University of South Bohemia in České Budějovice

Faculty of Fisheries and Protection of Waters

DIPLOMA THESIS

Year of defense: 2017

Author: VU THI TRANG

University of South Bohemia in České Budějovice

Faculty of Fisheries and Protection of Waters

Research Institute of Fish Culture and Hydrobiology

Laboratory of Germ Cells

DIPLOMA THESIS

"Utilization of genome editing technology to knock out *dnd1* gene in sturgeons"

Author: Vu Thi Trang, BSc

Supervisor: Assoc. Prof Dipl.-Ing. Martin Pšenička, Ph.D

Advisor: Abdul Rasheed Khanzai Baloch, MSc

Study program: Master study program

Field of study: Agricultural specialization/Fishery and Protection of Waters

Form of study: Full-time

Year of study: 2015 - 2017

České Budějovice, May 2017

Declaration

I declare that I have written my Diploma thesis on my own and that I have used only the sources listed in the bibliography. I declare that in compliance with Section 47b of the Act No. 111/1998 Coll., as amended, I agree with publishing of my Diploma thesis in its unabbreviated version or in an adapted version based on leaving out marked parts archived by the FFPW USB. The publishing is made through an electronic way in the part of the STAG database, which is accessible to public and managed by the University of South Bohemia in České Budějovice on its internet pages. When doing so, my copyright to the submitted text contained in this final thesis will be maintained. I also agree that supervisor's and reviewers' opinions as well as the record of the course and results of the final thesis defence are published through the same electronic way in compliance with the above- mentioned provisions of Act No. 111/1998 Coll.. I also agree that the text of my final thesis will be compared with the Theses.cz database which is administered by the National Registry of Theses and the system for plagiarism search.

Date

Student's signature

Acknowledgement

I am heartily grateful to my M.Sc thesis supervisor Assoc. Prof Dipl.-Ing. Martin Pšenička, Ph.D, for his consistent encouragement, proper guidance and positive criticism throughout the course of my studies, without which it was not possible to accomplish my M.Sc thesis project.

I would also like to thank Abdul Rasheed for his assistance, valuable comments and encouragement during my study period. Special thanks go to my colleagues and friends in Vietnam and Czech Republic for their encouragement and information sharing.

Finally, I would like to convey my warmest gratitude to my parents, my husband, my daughter and all members in my family for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of research and writing this thesis.

Author

VU THI TRANG

Diploma thesis assignment

Contents

1 Introduction

Primordial germ cells (PGCs) are embryonic precursors of gametes. They migrate from the place of their origin into genital ridge, where they develop into gonial cells, undergo proliferation, differentiation and recombination of genetic information during gametogenesis (Saito and Psenicka, 2015). The function of *dead end* (*dnd1*) gene has been described well in model species, and in zebrafish it is crucial for the migration and development of PGCs (Weidinger *et al.* 2003). At early stage of embryonic development, *dnd1* is maternally supplied and later the *dnd1* transcript is produced by germ stem cells until differentiation. However, little is known about its function in several other species.

Sturgeon, as representative of fish with holoblastic cleavage pattern of embryo, has a completely different origin of PGCs as well as deposits of maternally supplied *dnd1* in comparison with teleostean fishes (Saito *et al.* 2014). Therefore, it will be interesting to reveal more about the function/s of *dnd1* in these ancient species. Recently, by using antisense morpholino oligonucleotides we have found that the inhibition of translation of maternally supplied RNA led to un-directional migration and depletion of PGCs in sturgeons (Linhartová *et al.* 2015). In our present study, we aim to extend the state-of-art and target directly DNA in order to elucidate the function of *dnd1* transcribed from zygotic genome after migration of PGCs. For this purpose, we used CRISPR/Cas9, a cutting-edge genome editing technology that presents certain advantages over other genome editing technologies such as TALENs and ZFNs. This technology has been successfully used in various animal models, and in zebrafish, the CRISPR/Cas9 can generate gene knockout with very high efficiency (Hwang *et al.* 2013). It is assumed that knock out of *dnd1* will lead to sterilization of treated animals; hence they can be practically used as recipients for technology of surrogate production. In addition, this will be a pioneer study on utilization of recent techniques of genome editing technology, CRISPR/Cas9, in sturgeons.

2 Review of literature

Genome editing nucleases designed as a kind of genetic engineering in which DNA is inserted, removed or replaced in the genome of an organism using nucleases design, or molecular scissors. These nucleases create double strand breaks (DSBs) at the desired location in the genome. The targeted mutation can be occurred after the induced DSBs are repaired through Homologous Recombination (HR) or Non-Homologous End Joining (NHEJ). HR can act as an exchange intermediary between an endogenous genomic region and an exogenous DNA fragment through flanking homologous sequences, leading to DNA insertion, deletion or replacement. DNA repair by NHEJ often contain small deletion and insertion at the break sites. Therefore, the incorrect DSBs repair by NHEJ can be applied for gene disruption by introducing frame-shift mutations. Genome editing have numerous applications in industry, agriculture and human therapeutics. Currently, there are some engineered nucleases being used such as Meganucleases, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and CRISPR-Cas9 system.

Meganucleases, ZFNs and TALENs has been widely used for genome editing in a different kind of cells and organisms. Meganucleases were designed from natural restriction enzymes version that mostly have extended the DNA sequence identity. Using technical meganucleases was challenging for most academic researchers because the DNA recognition and separation of these enzymes are intertwined in a single domain. Conversely, ZFNs and TALENs are proteins and synthetic reactions including a DNA binding domain designed nuclease incorporated in a non-specific domain of FokI restriction enzymes. DNA binding domains of ZFNs and TALENs are distinct from FokI cleavage domain, thus making them much easier to change the DNA of the specific nucleases (Sander and Joung, 2014).

2.1 Zinc finger nucleases (ZFNs)

Zinc finger is a small protein structural motif with 30 amino acid that is characterized by coordination of one zinc atom using two His and two Cys residues in order to stabilize the fold (Sun and Zhao, 2013). With structurally diverse and presenting among proteins, zinc fingers perform a broad range of functions in various cellular processes, such as replication and repair, transcription, translation, metabolism, signaling, cell proliferation and apoptosis. They are typically function as interaction modules and bind to a wide variety of compounds,

such as nucleic acids, proteins and small molecules (Krishna *et al.* 2003). Zinc finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that facilitate targeted editing of genome by creating double-strand breaks in DNA at user-specified locations. Each ZFN consists of two functional domains (Gaj *et al.* 2013). The first one is DNA-binding domain where typically contain between three and six individual zinc finger repeats which can each recognize 9 or 18 bp. With a 18 bp sequence, it can define a unique sequence of the entire genome, allowing detection editing points. The second one is the non-specific DNA-cleaving domain comprised of the nuclease domain of FokI is responsible for the catalytic cleavage of DNA. When the DNA-binding and DNA-cleaving domains are fused together, a highly-specific pair of genomic scissors are created (Gaj *et al*. 2013).

ZFNs are useful to manipulate the genomes of many plants, animals and human including for instances: Tobacco (*Nicotiana sp*.) (Cai *et al.* 2009); Maize (*Zea mays*) (Shukla *et al.* 2009); Fruit fly (*Drosophila melanogaster*) *(Bibikova et al. 2002)*; Mouse (*Mus musculus*) (Cui *et al.* 2011) and Human (*Homo sapiens*) (Porteus and Baltimore, 2003), (Urnov *et al.* 2005), (Perez *et al.* 2008), (Kim *et al.* 2009). In Zebrafish (*Danio rerio*) (Doyon *et al.* 2008) designed ZFNs for golden and no tail/Brachyury (ntl) targeted genes. The result shown that more than a half of fish transmitted disrupted ntl alleles at frequencies averaging 20% when using ZFN mRNA injected. In other research of Meng *et al.* (2008), they used ZFNs technology to create targeted mutations in the zebrafish germ line. The gene sequences of ortholog of the vascular endothelial growth factor-2 receptor, kdr (also known as kdra) were recognized by engineered ZFNs. By another approach, Foley *et al.* (2009) used OPEN (Oligomerized Pool ENgineering) to generate successfully engineered ZFN pairs for five endogenous zebrafish genes: tfr2, dopamine transporter, telomerase, hif1aa, and gridlock. Each of these ZFN pairs caused expected insertions and deletions with high efficiency at targeted genome in zebrafish. Their study suggested that to introduce any interest targeted mutations gene in zebrafish, OPEN presents a reliable method.

2.2 Transcription Activator-Like Effector Nucleases (TALENs)

Transcription activator-like effector nucleases (TALENs) are restriction enzymes that can be engineered to bind practically with any desired DNA sequence and then cutting at specific sequences of DNA. This system works similarly to ZFNs (Gaj *et al.* 2013; Sun and

Zhao, 2013) and contain a non-specific FokI nuclease domain. They are made by fusing a TALE DNA-binding domain to a non-specific DNA cleavage domain FokI, which functions as dimers. The DNA-binding domains derived from TALE proteins which are secreted by *Xanthomonas* bacteria by way of their type III secretion system when they infect plant cells. Each TALE comprises of 33–35 amino acid and recognizes a single base pair (Sun and Zhao, 2013). Non-specific DNA cleavage domain from end of FokI endonuclease can be used to construct hybrid nucleases that are function in a yeast assay. These reagents are also found in plant cells and in animal cells. Compare with ZFNs, TALENs construction is simpler, potentially more reliable and in the few cases examined, shows fewer off-target effects (Clark *et al.* 2011).

Alongside ZFNs, TALENs recently are extensively efficient application in introduction of targeted alterations in a wide range of organisms. The first report about performing TALENs technology in modification fruit fly genome was conducted by Liu *et al.* (2012), in roundworm was studied by Wood *et al.* (2011). Besides that, there was some research of using TALENs in frog (Lei *et al.* 2012), in rat (Tesson *et al.* 2011), in pig (Carlson *et al.* 2012) and in human (Miller *et al.* 2011), (Hockemeyer *et al.* 2011). To check the activation ability of TALE nucleases in zebrafish, Sander *et al.* (2011) constructed four and two TALE nuclease monomers to a site in the two targeted endogenous zebrafish genes *gria3a* and *hey2*, respectively. The six TALE nuclease monomers fuse with the FokI cleavage domain and RNA encoding these different TALE nuclease pairs then injected into zebrafish embryos at one-cell stage. The authors found that high targeted insertions/deletions (indels) frequencies (from 11 to 33%) induced by four pairs of TALE nucleases. With the new approach, Bedell *et al.* (2012) used enhanced TALEN toolkit (GoldyTALEN modified scaffold) in zebrafish and the result shown that the specific locus DNA broke both in somatic and germline tissues at a high efficiency. The TALENs technology may have more advantages than ZFNs because of they can be engineered relative ease and their possible ability can be targeted to a wide range of sequences.

2.3 CRISPR/Cas9

CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-Associated Proteins 9) is a particularly unique technology that allows scientist to edit parts

of genome by removing, adding or altering segments of the DNA sequence. It is currently the simplest and precise method of genetic manipulation compare with previous DNA editing technique and has a wide range of potential applications. Ability to precisely edit and change any part of an organism's genome has been long sought by scientists and we are today closer to that goal than ever before. With the discovery of the CRISPR/Cas9 system scientists are now able to effortlessly and efficiently knock-out or knock-in any gene of interest. Given its ease of use, RNA-directed CRISPR/Cas9 genome-editing system offers a versatile platform and has potential to supplant the ZFNs and TALENs approaches.

In the mid-2000s, a few microbiology and bioinformatics laboratories began to investigate CRISPR, which had been described in 1987 by Japanese researchers as a series of short direct repeats interspaced with short sequences in the genome of *Escherichia coli* (Ishino *et al.* 1987). CRISPRs are segments of prokaryotic DNA containing short repetitions of base sequences. Each repetition is followed by short segments of spacer DNA from previous exposures to a bacterial virus or plasmid (Marraffini and Sontheimer, 2010). CRISPR/Cas9 was observed originally in bacteria and archaea as an adaptive microbial immune system that provides acquired immunity against foreign viruses and plasmids (Gasiunas *et al.* 2012). Adaptive immunity occurs in three stages. The first stage is insertion of a short sequence of the invading DNA as a spacer sequence into the CRISPR array. The second stage is transcription in which CRISPR arrays are transcribed and processed into small interfering CRISPR RNAs (crRNAs), each composed of a repeat portion and an invader-targeting spacer portion. The final stage is cleavage of foreign nucleic acid by Cas proteins at sites complementary to the crRNA spacer sequence (Barrangou and Marraffini, 2014). There are three major types of CRISPR Cas system classified base on Cas protein sequences and structures, namely type I, type II and type III to achieve nucleic acid recognition and cleavage (Makarova *et al.* 2011). While the type I and type III systems use a large complex of Cas proteins for crRNA-guided targeting (Nam *et al.* 2012), the type II system requires only a single protein for RNA-guided DNA recognition and cleavage (Jinek *et al.* 2012; Hsu *et al.* 2014). Therefore, type II is a property that proved to be extremely useful for genome engineering applications. In the type II CRISPR loci mostly consist of the cas9, cas1, and cas2 genes, as well as a CRISPR array and tracrRNA (Hsu *et al.* 2014).

Genome editing is carried out with a type II CRISPR system associated with cas9 is called CRISPR/Cas9.

Targeted genome editing using CRISPR/Cas9 technology has two components: an endonuclease and a single guide RNA (sgRNA). The endonuclease is the bacterial Cas9 nuclease protein from *Streptococcus pyogenes*. In the family of Cas9 nuclease, there are two DNA cleavage domains: the RuvC and HNH-like nuclease domains that cleave double stranded DNA leading to double strand breaks (DSB). While HNH is a single nuclease domain, RuvC domain is divided into three subdomains across the linear protein sequence, with RuvC I near the N-terminal region of Cas9 and RuvC II/III flanking the HNH domain near the middle of the protein (Hsu *et al.* 2014). The sgRNA is an engineered single-stranded chimeric RNA which combining the scaffolding function of the bacterial tracrRNA with the specificity of the bacterial crRNA that retains two critical features (Jinek *et al.* 2012). The first feature is the 20-nucleotide at the 5' end of the sgRNA acts as a homing device, which recruits the Cas9/sgRNA complex to a specific DNA target site, directly upstream of a protospacer adjacent motif (PAM), by Watson-Crick base pairing. The second one is double-stranded structure at the 3′ side of the guide sequence that binds to Cas9 (Jinek *et al.* 2012). This created a simple two-component system in which changes to the guide sequence (20 nucleotides in the native RNA) of the sgRNA can be used to program CRISPR/Cas9 to target any interested DNA sequence as long as it is adjacent to a PAM. In contrast to ZFNs and TALENs, to modify each target DNA site, they require substantial protein, the CRISPR/Cas9 system requires only a change in guide RNA sequence. The scientists adopted CRISPR/Cas9 technology to target, edit, or modify the genomes of cells and organisms. This application has been rapidly and widely used recently (Doudna and Charpentier, 2014).

PAM is a short sequence motif adjacent to crRNA-targeted sequence on the invading DNA which plays essential role in adaptation stage and interference in type I and type II systems (Shah *et al.* 2013). CRISPR/Cas9 system can be targeted towards any genomic region through the design of a sgRNA; however, the specificity of system depends on PAM located immediately downstream of the target sequence (Zhang *et al.* 2014). Recognition sequence of PAM differs depending on the species and the type of the bacteria from which the Cas9 nuclease is derived. The most commonly used type II CRISPR system uses the Cas9

nuclease from *S. pyogenes*. This is particular nuclease that recognizes 5'-NGG on the immediate 3' end of the sgRNA sequence. Mechanistic studies show that the PAM is critical for initial DNA binding, in case of absence of the PAM, the sequence is not recognized by Cas9 even target sequences fully complementary to the guide RNA sequences (Sternberg *et al.* 2014). Our CRISPR/Cas9 system utilizes the type II CRISPR system from *S. pyogenes*, which recognizes the 5'-NGG PAM sequence.

CRISPR/Cas9 technology offers several advantages such as: target design simplicity, efficiency modifications and multiplexed mutations. Therefore, this system has been widely used for genome editing in many kinds of animals. For example, in Drosophila, Bassett A *et al.* (2013) found that mutations at desired target genes up to 88% of injected embryos by using CRISPR/Cas9 technique. In addition, the research of Yu *et al.* (2013) and Port *et al.* (2014) also shown that Cas9/gRNA is an attractive tool for genome modifications in Drosophila. With the role as an important model organism for scientific research, zebrafish is a subject of numerous experimentations about gene editing by CRISPR/Cas9 system. For instance, the studies of Hwang *et al.* (2013) and Chang *et al.* (2013) together proved that microinjection of customizable sgRNA and Cas9–encoding mRNA into zebrafish embryos at one-cell stage is a rapid and simple manner with high capable modify the target genes *in vivo*. In another research, Hruscha *et al.* (2013) demonstrated that the efficient of mutation in zebrafish causing by CRISPR/Cas9 reached up to 86% and heritable. Besides, there were other studies about using CRISPR/Cas9 technique in zebrafish, such as the study of Irion *et al.* (2014), Ablain *et al.* (2015) and Shah *et al.* (2015). Similarly, editing genome by CRISPR/Cas9 were also conducted on rats and mice with studies of Li *et al.* (2013) and Wang *et al.* (2013), Yang *et al.* (2013), respectively.

Molecular genetics plays an important role in the discovery of the molecular mechanisms of disease. Generally, genetic modification in animal models are essential methods to understand human diseases both in gene functions and pathogenesis. Compared with other methods, scientists realize that the CRISPR/Cas9 system can be applied easily and efficiently for multiple genes editing in animal disease models. There were a lot of studies about human diseases by CRISPR/Cas9 approach and using mice as disease models. For liver cancer, Xue *et al.* (2014) have aimed to *phosphatase and tensin homolog* (*Pten*) and *p53* genes. These

genes were inhibited by a complex Cas9 and sgRNAs, which was designed by hydrodynamic injection to deliver a plasmid encoding to sgRNAs/Cas9. With contextual memory, Swiech *et al.* (2015) injected adeno associated viruses (AAVs) that encoding Cas9 and an sgRNA that targeting to the *methyl-CpG binding protein 2 (MeCP2)* gene. The multiple mutations possibility when using CRISPR/Cas9 system was applied by study of Weber *et al.* (2015). This system mutates in broad gene sets and causing hepatocellular carcinoma and intrahepatic cholangiocarcinoma in mice. The result revealed about gene function and functional annotation of genes that induce cancer in mice.

Application of CRISPR/Cas9 in non-model fish

Application of CRISPR/Cas9 technology in inducing targeted mutagenesis in fish has been coming common recently not only in model fish but also in non-model fish. Studies of gene function in non-model organism have so far been difficult to implement because lack of information and approaches available for eliminating gene function. However, there were studies by using CRISPR/Cas9 to produce gene function changes. Atlantic salmon (*Salmo salar*) is an important commercial species in aquaculture and salmon genetics is currently being attracted more attention (Davidson *et al.* 2010). Specific target of two genes *tyrosinase (tyr)* and *solute carrier family 45, member 2 (slc45a2)* have known to be associated in pigmentation in zebrafish and other species were used as targeted genes editing by CRISPR/Cas9 technology in Atlantic salmon (Edvardsen *et al.* 2014). These sequences were cloned into a CRISPR vector constructed for zebrafish (Jao *et al.* 2013) then the constructs were injected into Atlantic salmon embryos at one-cell stage together with Cas9 mRNA. Embryos mutation rates were examined at the 17 somite stage. The results showed that there was high degree of mutation in injected embryos with 40% and 22% mutation induction for *slc45a2* and *tyr*, respectively (Edvardsen *et al.* 2014). This study shows for the first time successfully use of CRISPR/Cas9 in editing genes in non-model animals as well as in a marine cold water species. In Nile tilapia (*Oreochromis niloticus*), the whole genome was sequenced together with available monosex populations which made study the genes contributed to sex determination much easier. The CRISPR/Cas9 system was applied in research of Li *et al.* (2014) to disrupt genes concerning to sex determination in tilapia. These genes including *nanos2, nanos3, dmrt1, and foxl2* were selected as target to show the ability

of CRISPR/Cas9 mediated mutation in this species. The resulting represented that the indel frequencies were 38% (*nanos2*), 49% *(nanos3*), 22% (*dmrt1*) and 42% (*foxl2*). Moreover, mutation in *dmrt1* and *foxl2* were transmitted efficiently to F_1 through the germline. Lampreys are jawless fish in the order *Petromyzontiformes* and placed with hagfishes in the class *Cyclostomata.* The sea lamprey *Petromyzon marinus* has been used largely in study the vertebrate development evolution among lamprey species. Its whole genome was sequenced which provides an important resource for rebuilding vertebrate origins and the evolutionary events (Smith *et al.* 2013). The study of Square *et al.* (2015) reported that CRISPR/Cas9-mediated interruption of the genes *tyrosinase* and *FGF8/17/18* in the sea lamprey with the high frequencies.

2.4 Sturgeons

The sturgeons are known as "living fossil" in literature, and these are distinguished by their elongated body, lack of scales and large size frequently ranging from 7-12 feet (2-3½ m) in length are common. According to International Union for Conservation of Nature (IUCN) (http://www.iucnredlist.org/news/sturgeons-highly-threatened), 63% of 27 species of sturgeon are on the red list of critically endangered species. Human's activities such as illegal fishing, overexploitation, breaking up of the migratory routes and pollution are the key factors that made almost all species to the brink of extinction. Moreover, sturgeons can live up to 100 years and do not reproduce every year, which means that they take many years to recover from any population declines. A ray of hope for this species is their ability of producing millions of eggs. If proper protection can be given in their reproductive place may gradually supplementing their population.

The functions of polyploidy in evolution and biology diversity has been long admitted in plants. However, the possibilities of genome duplication in vertebrate and their important roles in evolution have recently received more consideration. Sturgeon (order *Acipenseridae*) presents as an optimal taxonomic context for exploration of genome duplication events. Difference levels of ploidy occur among these species. Polyploidies could occur through chromosome duplication within a specie (autopolyploidy) or in combination by interspecific hybridization (allopolyploidy). In sturgeons, there were some proposed theories to explain about polyploidization events: from several whole genome duplication events occur

independently in different lineages (Ludwig *et al.* 2001) and multiple hybridization events (Fontana, 2002) to both autopolyploidy and allopolyploidy associations (Fontana *et al.* 2008). According to Fontana (1994), Fontana *et al.* (1998), Tagliavini *et al.* (1999) and Ludwig *et al.* (2001) supposed that sturgeons with approximately 120 chromosomes to be functional diploids and sturgeons with around 250 chromosomes are called functional tetraploid due to functional genome reduction events. According to Ludwig *et al.* (2001), there were 962 fish from 20 sturgeon species were used for determining ploidy in sturgeons by microsatellite markers approach. The result shown that all investigated fish can divided in different three groups of chromosomes number: the first group with around 120 chromosomes including all species which have from 110 to 130 chromosomes; the second group with around 250 chromosomes including all species which have between 220 and 276 chromosomes; and the last group is represented only by *Acipenser brevirostrum* that having around 360 chromosomes. It means that *A. brevirostrum* is the specie with the highest chromosome number and highest amount of DNA among all *Acipenseridae* species. The theory of genome duplication and following reduction in functional ploidy has been applied, even in the evolutionary diploid vertebrates. Diploidization is the repeated loss of chromosomes by a tetraploid organism to become diploid. Functional diploidization is one of the most exciting aspects of genome evolution that has still not explained entirely. It is the evolutionary process in which the whole-genome duplication tetraploid organism degenerates its gene content to become functional diploid but it still maintains twice as many as distinct chromosomes (Wolfe, 2001).

Sterlet (*Acipenser ruthenus*) inhabits exclusively in freshwater and representing as the smallest and the fastest reproductive cycle among sturgeons. The sterlet males and females can reach maturity in the second and third year of life, respectively, but mostly they mature at age 3-4 with males and 4-5 with females. It is only sturgeon species that inhabits on two continents, both Europe and Asia (Bemis and Kynard, 1997). There was some scientific research on sterlet started in the years 1950s. These research activities were mainly focused on morphological analyses, reproductive cycle, fecundity, nutrition, population age structure and ability migration. The 1990s were marked development in the field of biomarkers and some species were use as biomarkers to achieve early warning signals of environmental risks (van der Oost *et al.* 2003). Sterlet is exposed to contaminants from both water and sediments

since it is a bottom feeding species, therefore, it could play the role as good indicator of water and sediment pollution (Poleksic *et al.* 2010). However, its ability migration to large distance was the main problem in using sterlet as indicator fish. In recent decades, most natural of Eurasian sturgeon populations have dropped dramatically reaching historic low levels today. The investigation of Ludwig *et al.* (2009) was the first describe case hybridization between non-native Siberian sturgeons in Western Europe with native sterlets in the Upper Danube River. Meiotic gynogenesis in sterlet was conducted by Rekubratskii *et al.* (2003) using the method of insemination of the eggs with UV-irradiated spermatozoa and elimination of the second meiotic division by heat shock. Microsatellite markers were developed for lake sturgeon (May *et al.* 1997), for shovelnose sturgeon (McQuown *et al.* 2000) and Atlantic sturgeon (King *et al.* 2001) were used in cross-species amplification in sterlet (Ludwig *et al.* 2009).

2.5 Primordial Germ Cells (PGCs)

When the eggs are fertilized and divided several times to produce the cells of the early embryos, the cells that inherit specific molecules localized in a particular region of the egg cytoplasm become PGCs. PGCs are precursor to germ cells that give rise to gametes. In animals, PGCs arise far from the somatic cells of the developing gonad (somatic gonadal precursors - SGPs), therefore they have to actively migrate across the embryo to reach their site of function (Raz, 2004). In fish, PGCs also form at various locations by the maternal inheritance supplied germplasm and then migrate toward the place where the gonad will develop (Saito and Psenicka, 2015). PGCs form early in embryonic development and can be readily identified by morphology, embryonic position and gene expression profile. Therefore, it can be analyzed by live and fixed imaging approaches. Such approaches, combined with genetic analysis, PGC migration has begun to clarify in the cellular behaviors and molecular mechanisms (Richardson and Lehmann, 2010). Sturgeons (genus *Acipenser*) have developmental pattern similarly to that of amphibians, although their phylogenetic position is an out-group to teleost fishes. They are thereby great potential for comparative and evolutionary studies of development. Germplasm and PGCs can be seen by using injection technique for sturgeon eggs. The result demonstrates that the sturgeon's PGCs are specified by inheritance of maternally supplied germplasm and generated at the vegetal pole

of the egg with holoblastic cleavage and then they migrate on the yolky cell mass toward the gonadal ridge (Saito *et al.* 2014).

PGCs are the only cells in developing embryos have potential to transfer genetic information to the next generation, undergo proliferation and differentiate into functional gametes in sexually reproducing organisms (Yamaha *et al.* 2010). PGCs therefore are good candidates for induction of germ line chimerism through transplantation. Using PGCs transplantation to obtain donor-derived offspring, within and between species, has been demonstrated feasibility in some animal species including teleost fish (Saito T, 2010). The production of germ line chimerism biotechnology could provide important advantages such as: shortening the reproduction period of sexually late maturing species; reducing the space for culture when small fish species used as hosts; conserving germ cells for maintaining genetic resources; retaining target species without keeping adult fish (Yazawa *et al.* 2013; Linhartova *et al.* 2014). Among sturgeon species, the sterlet (*Acipenser ruthenus*) has been known with a small body size and the fastest reproductive cycle (sexually mature in 5 years). Therefore, it can be used as a *host* to produce gametes of critically endangered species (*donors*) with large body size and a long reproductive cycle such as beluga (maturating in approximately 20 years). Furthermore, a *sterile host* is prerequisite criteria for production of the *donor's* gametes only. For this idea, Linhartová *et al.* (2015) produced sterlet sterile host by using knockdown agent - antisense morpholino oligonucleotide to deplete dead end gene which lead to block the migratory activity of PGCs. The result has shown that no PGCs were present in the body cavities of morphants at 21 days after fertilization.

Formation and migration of PGCs in sturgeon

The zygotes of many species experience into fast cell cycles to produce a cluster of cells with the same size as original zygote without increasing the mass. This process is called embryonic cleavage. Depending mainly on the amount of yolk in the egg, the embryo cleavage form can be holoblastic or meroblastic. While holoblastic is characterized by complete cleavage that the whole egg divides into distinct and separate blastomeres, meroblastic is described by incomplete cleavage that the only a portion of the egg divides. The holoblastic type of cleavage is commonly seen in amphibians, echinoderms, flatworms, nematodes and mammals, whereas meroblastic is observed in most insects, cephalopod

molluscs, reptiles, birds and fish. Study about sturgeon embryogenesis has been provided a valuable point in comparison between teleost and amphibians because their phylogenetic is located an out-group of teleost. Sturgeon eggs have holoblastic cleavage and their embryos develop more similarly to *Xenopus* than teleost in many aspects (Bolker, 1993). In teleost embryo such as zebrafish undergoes meroblastic cleavage in which the animal half of egg divides to form three germ layers while the nutritive yolk part from the vegetal half will not differentiate into embryonic body.

The formation of PGCs is altered according to the forms of embryo cleavage from holoblastic to meroblastic. In *Xenopus*, germplasm aggregates after fertilization by accompany and reorganization of microtubules. In anuran and teleost, PGCs are particularized by inheritance of maternally supplied. The GFP-*nos*3 3'UTR mRNA which is known as efficient visualization of teleost PGCs was injected to animal or vegetal pole at 1 cell stage of sturgeon embryos to determine the region where PGCs were generated. The visualized at blastula stage showed that GFP expression was detected at the injected hemisphere only. PGCs-like cells were not observed in embryos that were injected mRNA into animal pole region. However, they expressed with stronger GFP fluorescence than surrounding cells in embryos treated with mRNA at the vegetal pole. This expression frequently occurred during development of embryos. Furthermore, PGCs-like cells were originally recognized near the yolk-rich endoderm after the closed neural tube stage. This result illustrated that sturgeon PGCs are specified in the vegetal pole as the same manner as anurans. Then they migrated toward the embryonic body (Saito *et al.* 2014). Sturgeon PGCs migrated after stage 22 on the yolk ball and via the mesenchyme they migrate toward to the gonadal ridge with a long distance from their origin position (Saito *et al.* 2014).

2.6 Dead end1 gene (*dnd1*)

The recent identification of several germ cell markers has helped to advance the research on germline development. There are dozens of genes essential for PGCs development which are known in the model invertebrates and lower vertebrates (Houston and King, 2000). For instance, in *Drosophila*, *oskar* acts as PGC specifier that is necessary for PGC formation (Ephrussi and Lehmann, 1992). Other one, *piwi* - an evolutionarily conserved gene is also crucial for PGC formation (Megosh *et al.* 2006). *Piwi* dispensable for PGC specification but

essential for subsequent PGC development in vertebrates, such as spermatogenesis in mouse (Deng and Lin, 2002), germ cell maintenance in zebrafish (Houwing *et al.* 2007) and PGC migration in medaka (Li *et al.* 2012). Among those, dead end1 gene (*dnd1*) is a vertebrate specific component of germplasm that encodes RNA-binding protein crucial for PGCs migration and survival which was first discovered in zebrafish (Weidinger *et al.* 2003). Its homologues have been identified in other vertebrates such as mouse, chicken and *Xenopus*. At early stages of embryonic development, *dnd1* is maternally supplied and later, *dnd1* transcript is produced by germ stem cells until differentiation. Following germline specification, *dnd1* is exclusively expressed in PGCs which is mediated through a common 3' untranslated region (UTR) dependent mechanism for the maternal localization determinants to the germline (Slanchev *et al.* 2009). In zebrafish *dnd1* protein is crucial for the migration and development of PGCs, it was shown to regulate the expression of two other germline specific genes, *nanos3* and *tudor domain* containing protein 7 (tdrd7), presumably by protecting them from miRNA-mediated degradation of 3'UTR (Mishima *et al.* 2006). To study the role of *dnd1* for proper development of gonad in teleost fish, several knockdown assays were conducted. For instance, in zebrafish, knockdown of *dnd1* by injection of morpholino oligonucleotides (MO) during early cleavages, which led to loss of PGC migration and their eventual death apparently without affecting somatic development (Weidinger *et al.* 2003). *Dnd1* knockdown in Atlantic cod resulted in the complete loss of *nanos3* positive PGCs at late somitogenesis (Škugor *et al.* 2014). *Dnd1* also serves as a critical PGC specifier in medaka fish *(Oryzias latipes)* where *dnd1* depletion specifically abolished PGCs, and its overexpression boosted PGCs (Hong *et al.* 2016).

In this study, we used the *dnd1* sequence of sterlet as a targeted sequence when we designed sgRNA, primers and it was the reference sequence for alignment with our sequences. The *dnd1* gene sequence of sterlet (*Acipenser ruthenus – Ardnd*) shares 97% identity with *dnd1* gene sequence of Russian sturgeon (*Acipenser gueldenstaedtii - Agdnd*) (Linhartová *et al*. 2015). The Medaka maturity fish had normal survival and development when they were disrupted *dnd1* gene by TALENs in embryonic stages (Wang and Hong, 2014). The research of Hong *et al.* (2016) used two morpholino oligos for *dnd1* depletion in medaka: MO *dnd1* targets the medaka *dnd* mRNA and MOddm is a MO*dnd* mutant derivative by introducing four mismatches which were directly microinjected into medaka

embryos at one-cell stage. Resulting showed that the complete absence of PGCs in all manipulated embryos (n = 333) when injection with 50 – 100pg of MO *dnd1* while there was no effect on the PGCs number when embryos were injected by MOddm. In *Xenopus*, *dead end* mRNA localizes to the vegetal pole of oocytes and inhibition translation of *dead end* in *Xenopus* embryos results to loss of PGCs at tadpole stages of development (Horvay *et al.* 2006). The function of *dnd1* gene in forming and development of PGCs was also investigated in mice (Bhattacharya *et al.* 2007), in rat (Northrup *et al.* 2011) and in chicken (Aramaki *et al.* 2009).

2.7 Germ cells transplantation in fish

Numerous assisted reproductive technologies such as cryopreservation of gametes and embryos, embryo transfer and germ cell transplantation have been introduced to create functional gametes and offspring from endangered or commercial species that are difficult to cultivate in captivity. Particularly interested in developing effective method of germ cell transplantation for fish due to increasing interest in declining fish stocks and loss of biodiversity due to overexploitation (Pukazhenthi *et al.* 2006). The success of this method mainly depends on the available recipients that are completely endogenous stem cells and the recipient gonads are also genetically compatible with donors. However, in fact most recipients present a little or no rejection when transplanted cells into them even if they come from unrelated relative with donors. This advantage makes potential to use domesticated strains or commercially valuable species as surrogated recipients in germ cell transplantation (Honaramooz *et al.* 2005; Yazawa *et al.* 2010).

The development of a germ cell transplantation technique in the study of male germ line was the first successfully reported in chicken where PGCs were transferred to the blood stream of developing embryos. The sexual maturity of germline chimeras then were able to produce derived donor offspring (Tajima *et al.* 1993). Then this technique was established to study in rodents. According to the research of (Brinster and Zimmermann, 1994), these authors used the method utilized a cell suspension collected from donor testis then injected to the seminiferous tubules of sterilize recipient mice. Spermatogonial stem cells presented in the injected cell suspension. They were able to colonize the recipient seminiferous tubules,

reestablishing spermatogenesis and maintaining donor spermatogenesis (Brinster and Zimmermann, 1994).

The transplantation of the germ cells is becoming a promising approach in preservation of endangered species including fish with different approaches have been described in teleost. Currently available methods have been used recipient fish at varying development stages and PGCs are optimal donor cells because of their potential to develop into either male or female gametes. For instance, intra-specific transplantation of PGCs into blastula-stage fish embryos were success in zebrafish and their F1 offspring generation showing donor-derived mutant phenotypes (Ciruna *et al.* 2002; Giraldez *et al.* 2005). In this method, the PGCs of the recipient were blocked by antisense morpholino oligonucleotide (MO) while other PGCs from donor embryos were harvested and injected into blastula stage of recipients. The resulting represented the complete replacement PGCs of donor in the recipients. Another method was developed by (Saito *et al.* 2008) has been used in xenogenic transplantation of PGCs. These authors indicated that a single PGC in pearl danio (*Danio albolineatus*) could be microinjected into the blastodisc of each host embryo zebrafish (*Danio rerio*) at blastula stage. Before microinjection, the host germ-line cells were blocked by an antisense dead end morpholino oligonucleotide. The hosts then matured to male and female chimeras that produced the donor gametes only. These chimeras could spawn naturally and their offspring were analyzed by morphology and DNA showed that offspring were of donor origin. This was possible because the PGCs transplantation of donor fish was performed before the period of sexual determination in recipient fish (Ciruna *et al.* 2002; Giraldez *et al.* 2005; Saito *et al.* 2008). The achievement of this technique not only indicated in using the donor and recipient with close relationship but also in species with phylogenetic distance. In the same research of (Saito *et al.* 2008) shown that when transplantation PGCs from goldfish (*Carassius auratus*) and loach (*Misgurnus anguillicaudatus*) into zebrafish blastulae, chimeras were successfully generated donor sperm. On the other hand, this investigation was unsuccessful in demonstration the normal development ovaries in females of xenogeneic chimeras. However, this technique can be used to produce millions of gametes in recipient gonads by using only one donor PGC. Therefore, this approach contributes to increase the reproduction potential of fish strains carrying valuable traits that not requirement the large amount of donor PGCs (Saito *et al.* 2008).

Another development stage in fish can be used for germ cell transplantation is newly hatched larvae. In this method, donor germ cells were isolated and transplanted into peritoneal cavities of newly hatched larvae by using microinjector. The first report of intraperitoneal transplantation of PGCs into hatchlings were represented by Takeuchi *et al.* (2003) which used wild trout as the recipient and GFP-transgenic trout as the donor. The eggs and sperms obtained from the mature chimeras were fertilized artificially, the resulting gametes produced normal offspring with phenotype similarly to donor-derived. In another study, the masu salmon (*Oncorhynchus masou*) hatchlings were used as recipients and transgenic *vasa-Gfp* rainbow trout (*Oncorhynchus mykiss*) were used as PGC donors. The mature chimeras then able to produce donor derived offspring xenogenic (Takeuchi *et al.* 2004). Spermatogonial stem cells are the early precursor for spermatozoa and responsible for the continuation of spermatogenesis in adult mammals. While PGCs transplantation has been obtained great successes, a question was raised, whether or not spermatogonial stem cells could be functionally in the same way as PGCs after transplantation. This question was investigated by Okutsu *et al.* (2006). Spermatogonial stem cells were isolated from adult male *vasa-Gfp* rainbow trout and injected into newly hatched larvae peritoneal cavities males and females. The recipient fish when reaching mature showed donor spermatozoa in males and were also able to fully functional eggs in females. Moreover, spermatozoa and eggs obtained from these recipients could produce the normal offspring. Germ cells transplantation technique using PGCs injected to embryos or newly hatched larvae required refined tools and long time period to produce feasible eggs and sperm. Therefore, the new approach in which germ cells were transplanted in adult fish were developed by Lacerda *et al.* (2006). In this method, spermatogonia were transplanted into Nile tilapia adult (*Oreochromis niloticus*) through urogenital papilla. The endogenous spermatogenesis of recipient fish was abolished by using the cytostatic drug, busulfan (1,4-butanediol dimethanesulfonate) association with high temperature at 35°C. Type A spermatogonial suspension were collected from donor testis and labeled with the PKH26-GL fluorescent lipophilic dye before being injected into recipient adult testis fish through the common spermatic duct. The recipient testis was analyzed after transplantation represented PKH26 labeled germ cells in the lumen of the seminiferous tubules. This method exposed the feasibility when using sexually mature fish as recipient in germ cells transplantation. A

subsequent study where the tilapia recipients were observed after 8 and 9 weeks post transplantation displayed that complete spermatogenesis and production of donor spermatozoa in testis of recipients (Lacerda *et al.* 2010). Moreover, the progeny of these recipients showed genotype of donor fish which came from different strain with Nile tilapia. The genotype of progeny was analyzed by microsatellites marker. The germ cells transplantation methods above mentioned have both advantages and disadvantages in each method. With the method of transplantation of PGCs into blastula embryo recipient sterilization by knock down dead end gene or transplantation of PGCs, spermatognia, oogonia into hatching triploid recipient which require refine tools like micro manipulation and long time required for donor-derived gametogenesis. However, the germline transmission rate induced by these methods reached to 100% and recipient can produce sperm or eggs (Okutsu *et al.* 2007; Saito *et al.* 2008). While, other method of transplantation of only spermatogonia into adult sterilization fish by busulfan treatment was not require refine techniques and the donor gametes could be determined in recipients shortly after transplantation. However, the germline transmission rate activated by this method was much lower than efficiency induced by other methods and obviously that the recipients show production of functional sperm only (Majhi *et al.* 2009a; Majhi *et al.* 2009b; Lacerda *et al.* 2010).

3 Material and Methods

3.1 Material

- pX330-U6-Chimeric_BB-CBh-hSpCas9 backbone (Addgene plasmid ID 42230)
- DNA oligos (Standard de-salted) for cloning of chosen guides
- PCR reagents
- Cloning enzymes: FastDigest BbsI, T7 DNA ligase.
- 10× T4 DNA ligase buffer.
- 10× Tango buffer (Thermo Scientific/Fermentas)
- DTT (DL-Dithiothreitol)
- Ultrapure water (RNAse/DNAse-free)
- *Escherichia coli* DH5α competent cells
- Luria Broth (LB) media
- Ampicillin
- Spin miniprep kit
- Plasmid Midi/Maxi Kit
- Restriction enzyme: BbsI, AgeI
- DNA extraction kit
- Gel extraction kit
- Standard gel electrophoresis reagents and apparatus

3.2 Methods

3.2.1 Design and cloning of genome engineering constructs

3.2.1.1 Design CRISPR (sgRNA oligos) sequence

In sturgeon, genomic information is not available yet. To speculate a position of exon(s) of the target gene (*dnd1*), we used zebrafish database. The position of exons is generally conserved among animals. By comparing amino acid sequence of zebrafish and sturgeon, we speculated on the position of exons on sturgeon gene. We used the first and the second exons for CRISPR design in order to maximize the possibility of genome editing.

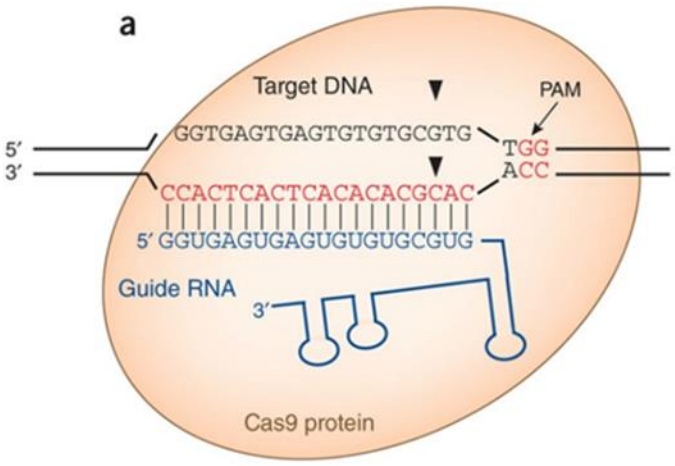

Fig. 1. sgRNA directs the Cas9 nuclease to a cleavage site in the genome

(http://studylib.net/doc/7162172/designing-and-making-sgrna-constructs)

Short guide RNA (sgRNA) directs the Cas9 nuclease to a cleavage site in the genome which must match with 20 nucleotides target sequence (protospacer sequence) in DNA genomic and must be followed by a protospacer adjacent motif (PAM) sequence of NGG. sgRNA sequence is not included the PAM site. The seed sequence plays important role for efficient cleavage that contains 12 nucleotides preceding the PAM. A matching between the seed sequence and non-target loci should be avoided when designing sgRNAs.

In our research, the DNA genomic target sequence and oligos sequences are as following:

Genomic sequence:	5’	GATGGGCAGAGAAAATA/TGG**AGG**	3’
Sense oligo:	5’	**CACC**GATGGGCAGAGAAAATATGG	3’
Antisense oligo:	3’	**AAAC**CCATATTTTCTCTGCCCATC	5’

The **AGG** is the PAM sequence. The overhang sequences 5’ – **CACC** – 3’ (sense oligo) and 3’ – **AAAC** – 5’ (antisense oligo) are complementary to the BbsI cut site. The **G** in the sense oligo corresponds to the first nucleotide of the sgRNA and is necessary for efficient U6-driven expression. This **G** is the first nucleotide in the 20 nucleotides targeting sequence. The sense oligo contains 20 nucleotide sequence is the exact same sequence as the genomic target sequence and it does not include the 3 nucleotides (NGG) of PAM sequence. The red slash (/) indicates the cutting site for Cas9.

3.2.1.2 sgRNA oligo duplex preparing

Dilute the oligos to a final concentration of 100 µM with distil water. Then mixing 1 µl forward oligo and 1 µl reverse oligo with 1 µl of 10x T4 ligation buffer and 7 µl of distil water. Total volume is 10 µl. Place the mixture in a thermocycler using following parameters: 37˚C in 30 minutes, 95˚C in 5 minutes and then ramp down to 25°C at 5˚C/min to phosphorylate and anneal the oligos. Then holding at 4˚C until ready to proceed. The annealed oligos then was diluted 200-fold by distil water to use at next step (Cong and Zhang, 2015).

3.2.1.3 Cloning of targeting constructs

We used pX330-U6-Chimeric_BB-CBh-hSpCas9 backbone (Addgene plasmid ID 42230 - *https://www.addgene.org/42230/*) as cloning backbone vector to insert the annealed oligos. The ligation reaction includes: 2 µl of backbone plasmid (total amount 100 ng), 2 µl of sgRNA oligo duplex (200-fold diluted), 2 µl of 10x Tango buffer, 1 µl of DTT (Dithiothreitol), 1 µl of ATP (Adenosine triphosphate), 1 µl of FastDigest BbsI, 0.5 µl of T7 ligase and 11.5 µl of distil water. The total volume is 20 µl. The mixture then incubating in a thermocycler with following paremeters: 37˚C in 5 minutes and 23˚C in 5 minutes, cycle the previous two steps for 6 cycles (total run time 1 hour), then holding at 4˚C until ready to proceed (Cong and Zhang, 2015).

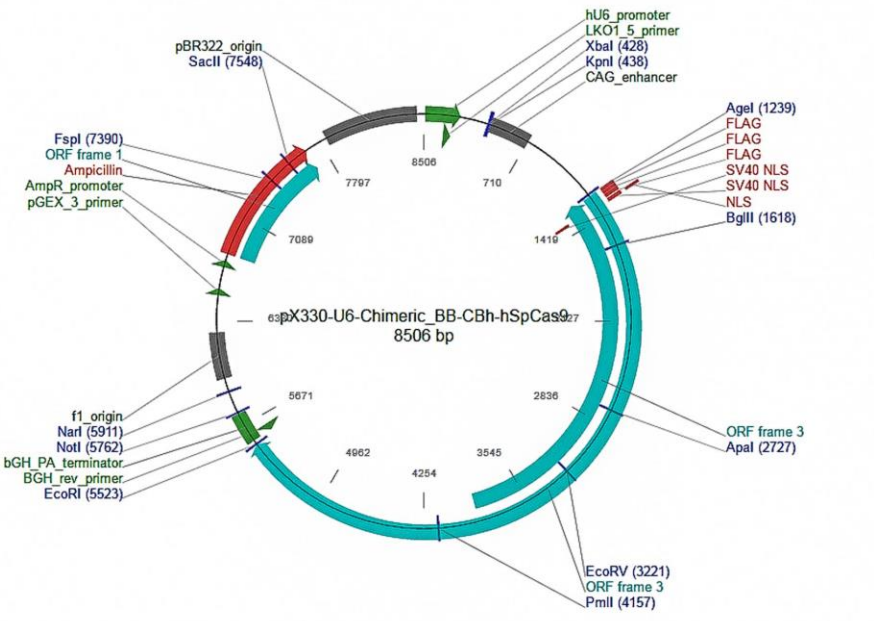

Fig. 2. pX330-U6-Chimeric_BB-CBh-hSpCas9 backbone

3.2.1.4 Transformation

We used *Escherichia coli* DH5α as competent cells for transformation. We added 5 µl DNA cloning of target constructs from last step into a vial of one shot competent cells and mixing gently. Then, the vial was incubated on ice in 30 minutes and heat-shock the cells for 30 seconds at 42°C without shaking. The vial was placed on ice in 2 minutes after removing from the 42°C bath. The aseptically S.O.C medium (attached with the kit) was warmed before adding to each vial with volume 250 µl/vial. The vial then tightly cap and horizontally shake at 37°C for 1 hour at 255 rpm. Spread the transformed cells (20 µl/plate) on a pre-warmed Ampicillin selection LB (Luria Broth) agar plates and incubating overnight at 37°C. The remaining transformation can be stored at 4°C and using the next day if desired. The day after, the single colony was transferred to 10 ml LB medium and incubated overnight at 37°C in shaking incubator, and subsequently the plasmid was extracted.

3.2.1.5 Plasmid purification

We used JetStar^{TM} 2.0 Miniprep Kit (Cat.no. 200050) for plasmid purification by following steps. Harvest the cells by centrifuging the overnight LB-culture at 4,000 rpm for 5–10 minutes. Remove a medium and adding 0.4 ml Cell Resuspending Buffer E1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) to the pellet and resuspend cells until homogeneous. Then mixing gently the homogeneous cell with 0.4 ml Lysis Buffer E2 (0.2 M NaOH, 1% (w/v) SDS) and keeping room temperature in 5 minutes. 0.4 ml Precipitation Buffer E3 (3.1 M Potassium acetate, pH 5.5) continues adding. The supernatant is harvested after centrifuging at 12,000 rpm in 10 minutes. Supernatant is loaded onto equilibrated column and draining by gravity. The column then is washed twice by Wash Buffer E5 (0.1 M Sodium acetate, pH 5.0, 0.8 M NaCl). Proceed to elute and precipitating DNA by 0.9 ml Elution Buffer E6 (0.1 M Tris-HCl, pH 8.5, 1.25 M NaCl) and 0.63 ml Isopropanol, respectively. The mixture then centrifuging at 12,000 rpm, 4°C in 30 minutes and DNA will be remained at column after discarding supernatant. DNA is washed by 1 ml 70% ethanol then eluting by distil water and storing at -20°C.

3.2.1.6 Double digestion ligated plasmid

Ligated plasmid was verified by double-digestion assay by using BbsI and AgeI restriction enzymes and visualized on 1% agarose gel. It is possible to screen for correct

insertion of the target sequence oligos by this digestion because a successful insertion will be destroyed the recognition site of BbsI. Therefore, after double digestion, clones with sgRNA insertion will show only linearized plasmid while clones without insertion will show two fragments with sizes approximately of 980 bp and 7520 bp on an agarose gel.

3.2.2 Microinjection

3.2.2.1 Preparation of embryos

In this study, we used embryos from sterlet (*Acipenser ruthenus*) and Russian sturgeon (*Acipenser gueldenstaedtii*), Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, University of South Bohemia in Ceske Budejovice, Czech Republic. Fish were held in tanks at 13°C. Ovulation and spermiation were induced by intramuscular injection of carp pituitary homogenized extract at a dose 40 $mg \times kg^{-1}$ body weight (b.w) for males and for females there are twice injection. The first injection at a dose 5 $mg \times kg^{-1}$ b.w and the second time at a dose 45 $mg \times kg^{-1}$ b.w, 12 hours after the first injection. Sperm and eggs were collected after 48 hours hormone injection with males and 18 – 20 hours after the second hormone injection with females. Eggs were fertilized with sperm in dechlorinated water at 15°C. The solution with 0.04% tannic acid or clay water were used to remove the stickiness of fertilized eggs. In order to manipulate embryos, the outer layer of chorion had to remove by using forceps under the microscope.

3.2.2.2 Microinjection

The needle puller (PC-10; Narishige, Tokyo, Japan) was used to make a glass micropipette from a glass needle. The plasmids or FITC was loaded into glass micropipette and microinjection was performed under a fluorescent stereo microscope Leica M165 FC (Leica, Wetzlar, Germany) together using with a micromanipulator M152 (Narishige) and microinjector FemtoJet (Eppendorf, Hamburg, Germany). In the treated groups, each embryo experienced in double microinjections. The first one for extracted single plasmid carrying target sequence, which was microinjected into animal pole of embryo to study the reverse genetics. To prepare this injection solution, we diluted plasmid with 10% of 0.2M KCl (9 µl of plasmid diluted with 1 µl of 0.2M KCl). Then solution was injected to animal pole of the fertilized eggs at the one to four-cell-stage at one to four hours after fertilization. At the same time, the second microinjection with 2.5% FITC-biotin-dextrans (molecular weight =

500,000) was injected into vegetal pole for labelling PGCs according to Saito and Pšenička (2015). In the control groups, there was only injected by 2.5% FITC into vegetal pole. The embryos were kept in petri dishes and water was replaced every 24 hours.

3.2.3 Microscopy

PGCs of sterlet chimeras were visualized and photographed using a fluorescent stereo microscope Leica M165 FC.

3.2.4 DNA extraction

The total DNA was extracted by using GenElute™ Mammalian Genomic DNA Miniprep G1N70 Kits (Sigma – Aldrich). The quality of isolated DNA was tested by 0.8% agarose gel electrophoresis and measured by the Nanodrop 2000c (Thermo Scientific).

3.2.5 PCR

The *dnd1* gene was amplified in a 25 µl volume with 12.5 µl of PCR master mix (Top Bio PPP Master Mix), 1 µl of each forward and reverse primer (10 µM/µl), 3-4 µl of genomic DNA (15-20 ng/µl) and fill up to 25 µl with nuclease free water. The primers used for amplification the *dnd1* gene were designed by ourselves. The mixture placed in a thermocycler using following parameters: denaturation at 95°C for 5 minutes, 35 cycles of amplification at 95°C for 30 seconds, 55°C for 30 seconds, 68°C for 1 minute and additional elongation at 68°C for 5 minutes. The polymerase chain reaction products were separated by 1.2% agarose gel and/or by capillary electrophoresis. To screen CRISPR/Cas 9 induced mutation (deletions/insertions), the PCR products were sequenced.

The sequence of primer is as following table 1:

Tab. 1. Sequence of primer

Name of primer	**Sequence of primer**		**Amplicon size**
dnd1a	Fw	GAGAGGGCAAGTTGTCTGGA	205 bp
	Rw	AAAACCTCACAGCCAGAGGAA	

3.2.6 Capillary electrophoresis

To confirm the presence or deletion/insertion occurring in the target gene, we used MCE-202 MultiNA microchip electrophoresis system for DNA analysis, in which the targeted gene

after amplifying by PCR was analyzed. DNA samples are separated by size. The MCE uses a microchip so that the size of DNA samples is verified and approximately quantitated. Simply load 9 µl PCR products into 0.2 ml PCR tubes without caps; the volume of mixture of separation buffer and SYBR gold nucleic gel stain was calculated based on the number of samples; the ladder was prepared by diluting 1 µl of reagent kit DNA-500 25bp DNA ladder in 49 µl TE 1X and marker 100 to perform analysis with high sensitivity and automatically. The volume of separation buffer and marker depend on the number of analyzed samples which were calculated by the software accompany with MCE system.

3.2.7 Sanger sequencing

Mutations in treated samples were further assessed by Sanger sequencing. In this study, we sent the samples to sequence in SEQme s.r.o., company in Dlouha 176, 26301 Dobris, Czech Republic. Before sequencing, the PCR products were run on 1.5% agarose gel and the expected bands were cut for DNA gel purification. We used TIANgel Mini DNA Purification Kit (Cat.no.DP208) for gel purification.

3.2.8 Sequences alignment and analysis

Gene sequences were checked their quality by Finch TV 1.4 software, then comparing and clustering sequence by ClustalW multiple alignment on BioEdit Sequence Alignment Editor Software.

3.2.9 Statistical analysis:

Difference between the number of PGCs in sterlet embryos in treated and control group was tested as the following procedure. At first normality was tested using Shapiro-Wilk test. If this condition was satisfied, Student’s unpaired t-test was used for determining whether there was any significant difference between control and treated group. If the condition was not satisfied, nonparametric test – Mann-Whitney U test was used. All statistical analyses were performed using the SPSS statistical software (version 23) for Windows.

4 Results

4.1 Competent cells and transformation

We prepared out own competent cells (*Escherichia coli* DH5α). These competent cells worked effectively when the pX330 vectors carried the sgRNA were transformed.

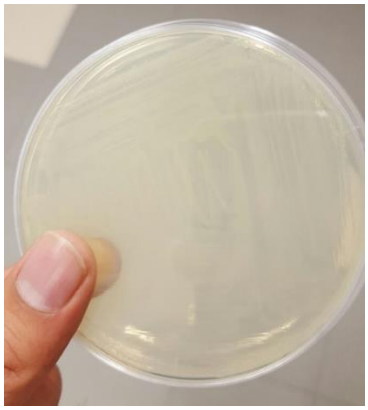

Fig. 3. Competent cells E. coli DH5α

Fig. 4. The recombinants of plasmid pX330 and sgRNA were amplified in E. coli DH5α

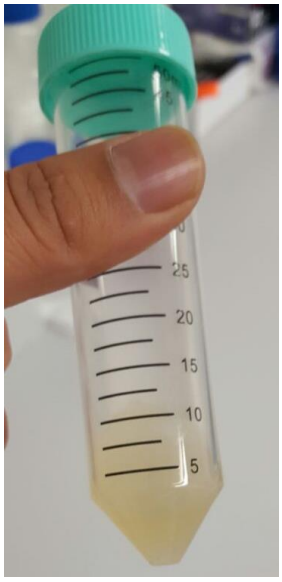

Fig. 5. Single colony growth in LB broth for plasmid purification

.

4.2 Plasmid purification

The plasmid pX330 that was ligated with sgRNA (shortly we called plasmid) was extracted by using JetStarTM 2.0 Miniprep Kit. The plasmid was then verified by double-digestion assay by using BbsI and AgeI restriction enzymes and visualizing on 1% agarose gel. The result represented that there was only one band of linearized plasmid. It illustrated that the sgRNA oligos were inserted into plasmid at the position of BbsI and destroyed the recognition site of BbsI on ligated plasmid. Therefore, only AgeI was function when using double digestion assay.

The concentration of plasmid purification was measured by spectrophotometer NanoDrop and 0.8% agarose gel electrophoresis. The result showed that the quality of plasmid with concentration from 8.3 ng/µl to 14.6 ng/µl was consistent for downstream application.

4.3 Malformation of embryos in Russian sturgeon

The sgRNA oligos were designed base on *dnd1* nucleotide sequence of sterlet which shares 97% identity with *dnd1* sequence of Russian sturgeon (Linhartová *et al*. 2015). Therefore, we applied microinjection plasmid to embryos of both sterlet and Russian. In Russian, there were 19 alive embryos in total 21 embryos were injected with plasmid and five samples without PGCs. The malformation of Russian embryos was observed in all samples while it was not detected in sterlet.

As shown in fig. 6, the Russian embryos treated with CRISPR/Cas9 exhibited abnormal development especially the region where PGCs migrate. These developmental deformities thus suggest the pleiotropic effect of *dnd1* gene as CRISPR/Cas9 affected factors responsible for embryonic development and subsequent organogenesis.

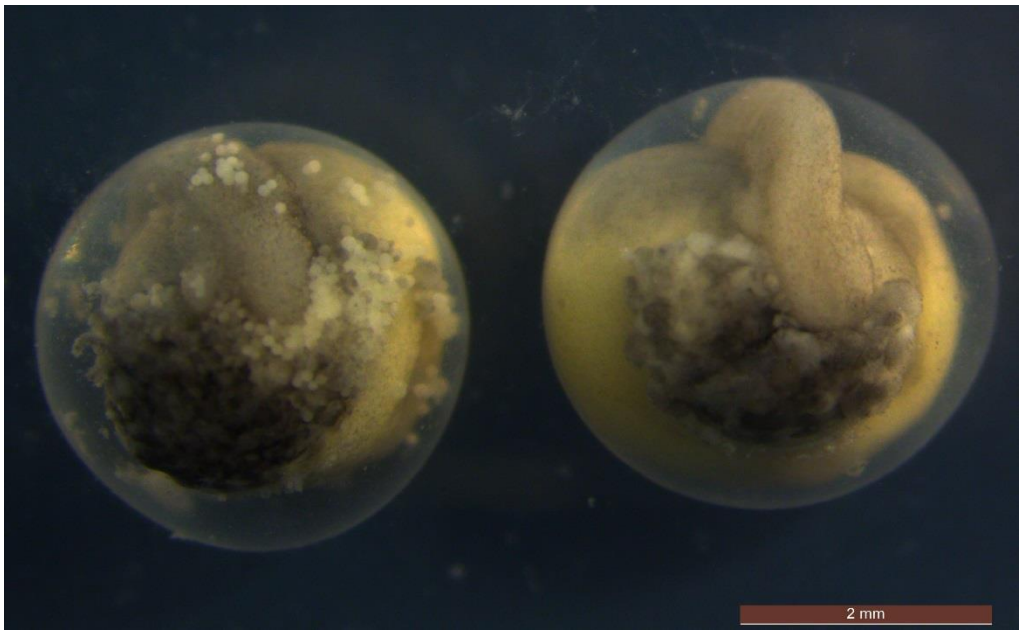

Fig. 6. Malformation of embryos after microinjection of CRISPR/Cas9

4.4 Primordial Germ Cells (PGCs) in sterlet

Tab. 2. Average number of PGCs in control and treated embryos in sterlet

Group	Control	Treated
Number of PGCs	22.5 ± 2.74	1.67 ± 1.30*

Note: data are presented as mean ± SD asterisk means significant difference compared to control and treated embryos (P<0.05). n control = 6, n treated = 12.

PGCs of sterlet chimeras were visualized and photographed using a fluorescent stereo microscope Leica M165 FC. The results presented that PGCs were detected in the body cavity of both the FITC - labeled control samples and the treated samples injected with CRISPR/Cas9 in animal pole together with FITC in vegetal pole. Table 2 showed that the number of PGCs in sterlet in treated embryos was significantly lower than in control embryos. These embryos were used for all downstream application.

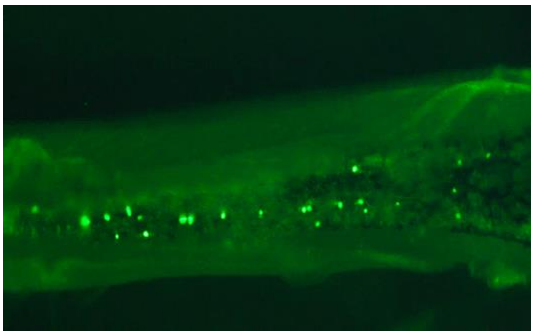

Fig. 7. PGCs in control sample injected with only FITC in vegetal pole

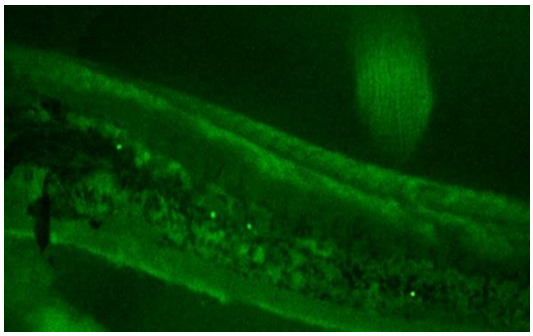

Fig. 8. PGCs in treated sample with double injected: CRISPR/Cas9 in animal pole and FITC in vegetal pole

4.5 DNA analysis

4.5.1 DNA extraction

The sterlet embryos (treated and control) were used for genomic DNA extraction. We used NanoDrop and 0.8% agarose gel to quantitative and qualitative of DNA extraction. The results represented that genomic DNA extraction was appropriate for PCR.

Tab. 3. Concentration of DNA measured by NanoDrop

Group	**Name of samples**	**ng/µl**
Treated with CRISPR/Cas9	T1	29.37
	T2	15.41
	T3	48.43
	T4	36.68
	T5	61.53
Control	C1	24.18
	C2	56.8

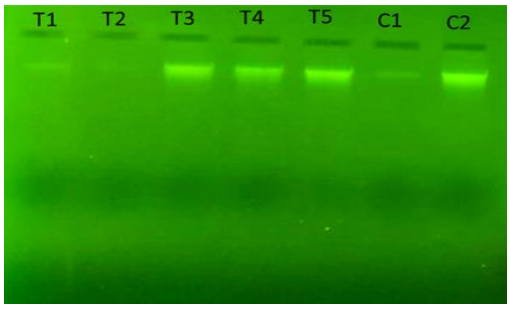

Fig. 9. Genomic DNA were visible by 0.8% agarose gel

4.5.2 PCR

After DNA genomic extraction, *dnd1* gene was amplified by *dnd1a* primer with expected amplicon size of 205 bp. The PCR products were visualized and analyzed by capillary electrophoresis. The results displayed that in control group, the bands represented similarly in every samples at the size around 200 bp. However, in treated samples with CRISPR/Cas9 group, the bands occurred in difference sizes, they were bigger or smaller than 200 bp by some nucleotides. An assumption was given, CRISPR/Cas9 system cut the targeted site then because of the randomness of NHEJ-mediated DSB repair which gives rise to small insertions/deletions in the target DNA. To clarify how many nucleotides were inserted or deleted in target DNA, we sequenced these samples.

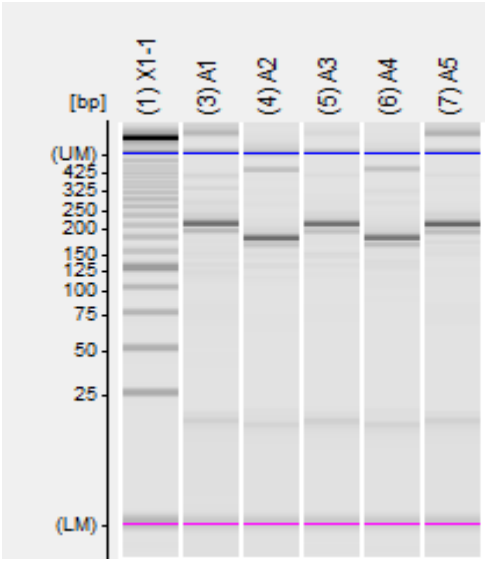

Fig. 10. PCR fragments in samples treated with CRISPR/Cas9

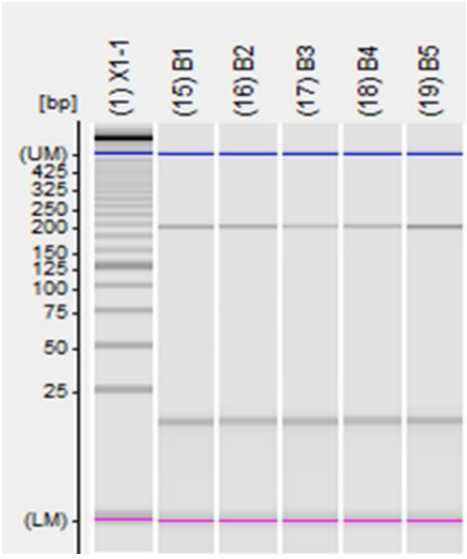

Fig. 11. PCR fragments in control group

4.5.3 Sequencing

PCR product from four samples in treated group and one sample in control group were sequenced. The fragment size 205 bp was expected, however, we got the shorter gene sequences around 160 bp. There were some reasons explains for obtaining unexpected fragments size. The first one is because of low DNA concentration after gel purification.

Tab. 4. Concentration of DNA after gel purification ready for sequencing measured by NanoDrop

Group	**Name of samples**	**ng/µl**
Treated with CRISPR/Cas9	T1	1.3
	T2	8.5
	T3	6.2
	T4	12.6
Control	C1	12.6

The second reason could be the contamination of the templates, primer stock or other sequencing reagents which caused to the total quality scores counts were generally low to moderate.

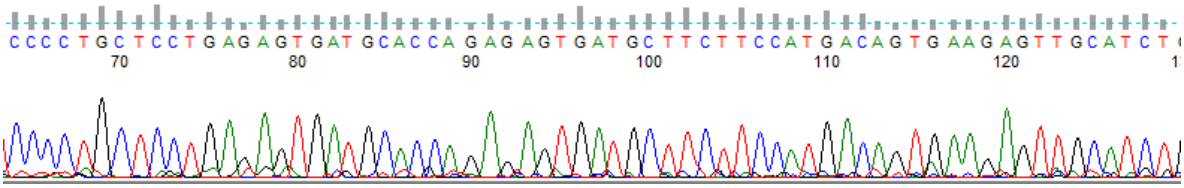

Fig. 12. Sequencing chromatograms sample T1

Nucleotide sequences T1, T2, T3, T4 and C1 were checked their quality by Finch TV and deleting weak or noisy trace peaks, then comparing and clustering sequences by ClustalW multiple alignment on BioEdit.

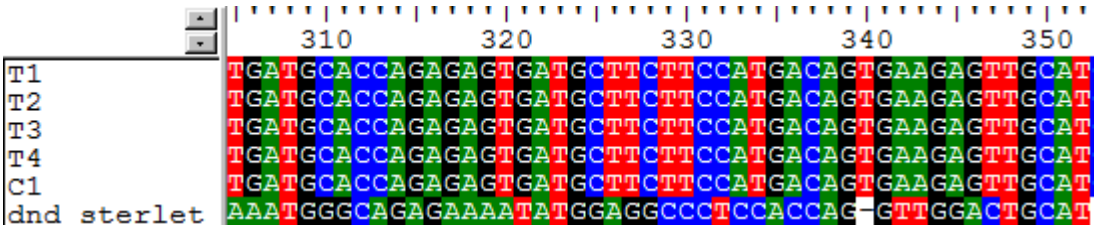

Fig. 13. Nucleotide sequence alignment among treated and control samples with reference Ardnd

(treated samples T1, T2, T3, T4; control sample C1; *dnd* sterlet - complete sequence)

In this study, we used full-length *dnd1* gene sequence of sterlet (*Ardnd*). The fig. 13 showned that there were no differences between nucleotide sequences of samples that were injected CRISPR/Cas9 (T1, T2, T3, T4) and control sample (C1). However, there were obvious differences among our obtained sequences (T1 to C1) with *Ardnd* in nucleotides sequence. The *Ardnd* fragment sequence was used as template sequence when we designed the primers for PCR. However, the sequences received after PCR sequencing were not similar with usage template. A raised theory is that we designed and used the primers that amplified for non-functional copy of *dnd1* gene because sterlets have duplicate genes, in particularly for *dnd1* gene there are two copies of *dnd1*, one copy is the functional gene that encodes *dnd1* protein while other one does not.

5 Discussion

In our present study, we used sterlet *dnd1* nucleotide sequence to design sgRNA and primers to amplify the targeted sequence. Protocol developed by Cong and Zhang (2015) was followed to prepare sgRNA oligo duplex and cloning of targeting constructs. Other step-wise protocols such as embryos preparation, microinjection and microscopy analysis were referred from experimentations of Saito *et al*. (2014) and Linhartová *et al*. (2015). In this study, we aimed to extend the state-of-art and target directly DNA in order to elucidate the function of *dnd1* transcribed from zygotic genome after migration of PGCs. It is assumed that knock out of *dnd1* will lead to sterilization of treated animals; hence they can be practically used as recipients for technology of surrogate production. We used CRISPR/Cas9 technique for this purpose; and it was function successfully in inducing the embryos without PGCs or decreasing significantly the number of PGCs between experiment and control group of sterlet. In this study, PGC played the role as a marker in order to determine CRISPR/Cas9 was action or not, because they could be visualized clearly and photographed easily by using a fluorescent stereo microscope. The samples with one to two PGCs detected and without PGCs then were analysed by sequencing. To elucidate that whether CRISPR/Cas9 could be induced mutation or not, it is necessary in designing the optimal primers and more samples should be sequenced for authentication. The genome duplication in sturgeon is one of the difficult challenges for designing specific primers and sgRNA, which can be functionally incorrect region. Linhartová *et al*. (2015) designed and constructed *dnd1* morpholino oligonucleotide (*dnd*-MO) to sterilize sterlet by *Ardnd* knockdown. The results shown that *dnd*-MO at concentration 250µM was important in depletion of PGCs development in sturgeon. Our study is the first research about application of genome editing technology in sturgeon, particularly in knock out *dnd1* gen in sterlet using CRISPR/Cas9 technique with ambition to make sterilization of treated fish. Sterilization fish has the potential as surrogate production that can generate offspring of other species by germ cells transplantation from donor to sterilize fish. This germline chimerism biotechnology could be shortening the reproduction period in species with late-maturation sexual; reducing the capacity for culture when small fish species used as hosts; conserving germ cells for maintaining genetic resources; retaining target species without keeping adult fish (Yazawa *et al*. 2013; Linhartova *et al*. 2014). For instance, sterlet is a smaller sturgeon species and shorter period of

maturation, it could be used as a host to produce gametes from donor sturgeon with large body size, long reproductive cycle and critically endangered species such as beluga. There are some methods of germ cells transplantation have been well developed for teleost, for example, single PGC transplantation into blastula (Saito *et al*. 2008); PGCs, spermatogonia and oogonia transplantation into newly hatched larvae (Okutsu *et al*. 2007) and transplantation of spermatogonia into adult fish (Majhi *et al*. 2009a; Majhi *et al*. 2009b; Lacerda *et al*. 2010). The recipient should be sterile to produce donor gametes only. Generally in fish, hybridization between species with distant relationship induces sterility by complication in pairing of their chromosomes (Piferrer *et al*. 2009). Another method of sterilization is by chemical treatment. For examples, the endogenous spermatogenesis of recipient fish was abolished by using the cytostatic drug, busulfan (1,4-butanediol dimethanesulfonate) association with high temperature at 35°C (Lacerda *et al*. 2006). These methods are not proven or not function methods of sturgeon sterilization. In addition, *dnd1* gene has been known as the best gene candidate in expression of germ plasm and responsible for the development and migration of PGCs (Weidinger *et al*. 2003). Therefore, using genome editing technology to inhibit *dnd1* gene function is an efficiency approach in inducing sterilization sturgeon. Sturgeon are very interesting species and frequently referred to as living fossils, therefore, an effective genome editing technology could provide us to reveal other amazing findings. In addition, they are used as ornamental fish, therefore, by application of genome editing technique such as ZFNs, TALENs and CRISPR/Cas9, we can modify for instance in their color in near future.

6 Conclusions and recommendations

In this study, we reported about the method of sterlet sterilization using CRISPR/Cas9 technology. We successfully established basic protocols and methods for CRISPR/Cas9 such as preparation of competent cells (*Escherichia coli DH5α*), construction of vector (pX330) carrying sgRNA and its transformation into competent cells. Less number and/or mis-migration of PGCs was observed in embryos that were treated with CRISPR/Cas9; however, sequencing did not provide us a reliable evidence for mutation of the targeted gene probably due to an unspecific PCR. Therefore, more authentication of *dnd1* knockout should be done in future by more specific PCR and repeated sequencing.

7 Bibliography

Ablain J., Durand Ellen M., Yang S., Zhou Y. & Zon Leonard I. (2015) A CRISPR/Cas9 Vector System for Tissue-Specific Gene Disruption in Zebrafish. *Developmental Cell* **32**, 756-64.

Aramaki S., Kubota K., Soh T., Yamauchi N. & Hattori M.A. (2009) Chicken dead end homologue protein is a nucleoprotein of germ cells including primordial germ cells. *J Reprod Dev* **55**, 214-8.

Barrangou R. & Marraffini L.A. (2014) CRISPR-Cas systems: prokaryotes upgrade to adaptive immunity. *Molecular cell* **54**, 234-44.

Bassett A R., Tibbit C., Ponting C P. & Liu J.L. (2013) *Highly Efficient Targeted Mutagenesis of Drosophila with the CRISPR/Cas9 System*. Cell Rep.4(1):220-8. doi:10.1016/j.celrep.2013.06.020.

Bedell V.M., Wang Y., Campbell J.M., Poshusta T.L., Starker C.G., Krug Ii R.G., Tan W., Penheiter S.G., Ma A.C., Leung A.Y.H., Fahrenkrug S.C., Carlson D.F., Voytas D.F., Clark K.J., Essner J.J. & Ekker S.C. (2012) In vivo genome editing using a high-efficiency TALEN system. *Nature* **491**, 114-8.

Bemis W.E. & Kynard B. (1997) Sturgeon rivers: An introduction to acipenseriform biogeography and life history. In: *Sturgeon Biodiversity and Conservation* (eds. by Birstein VJ, Waldman JR & Bemis WE), pp. 167-83. Springer Netherlands, Dordrecht.

Bhattacharya C., Aggarwal S., Zhu R., Kumar M., Zhao M., Meistrich M.L. & Matin A. (2007) *The mouse dead-end gene isoform α is necessary for germ cell and embryonic viability*. Biochem Biophys Res Commun. 2007 Mar 30;355(1):194-9. Epub 2007 Feb 2 doi:10.1016/j.bbrc.2007.01.138.

Bibikova M., Golic M., Golic K.G. & Carroll D. (2002) Targeted Chromosomal Cleavage and Mutagenesis in Drosophila Using Zinc-Finger Nucleases. *Genetics* **161**, 1169-75.

Bolker J.A. (1993) Gastrulation and mesoderm morphogenesis in the white sturgeon. *J Exp Zool* **266**, 116-31.

Brinster R.L. & Zimmermann J.W. (1994) *Spermatogenesis following male germ-cell transplantation*. Proc Natl Acad Sci U S A;91(24):11298-302.

Cai C.Q., Doyon Y., Ainley W.M., Miller J.C., DeKelver R.C., Moehle E.A., Rock J.M., Lee Y.-L., Garrison R., Schulenberg L., Blue R., Worden A., Baker L., Faraji F., Zhang L., Holmes M.C., Rebar E.J., Collingwood T.N., Rubin-Wilson B., Gregory P.D., Urnov F.D. & Petolino J.F. (2009) Targeted transgene integration in plant cells using designed zinc finger nucleases. *Plant Molecular Biology* **69**, 699-709.

Carlson D.F., Tan W., Lillico S.G., Stverakova D., Proudfoot C., Christian M., Voytas D.F., Long C.R., Whitelaw C.B.A. & Fahrenkrug S.C. (2012) Efficient TALEN-mediated gene knockout in livestock. *Proceedings of the National Academy of Sciences* **109**, 17382-7.

Chang N., Sun C., Gao L., Zhu D., Xu X., Zhu X., Xiong J.W. & Xi J.J. (2013) Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. *Cell Res* **23**, 465-72.

Ciruna B., Weidinger G., Knaut H., Thisse B., Thisse C., Raz E. & Schier A.F. (2002) Production of maternal-zygotic mutant zebrafish by germ-line replacement. *Proc Natl Acad Sci U S A* **99**, 14919-24.

Clark K.J., Voytas D.F. & Ekker S.C. (2011) A TALE of Two Nucleases: Gene Targeting for the Masses? *Zebrafish* **8**, 147-9.

Cong L. & Zhang F. (2015) Genome engineering using CRISPR-Cas9 system. *Methods Mol Biol*, 1862-1_10.

Cui X., Ji D., Fisher D.A., Wu Y., Briner D.M. & Weinstein E.J. (2011) Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nat Biotech* **29**, 64-7.

Davidson W.S., Koop B.F., Jones S.J., Iturra P., Vidal R., Maass A., Jonassen I., Lien S. & Omholt S.W. (2010) Sequencing the genome of the Atlantic salmon (Salmo salar). *Genome Biol* **11**, 2010-11.

Deng W. & Lin H. (2002) miwi, a Murine Homolog of piwi, Encodes a Cytoplasmic Protein Essential for Spermatogenesis. *Developmental Cell* **2**, 819-30.

Doudna J.A. & Charpentier E. (2014) The new frontier of genome engineering with CRISPR-Cas9. *Science* **346**.

Doyon Y., McCammon J.M., Miller J.C., Faraji F., Ngo C., Katibah G.E., Amora R., Hocking T.D., Zhang L., Rebar E.J., Gregory P.D., Urnov F.D. & Amacher S.L. (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol* **26**, 702-8.

Edvardsen R.B., Leininger S., Kleppe L., Skaftnesmo K.O. & Wargelius A. (2014) Targeted mutagenesis in Atlantic salmon (Salmo salar L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 generation. *PLoS One* **9**.

Ephrussi A. & Lehmann R. (1992) Induction of germ cell formation by oskar. *Nature* **358**, 387-92.

Foley J.E., Yeh J.R., Maeder M.L., Reyon D., Sander J.D., Peterson R.T. & Joung J.K. (2009) Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by Oligomerized Pool ENgineering (OPEN). *PLoS ONE* **4**, 9.

Fontana F. (1994) Chromosomal nucleolar organizer regions in four sturgeon species as markers of karyotype evolution in Acipenseriformes (Pisces). *Genome* **37**, 888-92.

Fontana F. (2002) A cytogenetic approach to the study of taxonomy and evolution in sturgeons. *Journal of Applied Ichthyology* **18**, 226-33.

Fontana F., Congiu L., Mudrak V.A., Quattro J.M., Smith T.I., Ware K. & Doroshov S.I. (2008) Evidence of hexaploid karyotype in shortnose sturgeon. *Genome* **51**, 113-9.

Fontana F., Tagliavini J., Congiu L., Lanfredi M., Chicca M., Laurente C. & Rossi R. (1998) Karyotypic characterization of the great sturgeon, Huso huso, by multiple staining techniques and fluorescent in situ hybridization. *Marine Biology* **132**, 495-501.

Gaj T., Gersbach C.A. & Barbas C.F. (2013) ZFN, TALEN and CRISPR/Cas-based methods for genome engineering. *Trends in biotechnology* **31**, 397-405.

Gasiunas G., Barrangou R., Horvath P. & Siksnys V. (2012) Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences* **109**, E2579–E86.

Giraldez A.J., Cinalli R.M., Glasner M.E., Enright A.J., Thomson J.M., Baskerville S., Hammond S.M., Bartel D.P. & Schier A.F. (2005) MicroRNAs regulate brain morphogenesis in zebrafish. *Science* **308**, 833-8.

Hockemeyer D., Wang H., Kiani S., Lai C.S., Gao Q., Cassady J.P., Cost G.J., Zhang L., Santiago Y., Miller J.C., Zeitler B., Cherone J.M., Meng X., Hinkley S.J., Rebar E.J., Gregory P.D., Urnov F.D. & Jaenisch R. (2011) Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotech* **29**, 731-4.

Honaramooz A., Behboodi E., Hausler C.L., Blash S., Ayres S., Azuma C., Echelard Y. & Dobrinski I. (2005) *Depletion of Endogenous Germ Cells in Male Pigs and Goats in Preparation for Germ Cell Transplantation*. J Androl.26(6):698-705. doi:10.2164/jandrol.05032.

Hong N., Li M., Yuan Y., Wang T., Yi M., Xu H., Zeng H., Song J. & Hong Y. (2016) Dnd Is a Critical Specifier of Primordial Germ Cells in the Medaka Fish. *Stem Cell Reports* **6**, 411-21.

Horvay K., Claussen M., Katzer M., Landgrebe J. & Pieler T. (2006) Xenopus Dead end mRNA is a localized maternal determinant that serves a conserved function in germ cell development. *Dev Biol* **291**, 1-11.

Houston D.W. & King M.L. (2000) Germ plasm and molecular determinants of germ cell fate. In: *Current Topics in Developmental Biology* (ed. by Gerald PS), pp. 155-IN2. Academic Press.

Houwing S., Kamminga L.M., Berezikov E., Cronembold D., Girard A., van den Elst H., Filippov D.V., Blaser H., Raz E., Moens C.B., Plasterk R.H.A., Hannon G.J., Draper B.W. & Ketting R.F. (2007) A Role for Piwi and piRNAs in Germ Cell Maintenance and Transposon Silencing in Zebrafish. *Cell* **129**, 69-82.

Hruscha A., Krawitz P., Rechenberg A., Heinrich V., Hecht J., Haass C. & Schmid B. (2013) Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development* **140**, 4982-7.

Hsu Patrick D., Lander Eric S. & Zhang F. (2014) Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell* **157**, 1262-78.

Hwang W.Y., Fu Y., Reyon D., Maeder M.L., Tsai S.Q., Sander J.D., Peterson R.T., Yeh J.R. & Joung J.K. (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* **31**, 227-9.

Irion U., Krauss J. & Nüsslein-Volhard C. (2014) Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development* **141**, 4827-30.

Ishino Y., Shinagawa H., Makino K., Amemura M. & Nakata A. (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *Journal of Bacteriology* **169**, 5429-33.

Jao L.E., Wente S.R. & Chen W. (2013) Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci U S A* **110**, 13904-9.

Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J.A. & Charpentier E. (2012) A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **337**, 816-21.

Kim H.J., Lee H.J., Kim H., Cho S.W. & Kim J.S. (2009) Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res* **19**, 1279-88.

King T.L., Lubinski B.A. & Spidle A.P. (2001) Microsatellite DNA variation in Atlantic sturgeon (Acipenser oxyrinchus oxyrinchus) and cross-species amplification in the Acipenseridae. *Conservation Genetics* **2**, 103-19.

Krishna S.S., Majumdar I. & Grishin N.V. (2003) SURVEY AND SUMMARY: Structural classification of zinc fingers. *Nucleic Acids Research* **31**, 532-50.

Lacerda S., Batlouni S.R., Costa G.M.J., Segatelli T.M., Quirino B.R., Queiroz B.M., Kalapothakis E. & França L.R. (2010) *A New and Fast Technique to Generate Offspring after Germ Cells Transplantation in Adult Fish: The Nile Tilapia (Oreochromis niloticus) Model*. PLoS One. 5(5):e10740. doi:10.1371/journal.pone.0010740.

Lacerda S.M.S.N., Batlouni S.R., Silva S.B.G., Homem C.S.P., França2 L.R. & (2006) Germ cells transplantation in fish: The Nile tilapia model. *Anim Reprod 3:146–159*.

Lei Y., Guo X., Liu Y., Cao Y., Deng Y., Chen X., Cheng C.H., Dawid I.B., Chen Y. & Zhao H. (2012) Efficient targeted gene disruption in Xenopus embryos using engineered transcription activator-like effector nucleases (TALENs). *Proc Natl Acad Sci U S A* **109**, 17484-9.

Li M., Hong N., Gui J. & Hong Y. (2012) Medaka piwi is Essential for Primordial Germ Cell Migration. *Current Molecular Medicine* **12**, 1040-9.

Li M., Yang H., Zhao J., Fang L., Shi H., Sun Y., Zhang X., Jiang D., Zhou L. & Wang D. (2014) Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. *Genetics* **197**, 591-9.

Li W., Teng F., Li T. & Zhou Q. (2013) Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat Biotech* **31**, 684-6.

Linhartová Z., Saito T., Kašpar V., Rodina M., Prášková E., Hagihara S. & Pšenička M. (2015) Sterilization of sterlet *Acipenser ruthenus* by using knockdown agent, antisense morpholino oligonucleotide, against *dead end* gene. *Theriogenology* **84**, 1246-55.e1.

Linhartova Z., Saito T. & Psenicka M. (2014) Embryogenesis, visualization and migration of primordial germ cells in tench (Tinca tinca). *Journal of Applied Ichthyology* **30**, 29-39.

Liu J., Li C., Yu Z., Huang P., Wu H., Wei C., Zhu N., Shen Y., Chen Y., Zhang B., Deng W.M. & Jiao R. (2012) Efficient and specific modifications of the Drosophila genome by means of an easy TALEN strategy. *J Genet Genomics* **39**, 209-15.

Ludwig A., Belfiore N.M., Pitra C., Svirsky V. & Jenneckens I. (2001) Genome duplication events and functional reduction of ploidy levels in sturgeon (Acipenser, Huso and Scaphirhynchus). *Genetics* **158**, 1203-15.

Ludwig A., Lippold S., Debus L. & Reinartz R. (2009) First evidence of hybridization between endangered sterlets (Acipenser ruthenus) and exotic Siberian sturgeons (Acipenser baerii) in the Danube River. *Biological Invasions* **11**, 753-60.

Majhi S.K., Hattori R.S., Rahman S.M., Suzuki T. & Strussmann C.A. (2009b) Experimentally induced depletion of germ cells in sub-adult Patagonian pejerrey (Odontesthes hatcheri). *Theriogenology* **71**, 1162-72.

Majhi S.K., Hattori R.S., Yokota M., Watanabe S. & Strüssmann C.A. (2009a) *Germ Cell Transplantation Using Sexually Competent Fish: An Approach for Rapid Propagation of Endangered and Valuable Germlines*. PLoS One. 4(7):e6132. doi:10.1371/journal.pone.0006132.

Makarova K.S., Aravind L., Wolf Y.I. & Koonin E.V. (2011) Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems. *Biology Direct* **6**, 38-.

Marraffini L.A. & Sontheimer E.J. (2010) CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet* **11**, 181-90.

May B., Krueger C.C. & Kincaid H.L. (1997) Genetic variation at microsatellite loci in sturgeon: Primer sequence homology in Acipenser and Scaphirhynchus. *Can. J. Fish. Aquat. Sci., vol. 54, no. 7, pp. 1542-1547.*

McQuown E.C., Brian L.S., Robert J.S., Jeff R., Gregory J.T. & Bernie M. (2000) Microsatellite Analysis of Genetic Variation in Sturgeon: New Primer Sequences for Scaphirhynchus and Acipenser. *Transactions Of The American Fisheries Society Vol. 129 , Iss. 6.*

Megosh H.B., Cox D.N., Campbell C. & Lin H. (2006) The Role of PIWI and the miRNA Machinery in Drosophila Germline Determination. *Current Biology* **16**, 1884-94.

Meng X., Noyes M.B., Zhu L.J., Lawson N.D. & Wolfe S.A. (2008) Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol* **26**, 695-701.

Miller J.C., Tan S., Qiao G., Barlow K.A., Wang J., Xia D.F., Meng X., Paschon D.E., Leung E., Hinkley S.J., Dulay G.P., Hua K.L., Ankoudinova I., Cost G.J., Urnov F.D., Zhang H.S., Holmes M.C., Zhang L., Gregory P.D. & Rebar E.J. (2011) A TALE nuclease architecture for efficient genome editing. *Nat Biotech* **29**, 143-8.

Mishima Y., Giraldez A.J., Takeda Y., Fujiwara T., Sakamoto H., Schier A.F. & Inoue K. (2006) Differential Regulation of Germline mRNAs in Soma and Germ Cells by Zebrafish miR-430. *Current Biology* **16**, 2135-42.

Nam K.H., Haitjema C., Liu X., Ding F., Wang H., DeLisa M.P. & Ke A. (2012) Cas5d protein processes pre-crRNA and assembles into a Cascade-like interference complex in Subtype I-C/Dvulg CRISPR-Cas system. *Structure (London, England : 1993)* **20**, 1574-84.

Northrup E., Eisenblatter R., Glage S., Rudolph C., Dorsch M., Schlegelberger B., Hedrich H.J. & Zschemisch N.H. (2011) Loss of Dnd1 facilitates the cultivation of genital ridge-derived rat embryonic germ cells. *Exp Cell Res* **317**, 1885-94.

Okutsu T., Shikina S., Kanno M., Takeuchi Y. & Yoshizaki G. (2007) Production of Trout Offspring from Triploid Salmon Parents. *Science* **317**, 1517-.

Okutsu T., Suzuki K., Takeuchi Y., Takeuchi T. & Yoshizaki G. (2006) Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. *Proc Natl Acad Sci U S A* **103**, 2725-9.

Perez E.E., Wang J., Miller J.C., Jouvenot Y., Kim K.A., Liu O., Wang N., Lee G., Bartsevich V.V., Lee Y.-L., Guschin D.Y., Rupniewski I., Waite A.J., Carpenito C., Carroll R.G., S Orange J., Urnov F.D., Rebar E.J., Ando D., Gregory P.D., Riley J.L., Holmes M.C. & June C.H. (2008) Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotech* **26**, 808-16.

Piferrer F., Beaumont A., Falguiere J.C., Flajshans M., Haffray P. & Kolombo L. (2009) Polyploid fish and shellfish: production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture;239:125–56.*

Poleksic V., Lenhardt M., Jaric I., Djordjevic D., Gacic Z., Cvijanovic G. & Raskovic B. (2010) Liver, gills, and skin histopathology and heavy metal content of the Danube sterlet (Acipenser ruthenus Linnaeus, 1758). *Environ Toxicol Chem* **29**, 515-21.

Port F., Chen H.M., Lee T. & Bullock S.L. (2014) *Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila*. Proc Natl Acad Sci U S A. 111(29):E2967-76. Epub 2014 Jul 7 doi:10.1073/pnas.1405500111.

Porteus M.H. & Baltimore D. (2003) Chimeric Nucleases Stimulate Gene Targeting in Human Cells. *Science* **300**, 763-.

Pukazhenthi B., Comizzoli P., Travis A.J. & Wildt D.E. (2006) Applications of emerging technologies to the study and conservation of threatened and endangered species. *Reprod Fertil Dev* **18**, 77-90.

Raz E. (2004) Guidance of primordial germ cell migration. *Current Opinion in Cell Biology* **16**, 169-73.

Rekubratskii A.V., Grunina A.S., Barmintsev V.A., Golovanova T.S., Chudinov O.S., Abramova A.B., Panchenko N.S. & Kupchenko S.A. (2003) [Meiotic gynogenesis in the stellate, Russian sturgeon and sterlet]. *Ontogenez* **34**, 121-31.

Richardson B.E. & Lehmann R. (2010) Mechanisms guiding primordial germ cell migration: strategies from different organisms. *Nature reviews. Molecular cell biology* **11**, 37-49.

Saito T G.-K.R., Fujimoto T, Kawakami Y, Arai K, Yamaha E. (2010) Inter-species transplantation and migration of primordial germ cells in cyprinid fish. *Int J Dev Biol* **54**, 1481 - 6.

Saito T., Goto-Kazeto R., Arai K. & Yamaha E. (2008) Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. *Biol Reprod* **78**, 159-66.

Saito T. & Psenicka M. (2015) Novel Technique for Visualizing Primordial Germ Cells in Sturgeons (Acipenser ruthenus, A. gueldenstaedtii, A. baerii, and Huso huso). *Biology of Reproduction* **93**, 96, 1-7.

Saito T., Pšenička M., Goto R., Adachi S., Inoue K., Arai K. & Yamaha E. (2014) The Origin And Migration Of Primordial Germ Cells In Sturgeons. *PLoS ONE* **9**, e86861.

Sander J.D., Cade L., Khayter C., Reyon D., Peterson R.T., Joung J.K. & Yeh J.R.J. (2011) *Targeted gene disruption in somatic zebrafish cells using engineered TALENs*. Nat Biotechnol;29(8):697-8. doi:10.1038/nbt.1934.

Sander J.D. & Joung J.K. (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotech* **32**, 347-55.

Shah A.N., Davey C.F., Whitebirch A.C., Miller A.C. & Moens C.B. (2015) Rapid reverse genetic screening using CRISPR in zebrafish. *Nat Meth* **12**, 535-40.

Shah S.A., Erdmann S., Mojica F.J.M. & Garrett R.A. (2013) Protospacer recognition motifs: Mixed identities and functional diversity. *RNA Biology* **10**, 891-9.

Shukla V.K., Doyon Y., Miller J.C., DeKelver R.C., Moehle E.A., Worden S.E., Mitchell J.C., Arnold N.L., Gopalan S., Meng X., Choi V.M., Rock J.M., Wu Y.-Y., Katibah G.E., Zhifang G., McCaskill D., Simpson M.A., Blakeslee B., Greenwalt S.A., Butler H.J., Hinkley S.J., Zhang L., Rebar E.J., Gregory P.D. & Urnov F.D. (2009) Precise genome modification in the crop species Zea mays using zinc-finger nucleases. *Nature* **459**, 437-41.

Škugor A., Slanchev K., Torgersen J.S., Tveiten H. & Andersen Ø. (2014) Conserved Mechanisms for Germ Cell-Specific Localization of nanos3 Transcripts in Teleost Species with Aquaculture Significance. *Marine Biotechnology* **16**, 256-64.

Slanchev K., Stebler J., Goudarzi M., Cojocaru V., Weidinger G. & Raz E. (2009) Control of Dead end localization and activity – Implications for the function of the protein in antagonizing miRNA function. *Mechanisms of Development* **126**, 270-7.

Smith J.J., Kuraku S., Holt C., Sauka-Spengler T., Jiang N., Campbell M.S., Yandell M.D., Manousaki T., Meyer A., Bloom O.E., Morgan J.R., Buxbaum J.D., Sachidanandam R., Sims C., Garruss A.S., Cook M., Krumlauf R., Wiedemann L.M., Sower S.A., Decatur W.A., Hall

J.A., Amemiya C.T., Saha N.R., Buckley K.M., Rast J.P., Das S., Hirano M., McCurley N., Guo P., Rohner N., Tabin C.J., Piccinelli P., Elgar G., Ruffier M., Aken B.L., Searle S.M.J., Muffato M., Pignatelli M., Herrero J., Jones M., Brown C.T., Chung-Davidson Y.W., Nanlohy K.G., Libants S.V., Yeh C.Y., McCauley D.W., Langeland J.A., Pancer Z., Fritzsch B., de Jong P.J., Zhu B., Fulton L.L., Theising B., Flicek P., Bronner M.E., Warren W.C., Clifton S.W., Wilson R.K. & Li W. (2013) *Sequencing of the sea lamprey (Petromyzon marinus) genome provides insights into vertebrate evolution*. Nat Genet. 2013 Apr;45(4):415-421e2. Epub 2013 Feb 24 doi:10.1038/ng.2568.

Square T., Romášek M., Jandzik D., Cattell M.V., Klymkowsky M. & Medeiros D.M. (2015) *CRISPR/Cas9-mediated mutagenesis in the sea lamprey Petromyzon marinus: a powerful tool for understanding ancestral gene functions in vertebrates*. Development. 2015 Dec 1;142(23):4180-7. doi:10.1242/dev.125609.

Sternberg S.H., Redding S., Jinek M., Greene E.C. & Doudna J.A. (2014) DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* **507**, 62-7.

Sun N. & Zhao H. (2013) Transcription activator-like effector nucleases (TALENs): A highly efficient and versatile tool for genome editing. *Biotechnology and Bioengineering* **110**, 1811-21.

Swiech L., Heidenreich M., Banerjee A., Habib N., Li Y., Trombetta J., Sur M. & Zhang F. (2015) In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat Biotechnol* **33**, 102-6.

Tagliavini J., Williot P., Congiu L., Chicca M., Lanfredi M., Rossi R. & Fontana F. (1999) Molecular cytogenetic analysis of the karyotype of the European Atlantic sturgeon, Acipenser sturio. *Heredity* **83**, 520-5.

Tajima A., Naito M., Yasuda Y. & Kuwana T. (1993) Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (Gallus domesticus). *Theriogenology* **40**, 509-19.

Takeuchi Y., Yoshizaki G. & Takeuchi T. (2003) Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. *Biol Reprod* **69**, 1142-9.

Takeuchi Y., Yoshizaki G. & Takeuchi T. (2004) Biotechnology: surrogate broodstock produces salmonids. *Nature* **430**, 629-30.

Tesson L., Usal C., Menoret S., Leung E., Niles B.J., Remy S., Santiago Y., Vincent A.I., Meng X., Zhang L., Gregory P.D., Anegon I. & Cost G.J. (2011) Knockout rats generated by embryo microinjection of TALENs. *Nat Biotech* **29**, 695-6.

Urnov F.D., Miller J.C., Lee Y.-L., Beausejour C.M., Rock J.M., Augustus S., Jamieson A.C., Porteus M.H., Gregory P.D. & Holmes M.C. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**, 646-51.

van der Oost R., Beyer J. & Vermeulen N.P. (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ Toxicol Pharmacol* **13**, 57-149.

Wang H., Yang H., Shivalila C.S., Dawlaty M.M., Cheng A.W., Zhang F. & Jaenisch R. (2013) One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering. *Cell* **153**, 910-8.

Wang T. & Hong Y. (2014) Direct gene disruption by TALENs in medaka embryos. *Gene* **543**, 28-33.

Weber J., Ollinger R., Friedrich M., Ehmer U., Barenboim M., Steiger K., Heid I., Mueller S., Maresch R., Engleitner T., Gross N., Geumann U., Fu B., Segler A., Yuan D., Lange S., Strong A., de la Rosa J., Esposito I., Liu P., Cadinanos J., Vassiliou G.S., Schmid R.M., Schneider G., Unger K., Yang F., Braren R., Heikenwalder M., Varela I., Saur D., Bradley A. & Rad R. (2015) CRISPR/Cas9 somatic multiplex-mutagenesis for high-throughput functional cancer genomics in mice. *Proc Natl Acad Sci U S A* **112**, 13982-7.

Weidinger G., Stebler J., Slanchev K., Dumstrei K., Wise C., Lovell-Badge R., Thisse C., Thisse B. & Raz E. (2003) dead end, a Novel Vertebrate Germ Plasm Component, Is Required for Zebrafish Primordial Germ Cell Migration and Survival. *Current Biology* **13**, 1429-34.

Wolfe K.H. (2001) Yesterday's polyploids and the mystery of diploidization. *Nat Rev Genet* **2**, 333-41.

Wood A.J., Lo T.W., Zeitler B., Pickle C.S., Ralston E.J., Lee A.H., Amora R., Miller J.C., Leung E., Meng X., Zhang L., Rebar E.J., Gregory P.D., Urnov F.D. & Meyer B.J. (2011) Targeted genome editing across species using ZFNs and TALENs. *Science* **333**, 23.

Xue W., Chen S., Yin H., Tammela T., Papagiannakopoulos T., Joshi N.S., Cai W., Yang G., Bronson R., Crowley D.G., Zhang F., Anderson D.G., Sharp P.A. & Jacks T. (2014) CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature* **514**, 380-4.

Yamaha E., Goto-Kazeto R., Saito T., Kawakami Y., Fujimoto T., Adachi S. & Arai K. (2010) Primordial germ cell in teleost fish with special references to its specification and migration. *Journal of Applied Ichthyology* **26**, 816-22.

Yang H., Wang H., Shivalila Chikdu S., Cheng Albert W., Shi L. & Jaenisch R. (2013) One-Step Generation of Mice Carrying Reporter and Conditional Alleles by CRISPR/Cas-Mediated Genome Engineering. *Cell* **154**, 1370-9.

Yazawa R., Takeuchi Y., Higuchi K., Yatabe T., Kabeya N. & Yoshizaki G. (2010) Chub mackerel gonads support colonization, survival, and proliferation of intraperitoneally transplanted xenogenic germ cells. *Biol Reprod* **82**, 896-904.

Yazawa R., Takeuchi Y., Morita T., Ishida M. & Yoshizaki G. (2013) The Pacific bluefin tuna (Thunnus orientalis) dead end gene is suitable as a specific molecular marker of type A spermatogonia. *Molecular Reproduction and Development* **80**, 871-80.

Yu Z., Ren M., Wang Z., Zhang B., Rong Y.S., Jiao R. & Gao G. (2013) Highly efficient genome modifications mediated by CRISPR/Cas9 in Drosophila. *Genetics* **195**, 289-91.

Zhang F., Wen Y. & Guo X. (2014) CRISPR/Cas9 for genome editing: progress, implications and challenges. *Human Molecular Genetics*.

http://www.iucnredlist.org/news/sturgeons-highly-threatened

http://studylib.net/doc/7162172/designing-and-making-sgrna-constructs

http://www.snapgene.com/resources/plasmid_files/mammalian_expression_vectors/pX330/

8 List of abbreviations

CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-Associated Proteins 9
crRNA	CRISPR RNA
sgRNA	Single/Short guide RNA
PAM	Protospacer Adjacent Motif
PGCs	Primordial Germ Cells
dnd1	dead end1 gene
DSBs	Double Strand Breaks
HR	Homologous Recombination
NHEJ	Non-Homologous End Joining
ZFNs	Zinc Finger Nucleases
TALENs	Transcription Activator-Like Effector Nucleases
MO	Morpholino Oligonucleotide
Ardnd	*dnd1* gene sequence of sterlet
Agdnd	*dnd1* gene sequence of Russian
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
Indels	Insertions/Deletions

9 List of figure and table

List of pictures

List of tables

10 Abstract

Thesis title: "Utilization of genome editing technology to knock out *dnd1* gene in sturgeons"

Author: Vu Thi Trang

Study program: Master study program

Field of study: Agricultural specialization/Fishery and Protection of Waters

Institute/Department: Laboratory of Germ Cells, Research Institute of Fish Culture and Hydrobiology, Faculty of Fisheries and Protection of Waters, University of South Bohemia in the Czech Budejovice.

Supervisor: Assoc. Prof Dipl.-Ing. Martin Pšenička, Ph.D.

Advisor: Abdul Rasheed Khanzai Baloch, MSc

In this study, for the first time we used CRISPR/Cas9 gene editing technology in sturgeons i.e., sterlets (*Acipenser ruthenus*). The sequences of sgRNA and primers were designed based on published *dnd1* sterlet sequence. Each pair of sgRNA oligos after ligation – ready duplex DNA fragment was cloned into vector pX330-U6-Chimeric_BB-CBh-hSpCas9 backbone and thereafter the transformation to competent cells *Escherichia coli DH5α* was done. The plasmid carried sgRNA was extracted for downstream applications. We diluted extracted plasmid with 10% of 2 M KCl and injection into animal pole of fertilized eggs of sterlets at one to four-cell-stage, 4 hours post fertilization (hpf). At the same time, second microinjection with 2.5% FITC-biotin-dextrans was injected into vegetal pole for labelling PGCs. In the control groups, the eggs were only injected by 2.5% FITC into vegetal pole. PGCs of sterlet were visualized and photographed using a fluorescent stereo microscope Leica M165 FC. To confirm the presence or deletion/insertion occurring in the target gene, we used MCE-202 MultiNA microchip electrophoresis system for DNA analysis, in which the targeted gene after amplifying by PCR was analyzed. Mutations in both treated and control embryos of sterlet were further assessed by Sanger sequencing of the PCR product.

In present study, we successfully established basic protocols such as preparation of competent cells, construction of vector carrying sgRNA and its transformation into

competent cells to carry out the CRISPR/Cas9 technology in sturgeons. Less number of PGCs was observed in embryos that were treated with CRISPR/Cas9; however, sequencing did not provide us a reliable evidence for mutation of the targeted gene probably due to an unspecific PCR. Therefore, more authentication of *dnd1* knockout should be done in future by more specific PCR and repeated sequencing.

Key words: CRISPR/Cas9, primordial germ cells, *dead end*, knockout, sterilization, sterlet.

Abstract (in Czech)

Název diplomové práce: "Využití technologie úpravy genomu pro knock-out genu *dnd1* u jeseter".

V této studii jsme poprvé použili technologii úpravy genomu CRISPR/Cas9 u jeseterů, tj. jesetera malého (*Acipenser ruthenus*). Sekvence sgRNA a primerů byla navržena na základě známé sekvence *dnd1* genu jesetera malého. Každý pár sgRNA oligonukleotidu byl po ligaci klonován do vektoru pX330-U6-Chimeric_BB-CBh-hSpCas9 backbone a poté transformován do kompetentních buněk *Escherichia coli DH5α.* Plasmidy, které nesly sgRNA byly extrahovány, naředěny 10% 2M KCl a injikovány do animálního pólu oplozené jikry jesetera malého ve stádiu 1-4 buněk (do 4 hodin po oplození). Ve stejný čas byla provedena injikace 2,5% FITC-dextranu do vegetativního pólu, kterým byly barveny primordiální gonocyty (PGC). Kontrolní skupina byla injikována pouze 2,5% FITC-dextranem do vegetativního pólu. PGC byly poté vizualizovány a fotografovány pomocí fluorescentního stereomikroskopu Leica M165 FC. PCR s použitím kapilárové elektroforézy MCE-202 MultiNA sloužilo pro potvrzení mutace (delece nebo inzerce) cílového genu. Mutace byla dále hodnocena pomocí Sanger sekvenování PCR produktu.

V této studii jsme úspěšně vyvinuli základní protokoly k provedení CRISPR/Cas9 technologie u jesetera, jako příprava kompetentních buněk, konstrukce vektoru nesoucího sgRNA, transformace do kompetentních bakterií, injikace do embryí. Menší počet PGC bylo pozorováno u embryí, které byly ošetřeny CRISPR/Cas9. Ovšem sekvenování nám neposkytlo uspokojivý důkaz mutace cílového genu pravděpodobně kvůli nespecifické PCR. Proto bude v budoucnu nutné provést potvrzení knockoutu více specifickou PCR a opakovaným sekvenováním.

Klíčová slova: CRISPR/Cas9, primordiální gonocyty, dead end, knockout, sterilizace, jeseter malý