Transposon-mediated targeted and specific knockdown of maternally expressed transcripts in the ascidian *Ciona intestinalis*

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Maternal mRNAs play crucial roles during early embryogenesis of ascidians, but their functions are largely unknown. In this study, we developed a new method to specifically knockdown maternal mRNAs in *Ciona intestinalis* using transposon-mediated transgenesis. We found that GFP expression is epigenetically silenced in *Ciona intestinalis* oocytes and eggs, and this epigenetic silencing of GFP was used to develop the knockdown method. When the 5′ upstream promoter and 5′ untranslated region (UTR) of a maternal gene are used to drive GFP in eggs, the maternal gene is specifically knocked down together with GFP. The 5′ UTR of the maternal gene is the major element that determines the target gene silencing. Zygotic transcription of the target gene is unaffected, suggesting that the observed phenotypes specifically reflect the maternal function of the gene. This new method can provide breakthroughs in studying the functions of maternal mRNAs.

Eggs store a wide variety of mRNAs. These maternal transcripts, or maternal mRNAs, play crucial roles in the developmental processes of multicellular organisms. In order to understand these early developmental stages, it is necessary to determine the functions of maternal mRNAs. Ascidians, a group of chordates, are a good model to investigate the role of maternal mRNAs in developmental processes. Ascidian eggs are typical mosaic eggs1, and the factors that determine cell fates and morphogenetic movement are prelocalized to specific parts of the egg2. Maternal mRNAs are potential candidates for these factors. Indeed, the maternal transcript of a gene named *macho1* determines the differentiation of muscle cells3. However, the functions of many ascidian maternal mRNAs remain unknown, mainly due to the limitation of techniques to investigate their functions.

In order to study maternal mRNAs, it is important to disrupt their functions. In ascidians, several approaches are currently used to disrupt maternally expressed genes. However, these approaches have disadvantages and are insufficient. For example, knockdown approaches, as represented by RNA interference (RNAi) and morpholino oligonucleotide (MO)—based knockdown, are convenient methods for disrupting maternal mRNAs of ascidians4,5. Generally speaking, RNAi has a disadvantage in that small RNAs can disrupt zygotic gene expression. Thus, it is difficult to determine whether the observed phenotype reflects the maternal or zygotic function of the gene if the target maternal gene has zygotic transcription. MOs are usually introduced into matured ascidian eggs to disrupt mRNA splicing or translation. Therefore, the functions of maternal genes that are already translated during oogenesis cannot be disrupted using MOs. Thus, it is important to establish a new method that efficiently and specifically disrupts ascidian maternal transcripts. Although forward genetics present one promising method, this approach requires extensive labor to isolate mutants. Screening maternal mutants takes one more generation than zygotic mutants, since it is necessary to create mutant females. Furthermore, if the mutation causes lethality during growth and development, maternal mutants cannot be obtained. This is also a disadvantage of knockout of *Ciona* genes using engineered nucleases6,7.

We recently established a method of germline transformation for *Ciona intestinalis* using a transposon *Minos*. With this method, we have created many transgenic lines that express green fluorescent protein (GFP) in a variety of tissues. By observing GFP expression in these lines, we noticed a curious phenomenon; namely, GFP expression in oocytes and eggs is epigenetically suppressed. Using this phenomenon, we established a new method to knockdown maternally expressed genes that does not affect zygotic mRNAs. Thus, we can specifically investigate...
the functions of maternal mRNAs even though some genes exhibit both maternal and zygotic expression patterns. This new method will provide breakthroughs in the study of maternal mRNA function in Ciona.

Results

GFP expression is epigenetically suppressed in Ciona intestinalis oocytes and eggs. Transgenic lines that express GFP in oocytes and eggs were created using the 5′ upstream regions of maternally expressed genes or by transposon-mediated enhancer detection that entraps enhancers for maternal gene expression. GFP expression was typically observed in only a few oocytes and eggs of these maternal GFP lines (Fig. 1a). The percentage of GFP-positive or GFP-negative eggs ranged from 0 to 100% among transgenic lines, even though the lines were created with the same transposon vector. Whole-mount in situ hybridization (WISH) showed that GFP mRNA was absent in GFP-negative eggs (Fig. 1b, c), suggesting that transcriptional or post-transcriptional regulation is a likely cause of maternal GFP suppression. Because Ciona oocytes and unfertilized eggs are diploid, these cells of GFP-transgenic lines must have the GFP gene. Indeed, when transgenic lines expressing GFP in both a maternal and zygotic fashion showed epigenetic GFP silencing in eggs, zygotic GFP expression was observed in animals that developed from GFP-negative eggs (Supplementary Fig. 1), suggesting that GFP-negative eggs contain an intact GFP gene. Thus, the absence of GFP expression in oocytes and eggs was caused by epigenetic gene silencing. In addition, zygotic GFP expression was comparable in animals derived from GFP-negative eggs and GFP-positive eggs, suggesting that suppressed GFP expression is specific for maternal expression but not zygotic GFP expression.

Knockdown of maternal Ci-pem mRNA. The aptly named gene posterior end mark (pem) encodes a maternal mRNA that localizes to the posterior end of eggs. Ciona intestinalis pem (Ci-pem) exhibits exclusive maternal expression throughout embryonic development15. Using the 5′ upstream region of Ci-pem, we created a transposon vector that drives GFP expression in oocytes and eggs. The 5′ upstream region of Ci-pem includes the 5′ untranslated region (UTR) and initiation codon of this gene. A fusion of the 5′ upstream region/5′UTR of a muscle gene Ci-TnI (which encodes Troponin I) with GFP was introduced next into the Ci-pem > GFP cassette (Fig. 2a). The Ci-TnI promoter drives GFP in muscle tissue but not in oocytes or eggs15. GFP expression from the Ci-TnI > GFP cassette was used as a marker to select transgenic animals during culture. Using this transposon vector, we created several transgenic lines expressing GFP in oocytes and eggs. Hereafter, these lines are called “pem > GFP lines”. As described above, GFP expression appeared in a mosaic pattern in oocytes and eggs in pem > GFP lines (Fig. 2b).

Progeny were obtained by crossing these pem > GFP lines with wild-type animals. When sperm from pem > GFP lines were crossed with wild-types eggs, the progeny showed normal embryogenesis and larval development (Fig. 2c). In contrast, when eggs of pem > GFP lines were crossed with wild-type sperm, many progeny exhibited abnormal embryogenesis (Fig. 2d). At the larval stage, their embryonic axis could not be recognized. The body of the abnormal larvae usually separated into two parts, one of which had vacuolated notochord cells (Fig. 2d). When the tissue differentiation of these animals was examined, the major tissues of the larval body, namely the epidermis, muscle, notochord, neural tissues and endoderm, had properly differentiated (Fig. 2e–i). However, their relative position was abnormal. In normal larvae, muscle and notochord cells were placed in the tail, while most endodermal cells localized in the trunk, separate from the muscle and notochord. In abnormal larvae, cells of the muscle, notochord and endoderm were clustered in the same position. This positional pattern looks like that of the vegetal hemisphere of 110-cell stage embryos. Because endodermal cells move toward the trunk by gastrulation that starts around the 110-cell stage, the abnormality of pem > GFP embryos is likely associated with defects in gastrulation.

The ratio of normal and abnormal embryos derived from pem > GFP eggs differed among transgenic lines (Table 1) and correlated with the ratio of GFP silencing. For example, all of the eggs of pem > GFP line 2, which did not express GFP, developed abnormally, while all of the eggs of pem > GFP line 9, which expressed GFP, developed normally. In concert to the correlation between GFP silencing and abnormal development, all of the normally developed larvae showed maternal GFP expression, while abnormal larvae never exhibited maternal GFP expression even though zygotic GFP expression from the Ci-TnI > GFP cassette was observed (Fig. 3a). Therefore, the abnormal development observed in pem > GFP lines is strongly associated with epigenetic GFP silencing in eggs.

To investigate the mechanisms by which embryos derived from pem > GFP lines become abnormal, we performed quantitative RT-PCR and WISH for Ci-pem mRNA (Figs. 3b–g). WISH showed that Ci-pem mRNA was reduced to undetectable level in the GFP-negative eggs of pem > GFP lines (Figs. 3b–f). Quantitative RT-PCR showed that the level of Ci-pem mRNA was reduced to approximately 2.3–21% of the level in wild-type eggs (Fig. 3g). The abnormal development observed in pem > GFP lines was ameliorated by introducing in vitro-transcribed Ci-pem mRNA (Fig. 3h), suggesting that abnormal development was specifically caused by loss of Ci-pem mRNA. We investigated the quantities of four maternal mRNAs,
including Ci-mT\(^{12}\), Ci-Nut\(^{13}\), Ci-Wnt5\(^{14}\), and Ci-POPK1\(^{15}\), in Ci-pem–knockdown eggs derived from pem > GFP line 2. None of these mRNAs were present at lower levels in Ci-pem–knockdown than in wild-type eggs (Supplementary Fig. 2a), suggesting that the effect of the knockdown is specific to this gene. These results suggest that Ci-pem is silenced through a mechanism associated with epigenetic silencing of GFP in Ciona eggs.

There was a possibility that the transposon vector might have been inserted into the genomic region near Ci-pem gene to disrupt its expression in the pem > GFP lines. To examine this possibility, we identified the insertions sites of three pem > GFP lines in which Ci-pem knockdown was observed (Supplementary Table 1). All integration sites in these lines are distant from the Ci-pem locus (the gene model of Ci-pem is KH.C1.755), suggesting that transposon insertion is not the cause of knockdown of Ci-pem.

Silencing of maternal mRNAs depends on their promoters and 5’ UTRs. To understand mechanisms how Ci-pem gene was silenced in pem > GFP transgenic lines, the elements in the transposon vector for pem > GFP lines (pMiCiTnIGCipemG) were exchanged and the effects of the variant vectors on gene knockdown were examined. We deleted the 5’ UTR of Ci-pem from the pMiCiTnIGCipemG vector (Fig. 4a), and three transgenic lines were established with this vector. None of the three lines showed the Ci-pem knockdown phenotype (Fig. 4b), suggesting the important role of the 5’ UTR for knockdown of this gene. Then, we exchanged the 5’ UTR of Ci-pem with that of another maternally transcribed gene encoding an Opsin-related protein, Ci-Nut\(^{13}\), and created three transgenic lines (Nut\(^{5’UTR}\) lines) with the vector (the marker cassette was exchanged with a cassette expressing DsRed, according to the result described below; Fig. 4c). Eggs of the Nut\(^{5’UTR}\) line did not exhibit significant reduction of Ci-pem mRNA (Fig. 4d,e). By contrast, the level of Ci-Nut mRNA was reduced to approximately 27% of the level in wild-type eggs (Fig. 4d). This reduction is less efficient than the knockdown of this gene observed in the Ci-Nut > GFP line, as described below (Fig. 5c). From these data, we conclude that the 5’ UTR is a major element for the selection of the target gene for silencing; however, it is possible that the promoter region also participates in efficient silencing.

Next, we tested the necessity of GFP for Ci-pem silencing. The reporter gene driven by the Ci-pem promoter was changed from GFP to Kaede (Fig. 4f), and four transgenic lines (pem > Kaede lines) were created with the vector. Among them, two lines showed silencing of Kaede expression in eggs (Supplementary Fig. 3a–d). Larvae derived from the Kaede-silenced eggs showed phenotypes characteristic of Ci-pem knockdown (Fig. 4g), suggesting that the reporter gene does not have to be GFP to achieve Ci-pem knockdown. Finally, the necessity of the marker cassette was investigated. The Ci-Tnl > GFP cassette was exchanged with a cassette expressing DsRed driven by the Ci-musashi Fr3 enhancer and Ci-TPO promoter (Fig. 4h)\(^{16}\). Neither the Fr3 enhancer nor the Ci-TPO promoter drove maternal expression. Among three transgenic lines created with this vector (DsRed\(^{marker}\) lines), two of them showed Ci-pem knockdown (Fig. 4i and Supplementary Fig. 3e–g), suggesting that the marker cassette does not affect maternal knockdown.

Knockdown of various maternal mRNAs. We tested whether maternal mRNAs other than Ci-pem can be knocked down using a similar experimental design. We chose two maternal genes as targets, namely Ci-mT and Ci-Nut. Ci-mT encodes a T-box transcription factor that shows maternal expression throughout embryonic stages\(^{12}\). To construct the knockdown vectors for these genes, the 5’ upstream region and 5’ UTR of the two genes were utilized (Fig. 5a,b). Ci-Nut shows zygotic expression in the neural tissues in

| Table 1 | Frequency of larvae derived from eggs of pem > GFP lines showing Ci-pem knockdown phenotypes |
|----------|---------------------------------|
| Line ID  | % of normal larvae | % of abnormal larvae | no. of larvae examined |
| 1        | 16                | 84                 | 39                   |
| 2        | 0                 | 100                | 191                  |
| 4        | 22                | 78                 | 296                  |
| 9        | 100               | 0                  | >100                 |

Figure 2 | Morphological defects seen in pem > GFP lines. (a) The transposon vector used to knockdown Ci-pem. Black arrowheads indicate inverted repeats (ITR) of Minos. UTR, untranslated region; NLS, nuclear localization signal sequence; Ter, transcription termination sequence. (b) GFP expression in unfertilized eggs of pem > GFP line 1. The egg in the upper right corner emitted GFP fluorescence, while the egg in the lower left corner did not. Bar, 100 μm. (c) A larva derived from sperm of pem > GFP line 4 and a wild-type egg. Bar, 100 μm. (d) A larva derived from an egg of pem > GFP line 4 and wild-type sperm. No, notochord. (e–i) Differentiation of major tissues in abnormal larvae derived from eggs of pem > GFP lines. (e) Epidermis (green). (f) Muscle (green). (g) Notochord (green). (h) Neural tissues (red). (i) Endoderm (En).
addition to maternal expression, and the 5’ upstream region of Ci-Nut can drive GFP in neural tissues like endogenous Ci-Nut. For this reason, the marker cassette was omitted from the Ci-Nut knockdown construct (Fig. 5b). Using these vectors, three transgenic lines, namely Tg[MiCiTnIGCimTG]1, Tg[MiCiNutG]3 and Tg[MiCiNutG]4, were established (Tg[MiCiNutG]3 and Tg[MiCiNutG]4 were previously described18). Among them, Tg[MiCiTnIGCimTG]1 and Tg[MiCiNutG]3 showed silencing of GFP expression in eggs. The Ci-mT and Ci-Nut expression levels in GFP-negative eggs were examined by WISH and quantitative RT-PCR. There was a dramatic reduction in Ci-mT and Ci-Nut in GFP-negative eggs of the corresponding lines (Fig. 5c and Supplementary Fig. 4). Ci-mT mRNA in Tg[MiCiTnIGCimTG]1 eggs was reduced to 4.1% of that in wild-type eggs, and Ci-Nut mRNA was reduced to 1.4% in Tg[MiCiNutG]3 eggs (Fig. 5c). Embryos derived from Ci-mT-knocked down eggs showed an abnormality in the tail (Fig. 5d), which was distinct from the phenotypes observed in Ci-pem knocked down embryos. This phenotype could not be rescued by introducing in vitro-transcribed Ci-mT mRNA. Ci-mT is probably translated during oogenesis, and the maternally supplied Ci-mT protein may be necessary for morphogenesis. Ci-Nut knocked down eggs showed normal embryogenesis and developed into normal larvae (Fig. 5e). This suggests that the knockdown method itself does not affect embryogenesis. The knockdown lines of Ci-pem, Ci-mT and Ci-Nut showed different phenotypes, suggesting that the phenotypes obtained from this method reflect the loss of the target gene. We investigated expression of four maternal transcripts in Ci-mT– or Ci-Nut– knockdown eggs by quantitative RT-PCR (Supplementary Fig. 2b,c). The knockdown effect was generally specific to the target mRNAs, although Ci-pem mRNA exhibited very weak reduction in both cases as supported by the statistical analysis.

Ci-Nut is both maternally and zygotically expressed13. Using this characteristic, we investigated whether zygotic expression of the target gene is affected by maternal gene silencing. Eggs in which maternal Ci-Nut mRNA was knocked down were fertilized with wild-type sperm, and expression of zygotic Ci-Nut was investigated using quantitative RT-PCR and WISH. Quantitative RT-PCR revealed that expression levels of Ci-Nut in tailbud embryos from maternal Ci-Nut– knockdown eggs did not differ significantly from the levels in wild-type embryos at the same stage (Fig. 5f), suggesting that zygotic expression occurred in embryos derived from maternal Ci-Nut– knockdown eggs. WISH of maternal Ci-Nut– knockdown embryos revealed that zygotic Ci-Nut expression begins in the neural tissues at the late gastrula stage, as in embryos derived from wild-type eggs, and that this expression continued until the tailbud stage.
We conclude that the knockdown of genes investigated in this analysis is specific to maternal transcripts as was observed with GFP (Fig. 3a).

**Discussion**

Here we established a new method to knockdown maternal mRNAs in the ascidian *Ciona intestinalis*. We named this method MASK, after maternal mRNA-specific knockdown. MASK has four advantages over previously reported methods for maternal mRNA disruption in ascidians. First, MASK is a reverse genetic approach and we can disrupt the function of a gene of interest without laborious screenings. Second, maternal expression can specifically be knocked down using MASK and the obtained phenotypes reflect the maternal function of the targeted gene. Third, this method utilizes genetic modification; once the transgenic lines have been established, we can obtain maternal mRNA-knocked down eggs and embryos without further experimentation.
Fourth, MASK is simple and easy and the only steps required to create the knockdown vector are isolation of the 5′ upstream region and 5′ UTR and their fusion with GFP/Kaede in Minos. With these advantages, MASK will be a powerful approach to study the function of maternal mRNAs in ascidian eggs.

The basis of MASK is epigenetic knockdown of GFP and Kaede, which occurs in oocytes and eggs. How do these reporter genes become targets of epigenetic silencing in eggs? A plausible explanation is that epigenetic gene modification occurs during oogenesis of Ciona to silence some endogenous genes, and the reporter genes may be accidental targets of the epigenetic regulation. Silencing occurs at the mRNA level, suggesting that transcriptional gene silencing or post-transcriptional degradation of mRNAs is a potential mechanism for silencing. MASK does not affect zygotically transcribed mRNAs. If silencing occurs at the post-transcriptional level, residual MASK factors could silence zygotically transcribed mRNAs. This mechanism resembles maternal gfp/gene silencing (MGS), which is another form of epigenetic silencing in Ciona that degrades both maternal and zygotic GFP mRNAs. Therefore, we favor the idea that transcriptional silencing is the mechanism of MASK. A portion of oocytes and eggs that escaped MASK often showed strong GFP expression (see Fig. 1a). Transcriptional silencing can explain this phenomenon; once silencing is canceled in some oocytes, continuous transcription of GFP causes the accumulation of GFP proteins in cells, resulting in strong fluorescence. We assume that the cis elements and 5′ UTRs of Ciona genes transcribed together with GFP and Kaede are regulated to make factors like microRNAs that suppress transcription of the endogenous gene. This silencing may be inhibited after fertilization, probably through epigenetic remodeling in chromosomes, causing zygotic transcription not to be affected. Similarly, transcriptional silencing may accidentally be inhibited in a portion of oocytes during oogenesis, and therefore silencing was seen in a mosaic fashion. MASK may be affected by the genomic context around the transposon vector insertion sites since the degree of silencing differs across transgenic lines created with the same knockdown vector. This suggests that production of the factor that generates MASK may depend on the genomic context around the insertion site. We identified some of the insertion sites of the knockdown vectors, although we did not identify differences between MASK-positive and -negative loci. The dependency of MASK on the genomic context suggests that some maternal genes might be resistant to MASK based on their genomic location.

Knockdown of maternally expressed genes with MASK will disclose the function of maternal genes in Ciona. Moreover, future studies will elucidate the detailed mechanisms of MASK and reveal the endogenous function of maternal gene silencing in Ciona intestinalis. Based upon previous reports of epigenetic silencing in other organisms, possible approaches to uncover the mechanism of MASK include, exchanging GC composition or codon usage of GFP/Kaede, mutating inverted repeats of Minos transposons, and characterizing trans factors that binds to the target gene to cause MASK. In addition, other chordates should be examined in future.
studies to determine whether MASK is more broadly applicable, which would facilitate the study of maternal mRNAs in other eukaryote lineages.

**Methods**

**Constructs.** The 5' upstream region and 5' UTR of genes were isolated by PCR using *C. intestinalis* genomic DNA as a template. The PCR fragments were digested with BamHI and subcloned into the BamHI site of pSPCeGFP or pSPKaeede. The fusion cassettes were PCR amplified, and subcloned into pMCiTnFG. For pMHiZtetPORProcExC, the Ci-pem-GFP cassette was subcloned into pMHiF3dTPORDesR using gateway technology (Invitrogen).

**Transgenic lines.** Tg[MgiCunGtl]3 and Tg[MgiCunGtl]4 were previously described. EF[MiTAcDSTOG74] is an enhancer detection line created by the method described in a previous report. The other transgenic lines were created by *Minos*-mediated transgenesis as previously described.

**Identification of insertion sites.** The insertion sites of *pem* > GFP lines were characterized by thermal asymmetric interlaced (TAIL) PCR, according to previous reports. Genomes isolated from sperm were used as templates for PCR. PCR fragments were subcloned into the pGEM-T (Easy) vector (Promega), and their sequences were determined. The presence of characterized insertion sites was confirmed by genomic PCR with specific primers designed near the insertion sites.

**Microinjection.** CI-pem cDNA was amplified by PCR and subcloned into pBS-RN30 to create pRN3CipemFL. mRNA was synthesized using the Megascript T3 kit (Ambion). The poly(A) tailing kit (Ambion), and Cap structure analog (New England Biolabs). Microinjection of mRNA was performed according to a previous report. The concentration of mRNA in the injection medium was adjusted to 500 ng/ml.

**Tissue differentiation.** Identification of epidermis, notochord and neural tissues in *C. intestinalis* larvae was investigated using marker transgenic lines that express GFP or Kaeede reporter genes under the control of cis elements of the Ci-Epil, Ci-Boroed and Ci-B2T genes. The splice of these transgenic lines was used to fertilize eggs of *pem* > GFP lines, and fluorescence was detected at the larval stage. To monitor muscle differentiation, GFP expression from the Ci-Tnl-GFP cassette in the Ci-pem knockdown vector was utilized. For endoderm differentiation, histochemical staining of alkaline phosphatase was performed as previously described.

**Gene expression.** Eggs of transgenic lines were divided according to the presence and absence of GFP fluorescence prior to sampling for the following experiments. Whole-mount in situ hybridization (WISH) was performed as previously described. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described using GAPDH as normalizing gene. A 0.5–1.0 egg or embryo equivalent quantity of cDNA was used as a template for quantitative reverse transcription-PCR (RT-PCR). Relative quantification of mRNA was carried out using standard curves created by cloned cDNAs. The primer sequences were 5'-gtcctcgtacagtttagccatgtcg-3' and 5'-gtcctcgtacagtttagccatgtcg-3' for *Ci-pem*, 5'-ggtacagccaggttcg-3' and 5'-ggtacagccaggttcg-3' for Ci-Mnt, 5'-cggattgccagtcg-3' and 5'-cggattgccagtcg-3' for *Ci-Tnl*, 5'-ggtacagccaggttcg-3' and 5'-ggtacagccaggttcg-3' for Genome Biol. 3, 525–528 (2011).

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