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Morpholino-Induced Knockdown of *fgf8* Efficiently Phenocopies the *Acerebellar* (*Ace*) Phenotype

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Homozygous *acerebellar* (*ace*) embryos lack their cerebellum and the midbrain–hindbrain boundary (MHB) organizer, and in addition have defects in forebrain and heart development (Brand *et al.*, 1996; Picker *et al.*, 1999; Reifers *et al.*, 1998, Shanmugalingam et al. (2000); Raible and Brand, 2001; Araki and Brand, unpublished data), suggesting that this may be because of functional redundancy between Fgfs. Because morpholinos might help to resolve such issues, we sought to phenocopy the known defects caused by absence of Ace/Fgf8 through injection of a morpholino against *fgf8* (MO-*fgf8*). In spite of the complexity of the *ace* phenotype, we find that MO-*fgf8* efficiently and uniformly phenocopies the *ace* mutant in MHB, forebrain, and heart development.

We designed an antisense morpholino against *fgf8*, covering the translational start codon (Fig. 1J). After injections into one- to eight-cell-stage wild-type embryos, we find that MO-*fgf8* effectively phenocopies the *ace* phenotype. We used between 0.5 and 4 μg/μl of MO-*fgf8* for the injection (Table 1), which delivers between 1.6 and 12.6 ng of MO-*fgf8* per embryo. Morphologically, embryos at the 24-h stage injected with 1–4 μg/μl lacked the cerebellum and the MHB organizer, as do *ace* mutants at the same stage (Fig. 1A–I). A morpholino with four mismatched base pairs against *fgf8* (control MO in Fig. 1J) had no effect, nor did it show any nonspecific effects at the same concentration (Table 1). To examine how closely the *ace* phenotype is mimicked, we stained the injected embryos with a probe for *pax2.1* which reveals several of the tissues where Fgf8 functions. *pax2.1* expression at the MHB is initially normal in *ace* mutants, but is not properly maintained (Reifers *et al.*, 1998; Lun and Brand, 1998). At 24 h, MO-*fgf8* injected embryos either lacked *pax2.1* expression at the MHB completely, or it was reduced to a small dorsal patch, as seen in *ace* mutants at the same stage (Fig. 1D–I and not shown; Reifers et al., 1998). Because *ace* mutants show defects also in forebrain and heart development, we analyzed with molecular markers whether MO-*fgf8* injection can phenocopy the *ace* mutant also in these tissues. Ten hours after fertilization, injected embryos have reduced and perturbed expression of *emx1*, an early telencephalic marker, as in *ace* mutants (Fig. 1K–M; Shanmugalingam *et al.*, 2000). Similarly, at 24 h expression of *pax2.1* in the optic stalk is reduced, and in the optic chiasm is reduced or missing (not shown), as found previously for *ace* mutants (Shanmugalingam *et al.*, 2000). Injected embryos stained with *nkx2.5*, an early marker for heart primordium, showed downregulation of *nkx2.5*, as is observed in *ace* mutants (Fig. 1N–P; Reifers *et al.*, 2000a). We did not detect any nonspecific effect of MO-*fgf8*. We conclude that MO-*fgf8* injection efficiently phenocopies the known loss-of-function phenotype of *acerebellar* in MHB, telencephalon, optic stalk, and heart development, thus validating the usefulness of this method. Because morpholino injection is thought to prevent translation (which we assume to be true, but have not tested, for *fgf8*), these findings also support (Reifers *et al.*, 1998) that *ace* is a null allele. To further test this notion, we examined whether the phenotype of homozygous *acerebellar* mutants can be enhanced by Mo-*fgf8* injection. Morphologically and after examining *pax2.1* expression in injected embryos at the tailbud, two-somite, five-somite, and 24-h stage, we did not observe a difference between *ace* homozygotes and their wild-type siblings in the same clutch (Table 1 and not shown), showing that the *ace* phenotype cannot be further enhanced and therefore most likely represents the null phenotype. Morpholinos against other *fgf* mRNAs alone or in combination with Mo-*fgf8* will help to resolve the problem of redundancy that is often found with signaling by Fgfs.

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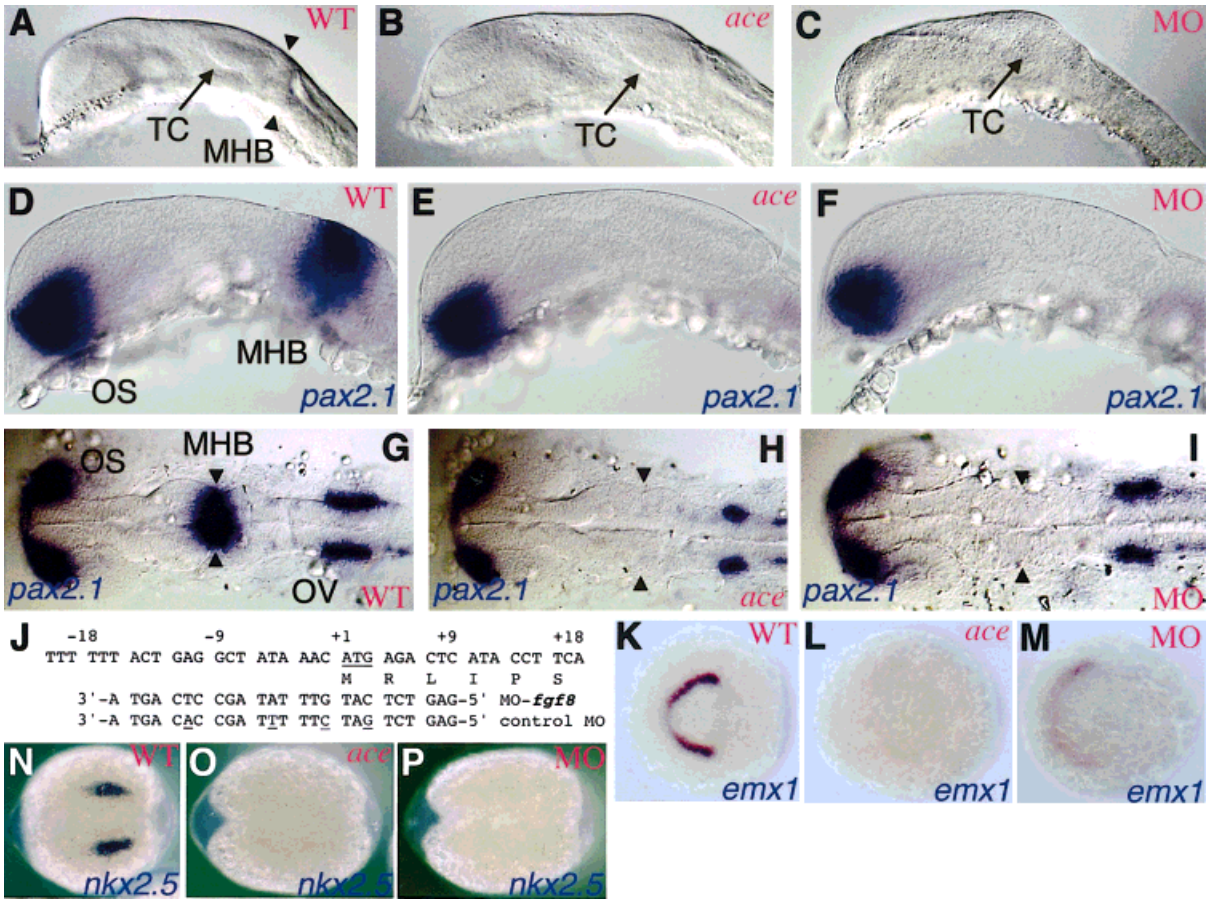

FIG. 1. Injection of a morpholino against *fgf8* phenocopies the *ace* phenotype. (**A–K**) MO-*fgf8* phenocopied *ace* phenotype in the midbrain–hindbrain boundary (MHB). Injected embryos or *ace* embryos lack the structure of MHB (arrowheads in A), the conspicuous constriction at MHB (compare arrowheads in G and H/I), and *pax2.1* expression at MHB. The embryos were injected with MO-*fgf8* at 2 μg/μl in this figure. The embryos in A, D, and G are wild type; the embryos in B, E, and H are *ace* mutants. The embryos in D–I were stained with probe for *pax2.1* mRNA. The pictures in A–F are lateral view, whereas the ones in G–I are dorsal view. In A–I, embryos were fixed at 24 h after fertilization. (**J**) The nucleotide/amino acid sequence around the initiation codon (underlined) of fgf8 cDNA and the sequences of the morpholino against *fgf8* (MO-*fgf8*) and its negative control (control MO) are shown. The control-MO contains four single nucleotide exchanges (underlined) that are predicted to lead to a strongly reduced target binding (Gene Tools, LLC, Corvallis, OR). (**K–P**) MO-*fgf8* phenocopied *ace* phenotype also in other tissues. The embryos in K and N are wild type; the embryos in L and O are *ace* mutant; the embryos in M and P are MO-*fgf8*-injected. The embryos were stained with an *emx1* probe and a *nkx2.5* probe in K–M and N–P, respectively. The pictures in K–P are dorsal views. Embryos in K–M were fixed at 10 h after fertilization; at 12 h in N–P. All embryos in this figure are oriented with rostral to the left. OS, optic stalk; OV, otic vesicle; TC, tectum. Methods: Crystallized morpholino oligos (Gene Tools, LLC) were dissolved in water at 40 μg/μl, and this stock solution was stored at = −20°C. The working solution consisted of 5 mM HEPES (pH 7.5), 0.1% phenol red and the appropriate concentration of morpholinos. Injection was performed between one-cell to eight-cell stage. The injected/control embryos were raised at 28°C.

Table 1
Dose Response to a Morpholino Against Fgf8

Morpholino	Concentration (μg/μl)	Dose (ng)	*n*	pax2.1 staining at MHB		
				Absent	Reduced	Normal
MO-Fgf8	0.5	1.6	30		20 (67%)	9 (30%)
	1	3.1	103	50 (49%)	53 (51%)	0
	2	6.3	101	60 (59%)	41 (41%)	0
	4	12.6	24	10 (42%)	14 (58%)	0
	4	12.6	7	7 (100%)	0	0
Injection into *ace*/*ace* homozygotes	2	6.3	63	58 (93%)	5 (8%)	0
Control MO	2	6.3	22	0	0	22 (100%)
	4	12.6	19	0	0	19 (100%)
Mock	0	0	23	0	0	23 (100%)

The readout was in situ hybridizations with *pax2.1* probe at 24 h after fertilization.

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