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Sterilization of sterlet *Acipenser ruthenus* by using knockdown agent, antisense morpholino oligonucleotide, against *dead end* gene

Zuzana Linhartová [a,*], Taiju Saito [a], Vojtěch Kašpar [a], Marek Rodina [a], Eva Prášková [a], Seishi Hagihara [b], Martin Pšenička [a]

[a] *Research Institute of Fish Culture and Hydrobiology, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic*

[b] *Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan*

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ABSTRACT

Sturgeons (chondrostean, acipenseridae) are ancient fish species, widely known for their caviar. Nowadays, most of them are critically endangered. The sterlet (*Acipenser ruthenus*) is a common Eurasian sturgeon species with a small body size and the fastest reproductive cycle among sturgeons. Such species can be used as a host for surrogate production; application is of value for recovery of critically endangered and huge sturgeon species with an extremely long reproductive cycle. One prerequisite for production of the donor's gametes only is to have a sterile host. Commonly used sterilization techniques in fishes such as triploidization or hybridization do not guarantee sterility in sturgeon. Alternatively, sterilization can be achieved by using a temporary germ cell exclusion–specific gene by a knockdown agent, the antisense morpholino oligonucleotide (MO). The targeted gene for the MO is the *dead end* gene (*dnd*) which is a vertebrate-specific gene encoding a RNA-binding protein which is crucial for migration and survival of primordial germ cells (PGCs). For this purpose, a *dnd* homologue of Russian sturgeon (*Agdnd*), resulting in the same sequence in the start codon region with isolated fragments of sterlet *dnd* (*Ardnd*), was used. Reverse transcription polymerase chain reaction confirmed tissue-specific expression of *Ardnd* only in the gonads of both sexes. *Dnd*-MO for depletion of PGCs together with fluorescein isothiocyanate (FITC)–biotin–dextran for PGCs labeling was injected into the vegetal region of one- to four-cell-stage sterlet embryos. In the control groups, only FITC was injected to validate the injection method and labeling of PGCs. After optimization of MO concentration together with volume injection, 250-μM MO was applied for sterilization of sturgeon embryos. Primordial germ cells were detected under a fluorescent stereomicroscope in the genital ridge of the FITC-labeled control group only, whereas no PGCs were present in the body cavities of morphants at 21 days after fertilization. Moreover, the body cavities of MO-treated and nontreated fish were examined by histology and *in situ* hybridization, showing gonads which had no germ cells in morphants at various stages (60, 150, and 210 days after fertilization). Taken together, these results report the first known and functional method of sturgeon sterilization.

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* Corresponding author. Tel.: +420 387 774 784; fax: +420 387 774 634.

E-mail address: linhartova@frov.jcu.cz (Z. Linhartová).

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1. Introduction

With the recent worldwide poaching of sturgeons for caviar, construction of dams, and environmental pollution, these fishes are listed in the International Union for the Conservation of Nature's Red List as the most endangered group of species in the world. Moreover, their artificial reproduction is complicated by late maturation and the inability of females to repeat reproduction every year [1]. Therefore, an efficient approach for their reproduction and conservation is needed. In recent years, techniques for surrogate production through germ line chimera by early germ cells transplantation into closely related species have been established in several teleostean species [2,3]. The sterlet (*Acipenser ruthenus*) is one of the most common and smallest Eurasian sturgeon species with the fastest reproductive cycle (sexual maturity of males 3–7 and females 5–9 years of age) in comparison with other sturgeons [1]. A prerequisite to generate germ line chimera, producing the donor's gametes only, is the sterilization of the host. Triploidization is the method of choice for practical use in species whose triploid individuals are sterile [4,5]. However, in sturgeons (evolutionary polyploids), all ploidy levels are probably fertile [6]; therefore, an alternative technique for sturgeon sterilization must be considered. The approach was to use a temporary exclusion of the germ cell–specific gene that is responsible for their development by a knockdown agent, from which the antisense morpholino oligonucleotide (MO) was applied. *Dead end* (*dnd*), a gene that encodes an RNA-binding protein crucial for migration and survival of primordial germ cells (PGCs), was selected. This gene has already been described in a number of model species, and knockdown of *dnd* by the MO interferes with PGC migration and results in their death [7,8].

2. Materials and methods

2.1. Ethics

All experiments were carried out in accordance with the Animal Research Committee of the Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic. Fish were maintained according to the principles based on the EU harmonized animal welfare act of Czech Republic, and principles of laboratory animal care and the national laws 246/1992 "Animal welfare" on the protection of animals were followed and respected.

2.2. Fish source, embryos preparation, and sample collection

Adult sterlet (*A ruthenus*) females and males, aged 5 to 9 years, were transferred from outdoor ponds into indoor recirculating aquaculture system during the spawning season March to June 2014. Fish were held in 4000-L tanks at mean water temperature of 13 °C. To induce spermiation, males were injected by a single intramuscular injection of carp pituitary extract at 4 mg/kg of body weight (BW) in 0.9% NaCl. Sperm was collected 48 hours after hormone injection and kept on ice at 4 °C until fertilization. Spermatozoa motility was assessed by light microscopy and was greater than 90%. Ovulation was stimulated with carp pituitary extract by intramuscular injection in two doses: the first dose, 0.5 mg/kg of BW and the second, 4.5 mg/kg of BW, 12 hours after the first injection. The ovulated eggs were collected from three females 18 to 20 hours after the second injection. The eggs were inseminated with sperm from two males in dechlorinated water at 15 °C. Stickiness of the fertilized eggs was removed by treating with 0.04% tannic acid. Eggs were dechorionated (outer layer) 1 hour after fertilization using forceps. Dechorionated eggs were transferred to 100-mL dechlorinated tap water with 0.01% penicillin and streptomycin in glass Petri dishes and incubated at 15 °C in an incubator. Temperature was regulated at 15 ± 1 °C throughout the experiment, and water was changed daily. Embryos were mainly used for injection of fluorescein isothiocyanate (FITC)–biotin–dextran for PGCs labeling, antisense MO for PGCs depletion, reverse transcription polymerase chain reaction (RT-PCR), histology, and *in situ* hybridization (ISH, described in the following).

Tissue samples used for determination of *dnd* gene expression by RT-PCR were obtained from adult sterlet males and females; they were rapidly dissected and washed in PBS (adjusted to 248 mOsm/kg, pH 8). For RNA extraction, tissues were frozen in liquid nitrogen and stored at −80 °C.

2.3. Isolation of Ardnd fragments (sterlet dnd) for comparison with full-length Agdnd (Russian dnd)

The *dnd* gene of Russian sturgeon (*Agdnd*; Hagihara, unpublished data) was used as a reference sequence for MO design. Full length of Russian *dnd* (*Agdnd*) was homology searched and identified from the database of transcriptome in a developing gonad by Hagihara (unpublished data). For MO design, the sequence of gene in the start codon region has to be known; therefore, only fragments of sterlet *dnd* (*Ardnd*) including the ATG region were identified in this study and compared with *Agdnd*. For this purpose, total RNA was isolated from stripped unfertilized eggs of sterlet using RNeasy Lipid Tissue Mini Kit (Qiagen, Prague, Czech Republic). First-strand complementary DNA (cDNA) was synthesized from 1 to 4 μg of total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany). The cDNA fragments of *Ardnd* gene were amplified by RT-PCR using primers (Table 1) designed according to the full-length sequence of *Agdnd* (Hagihara, unpublished data). Polymerase chain reaction was performed using a C1000 thermal cycle (Bio-Rad, Hercules, CA, USA) under following conditions: denaturation at 95 °C for 5 minutes, 35 cycles of amplification at 95 °C for 20 seconds, 60 °C for 30 seconds, 72 °C for 1 minute, and additional elongation at 72 °C for 10 minutes. Polymerase chain reaction products were separated by 1.2% agarose gel electrophoresis. The fragments were purified by NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Duren, Germany) and cloned into pCR 2.1-TOPO TA vector using TOPO TA Cloning Kit with competent *Escherichia coli* cells (Life Technologies, Prague, Czech Republic). Positive clones were amplified by PCR as follows: initial step at 95 °C for 2 minutes, 5 cycles at 95 °C for 30 seconds, 50 °C for 1 minute, 72 °C for 1 minute, followed by 30 cycles at 95 °C for 30 seconds, 50 °C for 45 seconds, 72 °C for 1 minute, and a final

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