THE GOOD FOOD INSTITUTE

Sustainable Seafood Initiative Research Proposal

Creating a cell line repository for seafood-relevant species

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Executive summary

Our oceans hang in a precarious balance. Overfishing and harmful fishing practices have damaged fragile marine habitats, destabilized ocean ecosystems, and severely depleted global fisheries. The aquaculture industry has expanded rapidly as wild fisheries have collapsed, but these systems often present unique risks and limitations. New approaches are urgently needed to meet the increasing global demand for seafood without further jeopardizing aquatic ecosystems or placing undue burden on other global resources.

Plant-based and cell-based seafood present novel solutions to address these challenges by presenting consumers with more sustainable, healthier, and more humane options without compromising on taste. In the last decade, the market has seen massive shifts in consumer demand and product innovation for alternatives to meat and dairy products. These trends are likely to reflect a similar forthcoming transformation within the seafood industry, and the rapidly growing unmet demand for seafood coupled with the looming collapse of many global fisheries is likely to accelerate this shift.

However, virtually no dedicated funding outside of a few companies' R&D budgets has been expended in the development of plant-based and cell-based seafood thus far, resulting in substantial knowledge gaps for new product development. This industry exhibits tremendous potential to benefit from concerted resource allocation toward developing publicly accessible data to guide innovators in this space.

One area of urgent need is information on the parameters that define high-quality meat from a number of seafood-relevant species. A deep understanding of the molecular and structural signatures that define consumer experiences like taste, aroma, and texture is critical for developing both plant-based and cell-based products that recapitulate these sensory experiences as well as nutritional, aesthetic, and processing qualities. While the terrestrial meat industry (beef, poultry, pork, etc.) has a long history of publicly funded meat science research, detailed molecular and structural characterization of seafood products are either nonexistent or must be laboriously scraped from the scientific literature. In many cases, the data that exist are inconsistent, use outdated methods, or are simply too disaggregated to meaningfully use for guiding product development.

This proposal first establishes the parameters that define various types of seafood and surveys the data that exist on these attributes across various species. What follows is a detailed research plan for generating data to fill the knowledge gaps and a framework for incorporating both existing and novel data into a publicly accessible database.

The proposal identifies specific partners (companies, institutions, or specific research labs) with the appropriate expertise to conduct the work. Fifteen exemplar species representing several classes of seafood-relevant aquatic organisms are suggested, and three scopes of work are proposed to reflect a range of possible budgets. A "1x" scope with a budget of approximately $30,000 and a 12-week project timeline represents the minimum work that will provide meaningful results to advance the industry. Work packages corresponding to five-fold and ten-fold higher budgets are also presented, along with several optional work packages. The project scopes outlined in this proposal should be viewed as examples from a menu of possible options. Some costs scale linearly with species number and are therefore purely variable costs, but most work streams within the project present savings for higher volumes. Thus, funders are at liberty to define the scope of work such that it aligns with their mandate and mission.

The proposed research will address a critical knowledge gap that is hampering the development of high-quality, sustainable plant-based and cell-based seafood products: namely, detailed characterization of the seafood products that these approaches aim to emulate. The resulting public resource will enable researchers and innovators to accelerate the development and widespread commercial adoption of plant-based and cell-based seafood.

The transition to plant-based and cell-based seafood can be further accelerated by concerted efforts to apply insights from the development, commercialization, and generation of demand for plant-based and cell-based versions of terrestrial animal agriculture products. While many of these insights can be translated directly to plant-based and cell-based seafood, the seafood sector does pose some unique technical challenges for both plant-based and cell-based approaches. Consumer research providing a more nuanced understanding of seafood purchasing behavior across diverse consumer segments and cultures is also needed, to enable refinement of marketing and product development strategies.

While plant-based and cell-based seafood products will ultimately be produced and supplied through the private sector, the underlying technologies and their path toward commercialization will require a robust innovation ecosystem. Given that virtually no dedicated funding outside of a few companies' R&D budgets has been expended in this area and that the estimated total global R&D expenditure to date across all forms of plant-based and cell-based seafood is on the order of $10-20 million, this industry exhibits tremendous potential to benefit from concerted public and private resource allocation. To accelerate the process from early product development through to widespread market adoption, activities must be coordinated across startup companies, multiple sectors of established industries, private and public funders and investors, governments, trade associations, and academic and other research institutions.

All of these entities – and any individual who envisions a future with sustainable oceans of abundance – should consider this a call to action to contribute to the development and growth of the plant-based and cell-based seafood industry.

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1 Significance

The global population will exceed 9 billion by 2050, indicating a growing need to produce food in a more efficient and sustainable manner [1]. Seafood represents a significant source of dietary protein, with global per capita consumption exceeding 20 kg/year and expected to grow in coming decades [2,3]. Plant-based and cell-based seafood products exhibit the potential to meet increasing consumer demand for seafood without the drawbacks of industrial fishing or aquaculture, but these approaches present technical challenges that must be addressed before they see widespread adoption.

Unlike cell-based endeavors for products like beef, pork, and poultry, the cell-based seafood industry cannot capitalize on the wealth of information generated by decades and billions of dollars' worth of publicly funded basic research involving cultivation of relevant cell types. But while the cell-based seafood industry starts at a relative deficit, it has the advantage of being able to leverage extraordinarily powerful new tools, techniques, and insights to their fullest. This allows a relatively modest research investment today to generate many of the same resources and materials that the mammalian cell culture field spent decades developing.

A major bottleneck for the development of cell-based seafood is a lack of proliferative and pluripotent cell lines, which can serve as stable cell sources for both basic cell biology research and for the refinement of manufacturing platforms for cell-based seafood. Researchers working on projects relevant for cell-based seafood currently tend to rely on primary cell culture, which introduces variability and necessitates that the researchers laboriously repeatedly source tissue and recreate cell cultures. Furthermore, translating findings between labs or companies is virtually impossible without consistent cell lines for validating reproducibility. Finally, many researchers who would potentially like to pursue projects that are relevant to cell-based seafood simply do not have access to the source tissue or adequate protocols for beginning new research in this area.

A cell line repository (CLR) that generates and curates stable cell lines derived from aquatic species will be critical for fostering research and innovation to advance cell-based seafood. The CLR can drastically reduce the need for repeated generation of primary cell cultures and will allow for the nascent cell-based seafood industry to work with stable, consistent cell lines during product development and basic research. In addition to populating the repository with cell lines derived from seafood-relevant species, this project will also establish standardized protocols for working with these cell lines. This will significantly reduce the barrier for entry and will encourage and empower academic researchers and startups to begin working with aquatic cell lines. Additionally, the CLR could be of service to conservation efforts, particularly those related to species that have become endangered as a result of fishery mismanagement.

In its initial stages, the CLR will utilize two strategies for obtaining cell lines. Existing cell lines that have been reported in the academic literature but are not otherwise commercially available will be added to the repository after thorough characterization and validation. This approach will allow the CLR to leverage existing research and make these cell lines more accessible to interested parties. The second approach is to generate new pluripotent cell lines derived from aquatic species with commercial value. Combining these strategies will reduce redundancy and ensure that the CLR is populated with cell lines from high-value species for which there is robust consumer demand. Once operational, the CLR will significantly reduce the barrier for entry for cell-based seafood researchers and companies by providing proliferative fish cell lines and standardized protocols for their cultivation.

2 Background

The scientific literature describing cell culture from aquatic species pales in comparison to that for terrestrial organisms (most notably mammalian and avian). This presents some practical challenges for creation of the Sustainable Seafood Initiative Cell Line Repository (SSI CLR), namely a lack of established protocols and a relatively shallow pool of potential partners with extensive fish cell expertise. Most studies using cell cultures derived from aquatic organisms have focused primarily on developmental biology or disease-modelling. Consequently, many of the existing stable aquatic cell lines represent tissue types that are not relevant for the production of cell-based meat or they exhibit various cell pathologies or atypical behavior. Nonetheless, there exists some valuable information, surveyed below.

2.1 Model Organisms

Research involving fish cell lines has primarily focused on two model organisms: zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Multiple cell lines exist for each of these species covering a wide array of cell types. While neither of these organisms are produced as food, much of the work performed on these cell lines may be applicable to other commercially relevant species. In particular, protocols for establishing immortal pluripotent cell lines and controllably differentiating them into desired cell types can provide a broad strategy for accomplishing similar milestones in less-studied species.

Perhaps the most significant finding obtained from studying zebrafish and medaka is that many of the phenotypic markers associated with pluripotent stem cells that have previously been characterized in other model organisms are conserved within fish lineages [4]. One of the best characterized medaka embryonic cell lines, MES1, was shown to display multiple characteristic markers of pluripotent stem cells. These included a round or polygonal cellular morphology, high alkaline phosphatase activity, a normal karyotype, and stable growth over many passages and a long (>1 year) duration [5].

Researchers characterized the pluripotency of the MES1 cell line molecularly by identifying fish orthologs of many genes associated with undifferentiated cell states. For example, oct4 and nanog expression (or orthologs thereof) were elevated in MES1 and down-regulated in differentiated medaka cells. Thus, as in many terrestrial vertebrates, these genes provide a molecular fingerprint of pluripotency that can be used to assay fish cell lines. Additional features of pluripotent animal cell lines translate to zebrafish and medaka including elevated telomerase activity, the ability to form embryoid bodies, and the ability to terminally differentiate into multiple cell types. MES1 was shown to be capable of differentiating into neuron-like cells and muscle cells *in vitro*. The pluripotency of model fish stem cells has also been characterized extensively *in vivo* through the study of chimeric embryos. In these studies, development of the chimeric embryo is monitored and the ability of the cell line to differentiate into cells descending from all three germ layers is observed.

Regarding the creation of a SSI CLR, the most relevant findings from the study of zebrafish and medaka are that much of the work involving pluripotent stem cells in terrestrial vertebrates is likely broadly applicable to much of the (if not the entire) vertebrate lineage. This sentiment is echoed below in the review of a handful of studies that aimed to create embryonic stem cells (ESCs) derived from fish species of commercial interest. Thus, while the body of research available on fish stem cells is quite small relative to that for terrestrial vertebrates, there is still extensive information relevant to future studies, rendering this a tractable project with a high likelihood of success if the appropriate partners can be secured.

2.2 Freshwater Species

Bluegill fry (Lepomis macrochirus) – The bluegill fry is a freshwater species native to North America. Commercial production is limited because this species is primarily caught and consumed by recreational fisherman. A stable cell line from this species has been isolated and is one of the few commercially available fish cell lines through ATCC (BF-2, ATCC CCL-91) [6]. This is an adherent fibroblast cell line commonly used for disease modeling. It may be possible to use this cell line to effectively produce (or study production of) the connective tissue components of cell-based seafood or as a source material for induced pluripotent stem cell generation.

Rainbow trout (Oncorhycnchus mykiss) – Trout are heavily aquacultured, with global production levels exceeding 800,000 tonnes in 2014 [7]. The global market is valued at over $2 billion and it is a popular fish for consumption in many Western countries. The rainbow trout is one of the most extensively studied species in fish cell culture. There are more than 10 proliferative trout cell lines in existence, covering a wide array of tissue types including gill, gonad, intestine, and early embryo [8]. The early embryo cell line, RTee, is most suitable for the SSI CLR as its pluripotency provides the most flexibility for cell-based seafood initiatives [9]. Information obtained from the study of other trout cell lines, such as optimal growth conditions, may be valuable for maintaining trout cells in various differentiated states [10].

Nile tilapia (Oreochromis niloticus) – Consumption of Nile tilapia has grown rapidly over the past two decades. Global production of this species via aquaculture exceeded 3 million tonnes in 2014 with China and Egypt as the main producers [11]. A Nile tilapia embryonic cell line, TES1, was recently established from middle blastula embryos and is currently the only reported proliferative cell line derived from this species [12]. This cell line exhibits high levels of alkaline phosphatase activity consistent with embryonic lineages. Additionally, molecular characterization indicates expression of several pluripotency markers, including oct4, sox2, myc and klf4. Upon treatment with retinoic acid, TES1 cells produced embryoid bodies and terminally differentiated into a variety of cell types, indicating an ability to descend into all three germ layers *in vitro [12]*. The pluripotency of TES1 was also validated *in vivo* through experiments involving chimeric embryos, which revealed differentiation into multiple tissue types descended from the mesoderm, endoderm, and ectoderm [12].

2.3 Marine Species

Atlantic cod (Gadus morhua) – Atlantic cod is a demersal marine species found in the Northern Atlantic Ocean [13]. Overfishing of this species in the northwest Atlantic led to severe fishery collapse in the early 1990s and many populations have still not fully recovered [14,15]. From a conservation perspective, Atlantic cod is considered vulnerable and the species has proven somewhat recalcitrant to aquaculture [16,17]. Consequently, cell-based cod will be a significant boon to environmental and conservation efforts for this species. An ESC-like cell line derived from Atlantic cod was established in 2010. The cells were sourced from a fertilized blastula and upon establishment of the cell line were assayed for ac-POU2 expression [18]. This transcriptional regulator is the cod ortholog of a marker of undifferentiated cells. While this cell line did demonstrate a degree of pluripotency, the ability to reliably control differentiation was limited. This cell line was capable of forming embryoid bodies; however, some spontaneous differentiation was also observed.

Japanese sea bass (Lateolabrax japonicus) – Japanese sea bass is found in the Western Pacific Ocean and in freshwater environments during spawning. Commonly used in sushi, this organism is fished and

farmed via aquaculture in Japan and China. A stable GFP-expressing ES cell line (LJES1) was established for this species from transfected embryos [19]. The cell line was stable over more than 20 passages and demonstrated characteristics of pluripotency including embryoid body formation and differentiation into multiple cell types upon treatment with retinoic acid. Differentiation into both muscle cells and neuron-like cells was observed. Pluripotency of this cell line was further demonstrated by injecting GFP-expressing ES cells into chimeric zebrafish embryos [20]. In surviving embryos, GFP expression was detected in multiple tissue types, indicating robust pluripotency of this cell line.

Asian sea bass/barramundi (Lates calcarifer) - Asian sea bass is a migratory fish found in both freshwater and marine environments. It is a common source of dietary protein in southeast Asia, Australia, and India with global production exceeding 70,000 tonnes in 2014 [21]. Aquaculture for this species is well established but multiple countries still import Asian sea bass due to high demand. An Asian sea bass ES-like cell line (termed SBES) was established from blastula stage embryos [22]. The cell line exhibited characteristic markers of an undifferentiated state, namely significant alkaline phosphatase activity and high levels of oct4 expression. Pluripotency for this cell line was demonstrated by its ability to differentiate into multiple cell types, including neuron-like cells, muscle cells, and beating cardiomyocytes.

Haddock (Melanogrammus aeglefinus) – Haddock are a major food fish found in the Northern Atlantic. Fishery mismanagement has resulted in their addition to the IUCN seafood red list and assignment of a 'vulnerable' conservation status [23]. An embryonic cell line derived from Haddock, HEW, was established in 2005 [24]. This cell line was reported as immortal, having survived nearly 100 passages over a period of four years. HEW also displayed increased telomerase activity, which is typically associated with proliferative cell lines.

Chinook salmon (Oncorhynchus tshawytscha) – Chinook salmon are migratory fish found in the Northern Pacific and in inland rivers during spawning. They are highly valued from a dietary perspective due to their high levels of polyunsaturated omega-3 fatty acids. Wild capture and aquaculture are both significant sources of Chinook salmon. Wild capture levels were near 8,000 tonnes in 2010 while aquaculture exceeded 13,000 tonnes [7]. Many populations of Chinook salmon are considered vulnerable or endangered, and climate change and fishery mismanagement pose significant threats to the species [25]. Multiple embryonic stem cell lines exist for Chinook salmon, many of which are commercially available. The most widely used cell line, CHSE-214, is commonly used as a vector for the propagation of fish viruses for use in disease modeling studies. Despite the widespread use of this cell line, there is limited documentation regarding its pluripotency or ability to predictably differentiate into desired cell types. Further characterization will be required to determine the feasibility of using this cell line in cell-based seafood research or production; however, CHSE-214 will be included in the SSI CLR due to the relative abundance of scientific literature documenting its use.

Red sea bream (Pagrus major) – Red sea bream is the second most commonly aquacultured marine organism in Japan, with production exceeding 65,000 tons in 2016 [26]. While this organism performs well in aquaculture, farms collapse due to disease with regular frequency. A stable red sea bream cell line surviving more than 60 passages has been established from fertilized embryos [27]. The cell line displayed high levels of alkaline phosphatase activity and a normal diploid karyotype. Treatment with retinoic acid resulted in differentiation into neuron-like and muscle-like cells, highlighting a degree of pluripotency with this cell line.

Japanese halibut (Paralichthys olivaceus) – Also known as olive flounder, Japanese halibut is one of the most commonly consumed flatfish species in Japan, China, and Korea. Wild capture and aquaculture are both significant contributors to the overall production of this species, with levels exceeding 11,500 and 44,000 tonnes in 2014, respectively [28]. An embryonic cell line, OFEC-17FEN, was reported for this species in 2018 derived from blastula-stage embryos [29]. This cell line has survived more than 20 passages while maintaining a normal karyotype. Molecular characterization of this cell line revealed that multiple self-renewal factors (oct4 and nanog) were expressed; however, researchers were unable to detect gene expression of a number of characteristic markers of pluripotency [29].

Turbot (Scophthalmus maximus) – Turbot are demersal marine fish found in the North Atlantic, Baltic, and Mediterranean seas. Prized for their taste, turbot are primarily produced through aquaculture with production levels exceeding 70,000 tonnes in 2014 [30]. The commercial value of the turbot aquaculture industry is valued at approximately $40 million. An embryonic stem cell line, TEC, was established from embryos obtained at the gastrula stage. This cell line was stable over 60 passages and viable through cryopreservation [31]; however, investigations into its pluripotency and ability to differentiate are lacking.

Gilt-head bream (Sparus aurata) – The gilt-head bream is a staple of piscine diets in the Mediterranean region. Sourcing of this organism for consumption is primarily through aquaculture, with production levels reaching 140,000 tonnes in 2016 [32]. A *bona fide* ES cell line, SaBE-1c, has been developed for this species exhibiting characteristics of an undifferentiated state including high alkaline phosphatase activity and elevated levels of telomerase activity comparable with other established immortal cell lines [33]. The pluripotency of this cell line was demonstrated both *in vitro* and *in vivo*. Treatment with retinoic acid resulted in differentiation of SaBE-1c into multiple cell types including muscle cells. SaBE-1c was also successfully incorporated into chimeric embryos, demonstrating an ability to differentiate into multiple cell types *in vivo [33]*.

2.4 Invertebrate Species

To date, there are no immortal cell lines in existence derived from marine invertebrates [34,35]. For some phyla, this may simply be due to a general lack of research on these species; however, in the case of marine crustaceans significant effort has been expended in an attempt to produce proliferative cell lines. Attempts at producing immortal shrimp cell lines have been ongoing for more than 25 years with no reported success to date [36].

There is a single embryonic cell line derived from the freshwater snail Biomphalaria glabrata [37], which is currently the only proliferative cell line derived from mollusks. This species is not consumed as food, though the protocols used to establish the Bge cell line may be applicable to other mollusk species with commercial significance. Cell lines are similarly lacking in other seafood-relevant aquatic invertebrate lineages including echinoderms and cnidarians.

2.5 Other Chordates

Sea tunicates are consumed in Korea and Japan and are often referred to as sea pineapples. There is a single report of a tunicate cell line; however, it is not derived from a species that is consumed as food [38].

Soft-shell turtles are valued for perceived nutritional and medicinal properties in a variety of Asian countries. Production of the most commonly consumed species, *Pelodiscus sinensis*, reached 3.6 million tonnes in 2014 [39]. The aquaculture of this species has been greatly impacted by disease outbreaks in recent years, leading to increased research into the development of continuous cell lines. A recent report described a continuous fibroblast cell line derived from soft-shell turtle heart tissue [40]. This cell line may be a starting point for further examination into pluripotent cell lines derived from this species, which will aid in conservation efforts and a reduction in farmed turtle suffering.

Table 1: Summary of existing relevant fish cell lines

Cell line	Species	Commercially available?	Genome sequenced?
BF-2	*Lepomis macrochirus*	Yes, ATCC	No
RTee	*Oncorhycnchus mykiss*	No	Yes
TES1	*Oreochromis niloticus*	No	Yes
Unnamed	*Gadus morhua*	No	Yes
LJES1	*Lateolabrax japonicus*	No	No
SBES	*Lates calcarifer*	No	Yes
HEW	*Melanogrammus aeglefinus*	No	Yes
CHSE-214	*Oncorhynchus tshawytscha*	Yes, ECACC, Sigma	Yes
SBES1	*Pagrus major*	No	No
OFEC-17FEN	*Paralichthys olivaceus*	No	Yes
TEC	*Scophthalmus maximus*	No	Yes
SaBE-1c	*Sparus aurata*	No	Yes

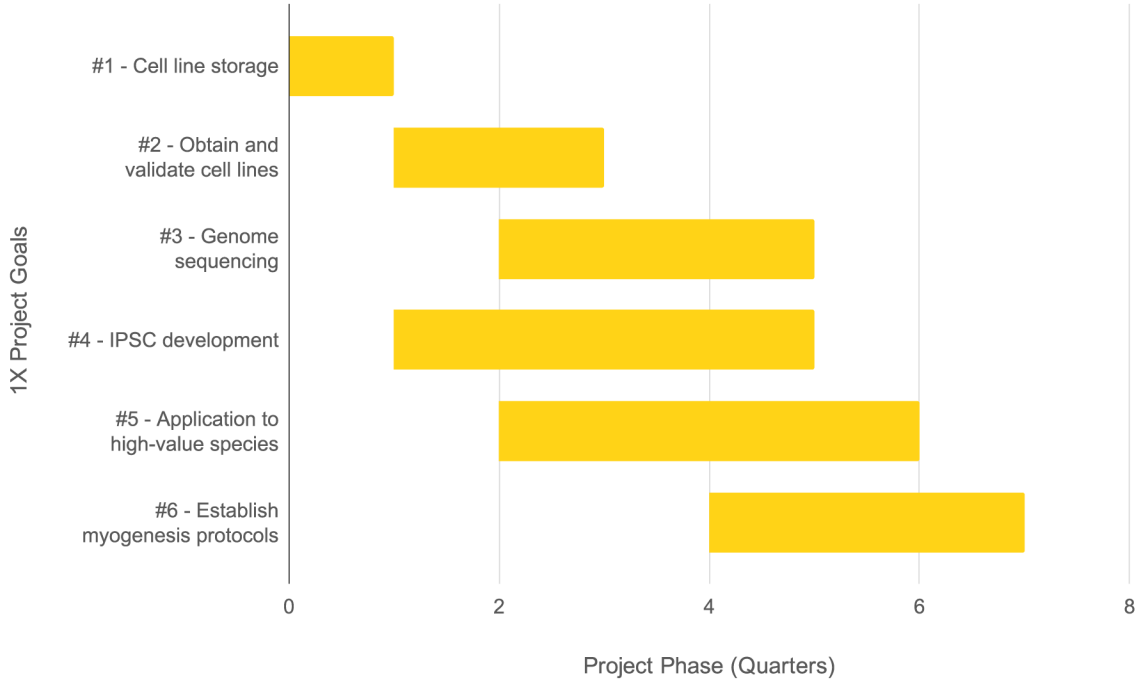
3 Research Proposal

Existing research on cell lines derived from aquatic species is sparse compared to the extensive work that has been done on terrestrial organisms. Of the limited work that has been done, the majority of it has a developmental biology or disease-modelling focus. Consequently, the creation of a CLR as part of a sustainable seafood initiative will require significant investment in the creation of new stable, proliferative cell lines from commercially relevant species.

The six project goals outlined below will generate the following publicly accessible resources:

- An aquatic species cell line repository containing 10 to 15 validated cell lines that can be shipped to academic or industry researchers upon request at a nominal cost.
- At least 7 established cell lines from previous work and at least three Induced Pluripotent Stem Cell (iPSC) lines established as part of this work package.
- Data demonstrating thorough characterization and validation of all cell lines in the repository, including fully annotated genome sequences for all species.
- Validated protocols for handling, growth conditions, and media requirements for all cell lines in the repository.
- Validated protocols for muscle cell differentiation from the ESC and iPSC cell lines.

Figure 1. Timeline projection for each major project goal associated with the 1X project scope. Each phase represents approximately one quarter (three months).



3.1 Project goal #1: Establish storage and distribution of cell lines within the CLR

Prior to obtaining or generating cell lines, logistical details related to cell line storage need to be addressed. Eukaryotic cell lines are typically stored cryogenically at temperatures below -130°C [41]. Thus, the CLR will require reliable access to liquid nitrogen storage facilities.

Identifying suitable collaborators for this project aim is crucial and selection of an ideal collaborator will be influenced by the scale and scope of the CLR project. Most post-secondary research institutions in North America and Europe have suitable infrastructure for storing cell lines, so academic research institutions with experience maintaining and storing cell lines are candidates for housing the CLR. There are also several for-profit institutions that specialize in cell banking that can serve this role. Finally, large biorepositories such as the American Type Culture Collection (ATCC) and the European Collection of Authenticated Cell Cultures (ECACC) could also be considered, though these organizations typically have to approve cell lines for submission and will require evidence of a certain volume of demand.

Selecting among these options (academic, private, biorepository) should account for the ease of distribution of cell lines. If storing at an academic institution, transfer of the cell lines to interested parties will likely require material-transfer agreements to be in place between the institution and the recipient. This potentially time-consuming process may be avoided if using a commercial option.

A sensible storage solution for the initial stages of the project may be to identify a collaborator at an academic institution to assist with cell line storage. The barrier for entry and costs will be lowest with this option. If demand for the cell lines within the SSI CLR expands beyond the capacity of an academic lab,

they can then be submitted to a biorepository that has a greater capacity for processing volumes. Regardless of which storage option is selected, a suitable backup storage site should also be selected to protect against loss due to cooling failures that may arise at a given location.

3.2 Project goal #2: Obtain and validate existing cell lines for inclusion in CLR

After establishing an appropriate storage facility, a reasonable effort should be made to obtain the cell lines described in the literature survey in Section 2. With the exception of CHSE-214 and BF-2, none of the cell lines are commercially available and will therefore require direct contact with the research authors. These cell lines are desirable candidates for inclusion because most have demonstrated a degree of pluripotency and all are derived from commercially important species. Successful addition of these cell lines to the CLR will streamline and simplify access for researchers or innovators pursuing cell-based seafood for one or more of these species.

One major challenge is that many of these existing cell lines were established several years ago and have not necessarily been used extensively since their initial publication. Prior to formal inclusion of these cell lines into the CLR, they should be thoroughly validated through methods such as karyotyping, assaying levels of enzymatic activity for key markers like alkaline phosphatase and telomerase, and next-generation DNA sequencing. The specific approach will differ for each cell line depending on the thoroughness of the associated scientific literature and how well they have been stored, characterized, and maintained. At a minimum, each of these existing cell lines should be validated in accordance with the guidelines established and practiced by existing biorepositories (e.g. ATCC). These organizations rely on morphological observations, karyotyping, and PCR-based screening to validate cell lines and ensure there is no cross-species or mycoplasma contamination.

These validation efforts could be contracted to a CRO for expediency and consistency, but it may also be possible to utilize the expertise of the researchers who developed the cell lines to perform these quality control experiments in-house.

3.3 Project goal #3: Genome sequencing of species included in CLR

High-resolution and rigorously annotated genome sequences are invaluable tools for researchers and will benefit many of the aims of the SSI CLR. Most of the existing fish cell lines considered candidates for inclusion in the SSI CLR are from species for which there is a complete genome sequence (Table 1). For existing cell lines that lack a full genome sequence (*Lateolabrax japonicus, Pagrus major*) and for future cell lines that may come from more obscure species lacking genome sequences, there are significant and tangible benefits to sequencing their genomes. Genome sequencing can be performed via Illumina and/or PacBio, both of which can provide sufficient coverage reads to assemble a complete genome sequence. Annotation of these genomes can be challenging if annotated reference genomes for related species are not available, but many of the initial genes of interest will likely be those that have homologs in conserved pathways in other organisms — for example, genes governing conserved developmental lineages.

3.4 Project goal #4: Develop a standardized methodology for iPSC development from aquatic vertebrates

The composition of meat varies depending on the species, tissue type, and individual specimen from which it was sourced, but most desirable meat products are primarily comprised of skeletal muscle. Skeletal muscle is typically 90% muscle fibers, with the remaining 10% being a mix of connective and adipose tissues [42]. For cell-based meat to be perceived as a suitable alternative to traditional meat, it will likely require the incorporation of tissues descended from multiple cell types. Consequently, cell lines within the CLR that are pluripotent and capable of differentiating into a variety of relevant cell types will be most valuable.

As discussed above, researchers have had some success in establishing proliferative embryonic cell lines from commercially relevant fish species. However, processes for establishing ESCs can differ significantly between species. Furthermore, ESC establishment requires access to fertilized embryos, which will be exceedingly difficult for many non-aquacultured species. An alternative method for generating pluripotent, proliferative cell lines from fish species would be invaluable for the SSI CLR.

A powerful technique for establishing pluripotent stem cells derived from adult tissues was established in 2006 and has since been used successfully in a variety of species across diverse branches of the animal kingdom. Induced pluripotent stem cells (iPSC) eliminate the need for embryonic tissue while providing many of the desired properties of ESCs. The underlying methodology for generating iPSCs has recently been applied to a number of terrestrial farmed animals, including pigs, cows, horses, sheep, goats, and rabbits [43].

The general strategy for iPSC generation relies on four so-called Yamanaka factors – oct4, sox2, myc and klf4 [44]. Stably or transiently introducing these proteins, or 'reprogramming factors', has been shown to initiate the transformation of recipient cells into iPSCs. Based on the work done with fish stem cells to-date, it appears reasonable to expect that each of these reprogramming factors is conserved within fish lineages. Thus, Yamanaka factors may be capable of reprogramming adult fish cells to generate fish iPS cells.

A primary challenge for using Yamanaka factors for fish iPSC development is the lack of well-established methods for transfecting cells. A variety of approaches have been utilized in terrestrial organisms, including the use of viruses, extrachromosomal plasmids, and microRNAs. Because of the lack of established extrachromosomal plasmids in fish species, reliance on viral delivery mechanisms may be most practical. Study of fish viruses is fairly common as they pose major threats to aquaculture systems. Correspondingly, there may be molecular tools available for using fish viruses for delivery of the reprogramming factors required for iPSC development. In cases where viral transfection is not possible or is prohibitively costly, chemical induction of pluripotency could be an alternative option. Other footprint-free methods that rely upon electroporation or direct injection to introduce mRNAs or proteins could also be explored as alternatives, which would also alleviate potential consumer perception concerns regarding genetically manipulated cell lines.

The main objective of this project goal should be to develop the most broadly applicable strategy for iPSC development in fish species. Because fish viruses may be specific to certain species or families, a chemical or footprint-free approach may be most suitable for elucidating a methodology that can be applied in a high-throughput manner. Ultimately, the best strategy for furthering the objectives of the

CLR will need to be determined empirically. Fortunately, there has been some preliminary work in this area with zebrafish cell lines. A recent study found that zebrafish cells could be reprogrammed to an iPSC-like state by viral-mediated expression of endogenous orthologs of the classical Yamanaka factors [45]. While the iPSC-like cells were not exhaustively validated, they showed elevated telomerase activity, an ability to form embryoid bodies, and a degree of pluripotency *in vivo*.

Establishing a generalized approach for fish iPSC development will advance the SSI CLR and cell-based seafood initiatives in a number of ways. While this approach may require more work up-front, it should ultimately shorten the amount of time and effort required before new cell lines can be generated and added to the SSI CLR. Additionally, because these new cell lines will all have been reprogrammed in a similar fashion, characterization protocols can be concurrently established in a standardized fashion. The end result will be fish iPSCs that have been validated for pluripotency and ability to differentiate, as well as reprogramming and validation methods that can be adopted by other researchers or commercial endeavors.

3.5 Project goal #5: Application of standardized methodology for IPSC development to a set of 3-5 fish species as a proof-of-concept

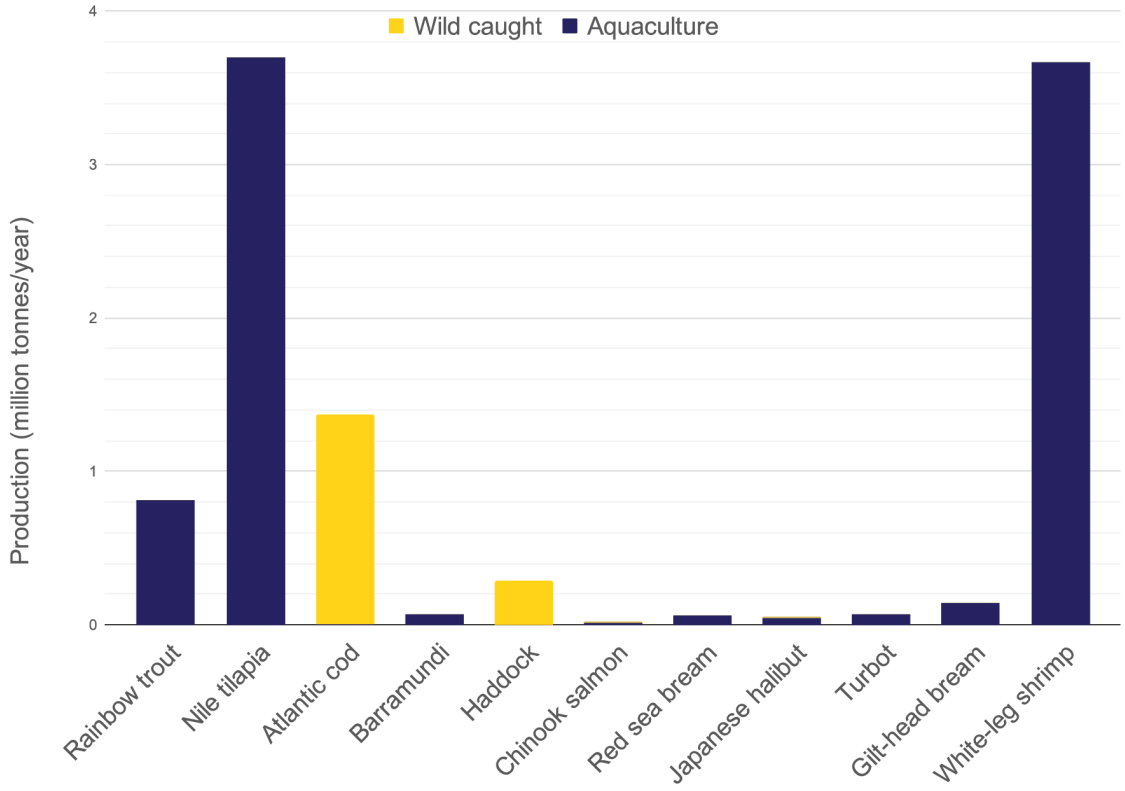
This project goal will be performed concurrently with project goal #4 and act as a proof-of-concept for the iPSC development protocols established (Figure 2). The rationale for assigning this aspect of the project its own milestone is to ensure that proper consideration is given to species selection. There are a number of factors to consider for inclusion in this stage of the project (and the CLR overall) including the likelihood of success, availability of suitable tissue, and potential to advance the cell-based seafood industries. Consultation with existing cell-based seafood companies, ocean conservation groups, or the existing seafood industry may help to identify the most impactful species to target. Below are some initial recommendations with justifications.

Alaskan pollock (Theragra chalcogrammus) – Alaskan pollock is the fifth most consumed type of fish in the U.S. [46]. It is commonly used in the fast food industry including in the McDonald's Filet-O-Fish [47]. Pollock is also commonly used in imitation crab products, suggesting cell-based production of this species may help to ease demand on crustacean fisheries as well. Despite its high consumption, there is no existing research on pollock stem cells. Development of iPSCs derived from this species and their inclusion in the CLR will provide a starting point for cell-based production of pollock.

Basa (Pangasius bocourti) – Basa is among the top ten most consumed types of seafood in the U.S. [46]. Aquaculture of this species has been criticized by a number of organizations due to pollution issues and the potential for escape and invasion of existing ecosystems [48]. The fact that this species is produced reliably via aquaculture suggests that obtaining fresh tissue for iPSC development should be relatively easy. Cell-based production of Basa has the potential to reduce reliance on these problematic aquafarms and have a substantial positive environmental impact.

Other species — Many of the previously established cell lines detailed above are from the early 2000s. Consequently, some of these may not have been maintained properly and may no longer be viable for use and inclusion in the SSI CLR. If this is the case, then several of the seafood-relevant species discussed above would make ideal candidates for inclusion in this stage of the project.

Figure 2. Production levels of commercially relevant fish species, which may influence selection of species to include in the iPSC line set. Data obtained from FAO, reflecting production levels between 2014-2016.



3.6 Project goal #6: Establishment of protocols for IPSC differentiation into muscle cells

Because meat is primarily comprised of muscle tissue, a set of protocols for differentiating the cell lines within the SSI CLR into muscle cells is highly valuable. A recent high-throughput screen of small molecules identified a cocktail of six compounds that reliably differentiated zebrafish ESC into muscle cells [49]. Moreover, this set of compounds was also able to differentiate mouse and human iPSCs into muscle cells, suggesting the mechanism of action is conserved broadly across diverse lineages. Applying this established methodology to the initial set of cell lines within the CLR will help to validate this approach for the production of muscle cells in a variety of fish species.

Using small molecules to trigger differentiation poses potential cost advantages but may be problematic if the compounds lack approval as food-safe ingredients or processing aids. In this case, while still suitable for research purposes, they may pose regulatory challenges if used for commercial product development. As a result, we will establish and validate a parallel strategy for myogenesis of fish iPSCs that does not rely on small molecules. There are a variety of options for this approach. For example, overexpression of the endogenous transcriptional regulator MyoD has been shown to promote myogenesis in human iPSCs [50] and other species. Alternatively, growth factors involved in a number of well characterized developmental pathways in other species may also be applicable to fish. A recent review [51] provides a detailed summary of the various approaches to ESC/iPSC myogenesis, many of

which could be options for the development of a secondary differentiation protocol. As these alternatives will require more involved research and development, only the small molecule-induced differentiation protocol is achievable within the minimal budget scope.

4 Prospective Partners

Due to the different expertise required for each goal of the project, this work will likely be pursued in collaboration with a handful of researchers or contract labs. Table 2 provides several suggested candidates for each aspect of the work along with a brief rationale. The funding scope of this project will also factor into which partners to approach. For example, contract labs are likely to complete work in a more timely and accountable fashion, but they often have higher costs than academic collaborators. Note that for expanded project scopes that will increase the number of species in the CLR and specifically target marine organisms that are not amenable to aquaculture, additional partners will be needed for access to freshly harvested tissue samples. Oceanographic research vessels often accept applications to host researchers on board, so a collaborator skilled in cell harvesting and isolation may be able to accompany a mission at sea to gather samples. Alternatively, establishing a relationship with recreational or commercial fishers may provide access to fresh tissue for hard-to-access species.

Table 2: Candidates for research collaborators or contract labs to conduct this work

Project contribution	Company or lab	Rationale
Cell banking and storage	QED Bio	Will create 50-vial MCB and assay for viability and sterility. Can also store cell lines with QED for off-site backups.
	sdix	Offer dual-site storage for cell banks.
	ImmunoPrecise	No minimums for storage, may be a good option for initial stages of CLR
	UTMB Health	A core facility equipped for cell storage.
	NIAID Cell culture core laboratory	Government facility offering storage located in the Eastern United States.
	UCSD Core Bio services	Clear fee schedule, relatively inexpensive but "bare bones".
	National Repository for Fish Cell Lines	Clear overlap with mandate of the SSI CLR. Location (India) may present some logistical challenges.
Genome sequencing	University of Washington PacBio	Offer library prep and have a non-profit rate. Certified PacBio service provider.
	UC Davis Genome Center	Offer library prep and can sequence via a variety of strategies.
	MOgene	Certified PacBio service provider, also offer bioinformatics services to help with data analysis (genome assembly). Offer sequencing via alternate methodologies as well.
	BGI	Full-service *de novo* sequencing of animal genomes. Service includes data analysis.

Project contribution	Company or lab	Rationale
	Novogene	Full-service *de novo* sequencing of animal genomes. Offer Illumina short-read or PacBio as strategies. Support project with bioinformatic analysis.
IPSC Develoment	Evercyte	Offer customer tailored cell lines as a service.
	REPROCELL	Utilize an RNA-based methodology, non-integrative and (purported) higher efficiency.
	ABM	Offer a variety of strategies, of which the protein-based is likely most suitable.
	Ute Hochgeschwender	Corresponding author on a 2013 study that looked at PSC induction in a wide array of species, including zebrafish.
	Erich Jarvis	Corresponding author on a 2013 study that looked at PSC induction in a wide array of species, including zebrafish.
	Yunhan Hong	Performed much of the existing work on fish ESC - including medaka and many farmed species.
	Songlin Chen	Performed work involving fish ESC and ESC-like development; experience creating biobanks (fish sperm).
Differentiation (myogenesis)	Thermo - CellModel	Offer reprogramming and differentiation services.
	Amy Wagers	Corresponding author on 2013 study that identified small molecules that differentiated zebrafish ESC to muscle cells.
	Leonard Zon	Corresponding author on 2013 study that identified small molecules that differentiated zebrafish ESC to muscle cells. Appears to work extensively with zebrafish, though as a model for human disease.
	Yunhan Hong	Differentiated fish cells upon treatment with retinoic acid both *in vitro* and *in vivo*.
	Songlin Chen	Differentiated fish cells upon treatment with retinoic acid both *in vitro* and *in vivo*.

5 Budget Proposal

Three scopes of work are described below. The "1X" scope represents a work package of the minimum scope for meaningfully advancing the cell-based seafood industry, while the "5X" and "10X" scopes describe projects requiring approximately five-fold and ten-fold larger budgets, respectively. While all of the proposed work exhibits tremendous potential to advance the field, the additional proposed work packages beyond the 1X funding level can in some cases be viewed as a menu of *a la carte* options. For example, a funder who has the capacity to support this work at the $2.5M level may wish to prioritize a greater species breadth, so they could choose to expand the CLR to house approximately 40 new species rather than support exploratory research for invertebrate species. The budget for cell storage is based on external-user pricing from the UCSD banking facility.

Cell storage

Category	Amount
Storage fees	$5,000
Service charges	$1,000
Distribution fees	$1,000
TOTAL	**$7,000**

This budget allows for 1-2 racks to be stored for two years, which is a sufficient amount of space for the early stages of the CLR. The service charges and distribution fees are user fees associated with freezing new additions to the CLR and distributing existing cell lines to interested parties. Researchers requesting cell lines will be charged a nominal fee to cover shipping, so this distribution fee accounts for handling time from the staff at the storage facility.

Obtaining and validating existing cell lines

Category	Amount
Shipping costs	$3,000
Honorarium to researchers	$5,000
Validation efforts	$10,000
TOTAL	**$18,000**

The budget for obtaining existing cell lines is based on projected shipping rates for the existing cell lines outlined within Section 2. The temperature-sensitive nature of the cell lines will require overnight shipping on dry ice, and many of these cell lines will be shipped internationally. This projection allows for a shipping budget of ~$350/cell line. The honorarium will be paid to researchers providing the cell lines to compensate them for their time and any costs associated with preparing samples for shipment, as a means of incentivizing their contributions to the CLR. Validation efforts will ensure that existing cell lines are still viable, free of contamination, and reflect the stated cell type and species. The cell lines will also be expanded in culture so that many vials are available for distribution to researchers.

Genome sequencing

Category	Amount
Data collection (PacBio) per species	$8,000
Annotation of sequences per species	$5,000
TOTAL	**$13,000**

The estimated cost of sequencing a fish genome is based on projections of a 2N copy number and a genome size of ~1 Gb. The budget item "annotation of sequences" is to account for salary paid to bioinformatics specialists who can annotate the genome and make sure it is accessible to researchers or companies interested in using the genome sequence for cell-based seafood projects. The cost listed is for a single genome, but the total cost for this budget item may increase depending on the species selected for iPSC development. If these species do not have annotated, sequenced genomes then they will contribute to increased costs associated with the genome sequencing aspect of this proposal.

Reprogramming/iPSC generation

This section of the budget is most speculative. Contract research organizations (CROs) charge approximately $20,000 for iPSC development via non-integrative methods (protein, microRNA). The vast majority of organizations working in iPSC development focus on derivation from human samples.

Because of their lack of experience with fish samples, it is likely that projections for time and cost based on existing workflows for human samples will be underestimates of the true cost. This has been taken into account for this budget by allowing for a 50% contingency, so a rate of $30,000 is estimated for each fish iPSC line plus an additional $10,000 contingency overall, which could perhaps be used to pay a specialty consultant with expertise in fish cell cultivation to advise on the work. Academic collaborators may offer a less expensive option, but the turn-around time would likely be substantially longer and results would not be contractually guaranteed, so a CRO is the recommended partner for this work.

Tissue/primary cell culture sourcing

This budget item will depend largely on the species chosen for inclusion in the iPSC-development stage of the project. There may be organizations working with primary cell cultures who would greatly benefit from an iPSC line and therefore may be willing to share cells from their primary cultures for little or no cost. For example, there are research institutes with extensive aquaculture research that routinely derive and expand primary cell cultures. If sourcing tissue from a live animal, costs will depend on transportation to the research facility as well as the cost of the animal traded as a commodity. In this budget, $10,000 is allocated for this aspect of the project.

Establishing protocols for differentiation into muscle cells

Adapting the myogenesis protocols established in zebrafish to other commercially relevant fish species should be relatively straightforward. Very similar protocols were successful in mouse and human iPSCs, suggesting a high degree of conservation. Ultimately the protocol may need to be modified to some degree for different species. The treated cells will be monitored and assayed to validate successful myogenesis. Demonstrating that the differentiation protocol works in the initial subset of species will likely be the most costly and time consuming, and this cost will likely decrease for subsequent species as consensus protocols are established. A rough estimate of cost is $20,000 per species for the first 2-3 species. This includes the cost of reagents, salary, and equipment use. Assuming the pilot stage of this project aim is successful, the cost for validating this differentiation protocol in subsequent species will likely decrease significantly, to approximately $10,000 per species. The initial phase of the CLR will likely reach ~10 species, which will result in a total cost of ~$130,000 for this aspect of the project.

TOTAL BUDGET

Category	Amount
Cell storage	$7,000
Obtaining and validating existing cell lines	$18,000
Genome sequencing	$13,000
Reprogramming/iPSC generation	$100,000
Tissue/primary culture sourcing	$10,000
Differentiation (myogenesis)	$130,000
TOTAL	**$278,000**

5.2 Project Outline: 5X Budget Scope ($1.25 million)

Increasing the initial budget by five-fold will enable the project to be expanded in multiple directions. In addition to the aims articulated above for the 1X scope of work, an undertaking of this scale will accelerate the creation of the CLR and expand its utility through five primary initiatives. Note that these areas are more fluid and can be substituted more freely than the work packages within the 1X scope. For example, depending on feedback from the research community, it may be preferable to develop immortalized cell lines of various cell types for relevant species rather than developing new differentiation protocols.

1. Increase investment in iPSC reprogramming efforts to accelerate timeline ($100,000)

Doubling the investment into the iPSC reprogramming research will help to accelerate the project aim, particularly if a CRO were employed for this aspect of the project. This funding will allow for additional staff to complete the work. While the timeline may not speed up proportionally with the increase in funding, it will likely shorten significantly. As this part of the project is the most susceptible to unforeseen delays due to the lack of prior research upon which to draw for this work, increasing the amount of resources devoted to this goal could preemptively avert potentially significant delays.

2. Create a portal for interested parties (startups, researchers, ocean conservation groups, etc.) to nominate species of interest for iPSC reprogramming and validation, thus expanding the number of iPSCs from 3 to 13 species ($300,000)

This approach will allocate sufficient funds for iPSC development for ten additional species. In order for species selection to be most valuable to cell-based seafood initiatives, a portal will be created for interested parties to nominate species. This process is akin to a short grant proposal where approved applications will be selected for iPSC generation and deposited into the CLR. The resulting iPSC will be distributed immediately to the requesting party for application in cell-based seafood production or research but also made available to others, as with all cell lines in the CLR. The nominating parties could potentially be relied upon to provide tissue or primary cell cultures for the species they have proposed. This outsourcing of species selection will ensure that money invested into cell line development will target species for which there is immediate interest and demand. Accepting community-nominated species for inclusion in new databases or resources has successful precedent in several life science fields and tends to encourage greater use of the resource.

3. Purchase a dedicated cryogenic freezer to accommodate the storage needs of the CLR ($150,000)

Cryogenic freezers capable of maintaining temperatures below -130°C range in price from $80,000 - $150,000. At this budget level, the CLR could afford to purchase and operate a dedicated cryogenic storage freezer. This will eliminate the contract cell storage needs and allow for the CLR to scale significantly without increasing costs beyond this initial investment. A dedicated freezer also reduces the risk of sample mishandling, misplacement, or cross-contamination from other researchers whose samples are stored within a shared cryostorage facility.

4. Develop serum-free media suitable for culturing and propagating fish ESC/IPSC ($100,000)

Each of the existing fish cell lines detailed in Section 2 were cultured in conditions containing 10-20% fetal bovine serum (FBS). FBS is not viable for cell-based seafood due to cost, inconsistency, contamination risks, and supply limitations, among other regulatory and ethical concerns. Thus, it is necessary to develop a growth medium that is serum-free. Serum-free media have become routinely used in recent years and existing serum-free formulations can be used as a starting point for achieving this project aim [52]. Multiple cell culture media companies perform custom media formulation services. However, this project would either require a negotiated contract that allows the resulting formulation to be published rather than held as trade secrets by the formulation company, which will command a high fee. Alternatively, there is some evidence to suggest that protocols might be developed for robust adaptation of cell lines within the CLR to serum-free conditions with no or minimal reformulation or supplementation of the medium. An existing fish cell line, channel catfish ovary (CCO), was successfully adapted to serum-free conditions over a period of 32 days by gradual reduction of FBS concentration, displaying no morphological changes during the transition [53]. This study could be used as a template for transitioning cell lines within the SSI CLR to serum-free growth conditions.

5. Fund research into establishing an alternative myogenesis protocol and standardized differentiation protocols into other cell types of interest ($300,000)

The 1X budget will establish a protocol for the differentiation (via small molecule induction) of pluripotent fish stem cells into muscle cells, which accounts for ~90% of meat. The 5X funding scope will fund research into an alternative method of myogenesis not reliant on small molecules. The alternative protocol may utilize various recombinant growth factors and correspondingly will likely require a higher level of investment as commercial sources of growth factors for aquatic species are not readily available. However, a differentiation approach not mediated by small molecules is more likely to be commercially viable as discussed above and it is more likely to recapitulate native differentiation in development, which may render it more valuable for studying the cell biology of this process.

The other aspect of this proposed project aim is elucidating differentiation pathways for directing fish stem cells into other cell types, such as adipose and connective tissues, that account for the other 10% of meat. This will be more exploratory research and likely require a multi-year collaboration with academic labs. If successful, this could help cell-based seafood initiatives create products that more closely mimic conventional fish meat. Fat is widely regarded as a key component of nutrition, flavor, and mouthfeel, while the connective tissue is critical for structure and texture in whole-tissue products like filets.

5.3 Project Outline: 10X Budget Scope ($2.5 million)

A budget of 10X the initial level will allow for increased investment in previously proposed project aims, as well funding for additional exploratory research with profound significance for the cell-based seafood field as a whole. These projects tackle areas that are so markedly nascent that they are unlikely to be attractive areas for commercial activity in the near future without a substantial catalytic public research investment.

1. Funding aimed at accelerating research into ESC/iPSC development from marine invertebrate species ($1,000,000)

There are no existing cell lines derived from marine invertebrates, leaving a huge gap for cell-based seafood organizations seeking product development in this area. Shrimp have the highest per capita rates of consumption of any seafood, and aquaculture of many shrimp species is notoriously environmentally destructive due to irreversible damage to mangroves and other natural coastal protective habitats. Likewise, development of proliferative cell lines derived from cephalopods will advance cell-based alternatives to squid and octopus seafood products. Cephalopods are regarded as some of the most intelligent organisms on the planet, raising ethical concerns about their consumption as meat.

At this budget level, sufficient funding could be provided to researchers attempting to develop cell lines for both crustaceans and mollusks. This will take the form of two or more multi-year grants for research in this area since collaborative partners need to be supported through multiple exploratory attempts. This work has the potential to significantly advance the viability of cell-based versions of these products, which otherwise may be many years from gaining commercial traction.

2. Funding for ten additional community-nominated targets for iPSC development ($250,000)

Increasing the funding available for iPSC generation from community-nominated targets will allow for the CLR to grow more rapidly. Making this resource more widely available will encourage more researchers and innovators to pursue cell-based seafood-related endeavors because it effective de-risks the riskiest phases of their work.

6 Conclusion

The cell-based meat industry has garnered significant attention for its tangible advances toward the development of consumer products that will fundamentally change agriculture for the better. Many of the benefits of shifting to cell-based production of meat also apply to the production of cell-based seafood. However, the amount of research and effort toward the production of cell-based seafood pales in comparison to the work that has been done involving terrestrial organisms, primarily due a lack of resources such as cell lines and standardized protocols.

The creation of the cell line repository proposed in this document will address major obstacles preventing the swift advancement of cell-based seafood. By curating existing cell lines, overseeing the development of new cell lines, and providing standardized protocols for working with them effectively, the SSI CLR will significantly reduce the barrier (and risk) for entry to researchers and startups interested in working in cell-based seafood.

All of the resources articulated in this proposal will have a catalytic effect on the cell-based seafood industry due to the feed-forward relationship between the availability of robust, well-characterized research materials and their rate of uptake by the scientific community. Once cell lines and corresponding protocols are available, more researchers will use them and more findings will be published that continue to increase the ease of working with them. Furthermore, once a certain research model gains traction, the life science industry is incentivized to develop even more tools, reagents,

protocols, training materials, and so forth to support that growing research community. Thus, basic research tools like fish-specific antibodies, growth factors, and gene expression panels will become commercially available, further reducing the barrier to entry for new researchers and innovators to contribute to the cell-based seafood field.

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The Good Food Institute is a 501(c)(3) nonprofit organization dedicated to creating a healthy, humane, and sustainable food supply. Our work is 100% powered by gifts and grant support. GFI's team of scientists, entrepreneurs, lawyers, and policy specialists are laser focused on using markets and food innovation to transform our food system away from industrial animal agriculture and toward plant-based and cell-based meat. To learn more, please visit GFI.org.

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