Effective heritable gene knockdown in zebrafish using synthetic microRNAs

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Although zebrafish is used to model human diseases through mutational and morpholino-based knockdown approaches, there are currently no robust transgenic knockdown tools. Here we investigate the knockdown efficiency of three synthetic miRNA-expressing backbones and show that these constructs can downregulate a sensor transgene with different degrees of potency. Using this approach, we reproduce spinal muscular atrophy (SMA) in zebrafish by targeting the \textit{smn1} gene. We also generate different transgenic lines, with severity and age of onset correlated to the level of \textit{smn1} inhibition, recapitulating for the first time the different forms of SMA in zebrafish. These lines are proof-of-concept that miRNA-based approaches can be used to generate potent heritable gene knockdown in zebrafish.
owing to its transparency, extraterine development, versatility for microscopy and suitability for large-scale experiments, zebrafish (Danio rerio) has become an attractive model for genetic and biomedical research. Several reverse genetic tools, such as Zinc-finger Nucleases, TALEN and CRISPR, have been recently developed that allow to interrogate gene function and to model human diseases. Nonetheless, zebrafish is lacking reliable and heritable spatiotemporal knockdown tools. Knockdown through injected morpholinos can only be used for the first few days of development and is difficult to control spatiotemporally.

Moreover, it was recently suggested that off-target effects of morpholinos may be more prevalent than previously thought. Several recent publications suggest that an alternative might come from microRNA (miRNA)-based knockdown. However, this approach would be to reduce SMN to a level observed in patients. Efforts have been made to reintroduce human SMN1 into the zebrafish. In addition, the use of tissue-specific promoters in the zebrafish has become an attractive model for genetic and biomedical research. Several studies suggest that artifical miRNA expression can be used effectively to address gene function and to generate useful transgenic models.

Spinal muscular atrophy (SMA) is an autosomal recessive human disease characterized by motoneuron loss, progressive muscle weakness and premature death. SMA results from a partial loss of Survival Motor Neuron (SMN) gene activity. SMN is encoded by a highly conserved gene, expressed ubiquitously and lethal when fully absent. In humans, and only in humans, this protein is encoded by a nearly identical genes, SMN1 and SMN2 (refs 19–21). SMN2 contains a mutation, which leads to inefficient splicing of exon 7 and to an unstable truncated protein (for review see refs 20,21). SMN2 is present in human in multiple copies with variable numbers between individuals. All SMA patients present full SMN1 loss of function, partially rescued by SMN2 expression. There are four types of SMA (I–IV) from severe to mild (OMIM 253300, 253350, 253400 and 271150), which are directly correlated to the amount of residual functional SMN protein. The zebrafish genome contains a single orthologue named smn1. In zebrafish, loss of function mutations of smn1 are early lethal. To mimic SMN in vivo, one has to downregulate the level of SMN instead of abolishing it. Efforts have been made to reintroduce human SMN2 into the smn1 loss of function background; however, to date this approach failed to recapitulate the hypomorphic forms of SMA in zebrafish. Another approach would be to reduce SMN to a level observed in patients. This has been achieved in zebrafish through morpholino injections, revealing a critical role of SMN in motoneuron development. However, this approach is limited to the first few days of the fish, is difficult to control spatiotemporally and needs to be repeated for each embryo to be studied.

Here we tested the knockdown efficiency of three miRNA-based backbones and show that they can be used to downregulate a gene of interest, but differ in potency. By targeting the smn1 gene, we used this approach to generate new transgenic zebrafish models of SMA. These models exhibit phenotype severity that is correlated to the level of smn1 inhibition. Thus, this strategy allows recapitulating the different forms of the disease by tuning the level of Smn protein. We achieved reduction of Smn protein by up to 90%, producing transgenic animals with early phenotypes, including motor neuron abnormalities, shorter size, abnormal swimming behaviour and premature death. Conversely, several transgenic lines with less potent smn1 reduction developed adult onset phenotypes, including abnormal swimming behaviour, scoliosis and considerable weight loss. This is the first time that a zebrafish model offers the possibility to investigate SMA at later stages. The ease with which these heritable knockdown models can be generated opens new avenues, not only to study SMA, but also to model other diseases in which gene dosage is critical. This approach would also be useful for the study of genes that are embryonically lethal in null alleles, haploinsufficient, when spatiotemporal inhibition is desired or when several genes need to be downregulated simultaneously, such as in models of polygenic disorders.

Results

Generation of pME-RNAi artificial-miR-expressing constructs. To select an appropriate vector for both in vitro artificial miRNA synthesis for zebrafish transient experiments and the generation of miRNA-expressing constructs for transgenesis, we generated three different plasmids compatible with the Tol2 kit components and containing minimal elements for RNA synthesis (named pME-RNAi651, pME-RNAi661 and pME-RNAi671; Fig. 1). Two constructs were generated starting from existing miR-expressing cassettes, based on the dre-miR30 and mmu-miR155 scaffold. The third plasmid, based on the hsa-miR218 sequence, was made in our laboratory. To test the ability of these pME-RNAi constructs to successfully trigger genetic knockdown, we took advantage of the transgenic fish line Bactin:mCherry:UTr, which has ubiquitous and homogeneous mCherry expression (Fig. 2). Starting from the different pME-RNAi empty backbones, we introduced compatible sequences (pre-miRs) that would lead to the formation of the same synthetic mature miRNA with perfect complementarity to a unique region in the 3’-untranslated repeat (UTR) of the mCherry mRNA (Fig. 1, Supplementary Figs 1 and 2).

miR-155 and miR-218 backbones lead to potent knockdown. pME-RNAi651, pME-RNAi661 and pME-RNAi671 constructs with green fluorescent protein (GFP) marker followed by a synthetic pre-miR directed against the 3’-UTR of mCherry were cloned downstream of the ubiquitin promoter in a mini-tol2-R4R2 multisite gateway-compatible destination plasmid (Supplementary Fig. 3). Destination clones were co-injected with transposase mRNA to promote DNA integration. GFP mosaic expression was obtained as expected and allowed the tracking of artificial miRNA expression (Fig. 2a,d,g). This approach allows rapid evaluation of mCherry knockdown efficiency within a single fish larva by comparing cells expressing synthetic miRs (GFP-positive) to cells expressing little or no miRNA (GFP-negative; Fig. 2a–l). All three pre-miR backbones led to a visible cell-specific downregulation of mCherry accumulation. However, the
Regulate mCherry expression by introducing miRNAs able to significantly down-reduce red fluorescence by 78%, while only one miR directed against the ORF (target site 3) was able to significantly down-regulate mCherry expression by 29% (Fig. 3q–u).

**Figure 1** | Schematics of pME-RNAi plasmids generated in this study. Three different miRNA-mediated backbones were cloned in a modified pME construct, which encodes Spectruminic resistance and minimal sequences for RNA synthesis (SP6/SV40). pME-RNAi651 is based on a mmu-miR155 backbone, pME-RNAi661 on a dre-miR30 backbone and pME-RNAi671 on a hsa-miR218 backbone. Two additional constructs, encoding dsRED or Cerulean reporters instead of GFP, were generated for the scaffold based on mmu-miR155 (named pME-RNAi652 and pME-RNAi653, respectively). Unique restriction sites BamHI, BglII and XhoI can be used to chain artificial miRNA-expressing cassettes.

**Targeting the 3′-UTR leads to potent knockdown.** We next tested the potential of the miR155 backbone to trigger knockdown when the open reading frame (ORF) was targeted (Fig. 3). In addition to the 3′-UTR target site, we defined three sites in the ORF of mCherry mRNA (Fig. 3a, Supplementary Figs 1 and 2). All four sequences, driven by the ubiquitin promoter, were injected along with transposase mRNA into zebrafish embryos expressing the reporter mCherry protein (Fig. 3a–p). Again, targeting the 3′-UTR led to a visible cell-specific mCherry knockdown (Fig. 3n–p), while two target sites in the ORF did not result in any obvious visible effect (Fig. 3e–j), and a third site inhibited mCherry expression only slightly (Fig. 3k–m). To confirm these observations, RNAs encoding each artificial miRNA were synthesized from pME-RNAi clones and injected at 150 pg per embryo. Artificial miRNA targeting the 3′-UTR region reduced red fluorescence by 78%, while only one miR directed against the ORF (target site 3) was able to significantly down-regulate mCherry expression by 29% (Fig. 3q–u).

Stable and heritable miRNA-mediated knockdown of dre-smn1. To test the potential of this approach to knockdown endogenous gene expression and to try to mimic SMA in zebrafish, synthetic miRNAs were designed to target the dre-smn1 gene (Fig. 4). Four targets were selected, with two in the smn1 3′-UTR and two in the ORF, named miRsmn1-1 to -4 (Fig. 4b and Supplementary Fig. 2). All miRsmn1-expressing constructs were based on the mmu-miR155 scaffold fused to dsRED (pME-RNAi652), and were cloned downstream of the ubiquitin promoter in a mini-tol2-R4R2 destination plasmid (Fig. 4a). To generate transgenic lines, the corresponding DNA constructs (that is, UBI:miRsmn1-1 to -4) were microinjected into one-cell stage hsa-miR218-2-GFP zebrafish embryos, a transgenic line that specifically expresses GFP in motor neurons34. Several founders were identified for each construct, and then sorted based on strong dsRED expression in F1 embryos. The highest expressing founders for UBI:miRsmn1-2 to -4 constructs were then outcrossed to establish F1 transgenic lines and were named as follows: UBI:miRsmn1-2-7, UBI:miRsmn1-3-2 and UBI:miRsmn1-4-1 (Fig. 4c). Two founders and two independent F1 transgenic lines were generated for the miRsmn1-1 construct, named UBI:miRsmn1-1-5 and #9 (Fig. 4c). F1 transgenic lines were further evaluated in regards to the number of transgene insertions. Each F1 transgenic line used gave birth to a ratio of close to 50% positive dsRED embryos when outcrossed with wild-type animals, suggesting the presence of a single insertion (or at least insertion(s) in a single chromosome). In order to analyse smn1 knockdown efficiency, F1 animals were incrossed and the brightest 20 embryos per cross were sorted for analysis. The larvae were killed at 72 hpf to extract whole-lysate proteins. Smn protein was revealed and quantified in each genetic background via western blot and image analysis (Fig. 4c,d). No significant difference was observed for artificial miRs targeting the smn1 ORF, while a strong reduction was detected for miRs targeting the 3′-UTR. Using image j band intensity measurement, Smn was reduced by 61% in UBI:miRsmn1-4-1, by 73% in UBI:miRsmn1-1-9 and by 93% in the UBI:miRsmn1-1-5 zebrafish line. Similar results were obtained at 6-days post fertilization (dpf), suggesting that smn1 knockdown was stable over time (Supplementary Fig. 4). These results show that artificial miRNA-expressing constructs can be used to generate transgenic zebrafish that undergo potent gene knockdown as we achieved the reduction of Smn protein by up to 90%.

smn1 knockdown transgenic fish show hallmarks of SMA. We next tested whether our transgenic lines presented signs of SMA. It has been shown previously via morpholino injection that transient smn1 knockdown leads to motor neuron malformations27. We started by examining primary motor neuron development to see whether this phenotype was recapitulated in miRsmn1-expressing fish. As above, F1 animals were incrossed and the 20 brightest embryos per cross were sorted out for analysis. Motor neuron axonal projections were examined at 30 hpf, 52 hpf and 5 dpf. There are four types of primary motor neurons, including Caudal Primary (CaP) motor neurons that project long axons ventrally (for review see ref. 32). We only quantified CaP motor neuron projection abnormalities. As expected, animals that express miRsmn1-2 and -3, which target the smn1 ORF, did not show any significant motor neuron projection aberration at 52 hpf when compared with controls. However, fish expressing artificial miRNA directed against the smn1 3′-UTR had CaP motor neuron abnormalities at 52 hpf, including short axons, abnormal branching, as well as pathfinding errors (Fig. 5 and Fig. 6a). F2 transgenic UBI:miRsmn1-1-5 embryos, which present ∼90% Smn1 reduction, were the most affected at 52 hpf with 2.8 (± 0.4) abnormal CaP motor neurons.
neuron projections per side analysed. UBI:miRsmn1-1#9 and UBI:miRsmn1-4#1, with 70% and 60% Smn1 reduction, respectively, presented with 1.4 (±0.3) and 1.1 (±0.3) aberrant CaP motor neuron projections per side. In addition, anti-SV2 and anti-α-tubulin immunostaining performed at 52 hpf on F2 transgenic animals confirmed the in vivo results presented above (Supplementary Figs 5 and 6).

In addition to motor neuron phenotypes, smn1 miR-mediated knockdown fish died prematurely (Fig. 6b). As for motor neuron phenotypes, F2 UBI:miRsmn1-1#5 larvae were the most affected, with no larvae that survived beyond 11 days. No other obvious anatomical abnormality was detected in any miRNA-expressing transgenic lines, except for UBI:miRsmn1-1#5 F2 larvae, which were significantly shorter starting from 52 hpf (Fig. 6c). The absence of other malformations suggests that the level of artificial miRNA expression in the line tested does not induce toxicity such as observed after siRNA injection10.

As SMA impairs motor function in human, swimming behaviour was investigated. Animals were loaded into 24-well plates and spontaneous swimming behaviour was recorded for 10 min. No significant difference was detected with the different transgenic lines, except for F2 UBI:miRsmn1-1#5 animals, which showed strong motor impairment starting at 5 dpf (Supplementary Fig. 7 and Supplementary Movie 1). Compared with control, overall distance swum by these larvae, as well as overall speed, was dramatically reduced (Supplementary Fig. 7b,c). Thus, in addition to motor neuron abnormalities, F2 UBI:miRsmn1-1#5 animals had poor motor function.

*hsa-SMN1 rescues smn1 knockdown phenotypes.* In order to validate these new zebrafish SMA models, we investigated the potential of human SMN1 to rescue the phenotypes presented above. First, we injected hsa-SMN1 mRNA into F2 UBI:miRsmn1-1#5 embryos at 200 and 400 pg. As before, the brightest 20 embryos per condition were selected for further analysis. Both concentrations partially rescued the CaP motor neuron phenotype observed at 52 hpf in F2 UBI:miRsmn1-1#5 animals. The number of abnormal CaP motor neuron projections in F2 UBI:miRsmn1-1#5 was reduced from 2.8(±0.4) to 1.7(±0.2) and 1.6(±0.4) after 200 and 400 pg hsa-SMN1 mRNA injection, respectively (Fig. 7a). Similarly, hsa-SMN1 mRNA partially rescued the reduced length of F2 UBI:miRsmn1-1#5 animals (Fig. 7b). Larvae were killed at 60 hpf to extract whole-lysate protein. Western blot analysis against SMN1 confirmed that hsa-SMN1 mRNA was translated into detectable SMN1 protein (Fig. 7c). These results demonstrate that the observed phenotypes were specific to smn1 knockdown. However, because it has been...
shown that an excess of artificial small RNA can flood the endogenous miRNA pathway and lead to toxicity by repressing miR-430 maturation, a possible role of miR-430 was investigated\(^{10,15,16}\). A synthetic form of miR-430 was injected at \(|B| 100–150\) pg. No significant effect was detected on motor neuron development and overall length of 52 hpf F2 UBI:miRsmn1-1#5 animals (Fig. 7a,b).

In addition to these transient experiments, a transgene carrying hsa-SMN1 under the control of the ubiquitin promoter was introduced into the F1 UBI:miRsmn1-1#5 transgenic line as described in Supplementary Fig. 8. A founder F0 UBI:miRsmn1-1#5; UBI:hsa-SMN1(clmc2:GFP) was outcrossed with F1 UBI:miRsmn1-1#5. Embryos were separated into two batches, one injected with 200–400 pg hsa-SMN1 RNA to serve as positive control and one kept without treatment (Supplementary Fig. 8). Embryos were then sorted as described schematically in Supplementary Fig. 8 to obtain untreated UBI:miRsmn1-1#5 embryos (negative control), UBI:miRsmn1-1#5 embryos treated with 200–400 pg hsa-SMN1 RNA (positive control) and UBI:miRsmn1-1#5; UBI:hsa-SMN1(clmc2:GFP) animals. Confirming the transient experiments, both the presence of hsa-SMN1 RNA or the integrated UBI:hsa-SMN1(clmc2:GFP) transgene led to a reduction in the CaP motor neuron phenotype at 52 hpf. However, while injected mRNA only partially rescued smn1 knockdown, transgenic hsa-SMN1 expression resulted in complete rescue (Figs 7d and 8). Larvae expressing hsa-SMN1 were also indistinguishable from wild type in terms of overall anatomy and length at 52 hpf. Finally, the presence of the transgene restored a normal survival rate, while injection of mRNA did not extend the life of the affected larvae (Fig. 7e). These results confirm that the defects observed in UBI:miRsmn1-1#5 were specific to smn1 knockdown, and that Smn protein is critical to early motor neuron development in zebrafish and also for survival, presumably independently of its early role in motor neuron formation.

smn1 knockdown recapitulates the hypomorphic forms of SMA. We generated several independent transgenic lines presenting phenotypes with different degrees of severity, proportional to the level of smn1 reduction (Figs 4–6). There are four types of SMA with outcomes linked to the amount of residual functional SMN. Depending on the type of the disease, patients present with early to late/adult motor neuron disease along with very short- to normal lifespan. Thus, our miR-mediated knockdown approach allows to better recapitulate these conditions. For example, F2 UBI:miRsmn1-1#5 animals are comparable to the severe form of the disease with early phenotypes, and death early in life. Interestingly, F1 UBI:miRsmn1-1#5, which did not show increased death at the larval stage or strong early motor neuron phenotype, developed adult phenotypes starting from 3 months post fertilization, including marked weight loss, scoliosis and abnormal swimming behaviour (Supplementary Fig. 9 and Supplementary Movie 2). It is noteworthy that double transgenic UBI:miRsmn1-1#5; UBI:hsa-

Figure 3 | Evaluation of the effect of target sequence/position on knockdown efficiency in 52 hpf zebrafish embryos. (a) Schematic representation of mCherry mRNA produced in the \(\text{bactin:}\text{mCherry:sUTR}\) zebrafish line, as well as in artificial miRNA targets. (b–p) Embryos with ubiquitous mCherry expression were injected with transposase and 30 pg of toto-flanked miRNA-expressing constructs (mmu-miR5 backbone), which were designed to produce artificial miRNA targeting different sites of the mCherry mRNA. A minimum of 10 larvae per condition were analysed in order to estimate knockdown efficiency. (q–u) Homozygous bactin:miRcherry:UTR embryos were injected with corresponding RNA (150 pg), synthesized via linearized pME-RNAi clones, and raw red fluorescence was quantified, normalized and presented as percentage. The means of 10 embryos \(\pm\) s.d. Different from control at *\(P = 0.05\), **\(P = 0.00001\). Scale bar, 50 \(\mu\text{m}\).
SMN1(cmlc2:GFP) animals—used in the rescue experiment above—did not show any obvious defect at adult stages, confirming the specificity of these adult phenotypes. Similar to intermediate forms of SMA, F2 UBI:miRsmn1-1#9 and F2 UBI:miRsmn1-4#1 showed mild early phenotypes with better survival outcome than F2 UBI:miRsmn1-1#5 (Fig. 6). However, no sorted fish survived more than 3 months and reached the adult stage. Thus, using miR-mediated knockdown, it is possible to mimic the different hypomorphic states of SMA in zebrafish.

**Discussion**

We showed here that the use of synthetic miRNA is a viable approach to knockdown a gene of interest in zebrafish. We first generated pME-RNAi clones that present the advantage of allowing RNA synthesis for rapid knockdown validation and that are also compatible with the Tol2 kit (Fig. 1)23,29. We tested three pri-miR backbones and showed that they are able to trigger RNAi. Surprisingly, in our hands, backbones based on pri-dre-miR30, which was previously used in zebrafish studies, had a very limited effect (Fig. 2)7,8. However, only one target was used to test and to compare the different constructs. More experiments may have to be performed to definitively conclude on the efficiency of each backbone to trigger knockdown. We suspected that each pri-miR sequence would behave differently depending on the artificial miRNA sequence that has to be processed. The sequence used in our experiments may not be compatible with the pri-dre-miR30 sequence. Unfortunately, it is currently difficult to predict which backbone/pri-miR would be the most appropriate to process a particular artificial miR sequence. Nonetheless, pri-dre-miR30 still presents limitations in terms of use. The restriction site used to insert artificial pre-miRs—that is, 2 × BbsI—does not allow directional cloning and, more problematically, is present in several fluorescent markers, hampering the use of mCherry or dsRED markers for instance.

In addition to backbone-dependent limitations, our data also suggest that the choice of the target sequence is critical. For example, it is difficult to design an efficient synthetic miR that targets the ORF, and those that triggered an RNAi response only showed poor knockdown. Interestingly, the literature tends to confirm this phenomenon, not only in zebrafish but also in mouse.

**Figure 4** | Generation and analysis of zebrafish transgenic lines expressing artificial microRNAs against different smn1 target sites. (a) Schematic representation of miR-RNAi construct used to release artificial miRNA against smn1, based on the mmu-miR155 scaffold. (b) Schematic view of dre-smn1 transcript and corresponding target sites. Artificial anti-smn1 miRNA (miRsmn1) target sequences are annotated from 1 to 4. (c) Western blot analysis of whole 72hpf-transgenic larval lysate revealing endogenous SMN protein. (d) Western blot band quantification. SMN band intensity is expressed as the percentage of control. UBI:miRsmn1-X, with X between 1 and 4, corresponds to smn1 target position number.

**Figure 5** | Representative images of 52 hpf zebrafish larvae expressing GFP in motor neurons ± smn1 knockdown. Lateral views of (a) control (empty vector) and (b–e) transgenic zebrafish larvae expressing artificial miR (targeting smn1 3′-UTR). Compared with control animals, transgenic larvae expressing miRsmn1 present abnormal motor neuron development, including abnormal branching, short axons as well as pathfinding errors (white arrowheads). Transgenic animals expressing artificial miRNA against the ORF (targets 2 and 3) do not show obvious motor neuron abnormality. Scale bar, 50 μm.
Figure 6 | Phenotype severity is correlated with the level of SMN1 inhibition. (a) Number of ventral motor neuron (CaP) abnormalities observed per side of 52 hpf larvae (short axons, abnormal branching, pathfinding error and/or absence of cell body). Raw red fluorescence evaluation of each population (reflecting transgene expression) is shown in Supplementary Fig. 4a. (b) Survival assay of different transgenic lines expressing artificial miRNA against dre-smn1. (c) Animal size comparison between 52 hpf control (empty vector) and 52 hpf UBI:miRsmn1-1#5 transgenic lines (compared with control, other miRsmn1-expressing lines do not present significant difference). The means of 20 larvae ± s.e.m. Different from control at *P ≤ 0.04, **P = 0.01, ***P = 0.0001.

Figure 7 | Phenotypic rescue of UBI:miRsmn1-1#5 transgenic line. (a) Number of ventral motoneuron (CaP) abnormalities observed per side of 52 hpf larvae. miRsmn1-5-expressing animals were injected with either hsa-SMN1 RNA or miR-430 to test their capacity to rescue the observed motoneuron phenotype. (b) Animal size comparison between 52 hpf control (empty vector) and 52 hpf UBI:miRsmn1-1#5 transgenic lines ± hsa-SMN1 RNA or miR-430 injection. (c) Western blot analysis of whole 56 hpf UBI:miRsmn1-1#5 larvae lysate (± hsa-SMN1 RNA) revealing dre-SMN and hsa-SMN protein. (d) Number of ventral motor neuron (CaP) abnormalities observed per side of 52 hpf larvae. Rescue experiment comparing RNA injection versus ubiquitous transgenic expression of hsa-SMN1 (see Supplementary Fig. 5 for strategy used). (e) Survival assay comparing control (empty vector) versus UBI:miRsmn1-1#5 ± hsa-SMN1 RNA injection or ubiquitous transgenic expression of hsa-SMN1. Means of 20 larvae ± standard error of the mean. Different from control at *P ≤ 0.04, **P = 0.02, ***P = 0.001, ****P = 0.0001.
Using transgenic artificial miRNA expression, we generated several lines that present heritable and potent (up to 90%) smn1 gene knockdown. To our knowledge, this is the first evidence that miR-mediated knockdown in zebrafish can lead to the generation of useful transgenic animals for biomedical research. Indeed, several attempts demonstrated that heritable gene inhibition using artificial miR could be achieved, but with a level of inhibition that is not potent enough to suit traditional genetic studies. A reason that might explain these results is that researchers often focused on the ORF to select target sequences. As explained above and supported by our results, the ORF is difficult to target in zebrafish or mouse. Our success may result from the use of the smn1 3'-UTR, or, alternatively, smn1 might be exceptionally sensitive to this knockdown approach.

Using the technique described here, we generated new transgenic models of SMA. There are four different types of SMA in human, with onset and severity correlated to the residual SMN protein level. To date there is no zebrafish model that recapitulates these hypomorphic states, and while an smn1 mutation has been recently identified in zebrafish, total absence of SMN does not reflect SMA conditions. Efforts are now under way to introduce human SMN2, as was performed in the mouse. However, even if the corresponding models might be useful to study factors that would act on SMN2 stability and/or synthesis, they failed to mimic the different hypomorphic forms of SMA in zebrafish. Using synthetic miRNA expression against smn1, one can now easily generate allelic series of transgenic lines that better mimic SMA in zebrafish. We have already generated animals with very early phenotypes to animals with adult onset, offering new possibilities to investigate SMA biology. It is also now straightforward to interrogate this disorder tissue specifically by restricting knockdown to muscle or to specific neuronal cell populations.

There is no cure for SMA, and patients are still awaiting palliative treatment that would improve their day-to-day life, especially with respect to motor function. Beneficial drugs might already exist in the current pharmacopeia. Finding such drugs is a challenge as there is no SMA model suitable for large-scale drug screening; with the exception of cell lines that can be used to find drugs increasing human SMN2 gene activity. Indeed, mouse models are not compatible with large-scale drug screening experiments. Zebrafish is, but not the current, SMA model. For example, homozygous null smn1(-/-) animals are not viable, while heterozygous fish are phenotypically normal. Consequently, to generate homozygous larvae to screen, one has to cross heterozygous fish, which will result in 75% larvae with no phenotype and that are impossible to sort out before analysis. This drawback makes large-scale screening experiments difficult and also hampers the workflow of traditional research experiments. Finally, incorporation of human SMN2 failed to rescue smn1(-/-) lethality.

MIR-mediated knockdown zebrafish models of SMA would be a valid option for drug screening. Indeed, this approach allows to generate animals that are relevant to SMA and that present the advantage of expressing a fluorescent marker, allowing sorting out the affected embryos before experiments. Moreover, the most affected embryos in our study (F2 UBI:miRsmn1-1#5) presented with strong motor phenotypes (Supplementary Fig. 7, Supplementary Movie 1). Such a motor phenotype is relatively easy to use in large-scale experiments, and presumably relevant for seeking beneficial drugs that would improve patient lives.

In conclusion, we demonstrate here that promoter-driven synthetic miRNA expression can be used to trigger potent heritable gene silencing in zebrafish. It is probable that

Figure 8 | hsa-SMNI rescues the dre-smn1 knockdown motor neuron phenotype. Representative images of 52 hpf zebrafish larvae expressing GFP in motor neurons. Lateral views of (a) control (empty vector) transgenic animals. (b-d) transgenic UBI:miRsmn1-1#5 zebrafish line (presenting ~90% downregulation of SMN), with hsa-SMNI RNA injection (c) or hsa-SMNI ubiquitous expression via integrated transgene (d; see Supplementary Fig. 7 for strategy used). Scale bar, 50 μm.
miRNA-mediated knockdown will never be able to reproduce a complete null condition. However, miRNA-mediated knockdown may in some scenarios, such as for SMA disease, be a more flexible option for interrogating gene function. Indeed, the ease to restrict their effect tissue specifically as well as the possibility to target specific isoforms or to generate hypomorphic states would enrich the study of gene function and human diseases in which gene dosage is critical. For example, many gene knockouts are embryonically lethal but fully viable as heterozygotes. It has also been reported that complete loss of function often leads to molecular compensation, hiding the role of the deleted gene.\(^2\) Partial knockdown of protein function may also mimic chemical treatments more closely than complete ablation of protein function. Finally, it is relatively easy and inexpensive to generate such models. In the future, this approach may also be used to study diseases with polygenic inheritance—that is, cases where more than one gene has to be reduced in expression to generate a phenotype.\(^2\)

Methods

Zebrafish maintenance and transgenic lines. Adult Zebrafish and embryos were maintained by standard protocols approved by the University of Sydney Animal Ethics Committee.

Generation of pDONR224 plasmid. To2k2t-compatible pME (Middle Entry) clones are usually generated via pDONR224 plasmid and/or present kanamycine resistance gene. The kanamycine resistance gene contains a BsmBI site, hampering the use of the miRNA-expressing cassettes presented in Fig. 1. To generate a compatible DsRed plasmid backbone, we started from pDONR223 plasmid (gift of Dr David Hill), which presents spectrinmycin resistance. To eliminate an XhoI site that would interfere with miRNA-chaining procedures, pDONR223 was digested with XhoI and depolymerized (Antarctic phosphatase). Oligos 78-Forw-XhoIDELETE and 79-Rev-XhoIDELETE were annealed and cloned into linear pDONR223 to generate a DsRed backbone (primers are listed in Supplementary Table 1). To introduce a polyA signal sequence downstream of the attL2 recombination site, pDONR223-xhoI was digested by EcoRV and depolymerized. A SV40-polyA signal sequence was amplified from pCS2-sp using oligonucleotides 72-SV40-Forw and 73-SV40-Rev. The PCR product was purified, digested with EcoRV and inserted into linearized pDONR223-xhoI to generate pDONR224.

pME-RNAi651, 652 and 653 plasmid generation. mmu-miR155-based plasmid: pCDNA 6.2 GV/EmGFP-mir, containing artificial miRNA-expressing mmu-miR155 backbone, was provided by Dr Donald Love.\(^9\) The gfpH/mmu-miR155 cassette was transferred into pDONR224 using BP clonase reaction (following the manufacturer’s instructions). The resulting plasmid was named pME-RNAi641. eGFP in pME-RNAi642 and pME-RNAi643 for the two different 3′-UTR sequences of a gene of interest in zebrafish. To integrate DNA constructs into the zebrafish genome, 1 ml of a mix containing 30 ng oligo of DNA of interest plus 25 ng oligo of Transposase mRNA and phenol red were injected at the one-cell stage.

miRNA target site selection. To select target sequences, we mainly used the BLOCK-TT RNAi Designer website from Invitrogen that is optimized for artificial miRNA-mediated knockdown (http://maidesigner.invitrogen.com/miexpress/ BLOCK-TT/chooseOptiDesignionOption/miRнымииsnpID#:9854414918167072314). Among other tools and websites, we also used GenScript’s siRNA Design Center (www.genscript.com/ design_center.html), siSearch (sidirect2.rnai.jp/), IDT SciTools RNAi Design (http://sg.idtdna.com/ScitoTools/Applications/RNAi/RNAi.aspx), Target Finder supplied by Ambion (http://www.ambion.com/techlib/misc/sirNA_finder.html), Dharmaco RNAi design centre (http://dharmaco.goldfischeciences.com/design center/) and siSPOUT tools (https://sispot.icts.uio.esc/). Targets can be obtained at www.targetscan.org/fish_62/ is also useful to obtain complete and accurate 3′-UTR sequences of a gene of interest in zebrafish.

Oligo-miRNAs (artificial pri-miRNA) cloning. The different pME-RNAi plasmids were digested with BsmBI or BssI and gel-extracted/purified, Linearized pME-RNAi backbone was stored at −20 °C at 300 ng ml\(^{-1}\). To insert a specific artificial pri-miRNA, 10 ng of linearized pME-RNAi construct was mixed with 4 μl of the 1:5,000-diluted ds-oligos (artificial pri-miR) and ligated following the manufacturer’s instruction (NEB T4 DNA ligase).

DNA injection and selection of transgenic lines. To integrate DNA constructs into the zebrafish genome, 1 ml of a mix containing 30 ng oligo of DNA of interest plus 25 ng oligo of Transposase mRNA and phenol red were injected at the one-cell stage.

Western blot analysis and quantification. For each condition, 20 embryos were dechorionated using pronase (1 mg ml\(^{-1}\), 10 min on ice) and rinsed in calcium-free Ringer’s solution. Yolks were separated from embryos by pipetting up and down using Pasteur pipettes. Samples were centrifuged 5 min at 200g. Pellets were washed in cold PBS and centrifuged for 5 min at 200g. 4°C. Pellets were resuspended in 20 μl of cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Roche). Samples were centrifuged 20 min at 2,000g, 4°C. Supernatants were collected and stored at −80 °C for western blot analysis. The following antibodies were used: Anti-SMN1 (MANSMN12, 2E6, 1:5,000; Glenn E. Morris, Developmental Studies Hybridoma Bank) and Anti-β-Actin antibody (Sigma-Aldrich, Catalog number A5441-2ML, 1:5,000). Western blot band intensity measurements were performed in ImageJ and normalized using -Actin band intensity. Uncropped blots are shown in Supplementary Fig. 11.

Immunohistochemistry. Dechorionated zebrafish embryos/larvae were fixed in 4% paraformaldehyde for 3 h at room temperature or overnight at 4°C. Animals were washed three times in PBT (1× PBS + 0.8% Triton X) and then gradually transferred into 100% methanol, via a 5-min step at 50/50% PBT/methanol. Animals were stored in −20°C if required. Animals were then rehydrated for 5 min in 50/50% PBT/methanol and three times in PBT for 5 min. Larvae were treated by collagenase (Sigma no. C9891, 1 mg ml\(^{-1}\)) in PBT for 10–30 min depending on the stage at room temperature. Larvae were washed three times for 5 min in H\(_2\)O and then treated with acetic for 20 min at 20°C. Animals were washed for 3 × 5 min in PBT. Animals were fixed again in 4% formaldehyde for 20 min and washed three times in PBT. Larvae were then incubated for 1–2 h in blocking solution (500 μl of 10% goat serum, 1% dimethylsulphoxide (DMSO), 1 ×
PBT) at room temperature under gentle agitation. Four hundred microlitres of the blocking solution were removed and one hundred microlitres containing primary antibody (anti-Acetylated-Tubulin (Sigma-Aldrich no. T7451) and presynaptic anti-SV2 (Developmental Studies Hybridoma Bank) at 1:250 final dilution, anti-GFP (AMS Biotechnology no. TP401) at 1:1,000, and incubated overnight at 4 °C under slight agitation. Animals were rinsed three in PBT and washed for 4 × 30 min in PBT. Larvae were incubated for 1–2 h in 500 μl of the blocking solution (5% of bovine serum, 1% DMSO, 1 × PBT) at room temperature. Four hundred microlitres were taken out, and hundred microlitres containing secondary antibodies (Life Technologies Alexa Fluor 488 no. A-11034 and Alexa Fluor 594 no. A-21201) were added and incubated overnight at 4 °C. Samples were washed five in PBT and analysed and/or scanned using confocal microscopy.45

Generation of hsa-SMN1 plasmids. Using human cDNA as template, the ORF and a small 3′-UTR part of hsa-SMN1 were amplified with the forward primer (63-hsa-smm-atg) and reverse primer (65-hsa-smm-reverse; Supplementary Fig. 1). The 976-bp PCR product was digested with EcoRI and XhoI and ligated in EcoRI/XhoI-digested and gel-purified pCS2 þ vector. The corresponding plasmid was named pCS2 þ hsa-SMN1. In order to generate a tol2kit-compatible pME vector containing hsa-SMN1, pCS2 þ hsa-SMN1 was digested by EcoRI/BglII and the hsa-SMN1 cassette was gel-purified. This cassette was cloned into an EcoRI/BamHI-opened and gel-purified pME-MCS (237, from the tol2kit) plasmid, and the final construct was named pME-hsa-SMN1. pME-hsa-SMN1 was then combined with p5E-UBI (containing the ubiquitin promoter, a gift from Dr Benjamin Hogan) and p3E-polyA (302) in a pDestTol2CG2 (395) destination vector carrying Hoppel and Fjose, A., Larsen, N., Helvik, J. V. & Drivenes, O. Treatment with morpholino-induced and mutant phenotypes in zebrafish. Dev. Cell 32, 97–108 (2015).

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Author contributions
J.G. designed and performed the research. J.G. processed the data. S.R. provided hsa-miR218-2-GFP transgenic line. J.G. and T.B. wrote the manuscript.

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