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Transgenic studies on homeobox genes in nervous system development: spina bifida in *Isl1* transgenic mice

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Abstract

To develop in vivo assays for homeobox gene function in neural development, we generated transgenic mice in which the expression of a homeobox gene is altered only within the nervous system, in neurons or neuronal precursor cells. Transgenic expression of *Hoxc8* did not result in gross abnormalities, while a *Hoxd4* transgene caused death shortly after birth. In neural progenitor cells, the motoneuron-specific homeodomain transcription factor *Isl1* induced early developmental defects, including absence of anterior neural structures, profound defects in the neuroepithelium and defective neural tube closure. A fraction of *Isl1* transgenic mice exhibited spina bifida. *Isl1* transgene expression was also associated with decreased proliferation and increased *Pbx1* expression in the ventral neural tube. Our results suggest a function for some homeobox genes in development of the nervous system, and that cell-type- and region-specific transgenic models will be useful to identify the cellular and molecular targets of homeobox transcription factors in nervous system development.

Keywords

Binary transgenic system; Transactivator; VP16; Hox genes; *Hoxd4*; *Hoxc8*; *Isl1*; Motoneurons; Ventral neural tube; Mesoderm; Cell proliferation; *Pbx1*; Transcriptional target; Spina bifida; Neural tube closure

Introduction

During embryonic development, the differential expression of various transcription factors is thought to play a role in patterning the nervous system (Hoch et al. 2009; Tanabe and Jessell 1996). One family of such transcription factors are the homeodomain-containing proteins.

The expression of genes encoding these proteins has been implicated in (1) specifying positional information to the cell, (2) defining particular neuronal subtypes, (3) regulating cell proliferation and migration, and (4) axonal outgrowth and target innervation (reviewed in (Keynes and Krumlauf 1994; Dasen and Jessell 2009). Homeobox genes of the Hox class are expressed in distinct domains in the nervous system, and also in the mesoderm, which is known to influence neuronal patterning (Lloret-Vilaspasa et al. 2010; Amirthalingam et al. 2009; Guidato et al. 2003; Omelchenko and Lance-Jones 2003; Ensini et al. 1998). In this study, we sought to investigate whether Hox genes play a role in neuronal development independent of their mesodermal expression. Our experimental strategy was to generate transgenic mice in which, through the use of nervous-system-specific promoters/enhancers, expression of a given homeobox gene would be altered selectively in neurons, or their progenitors, without changes of expression in the mesoderm.

As models, we chose two homeobox genes that are involved in anterior-posterior patterning, *Hoxc8* and *Hoxd4*, both of which are expressed in neurectodermal as well as mesodermal derivatives (Shashikant et al. 1991). *Hoxc8* is normally expressed in the thoracic region of the spinal cord, and more posterior in the mesoderm (Utset et al. 1987; Breier et al. 1988; LeMouellic et al. 1988; Awgulewitsch and Jacobs 1990). In the skeleton, *Hoxc8*-deficient mice exhibit anterior homeotic transformations in the lower thoracic region (LeMouellic et al. 1992) conversely, mice with anteriorized expression of a *Hoxc8* transgene display posterior transformations in anterior regions of their skeletons (Pollock et al. 1992). These findings demonstrate that *Hoxc8* specifies identities of skeletal elements. Furthermore, we have shown that overexpression of *Hoxc8* under control of its own promoter inhibits cartilage differentiation at later stages of development (Yueh et al. 1998) and promotes proliferation of cartilage precursor cells. Consistent with this, inhibition of *Hoxc8* expression in primary chondrocytes, by virtue of morpholino anti-sense oligonucleotides, reduces cell proliferation (Kamel et al. 2009). *Hoxc8* is normally expressed in the ventral spinal cord, in presumptive motor neurons or their precursors, as well as in the dorsal root ganglia and sympathetic ganglia (Awgulewitsch and Jacobs 1990; Tiret et al. 1998; Ensini et al. 1998). Motor-neurons in spinal segments C7 and T1 exhibited enhanced apoptosis in *Hoxc8* loss-of-function mutants (Tiret et al. 1998), indicating a role of *Hoxc8* in survival of differentiated cells in the neural tube. *Hoxc8*-deficient mice die at birth, but the few survivors exhibit abnormal motoneuron innervation of the forelimbs and thoracic region (Tiret et al. 1998) supporting a role for *Hoxc8* in neuronal patterning (Vermot et al. 2005). Thus, our prediction was that *Hoxc8* overexpression in neuronal cells might lead to increased proliferation and survival, and, where expression is anterior to the normal *Hoxc8* domain, to posterior transformation of the patterns of neuronal projections.

Similarly, *Hoxd4* was shown to be required for proper patterning of the cervical region of the skeleton (Horan et al. 1995a; Folberg et al. 1999). Ectopic expression of *Hoxd4* induced homeotic transformations of the occipital bones of the skull and brain deformities (Lufkin et al. 1992). Although *Hoxd4* is expressed in the developing neural tube (Tuggle et al. 1990; Watrin and Wolgemuth 1993; Nolte et al. 2003), overt neurological abnormalities were not observed in *Hoxd4*-deficient mice (Horan et al. 1995a, b). Analogous to *Hoxc8*, we would expect posterior transformations when *Hoxd4* is misexpressed in regions anterior to its endogenous expression domain, and possibly neurological abnormalities when misexpressed throughout the nervous system. Taken together, these experiments indicate that Hox gene transgenesis is an effective approach to assess functional roles of these homeobox genes.

Dorso-ventral patterning in the spinal cord is associated with region-specific expression of transcription factors of the Pax, Nkx, and LIM homeobox gene families (reviewed in (Tanabe and Jessell 1996). One such gene is the divergent homeobox gene *Isl1* (*Isl1*) (Karlsson et al. 1990) which encodes a LIM-homeodomain protein required for the

generation of motoneurons and a subset of interneurons (Pfaff et al. 1996). It is expressed as progenitors exit the cell cycle and become postmitotic (Ericson et al. 1992). At later stages of development, Islet-1 is also required for the generation of sensory neurons that innervate the skin (Sun et al. 2008). We hypothesized that expression of *Isl1* in neuronal progenitor cells would prematurely drive them into differentiation, and that dorso-ventral patterning could be perturbed in our transgenic system.

In the great majority of cases, Hox-transgenic mice die perinatally (Zhang et al. 1994) (and discussion in (Gardner et al. 1996), leaving little material for analysis, and necessitating the generation of multiple independent transgenic individuals. To avoid the genetic variability inherent in using transgenic founder animals, we used our previously developed binary transgenic mouse system based upon the viral transactivator VP16 (Byrne and Ruddle 1989; Gardner et al. 1996; Rundle et al. 1998). This system consists of transactivator (TA) mouse lines that harbor the VP16 transgene under the control of a specific promoter, and transresponder (TR) mouse lines, in which the gene of interest is under control of the Herpes Simplex Virus immediate early 4 gene promoter. This promoter is quiescent in the absence of VP16 (Rundle et al. 1998). Therefore, a given TR transgene is only transactivated in progeny that inherit both a TA and a TR transgene (Rundle et al. 1998) (Fig. 1a), and is transactivated only in those cells where VP16 is expressed. To monitor promoter activity in directing VP16-expression, TA mice can be crossed to IE-LacZ transresponder (TR) strains, which will lead to β -galactosidase staining in cells where VP16 is expressed (Gardner et al. 1996). We previously validated this system for transactivators driven by the promoter from the *Hoxc8* gene (Gardner et al. 1996; Rundle et al. 1998), which directs VP16 expression in posterior mesoderm and spinal cord (Fig. 1b, c), and reported that *Hoxc8*-VP16-driven transgenic expression of *Hox8*, *Hoxd4* and *Isl1* TR genes causes skeletal defects (Yueh et al. 1998; Kappen et al. 2004; Muller et al. 2003). Because we had these TR lines already at our disposal, the work reported here was focused on generating neuronal cell-specific homeobox transgenic mice.

To direct VP16 expression specifically to differentiating neurons, we used the murine neurofilament light chain gene promoter (Yaworsky et al. 1997). In parallel, we sought to direct transgene expression to yet undifferentiated neuron progenitor cells. We therefore generated new transactivator transgenic mice by using the enhancer from the second intron of the rat nestin gene (Zimmerman et al. 1994; Yaworsky and Kappen 1999). We here report comparative studies in which *Hoxc8*, *Hoxd4*, and *Isl1* transgenes were expressed under control of the same regulatory elements with very different biological outcomes. Our results provide evidence that each of these homeodomain transcription factors acts on neuronal development in a unique fashion.

Materials and methods

Generation of transgenic constructs

The plasmid pMinVP16 (Yueh et al. 2000) harbors a 2.5 kb PstI fragment from the plasmid pNFL-TSV (Yaworsky et al. 1997), which contains 96 bp of upstream promoter from the mouse neurofilament light chain gene including the transcription initiation site, linked to the Herpes Simplex Virus 1 VP16 gene and SV40 intron and polyadenylation site (Byrne and Ruddle 1989). The nestin-VP16 transactivator constructs were generated by ligating a 1.8 kb BstYI fragment harboring the second intron of the rat nestin gene (Zimmerman et al. 1994; Yaworsky and Kappen 1999) into the pMinVP16 plasmid. The structures of these constructs have previously been published (Yueh et al. 2000). The insert DNA was released by NotI-ClaI restriction digestion.

The neurofilament promoter NFL-VP16 constructs and mice have been previously described (Byrne and Ruddle 1989; Yaworsky et al. 1997).

The IE-Hoxc8, IE-Hoxd4 and IE-Isl1 transresponder constructs have been previously described (Rundle et al. 1998) and contain the respective cDNA downstream of the 360 bp IE promoter from the herpes simplex virus ICP4 gene (Byrne and Ruddle 1989).

A graphical representation of the transgenes employed in this study is given in Fig. 2.

Generation and identification of transgenic mice

Linearized DNA fragments were purified using Qiagen columns (Chatsworth, CA). The purified DNA was diluted to a final concentration of 2 µg/ml in an injection buffer of 10 mM Tris–Cl pH 7.3, 0.25 mM EDTA. DNA microinjection followed the standard protocols of Hogan et al. (Hogan et al. 1994), using FVB mice as embryo donors and CD-1 mice as foster mothers. Tail biopsies or embryonic yolk sacs were obtained as a source of genomic DNA from potential transgenic mice and purified according to Laird et al. (Laird et al. 1991). The presence of the transgenes was initially determined by the polymerase chain reaction (PCR), using primers as previously described (Rundle et al. 1998). The VP16 transgene was detected using the primers: 5'-CTATGTACCATGCTCGATACCTG GA-3' and 5'-CCATAGGATCTCGCGGGTCAAAA AT-3' resulting in a 494 bp product (Yaworsky et al. 1997); Southern blot analyses were subsequently performed to confirm the integrity of the transgene and to estimate transgene copy number using standard protocols (Sambrook et al. 1989).

For the differential display screen, transgenic mice were generated with a construct that contained the Isl1-SV40 transgene (Muller et al. 2003) directly linked to the Hoxc8 promoter (Gardner et al. 1996). Microinjection was performed as described above, and embryos were isolated at E10.5. Genotyping was performed on yolk sac DNA, and whole Isl1 transgenic founder embryos were used for extraction of RNA.

Detection of β -galactosidase activity

The expression of VP16 from the transactivator transgenes was detected by crossing transactivator mice to IE-LacZ transresponder mice (Gardner et al. 1996). Embryos were isolated at different timepoints and processed for staining as described previously (Yaworsky and Kappen 1999). Sections were cut from paraffin-embedded specimen at 10 µm thickness.

Identification and cloning of Pbx1a

Individual founder embryos that were Hoxc8-Isl1 transgenic and nontransgenic littermates were isolated on gestational day 10.5, RNA was isolated and subjected to Differential Display assay (Liang et al. 1993; Liang and Pardee 1992). PCR-mediated cloning of full-length cDNA was accomplished using RACE (Frohman 1993).

Immunohistochemistry and in situ hybridization

Immunohistochemistry and in situ hybridizations were performed on frozen sections as described previously (Salbaum and Ruddle 1994).

Detection of transgene expression by RT-PCR

The expression of the VP transgene and respective homeobox transresponder transgenes was assayed by RT-PCR as previously described (Rundle et al. 1998).

Statistical analyses

The observed frequency of transgenic genotypes was compared to that expected for normal Mendelian inheritance using a χ^2 test with 3 degrees of freedom. For comparisons between 2 groups, Fisher's exact tests were employed for categorical data, and T-tests for scaled data.

Results

To direct transgene expression into neuronal progenitor cells without affecting expression of the endogenous homeobox gene in the mesoderm, we sought to generate transgenic mice in which VP16 is expressed under control of the neural enhancer from the rat nestin gene. We first added this enhancer to the NFL-TSV transgene that we had previously used to generate mice with VP16 expression under control of the promoter from the mouse neurofilament light chain gene (Yaworsky et al. 1997). Transgenic founders were identified (Table 1) and crossed to IE-LacZ reporter transresponders to assay for VP16 expression at midgestation, but none of the embryos displayed evidence of VP16 expression. We then reduced the size of the NFL promoter to 96 bp that include the transcription start site and added the nestin gene enhancer in various configurations (Yueh et al. 2000). Five additional founders for two of the constructs (334 and 339) also failed to express VP16 in the neuroepithelium (Table 1). Out of 88 potential founder animals screened, 7 were identified as positive for the Nes-TSV. 411 construct, but only 2 founders sired offspring that expressed the transgene (Table 1), as measured by LacZ activity in double transgenic progeny (Fig. 3a, b). Both of these founders appeared to be chimeras, as indicated by deviation of transgene transmission from Mendelian ratios (data not shown). Only founder animal 73 produced live transgenic offspring, which were used to establish the stable Nestin-VP16 transactivator transgenic line. This line, in a pattern that coincides with nestin protein distribution (Zimmerman et al. 1994; Lendahl et al. 1990), was able to direct VP16 expression to neuroepithelium (Fig. 3c). To restrict transresponder transgene expression exclusively to differentiated neurons, we used transgenic mice in which VP16 expression is under control of the promoter from the mouse neurofilament light chain gene (NFL) (Yaworsky et al. 1997). These transgenic transactivator mouse strains are called NFL-VP16 and transactivate an IE-LacZ reporter gene in cells committed to neuronal differentiation (Fig. 3d, e).

In order to achieve Hoxc8 transgene expression in neuronal cells without affecting the mesoderm, we first crossed the NFL-VP16 transactivator mice to transresponder mice carrying IE-Hoxc8 transgenes and examined progeny at weaning for the presence of the respective transgenes. Offspring that inherited both TA and TR transgenes (TA/+ TR/+ double transgenics) were produced with a frequency close to the expected Mendelian ratio of 25 % (Table 2A). Inspection of these mice and their behavior revealed no observable abnormalities or neurological deficits. The mice were fertile and apparently unaffected by the activation of the Hoxc8 transresponder gene even when the transgene dosage was increased (TA/+ TR/TR; data not shown). We used two independent TR lines, IE-Hoxc8.45 and IE-Hoxc8.254; the latter transgene produces stronger transgene expression (Rundle et al. 1998). We obtained comparable results (Table 2A), providing evidence that the lack of an overt phenotype is not a mouse line-dependent effect.

Interestingly, crossing Nestin-VP16 transactivators to IE-Hoxc8 transresponders also produced double transgenic offspring with close to Mendelian frequency (Table 2B). Again, these mice were viable, fertile and healthy without overt signs of neurological abnormalities. Moreover, we crossed double transgenic animals (TA/+ TR/+) to homozygous transresponders (TA-/- TR+/+) to obtain offspring homozygous for the transresponder locus (TA+/- TR+/+) that would have a dose-dependent increase in IE-Hoxc8 transresponder gene expression (Rundle et al. 1998; Yueh et al. 1998). These mice were also obtained with frequencies of normal Mendelian inheritance (Table 2B) and were apparently

normal. Taken together, these results indicated that ectopic expression of *Hoxc8* exclusively in neurons or their precursors was insufficient in grossly altering neuronal patterning.

In contrast, when IE-*Hoxd4* transresponder mice were bred to the same Nestin-VP16 transactivator line used before, we recovered only few double transgenic (TA+/-TR+/-) offspring by weaning age (Table 2C). This was due to postnatal death before weaning: including genotype results from pup carcasses, the pre-weaning rate of demise was significantly higher ($P = 0.0254$) for double transgenic offspring (5 out of 7: 71 %) than for single transgenic and control animals (7 out of 30: 23 %). These data suggested that *Hoxd4* expression in neural progenitors and ectopic anterior regions of the neural tube induced developmental defects incompatible with postnatal survival. The two surviving double transgenic mice were smaller than their litter-mates and exhibited unusual movement and activity, but numbers of surviving animals were too small for behavioral analyses. These results indicated that expression of *Hoxd4* in neural progenitors was detrimental to nervous system development, resulting in death in the most severe cases.

In the next set of experiments, we mated IE-*Isl1* transresponder mice to our Nestin-VP16 transactivator line. Here, we observed a significant reduction of double transgenic progeny by weaning age (Table 2D) for 3 out of 4 independent transresponder mouse lines. Surviving mice had no gross abnormalities. In additional crosses with the IE-*Isl1.50* line, we observed significantly greater loss ($P < 0.05$) of double transgenic offspring: while from 30 controls, 25 were alive at weaning age, only 1 of 21 double transgenic pups survived the first week but died before weaning without an obvious phenotype. Ten double transgenic offspring from IE-*Isl1.5* transresponders did not survive past the first postnatal day. The IE-*Isl1.81* transresponder line produced no live double transgenic progeny at all in crosses with the Nestin-VP16 transactivator (Table 2D).

We therefore investigated whether activation of the *Isl1* transgene in neuronal progenitors was detrimental to prenatal development. We examined and genotyped embryos from the crosses of Nestin-VP16 transactivators to the IE-*Isl1.81* transresponder line at progressively earlier stages of development, ranging from E17.5, E15.5, E13.5, E12.5, E11.5, E10.5 to E9.5. From embryonic day 10.5 (E10.5) onward, no double transgenic embryos were obtained (not shown). However, at E9.5, double transgenic embryos were identified with the expected Mendelian frequencies. These results established that ectopic expression of *Islet-1* in neural precursor cells is associated with lethality between E9.5 and E10.5.

Compared to their single transgenic control litter mates (Fig. 4a), which were normal, embryos double transgenic for VP16 and *Isl1* were more immature and much smaller (Fig. 4b). In addition to the absence of a head (Fig. 4c), there was developmental delay (Fig. 4d) and failure to close the neural tube. Histo-logical sections through the embryos revealed improper folding of the neuroepithelium (Fig. 4e). Staining with a nestin-specific antibody confirmed that neuronal progenitor cells were present (not shown). The neuroepithelial layer was also substantially thinner with fewer cells than in normal embryos, possibly indicative of decreased cell proliferation. Taken together, these data strongly suggest that the lethal phenotype observed in these embryos is due to ectopic *Islet-1* expression in progenitor cells in the developing nervous system.

Due to the severity of malformations, considerable developmental delay and early lethality, however, it was difficult to investigate the exact nature and cellular or molecular basis of the defects. Specifically, we could not address whether overexpression of *Islet-1* in progenitors might be associated with precocious motoneuron differentiation, because even in normal embryos at E9.5, differentiation of progenitors into the different types of mature motoneurons is not complete (Pfaff 2008). We therefore turned to the third transgenic

model in which we achieved ectopic Islet-1 expression in the developing nervous system: Isl1 transgenic mice in which the transresponder gene is transactivated under control of the Hoxc8 promoter (Yueh et al. 1998; Muller et al. 2003) (and Fig. 1b, c). We previously showed that these mice exhibit caudal growth defects and reduced number of caudal vertebrae. This was associated with increased apoptosis in the mesoderm, indicating that increasing Isl1 levels above its normal expression in posterior mesoderm (Kappen and Salbaum 2009) is incompatible with cell survival. We also reported that expansion of Isl1 expression into regions of the neural tube more dorsal to endogenous Isl1 domains, as mediated by the Hoxc8 promoter (see Fig. 1b, c), was not associated with any detectable alterations in dorso-ventral patterning (Muller et al. 2003). However, in some animals, we observed failure of neural tube closure, which suggested to us that Isl1 overexpression might have previously undetected effects on cell proliferation in the neuroepithelium.

We therefore performed additional crosses employing the Hoxc8-VP16 transactivator line T239 (Rundle et al. 1998) and the IE-Isl1.50 transresponder (Rundle et al. 1998) mouse line, to determine the frequency of occurrence of neural tube defects and to examine cell proliferation specifically in the neuroepithelium in response to Islet-1 overexpression. First, we recapitulated the caudal growth phenotype that we previously reported (Muller et al. 2003). Figure 5 shows that presence of the VP16 transactivator alone, in either hemi- or homozygous form (TA/+ and TA/TA, respectively), does not affect caudal development. In contrast, the majority of double transgenic offspring (TA/+ TR/+) exhibited tail reduction, and with increasing transgene dosage (TA/TA TR/+), we found increased numbers of dead-born pups ($P < 0.05$). This was associated with increased Isl1 expression in the caudal region in E10.5 transgenic embryos (data not shown). Complete absence of the tail was incompatible with survival, indicative of possibly more extensive malformations that may involve internal organs.

A few of the progeny displayed spina bifida (Fig. 6a, b); skeletal preparations confirmed splaying of the neural arches in such pups (data not shown). These observations prompted us to determine the frequency of this phenotype in Isl1-transgenic embryos prior to birth. On E11.5, almost 10 % (9 out of 97) of the Isl1-transgenic embryos displayed spina bifida (Fig. 6c, d). While the location of the spinal opening was not always in the same anterior-posterior position, it was consistent in all cases with the domain of Hoxc8-promoter-driven VP16 expression, and hence Isl1 transgene activation. No spina bifida was observed in non-transgenic or single transgenic progeny, demonstrating that the neural tube closure defect is indeed induced by the Isl1-transgene.

In order to obtain insight into the cellular basis for this phenotype, we performed histological analyses. Earlier experiments had not provided any evidence of abnormal dorso-ventral patterning of the neural tube (Muller et al. 2003) and had also indicated that the notochord was present in double transgenic embryos. The presence of the notochord, even in areas affected by spina bifida was confirmed here (Fig. 7, compare Panels a to c and b to d, respectively). However, staining for Proliferating Cell Nuclear Antigen (PCNA) suggested the possibility of altered cell proliferation. We therefore counted PCNA-positive nuclei and determined the fraction of dividing cells within defined areas: dorsal neuroepithelium, ventral neural tube, and in somites (Fig. 7, in magnification). As expected from our earlier finding of increased apoptosis in mesodermal cells of Isl1-transgenic embryos (Muller et al. 2003), somite areas in double transgenic embryos contained a smaller fraction of PCNA-positive cells, indicative of decreased cell division. Ventral areas of the neuroepithelium also displayed significantly reduced PCNA staining, while PCNA staining in dorsal areas was moderately reduced at E11.5 (Fig. 7e) and similar to control specimen at E12.5 (Fig. 7f). At E11.5, however, PCNA staining in somite and ventral neural tube areas was still significantly reduced. These results indicate that activation of transgenic Isl1 expression had

a greater inhibitory effect on cell proliferation in somites and ventral neural tube than in dorsal neural tube. Thus, it is conceivable that the neural tube defects in *Isl1*-transgenic embryos arise from the combination of altered cell proliferation in both mesodermal and neuroectodermal derivatives.

One of the original objectives of this project was to use *Isl1*-transgenic embryos for the identification in vivo of the downstream targets of the *Islet-1* transcription factor. Because the *NFL-VP16* and the *Nestin-VP16* transactivators turned out to be unsuitable (due to lack of phenotype and too strong a phenotype, respectively), we generated transgenic mice in which the *Isl1* transgene is directly linked to the *Hoxc8* promoter. Transgenic founder animals were rarely viable after birth, but *Isl1*-transgenic embryos were readily recovered (Salbaum, unpublished observations). Screening for differential gene expression identified several de-regulated transcripts (Salbaum 1998), including the *Pbx1a* mRNA isoform derived from the *Pbx1* gene. Originating from a 3'-end expression clone initially termed A67, contiguity between A67 and the *Pbx1a* mRNA sequence was established through 4 rounds of 5'-RACE, and later confirmed by congruence with the mouse genome sequence. Quantification of expression changes, based on the initial A67 sequence, was performed by PCR-mediated incorporation of ^{32}P -dATP into PCR products (Rundle et al. 1998), and revealed significantly higher *Pbx1a* expression in *Isl1*-transgenic embryos compared to wildtype (Fig. 8a). In order to define the sites of expression in the developing embryo relative to the expression of *Islet-1* itself (Fig. 8b), we performed in situ hybridizations for *Pbx1a* using the A67 clone to generate a specific probe. We observed a strong signal in the ventral spinal cord, coinciding with the domain of *Islet-1* expression. Thus, given the colocalization, and the positive correlation of elevated *Pbx1a* expression to increased expression of *Isl1* in *Isl1*-transgenic embryos, these data suggest that *Pbx1a* could be a target for transcriptional regulation by *Islet-1*.

Discussion

Our results for neuronal transgenic activation of *Hoxc8* revealed that ectopic expression of *Hoxc8* exclusively in neurons or their precursors was insufficient in grossly altering neuronal patterning. This was unexpected, since activation of these same IE-*Hoxc8* transgenes in the developing skeleton induced profound defects in cartilage differentiation (Yueh et al. 1998). Thus, even though these transgenes have the capacity to interfere with normal development when overexpressed, we did not observe neurological deficits in the double transgenic progeny for the *Hoxc8*-transresponder with either *NFL*-promoter- or *Nestin*-enhancer-driven transresponder activation. In contrast, *Hoxd4* transactivation by the *Nestin-VP16*-transactivator resulted in perinatal death in most of the double transgenic progeny. Some compromised offspring appeared not to be able to suckle, as they had air in their stomachs rather than milk (data not shown).

Possible explanations for the absence of overt phenotype in the *Hoxc8*-transgenic offspring are: (1) The neuron-specific transactivators are too weak for achieving sufficient duration or level of expression of the transgene to induce functional perturbations. This could be the case for the *NFL-VP16* strain, as we found only moderate activation of the *LacZ*-transresponder previously (Yaworsky et al. 1997). However, this argument is unlikely to apply to the *Nestin-VP16*-transactivator, because the *nestin* enhancer is active in proliferating progenitor cells, and exhibits strong activity by virtue of the potentiator element (Yaworsky and Kappen 1999). Also, since we obtained detrimental outcomes from *Hoxd4*-transgene activation under control of the *Nestin-VP16*-transactivator, we are inclined to consider transactivator strength as sufficient. (2) Co-factors required for *Hoxc8* action might be missing. For example, in the developing hindbrain, *Hox* proteins have been shown to cooperate with the homeodomain factor *Pbx* (Pöpperl et al. 1995). In vitro experiments

have demonstrated that the Pbx1a isoform cooperates with paralogous groups Hox1-Hox10 but not Hox11-Hox13 proteins (Chang et al. 1996), and that it has transactivation activity when complexed with a Hox protein (Di Rocco et al. 1997). But Pbx1a is also able to recruit co-repressors (Asahara et al. 1999). Our in situ hybridization results demonstrate Pbx1a expression in ventral neural tube in motoneuron precursors, where Hoxc8 is also normally expressed (Ensini et al. 1998); lack of cofactors is thus an unlikely explanation for lack of a phenotype in our Hoxc8 transgenic mice. (3) It is conceivable that expression of Hoxc8 in differentiated neurons after their exit from the cell cycle may be too late to affect their anterior-posterior patterning. However, in progenitor cells, Hoxc8 would be expected to alter patterning, at least at the molecular level: In the chicken, extending Hoxb8 activity by removal of microRNA miR-196 inhibited the generation of Isl1-expressing postmitotic motoneurons, but had no effect on Isl1-positive interneurons (Asli and Kessel 2010). The authors concluded that the target of Hoxb8 was a transient progenitor stage before motoneuron cell cycle exit. It is noteworthy that miR-196 also targets Hoxc8 (McGlinn et al. 2009). Also in chicken, localized ectopic expression of Hoxc8 altered the identity of rostral brachial motoneurons; this was before the cells in the lateral motor columns segregate into distinct pools (Dasen et al. 2005). As it is currently unknown to what extent this would have resulted in functional impairment, it is possible that our visual inspections may have missed subtle abnormalities. Typically, with broader domains of misexpression of Hox genes that reached into the hindbrain, patterning defects were observed in mice, and they were usually severe, compromising viability (Zhang et al. 1994; Carpenter et al. 1993; Mark et al. 1993; Studer et al. 1996).

Recent evidence indicates that the action of Hox transcription factors on neuronal patterning can be predominantly activating, such as found for Hoxc8, or repressive, such as in the case of Hoxc9 (Jung et al. 2010). In this regard, it is interesting that paralogous group 4 homeodomain activator function is required for medial motoneurons innervating pectoral, anterior latissimus dorsi and flexor carpi ulnaris muscles, but repressive action was detected on Hoxa7 expression in lateral motor column (Dasen et al. 2005). Such differential action might be one explanation for our finding of lethality with ectopic Hoxd4 (but not Hoxc8) expression. This would also imply that the activity of these two homeodomain transcription factors is regulated by different upstream and co-factors. Further studies in both Hoxc8 and Hoxd4 transgenic mice will be needed to evaluate this possibility. Taken together, our results indicate that Hoxd4 and Hoxc8, even when activated by the same transactivator during development of the nervous system, produce different outcomes. Overexpression of Hoxc8 in neurons or their progenitors appeared to induce no obvious detrimental effects, while Hoxd4 transactivation was incompatible with postnatal survival.

Transactivation of Isl1 in neuronal progenitor cells was also lethal, albeit at earlier stages in development. Embryos displayed defects reminiscent of abnormal gastrulation, as head structures were not readily detectable and embryos were developmentally delayed. Folding of neurectoderm was abnormal, too, and the notochord appeared to be very small or absent. This phenotype argues not only for strong transactivation by the Nestin-VP16-transactivator, but also for very early activity, suggesting that Isl1 may be transactivated as soon as neuroepithelial progenitor cells are present. There is no evidence to date that the nestin enhancer used in this transgenic strain is active in other cell types at the time the defects arise (Yaworsky, unpublished observations). In addition to being malformed, the Isl1-transgenic embryos also exhibited substantially reduced growth, which could be due to impaired cell proliferation. While the early defects in Nestin-VP16/IE-Isl1 double transgenic embryos precluded a detailed investigation, the Hoxc8-VP16/IE-Isl1 double transgenics provide evidence to support this proposition: PCNA staining in those posterior regions of the neural tube, where VP16-driven transactivation would be expected to be present, was reduced in Isl1-transgenics, most profoundly in somites and the ventral neural tube. This

indicates that these cells types, or their progenitors, are sensitive to Isl1; their response is also dosage-sensitive, as increasing transgene dosage is associated with greater reduction of tail length, and increasing lethality of the neonates. Thus, growth of the embryo -in Nestin-VP16-transactivated Isl1 transgenic embryos- and growth of caudal structures -in Hoxc8-VP16-transactivated Isl1-transgenic embryos- are inhibited by increased Islet-1 expression, suggesting that high Islet-1 levels may interfere with cell proliferation. It remains to be established whether this is a direct function of Islet-1, or whether the inhibitory action is mediated by Isl1-regulated targets.

Few transcriptional targets of Isl1 have been identified to date. Islet-1 was originally identified as an insulin-enhancer binding protein (Karlsson et al. 1990). In pancreatic cells, it also regulates the genes encoding amylin (Wang and Drucker 1996), proglucagon (Wang and Drucker 1995) and somatostatin (Leonard et al. 1992), and recently, MafA (Du et al. 2009) and Arx (Liu et al. 2011) were identified as Islet-1 targets. The Mef2c (Dodou et al. 2004) and the Nkx2.5 (Takeuchi et al. 2005) genes have been reported to be direct target of Islet-1 in cardiac cells. Numerous genes have been identified as potential targets in sensory neurons (Sun et al. 2008). Intriguingly, expression of Pbx1 was unchanged in dorsal root ganglia lacking Isl1 (Sun et al. 2008). We here report increased expression of Pbx1a in Isl1-transgenic embryos, and that Pbx1a is expressed in motoneurons at the same time as Isl1. These data suggest that Pbx1 could be a direct target of Islet-1. It is currently unclear whether the cell proliferation defects in our Isl1-transgenic embryos could be mediated by overexpression of Pbx1.

Pbx1-deficiency does not alter Isl1 expression in motoneurons (Kim et al. 2002). In Pbx1 null mutants, organ size of the developing pancreas (Kim et al. 2002) and spleen (Brendolan et al. 2007) was reduced, indicative of reduced cell proliferation. From these studies, it would be expected that elevated Pbx1 expression would be associated with increased cell proliferation. Consistent with this, Pbx1 was found to enhance Hox-gene induced cellular transformation and proliferation (Krosl et al. 1998). But increased apoptosis was also observed in transgenic mice expressing an E2A/Pbx1 fusion transgene (Dedera et al. 1993), and Pbx1 clearly limited hematopoietic stem cell expansion (Krosl et al. 2003). Thus, increased Pbx1 expression could be associated with the increased apoptosis in mesodermal cells and with reduced cell proliferation in somites and ventral neural tube in Isl1-transgenic embryos.

The interesting implication of these results is that transgenic activation of the Isl1 transgene not only affects cells in which it becomes ectopically expressed, such as somitic cells, but also those cells for whose development Islet-1 is critically required, namely the motoneurons in the ventral neural tube. Although we did not observe abnormalities in neural tube patterning (Muller et al. 2003), as assessed by Pax-7 and Nkx2.2 that mark neuronal subpopulations in the spinal cord, there remains a possibility that motoneuron differentiation could be altered in the Isl1 transgenics. Hindlimb gait anomalies and paralysis were observed in some founder animals from the direct Hoxc8-Isl1 construct (Salbaum, unpublished observations), and in some Hoxc8-VP16/IE-Isl1 double transgenic live offspring (Yaworsky and Kappen, unpublished observations). However, not all of the progeny displayed motor impairments, which may indicate that the phenotype is incompletely penetrant. The incomplete penetrance of spina bifida in Isl1-transgenic mice is consistent with this notion. This would imply that Isl1 not only impairs proliferation and possibly differentiation of cells when it is ectopically expressed, but also that the precise levels of Isl1 expression influence the phenotypic outcome.

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Fig. 1.

VP16-driven binary transgenic system for activation of homeobox transgenes. **a** Schematic overview. The system consists of two parental mouse strains: transactivator mice (TA) and transresponder mice (TR). The transactivator carries the Herpes Simplex Virus VP16 gene under control of a cell-type-specific enhancer/promoter. The transresponder strain carries the transgene of interest linked to a VP16-responsive viral immediate early (IE) gene promoter. This promoter is silent in mice in the absence of VP16. When the parental strains are crossed, double transgenic progeny will inherit both the TA and TR transgene (TA + TR). In these mice, VP16 transactivates the transresponder gene and the transgene of interest (GENE) becomes expressed in cells where the promoter/enhancer of choice is active. **b** Double transgenic embryo that expressed VP16 under control of the Hoxc8 promoter, and therefore displays VP16-mediated activation of expression of the IE-LacZ transresponder gene in the region where the Hoxc8 promoter is normally active at E10.5. **c** Section from a paraffin-embedded stained embryo shows β -galactosidase activity in mesodermal derivatives, such as the somites, and the neuroepithelium (Gardner et al. 1996)

Fig. 2.

Constructs for transgenes. **a** The enhancer from the rat nestin gene (Yaworsky and Kappen 1999) was linked to the neurofilament light chain gene promoter (Yaworsky et al. 1997) upstream of the VP16 coding region, an SV40 intron and a polyadenylation site. **b, c** The rat nestin gene enhancer was ligated in both directions (**b** and **c**, respectively) to the minimal promoter region (96 bp) from the neurofilament gene upstream of the same VP16 construct (Yueh et al. 2000) as in **a**. **d** The rat nestin enhancer downstream of the VP16 construct in orientation opposite to the minimal NF promoter. **e** The promoter from the Hoxc8 gene (Gardner et al. 1996) was ligated upstream to the Isl-1 cDNA (Muller et al. 2003), followed by the same SV40/polyA fragment as in **a**

Fig. 3.

Neuron-specific transgene activation by neuron-specific transactivators. Embryos were isolated at E10.5 and genotyped by PCR on genomic DNA isolated from their associated yolk sacs, respectively. Staining for β -galactosidase activity was performed on whole mount specimens. TA-negative embryos did not exhibit any staining in the neural tube; staining of the apical ectodermal ridge is an inherent property of the IE-LacZ strain (Gardner and Kappen 2000). **a, b** Progeny from a cross of Nestin-VP16 transactivators crossed to IE-LacZ transresponders. Staining with X-gal reveals β -galactosidase expression in the developing nervous system of double transgenic (TA + TR) progeny from transgenic founder animals #73 (**a**) and #2 (**b**). **c** Histological section through an embryo derived from founder #2 demonstrating specific localization of β -galactosidase activity in neuroepithelial cells, predominantly in ventricular zone cells. **d** Progeny from a cross of an NFL-VP16 transactivator to an IE-LacZ transresponder mouse. **e** Histological section demonstrating neuron-specific localization of β -galactosidase activity

Fig. 4.

Nestin-VP16-driven *Isl1* transresponder activation. Nestin-VP16 transactivators were crossed to IE-*Isl1* transresponder mice, embryos were isolated at E10.5 and genotyped by PCR on yolk sac DNA. **a** Schematic of an E10.5 embryo for the purpose of size comparison. Note the size bar to the right, representing the length of the embryo in **b**. **b** Double transgenic (TA + TR) embryo was much smaller than normal size and lacked head development. **c** Additional independent double transgenic embryo was also much smaller and anteriorly malformed. **d** Third double transgenic embryo exhibited substantially retarded development. **e** Histological section through a fourth double transgenic embryo was stained with Hematoxylin/Eosin and reveals abnormal folding of the neuroepithelium, which is also thinner than normal. Staining of adjacent sections with antibodies specific for Proliferating Cell Nuclear Antigen (PCNA) and nestin (not shown) indicated that the epithelial cells are proliferating neural precursor cells. Magnifications are not equivalent

Fig. 5.

Hoxc8-VP16-driven Isl1 transresponder activation. Hoxc8-VP16 transactivators were crossed to IE-Isl1 transresponder mice, in two types of crosses: TA/+ $\times$ TR/+, or $\times$ TA/+ TR/+. Pregnancies were allowed to go to term, and tail size was determined on neonates, with confirmation of assessment on day 3 after birth, or on the carcass. Genotyping was performed on DNA from the tail of weaning age progeny, or from the carcass on the day of discovery. Bars represent the number of live offspring (*green*), and of dead neonates (*red*). TA-only containing newborns had all normal tail length, one pup homozygous for the TA locus was recovered dead. Neonates inheriting both a TA and TR locus exhibited shortened tails, and some deaths. More offspring with shorter tails were found with higher TA transgene dosage, and more deaths, correlated to tail length; all tailless offspring were recovered dead. No abnormalities or deaths were found in progeny that inherited only the TR locus from the first type of cross

Fig. 6. Spina bifida in Isl1-transgenic mice. **a** Neonate from a cross of Hoxc8-VP16 transactivator to IE-Isl1 transresponder with shortened tail and open back. **b** Close-up of the open site, with splaying of the neural arches. **c** Scanning electron micrograph of an E11.5 Isl1-transgenic embryo with open neural tube in the caudal region. **d** Independent Isl1-transgenic embryo with spina bifida just posterior to the hind limbs

Fig. 7.

Staining with PCNA-specific antibody. Sections were cut from paraffin-embedded control and Isl1-transgenic embryos and processed together in the same incubation chamber. **a** Control embryo at E11.5; section at the hind limb level. Areas for cell counting are marked by a rectangle, and magnified pictures are provided for: dorsal neural tube, ventral neural tube and somite. **b** Control embryo at E12.5; section behind the hind limb level. **c** Isl-1 transgenic littermate at E11.5; level comparable to A. **d** Isl1-transgenic embryo at E12.5; level comparable to B, with spina bifida. **e, f** Quantification of results in three independent embryos for each area, genotype, and time point, respectively, plotted as % of cells with PCNA positive nuclei (average $\pm$ standard deviations). *Open bars* control embryos; *filled bars* Isl1-transgenic embryos; ****** $P < 0.005$. **e** Quantification of staining for E11.5 embryos in somite, ventral neural tube and dorsal neural tube; **f** Quantification of staining for E12.5 embryos in somite, ventral neural tube and dorsal neural tube

Fig. 8.

Pbx1a as a potential target gene of Islet-1 in motorneurons. Sequence A67 was identified in a differential display screen between wildtype (WT) and Isl1-transgenic embryos. **a** A67 expression was higher in Isl1-transgenic embryos; incorporation of ^{32}P into A67 PCR product was normalized to incorporation of ^{32}P -ATP into PCR product from β -actin mRNA. $N = 4$ independent embryos for each group. **b** Immunohistochemistry for Islet-1 demonstrates expression in motorneurons and dorsal root ganglia on a frozen section from a normal E10.5 mouse embryo. **c** An in situ hybridization probe for A67 detects expression in motorneurons in a normal embryo at E10.5 (the section is from a slightly more posterior level than in panel **b**). Some expression was also detected in mesoderm at the hindlimb level, an area known to express Isl1 mRNA (Kappen and Salbaum 2009). Sequencing revealed that A67 was the Pbx1a isoform derived from the Pbx1 gene

Table 1

Generation of Nestin-VP16 transactivator transgenic mice

Construct	Founder mice	Transgene positive	Transgene-expressing
Nestin-NFL-VP16	74	3	0
Nestin-VP16.334	49	4	0
Nestin-VP16.339	45	1	0
Nestin-VP16.411	88	7	2

Four different Nestin-VP16 constructs (structures are depicted in Fig. 2) were microinjected into fertilized mouse oocytes to generate transgenic mice. The presence of the VP16 transgene was ascertained by PCR on tail DNA. To assay for VP16 expression, each transgenic founder animal was mated to an IE-LacZ transresponder mouse, and embryos were stained for β -galactosidase activity at E10.5. Activity in the expected cell types was only detected in progeny from 2 founders; both of these turned out to be chimeras, as evident from non-Mendelian ratios of transgene transmission

Table 2

Outcomes from crosses of transactivator and transresponder transgenic lines

TR gene	Total	Genotype		P		
		+/+	TA/+	TR/+	TA + TR	
A: NFL-VP16 transactivator × IE-Hoxc8 transresponder crosses						
IE-c8.45	39	11 (28.2 %)	9 (23.1 %)	12 (30.8 %)	7 (17.9 %)	n.s.
IE-c8.254	26	6 (23.1 %)	4 (15.4 %)	9 (34.6 %)	7 (26.9 %)	n.s.
B: Nestin-VP16 transactivator × IE-Hoxc8 transresponder crosses						
IE-c8.45	24	8 (33.3 %)	7 (29.2 %)	4 (16.7 %)	5 (20.8 %)	n.s.
IE-c8.254	64	18 (28.1 %)	7 (10.9 %) ^a	26 (40.6 %) ^a	13 (20.3 %)	<0.05 ^a
C: Nestin-VP16 transactivator × IE-Hoxd4 transresponder crosses						
IE-d4.70	37	14 (2)	9 (2)	7 (3)	7 (5)	n.s.
D: Nestin-VP16 transactivator × IE-Isl1 transresponder crosses						
IE-Isl.16	31	10 (32.3 %)	7 (22.6 %)	7 (22.6 %)	7 (22.6 %)	n.s.
IE-Isl.5	87	38 (43.7 %) ^b	12 (13.8 %) ^b	35 (40.2 %) ^b	2 (2.3 %) ^b	<0.05 ^b
IE-Isl.50	96	36 (37.5 %) ^b	17 (17.7 %)	41 (42.7 %) ^b	2 (2.1 %) ^b	<0.05 ^b
IE-Isl.81	46	12 (26.1 %)	19 (41.3 %)	15 (32.6 %)	0 (0 %) ^b	<0.05 ^b

All progeny were genotyped by PCR at 3–4 weeks of age; in case of postnatal death, carcasses were genotyped. The numbers of animals screened are given, with percentages in brackets. TA Transactivator, TR Transresponder

A: Activation of Hoxc8 transgene expression under control of the neurofilament light chain gene promoter (NFL-VP16 transactivator). Two transresponder strains were used, IE-Hoxc8.45 and IE-Hoxc8.254. The inheritance of all transgenes followed the expectation of Mendelian inheritance, e.g. 25 % for each possible genotype. The viability of double transgenic animals at 4 weeks of age (and beyond) suggested that ectopic expression of Hoxc8 in anterior regions of the nervous system was compatible with survival

B: Activation of Hoxc8 transgene expression under control of the nestin gene enhancer (Nestin-VP16 transactivator). Transgenic offspring were produced in close to Mendelian ratios with the IE-Hoxc8.45 transresponder.

^aIn crosses involving the IE-Hoxc8.254 transresponder, there was reduced transmission of the TA to TA-only positive progeny, but this did not affect double transgenic offspring. Double transgenic progeny from all these crosses were behaviorally normal, viable and fertile, indicating that overexpression of Hoxc8 in neuronal progenitor cells is compatible with survival

C: Activation of Hoxd4 transgene expression under control of the nestin gene enhancer (Nestin-VP16 transactivator). The transresponder line IE-Hoxd4.70 was used. Genotypes were determined at weaning age for surviving progeny, and from carcasses recovered at any time after birth (numbers in bold). In all genotypes, a few pups were found dead, associated with generally smaller litter sizes in this line

D: Activation of IE-Isl1 transgene expression by the Nestin-VP16 transactivator. Four independent TR lines were used: IE-Isl1.5, IE-Isl1.16, IE-Isl1.50, and IE-Isl1.81. Mendelian ratios were found for transmission of the IE-Isl1.16 transresponder transgene; progeny appeared normal and healthy.

^bWe found deviation from Mendelian ratios, and very few, if any, double transgenic offspring with the three other transresponder lines (~2 vs. 25 % expected; none for IE-Isl1.8). These data indicated that activation of the Isl1 transresponder gene in neural progenitors was incompatible with survival