduction in anterior forebrain (AFB) tissue rostral to the zona limitans intrathalamica (ZLI) at 8.5 dpc. The presumptive AFB is initially induced, but this territory is reduced at a later stage of development in Hesx1−/− mutants. Chimeric analysis has shown that this forebrain phenotype is a consequence of the requirement of Hesx1 within the anterior neural ectoderm, and not due to disturbances in the anterior visceral endoderm or anterior definitive endoderm (Martinez-Barbera et al., 2000). Hesx1 mutants also show defects in dorsal forebrain commissural structures, eye abnormalities and pituitary dysplasia (Dattani et al., 1998). A comparable phenotype is observed in a human congenital disorder called septo-optic dysplasia (SOD). Indeed, mutations in HESX1 are associated with familial cases of SOD and other forms of hypopituitarism in humans (Dattani et al., 1998; Dattani, 2004; Sobrier et al., 2006). In Xenopus, overexpression of the Hesxl orthologue, Xanf1, results in enlargement of the neural plate (NP) at the expense of epidermis and neural crest (NC) (Ermakova et al., 1999). Thus, it is now established that Hesx1 is essential for normal AFB development in vertebrates. However, little is known about how Hesx1 performs its function.

Experiments in Xenopus, zebrafish, chick and mammals have provided compelling evidence indicating that suppression of posteriorising signals is an essential requisite for the formation of anterior neural tissue (Kimura et al., 2000; Perea-Gomez et al., 2001; Kudoh et al., 2002; Wilson and Houart, 2004; Stern, 2005). Among them, the Wnt/β-catenin pathway plays a key role in anterior-posterior patterning of the NP [(Yamaguchi, 2001; Wilson and Houart, 2004; Markkula, 2006) and references therein]. It is thought that the action of Wnt signalling inhibitors and activators create a gradient along the neural ectoderm (high caudally and low rostrally) that is required for normal anterior-posterior regionalisation of the NP in distinct subdivisions (Leyns et al., 1997; Glinka et al., 1998; Hsieh et al., 1999; Kiecker and Niehrs, 2001; Nordstrom et al., 2002). Normal AFB development is particularly sensitive to Wnt/β-catenin signalling (Fredieu et al., 1997; van de Water et al., 2001; Houart et al., 2002). For instance, the zebrafish masterblind mutants, which carry a mutation in Axin1 that abolishes binding to Gsk3β causing Wnt/β-catenin signalling overactivation, show a phenotype in which telencephalon and eyes are reduced or absent, and dorsal diencephalic fates are expanded rostrally (van de Water et al., 2001; Heisenberg et al., 2001). Similarly, lack of Tcf3/Headless repressor function leads to the loss of anterior neural tissue (Kim et al., 2000). In chick, it has been shown that posterior forebrain character can be induced in explants, which would normally acquire an AFB fate, through the direct caudalisising activity of Wnt proteins (Nordstrom et al., 2002). In mouse, both overexpression of Wnt8 and lack of Wnt/β-catenin antagonists lead to suppression of anterior neural fates (Popperl et al., 1997; Mukhopadhyay et al., 2001; Satoh et al., 2004). Therefore, it is now established that Wnt/β-catenin signalling must be modulated during vertebrate development to allow normal anterior-posterior patterning of the NP; in particular, Wnt/β-catenin signalling needs to be suppressed for the AFB to develop.

The transcriptional repressors Hesx1, Six3 and Rx (Rx) show overlapping expression patterns in the anterior neural ectoderm during early mouse development (Oliver et al., 1995; Thomas and Beddington, 1996; Mathers et al., 1997). Moreover, Six3 and Rx mutants show defects in the forebrain, such as reduced telencephalon and eyes, which are very similar to those observed

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in Hesx1−/− mutants (Mathers et al., 1997; Martinez-Barbera et al., 2000; Lagutin et al., 2003). A mechanism has been postulated in which Six3 is thought to directly repress the Wnt1 promoter and thereby reduce Wnt/β-catenin signalling in the AFB. Whether or not Hesx1 and Rax perform a similar function during forebrain formation is an important question that remains unanswered.

To further understand the function of Hesx1 in forebrain development, we have analysed in detail the neural patterning of Hesx1 mutant embryos and performed a genetic fate map of Hesx1-expressing cells in normal embryos and Hesx1 homozygous mutants. Our data indicate that in the absence of Hesx1 there is a posterior transformation of the AFB. Contrary to the observations in the Six3 mutants, HESX1 appears not to directly repress the Wnt1 locus, although absence of Hesx1 leads to an anteriorisation of Wnt1 expression and ectopic activation of the Wnt/β-catenin targets Sp5 and Axin2 in the AFB. Gain-of-function experiments suggest that Hesx1 alone cannot anteriorise posterior neural tissue, but can rescue the forebrain defects of the Hesx1-deficient mutants.

**MATERIALS AND METHODS**

**Generation of the Hesx1Cre+/- mouse line**
The Hesx1-Cre targeting vector was generated by replacing the Hesx1 coding region with Cre recombinase (Fig. 4). CCE embryonic stem cells (129/SvEv) (kindly provided by E. Robertson, Wellcome Trust Centre for Human Genetics, Oxford, UK) were electroproporated and 500 colonies were picked, expanded and screened as described (Dattani et al., 1998). Two correctly targeted clones were isolated and injected into blastocysts from C57BL/6J mice. The PGK-Neo cassette was excised by crossing heterozygous mice with the ACTB-FLPe strain (Rodriguez et al., 2000). Hesx1Cre+/- mice were maintained on the C57BL/6J background and to date have been backcrossed a total of eight times. Hesx1Cre+/- embryos showed the forebrain and pituitary defects observed in Hesx1−/− mutants. It is worth noting that the severity of the phenotype of the Hesx1-deficient embryos (Hesx1Cre+/-) has increased compared with the initial analysis (Dattani et al., 1998). No viable homozygous mice are now identified at weaning and eye defects are fully penetrant with variable expressivity. We think this is a consequence of maintaining the colony on the C57BL/6J, whereas it was previously kept on a C57BL/6 inbred colony maintained at the National Institute for Medical Research, UK.

**Generation of the R26Cond-Hesx1+/– mouse line**
The components of the Rosa26 (R26) targeting vectors were a gift (Soriano, 1999; Srinivas et al., 2001). To avoid possible interference with the translation of Hesx1 owing to the presence of an ATG within the loxP sites, we generated a BigT-Tin-loxP by inverting the orientation of both loxP sites (Ivanova et al., 2005). After electroporation, a total of 300 clones were picked, expanded and frozen (see Fig. S6 in the supplementary material). Five correctly targeted clones were identified and two were used for blastocyst injection (C57BL/6J background). R26Cond-Hesx1+/– heterozygous mice were backcrossed three times with C57BL/6J mice and then intercrossed to generate R26Cond-Hesx1+/– Hesx1 homozygous mice, all of which were viable and fertile.

**Genetic crosses**
Hesx1Cre+/- mice were crossed with R26Cond-lacZ/Cond-lacZ reporter animals to generate the Hesx1Cre+/–, R26Cond-lacZ+/– compound mice, then backcrossed to R26Cond-lacZ/Cond-lacZ mice to obtain Hesx1Cre+/–, R26Cond-lacZ/Cond-lacZ mice and embryos. Hesx1Cre+/-, R26Cond-lacZ/+ and Hesx1Cre+/-, R26Cond-lacZ/+ embryos were generated from crosses between Hesx1Cre+/-, R26Cond-lacZ/Cond-lacZ and Hesx1+/– mice. Hesx1+/– mice carry a null allele and have been described previously (Dattani et al., 1998).

**Genotyping of mice and embryos**
Embryos and neonates were genotyped by PCR on DNA samples prepared from tail tips, yolk sacs or whole embryos. Primer sequences and PCR protocols are available on request.

**RESULTS**

**Posterior transformation of AFB in Hesx1-deficient embryos**
We have previously shown that in the absence of Hesx1 there is a lack of AFB tissue at 8.5 dpc, but the reasons underlying this phenotype are not known (Martinez-Barbera et al., 2000). To find out the cellular basis of the early forebrain defects, we analysed neural patterning, proliferation and apoptosis of Hesx1+/– in comparison with Hesx1+/– and wild-type embryos from 7.5 to 9.5 dpc.

At presomitic stages, the expression domains of Six3 (AFB marker), Ato (Dmbx1) and Pax2 (posterior forebrain and midbrain markers at these stages), and Hoxb1 (hindbrain marker) were identical in all genotypes (Fig. 1A,B and data not shown) (Oliver et al., 1995; Nornes et al., 1990; Hunt and Krumlauf, 1991; Gogoi et al., 2002). At the 0- to 1-somite stage, Pax6 is expressed in the prospective forebrain region of the developing mouse embryo (Walther and Gruss, 1991). Pax6 expression in the anterior NP was significantly reduced in Hesx1+/– mutants when compared with Hesx1+/+ or wild-type littermates at this stage, thus confirming that the prospective forebrain region is reduced only at the early somite stage (Fig. 1C) (Martinez-Barbera et al., 2000). The possibility that prospective forebrain was being specified was analysed using the neural crest (NC) markers Pax3 and Foxd3. These markers are expressed on the margin of the anterior NP at the 2- to 3-somite stage in wild-type embryos, but the most rostral region is free of Pax3 and Foxd3 transcripts (Fig. 1D,F,H) (Goulding et al., 1991; Labosky and Kaestner, 1998). By contrast, their expression domains were anteriorised in the 2- to 3-somite stage Hesx1+/– mutants, and the region devoid of Pax3 and Foxd3 expression was clearly reduced when compared with Hesx1+/– or wild-type embryos (Fig. 1E,G,I,K). It is important to note that these changes were observed prior to a gross lack of neural tissue, although there was a slight change in the shape of the anterior NP (Figs 1 and 2; see below). NC
Fig. 1. Posterior transformation of the AFB in Hesx1−/− mutants at early somite stages. (A,B) Dorsal view of the Pax2 expression domain in wild type (A) and Hesx1−/− mutant (B) at presomitic stages. No significant differences are observable. (C) Dorsal view of the Pax6 expression domain in wild type (left) and Hesx1−/− mutant (right) at the 0- to 1-somite stage. Note the reduction in Pax6 expression in the anterior NP (arrowheads). (D-G) Dorsal (D,E) and frontal (F,G) views of Pax3 expression domain in wild type (D,F) and Hesx1−/− mutant (E,G). Arrowheads indicate the rostral limit of Pax3 expression. (H-K) Dorsal (H,I) and frontal (J,K) views of Foxd3 expression domain in wild type (H,J) and Hesx1−/− mutant (I,K) at the 2- to 3-somite stage. Foxd3 expression domain is anteriorised in the Hesx1−/− mutant (I,K). Arrowheads indicate the rostral limit of Foxd3 expression. Anterior is to the top in A,E,H,I.

Posterior cell fate transformation in Hesx1 mutants

When the AFB is lost in Hesx1 mutants, we analysed the expression of Hesx1-null mutants (Fig. 2C-F). Reduced or absent in this stage, the (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997). At the ridge (ANR) occurs at the 4- to 5-somite stage in wild-type embryos. Likewise, compared with wild-type littermates at the 3- to 4-somite stage (Fig. 2K,M and data not shown). Strikingly, not expressed in the most anterior region of the NP of the 2- to 3-somite stage wild-type embryo (Fig. 2K,M and data not shown).

Wnt/β-catenin ectopic activation in the AFB of Hesx1−/− mutants

We analysed the expression patterns of Wnt1 and Fgf8 because they are essential signals required for normal expansion and patterning of midbrain and AFB, respectively (McMahon and Bradley, 1990; Echelard et al., 1994; Meyers et al., 1998; Storm et al., 2006). In the 3- to 4-somite wild-type embryo, there was a segment of the NP that expressed Wnt1 (prospective midbrain), but the most anterior part of the NP was devoid of Wnt1 transcripts (Fig. 2A). By contrast, in Hesx1−/− mutants, the Wnt1 expression domain was expanded rostrally throughout the edge of the NP until its most anterior tip (Fig. 2B). This anteriorisation was not observed at earlier stages, but Wnt1 expression is barely detectable at the 2-somite stage (Echelard et al., 1994). The onset of Fgf8 expression in the anterior neural ridge (ANR) occurs at the 4- to 5-somite stage in wild-type embryos (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997). At this stage, the Fgf8 expression domain in the ANR was either reduced or absent in Hesx1-null mutants (Fig. 2C-F).

To check whether Wnt/β-catenin signalling was ectopically activated within the prospective AFB, we analysed the expression of the Wnt/β-catenin targets Axin2 and Sp5 (Jho et al., 2002; Takahashi et al., 2005; Weidinger et al., 2005). Normally, Axin2 and Sp5 are not expressed in the most anterior region of the NP at the 2- to 3-somite stage wild-type embryo (Fig. 2K,M and data not shown). Strikingly, Axin2 expression was anteriorised in the mutants as compared with wild-type littermates at the 3- to 4-somite stage (Fig. 2G,H). Likewise, Sp5 transcripts were detected all over the anterior NP of Hesx1−/− mutants at the 2- to 3-somite and 5- to 6-somite stages (Fig. 2I-N). Taken together, this marker analysis suggests that the lack of Hesx1 leads to the ectopic activation of Wnt/β-catenin signalling in the prospective AFB prior to the onset of Fgf8 expression in the ANR.

Rostral expansion of dorsal diencephalic markers in Hesx1−/− mutants

The anterior expansion of posterior neural markers was also evident in Hesx1 mutant embryos at the 8-to 10-somite stage, although at these stages there was a marked reduction of AFB tissue. Pax3 and Foxd3 expression domains did not reach the most anterior region of the developing forebrain in the wild type and heterozygous Hesx1 mutants, but did so in Hesx1−/− mutants (Fig. 3A-D) (Goulding et al., 1991; Labosky and Kaelin, 1998). Wnt1 is normally expressed in the midbrain and posterior diencephalon, whereas Wnt3a is expressed posteriorly throughout the region of the anterior NP in the Hesx1−/− mutants (arrowheads indicate the rostral limit of Wnt3a expression).
expressed only in the dorsal diencephalon (Parr et al., 1993). At these stages, Wnt1 and Wnt3a were also clearly anteriorised and their expression domains were enlarged (Fig. 3I-P). Therefore, the Wnt1 expression domain in wild type (G) and Hesx1–/– mutant (H). Pax2 expression around the midbrain-hindbrain boundary is normal (arrowheads), but there is no Pax2 expression in the prospective dorsal diencephalon (bracket). (A,B) Frontal views of Wnt3a expression in the AFB of the wild type (E) and Hesx1–/– mutant (F). En1 expression domain around the midbrain-hindbrain region is normal in Hesx1–/– mutants when compared with wild-type littermates. (G,H) Lateral view of the Pax2 expression domain in wild type (G) and Hesx1–/– mutant (H). Pax2 expression in the AFB region is clearly anteriorised and two heterozygous (Cre/+ ) ES cell clones cut with EcoRI and hybridised with an external probe (brown line in A). (C) Representative example of PCR genotyping of DNA samples from wild-type, Hesx1Cre/+ and Hesx1Cre/Cre embryos. (D) Genotype of the Hesx1Cre/Cre mutant showing lack of AFB tissue (telencephalon and eye).

A reduction in cell proliferation in the anterior NP and/or an increase in cell death might also contribute to the forebrain defects observed in Hesx1–/– deficient embryos. Proliferation and apoptosis in the NP were analysed using an anti-phosphorylated histone H3 antibody and TUNEL staining, respectively. No significant differences were observed in the mitotic indices between Hesx1–/– and wild-type littermates at 8.5 dpc (see Fig. S1I-L in the supplementary material). However, at 9.5 dpc, Hesx1–/– mutants exhibited exacerbated apoptosis within neural tissue at the level of the posterior forebrain-midbrain boundary. Cell death in the roof plate of the telencephalon was reduced as compared with that in heterozygous or wild-type littermates (see Fig. S1C-F in the supplementary material). Since enhanced apoptosis occurs in a domain of the forebrain where Hesx1 is never expressed and after the onset of forebrain defects, we conclude it is an indirect effect of the abnormal development of the forebrain in Hesx1 mutants. Altogether, these data indicate that the reduction of AFB tissue in Hesx1 mutants from 8.5 dpc onwards is caused by the posteriorisation of AFB precursors at early somite stages.
Cell fate analysis of Hesx1-expressing cells in normal and Hesx1-deficient embryos

We carried out genetic fate mapping of Hesx1-expressing cells using a novel Hesx1-Cre mouse line (Fig. 4) and the R26-floxstop-lacZ reporter line (hereby called R26-Cond-lacZ) (Soriano, 1999). X-Gal staining of Hesx1Cre/+;R26Cond-lacZ embryos, which are phenotypically normal, revealed lacZ expression in all cell types where Hesx1 is normally expressed and their descendants (see Fig. S2 in the supplementary material). This analysis revealed that the normal fate of Hesx1-expressing cells within the NP is to colonise the AFB.

This fate analysis was also performed on Hesx1-deficient embryos. By comparing Hesx1Cre/+;R26Cond-lacZ and Hesx1Cre/–;R26Cond-lacZ compound embryos, differences in the fate of lacZ-expressing cells between genotypes could be attributed to the loss of Hesx1, as Cre and lacZ dosage is the same in both.

First, we analysed the Cre expression pattern in Hesx1Cre/+ and Hesx1CreCre embryos. ISH on Hesx1Cre/Cre embryos at 8.5 dpc revealed a pattern of Cre expression that was very similar to the Hesx1 expression domain in wild-type embryos (see Fig. S3A–C in the supplementary material). No differences in Cre expression were observed between Hesx1Cre/+ and Hesx1CreCre embryos, although the intensity of the expression domain was weaker in embryos carrying just one copy of Cre (see Fig. S3D–F in the supplementary material). RT-PCR on Hesx1Cre/+ and Hesx1CreCre embryos from 7.5-9.5 dpc detected Cre expression at all stages analysed, but no expression was detected in the brain of 11.5-dpc embryos (data not shown). qRT-PCR revealed no differences in Cre expression between Hesx1Cre/+ and Hesx1CreCre embryos at the 2-somite stage, but a reduction in Cre expression was observed in Hesx1Cre/– embryos at the 5-somite stage, possibly reflecting the ongoing posterior transformation of the AFB (see Fig. S3F in the supplementary material). These data suggest that there is no ectopic expression of Cre within the NP of Hesx1-deficient embryos.

No significant differences in X-Gal staining were observed from the onset of gastrulation to the 2- to 3-somite stage between Hesx1Cre/+;R26Cond-lacZ and Hesx1CreCre embryos. ISH on Hesx1Cre/Cre embryos at 8.5 dpc revealed a pattern of Cre expression that was very similar to the Hesx1 expression domain in wild-type embryos (see Fig. S3A–C in the supplementary material). No differences in Cre expression were observed between Hesx1Cre/+ and Hesx1CreCre embryos, although the intensity of the expression domain was weaker in embryos carrying just one copy of Cre (see Fig. S3D–F in the supplementary material). RT-PCR on Hesx1Cre/+ and Hesx1CreCre embryos from 7.5-9.5 dpc detected Cre expression at all stages analysed, but no expression was detected in the brain of 11.5-dpc embryos (data not shown). qRT-PCR revealed no differences in Cre expression between Hesx1Cre/+ and Hesx1CreCre embryos at the 2-somite stage, but a reduction in Cre expression was observed in Hesx1Cre/– embryos at the 5-somite stage, possibly reflecting the ongoing posterior transformation of the AFB (see Fig. S3F in the supplementary material). These data suggest that there is no ectopic expression of Cre within the NP of Hesx1-deficient embryos.

Fig. 5. Descendants of Hesx1-expressing cells in the anterior NP colonise posterior regions of the neural tube and the first branchial arch. All embryos were X-Gal stained. (A,B) Higher numbers of lacZ-expressing cells reach more posterior regions of the NP in the Hesx1Cre/+;R26Cond-lacZ mutant (B, arrowhead) as compared with a Hesx1Cre/+;R26Cond-lacZ embryo (A) at the 6- to 7-somite stage. (C,D) This is accentuated in an 8- to 10-somite Hesx1Cre/+;R26Cond-lacZ mutant (D) as compared with an Hesx1Cre/+;R26Cond-lacZ embryo (C). Arrowhead in C indicates the boundary between anterior and posterior forebrain. (E-H) Lateral (E,F) and dorsal (G,H) views of a Hesx1Cre/+;R26Cond-lacZ embryo (E,G) and a Hesx1Cre/+;R26Cond-lacZ mutant (F,H). The brain was partially dissected to improve X-Gal staining. lacZ-expressing cells barely colonise the posterior forebrain of the Hesx1Cre/+;R26Cond-lacZ embryo (arrowheads in E,G), but massively populate this region in the Hesx1Cre/+;R26Cond-lacZ mutant (black arrowheads in F,H). Note that the Hesx1Cre/+;R26Cond-lacZ mutant depicted in F,H shows asymmetric telencephalic development with a very small right telencephalic vesicle. This is concomitant with a more pronounced degree of colonisation of lacZ-expressing cells on the right side of the posterior forebrain (white arrowhead).

(l,J) Hesx1Cre/+;R26Cond-lacZ embryo (l) and Hesx1Cre/+;R26Cond-lacZ mutant (J) at 9.5 dpc. Note the presence of lacZ-expressing cells within the first branchial arch in the Hesx1Cre/+;R26Cond-lacZ mutant only (J). (K-N) Frontal sections of a Hesx1Cre/+;R26Cond-lacZ embryo (K,M) and a Hesx1Cre/+;R26Cond-lacZ mutant (L,N) at 10.5 dpc. Many more lacZ-expressing cells are localised in the frontonasal mass (arrowheads in L) of the Hesx1Cre/+;R26Cond-lacZ mutant embryos as compared with the Hesx1Cre/+;R26Cond-lacZ embryo (K). lacZ-expressing cells within the first branchial arch are only observed in the Hesx1Cre/+;R26Cond-lacZ embryo. ba-l, first branchial arch; dd, dorsal dienephalon; dt, dorsal telencephalon; op, olfactory placode; rp, Rathke's pouch; vt, ventral telencephalon.
At 12.5 dpc, most of the lacZ-expressing cells colonised the telencephalic vesicles, eyes, hypothalamus and ventral diencephalon in the Hesx1(Cre/+); R26-Cond-lacZ/+ embryos, and only sporadic stained cells were observed within the frontonasal mass and the posterior forebrain (Fig. 5E,G). By contrast, Hesx1(Cre/+); R26-Cond-lacZ/− embryos showed an abundance of lacZ-expressing cells in the posterior forebrain and frontonasal mass, in addition to the ectopic lacZ-expressing cells within the first branchial arch (Fig. 5F,H and data not shown). We noticed that Hesx1-deficient embryos at 11.5-12.5 dpc had more lacZ-expressing cells localised in the AFB (Fig. 5E,F).

Since Cre expression is not activated ectopically within the NP, this finding might be due to the fact that in these embryos the AFB derives predominantly from the most rostro-medial (RM) region of the early NP, as more lateral regions change fate to posterior forebrain and NC (Figs 1 and 2). The RM region of the NP contains a higher density of lacZ-expressing cells than more lateral regions (see Fig. S2B and Fig. S4F in the supplementary material). By contrast, the AFB of Hesx1(Cre/+); R26 Cond-lacZ embryos derives from regions of the early anterior NP, containing both high and low densities of lacZ-expressing cells.

Often, asymmetric development of the telencephalic and optic vesicles was observed in Hesx1(Cre−/+); R26-Cond-lacZ/+ mutants (~75%, n=134), in agreement with previous observations (Dattani et al., 1998). In these embryos, one side developed better than the other, with the right side commonly being the most severely affected (95%) (Fig. 5H). The greater reduction of telencephalic and eye tissue was concomitant with an increased colonisation of X-Gal-positive cells in the posterior forebrain of the same side. This suggests that more cells that would normally colonise the telencephalon and eyes have reverted their fate to posterior forebrain in the most-affected side. The reasons underlying the asymmetry defects in Hesx1-deficient embryos remain unknown at present, as Hesx1 is not expressed asymmetrically within the NP. X-Gal staining on whole brains followed by ISH with Shh and Pax6 revealed that lacZ-expressing cells did not cross the forebrain-midbrain boundary, which is marked by the posterior limit of Pax6 expression in the forebrain (Inoue et al., 2000) (see Fig. S5 in the supplementary material).

Taken together, these data indicate that a lack of Hesx1 expression causes a transformation of the AFB primordium, which acquires a posterior forebrain identity and generates NC.

**Hesx1 might not be sufficient to change posterior neural fates**

If a lack of Hesx1 leads to a posteriorisation of the forebrain, could Hesx1 misexpression alter posterior fates when ectopically expressed? To address this issue we generated a R26-Cond-Hesx1 mouse line by gene targeting in ES cells (see Fig. S6 in the supplementary material). In this mouse, Hesx1 expression is activated upon Cre-mediated excision of the stop cassette.

R26-Cond-Hesx1 mice were crossed with the β-Actin-Cre transgenic line, in which Cre expression is driven by the β-Actin (Actb) promoter (Meyers et al., 1998). R26-Cond-Hesx1; β-Actin-Cre compound embryos expressed Hesx1 transcripts ubiquitously (except in the heart where β-Actin is not active), whereas R26-Cond-Hesx1; β-Actin-Cre and wild-type littermates expressed Hesx1 only within the endogenous domain in the ventral forebrain and/or Rathke’s pouch (see Fig. S6E in the supplementary material). Western blot analysis of whole heads demonstrated that R26-Cond-Hesx1; β-Actin-Cre compound embryos contained more HESX1 protein than R26-Cond-Hesx1; β-Actin-Cre or wild-type littermates (see Fig. S6F in the supplementary material). Protein levels were even higher when embryos contained two copies of the R26-Cond-Hesx1 allele in R26-Cond-Hesx1; β-Actin-Cre compound embryos (data not shown).

Marker analysis revealed no significant differences in the expression of Six3, Pax3, Foxd3, Wnt1 and Fgfb between R26-Cond-Hesx1; β-Actin-Cre, R26-Cond-Hesx1; β-Actin-Cre, single mutants and wild-type littermates (data not shown). Therefore, it seems likely that Hesx1 is not sufficient to confer anterior identity to posterior neural tissues. However, a proportion of compound embryos overexpressing Hesx1 had exencephaly (10% of R26-Cond-Hesx1; β-Actin-Cre, n=65; and 80% of R26-Cond-Hesx1; β-Actin-Cre, n=35) (see Fig. S6E in the supplementary material). The reasons underlying the exencephaly are at present unknown, but this phenotype is background-dependent and not detected in a mixed C57BL6/J;CD1 background (data not shown).

**AFB is likely to exhibit a differential sensitivity to Hesx1 levels**

We used the R26-Cond-Hesx1 mouse line in an attempt to rescue the forebrain defects of the Hesx1 homozygous mutants. Controlling the Hesx1 dosage from the R26 locus, by adding either one or two
copies on a Hesx1-null background, might reveal distinct requirements for HESX1 protein levels for normal development of specific AFB structures. Hesx1Cre/+ mice were intercrossed and 12.5-dpc embryos were subject to morphological and histological analyses. Table 1 summarises the results of all genotypes determined at weaning in the offspring of Hesx1Cre/+ ;R26Cond-Hesx1 heterozygotes. As expected, no Hesx1Cre/Cre animals were obtained (see Materials and methods). However, five mice of Hesx1Cre/+ ;R26Hesx1/Hesx1 genotype were identified. All exhibited eye abnormalities, except for one Hesx1Cre/Cre;R26Cond-Hesx1/Cond-Hesx1 mouse that had normal eyes. These data suggest that the addition of HESX1 from the R26-Cond-Hesx1 allele can rescue the perinatal lethality observed in the Hesx1 homozygous mutants.

To assess the degree of rescue of AFB structures, both Hesx1Cre/Cre;R26Cond-Hesx1/+ mice were intercrossed and 12.5-dpc embryos were subject to morphological and histological analyses. Hesx1Cre/Cre embryos obtained from Hesx1Cre/+ ;R26Cond-Hesx1/+ intercrosses showed reduced or absent telencephalon, but eye defects were fully penetrant with variable expressivity ranging from unilateral microphthalmia to bilateral anophthalmia (n = 7) (Fig. 6B) (Dattani et al., 1998). By contrast, Hesx1Cre/+ ;R26Cond-Hesx1/+ embryos showed a significant improvement in AFB development (Fig. 6C,D). The size and shape of the telencephalic vesicles in Hesx1Cre/+ ;R26Cond-Hesx1/+ embryos was comparable with wild-type littermates in nine out of 12 embryos. However, there was no major improvement in eye development. Of the embryos recovered, all had abnormal eyes (n = 12), and either showed bilateral anophthalmia (n = 10) or unilateral microphthalmia (n = 2). Eye phenotype rescue was accentuated in Hesx1Cre/+ ;R26Cond-Hesx1/Cond-Hesx1 compound embryos (Fig. 6E,F). Bilateral anophthalmia was observed in only three out of ten embryos. From the remaining seven embryos, three had normal eyes, two had bilateral microphthalmia and two unilateral anophthalmia. Telencephalic development was normal in nine out of ten embryos analysed. No phenotypic defects were seen in embryos of all other genotypes. The results from these experiments allow us to draw two conclusions. First, that Hesx1 expression from the R26-Cond-Hesx1 allele can rescue the forebrain defects in Hesx1-deficient embryos. Second, that the telencephalon and eyes might exhibit a distinct sensitivity to HESX1 dosage.

The rescue of the forebrain defects was analysed at the molecular level in 8.5-dpc embryos. Hesx1 expression was restricted to the AFB in Hesx1Cre/Cre;R26Cond-Hesx1/Cond-Hesx1 and wild-type littermates and levels of expression were comparable (Fig. 7A,B). Foxd3 expression was anteriorised in the Hesx1-deficient embryos (Fig. 3C,D), but the Foxd3 expression domain in Hesx1Cre/+ ;R26Cond-Hesx1/Cond-Hesx1 was similar to that of wild-type embryos (Fig. 7C,D). Finally, the anteriorisation of Axin2 expression in the Hesx1 alleles (Fig. 2G,H) was reverted in Hesx1Cre/+ ;R26Cond-Hesx1/Cond-Hesx1 compound embryos, and an Axin2-free region of the anterior NP was evident (Fig. 7E,F). Overall, these experiments suggest that the rescue of the morphological forebrain defects correlates with an improvement of the neural patterning of the anterior NP.

**DISCUSSION**

It has been previously established that Hesx1 is essential for normal forebrain formation in mouse and humans, but the mechanisms underlying the forebrain defects of Hesx1-deficient embryos have remained elusive (Dattani et al., 1998; Martinez-Barbera et al., 2000). In this study we demonstrate that lack of Hesx1 leads to a fate transformation of anterior to posterior forebrain. Cells that normally colonise the telencephalon, eyes, hypothalamus and ventral diencephalon are found in more-posterior regions, such as the dorsal thalamus and pretectum in Hesx1−/− mutants. Gain-of-function experiments indicate that Hesx1 neither promotes AFB identity when misexpressed in the posterior NP, nor increases the size of the AFB when overexpressed in its endogenous expression domain. Moreover, our experiments suggest that the eyes might require higher levels of HESX1 protein than the telencephalon for normal

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**Table 1. Genotype of mice resulting from Hesx1Cre/+;R26Hesx1+/+ intercrosses**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of embryos Obtained</th>
<th>Expected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesx1Cre/+;R26Hesx1/+</td>
<td>8</td>
<td>7-8 (6.25)</td>
</tr>
<tr>
<td>Hesx1Cre/+;R26Hesx1/+</td>
<td>23</td>
<td>15 (12.5)</td>
</tr>
<tr>
<td>Hesx1Cre/+;R26Hesx1/+</td>
<td>9</td>
<td>7-8 (6.25)</td>
</tr>
<tr>
<td>Hesx1Cre/+;R26Hesx1/+</td>
<td>14</td>
<td>15 (12.5)</td>
</tr>
<tr>
<td>Hesx1Cre/+;R26Hesx1/+</td>
<td>39</td>
<td>30 (25)</td>
</tr>
<tr>
<td>Hesx1Cre/+;R26Hesx1/+</td>
<td>19</td>
<td>15 (12.5)</td>
</tr>
<tr>
<td>Hesx1Cre/+;R26Hesx1/+</td>
<td>0</td>
<td>7-8 (6.25)</td>
</tr>
<tr>
<td>Hesx1Cre/+;R26Hesx1/+</td>
<td>5</td>
<td>15 (12.5)</td>
</tr>
<tr>
<td>Hesx1Cre/+;R26Hesx1/+</td>
<td>4</td>
<td>7-8 (6.25)</td>
</tr>
<tr>
<td><strong>Total number of embryos</strong></td>
<td><strong>121</strong></td>
<td></td>
</tr>
</tbody>
</table>

Mice were genotyped at weaning.
development. This study has revealed that one of the functions of Hesx1 is to prevent the Wnt/β-catenin signalling pathway from being activated within the AFB.

**Normal fate of Hesx1-expressing cells**

The fate of Hesx1-expressing cells in our study is in agreement with previous fate mapping studies in the mouse. Soon after the onset of gastrulation, the anterior visceral endoderm is displaced towards the extraembryonic region by the definitive endoderm, which is formed at the tip of the primitive streak (Lawson and Pedersen, 1987; Thomas and Beddington, 1996). X-Gal staining of Hesx1Cre/+;R26Cond-lacZ embryos detected patches of blue cells in the visceral endoderm of the extraembryonic region from late streak stages. It was noticeable that lacZ-expressing cells remained clumped together and, as previously described, not much intermingling was observed (Gardner and Cockroft, 1998). Hesx1 is expressed in the axial levels of the anterior definitive endoderm, an area containing the precursors of some anterior foregut derivatives (Lawson and Pedersen, 1987; Thomas et al., 1998). In fact, lacZ-expressing cells colonised the anterior foregut in Hesx1Cre/+;R26Cond-lacZ embryos at 8.5 dpc and were found in its derivatives, including pharyngeal endoderm, thyroid gland, liver and ventral pancreas.

Strong X-Gal staining was detected in the Rathe’s pouch from 9.0 dpc onwards. Interestingly, blue cells mainly colonised the anterior and intermediate lobes, both of which are derived from the oral ectoderm, but blue cells were rarely observed in the posterior pituitary, which has neural origin from a recess in the floor of the hypothalamus (see Fig. S4E in the supplementary material).

Cell fate analysis of lacZ-expressing cells within the NP showed that the majority of the labelled cells colonised the forebrain region anterior to the ZLI, i.e. telencephalon, eyes, hypothalamus and ventral diencephalon. Only scattered cells ended up in the dorsal thalamus and pretectum and, very rarely, lacZ-expressing cells crossed the forebrain-midbrain boundary. Therefore, Hesx1 marks only prospective forebrain, in particular the AFB.

**Anterior to posterior transformation of the forebrain in Hesx1-deficient embryos**

A main finding of this study is that the absence of Hesx1 brings about a posterior transformation of the AFB, which is evidenced by rostral expansion of the expression domains of the NC markers Foxd3 and Pax3, prior to an overt morphological defect of the anterior NP. Numerous fate mapping studies have demonstrated that premigratory NC cells are dorsal cell types that form at the border of the NP, from mid-diencephalon to spinal cord levels, but not rostral to the mid-diencephalon (Couly and Le Douarin, 1987; Sechrist et al., 1995; Muhr et al., 1997). However, cells within this NC-free region can be induced to form NC when exposed to caudalising signals in explant experiments (Muhr et al., 1997). We believe that the Hesx1 expression domain in the anterior NP corresponds to this crest-free area. For instance, X-Gal staining of Hesx1Cre/+;R26Cond-lacZ embryos, which are phenotypically normal, showed that the majority of lacZ-expressing cells colonised the AFB and did not form NC. By contrast, lacZ-expressing cells populated the posterior forebrain in Hesx1Cre/+;R26Cond-lacZ mutants, thus reaching the area of the NP that yields migratory NC of the first branchial arch and the frontonasal mass (Inoue et al., 2000; Creuzet et al., 2005).

The expression domains of Wnt3a, Wnt1, Atoh1 and Pax6 were expanded rostrally and lacZ-expressing cells were found throughout the dorsoventral axis of the dorsal thalamus and pretectum. This suggests that in the absence of Hesx1, cells initially specified to AFB fates colonise the entire segment of the posterior forebrain, not only NC-generating regions. However, the size of the posterior forebrain is not affected in Hesx1-deficient embryos at 12.5-18.5 dpc, possibly because the increased cell death in the posterior forebrain compensates for the excess of cells populating this region.

Although required for the maintenance of AFB identity, Hesx1 cannot alter posterior fates in caudal neural tissue when misexpressed in the mouse embryo. To do so, Hesx1 might require specific co-factors only present in anterior and not posterior neural tissue. (see Fig. S6 in the supplementary material) (Ermakova et al., 1999). The partial rescue of the forebrain defects and attenuation of the posterior transformation observed in Hesx1Cre/+;R26Cond-Hesx1 and Hesx1Cre/+;R26Cond-Hesx1 compound embryos suggest that only the AFB is competent to respond to HESX1. Overall, these data indicate that Hesx1 is required for AFB development but, on its own, it is not sufficient to promote AFB fates.

The genetic rescue experiments have revealed that whereas one copy of R26-Cond-Hesx1 in a Hesx1-null background is sufficient to improve telencephalic development, two copies of the R26-Cond-Hesx1 allele are required to rescue both telencephalon and eye development. Although we cannot rule out the possibility that telencephalic and eye precursors could express different levels of Hesx1 from the R26 locus, we think that our experiments point to a differential sensitivity of the eye and telencephalon to Hesx1 levels. Fate mapping studies in the mouse embryo have shown that telencephalic precursors reside in the rostral-lateral regions of the anterior NP and that the eye field is located caudal to this and more medially (Inoue et al., 2000). It is worth noting that Hesx1 expression is higher in the area of the NP that is thought to correspond to the eye field (Thomas and Beddington, 1996). In this scenario, eye development is expected to be more sensitive than telencephalic development to HESX1 levels, and indeed, Hesx1-deficient embryos show a higher penetrance of eye versus telencephalic abnormalities. In agreement with this concept, mutant mice bearing a single amino acid change in position 26 that yields a HESX1 protein with reduced repressing activity (Carvalho et al., 2003), display eye abnormalities but have normal telencephalic development (unpublished results). This suggests that Hesx1 might have a dual role within the anterior NP: to promote anterior versus posterior forebrain development and to segregate telencephalic and eye field identities (Stigloher et al., 2006; Mathers et al., 1997).

**Hesx1 antagonises caudalising signals within the NP**

Our data strongly suggest that the mechanism underlying the posterior transformation of Hesx1−/− mutants is the ectopic activation of Wnt/β-catenin signalling within the prospective AFB at early somite stages. Experiments in Xenopus and zebrafish have indicated that the eye is the most sensitive region of the NP to Wnt/β-catenin activation (Fredieu et al., 1997; van de Water et al., 2001). When zebrafish embryos were exposed for a short time to LiCl, an inhibitor of Gsk3β that enhances Wnt/β-catenin signalling, only eye development was disrupted and the treated embryos displayed microphthalmia or anophthalmia. However, when embryos were treated with LiCl for longer, they developed telencephalic as well as eye defects. An excess of Wnt/β-catenin signalling might explain the reduction in the AFB markers Six3 and Pax6 and the concomitant rostral expansion of the NC markers Pax3 and Foxd3.

It seems likely that Hesx1 and Six3 might work in parallel in the maintenance of AFB identity, but through distinct mechanisms. Hesx1 can bind to Wnt1 regulatory elements in in-vitro assays, but
it cannot repress reporter vectors containing Wnt1 regulatory elements (see Fig. S7 in the supplementary material). Moreover, expansion of the Sp5 expression domain occurs prior to Wnt1 relocalisation in Hesx1−/− mutants. Therefore, in contrast to the Six3−/− mutants, ectopic Wnt1 expression within the prospective AFB might not be the primary reason for the forebrain defects; rather, the excess of Wnt1 might contribute to the final morphological defects later in development.

The possibility exists that Hesx1 may antagonise Wnt/β-catenin signalling by modulating the activity of some component(s) of the pathway causing a cell-autonomous inhibition within Hesx1-expressing cells. Alternatively, or in addition, Hesx1 might repress one or more Wnt/β-catenin target genes directly, thus preventing the acquisition of posterior fates. Chimeric experiments suggest that Hesx1 is required cell-autonomously within the NP (Martinez-Barbera et al., 2000). However, Hesx1 function can be partially compensated for, as most Hesx1−/− mutants show some AFB development. Moreover, in chimeric embryos, Hesx1-deficient cells, as well as wild-type cells, can also colonise the AFB. The possibility exists that a subset of Hesx1−/− cells might be more susceptible than Wnt/β-catenin signalling and acquire a posterior fate, but this might be difficult to observe in chimeric embryos, where the bulk of wild-type cells can colonise the same regions of the neural tube (Martinez-Barbera et al., 2000). Notably, the forebrain defects were completely rescued only in embryos with a high degree of chimerism, and the AFB was mainly populated with wild-type cells (Martinez-Barbera et al., 2000). Future research will elucidate the molecular function of Hesx1.


References


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