European Journal of Pharmacology 768 (2015) 77–86

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Review

Zebrafish models of cardiovascular diseases and their applications in herbal medicine research

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ARTICLE INFO

Article history:
Received 8 July 2015
Received in revised form
2 October 2015
Accepted 16 October 2015
Available online 19 October 2015

Keywords:
Zebrafish
Chinese herbal medicine
Cardiovascular
Animal model
Review

ABSTRACT

The zebrafish (*Danio rerio*) has recently become a powerful animal model for cardiovascular research and drug discovery due to its ease of maintenance, genetic manipulability and ability for high-throughput screening. Recent advances in imaging techniques and generation of transgenic zebrafish have greatly facilitated *in vivo* analysis of cellular events of cardiovascular development and pathogenesis. More importantly, recent studies have demonstrated the functional similarity of drug metabolism systems between zebrafish and humans, highlighting the clinical relevance of employing zebrafish in identifying lead compounds in Chinese herbal medicine with potential beneficial cardiovascular effects. This paper seeks to summarise the scope of zebrafish models employed in cardiovascular studies and the application of these research models in Chinese herbal medicine to date.

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1. Introduction

The zebrafish (*Danio rerio*) is small tropical freshwater pet fish that has been traditionally used for developmental biology, embryology and toxicology research (Streisinger et al., 1981). Due to a

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http://dx.doi.org/10.1016/j.ejphar.2015.10.031
0014-2999/

number of advantages of zebrafish over other animal models (Bahramsoltani et al., 2009), the use of zebrafish models has been extended to drug discovery and pathophysiological and pharmacological studies of human diseases (Hung et al., 2012). Adult zebrafish are only 3–4 cm long, making them suitable for laboratories with limited space. An adult female zebrafish produces several hundred eggs at weekly intervals. The eggs are fertilised externally by the male fish and the embryos develop rapidly and independently of the mother. A recognisable vertebrate body with a primitive vascular system and a beating heart, consisting of the sinus venosus, atrium, ventricle and bulbus arteriosus connected in series, can be observed 24 h post fertilisation (hpf). By 120 hpf, other organs such as the brain, liver, pancreas and kidney are fully developed (Stainier and Fishman, 1994). Moreover, the optical transparent embryos allow direct visual observation of organ development with minimal invasive manipulations. These features have made zebrafish an efficient research model suitable for high-throughput studies.

The cardiovascular and nervous systems of the zebrafish are anatomically and physiologically similar to those of mammals. Its genomic resemblance to humans is striking, with approximately 87% similarity (Lieschke and Currie, 2007). The high degree of conservation in pharmacological responses observed between zebrafish and human indicates similarity of amino acid sequences of the proteins and drug binding sites (Milan et al., 2003). A wide range of cardiovascular mutant phenotypes have been identified so far using forward genetic approaches (Beis et al., 2005; Chen and Fishman, 1996; Stainier and Fishman, 1994). Because of the accessibility and high sensitivity of the fertilised eggs to small molecule treatment, recent studies have focused on a reverse genetic approach by direct manipulation or modification of the gene of interest in zebrafish embryos using various tools such as morpholinos oligonucleotide knockdown (Nasevicius and Ekker, 2000), transcription activator-like effector nucleases (Cade et al., 2012), zinc-finger nucleases (Foley et al., 2009) and the clustered, regularly interspaced, short palindromic repeats associated (Cas) system (Hwang et al., 2013). Overall, zebrafish provide an effective and accessible *in vivo* system, which combines biological complexity with the ability for higher-throughput screening.

Chinese herbal medicine (CHM) is one of the main modalities of traditional Chinese medicine (TCM) and has been used for the treatment of cardiovascular diseases (CVD) over thousands of years. Although significant progress has been made in CHM research (Wang et al., 2015a; Zhong et al., 2015), the mechanisms of action underlying many CHMs remain unclear. CHM is frequently prescribed as a multi-herb formulation yielding pleotropic pharmacologic actions. Chinese herbs have also been regarded as an important resource of new lead compounds in drug discovery (Li et al., 2008). With the increasing popularity of CHM, detailed and rigorous pharmacological and toxicological research on complex Chinese herbal formulations and their bioactive ingredients using robust and high-throughput models are needed.

Complementing the traditional use of rodent models, the use of zebrafish in pharmacology studies and drug discovery has gained popularity in recent years, due to its ease of maintenance, genetic manipulability and ability for high-throughput screening. In this article, we will review the zebrafish models currently used in cardiovascular studies and the applications of these models in CHM research.

2. Genetic techniques used in zebrafish

With the ease of gene manipulation and a high degree of similarity to human genome (Lieschke and Currie, 2007), zebrafish has proven to be a worthy model in elucidating genetic program underlying cardiovascular development and pathogenesis. Over hundreds of mutant genes related to cardiovascular development and diseases have been identified from large-scale mutagenesis screening using classic/forward genetics methods. In this approach, random mutations are induced by irradiation or chemical treatment using ethylnitrosourea (ENU, a methylating agent) and the subsequent F2 and F3 generations are screened for developmental/diseased phenotypes. Genes responsible for these phenotypes are then identified by positional cloning (Amsterdam, 2006). For example, mutation named as *pandora* was identified to affect the development of heart valves (Dooley and Zon, 2000), while mutations of *futka* and *titin* were identified for their role in heart tube formation and cardiac contractility, respectively (Gerull et al., 2006; Knoll et al., 2007). Although this forward genetic approach has been successful in broadening our insight into the genetic regulation of disease development and providing models for further investigation of therapies for these diseases, recent studies have been largely conducted using reverse genetic methods.

Reverse genetics refers to the study of the role of a known gene. This is achieved by assessing a specific genotype by loss-of-function or other manipulation of a gene or known sequence. Gene 'knock-down' can be achieved by morpholino antisense oligonucleotides injection into fertilised egg. The morpholino can be designed to any mRNA sequence of interest, and once injected into a fertilised egg, it acts as RNA mimicking and binding to the target mRNA to prevent translation or induce aberrant splicing for 3–5 days (Nasevicius and Ekker, 2000). This method provides an easy, fast and relatively cheaper way to evaluate the candidate gene of interest. However, this method suffers from several disadvantages, such as short knock-down period, off target effects and incomplete loss-of-function of the target. Unlike morpholino injection, permanent loss-of-function is achieved by mutation of target gene of interest using techniques such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs).

ZFNs are widely used reagents for genome manipulations and have been shown to induce target knockout in zebrafish (Doyon et al., 2008). ZFNs are artificial proteins consisting of a zinc finger array fused to a non-specific nuclease domain. It works *via* breaking down of double-strand DNA at specific genomic loci and an introduction or deletion results from the normal cellular repair machinery pathway at the site of the breaks (Foley et al., 2009; Maeder et al., 2008). TALENs, on the other hand, could provide advantages over ZFNs with the ability to target a larger range of DNA sequences (Sander et al., 2011). Although these methods are more time-consuming (over several generations of zebrafish), the permanence of these mutations allows phenotypic assessment at later stage of the development, which cannot be achieved with the morpholinos injection. As a result, several stable transgenic zebrafish lines have been established and widely used for cardiovascular research. For example, Flk1:GFPnls transgenic expression of green fluorescent protein (GFP) in the nucleus of endothelial cells and GATA1:dsRed transgenic expression of fluorescent proteins in erythrocytes (Fig. 1) have been used in the study of vascular anatomy and visualisation of blood flow respectively (Dooley and Zon, 2000; Lawson and Weinstein, 2002).

3. Zebrafish as a tool for cardiovascular research

Owing to its unique characteristics and the availability of advanced genetic technology, zebrafish has become an attractive model for the study of the pathogenesis and biological/pharmacological activities of drugs for CVDs. Similar to other animal models, wild-type zebrafish do not spontaneously develop CVDs mimicking the human's conditions; most of the current models rely on chemical interventions or genetic modifications. In this