

Identification of zebrafish magnetoreceptor and cryptochrome homologs

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Magnetoreception is essential for magnetic orientation in animal migration. The molecular basis for magnetoreception has recently been elucidated in fruitfly as complexes between the magnetic receptor magnetoreceptor (MagR) and its ligand cryptochrome (Cry). MagR and Cry are present in the animal kingdom. However, it is unknown whether they perform a conserved role in diverse animals. Here we report the identification and expression of zebrafish MagR and Cry homologs towards understanding their roles in lower vertebrates. A single *magr* gene and 7 *cry* genes are present in the zebrafish genome. Zebrafish has four *cry1* genes (*cry1aa*, *cry1ab*, *cry1ba* and *cry1bb*) homologous to human *CRY1* and a single ortholog of human *CRY2* as well as 2 *cry*-like genes (*cry4* and *cry5*). By RT-PCR, *magr* exhibited a high level of ubiquitous RNA expression in embryos and adult organs, whereas *cry* genes displayed differential embryonic and adult expression. Importantly, *magr* depletion did not produce apparent abnormalities in organogenesis. Taken together, *magr* and *cry2* exist as a single copy gene, whereas *cry1* exists as multiple gene duplicates in zebrafish. Our result suggests that *magr* may play a dispensable role in organogenesis and predicts a possibility to generate *magr* mutants for analyzing its role in zebrafish.

magnetoreception, MagR, cryptochrome, ortholog

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INTRODUCTION

Migration or navigation over long distances is a common behavior in diverse animals (Bauer and Klaassen, 2013). Migration by the chum salmon for mating and spawning, for instance, covers up to 4,000 km from the British Columbia's coast to the river Grays freshwater area (Yano et al., 1997). The Arctic Tern annually migrates an estimated distance of over 40,000 km between the northern and southern hemispheres for breeding and wintering (Egevang et al., 2010; Newton, 2010). Mechanisms underlying orientation

and migration have attracted considerable interest. Although it has been proposed that orientation and/or migration is dependent on many factors such as the Sun, the Moon (Dacke et al., 2003), polarised light (Cronin et al., 2006) and olfaction, an essential role of the Earth's magnetic field in orientation/migration has received increasing attention and wider acceptance (Kimchi et al., 2004). Extensive behavioral experiments show that animals have the ability to sense the earth's magnetic field for orientation and migration (Wiltschko and Wiltschko, 2006). This ability is called magnetoreception (MR) and is present in diverse animals ranging from invertebrates to vertebrates. Indeed, it has been documented that MR is necessary for orientation in diverse organisms such as the nematode (Vidal-Gadea et al.,

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2015), fruitfly (Gegear et al., 2008), pigeon (Mora et al., 2004), bat (Holland et al., 2006) and woodmice (Mather and Baker, 1981). Therefore, MR appears to represent a conserved sense underlying orientation and migration across animal phyla.

The molecular basis of MR has recently been established in *Drosophila* (Qin et al., 2016). In this organism, a flavo-protein called cryptochrome (Cry) and a compass protein called magnetoreceptor (MagR) form complexes essential for perceiving geomagnetic information, whereas Cry-deficient *Drosophila* does not show magnetosensitive behaviors (Gegear et al., 2008). The Cry-MagR complexes are capable of activation by magnets and sensing the surrounding magnetic field. Besides *Drosophila*, Cry is also considered to be a potential magnetoreceptor in many other species (Heyers et al., 2007; Liedvogel and Mouritsen, 2010).

Besides its essential role in animal orientation and migration, MR is also involved in certain physiological processes (Lai, 1996; Walleczek and Liburdy, 1990). In human, magnetotherapy is widely used to treat a variety of diseases including pain, musculoskeletal trauma, vascular and endocrine disorders, and has even led to the concept and practice of magnetomedicine (Markov, 2015; Wang and Wu, 1997). Understanding of mechanisms underlying magnet biology and physiology will provide invaluable information for magnetomedicine.

Laboratory fishes such as zebrafish (*Danio rerio*) and medaka are excellent vertebrate models for developmental biology and functional genomics. This study aimed at the identification of zebrafish *magr* and *cry* homologs as a prelude for magnet biology in this organism. We show that zebrafish has a single *magr* gene and 5 *cry* genes as well as

2 *cry*-like genes. All the zebrafish *magr* and *cry* genes exhibited ubiquitous RNA expression throughout life. Moreover, *magr* depletion through morpholino-mediated gene knockdown did not show strong impact on organogenesis and survival, suggesting the possibility of generating *magr* knockout zebrafish.

RESULTS

Identification of zebrafish *magr* and *cry* genes

In the zebrafish genome, there is a single *magr* gene predicting a protein of 129 amino acid residues. Multiple sequence alignment revealed that the predicted zebrafish MagR is maximally identical to MagR from other organisms including human (84%) and *Drosophila* (74%; Figure S1 in Supporting Information). A phylogenetic analysis clustered this protein with known MagR proteins (Figure 1A). Moreover, the zebrafish *magr* possesses a conserved genomic organization of human *MAGR* gene in that all exons of the coding sequence (CDS) share the same lengths (Figure 2A). Most importantly, a clear syntenic relationship exists between zebrafish and human in the *magr*-bearing chromosomal region (Figure 3). Thus, zebrafish *magr* is orthologous to the human *MAGR*.

A total of 7 *cry* genes were identified in the zebrafish genome, namely, *cry1aa*, *cry1ab*, *cry1ba*, *cry1bb*, *cry2*, *cry4* and *cry5*. Protein sequence alignment revealed that the protein products predicted by *cry1aa*, *cry1ab*, *cry1ba* and *cry1bb* are maximally identical to Cry1 of other organisms including human (over 75%) and *Drosophila* (over 37%; Figure S2 in Supporting Information); zebrafish Cry2 is maximally identical to Cry2 of other organisms including

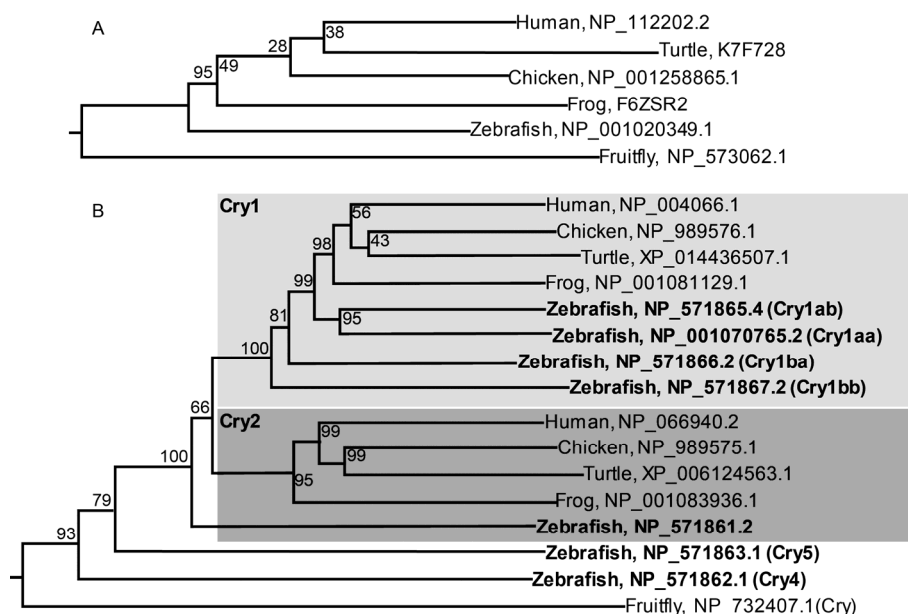


Figure 1 Phylogenetic tree of MagR and Cry proteins. The tree was generated by Neighbor-Joining algorithm. A, MagR proteins. B, Cry proteins. Sequence accession numbers are given following organisms. All zebrafish Cry proteins are remarkably in bold. Cry1 and Cry2 proteins are grouped in color.

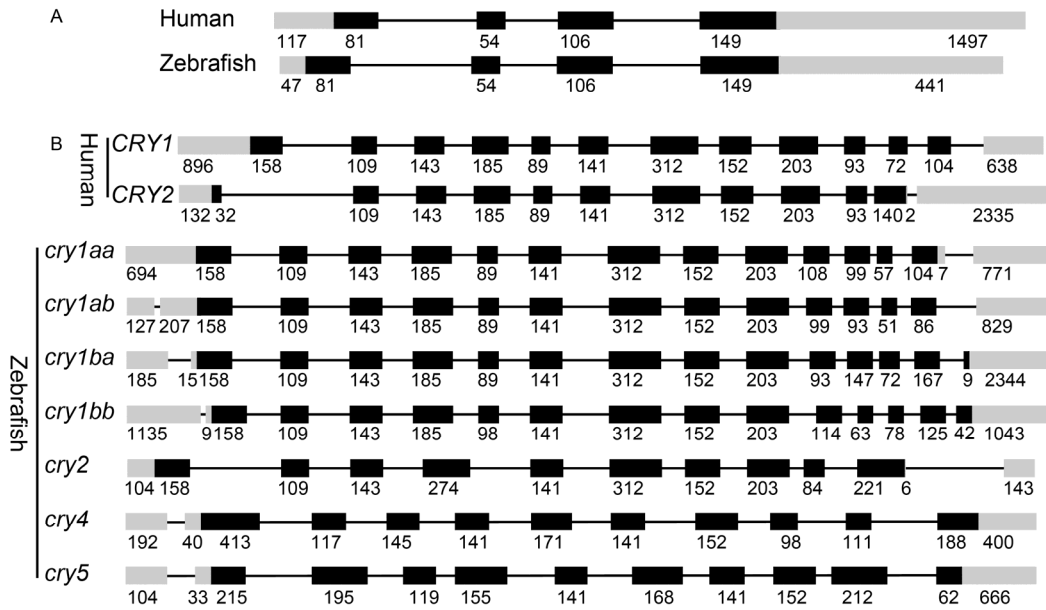


Figure 2 Gene structures of *magr* and *cry* genes in human and zebrafish. A, *magr* gene. B, *cry* genes. Exons are numbered with their sizes being given in bp. Notably, all *cry* genes, except for zebrafish *cry4* and *cry5*, have highly conserved exon-intron structures. Grey box, UTR (untranslated region); black box, CDS; line, intron.

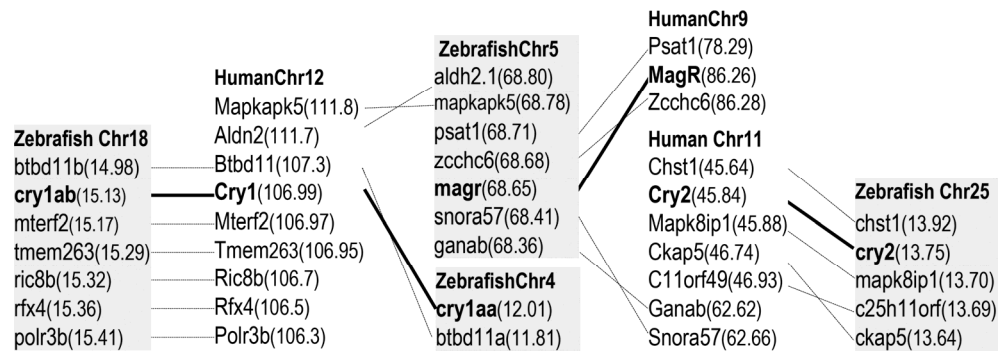


Figure 3 Chromosomal synteny of *magr* and *cry* genes. Shown are gene-bearing chromosomal regions that clearly have syntenic relationships between zebrafish and human. Parenthesis, chromosomal positions in million base pairs.

human (79%) and *Drosophila* (40%; Figure S2 in Supporting Information). The phylogenetic analysis displayed that Cry1 or Cry2 of different organisms were clustered together (Figure 1B). We also conducted splice site analysis of *cry* genes in zebrafish and human (Figure 2B). Notably, conserved exon structures were found in human *CRY1* and all zebrafish *cry1* genes (*cry1aa*, *cry1ab*, *cry1ba* and *cry1bb*) with the first 9 exons of CDS sharing the same lengths, except for exon 5 in *cry1bb*. Similarly, human *CRY2* and zebrafish *cry2* also possess certain coding exons (exon 2, 3 and exon 5–9) of the same lengths, which could also be found in *cry1* genes. Most importantly, zebrafish *cry1ab* and *cry1aa* exhibited syntenic relationship with the human *CRY1*, while zebrafish *cry2* showed syntenic relationship with the human *CRY2* (Figure 3). Therefore, zebrafish has two *cry1* orthologous (*cry1aa* and *cry1ab*) and two *cry1* homologous (*cry1ba* and *cry1bb*) to human *CRY1* and one

ortholog of human *CRY2*. On the contrary, zebrafish *cry4* and *cry5* were separated from any other *cry* genes in the phylogenetic analysis. They also have entirely different gene structures from any other *cry* genes. No syntenic relationship was found between zebrafish *cry4* or *cry5* and human *CRY* genes. Hence, *cry4* and *cry5* are paralogs of other zebrafish *cry* genes.

RNA expression patterns

RT-PCR was run to analyze RNA expression profiles of *magr* and *cry* genes. In adult zebrafish, transcripts of *magr* and all *cry* genes were examined in organs of the ectoderm (brain, eye and skin), mesoderm (heart, kidney and muscle), endoderm (liver) and female ovary. The expression of *magr* was detected in all organs (Figure 4). In developing zebrafish embryos, *magr* RNA was detectable already at the 2-cell stage and until hatching (Figure 4), suggesting its

maternal supply and continuous zygotic expression throughout embryogenesis. The transcripts of *cry* genes were highly abundant in the brain, eye and ovary but detectable also in the skin, heart and liver (Figure 4). In developing embryos, the transcripts of *cry1ab*, *cry1ba*, *cry1bb*, *cry2*, *cry4* and *cry5* were detected already at the 2-cell stage and also until hatching (Figure 4), suggesting their maternal inheritance and continuous zygotic expression throughout embryogenesis. In contrast, *cry1aa* exhibited a different expression pattern. Its RNA was absent at the 2-cell stage embryos and became barely detectable at the morula stage, but it was easily detectable from gastrula stage onwards (Figure 4), implying that *cry1aa* lacks maternal inheritance and commences zygotic expression from morula stage onwards. Taken together, a ubiquitous and high level RNA expression of *magr* accompanies differential expression patterns of *cry* genes in adult organs and developing embryos of zebrafish.

Dispensability of *magr* for normal organogenesis

To examine the role of *magr* in development, MO*magr*, which is a morpholino designed to prevent the translation of *magr* mRNA, was microinjected into zebrafish embryos at the 1-cell stage. Survival rate and phenotype of the injected embryos were observed for up to 72 hpf. MO*magr*-injected

embryos exhibited a survival rate similar to that of control embryos (Table 1). MO*magr* did not affect organogenesis, as MO*magr*-injected embryos resembled control embryos in normal development, evidenced by scheduled development of major organs such as the eye, somites, heart and brain in MO*magr*-injected embryos (Figure 5A–C'). Furthermore, we examined RNA expression of ectodermal marker genes (*fgf8a* and *otx2*), mesodermal marker genes (*gata4*, *gata5*, *has2* and *ntl*) and endodermal marker gene (*sall4*) at 9, 24 and 48 hpf and these genes displayed no obvious differential expression between MO*magr*-injected embryos and control embryos (Figure 5D). In addition, the abundant expression of *magr* and *cry* genes in eye and brain indicates their potential role in the development of nerve system. We detected the expression level of three early neuronal markers *sox2*, *elavl3*, and *gad1b* (Schmidt et al., 2013), and found that the expression of those marker genes between MO*magr*-injected and control embryos showed no obvious difference in 9, 30, and 48 hpf (Figure S4 in Supporting Information). Therefore, *magr* appears to be dispensable for embryonic survival and early organogenesis of zebrafish.

DISCUSSION

The interest in magnet biology is twofold. One is to under-

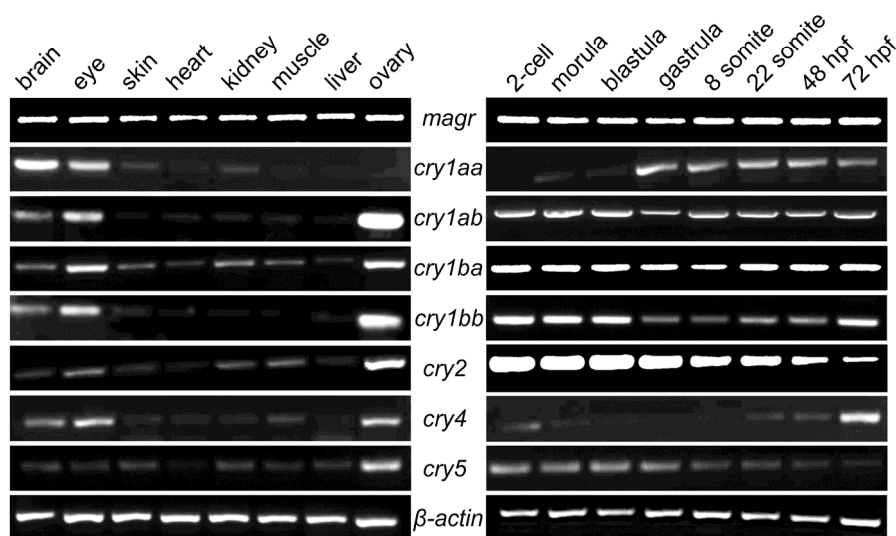


Figure 4 RNA expression of zebrafish *magr* and *cry* genes. left panel, Adult organs; right panel, Developing embryos. *magr* shows a high level of ubiquitous RNA expression in adult organs and developing embryos. All *cry* genes display RNA expression in the brain and eye as well as developing embryos.

Table 1 Development of MO*magr*-injected zebrafish embryos^{a)}

Injection	Embryos samples	Embryos survived, <i>n</i> (%) [*]			
		9 hpf	30 hpf	Total fry	Normal fry ^{**}
No	90	86 (96)	84 (93)	84 (93)	82 (98)
MO <i>magr</i>	85	71 (84)	71 (84)	70 (82)	68 (97)

a) Embryos were noninjected or injected at the 1-cell stage with 2 ng of MO*magr* and observed at indicated stage. *, % survival was obtained by comparisons between embryos sampled and embryos survived to stages of observation. **, % normal fry was obtained by a comparison between numbers of normal fry and total fry at hatching.

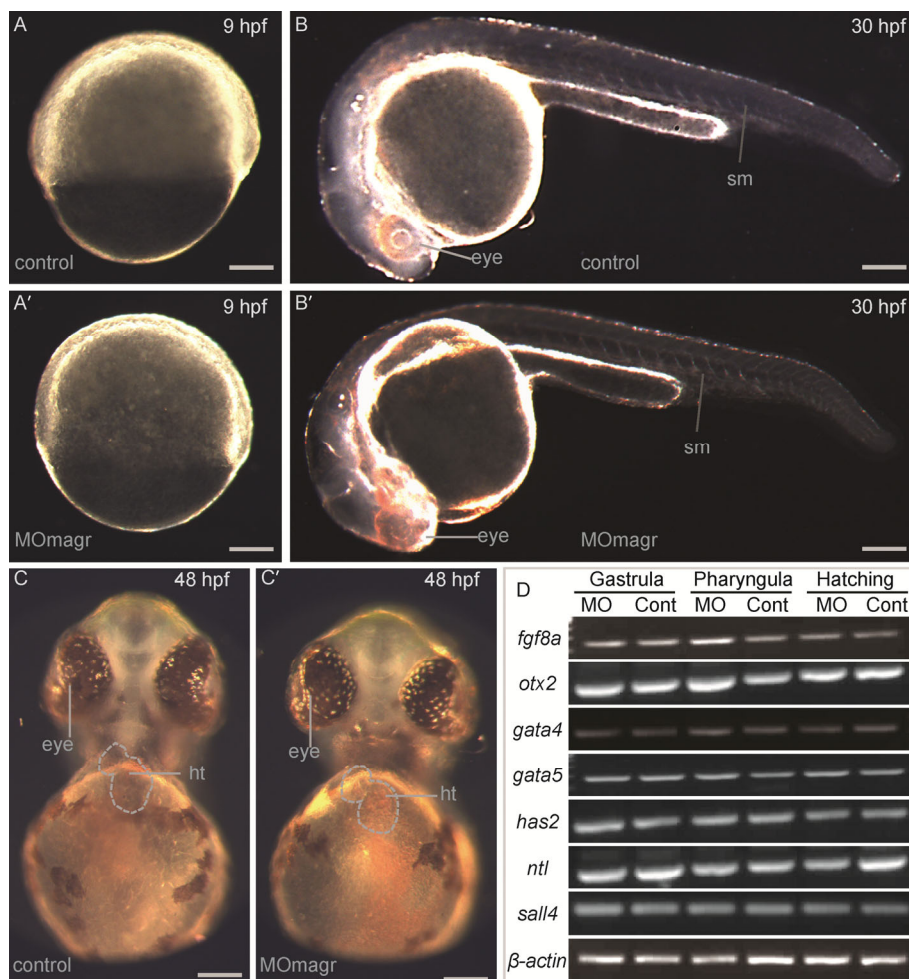


Figure 5 Normal development of *magr*-depleted embryos. Embryos were microinjected at the 1-cell stage with 2 ng of MOMagr and observed at 9, 30 and 48 hpf. A–C, Control embryos without injection; A'–C', MOMagr-injected embryos; Showing normal organogenesis as evidenced by normal gastrulation (A and A') and organs such as eyes, somites and heart (sm, B and B'; ht, C and C'). Scale bars, 200 μ m. D, RNA expression of three germ layer markers during embryogenesis. RNA expression of ectodermal marker genes (*fgf8a* and *otx2*), mesodermal marker genes (*gata4*, *gata5*, *has2* and *ntl*) and endodermal marker gene (*sall4*) displayed no obvious difference at gastrula (9 hpf), pharyngula (24 hpf), and hatching (48 hpf) stages between MOMagr-injected embryos (MO) and control embryos (Cont).

stand basic sciences via elucidating mechanisms underlying fundamental phenomena in nature such as animal migration. The other is to benefit human healthcare via elucidating mechanisms underlying the physiological role of magnetic fields towards the better practice of magnetomedicine. Zebrafish is an excellent model for developmental biology, functional genomics, and human diseases. In this study, we present data on the MagR-Cry system of zebrafish as a prelude to making use of this organism for magnet biology and physiology in vertebrates. We show that all the three human genes, namely *MAGR*, *CRY1*, and *CRY2*, have genuine orthologs in zebrafish.

We studied the expression pattern of *magr* and *cry* genes during developmental stages and in adult tissues of zebrafish. Base on previous studies, magnetic proteins are expected to express only in the eyes and brain. However, our RT-PCR result reveals that *magr* is expressed in all eight organs of zebrafish. We investigated human *MAGR*,

CRY1 and *CRY2* expression in the expression atlas of EMBL and discovered that human *MAGR*, *CRY1* and *CRY2* also express in the same eight organs. Our explanation about the ubiquitous expression of *magr* and *cry* genes is that MagR-Cry complexes act as a magnetoreceptor in brain and eye, while MagR or Cry could have other biological functions in others organs, such as mitochondrial and cytosolic iron-sulfur cluster biogenesis (Muhlenhoff et al., 2011; Sheftel et al., 2012).

Using bioinformatics technique, we confirm that *magr* and *cry2* are orthologs of human *MAGR* and *CRY2*; *Cry1aa* and *cry1ab* are the orthologs of human *CRY1*; *Cry1ba* and *cry1bb* are the homologs of human *CRY1*; the *cry*-like genes, *cry4* and *cry5*, are the paralogs of *cry* genes in zebrafish. In the representative species we have investigated, only one highly conservative *magr* gene exists in their genomes, implying that the *magr* gene keeps the original function. However, *cry* gene has been duplicated during the

process of evolution, especially in zebrafish. The 7 *cry* genes found in zebrafish exhibit divergences in protein sequences, gene structures and expression patterns, implying that these genes may have different functions. In light of the previous study, there are two main functions of Cry proteins in animals. One function is to regulate circadian rhythms as components of the circadian pacemakers (Miyamoto and Sancar, 1998; Sancar, 2004; van der Horst et al., 1999; Zhu et al., 2008). The other is to act as a candidate light-dependent magnetoreceptor (Gegear et al., 2010; Maeda et al., 2012; Moller et al., 2004; Mouritsen et al., 2004). Interestingly, the *Drosophila* circadian clock hinges on the Cry-dependent magnetic sensitivity (Yoshii et al., 2009), suggesting that ancestry *cry* gene possesses both functions. In Zebrafish *cry1aa*, *cry1ab*, *cry1ba* and *cry1bb*, can function in circadian rhythms while *cry2* and *cry4* cannot (Kobayashi et al., 2000; Liu et al., 2015), suggesting that *cry2* and *cry4* might function as magnetoreceptors. It has been demonstrated that magnetosensitivity can be found in human and cockroaches Cry2 (Bazalova et al., 2016; Foley et al., 2011), and in pigeon and garden warbler Cry4 (Liedvogel and Mouritsen, 2010; Qin et al., 2016). We conducted protein sequence alignment between zebrafish Cry proteins with pigeon Cry4 and found that zebrafish Cry4 is maximally identical to pigeon Cry4 (70%). Thus *cry2* and *cry4* could be good target genes in future investigation of zebrafish magnet biology.

Previous study reported that knocked down *magr* (*iscal*) results in profound anemia without affecting erythroid specification in zebrafish embryos (Nilsson et al., 2009), but no apparent effect on organogenesis and survival has been elucidated. In our gene knockdown experiments, MO*magr* zebrafish did not display obvious abnormalities during early development when compared with control. The expression level of endoderm, mesoderm and ectoderm marker genes showed no evident difference between MO*magr* and control embryos (Figure 5D). In addition, we found that the expression levels of its ligand *cry* genes also present no obvious variation (Figure S3 in Supporting Information). CRY is reported to be a deep brain circadian photoreceptor in fruit fly (Emery et al., 2000), and sponge eyes produce Cry proteins to communicate information of the light environment to the nervous system (Rivera et al., 2012), suggesting that Cry genes may play potential roles in neural system during embryogenesis. However, the expression level of early neuronal markers *sox2*, *elavl3*, and *gad1b* between MO*magr*-injected and control embryos showed no significant difference. These results suggest that *magr* is dispensable for early organogenesis and that generating *magr* mutant to analyze its role in zebrafish is applicable. Such mutants could be used to study the physiological role of magnetic fields in zebrafish.

Magnetic fields have impacts on organismal life on the Earth and utility for treating diseases in magnetomedicine (Zhen and Xing-de, 2006). Transcranial magnetic stimula-

tion based on the use of pulsed magnetic field, for example, is effective to treat disorders including multiple sclerosis, myelopathy, epilepsy, and stroke (Lefaucheur, 2005; Rossi et al., 2009). The molecular basis and mechanism of magnetomedicine remain to be elucidated. The availability of animal models will be invaluable to study the molecular basis and mechanism for magnet biology and magnet medicine. Our results that the MagR and Cry components are conserved from zebrafish to human demonstrate the suitability of zebrafish as a model organism for magnet biology and magnet medicine in vertebrates.

MATERIALS AND METHODS

Fish

Work with fish was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Advisory Committee for Laboratory Animal Research in Singapore and approved by this committee (Permit Number: 27/09). Zebrafish were maintained under an artificial photoperiod of 14-h/10-h light/darkness at 28°C and staged as described (Kimmel et al., 1995).

Phylogenetic, splice site and conserved syntenic analysis

The *magr* and *cry* genes of zebrafish (*Danio rerio*), human (*Homo sapiens*), chicken (*Gallus gallus*), Chinese soft turtle (*Pelodiscus sinensis*), frog (*Xenopus tropicalis*) and fruitfly (*Drosophila melanogaster*) were retrieved from NCBI (<http://www.ncbi.org/index.html>) or Ensembl (<http://www.ensembl.org/index.html>). Exon boundaries of the coding regions within *magr* and *cry* genes were confirmed on NCBI. Multiple sequence alignments of the MagR and Cry proteins were generated using Clustal W (Thompson et al., 1994). The phylogenetic tree was constructed by Neighbor-Joining (NJ) with Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6.0) (Tamura et al., 2013). The phylogeny was tested by the bootstrap method with 500 replications. The substitution model was the Jones-Taylor-Thornton (JTT) model. Numbers indicate bootstrap values. Using SyMap 4.0 (Soderlund et al., 2006) and biomaRt function in Ensembl, we determined the orthologs and co-orthologs of zebrafish to human genes in the chromosomal regions flanking *CRY1*, *CRY2* and *MAGR*.

RNA extraction and PCR

Total RNA was extracted from zebrafish embryos and 3-month-old adult female zebrafish organs by using the TRIzol reagent from Invitrogen (USA) for cDNA synthesis as described (Fleige and Pfaffl, 2006). PCR was run in a 20 μ L volume containing 10 ng of cDNA reaction and primers listed in Table S1 in Supporting Information. PCR was run for 25 cycles (β -actin) and 32 cycles for other genes of 10 s at 95°C, 30 s at 60°C and 30 s at 72°C. The PCR products were analyzed on 1.0% agarose gels and documented on the

Synoptics Bioimaging system (UK). All RT-PCR reactions were repeated three times.

Morpholino oligo and microinjection

MOMagr (AGAAGGCUUUAGGAAAGUUGAACAU), a morpholino oligo designed to block the translation of zebrafish *magr* mRNA, was synthesized by GeneTools (USA) and dissolved in water at 1 mmol L⁻¹. Zebrafish embryos at the one-cell cell stage were injected with MOMagr at 2 ng per embryos using a microinjector (Eppendorf, Femtojet). Injected embryos and control embryos without injection were reared in zebrafish egg water and regularly observed for survival and phenotypes.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

- Figure S1** Sequence alignment of MagR proteins.
- Figure S2** Sequence alignment of Cry proteins.
- Figure S3** RNA expression of cry genes during embryogenesis.
- Figure S4** RNA expression of early neural marker genes.
- Table S1** Zebrafish Genes and primers used for RT-PCR analyses

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