

2004 TAIWAN INTERNATIONAL SCIENCE FAIR

CATEGORY : Microbiology

**PROJECT : A Novel Approach of Gene
Knockdown in Zebrafish through
Targeted mRNA Cleavage**

AWARDS : Microbiology First Award

SCHOOL : Raffles Junior College

FINALISTS : Ben Yee Ming Leslie

COUNTRY : Singapore

Category: Microbiology

Title: A Novel Approach of Gene Knockdown in Zebrafish through Targeted mRNA Cleavage

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Background:

The zebrafish (*Danio rerio*) is a popular model organism used for elucidating complex developmental pathways and processes. Although the Zebrafish Genome Project is nearing completion, the function of most genes remains to be discovered. In zebrafish, where an efficient gene targeting method is still absent, costly Morpholinos (MOs) have been widely used to 'knockdown' specific genes.

Purpose of research:

The initial aim of the project was to test the ability of modified oligonucleotides containing locked nucleic acid (LNA) analogues in knocking out specific genes of interest. The zebrafish *mitfa* gene, which encodes a regulator protein affecting pigment cell fate, was targeted in heterozygous (*nac/+*) individuals. During the course of research, a novel approach of gene targeting was proposed based on the simultaneous introduction of LNA-DNA chimeras (from here on, LNA) capable of forming single base mismatches when bound to the targeted sequence and Cell, a "mismatch-cleaving" endonuclease. Results obtained pointed towards the possibility of targeted gene knockdown through Cell-mediated cleavage of LNA-mRNA heteroduplexes, whose investigation became the subsequent aim of this project.

Procedures:

Modified oligonucleotides containing varying numbers and positions of LNA residues were designed based on the *mitfa* cDNA sequence (AF119220), and ordered from Exiqon (Denmark). LNAs (0.2 μ M) were co-injected with 0.5 μ l of Cell (Transgenomic) per 20 μ l of injection mix into 1- to 8-cell stage zebrafish embryos, which were then observed for pigmentation loss at 36, 48 and 72 hours post fertilisation (hpf) through a microscope. RNA was also isolated from pooled 72 hpf zebrafish embryos and treated with the oligonucleotide and/or Cell before determining the integrity of the amplified mRNA via RT-PCR.

Results and Data:

Firstly, 63.2% of the embryos co-injected with Cell and the LNA-NT (complementary to the non-transcribing strand of the *mitfa* gene) displayed severe pigmentation loss at 55hpf, with 25.8% of them possessing pigmentless phenotypes. Co-injection of Cell and DNA oligonucleotides isosequential to the LNA-NT produced similar, but less pronounced effects. The pigmentation was restored in all injected embryos by 80hpf. This transient

loss of pigmentation was not observed when the LNA and Cell were injected individually, or when an LNA-T (complimentary to the transcribing strand) was co-injected with Cell.

RT-PCR performed with primers flanking the proposed binding site showed no amplification from *mitfa* mRNA treated with the LNA-NT and Cell *in vitro*, suggesting cleavage of the LNA/mRNA chimera. Irregular amplification was detected when the mRNA was treated with only the LNA, while amplification was unaffected if the RNA was incubated with only Cell.

Conclusion:

The transient loss of pigmentation observed in embryos co-injected with Cell and oligonucleotides capable of binding to the *mitfa* mRNA has pointed to targeted depletion of the *mitfa* gene product. *In vitro* analysis of mRNA treated with the oligonucleotides and Cell through RT-PCR indicated an efficient, specific Cell-mediated gene knockdown mechanism. This was further supported by the observed lack of pigmentation loss in embryos co-injected with Cell and oligonucleotides isosequential to the targeted *mitfa* mRNA. Thus, cleavage of mismatches in LNA-mRNA heteroduplexes by the mismatch-cleaving endonuclease Cell presents a potential alternative to Morpholinos for targeted gene knockdown in zebrafish, with many useful advantages, including reduced cost and increased versatility.