Albinism as a Visual, In Vivo Guide for CRISPR/Cas9 Functionality in the Sea Urchin Embryo

Understanding reproduction and development benefits enormously from a comparative approach. Sea urchins are important for many types of studies, including cell fate determination, morphogenesis, and gene regulatory networks. The CRISPR/Cas9 system of targeted DNA modification provides experimental opportunities for studying animal function in otherwise non-traditional genetic systems, such as the sea urchin—indeed, CRISPR/Cas9 was recently used to inform how signaling networks lead to left-right asymmetry (Lin and Su, 2016). Here, we streamlined the guide RNA (gRNA) construction step by targeting a non-essential gene that enables quick assessment of successful CRISPR/Cas9 function in the embryo, thus allowing for a simple visual readout of functional gene disruption.

Echinoderms produce a variety of pigments referred to as echinochromes, with key enzymes in the synthetic pathways recently documented (Calestani et al., 2003; Barsi et al., 2015). The enzyme Polyketide synthase 1 (PKS1) is essential for the final pigment product, and knockdown of this enzyme by morpholino antisense oligonucleotides (MASO) resulted in albino embryos—albeit transiently by virtue of the MASO lifespan. Here, we targeted the PKS1 gene using CRISPR-Cas9. We performed a rapid-throughput screen of gRNA constructions, and found that the CRISPRScan approach to identify quality gRNA sites and to synthesize the gRNAs (http://www.crisprscan.org/) (Moreno-Mateos et al., 2015) were well suited to this application.

Construction of gRNAs is the rate-limiting step for widespread utilization of CRISPR-directed gene inactivation, and the CRISPRScan approach is unique in that no cloning is necessary. The tail sequence of the gRNA is required to form the double-stranded RNA hairpins recognized and bound by Cas9, and is constant for each gRNA of the type II CRISPR/Cas9 system (Jinek et al., 2012). The targeting site, however, is variable and shorter (18–20 nucleotides). The standard methods to make gRNA DNA templates, by using complementary primers and cloning into a T7 vector, takes over a week. In this more efficient approach, one can make the DNA template for a guide RNA for less than US $20 in a matter of hours, by designing targeting sites with a constant sequence that anneals to the complementary tail sequence and adding the sequence for the T7 promoter to the 5’ end of the targeting sequence. The CRISPRScan website provides an effective algorithm for gRNA targeting with an output that includes the T7 site for direct transcription of the gRNA and the annealing site for the tail DNA primer (also see the Help section at CRISPRScan.org).

We tested three gRNAs to PKS1; the period from DNA ordering to analysis of phenotypes took less than 1 week. The RNA constructs were injected into fertilized sea urchin eggs (Cheers and Ettensohn, 2004), followed by assessment of Cas9 entry into the nucleus using mRNA synthesized from the plasmid pCS2-3xFLAG-NLS-SpCas9-NLS (a gift from Yonglong Chen; Addgene plasmid #51307), which encodes Cas9 (codon optimized for mammalian cells) along with two nuclear localization sequences (one N-terminal and one C-terminal of the Cas9 sequence) and three FLAG-tags at the amino terminus (Guo et al., 2014). Cas9 was detectable in all nuclei (Fig. 1), but its expression exhibited no discernible disruptive phenotype for long-term embryo culture. Adding a combination of targeting gRNAs for PKS1 (see Fig. 1 and Supplemental Material) with Cas9 did not impair embryo development, although the resulting larvae lacked pigment—thus pheno-copying the MASO knockdown of PKS1 mRNA translation (Calestani et al., 2003). This effect was highly penetrant and efficient: Of the 121 gRNA embryos analyzed, 119 were devoid of any pigment; one embryo had two pigmented cells while the other was indistinguishable from controls.

A second gene essential for pigment formation was targeted to further test the efficiency of this approach as well as the penetrance of the phenotype. The transcription factor Glial cells missing (GCM) is essential for transcribing PKS1 (Ransick and Davidson, 2012). The same procedure was followed for gRNA design and synthesis, with similar efficiency from the >100 embryos analyzed (Fig. 1). Again, the resulting larvae perfectly pheno-copied the MASO morphants (Ransick and Davidson, 2012).

Pigment cells are progeny of the veg2 tier of cells formed at the ~64-cell stage. Therefore, albino larvae must have experienced either disruption of PKS1 in all cells at that stage or in their precursor cells. Given the low variation of mutated genomic DNA sequences (Supplemental Material), we surmise that the targeted gene disruption occurred in all cells of the embryo between the 2–4 cell stage.

In conclusion, the CRISPR/Cas9 system is highly effective in early sea urchin embryos. We presented a quick and easy screen of gRNAs for gene disruption that employed protocols for simple, rapid, and cost-efficient gRNA construction and targeted genes predicted to yield a phenotype that does not alter the developmental program or cell fate in the embryo. Investigators can now build on this approach by co-introducing gRNAs that target their gene of interest, along with the gRNA for pigment synthesis used here (Supplemental Material), thus leveraging albinism to rapidly sort which CRISPR/Cas9-positive embryos to analyze for their experimental gene disruption.

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REFERENCES


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