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Review

It's time to swim! Zebrafish and the circadian clock

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ABSTRACT

The zebrafish represents a fascinating model for studying key aspects of the vertebrate circadian timing system. Easy access to early embryonic development has made this species ideal for investigating how the clock is first established during embryogenesis. In particular, the molecular basis for the functional development of the zebrafish pineal gland has received much attention. In addition to this dedicated clock and photoreceptor organ, and unlike the situation in mammals, the clocks in zebrafish peripheral tissues and even cell lines are entrainable by direct exposure to light thus providing unique insight into the function and evolution of the light input pathway. Finally, the small size, low maintenance costs and high fecundity of this fish together with the availability of genetic tools make this an attractive model for forward genetic analysis of the circadian clock. Here, we review the work that has established the zebrafish as a valuable clock model organism and highlight the key questions that will shape the future direction of research.

1. Introduction

1.1. Background

An immense amount of progress has been made recently in our understanding of the basic mechanisms and function of the vertebrate circadian clock. The major catalysts for this progress have been the success of the genetic dissection of the clock mechanism in *Drosophila* [1] together with the fundamental similarities between vertebrate and fruit fly clocks. Furthermore, by applying powerful mouse genetics tools, detailed insight into the precise function and organization of vertebrate circadian clock components has been gained [2]. It is therefore not intuitively obvious what we could gain at this point from studying the circadian clock molecular mechanism in an alternative vertebrate model such as the zebrafish. This review attempts to give an overview on the current knowledge that has been gathered in zebrafish and show how the biology of this model actually makes it eminently well suited to explore the circadian clock.

1.2. Zebrafish as a model

The original interest in zebrafish as a vertebrate model system came not from the circadian clock – but from the field of embryology and developmental biology [3,4]. Several aspects of its basic biology make it inherently ideal for these areas of study. For one, it has small, completely transparent embryos that develop rapidly in an egg-shell or chorion. Development from the single fertilized cell to a moving, recognizable vertebrate embryo with a central nervous system and most organ systems normally takes just 24 h. Furthermore, the embryos develop externally and so the whole developmental process can be watched non-invasively in a petri dish under the microscope. Combined with the ability to establish transgenic lines expressing fluorescent reporter genes, the zebrafish is widely regarded as an excellent model for live imaging in vivo [5] (Fig. 1).

Another major advantage of the zebrafish is its proven utility for large-scale forward genetic screens [3,4]. Again, certain features of its biology make it a more attractive model than mice for such experiments. Firstly, reproduction is relatively simple to establish in the lab. If a single pair of male and female zebrafish is left together overnight in a small tank of water, soon after "lights on" the following morning, the fish typically lay hundreds of eggs. Secondly, the adults are hardy, small (2–3 cm long), reach sexual

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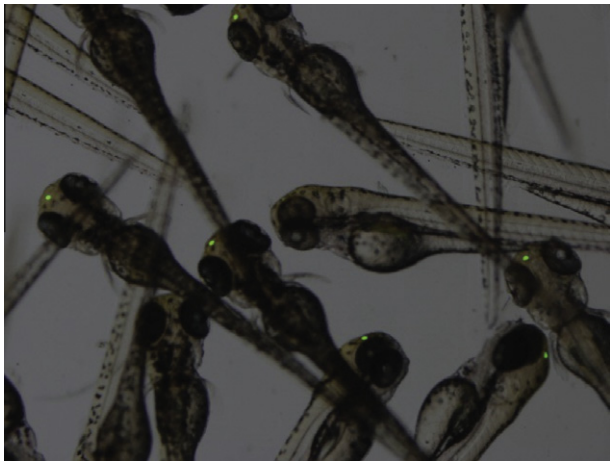

Fig. 1. A group of living transgenic zebrafish larvae visualized with fluorescence microscopy. Three day old transgenic (*aanat2*:EGFP)Y8 zebrafish larvae exhibit specific EGFP expression in the pineal gland as early as 24 h post fertilization (hpf). EGFP is expressed under the regulation of the pineal specific *aanat2* promoter and the PRDM. This line was generated in order to study the regulation of *aanat2* and of mechanisms underlying specific gene expression in the pineal gland [90]. Their optical transparency makes early life stages of zebrafish perfectly well suited for in vivo imaging applications.

maturity after 2–3 months and can be raised at high density at very low cost. Various protocols have now been established for mutagenesis including the use of chemical mutagens and retroviral insertion and also for the subsequent mapping of the mutated gene [5].

The last few years have seen the use of zebrafish expanding from its traditional user base and being applied to study diverse biomedically relevant aspects of biology including behaviour and physiology [6,7]. This is primarily the result of its low cost, its proven utility for forward genetics, the many similarities in basic physiology between mammals and fish, and, for the early developmental stages, fewer ethical concerns. Furthermore, the impressive capacity of most zebrafish tissues to regenerate following injury has attracted considerable attention from research aimed at understanding and treating certain human diseases such as heart and neurodegenerative diseases and cancer [7,8].

1.3. Chronobiology and the zebrafish

The attention of chronobiologists originally turned to zebrafish many years ago [9,10]. This was during the "dark ages" of our knowledge of the molecular basis of the vertebrate clock. At that stage our understanding of the workings of the *Drosophila*, *Neurospora* and cyanobacterial circadian clocks was far from complete. Furthermore, no vertebrate circadian clock genes had been cloned and so the nature of the molecular mechanism of the circadian clock in vertebrates was a complete mystery. Given the clear success of using forward genetics to identify the first clock mutants and genes in non-vertebrates, and the difficulties to perform large scale genetic screening on mice, the zebrafish seemed an ideal model to apply in the quest to identify and characterize the first clock mutants in a genetically tractable vertebrate species. Furthermore, given its extensive characterization in many developmental biology studies, it held great promise as a model to trace the origin of the circadian clock during embryogenesis. However, with the availability of the first molecular tools to study the core circadian clock, it soon became apparent that zebrafish offered many more advantages. Notably, the peripheral clocks of this species are directly entrainable by light [11]. This contrasts with the situation in mammals but resembles the peripheral clocks of *Drosophila* [12]. This direct light sensing property was also encountered in cell lines derived from zebrafish embryos [11].

Another major attraction of the zebrafish relates to its pineal gland. In mammals, light input to the clock is perceived uniquely by a subset of intrinsically photosensitive retinal ganglion cells in the eye. This photic information reaches the central oscillator in the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract, induces transcriptional changes in clock genes (e.g., *period1* and *period2*), and so synchronizes rhythmic neuronal activity [13,14]. Signals from the SCN then regulate the activity of many other targets, including melatonin synthesis in the pineal gland [15]. In non-mammalian vertebrates, including zebrafish, the pineal gland contains all elements required for photic entrainment and circadian rhythm generation: it is photoreceptive and contains an intrinsic circadian oscillator [16,17]. Fish pineal cells are classical photoreceptor cells with structural and functional similarities to retinal photoreceptors. Pineal and retinal photoreceptor cells share a similar set of genes, or, in certain cases, paralogs [18]. The fish pineal contains an intrinsic circadian clock that drives rhythmic synthesis of the hormone melatonin. Melatonin levels are high at night and low during the day as a result of regulated transcription and stability of serotonin-*N*-acetyltransferase (AANAT), the key enzyme for melatonin synthesis. Zebrafish, like other teleosts have two aanat genes: *aanat1* that is expressed predominantly in the retina and *aanat2* that is expressed in the pineal gland and to a limited extent in the retina [18,19]. Activity of this enzyme is dictated by the circadian clock and also shows a rapid suppression in response to illumination during the night [20,21]. Thus the pineal gland is considered to serve as central pacemaker: transducing environmental light information into a neural and a neuroendocrine signal. Many studies now focus on identifying the control mechanisms directing the first appearance of rhythmic melatonin synthesis during development, its regulation by the clock and light as well as pineal-specific patterns of gene expression.

2. Clock genes in zebrafish

2.1. The circadian clock mechanism in vertebrates

There are many fundamental similarities between the well-studied *Drosophila* circadian clock and the vertebrate clock mechanism (see Fig. 2) [1,2]. At the core of both clocks is a transcription-translation feedback loop that cycles in a period of approximately 24 h [22,23]. More specifically, in the case of vertebrates this regulatory loop consists of positive elements (Clock and Bmal) that drive the expression of negative elements (Period (Per) and Cryptochrome (Cry)) that in turn feedback to down-regulate their own expression and so allow the start of a new cycle of the feedback loop. The bHLH PAS domain transcription activators Clock and Bmal bind as heterodimers to E box elements located in the promoters of the *per* and *cry* genes and thereby induce their transcription. After translation, dimerization and translocation to the nucleus, the Per and Cry proteins physically interact with and thereby inhibit the transcriptional activation driven by the Clock:Bmal complex.

Control of translation, post-translational modifications, stability, turn over and sub-cellular localization all contribute to timing this feedback loop. Furthermore, the existence of an additional feedback loop that directs the rhythmic expression of the Bmal transcript tends to confer robustness and stability on the core loop [24].