Gene knockdown using morpholino nucleotides in zebrafish (*Danio rerio*) to investigate the function of Nf1 and FoxP4 in cardiovascular development

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Congenital cardiovascular defects are the most common form of birth defects in man occurring in almost 1% of live births[1]. The etiology of congenital heart disease includes both genetic and environmental factors, though little is known about the influence of environment as it relates to genetic susceptibility during cardiac development. The identification of specific genes and environmental exposures that influence cardiovascular development is of significant interest. Zebrafish (*Danio rerio*) embryos are transparent, and the developing cardiovascular system can be easily visualized and monitored during critical phases of formation, which occur between fertilization and 72 hours post fertilization (hpf). Expression of specific genes can be disrupted by injection of fertilized oocytes with antisense morpholino-modified oligonucleotides that impair expression of specific targets[2]. Signaling via the small G-protein Ras is required for proper formation of the heart and valves, and levels of Ras are regulated by the Ras GTPase activating protein (GAP) encoded by the type 1 Neurofibromatosis gene (Nf1)[3]. Ras signaling is also modified by numerous external stimuli including ß-adrenergic signaling. In this study, the zebrafish homologue of Nf1 was sought and its function during zebrafish cardiac development in the presence or absence of ß-adrenergic stimulation was analyzed. In addition, unpublished work has identified a novel transcription factor that is expressed in the early embryo that is required for midline fusion of the bilateral embryonic heart tubes. Disruption of this gene, FoxP4, in mice results in cardia-bifida (E. Morrisey, personal communication). A zebrafish homologue of FoxP4 was sought and disruption of its function in zebrafish so as to determine gene function in a model system in which morphogenetic movements of cardiovascular development could be observed was carried out. Future studies would address whether modest diminution of gene expression would sensitize embryos to external toxins and the evolution of congenital heart disease.

In silico database analysis was used to identify zebrafish homologues of Nf1 and FoxP4, which were named zNf1 and zFoxP4, respectively. Neither zebrafish gene has been previously described or analyzed. Morpholino antisense nucleotides were generated that were expected to result in impairment of expression of each gene, and control morpholinos to Smad5 (a member of the TGFß signaling cascade) which produces a known phenotype characterized by a shortened tail and "ventralized" appearance[4] were also prepared. Negative control morpholinos similar to the Smad5 morpholinos but with 5 mismatched residues which have previously been shown to have no effect, were also utilized. A zebrafish colony was established at the Mount Desert Biological Laboratory and conditions that were successful for the production of fertilized eggs were identified. These eggs were collected shortly after fertilization and used for microinjection of morpholino nucleotides using pulled glass needles and a Femptojet microinjection apparatus (Millipore). Injection volume (0.5 nl) was monitored by including phenol red (0.5%) in the injectate and calibrated using a micrometer. Morpholinos (5-10 ng/nl) were diluted in E3 medium. Some developing embryos were incubated in isoproterenol (1mM) and heart rate was evaluated at 24 and 48 hours. Cardiovascular development was monitored under the dissecting microscope and videotaped at 12, 24, 48 and 72 hours.

A zebrafish colony that produced fertilized eggs capable of normal development was successfully established. This required significant modification of available housing and fish tank facilities to allow for the appropriate stable temperature, water composition and feeding schedule. A daily mixture dried fish food alternating with ample amounts of previously frozen brine shrimp was successful in producing fish that laid eggs. Fish were housed in small tanks with screened bottoms such that the eggs would fall through the screen after being laid and would not be eaten by the fish. Housing of male and female fish together produced fertilized eggs that developed normally in the laboratory.

During control experiments involving ~500 fertilized egg injections, the necessary injection skills and equipment were established. In early experiments, few or no injected eggs matured normally. However, after significant practice and experience, >80% of injected eggs developed with normal cardiovascular morphology and function as observed at 72 hpf. Having established all of the necessary equipment for holding and manipulating eggs and for microinjection, subsequent incubation and photography, an additional 166 fertilized eggs in four separate experiments were collected during the available time. Injections included 104 eggs with zNf1 morpholinos, of which 40% showed variable abnormalities including slow heart rate and reduced overall size. 9 uninjected eggs developed normally as did 28 eggs injected with morpholinos to zFoxP4. 16% of 25 eggs injected with a negative control morpholino (related to the Smad5 morpholino but with mismatches) were also abnormal with small size suggesting a non-specific defect.

Additional experiments involved treatment of 34 embryos at 12 hpf with or without isoproterenol (1mM) and examination of heart rate after video-capture microscopy at 24 and 48 hours. Heart rate was not significantly altered in treated embryos (189 +/- 44, 170 +/- 13, 171 +/-13 bpm at 12, 24, 48 hours, respectively), although the heart rate dropped further in untreated embryos at 24 hours compared to treated embryos (187 +/- 8, 143 +/- 18 in untreated embryos at 12 and 24 hours respectively, $p<0.001$). All treated and untreated embryos developed normally.

These data suggest that further analysis of zNf1 function during embryogenesis is warranted. The high rate of developmental defects in zNf1 morpholino injected embryos cannot be attributed to a specific effect without further controls and greater numbers and analysis. However, these studies encourage the production of additional zNf1 morpholino oligonucleotides and mismatched controls for further evaluation. On the other hand, no significant effects were seen with zFoxP4 suggesting that this gene is not required for cardiovascular development, perhaps owing to functional redundancy of other genes. Indeed, we have identified an alternative or additional potential zebrafish FoxP4 homologue in the database. Studies with isoproterenol suggest that this drug at 1mM concentration is well tolerated and does not itself cause cardiovascular developmental defects. Interestingly, heart rate did not increase suggesting that higher doses may be needed to alter intracellular signaling (including Ras levels), or that the embryonic zebrafish heart lacks functional ß-adrenergic receptors. Future studies will address these possibilities.

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