Fakulta rybářství
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Utilization of CRISPR/Cas9 and novel germ cells labelling technique for surrogate production in sturgeons

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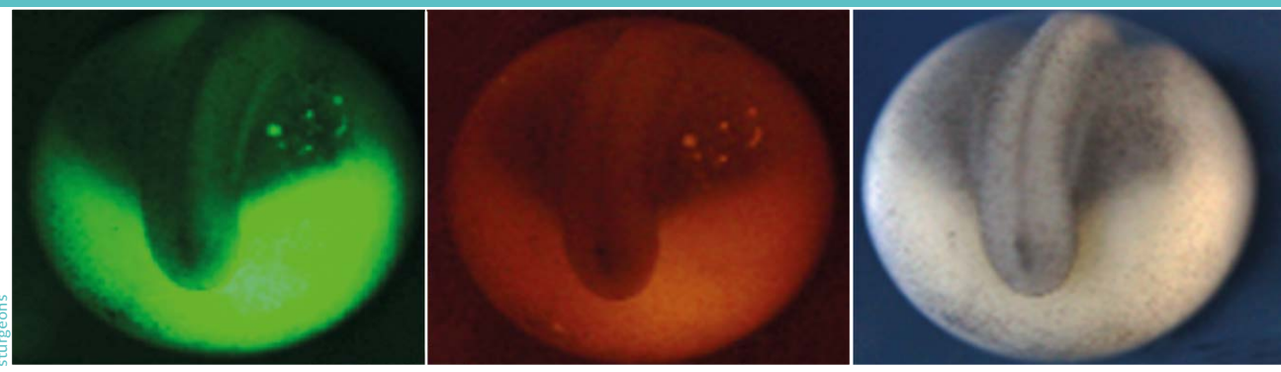

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Czech Republic, Vodňany, 2019

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

At present more than 97,000 species are at the red list of IUCN (The International Union for Conservation of Nature), and reportedly more than 27,150 species are at risk of extinction (IUCN, 2018). It is therefore, becoming challenging to decide how to allocate scarce resources to restore these species (McDonald-Madden et al., 2011). One of these resources is scientific effort aiming to enhance knowledge about status of critically endangered species. The IUCN is a useful tool that guides allocation of scientific efforts (Rodrigues et al., 2006; Brito, 2008; Brooks et al., 2008; De Lima, Bird & Barlow, 2011) and also shows current state of knowledge and level of threat of several species (Jaric et al., 2017).

Main reasons that led several fish species in 'threated' or in another category of IUCN are anthropogenic activities. Such activities result to alterations to freshwater ecosystems that led in dramatic decline in populations of several native fish species (Dudgeon et al., 2006; Ricciardi & Ramussen, 1999). To tackle this situation, resource managers turned towards the hatcheries to take steps to prevent extinction of several species. As a conservation tool, resource managers often include production and release of captive-reared individuals for purpose of conservation (Hartman & Preston, 2001; Radonski & Loftus, 1995). Various programs have been implemented for restoration of self-sustaining populations of fish species and most importantly, re-establishment of survival of hatchery released individuals as it is quite often that survival of hatchery released of endangered fish species is limited (Durst, 2009; Marsh, 2004; Miller, 1961; Minckley & Deacon, 1968; Zelasko & Bestgen, 2010).

1.2. Sturgeons and primordial germ cells

Sturgeons (Acipenseridae) are ancient fish species that are often called as "living fossils" in literature. Sturgeons' fossil record dates back to Upper Cretaceous (Grande and Bemis, 1991). When DNA from mitochondria was analysed, it suggested that sturgeons diverged from ancient, pre-Jurassic teleost lineage ~200 Mya (Millions years ago) (Bemis and Kynard, 1997). Sturgeons are known for their caviar; however, unfortunately, due to worldwide poaching, construction of big dams and habitat destruction led them at verge of extinction. Sturgeons' 85% species are listed as critically endangered in the IUCN. Furthermore, artificial reproduction of sturgeons is also complicated as they mature late and inability of female sturgeons to repeat their reproductive cycle each year (Dettlaf et al., 1993).

Factors that affect dwindling populations of sturgeons include water pollution and interference in their natural habitats. Other significant factors that affect sturgeon populations are recreational fishing, water divergence, hybridization, reduced food supply and salt water intrusion (Ludwig et al., 2001; Zhang et al., 2011). Spawning and eggs/larvae habitats have been eliminated and/or reduced due constructions of dams (Hildebrand et al., 1999). Lake sturgeons were abundant once in great lakes until overfishing in 1800's and early 1900's. Their reproductive traits make their rehabilitation more difficult, these traits include delayed maturation and periodic interrupted spawning cycles. Shovelnose sturgeon (*Scaphirhynchus platorynchus*) is important in the USA due to ban on imported caviar. In upper Mississippi river, sturgeons from upstream pools had greater lengths, weights and ages than those from downstream pools. Interestingly, mortality in upstream pools was also lower (Koch and Quist, 2009). It takes time and also cost-intensive to recover their populations. On other hand, demand of caviar continues to outstrip supply all over world.

Among sturgeons, sterlet usually matures from 3–7 years (males), while for females it takes from 5–9 years. These characteristics provide advantage as it reduces time for genetic breeding cycle; therefore helping in establishing techniques to manipulate their genome. At

the same time, sterlet (tetraploid) is sturgeon with lowest polyploidy genome, thus making it easier and convenient to study evolution and functions of paralogous genes. In short, sterlet, can be called as model or ideal species in sturgeons (Chen et al., 2018; Dettlaf et al., 1993).

Primordial Germ Cells (PGCs) are origin of all germ cells in developing embryos that are specified early in developmental period and their migration is guided by SDF1A and SDF1A receptors (GPCR chemokine receptor 4b). In several animal species, PGCs formation and migration patterns have been investigated thoroughly and results have found that these patterns vary among studied species. PGCs formation is altered according to cleavage from holoblastic to meroblastic (fig. 1; taken from Saito et al., 2014). Previously in our lab, we have shown that injection technique in sturgeons can help to visualize germplasm and PGCs. Our colleagues have already shown that PGCs are generated at vegetal pole (VP) of sturgeon eggs and PGCs migrate on yolky cell mass towards gonadal ridge. It has been shown that migratory mechanism is well conserved between sturgeon and other remotely related teleosts like goldfish (Saito et al., 2014). Research on PGCs transplantation in sturgeons is useful to get valuable insights to understand gametogenesis, sex differentiation and PGCs evolution in vertebrates. Pšenička et al. for first time isolated and transplanted female and male early-stage germ cells in sturgeon to produce a germline chimera as a surrogate parents and gene-banking tool (Pšenička et al., 2015).

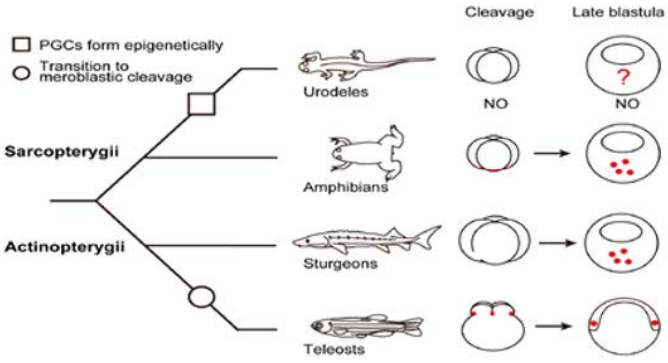

Figure 1. *Schematic representation of embryo cleavage patterns and formation of PGCs, and migration patterns of PGCs in selected vertebrates (figure is adapted from Saito et al., 2014).*

1.3. Dead end (dnd1) protein

In last decade, *dnd1* was discovered in zebrafish and then it was later discovered in other vertebrates such as Xenopus, mouse, human and chicken (Weidinger et al., 2003). *Dnd1* expresses exclusively in vertebrates germline, therefore indicating that it plays a conserved role in germline development. Dnd1 protein binds with U-rich sequences at miR430 binding sites, and in this way, it prevents access of miRNAs (Kedde et al., 2007). Several studies have authenticated that dnd1 protein is essential for migration of PGCs and PGCs migration follows complex path by several developing tissues. Disruption of PGCs migration affects fertility. Very recent study of Gross-Thebing and colleagues revealed that dnd1 protein deficient PGCs in zebrafish transdifferentiate into somatic cells (Gross-Thebing et al., 2017), and in zebrafish, this protein is localized in perinuclear germ granules within PGCs (Weidinger et al., 2003). Scientists presumed that dnd1 proteins carry RNA molecules into the nucleus to trigger germline specification (Taguchi et al., 2014). PGCs face significant challenges during their

migration period, and their protection is necessary to make sure that these cells differentiate into gametes and thus fertility of organism is ensured (Gross-Thebing et al., 2017). PGCs polarization and motility is dependent on dnd1 protein and when *dnd1* is knocked-down by using morpholino approach, migration of PGCs becomes abnormal, therefore leading to ablation of PGCs in zebrafish (Weidinger et al., 2003).

Various studies have showed that *dnd1* is a conserved germ cells molecular marker in several fish species. In testes and ovary of Pacific Bluefin tuna and turbot, differential expression of *dnd1* have been reported (Lin et al., 2013; Yazawa et al., 2013). *Dnd1* was seen to be determinant for PGCs specification in *Oryzias celebensis*; expression of *ocdnd1* was shown in gonads, and specific expression was observed in spermatogonia (Zhu et al., 2018). In common carp (*Carassius gibelio*), expression of *dnd1* is still unclear, and in gibel carp, the dnd1 contains RRM (RNA recognition motif) that is conserved in genomic organization. Expression of *dnd1* have been reported in ovary and testes of gibel carp, therefore making it potential molecular marker for gametogenesis (Li et al., 2016). Dnd1 in Olive flounder (*Paralichthys olivaceus*) i.e., *Podnd1* was studied, and was recognized as germ cell specific marker; its expression was seen in testes and ovary of olive flounder. Localization of was found to be similar like zebrafish in this species (Wang et al., 2015). *Acipenser sinensis* dead-end homolog (*Asdnd1*) was recently characterized where its expression was detected in the gonads of immature Chinese sturgeons of both the sexes (Yang et al., 2015). In order to apply surrogate production, it is essential that host should be sterile/free of germ cells. For germ cell transplantation for surrogate production in sturgeon, sterlet that matures fast when compared with other sturgeon species should be used as host. To produce sterile host for aforementioned purpose, previously in our lab, *dnd1* was knocked-down by using morpholino oligonucleotide (Linhartová et al., 2015). A table comprising of summary of *dnd1* and its function in fish is given below (table 1);

Table 1. *Summary of functions of dnd1 found in fish.*

Species	Function and applications of dnd1 protein
Zebrafish	Survival and migration of PGCs; first time identified in zebrafish; *Zdnd* associates with 3′ UTR of GMNN mRNA; Possess ATPase activity that is necessary for PGCs formation;
Medaka	Critical for PGCs specification; its depletion abolishes PGCs; overexpression boosts PGCs by increasing their precursors; not found in sperms, however, persists throughout oogenesis.
Atlantic Cod	Transient inhibition does not block PGCs development until hatching
Loach, Goldfish, Medaka and Sterlets	Inactivation of *dnd1* in these species cause the sterilization
Turbot	*Smdnd* (*Scophthalmus maximus dnd1*), present in presumptive PGCs, in adult male and female germ cell, can also be used germ cell marker
Atlantic Salmon	Knockout of *dnd1* produces germ cell free salmon in K0.
Rare minnow	It is expressed in gonads of both sexes and is restricted to germ cells;
Pacific Bluefin tune	Molecular marker for ASGs
Rainbow trout	*Dnd1* knock down by dnd-AMO led germ cell deficient larvae.

The microRNAs (miRNAs) are found to inhibit genes expression, and also control normal development and cancer (Kedde et al., 2007). The role of dnd1 protein has been found in protection of mRNA from miRNAs mediated repression and it has thus been confirmed that

expression of *dnd1* counteracts role of several miRNAs in zebrafish PGCs (Kedde et al., 2007). Dnd1 protein abolishes interaction between miR221 and 3'UTR of the p27 mRNA that led downregulation of p27 mRNA in human cells. The *nanos1* expresses in germline development and this gene has been discovered to be resistant against repression caused by miRNAs in germline (Mishima et al., 2006), this is because dnd1 protein binds at its 3'UTR (Kedde et al., 2007). *Dnd1* in zebrafish (*Zdnd*) associates with 3'UTR of the GMNN mRNA and it regulates expression of *Zdnd*. The Zdnd does binds with eight mRNAs by SNAAP assay, which was authenticated by performing RT-PCR (Chen et al., 2010).

Consistently with aforementioned studies, in olive flounder the *nanos3* 3'UTR is responsible for localization of mRNA in PGCs (Li et al., 2015). Regulation of germline and somatic cell distinction in zebrafish happens by combination of germ cell specific RNA binding proteins and miRNAs. Repression mediated by miR430 counteracted in zebrafish when *dnd1* over-expresses in somatic cells, and interestingly, translation repression of *dnd* has been found to be intendent to *nanos3* (Kobayashi et al., 2017).

Dnd1 protein can be potentially used in conservation of critically endangered fish species as well as in the aquaculture. Fish species reared in aquaculture facilities when escape can pose great threat to the integrity of wild stocks; thus, by targeting the *dnd1*, sterile stocks for aquaculture can be produced. In this way, aforementioned problem can be mitigated (Wargelius et al., 2016). Advantage of producing sterile stock is that gene flow from domesticated fish will not occur to wild stocks (Glover et al., 2012). Concluding the role of dnd1 protein, as it is not essential for PGCs survival, and targeting it by using various technologies such as MO or novel genome editing technology *i.e.,* CRISPR/Cas9, can be effective to achieve sterility in fish. It in turn can be applied in farming of fish in order to make sure that no genetic containment of wild stocks once farmed fish escape accidently, and also sterile fish can be used for surrogate production of IUCN-red listed species. Further, this gene can be potentially used as specific marker of the germ cells in several species of fish.

1.4. Germ cell transplantation

Various technologies regarding assisted reproduction have been designed and these technologies help to produce functional gametes and offsprings of critically endangered and/or endangered fish species. Cryopreservation of gametes, nuclear transfer (NT), germ cell transplantation (GCT), embryo transfer and induction of multiple ovulations approaches have been documented that fall in the sphere of assisted reproductive technologies (Pukazhenthi et al., 2006). Scientific community especially from aquaculture and conservation biology are focusing on GCT approach that gives opportunity to sustain and/or prevent loss of genetic biodiversity of fish species due to over exploitation and environmental degradation. GCT depends on availability of recipients that should be sterile/germ cell free (Ma et al., 2011; Lacerda et al., 2010; Saito et al., 2011). So far, several methods have been reported to remove/eliminate endogenous germ cells in various fish species in order to prepare them as host for surrogate production. In mammals, these methods include use of Busulfan (Ogawa et al., 1999; Brinster et al., 1994; Honaramooz et al., 2005), irradiation (Van den Aardweg et al., 1983), hyperthermia treatment (Rockett et al., 2001) and cold ischemia (Yong et al., 1988). In fish species, several methods have been developed to sterile the host fish species, these include use of triploid animals in salmonids (Okutsu et al., 2007). In sturgeons, sterile host have been produced by targeting *dnd1* by using MO and CRISPR/Cas9; PGCs have also been removed by UV irradiation (Linhartová et al., 2015; Saito et al., 2018). Schematic representation of GCT in sturgeons is shown in figure 2. Our colleagues have already demonstrated that visualization of PGCs *in vivo* is possible by simply injecting into vegetal pole of sturgeon embryos with

fluorescein isothiocyanate (FITC)-dextran (Saito and Pšenička, 2015). It is important that gonads of recipients should also be genetically compatible with gonads of donors (Saito et al., 2011; Ogawa et al., 1999; Shinohara et al., 2002; Wong et al., 2011; Brinster, Averbock, 1994). Studies have shown that recipients from various fish species do not present any or very little rejection to transplanted cells from unrelated species (Honaramooz et al., 2005; Rodriguez-Sosa et al., 2006; Herrid et al., 2006; Majhi et al., 2009; Yazawa et al., 2010).

Transplantation methods also vary like micro-injection of germ cells into blastodisc of blastula stage of embryos (Saito et al., 2011), micro-injection in coelomic cavity of hatchlings (Wong et al., 2011; Okutsu et al., 2007) and interestingly, some studies have shown direct micro-injection in the gonads of adults by intra-papillar intervention (Lacerda et al., 2010; Majhi et al., 2009). Thus, taking advantage and possibilities to use model fish species as recipients in aforementioned technology. On the other hand, different level of skills and equipment are required to perform GCT, and it may affect technique when some methods are not applicable in areas, which are difficult to access easily and conservation of endangered fish species is indispensable. Taking aside advantages and disadvantages of before mentioned methods, GCT done by using any method and at any developmental stage produced donor-derived functional gametes.

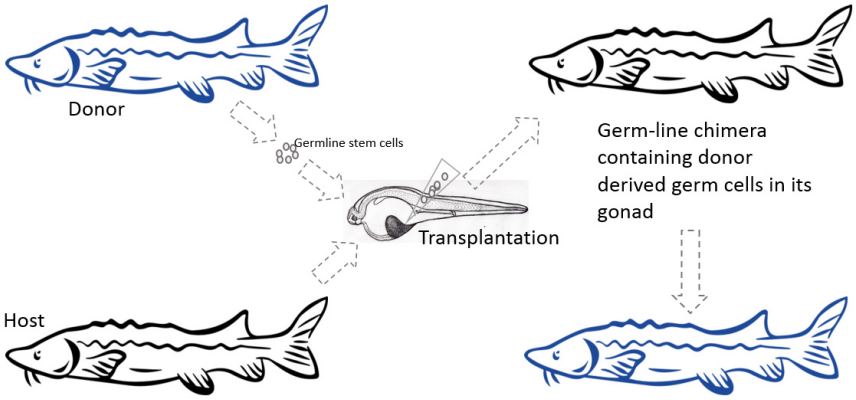

Figure 2. *Schematic representation of GCT in sturgeons.*

1.4. Genome Editing Techniques

Genome editing also referred as genome editing with engineered nucleases is a type of genetic engineering where DNA is inserted, deleted or replaced in genome of an organism by using molecular scissors. Genome editing technology helps to study the reverse genetics of the functions of genes. After success of recombinant DNA technology in 1972, genetic engineering has achieved huge success in biotechnology (Singer, 1979; Thurtle-Schmidt and Lo, 2018). Until now, several molecular methods and genetic mechanisms have been discovered and studied in details in various living organisms. These developments thus allow bio-scientists to create organisms including microorganisms and interestingly also in higher organisms.

Available tools for editing of genome include Zinc Finger Technology (ZFN), TALEN (Transcription activator-like effector nucleases) Technology and CRISPR/Cas9 technology. During last century, scientists discovered that protein domains like 'zinc fingers' with FokI

endonuclease domains target at specific sites, therefore naming it as zinc finger nucleases (ZFNs) that cleave DNA with high precision (Kim et al., 1996). Efforts were continued in genome editing field and it led to development of TALENS and CRISPR/Cas9. When using TALENS to target specific site, its design requires re-engineering of new proteins for each new targets. This technology interested and excited molecular biologists as it is easier and simpler where scientists use "protein-DNA code' that is compatible to modular DNA binding TALE repeat domains. TALENs have been extensively used in different animal as well as in plant species (Joung and Sander, 2013).

Model fish species especially zebrafish has played important role in testing of new protocols of genome editing because of its ease of using. High number of genes have been studied after application of genome editing in various species of fish, mainly those genes that are involved in fish reproduction (Zhu and Ge, 2018). In medaka, the *dmy* have been identified whose functions are found to be equivalent to the SRY in mammals (Matsuda et al., 2002). In tilapia, by using CRISPR/Cas9, *amhy* was knocked-out in XY fish and it led to male to female reversal of sex (Li et al., 2015).

PGCs are the precursors of germ cells (sperms and eggs) in the embryos; and studies have found relation of few genes with the formation and maintenance of PGCs, these genes include the *dnd*, *nanos2* and *nanos3*. *Dnd1* was knocked out by CRISPR/Cas9 in Atlantic salmon (Wargelius et al., 2016); and in the zebrafish, *nanos1* have been reported to be essential for migration of the PGCs, and by using CRISPR/Cas9 in tilapia, mutations of *nanos2* and *nanos3* in the F0 founder tilapia was not reported to be transmitted to F1 generation because it caused germ cell loss (Li et al., 2014). Besides aforementioned genes that are involved in reproduction or development, several other genes have also been studied in fish (Zhu and Ge, 2018).

The genome editing technologies have been used in aquaculture according to the needs. Various traits important from aquaculture point of view such as reproductive performance, growth, feed conversion ratio, resistance to numerous diseases, and also tolerance to stressors from environments i.e., salinity, oxygen and temperature, have been or could be the potential targets of genome editing (Abdelrahman et al., 2017). CRISPR/Cas9 was used to disrupt the *mstnba* (inhibitor of the skeletal muscle growth) in common carp, and the F0 showed increased muscles (Zhong et al., 2016). Concluding the use of genome editing technologies in fisheries; high efficiencies of these technologies can help us to enter into new era, which will witness high adoption of these powerful and cost-effective technologies in various fish species from economical important to the model fish species.

In our present study, we have used CRISPR/Cas9 genome editing technology; it has been described in detail.

1.4.1. CRISPR/Cas9

Targeted genome editing by using engineered endonucleases has attracted scientific community, and further excitement was evidenced by emergence of CRISPR/Cas9 technology. CRISPR/Cas9 system is adaptable immune system used by various bacteria and archaea to protect them from foreign invading nucleic acids like plasmids and viruses (Fineran and Charpentier, 2009; Wiedenheft et al., 2012). CRISPR/Cas9 system can efficiently insert sequences from phages or other foreign DNAs in CRISPR repeats and these are then encoded as array within genome of host.

CRISPR RNAs are responsible to process CRISPR repeat arrays and each harbours variable sequence, which is then transcribed from invading foreign phages/DNAs. RNA called trans-activating CRISPR RNA (transRNA) does hybridization with the tracRNA, and these RNAs

complexes with the Cas9 enzyme; the endonuclease that cleaves target DNA (Deltcheva et al., 2011). tracRNA directs Cas9 enzyme to cleave complementary target DNA sequences; it happens when they are adjacent to PAM (proto-spacer adjacent motifs). It is essential that section of DNA that is to be cleaved must be incorporated into CRISPR locus. Power of CRISPR/Cas9 system has been harnessed and genome editing in various living organisms have been done successfully. Two components are very much important for this technology to be functional. These components include Cas9 endonuclease and guide RNA (gRNA). Pre-requisite of gRNA working is to have 20 nucleotides at 5′ end in order to direct Cas9 to specific DNA target. It is also important to note that target site has to lie immediately at 5′ of PAM sequence. Genome editing in various organisms can be done just by altering 20 nucleotides of gRNA to corresponding sequence. Figure 3 shows how CRISPR/Cas9 functions.

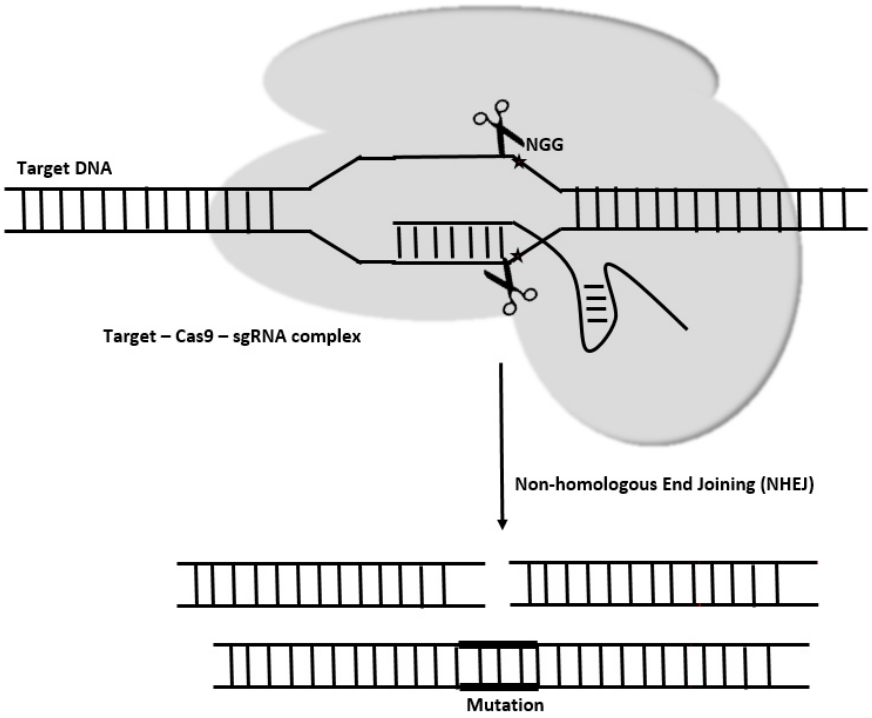

Figure 3. *How CRISPR/Cas9 genome editing system functions.*

Cas9 enzyme induces DSBs (Double Stranded Breaks), which are then used to introduce NHEJ (Non-homologues end joining) mediated insertions/deletions mutations. After DNA is broken, NHEJ is activated, and this pathway repairs damaged DNA in absence of template DNA, therefore introducing indels, which results un-predictable genetic modifications (Barnes, 2001). Advantage presented by this technology over ZFNS and TALENS that it makes DSBs at multiple sites in parallel utilizing Cas9. This technology is also easy to work with, as it is simpler and takes short time from microinjection to analysis of results with special attention to sturgeons (figure 4). Several successful studies have been conducted by using Cas9 and multiple gRNAs to induce large and small changes/indels between double stranded breaks (Cong et al., 2013; Xiao et al., 2013) in order to introduce mutations in different numbers of genes in various organisms. When compared with TALENS and ZFNs, genome editing by using CRISPR/Cas9 largely depends upon DBS at targeted site. However, on contrary, when DNA

template is provided, DBS are repaired by HDR (Homology directed repair); DSB facilitates frequency of homologous recombination (Liang et al., 1996; Urnov et al., 2005).

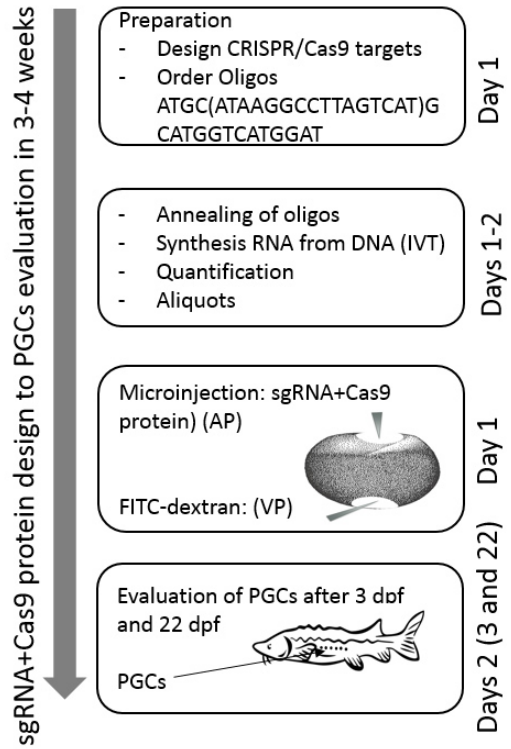

Figure 4. *Streamline CRISPR/Cas9 workflow. (sgRNA+Cas9 protein into Animal Pole-AP; FITC-dextran into Vegetal Pole-VP).*

1.5. Objectives of the thesis

In our present study, we aimed to use cutting-edge genome editing technology *i.e.,* CRISPR/Cas9 to knockout *dnd1* (dnd1 protein responsible for formation and migration of PGCs). We also for the first time used nanoparticles to label germ cells in any species, *i.e.,* sturgeons.

The goals of present study were to:

1. Knockout *dnd1* in sturgeons by using CRISPR/Cas9 genome editing technology.
2. Compare CRISPR/Cas9 with knockdown agent, antisense morpholino oligonucleotide, and elimination of germ cells by UV irradiation.
3. Label Primordial Germ Cells (PGCs) in sturgeons by using Iron Oxide Nanoparticles.

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CHAPTER 2

DEAD-END (DND) PROTEIN IN FISH-A REVIEW

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Dead-end (dnd) protein in fish—a review

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Abstract *Dead end* (*dnd*) is a germ plasm-specific maternal RNA discovered in zebrafish and then in other vertebrates. Dnd protein is essential for migration and motility of primordial germ cells (PGCs), only cells destined to transfer genetic information to offspring. PGCs arise far from somatic cells of developing gonads and they must migrate to their site of function. Migration of PGCs follows complex path by various developing tissues as their disruption impacts on the fertility. Recently, it has been found that dnd is not required for survival of PGCs and dnd-deficient zebrafish PGCs transdifferentiate into the somatic cells. In fish, targeting *dnd* causes removal of PGCs that ultimately affects sex differentiation. Sterility in various fish species can be achieved by knockdown or knockout of *dnd*. In our review, we have discussed *dnd* as a germ cell-specific molecular marker in fish, its interaction with miRNAs, and its use in aquaculture and fish conservation.

Keywords Dnd protein · Aquaculture · PGCs · Sterilization · Surrogate production

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Introduction

The RNA of *dead end* (*dnd*) is expressed in germline of different vertebrate classes, which was originally discovered in zebrafish, thereafter in several other vertebrates such as human, chicken, *Xenopus*, and mouse (Weidinger et al. 2003). Exclusive expression of *dnd* in the vertebrates' germline indicates that it has a conserved role in development of germline. Proteins in dnd family are RNA binding proteins (RBPs) consisting of two RNA recognition motifs (RRMs) in their C-terminus; a double-stranded RNA binding domain (DSRM) is also present at C-terminus end; however, function of which has not been evaluated. Dnd in zebrafish binds to U-rich sequences at miR430 binding sites, thus prevents access of miRNAs and subsequently degradation of dnd target RNAs (Kedde et al. 2007). Dnd interacts with CNOT deadenylase complex in order to mediate mRNA decay (Suzuki et al. 2016). Recently, Gross-Thebing and colleagues discovered that in zebrafish dnd-deficient primordial germ cells (PGCs) transdifferentiate into somatic cells and gain somatic gene expression profile. Further, interestingly, dnd protein was found not to be essential for the survival of PGCs in zebrafish (Gross-Thebing et al. 2017). Dnd is localized in perinuclear germ granules within PGCs in zebrafish (Weidinger et al. 2003); in mice, it is co-localized with nanos2 in P-bodies (Suzuki et al. 2016). Dnd in *Xenopus* is initially localized to germ plasm in cortex and then moves from cortex to perinuclear region with germplasm and enters into nucleus. It is assumed that dnd carries RNA into nucleus to trigger germline

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specification (Taguchi et al. 2014). Essentiality of *dnd* for embryonic viability was determined in mice (Zechel et al. 2013), and its knockdown by antisense oligonucleotide (AS-oligo) led to ventralization in *Xenopus* embryos (Mei et al. 2013).

In vertebrates, PGCs migrate from site of specification towards developing gonads (Starz-Gaiano and Lehmann 2001). During PGCs migration, significant challenges are imposed and protecting of PGCs from adapting somatic cell fate ensures their arrival at target site, differentiate into the gametes and fertility of the organism (Gross-Thebing et al. 2017). Dnd is essential for polarization and motility of PGCs, knocking down of *dnd* by using morpholino caused abnormal migration of PGCs and elimination of germ cells in zebrafish (Weidinger et al. 2003). Conserved *HRAAAMA* motif is found in dnd and Mg^{2+}-dependent ATPase activity of zebrafish dnd is attributed to this motif; the aforementioned activity is required for viability and formation of PGCs (Liu and Collodi 2010). However, mutation in mouse *dnd* did not prevent the formation of PGCs (Youngren et al. 2005). Medaka fish (*Oryzias latipes*) is becoming popular to study developmental biology in vertebrates (Wittbrodt et al. 2002; Hong et al. 1996). PGCs were abolished when dnd was depleted and over expression of *dnd* caused higher number of PGCs; therefore, results provide insights in understandings of formation of PGCs and manipulation in medaka as a lower vertebrate model (Hong et al. 2016). *Oryzias celebensis dnd* (*Ocdnd*) was found to be determinant of specification of PGCs and *Ocdnd* RNA showed germ cell-specific expression in gonads. In the testis, *Ocdnd* RNA was detected in spermatogonia and meiotic cells; its expression decreased during spermatogenesis; however, *Ocdnd* RNA was found to be present throughout the oogenesis (Zhu et al. 2018). When MO was used against *dnd* in loach (*Misgurnus anguillicaudatus*, Cypriniformes: Cobitidae) and goldfish (*Carassius auratus*), sterilized embryos developed into both sexes (Fujimoto et al. 2010; Goto et al. 2012), contrary to medaka and zebrafish where all male progeny were produced in treated embryos (Slanchev et al. 2005; Kurokawa et al. 2007), therefore suggesting the importance of PGCs in sex determination. In sturgeon, sterilization was achieved by using MO approach; however, sex differentiation after PGCs removal is not clear yet (Linhartová et al. 2015). In salmonids, *dnd* was targeted by MO to study suitability of germ cell-deficient recipients for xenogeneic transplantation. The spermatogonia from rainbow trout (*Oncorhynchus mykiss*) were transplanted into germ cell-deficient masu salmon (*Oncorhynchus masou*) that when matured produced trout gametes (Yoshizaki et al. 2016). Functions of dnd have been studied in other fish species such as Atlantic salmon (Wargelius et al. 2016), turbot (Lin et al. 2013), olive flounder (Wang et al. 2015), bluefin tuna (Yazawa et al. 2013), rare minnow (Duan et al. 2015), etc. In our present review, we have focused on the role of dnd protein in migration and specification of germ cells, knockdown/out of *dnd* in fish conservation, and population control and interaction of dnd protein with miRNAs.

Dnd as conserved molecular marker of germ cells in fish

In various vertebrates, *dnd* has been identified and characterized as germ cell marker. In *Xenopus*, expression of *Xdnd* is restricted in the ovary (Horvary et al. 2006) and in mouse, *dndα* is exclusively expressed in germ cells of the testis (Bhattacharya et al. 2007). *Ter* mutation in *dnd* of mouse led to loss of germ cells and testicular germ cell cancer (Youngren et al. 2005); therefore, indicating a conserved role *dnd* in the development of germline from fish to mammals. Differential expression of *dnd* has also been revealed in the testes and ovary of turbot (Lin et al. 2013) and Pacific bluefin tuna (Yazawa et al. 2013). In *Oryzias celebensis*, *dnd* was evidenced to be determinant for specification of PGCs. Specific expression of *Ocdnd* RNA was shown in gonads; strong expression of *Ocdnd* was found to be in spermatogonia; however, its expression decreased during spermatogenesis. Nevertheless, *Ocdnd* RNA was present throughout the oogenesis. Therefore, suggesting that *dnd* is a conserved PGC specifier in genus *Oryzias* (Zhu et al. 2018). Expression patterns of *dnd* in gibel carp (*Carassius gibelio*) are still unclear. *Dnd* in gibel carp contains an RRM that is highly conserved in the genomic organization. RT-PCR and Western blot confirmed that this gene is intensively expressed in the testis and ovary of gibel carp, thus making *dnd* a conserved germ cell marker during gametogenesis (Li et al. 2016). Cloning and expression profile of *dnd* was reported and RT-PCR results revealed expression of *dnd* in gonads of both the sexes that was restricted to germ cells (Duan et al. 2015).

Turbot (*Scophthalmus maximus*) is a species of flatfish, native to marine or brackish waters of the North

Atlantic, Baltic Sea, and Mediterranean Sea. This species is an important aquaculture fish that is acquired through aquaculture and trawling (Lei 2003). *Dnd* homolog in turbot (*Smdnd*) was identified and expression pattern was investigated during embryogenesis and gonadal development. Phylogenetic analysis found that *Smdnd* was closely related to its teleost counterparts. Results of RT-PCR and in situ hybridization (ISH) determined *Smdnd* transcripts exclusively in germ cells of male and female including presumptive PGCs. Therefore, it suggested that *Smdnd* can be potentially used as a germ cell marker in turbot (Lin et al. 2013). In the channel catfish (*Ictalurus punctatus*), *nanos* and *dnd* have been used as the PGC marker, and in order to achieve transgenic sterilization, the *dnd* was knocked down (Su et al. 2015). Olive flounder (*Paralichthys olivaceus*) is an economically important marine fish; the *dnd* of this fish, i.e., *Podnd* was identified as a germ cell-specific marker. Expression of *Podnd* transcript was detected in the ovary as well as testis. qRT-PCR showed that expression of *Podnd* transcript was higher in the ovary when compared with the testis in olive flounder. Results of *tissue section in situ hybridization* (SISH) showed the expression of *Podnd* only in germ cells at different developmental stages and expression was not detected in surrounding somatic cells. Strong signals were detected in oogonia and oocytes in ovarian section; and in the testis, clear signals were detected in spermatogonia, followed by spermatocytes and no signals were seen in the spermatid and spermatozoon. In olive flounder, the localization and migration of *Podnd* were found to be similar like in zebrafish (Wang et al. 2015). *Asdnd* (*Acipenser sinensis dead-end* homolog) was identified and characterized. Transcripts of *Asdnd* showed to originate from maternal parent and expressed in the gonads of immature Chinese sturgeon of both the sexes; therefore, *dnd* could be used a germ cell marker in these ancient fish species (Yang et al. 2015).

Use of dnd protein in aquaculture and fish conservation

Introgression of the farmed fish escaping into wild stocks is a serious threat to the genetic integrity of wild populations. The aforementioned problem can be addressed by producing sterile fish for aquaculture purposes (Wargelius et al. 2016). Most used methods to achieve sterilization include triploidization, hybridization, and generation of new lines by using advanced biotechnological methods (Golpour et al. 2016). Sterile stocks can prevent gene flow of domesticated fish into wild fish populations (Glover et al. 2012). Genes such as *nanos*, *vasa*, and *dnd* are found to be associated with migration of PGCs, colonization in genital ridge, and gamete formation in fish (Su et al. 2015). The dnd binds to 3′UTR (untranslated region) of germ cell-specific RNA; therefore, in this way, it protects them against microRNA-mediated degradation. Protected RNAs thereafter contribute to maintain the fate of PGCs (Kedde et al. 2007). Inactivation and knockout of *dnd* have already been done to produce sterile fish stocks; detailed description of some fish species is given below.

Sturgeon (Acipenseridae)

Sturgeons are commonly known as living fossils that have existed for a minimum of 200 million years and are famous for their caviar (Bemis and Kynard 1997). Their value as a source of caviar has led sturgeons to be target of intensive legal and illegal fisheries, thus resulting in collapse of certain sturgeon species and stocks (Pikitch et al. 2005; Billard and Lecointre 2001). Sterlet (*Acipenser ruthenus*) has the shortest reproductive cycle among sturgeons. Therefore, this species can be used as host for surrogate production. In order to prepare sterile sterlet host, *dnd* was knocked down by using antisense morpholino oligonucleotide (MO). Tissue-specific fluorescein isothiocyanate (FITC)-biotin-dextran was used to label PGCs and under fluorescent stereomicroscope, no PGCs were observed in body cavities of morphants at 21 days post fertilization (Linhartová et al. 2015). Thus, suggesting the role of *dnd* knockdown in conservation of critically endangered fish species by using surrogate production technology.

Transgenic sterilization in channel catfish (*Ictalurus punctatus*) and common carp (*Cyprinus carpio*)

Genetically engineered catfish can potentially be better disease resistant and have higher growth rate and, importantly, higher nutritional values when compared with non-transgenic catfish (Dunham 2009). However, it should be ensured that genetically engineered fish does not establish permanent population in ecosystem; therefore, genetically engineered repressible transgenic sterilization should be used (Su 2012; Su et al. 2015; Thresher et al. 2009). *Dnd* and *nanos* genes were targeted as PGCs

marker, where PGCs migration and gametogenesis was prevented, therefore leading to sterility in channel catfish. This process was then repressed so that fertile brood stock can be produced that in turn generates sterile offspring (Li et al. 2018). Common carp is a freshwater fish that is grown worldwide. Annual global production of common carp is 3.4 million tons, accounting 14% of fresh water aquaculture production in the world (FAO 2012). However, in several countries, common carp is considered as an invasive species. Negative effects posed by the populations of common carp have been found in Australia (IACRC 2013; Industry and Investment NSW 2010) as well as in other countries (Lowe et al. 2000). Common carp along with other non-indigenous fish species in the USA alone caused the loss of one billion dollars (Pimentel et al. 2005). Therefore, transgenic technologies should be used to control enhancing populations. Knockdown constructs were designed to knockdown expression of *dnd* and *nanos* in order to produce sterile fish. Knockdown constructs were electroporated into the embryos of common carp; the constructs appeared to be promising for transgenic sterilization of common carp (Su et al. 2014).

Pacific bluefin tuna (*Thunnus orientalis*)

With increase in human population and great demand in tuna consumption, farming of Pacific bluefin tuna (*Thunnus orientalis*) and Atlantic bluefin tuna (*Thunnus thynnus*) has also increased significantly (Ottolenghi et al. 2004). Maintenance of Pacific bluefin tuna brood stock at captivity is a major bottleneck, as it takes minimal 3 years for fish to reach sexual maturity (Seoka et al. 2007; Masuma et al. 2011). Use of *dnd* gene was examined as a convincing candidate molecular marker in order to identify PGCs, type A spermatogonia (ASG) in Pacific bluefin tuna. Spermatogonial transplantation technique was established to produce donor-derived gametes in surrogate fish, with prime goal of producing bluefin tuna. Pacific bluefin tuna *dnd* (*BFTdnd*) was used as convincing molecular marker to identify germ cells with high transplantability and transplantation of the dissociated testicular cells from juvenile, immature, and mature Pacific bluefin tuna was performed by using mackerel as surrogate recipient. *BFdnd* was identified as potential tool to recognize transplantable ASG for spermatogonial transplantation (Yazawa et al. 2013).

Zebrafish (*Danio rerio*)

Zebrafish is a freshwater fish, native to Himalayan region and widely used vertebrate model organism in biological research. *Dnd* in zebrafish was knocked out in order to prepare sterile host and these sterile host were used as recipient of germ cell by intraperitoneal transplantation of the testicular cells which were prepared from the *vasa-gfp* zebrafish. Results showed that transplanted germ cells into sterile fish become functional sperm; the progeny test further confirmed that the sterile recipient derived sperms entirely from donor cells (Li et al. 2017). This therefore supports that *dnd*-knockout recipients can be used for spermatogonial transplantation of endangered as well as farmed fish species.

Atlantic salmon (*Salmo salar* L.)

Atlantic salmon (*Salmo salar* L.) are cultured in open sea cages and their escape is a serious threat due to the genetic integrity with wild populations. Sterilization of salmon in aquaculture can solve this problem. Wargelius et al. (2016) for the first time used CRISPR/Cas9 to target *dnd* in any fish species to generate germ cell free fish (Wargelius et al. 2016). In Atlantic salmon, germ cells are not required for gonadal sex differentiation due to fact that somatic sex index is maintained in female and male germ cell free fish. Using sterile fish in aquaculture can potentially prevent the gene flow into wild populations from the domesticated ones (Glover et al. 2012).

Dnd and microRNAs with special focus in fish

The microRNAs (miRNAs) inhibit expression of genes and control processes in normal development and cancer (Kedde et al. 2007). The miRNAs constitute a family of approximately 22 nucleotides that are extensively expressed in metazoans (Pillai et al. 2007; Lee et al. 1993). Seed sequence of 6–8 nucleotides is used by miRNAs to bind with 3′UTR of mRNAs to inhibit their expression (Fig. 1). Role of dnd protein regarding protection of mRNA from miRNAs mediated repression has been unraveled where it was confirmed that *dnd* expression counteracts functions of various miRNAs in PGCs of zebrafish. Dnd protein binds to mRNAs and prohibits miRNAs from associating with their specific targets (Kedde et al. 2007). When dnd protein was introduced in human cells, it abolished interaction between miR221

and 3′UTR of p27 mRNA (cyclin-dependent kinase inhibitor 1B) that caused downregulation of p27 mRNA expression by miR221. Cheng et al. (2017) knocked-down *dnd* in MCF-7 cells and confirmed that dnd antagonized miR-221 inhibitory effects on expression of Bim, a hall marker for apoptosis (Cheng et al. 2017). *Nanos1* is expressed in germline development that is resistant to miRNA repression in germline (Mishima et al. 2006) due to binding of dnd at its 3′UTR (Kedde et al. 2007). Geminin (GMNN) encoded by *GMNN* that inhibits DNA replication. SNAAPs (specific nucleic acids associated with proteins) were used to identify potential mRNA targets of zebrafish dnd (*Zdnd*) proteins. Zdnd protein has been found to be associated with 3′UTR of GMNN mRNA and regulates its expression. Zdnd binds with eight mRNAs by SNAAP assay that was confirmed by RT-PCR (Chen et al. 2010). In olive flounder, *nanos3* 3′UTR was found to be responsible for the localization of mRNA in PGCs (Li et al. 2015). *Nanos3* 3′UTR contains non-canonical miR-430 binding sites and U-rich regions. Functional regions of *nanos3* 3′UTR were further investigated in olive flounder by using truncated and mutated *nanos3* 3′UTR that were fused to chimeric RNAs. These were microinjected into the eggs of zebrafish. Results demonstrated that 68-bp functional element in *nanos3* 3′ UTR of olive flounder played an important role in protecting and degrading of RNA. Overall, it was found that two GCACs (miR-430 binding site) were sites for binding of miR-430, and U-rich region was found to be the binding site of dnd protein that antagonizes miR-430-mediated mRNA silencing (Li et al. 2015). The GCAC and U-rich region (dnd binding site) were found within 68-bp functional element; however, mechanism that controls this expression needs further studies. Conserved sequence GCACUU in 3′UTR is present in several teleost fish species such as tilapia, stickleback, and zebrafish; however, it is absent in cod, salmon, and olive flounder (Škugor et al. 2014). In the zebrafish, regulation of germline and somatic cell distinction is done through combination of miRNAs and germ cell-specific RNA binding proteins. miR-430-mediated repression in zebrafish was found not to be counteracted by over expression of dnd protein in somatic cells, and repression of translation by this protein was independent to nanos3, another germline-specific translation repressor, in zebrafish. Therefore, it was confirmed that dnd functions as repressor of translation of specific mRNAs in order to control the development of PGCs in this model fish (Kobayashi et al. 2017). The stability of mRNA was not affected when dnd, nanos2, and nanos3 repressed translation of target mRNAs; further studies needed to be conducted mechanisms underlying how dnd represses translation without affecting the stability of mRNAs.

Dnd and PGCs migration

PGCs are specified early in development by induction or by inheritance of maternally provided determinants (Strome and Updike 2015). Germ cells along with other somatic cells migrate from place of their origin towards site where they form gonads. It is also important that the fate of germ cells should be preserved so that they do not undergo partial transdifferentiation leading to formation of germ cell tumor (Gobel et al. 2000; Schneider et al. 2001). Molecular mechanisms such as regulating transcription silencing machinery and chromatic modification and protein stability are required to maintain fate of germ cells (Lai et al. 2012; Robert et al. 2015; Strome and Updike 2015). PGC migration is guided by SDF1A molecules and their receptors, i.e., GPCR chemokine receptor 4b (CXCR4B) which are expressed in PGCs (Doitsidou et al. 2002). CXCR7B another important receptor for SDF1A is also required for migration of PGCs that functions in somatic cells and is distributed uniformly throughout the zebrafish embryo. *Dnd* plays a role in PGCs polarization and migration, in a part, when MO was used to block its activity and it functions by regulating zebrafish E-cadherin during PGCs individualization (Fig. 2). Formation of PGCs in zebrafish

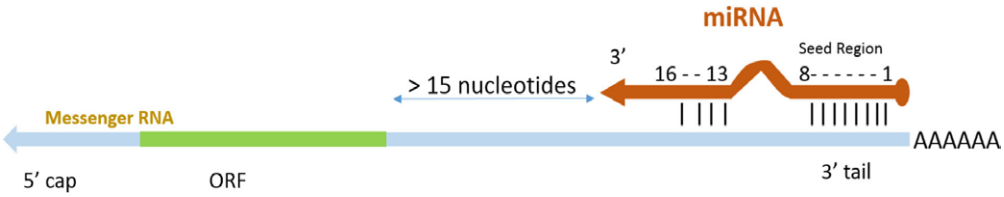

Fig. 1 miRNA post-transcriptional gene regulation. miRNA MicroRNA, ORF open reading frame

Fig. 2 E-cadherin molecule and SDF1A expression

occurs at early embryonic stages (3 hpf); however, these PGCs are not formed at a single embryonic position (Raz 2003).

Recently, Gross-Thebing et al. (2017) discovered that function of dnd protein is not required for survival of PGCs in zebrafish. Dnd-depleted PGCs survived; however, they lacked expression of *vasa* and *nanos3* (Gross-Thebing et al. 2017). Complete removal of PGCs in medaka and zebrafish by using antisense MO against dnd caused the production of all male progeny (Slanchev et al. 2005; Kurokawa et al. 2007); however, fish such as loach and gold fish when were treated with same approach, treated embryos developed into both the sexes (Fujimoto et al. 2010; Goto et al. 2012), in sturgeon targeting *dnd* produced germ cell free host for surrogate production (Linhartová et al. 2015). RT-PCR and in situ hybridization showed that transcripts of *Smdnd* (*turbot*, *Scophthalmus maximus*) could be detected in germ cell including presumptive PGCs (morphologically undistinguishable stage of PGC in very early development) and adult male and female germ cells. Transient inhibition of *dnd* mRNA translation in Atlantic cod did not block PGCs development until hatching, therefore suggesting that ablation of *dnd* might have indirect significant effects including suppression of the reproductive functions (Škugor et al. 2014). These morphants also displayed lowered expression of genes, which encode proteins involving in cholesterol, lipid, retinoid, and steroid metabolism.

Conclusions

Dnd has been studied in various classes of vertebrates, and its expression in germline shows a conserved role in germline development as it is required for successful PGCs migration during the embryonic development. Recently, it is discovered that dnd protein is not required for survival of PGCs. Targeting *dnd* by MO or CRISPR/Cas9 can serve as tool for sterility induction in fish with applications in fish farming to ensure no genetic contamination of wild stocks after accidental escape of farmed fishes and to produce germ cell free recipients for surrogate production fish. *Dnd* has also been identified as potential germ cell-specific molecular marker in various fish species; however, expression patterns and function implication of *dnd* should be further investigated in diverse fish species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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CHAPTER 3

DND1 KNOCKOUT IN STURGEONS BY CRISPR/CAS9 GENERATES GERM CELL FREE HOST FOR SURROGATE PRODUCTION

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My share on this work was about 80%.

animals

MDPI

Article

Dnd1 Knockout in Sturgeons By CRISPR/Cas9 Generates Germ Cell Free Host for Surrogate Production

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Simple Summary: Sturgeons, also called archaic giants, are critically endangered fish species due to overfishing for caviar and interference in their natural habitats. Some sturgeon species have life spans of over 100 years and sexual maturity is attained between 20 to 25 years. Sterlet (*Acipenser ruthenus*) has fastest reproductive cycle; thus, this species can be used for surrogate production in sturgeons. Primordial germ cells are the origin of all germ cells in developing embryos. *Dnd1* is essential for formation and migration of primordial germ cells and its inactivation results in sterility in fish. In our study, we have used a cutting-edge genome editing technology known as CRISPR/Cas9 to knockout *dnd1* and to prepare a sterile sterlet host. CRISPR/Cas9 knocked-out embryos lacked primordial germ cells and can be used as a sterile host for surrogate production in sturgeons.

Abstract: Sturgeons also known as living fossils are facing threats to their survival due to overfishing and interference in natural habitats. Sterlet (*Acipenser ruthenus*) due to its rapid reproductive cycle and small body size can be used as a sterile host for surrogate production for late maturing and large sturgeon species. Dead end protein (dnd1) is essential for migration of Primordial Germ Cells (PGCs), the origin of all germ cells in developing embryos. Knockout or knockdown of *dnd1* can be done in order to mismigrate PGCs. Previously we have used MO and UV for the aforementioned purpose, and in our present study we have used CRISPR/Cas9 technology to knockout *dnd1*. No or a smaller number of PGCs were detected in crispants, and we also observed malformations in some CRISPR/Cas9 injected embryos. Furthermore, we compared three established methods to achieve sterility in sterlet, and we found higher embryo survival and hatching rates in CRISPR/Cas9, UV and MO, respectively.

Keywords: Acipenser; caviar; conservation; genome editing; morpholino oligonucleotide; PGCs

1. Introduction

Sturgeons are an ancient fish species that have existed for at least 200 million years and are famous for their caviar [1]. Their value as a source of caviar has led sturgeons to be target of intensive legal and illegal fisheries, therefore resulting in the collapse of several sturgeon species and stocks [2,3]. Natural populations of these archaic giants have been declining due to certain factors such as water pollution and interference in their natural habitats. Other prominent impacts on the sturgeon population are hybridization, introduced species, water divergence, reduced food supply and saltwater intrusion [4,5]. Damming of rivers has also been proved to be detrimental to sturgeon populations because it reduces

and/or eliminates spawning and egg/larvae habitats [6]. According to the International Union for the Conservation of Nature (IUCN 2010), 85% of sturgeon species are at the verge of extinction. Sturgeons are generally a long living species and exhibit late onset of maturity, and a slow growth rate with infrequent reproduction [2]. Some sturgeon species have a life span over 100 years and sexual maturity is attained between 20 to 25 years or even later in females [3]. Sterlet (*Acipenser ruthenus*) has the fastest reproductive cycle; males mature over 3 to 7 years and females over 5 to 9 years [7], therefore making it convenient to produce donor-derived gametes of large and long maturing sturgeons. Surrogate production by the generation of germ line chimera through germ cell transplantation in various closely related species has been established in several fish species [8–16]. A germ cell free host is a pre-requisite for surrogate production, and host gonads lacking germ cells could improve transplantation efficiency, as the niche is not occupied by the endogenous germ cells and germ-line chimera only produce donor derived gametes. Triploidization is practically used in species whose triploid individuals are sterile [17–19]. Nevertheless, sturgeons are evolutionary polyploids and sterility of triploids is not yet well proved [20], therefore alternatively, other methods should be used to achieve sterility in sterlet.

Inactivation of mRNAs such as *dead end* (*dnd1*) that is essential for formation and migration of PGCs should be done to achieve sterility experimentally, as was already shown in zebrafish, medaka, loach, goldfish and sturgeons [21–24]. Dnd1 in vertebrates binds to 3′-UTRs (untranslated regions) of germ cells specific RNAs, thus it protects them against miRNA-mediated degradation so that these RNAs can contribute to maintain the fate of PGCs [25,26]. Loss of *dnd1* in mouse also results in germ cell free sterile gonads [27]; however, in mammals, zygotic transcription replaces maternal RNA at the 1 cell stage, while in fish it occurs later during mid-blastula transition [28]. This feature affects the ability of some maternal RNAs to maintain germ cells like *piwil1*, *piwil2* and *vasa* that start germ cells formation in homozygous mutant fish for aforementioned genes; however, the loss of germ cells occurs at later stages [29–31]. Certain studies in fish have assayed function of *dnd1* when both maternal and zygotic *dnd1* mRNA is lost during early development [21,22,32]. Zygotic gene expression is not turned on until the onset of gastrulation in fish; and zygotic *dnd1* RNA in salmon was found to be required for germ cells migration to gonads [33]. In tilapia, CRISPR/Cas9 KO of *nanos3* gene resulted in germ cell free gonads, therefore further strengthening the notion that maternally contributed factors cannot rescue germ cell development and survival [34]. Loss of germ cells in fish affects the somatic sex of gonad differently since PGCs loss leads to an all-male phenotype in medaka, zebrafish and tilapia, while both males and females develop in germ cell-free loach and goldfish [21–23,34,35]. In mice, *dnd1* knock out leads to all-male offspring. It is still unclear whether or not the absence of germ cells is relevant for the differentiation of sex in sturgeons.

The latest techniques of genome editing can insert, delete and/or alter DNA sequences of cells and organisms, thus enabling scientists to dissect functions of specific genes [36]. In CRISPR/Cas9, RNA guided endonucleases (Cas9) from microbial adaptive immune system CRISPR can be easily targeted to virtually any genomic location of choice by short RNA guide. This genome editing system presents advantages over other genome editing technologies (ZFNs and TALENs) such as high efficiency, convenience and cost-effectiveness [37,38]. The CRISPR/Cas9 system therefore, is suitable to be applied in new fish models to generate sterile host by targeting the germ-cell specific candidate gene approach [26]. Previously we have used morpholino oligonucleotide (MO) against *dnd1* in sterlet to achieve sterility [24] and depleted PGCs from sturgeons' embryos by UV irradiation [39]; however, it needs to inject many embryos with MO, and dechorionation is required for UV irradiation, which is laborious. In our present study, we have knocked out *dnd1* in sterlet by applying CRISPR/Cas9 technology.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments were conducted in accordance with the Animal Research Committee of Faculty of Fisheries and Protection of Waters in Vodňany, University of South Bohemia in České Budějovice, Czech Republic. All experimental fish were maintained according to principles based on the European Union (EU) Harmonized Animal Welfare Act of the Czech Republic, and Principles of Laboratory Animal Care and National Laws 246/1992 "Animal Welfare" on the protection of animals were followed. Experiments were approved by the Ministry of Agriculture of the Czech Republic (reference number: MSMT-12550/2016-3).

2.2. Fish Source, Preparation of Embryos and Collection of Samples

During the spawning season (March to June 2017), females and males of adult sterlet (*Acipenser ruthenus*) of five to nine years of age were transferred from outdoor ponds into recirculating aquaculture system installed indoors. Fish were kept in tanks of 4000-L and water temperature was increased to 15 °C. In order to induce spermiation, male sterlet were injected with single intra-muscular injection of carp pituitary extract at 4 mg/kg body weight (BW) in 0.9% NaCl. Sperm collection was done 48 h after hormonal injection and were kept at ice at 4 °C until fertilization. Light microscopy was used to assess motility of spermatozoa that was found to be more than 80% and were used for the fertilization. Carp pituitary extract was used to stimulate the ovulation by intra-muscular injection in two doses, i.e., first dose at 0.5 mg/kg BW and second at 4.5 mg/kg BW, 12 h after the first injection. Collection of ovulated eggs was done from three different females from 18 to 20 h after the second injection, and eggs were inseminated with sperm from two different males at 15 °C in dechlorinated water. To remove stickiness, eggs were three times rinsed in 0.04% tannic acid. One hour later after the fertilization, chorion membrane (outer layer of eggs) was removed by using forceps. Chorion removed eggs were then transferred into 100 mL dechlorinated tap water with 0.01% penicillin and streptomycin in the glass petri dishes. Embryos were incubated at 15 °C in incubator. Temperature regulation was done at 15 ± 1 °C throughout the experimental period and water was changed on daily basis. Embryos were used for injection of sgRNAs and Cas9 complex to knockout *dnd1*, isothiocyanate (FITC)-biotin-dextran (molecular weight = 500,000) in order to label PGCs [40], antisense MO for the depletion of PGCs [24], UV irradiation for PGCs removal [39] and polymerase chain reaction. Embryos were kept at −80 °C for the extraction of DNA for downstream applications.

2.3. Cas9 Protein and sgRNAs

Five single guide RNAs (sgRNAs) were designed to target *Acipenser ruthenus dnd1* (*Ardnd1*) gene. Target sites were, sgSRNA1: GGGGGGAATGCAGTCCAACC; sgRNA2: GGGGGAATGCAG TCCAACC; sgRNA3: TTCAATCATTTTCTTTCTTA; sgRNA4: TGGTTTAAAACCGTAAAGAT and sgRNA5: ATTTTCTGAGTCCATGTTTC. Oligos for sgRNAs were ordered from Macrogen Company (Macrogen Inc., Amsterdam, the Netherlands) and were annealed according to references [41,42] (Figure S1). In vitro transcription (IVT) using HiScribeTM T7 High Yield RNA synthesis kit (NEB) was used to generate sgRNAs, according to manufacturers' instructions. Synthesized sgRNAs were treated with DNAse to remove any remaining DNA traces and mySPEC spectrophotometer (VWR® mySPEC spectrophotometer) was used for sgRNAs quantification. All sgRNAs were diluted and aliquoted. Cas9 protein was purchased from PNA Bio and was re-suspended as per manufacturers' instructions, aliquoted and stored at −80 °C.

2.4. Microinjection of sgRNA and Cas9 Complex

Approximately 50 fertilized eggs of sterlet were aligned in each petri dish; in total 600 embryos were injected. Embryos at the 1 cell stage were injected with prepared complex of mixture all five sgRNAs and Cas9 protein (gRNAs+Cas9 ribonucleoprotein complex) in animal pole using a glass

capillary needle. 1% FITC-biotin-dextran (molecular weight = 500,000) was co-injected in vegetal pole to label PGCs, and in the control group only 1% FITC-biotic-dextran was injected according to Saito et al. [43]. Glass micropipette was drawn from a glass needle (Drummond, Tokyo, Japan) using a needle puller (PC-10; Narishige, Tokyo, Japan). Microinjection was done under fluorescent stereomicroscope Leica M165 FC (Leica, Wezlar, Germany) with a pressure of 100 hPa for ~1 second. After microinjection of sgRNAs+Cas9 ribonucleoprotein complex, the survival rate and number of FITC labeled PGCs were examined at four days post fertilization (dpf). At 22 dpf, larvae were taken from each group, euthanized by using tricaine solution overdosing, body cavity of larvae was opened, dissection of the gut was performed and the position of PGCs were observed and their number was counted precisely. Embryos were kept at 15 °C, hatched larvae were fed with Tubifex. Embryo development was recorded with steromicroscope Leica M165 FC with camera (Leica DFC425C). Embryos and larvae from each group were kept in −80 °C for downstream applications.

2.5. Preparation of Genomic DNA

Control sterlet embryos and sgRNA+Cas9 ribonucleoprotein injected embryos were individually collected after 4 dpf and 22 dpf of injection. Total genomic DNA was isolated using a PureLink Genomic DNA Mini Kit (Invitrogen). Primers (F: GAGAGGGCAAGTTGTCTGGA; and R: AAAACCTCACAGCCAGAGGAA) were used to amplify the region of *Ardnd1* gene spanning the target sites. Mutation detection assay was performed according to [44,45] and Multina-500 (Shimadzu) according to reference [46] was used to detect the mutations.

2.6. Capillary Electrophoresis and Mutation Detection Assay

In order to perform the mutation detection assay (HMA; heteroduplex mobility assay), PCR on the genomic DNA was run over 35 cycles using KOD FX Neo (TOYOBO) and PCR products were analyzed using a microchip electrophoresis system (DNA-500 reagent kit and MCE-202 MultiNA; Shimadzu) according to Shigeta et al., 2016 [47].

2.7. Statistical Analysis

Statistical significance of treatments on number of PGCs was analyzed by Wilcoxon test and Kruskal-Wallis test. If there was a significant difference, thereafter we used Dunn's Post-Hoc test to find out which groups were different ($p < 0.05$). Statistical tests were performed using R programming language software (Version 3.5.1, 2018).

3. Results

3.1. sgRNA/Cas9 Ribonucleoprotein

In our present study, no significant difference was observed in survival and hatching rates of embryos injected with different concentrations of gRNAs and the Cas9 protein (Figure 1A"). PGCs in FITC labeled control group and gRNAs+Cas9 ribonucleoprotein complex injected group with different concentrations of sgRNAs and Cas9 protein were counted at 4 dpf and 22 dpf. At 4 dpf, almost no PGCs and/or a smaller number of PGCs were observed compared to the control group (Table 1, Figure 1A'). After 22 dpf, larvae from the gRNAs+Cas9 ribonucleoprotein complex injected group and control groups were euthanized and dissected and the number of PGCs was analyzed in body cavity. PGCs were found to be colonized where the genital ridge presumed to be localized (Figure 1B). gRNAs+Cas9 ribonucleoprotein complex injected group contained significantly lower number of PGCs than control group, and moreover, PGCs were not detected in dissected body cavities of crispants that were lacking PGCs at 4 dpf (Table 2, Figure 1B'). Intriguingly, eggs from one batch were found to be malformed such as having a cloudy structure at the region where the PGCs were supposed to have originated, when injected with sgRNA+Cas9 ribonucleoprotein (Figure 1C). These embryos did not hatch and ultimately died in chorion membrane.

Table 1. Different concentrations of sgRNAs and Cas9 protein.

Treatment	Stage	No. of Embryos/Larvae	Min. No. of PGCs	Max. No. of PGCs	Mean	SD	*p* ***
Control	Neurula	29	12	86	44.14	21.04	0.006679
	22 dpf	14	12	41	26.57	10.30	
150/200 **	Neurula	30	0	20	7.23	5.85	0.00616
	22 dpf	20	0	12	5.90	4.78	
200/200 **	Neurula	21	0	19	6.90	6.94	0.9672
	22 dpf	19	0	12	3.68	4.06	
250/150 **	Neurula	21	0	21	7.23	6.70	0.6031
	22 dpf	18	0	15	5.22	4.85	

** sgRNA/Cas9 Concentrations in ng/µl; *** Wilcoxon test

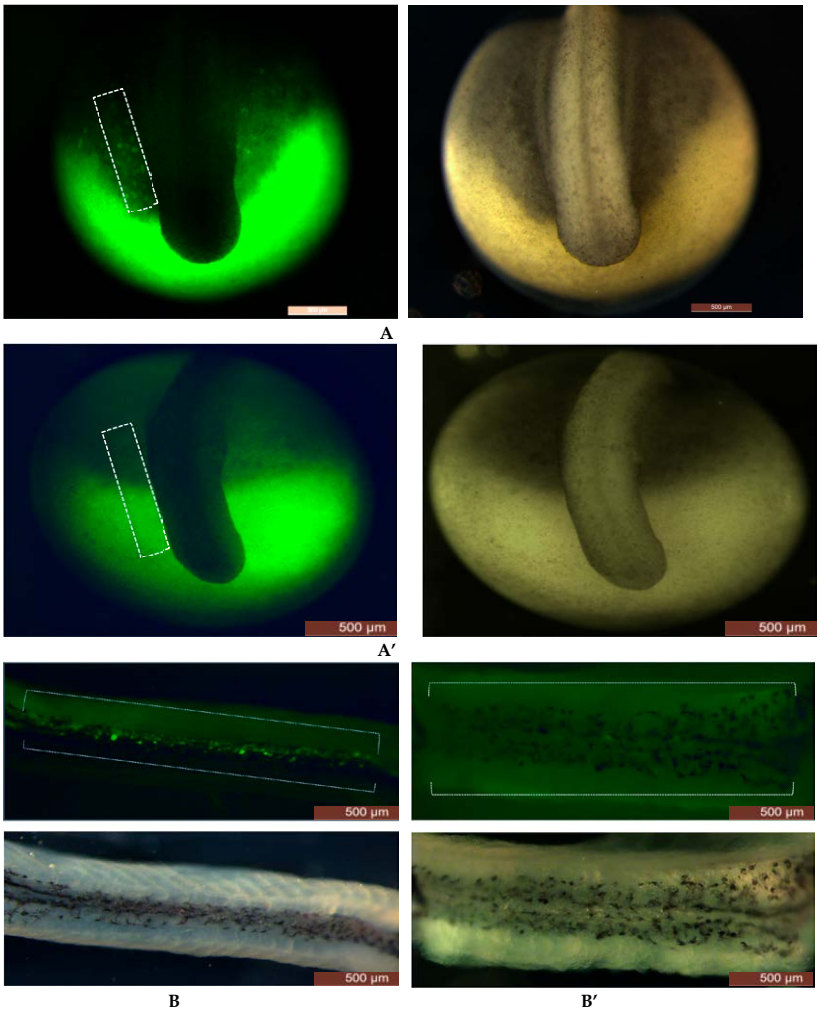


Figure 1. *Cont.*

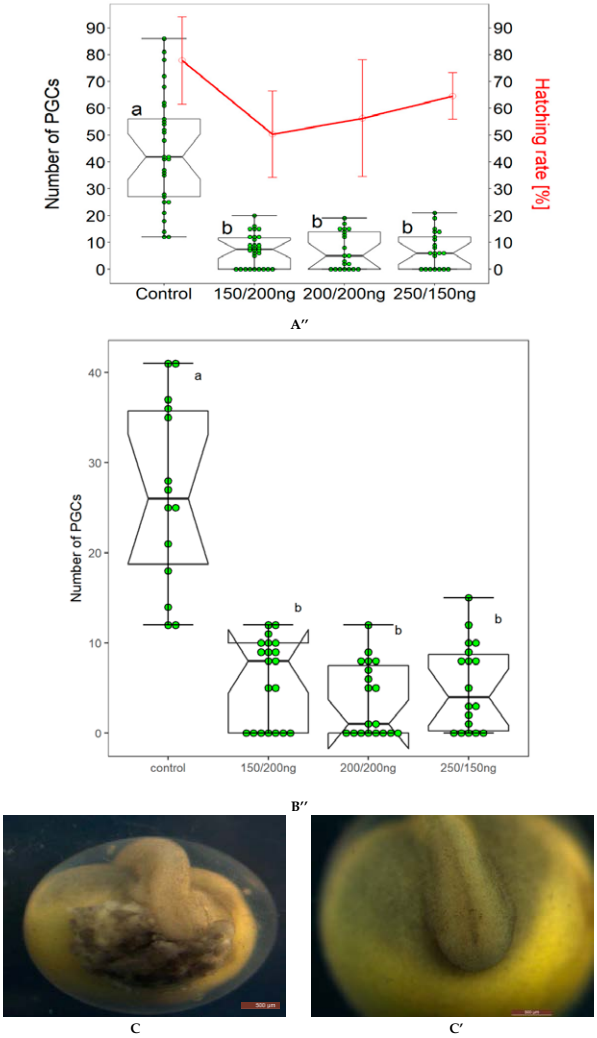


Figure 1. Number of FITC-labeled PGCs reduced from neurula stage to tail bud stage after embryos were injected with RNPs (sgRNAs+Cas9 protein). (**A**) Control group and RNPs injected group with FITC-dextran to label the PGCs at tail bud stage [40], respectively. Embryos injected with RNPs (**A′**) are lacking the FITC-labeled PGCs around surround the region where tail develops; however, in control group (**A**) FITC-labeled PGCs can be easily seen. (**A″**): Different concentrations of sgRNAs and Cas9 protein were used to find optimal concentrations; however, no significant difference of sgRNAs/Cas9 concentrations was found on FITC-labeled PGCs (box graphs). Hatching rates were also found to be insignificant (line graph). (**B**,**B′**): In fluorescent image, many FITC-labeled PGCs can be seen in gonadal region (under a white broken line) in the control group (**B**); however on the contrary no FITC-labeled PGCs can be seen at the same region when injected with RNPs complex (B′; under white broken line). Number of FITC-labeled PGCs were observed by opening the body cavity of larvae, FITC-labeled PGCs were found to be in lower number when different concentrations of sgRNAs/Cas9 were used; while in the control group, FITC-labeled PGCs were found in a higher number (**B″**). (**C**) Malformed embryo at 4 dpf after the injection of RNPs (in one batch of eggs from one female) and the non-injected control group (**C′**). The boxes with different letter show the significant difference ($p < 0.05$).

3.2. Comparison of CRISPR/Cas9, Dnd1-MO and UV Irradiation

We have already successfully used the *Dnd1-MO* technology and UV irradiation to achieve sterility in sterlet. In our present study, we compared two aforementioned methods with CRISPR/Cas9. *Dnd1-MO* technology was applied according to Linhartova et al., [24]. *Dnd1-MO* caused complete removal of PGCs from the injected embryos (Table 2, Figure 2A); however, average lower survival and hatching rates were found in embryos and larvae at 4 and 22 dpf (Figure 2A) as compared to the control. We then studied the effect of UV irradiation on number of FITC-labeled PGCs by exposing the embryos at 240mJ/cm^2 (UV240) according to Saito et al., [39]. In group of embryos that were exposed to UV240, few ectopic FITC labeled PCCs were detected in some exposed embryos (Table 2, Figure 2A). However, most of exposed embryos were lacking the FITC-labeled PGCs in the surrounding region of the tail bud, where they were expected to accumulate. Embryos that were exposed to UV240 developed normally and grew up healthy until 22 dpf when they were euthanized to count number of PGCs in body cavity (Figure 2B). In the CRISPR/Cas9 experiment, number of PGCs was counted at 4dpf and 22 dpf. FITC-labeled PGCs were rarely found in genital ridges of CRISPR/Cas9 treated embryos. Compared to three aforementioned methods, number of FITC-labeled PGCs at 22 dpf in control group increased when compared those in neurula stage. No significant difference in hatching and survival rates of embryos were found in the three compared methods.

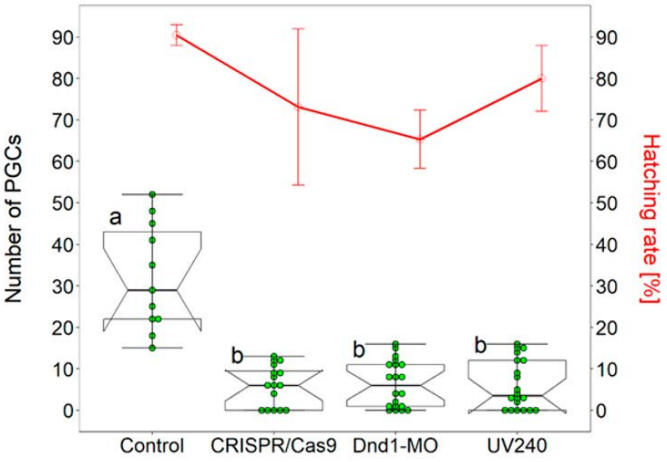
Table 2. Comparison of CRISPR/Cas9, *Dnd1*-MO and UV240 methods.

Treatment	Stage	No. of Embryos/Larvae	Minimum No. of PGCs	Maximum No. of PGCs	Mean	SD	*p*
Control	Neurula	11	15	52	32.00	12.88	0.0004467
	22 dpf	8	51	92	71.88	16.78	
CRISPR/Cas9	Neurula	16	0	13	6.00	4.84	0.008104
	22 dpf	11	0	5	1.18	2.04	
Dnd1-MO	Neurula	20	0	16	3.30	5.59	0.7032
	22 dpf	9	0	12	5.56	4.77	
UV240	Neurula	20	0	16	6.05	5.96	0.3819
	22 dpf	19	0	18	4.89	6.13	

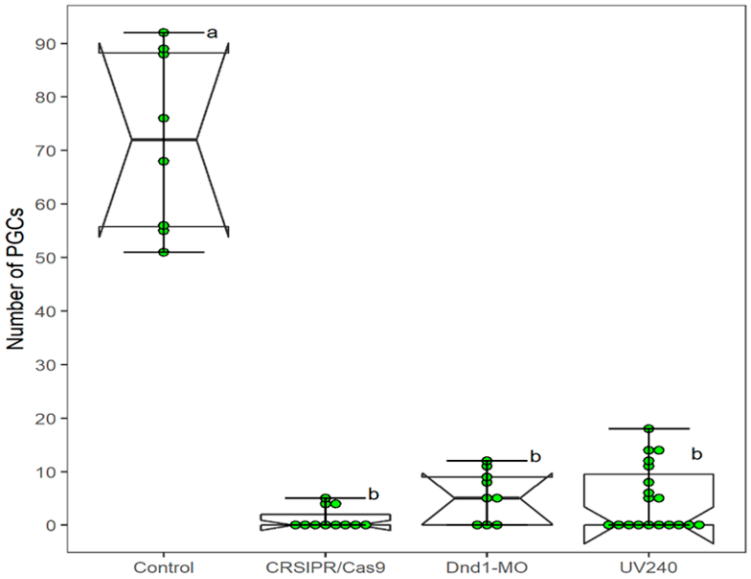
Wilcoxon test.

3.3. Capillary Electrophoresis and Mutation Detection Assay

Normally developed embryos lacking PGCs were collected to examine the efficiency of disruption at on-target sites at 4 dpf. By using capillary electrophoresis some samples were found to have different band sizes (Figure 3) when compared with non-injected embryos. In mutation detection assay, different bands formation was detected in aforementioned samples, indicating that disruption of target sites occurred for *Ardnd1* (Figure 3). As expected, several shorter bands were observed in embryos injected with mixture of sgRNA+Cas9 ribonucleoprotein. Injection of sgRNA+Cas9 ribonucleoprotein presumably induced frameshift mutations and/or small deletions in the *dnd1* gene [33,46–48].



A



B

Figure 2. Three different methods to achieve sterility in sterlet (*Acipenser ruthenus*) have been established and were compared. (**A**): The control and embryos injected with CRISPR/Cas9, *Dnd1*-MO and UV240 exposed embryos; injected with FITC-dextran to label PGCs. Number of PGCs were counted in all three treated groups i.e., CRISPR/Cas9, *Dnd1*-MO and UV240, and in control group. FITC-labeled PGCs were found to be reduced at the neurula to tail bud stage (box plots). Hatching and survival rates were found to be lower in *Dnd1*-MO embryos (line graph) when compared to the control group. In CRISPR/Cas9 and UV240 irradiation method, hatching and survival rates of embryos did not significantly vary (line graph). The survived embryos from all three treated groups were kept and FITC-labeled PGCs were observed at 22 dpf. Number of FITC labeled PGCs continued to be absent in CRISPR/Cas9, *Dnd1*-MO and UV240 groups, while larvae from the control group have many FITC-labeled PGCs in the gonadal region (**B**). Bars with different letters signify a significant difference ($p < 0.05$).

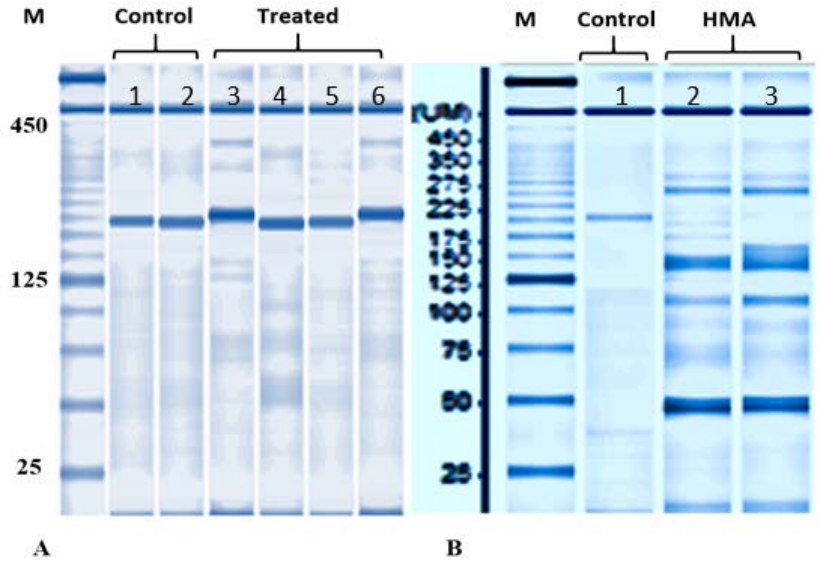


Figure 3. Genotyping by HMA assay in the un-injected and injected sterlet embryos. PCR products encompassing sgRNAs target sites were analyzed using a microchip electrophoresis system. (**A**) Bands from DNA of two injected embryos (sample number 3 and 6) were found to have different size; (**B**) HMA of two embryos having different bands (sample number 2 and 3). The sample 2 and 3 in Figure 3B were numbered as 3 and 6 in Figure 3A. Heteroduplex bands and multiple short bands are shown in sgRNA+Cas9 RNP injected embryos (crispants; B: sample 2 and 3).

4. Discussion

The majority of the genome editing studies have been conducted on polyploid species in plants such as rice (*Oryza sativa*), sugarcane (*Saccharum* spp. *hybrids*), *Camelina sativa* and *Arabidopsis thaliana* [49–51]. In fish model species, zebrafish, the CRISPR/Cas9 research with codon optimization of Cas9 mRNA showed induction of mutation with 75 to 99% rates in endogenous genes [52]. However, same method does not induce more than 55% mutation in sterlet, which might be due to different characteristics of embryos such as size, incubation temperature, cleavage pattern and ploidy level [53]. Interestingly, in sterlet when Cas9 protein instead of Cas9 mRNA was injected, mutation and survival rate of embryos increased by more than 90% [53]. Our present study was aimed to disrupt the *Ardnd1* to achieve sterility in sterlet (*Acipenser ruthenus*) to prepare a host for surrogate production in critically endangered sturgeon species. This study is consistent with Chen et al., [53], suggesting that Cas9 endonuclease can be efficiently applied in sturgeon having a higher ploidy level such as hexaploid or octoploid genomes.

Microinjection is an extensively used method of gene transfer in fish species due to its low cost, ease of visualization and high efficiency [54]. We have established this method in our lab to micro-manipulate sturgeon embryos. The sgRNA+Cas9 ribonucleoprotein complex was microinjected into 1 cell stage of sterlet eggs and this was repeated several times. Survival and hatching rates of embryos varied from batch to batch of eggs and also from different females; however, the mutation rate from phenotypic evidences was stable therefore proving the reliability of CRISPR/Cas9 in sterlet. Consistent with Chen et al., [53], low toxicity to embryos was found when different concentrations of sgRNAs and Cas9 endonuclease were injected. Some malformations in embryos in region where PGCs should originate were detected at 4 dpf in one batch of eggs from one female (Figure 1C); this is possibly because of double injection i.e., injection of sgRNA+Cas9 ribonucleoprotein complex into animal pole and FITC to label PGCs in the vegetal pole of embryos, and more likely due to off-target effects. The malformed embryos eventually died in the chorion membrane.

As expected, the number of PGCs was reduced in CRISPR/Cas9 treated embryos when compared with control embryos, however, in contrast, a few embryos with PGCs were still detectable in larvae until 22 dpf. Possible explanation can be mosaicism and/or physiological differences among embryos. Favoring the latter explanation, there might be differences in FITC labeling efficiency among embryos due to different chorion thickness, amount and/or depth/position germ plasm in embryo and also the depth/position of germ plasm in embryo [39]. Moreover, studies have showed that maternally supplied germ plasm and PGCs number vary among embryos within the same species [55,56], and the sturgeons are consistent with these findings [40,43].

We observed a healthy development of embryos after double injection until 22 dpf. Nevertheless, to guarantee the success of production of gametes via surrogacy, it is essential to check the development of sturgeons until the adult stage. It is important to confirm that complete gonads are formed in both of the sexes. In medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), complete removal of PGCs by using MO against *dnd1* led to production of all-male population in treated embryos [21,35]. However, in other fish species such as in loach (*Misgurnus anguillicaudatus*) and goldfish (*Carassius auratus*), sterilized embryos developed into both the sexes [22,23]. Linhartová and colleagues used AMO-*dnd1* in sturgeons to generate sterile host for surrogate production; however, sex determination after PGCs removal is still unclear [24]. Therefore, sex determination should not be biased in sturgeons when preparing the host for surrogate production. We have already established methods in our laboratory to prepare a germ cell free sterlet host. In our preset study, we compared three different methods to find out which method is more suitable in terms of embryo survival and hatching rates, and the removal of PGCs. Average lower survival and hatching rates were found as compared to CRISPR/Cas9 and UV240 irradiation when *Dnd1*-MO was injected as it is toxic to embryos [24].

Several previous studies in fish species have studied the functions of *dnd1* when both maternal as well as the zygotic mRNA are lost during early development. When CRISR/Cas9 was used against *dnd1* in Atlantic salmon, results showed that knockout (KO) fish were germ cell free in the F0 [33]. Thus, in the aforementioned studies, for the first time it has been revealed that CRISPR/Cas9 mediated knockouts of *dnd1* gene caused complete loss of the germ cells in F0 generation. Interestingly, maternal RNA was unable to compensate for loss of zygotic gene in F0 generation [33]. In sturgeons, the F0 mosaic embryos having maternal RNA compensating for zygotic gene loss could be targeted in the next generation to produce embryos having a complete loss of germ cells.

Host derived sperm or egg generation is not permitted in surrogate production technique to avoid the possibility of hybrids among offspring and consequent genetic contamination of target species. Due to complications in chromosomal pairing, interspecific hybrids of distantly related species are generally sterile. Nevertheless, hybrids of sturgeons from various species crosses tend to be viable and also fertile, even with different ploidy levels [57]. Therefore, 100% sterilization of the host is crucial in the surrogate production of sturgeons. We continue our work in this field to meet the promise that surrogate production in sturgeons is becoming feasible at a greater rate [24,39,43,58]. We trust that this cutting-edge technology, CRISPR/Cas9, will certainly be an invaluable tool for surrogate production of these IUCN red listed species.

5. Conclusions

In our present study, we have used CRISPR/Cas9 genome editing technology to achieve sterility in sterlet (*Acipenser ruthenus*) in order to prepare a host for surrogate production in sturgeons. The *dnd1* is essential for the formation and migration of PGCs in vertebrates, and its knock out in sterlet led to mismigration and/or absence of PGCs in the sterlet embryos, thus these sterile embryos can be potentially used as hosts for surrogacy in sturgeons.

Thereafter, we also compared three methods *viz.*, *Dnd1*-MO, UV irradiation and CRISPR/Cas9 that have been established in our lab to sterile sterlet. In terms of removal of PGCs, no significant difference was found among the compared methods; however, higher survival and hatching rates were found in CRISPR/Cas9, UV and MO, respectively.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/9/4/174/s1, Figure S1: Generation of sgRNAs by overlap PCR.

Author Contributions: Conceptualization and methodology, A.R.B., R.F., M.R., M.P.; Data collection, A.R.B., M.F. Data processing and statistical analysis, A.R.B., T.T., M.P.; Funding acquisition, M.P.; All authors have been involved in developing, writing and commenting on the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest: No conflict of interest exits among the authors.

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CHAPTER 4

DELIVERY OF IRON OXIDE NANOPARTICLES INTO PRIMORDIAL GERM CELLS IN STURGEON

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My share on this work was about 80%.

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MDPI

Communication

Delivery of Iron Oxide Nanoparticles into Primordial Germ Cells in Sturgeon

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Abstract: Nanoparticles are finding increasing applications in diagnostics, imaging and therapeutics in medicine. Iron oxide nanoparticles (IONs) have received significant interest of scientific community due to their distinctive properties. For the first time, we have delivered IONs into germ cells in any species. Our results showed that sturgeon primordial germ cells (PGCs) delivered with IONs could be detected until seven days post fertilization (dpf) under fluorescent microscope and at 22 dpf by micro-CT. Delivery of IONs into cells could be helpful for studying germ cell biology and the improvement of germ cell-based bio-technologies as isolation of PGCs using magnetic activated cell sorting or application of hyperthermia for a host sterilization purpose. Intriguingly, in our study, we did not find any toxic effects of IONs on the survival and hatching rates of sturgeon embryos when compared with embryos injected with FITC-dextran only.

Keywords: acipenser; caviar; hyperthermia; iron oxide nanoparticles; micro-CT; sterilization

1. Introduction

Nanoparticles (NPs) are an agglomeration of atoms and molecules ranging from 1 nm to 100 nm, and can be composed of one or more species of the atoms [1]. As a result of their comparable comparable to viruses, cells, genes and proteins, the NPs opened new research avenues in interacting with fundamental biological processes [2]. The NPs are generally classified based on their morphology, composition, agglomeration, dimensionality and uniformity [3]. NPs are made-up of metal, metal oxides, carbon and organic matter, and possess unique magnetic, optical and chemical properties. NPs have been studied thoroughly in different research fields and have generated intense scientific interest in biomedical, optical and electronic fields because of their potential applications [4]. Characterization of NPs have been improved that in-turn has enhanced their applications [5]. Broadly, NPs are divided into two groups, i.e., ultrafine NPs and engineered NPs, which are present in nature and produced in a controlled way, respectively [6].

The engineered magnetic nanoparticles (MNPs) are composed of iron, cobalt or nickel oxides; these particles exhibit special properties like the higher magnetic moment and higher surface to volume ratio; thus enabling them to be potentially manipulated by an external magnetic field [7]. MNPs that are composed of ferromagnetic material, i.e., iron oxide nanoparticles (IONs), made-up of magnetite (Fe_2O_4) and maghemite (γ-Fe_2O_3) combine the ideal bio-compatibility with superparamagnetic properties, therefore allowing widespread biomedical uses like hyperthermia, targeted drug delivery, biosensors, photoablation therapy, and also in the theranostics applications [8,9]. Moreover, the IONs are also being used in the MRI (magnetic resonance imaging) contrast, and labelling of biological materials [10].

In these applications, iron is not the choice; however, the iron oxides used are more amenable to buffer aqueous environments present in the biological systems. Nevertheless, iron presents advantages over its oxides of being a softer magnet; therefore, it is capable of maintaining its superparamagnetism at larger sizes [11]. The superparamagnetic IONs can be used to improve the treatment of cancer by generating local heat called hyperthermia when exposed to the alternate magnetic field. In hyperthermia as a medical treatment, raising temperature to approximately 43 °C for 30 min to 60 min can trigger apoptosis [12,13]. Tumors as compared to normal cells are more susceptible to hyperthermia due to faster cell division, low pH, increased hypoxia and limited temperature regulation because of poor fluid transfer [14,15]. Additionally, IONs have been shown to be less toxic when compared with other metal oxide NPs [16]. Development of these non-invasive, highly sensitive techniques can be helpful to label any cells (here in our study, germ cells for the first time in any species) will certainly provide knowledge about poorly understood mechanisms [17,18].

Sturgeons, also called as archaic giants are ancient fish species, which have existed for a minimum of 200 million years [19]. As a result of the high value of caviar, the sturgeons became a target of intensive legal and illegal fisheries, therefore resulting in the collapse of several sturgeon species and stocks [20,21]. Natural populations of these living fossils have been declining especially because of the water pollution and interference in their natural habitats. Hybridization, water divergence, reduced food supply and saltwater intrusion are other prominent reasons affecting the populations of sturgeons [22,23]. Moreover, damming of rivers also resulted in the reduction and/or elimination of spawning and egg/larvae habitats of sturgeons [24]. According to the International Union for Conservation of Nature (IUCN) 2010, 85% of sturgeon species are at the verge of extinction. Some sturgeon species have a life span of over 100 years and they attain sexual maturity between 20 to 25 years [21]. However, amongst, the sterlet (*Acipenser ruthenus*) has the fastest reproductive cycle; males mature from three to seven years and females from five to nine years [25]. Thus, this sturgeon species provides opportunities to study the germ cell fate in sturgeons.

The primordial germ cells (PGCs) are the origin of all germ cells in developing embryos that will generate gametes, i.e., spermatozoa and oocyte [26,27]. The formation, migration and proliferation of PGCs are essential for gametogenesis in sexually mature individuals [28,29]. The elucidation of PGCs development in fish species will be helpful to provide fundamental insights regarding gonadal development, sex determination, sexual differentiation [30–32] and also a promising technique to manipulate fish reproduction [33,34].

Despite the importance of these amazing ancient fish species, so far not many studies have been conducted regarding the development and PGCs tracking in embryos [27,35]. Previously, our research group have already investigated PGCs development in sturgeon embryos; where visualization of PGCs in sturgeons embryos was done by injecting with a fluorescent tracer dye conjugated to a high-molecular-weight dextran (fluorescein isothiocyanate [FITC]-dextran) [27]. In our present study, however, for first time we have used IONs to label the germ cells in any species; here sturgeons, the IUCN red-listed species, and this study thus can shed light on the interactions of NPs with any cell precisely.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments were conducted in accordance with the Animal Research Committee of the Faculty of Fisheries and Protection of Waters in Vodňany, University of South Bohemia in České Budějovice, Czech Republic. All experimental fish were maintained according to principles based on the European Union (EU) harmonized animal welfare act of Czech Republic, and principles of laboratory animal care and national laws 246/1992 "Animal Welfare" on the protection of animals were followed. Experiments were approved by the Ministry of Agriculture of the Czech Republic (reference number: MSMT-6406/2019-2).

2.2. Fish Source, Preparation of Embryos and Sample Collection

During the spawning season (February to April 2019), females and males of adult sterlet (*Acipenser ruthenus*) of five to nine years of age were transferred from outdoor ponds into the recirculating aquaculture system installed indoor. Fish were kept in tanks of 4000-L at a mean water temperature of 15 °C. To induce spermiation, male sterlet were injected with the single intra-muscular injection of carp pituitary extract at 4 mg/kg body weight (BW) in the 0.9% NaCl. Sperm collection was done 48 h after injection of hormones and kept at ice at 4 °C until fertilization. Light microscopy was used to assess the motility of spermatozoa that was found to be more than 80% and then were used for the fertilization. In order to stimulate ovulation, the carp pituitary extract was used by intra-muscular injection in two doses (first dose at 0.5 mg/kg BW and second at 4.5 mg/kg BW 12 h after the first injection). Ovulated eggs were collected from three different females from 18 to 20 h after the second injection, and these eggs were inseminated with sperm from two males at 15 °C in the dechlorinated water. In order to remove stickiness, eggs were rinsed three times in 1% tannic acid. One hour later after the fertilization, the chorion membrane (outer layer of eggs) was removed by using forceps. Chorion removed eggs were then transferred into 100 mL dechlorinated tap water with 0.01% penicillin and streptomycin in glass petri dishes. Embryos were incubated at 15 °C in an incubator. Temperature regulation was done at 15 ± 1 °C throughout the experimental period and changing of water was done on a daily basis. Embryos were used for injection of IONs mixed with fluorescein isothiocyanate (FITC)- dextran (molecular weight = 500,000) or FITC-dextran only as control in order to label the PGCs. The procedure was repeated two times so that a total of 600 sterlet embryos from six different females were used for the injection of IONs/FITC-dextran. The same number of embryos were used as the control group injected with FITC-dextran only, the remaining embryos were kept as non-injected controls for further incubation to assess hatching and survival rates.

2.3. Microinjection of Iron-Oxide Nanoparticles

Injection ready IONs with Rhodamine B [(10 nm) (IRB-10-02)] were bought (Ocean NanoTech, LLC, San Diego, CA, USA). Glass micropipette was drawn from the glass needle (Drummond, Tokyo, Japan) using the needle puller (PC-10; Narishige, Tokyo, Japan). IONs/ 1% FITC-dextran and/or just 1% FITC-dextran were loaded into the glass capillary and thereafter injected into the vegetal pole of sterlet embryos at 1–2 cell stage at 1–4 h post fertilization (hpf) according to Saito et al. [35]. Microinjection of the embryos was performed under the fluorescent stereomicroscope Leica M165 FC (Leica, Wetzlar, Germany) using the automatic micro-injector (Eppendorf, FemtoJet 4×, Hamburg, Germany) with a pressure of ~100 hPa for 1 s. Each embryo was injected with ~50 nL. Survival and hatching rates, and number of IONs and FITC-labelled PGCs at 4 and 5 dpf were examined in all injected groups from different females. Hatched larvae were fed with artemia and at 22 dpf, larvae from each group were anaesthetized by the tricaine solution and the body cavity was opened, gut was dissected and PGCs position were checked in all injected larvae.

2.4. Micro-CT Imaging

Paraformaldehyde (PFA)-fixed samples at 22 dpf were post-fixed in the Karnovsky fixative at least overnight and kept in 70% EtOH. Samples were mounted without further contrast staining in 1% low melting temperature agarose in 200 µL pipette tips, and scanned using the MicroXCT system (Zeiss/Xradia, Berlin, Germany) at the Department of Theoretical Biology, University of Vienna, Austria. X-ray projections were taken with the 10× detector objective, tungsten source at 40 kVp (4 W), and pixel size of 2.1–2.5 µm. Reconstructed virtual sections were analysed in Amira 2019.2 (FEI software, Thermo Fisher Scientific, Berlin, Germany). The entire larvae were visualized either by the volume rendering or maximum intensity projection.

2.5. Statistical Analysis

The statistical significance of the injection of IONs/FITC-dextran and FITC-dextran on the number of PGCs was analysed by the Wilcoxon rank-sum test. Logistic regression with post hoc Tukey's test was used for the analysis of survival rates of the embryos. Statistical tests were performed using the R software (Version 3.5.2; R foundation for Statistical Computing, Vienna, Austria) with a significance level of p-value < 0.05.

3. Results

3.1. Fertilization, Hatching and Survival Rates

We used eggs for the injection of IONs and FITC-dextran from six different sterlet females and the fertilization rate of eggs was found to be 95.8 ± 1.8. Hatching and survival rates of sturgeon embryos when injected with IONs are most important indices that help to evaluate the toxicity of IONs. Therefore, we evaluated hatching and survival rates of embryos when injected with FITC-dextran only, IONs/FITC-dextran and un-injected embryos from all six different females (Figure 1). Our data indicates that IONs did not present any toxic effects on the aforementioned parameters when compared with the FITC-dextran injected embryos; however, a significant difference was found when both injected groups were compared with the uninjected group.

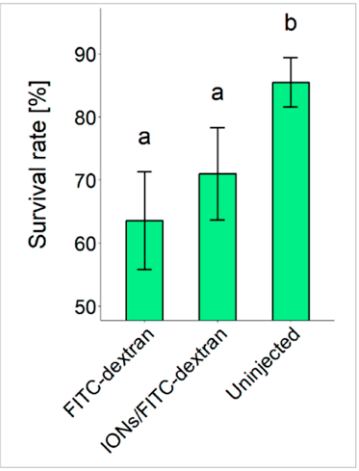

Figure 1. Survival rates of sturgeon embryos injected with FITC-dextran only, IONs/FITC-dextran and uninjected embryos. Different letters (a and b) above the SD bars represent statistical significance.

3.2. Delivery of IONs into PGCs

We injected IONs mixed with FITC-dextran into the vegetal pole of 1–2 cell stage sturgeon embryos in order to deliver IONs into PGCs according to Saito and Pšenička [27]. PGCs loaded with IONs/FITC-dextran and only FITC-dextran were visualized and they appeared around the margins of tail bud at 4 dpf (Figure 2A). PGCs were also detected at 5 dpf and tracked their migratory pattern that was found to be at the final positon where gonads develop (Figure 2B). The number of PGCs in the embryos injected with FITC-dextran only as the control were found to be in a higher number and significantly differed from those that were injected with IONs/FITC-dextran (Figure 2C).

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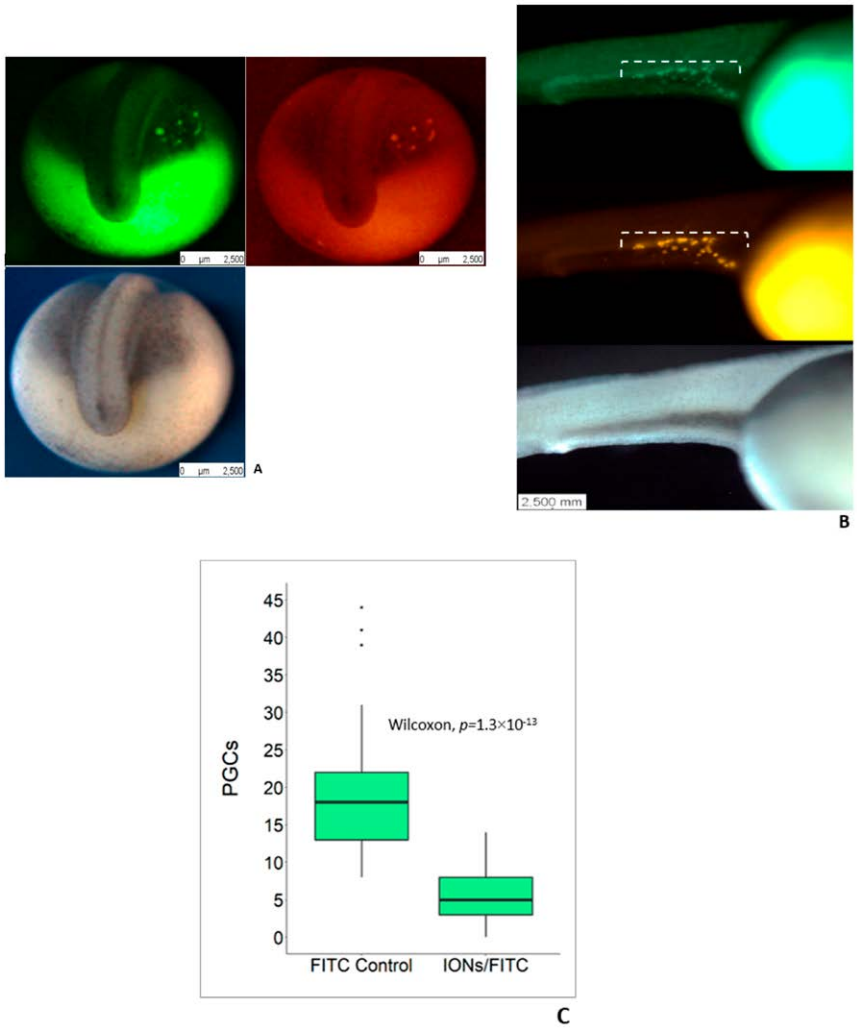

Figure 2. Labelling of primordial germ cells (PGCs) in sturgeons by using IONs and their visualization under fluorescent stereomicroscope. (**A**) IONs/FITC-dextran injected embryos to deliver IONs into PGCs at the tail bud stage. Visualization of PGCs in IONs/FITC-dextran injected embryos at 4 dpf; left: FITC-dextran labelling; right: IONs delivered into PGCs; bottom: Bright field view. (**B**) Visualization of PGCs in IONs/FITC-dextran injected group at 5 dpf. PGCs can be clearly seen under white broken line; top: FITC-dextran labelling; middle: IONs delivered into PGCs; bottom: Bright field view. (**C**) Graph shows a significant difference in the number of PGCs in FITC-dextran injected group and IONs/FITC-dextran injected group; number of PGCs were counted at 4 dpf, (Figure 2c, $p < 0.05$).

3.3. Micro-CT Imaging

Computed tomography is used for in vivo imaging as it is non-invasive, fast, provides high resolution and cost-effective, and is often employed in research as a micro-CT. IONs injected into sturgeon embryos tend to accumulate in the PGCs due to their enhanced retention capability [36], thus labelling them. The micro-CT imaging showed the PGCs labelled with IONs at 22 dpf (Figure 3; Video S1 in Supplementary Materials).

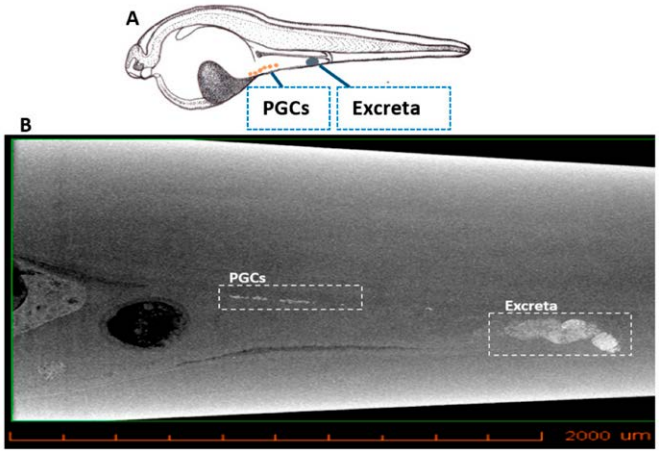

Figure 3. X-ray microtomographic (Micro-CT) section of sturgeon larvae after injection with IONs. (**A**) Illustration showing the position of IONs delivered into PGCs and excreta in sturgeon larvae. (**B**) Micro-CT shows the position of PGCs (in whit dotted box) during their migration towards the position where gonads are formed. Excreta of larvae can also be seen in the white dotted box.

4. Discussion

In our present study, we opted to take the opportunity to use IONs, for the first time to label germ cells in any species. Labelling PGCs by injecting IONs is a non-transgenic approach that provides significant experimental advantages to investigate the biology of germ cells in sturgeons. Transgenic strains such as zebrafish, medaka and trout carrying fluorescent protein in their germ cells have already been produced [37–39]. However, on the other hand, sturgeons mature late; their reproduction occurs from five to 25 years, it thus requires 10 to 50 years (at least two generations) to establish transgenic strains. Secondly, the selection and maintenance of transgenic sturgeons will need more keeping space because of big body sizes. In this study, the IONs were injected into the vegetal pole of sterlet embryos from six different females, and no toxic or adverse effects on embryo hatching and survival rates were found after IONs injection. Additionally, we also injected polystyrene NPs into the sturgeon embryos and did not find any toxic effects on survival and hatching rates (data not shown here). It was presumably the no vital somatic tissues in the embryo that were affected with the IONs. Embryos injected with IONs/FITC-dextran and only FITC-dextran were visualized under a fluorescence stereomicroscope to count the number of IONs labelled PGCs from 4 dpf to 7 dpf. We observed a significantly lower number of PGCs labelled with IONs as compared to the control group injected with FITC-dextran only. This could be due to an adverse effect of IONs towards PGCs migration and also FITC-dextran labelling is more effective because molecules of FITC-dextran have a better dispersion capability and thus they have higher labelling efficiency than 10 nm nanoparticles. The injection of IONs did not cause any malformations in embryos. Nevertheless, in zebrafish, when ecological effects of IONs were studied by exposing them against different concentrations, IONs caused developmental toxicity, mortality, malformations and delay in hatching [40].

Similarly to FITC-dextran labelling, according to Saito and Pšenička [27], when the IONs were injected into the vegetal pole, they labelled the PGCs and yolk in the sturgeon embryos; however, yolk is excreted as a faecal material of endogenous nutrition while only PGCs remained labelled. The IONs/FITC-dextran injected embryos were kept until 22 dpf in order to assess the labelling and migratory pattern of PGCs after the first exogenous nutrition intake. However, when the body cavity of euthanized larvae was opened to visualize IONs/FITC-dextran labelled PGCs, only FITC-dextran labelling could be observed under the fluorescent stereomicroscope and the fluorescent signal from IONs (rhodamine B) were lost. Nevertheless, IONs labelled PGCs and excreta could be traced by using

micro-CT; and the PGCs were found to be localized at a position where gonadal formation has been described [27], which confirmed the presence of IONs at the transition to exogenous nutrition.

IONs have been studied in connection with hyperthermia to activate cell death [41] by heating up tissues for cancer therapy, and besides, IONs are also released by the cell in vivo as they show no toxic effects [42]. Germ cells labelling in vivo by using IONs can be beneficial to study the interactions of IONs with cells precisely, that will in-turn help how to treat tumors by enhanced generated heat locally with minimal damage to nearby cells or tissues [43]. This application of IONs thus can be applied as an alternate approach to achieve sterility in sturgeon for surrogate production.

Elucidation of the phenomenon of PGCs formation in sturgeons have already been investigated thoroughly by Saito et al. [27] and Saito and Pšenička [35]. They showed that PGCs migration is divided into two phases, i.e., active and slow migration, at different developmental stages. The study was also supported by PGCs localization near mesentery around the hatching stage in Adriatic sturgeons, and PGCs are surrounded by cytoplasmic extensions from somatic cells [44] and thus the fluorescent signal is difficult to be observed. Our present results are consistent with the aforementioned studies and suggest that it will be more convenient to study the interactions of PGCs with somatic cells in sturgeons by using micro-CT after IONs labelling.

The sturgeon PGCs are extremely important cells securing the reproduction of this critically endangered species. However, the number of PGCs in an embryo is very low and handling them is very difficult [35]. An efficient isolation method could enable their research and use in surrogate production technologies. Magnetic-activated cell sorting can be applied when IONs are loaded into PGCs and when the fluorescent signal is difficult to trace under tissues. Efforts and results from our research team have been trying to meet the promise that techniques for "surrogate production" in IUCN red-listed sturgeons are becoming more practicable and convenient [35,45–49].

5. Conclusions

We have developed a novel method where IONs were used to label and visualize PGCs in sterlet. PGCs visualization has a great potential for investigation of PGCs development, including their migration and proliferation patterns in developing embryos and hatched larvae. This latest technique can be helpful to study germ cell biology, and consequently the improvement of germ cell based biotechnologies such as PGCs isolation and hyperthermia application for sterilization of host for sturgeon surrogate production.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/9/8/333/s1, Video S1: The micro-CT imaging showed the PGCs delivered with IONs at 22 dpf.

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CHAPTER 5

GENERAL DISCUSSION

Sturgeons are commonly known for caviar and their fossil record dates back to Upper Cretaceous (Grande and Bemis, 1991). Analysis of mitochondrial DNA suggests that these fish species diverged from ancient, pre-Jurassic teleost lineage approximately 200 Mya (Bemis and Kynard, 1997). Sturgeons' 85% species are listed as critically endangered in red list of the IUCN. Introduced species, water divergence, reduced food supply, and mainly construction of dams affected sturgeon populations (Ludwig et al., 2001; Zhang et al., 2011). Reproductive traits of sturgeons make their rehabilitation more difficult; these traits include delay in sexual maturation and periodic interrupted spawning cycles. Sturgeons are generally long living and have late onset of sexual maturity accompanied by slow growth rate with reproduction that is infrequent (Pikitch et al., 2005). It has been reported that some species of sturgeons exhibit life span over 100 years and their sexual maturity is attained between 20 to 25 years or sometimes even more in females (Billard and Guillaume, 2001). However, among sturgeon species, sterlet (*Acipenser ruthenus*) has shortest sexual maturation period. This species usually matures from 3 to 7 years (males); while the females take from 5 to 9 years. Therefore, these characteristics give advantages to use sterlet in sturgeon surrogate production. Besides, fast maturation and short reproductive cycle make sterlet more convenient to be used in establishing techniques to manipulate genome (Chen et al., 2018; Dettlaf et al., 1993).

PGCs in developing embryo arise elsewhere and then these cells migrate towards gonadal ridges (Saito et al., 2014). In animal species, formation and migration patterns of PGCs vary, and PGCs formation is altered according to cleavage from holoblastic to meroblastic. During process of PGCs migration, various challenges are imposed, including their route, and reach at specific target. However, during course of migration, PGCs are exposed to various signals, these signals direct differentiation of somatic cells. Thus, protection of PGCs during this journey makes sure their arrival at target site and differentiate into gametes and then the fertility of organism is ensured. Furthermore, preservation of fate of germ cells is very important as it prevents germ cell tumor formation that is result of germ cells, which have become subject of partial transdifferentiation (Göbel et al., 2000; Schneider et al., 2001). Maintenance of PGCs fate needs strong molecular mechanisms, these mechanisms include, transcription silencing machinery should be regulated, modification of chromatic should be done, and importantly, regulation of translation of mRNA and stability of protein is essential (Strome and Updike, 2015; Wolke et al., 2002; Lai et al., 2012; Robert et al., 2015). PGCs maintain their germline fate during course of their migration through developing embryo; and it has been documented before that dnd1 protein is essential for their survival; however, recent studies of Gross-Thebing and colleagues have shown that dnd1 protein does maintain fate of PGCs in a way where it inhibits somatic differentiation. Thus, they found that PGCs deficient and/or lacking dnd1 protein transdifferentiate into somatic cells. These cells further achieve expression of somatic gene profile as well as morphology, and it is possible to visualize germline into soma trans-differentiation *in vivo* (Gross-Thebing et al., 2017). These results, therefore, indicate mechanisms that control maintenance of germ cell fate, and interestingly, it is also relevant to teratoma formation (Gross-Thebing et al., 2017). Studies have found that migratory mechanism of PGCs is conserved between sturgeons and other related teleosts (Saito et al., 2014). Previously, colleagues from our lab have demonstrated that in sturgeons injection technique where it was confirmed that PGCs in sturgeons are generated at vegetal pole (VP), and these cells then migrate on yolky cell mass in direction of gonadal ridge. In sturgeons, germ cell transplantation from donor that matures late into recipient maturing fast having small body size can be a potential tool to save sturgeons (Pšenička et al., 2015). For the first time male and female early stage germ cells were isolated and transplanted in

sturgeons in order to produce a germline chimera for purpose of surrogate parents as well as gene banking tool (Pšenička et al., 2015).

In second chapter of thesis, importance of dnd1 protein in fish have been reviewed. *Dnd1* expresses exclusively in vertebrates' germline, thus showing that it plays a conserved role in germline development (see Baloch AR., et al., 2019). Various studies confirmed that dnd1 protein is essential for PGCs migration, and migration of these cells follows complex path by various developing tissues. It is important to note that disruption of PGCs migration affects fertility (Weidinger et al., 2003; Baloch, AR., 2019; Wargelius et al., 2016). *Dnd1* is conserved germ cell marker in various fish species. In ovary and testes of Pacific Bluefin tuna and turbot, its differential expression have been reported (Lin et al., 2013; Yazawa et al., 2013). Expression of *dnd1* in *Oryzias celebensis* (*Ocdnd1*) was detected in gonads, and specific expression was observed in spermatogonia (Zhu et al., 2018). In gibel carp, *dnd1* expresses in testes and ovary, therefore suggesting as a potential molecular marker for gametogenesis (Li et al., 2016). Various studies have been conducted on the miRNAs (microRNAs), and they have been found to inhibit gene expressions, and in this way, they control processes in normal development as well as cancer. Studies of Kedde and colleagues confirmed role of dnd1 protein in protection of mRNA from miRNAs mediated repression, where it was shown that expression of *dnd1* does counteract functions of several miRNAs in zebrafish PGCs; dnd1 protein binds with mRNAs, and does not allow/prohibits miRNAs from binding with their specific targets (Kedde et al., 2007). In model fish species, zebrafish, germline regulation and somatic cell distinction has been found to be done by combination of miRNAs and germ cell-specific RNA binding proteins; and repression that is mediated by miR-430 was observed in zebrafish, and interestingly, it was not counteracted by up-regulation and/or over expression of *dnd1* in somatic cells. Furthermore, it was also seen that translation repression of the dnd1 protein was not dependent to *nanos3*, which is documented as another repressor of translation in zebrafish (Kobayashi et al., 2017). Atlantic salmon (*Salmo salar* L.) cultured in open sea cages, and their escape can pose great threat to the genetic integrity with wild populations; thus their sterilization can mitigate this problem. In summary, *dnd1* has been described as germ cell-specific molecular marker, its interactions with miRNAs have studied in detail, and importantly, its further detailed studies can be helpful in aquaculture and fish conservation.

In third chapter of thesis, we have described use of CRISPR/Cas9 to achieve sterility in sterlet. CRISPR/Cas9 is a latest genome editing technology that can delete, insert and/or can alter sequences of cells and organisms, therefore, it enables scientists to disrupt genes of choice to dissect their functions (Hsu et al., 2014). In CRISPR/Cas9, RNA guided Cas9 can be easily targeted to any target on genome with help of short RNA guide (Nakayama et al., 2014). Sterlet among sturgeon species can be used as host for surrogate production in sturgeons (Linhartová et al., 2015). We used CRISPR/Cas9 to knock-out *dnd1*, and in CRISPR/Cas9 injected embryos, no or less number of PGCs were detected in crispants. Number of PGCs were analysed on 4 dpf, and at 22 dpf, we euthanized larvae and opened body cavity and PGCs numbers were counted. However, interestingly, a small number of PGCs could be seen in larvae when body cavity was opened. It can be due to mosaicism or physiological differences among injected embryos. Healthy development of embryos until ~3 weeks post fertilization was recorded after double injection. However, it is important to make sure success of gamete production by surrogacy, it is essential to check sturgeons development till they become adult. It is significant to ensure that gonads are formed in both sexes. When DNA from CRISPR/Cas9 injected embryos was extracted and PCR results showed different band patterns in capillary electrophoresis when HMA was performed. These embryos were presumably induced with frameshift mutations or small deletions in *dnd1* gene (Wargelius et al., 2016). When sterlet embryos were injected with CRISPR/Cas9, some of injected embryos

exhibited malformations where PGCs ought to originate, that was evident in one batch of eggs from one female. Possible explanation for this can be the off-target effect and double injections *i.e.,* CRISPR/Cas9 and FITC-dextran into animal and vegetal pole to knockout *dnd1* and label PGCs, respectively. We have already established other techniques (knockdown by MO and UV irradiation) to remove germ cells from host (Linhartová *et al.,* 2015; Saito *et al.,* 2018). Thus, we aimed to compare all techniques in our present study. We found higher embryo survival and hatching rates in CRISPR/Cas9, elimination of PGCs by UV irradiation and knockdown of *dnd1* by using MO respectively. Lower number of survival and hatching in MO injected group was because it is toxic to the embryos (Linhartová *et al.,* 2015). It is noteworthy that in sturgeons embryos having *dnd1* knocked out, their maternal mRNA can possibly compensate for zygotic gene loss, and these can be targeted in coming generation to produce embryos full loss of germ cells. Sturgeon hybrids from different species crosses tend be fertile, interestingly, with different ploidy levels (Havelka et al., 2011). Thus, complete sterilization with 100% surety of host is essential in sturgeon surrogate production. We believe that this cutting-edge genome editing technology will be invaluable tool for surrogate production of the IUCN red-listed species.

In fourth chapter of thesis, we have discussed novel method to label PGCs in sturgeons by using Iron Oxide Nanoparticles (IONs). Nanoparticles (NPs) are dispersion of particles or solid particles having nanoscale sizes, and these can be divided as nanopores, nanotubes, quantum dots, nanoshells, nanorods and so on (Heera and Shanmuga, 2015). NPs have been studied in various fields and have generated intensive scientific interest in the biomedical, optical and electronic fields (Hassan, 2015). In broad spectrum, NPs are divided into ultrafine and engineered NPs that are present in nature and produced in controlled way, respectively (Oberdörster et al., 2005). Engineered magnetic nanoparticles comprises of cobalt, iron, or nickel oxides; these particle show unique properties such as enhanced magnetic moment and higher surface to volume ratio; therefore can be effectively manipulated by supply external magnetic field (Cardoso et al., 2018). Magnetic nanoparticles (IONs) are composed of magnetite and maghemite, these both combine ideal biocompatibility with superparamagnetic properties, thus in this way allowing a wide range of biomedical uses/applications such as targeted drug delivery, hyperthermia and biosensors (Khanna et al., 2018). Furthermore, IONs are also being used in MRI (Magnetic Resonance Imaging) contrast, and also labelling of materials. Supermagnetic IONs have potentialities that can be used in order to improve cancer treatment in a ways to generate local heat when injected IONs can be exposed to alternate magnetic fields. IONs have been found to be less toxic to cells when were compared with other metal oxide nanoparticles (Bednarikova). Therefore, development of such methods which are non-invasive can be very helpful to label any cells; and also it will further provide knowledge regarding poorly understood mechanisms (Guzman, 2007; Hoehn et al., 2002). Taking advantages of IONs applications, we opted to use them to label PGCs in sturgeons. We injected IONs combined with FITC-dextran into the vegetal pole of sturgeons embryos to label PGCs. Zebrafish, trout and medaka as transgenic strains carry fluorescent protein in their germ cells have been produced previously (Krøvel et al., 2002; Yoshizaki et al., 2000). In our present study, we injected IONs in the vegetal pole of sturgeon embryos from six different females, and injection of IONs in sturgeons did not affect on hatching and survival rates of injected embryos. Zebrafish when exposed to nanoparticles in order to study ecological effects, IONs were used in different concentrations, used IONs caused developmental toxicity, malformations, mortality, and delay in hatching (Zhu et al., 2012). In order to make sure, whether labelling of PGCs with IONs can affect survival and migration of PGCs; we also injected embryos with FITC-dextran only to compare with IONs injected groups. Interestingly, at 4 dpf and 5 dpf, significantly less number of FITC-dextran labelled PGCs in FITC/IONs injected group

were observed when compared with PGCs that were labelled with FITC only. Injected embryos were kept till 22 dpf in order to assess migratory pattern of PGCs. Interestingly, when these larvae were euthanized and their body cavity was opened, no labelling of PGCs with IONs could be observed under fluorescent stereomicroscope. However, on contrary, these samples were used for assessing the presence of IONs; IONs could be traced by suing micro-CT, where these were found to be localized at the positon where formation of gonads occurs (Saito and Pšenička, 2015).

Phenomenon behind formation of PGCs in sturgeons have been investigated already, and it have been demonstrated that PGCs migration is divided into two main phases i.e., slow but active migration at various developmental stages (Saito and Pšenička, 2015). This is first study of its kind where germ cells of any species have been labelled by using nanoparticles.

Efforts and results from our lab have been trying to meet promise that techniques for ''surrogate production'' in IUCN red-listed sturgeons are becoming more convenient and practicable. PGCs labelling in sturgeon using IONs described here will be of value to monitor successful induction of sterility by applying hyperthermia after IONs are inserted into PGCs.

In this thesis, following conclusions and suggestions have been drawn;

- Novel genome editing technique (CRISPR/Cas9) have been successfully used to knockout *dnd1* in sterlet for sturgeon surrogate production.
- CRISPR/Cas9, UV-irradiation and MO were compared; higher embryo survival and hatching rates were found in CRISPR/Cas9, UV and MO, respectively.
- CRISPR/Cas9 can be applied in sturgeons where other functionally important genes can be targeted to study reverse genetics.
- For the first time, IONs are used to label PGCs in sturgeons.
- Under fluorescent stereomicroscope, IONs labelled PGCs were detected until 8 dpf. IONs can be traced in larvae at 22 dpf by micro-CT.
- Precise labelling of cells with IONs can be helpful to study germ cell biology and improvement of germ cell-based bio-technologies as isolation of PGCs using magnetic activated cell sorting or application of hyperthermia for host sterilization purpose.

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ENGLISH SUMMARY

Utilization of CRISPR/Cas9 and novel germ cells labelling technique for surrogate production in sturgeons

Abdul Rasheed Khanzai Baloch

Sturgeons are commonly known as living fossils or ancient giants that diverged from ancient pre-Jurassic teleost lineage approximately ~200 million years ago (Mya). Sturgeons' 85% species are listed as critically endangered in the International Union for Conservation of Nature (IUCN). Water divergence, reduced food supply, interference in their natural habitats and mainly construction of dams affected their populations. Sturgeons' reproductive traits such as delay in sexual maturation and periodic interrupted spawning cycles make their rehabilitation more difficult. However, among sturgeon species, the sterlet (*Acipenser ruthenus*) has shortest sexual maturation period. Therefore, it can be used as a host in surrogate production in sturgeons.

Dnd1 was discovered as germ-plasm specific maternal RNA that exclusively expresses in vertebrate germ-line. It thus indicates that *dnd1* plays a conserved role in germline development. Various studies have confirmed that dnd1 protein is essential for Primordial Germ Cells (PGCs) migration; and disruption of PGCs migration affects fish fertility. *Dnd1* deficient PGCs in zebrafish transdifferentiate into somatic cells. *Dnd1* have been detected as conserved germ cell molecular marker in fish species. In *Oryzias celebensis, dnd1* expression was specifically detected in spermatogonia. In common carp, its expression is unclear, while in gibel carp, it expresses in ovary and testes. In olive flounder, *dnd1* is recognized as germ cell specific marker and it expresses in ovary and testes of this fish. *Dnd1* in Chinese sturgeon was characterized and its expression was detected in gonads of both sexes. Previously our colleagues used morpholino oligonucleotide to knock down *dnd1* in sterlet to produce germ cell free host for surrogate production. *Dnd1* targeting can also be used for fish conservation purpose, like fish species raised in aquaculture when escape into wild threat integrity of wild stocks, and this problem can be mitigated by production of sterile stocks.

CRISPR/Cas9, a cutting-edge genome editing technology is being used in different research fields; here we thus aimed to harness the power of aforementioned technology to knock out *dnd1* in sterlet. Among sturgeons, sterlet has shortest sexual maturation period, therefore it can be used as sterile host for surrogate production for huge and late maturing sturgeon species. No or less number of PGCs were observed in CRISPR/Cas9 injected embryos as compared to control group injected with FITC-dextran only in order to label PGCs. Furthermore, we compared three different sterilization techniques *viz.,* CRISPR/Cas9 and morpholino oligonucleotide (MO) targeting *dnd1* and ultraviolet irradiation to eliminate PGCs in sterlet. Our data showed higher hatching and survival rates in CRISPR/Cas9, UV irradiation, and MO knockdown groups, respectively. Interestingly, some embryos treated with CRISPR/Cas9 displayed malformations. We presume that malformations were due to off-target effects and/or due to double injections *i.e.,* injection of CRISPR/Cas9 at animal pole to knock-out the *dnd1* and FITC-dextran at vegetal pole.

Taking advantages of Iron Oxide nanoparticles (IONs) applications in various burgeoning research fields, we opted to use them to label PGCs in sturgeons. We injected IONs combined with FITC-dextran into vegetal pole of sturgeon embryos, and have successfully labelled PGCs. Injection of IONs in sturgeons did not affect hatching and survival rates of embryos. Interestingly at 5 dpf, significantly less number of FITC-dextran labelled PGCs in FITC-dextran/IONs injected group were observed when compared with PGCs that were labelled with FITC-

dextran only. Less number of PGCs in IONs injected group presumably could be because of interference posed by IONs to PGCs during the course of their migration. This is first study of its kind where germ cells of any species have been labelled by using nanoparticles.

In conclusions, this thesis provides information regarding role of Dnd1 protein as potential germ-cell molecular marker in various fish species, and its use for conservation of fish species. *Dnd1* knockout sterlet can be potentially used as sterile host for surrogate production in sturgeons. Moreover, labelling of PGCs in sturgeons by using IONs can thus open new avenues to study interactions of nanoparticles with cells that will ultimately help in hyperthermia where cells/tissues are exposed to electromagnetic field increasing temperatures to activate their death. After insertion of IONs to PGCs in sturgeon embryo, it could be possible to isolate PGC using a magnetic field or to apply hyperthermia for host sterilization purpose.

CZECH SUMMARY

Použití CRISPR/Cas9 a nové techniky značení zárodečných buněk pro náhradní reprodukci u jeseterů

Abdul Rasheed Khanzai Baloch

Jeseteři, obvykle také známí jako žijící fosilie nebo starověcí giganti, se odštěpili od starověké pre-Jurské line kostnatých ryb přibližně před 200 miliony let. Osmdesát pět procent druhů jeseterů je podle Mezinárodního svazu ochrany přírody (IUCN) řazeno mezi kriticky ohrožené. Znečištění vod, redukce přirozené potravy, zásahy do jejich stanovišť, a především přehrazování řek vážně ovlivnilo jejich populace. Charakteristiky reprodukce jeseterů, jako například pozdní pohlavní dospělost a periodicky přerušovaný reprodukční cyklus komplikují jejich obnovu. Jeseter malý (*Acipenser ruthenus*) patří k jeseterům s nejkratší dobou dospívání. Proto by mohl být použit jako recipient pro náhradní reprodukci jeseterů.

Dead end gen (*dnd1*) byl objeven jako maternální RNA se specifickou expresí výhradně v zárodečné linii obratlovců. To ukazuje, že dnd1 hraje konzervovanou roli ve vývoji zárodečné linie. Různé studie potvrdily, že protein Dnd1 je nezbytný pro migraci primordiálních gonocytů (PGC) a že narušení migrace PGC ovlivňuje plodnost ryb. PGC u zebřičky pruhované deficientní na Dnd1 protein se transdiferencují do somatických buněk. Dnd1 byl detekován jako konzervovaný molekulární marker zárodečných buněk u ryb. U *Oryzias celebensis* byla exprese Dnd1 specificky detekována ve spermatogoniích. U kapra obecného je exprese Dnd1 nejasná, zatímco u karase se exprimuje ve vaječnících a testes. U platýse *Paralichthys olivaceus* je Dnd1 rozpoznán jako marker specifický pro zárodečné buňky a exprimuje se ve vaječnících a varlatech této ryby. Dnd1 u jesetera čínkého byl také charakterizován a jeho exprese byla detekována u gonád obou pohlaví. Naši kolegové v minulosti využili antisense morfolino oligonukleotid (MO) způsobující knock-down *dnd1* u jesetera malého, aby produkovali recipienty bez zárodečných buněk pro techniku náhradní reprodukce. Dnd1 lze také použít pro účely ochrany druhů ryb chovaných v akvakultuře. Pokud uniknou do volné přírody, mohou ohrozit integritu volně žijících populací. Tento problém může být zmírněn vypnutím *dnd1* genu pro produkci sterilních populací.

CRISPR / Cas9, technologie pro úpravu genomu, se používá v různých oblastech výzkumu; zde jsme se proto snažili využít možnosti výše zmíněné technologie ke knock-outu *dnd1* u jesetera malého pro vytvoření sterilního recipienta využitelného v náhradní reprodukci jeseterů. U embryí injikovaných CRISPR/Cas9 byl pozorován nulový, nebo menší počet PGC ve srovnání s kontrolní skupinou injikovanou pouze fluorescein isothiocyanatem konjugovaným s dextranem (za účelem značení PGC). Dále jsme srovnávali tři různé techniky: CRISPR/Cas9, UV záření a MO. Naše údaje ukázaly vyšší míru vykulení a přežití ve skupinách CRISPR/Cas9, následovalo UV a jako poslední MO. Je zajímavé, že některá embrya ošetřená CRISPR/Cas9 vykazovala malformace, předpokládáme tedy, že malformace byly způsobeny účinky off-targetu a/nebo v důsledku dvojitých injikací, tj. injikací CRISPR/Cas9 do animálního pólu a FITC-dextran do vegetativního pólu.

Vzhledem k výhodám aplikace nanočástic v různých rozvíjejících se výzkumných oborech jsme se rozhodli je použít ke značení PGC u jeseterů. Injikovali jsme nanočástice oxidu železa v kombinaci s FITC-dextranem do vegetativního pólu embryí a úspěšně jsme takto PGC označili. Injikace nanočástic do jeseterů neovlivnila míru vylíhnutí a přežití embryí. Je zajímavé, že 5 dní po oplození bylo ve skupině injikovaných s FITC-dextranem a nanočásticemi pozorováno signifikantně nižší množství značených PGC ve srovnání s PGC, které byly označeny pouze FITC-dextranem. Jedná se o první studii svého druhu, ve které byly zárodečné buňky označeny nanočásticemi.

Tato práce poskytuje informace o úloze proteinu Dnd1 jako potenciálního molekulárního markeru zárodečných buněk u různých druhů ryb a jeho využití pro ochranu ohrožených i kriticky ohrožených druhů ryb. Dnd1 knock-out u jesetera malého může být potenciálně použit jako metoda pro vytvoření sterilního hostitele pro náhradní reprodukci jeseterů. Navíc značení PGC u jeseterů pomocí nanočástic může otevřít nové cesty ke studiu interakce nanočástic s buňkami, které mohou v kombinaci s hypertermií, při které jsou buňky nebo tkáně vystaveny elektromagnetickému poli způsobujícímu lokální zvýšení teploty, aktivovat buněčnou smrt. Po vložení nanočástic do PGC embrya jesetera by mohlo být možné izolovat PGC pomocí magnetu nebo aplikovat hypertermii pro účely sterilizace recipientů.

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LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Baloch, A.R., Franěk, R., Saito, T., Pšenička, M., 2019. Dead-end (dnd) protein in fish-a review. Fish Physiol Biochem. Online. (IF 2018 = 1.729)

Baloch, A.R., Franěk, R., Tichopád, T., Fučíková, M., Rodina, M., Pšenička, M., 2019. Dnd1 knockout in sturgeons by CRISPR/Cas9 generates germ cell free host for surrogate production. Animals 9: 174. (IF 2018 = 1.832)

Baloch, A.R., Fučíková, M., Shah, M.A. Metscher, B., Franěk, R., Tichopád, T., Rodina, M., Pšenička M. 2019. Delivery of iron oxide nanoparticles into primordial germ cells in sturgeon. Biomolecules 9: 333. (IF 2018 = 4.694)

Pan, Y., Wang, M., **Baloch, A.R.,** Zhang, Q., Wang, J., Ma, R., Xu, G., Kashif, J., Wang, L., Fan, J., Cui, Y., Yu, S., 2019. FGF10 enhances yak oocyte fertilization competence and subsequent blastocyst quality and regulates the levels of CD9, CD81, DNMT1, and DNMT3B. Journal of Cellular Physiology 234: 17677-17689. (IF 2018 = 3.923)

Wang, M., Wang, Y., **Baloch, A.R.,** Pan, Y., Tian, L., Xu, F., Chen, S., Zeng, Q., 2018. Chuzan Virus in Yaks, Qinghai-Tibetan Plateau, China. Emerging Infectious Diseases 24: 2371-2373. (IF 2018 = 7.422).

Ji, S., Li, W., **Baloch, A.R.,** Wang, M., Cao, B., 2015. Improved production of sublancin via introduction of three characteristic promoters into operon clusters responsible for this novel distinct glycopeptide biosynthesis. Microbial Cell Factories 14: 17. (IF 2014 = 4.25)

Xu, F., Pan, Y., **Baloch, A.R.,** Tian, L., Wang, M., Ding, L., Zeng, D., 2014. Hepatitis E Virus Genotype 4 sequences in Bos grunniens (Yak), Northwestern China. Emerging Infectious Diseases 20: 2182–2184. (IF 2013 = 7.325)

Manuscripts

Franěk, R., **Baloch, A.R.,** Kašpar, V., Saito, T., Fujimoto, T., Arai, K., Pšenička M. Isogenic lines in fish – a critical review. Reviews in Aquaculture (under review).

Abstracts and conference proceedings

Baloch, A.R., Franěk, R., Saito, T., Pšenička, M., 2019. Dnd1 knockout in sterlet (*Acipenser ruthenus*) generates germ cell free host for surrogate production. 4th International Conference on Agriculture, Food and Animal Sciences (ICAFAS-2019). January 21-22, 2019. SAU Tandojam, Pakistan. (Oral Presentation)

Pšenička, M., **Baloch, A.R.,** Saito, T., 2019. Germ cells technologies in sturgeons. 4th International Conference on Agriculture, Food, and Animal Sciences. January 21–22, 2019, Tandojam, Pakistan. (Invited presentation)

Baloch, A.R., Franěk, R., Pšenička, M., 2018. Targeting *dnd1* in sterlet (*Acipenser ruthenus*) by CRISPR/Cas9 generates phenotypic abnormalities. Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation. June 17-19, 2018. Arendal, Norway. (Oral Presentation)

Baloch, A.R., Franěk, R., Pšenička, M., 2018. Targeting dnd1 in sterlets (*Acipenser ruthenus*) by CRISPR/Cas9 generates germ cell free host for surrogate production. "Precision CRISPR: Drug Discovery & Gene Therapy. Oct, 2018, London, England. (Poster Presentation)

Baloch, A.R., Franěk, R., Saito, T., Pšenička, M., 2018. Targeting *dnd1* in sterlet (*Acipenser ruthenus*) by CRISPR/Cas9 generates phenotypic abnormalities. 11th International Symposium on Reproductive Physiology of Fish. June 3-8, 2018. Manaus, Brazil. (Poster)

Franěk, R., Tichopád, R., **Baloch, A.R.,** Marinović, Z., Lujić, J., Urbányi, B., Horváth, Á., Kašpar, V., Pšenička, M., 2018. Generation and management of isogenic lines of common carp using manipulation with germ stem cells. Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation. 17–19 June, 2018, Arendal, Norway.

Franěk, R., Tichopád, R., **Baloch, A.R.,** Marinović, Z., Lujić, J., Urbányi, B., Horváth, Á., Kašpar, V., Pšenička, M., 2018. Cryopreservation and transplantation of common carp germ stem cells into goldfish. 11th International Symposium on Reproductive Physiology of Fish. Book of Abstracts. 3–8 June, 2018, p. 131.

Baloch, A.R., et al., 2017. Targeting *dnd1* in sturgeons by CRISPR/Cas9 generates phenotypic abnormalities. 6th International Workshop on the Biology of Fish Gametes. Sept 2017. Czech Republic. (Poster)

Bláhová, Z., **Baloch, A.R.,** et al., 2017. Knocking out of delta-6 desaturase by CRISPR/Cas9 in common carp (*Cyprinus carpio* L.). Euro Fed Lipid Congress. Sweden 2017. (Poster)

TRAINING AND SUPERVISION PLAN DURING STUDY

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Research department	2015–2019 – Laboratory of Germ Cells of FFPW
Supervisor	Assoc. Prof. Martin Pšenička
Period	14th December 2015 until 15th September 2019

Ph.D. courses	**Year**
Pond aquaculture	2016
Applied hydrobiology	2016
Basic of scientific communication	2017
Ichthyology and fish taxonomy	2017
English language	2018

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	
Utilization of genome editing techniques for surrogate production in fish	2016
Utilization of genome editing techniques for surrogate production in fish	2017
Utilization of genome editing techniques for surrogate production in fish	2018
Utilization of genome editing techniques for surrogate production in fish	2018

International conferences	**Year**
Baloch, A.R., Franěk, R., Saito, T., Pšenička, M., 2019. Dnd1 knockout in sterlet (*Acipenser ruthenus*) generates germ cell free host for surrogate production. 4th International Conference on Agriculture, Food and Animal Sciences (ICAFAS-2019). January 21–22, 2019. SAU Tandojam, Pakistan. (Oral Presentation)	2019
Pšenička, M., **Baloch, A.R.,** Saito, T., 2019. Germ cells technologies in sturgeons. 4th International Conference on Agriculture, Food, and Animal Sciences. January 21–22, 2019, Tandojam, Pakistan. (Invited presentation)	2019
Baloch, A.R., Franěk, R., Pšenička, M., 2018. Targeting *dnd1* in sterlet (*Acipenser ruthenus*) by CRISPR/Cas9 generates phenotypic abnormalities. Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation. June 17–19, 2018. Arendal, Norway. (Oral Presentation)	2018
Baloch, A.R., Franěk, R., Pšenička, M., 2018. Targeting dnd1 in sterlets (*Acipenser ruthenus*) by CRISPR/Cas9 generates germ cell free host for surrogate production. "Precision CRISPR: Drug Discovery & Gene Therapy. Oct, 2018, London, England. (Poster Presentation)	2018
Baloch, A.R., Franěk, R., Saito, T., Pšenička, M., 2018. Targeting *dnd1* in sterlet (*Acipenser ruthenus*) by CRISPR/Cas9 generates phenotypic abnormalities. 11th International Symposium on Reproductive Physiology of Fish. June 3–8, 2018. Manaus, Brazil. (Poster)	2018
Franěk, R., Tichopád, R., **Baloch, A.R.,** Marinović, Z., Lujić, J., Urbányi, B., Horváth, Á., Kašpar, V., Pšenička, M., 2018. Generation and management of isogenic lines of common carp using manipulation with germ stem cells. Sustaining iconic diadromous fishes: The potential and pitfalls of cultivation. 17–19 June, 2018, Arendal, Norway.	2018
Franěk, R., Tichopád, R., **Baloch, A.R.,** Marinović, Z., Lujić, J., Urbányi, B., Horváth, Á., Kašpar, V., Pšenička, M., 2018. Cryopreservation and transplantation of common carp germ stem cells into goldfish. 11th International Symposium on Reproductive Physiology of Fish. Book of Abstracts. 3–8 June, 2018, p. 131.	2018
Baloch, A.R., et al., 2017. Targeting *dnd1* in sturgeons by CRISPR/Cas9 generates phenotypic abnormalities. 6th International Workshop on the Biology of Fish Gametes. Sept 2017. Czech Republic. (Poster)	2017
Bláhová, Z., **Baloch, A.R.,** et al., 2017. Knocking out of delta-6 desaturase by CRISPR/Cas9 in common carp (*Cyprinus carpio* L.). Euro Fed Lipid Congress. Sweden 2017. (Poster)	2017

Foreign stays during Ph.D. study at RIFCH and FFPW	Year
Internship at Fischucht Röhnforelle GmbH & Co (FRG), Germany.	2016
Internship at Center for Medical Genetics, IRC, VIB, Ghent University, Belgium.	2017
Internship at the Departamento de Ciencia Animal, Universitat Politècnica de València, Spain.	2017
Pedagogical activities	**Year**
International Summer School – 1	2016
International Summer School – 2	2018
Delivered lecture on "Fisheries and Aquaculture in Pakistan" in Ceske Budejovice	2016
Consultant of MSc student Vu Thi Trang	2017

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EDUCATION

2015 – present	Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic
2011–2014	M.Sc., College of Veterinary Medicine, Northwest A&F University, Yangling, China
2006–2011	DVM (Doctor of Veterinary Medicine), Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam, Hyderabad, Sindh, Pakistan
2003–2005	F.Sc., BISE, Hyderabad, Sindh, Pakistan

WORKSHOPS / TRAINING SCHOOLS

02/03–05/03 2016	Second ITN-IMPRESS Training School. Personal Development and Career Plan. Valencia, Spain.
07/03–11/03 2016	5th Aquagamete Training School. Cryopreservation of fish germ cells. Valencia, Spain.
06/06–10/06 2016	6th Aquagamete Training School. Molecular basis of fish gamete quality: genomic tools. Rennes, France.
18/10–26th/10 2016	3rd ITN-IMPRESS Training School. Brookstock management / Entrepreneurship, commercialization and intellectual property rights. Vodnany, Czech Republic.
12/06–21/06 2017	4th ITN-IMPRESS Training School. Chanteuges, Loire, France.

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23/06–23/07 2017	Professor Kris Vleminckx. Internship at the Department of Biomedical Molecular Biology, Center for Medical Genetics, IRC, VIB, Ghent University, Ghent, Belgium.
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a ochrany vod
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