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FISH STEM CELL TECHNOLOGY IN AQUACULTURE

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ABSTRACT

Stem cells are a class of undifferentiated cells, have the potential to reproduce themselves by mitotic cell division, generate progeny destined to differentiate into functional cell types, persist for a long time and its behavior is regulated by the micro environment. Aquaculture's primary goal is to produce fresh and marine fish species in order to supply world's protein needs. Because of global climate change, depletion of water bodies, antibiotics misuse, environmental pollutants, competition with agriculture and lack of fish meal and oil for use in fish feeds the production of aquaculture will not be sustainably in the future. These impacts can be avoided by using stem cell technologies such as; surrogate broodstock, endangered fish protection and production, fish meat production from stem cells, monosex fish production and gene transfer studies. This review aims to provide information regarding fish stem cells application technologies for sustainability of aquaculture.

Keywords: Cell transplantation, endangered fish, surrogate broodstock, gene transfer

INTRODUCTION

Stem cells technologies continue to accelerate in all over the world. Publications of articles in this area are also steadily increasing in the last 4 decades. When these articles examined there is no standard definition of stem cells. However, the two defining characteristics of a stem cell are self-renewal by mitotic division and the ability to differentiate into a specialized adult cell type (Çek et al., 2016). Latest uses of the term 'stem cell'' are reviewed by Slack (2018). The author claimed that, defining stem cells was slippery and difficult, but defining stem cell behavior was relatively easy. Because, stem cells did not exist outside their microenvironment and that stem cell behavior was an emergent property of a multicellular system rather than of a single cell. Four properties of stem cells was defined by Clevers, (2015) and Slack (2018). These were reproducing of themselves, generating progeny designated to differentiate into functional cell type, persisting for long time and behavior regulation by the immediate environment. Stem cell definition and behavior are given in Figure 1.

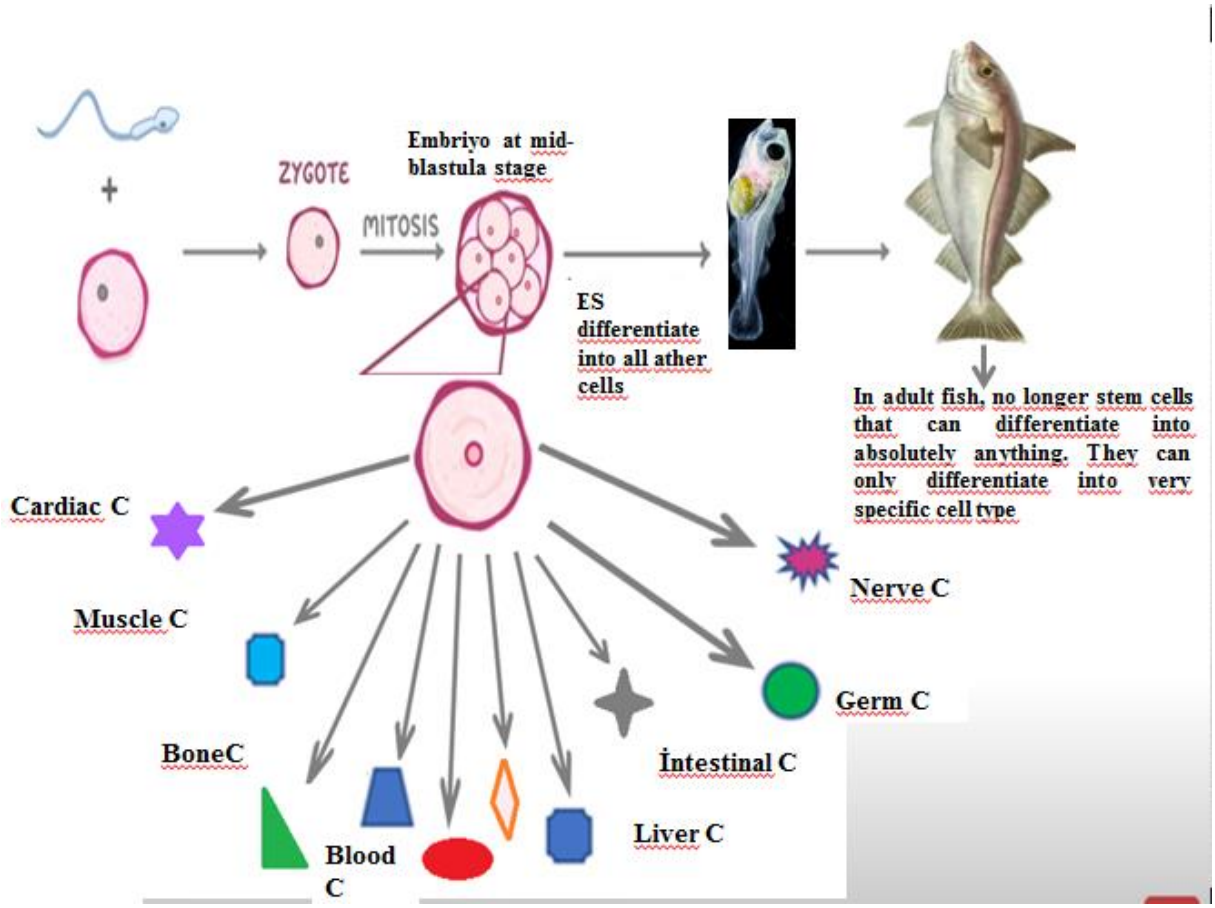

FIGURE 1: Definition of Fish Stem cells. Embryonic stem cells are able to generate a mature fish. After fertilization, cells start to divide by mitosis and increase in number. As a result generating an embryo at mid-blastula stage, only one cell in this embryo has the power to generate an adult fish as it is seen in the figure.

The global demand for marine and freshwater aquaculture products is the largest of all animal food products and the aquaculture is the fastest growing food production sector in the world (Maulu et al., 2021). China is contributing to more than half of global aquaculture water consumption and greenhouse gas emissions, followed by India and Indonesia (Jiang et al., 2022). Global aquaculture production has increased by 500% since the late 1980 (FAO, 2018). According to FAO (2020), aquaculture's contribution to global fish production has continued to increase, reaching 82.1 million tons /46%) out of the estimated 179 million tons of global production. Furthermore, aquaculture production is expected to grow from the current 46% to 53% in 2030 (FAO, 2020). However, global climate change, environmental pollutants, competition with agriculture and lack of fishmeal and oil for use in fish feeds the production of aquaculture will not be sustainably in the future. Very few trials have been performed to measure the effects of climate change on aquaculture production (Reverter et al., 2020; Engelhard et al., 2022). However, it is extremely difficult to validate these measurements (Reid et al., 2019; Naylor et al., 2021). The negative effects are direct and indirect. Of these are rising temperature, sea level rise, changes in rainfall patterns, changes in sea pH, and extreme climatic events (Reid et al., 2019; Elsheikh, 2021). While some regions may experience short-term benefits from climate change overall global productions are predicted to decrease 10% by 2050 (Barange et al., 2014) and the aquaculture's long-term viability is challenged by the consequences of pollution, global warming and lack of fish feeds.

For sustainable aquaculture production, a more open approach on cutting edge technologies is inevitable, as well as application of these technologies are needed for resilient aquaculture production. Of these technologies stem cell breakthrough could offer new means of sustainable aquaculture production. Cellular aquaculture production, the production of fresh meat from stem cells such as muscle and fat cells using cell culture techniques has been proposed as a novel approach to complement the conventional marine and freshwater fish production.

Yoshizaki et al (2011, 2012) have succeeded in producing the first-ever mass proliferation of rainbow trout germline stem cells in vitro, a technique that could lead to mass production and preservation of endangered fish species. This technology makes possible the production of donor-derived oogonia and spermatogonia in surrogates and encompasses transplanting germ cells of a donor into recipients of a different strain or different species (Yoshizaki and Yazawa, 2019).

Recently genome editing (GE) technology is applied to improve aquaculture production. Genetically modified organism (GMOs) has been produced for enhanced growth rate, increased production, disease resistance, improved reproduction and living in different environmental conditions including global warming. Devlin et al., (1994) indicated that salmon transgenic for a homologous growth hormone (GH) gene averaged more than 11-fold heavier than non-transgenic controls.

In this review the application of these technologies, in the field of sustainable aquaculture will be revised.

GERM STEM CELL TRANSPLANTATION IN AQUACULTURE

Primordial germ cells (PGCs) are undifferentiated stem cells that move into gonadal anlage (gonocytes) during embryogenesis and after reaching gonadal sides, they differentiate into a mature egg in females or a mature sperm in males (Çek, 2006; Çek et al., 2016). These cells are the only embryonic cells that carry genetic information from one generation to the next. Therefore, these cells are extremely important in protection and preservation of gametes, germ cell transplantation in the technology for surrogate production of donor-derived gametes (Yoshizaki et al., 2012; Shang et al., 2015; 2018; Çek-Yalnız and Yaraş, 2019; Yaraş and Çek-Yalnız, 2021). In Salmonidae, Yoshizaki et al (2002) first developed germ stem cell transplantation technology. In this technology, there are three methods for PGCs transplantation in fish. In the first one, PGCs are obtained from donor embryos and transplanted into blastula stage, at side of the blastodisc where these cells divide prior to settling at the gonadal anlage of the recipient that have had endogenous PGCs development blocked by the injection of a dead end antisense morpholio oligonucleotide (MO) (Lacerda et al., 2013). In this technique, the recipient's germ line is completely replaced by the donor' PGCs. The PGCs transplanted fish must be reared until they are sexual mature and are able to produce donor gametes (Figure 2a). In the second method, PGCs are transplanted into newly hatched larvae. These donor-derived germ cells are injected in the coelomic cavity during the time-period in which sexual differentiation has not formed yet and, PGCs are still actively migrating to the gonadal anlage. Transplanted germ cells are able to move and colonize in the gonadal anlage of the recipient. After maturation stage, recipient fish are able to produce donor gametes (Figure 2b). In the last method, germ cells are transplanted in sexually mature fish. Spermatogonial or oogonial germ cells are directly transferred into matured testis and ovaries respectively. Where they generate viable donor gametes (Figure 2c).

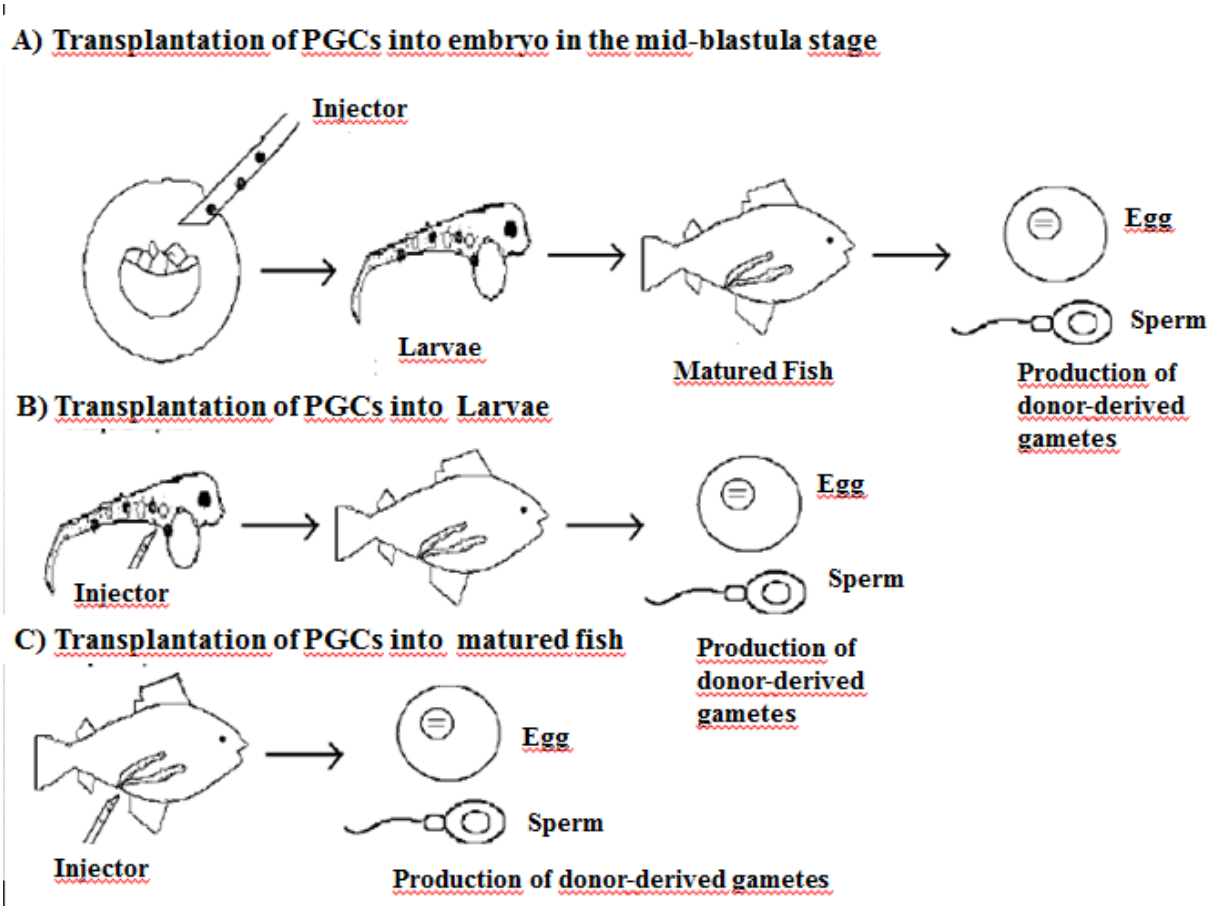

FIGURE 2: Germ cell transplantation techniques used in fish. A) PGCs transplantation in embryos. Donor derived PGCs are microinjected into embryo at mid-blastula stage. These embryos must be grown until they are matured and produced donor-derived gametes. B) PGCs transplantation into newly hatched larvae. At this stage, endogenous PGCs are still migrating to the gonadal anlage. Immunolgical development has been developed and the larvae cannot reject PGCs. C) Germ cell transplantation in adult fish, which have been previously sterilized. This fish generate viable donor gametes. Modified from Lacerda et al., 2013; Çek et al., 2016).

This technology is expected to be able to produce large bodied commercially important fish species like Bluefin tuna (*Thunnus orientalis*) from a small chub mackerel. It is quite difficult to maintain Bluefin tuna in hatchery condition because of its 500kg body weight. It is required 5 years to sexually mature. In contrast, it is quite easy to handle and maintain adult chub mackerel in the hatchery conditions. It belongs to same family, to the *Scombridae* and weighs only 300 g and reach sexual maturity in one year (Yoshizaki et al., 2019). Therefore, if the chub mackerel were able to produce gametes of Bluefin tuna, the space, labor, feed expenses, handling stress and cost required for maintenance of the broodstock would be minimized. In addition, this technology in fishes has potential for endangered species conservation, protection and propagation. Because it is much easier to preserve germ cells by cryopreservation than eggs and sperm. Sperm cryopreservation has been done for decades but oocytes and egg cryopreservation is not yet successful. PGCs, spermatogonial stem cells and oogonial stem cells are small and do not contain much lipid or egg yolk. Therefore, they are much easier to keep them in lıquid nitrogen for a long time and then they can be transplanted into suitable recipients or culture in vitro to produce viable gametes and protect endangered

species (Morinovic et al., 2018; Lujić et al., 2017 and 2018). Zebrafish has been used as a model fish species in many studies (Yaraş and Çek-Yalnız, 2023). Franék et al., (2022) studied on zebrafish and find out that, only germ cell depleted recipients retained reproductive characteristics of the donor strain. In their study, adult germ line chimera rate and their reproductive output were best in germ cell-depleted recipients with normal somatic.

The remarkable of surrogate broodstock technology is that female recipient produce functional eggs derived from donor cells after the transplantation of male stem cells prepared from the donor testis (Okutsu et al., 2006) and male recipients produce functional sperm derived from the donor after the transplantation of female stem cells prepared from the donor ovary (Yoshizaki et al., 2010).

Ryu et al., (2022) reviewed 70 publications regarding to germ stem cell transplantation and concluded that this technology had not actively been utilized for commercial purposes, what barriers need to be overcome, and what potential solutions could advance its applications in aquaculture was discussed in detail

STEM CELL-BASED FISH MEAT PRODUCTION IN AQUACULTURE

Cell based fish meat production has been acknowledged by many names, like cellular meat, cell culture meat, engineered meat, factory-grown meat, in-vitro meat, fake meat, clean meat, neat meat, synthetic meat, lab-grown meat, and artificial meat (Azhar et al., 2023). Hallman, (2021) suggested to use the term Cell-based Seafood to label seafood products produced from the cells of fish. Because cell-based seafood products more positively than cell-cultured and were slightly more inclined to want to taste and purchase. Morris Benjaminson produced the first stem cell-based fish in 2000 (Benjaminson et al., 2002). Stem cell-based fish meat production is expected to solve global temperature rise, depletion of water bodies, antibiotics misuse, environmental pollutants, and competition with agriculture and even fish welfare challenges. Nevertheless, considerations for cell-based seafood productions are numerous, differing in many respects from those of cell-based terrestrial animals. While cell-based seafood shares some common characteristics with terrestrial-based analogs in science and human considerations, sustainability is a more substantial driver, as cell-based seafood could lead to greater preservation of marine environments by reducing fishing pressure (Rubio et al., 2019).

More than100 ventures are competing to commercialize cell-based meat production in the world. Of these 30 are trying to produce cell-based seafood (TABLE 1).

The production of stem cell-based seafood requires the extraction, isolation of muscle and lipid cells from fish, mollusks and/or crustaceans followed by their generation in ideal conditions inside a bioreactor (Figure 3). Cells are typically grown on an edible scaffold that is designed to give them the structure and texture of wild-caught and hatchery cultured fish meat. The expected ideal result is that cultured cell-based meat becomes indiscernible from wild-caught and hatchery cultured fish meat.

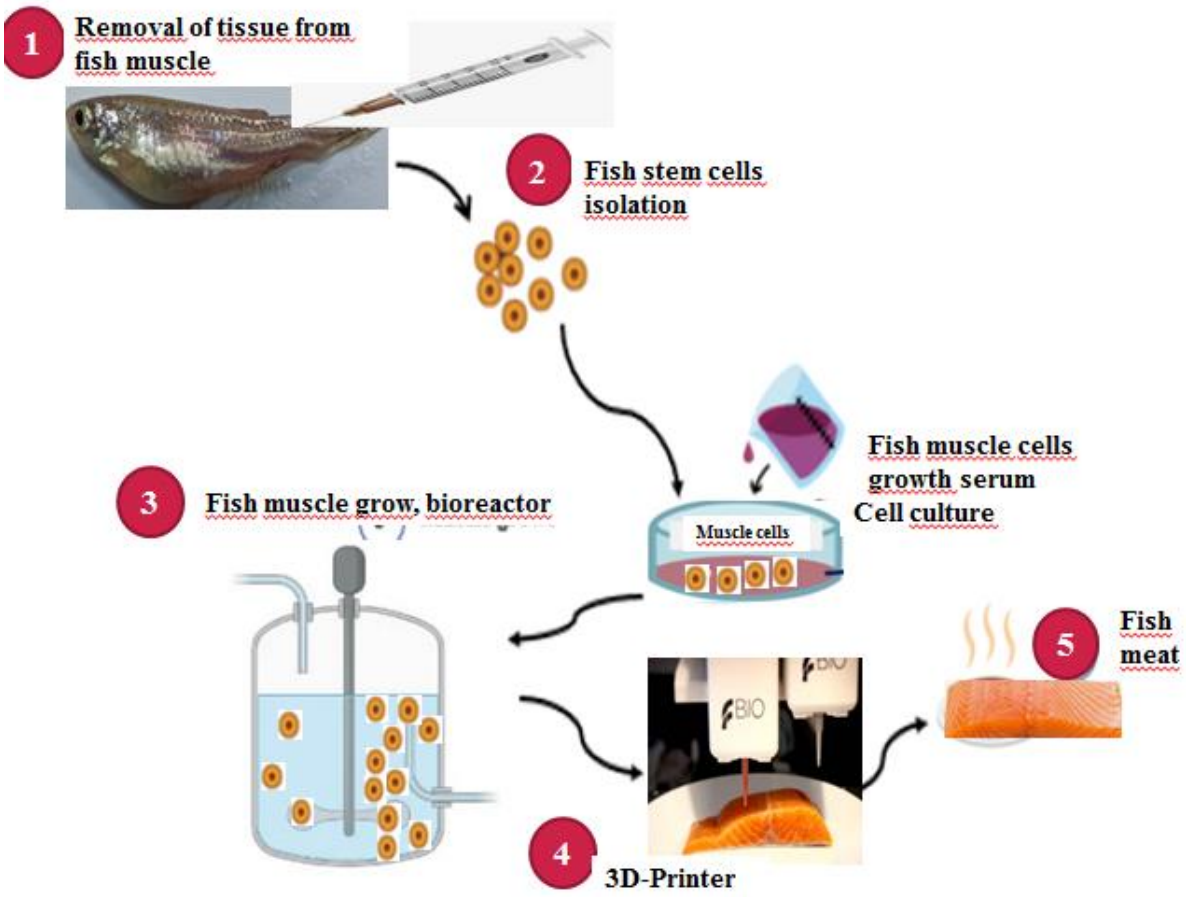

FIGURE 3: Illustration of Cell-based fish meat production. Modified from https://blogs.ifas.ufl.edu/fshndept/2022/04/11/alternative-protein-sustainable-cell-based-seafood/.

However, a research by Halpern et al., (2021) suggested that the links between cell-based seafood and marine conservation might be more tenuous than they first appear. Specifically, getting consumers to make the switch to lab-grown fish. The researchers identified some very important key issues regarding to cell-based seafood to be solved by the industry. These are; the muscle tissue has multiple cell types with different proliferative and differentiation capabilities and the correct identification of the proportion of such cells for co-culture is a challenging issue. Another issue is that cell lines for crustacean and mollusk are not available. The traditional cell culture method is dependent on a serum that is very expensive, inconsistent and unsustainable component in the culture media and will be a major challenge in the large-scale production of stem cell-based meat. Serum free culture may be a future direction to improve in vitro meat production (Schepici et al., 2022). Stem cells itself is the major technical challenge for in vitro meat production. Meat structure is mostly dependent on the scaffold and the fully development of scaffold is also one of the major technical challenges. Markers (to separate muscle cells and progenitor cells) for identification of cell-surface proteins are another difficult task. Consumers may resist the artificial produced meat because of lack of knowledge about the novel technique. Finally, cell-based meat should meet the desired texture, color and appearance in natural meat (Goswami et al., 2022)

TABLE 1 List of Companies Working on Cell-Based Seafood Production

No	Company Name	Location	Seafood Type	Date Founded
1	Thai Union Group	Thailand	Tuna and mahi-mahi	1977
2	ArtMeat	Russia	Sturgeon	2015
3	Memphis Meats (UPSIDE Foods)	USA	Sea foods	2015
4	Finless Foods	USA	Bluefin tuna and carp	2016
5	WildType	USA	Salmon	2016
6	BlueNalu	USA	Mahi-mahi and Bluefin tuna, to start.	2017
7	SeaFuture	Canada	Fish	2017
8	**Biftek.co**	**Türkiye**	**Currently only beef, no seafood Founder: Can Akçalı and Erdem Erikçi**	**2018**
9	Avant Meats	Hong Kong	fish maw and undisclosed fish fillets	2018
10	Shiok Meats	Singapore	crustaceans- shrimp, crab and lobster have already been unveiled	2018
11	Magic Caviar	Netherlands	Caviar	2019
12	Cell MEAT	South Korea	Shrimp, lobster and other high-value seafood varieties.	2019
13	Cell Ag Tech	Canada	White fish	2019
14	SoundEats	USA	White Fish	2019
15	Umami Meats	Singapore	Exotic, crab, shrimp	2020
16	CellX	China	Seafood1/3 world ‘demand	2020
17	Cultured Decadence	USA	Crustacea	2020
18	Bluu Biosciences	Germany	Salmon, trout and Carp	2020
19	Another Fish	Canada	Fish	2021
20	Bluefin Foods	USA	Bluefin tuna	2021
21	Fisheroo	Singapore	Fish	2021
22	SeaWith	South Korean	Fish	2021
23	Sea-Stematic	South Africa	Sea foods	2021

Singapore is the first country in the world, which allowed stem cell-based meat product for safe consumption and commercial sale (Waltz, 2021). The stem cell-based chicken products developed by the company Eat Just were available on the market in Singapore in 2020 (Ye et al., 2022). The USA is the second country allowing stem cell-based products as safe consumption and sale. Wang et al., (2023), recently reviewed three-Dimensional Scaffolds for stem cell-based meat production. The authors concluded that, cell-based meat production could not completely replace aquaculture.

GENE TRANSFER IN AQUACULTURE BY EDITING GERM STEM CELL LINE

Gene transfer technology has allowed the transfer of genes from one fish species to another in order to create new lineages of organism with improvement in characteristics particularly important to aquaculture (Levy et al., 2000; Yang et al., 2022). Genetically modified organism (GMOs) has been produced for enhanced growth rate, increased production, disease resistance and living in different environmental conditions including global warming. Devlin et al., (1994) indicated that salmon transgenic for a homologous growth hormone (GH) gene

averaged more than 11-fold heavier than non-transgenic controls. There are several gene transfer technologies such as: Antisense Morpholino Oligomers (MO), Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALLENS), Dre-Rox, Cre-Lox, Clustered Regularly Interspaced Short Palindromic Repeats (Crispr/Cas9). However, this section of the review summaries only gene editing in germ stem cell technology. Genome editing was first started with model fish, zebrafish and Medaka, then technology followed with economically important aquaculture species. GE may be the next important technology for the second quarter of the century, offering potential solutions to some of the aquaculture sector's biggest sustainability challenges (Houston et al., 2022).

Surrogate broodstock technology makes possible the production of donor-derived sperm and eggs in surrogates, and incorporates transplanting PGC cells of a donor into recipients of a different strain or different species. In this technology, the efficient and reliable production of offspring carrying superior genetic traits is possible. Germ stem cells can be taken from a single selected donor fish and transplanted into many recipient fish (Yoshizaki et al., 2016, 2019; Divyanand et al., 2023). Therefore, this technology enables the generation of males that lack germline stem cells of their own, but can receive germ cells from superior donor males (Yoshizaki et al., 2019; Jin et al., 2021). In this technology, a single superior male can produce many larvae. Surrogate mother generated in Salmon using a knockout of the *dnd* gene with CRISPR/Cas9. In the gene edited sterile male surrogate fish, seminiferous tubes are usually intact, but no sperms. Male germ cells can be transplanted into these seminiferous tubes in order to produce superior donor male germ cells. Genome editing in combination with germline cloning by surrogate males is able to increase the selection intensity (Yang et al., 2022).

Genome editing (to knockdown or knockout of dnd gene) require individual microinjection of the antisense oligonucleotide (ASO) and guide RNA, together with Cas9 protein into fertilized eggs under a microscope. However, this is not a practical method because gametes cannot be obtained from sterile individuals produced by this method. In addition, microinjection into the fertilized egg is required every time to produce sterile fish (Yoshizaki et al., 2019).

PGCs can be transplanted into the peritoneal cavity of embryo in which sterility was induced by knockdown of dead end, triploidy or hybridization. Currently there is no direct genome editing in aquaculture species. It has been reported in chicken where edited PGCs were transplanted into recipient and produced homozygous F1 and F2 progeny.

The most widely applied GE method in aquatic species is microinjection in the embryo at the blastula stage, and this typically results in highly mosaic F0 fish (Gratacap et al., 2019). This method is not stable for aquaculture species. Firstly, multiple generations of breeding may be required in order to achieve fully homozygous fish, and this is difficult particularly for species with long generation intervals, such as Atlantic salmon. Secondly, due to substantial variation in regulatory environments and customer preferences, introducing edits into the core-breeding nucleus may be a risky approach, and editing in dissemination or multiplication lines may be preferable (Houston et al., 2022). These obstacles may be overcome by editing PGCs and

combining with sterilized surrogate broodstock (Yoshizaki et al., 2019; Jin et al., 2021). Moreover, GE in PGCs/Germ Cells causing sterility of the offspring prevents any potential escape fish interbreeding with wild stock. Yoshikawa et al., (2020) used GE techniques, knock downed dead end 1 gene in grass puffer fish, and produced functional gametes of tiger puffer fish. Previously, Hamasaki et al., (2017) used triploidization for sterilization of grass puffer fish. However, triploidization leads to a reduction in survival rates and triploid rate itself is not very high in some fish species, especially in marine species. Xu et al., (2023) produced sterile coho salmon and sablefish for the first time. The authors used morpholino oligonucleotides to temporarily silence dead end (dnd) gene.

The efficiency of GE technology in aquaculture species can be improved by using GSCs transplantation to surrogate broodstock. First GSCs are taken from embryo (Primordial germ cells) and/or juvenile ovaries (oogonial stem cells) or testes (Spermatogonial stem cells). Then these cells isolated and cultured in petri-dishes in controlled sterilized laboratory conditions. Then GSCs could be engineered to express the CRISPR/Cas9 system, with gRNA delivered by viral vectors or transposons. Subsequently, the edited GSCs pool can be transplanted into surrogate broodstock (are lacking endogenous germ cells) which previously been sterilized by knockout of the *dnd* gene with CRISPR/Cas9 (FIGURE 4), (Jin et al., 2021). In this method, germ line stem cells from a donor carrying superior traits can be transplanted into a large number of broodstock fish to generate a large number of females and males that produce gametes carrying genes associated with the superior traits.

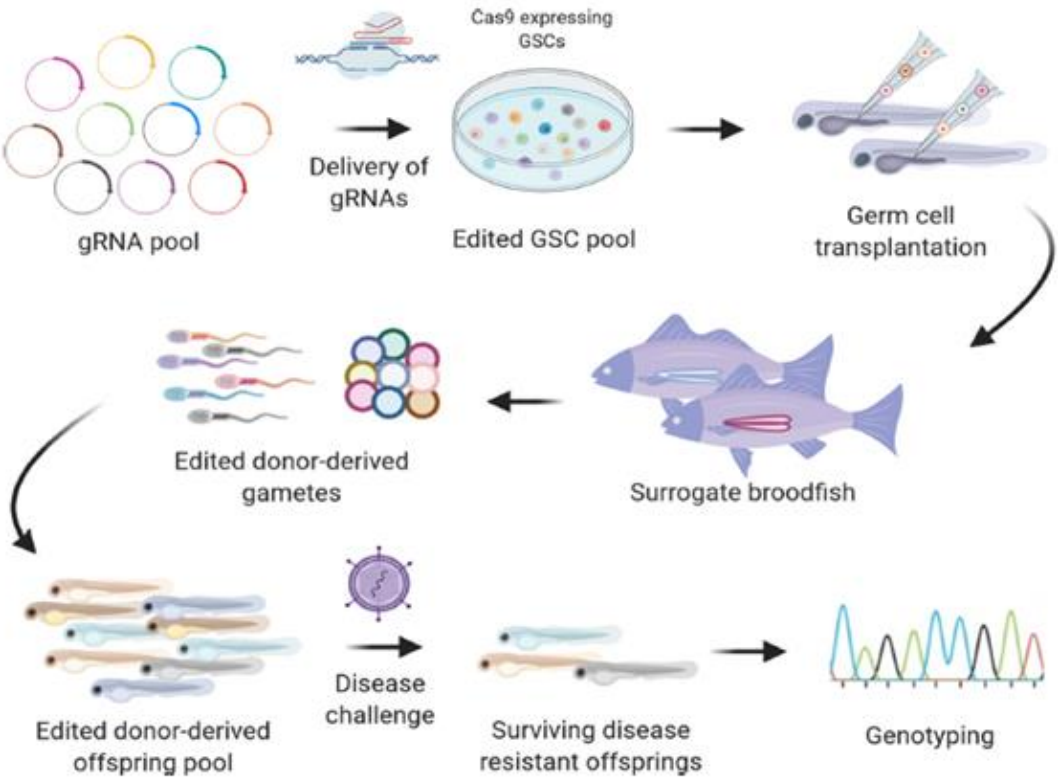

FIGURE 4: Schematic overview of early-life in vivo genome-wide CRISPR screening for disease resistance by targeting GSCs and subsequent transplantation using surrogate technology in fish (Jin et al., 2021).

CRISPR/Cas9 technology has significant applications, in order to increase aquaculture production. In Japan the sale of GE some fish species have targeted mutations in order to improve growth rate has currently been approved (Japan embraces CRISPR-edited fish, 2022). Recently GE technology has focused on improving growth rate in aquaculture. However, in near future, traits such as disease resistance, animal welfare, body morphology and color, strength to undesirable environmental conditions (like global warming) and eventually specific traits are likely to gain significant attraction.

CONCLUSION

Aquaculture is playing an increasingly important role in achieving global food security. Stem cell based fish meat production; germ stem cell transplantation and GE in surrogate mother are the novel technologies for increasing aquaculture production.

The future of stem cell –biased meat production depends on the development and depiction of appropriate muscle cell lines from commercially important and highest income-generating aquaculture species. Internationally available serum and/or serum free culture media, optimization of cell culture conditions, mass production of cells in bioreactors, consumer acceptance are challenges that need to be overcome in next decade. Nevertheless, stem cell-based meat production cannot completely replace aquaculture. Because of public prejudice and currently high price is not affordable.

Germ stem cell transplantation technology can be applied for mitigating reproductive problems in aquaculture fish species. This technology has a potential to enhance aquaculture production. However, currently germ stem cell transplantation technology is not actively been utilized for commercial purposes. Its procedures such as, donor germ stem cell isolation, culture, recipient preparation, apparatus, skills and scientists for transplantation must be standardized for each commercially important fish species.

Despite its success in improving growth rate, controlling sex, color and disease resistance of some economically important fish species, the application of genome editing in aquaculture species is just still its infancy, and is still scarce and there is several technical challenges and regulatory and ethical issues as well. One of the reason for this is that public have not been convinced yet and global regulations on GE is unclear. Another reason, GE is not integrated into selective breeding programmers. The application of the GE in fish farms is quite difficult and requires expertise personnel, and cutting edge technology.

Challenges of these novel technologies are summarized above. The potential economic benefits of stem cell-based meat production, germ stem cell transplantation and GE in surrogate broodstock technology to aquaculture are obvious. Nevertheless, there are some common challenges in the application of these technologies to aquaculture. Such as; the complexity of application methods, availability of internationally accepted procedures and consumer resistance to consume meat produced by these technologies. These technologies have the principal goal of producing food for human consumption; thus, the design of cell-based meat production, germ stem cell transplantation and GE in surrogate broodstock constructs must take into consideration the potential risk to consumer health, as well as

marketing strategies and product acceptance in the market. Nonetheless, in next decade, meat produced by these technologies will be on the market in many countries.

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