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Research Paper

Specific RNAi Mediated Gene Knockdown in Zebrafish Cell Lines

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KEY WORDS

RNA interference, RNAi, Danio rerio, lamin A, lamin B2, Eg5

ABBREVIATIONS

RNAi	RNA interference
miRNA	microRNA
siRNA	small interfering RNA

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ABSTRACT

Here we demonstrate highly efficient RNA interference in ZFL, SJD and ZF4 cell lines derived from adult and embryonic zebrafish *Danio rerio*. Microinjection of siRNAs resulted in silencing in almost 100% of cells while transfection using cationic liposomes led to silencing in 30%. Use of siRNAs against zebrafish lamin A, lamin B2 and the motor protein Eg5, led to knockdown of the target genes with the specific phenotypes expected from prior studies in mammalian cells. In contrast injection of lamin A, GL2 and eGFP siRNAs into zebrafish embryos resulted in morphological defects, abnormal development and early death of most embryos. The results indicate unspecific responses to siRNAs in the embryo but a fully developed and active RNAi machinery in cell lines.

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INTRODUCTION

RNA interference (RNAi) is a powerful method to transiently knockdown specific gene products. The effect relies on a highly conserved cellular response to double-stranded RNA (dsRNA). Introduction of dsRNAs leads to a series of events resulting in degradation of the homologous endogenous mRNA in a sequence specific manner through siRNAs, or translational inhibition by miRNAs. Since the discovery of this posttranscriptional gene silencing mechanism in *C. elegans* in 1998,[1] RNAi has been employed as a research tool in a variety of species. These include model organisms such as *Arabidopsis thaliana, C. elegans, Drosophila melanogaster* and mice as well as murine and human cell lines.[2-4]

In contrast, attempts to explore RNAi mediated gene knockdown in the zebrafish embryo (*Danio rerio*) yield conflicting results. A number of groups have attempted to establish RNAi mediated gene silencing in zebrafish by injection of dsRNAs into embryos. Two studies report only unspecific effects after injection of either long dsRNA or siRNAs into the embryo. Thus, defects such as a truncated tail, loss of eye and brain structures, enlarged heart cavities and growth retardation were observed after injection of both types of dsRNA regardless of the target genes against which the RNAi was directed.[5,6] Other groups have used dsRNAs specific for the targets ntl, flh, pax2.1[7] and Zf-T and pax6.[8] Unspecific effects increased with increasing dsRNA concentrations[7] while documentation of specific effects depended on a statistical interpretation of the data. Specific silencing of the zebrafish M2 muscarinic acetylcholine receptor was reported in the developing embryo after injection of long dsRNA targeting the M2 mRNA.[9] Silencing of the Duchenne muscular dystrophy gene (dmd) after injection of specific siRNAs into the yolk of two-cell zebrafish embryos was reported,[10] but the phenotypes seen in this study resemble those reported by others as unspecific.[5,6] Specific downregulation by translational inhibition of target genes in the developing embryo was shown with siRNAs directed against modified 3'UTR regions. These regions normally serve as miRNA target regions.[11] The combined data on RNAi in embryos can be summarized by the statement in a review published this year that after RNAi "the ratio of specific phenotypes was generally low and variable and there have been studies reporting substantial unspecific effects of RNAi on the development of the zebrafish."[12]

In organisms, such as the fruit fly *Drosophila melanogaster* or the mouse *Mus musculus*, miRNAs are expressed already at very early stages of embryonic development[13,14] and essentially regulate gene expression. In the zebrafish dicer mRNA and the active enzyme are present in the fertilized egg and the early embryo[15] and dicer is essential for embryonic development after day 11.[16] Mutant zebrafish that cannot produce mature miRNAs undergo abnormal morphogenesis during gastrulation, brain formation, somitogenesis and heart development. Microinjection of the early miRNA, miR-430, into 2-cell embryos rescues at least the brain defects suggesting that the miRNA/RNAi pathway is already

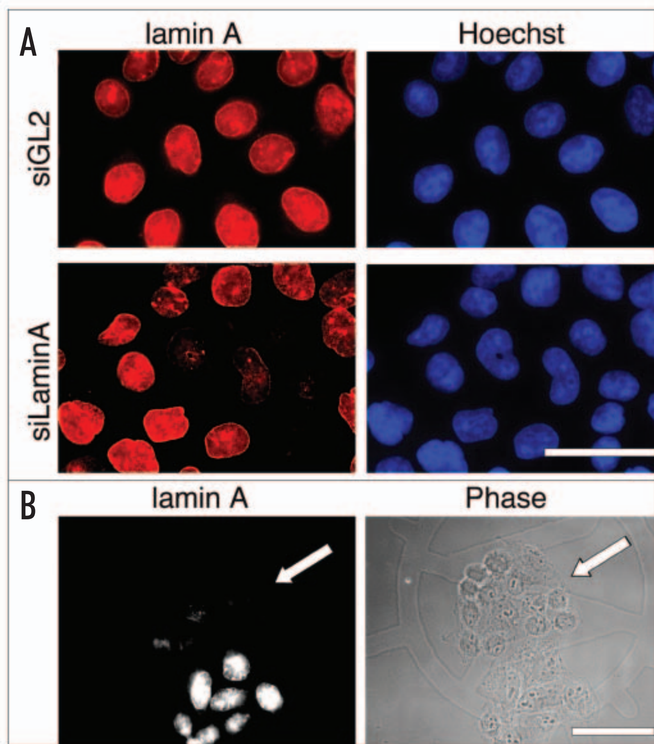

Figure 1. (A) Transfection of zebrafish ZFL cells with either control firefly luciferase siRNA (siGL2) (top panel) or with *Danio* lamin A siRNA (siLamin A) (bottom panel) using oligofectamine. Cells were fixed 44 hours after transfection and stained with lamin A antibody. Cells transfected with an siRNA against GL2 display lamin A protein in the nuclei of all interphase cells (top left). In contrast transfection with the lamin A siRNA led to silencing in a maximum of 30% of the cells (bottom left). (B) Silencing of lamin A by injection of lamin A siRNA. Cells in the top group (arrow) were injected, whereas those in the lower group were not. Cells were fixed and stained with lamin A specific antibody 44 hours after injection. Note the nearly complete loss of lamin A in the cells injected with the lamin A siRNA. Bars: 25 µm.

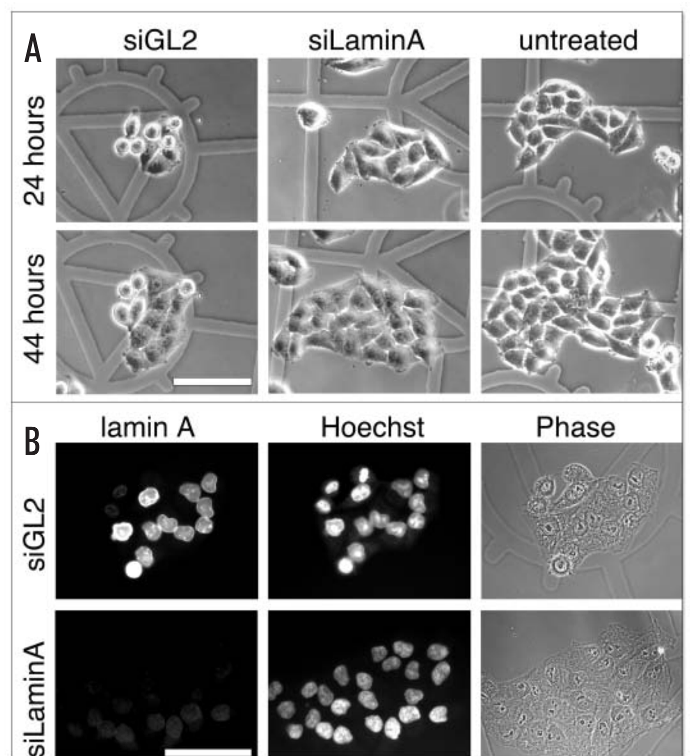

Figure 2. Injection of lamin A siRNA into the cytoplasm of ZFL cells led to sequence specific gene knockdown without affecting proliferation or cell viability. (A) Cells were injected with an unspecific control siRNA (siGL2, left) or with lamin A siRNA (siLaminA, middle) and photographed 24 and 44 hours after injection. Cell growth after treatment with either siRNA was comparable to that of the uninjected cells (right panel). (B) After 44 hours the cells were fixed and stained with lamin A specific antibody. Injection of lamin A siRNA led to a successful knockdown of the target gene (left bottom panel) whereas the GL2 siRNA did not alter the lamin A protein level or localization (left top panel). The middle and right panels show the same cells stained for Hoechst and in phase microscopy. Bar: 50 µm

active at this early developmental stage (2 to 3 hours post fertilization).[17] The morphology of these mutant fish show significant similarities to the unspecific effects on morphology observed after injection of dsRNAs into zebrafish embryos[5,6] indicating that the exogenous and endogenous dsRNA might possibly compete for components of the RNAi machinery.

To see whether RNAi is possible in the zebrafish we decided to test zebrafish cell lines. Using two different siRNA delivery techniques we knocked down lamin A and lamin B2, as well as the kinesin related motor protein Eg5 and exogenous GFP. These experiments document functional RNAi in three zebrafish cell lines. The specific phenotypes observed were identical to those previously reported for homologous siRNAs in mammalian cell lines. Unspecific effects on cell viability and proliferation were not observed.

MATERIALS AND METHODS

Cell lines and cell culture. The zebrafish cell lines ZFL (CRL-2643, adult dorsal fin fibroblast), SJD (CRL-2296, adult liver epithelium) and ZF4 (CRL-2050, fibroblast cell line derived from 24 hour embryos) were purchased from the American Type Culture Collection (ATCC) and maintained according to the supplier's guidelines. Cells were propagated at 28°C in DMEM supplemented with 10% FCS in 5% CO_2.

siRNAs and Delivery. Liposome based transfection of siRNAs was performed as described.[18] 15,000 to 30,000 cells per well were seeded on coverslips in 24 well plates 24 hours prior to transfection. Transfection with oligofectamine (Invitrogen) followed the manufacturer's protocol. Chemically synthesized siRNAs (Dharmacon, Lafayette, CO) contained 3'-dTdT overhangs. siRNA sequences and target accession numbers are shown in Table 1.

For microinjection of siRNAs into cell lines, cells were seeded in their standard culture medium on CellLocate coverslips (Eppendorf, Hamburg) 24 hours prior to injection. A 6 µM solution of siRNAs in buffer A (100mM potassium acetate, 2mM magnesium acetate, 30mM HEPES, adjusted to pH 7.4 with KOH) was injected into the cytoplasm using Femtotips (Eppendorf) and an Eppendorf Micromanipulator.

For embryos a 50 µM solution of siRNAs in buffer A, or buffer A alone, was injected into the yolk of one or two cell embryos. After 24 and 48 hours embryos were dechorionated and examined by bright field microscopy.

Antibodies and indirect immunofluorescence microscopy. Clone R27 is specific for vertebrate A type lamins,[19,20] while clone X223 is specific for lamin LII/B2 of amphibia and higher vertebrates.[21,22] Cells were fixed in methanol at -10°C for ten minutes and rinsed with PBS. Supernatants from the lamin A and lamin B2 clones were diluted in PBS containing 0.5% BSA and applied to cells for one hour at 37°C. The monoclonal α-tubulin antibody (clone DM1A, Sigma-Aldrich Corporation, Munich) was applied under the same conditions. After washing three times with PBS, rhodamine conjugated goat anti mouse antibodies (Dianova, Hamburg) were applied for 45 minutes at 37°C. After a further washing step DNA was visualized with 1 µM Hoechst 33342 dye in PBS for 4 minutes at room temperature. Coverslips were washed and mounted in Mowiol.

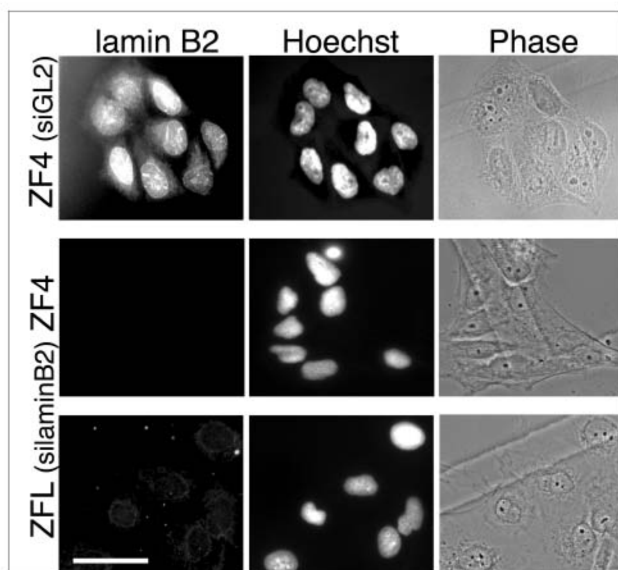

Figure 3. siRNA mediated silencing of lamin B2 in zebrafish cell lines of adult and embryonic origin. ZF4 cells were microinjected with control siGL2 siRNA (top row) or with Lamin B2 siRNA (middle). siGL2 injected ZF4 cells showed normal levels of expression of lamin B2 while those injected with laminB2 siRNA displayed a nearly complete knockdown of the lamin B2 protein 44 hours after injection. ZFL cells (lower panel) showed a strong reduction in lamin B2 protein levels 44 hours after injection of siRNA against lamin B2. Bar: 50 µm.

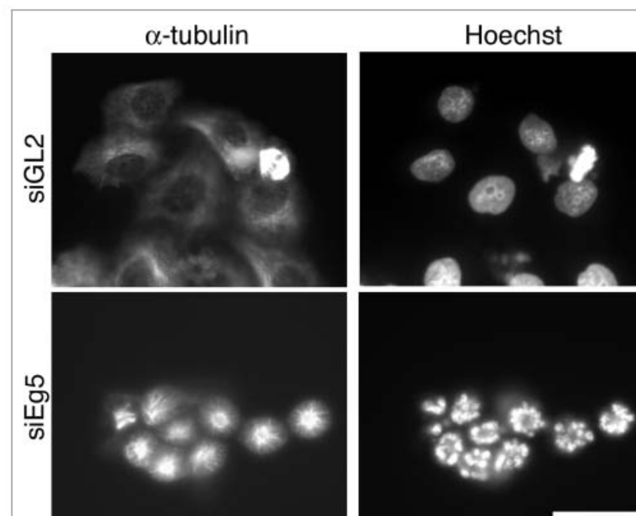

Figure 4. Injection of Eg5 siRNA into ZFL cells led to monopolar mitotic spindles after 44 hours. Microtubules were stained with an α-tubulin antibody. Control cells were injected with siGL2 (top panel). A single bipolar mitotic cell is visible in the group of cells. Injection of Eg5 siRNA led to an increased number of cells arrested in mitosis, which displayed monopolar mitotic spindles (bottom panel). Staining of the same cells with Hoechst dye is shown in the right panels. Bar: 50 µm.

Table 1 **siRNA sequences.**

Target gene	accession no.	siRNA sequence
lamin A	NM_152971	s 5'-AGAAGCAGCUGCAGGAUGAdTdT as dTdTUCUUCGUCGACGUCCUACU-5'
lamin B2	NM_131002	s 5'-GUCUCUAUAUGAGGCUGAAdTdT as dTdTCAGAGAUAUACUCCGACUU-5'
Eg5 (knsl1)	NM_173261	s 5'-AAGGAUAAGCGUGCACGUGdTdT as dTdTUUCCUAUUCGCACGUGCAC-5'
GL2 (control) firefly luciferase	X65324	s 5'-cguacgcggaauacuucgadTdT as dTdTGCAUGCGCCUUAUGAAGCU-5'
eGFP	U55762	s 5'-gaacggcaucaaggugaacdTdT as dTdTCUUGCCGUAGUUCCACUUG-5'

RESULTS AND DISCUSSION

Cell lines. The *Danio rerio* genes lamin A, lamin B2 and Eg5 were chosen as the endogenous targets to be silenced. SiRNA sequences and positions (Table 1) were selected according to known requirements for efficient siRNA design.[23-25] In a first set of experiments zebrafish cells were transfected with lamin A siRNA according to standard protocols.[26] A variety of different conditions and different transfection reagents were tried. However, the maximum transfection efficiency that could be achieved was around 30% (Fig. 1A). The relatively low efficiency of siRNA delivery with oligofectamine was reflected in a low number of silenced cells. Therefore in a second set of experiments siRNAs were delivered by microinjection into the cytoplasm. Microinjection of the siRNA against lamin A into the cytoplasm of SJD cells resulted in a very efficient knockdown of the target gene (Fig. 1B).

Microinjection of siRNAs into the cells did not alter the viability or growth rate. A small percentage (<5%) died directly after the injection process due to mechanical rupture. The surviving injected cells maintained the generation time of 24 to 26 hours, seen in untreated cells (Fig. 2). Cells injected with lamin A or GL2 siRNA displayed doubling rates approximately equal to that of the non-injected control cells (Fig. 2A). For example a group of ten cells that were injected with GL2 siRNA had expanded to twenty cells after 25 hours and to 40 cells after 48 hours, indicating no negative effects of siRNAs in the cytoplasm. The siRNA for lamin A also did not affect the cell viability or cell growth (Fig. 2A). Indirect immunofluorescence microscopy with lamin A specific antibody shows that injection of the control siRNA (siGL2) did not alter lamin A levels (Fig. 2B). In contrast, injection of lamin A siRNA into SJD cells led to a nearly complete knockdown of the lamin A protein in all injected cells within 44 hours (Fig. 2B). Effects other than specific reduction of the target protein were not observed. Results obtained after microinjection of lamin A siRNA into zebrafish cell lines are identical to those obtained on human HeLa cells.[18,27] Lamin A is non-essential both in HeLa and in zebrafish cell lines.

The siRNA for lamin B2 was injected into the ZF4 cell line, derived from fibroblasts of 24 hour embryos, and into the fibroblast cell line ZFL originating from dorsal fin fibroblasts from adult male fish. ZF4 cells are lamin A negative but lamin B2 positive while ZFL cells are lamin A and lamin B2 positive. After microinjection of the siRNA against lamin B2 into ZF4 and ZFL cells the protein level of the target gene was knocked down very efficiently (Fig. 3, middle and bottom panels). Indirect immunofluorescence with antibodies specific for lamin B2 revealed that ZF4, the zebrafish cell line of embryonic origin, was able to perform functional RNAi against the target lamin B2 (Fig. 3, middle). Injection of the control siRNA (siGL2) did not show any effect (Fig 3, top). siRNA against lamin B2 was also injected into ZFL cells. Efficient downregulation was observed 44 hours after microinjection (Fig. 3, bottom). The lethal phenotype expected from results with mouse and human cell lines[27] after downregulation of the lamin B2 gene was also observed in the ZFL and ZF4 cell lines. Cells grew normally for 44 hours after injection of lamin B2 siRNA but started to die after 72 hours (data not shown). Thus, also with the lamin B2 siRNA, results obtained with the zebrafish cell lines parallel those seen in HeLa cells.[27] Lamin B2 is an essential protein both in HeLa and in zebrafish cell lines.

Finally, the *Danio* homologue of the kinesin related motor protein Eg5 was targeted by an siRNA. Injection of the fish specific Eg5 siRNA into ZFL cells resulted in a striking phenotype. Cells were forced into mitotic arrest and displayed monopolar mitotic spindles which were revealed by staining with an α-tubulin antibody at 44 hours post injection (Fig. 4). Cells

subsequently underwent apoptotic cell death. The phenotype seen after microinjection of Eg5 siRNA into the zebrafish cells is identical to that reported for human HeLa cells after injection of homologous siRNAs.[27]

Coinjection of GFP mRNA with control non-cognate siGL2 siRNA resulted, as expected, in GFP fluorescence in all injected cells. In contrast, coinjection of GFP mRNA and the corresponding GFP siRNA inhibited fluorescence in the injected cells (data not shown). Thus siRNA inhibition in zebrafish cell lines functions not only on endogenous targets but also on exogeneous targets introduced by microinjection.

Our RNAi experiments on cell lines are summarized in Table 2. They demonstrate highly efficient RNA interference for three different target genes—lamin A, lamin B2 and the motor protein Eg5—in three different zebrafish lines of adult and embryonic origin. The phenotypes seen with these siRNAs in the zebrafish cell lines are identical to those observed with the equivalent siRNAs in human cell lines.

Embryos. We also investigated the ability of siRNAs to inhibit specific gene functions in zebrafish embryos using sequences targeted to the lamin A gene as well as to eGFP and to GL2 (firefly luciferase) as controls. The lamin A and the GL2 siRNAs had the same target sequences as those used in the cell lines (Fig. 5). Injection of a 50 μM siRNA solution of each siRNA into the yolk of one or two cell embryos resulted in general growth arrest during gastrulation as well as in various unspecific defects at 24 hours post-fertilization, although embryonic development was unaffected before the midblastula stage. Only three of 30 embryos injected with the eGFP siRNA (Fig. 5B) developed a beating heart and none reached the hatching stage. Two of 34 embryos injected with the lamin A siRNA (Fig. 5C) developed a beating heart, and one embryo hatched and developed to a healthy juvenile. All other embryos displayed severe defects including truncated tails, loss of eye and brain structures, enlarged heart cavities and growth retardation (Fig. 5B, C and D). The same defects were observed after injection of the siRNA targeting GL2 (Fig. 5D). In contrast, injection of buffer into the yolk of one or two cell embryos did not influence growth, morphology or viability of the embryos (Fig. 5E). For lower siRNA concentrations (10 or 25 μM) the level of unspecific effects was reduced, but no specific effects on target gene expression were observed.

Our results with eGFP, lamin A and GL2 siRNAs show unspecific effects on zebrafish embryo development in agreement with a variety of other studies looking at RNAi in the zebrafish embryo (see references cited in introduction and in ref. 12). Successful siRNA mediated silencing of the dystrophin gene has been reported in zebrafish embryos.[28] However, the observed phenotype was very similar to that reported here after injection of control siRNAs against eGFP and GL2 or of the siRNA targeting the lamin A transcript and also resembles those reported by others as unspecific.[5,6] Dodd et al.[28] used an eGFP siRNA as control. They did not report unspecific effects with this siRNA although in our study, using a different eGFP siRNA under comparable experimental conditions, unspecific effects are clearly present (Fig. 5C).

The unspecific effects of siRNAs on embryonic development seen in this and other studies indicate that siRNAs in the zebrafish have an unspecific effect. Thus currently RNAi is not a useful technique for studying gene function in zebrafish embryos and the morpholino technique where modified oligonucleotides block translation of the corresponding mRNAs[12] is clearly preferable.

In summary this study demonstrates for the first time that the cellular RNAi machinery is active in *Danio* cell lines, and that RNAi targeting of specific genes in cell lines is possible. However, the question as to why only unspecific effects are seen when the same siRNAs are injected into the zebrafish embryos remains open. Two explanations seem possible. First competition between the RNAi and the miRNA pathways for the argonaute proteins, which form the RNA silencing effector complex, could occur. This is supported by the results of Giraldez et al., who observed developmental defects in zebrafish embryos lacking miRNA processing.[17] The striking similarity of the reported defects, and those observed in our experiments, indicate that a block of miRNA function may be caused by siRNA mediated titration of components of the RNA silencing machinery. Alternatively the unspecific effects in the embryo might be caused by an interferon response to exogenous dsRNA since a type-1 interferon response has been reported after injection of dsRNAs into fish embryos[29] and is thought to be part of the antiviral mechanism in fish.[30]

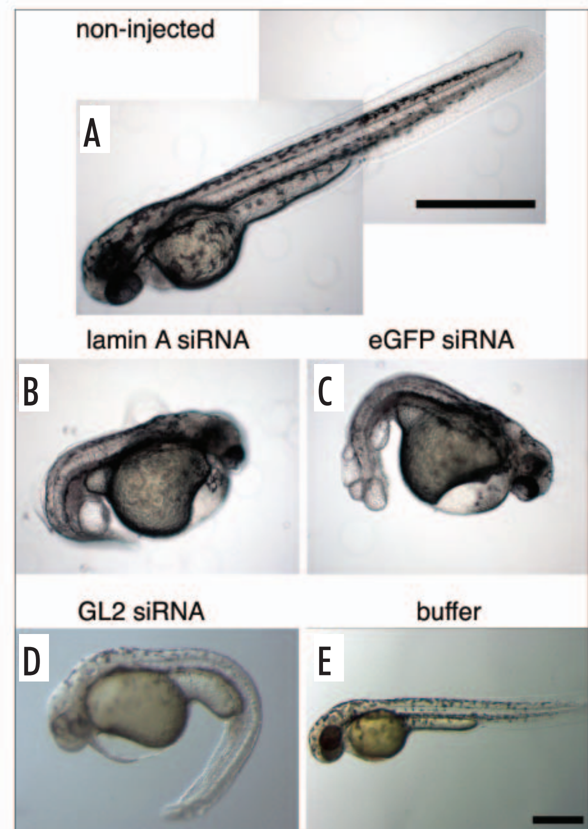

Figure 5. Light micrographs of siRNA injected embryos 48 hours post fertilization showed severe unspecific defects. (A) The uninjected embryo displayed the typical morphology of a 48 hours post fertilization embryo. (B) Injection of a control siRNA targeting eGFP led to morphological abnormalities of the embryo. (C) The same defects, including tail truncation, enlarged heart cavity and growth retardation were observed after injection of the lamin A siRNA. (D) A second control siRNA targeting GL2 resulted in the same morphological defects after injection. (E) Buffer A alone did not disturb the embryonic development. For details see text. Bars: 0.1 mm

Table 2 **RNAi in zebrafish cell lines: targets, cells and effects**

Gene	Essential	Effect	cell lines used	Effect in studies with mammalian cells
lamin A	no	loss of lamin A protein	SDJ, ZFL,	loss of lamin A protein[18]
lamin B2	yes	loss of lamin B2 protein, cell death	SDJ, ZFL, ZF4	loss of lamin B2 protein, cell death[27]
Eg5	yes	monopolar mitotic spindles	SDJ	monopolar mitotic spindles[27]
GL2 (control)	no	no effect	SDJ, ZFL, ZF4	no effects [18,27]
mGFP	no	reduced GFP	SDJ, ZFL, ZF4	

SDJ: (adult, caudal fin fibroblast), ZFL: (adult, liver epithelium), ZF4: (embryonic fibroblast). Gene silencing was monitored by indirect immunofluorescence (lamins, Eg5) or by GFP fluorescence. A stop in growth or cell death was taken to indicate that the protein is essential. For further description see text.

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