

### Homozygous knockout of the *piezo1* gene in the zebrafish is not associated with anemia

The human erythrocyte travels nearly 300 miles through 170,000 circuits of the circulatory system during its 120-day lifespan. This prolonged voyage subjects the red cell membrane to high and varied shear forces, to compression and stretching when traversing sinusoidal capillary beds, to osmotic shrinkage and swelling when passing through the renal medulla, to oxidative stress during repeated cycles of deoxygenation and re-oxygenation, to assault from the complement system, and to gradual loss of surface area from microvesicle shedding and from macrophage-mediated erythrophagocytosis. Volume regulatory ion transport systems help red cells adapt to these demands, and the transporters and channels that regulate red cell volume are controlled, at least in part, by membrane mechanosensors, including the cation channel, PIEZO1.<sup>1</sup> Gain-of-function mutations in *PIEZO1* cause autosomal dominant dehydrated stomatocytosis (DHSt), also known as xerocytosis, characterized by increased cell volume and Na<sup>+</sup> content, decreased K<sup>+</sup> content, and elevated MCHC,<sup>2,4</sup> with often fully compensated anemia. The *PIEZO1* mutants characterized to date in DHSt patients have been associated with delayed inactivation after channel opening.<sup>5</sup> *Piezo1* loss-of-function in the mouse is lethal at mid-gestation due to defective vasculogenesis,<sup>6,7</sup> so the role of PIEZO1 in the mature circulating erythrocyte cannot be studied in the *Piezo1* global knockout mouse.

Morpholino-knockdown of *Piezo1* expression in the zebrafish (*Danio rerio*) was reported to result in severe anemia, as evidenced by near absence of o-dianisidine staining of 2-day post-fertilization (dpf) embryonic yolk sac and by a 60% reduction in estimated red cell number at 3 dpf. Surviving red cells were noted to be swollen and spherocytic, fragile and dysmorphic.<sup>8</sup> The evidence supporting the specificity of *piezo1* knockdown in generation of the anemic phenotype reported by Faucherre *et al.*<sup>8</sup> was strengthened by reproduction of the observation in three different zebrafish strains using two different *piezo1* morpholinos. The effective working doses of morpholino oligomers were titrated to maintain absence of gross developmental defects. These rather high doses injected at the one-cell stage were 8 ng for translation-blocking MO1 (complementary to the *piezo1* start codon) and 10 ng for splice-blocking MO2 (complementary to the intron 1/exon 2 splice site). In addition, combined injection of the indi-

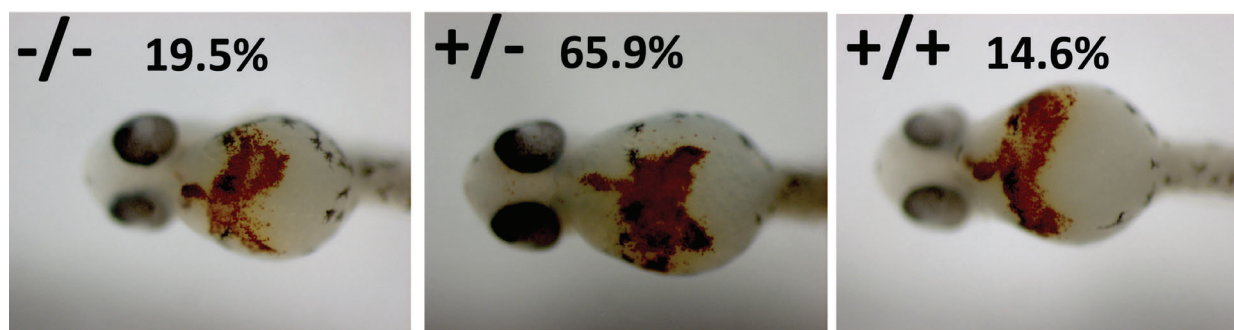
vidually ineffective doses of 4 ng MO1 and 5 ng MO2 decreased embryonic yolk sac hemoglobinization, further supporting a specific effect of *piezo1* knockdown.

However, recent reports have highlighted substantial differences between the zebrafish phenotypes produced by morpholino oligomer knockdowns and the phenotypes of genomic inactivation of the same genes in as many as 80% of the genes examined.<sup>9,11</sup> In particular, a morpholino knockdown of *piezo1* in zebrafish expressed a phenotype of fin blistering and defective gastrulation that was reasonably interpreted as supporting a role for Piezo1 in maintenance of the ability of epithelial monolayers at steady state to extrude dying cells.<sup>12</sup> However, the fin blistering phenotype was not present in an independent zebrafish line carrying a zinc finger (ZFN)-induced 5 nt deletion in the *piezo1* gene, encoding a predicted frameshift in *piezo1* exon 8, followed by termination in early exon 9.<sup>9</sup> The erythroid phenotype of that *piezo1*<sup>-/-</sup> strain was not investigated in that work.

We have now examined the erythroid phenotype in this zebrafish strain carrying a ZFN genomic knockout of *piezo1*. Genotyping was performed as previously described.<sup>9</sup> In contrast to the anemic phenotype observed in zebrafish subjected to morpholino knockdown of *piezo1*,<sup>18</sup> the genomic ZFN knockout of *piezo1* did not segregate either with anemia in the 3-dpf embryo or with dysmorphic erythrocyte morphology in the adult fish.

As shown by o-dianisidine staining of embryonic yolk sac for one clutch of zebrafish progeny arising from the mating of two *piezo1*<sup>-/-</sup> parents (Figure 1), normal hemoglobinization was uniformly evident among 41 embryos in a near Mendelian distribution of *piezo1* genotypes among *piezo1*<sup>-/-</sup> (homozygous knockouts, 19.5%), *piezo1*<sup>+/-</sup> (heterozygote knockouts, 65.9%), and *piezo1*<sup>+/+</sup> (WT, 14.6%).

cDNA sequencing of pooled embryos confirmed that the ZFN mutant *piezo1* allele is transcribed (Figure 2A). The previously reported ZFN-induced genomic lesion indeed produced the expected exon 8 frameshift in the transcribed *piezo1* mRNA, encoding the missense frameshift *piezo1* mutant polypeptide, ΔS349L350fs355X. The frame-shifted sequence continues for 7 residues of neo-sequence before encountering an out-of-frame nonsense codon early in exon 9. The presence of this identical mutation was documented by sequencing in multiple individual embryos and adults of *piezo1*<sup>-/-</sup> genotype. To exclude compensatory exon-skipping in the ZFN mutant fish that might have rescued expression of (modified) Piezo1 polypeptide (encoding in its wild-type form a complex



**Figure 1.** Loss of *piezo1* does not result in anemia. No defect in hemoglobinization is evident in 3-dpf zebrafish embryos of the indicated *piezo1* genotypes stained with o-dianisidine as previously described. The percentage values are derived from 41 embryos from a single clutch.

A

Exon 8 -><- Exon 9

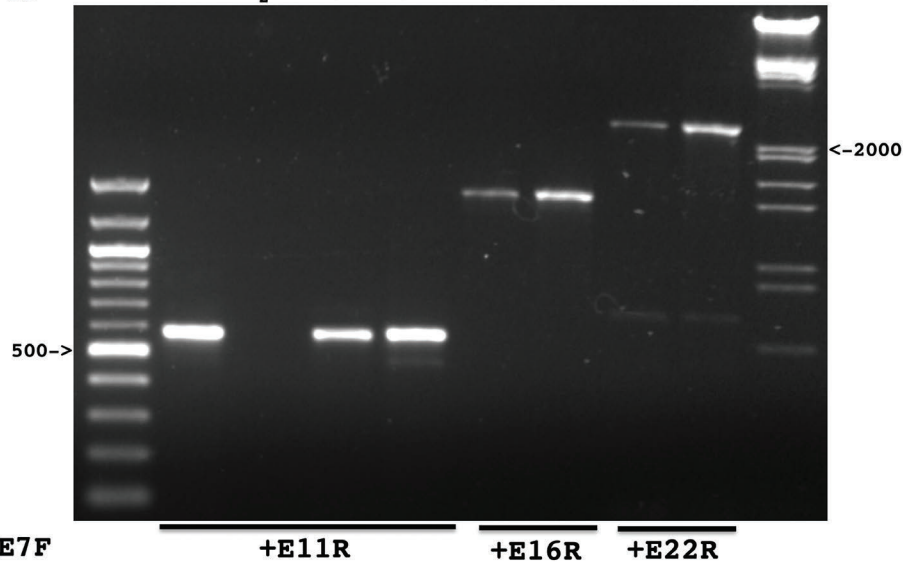
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RefSeq      TACATCTCTGTGACCATTGTGCTGAAAATCAACAGCAACAGTCTAGGAGATGCAGACAAGGCCCGTGAGGGTGAAGCTGTGAAAACAGTG 1612
Ref-Pro 336 Y I S V T I V L K I N S N S L G D A D K A R E G E A V K T V 365
piezo1+/+ TACATCTCTGTGACCATTGTGCTGAAAATCAACAGCAACAGTCTAGGAGACGCAGACAAGGCCCGTGAGGGTGAAGCTGTTAAAACAGTG
piezo1-/- TACATCTCTGTGACCATTGTGCTGAAAATCAACAGCAAC-----AGGAGRTGCAGACAAGGCCCGTGAAGGGTGAAGCTGTGAAAACAGTG
KO-Pro      Y I S V T I V L K I N S N R R C R Q G P *
  
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B

**Piezo1**

**Genotype** Stnd WT H<sub>2</sub>O -/- +/+ -/- +/+ -/- +/+ Stnd



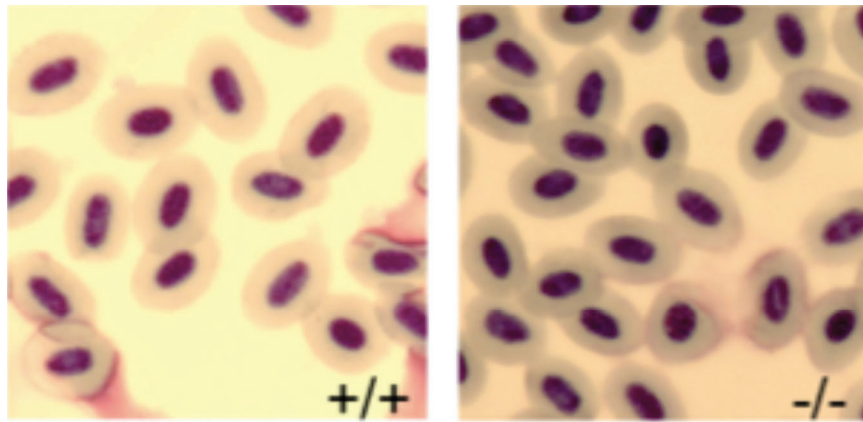
**Figure 2.** Presence of the predicted mutation and absence of exon-skipping in the *piezo1*<sup>-/-</sup> zebrafish. (A) Aligned *piezo1* cDNA sequences across the Exon 8-Exon 9 splice junction taken from pooled embryos of either *piezo1*<sup>-/-</sup> or *piezo1*<sup>+/+</sup> genotypes at 3-dpf. Frameshift terminator codon of mutant is in red. Synonymous SNPs are shown in alternate colors. (B) RT-PCR (31 cycles) amplification of zebrafish *piezo1* from pooled embryos of the indicated *piezo1* genotype using a forward primer from Exon 7 (E7F) and reverse primers from Exon 11 (+E11R), Exon 16 (+E16R), or Exon 22 (+E22R). "H<sub>2</sub>O" lane (lacking added RNA) and "WT" lane (RNA sample from unrelated embryos) were amplified with E7F and E11R. Size standards are 100 bp ladder (far left lane) and EcoRI/HindIII phage  $\lambda$  digest (far right lane).

polytopic membrane protein of 2538 aa), we performed RT-PCR with pooled embryo RNA to amplify and sequence *piezo1* cDNA fragments. The amplicons encompass *piezo1* exons 7-11, 7-16, and 7-22 (Figure 2B) and exons 42-51 (*data not shown*). Amplicon lengths from *piezo1*<sup>-/-</sup> and *piezo1*<sup>+/+</sup> genotypes were indistinguishable, and the exon 7-11 amplicon sequences revealed no additional mutations downstream of the ZFN-induced deletion mutation. (Oligonucleotide sequences are available upon request). These results demonstrate the absence of detectable exon skipping or intron retention between exons 7-22 and between exons 42-51 in the setting of the 5 bp deletion in the ZFN knockout allele. The data allow a further conclusion to be drawn that open reading frame restoration does not occur before M438 in exon 11 of the mutant *piezo1* mRNA. Semi-quantitative RT-PCR (27-29 cycles, n=12) revealed mutant PIEZO1 transcript levels 35.0±1.7% of wild-type levels, consistent with a modest degree of nonsense-mediated decay.

Faucherre *et al.* reported in their *piezo1* morphants an erythroid phenotype of fragile, spherocytic, dysmorphic cells characterized by occasional uniform attenuation, blurring, and occasional disruption of the equatorial microtubule ring that characterizes fish red cells.<sup>8</sup> However, Figure 3 shows the normal appearance of peripheral smears of circulating red cells obtained from adult *piezo1*<sup>+/+</sup>

and *piezo1*<sup>-/-</sup> zebrafish, without evidence of spherocytosis or dysmorphic changes. The x-y dimensions of WT and knockout adult erythrocytes were subjected to image analysis, as described previously.<sup>13,14</sup> The calculated aspect ratios of the elliptical adult red cells from *piezo1*<sup>+/+</sup> (n=634) and *piezo1*<sup>-/-</sup> fish (n=764) were indistinguishable at 1.40 ( $P>0.1$ ). The cross-sectional area of *piezo1*<sup>-/-</sup> red cells was 7.6% less than that of *piezo1*<sup>+/+</sup> red cells, inconsistent with the increased cell volume previously reported in the *piezo1* morphants.<sup>8</sup> The reasons for the lack of agreement between the erythroid phenotypes of reported zebrafish *piezo1* morphants<sup>8</sup> and the ZFN knockout of *piezo1*<sup>9</sup> are unclear. Possible considerations include use in the morphant experiments of uninjected embryos as negative controls, rather than embryos injected with scrambled or sense morpholinos. In addition, the fraction of total injected embryos represented by embryos that showed the anemic phenotype was not reported, and *piezo1* rescue of the anemic phenotype was not presented.

The 25 nt *piezo1* morpholinos,<sup>8</sup> MO1 and MO2, are not predicted to interfere with transcription or splicing of the four currently annotated *piezo2*-like genes. However, MO1 also shares perfect matches over 15 contiguous nt with predicted exonic sequences in *ogt1* (GlcNAc transferase), *rhd12* (rhomboid protease), and LOC101886676 (encoding an HMG domain protein), and so may be non-specific



**Figure 3.** No gross morphological differences in red cells are evident with loss of *piezo1*. Wright-Giemsa stain of peripheral blood smears from adult zebrafish of the indicated *piezo1* genotype. The cell aspect ratios (long axis diameter divided by short axis diameter) are indistinguishable, but the cross-sectional area of *piezo1*<sup>-/-</sup> red cells is 7.6% lower than that of *piezo1*<sup>+/+</sup> red cells.

at the relatively high dose of 8 ng. MO2 also perfectly matches sequences of 16-19 nt in length found in nearly all zebrafish linkage groups, as well as in exons of *vegfc*, *ttna*, and *ttnb*. Thus, the modest 50% reduction in exon 1-2 splicing of *piezo1* mRNA produced by 10 ng MO2<sup>8</sup> may be non-specific.

Of course, provisos also apply to the non-anemic phenotype of the ZFN knockout. Basal exon-skipping has been proposed to be a widespread mechanism for generation of genetic pleiotropy, active even in the absence of frameshift and termination mutations.<sup>15</sup> Thus, the mRNA transcript of the ZFN-knockout allele might undergo exon-skipping between exon 8 and a locus between exons 22 and 42, but the resulting large internal deletion probably would not encode a functional PIEZO1 polypeptide. Alternatively, *piezo1* mRNA translation might undergo re-initiation at a downstream initiation codon, such as M438 in exon 11 (corresponding to mPIEZO1 L424), or at a methionine further downstream, or at an atypical translational re-initiation codon. (The functional consequences of N-terminal PIEZO1 deletions have not been reported, but deletion of the C-terminal 303 amino acid residues of human PIEZO1 prolongs channel inactivation<sup>5</sup>). Additional possible downstream rescue mechanisms include activation of cryptic splice sites, RNA editing, ribosomal frameshifting, or possible ZFN-triggered DNA repair processes that might generate additional polymorphisms such as the synonymous SNPs evident in Figure 2A. Lastly, *in vivo* selective pressure might up-regulate uncharacterized compensatory suppressor mutations, as recently reported.<sup>11</sup>

After completion of our studies, Cahalan *et al.*<sup>16</sup> recently reported creation of a mouse line in which the *Piezo1* gene underwent Cre-mediated deletion of exons 20-23 in hematopoietic cells. The resulting mice are remarkable for the absence of anemia or reticulocytosis, and their morphologically normal discoid erythrocytes are without spherocytosis. However, the discocytes are mildly macrocytic with slightly reduced MCHC and correspondingly increased susceptibility to osmotic lysis. Intravascular hemolysis was suggested by reduction in serum haptoglobin. This recently reported phenotype of mouse red cells lacking functional PIEZO1 is consistent with our observations that the ZFN-knockout of *piezo1* in zebrafish is compatible with a grossly normal erythroid phenotype, with-

out severe spherocytic anemia.

Subsequent to our submission, two recent studies have described patients with autosomal dominant lymphatic dysplasia due to homozygous loss-of-function mutations in PIEZO1. These patients exhibit asymptomatic, fully compensated mild hemolytic anemia of incomplete penetrance.<sup>17,18</sup>

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