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490. Removing the dopaminergic inhibition with CRISPR-Cas9 to study the progression of sexual maturation in zebrafish

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Abstract

In fish, dopaminergic neurones originating from the preoptic area innerve the region of the pituitary gland that harbors gonadotropin cells. The innervation inhibits the release of gonadotropins that are essential for sexual maturation. The dopaminergic inhibition, mediated by dopaminergic receptors, is a major bottleneck in the propagation of many fish species in aquaculture. In this study, we aimed to knock-out one of the three dopamine receptors paralogue (*drd2b*) that mediates the inhibitory control of gonadotropins in zebrafish *Danio rerio* to study the progression of sexual maturation and potential occurrence of side-effects. Here, *drd2b* heterozygote or mosaic mutants were produced with an efficiency up to 73%. Knocking-out *drd2b* could be lethal since *drd2b* is involved in crucial processes during early ontogeny. If future studies confirm that inactivating *drd2b* is lethal, it may be useful to produce inducible and/or spatially restricted mutants to study sexual maturation.

Introduction

In fish, oogenesis is controlled by two pituitary gonadotropins (Gths); the follicle stimulating hormone (Fsh) and the luteinizing hormone (Lh). Fsh and Lh regulate oogenesis consisting of the succeeding developmental stages of oogonia proliferation, oocyte growth, oocyte maturation and ovulation (reviewed by Lubzens *et al.*, 2010). In fish, Fsh activity is essential for oocyte growth and storage of lipids and yolk. When ending the growth period, Lh induces oocyte maturation and ovulation after which oocytes are released to the external environment and fertilized.

In fish, Gths production and release are under the dual control of gonadotropin releasing hormone (GnRH) and dopamine (DA) (reviewed by Dufour *et al.*, 2009). Dopaminergic neurons originating from the *nucleus anteroventralis* of the preoptic area (NPOav) directly innerve the region of the pituitary gland that harbors the gonadotropic cells, the *proximal pars distalis* (Jolly *et al.*, 2016). The dopaminergic innervation in the pituitary downregulates Gths release by counteracting the stimulatory effects of GnRH in many teleosts (reviewed by Dufour *et al.*, 2009). The effect of DA is mediated by D2 dopaminergic receptors (D2r) (reviewed by Dufour *et al.*, 2009) that are expressed in the pituitary (Jolly *et al.*, 2016). In zebrafish, one of the D2r paralogue, *drd2b*, is involved in mediating the inhibitory control of gonadotropins (Fontaine *et al.*, 2013).

Dopaminergic inhibition of the reproductive axis is present in many fishes belonging to the salmonids, cyprinids, silurids, percomorphs, elopomorphs but also in one chondrostean fish (reviewed by Dufour *et al.*, 2009). The inhibitory role of DA is one of the major bottlenecks in the propagation of many fish species in aquaculture. Treatment with pituitary extracts to circumvent the pituitary, but also with GnRH in combination with D2r antagonists to counteract dopaminergic inhibition, are used to induce sexual maturation and ovulation. Long term hormonal treatment can impact egg quality and thus it is essential to gain insights about the mechanisms of dopaminergic inhibition to gain control over it. Thus, we explored the feasibility of producing *drd2b* knock-out mutants with CRISPR-Cas9 in zebrafish as model species to study the progression of sexual maturation.

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Materials & methods

An overview on how CRISPR-Cas9 induces targeted mutations is presented in Figure 1. In this study, two different protocols, applied by Utrecht University and Wageningen University & Research respectively, were used to explore the feasibility of producing *drd2b* mutants with CRISPR-Cas9. While Utrecht University follows the protocol of Essner *et al.* (2016), Wageningen University & Research used a combination of the Shawn Burgess lab protocol (Varshney *et al.*, 2015) and Hubrecht lab protocol. CRISPR-Cas9 activities were covered by GGO permits (Wageningen University & Research: IG 19-08; Utrecht University: IG 02-115).

sgRNA. At Utrecht University, the functional sgRNA was generated by mixing crRNA that was designed on the target sequence and the generic tracrRNA that anchors to the Cas9. Two crRNAs (AS and AX) were designed on the first exon of *drd2b* by using the IDT (Integrated DNA technologies, Coralville, United States) online tool after entering the *drd2b* sequence of *Danio rerio* (NM_197936.1). At Wageningen University & Research, the sgRNA was designed as follows (Figure 2): (1) the target sequence (20 nt) of the fourth exon of *drd2b* was designed by using the online tool of UCSC Genome Browser (Santa Cruz, United States); (2) the oligo A was ordered after adding the T7 promoter sequence (17 nt) and the first 20 nt of the gRNA to the respective 5' and 3' of the target sequence; (3) the generic oligo B (80 nt) that was complementary (20 nt) to the last part of oligo A was ordered; (4) both oligos were annealed and extended to produce a 117 nt double stranded DNA fragment that was *in vitro* transcribed to generate the sgRNA (Exon4).

Microinjections. At Utrecht University, the CRISPR-Cas9 solution was made by: (1) mixing and heating at 95 °C for 5 min 1 µl crRNA, 1 µl tracrRNA and 31.3 µl buffer to create the 3 µM sgRNA solution; (2) adding 0.5 µl Alt-R *S.p.* Hifi Cas9 Nuclease V3 (IDT, Coralville, USA) diluted in 9 µl Cas9 working buffer and 0.5 µl Phenol Red to a working concentration of 0.5 µg/µl; and (3) combining 3 µl of sgRNA with 3 µl diluted Cas9

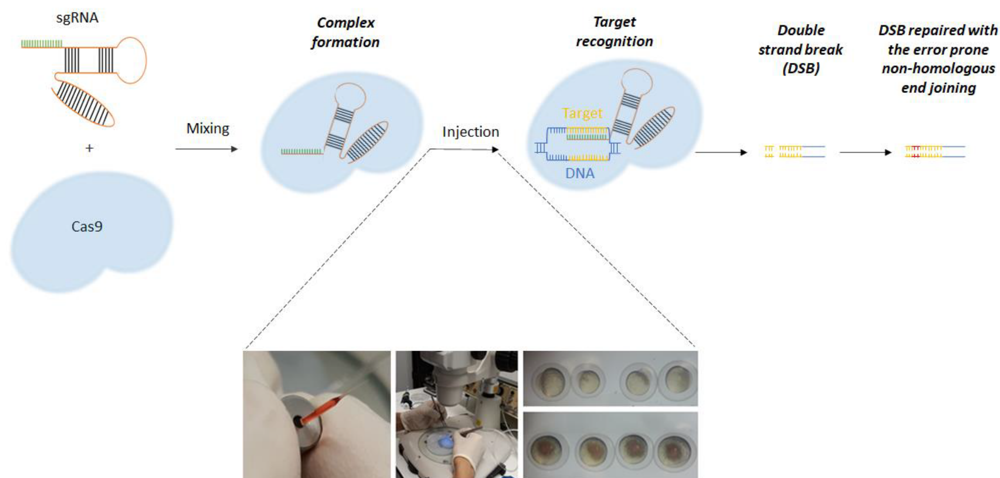

Figure 1. Overview of how the CRISPR-Cas9 system induces targeted mutations. CRISPR-Cas9 consists of two components: (1) a sgRNA that contains a guide sequence (designed by the user) which is complementary to the target sequence and (2) a scaffold sequence that is necessary for the Cas9 protein binding. Once mixed together, the sgRNA and Cas9 form a complex that binds to the target to induce a double strand break (DSB) within the DNA. The DSB will be repaired by the error prone non-homologous end joining (NHEJ) pathway that causes indels at the DSB site.

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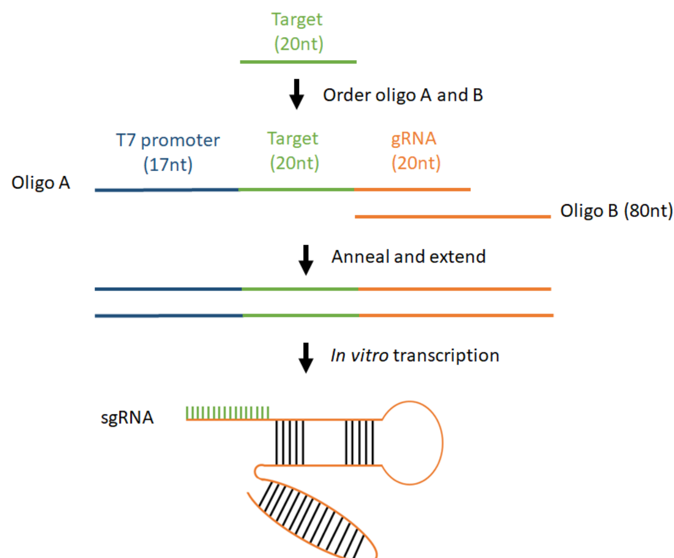

Figure 2. *In vitro* transcription of sgRNA (adapted from Varshney *et al.* 2015).

protein. At Wageningen University & Research, the CRISPR-Cas9 complex was made by mixing 600-900 ng sgRNA, 0.8 µl Alt-R *S.p.* Cas9 Nuclease V3 (IDT) at 5 ng/µl in 400 mM KCl, 0.3 µl Phenol Red and 400 mM KCl to a final volume of 6 µl. Injection mixes were loaded, calibrated under the binocular to inject 1 nl in at the 1-cell stage in zebrafish. Larvae were sampled at 2 days post hatching (dph) for DNA extraction to screen for mutation. Primers, that screened for the mutations were designed and PCR products sent for sequencing to check for mutation efficiency that was calculated as followed: (the number of zebrafish mutants confirmed by sequencing/ the total number of zebrafish sequenced) × 100%.

Results

An overview of the results is presented in Table 1. In zebrafish, ~300 eggs that were injected with CRISPR-Cas9 had a survival rate ranging from 6 to 40%. Screening showed that WT sequences had one clear peak for each nt in the *drd2b* sequence, while mutants had several peaks (two or more) for each nt around and after the protospacer adjacent motif (PAM) sequence (Figure 3); results suggesting that mutants were heterozygotes or mosaics. CRISPR-Cas9 induced mutations with an efficiency up to 73%.

Discussion

In zebrafish, *drd2b* mutants were produced but they were heterozygotes or mosaics. It is possible that homozygote mutants did not survive because knocking-out *drd2b* would be lethal. This hypothesis is

Table 1. Number of eggs injected with CRISPR-Cas9, survival rates and mutation efficiency for each target in zebrafish.

Target	Number of eggs injected	Survival rate (%)	Efficiency (%)
Exon 1, AS	300	6	33
Exon 1, AX	300	10	0
Exon 4	300	40	73

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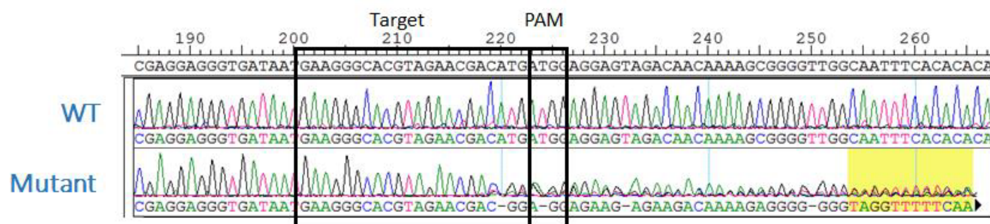

Figure 3. *Drd2b* sequences of the wild type (WT) and mutant in zebrafish *Danio rerio*.

supported by the study of Boehmler *et al.* (2004) who suggested that *drd2b* is involved in crucial processes (e.g. motor behaviour). Unfortunately, we could not confirm that knocking-out *drd2b* is lethal since the controls also died in this experiment. It is important to note that mutation in a seemingly crucial gene is not necessarily lethal (Ziv *et al.*, 2012). Future studies should investigate whether viable homozygous *drd2b* mutants can be produced. If knocking-out *drd2b* turns out to be lethal in zebrafish larvae, it might be useful to produce inducible and/or spatially restricted *drd2b* mutants to study the progression of sexual maturation. Gained knowledge could help to develop methodology to sexually mature fish with natural triggers rather than with artificial stimulators. The authors acknowledge Wageningen Livestock Research for funding this study as 'innovation challenge'.

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