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Identification of medaka magnetoreceptor and cryptochromes

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Magnetoreception is a hallmark ability of animals for orientation and migration via sensing and utilizing geomagnetic fields. Magnetoreceptor (MagR) and cryptochromes (Cry) have recently been identified as the basis for magnetoreception in *Drosophila*. However, it has remained unknown whether MagR and Cry have conserved roles in diverse animals. Here we report the identification and expression of *magr* and *cry* genes in the fish medaka (*Oryzias latipes*). Cloning and sequencing identified a single *magr* gene, four *cry* genes and one *cry*-like gene in medaka. By sequence alignment, chromosomal synteny and gene structure analysis, medaka *cry2* and *magr* were found to be the orthologs of human *Cry2* and *Magr*, with *cry1aa* and *cry1ab* being coorthologs of human *Cry1*. Therefore, *magr* and *cry2* have remained as single copy genes, whereas *cry1* has undergone two rounds of gene duplication in medaka. Interestingly, *magr* and *cry* genes were detected in various stages throughout embryogenesis and displayed ubiquitous expression in adult organs rather than specific or preferential expression in neural organs such as brain and eye. Importantly, *magr* knockdown by morpholino did not produce visible abnormality in developing embryos, pointing to the possibility of producing viable *magr* knockouts in medaka as a vertebrate model for magnet biology.

magnetoreception, MagR, cryptochrome, magnetogenetics

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INTRODUCTION

Dozens of experiments have now shown that diverse animal species, including bees, salmons, sea turtles and birds, have the ability to sense and utilize the Earth's magnetic field for orientation and migration over long distances (Wiltschko and Wiltschko, 1988; Wiltschko and Wiltschko, 2005). This ability is called magnetoreception (MR). However, the nature of MR still remains a contested topic. Several models have been proposed to illustrate its mechanism (Johnsen and Lohmann, 2008). Among these models, the most widely accepted one is the chemical compass model, which was first proposed by Schulten (Schulten and Weller, 1978; Mohseni et al., 2014), then later modified by others (Ritz et al., 2000; Möller et al., 2004; Maeda et al., 2008; Cai and Plenio, 2013). According to this model, cryptochromes (Cry), which belong to the DNA photolyase/cryptochrome family (Todo et al., 1996; Cashmore et al., 1999; Todo, 1999), play an essential role in MR. Cry was first found to be a crucial part of the circadian system and later reported as necessary for the magnetosensitive behavior in *Drosophila* (Gegear et al., 2008; Gegear et al., 2010). Recently, Xie and his colleagues have identified a crucial partner protein *Drosophila* CG8198, the homologue of bacterial iron-sulfur cluster assembly *Isca1,* complementary to Cry that enables polarity sensing of magnetic fields. They named it as magnetoreceptor (MagR) and proposed the Cry/MagR system, in which the protein complex of Cry and MagR mediates MR (Qin et al., 2016).

Besides its function in orientation and migration, MR may also contribute to biomedical research. For example, in

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neuroscience, magnetogenetics was recently reported as a promising noninvasive technique to control gene expression and cellular activity *in vivo* (Etoc et al., 2013; Etoc et al., 2015; Long et al., 2015; Stanley et al., 2015; Stanley et al., 2016; Wheeler et al., 2016). It shows many advantages over the currently widely used techniques such as deep-brain stimulation and optogenetics (Wichmann and Delong, 2006; Zhang et al., 2011), which require surgical implantation of a wire electrode or optical fiber (Kringelbach et al., 2007; Häusser, 2014). More importantly, the technique that combines the genetic targeting of MagR with remote magnetic stimulation has been reported (Long et al., 2015). The study of MR will provide a solid basis for this emerging and promising technique.

The medaka fish (*Oryzias latipes*) is an excellent vertebrate model for developmental biology and functional genomics. As a prelude to medaka magnet biology, our study aimed at the identification of magnetoreceptor and cryptochrome homologs in this organism. We identified a single *magr* gene, four *cry* genes and one *cry*-like gene. All these genes exhibited ubiquitous RNA expression throughout lifetime. Moreover, we provided evidence that morpholino-based *magr* gene knockdown did not affect medaka survival, pointing to the possibility to produce *magr* knockout animals in medaka as a vertebrate model for magnet biology.

RESULTS

Identification of medaka *magr* and *cry* genes

Using the published fruit fly MagR (*Drosophila* CG8198) sequence as the protein query to BLAST against National Center for Biotechnology Information (NCBI) non-redundant and Ensembl protein database, we identified one single medaka *magr* gene predicting a protein of 129 amino acid residues. The putative amino acid sequence was aligned with MagR from other species, after which a phylogenetic tree was constructed (Figure 1A). The topology is similar to a tree constructed previously for zebrafish (Zhou et al., 2016). Protein sequence alignment revealed that medaka MagR has a high degree of identity with human (85%) (Figure S1 in Supporting Information). Besides, medaka *magr* possesses a conserved exon/intron structure with human *MAGR* (Figure 2A). More importantly, there is a clear syntenic relationship between medaka and human in the *magr*-bearing chromosomal region (Figure 3). Thus, medaka *magr* is orthologous to the human *MAGR*.

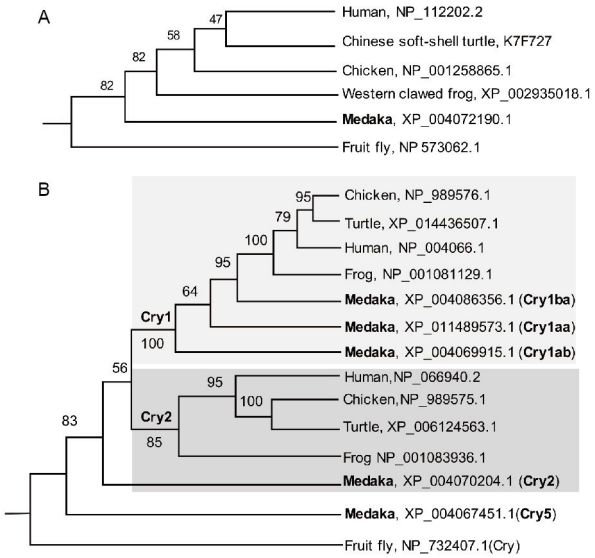

Figure 1 Phylogenetic tree by Neighbor-Jointing algorithm. A, MagR proteins. B, Cry proteins. Cry1 form one clade and Cry2 form another clade. Medaka Cry5 does not cluster with any other Cry proteins. Bootstrap values are given. Sequence accession numbers follow organisms. All medaka Cry proteins are shown in bold.

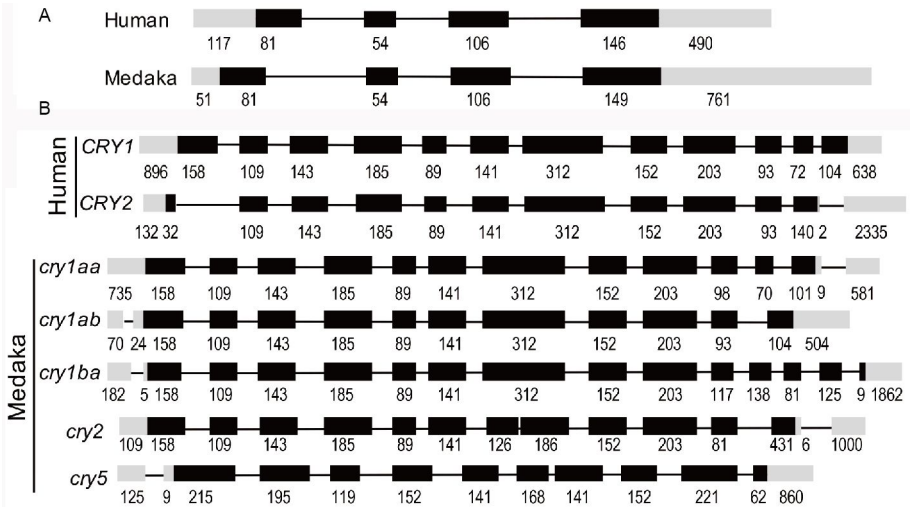

Figure 2 Gene structures of *magr* and *cry* in human and medaka. A, *magr* gene. B, *cry* genes. Exons are numbered with their sizes in bp. Notably, all *cry* genes except medaka *cry5* have highly conserved exon-intron structures. Exons are not drawn to scale. Light grey column, UTR; black column, CDS; line, intron.

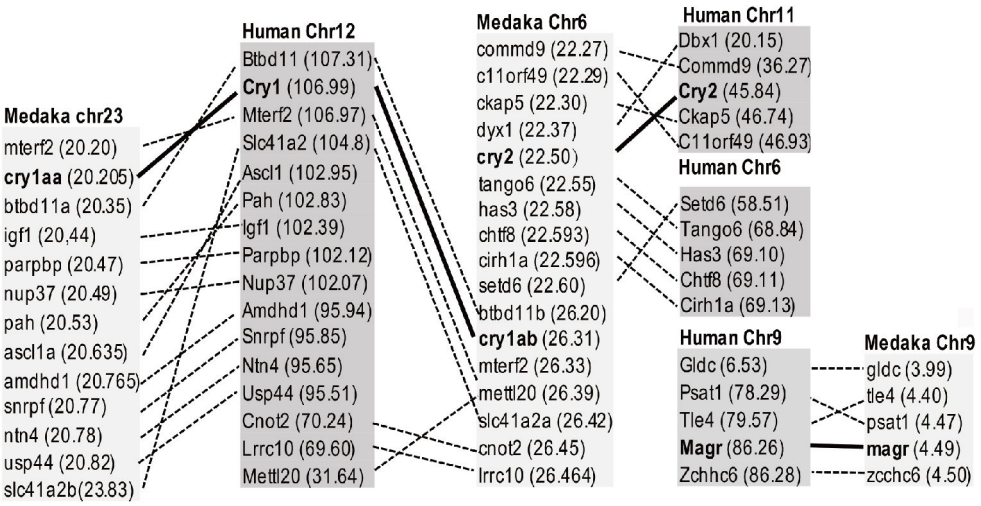

Figure 3 Chromosomal synteny of *magr* and *cry* genes. Shown are genes and chromosomal regions that clearly have syntenic relationships between medaka and human. Chromosomal positions are in parenthesis in million base pairs. Notably, *cry1ab* and *cry2* reside on the same chromosome, namely chromosome 6, within a small region only 4 Mb long in medaka.

Similarly, we also identified four medaka *cry* genes and one *cry*-like gene, namely *cry1aa, cry1ab*, *cry1ba*, *cry2* and *cry5*. By protein sequence alignment, medaka Cry1aa, Cry1ab and Cry1ba displayed higher degrees of conservation with human CRY1 (83%, 84% and 81%, respectively) than human CRY2 (81%, 80% and 80%, respectively); medaka Cry2 showed an identity of 75% to both human CRY1 and CRY2 (Figure S2 in Supporting Information). Phylogenetic analysis indicated that vertebrate Cry1 and Cry2 formed a sister clade (Figure 2A), implying they shared a common ancestry. By exon/intron structure analysis, conserved exon structures were found in medaka *cry1aa, cry1ab, cry1ba* and human *CRY1* and *CRY2* with eight exons of the same length at the 5′ end (Figure 2B). In addition, medaka *cry2* also displayed a similar structure, with the only difference of one 312 bp exon splitting into two exons of 126 and 186 bp. Furthermore, comparisons of chromosome locations revealed clear syntenic relationships between the *cry1*-bearing regions of human chromosome 12 and medaka chromosomes 6 and 23, suggesting that the presence of *cry1aa* and *cry1ab* in medaka fish was the consequence of whole genome duplication (Figure 3). In contrast, medaka *cry5* gene was separated with others *cry* genes on the evolutionary trees. Besides, *cry5* exhibited an entirely different gene structure with other *cry* genes and no syntenic relationship was found between medaka *cry5* and human *CRY* genes. Moreover, *cry1ba* still has not been mapped on any chromosomes. Taken together, we could conclude that medaka has two *cry1* orthologs (*cry1aa* and *cry1ab)* for human *CRY1* and one *cry2* ortholog for human *CRY2*.

RNA expression pattern

RT-PCR analysis was undertaken to examine *cry* and *magr* expression in developing embryos and adult organs (Figure 4). Embryos of nine different stages and 10 adult organs of ectoderm (brain, eye and skin), mesoderm (heart, kidney and muscle), endoderm (liver) and gonad (ovary and testis) were chosen. Interestingly, *magr* and *cry* were easily detected in early developing embryos before midblastula stage when bulk zygotic gene transcription takes place (Aizawa et al., 2003), demonstrating that they were maternally supplied. Furthermore, *magr* and *cry* genes were detected throughout embryogenesis and ubiquitously expressed in adult organs with differential expression patterns. Notably, *magr* and *cry5* both exhibited a high level of RNA expression in ovary.

magr is dispensable for organogenesis

We analyzed the effect of *magr* depletion on medaka development by injection of MOmagr, a Morpholino oligo that targets the medaka *magr* mRNA, to one-cell stage embryos. When injected at 2 ng, there was no apparent difference in survival rates between morphant and wildtype (Table 1). Normal development was observed for most MOmagr injected embryos (Figure 5). A higher dose at 8 ng didn't significantly increase the number of abnormally developing embryos and embryonic lethality, only leading to certain developmental retardation noticeable after epiboly (Figure S3 in Supporting Information), which might be the side effect of high dose morpholino injection. We chose seven marker genes for pluripotency and germ layers to examine their expressions in 2 ng MOmagr knockdown and control embryos at different stages by RT-PCR (Figure 6). For the seven detected genes, namely *nanog, oct4*, *cyt1, fgf8a, gata4*, *pax6a* and *otx2*, no obvious differential expressions between knockdown and control groups were detected. Therefore, *magr* appears to be dispensable for medaka survival and organogenesis.

Table 1 Development of MOmagr-injected medaka embryos[a)]

Injection	Embryo sample	Embryos survived, *n*(%)*					
		18 hpf	1 dpf	3 dpf	5 dpf	Total fry	Normal fry**
NO	76	73(96)	71(93)	70(92)	70(92)	70(92)	70(100)
MOmagr	80	72(90)	72(90)	70(88)	69(86)	68(85)	65(96)

a) Embryos were non-injected or injected at the 1-cell stage with 2 ng of MOmagr and observed at indicated stage. hpf, hours post fertilization. dpf, days post fertilization. *, % 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

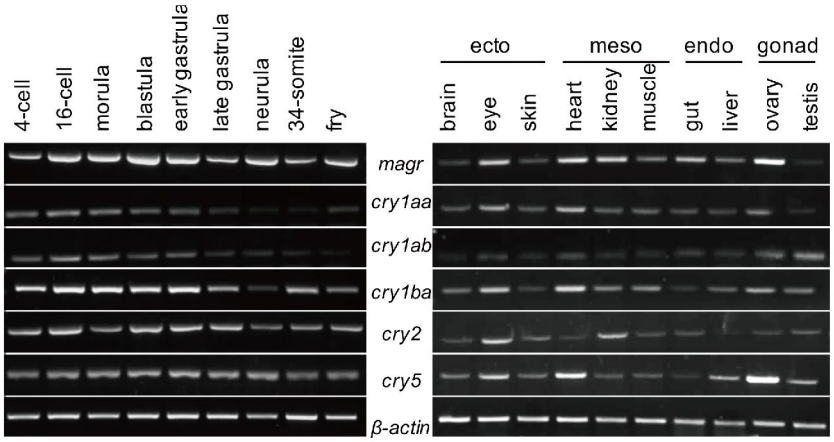

Figure 4 Expression of medaka *magr* and *cry* RNAs by RT-PCR. Left panel, Embryos at different stages. Right panel, Adult organs. *magr* and *cry* genes are maternally expressed and show a ubiquitous RNA expression in adult organs. ecto, ectoderm; meso, mesoderm; endo, endoderm.

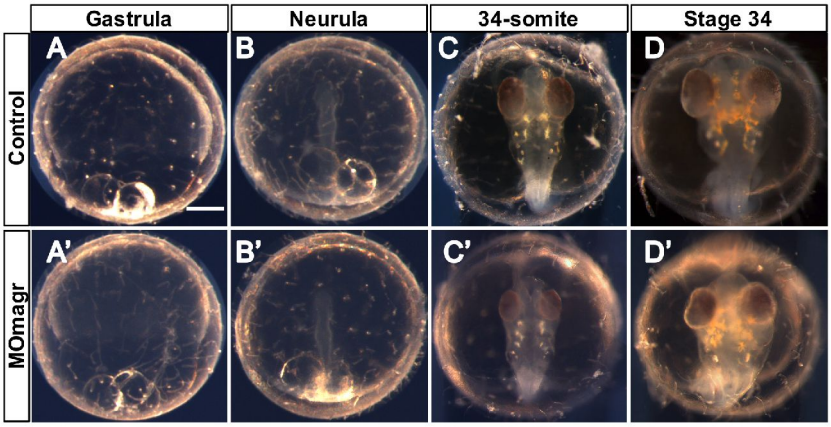

Figure 5 (Color online) *magr* knockdown by normal dose MOmagr has little effect on medaka organogenesis. Embryos were injected at the 1-cell stage with 2 ng of MOmagr and phenotypically observed during embryogenesis. A–D, Non-injected control embryos. A'–D', MOmagr-injected embryos. Scale bar 200 μm.

DISCUSSION

The discovery of Cry/MagR magnetosensor system is a huge step towards fully understanding the molecular mechanism of animal MR. In this present study, we present preliminary data on the Cry/MagR system of medaka as a prelude to making use of this organism for magnet biology.

In medaka and other representative species we have chosen, we identified one single *magr*. Compared with the recent study on zebrafish *magr*, medaka MagR not only shows an high identity to human and zebrafish MagR (85% and 87%, respectively), but also displays the same exon/intron structure with human and zebrafish (Zhou et al., 2016), implying that *magr* may reserve its original function among these animals. Furthermore, we found four *cry* genes and one *cry*-like gene in medaka, indicating the gene duplication events and the divergent roles of these genes. The ancestral *cry* occurred likely before the first round of vertebrate genome duplication (Liu et al., 2015). Fish has undergone one additional whole genome duplication event after divergence from the tetrapod vertebrate 450 Ma ago, leading to an additional duplication

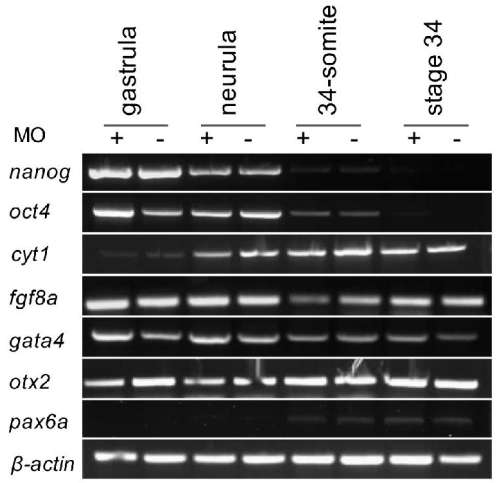

Figure 6 RT-PCR analysis of selected marker genes in MOmagr knockdown and control embryos. +,embryos were injected at the 1-cell stage with 2 ng of MOmagr; −, non-injected embryos.

of genes (Amores et al., 1998). Based on their phylogenetic positions and their functions in regulating circadian rhythm, animal Cry proteins can be divided into two types (Yuan et al., 2007). One is mammalian-like Cry, or repressive Cry. This type of Cry can inhibit CLOCK:BMAL1-mediated transcription, functioning mainly as negative regulators of the clockwork's transcriptional feedback loop in a light-independent manner (Griffin et al., 1999; Kume et al., 1999). The other type is *Drosophila*-like Cry, or non-repressive Cry. This type of Cry does not inhibit CLOCK:BMAL1-mediated transcription, but functions instead as a light-dependent suppressor of PER/TIM-mediated inhibition of transcription (Ceriani et al., 1999). Former studies on zebrafish have shown that there are six Cry proteins, with Cry1aa, Cry1ab, Cry1ba and Cry1bb being mammalian-like, and Cry2 and Cry4 being *Drosophila*-like (Kobayashi et al., 2000; Liu et al., 2015; Zhou et al., 2016). The evolutionary relationship between zebrafish *cry* genes and other animals were also explored, and different nomenclature systems were introduced in the above studies. Based on the phylogeny, Huag et al. has also proposed their new nomenclature for *cry* recently (Haug et al, 2015). Our study follows Ensembl's nomenclature system and is consistent with Zhou's study (Zhou et al., 2016). Compared with zebrafish, medaka has only three *cry1* genes (*cry1aa, cry1ab, cry1ba*) but no *cry4* gene. *cry1bb and cry4* may be lost due to subsequent gene losses after the third round of teleost genome duplication (Liu et al., 2015). The topology of our evolutionary tree for *cry* is also similar to Zhou's result (Zhou et al., 2016). *cry1* genes and *cry2* gene are clustered with *cry1* genes and *cry2* genes from tetrapods, respectively. Besides, *cry5* appears to diverge earlier than *cry1 and cry2* in the phylogenetic trees, and it has been reported to be clustered with 6-4 photolyases in previous phylogenetic studies (Oliveri et al., 2014), which can catalyze light-dependent DNA repair (Todo et al., 1993). Therefore, these results indicate that medaka *cry1aa*, *cry1ab*, *cry1ba* might be mammalian-like *cry* genes, and *cry2* might be *Drosophila*-like genes, whereas *cry5* belongs to photolyase genes. Furthermore, it has been reported that human CRY2, pigeon Cry4, garden warbler Cry3 and cockroaches Cry2 are involved in MR (Liedvogel and Mouritsen, 2010; Foley et al., 2011; Bazalova et al., 2016; Qin et al., 2016). Specifically, pigeon Cry4 and human CRY2 have shown the ability to form complexes with MagR and can be co-purified (Qin et al., 2016). Hence, medaka *cry2* might be a potential target for future medaka magnet biology study.

Further analysis by RT-PCR displayed that medaka *magr* and *cry* genes are maternally expressed, detectable throughout embryogenesis, and exhibited ubiquitous expression in adult organs rather than specific or preferential expression in neural organs such as brain and eye. A similar result was reported in zebrafish (Zhou et al., 2016). This implies the diverse roles of *magr* and *cry*. Unlike *cry*, which has already been reported to have other important functions such as circadian rhythm resetting and photosensitivity (Zhu et al., 2008; Chaves et al., 2011), the potential function of *magr* is to be explored. Since *magr* is the homologue of bacterial iron-sulfur cluster assembly *Isca1*, which is evolutionarily highly conserved and found in prokaryotic and eukaryotic organisms for the biogenesis of iron-sulfur cluster across species (Zheng et al., 1998; Schwartz et al., 2001; Vinella et al., 2009), it is predictable that *magr* may also have similar roles. For example, Nilsson et al. have reported that *Isca1* knockdown in zebrafish results in anemia (Nilsson et al., 2009). Moreover, the inhibition of *magr* expression in fruit fly has been reported to disrupt circadian behavior (Mandilaras and Missirlis, 2012). In medaka, we show that morpholino knockdown of *magr* did not affect survival rates of the medaka embryos at both 2 and 8 ng dose since most *magr* knockdown embryos survived and developed into fry. The retardation shown at 8 ng dose could be the nonspecific effects of high dose morpholino injection (Heasman, 2002).

In summary, we identified *magr* and *cry* genes and found the orthologue of human *MAGR, CRY1* and *CRY2* genes in medaka. Besides, *magr* knockdown by morpholino did not produce visible abnormality in developing embryos. The meaning of our study is two-fold. On one hand, even though human are widely assumed not to have magnetic sense (Phillips et al., 2010), there is consistent evidence of the influence of geomagnetic field on the light sensitivity of human visual system (Thoss et al., 2000; Thoss et al., 2002). It has also been verified that human CRY2 has the molecular capability to function as a light-sensitive magnetosensor (Foley et al., 2011). Besides, Xie's group has reported the co-purification of MAGR and CRY2 in human (Qin et al., 2016). Therefore, using medaka as a vertebrate model for magnet biology will not only boost our knowledge of animal MR but also cast light on understanding human MR. On

the other hand, magnetogenetics that combine the genetic targeting of MagR with remote magnetic stimulation has been developed as a promising method for non-invasive neuron stimulation, which might replace the invasive approaches such as optogenetics and deep-brain stimulation in neurobiology research (Long et al., 2015). Thus, our study of medaka magnet biology could provide a good model and help advance the technology of magnetogenetics.

MATERIALS AND METHODS

Fish

Work with medaka fish followed guidelines on the Care and Use of Animals for Scientific Purposes as outlined by the National Advisory Committee for Laboratory Animal Research in Singapore. Medaka strain Hd-rR was maintained under an artificial photoperiod of 14-h/10-h light/darkness at 26°C as described (Hong et al., 2011). Embryogenesis was staged as described (Iwamatsu, 2004).

Gene identification

BLAST searches by using protein sequences of the fruit fly MagR and Cry as queries against the NCBI non-redundant and Ensembl protein database led to the identification of putative genes orthologous to *magr* and *cry*. Six representative species were interrogated for each gene, namely, fruit fly (*Drosophila melanogaster*), human (*Homo sapiens*), chicken (*Gallus gallus*), Chinese soft-shell turtle (*Pelodiscus sinesis*), medaka (*Oryzias latipes*) and African clawed frog (*Xenopus laevis)* for *cry* or western clawed frog (*Xenopus tropicalis*) for *magr*.

Sequence Analysis

Multiple sequence alignment was conducted by using the Vector NTI suite 11 (Invitrogen, USA). Phylogenetic trees were constructed by using the MEGA6 package from a matrix of pairwise genetic distances according to the neighbor-joining (NJ) algorithm, and 1,000 trials of bootstrap analyses were used to provide confidence estimates for tree topologies. Genomic organization and chromosomal locations were explored by comparing the cDNA and corresponding genomic sequences in NCBI and UCSC genome browser. SyMap 4.0 (Soderlund et al., 2011) and biomart function in Ensembl were used to determine the orthologues of medaka to human genes in the chromosomal regions flanking *MAGR, CRY1*, and *CRY2*.

RNA isolation and RT-PCR analysis

Total RNA from medaka embryos and adult organs was isolated by using the Trizol reagent (Invitrogen, USA). All organs were from 3-month-old adult female adult except for testis. Synthesis of cDNA templates was primed with oligo(dT)$_{25}$ by using M_MLC reverse transcriptase (Promega, USA) (Hong et al., 2004). PCR was performed by using primers listed in Tables S1 and S2 in Supporting Information. β-actin was amplified from the same set of cDNA samples as an internal control. PCR was run in a 20-μL volume containing 10 ng of cDNA reaction for 26 cycles (β-actin) and 30 cycles (*magr, cry* and other selected marker genes) of 30 s at 95°C, 10 s at 55°C and 60 s at 72°C. The PCR products were separated on 1% agarose gels and documented with a bioimaging system (Synoptics, UK).

Morpholino oligo

MOmagr (5′-CGCTCGCACTAAGGAGGCAGACAT-3′), a Morpholino oligo designed to block the translation of medaka magr mRNA, was synthesized by GeneTools (Philomath, USA) and dissolved in water at 1 mmol L^{-1} as stock solution.

Microinjection

Medaka embryos at the one-cell cell stage were injected with 2 and 8 ng MOmagr per embryos. Injected embryos and control embryos without injection were reared in medaka embryo rearing medium 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 from six representative species.

Figure S2 Sequence alignment of Cryptochromes (Crys) from six representative species.

Figure S3 *magr* knockdown by high dose MOmagr has little effect on medaka organogenesis.

Table S1 Primers used for RT-PCR analysis

Table S2 Primers used for MOmagr knockdown RT-PCR analysis

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