The Serine-Threonine Protein Kinase PAK4 Is Dispensable in Zebrafish: Identification of a Morpholino-Generated Pseudophenotype

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Abstract

TALEN-based inactivation of the zebrafish *pak4* gene resulted in embryos and adult fish that appear normal and fertile. This is in contrast to our previously published studies which were based on the use of antisense morpholino oligonucleotides (MOs). We have excluded potential explanations such as gene duplication, alternate splicing, cryptic initiation of translation, and translation-independent RNA function. Our conclusion is that *pak4* is dispensable in zebrafish, and that even when corroborated by robust controls, such as RNA rescue, MOs may elicit misleading pseudophenotypes that do not correspond to results obtained by genetic mutations, and should thus be used with caution.

Citation: Law SHW, Sargent TD (2014) The Serine-Threonine Protein Kinase PAK4 Is Dispensable in Zebrafish: Identification of a Morpholino-Generated Pseudophenotype. PLoS ONE 9(6): e100268. doi:10.1371/journal.pone.0100268

Editor: Bruce Riley, Texas A&M University, United States of America

Received April 14, 2014; Accepted May 26, 2014; Published June 19, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data included in manuscript.

Funding: Funding provided by Intramural Research Program, National Institute of Child Health and Human Development. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The optical clarity, rapid, in vitro embryonic development and relatively short generation time of zebrafish (*Danio rerio*) have led to the extensive use of this species for the characterization of gene function in vertebrate development. Classical genetic screens of mutagenized zebrafish have been highly successful in the identification of genes required for primary embryonic axis formation and numerous aspects of tissue and tissue differentiation [1,2], yielding important insights with broad significance for developmental biology and human health. However, the majority of gene function studies in zebrafish have been based on the use of antisense oligonucleotides with degradation-resistant morpholino phosphorodiamidate backbones (MOs). These have been shown to interact specifically with complementary RNA targets, resulting in inhibition of translation, RNA splicing, and other steps in post-transcriptional gene regulation [e.g. Lim et al., 2012 [3]]. However, it has become evident that in addition to specific inhibition of the cognate target genes, MOs can also have side effects. Ekker and Larson [4] reported that 15–20% of tested MOs show non-specific toxicity on developing embryos, with the most common artifact being induction of apoptosis in one or more tissues, frequently but not exclusively in the brain [reviewed by Eisen and Smith, 2008 [5]]. Ekker and colleagues showed this was due at least in part to activated expression of an N-terminal truncated isoform of the transcription factor p53, and that the MO-induced apoptosis artifact could be suppressed by adding an antisense p53 MO to the injection cocktail [6]. In addition to cell death, MO-induced p53 can also elicit patterned gene expression via activation of pro-apoptotic factors, that can be mistaken for developmental regulation [7]. In many cases, legitimate effects by MOs on gene expression have been distinguished from artifacts by using a rescue control in which a synthetic mRNA, engineered to lack the MO recognition sequence, is co-injected. If the MO-induced phenotype can be reverted to the control pattern, the knockdown has been deemed to result specifically from the loss of target gene expression. We used MOs, in combination with both of these types of controls, to analyze the developmental function of the serine/threonine protein kinase PAK4 in zebrafish [8].

PAK4 is one of six related proteins that mediate signal transduction via Rho-class GTPases to control a plethora of biological processes, including cytokineskeletal dynamics, cell polarity and migration [9]. The family can be subdivided into two groups, PAK1-3, which are tightly regulated by interaction with GTPases, and PAK4-6, which bind specifically to GTPases, but have kinase activity that does not depend on this binding. In addition, some functions of the Group II PAKs may be independent of protein kinase activity [10,11]. In mouse, loss of *pak4* function via gene targeting results in a complex lethal phenotype including disruption of placental and epiblast vasculature, heart failure, defective neurons and folding of caudal neural tube. Some aspects of this phenotype might be secondary effects of vascular failure [12,13]. The loss of function phenotype we observed in zebrafish is also lethal, but differs in many respects from that found in mouse. The zebrafish egg contains high levels of *pak4* mRNA, but PAK4 protein is not detectable until the high stage of cleavage, approximately three hours after fertilization. Consistent with this maternal mRNA storage, we found that loss of *pak4* expression in zebrafish behaved as a maternal effect: disruption of zygotic expression alone, via splice-inhibitory MOs, had no effect on...
development, whereas inhibition of translation using a MO targeting the 5’-end of the open reading frame, alone or in conjunction with splice MOs, resulted in complex defects on the formation of primary myelopoietic cells, vasculature, and somite formation. All of these defects were shown to be independent of the p53-mediated interference noted above, and could be efficiently rescued by the injection of synthetic mRNA lacking the MO target sequence [8].

Because of the uncertainties associated with MOs, and to provide a genetically defined platform for further analysis of pak4 function in zebrafish, we used TALEN-based gene targeting to establish germ-line loss-of-function mutations. TALENs (transcription activator-like effector nucleases) are recombinant proteins based on the TALE transcription factors of *Xanthomonas* bacteria. These have been used to generate site-specific double strand breaks in the genomes of several metazoan species, including zebrafish. These breaks are usually repaired by non-homologous end joining, which is error prone, frequently leading to small insertions or deletions (indels) at the repair site. When such indels result in a translational frame shift, loss of function for the encoded protein may be inferred. This strategy was used to generate frame shifts just downstream from the N-terminal methionine codon in the *pak4* transcript, resulting in complete loss of this protein. In contrast to the results with MOs, *pak4* null zebrafish were found to be normal and fertile, in both F2 and F3 homozygous and compound heterozygous mutant individuals. We conclude that *pak4* function is dispensable in zebrafish, and that the antisense MO approach, even with robust RNA rescue controls, may be subject to misleading results.

**Materials and Methods**

**Animals and Ethics Statement**

Zebrafish of EK background were used in this study. Embryo handling and care was carried out using standard procedures [14]. All experiments using zebrafish were performed according to the NICHD IACUC-approved animal study protocol # 12-039. This ASP is not used for other projects.

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**Figure 1. TALEN design and results of indel mutations in zebrafish pak4.** (A) Alignment of wild type *pak4* sequence with mutated PCR amplicons. A pair of TALEN was designed overlapping (left arm) and downstream (right arm) the start ATG codon. The target sequences of the TAL Effector DNA-binding domains are highlighted in red. The start codon and two polymorphic nucleotides found within the right arm target are underlined. wt, wild type EK, Δ2, Δ4 and Δ5, alleles with 2-, 4- and 5-nucleotide deletion, respectively. Ins6, allele with 6-nucleotide insertion. (B) Results of immunoblotting for *pak4* protein. Total protein from 100 embryos at shield stage was collected, immunoprecipitated and analyzed using a custom-made zebrafish *pak4* anti-peptide antibody. wt, wild type EK, Δ2/Δ4, F3 mutants of mixed genotypes *pak4*Δ2/Δ2, *pak4*Δ4/Δ4 and *pak4*Δ2/Δ4. *pak4* protein with a predicted molecular weight of 72 kDa was detected in the wild type embryos but not in the mutants. Duplicate membrane probed with antibody to α-tubulin was used as loading control.

doi:10.1371/journal.pone.0100268.g001
Design and Generation of TALENs

The TALEN reagents used in this study were designed and constructed by Cellectis (Paris, France). Briefly, candidate TALEN target sequences in the zebrafish pak4 gene were identified (TALEN Hit Search Report), and from these we selected one immediately downstream from the start methionine codon, in order to achieve maximal disruption of the pak4 coding sequence (see Figure 1A for positions and target sequences). TALEN expression constructs were generated in the pTAL.T7 vector backbone using the “Unit Assembly” method. Sequences of repeat-variable di-residues (RVDs) in the TAL Effector DNA-binding domains were NN-NG-NG-HD-NI-NN-HD-NI-NI-NN-NI-NI-NI-NG for the TALEN left arm and NG-HD-NN-NI-NI-NN-NN-NG-NN-HD-NG-NN-NI-NN-NI-NI-NG for the TALEN right arm. Both TALENs had nuclear localization signal sequences at the N-termini. Initial validation test for cleavage activity were performed by using the single strand annealing (SSA) assay on episomal target sequences in yeast by Cellectis. The TALEN arms were subcloned into the pCS2 vector for expression in zebrafish embryos. TALEN RNAs were synthesized in vitro by using a mMachine mMessage Transcription Kit (Ambion, USA) and injected into 1-cell stage embryos. Total protein at shield stage was then extracted for Western blot analysis to confirm successful translation (data not shown). TALEN mRNAs (25, 50, 125 and 250 pg of each arm) were injected at 1-cell stage. To detect indel mutations in the TALEN target region, High Resolution Melting Curve Analysis (HRMA) was performed. Double-stranded DNA content was monitored continuously via EvaGreen dye fluorescence. The melting curve profile of each sample was compared to that of control zebrafish EK DNA. When a significant deflection of the melting curve was observed, indicating heterogeneity in the sample, an extended PCR amplicon (primers F14 and R3) was cloned into the pGEM-T Easy vector (Promega) according to the manufacturer’s instructions, and sequenced (Macrogen, USA). The TALEN dosage showing the highest indel mutation efficiency, and less than 50% morphological toxicity at 24 hpf, was used for subsequent injection to generate TALEN mutant fish.

Genotype Analysis

Genomic DNA from tail fin biopsies or whole embryos was prepared by the hot alkaline lysis method described above. Wild type- and mutation-specific forward primers (wt-F, Δ2-F and Δ4-F; see Table 1 for sequences) were designed at the mutation site and used for PCR with reverse primer R3. Long amplicons (primers F14 and R3) were cloned and sequenced for verification.

Whole Mount In Situ Hybridization

In situ hybridization of whole mount embryos at 7-somite and 24 hpf was carried out according to published procedures [15]. Antisense digoxigenin (DIG)-labeled riboprobes were synthesized using the DIG RNA Labeling kit (Roche) according to the manufacturer’s instructions.

RNA Isolation and RT-PCR

Total RNA from whole embryos was extracted using guanidine thiocyanate and phenol/chloroform procedures as previously published [16]. Total cDNA was synthesized by using the Superscript III Reverse Transcriptase (Life Technologies) and oligo (dT)20 primer according to the manufacturer’s instructions.

Table 1. PCR Primers.

| Name  | Sequences (5’ - 3’) |
|-------|---------------------|
| F13   | TTGCTTCTAGGTCTGATGCC |
| R17   | ATCACTGTGGACAGATGCTC |
| F1    | TCAAGGGGCTTTCTACCTGA |
| R2    | CCTCCGAGTCTACATGCGGTTTGTCTC |
| R3    | CAGTAGTATAGCATGTGGATC |
| F17   | TACGGCCACTTAGCGGTTT |
| MO1F  | ACTCTCTACAGGTCTGATGGC |
| MO3R  | CTCTGGCAGAGTACCTTTTCAGAG |
| F7    | TCTCACGGACGATTTGAG |
| F2    | GAAATTCCTTCTGCGAGGAGCATGTTGCA |
| F14   | TAAATGACCTCGTCTCCTGG |
| wt-F  | CAGCAAGAAGAAAAAGCAAG |
| Δ2-F  | CAGCAGAAGAAAAAGCAACA |
| Δ4-F  | CAGCAGAAGAAAAAGCAACC |
| β-actin-F | GAGGAGACCCCGTCTCGTGCAC |
| β-actin-R | GATGGCTGGAACAGGGGCCTCTG |

Table 1. PCR Primers. 

doi:10.1371/journal.pone.0100268.t001
Gene-specific primers were designed and used in PCR detection and amplification of pak4 or β-actin mRNA target regions (see Table 1 for primer sequences).

Antisense Morpholino (MO) Injection
A cocktail containing two splice-blocking (1.5 ng each) and one translation-blocking (3 ng) MOs targeting zebrafish pak4 was injected into 1- or 2-cell stage zebrafish embryos as previously described [8]. [Note that corrected MO sequences have been reported as a corrigendum to the original article. The correct sequences are: MO-1, 5′-GCT TTA TAT TCT GAT TAC CTG ACC G-3′; MO-2, 5′-GTT CTC CCA GAG TCA CTT TGA GAG C-3′; MO-3, 5′-GGG CAT GAG ACC TGT AGT GAG AAG T-3′]. A standard control MO (5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′) as well as the pak4-specific MOs were purchased from Gene Tools and prepared for injection according to the manufacturer’s instructions.

Cloning of PAK4-GFP Fusion Constructs
Forward and reverse primers F1 and R2, annealing to 5′-UTR and translation stop codon of zebrafish pak4, respectively, were used to amplify pak4 cDNA by RT-PCR using RNA extracted from whole embryos at 60% epiboly from control EK, pak4D2/D2 and pak4D4/D4 mutant fish. PCR products were cloned upstream of eGFP, in frame, in pCS2 vector using the Gibson Assembly kit (New England Biolabs, USA) to generate mRNAs encoding PAK4-eGFP fusion proteins. All constructs were verified by DNA sequence analysis.

Immunoprecipitation and Western Blot Analysis
A custom-ordered anti-PAK4 peptides antibody raised in rabbit was used in immunoprecipitation and Western blotting as previously described [8]. A mouse monoclonal anti-GFP antibody (SC-9996, Santa Cruz, USA) was used for detecting enhanced GFP fusions of PAK4 in Western blotting.

Microscopy
Live and fixed embryos, for both fluorescence and bright field, were mounted in 3% methylcellulose and 100% glycerol, respectively, and imaged and documented with Leica MZ16F microscope and Leica DFC500 camera.

Results
Generation and Identification of pak4 Mutant Alleles
Two TALEN arms were designed targeting exon 2 of the zebrafish pak4 gene, centered just downstream of the ATG start codon (Figure 1A). TALEN RNAs (50 pg left +50 pg right) were injected to produce pak4 mutant alleles as described in Materials and Methods. Bacterial clones of PCR-amplified genomic DNA isolated from pooled (5) 24 hpf TALEN-injected embryos revealed indel mutations in the target region, ranging from 1 to 9 nucleotides deletion or insertion. Sibling embryos were raised to 2 months and screened individually by HRMA on fin biopsy genomic DNA, which identified a total of 18 out of 64 fish carrying somatic indel mutations. These carriers were out-crossed with wild type EK fish and the offspring (F1) were raised at a reduced culturing density (6 fish per 1.8 liter) in order to accelerate growth, allowing breeding as early as 6 weeks. Genotype analysis of the 1-month-old F1 fish revealed an average of 10% germ-line transmission of mutant alleles, with the same mutant allele present in all individuals derived from a single G0 parent. A total of four mutations were identified: deletion of 2, 4 and 5 nucleotides (D2, D4 and D5) and insertion of 6 nucleotides (ins6; Figure 1A). The D2 and D4 mutant alleles were identified first, and for this reason we focused on these lines for subsequent analyses. The pak4D2 and pak4D4 mutant alleles both generate frame shifts at amino acid residue #9, followed by missense polypeptides of 9 and 83 amino acid residues. No additional mutations were detected in the pak4 genome in subsequent analyses.

Figure 2. Morphological phenotype of live pak4D2/D4 mutants.
(A) wild type and F3 mutant fish at 2 dpf. (B) wild type and F3 mutant fish at 6 months. Fish were first photographed for documentation followed by fin biopsy genotyping for confirmation. (C) Whole mount in situ hybridization of wild type and mutant embryos at 7-somite for scl. Lateral (left) and dorsal (right) views are shown. Red and blue arrowheads indicate anterior and posterior blood domains, respectively. (D) Whole mount in situ hybridization of wild type and mutant F3 embryos at 30 hpf for mpo. wt, wild type EK. In panels A, C and D, Δ2/Δ4 refers to the mixed population of homozygous mutant and trans-heterozygous mutant embryos. Panel B shows individuals of each defined genotype, all of which were normal and fertile.

Gene-specific primers were designed and used in PCR detection and amplification of pak4 or β-actin mRNA target regions (see Table 1 for primer sequences).
in the NCBI database. Wild type zebrafish PAK4 is 663 amino acids, 72 kDa [8].

In the F1 generation we identified 7 pak4D2 heterozygous carriers, all of which turned out to be female, while only a single male was identified as carrying the pak4D4 allele. We crossed the single D4 male with the D2 females to obtain a pool of F2 offspring yielding the expected Mendelian proportion of 25% trans-heterozygous pak4 mutants (pak4D2/pak4D4), determined by DNA sequence genotyping. Juvenile pak4D2/D4 individuals were raised and in-crossed to obtain an F3 generation comprising a mix of homozygous and trans-heterozygous mutant genotypes, which was used in most experiments. For brevity, we refer to these populations as “pak4D2/D4”.

Characterization of the pak4 Knockout

Based on the abundance of pak4 mRNA in zebrafish eggs and the observation that a translation-inhibiting MO, but not splice-blocking MOs, disrupted development, we concluded that pak4 may function as a maternal-effect gene [8]. To eliminate the possibility of maternal PAK4 protein contribution to eggs from heterozygous mothers, we carried out our genetic analyses on F3 offspring of F2 compound heterozygous null individuals. To verify the loss of pak4 expression, total protein from pak4D2/D4 embryos (F3) at shield stage was extracted for immunoprecipitation and Western blot analysis using the zebrafish PAK4 custom antibody as previously reported [8]. As shown in Figure 1B, the pak4D2/D4 embryos expressed no detectable PAK4 protein, indicating that both deletions represent null alleles for this gene. However, pak4D2/D4 embryos at 2 dpf (Figure 2A), as well as sexually mature adults (6 months old) exhibited no signs of physical abnormalities (Figure 2B). This was in contrast to the morphology of maternal-zygotic(MZ) pak4 MO knockdown, which included reduced head and tail size, kinked body axis and lethality by 6–7 dpf [8].

In addition to physical defects, pak4 knockdown also resulted in the inhibition of primitive myelopoiesis. Primitive erythropoiesis was unaffected, as was definitive hematopoiesis [8]. It seemed possible that transient loss of myeloid cell types might not be evident from gross morphology, so we carried out whole mount in situ hybridization of the two hematopoietic markers stem cell leukemia (scl) and myeloperoxidase (mpo) in the pak4D2/D4 embryos. Figure 2 shows that scl (C) and mpo (D) were expressed in the pak4D2/D4 embryos in normal patterns as compared to the wild type counterparts at 7-somite and 30 hpf, respectively. We conclude that unlike in pak4 morphants, primitive myelopoiesis is normal in pak4 null zebrafish.

In gene-inactivation experiments it is important to verify that there exists only a single copy of the target gene in the relevant haploid genome. As previously described [8], both of the most recent versions of the EnsEMBL zebrafish genome builds (Zv8 and Zv9) were exhaustively searched for pak4 and pak4-like sequence using different BLAST search engines. Similar searches were repeated in the present study with the same result, i.e. only a single copy of pak4 could be identified in the sequenced zebrafish genome (ENSDARG00000018110; Zv9). We have also searched zebrafish
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Figure 4. Testing for possible alternative translational initiation downstream from the mutation site. (A) Construction of the PAK4-eGFP fusions. The 5'-UTR and open reading frame of pak4 were cloned upstream of an eGFP cassette. The TALEN mutation site is indicated by the red arrow. (B) Expression of the fusions in zebrafish embryos. PAK4-eGFP RNAs were synthesized by in vitro transcription and injected into one-cell stage embryos. Green fluorescence was monitored at 24 hpf. Only the wild type construct exhibited fluorescence. (C) Western blot analysis of PAK4-eGFP RNA-injected embryos. Embryos at 24 hpf were dechorionated and used for total protein extraction and Western analysis with a GFP-specific antibody. eGFP RNA was injected into embryos as a positive control. UI, uninjected wild type EK. wt, wild type PAK4-eGFP. Δ2, PAK4Δ2-eGFP. Δ4, PAK4Δ4-eGFP. Ponceau Red staining of the Western blot membrane is shown as a loading control.

doi:10.1371/journal.pone.0100268.g004
was used as an indicator for myelopoiesis. As shown in Figure 5A and B, morphants expressed a reduced level of mpo (MO) as we reported previously [8]. Co-injection of wild type pak4 RNA (MO+wt) rescued mpo expression, whereas the mutant forms, PAK4Δ2-eGFP (MO+Δ2) and PAK4Δ4-eGFP (MO+Δ4), did not. It is therefore unlikely that the discrepancy between pak4 MO knockdown and gene inactivation by TALENs can be explained by non-coding RNA function. We conclude that the MOs used in these and previous experiments result in a highly specific but nevertheless spurious phenotype that is both independent of p53-mediated apoptosis [8] and reversible by co-injection of pak4 mRNA.

What could be the basis of such an effect? One possibility is that certain MOs might induce some form of stress on embryos that could conceivably, in concert with gene-specific knockdown, result in a phenotype that might not appear in mutant embryos, since they would lack the second hypothetical stress from the MOs. We attempted to test this notion by subjecting the pak4 mutant embryos to conditions that might lead to such a stress. First, we injected “standard control” MO (Gene Tools, USA) into wild type and mutant embryos (pak4Δ2/Δ4, F3) at the 1-cell stage, at the same dose as used for the pak4 MO cocktail (6 ng). As shown in Figure 6A, this had no effect: both sets of embryos developed normally into feeding larvae. Second, we tested thermal stress. Zebrafish embryos develop normally between 23°C and 33°C. The recommended optimal incubation temperature is 28.5°C [14]. Exposure to elevated temperature results in the induction of heat shock chaperone proteins [18], leading to increased mortality and developmental abnormalities. Wild type and mutant embryos (Δ2/Δ4, F3) were incubated at 35°C from blastula stage to 24 hpf. The mutant samples exposed to high temperature developed more rapidly, but had no observable morphological phenotype at 24 hpf (Figure 6B). Identical results were obtained with wild type embryos. By 3 dpf, 25–30% of both mutant and wild type embryos receiving the 33°C treatment showed abnormalities, such as curled tail, short body axis and edema (data not shown) indicating that thermal stress did in fact disrupt development. Thus neither MO injection per se, nor heat stress, was able to elicit the MO knockdown phenotype in pak4 mutant embryos. It is possible that

Figure 5. Testing the ability of mutant RNAs to rescue the MO knockdown phenotype. (A) Whole mount in situ hybridization of wild type and mutant embryos at 30 hpf for mpo. (B) MZpak4 knockdown-rescue data. Percentages of embryos showing undetectable, reduced or normal staining as compared to control for mpo expression at 30 hpf in (A). UI, uninjected wild type EK. MO, 6 ng pak4 MO cocktail. MO+wt, 6 ng pak4 MO cocktail plus 800 pg wild type PAK4-eGFP RNA. MO+Δ2, 6 ng pak4 MO cocktail plus 800 pg PAK4Δ2-eGFP RNA. MO+Δ4, 6 ng pak4 MO cocktail plus 800 pg PAK4Δ4-eGFP RNA. Only the wild type pak4 RNA resulted in significant rescue of mpo expression.

doi:10.1371/journal.pone.0100268.g005
Discussion

Loss of *pak4* in mouse by gene targeting resulted in an embryonic lethal phenotype with multiple tissue defects including placental and epiblast vasculature, myocardial walls, spinal cord motor neurons and interneurons, caudal neural tube [12,13]. It is surprising that in zebrafish this gene appears to be dispensable. It is reasonably clear that the locus we have studied in fish is the closest homolog to the mammalian *pak4* [8], but are these genes actually functional homologs? The genetics would seem to indicate that they are not. If there is an essential function in zebrafish that correspond to what PAK4 does in mammals, it must be performed by some other gene, or genes. It may be that PAK4, and the other Group II PAK proteins (5, 6a, 6b) have evolved different functions, or even lost essential functions altogether, during the mammalian-teleost divergence period. Such a notion is relevant to the practice of using zebrafish as a model system for the functional study of genes identified by mammalian genetics. As more loss-of-function data become available from gene targeting in fish, it will be interesting to see how common it is to find functional divergence compared to mammals, and if this might be predicted in some way a priori.

MOs have been in widespread use for the identification of primary gene function in zebrafish, *Xenopus* as well as other organisms [19,20]. While this approach affords rapid and relatively inexpensive assays, it comes with serious disadvantages that are becoming increasingly evident. Off-target effects on pro-apoptotic genes have been widely reported and reviewed [6,7]. Many studies have incorporated controls using MOs targeting the pro-apoptotic transcription factor p53 in an attempt to exclude such artifacts. Additional controls have included the use of multiple MOs targeting the same mRNA, or using injection of MO-resistant modifications of the targeted mRNA to achieve a phenotypic rescue [5].

However, as gene targeting methods have become established in zebrafish, some MO-induced phenotypes have been confirmed by loss of function alleles. For example AHR2 [21,22,23], WASp [24] and cas [25,26] mutant zebrafish closely mirror the MO phenotypes. However, other examples are beginning to emerge in which MO-induced phenotypes have been validated by stringent controls, but nevertheless do not coincide with stable loss-of-function genetic mutations. The first such report concerned

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Figure 6. Testing for stress contribution to the *pak4* MO phenotype. (A) Injection of standard control MO (6 ng) into wild type and *pak4* mutant embryos. Live embryos at 3 dpf were anesthetized and photographed. Images shown are representatives of 92 out of 98 for wild type and 17 out of 17 for mutant. (B) Heat exposure of embryos. Wild type and F3 mutants were exposed to 35°C from blastula stage to 24 hpf. Control samples (28.5°C) were incubated for 5 hours longer than the heated embryos in order to compensate for different developmental rates.

doi:10.1371/journal.pone.0100268.g006
Fmr1, the zebrafish homolog of FMR1, a highly-conserved gene that is mutated in human Fragile X Syndrome. Tucker et al. [27] used MOs to knock down Fmr1, reporting neuronal and craniofacial abnormalities, both of which could be rescued in RNA injection controls. Subsequently, den Broeder et al. [28] reported on two zebrafish lines independently isolated from a TILLING screen with inactivating mutations in Fmr1. Both lines yielded normal, fertile progeny, in particular lacking any sign of the defects noted in the earlier study using MOs. A second example came from analysis of barx1, a homeodomain gene homologous to the human BARX1 locus. Sperber and Dawid [29] reported MO knockdown of this gene resulted in reduced chondrogenesis in pharyngeal arch derivatives. Again, this phenotype could be rescued by co-injection of suitably modified barx1 mRNA. From another TILLING screen, Nichols et al. [30] identified two alleles with premature stop codons within the homeobox domain and thus likely to result in loss of function. These lines showed a number of defects in craniofacial morphology, but had none of the abnormalities reported with the MO knockdowns. A third case is the novel gene inka, which we identified using Xenopus and have also studied in mouse and zebrafish. MO knockdowns in frog and fish both resulted in severe craniofacial dysmorphology. These experiments were controlled by the use of multiple MOs, and parallel analysis in both species; mRNA injection controls were infeasible due to the highly disruptive nature of inka overexpression [31]. However, targeting the inka gene in mouse resulted in normal, fertile inka null adults [32]. Furthermore, inka null alleles in zebrafish have been identified by the TILLING procedure. Homozygous inka null zebrafish are, like the mouse, apparently normal and fertile (unpublished data). None of the TILLING-based studies carried out extensive tests for alternate expression modes that might account for the difference in phenotype compared to MOs, however the most likely interpretation in all three cases is that the MOs induced phenotypes unrelated to the target genes. It is interesting to note that craniofacial defects were prominent in all of these examples.

We have now described yet another example in which a MO phenotype, validated in this case by RNA rescue controls, fails to correspond to the phenotype of stable genetic mutations. In addition to the defects in myelopoiesis, we also observed other abnormalities in pak4 morphants, including somite morphology, which like the myelopoiesis defect was not p53-dependent and could be rescued with pak4 mRNA, and also defects in craniofacial cartilage, which did show a response to p53 inhibition ([10] and unpublished observations). In the Fmr1 and barx1 studies noted above, p53-dependence was not investigated, so it is possible that some aspects of the knockdown phenotypes might have been promoted by apoptosis. There have been anti-apoptotic functions attributed to pak4 [33,34,35,36], so it is conceivable that the p53-independent effects we observed could result from apoptosis mediated by other pathways nevertheless responsive to pak4 overexpression. Even if such a scenario could be supported, the fact remains, however, that mutating the pak4 gene did not elicit any of the defects found with pak4 MOs.

In conclusion, MOs can be useful in confirming the identity of genes responsible for mutant phenotypes, or other experiments in which the loss of function phenotype has been established independently. However, when these agents are used as a primary tool to determine gene function, extensive controls are essential. Ideally, these should include both rescue by mRNA injection, and the demonstration that non-overlapping MOs yield identical phenotypes. To date, few reports based on MO experiments have met these criteria. Hence, it is likely that additional instances of incongruity between MO and genetic mutant phenotypes will emerge.

Acknowledgments

We thank Benjamin Feldman (NICHD) for professional consultation and support on TALEN and HRMA, the Burgess, Chitnis, Dawid and Weinstein laboratories in the Program on Genomics of Differentiation, NICHD for discussion and materials, John Gonzales for zebrafish husbandry, and Yoko Ogawa and Valerie Virta for helpful discussions.

Author Contributions

Conceived and designed the experiments: SHWL TDS. Performed the experiments: SHWL TDS. Analyzed the data: SHWL TDS. Contributed reagents/materials/analysis tools: SHWL TDS. Contributed to the writing of the manuscript: SHWL TDS.

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