miR-218 is essential to establish motor neuron fate as a downstream effector of Isl1–Lhx3

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While microRNAs have emerged as an important component of gene regulatory networks, it remains unclear how microRNAs collaborate with transcription factors in the gene networks that determine neuronal cell fate. Here we show that in the developing spinal cord, the expression of miR-218 is directly upregulated by the Isl1–Lhx3 complex, which drives motor neuron fate. Inhibition of miR-218 suppresses the generation of motor neurons in both chick neural tube and mouse embryonic stem cells, suggesting that miR-218 plays a crucial role in motor neuron differentiation. Results from unbiased RISC-trap screens, in vivo reporter assays and overexpression studies indicated that miR-218 directly represses transcripts that promote developmental programs for interneurons. In addition, we found that miR-218 activity is required for Isl1–Lhx3 to effectively induce motor neurons and suppress interneuron fates. Together our results reveal an essential role of miR-218 as a downstream effector of the Isl1–Lhx3 complex in establishing motor neuron identity.
Throughout the development of the central nervous system (CNS), a vast number of neuronal types are produced with striking precision. Understanding the intricate gene regulatory networks, which establish the unique identity of each neuronal cell type and eventually lead to the great cellular complexity in the CNS, is an important topic in neurobiology. In the developing spinal cord, neuronal cell fate specification is initiated by the integration of morphogen gradients that direct the patterning of progenitor domains, each of which gives rise to a specific neuronal type. The boundaries of the progenitor domains are sharpened by cross-repressive interactions between transcription factors that are expressed in neighbouring progenitor domains. As progenitor cells exit the cell cycle, transcription factors that promote the differentiation of distinct interneuron types and motor neurons are upregulated. Two LIM-homeodomain factors, LIM homeobox 3 (Lhx3) and Islet-1 (Isl1) are co-expressed in differentiating motor neurons, while Lhx3, but not Isl1, is expressed in newly born V2 interneurons. Isl1 and Lhx3 form a hexameric Isl1–Lhx3 transcriptional complex with nuclear LIM interactors (NLI; Fig. 1a). The co-expression of Isl1 and Lhx3, along with endogenous factors, triggers the generation of motor neurons in various cellular contexts, such as the dorsal spinal cord, embryonic stem cells (ESCs) and induced pluripotent stem cells. This potent activity of Isl1–Lhx3 that drives motor neuron fate specification is partly attributed to the fact that Isl1–Lhx3 directly binds and robustly upregulates a wide range of terminal differentiation genes, including a battery of cholinergic pathway genes that enable cholinergic neurotransmission. Another critical factor is that the Isl1–Lhx3 complex inhibits the acquisition of non-motor neuron fates, as evident from the observation that Isl1–Lhx3 differentiate into interneurons, however, the mechanisms by which Isl1–Lhx3 represses interneuron differentiation or progenitor fate remain unknown.

MicroRNAs (miRNAs) are small RNA molecules that bind to target mRNAs and prevent translation or trigger the degradation of their target transcripts. A growing body of research has established that miRNAs serve as a crucial constituent of gene regulatory networks. Recent studies of miRNAs in the developing spinal cord have revealed that miR-124 and miR-17-3p play a role in neuronal differentiation and progenitor domain patterning, respectively, and that miR-9 is involved in fine-tuning the differentiation of motor neuron subtypes. However, it remains unclear how miRNAs are interconnected with cell fate–specifying transcription factors in the regulatory networks that determine neuronal cell fates in CNS development.

In this study, we investigated the role of miRNAs in the gene networks that specify motor neuron fate. We found that a single miRNA, miR-218, is highly and directly upregulated by Isl1–Lhx3 at the onset of motor neuron differentiation and that miR-218 is specifically expressed in motor neurons throughout spinal cord development. We also found that miR-218 is essential for the generation of motor neurons both in vitro and in vivo. Our RISC-trap screen revealed many miR-218 target transcripts whose primary function is to promote interneuron or neural progenitor characteristics in the spinal cord. Consistently, miR-218 was needed for Isl1–Lhx3 to efficiently suppress interneuron programmes and promote motor neuron fate in neural progenitors. Together, our results suggest that miR-218 functions as a crucial downstream effector of the Isl1–Lhx3 complex in establishing motor neuron identity by downregulating genes that promote non-motor neuron fates. Our study highlights an intricate gene regulatory network in which cell fate–specifying transcription factors cooperate with downstream miRNAs to define the gene expression profile for an appropriate cell fate.

Results

miR-218 is upregulated during motor neuron differentiation. To identify miRNAs that play a role in promoting motor neuron cell fate, we took advantage of Isl1–Lhx3–ESCs, which serve as a robust model of motor neuron differentiation. Islet-1–Lhx3–ESCs express an Isl1–Lhx3 fusion protein on doxycycline (Dox) treatment, which forms the Isl1–Lhx3 hexamer complex with endogenous NLI (Fig. 1a,b). On treatment with Dox and retinoic acid (RA) following the formation of embryoid bodies (EBs), Isl1–Lhx3–ESCs differentiate into motor neurons, which express numerous motor neuron markers and form neuromuscular junctions with myotubes. To systemically monitor the expression pattern of miRNAs during motor neuron differentiation in Isl1–Lhx3–ESCs in an unbiased manner, we determined the expression profiles of miRNAs in RA-treated Isl1–Lhx3–ESCs, incubated either with or without Dox (that is, expression of Isl1–Lhx3), using a TaqMan miRNA array. Pairwise comparison of miRNA arrays revealed that 18 miRNAs are induced more than threefold (Table 1, Supplementary Data 1). Interestingly, a single miRNA, miR-218, showed a remarkable ~71-fold induction, while the next highest induced miRNA, miR-382, was upregulated by ~9-fold. In addition, miR-218 was the fifth highest expressed miRNA in Dox-treated conditions, indicating that miR-218 is abundantly present in embryonic motor neurons. The expression levels of other miRNAs that are implicated in the development of motor neurons, such as miR-9 (refs 24–26), miR-124 (refs 21,22) and miR-17-92 cluster, were unaltered between Dox-treated versus Dox-untreated conditions. Our independent TaqMan quantitative PCR (qPCR) analyses confirmed the robust upregulation of miR-218 in Dox-treated conditions (Fig. 1c). These results suggest that miR-218 may play a role in motor neuron cell fate specification downstream of the Isl1–Lhx3 complex.

Islet-1–Lhx3 directly upregulates miR-218-1 and miR-218-2 genes. miR-218 is an evolutionarily conserved miRNA that is encoded in introns of Slit2 and Slit3 genes, which produce miRNA precursor hairpins pri-miR-218-1 and pri-miR-218-2, respectively (Supplementary Fig. 1a–c). To test whether the Isl1–Lhx3 complex directly regulates the expression of miR-218 genes, we analysed the genome-wide binding pattern of Isl1–Lhx3 using two independent chromatin immunoprecipitation (ChIP)-seq data sets. Interestingly, Isl1–Lhx3-bound ChIP-seq peaks were found in the introns of both Slit2 and Slit3 genes (Fig. 1d, Supplementary Figs 2 and 3). Our ChIP analyses in Isl1–Lhx3–ESCs further confirmed that Isl1–Lhx3 binds to the ChIP-seq peaks in Slit2 and Slit3 genes (Fig. 1e). Next, the ChIP assays in E12.5 mouse spinal cord using anti-Isl1 and anti-Lhx3 antibodies revealed that both Isl1 and Lhx3 are recruited to the ChIP-seq peaks in Slit2 and Slit3 genes in vivo (Fig. 1f).

The binding of Isl1–Lhx3 to miR-218-1 and miR-218-2 loci suggests that the upregulation of mature miR-218 by Isl1–Lhx3 is attributed to the direct induction of both miR-218 genes. Indeed, miR-218-1 and miR-218-2 pri-miRNAs were markedly upregulated during Isl1–Lhx3-directed motor neuron differentiation in ESCs (Fig. 1g). To further determine whether the upregulation of miR-218 genes is a direct outcome of transcriptional activation of the genes by Isl1–Lhx3 or an indirect outcome of motor neuron differentiation, we expressed Isl1–Lhx3 in ESCs without triggering motor neuron differentiation. When Isl1–Lhx3–ESCs are cultured in a monolayer without RA, the ESCs do not differentiate into neurons. In this condition, we treated Isl1–Lhx3–ESCs with Dox (that is, expression of Isl1–Lhx3), collected cells at multiple time points and monitored the miR-218 levels.
Interestingly, Isl1–Lhx3 expression still led to a drastic upregulation of miR-218 independently of miR-218 (Fig. 1h). These results suggest that Isl1–Lhx3 directly induces the expression of miR-218 independently of motor neuron differentiation.

Together our data demonstrate that the Isl1–Lhx3 complex upregulates both miR-218-1 and miR-218-2 genes by directly binding to both genes during motor neuron differentiation (Fig. 1i).

**miR-218 is specifically active in developing motor neurons.** The robust upregulation of miR-218 in ESC-derived motor neurons prompted us to investigate the in vivo expression pattern of miR-218 in developing embryos. In situ hybridization analyses revealed that miR-218 is specifically upregulated in motor neurons during cell fate specification (Fig. 2a–c, Supplementary Fig. 4a). miR-218 maintains its motor neuron-specific expression pattern in the spinal cord throughout embryonic development (Fig. 2c, Supplementary Fig. 4a).

To determine where endogenous miR-218 actively suppresses target gene expression in vivo, we used miRNA sensor plasmids, in which a cytomegalovirus/chicken β actin (CAG) promoter drives the expression of a destabilized nuclear green fluorescent protein (GFP) with a half-life of 4 h (d4EGFP) that is linked with complete complementary miRNA response elements (MREs; Fig. 2d)21. The miRNA sensor plasmids also have another CAG actin (CAG) promoter directing the expression of monomeric nuclear RFP (mRFPn). To assess the endogenous activity of miR-218 in embryonic spinal cord, we electroporated chick neural tubes with either miRNA sensor vector or miR-218 sensor, in which...
miR-218 MREs are inserted into the 3′ untranslated region (3′UTR) of the dAEGFP. We then monitored the expression levels of GFP compared with red fluorescent protein (RFP), which labels all transfected cells, 3 days after electroporation. In ovo electroporation of miRNA sensor vector resulted in the expression of GFP and RFP in similar ratios throughout the spinal cord (Fig. 2e,h). In contrast, the miR-218 sensor showed a drastic downregulation of GFP specifically in motor neurons, compared with interneurons (Fig. 2f,h), indicating that endogenous miR-218 actively represses target genes with miR-218 MREs in developing motor neurons.

It is possible that both strands of a miRNA precursor hairpin are expressed and actively repress mRNA targets27. To test whether the complementary miR-218-3p ‘star’ strand is also active in motor neurons, we generated a miR-218-star sensor. We did not observe any regional differences between motor neurons and interneurons in GFP/RFP pixel intensity in chick spinal cord electroporated with the miR-218-star sensor (Fig. 2g,h), suggesting that the miR-218-star strand is not functional in the developing spinal cord.

Together, our results demonstrate that miR-218, but not miR-218-star, is selectively expressed and actively represses its target genes with miR-218 MRE in motor neurons.

miR-218 is essential for motor neuron generation from ESCs.

To further investigate whether miR-218 is important for motor neurogenesis, we sought a cellular context in which miR-218 might be inhibited more efficiently. ESCs differentiate into motor neurons when embryoid bodies are formed and treated with RA and a sonic hedgehog agonist30. We first monitored miR-218 levels in this motor neuron differentiation paradigm. The miR-218 level was low in ESCs and embryoid bodies, but miR-218 was upregulated ~27-fold when ESCs acquire motor neuron characteristics (Fig. 3f), consistent with the motor neuron-specific expression of miR-218 in the developing spinal cord (Fig. 2b,c).

To test whether robust upregulation of miR-218 is important for the specification of motor neuron fate, we generated a mouse ESC line, in which Dox induces the expression of miR-218 sponge inhibitor linked to the GFP gene, as well as a control ESC line that expresses scrambled sponge gene, whereas the control scrambled sponge inhibitor has scrambled (scrm) bulge miR-218 MRE sequences (Fig. 3a). To enhance the inhibition of endogenous miR-218, we combined the miR-218 sponge inhibitor with 2′-O-methylated (2′OMe) antisense RNA inhibitors, which function as specific miRNA inhibitors29.

Together, our data provide in vivo evidence that miR-218 plays an important role in the specification of motor neurons.

miR-218 is important for motor neuron fate specification.

Next, to inhibit the action of endogenous miR-218, we designed miRNA bulge sponge inhibitors that function as stable and competitive miRNA inhibitors both in vitro and in vivo26,28. The miR-218 sponge inhibitor has multiple repeats of bulged miR-218 MRE sequences that are cloned into the 3′UTR of a CMV promoter-driven LacZ gene, whereas the control scrambled sponge has scrambled (scrm) bulge miR-218 MRE sequences (Fig. 3a). To enhance the inhibition of endogenous miR-218, we combined the miR-218 sponge inhibitor with 2′-O-methylated (2′OMe) antisense RNA inhibitors, which function as specific miRNA inhibitors29.

Table 1 | Selected miR-218 RISC-trap target mRNAs.

| microRNA | Fold change | P value | Dox + Exp. Rank |
|----------|-------------|---------|----------------|
| miR-218  | 70.76       | 0.006   | 5              |
| miR-382  | 8.89        | 0.034   | 87             |
| miR-203  | 6.57        | 0.045   | 142            |
| miR-200a | 4.30        | 0.043   | 114            |
| miR-376c | 4.15        | 0.047   | 82             |
| miR-411  | 3.28        | 0.048   | 34             |
| miR-26b  | 3.27        | 0.044   | 54             |
| miR-28   | 3.27        | 0.049   | 110            |
| miR-495  | 3.24        | 0.046   | 82             |
| miR-106b | 3.23        | 0.047   | 32             |
| miR-224  | 3.23        | 0.049   | 166            |
| miR-34b-3p| 3.22        | 0.049   | 120            |
| miR-539  | 3.20        | 0.050   | 56             |
| miR-29a  | 3.20        | 0.049   | 111            |
| miR-362-3p| 3.18        | 0.046   | 222            |
| miR-379  | 3.06        | 0.021   | 72             |
| miR-145  | 3.05        | 0.022   | 132            |
| miR-350  | 3.03        | 0.025   | 210            |

A list of miRNAs that exhibit a significant induction by Dox treatment (>3-fold, P<0.05), as determined by TaqMan miRNA arrays. Expression rank (Exp. Rank) describes the rank of relative expression levels of each miRNA in Dox-treated conditions.
**Figure 2 | miR-218 is expressed and functional in embryonic motor neurons.** (a) Illustration of the developing spinal cord. Stereotypical locations of progenitor cells, interneurons (INs) and motor neurons (MNs) in mouse E11.5 and chicken HH St.27 embryos are shown. (b) miR-218 is specifically expressed in motor neurons of mouse and chick embryos, as shown by in situ hybridization with a probe detecting mature miR-218. (c) miR-218 is induced at the onset of motor neuron differentiation and continues to be expressed in motor neurons throughout mouse embryonic development, as shown by in situ hybridization with a probe detecting mature miR-218. Scale bars represent 100 μm. (d) Illustrations of miRNA sensor plasmids. The multimerized MRE for miR-218 or miR-218* was inserted between the destabilized EGFP (d4EGFP) gene and polyA (pA) sequences. The expression of both d4EGFP and mononuclear RFP (mRFPnCAG) is driven by two separate, ubiquitously active CAG promoters. (e-g) The expression pattern of GFP and RFP in the chick spinal cord electroporated with miRNA sensor vector (e), miR-218 MRE sensor (f) or miR-218* MRE sensor (g). Only electroporated side of the spinal cord is shown. GFP expression is regulated by endogenous miRNA that binds to the MREs present in 3’UTR of the GFP gene, while RFP expression depicts the electroporated cells. The areas of IN and MN are magnified. The miR-218 MRE sensor shows drastically downregulated GFP expression in motor neuron area (bracket in f), indicating that endogenous miR-218 in motor neurons suppresses the expression of GFP. (h) Quantification of relative pixel intensity of GFP/RFP in motor neurons, as quantified using ImageJ programme. Error bars represent the s.d. *** * p<0.0001 in two-tailed Student’s t-test. n = 5 embryos. (i) Analyses using serial sections from the same chicken embryo electroporated with Isl1–Lhx3. In situ hybridization for miR-218 and immunohistochemical analyses with Hb9 antibody reveals that the expression of miR-218 was highly induced by Isl1–Lhx3 in the dorsal spinal cord, where Hb9+ ectopic motor neurons are formed. +, electroporated side; −, unelectroporated control side.

**Identification of direct miR-218 target mRNAs.** To understand the mechanisms by which miR-218 contributes to motor neurogenesis, it is important to determine miR-218 target mRNAs. To this end, we employed a RISC-trap assay, which effectively detects the interactions between a miRNA and its target transcripts, even when the target mRNAs are present at low abundance31. miRNAs regulate target mRNAs via a miRNA-induced silencing complex (miRISC), in which a mature miRNA directly binds to Argonaute that interacts with GW182, leading to destabilization of the mRNA transcripts (Supplementary Fig. 6a). We expressed miR-218 and a dominant negative form of Flag-tagged GW182 (Flag-dnGW182), which is incorporated into miRISC and stabilizes the RISC–miRNA–mRNA interaction, in HEK293T cells. We then immunopurified RISC using anti-Flag antibodies and sequenced co-purified mRNAs on a HiSeq platform. To identify miR-218-specific target transcripts, the RISC-trap data sets for miR-218 were compared against previously generated RISC-trap data sets of three miRNAs, miR-181, miR-124 and miR-132 (ref. 31). This analysis revealed a high confidence list of 1,178 target mRNAs, which satisfied the criteria for the significant enrichment in at least one pairwise comparison among RISC-trap data sets (Fig. 4a, Supplementary Data 2). Our list of miR-218 targets included previously identified miR-218 target transcripts, such as Rictor, CDK6 and GLCE (Fig. 4a, Supplementary Data 2), validating our RISC-trap analyses. In addition, the list revealed many novel miR-218 target mRNAs with high fold enrichments (Fig. 4a, Supplementary Data 2).

Direct MRE search analyses using our miR-218 target list from the RISC-trap revealed that the majority of targets contained miR-218-binding motifs (Fig. 4b). The RISC-trap target mRNAs...
miR-218 targets neural progenitor and interneuron mRNAs. A subset of miR-218 target genes from the RISC-trap screen are particularly relevant in the context of motor neuron development considering their expression pattern and previously reported functions in the developing spinal cord. These target mRNAs include genes that are important for the differentiation and development.
FoxP2 and Sox21.

such as Lhx1, BCL11A, SLC6A1, FoxP2, Pou4f1, Prdm13, Sox21 and Bmpr1b, as well as genes that play roles in spinal neural progenitors, including Tead1.

These target mRNAs include genes that are important for the differentiation and function of spinal interneurons, such as Lhx1, BCL11A, Lhx1 and FoxP2. We performed independent RISC-trap experiments followed by qRT–PCR analyses validated selected miR-218 target genes enriched against miR-181 RISC-trap. RFT1 is a miR-181 target mRNA identified in the RISC-trap screen. The qPCR results were enriched and BCL11A, Lhx1 and FoxP2 were also substantially enriched in miR-218 RISC-trap over miR-181 RISC-trap samples (Fig. 4d). Conversely, RFT1, a miR-181 target mRNA identified in the RISC-trap screen, was enriched in miR-181 RISC-trap over miR-218 RISC-trap.

The functional relevance and the presence of evolutionarily conserved miR-218 MREs on miR-218 target candidates led us to further validate five target mRNAs—Tead1, SLC6A1, BCL11A, Lhx1 and FoxP2. We performed independent RISC-trap experiments with miR-218 and miR-181 in HEK293T cells, and quantified the enrichment levels of the selected targets using qRT–PCR analyses (Fig. 4d). Tead1 and SLC6A1 were strongly enriched and BCL11A, Lhx1 and FoxP2 were also substantially enriched in the miR-218 RISC-trap samples, compared with the miR-181 RISC-trap samples (Fig. 4d). Conversely, RFT1, a miR-181 target mRNA identified in the RISC-trap screen, was enriched in miR-181 RISC-trap over miR-218 RISC-trap (Fig. 4d). This differential enrichment pattern of mRNA target transcripts in this RISC-trap assay was highly correlated with the RISC-trap data sets (Table 3).

Each of the Tead1, SLC6A1, BCL11A, Lhx1 and FoxP2 mRNAs has at least two putative miR-218 MREs in the 3′UTR region (Fig. 5a, Table 3, Supplementary Fig. 8). To test whether miR-218 regulates the expression of target transcripts via a miR-218 MRE, we constructed luciferase reporters, in which the 3′UTR region of each target containing one evolutionarily conserved miR-218 MRE is inserted between a luciferase gene and poly adenylation sequences (Fig. 5a–c). We then transfected luciferase reporters with either miR-218 or miR-181 in HEK293T cells, and monitored the luciferase expression levels. The expression of miR-218 led to significant repression in all five luciferase reporters linked with the 3′UTR of miR-218 targets, compared with miR-181 expression (Fig. 5c). The mutation in miR-218 MRE sequences completely eliminated miR-218-dependent repression of the reporter activity (Fig. 5c, Supplementary Fig. 8), indicating that a miR-218 MRE in the 3′UTR of target mRNAs triggers the miR-218-directed target gene suppression.

Together, our results suggest that miR-218 inhibits the expression of Tead1, SLC6A1, BCL11A, Lhx1 and FoxP2 by directly binding to their 3′UTR regions.

Figure 4 | Identification of direct miR-218 target mRNAs using RISC-trap screen. (a) RISC-trap screens identified direct target genes for miR-218, when analysed against RISC-trap screens for miR-181, miR-132 and miR-124 (ref. 31). All identified miR-218 targets are sorted in a heatmap by fold change and biological replicates, compared to miR-<sub>181</sub> RISC-trap (false discovery rate < 0.05, fold enrichment ≥ 4). (b, c) Analyses for miR-218 MREs in 1178 genes targeted by miR-218. (b) Percent distribution of miR-218 MREs was classified by the inclusion of at least one MRE motif in the order of 8mer > 7mer > 6mer > pivot. Each transcript was counted only once. (c) Percent distribution of miR-218 7mer MRE motifs per target in the 5′UTR, ORF and 3′UTR. (d) Independent RISC-trap experiments followed by qRT–PCR analyses validated selected miR-218 target genes enriched against miR-181 RISC-trap. (e) Schematic model of the expression patterns of selected RISC-trap miR-218 target mRNAs. These target mRNAs include genes that are important for the differentiation and function of spinal interneurons, such as Lhx1, BCL11A, SLC6A1, FoxP2, Pou4f1, Prdm13, Sox21 and Bmpr1b, as well as genes that play roles in spinal neural progenitors, including Tead1, FoxP2 and Sox21.
miR-218 represses target genes in motor neurons via 3'UTR.

To test whether endogenous miR-218 in motor neurons down-regulates each target mRNA via 3'UTR in vivo, we generated miRNA sensor plasmids in which the partial 3'UTR of Tead1, SLC6A1, BCL11A, Lhx1 and FoxP2 is cloned downstream of dEeGFP, and monitored the expression ratios of GFP and RFP in motor neurons and interneurons in the developing spinal cord 3 days after in ovo electroporation (Fig. 5d). The control miRNA sensor showed the same GFP/RFP ratio in interneurons and motor neurons (Fig. 5e, k). In contrast, the miRNA sensors with the 3'UTR of Tead1, SLC6A1, BCL11A, Lhx1 or FoxP2 transcripts showed varying degree of downregulation of GFP in motor neurons compared with interneurons. The 3'UTRs of Tead1, SLC6A1 and BCL11A directed ~45% downregulation of GFP in motor neurons, whereas the 3'UTRs of Lhx1 and FoxP2 led to 20% and 10% knockdown of GFP, respectively (Fig. 5f–k). These data suggest that endogenous miR-218 in developing motor neurons is capable of suppressing the expression of Tead1, SLC6A1, BCL11A, Lhx1 and FoxP2 via their 3'UTRs containing miR-218 MREs. 

The partial 3'UTRs of each target gene were sufficient to respond to miR-218 in our luciferase and miRNA sensor analyses, showing that each miR-218 target transcript has at least one functional miR-218-binding site. However, it is notable that each of the selected miR-218 targets contains multiple 218 MREs throughout the gene body (Table 3, Supplementary Fig. 8a–e). For example, the FoxP2 gene has three different miR-218 MREs in the 3'UTR, while the 3'UTR region that we tested contains only one miR-218 MRE. Thus, miR-218-directed suppression of the target genes in our reporter assays likely represents only a fraction of responsiveness of gene repression to miR-218 for each gene.

Table 2 | Gene ontology cluster analysis.

| Gene ontology analysis term                  | % Genes   | P value |
|---------------------------------------------|-----------|---------|
| Transcription 3.65*                         | 16.50     | 7.90E-04|
| Regulation of transcription                 | 14.80     | 7.00E-06|
| Transcription, DNA dependent                | 10.90     | 2.00E-02|
| Cell cycle 7.27*                            | 8.10      | 9.50E-11|
| Cell cycle                                  | 4.30      | 1.00E-07|
| Mitotic cell cycle                          | 3.90      | 1.70E-08|
| DNA stress response 3.66*                   | 5.00      | 9.40E-05|
| Cellular response to stress                 | 4.00      | 4.70E-06|
| DNA repair                                  | 2.70      | 2.00E-03|
| Neuron development 2.09*                    | 3.50      | 3.50E-04|
| Cell projection organization                | 3.50      | 7.70E-03|
| Neuron development                          | 2.80      | 7.40E-03|

*GO cluster score.
Biological process terms from selected significantly enriched clusters.

Table 3 | Selected miR-218 RISC-trap target mRNAs.

| miR-218 target mRNA | RISC-trap fold change | # 218 MREs |
|---------------------|-----------------------|------------|
| Tead1               | 37.3                  | Human-10   |
| versus miR-181:     |                      |            |
| versus miR-132:     |                      |            |
| SLC6A1 (GAT1)       | 14.5                  | Mouse-7    |
| versus miR-181:     |                      |            |
| versus miR-132:     |                      |            |
| versus miR-132:     | 13.7                  | Human-4    |
| BCL11A (Ctip1)      | 17.9                  | *Chicken-2 |
| versus miR-181:     |                      |            |
| versus miR-181:     | 4.8                   | Human-6    |
| versus miR-132:     | 6.8                   | Mouse-4    |
| versus miR-132:     | 5.8                   | Chicken-6  |
| Lhx1                | 4.2                   | Human-2    |
| versus miR-181:     |                      |            |
| versus miR-132:     | 4.9                   | Mouse-1    |
| versus miR-132:     | 4.3                   | Chicken-1  |
| FoxP2               | 5.9                   | Human-4    |
| versus miR-181:     |                      |            |
| versus miR-132:     | 4.9                   | Mouse-6    |
| versus miR-132:     | 4.8                   | Chicken-4  |
| Prdm13              | 4.3                   | Human-1    |
| versus miR-181:     |                      |            |
| versus miR-132:     | 5.9                   | Mouse-1    |
| Sox21               | 6.5                   | *Chicken: no seq |
| versus miR-181:     |                      |            |
| versus miR-132:     | 6.5                   | *Chicken: no seq |
| Pou4f1              | 4.4                   | Human-2    |
| versus miR-181:     |                      |            |
| versus miR-132:     | 4.8                   | Mouse-3    |
| versus miR-132:     | 5.4                   | *Chicken: no seq |
| BMPR1b              | 5.2                   | Human-2    |
| versus miR-181:     |                      |            |
| versus miR-132:     | 5.3                   | Mouse-2    |
| versus miR-132:     | 4.8                   | *Chicken-1 |

RISC-trap fold change shows enrichment folds in miR-218 RISC-trap against RISC-trap screens with miR-181, miR-132 or miR-124. The number of miR-218 MREs shows the total number of miR-218 MREs in each gene from human, mouse and chicken. Note that for some genes, the full-length sequences of chicken gene are unavailable.
*Only partial or provisional sequence available.

miR-218 suppresses interneuron differentiation. To test whether miR-218 is capable of suppressing spinal interneuron fate in the developing spinal cord, we misexpressed miR-218 in the dorsal spinal cord using in ovo electroporation of the miR-218 expression construct (Fig. 6a,b). miR-218 did not trigger ectopic generation of motor neurons in the dorsal neural tube (Fig. 6b,d), suggesting that miR-218 alone is not sufficient to drive motor neuron differentiation. However, miR-218 expression resulted in a substantial reduction of Lhx1+, Pax2+ or FoxP2+ interneurons, whereas it did not make a significant change in the number of Ngn2+ cells or Olig2+ motor neuron progenitors (Fig. 6c,d). These results are consistent with our finding that Lhx1 and FoxP2 are miR-218 targets. In addition, sequence analyses uncovered that Pax2 also has an evolutionarily conserved, strong miR-218 MRE in the 3'UTR (Supplementary Fig. 9a,b).

To further test the action of miR-218 in spinal interneuron differentiation, we generated ESC lines, in which either miR-218 or miR-control is constitutively expressed, and differentiated these ESCs to spinal interneurons by treating embryoid bodies with RA (Fig. 6e–g). In this condition, ESCs express spinal interneuron genes and acquire spinal interneuron characteristics13,30. miR-218 effectively blocked the expression of Pax2 and FoxP2, while the level of neuronal differentiation was comparable between miR-218 and miR-control ESCs as assessed by Tuj1 marker (Fig. 6g, Supplementary Fig. 9c). Similar to the spinal cord, miR-218 was insufficient to trigger motor neuron generation (Supplementary Fig. 9c).

Together, our data show that miR-218 serves as an efficient inhibitor of spinal interneuron differentiation, while miR-218 alone is incapable of inducing motor neuron fate.

miR-218 is needed for motor neuron generation by Isl1–Lhx3. Given our finding that miR-218 is upregulated by the Isl1–Lhx3 complex and directly targets a number of genes that control spinal interneuron differentiation, we hypothesized that miR-218
a miR-218 3′ DUGACCAUCACACCAUCACAC 5′
Tead1 hsa 5′ GCAUUCACUUCCACACACACACACACAC 3′
Tead1 opp 5′ GUGUCACACACUCAUCACACAC 3′
Tead1 bta 5′ AUAUCUCACACACACACACACAC 3′
miR-218 3′ DUGACCAUCACACCAUCACAC 5′
SLC6A1 hsa 5′ ACCAUUCAUCUUCAAACACAC 3′
SLC6A1 muu 5′ UCUUCAUCUUCAAACACAC 3′
SLC6A1 gga 5′ AAUCUCACACACACACAC 3′
miR-218 3′ DUGACCAUCACACCAUCACAC 5′
BCL11A hsa 5′ AAUUAACACACACACACACACAC 3′
BCL11A muo 5′ AGUACACACACACACACACAC 3′
BCL11A gga 5′ AUAUUCACACACACACACAC 3′
miR-218 3′ DUGACCAUCACACCAUCACAC 5′
Lhx1 hsa 5′ AAUUAACACACACACACACACAC 3′
Lhx1 muu 5′ AAUUAACACACACACACACACAC 3′
Lhx1 gga 5′ AAUUAACACACACACACACACAC 3′
miR-218 3′ DUGACCAUCACACCAUCACAC 5′
FoxP2 hsa 5′ ACAUAAUCACACACACACACACAC 3′
FoxP2 muu 5′ ACAUAAUCACACACACACACACAC 3′
FoxP2 gga 5′ ACAUAAUCACACACACACACACAC 3′

b CMV Luciferase
Target 3′ UTR with miR-218 MRE

Figure 5 | 3′ UTR region of Tead1, SLC6A1, BCL11A, Lhx1 and FoxP2 mediates miR-218-directed gene repression via miR-218 MRE. (a) Evolutionarily conserved miR-218 MREs in the 3′ UTRs of selected RISC-trap target as identified by TargetScan. (b) Illustration of miRNA luciferase reporter plasmids, in which the partial 3′ UTR of miR-218 targets is cloned downstream of the luciferase gene. (c) Luciferase assays using luciferase reporters linked with the 3′ UTR of miR-218 targets in HEK293T cells. miR-218 inhibits the 3′ UTRs in a miR-218 MRE-dependent manner. Each reporter was transfected with either miR-218 or miR-181. WT, luciferase reporters linked to the wild-type 3′ UTR sequences of each gene; Mut, luciferase reporters linked to the mutated 3′ UTR, in which the miR-218 MRE is mutated to eliminate the binding of miR-218. RLU, relative luciferase unit. Error bars represent the s.d. **P < 0.0001 in two-tailed Student’s t-test. n = 3. (d) Illustration of miRNA sensor plasmids, in which the partial 3′ UTR of miR-218 targets is cloned downstream of d4EGFP. The expression of both d4EGFP and monouclear RFP (mRFPn) is driven by two separate, ubiquitously active CAG promoters. (e-j) The in vivo miRNA sensor analyses in the developing chick spinal cord electroporated with each miRNA sensor as indicated. Only the electroporated side of the spinal cord is shown. GFP expression is regulated by the 3′ UTR of miR-218 target genes containing a miR-218 MRE, while RFP is ubiquitously expressed in all electroporated cells. Interneuron and motor neuron regions are magnified. The miRNA sensors show significant downregulation of GFP in motor neuron area compared with interneuron area. (k) Quantification of relative pixel intensity of GFP/RFP in motor neurons, as quantified using ImageJ programme. Error bars represent the s.d. ***P < 0.0001 and **P < 0.005 in two-tailed Student’s t-test. n = 6–8 embryos.

is a critical downstream effector of the Isl1–Lhx3 complex in suppressing non-motor neuron genes, thus ensuring the proper acquisition of motor neuron cell identity. To determine whether the Isl1–Lhx3 complex represses the differentiation of interneurons in vivo, we misexpressed Isl1–Lhx3 in the chick neural tube and monitored the differentiation of Pax2⁺ and Lhx1⁺ interneurons. Isl1–Lhx3 substantially suppressed the formation of Pax2⁺ and Lhx1⁺ interneurons in the developing spinal cord, compared with the unelectroporated control side (Fig. 7a–d), similar to its effect on interneuron genes in differentiating ESCs13. Notably, the ectopic miR-218/Hb9-expressing cells in the dorsal neural tube are mutually exclusive with Pax2⁺ cells (Supplementary Fig. 10), suggesting that Isl1–Lhx3 directs a complete fate transition from interneurons to motor neurons by strongly suppressing interneuron differentiation in the dorsal spinal cord. Next, to determine whether miR-218 is required for Isl1–Lhx3 to effectively trigger motor neuron differentiation, we electroporated Isl1–Lhx3 with either miR-218 sponge inhibitor or scrambled sponge control, and assessed the formation of ectopic motor neurons. Inhibition of miR-218 action suppressed the Isl1–Lhx3-directed differentiation of motor neurons by ~50% (Fig. 7e,f), while significantly increasing the proportion of Lhx1-expressing cells among Isl1–Lhx3-electroporated cells (Supplementary Fig. 11a,b). Likewise, the miR-218 sponge
inhibitor reduced the efficiency of motor neuron production by the co-expression of Isl1 and Lhx3 by ~35% (Supplementary Fig. 11c,d). These results demonstrate that the activity of miR-218, which is induced by Isl1–Lhx3, is crucial for Isl1–Lhx3 to effectively suppress interneuron fate and to establish motor neuron identity.
neurons in the dorsal spinal cord. The magnified images show that the Hb9
Lhx3 expression resulted in a decrease in Pax2
was quantified by the ratio of ectopic Hb9
Isl1–Lhx3 in triggering ectopic motor neurons in dorsal neural tube. (2
sponge inhibitor (Scrm Spg) in the chick neural tube.
(showing that Isl1–Lhx3 drives motor neuron formation at the expense of interneurons.
suggesting that Isl1–Lhx3 drives motor neuron formation at the expense of interneurons.
embryos. (model of the Isl1–Lhx3 and miR-218 gene regulatory network in motor neuron development.
During CNS development, neural progenitors undergo dramatic
leads to the aberrant upregulation of interneuron genes, and
neurons. Inactivation of
Isl1
choosing their cell fates and have potential to generate non-motor
non-motor neuron fates (Fig. 7g). The generation of motor
neuron progenitors, which give rise to motor neurons, has been
attributed to the action of a sonic hedgehog morphogen gradient and the cross-repressive interactions between progenitor
transcription factors, such as Olig2, Irx3 and Nkx2.2 (refs 1,2,6,46,47).
However, motor neuron progenitor cells maintain plasticity in
choosing their cell fates and have potential to generate non-motor
neurons. Inactivation of Isl1, Hb9 or LMO4 in motor neurons
leads to the aberrant upregulation of interneuron genes, and
Olig2-lineage cells produce ventral interneurons in addition to
motor neurons10,14,23,48,49. Thus, the mechanisms to suppress
alternative cell fates need to operate continuously to block the
erroneous gene expression during motor neuron differentiation.
Our study demonstrates that miR-218 is induced during motor
neurogenesis and plays an essential role to ensure the choice of
motor neuron fate by suppressing interneuron genes. In addition,
miR-218 is likely important to promote the timely transition of
progenitor cells to postmitotic neurons by repressing target
transcripts that promote neural progenitor characteristics.

Discussion
During CNS development, neural progenitors undergo dramatic
to differentiation into diverse types of
postmitotic neurons with distinct molecular and morphological
phenotypes. One of the fundamental challenges in development is
to understand the molecular mechanisms that drive this drastic
and thorough transformation of the gene expression profile as
neural progenitors acquire a specific neuronal identity. In the last
decade, there has been an increase in our understanding of the
role of miRNAs in neuronal development, where miRNAs have
been shown to be important regulators of neuronal differentiation
in numerous model systems1,23,27,44,45. However, the role of
miRNAs in directing the differentiation of distinct neuronal cell
types remains ambiguous. Here we report that miR-218 acts as an
essential regulator of motor neurogenesis as a direct downstream
target of the Isl1–Lhx3 transcription complex (Fig. 7g).
Our comprehensive analyses of miR-218 expression, function
and direct targets provide strong evidence that miR-218 plays
a crucial role in motor neuron differentiation by repressing

Figure 7 | miR-218 is required for efficient generation of motor neurons by Isl1–Lhx3. (a,c) Immunohistochemical analyses in the chick neural tube
electroporated with Isl1–Lhx3. Isl1–Lhx3 suppresses the generation of Pax2+
or Lhx1+ interneurons, while promoting the formation of ectopic Hb9+ motor
neurons in the dorsal spinal cord. The magnified images show that the Hb9+
neurons are largely exclusive with Pax2+ or Lhx1+ interneurons,
suggesting that Isl1-Lhx3 drives motor neuron formation at the expense of interneurons. (+), electroporated side; (−), unelectroporated control side. (b,d) Quantification of the number of Pax2+ or Lhx1+ interneurons on the electroporated ( +) and unelectroporated (−) sides of the spinal cord. Isl1–
Lhx3 expression resulted in a decrease in Pax2+ or Lhx1+ interneurons. Error bars represent the s.d. ***P<0.0001 in two-tailed Student’s t-test. n = 3
embryos. (e,f) The analyses of ectopic motor neuron formation by Isl1–Lhx3 in the presence of either miR-218 sponge inhibitor (miR-218 Spg) or scrambled
sponge inhibitor (Scrm Spg) in the chick neural tube. (+), electroporated side; (−), unelectroporated control side. miR-218 inhibition reduces the efficiency of
Isl1–Lhx3 in triggering ectopic motor neurons in dorsal neural tube. (f) The effect of miR-218 inhibition on Isl1–Lhx3-induced motor neuron differentiation
was quantified by the ratio of ectopic Hb9+ motor neurons (MNs) over Lhx3-expressing transfected cells (Elect cells). Error bars represent the s.e.m.
***P<0.0001 in two-tailed Student’s t-test. n = 5 embryos. (g) Model of the Isl1–Lhx3 and miR-218 gene regulatory network in motor neuron development.
While triggering the expression of many motor neuron-specific genes required for motor neuron differentiation and maturation, Isl1–Lhx3 also directly
induces the expression of miR-218-1 and miR-218-2, which are crucial to suppress unwanted interneuron genes in developing motor neurons.
Interestingly, miR-218 does not appear to play an instructive role in motor neuron fate determination on its own, given that the misexpression of miR-218 alone is not sufficient to induce the formation of motor neurons in the spinal cord and differentiating ESCs. These results indicate that the activity of Isl1–Lhx3 to inhibit interneuron fate\(^{13}\) can be uncoupled from its activity to induce motor neuron generation. Our results also suggest that the miR-218-mediated action of Isl1–Lhx3 to suppress interneuron genes is important for Isl1–Lhx3 to efficiently trigger motor neuron differentiation. Together, our results support a model where miR-218, a downstream effector of Isl1–Lhx3, represses transcripts that promote interneuron and progenitor characteristics in differentiating motor neurons to ensure precise differentiation and the development of proper cellular phenotypes (Fig. 7g).

miR-218 expression is detected at the onset of motor neuron differentiation and the expression is maintained exclusively in motor neurons throughout embryonic spinal cord development. Thus, miR-218 is likely to have roles in mature motor neurons in addition to the establishment of motor neuron fate. In this regard, it is interesting to note that many target transcripts identified in the unbiased RISC-trap screen suggest a potential function for miR-218 in regulating neurite morphogenesis and synapse development. The miR-218 targets from our RISC-trap screen include PTEN, NrCAM, CNTNAP2, EphA7, VCAN, MACF1, Clasp2, Robo1 and Robo2 (Data set2 and in this category include PTEN, NrCAM, CNTNAP2, EphA7, VCAN, MACF1, Clasp2, Robo1 and Robo2 (Data set2 and in this category). Together, our results strongly suggest a potential role for miR-218 in regulating neurite morphogenesis and synapse development and how they are interconnected with cell fate-determining transcription factors. The development of a unique cellular identity requires both activation and repression of genes, thereby building a gene expression profile that determines specific molecular and morphological characteristics. Our study suggests that employing miRNAs as downstream effectors of transcription activators to induce gene repression could be a prominent strategy in cell fate specification.

**Methods**

**DNA and RNA constructs.** Mammalian expression constructs for Isl1, Lhx3, Isl1–Lhx3, Isl1–Lhx3–ESC, and GFP were previously described. The miRNA sensor plasmid was previously described\(^{13}\). miRNA sensor MREs were cloned into the 3’UTR of a CMV-LacZ reporter. The sequences ordered as synthetic genes are listed in Supplementary Table 1. The miRNA array analyses was performed with TaqMan Array Rodent MicroRNA Card A (Life Technologies). The miRNA Array analyses 380 miRNAs and contains five endogenous controls and one negative control assay. RNA extraction and complementary DNA amplification for TaqMan miRNA array, miRNA and pri-miRNA assays were performed according to manufacturer’s instructions (http://www.lifetechnologies.com/us/en/website-overview/ab-welcome.html).

**In ovo electroporation and immunohistochemistry.** In ovo electroporation was performed as described\(^{15}\). In brief, the constructs were injected into the lumen of the neural tube of HH stage 12 chick embryos, which were then electroporated. The embryos were harvested at HH stage 24–27, fixed in 4% paraformaldehyde and cryosectioned at 12 μm. Immunohistochemistry was performed using 0.1% Fish gelatin (Sigma) blocking buffer with overnight incubation at 4 °C, using the following primary antibodies: mouse anti-HH9/MinR2 (DSHB, 5C10; 1:300), rabbit anti-Pax2 (Life Technologies, Zymed 71-6000; 50% glycerol 1:500), rabbit anti-Lhx3/Lim1-2 (ref. 11; Homemaded 1:3000), rabbit anti-FoxP2 (Abcam, ab16046; 1:1000), rabbit anti-Lhx3 (ref. 11; Homemaded 1:2000), rabbit anti-Olig2 (ref. 13; Homemaded 1:1000), rabbit anti-Ngn2 (refs 64,65; Homemaded 1:5000), chicken anti-GFP (Aves Labs, GFP-1020; 1:2000), mouse anti-Tuj1 (Covance, MMS-435P, 1:2000). The n in quantification of chick electroporation data indicates the number of embryos included in the analyses. At least two to six sections per embryo were used for quantification. The methods used for statistical analyses are listed within each figure legend.

For the quantification of pixel intensity in miRNA sensor analyses, in ovo electroporation of miRNA sensor plasmids (0.1 μg in 0.5 μl) was performed as described above. Unregistered images were acquired on a Zeiss Axio Imager.Z2 microscope, maintaining the same exposure time ratio of GFP and RFP for each section. Pixel intensity was determined using ImageJ programme (Supplementary Methods).

**Luciferase reporter assays.** HEK293T cells were cultured in DMEM media supplemented with 10% fetal bovine serum. Cells were plated in 48-well plate and were electroporated for 24 h and transient transfections were performed using electroporation 2000 (Invitrogen). An actin-β-galactosidase plasmid was co-transfected for normalization of transfection efficiency and 20 nM of miRDIAN microRNA
in situ hybridization assay. For in situ hybridization analysis, embryos were harvested at indicated stage, fixed in 4% formaldehyde, embedded in optimal cutting temperature compound, sectioned, and probed with biotinylated nucleic acid–modified miR-218 or miR-218* oligonucleotide probe (Exonq) was labelled with digoxigenin according to the suppliers protocol (Roche) and used for in situ hybridization as described.69

RISC-trap and quantitative RT-PCR. RISC-trap experiments and data analyses were performed as previously described, except that reads for each gene were counted by HTSeq (Simon.Huber.2013 HTSeq – A Python framework to work with high-throughput sequencing data_BioStar0298243). For independent miR-218 and miR-181 RISC-trap assays, transfections and immunoprecipitations were performed as previously described41 and Maxima H Minus (Thermo Scientific) used for reverse transcription. The levels of mRNA were determined with quantitative RT–PCR using SYBR-Green kit (Invitrogen) and Mx3000P (Stratagene).

GO and MRE analyses. The GO functional analysis was carried out with the online tool DAVID using the default configurations67. For the MRE-directed chromatin/antibody complex with protein A agarose beads, the beads were washed with NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5) sequentially. Then, cells and cells were dissociated and subjected to ChIP assays. Cells were washed with NaCl, 150 mM NaCl), TSE II (same components as in TSE I except 500 mM NaCl) and TSE III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM NaCl, 1 mM EDTA, 10 mM MgCl2). Supernatant was collected after quick spin and incubated with anti-Flag gm l.C. After pull down of Flag–Isl1–Lhx3/GFP antibody. For motor neurons, differentiation of miR-218 and miR-Control ESCs, EBs were treated with all-trans RA (0.5 μM) for 4 days. ChIP assays. ChIP was performed as described previously,11,46,69 in Isl1–Lhx3–ESCs and mouse embryonic spinal cords. Isl1–Lhx3–ESCs were cultured on 0.1% gelatin-coated dishes in the ESC growth medium. miR-218 and miR-181 treated reporter relative luciferase units. All transfections were repeated independently at least three times. Data are represented as the mean of triplicate values obtained from representative experiments. Error bars represent s.d.

ChIP-seq data analysis. ChIP-seq data sets from previous publications17,18 were analysed using MACS57. For the analysis of peak location, we collected gene annotation from the UCSC genome browser (mm9).
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Author contributions

K.P.T. and S.-K.L conceived and designed the project. K.P.T. planned and performed most of the experiments. S.L., H.N. and H.-C.H designed and performed ESC experiments in Fig. 1d–h, Fig. 3f–j and Fig 6e–g. A.C.C., R.S. and R.H.G. developed the RISC-trap assay and provided RISC-trap data. R.S. performed the bioinformatics analyses. S.M.G. provided preliminary miR-218 expression data. J.-s.K. provided additional bioinformatics analyses. J.W.L. and S.-K.L. provided advice and editing. R.S. performed the bioinformatics analyses. J.W.L. and S.-K.L. supervised the study. K.P.T. and S.-K.L. wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: A.C.C. is the inventor on a patent application on the described RISC-trap methodology. All other authors declare no competing financial interests.

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