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Reverse genetics tools in zebrafish: A forward dive into endocrinology

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ABSTRACT

The zebrafish is a powerful genetic model organism. In recent years, zebrafish has been increasingly used to model human diseases. Due to a number of recent technological advancements, the genetic tool box is now also stocked with sophisticated transgenic and reverse genetic tools. Here, we focus on both commonly used and recently established reverse genetic and transgenic tools available in zebrafish. These new developments make the zebrafish an even more attractive animal model in comparative endocrinology.

1. Introduction

The last three decades have seen the establishment of the zebrafish as a major vertebrate model organism for studies of human diseases. The zebrafish made its first splash as a model for genetic control of vertebrate development due to its favorable biological characteristics such as high fecundity, transparency and external development of embryos (Streisinger et al., 1981). Zebrafish genetics entered the picture and gained prominence with the two first large-scale mutagenesis screens performed in any vertebrate species (Driever et al., 1996; Haffter et al., 1996). At this time in the 1990s and in the following years, zebrafish genetics was nearly exclusively confined to forward genetic studies, exploiting the huge number of available mutant strains, many of which proofed to be relevant for our understanding of mechanisms of pathogenesis of a number of human diseases (Fishman Genomics, 2001; Lieschke and Currie, 2007). Genetic studies of human disorders benefit from the investigation of specific target genes of interest. In contrast to forward genetics, the lack of tools to manipulate specific genes of interest hindered the realization of the full potential of the zebrafish for modeling human diseases (Skromne and Prince, 2008). However, recent technological advances, mainly in antisense morpholino mediated gene knockdown, transgenics using the Tol2 system and the induction of heritable genetic alterations with zinc finger nucleases, TALENs and TILLING technology heralds a new era in zebrafish reverse genetics.

The strengths of zebrafish have been largely realized in the study of developmental biology, as is also the case for endocrine systems in zebrafish (Lohr and Hammerschmidt, 2011). Studies on zebrafish endocrine systems have revealed that the endocrine systems between humans and zebrafish are remarkably well conserved (McGonnell and Fowkes, 2006). Significantly, studies on zebrafish endocrine systems show that the development of endocrine systems mostly complete within 5 days post fertilization (dpf) in zebrafish. In other words, it is possible to model the human endocrine systems in developing zebrafish, taking full advantage of the strengths of this animal model.

In this review, we discuss several reverse genetics approaches that have been established in zebrafish as well as emerging techniques for targeted gene knockout including TALENs, and their application for studies of human endocrine systems.

2. Reverse genetic approaches in zebrafish

The mouse model has been a dominant system to study disease related genes because of conservation of gross anatomy with humans and sophisticated gene manipulation techniques made possible by homologous recombination (Thomas and Capecchi, 1987). Although application of gene targeting by homologous recombination has been explored in zebrafish, these attempts have only met with little success, mainly due to the absence of proper embryonic stem cells (Sun et al., 1995). However, other methods to manipulate gene function have gained prominence in the zebrafish. Morpholino antisense oligonucleotides and TILLING are two main reverse genetics methods, and now artificial endonuclease en-

Abbreviations: TALEN, transcription activator-like effector nucleases; ZFN, zinc finger nucleases; TILLING, targeted induced local lesions in genomes; UAS, upstream activating sequence.

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zymes, ZFNs and TALENs, are emerging as a new technique to study specific genes of interest.

2.1. Morpholino knockdown

RNAi mediated gene knockdown technique is widely used in a variety of model systems from cell cultures to mammalian systems. Although there have been a few successful efforts to apply this technique in the zebrafish, RNAi mediated gene knockdown is not widely applicable in the zebrafish (Kelly and Hurlstone, 2011; Oates et al., 2000). Instead, knockdown of gene function by morpholino antisense nucleotides is the most widely used reverse genetics technique to study genes of interest in the zebrafish (Bedell et al., 2011). Morpholinos are chemically synthesized nucleotides with morpholine rings, thereby resistant to breakdown by nucleases (Nasevicius and Ekker, 2000). Usually morpholino sequences are designed to bind in the vicinity of the start codon to block initiation of translation or to splice acceptor sequences to cause abberantly spliced mRNAs.

Morpholinos are injected into freshly fertilized eggs and effectively block mRNA translation or splicing of target genes up to 5dpf, before becoming too diluted to efficiently interfere with translation. At this stage of development, most organs, including endocrines systems, are fully functional in zebrafish (Lohr and Hammerschmidt, 2011). The biggest advantage of morpholinos is their ease of use and quick read-out. Together with the high fecundity of zebrafish, morpholinos can be injected into several hundreds of embryos in one experiment and resulting phenotypes are readily observed in those embryos a few days later (Fig. 1). Furthermore, co-injection of a combination of up to three morpholinos can be performed to achieve double and triple knockdowns of genes of interest (McNulty et al., 2005). In addition, concentrations of morpholinos injected can be titrated in order to investigate dose-dependent resulting phenotypes. One exemplary application to the field of endocrinology is the downregulation of the zebrafish *irx3a* orthologue (Ragvin et al., 2010). This transcriptional regulator is expressed in the kidney, hypothalamus and endoderm derived tissues. Morpholino-based downregulation of *irx3a* increased the mRNA expression level of ghrelin while decreased that of insulin, demonstrating an involvement of irx3a in the regulation of - and -cells of the pancreas.

Though highly efficient, knockdown experiments using morpholinos require optimization of the injection dose and careful observation of resulting phenotypes as there is a potential risk of off-target effects, which may cause unspecific phenotypes (Eisen and Smith, 2008). Also, the effectiveness of the downregulation needs to be carefully assessed, e.g. by demonstrating the reduction or absence of the target protein in western blots. Keeping these experimental precautions in mind, morpholino-based knockdown is one of the most advantageous techniques the zebrafish has to offer. In addition to the conventional knockdown of translation of target mRNAs, morpholinos can also be used to block the maturation of microRNAs (miRNAs) as well as to inhibit their binding to the target mRNAs, facilitating assessment of target genes of miRNAs (Staton and Giraldez, 2011).

2.2. TILLING

TILLING (targeted induced local lesions in genomes) is a combined method of forward and reverse genetics based on chemical mutagenesis to isolate mutants harboring point mutations in genes of interest (Wienholds et al., 2003). Firstly, male fish are mutagenized by ENU (N-Nitroso-N-ethylurea), a chemical mutagen, and subsequently, mutations in target genes are sought by sequencing the target regions from genomes extracted from the mutagenized individuals (Fig. 2A). Although TILLING is a technically efficient method to obtain fish with mutations in genes of interest, since it requires a large-scale sequencing of a large pool of individual fish, it is difficult to conduct as a routine technique in most individual laboratories. Therefore, community-based zebrafish mutation projects by TILLING were established by several consortia (e.g. Sanger Institute) and the information and resources are shared via the zebrafish community database (http://zfin.org/).

2.3. ZFNs

Zinc finger nucleases (ZFNs) are artificial endonuclease enzymes originally introduced as a hybrid restriction enzyme (Kim et al., 1996). ZFNs consist of two domains. One is the zinc finger motif domain that recognizes a specific sequence of genomic DNA and the other is the *Fok*I restriction enzyme domain that cuts double-stranded DNA (Ekker, 2008). A DNA double-stranded break

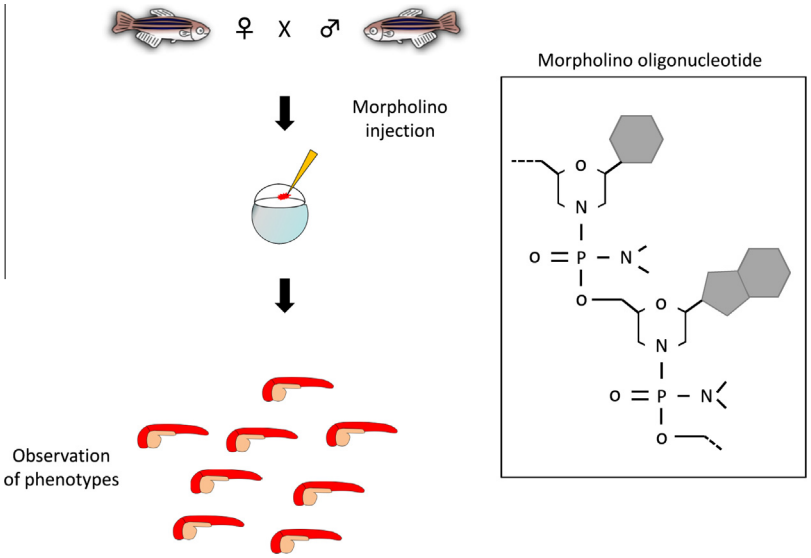

Fig. 1. Overview of a knockdown experiment by morpholino antisense nucleotides. A chemical structure of a morpholino is shown. Morpholinos are normally injected into fertilized embryos at the one cell stage. Effects of knockdowns of target genes are readily observed in these embryos up to 5dpf.