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Short Report

Functional analysis of FGF3 during zebrafish inner ear development

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ABSTRACT FGF3 has been involved in the development of the inner ear in a number of species including mouse, chicken and *Xenopus*. Initial experiments using antisense oligonucleotides in explants of chicken embryos indicated a role for FGF3 for proper formation of the otic vesicle (Represa *et al.,* 1991). However, mice carrying a mutant FGF3 allele exhibited normal formation of the otic vesicle and only showed defects during later differentiation of the inner ear (Mansour *et al.,* 1993). Implantation of beads incubated with FGF3 into *Xenopus* embryos led to the formation of ectopic vesicles (Lombardo *et al.,* 1998). To further resolve the functions of FGF3 during inner ear development we recently performed gain-of-function experiments in chicken embryos by ectopically expressing FGF3 via a Herpes simplex-derived viral vector (Vendrell *et al.,* 2000). Embryos infected by the virus developed ectopic otic vesicles confirming the involvement of FGF3 during formation of the otic vesicle in avians. To further clarify the role of FGF3 during inner ear development in vertebrates, we have now addressed its potential function in zebrafish.

To analyse the function of FGF3 for otic development we chose a loss-of-function approach using antisense morpholino oligomers designed to block translation of *fgf3* transcripts. Morpholinos have been shown to cause developmental defects that closely mimic phenotypes caused by null mutations in the same genes (Nasevicius and Ekker, 2000). In the light of the contrasting evidence for the involvement of FGF3 during formation of the otic vesicle we were particularly interested to analyse potential defects during this process. We therefore monitored formation of the otic vesicle in injected and control embryos using a Pax2 antibody which stains the otic placode and vesicle during zebrafish development (Püschel *et al.,* 1992).

Morpholino oligomers were obtained from Gene Tools Inc. and diluted as described (Nasevicius and Ekker, 2000). Approximately 4 ng were injected into the yolk of embryos. The morpholino sequence was as follows: 5´-ATTGTGGCATGGCGGGATGTCGGCA-3´. Next to uninjected control embryos a standard control oligo with a 3´ conjugated fluorescein was injected. Embryos were analysed after 16 or 21 hours by staining with a polyclonal primary antibody against Pax2 (Berkeley Antibody Company, diluted 1:200). The diameter of the otic vesicle was measured and compared to the diameter of the pigmented retina to avoid errors caused by variations between the developmental stages of different embryos.

Analysis of FGF-3-injected embryos (n=36) revealed no apparent abnormalities compared to uninjected controls (n=10) after 16 hours. However, at 21 hours approximately only 20% of the embryos injected with FGF3 morpholinos had survived (n=44) and showed a variety of defects compared to control- (n=53) and uninjected (n=13) embryos. The majority of embryos (n=36) were developmentally arrested (see Fig. 1) or displayed a general necrosis which was likely to cause death of the embryos. Embryos were stained with a Pax-2 antibody to better visualise the structure of the otic vesicle and to analyse it by light microscopy. Upon visual inspection of the embryos we observed an apparently normally formed otic placode or vesicle in FGF3-, control- and uninjected embryos (see Fig. 1). To monitor a potential minor size difference between the vesicles we measured their diameters in parallel to the embryonic body axis. To avoid potential errors caused by a possible generalised developmental delay in FGF3-injected embryos we also measured the diameter of the pigmented retina and used this value as a correction factor. The pigmented retina is easily visualised in the embryo and not expected to be affected by a loss or lack of FGF3. In 10 randomly chosen embryos from the FGF3- (without obvious signs of necrosis) and uninjected group we observed no significant differences between the diameters of the otic vesicles (Student´s *t*-test; p=0,127).

During the course of our studies two groups have reported similar results upon injection of FGF3 morpholinos into zebrafish embryos. In one case (Fürthauer *et al.,* 2001) injected embryos were reported to appear morphologically normal until early somitogenesis. However, by 15 hours of development embryos started to appear necrotic and died. Necrosis of FGF3-injected embryos has also been reported in a further study (Phillips *et al.,* 2001), although in this case embryos showed a better survival rate and were analysed at 32 hours. In our experiments we have also observed a high number of dead animals or necrosis in embryos analysed at 21 hours. Therefore, a complete block of FGF3 function appears to be lethal for embryonic development of the zebrafish. In mice carrying a mutant FGF3 allele a reduced number of postnatal homozygous mutants has been observed (Mansour *et al.,* 1993). The cause for this reduction is at present unclarified and may be due to developmental defects leading to embryonic death.

Most importantly, in one of the aforementioned studies in 80-90% of FGF3-injected embryos otic vesicles appear smaller than normal, although no quantification of the results are reported (Phillips *et al.,* 2001). In contrast, we have observed no reduction of otic vesicle size in FGF3-injected embryos in the present study. This discrepancy may be due to a number of reasons. First, we examined our embryos 11 hours before the ones in which inner ear phenotypes have been reported. Since the observed size reduction is not very dramatic this phenotype might only be detected in older embryos. Formation of the otic vesicle in FGF3 mutant mice also appears unaffected at the otic

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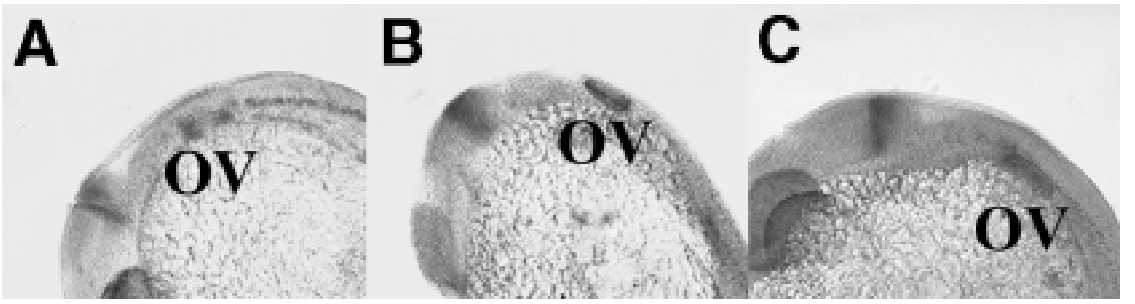

Fig. 1. Zebrafish embryos at 21 hours of development stained with *Pax2* antibodies to visualize the otic vesicle (OV). (A,B) *Embryos injected with morpholinos directed against FGF3.* **(C)** *Uninjected control embryo. Note the developmental retardation of the embryo in (B) compared to the control embryo (C).*

vesicle stage and begins to differ from wild-type mice at embryonic day 10.5 when mutants fail to induce the endolymphatic duct (Mansour *et al.,* 1993). In zebrafish otic vesicles enlarge as a simple ovoid structure until about 24 hours of development (Haddon and Lewis, 1996). The vesicle then starts to undergo a complex process of morphogenesis. The size reduction of otic vesicles observed at 36 hours of development (Phillips *et al.,* 2001) may well fit with the developmental timepoint in FGF3 mutant mice, when a phenotype is also observed upon onset of morphogenesis of the otic vesicle (induction of the endolymphatic duct). Alternatively, the morpholinos used in the present study may have caused a less efficient reduction of the FGF3 protein level. It is unlikely that this potential difference may be caused by the usage of different morpholinos since our sequence shares 96% identity with the morpholino applied in both of the other studies. Moreover, since we see a high amount of death or developmental arrest indicating a more severe loss-of-function phenotype, it is unlikely that in all better developed embryos insufficient blockage of FGF3 protein production has occurred and the inner ear phenotype might have been missed. Thus, although the reduction of otic vesicle size in FGF3-injected embryos has also been observed in non-necrotic healthy-looking animals (Phillips *et al.,* 2001) the inner ear phenotype might have been caused indirectly by a minor generalised developmental arrest of the embryos. The variety of phenotypes observed upon inactivation of FGF3 in zebrafish has also been reported in FGF3 mutant mice. Within homozygous mutant embryos a high variation in both penetrance and expressivity of the inner ear phenotype has been reported. This observation has been explained by the existence of parallel signalling pathways leading to redundancy. Indeed, zebrafish lacking FGF3 and FGF8 develop a severe inner ear phenotype resulting in a complete loss of the otic placode (Phillips *et al.,* 2001). Since additional FGFs, including FGF2, FGF8, FGF10 and FGF19 (Ladher*et al.,* 2000) are involved in early inner ear development of other vertebrate species a complex and at least partially redundant network of these factors appears to assure proper formation of the otic vesicle.

In summary, we have provided evidence for an essential function of FGF3 during zebrafish development. The exact function of FGF3 during zebrafish inner ear development requires further studies of the FGF3 morpholino knockdown in combination with loss-of-function alleles of other FGFs potentially involved during this process. Additionally, gain-of-function experiments by overexpression of FGFs during zebrafish development may also provide further insights in their exact roles during formation of the otic vesicle in vertebrates.

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