Abstract: Minichromosome maintenance protein 5 (MCM5) is a critical cell cycle regulator; its role in DNA replication is well known, but whether it is involved in the regulation of organogenesis in a cell cycle-independent way, is far from clear. In this study, we found that a loss of mcm5 function resulted in a mildly smaller liver, but that mcm5 overexpression led to liver bifida. Further, the data showed that mcm5 overexpression delayed endodermal migration in the ventral–dorsal axis and induced the liver bifida. Cell cycle analysis showed that a loss of mcm5 function, but not overexpression, resulted in cell cycle delay and increased cell apoptosis during gastrulation, implying that liver bifida was not the result of a cell cycle defect. In terms of its mechanism, our data proves that mcm5 represses the expression of cxcr4a, which sequentially causes a decrease in the expression of itgb1b during gastrulation. The downregulation of the cxcr4a-itgb1b cascade leads to an endodermal migration delay during gastrulation, as well as to the subsequent liver bifida during liver morphogenesis. In conclusion, our results suggest that in a cell cycle-independent way, mcm5 works as a gene expression regulator, either partially and directly, or indirectly repressing the expression of cxcr4a and the downstream gene itgb1b, to coordinate endodermal migration during gastrulation and liver location during liver organogenesis.

Keywords: mcm5; cell cycle; cxcr4a; endodermal migration; liver bifida

1. Introduction

The minichromosome maintenance (MCM) 2–7 complex, the crucial component of the DNA replication licensing system [1], acts as the eukaryotic DNA replicative helicase during the cell cycle, in normal development and tumorigenesis [2,3]. Previous studies demonstrated that Mcm2–7 proteins are loaded onto DNA in 20-fold excess over the number of replication origins [4], but that normal replication is still maintained when Mcm2–7 proteins are decreased [5]. This raises the possibility that an additional role of MCM family members exists besides their role in normal DNA replication regulation. Indeed, early research has demonstrated that the excess of MCM complex, allows dormant DNA replication origins to be recruited into the replication license during the cell cycle, in normal development and tumorigenesis [2,3]. Previous studies demonstrated that Mcm2–7 proteins are loaded onto DNA in 20-fold excess over the number of replication origins [4], but that normal replication is still maintained when Mcm2–7 proteins are decreased [5]. This raises the possibility that an additional role of MCM family members exists besides their role in normal DNA replication regulation. Indeed, early research has demonstrated that the excess of MCM complex, allows dormant DNA replication origins to be recruited into the replication license during the cell cycle, in normal development and tumorigenesis [2,3].

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MCM family, are involved in gene expression regulation by binding with the genomic DNA within highly transcribed genes.

MCM5 is a member of the family of microchromosomal maintenance proteins and is highly conserved in eukaryotes [8]. During zebrafish development, mcm5 is abundantly expressed in the proliferating cells [9]; mcm5 loss of function results in overt cell cycle arrest and apoptosis in the head and eyes, suggesting the critical role of MCM during the cell cycle. Recently, we also found MCM5 to be involved in FMN migration [10], but the role of MCM5 in liver development was not addressed. In our current study, we used mcm5 overexpression and loss of function to study the specific role of MCM5 in liver development. The data demonstrated that mcm5 loss of function ubiquitously gave rise to mild cell cycle defect and apoptosis during gastrulation, but did not disrupt endodermal convergence to the dorsal side and the final liver location. On the contrary, mcm5 overexpression led to liver bifida [11], where ‘liver bifida’ means both the left and right side of the embryo displays liver development (as is seen in cardia bifida [12]), resulting from delayed endodermal migration in the ventral–dorsal axis (V-D axis) during gastrulation. As its mechanism via a cell cycle- and cell apoptosis-independent way, mcm5 repressed the transcription of cccr4a and subsequently downregulated the expression of itgblb, which finally delayed endodermal migration and induced liver bifida. Our study directly identifies the specific role of MCM5 in endoderm migration and in the final liver location as being partially through its regulation of the expression of cccr4a and itgblb but not via cell cycle control. Our results also indirectly explain why an excess of MCM complex is found within highly transcribed genes [7], where it should regulate the expression of critical genes to coordinate organ development.

2. Materials and Methods

2.1. Fish Maintenance

Wild type (AB), transgenic line Tg(fabp10:EGFP), Tg(sox17:EGFP) [13], cccr4a µ20 [13], and mcm5 mutant [10] fishes were maintained in standard conditions at 28.5 °C. The developmental stages were characterized as previously described [14].

2.2. Plasmid Construction

The total RNA was extracted following the manufacturer’s instructions (TRIzol, Ambion, Austin, TX, USA). cDNA was prepared using a Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA) according to the manufacturer’s instructions. mcm5 and cccr4a CDS were individually amplified by PCR (PrimeSTAR Max Premix, Takara, Kusatsu, Japan) and cloned into the vector PCS+ (5x In-Fusion HD Enzyme Premix, Takara).

2.3. Generation of the Tg(Hsp70l:mcm5-T2A-mCherry) Transgenic Line

To generate the Tg(Hsp70l:mcm5-T2A-mCherry) transgenic line, first, the transgenic plasmid was produced via the method described in [15]. After constructing the plasmid, it was co-injected with I-SceI and I-SceI buffer in the cell body at the one-cell stage. When the embryos grew to 4 dpf, those embryos displaying eye fluorescein were screened out and grown for alternate experiments (Founder, Fo), and the remaining embryos were then crossed with a wild-type fish (AB strain) to produce the F1 embryos.

2.4. MO and mRNA Injection

Morpholino oligos (MO) for mcm5 (ATG MO, 5′-ATAGTTTCGATAAGTGCTGTC GATG-3′), cccr4a (ATG MO, 5′-GGTGTTTGATTGTCTGACCTTCATG-3′), and control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′) were obtained from Gene Tools [9,11,16]. mcm5 mRNA, mCherry mRNA, and cccr4a mRNA were synthesized in vitro using the mMESSAGE mMACHINE Kit (AM1340, Ambion). The concentration of MO was as follows: mcm5 MO, 300 µM; cccr4a MO, 500 µM; control MO, 500 µM. The concentration of
the mRNA injection was as follows: *mcm5* mRNA, 60 ng/µL; *mCherry* mRNA, 65 ng/µL; *cxcr4a* mRNA, 15 ng/µL. All of the MOs and mRNAs were injected at the 1–4-cell stage.

2.5. Real-Time qRT-PCR and Primers

Real-time q-PCR was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and the CFX96 Real-Time System (BIO-RAD, Hercules, CA, USA), according to the manufacturers’ instructions. A transcription of beta-actin was used for normalization. The primers are listed in Table S1.

2.6. Whole-Mount In Situ Hybridization

In situ hybridization was performed as described previously [17]. The probes *sox17*, *spaw*, *lft1*, *fabp10*, and *papc* were used [17]. The CDs of *vmhc* and *mcm5* were amplified via PCR and cloned into the pGEM-T easy vector system; then the plasmid was linearized, and the *vmhc* antisense probe and the *mcm5* antisense probe were synthesized. To synthesize probes *yap1*, *uwtri*, *itgb1b*, *itgb1a*, *cxcr4a*, and *cxcl12b*, the CDs for individual genes were amplified via PCR (an additional T7/Sp6 promoter was added to the start end of the Reverse Primer for each gene); part of the PCR product was used for sequencing to evaluate whether the right PCR product was obtained, while the remainder was used as a template to synthesize the probes [17].

2.7. Immunostaining

The embryos were fixed overnight with PEM at 4 ºC, washed with PBS (5 min, 3×), and blocked with PBSTN (4% BSA, 0.02%NaN₃, in PT) for 2 h at 4 ºC. Then, the H3p primary antibody was diluted with PBSTN to 1:100 and incubated on a shaker at 4 ºC overnight. The embryos were washed with PT (0.3% Triton-X-100, in 1X PBS) for at least 20 min, 8 times. The secondary antibody (GeneTex 26800, Irvine, CA, USA) was diluted with PBSTN to 1:500 and incubated overnight at 4 ºC in the dark. Embryos were prepared for imaging by washing with PT for 30 min, at least 8 times.

2.8. Cell Apoptosis Staining

Embryos were dechorionated and fixed in 4% paraformaldehyde overnight at 4 ºC, then washed with PBST buffer three times and permeabilized with methanol overnight. After washing three times with PBST (5 min each time), an In Situ Cell Death Detection Fluorescein kit (Roche 11684795910, Basle, Switzerland) was used to examine cell apoptosis according to the manufacturer’s instructions.

2.9. Cell Cycle Analysis

Twenty embryos were used for cell cycle analysis for each group. Briefly, embryos were washed with pre-cooled PBS, incubated, and blown in 1 mL of PBS with 0.25% trypsin to obtain single-cell suspensions. The cell suspensions were passed through a cell strainer (FALCON, NO.352340, Corning, NY, USA), followed by centrifugation at 4 ºC for 2 min at 1000 g and the careful removal of the supernatant (2 times). The cells were resuspended in 0.25 mL of PBS and fixed with 0.75 mL of cold ethanol at 4 ºC overnight. Then, the fixed cells were precipitated and resuspended in 500 µL of propidium iodide solution (0.1 mg/mL propidium iodide, 0.1% sodium citrate, 100 lg/mL RNase A, and 0.0002% Triton X-100), followed by analysis using a NovoSampler Q NO.2020098.

2.10. Statistical Analysis

All data were analyzed using Novoexpress, ImageJ, and statistical software in GraphPad Prism 8 for Windows (GraphPad Software). Data are represented with significance values (*p*) denoted by *p* < 0.05, **p** < 0.01, and ***p*** < 0.001. Experiments were performed at least three times for each condition.
3. Results

3.1. Mcm5 Overexpression Gives Rise to Liver Bifida

Since the quantity of MCM5 is largely more than that required for DNA replication [1], and mcml5 is also expressed ubiquitously in proliferating cells, including in the liver of zebrafish [9] (Figure S1), we propose that MCM5 plays an extra role beside DNA duplication regulation during liver development. Since in mcml5 morphants, the translation of the maternal mcml5 mRNA would be knocked down [16], to fully address the role of MCM5 in liver development, we analyzed the phenotypes in our mcml5 mutant [10], mcml5 morphants, and those embryos overexpressing mcml5 mRNA. The general phenotype in the mcml5−/− embryos was similar to that in a previously published mcml5−/− allele [9]; the eyes and heads were smaller, and the length of embryo bodies was shorter (Figure S1). Regarding liver development, the mcml5 morphants and the mcml5 mutants displayed a mildly smaller liver than the control at 3 dpf (Figure 1A–C; Figure S2). When mcml5 was overexpressed (Figure S3), a large number of embryos displayed liver bifida (Figure 1E–I). To confirm the specific role of mcml5 overexpression, the transgenic line Tg(Hsp70l:mcm5-T2A-desRed) was generated (Figure S4) and was used to induce the expression of mcml5 RNA via heat-shock at 40% epiboly (Figure S5); the phenotype of liver bifida was also observed in the treated embryos (Figure 1D,I). In contrast, in the controls, an injection of mCherry mRNA (65 pg) (Figure S6) did not lead to liver bifida (Figure 1). To rule out the influence of the transgenic background of the embryos in these experiments, the expression of fabp10 in the wild-type embryos, the mcml5−/− embryos, and the embryos injected with mcml5 mRNA was examined. The data showed that embryos injected with mcml5 mRNA displayed liver bifida and liver reversal (Figure 1K–N), but no liver localization defects were observed in the mcml5−/− embryos or in the controls (Figure 1 J,N). In addition, no heart bifida was observed in embryos injected with mcml5 mRNA (Figure S7A–C). These findings show that mcml5 overexpression specifically results in liver bifida.

3.2. MCM5 Represses Endodermal Migration during Gastrulation

During gastrulation, the liver progenitors migrate to the dorsal midline under the mesoderm and are then established as the left-sided liver at a late stage [18]. Any disturbance to the endodermal migration [11] or left–right asymmetric cascade [17,19] will cause randomized endodermal organ location, including liver bifida and liver reversal. To identify why liver bifida occurs in embryos overexpressing mcml5 mRNA, the KV morphogenesis, midline, Nodal/spaw, and endodermal progenitor migration were examined. No differences were observed regarding the expression of sox17, Nodal/spaw, and lft1 between treated embryos and the controls (Figure S8); however, endodermal migration (Figure 2A–C), but not mesodermal migration (Figure 2D–G), was slower at the V-D axis in embryos injected with mcml5 mRNA. Further, the location of the GFP-labelled cells in Tg(sox17:GFP) embryos was analyzed at the two-somite stage (Figure 2H–I). In control embryos, no GFP-labelled cells were localized in the regions “−4” and “4” (Figure 2H,J, blue line shown), but about 10 and 12 GFP-labelled cells were localized in regions “−4” and “4”, respectively, in embryos injected with mcml5mRNA (Figure 2I,J, red line shown). These results further confirmed that endodermal migration was reduced in embryos overexpressing mcml5 mRNA, and this is possibly the reason for liver bifida during liver morphogenesis.
3. Results

3.1. Mcm5 Overexpression Gives Rise to Liver Bifida

In our observations, 99.58% of the Tg(lfabp10:GFP) control embryos displayed a left-sided liver (A,I, n = 293). In the mcm5 morphants or mcm5 mutants, the liver became mildly smaller (B,C) than that in the control embryos; 99.29% of the mcm5 morphants (B,H, n = 140, p > 0.05) and 99.08% of the mcm5 mutants (C,I, n = 103, p > 0.05) displayed a left-sided liver. In embryos with a forced expression of mcm5, the proportions of embryos with a left-sided liver, liver bifida, and a right-sided liver were 66.67% (E,I, n = 156, p = 0.00025), 23.71% (F,G,I, n = 156, p = 0.0026), and 9.62% (H,I, n = 156, p = 0.0064), respectively. Similarly, in embryos with a forced expression of mcm5 by heat-shock at 40% epiboly, the proportion of embryos with liver bifida and a right-sided liver was increased when compared with the control embryos (D,I, n = 206, p = 0.0035). In total, 97.4% of the embryos injected with mCherry mRNA displayed a left-sided liver (I, n = 229, p = 0.35). (J–N) In the wild-type embryos, the rate of left-sided expression of lfabp10 was 99.97% (J,N, n = 92), while 99.89% of the mcm5 mutants displayed left-sided expression of lfabp10 (J, n = 91, p = 0.93). In total, 61.63% (K,N, n = 86, p = 0.0009), 24.42% (L,N, n = 86, p = 0.0006), and 13.95% (M,N, n = 86, p = 0.0017) of embryos injected with mcm5 mRNA displayed a left-sided, both-sided, and right-sided expression of lfabp10, respectively.

3.3. Overexpression of mcm5 did Not Result in Cell Cycle Progress Defect

The cell cycle and cell differentiation must be coordinated properly during organogenesis [20], as disrupting the cell cycle results in cell fate determination defects [21]. Therefore, we evaluated the causation between the cell cycle and endodermal migration in embryos injected with mcm5 mRNA. Specifically, 44.2%, 35.4%, and 19.8% of cells in the control embryos stayed in the G1 phase, S phase, and G2/M phase, respectively (Figure 3(Bb1)); 51.3%, 33.5%, and 14.6% of cells in mcm5 morphants stayed in the G1 phase, S phase, and G2/M phase, respectively (Figure 3(Bb2)); 44.8%, 35.6%, and 18.3% of cells in embryos injected with mcm5 mRNA stayed in the G1 phase, S phase, and G2/M phase, respectively (Figure 3(Bb3)). In total, 55.77% of cells stayed in the S/G2/M phase at the bud stage in control embryos (Figure 3B,C); in mcm5 morphants and mcm5-overexpressing embryos, 48.63% and 55.15% of cells, respectively, stayed in the S/G2/M phase (Figure 3B,C). This result indicates that mcm5 loss of function, but not mcm5 overexpression, led to more cells staying in the G1 phase, meaning that only mcm5 loss of function results in mild cell cycle delay. Since cell cycle delay results in decreased cell proliferation and increased cell apoptosis [16], we checked whether cell proliferation and cell apoptosis were disturbed in the treated embryos. Immunostaining experiments showed that the number of H3P-positive cells in mcm5 morphants was increased (Figure 3E,G), while the number of H3P-positive cells in mcm5-overexpressing embryos (Figure 3F,G) was similar to that in the control embryos.
Meanwhile, the proportions of apoptotic cells in control embryos and in embryos treated with camptothecin, mcm5 MO, and mcm5 mRNA were 0.25%, 1.75%, 0.85%, and 0.27%, respectively, meaning that only mcm5 morphants—and not embryos overexpressing mcm5 mRNA, displayed increased cell apoptosis (Figure 3H–I). These two sets of experiments showed that the liver bifida observed in embryos injected with mcm5 mRNA was not the result of cell cycle delay.

![Figure 2. A forced expression of mcm5 depressed endodermal migration. (A–C) Compared with that in the control (A, n = 109), the migration of endodermal cells in mcm5 morphants was normal (B, n = 127), but the endodermal migration in embryos injected with mcm5 mRNA was slowed down (C, n = 111, p = 0.0014). (D–G). Papc staining showed no difference in the width of the notochord (blue frame shown) between the control (D, n = 41; G, n = 11) and embryos injected with mcm5 mRNA (E, n = 38; G, n = 10, p = 0.3), but the width of the notochord was mildly decreased in mcm5 morphants (E, n = 38; G, n = 12, p = 0.015). The width of the presomitic mesoderm (white frame shown) in mcm5 morphants was also mildly decreased (E, n = 38; G, n = 12, p = 0.12), but it was normal in embryos injected with mcm5 mRNA (E, n = 38; G, n = 10, p = 0.69). (H–J) In Tg(Sox17:GFP) embryos, quantitative analysis showed no GFP-labeled cells in the “−4” area or “4” area in control embryos (H, J, n = 5), while about 10 and 12 GFP-labeled cells were observed in the “−4” area (I, J, n = 5, p = 0.00001) and the “4” area (I, J, n = 5, p = 0.00007), respectively, in embryos injected with mcm5 mRNA.](image)

### 3.4. Cxcr4a-itgb1b Cascade Mediates MCM5 to Regulate Endodermal Migration

Hippo signaling and the cxcr4a-itgb1b cascade were reported to be involved in regulating endoderm cell migration during gastrulation [11,22,23]. In situ and q-PCR experiments showed that Yap1, wnt1, last2, and itga6 were not significantly affected in embryos injected with mcm5 mRNA (Figure S9), while the expression levels of itgb1a and itgb1b were decreased (Figure 4A–D), especially that of itgb1b (Figure 4E). In addition, the downregulation of the MCM5 function by means of a mcm5 MO injection increased the transcription of itgb1b (Figure 4E). These data showed the possibility that mcm5 negatively regulates itgb1b transcription during gastrulation. Next, we examined the expression of cxcl12b/cxcr4a in embryos injected with mcm5 mRNA. Interestingly, cxcr4a was greatly downregulated in the mcm5-mRNA-injected embryos (Figure 4 F,G,J), but cxcl12b was mildly upregulated (Figure 4H–J). This result was confirmed by the observation that the expression levels of cxcr4a and cxcl12b were increased and decreased, respectively, in mcm5 morphants (Figure 4). These results suggest the possibility that the downregulated cxcr4a-itgb1b delayed endodermal migration at the V-D axis when mcm5 was overexpressed. To investigate this hypothesis, we examined whether restoring the cxcr4a function could rescue the phenotype in the embryos injected with mcm5 mRNA. We titrated a concentration of cxcr4a mRNA and co-injected mcm5 mRNA and cxcr4a mRNA (15 ng/µL) into
Tg(Sox17:GFP)/Tg(fabp10::GFP) transgenic embryos; then, we analyzed the endodermal migration and the liver location. At the two-somite stage, an cxcr4a MO injection greatly delayed endodermal migration ([23], Figure 4L), and an injection of cxcr4a mRNA, rescued endodermal migration delay in embryos injected with mcm5 mRNA (Figure 4M,N). Further, liver bifida and reversed liver rates were decreased (Figure 4P,Q). These data suggest that cxcr4a rescued the liver bifida phenotype in embryos overexpressing mcm5, and that cxcr4a, at least partially, mediated mcm5 to regulate endodermal migration during gastrulation.

**Figure 3.** mcm5 loss of function, but not overexpression, leads to cell cycle arrest and cell apoptosis at the bud stage. (A–C) Flow cytometry analysis showed that 44.23% of cells in control embryos were in the G1 phase at the bud stage (Bb1,C, n = 3), 51.37% of cells in mcm5 morphants were in the G1 phase (Bb2,C, n = 3, p = 0.023), and 44.85% of cells in embryos injected with mcm5 mRNA were in the G1 phase (Bb3,C, n = 3, p = 0.1). Specifically, 44.2%, 35.4%, and 19.8% of cells in control embryos stayed in the G1 phase, S phase, and G2/M phase, respectively (Bb1); 51.3%, 33.5%, and 14.6% of cells in mcm5 morphants stayed in the G1 phase, S phase, and G2/M phase, respectively (Bb2); 44.8%, 35.6%, and 18.3% of cells in embryos injected with mcm5 mRNA stayed in the G1 phase, S phase, and G2/M phase, respectively (Bb3). (D–G) The proportion of H3p positive cells in the control embryos, mcm5 morphants, and embryos injected with mcm5 mRNA were 11.19% (D,G, n = 14), 12.76% (E,G, n = 12, p = 0.0067), and 11.23% (F,G, n = 16, p = 0.75), respectively. (H–L) The proportion of apoptotic cells in the camptothecin-treated embryos, control, mcm5 morphants, and embryos injected with mcm5 mRNA were 1.75% (H,L, n = 3, p = 0.000024), 0.20% (I, L, n = 6), 0.76% (J, L, n = 8, p = 0.0042), and 0.22% (K, L, n = 9, p = 0.39), respectively.
Figure 4. The downregulation of the Cxcr4a-itgb1b cascade slowed down endodermal migration in embryos injected with mcm5 mRNA. (A–E) Compared with that in control embryos (A, C), the expression of itgb1a and itgb1b was decreased in embryos injected with mcm5 mRNA (B, D). Quantitative PCR results showed that the expression level of itgb1a mRNA in embryos injected with mcm5 mRNA was 0.9-fold to that in the control (E, p = 0.001), but it was no different in the mcm5 morphants or in the control (E, 0.95-fold, p = 0.67). The itgb1b level in embryos injected with mcm5 mRNA was 0.38-fold to that in the control (E, p = 0.001), while the level in mcm5 morphants was 1.20-fold to that in the control (E, p = 0.03). (F–J) In embryos injected with mcm5 mRNA, the expression levels of cxcr4a and cxcl12b were decreased (F, G) and increased (H, I), respectively. Quantitative PCR indicated that the expression levels of cxcr4a and cxcl12b in embryos injected with mcm5 mRNA were also downregulated (J, 0.19-fold relative to the control, p = 0.0009) and upregulated (J, 1.35-fold relative to the control, p = 0.0002), respectively. In mcm5 morphants, the expression levels of cxcr4a and cxcl12b were upregulated (J, 1.24-fold relative to the control, p = 0.0003) and downregulated (J, 0.9-fold relative to the control, p = 0.02), respectively. (K–M) Compared with that in the control (K, n = 25, 92%), GFP-labeled endodermal migration in cxcr4a morphants was slowed down (L, n = 122, 88.5%, p < 0.01), but mcm5 mRNA and cxcr4a mRNA co-injection restored endodermal migration (M, n = 98, 73.5%, p < 0.05). Quantitative analysis for the selected area (shown in K) showed that migration of the delayed endodermal cells in the “−4” area and the “4” area in embryos injected with mcm5 mRNA was restored by co-injection with cxcr4a mRNA (N, n = 5). (O–R) An injection of cxcr4a mRNA restored the liver location in embryos injected with mcm5 mRNA. An injection of cxcr4a mRNA (30 ng/µL or more) resulted in a small proportion of the embryos displaying liver bifida (R, last column shown, n = 181, p < 0.05). Co-injection with a low dose of cxcr4a mRNA (15 ng/µL) and mcm5 mRNA resulted in 10.1% and 4.7% of embryos displaying liver bifida and a right-sided liver, respectively (P, Q). Co-injection with cxcr4a mRNA and mcm5 mRNA restored the liver location phenotype in embryos injected with mcm5 mRNA (R, columns 2 and 3, n = 267, p < 0.05).

4. Discussion

It is well known that the MCM2–7 complex acts as the eukaryotic DNA replicative helicase during DNA replication in normal development and tumorigenesis [2,3]. As one component of the MCM2–7 complex, MCM5 also plays a critical role in the unwinding of the duplex DNA during DNA replication [24]. In early zebrafish development, the loss of mcm5 function disturbs the cell cycle, and gives rise to cell apoptosis and endomesodermal delamination defects, thus clarifying the causality between the cell cycle and cell fate.
determination [16]. This result confirms that the specific role of MCM5 during early embryonic development is a cell cycle regulatory role. On the other hand, previous studies demonstrated that Mcm2–7 proteins are loaded onto DNA in 20-fold excess over the number of replication origins [4]. These facts support the hypothesis that mcm5 has an additional critical role besides regulating DNA replication within proliferating cells. Indeed, recent research suggests that most of the extra MCM family members are within highly transcribed genes [7], which implies a possible role of MCM family members (including MCM5) in gene transcription regulation. In our current study, we found that mcm5 loss of function resulted in a mildly smaller liver at 3.5dpf. Further, overexpression of mcm5 induced by the injection of mcm5 mRNA led to liver bifida (Figure 1F,G). To rule out the non-specific role of an injection of mcm5 mRNA, mCherry mRNA was synthesized and the transgenic line Tg(Hsp70l:mcm5-T2A-desRed) was generated. An injection of mCherry mRNA didn’t lead to liver bifida but heat-shock induced overexpression of MCM5, resulting in liver bifida (Figure S5; Figure 1D,I) similar to the phenotype seen in embryos injected with MCM5 mRNA. These results partially prove that overexpression of MCM5 leads to liver bifida.

To confirm the role of MCM5 in liver development, we tried to find out how overexpression of MCM5 specifically induces liver bifida. The data showed that mcm5 loss of function upregulated the expression of cxcr4a and itgb1b, but did not upregulate the expression of itgb1a (Figure 4,E). In addition, we did not find an obvious liver developmental phenotype in the embryos with a loss of mcm5 function (Figure 1B,C). On the contrary, the overexpression of mcm5 downregulated the expression of cxcr4a and itgb1b (Figure 4,E) and subsequently gave rise to delayed endodermal migration (Figure 2C,H–J) and liver bifida (Figure 1E–H). Also, the expression of itgb1a was not significantly changed in the treated embryos. To further confirm that the downregulation of cxcr4a-itgb1b results in liver bifida in embryos overexpressed with mcm5, an injection of cxcr4a mRNA was used to rescue the liver bifida phenotype in embryos overexpressed with mcm5. The results showed that cxcr4a mRNA partially rescues the liver phenotype in embryos overexpressed with mcm5 (Figure 4M,O–R). These three sets of experiments suggest the possibility that mcm5 negatively regulates the expression of cxcr4a and itgb1b, which subsequently regulates itgb1b expression and endodermal migration (as shown in role “2”). This model only clarifies one role of mcm5 in regulating gene expression; far more of mcm5’s function awaits discovery through future research.

To exclude the possibility that the forced expression of mcm5 mRNA leads to cell cycle delay and cell apoptosis, indirectly delaying endodermal migration, we evaluated the cell cycle situation in embryos injected with mcm5 mRNA. The injection of mcm5 mRNA did not give rise to cell cycle delay (Figure 3B,C,F,G) or cell apoptosis (Figure 3K,L), thereby excluding this possibility. On the contrary, mcm5 loss of function (injection with mcm5 MO) resulted in cell cycle delay and increased apoptosis (Figure 3J,L), which is consistent with our early research [16]. Thus, according to our previous study and our current data, we propose a hypothesis and model of how mcm5 regulates organ development (Figure S10). MCM5 works as a dual-function factor during early development. During gastrulation, most of the cells are in the cell cycle with high proliferation potentiality. On the one hand, ORC acts to recruit Cdc6 and Cdt1 to the DNA replication origins, which together to recruit the MCM complex to the origins for the pre-RC formation [25]. Here, mcm5 works as a subunit of the hexameric helicase to unwind the DNA helix, subsequently initiating DNA replication, thereby ensuring that DNA integrates during cell proliferation (as shown in role “1”). On the other hand, in a cell-cycle independent manner, it is possible that mcm5 works as a critical transcription regulator to repress cxcr4a expression, which subsequently regulates itgb1b expression and endodermal migration (as shown in role “2”). This model only clarifies one role of mcm5 in regulating gene expression; far more of mcm5’s function awaits discovery through future research.

Recently, the role of cell cycle regulators in embryonic development and disease has been widely reported, including for the members of the MCM family [2,26–28]. In mice,
mutations in MCM2-MCM7 cause genomic instability and render female mouse embryos markedly more susceptible to embryonic lethality [29]; this role is dependent on DNA replication and the cell cycle. In zebrafish, mcm2 was found to work as the target gene of foxn1 to regulate thymic epithelial cell proliferation [30]; this study only identified mcm2 as the proliferation regulator during thymus development, in a manner that is also cell cycle-dependent. More recently, Philipp’s group discovered that MCM2 binds to the transcription start sites of cilia-inhibiting genes to block RNA polymerase II-mediated transcription [31], to coordinate ciliogenesis, and the subsequent organ LR patterning. Their work also showed that MCM7 is involved in ciliogenesis via a subset of genes and pathways distinct from those of MCM2 [31], implying different roles of individual members of the MCM family in embryonic development. In our study, we identified the possibility that mcm5 works as a transcription regulator, coordinating endodermal migration by repressing the activity of the Cxcr4a-Itgb1b cascade, instead of via cell cycle control. While regulating gene transcription, MCM3 is reported to be associated with MCM5, and this interaction is required for the MCM5/Stat1 complex to activate the downstream gene transcription [32]. Accordingly, it is possible that mcm5 acts as a part of a complex to regulate cxcr4a transcription. If MCM5 works as part of a complex, manipulation of the expression of the complex’s elements would also have the same function. This hypothesis needs far more investigation to be clarified and confirmed. In addition, far more research is required to clarify whether the role of mcm5 is direct or indirect.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/biom12020286/s1, Figure S1: Expression of mcm5 in wild-type and mcm5 mutant embryos at different stages. Figure S2: Liver phenotype in mcm5 mutants and mcm5 morphants. Figure S3: The overall phenotypes of the embryos injected with different mRNA. Figure S4: The construction of Tg(Hsp70l:mcm5-T2A-desRed) and overexpression of mcm5 induced by heat-shock. Figure S5: Heat-shock induced the expression of mcm5 in Tg(Hsp70l:mcm5-T2A-mCherry) embryos. Figure S6: The expression of mCherry after injection of mCherry mRNA. Figure S7: mcm5 mRNA injection did not lead to heart bifida. Figure S8: The expression of left–right-patterning-related genes. Figure S9: Yap signaling was not disturbed greatly in embryos injected with mcm5 mRNA. Figure S10: The model for mcm5 regulating DNA replication and endodermal migration in early zebrafish development. Table S1: Primers for q-PCR.

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