Film Seeding for Bovine Myoblast Cell Optimization by Characterizing Growth

A thesis submitted by

Luke Isayiw

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Abstract:

The growing population and increasing demand for animal protein necessitate sustainable solutions to reduce the impact of traditional livestock farming. Cultivated meat (CM) offers a promising alternative. This study characterizes the growth and differentiation of immortalized bovine satellite cells (iBSCs) on various edible film scaffolds to optimize conditions for CM. The metabolic activity, cell adhesion, and differentiation of iBSCs cultured on gelatin, zein, alginate, cellulose, chitosan, and soy films were evaluated. Metabolic activity was found to level off after 10 days, with a significant increase between 3 and 10 on gelatin, zein, chitosan, cellulose, and soy. Gelatin and zein films exhibited the highest metabolic activity. Cyquant assay quantified cell numbers over 10 days, showing an increasing trend in contrast to the leveling off in metabolic activity. Protein-based films outperformed polysaccharide films regarding cell adhesion, attributed to the affinity of mammalian cell integrins for protein ligands. Cellulose films were exhibit differentiation.

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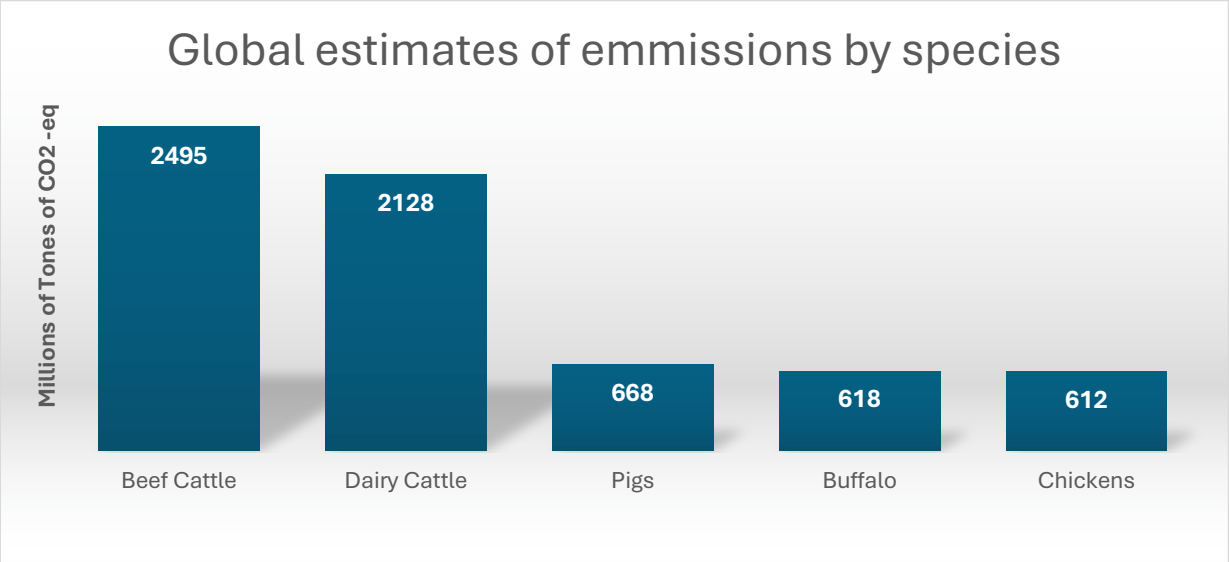

Figure 1: Global estimates of greenhouse gas emission by species. Cattle are split in to beef and dairy with beef with greater emissions than dairy, both are over four times greater than the next animal emissions. Emissions are calculated from both edible and non-edible products and services. Data from: Gerber PJ, Food and Agriculture Organization of the United Nations. Tackling climate change through livestock: a global assessment of emissions and mitigation opportunities. Rome: Food and Agriculture Organization of the United Nations; 2013. xxi, 115 pages p.

In the shadow of an escalating global population projected to reach nearly 10 billion by 2050, the demand for agricultural outputs, particularly animal proteins like beef, is experiencing unprecedented growth (1). The livestock sector, notably cattle farming, as seen in **Figure 1**, stands at the heart of this dilemma, contributing to some of the most pressing environmental issues today. Livestock production significantly uses natural resources, including land and water, contributing to deforestation, water scarcity, pollution, and biodiversity loss (2). Depending on rainfall, every 1lb of beef consumed takes between 200-1000L of water and 0.01 acres of land (3,4). This sector is also a substantial contributor to global greenhouse gas (GHG) emissions, with an estimated 7.1 gigatons of CO2 equivalent per annum, which accounts for 14.5% of all

anthropogenic emissions. The cattle industry alone is responsible for 65% of these emissions, underscoring a critical area for environmental mitigation efforts (5).

Cattle farming's environmental footprint extends beyond emissions; as one of the most significant users of land and freshwater resources, intensive cattle production has led to substantial deforestation, water scarcity, and loss of biodiversity (2). In Latin America, one-third of the beef GHG emissions are a result of deforestation (5). These activities exacerbate ecological degradation and pose a significant risk to global sustainability.

Moreover, the increase in demand for meat products has highlighted the increasing negative impacts of traditional livestock farming and the urgent need for solutions to reduce the sector's environmental impacts without compromising productivity and food security. To further elaborate on the topic, we can delve into the potential solution of cultivated meat, discussing the current state of commercialization, consumer acceptance, and challenges associated with this innovative approach. Additionally, we can examine the advancements in cell culture technology and scaffolding materials needed for cultivated meat production.

Cultivated Meat:

Cultivated meat (CM), also known as cell-based or lab-grown meat, is an innovative approach that aims to harness in vitro cell culture technology with animal cells, generally skeletal muscle-derived cells, to grow and differentiate to form consumer meat products. CM aims to create a product that reproduces the taste, texture, and nutrition of conventional meat while addressing the sustainability and environmental impacts of traditional livestock farming (6). The industry is relatively new and, as of 2021, had 70 companies working towards bringing CM to all market levels (6). Companies like Upside Foods (formerly known as Memphis Meats), JUST, and Modern Meadows have started releasing products such as meatballs, cookie dough,

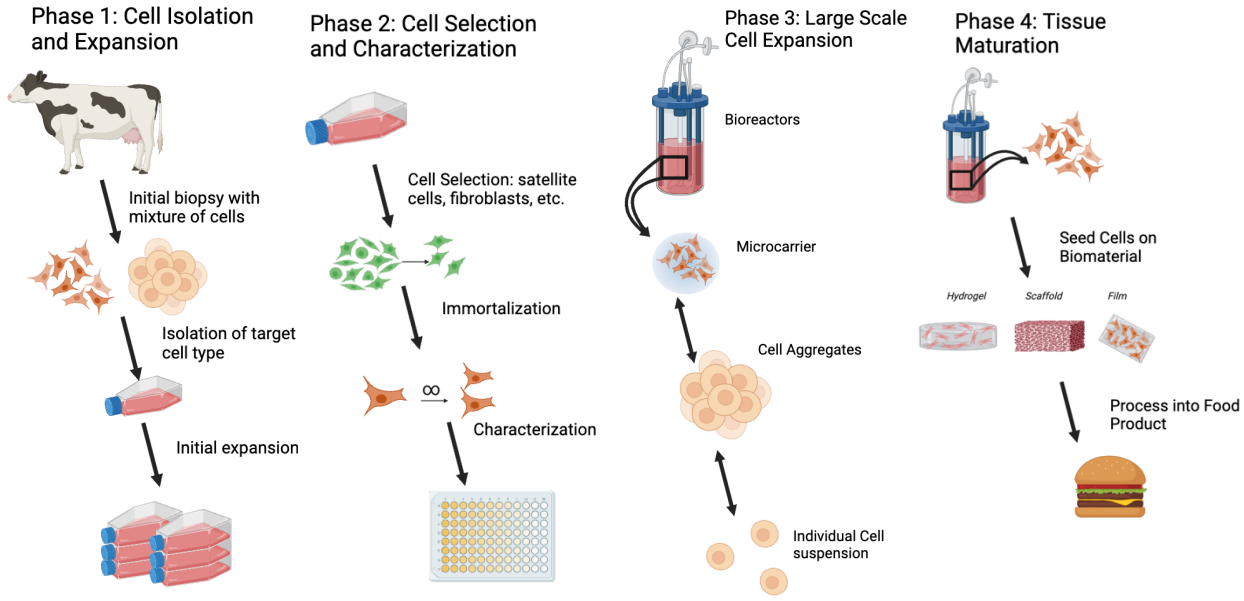

Figure 2: Overview of the 4 stages of the cultivated meat (CM) process. Phase 1 involves the initial isolation and lab expansion of target cell types. Phase 2 involves the characterization and immortalization of the isolated cell type. Phase 3 involves the large industrial-scale expansion of the cell line, typically achieved in bioreactors. Phase 4 involves the maturation, seeding onto scaffolds, and downstream processing into a marketable product.

chicken nuggets, and steak chips. Still, they have yet to be readily available in supermarkets. These companies, while making strides in the field of CM, are private, and thus, knowledge of technologies and feasible strategies are being kept unavailable to the public and other researchers (7). There are numerous academic approaches and methods for change management, but there is a need for greater consistency and comparison between these methods to address the difficulties.

On a large scale, the process for CM can be broken up into four stages: cell isolation and expansion, cell selection and characterization, large-scale multiplication, and tissue maturation (**Figure 2**). The initial stage involves the isolation and expansion of relevant cells, including muscle cells, fat cells, and fibroblasts, from a primary animal biopsy. Following this, cell selection and characterization ensure that only the best cells are used for further development. Cells are selected due to their ability to differentiate into muscle fibers and adipocytes and fast

doubling times (6,8). The process then enters the stage of large-scale multiplication in bioreactors. Advances in bioreactor designs, particularly the introduction of perfusion bioreactors and the use of microcarriers, have significantly improved the efficiency of this stage, providing an optimal environment for exponential cell expansion. After the isolation and expansion of suitable cells, the final stages of cell-based food production involve seeding scaffolds and tissue maturation. These steps are crucial, as the choice of scaffold material directly influences feasible methods for large-scale tissue growth, making this an essential focus of our research. While many questions remain to be answered at each stage to create a feasible product, this research aims to tackle the issue of tissue maturation on scaffold materials (6).

Edible Film Biomaterials:

The market product of CM will likely involve some form of scaffolding material or microcarrier as an essential part of cell growth and process scaling. Scaffolding materials play a crucial role in providing structural support and enhancing the organization of cells during cultivation. To better quantify the effects of different materials, the approach is to evaluate them as 2D films, which allows for precise control and measurement of cell behavior and material properties. Choosing materials for CM needs to consider several factors. The properties, such as mechanical strength, biocompatibility, and degradation rate, are crucial for their effectiveness in supporting cell growth. Optimization of these properties can enhance the performance of the films as scaffolds in CM production (9). With other factors involved, such as media formulation and cell type, edible films can offer a simple yet effective method for optimizing scaffolding materials used in CM(10). These edible films not only support cell adhesion and proliferation but also contribute to sustainability by using biodegradable and renewable resources. Using edible films is an approach to a more suitable and cost-effective practice, potentially reducing reliance

on synthetic materials and aligning with environmental goals. Here, we explore the possibilities of edible options, namely proteins, which are promising due to their film-forming properties and mechanical strength, and polysaccharides for their excellent barrier properties against oxygen, enhancing the cultivated meat product's structural integrity and shelf life (11).

Immortalized Cells:

Primary bovine cells have limited proliferative capacity and can only undergo a finite number of doublings. The exact number can vary depending on the specific cell type and culture conditions, but primary bovine cells can generally undergo approximately 20 to 40 doublings (12). The development of an immortalized bovine cell line is pivotal to advancing cellular agriculture, particularly continuous growth capability is essential for scaling up production to meet the growing demand for cultured meat, reducing reliance on animal-derived primary cells, and minimizing ethical and environmental impacts. Immortalized cell lines offer a consistent and renewable source of cells that can continuously proliferate without undergoing senescence, thereby ensuring a stable supply of cells for meat production (13,14). Moreover, using immortalized cell lines enhance the efficiency and cost-effectiveness of the production process, as these cells provide a uniform and controlled environment for optimizing growth conditions. Immortalized bovine cell lines are integral to achieving cultured meat products' commercial viability and sustainability by enabling sustained exponential growth and maintaining high cell densities.

Immortalized cells for other animals have been developed and explored greatly in other animals. C2C12 cells are mouse muscle-derived cells that can be maintained as proliferating myoblasts and induced to differentiate into myotubes, making them useful for studying muscle differentiation and myogenesis (15). C2C12s are useful when first conducting feasibility studies

for myoblasts, but as a market-ready product is approached in the cultured meat industry, the use of cells needs to be shifted to reflect consumer acceptance. Recently, a cell line of bovine satellite cells was immortalized by using constitutive expression of telomerase reverse transcriptase (TERT) and Cyclin-dependent kinase 4 (CDK4) (8). Coupled with the preliminary approval of TERT-immortalized cells for cultured chicken application, more research on the growth of this cell line is necessary.

Growth and differentiation media have also been developed in tandem with the development of immortalized cell lines for cultivated meat. Some key aspects are lower cost, food safety, and removing animal-derived components. For instance, significant efforts have been made to replace fetal bovine serum (FBS) with chemically defined media, reducing costs and addressing ethical and safety concerns associated with animal-derived products (16,17). Additionally, the use of small molecules such as LDN-193189, a BMP inhibitor, has been shown to enhance myogenic differentiation, thereby improving the efficiency of cell culture processes. LDN-193189, specifically, inhibits BMP signaling pathways which are known to regulate muscle differentiation, thereby promoting the formation of myotubes from myoblasts (18). This is particularly relevant for scaling up cultured meat production, as it ensures a more consistent and robust differentiation process, leading to higher yields of muscle tissue. As such, the co-development of optimized media and the application of molecules like LDN-193189 are integral to the successful commercialization of cultured meat products

Specific Aims:

The cultivated meat (CM) industry strives to replicate the taste and texture of natural meats while mitigating the environmental impact of traditional livestock practices. By leveraging tissue engineering techniques with edible scaffolding materials, this research employs a high-throughput methodology to comprehensively analyze material viability through analysis of cell growth, confluence, and differentiation on a range of scaffold materials. Work by Xiang et al. has previously explored edible biomaterials with C2C12s and primary bovine cells with a single density and cultivation time (11). Using the work as a basis, films of alginate, gelatin, soy, zein, cellulose, and chitosan will explore the creation of a full factorial design of experiment (DoE) model to optimize the growth of immortalized bovine satellite cells (iBSCs). The overall objective of this study is to develop optimized conditions for iBSC growth and differentiation using various scaffold materials. This objective will be addressed through the following specific aims:

Specific Aim 1: Characterize the growth of iBSCs through analysis of scaffolding materials, seeding density, and cultivation time. From the characterization, a growth model for iBSCs will be created with an emphasis on optimizing key features: cell viability, growth rate, adhesion, and confluency.

The hypothesis is that each scaffolding material has unique seeding densities and cultivation times that influence optimal iBSC growth. Using a high-throughput screening to evaluate the effectiveness of the material using a metabolic activity test. Then, confirm the ideal ranges through dsDNA quantification. The results from these experiments will be used to create the initial stages of a growth model, producing key ranges with ideal growth conditions for each material.

Specific Aim 2: Using the optimized growth conditions of cultivation time and seeding density for each material, characterize cell differentiation over time in cell culture.

The hypothesis is that the optimized growth condition will enhance cell differentiation and provide ideal conditions for future research. Building on the conditions identified for each material in Aim 1, films will be evaluated for cell coverage and differentiation using fluorescent microscopy. This will provide insight into the differentiation capacities of iBSCs on each material and facilitate cost-effective conditions for differentiation.

Design Goals:

Independent Variables:

Film Material	Gelatin (Control), Alginate, Cellulose, Chitosan, Soy, Zein
Seeding density	100 cell/cm^2 – 1,000,000 cell/cm^2
Cultivation time	1-14 days
Differentiation time	7-14 days

Dependent Variable:

Growth	RFU comparison of metabolic activity
Cell Number	Maximized
Cell Adhesion	0-100%
Confluence	Region of 80%
Myotube Formation	0-100%

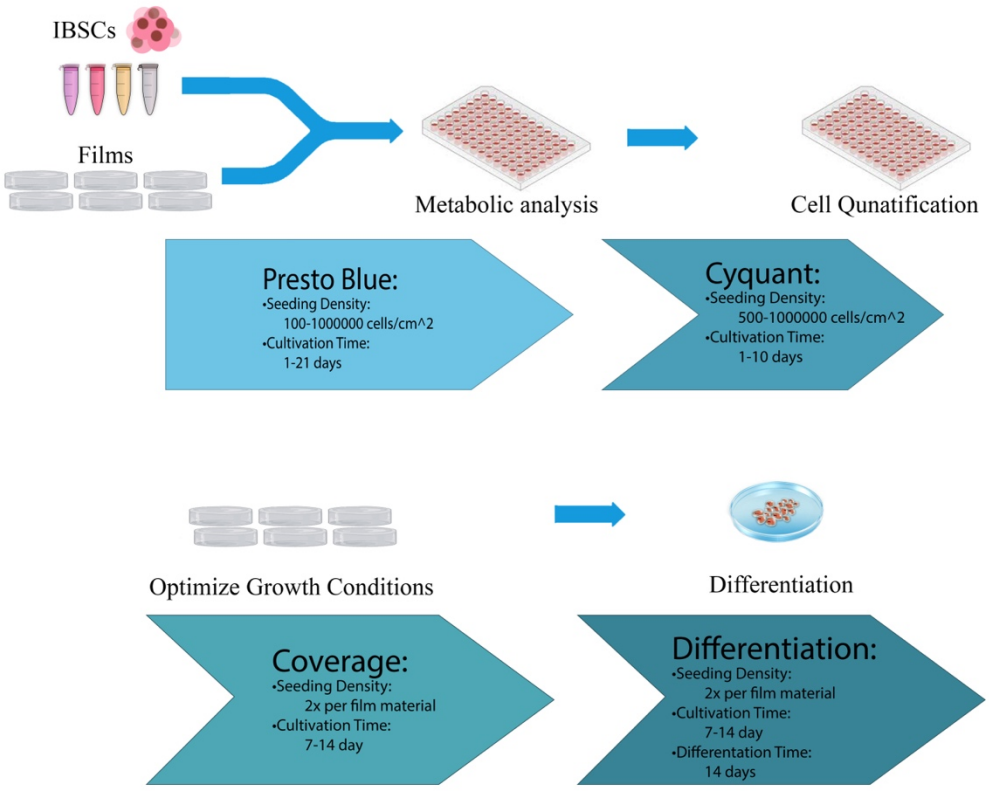

Figure 3: Design Goals: Phase 1 includes metabolic analysis with PrestoBlue using a wide range of seeding densities and cultivation times to assess the highest growth regions. Phase 2 includes cyqunat analysis with a focused range of seeding densities and cultivation times to quantify cell number and adhesion ratio. Phase 3 includes coverage analysis and differentiation to determine optimal growth conditions for differentiations

Methods:

Film Making:

Gelatin: The gelatin films used in this work were prepared using a method reported in literature *(11,19)*. Gelatin (Gelatin from bovine skin; Sigma-Aldrich, USA) solutions of 5 wt% were prepared by dissolving gelatin powder in water at 65°C for 1 hour. The gelatin solution was cooled to room temp, and 1%wt microbial transglutaminase (Sigma-Aldrich, USA) was added, mixing thoroughly. The solution was then added to molds, crosslinked at 37°C for 1 hour, and dried at room temperature overnight. The films were then sterilized with ethanol before UV exposure.

Zein: The zein films used in this work were prepared using a method reported in literature *(11,20)*. Zein (Sigma-Aldrich, USA) solutions of 7.5wt% were prepared in 70% ethanol. The solution was then cast into films and dried overnight. The films were then steam sterilized (autoclaved) at 121°C for 15 minutes.

Soy: The soy films used in this work were prepared using a method adapted from literature (11,21). Soy protein isolate was used to prepare soy films. *15% soy protein isolate* was dissolved in 1% sodium hydroxide at room temperature overnight. The solution was then added to molds and dried at room temperature overnight. The films were then steam sterilized (autoclaved) at 121°C for 15 minutes and subsequently neutralized with a 7.4pH HEPES (Sigma-Aldrich, USA) buffer overnight.

Alginate: The alginate films used in this work were prepared using a method reported in literature*(22)*. Briefly, alginate films were prepared by dissolving 5 wt% sodium alginate (Sigma-Aldrich, USA) in water. Films were dried overnight. Then the films were steam sterilized (autoclaved) at 121°C for 15 minutes and subsequently crosslinked by submerging in 4wt%

calcium chloride (Sigma-Aldrich, USA) for 5 minutes, then rinsed with DPBS (ThermoFisher, USA).

Chitosan: Chitosan (derived from mushroom) films were prepared by 4wt% in 2wt% acetic acid (Sigma-Aldrich, USA) at room temperature overnight. The solution was then cast into films and dried overnight. The films were then steam sterilized (autoclaved) at 121°C for 15 minutes and subsequently crosslinked by submerging in 1M sodium bicarbonate for 30 minutes and washed with DPBS.

Cellulose: The cellulose films used in this work were prepared using a method adapted from literature (11,23). Briefly, cellulose nanofibers (CNF, Cellulose lab, Canada) were diluted to a 1wt% aqueous solution and cast into films. The films were then dried overnight and were then steam sterilized (autoclaved) at 121°C for 15 minutes

Cell Culture:

Immortalized bovine satellite cells (iBSCs), developed by Stout et al. 2023 *(8)*, were used due to their retainment of myogenic differentiation post-immortalization. BSC growth medium (BSC-GM) made up of DMEM + Glutamax supplemented with 20% fetal bovine serum (FBS: ThermoFisher #26140079, MA, USA), 1% Antibiotic-Antimycotic (ThermoFisher, USA), 1 ng/mL human FGF-2 (ThermoFisher, USA), 2.5 ug/mL Puromycin (ThermoFisher, USA) was used. For regular cell maintenance, cells were cultured at 37C in 5% CO2 to confluency, passaged using 0.25% trypsin-EDTA (ThermoFisher #25200056), counted with NC-200 automated cell counter (Chemometec, Allerod, Denmark) and either seeded at 5,000 cells/cm^2 or frozen in FBS with 10%Dimethylsulfoxide (DMSO, Sigma #D2650). Cells were cultured to confluency for scaffold seeding and then serially diluted to the appropriate concentrations and seeding densities. Seeding densities for phase 1: 100, 500, 1000, 2000, 5000, 10000, 50000,

100000, 500000, and 1000000 cell/cm^2. Seeding for phase 2: 500, 5000, 10000, 50000, and 1000000 cell/cm^2. Seeding densities for phase 3 are unique based on each material based on phase 1 and 2 results.

Metabolic Analysis:

iBSCs were seeded on films according to the above phase 1 densities. The metabolic activity on the films was then determined at 1, 3, 7, 10, and 14 days via a PrestoBlue HS viability assay (ThermoFisher, USA) according to the manufacturer's instructions. Briefly, the cells cultured in the films were incubated in 200uL of 1:10 PrestoBlue reagent to growth media for 1 hour at 37°C in 5% CO2. Following the incubation, 100uL from each well was transferred into a new plate and measured with excitation at 560nm and emission at 590nm using a microplate reader (Biotek Synergy, USA). Assays were performed in triplicates. Data from the PrestoBlue assay will be analyzed in terms of peak fluorescence. The selection for seeding density ranges will be based upon achieving confluency by 14 days, identified by leveling-off of peak fluorescence.

Cyquant dsDNA Analysis:

iBSCs were seeded at densities that meet the initial screening requirements of the PrestoBlue assay and cultured with the same conditions. Cells were allowed to attach to films for 1, 3, 7, 10, and 14 days, after which the media will be aspirated out along with any unattached cells. The total number of cells was determined using a CyQunat cell counting assay (ThermoFisher, C35006). The manufacturer's direction will be followed for the assay. Briefly, 200uL of reagent was added to each well and incubated for 30 minutes. Then, 100uL from each well was transferred to a new plate, and the fluorescence was read with an excitation of 485 nm

and an emission of 530 nm at room temperature. Cell adhesion ratio will be determined using the day 1 timepoint.

Differentiation:

iBSCs were initially screened for the best differentiation media. Cells below passage number 40 were seeded at 5,000 cells/cm^2 in a TCP and grown for 7 days until confluent, and then differentiation media was added for 14 days. Each condition will be assessed in triplicate. Differentiation media preparations:

Serum Free Media: The media used in this work was adapted from literature (17). Briefly, Neurobasal (Invitrogen #21103049, Carlsbad, CA, USA) and L15 (Invitrogen #11415064) basal media (1:1) supplemented with 1% antibiotic/antimycotic, 10 ng/mL insulin-like growth factor 1 (IGF-1; Shenandoah Biotechnology #100-34AF-100UG, Warminster, PA, USA) and 100 ng/mL epidermal growth factor (EGF; Shenandoah Biotechnology #100-26-500UG) were combined. The media was changed every 48 hours.

2% Horse Serum Media: DMEM supplemented with 2% Horse Serum and 1% antibiotic-antimycotic. This was used both in a starvation capacity and regularly changing every 48 hours.

LDN Media: DMEM supplemented with 2% Horse Serum, 1% antibiotic-antimycotic, and 1% LDN-193189. The media was changed every 48 hours.

The wells will then be stained, imaged, and analyzed using ImageJ software for the media that provides the best differentiation. That media will then be best used in conjunction with the ideal growth conditions for each unique film to access differentiation on the films. This will be accomplished in triplicate.

Immunofluorescence:

A procedure adapted from literature *(11)*. Briefly, after culturing and differentiating for 14-28 days. Cells were fixed with 4% paraformaldehyde for 30 min, washed in DPBS, permeabilized with 0.5% Triton X-100 in DPBS, and blocked with 3% bovine serum albumin for 1 hour. Films were then incubated overnight at 4°C with 3ug/mL primary myosin heavy chain (MHC) antibodies in the blocking solution. Then, the films were incubated with Alexa Flour *594 goat anti-mouse IgG* secondary antibodies and counterstained with Alexa Flour 488 phalloidin and DAPI before fluorescent imaging.

Another procedure adapted from literature was used for zein films (24). Briefly, after culturing and differentiating for 14-28 days. Cells were fixed with 4% paraformaldehyde for 30 min and washed in DPBS. Treated with 75mg/mL Sudan Black B (Sigma-Aldrich, USA) dissolved in 45% ethanol solution and incubated overnight at 4°C. Films were then washed with DPBS, permeabilized with 0.5% Triton X-100 in DPBS, and blocked with 3% bovine serum albumin for 1 hour. Films were then incubated overnight at 4°C with 3ug/mL primary myosin heavy chain (MHC) antibodies in the blocking solution. Then, the films were incubated with were incubated with Alexa Flour 594 goat anti-mouse IgG secondary antibodies and counterstained with Alexa Flour 647 phalloidin and DAPI before fluorescent imaging.

Myotube Characterization:

To calculate the myotube fusion index, the number of nuclei within each multinucleated myotube will be counted; a minimum of three nuclei will be used as a threshold to account for occasional binucleation and averaged, then divided by the total number of nuclei in the entire cell population *(25)*. The result will then be expressed as a percentage. A higher percentage indicates a greater degree of myotube fusion and is often associated with more mature and functional muscle tissue in vitro.

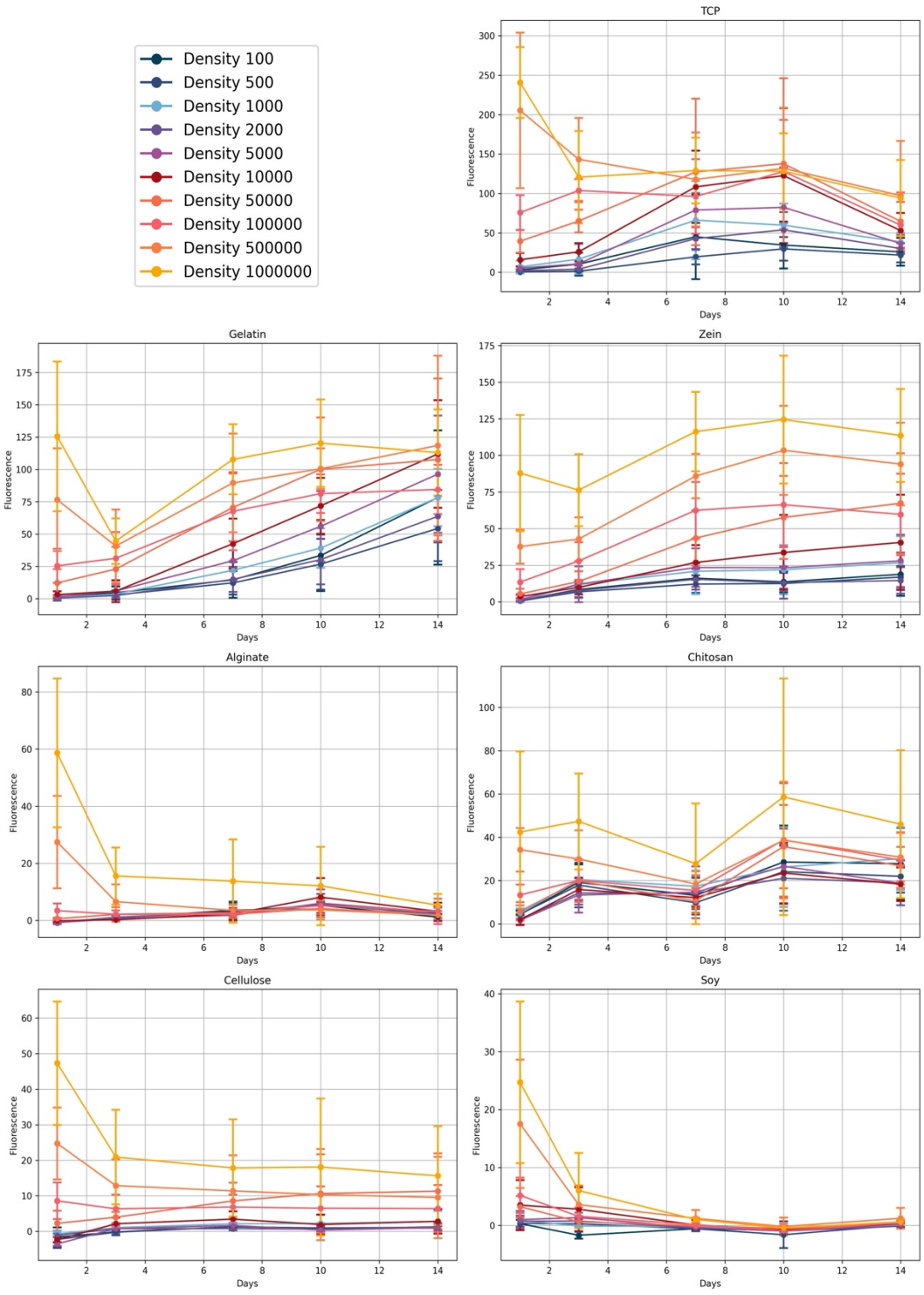

Figure 4: Presto Blue Data for iBSCs on film materials. TCP was used as the positive control, and gelatin was used as the positive control. Presto Blue was incubated for 1 hour, and time points were taken at 1, 3, 7, 10, and 14 days. Data is presented as the mean (n=9) +/- standard deviation.

Statistical Analysis:

In Python, a Shapiro-Wilk normality test was used to test the data for normalcy. Then, a one-way analysis of variance (ANOVA) was used to compare with Tukey's post-hoc analysis at a 0.05 significance level.

Results:

Phase 1: Metabolic Analysis

First, different seeding densities of iBSCs were examined. An initial screening showed no increase in metabolic activity after 14 days and no difference in seeding volume. From this, a 14-day study examining all conditions was conducted. The data was then plotted in **Figure 3**. A one-way ANOVA test assessed the significance across days and densities. The results are displayed in **Table 1** (Density-Wise Significance). Across all materials examined, the data trend in metabolic activity leveled off after 10 days, with a statistical significance between days 3 and 10. The positive control, tissue culture plastic (TCP), immediately leveled off, showing little significance across 14 days. The positive control, gelatin, and zein films leveled off on day 7. Chitosan and cellulose films showed an increasing trend over 14 days but not a significant trend. Alginate and Soy showed a decreasing trend over the 14 days with limited significance across seeding densities. Notably, the soy and alginate films showed signs of degradation over the 14-day period.

The data was normalized to the positive control to compare materials, as seen in **Figure 4**. At lower densities, the metabolic activity varied widely, decreasing as the density increased. The trends showed that gelatin and zein films had the highest metabolic activity of the film

TCP Density-Wise Significance

	1	3	7	10	14
100	A	A	A	A	A
500	A	A	B	B	B
1000	A	AB	B	B	B
2000	A	B	C	C	C
5000	A	B	C	C	D
10000	A	AB	B	B	B
50000	A	A	A	A	A
100000	A	A	A	A	A
500000	A	A	A	A	A
1000000	A	B	B	B	B

Chitosan Density-Wise Significance

	1	3	7	10	14
100	A	B	B	B	B
500	A	A	A	B	B
1000	A	B	B	B	B
2000	A	B	B	B	B
5000	A	B	B	B	B
10000	A	B	B	B	B
50000	A	A	A	B	B
100000	A	A	A	A	A
500000	A	A	A	A	A
1000000	A	A	A	A	A

Gelatin Density-Wise Significance

	1	3	7	10	14
100	A	A	A	A	B
500	A	A	AB	BC	C
1000	A	A	B	C	D
2000	A	A	A	BC	C
5000	A	A	A	BC	C
10000	A	A	B	C	D
50000	A	A	B	B	B
100000	A	A	B	B	B
500000	A	A	A	A	A
1000000	A	A	A	A	A

Cellulose Density-Wise Significance

	1	3	7	10	14
100	A	A	A	A	A
500	A	A	B	B	B
1000	A	B	B	B	B
2000	A	B	B	B	B
5000	A	B	B	B	B
10000	A	B	B	B	B
50000	A	A	A	A	A
100000	A	A	A	A	A
500000	A	A	A	A	A
1000000	A	A	A	A	A

Zein Density-Wise Significance

	1	3	7	10	14
100	A	A	A	A	A
500	A	A	B	B	B
1000	A	A	B	B	B
2000	A	A	B	B	B
5000	A	AB	ABC	BC	C
10000	A	AB	B	B	B
50000	A	AB	B	B	B
100000	A	AB	B	B	B
500000	A	AB	BC	BC	C
1000000	A	AB	B	B	B

Soy Density-Wise Significance

	1	3	7	10	14
100	A	B	B	B	C
500	A	A	A	A	A
1000	A	A	A	A	B
2000	A	A	B	B	C
5000	A	A	AB	B	C
10000	A	A	A	A	C
50000	A	AB	B	B	B
100000	A	AB	B	C	C
500000	A	AB	B	B	B
1000000	A	AB	B	B	C

Alginate	Density-Wise Significance				
	1	3	7	10	14
100	A	B	B	B	C
500	A	B	B	B	B
1000	A	B	B	B	B
2000	A	B	B	B	B
5000	A	B	B	B	B
10000	A	A	A	A	A
50000	A	A	A	A	A
100000	A	A	A	A	A
500000	A	A	B	B	B
1000000	A	A	B	B	B

Table 1: Density-wise significance by film material of PrestoBlue fluorescence. TCP was used as the positive control, and gelatin was used as the positive control. The data suggests a significant increase in fluorescence between days 3 and 10 across all materials. Data was analyzed using a Shapiro-Wilk normality test and then an ANOVA test with n=9 and $p < 0.05$. Letters A-C are used to group densities, with a common letter showing that groups are not significant from other densities—each row is unique.

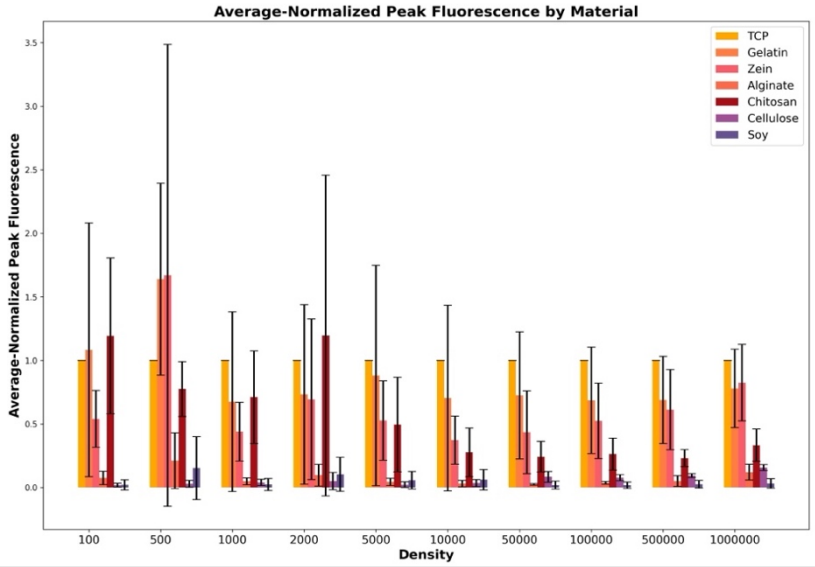

Figure 5: Average normalized peak fluorescence by film material of PrestoBlue fluorescence. Lower densities are highly variable, while at higher densities, data suggests similar growth on zein and controls (gelatin, TCP). Chitosan proved to be next best with approx. 30% fluoresces, and alginate, cellulose, and soy below 10% Data was normalized with respect to the positive control (TCP) and displayed as the mean (n=5), +/-SD.

materials, with some chitosan densities. Across a majority of densities, gelatin, zein, and chitosan had significantly higher metabolic activity than cellulose, alginate, and soy films. It was determined that at least 7 days of growth was optimal.

The statistical significance between densities was compared for each material, and the results are displayed in **Table 2**. The 7-14 days range was focused on determining ranges of significance seeding densities. The relationship in densities of the control groups, TCP, was analyzed but provided limited information without comparison to the other experimental groups. The alginate and soy films showed no significance in terms of density affecting metabolic activity over 14 days. Chitosan and cellulose films showed trends that densities above 100000 cells/cm2 had increased metabolic activity. Gelatin films showed several groups of densities significant from one another, notably 100 cells/cm^2-5000 cells/cm^2, 10000 cells/cm^2-100000 cells/cm^2, 500000 cells/cm^2, and 1000000 cells/cm^2. Zein films showed similar significance with a grouping of 100 cells/cm^2-2000 cells/cm^2, 5000 cells/cm^2-10000 cells/cm^2, 500000 cells/cm^2, and 1000000 cells/cm^2 displaying different levels of metabolic activity. Based on the ranges of significance determined from this analysis, it was determined to move forward with seeding densities of 500 cells/cm^2, 5000 cells/cm^2, 10000 cells/cm^2, 50000 cells/cm^2, and 1000000 cells/cm^2.

Density-wise significance by film material of PrestoBlue fluorescence. TCP was used as a positive control to verify cell growth, and gelatin was also used as the positive control to verify film interactions. The data suggests a significant increase in fluorescence between days 3 and 10 across all materials. Data was analyzed using a Shapiro-Wilk normality test and then an ANOVA test with $n=9$ and $p < 0.05$. Letters A-C are used to group days insignificant from each other with each row being unique.

TCP Day-Wise Significance

	100	500	1000	2000	5000	10000	50000	100000	500000	1E+06
1	A	A	A	A	A	AB	B	B	BC	C
3	A	A	A	A	A	A	B	B	B	B
7	A	A	A	A	A	A	A	A	A	A
10	A	A	A	A	A	A	A	A	A	A
14	A	A	A	A	A	A	A	A	A	A

Gelatin Day-Wise Significance

	100	500	1000	2000	5000	10000	50000	100000	500000	1E+06
1	A	A	A	A	A	A	A	A	BC	C
3	A	A	A	A	A	A	AB	BC	BC	C
7	A	A	A	A	A	AB	BC	BC	CD	D
10	A	A	AB	AB	ABC	CD	CD	CD	DE	E
14	A	A	A	A	A	A	A	A	A	A

Zein Day-Wise Significance

	100	500	1000	2000	5000	10000	50000	100000	500000	1E+06
1	A	A	A	A	A	A	A	A	B	C
3	A	A	A	A	AB	AB	AB	B	B	C
7	A	A	A	A	AB	AB	BC	C	D	E
10	A	A	A	A	AB	AB	BC	C	D	D
14	A	A	A	A	A	AB	BC	BC	CD	D

Alginate Day-Wise Significance

	100	500	1000	2000	5000	10000	50000	100000	500000	1E+06
1	A	A	A	A	A	A	A	A	A	B
3	A	A	A	A	A	A	A	A	A	A
7	A	A	A	A	A	A	A	A	A	A
10	A	A	A	A	A	A	A	A	A	A
14	A	A	A	A	A	A	A	A	A	A

Chitosan Day-Wise Significance

	100	500	1000	2000	5000	10000	50000	100000	500000	1E+06
1	A	A	A	A	A	A	A	AB	B	B
3	A	A	A	A	A	A	A	A	A	A
7	A	A	A	A	A	A	A	A	A	A
10	A	A	A	A	A	A	A	A	A	A
14	A	A	A	A	A	A	A	A	A	A

Cellulose Day-Wise Significance

	100	500	1000	2000	5000	10000	50000	100000	500000	1E+06
1	A	A	A	A	A	A	A	AB	B	B
3	A	A	A	A	A	A	A	A	A	A
7	A	A	A	A	A	A	A	A	A	A
10	A	A	A	A	A	A	A	A	A	A
14	A	A	A	A	A	A	A	A	A	A

Soy Day-Wise Significance

	100	500	1000	2000	5000	10000	50000	100000	500000	1E+06
1	A	A	A	A	A	A	A	A	A	A
3	A	A	A	A	A	A	A	A	A	A
7	A	A	A	A	A	A	A	A	A	A
10	A	A	A	A	A	A	A	A	A	A
14	A	A	A	A	A	A	A	A	A	A

Table 2: Day-wise significance by film material of PrestoBlue fluorescence. Target growth range was 10-14 days. Alginate, soy, and TCP showed no significant trends over 15 days. Cellulose and chitosan data suggested to densities over 100000cells/cm^2 having increased growth, and Zein showed 3 regions of significant difference in growth: <2000 cells/cm^2, 5000- 100000 cells/cm^2, and >500000 cells/cm2. Gelatin showed similar trends to zein. Data was analyzed using a Shapiro-Wilk normality test and then an ANOVA test with n=9 and $p < 0.05$. Letters A-E are used to group densities, with a common letter showing that groups are not significant from other densities—each row is unique.

Phase 2 Cyquant:

With the narrowed scope from Phase 1, the number of cells on several film materials was quantified using a Cyquant assay over a 10-day growth period. The data was then plotted in **Figure 5**. The results from the day 1 timepoint were used to calculate the adhesion (**Figure 6**). The minimum of the detection range was calculated to be 122 cells. No significant differences were observed across seeding densities and days, indicating each material has roughly the same number of cells independent of the number of cells seeded. There was a trend toward higher cell numbers for higher seeding densities. Cells cultured on TCP (positive control) and Gelatin films (positive control) were observed to have the most cells on average at each time point. Zein films

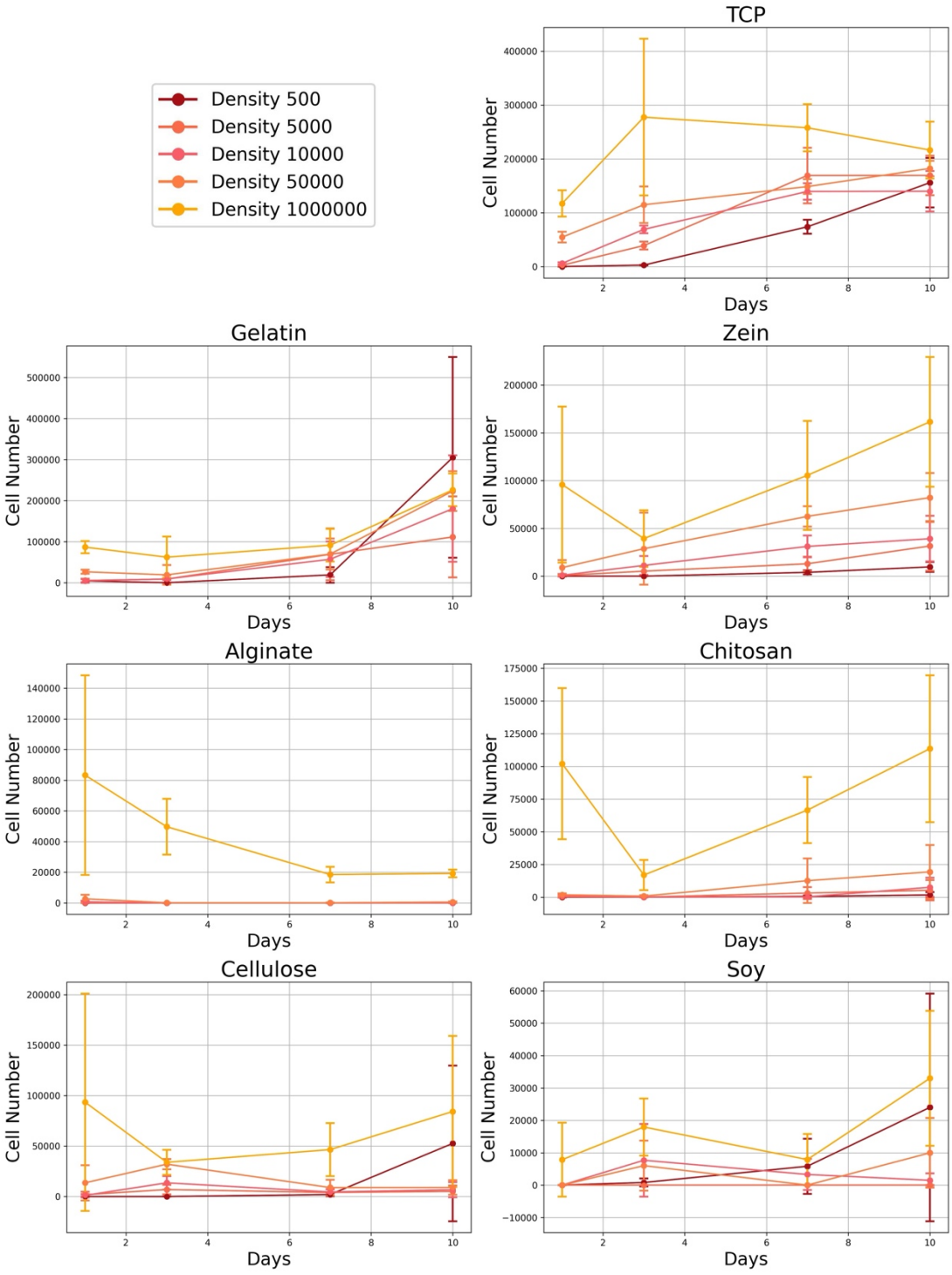

Figure 6: Cyqunat data for iBSCs. Cell number was calculated by creating a standard curve with known cell numbers. The minimum detection achieved was 122 cells. TCP displayed a maximum cell number by day 7 and leveled off after. Alginate displayed a decreasing trend, suggesting cell detachment. All other films displayed a positive trend across all 10 days. Data is presented as the mean (n=3) +/- SD.

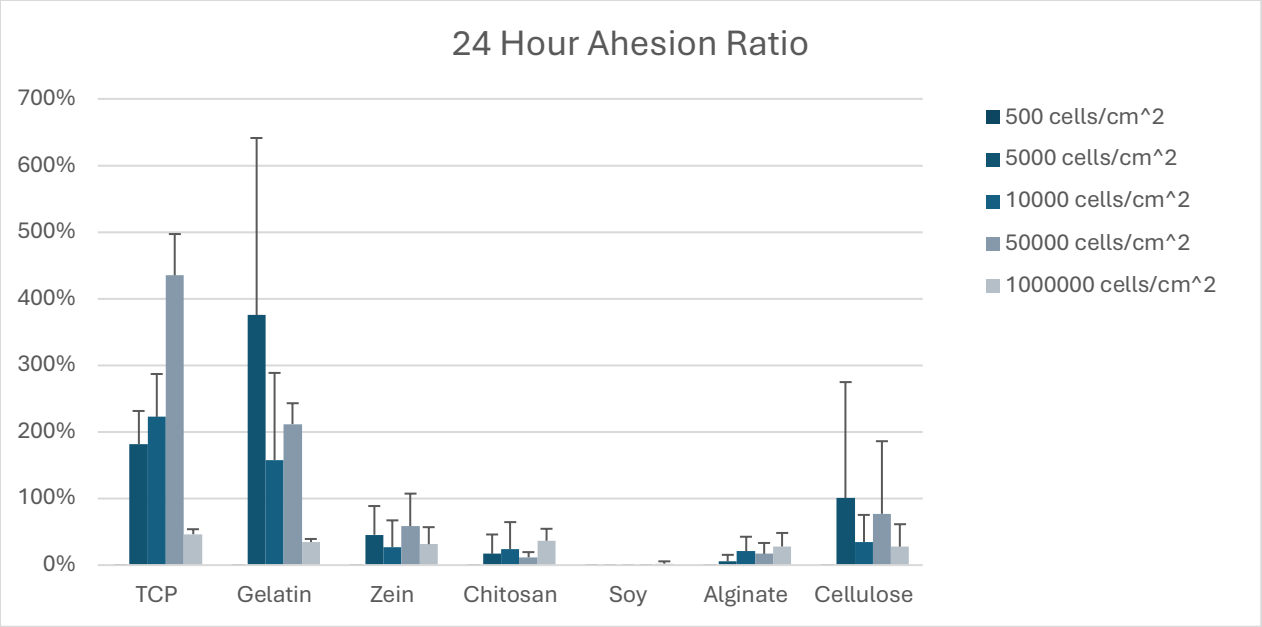

Figure 7: iBSC adhesion ratio on film materials after 24hrs. Across all materials, the data suggests the adhesion ratio increased with seeding density until 50000 cells/cm^2 and dropped off after. Controls, TCP and gelatin, displayed the best adhesion. Alginate and soy displayed the worst below 20%, with zein, chitosan, and cellulose all similarly above 20%. The adhesion ratio was calculated by dividing the number of cells adhered to the films by the number of cells seeded on the film. Data is presented as the mean +/- SD for n=3, the minimum detection range was 122 cells.

were observed to have a substantial number of cells when compared to the rest of the films, which all had roughly the same cell numbers. TCP was the only material observed to have a significant difference in cell number across days. All other materials showed an increasing trend in cell numbers across 10 days. Generally, protein-based films have better cell adhesion due to mammalian-expressed cell integrins binding to protein ligands rather than polysaccharides (11,26). Zein protein followed this trend with increased cell adhesion, but soy protein was not significantly better than any of the polysaccharides.

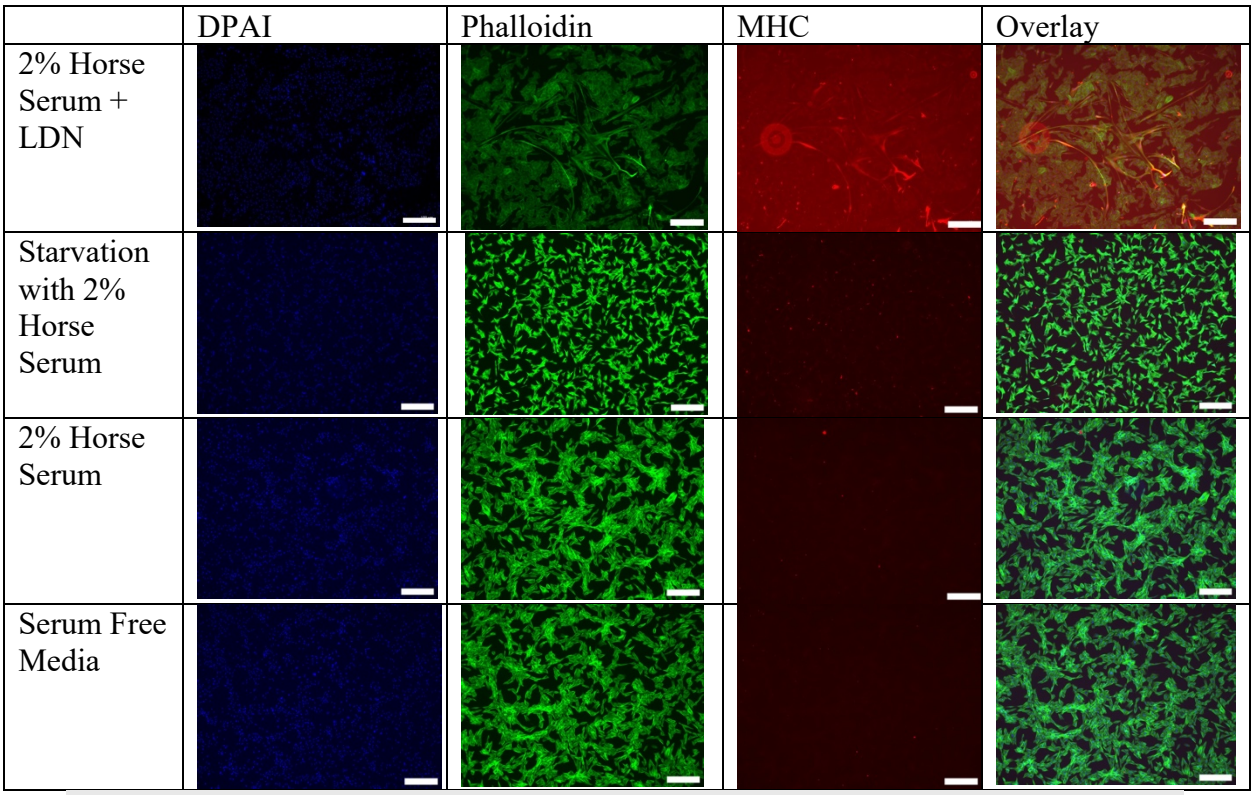

Figure 8: Assessments of differentiation media for iBSCs differentiation on TCP. 2% Horse Serum + LDN was the only formulation displayed, and myosin heavy chain (MHC) synthesis for myotube formation. Differentiated myotubes were marked by immunostaining for MHC, and nuclei were stained with DAPI. Color Code: blue=DAPI, green=phalloidin, red = MHC. Scale bars are 500um.

Phase 3 Differentiation:

Several differentiation media were first assessed to determine which would lead to the most differentiation of iBSCs. iBSCs were cultured for 7 days until confluency and then differentiated for 14 days using 1 of 4 differentiation media: 2% horse serum, 2% horse serum+ LDN, starvation with 2% horse serum, and serum-free media. After differentiation, the results were stained and images (**Figure 7**). Out of the 4 types of differentiation media, the 2% horse serum + LDN was the only one that exhibited any differentiation by 14 days and was selected as the media to use to assess differentiation on the films.

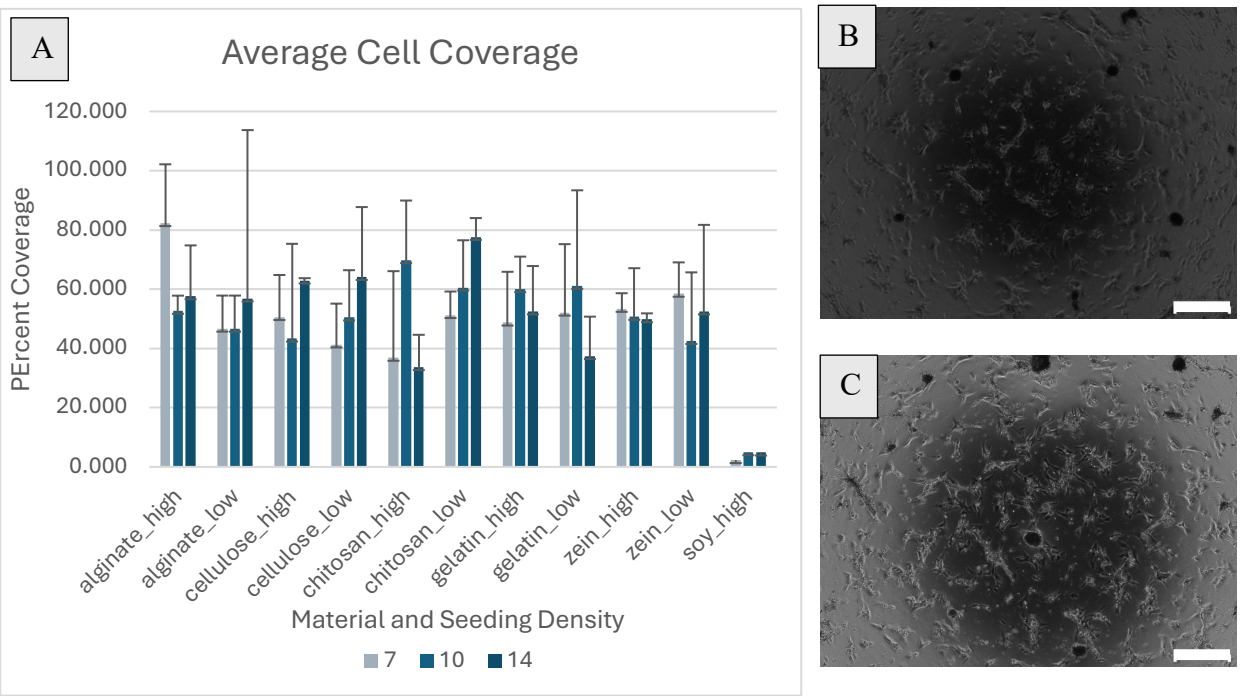

Figure 9: A) Cell coverage after 7, 10, and 14 days of growth. High denotes the higher seeding density for that material, and low denotes the lower seeding density. Alginate and Zein displayed the highest coverage after 7 days of growth at both seeding densities. Cellulose and Chitosan displayed the highest coverage on day 10 for higher seeding density and on day 14 for lower seeding density. Soy coverage was only determined at the high seeding density and saw the best coverage at 10 days. The coverage percentage was determined in ImageJ by calculating a minimum fluorescence threshold and taking that as a percentage of the total film area. Data is presented as the mean +/- SD for n=3 B&C) Brightfield microscopy of iBSC coverage on TCP after 14 days. Overconfluent cells tend to form clumps. Scale bar is 500um

To achieve differentiation, 80% confluency is needed, and this was assessed with two seeding densities for each film material over 7, 10, and 14 days. Based on the results from Phase 1 and Phase 2, the seeding density for gelatin was determined to be 10000 cells/cm^2 and 50000 cells/cm^2; zein seeding density was 10000 cells/cm^2 and 1000000 cells/cm^2; and alginate,

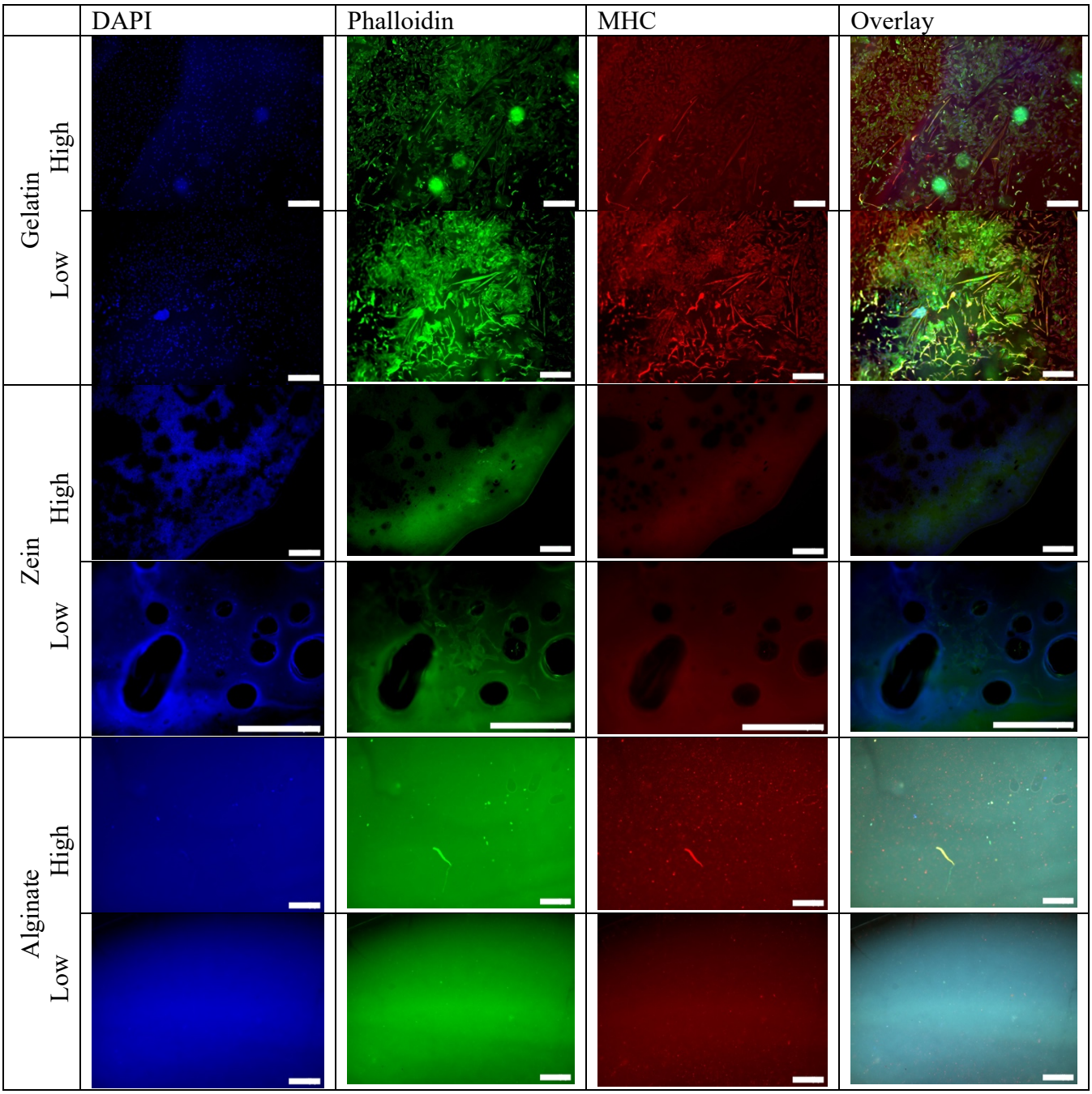

Figure 10: Differentiation of iBSCs on films with an optimal growth range of 7 days and then differentiated for 14 days, gelatin films (control). At both seeding densities, gelatin clearly displayed differentiation. Zein film's autofluorescence led to no clear sign of differentiation. Alginate showed no differentiation or cell attachment. Differentiated myotubes were marked by immunostaining for MHC, and nuclei were stained with DAPI. Color Code: blue=DAPI, green=phalloidin, red = MHC. Scale bars are 500um.

cellulose, chitosan, and soy seeding densities were determined to be 50000 cells/cm^2 and 1000000 cells/cm^2. Soy films were observed to be degrading in media. Thus, only a high seeding density, 1000000 cells/cm^2, was selected to minimize cultivation time and film degradation. In ImageJ, the coverage ratio was determined by calculating a minimum fluorescence threshold using the fluorescence of the control film, respectively. The threshold was used to determine the percentage of cell coverage compared to the total film area using a sample size of n=3 (**Figure 8**). Images of the films were randomly taken to represent the entire film accurately.

Zein films seeded at 10000 cells/cm^2 and 1000000 cells/cm^2 had the highest film coverage after 7 days of cell growth. Alginate films showed the best coverage at 50000 cells/cm^2 and 1000000 cells/cm^2 seeding densities on day 7 and appeared to decrease on day 10 and then on day 14 again. Cellulose and Chitosan had similar trends, with the lower 50000 cells/cm^2 seeding density presenting great coverage on day 14 and seeding at 1000000 cells/cm^2 had more coverage on day 10. Soy films showed degradation over the coverage analysis and resulted in data from the higher seeding density of 1000000 cells/cm^2, which had the best coverage on day 10. Gelatin was used as a control and seeded at 10000 cells/cm^2 and 50000 cells/cm^2, displaying the optimal cell coverage at all time points.

Differentiation was assessed at a high and low seeding density after 7, 10, or 14 days of growth. The seeding densities were chosen from the phase 1 and phase 2 results, and the cultivation time was chosen based on maximized film coverage. All films were stained for myosin heavy chain (MHC), phalloidin, and DAPI. Zein was also pretreated with Sudan Black B to reduce the autofluorescence seen on zein films in the coverage analysis. Gelatin films were seeded at optimal high and low seeding densities for all possible cultivation times and used as controls. Zein and alginate films were cultivated for 7 days and differentiated for 14 days, as

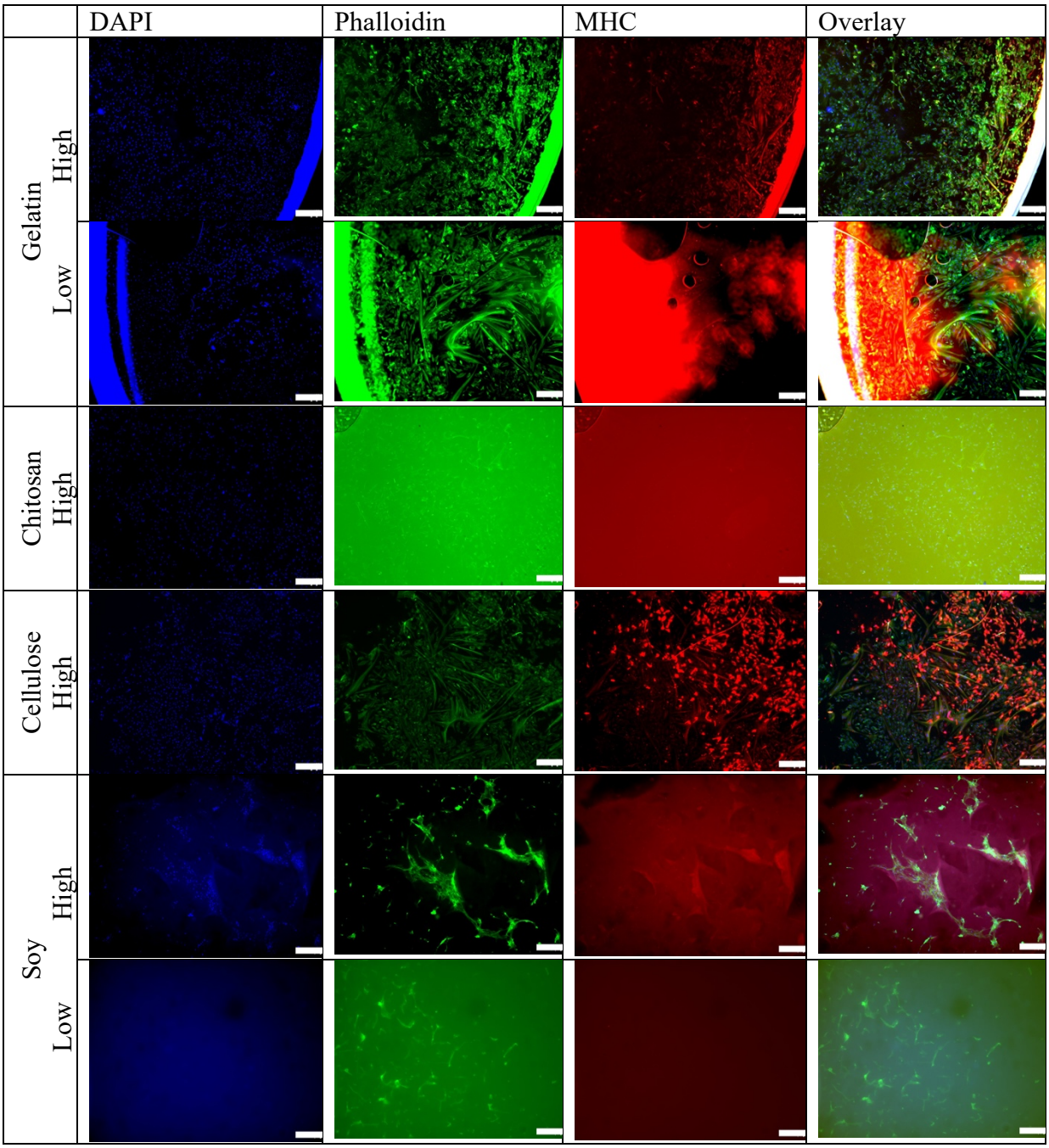

Figure 11: Differentiation of iBSCs on films with an optimal growth range of 10 days and differentiated for 14 days, gelatin films (control). At both seeding densities, gelatin displayed differentiation. Chitosan films were seeded at high seeding density and only displayed cell attachment. Cellulose films were seeded at high densities and exhibited differentiation. Soy was seeded at both high and low densities and only exhibited cell attachment that was under the 80% necessary for differentiation. Differentiated myotubes were marked by immunostaining for MHC, and nuclei were stained with DAPI. Color Code: blue=DAPI, green=phalloidin, red = MHC. Scale bars are 500um.

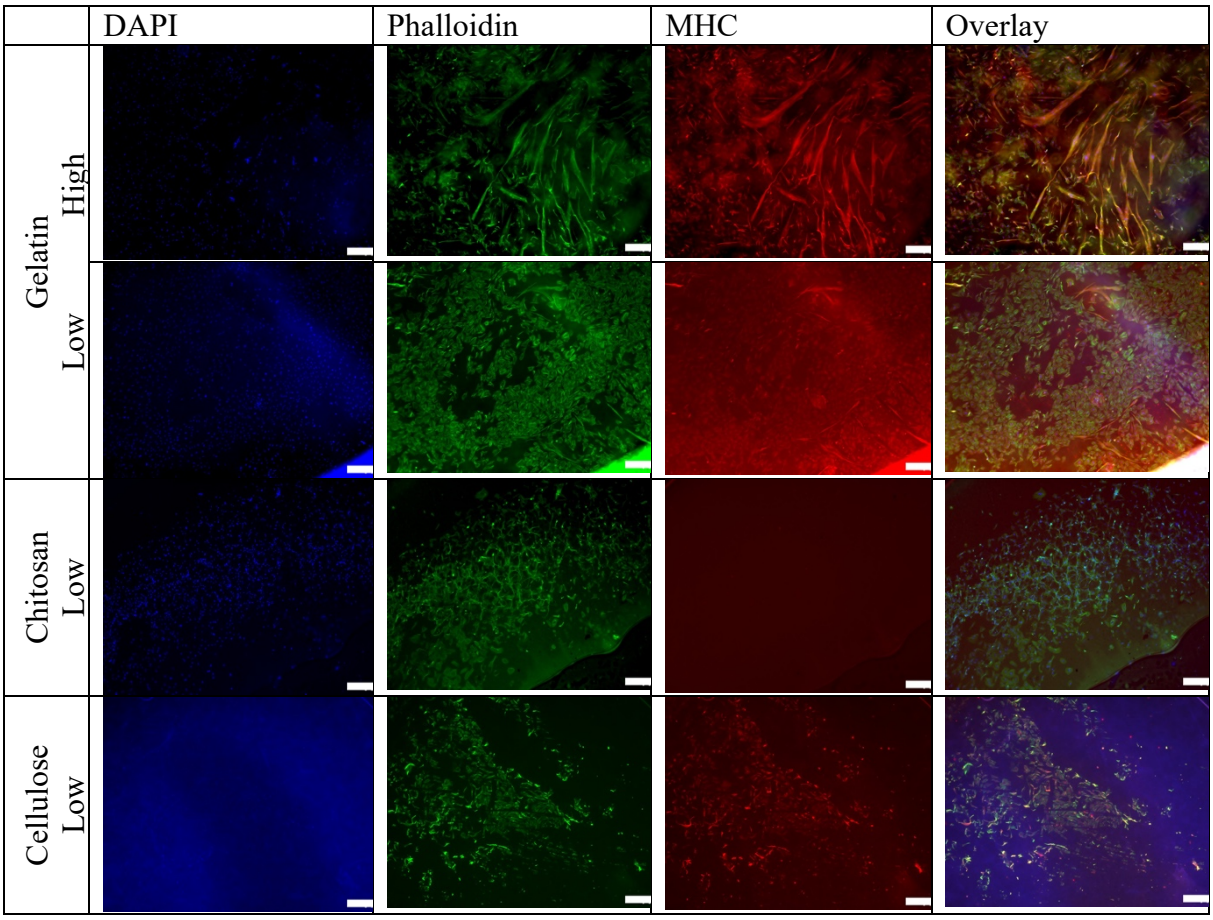

Figure 12: Differentiation of iBSCs on films with an optimal growth range of 14 days and differentiated for 14 days, gelatin films (control). At both seeding densities, gelatin displayed differentiation. Chitosan films were seeded at low seeding density and only displayed cell attachment. Cellulose films were seeded at low densities and only displayed cell attachment. Differentiated myotubes were marked by immunostaining for MHC, and nuclei were stained with DAPI. Color Code: blue=DAPI, green=phalloidin, red = MHC. Scale bars are 500um.

shown in **Figure 9**. Gelatin films differentiated at both seeding densities but exhibited more myotube formation at the lower seeding density. Zein film's autofluorescence prevented the ability to determine myotube formation but allowed for the visualization of cell attachment. Alginate films showed no differentiation or cell attachment, suggesting that the longer time in culture led to the continued trend of declined cell adhesion. Soy films at both high and low densities, chitosan films at high densities, and cellulose at high densities were cultivated for 10 days before 14 days of differentiation (**Figure 10**). Gelatin films were used as control films at

both high and low densities and exhibited differentiation at both densities. Chitosan and soy films exhibited no differentiation, but both still had cell coverage. Cellulose films exhibited minimal differentiation. Chitosan and Cellulose films seeded with low densities were cultivated for 14 days and then differentiated for 14 days (**Figure 11**). Gelatin films were used as control films at both high and low densities and exhibited differentiation at both densities. The chitosan and cellulose films at low seeding density did not exhibit any differentiation but maintained cell coverage.

The fusion index was calculated after differentiation was complete for all films. Since zein, chitosan, alginate, and soy exhibited no differentiation at any stage, the fusion index for those films was 0. The gelatin film fusion index was calculated across 7, 10, and 14 days at both seeding densities and was found to be about 12.5% across all days and densities. This was unsurprising because all films were cultivated in differentiation media for 14 days, and all were confluent after their respective growth time. The cellulose film fusion index was calculated after 10 days of growth seeding at a high density of about 10% and after 14 days of growth with a low seeding density of 0%. The cellulose films displayed areas with 80% coverage but were not completely covered by cells like the gelatin films were. This is likely also the case for chitosan, alginate, and soy films as well. Zein films lead to inconclusive results resulting from the autofluorescence of the films.

Discussion:

In this research, the growth and differentiation conditions for immortalized bovine satellite cells (iBSCs) on edible film materials were characterized by modeling their growth based on a process adapted from Xiang et al. (11). While the comprehensive analysis process for

various film materials provided insight into metabolic activity, cell adhesion, and differentiation for culturing iBSCs it did not explore surface roughness, tensile strength, and purity of the films.

The initial screening of a large array of seeding densities of iBSCs across multiple film materials revealed a distinct trend where metabolic activity leveled off after 10 days, with a notable statistical significance between days 3 and 10. For this experiment phase, confluency was assumed to be achieved close to the point where the metabolic activity leveled off. Tissue culture plastic (TCP), used as a positive control, showed minimal change in metabolic activity throughout the 14 days. Highlighting the ideal growth conditions leading to short growth time until confluency. In contrast, the positive control, gelatin, and zein films demonstrated a leveling off by day 7, highlighting their potential for promoting cell growth but still reaching confluency. Chitosan and cellulose films exhibited a non-significant increasing trend, which suggested growth but not confluency, while alginate and soy films showed a decreasing trend and signs of degradation, indicating their unsuitability for long-term cultures.

Further analysis by normalization to the positive control, TCP, highlighted that gelatin and zein films had the highest metabolic activity, especially at lower seeding densities. This suggests that protein-based films are more conducive to iBSC growth compared to polysaccharide-based films like alginate and soy. This can be explored by most protein film solutions being relatively stable in water due to their intramolecular forces. Polysaccharides also lack Arg-Gly-Asp (RGD) binding motifs, decreasing cell adhesion and inhibiting growth (11,27). The statistical analysis further indicated that seeding densities significantly influenced metabolic activity, particularly for gelatin and zein films, which showed varied metabolic responses across different densities. These findings informed the selection of optimal seeding densities for subsequent phases: 500 cells/cm^2, 5000 cells/cm^2, 10000 cells/cm^2, 50000 cells/cm^2, and

1000000 cells/cm². These findings informed the selection to decrease cultivation time to a maximum of 10 days.

In the next phase, a Cyquant assay was used to quantify cell numbers on film materials over a 10-day period. The assay showed that cell numbers presented an increasing trend across seeding densities and days but were insignificant. TCP and gelatin films exhibited the highest cell numbers, reinforcing their nature as ideal controls for cell growth. Zein films also supported substantial cell numbers, while alginate, cellulose, chitosan, and soy showed large variations but lower cell counts. TCP was the only material with a significant increase in cell numbers over time. The protein-based films' better cell adhesion can be attributed to the affinity of mammalian cell integrins for protein ligands, a trend also seen with zein but not soy, which performed similarly to polysaccharides (28).

The differentiation potential of iBSCs was assessed using various media, with 2% horse serum + LDN emerging as the most effective, leading to differentiation on TCP within 14 days. This aligns with other literature that has shown LDN to block growth pathways leading to forced differentiation (16,18,29). Achieving 80% confluency was essential for successful differentiation. Using optimized seeding densities derived from earlier growth optimization with metabolic activity and cell number, a film coverage test was conducted. From this, the ideal cultivation time was determined for both a high and low seeding density. As seen in **Figure 8**, after iBSCs reach confluency, the cells tend to clump, reducing the surface coverage and differentiation ability. The gelatin films used as control showed differentiation capacity across all cultivation conditions, signifying the ability of the methods used to lead to differentiation. Overall, the protein films were unable to differentiate conclusively. Zein had shown similar results to zein in the previous phase 1 and 2 trials, but one of the major limitations at this stage

was the autofluorescence. The autofluorescence exhibited during the coverage analysis by zein led to the adoption of a Sudan Black B treatment and the use of Alexa Flour 647 phalloidin over Alexa Flour 488 phalloidin (24). To fully understand the growth of iBSCs on zein, a better methodology for immunofluorescent microcopy needs to be developed first. Differentiation was unable to be assessed on zein films because of the autofluorescence. Soy also did not exhibit any differentiation. Over the three phases, there were several issues with degradation, which led to poor cell adhesion and limited growth thereafter. The soy formulation needs to be optimized past initial short-term cell adhesion testing to be able to not degrade over the course of a 28-day experiment before it becomes a viable option. The lack of differentiation in the protein films was unexpected, with many sources of literature reporting that intramolecular forces and stability in the solution (11,27).

The polysaccharides, which exhibited lower metabolic activity and cell numbers than the proteins, showed myotube formation. Cellulose seeded at a high seeding density of 1000000 cells/cm^2 exhibited differentiation after 10 days of cultivation and 14 days of differentiation, while the lower seeding density of 50000 cells/cm^2 did not exhibit differentiation after 14 days of cultivation and 14 days of differentiation. Since cellulose films demonstrated the ability to differentiate, this implies a longer cultivation time may be needed. Cellulose nanofibers were also used which potentially could have increased cell alignment and promotion of differentiation. Without further analysis of the surface roughness and structure, the effects cannot be confirmed. Chitosan films did not exhibit differentiation and maintained minimal cell coverage throughout all the studies.

Conclusion:

The optimization of immortalized bovine satellite cells (iBSCs) growth and differentiation on various edible films is needed to provide a basis for further exploration of the potential for cultured meat solutions. This study analyzes and compares several protein-based films and polysaccharide-based films. Protein films outperformed polysaccharide films like alginate and soy regarding metabolic activity, cell adhesion, and overall growth but were not substantially better when compared in terms of differentiation. These results underline the importance of selecting suitable materials to enhance cell cultivation in cultured meat applications.

Metabolic activity levels off after 10 days, with statistical analysis, highlighting that gelatin and zein films are more conducive to iBSC growth compared to polysaccharide-based films, aligning with the hypothesis that protein films provide better support for cell growth due to their stability in aqueous environments and their affinity for mammalian cell integrins. The Cyquant assay further confirmed this understating. For the differentiation experiments, achieving 80% confluency was critical for successful differentiation, and 2% horse serum + LDN medium was the most effective for iBSC differentiation.

Cellulose films produced significant results for the differentiation analyses which is hypothesized to be due to the increased alginate from the use of nanofibers and surface structure. Certain challenges, such as the autofluorescence properties of zein, impeded clear visualization of differentiation; and film stability over the 28-day experiments for soy and alginate. Additionally, cell clumping observed on certain materials suggests that further optimization of seeding densities, cultivation time, and film properties is needed to enhance cell differentiation.

Future Work:

Future research should investigate modifications to reduce autofluorescence in zein, possibly by pretreating with substances like Sudan Black B and developing hybrid films that balance mechanical strength and cell adhesion. Advanced differentiation techniques should be explored, including testing additional media formulations, other methods for blocking growth, and alternative stimuli such as mechanical stretching. Scaling up production protocols in bioreactors using microparticles and assessing the feasibility and economic implications of large-scale production with different scaffold materials is also crucial. Long-term stability and degradation studies are necessary to evaluate scaffold stability over extended periods and their effects on cell viability and differentiation. The surface roughness and patterning are other aspects that could be explored to improve differentiation and fiber alignment. Additionally, exploring other immortalized cell lines or primary cells for cultured meat applications and comparing their performance with iBSCs will help identify the best candidates for specific products.

References:

1. Saturna Capital. Feeding 10 billion people in a climate-changing world [Internet]. 2020 May p. 20. (From The Yardarm). Available from: https://www.saturna.com/sites/saturna.com/files/files/2020/FromTheYardarm-2020_05-Food-and-Climate-Change-web.pdf

2. Steinfeld H, Gerber P, Wassenaar TD, Castel V, Rosales MM, de Haan C. Livestock's long shadow: environmental issues and options [Internet]. Rome: Food and Agriculture Organization of the United Nations; 2006. Available from: https://www.fao.org

3. Rotz CA, Isenberg BJ, Stackhouse-Lawson KR, Pollak EJ. A simulation-based approach for evaluating and comparing the environmental footprints of beef production systems. J Anim Sci. 2013 Nov;91(11):5427–37.

4. Broom DM. Land and Water Usage in Beef Production Systems. Animals. 2019;9(6).

5. Gerber PJ, Food and Agriculture Organization of the United Nations. Tackling climate change through livestock: a global assessment of emissions and mitigation opportunities [Internet]. Rome: Food and Agriculture Organization of the United Nations; 2013. Available from: https://www.fao.org

6. Bomkamp C, Skaalure SC, Fernando GF, Ben-Arye T, Swartz EW, Specht EA. Scaffolding Biomaterials for 3D Cultivated Meat: Prospects and Challenges. Adv Sci (Weinh). 2022 Jan;9(3):e2102908.

7. Hong TK, Shin DM, Choi J, Do JT, Han SG. Current Issues and Technical Advances in Cultured Meat Production: A Review. Food Sci Anim Resour. 2021 May;41(3):355–72.

8. Stout AJ, Arnett MJ, Chai K, Guo T, Liao L, Mirliani AB, et al. Immortalized Bovine Satellite Cells for Cultured Meat Applications. ACS Synth Biol. 2023 May 19;12(5):1567–73.

9. Han ES, Wu Y, McCarter R, Nelson JF, Richardson A, Hilsenbeck SG. Reproducibility, Sources of Variability, Pooling, and Sample Size: Important Considerations for the Design of High-Density Oligonucleotide Array Experiments. The Journals of Gerontology: Series A. 2004 Apr 1;59(4):B306–15.

10. Post M, Levenberg S, Kaplan DL, Genovese N, Fu J, Bryant CJ, et al. Scientific, sustainability and regulatory challenges of cultured meat. Nature Food. 2020 Jul 16;1(7):403–15.

11. Xiang N, Yao Y, Yuen JSK, Stout AJ, Fennelly C, Sylvia R, et al. Edible films for cultivated meat production. Biomaterials. 2022 Aug 1;287:121659.

12. Reiss J, Robertson S, Suzuki M. Cell Sources for Cultivated Meat: Applications and Considerations throughout the Production Workflow. Int J Mol Sci. 2021 Jul 13;22(14).

13. Nawaz MA, Mesnage R, Tsatsakis AM, Golokhvast KS, Yang SH, Antoniou MN, et al. Addressing concerns over the fate of DNA derived from genetically modified food in the human body: A review. Food and Chemical Toxicology. 2019 Feb 1;124:423–30.

14. Parlasca MC, Qaim M. Meat Consumption and Sustainability. Vol. 14, Annual Review of Resource Economics. Annual Reviews; 2022. p. 17–41.

15. Crespi CL, Thilly WG. Continuous cell propagation using low-charge microcarriers. Biotechnology and Bioengineering. 1981;23(5):983–93.

16. O'Neill EN, Cosenza ZA, Baar K, Block DE. Considerations for the development of cost-effective cell culture media for cultivated meat production. Comprehensive Reviews in Food Science and Food Safety. 2021 Jan 1;20(1):686–709.

17. Stout AJ, Mirliani AB, Rittenberg ML, Shub M, White EC, Yuen JSK, et al. Simple and effective serum-free medium for sustained expansion of bovine satellite cells for cell cultured meat. Communications Biology. 2022 Jun 2;5(1):466.

18. Shi S, Hoogaars WMH, de Gorter DJJ, van Heiningen SH, Lin HY, Hong CC, et al. BMP antagonists enhance myogenic differentiation and ameliorate the dystrophic phenotype in a DMD mouse model. Neurobiology of Disease. 2011 Feb 1;41(2):353–60.

19. Liu F, Majeed H, Antoniou J, Li Y, Ma Y, Yokoyama W, et al. Tailoring physical properties of transglutaminase-modified gelatin films by varying drying temperature. Food Hydrocolloids. 2016 Jul 1;58:20–8.

20. Escamilla-García M, Calderón-Domínguez G, Chanona-Pérez JJ, Farrera-Rebollo RR, Andraca-Adame JA, Arzate-Vázquez I, et al. Physical and structural characterisation of zein and chitosan edible films using nanotechnology tools. Int J Biol Macromol. 2013 Oct;61:196–203.

21. Cao N, Fu Y, He J. Preparation and physical properties of soy protein isolate and gelatin composite films. Food Hydrocolloids. 2007 Oct 1;21(7):1153–62.

22. Rhim JW. Physical and mechanical properties of water resistant sodium alginate films. LWT - Food Science and Technology. 2004 May 1;37(3):323–30.

23. Yang X, Jungstedt E, Reid MS, Berglund LA. Polymer Films from Cellulose Nanofibrils—Effects from Interfibrillar Interphase on Mechanical Behavior. Macromolecules. 2021 May 11;54(9):4443–52.

24. Foster O, Shaidani S, Theodossiou SK, Falcucci T, Hiscox D, Smiley BM, et al. Sudan Black B Pretreatment to Suppress Autofluorescence in Silk Fibroin Scaffolds. ACS Biomater Sci Eng. 2023 Jun 12;9(6):3193–205.

25. Zhang H, Wen J, Bigot A, Chen J, Shang R, Mouly V, et al. Human myotube formation is determined by MyoD-Myomixer/Myomaker axis. Sci Adv. 2020 Dec;6(51):eabc4062.

26. Sun QS, Dong J, Lin ZX, Yang B, Wang JY. Comparison of cytocompatibility of zein film with other biomaterials and its degradability in vitro. Biopolymers. 2005 Aug 5;78(5):268–74.

27. Stevens B, Yang Y, Mohandas A, Stucker B, Nguyen KT. A review of materials, fabrication methods, and strategies used to enhance bone regeneration in engineered bone tissues. J Biomed Mater Res B Appl Biomater. 2008 May;85(2):573–82.

28. Elli L, Dolfini E, Bardella MT. Gliadin cytotoxicity and in vitro cell cultures. Toxicology Letters. 2003 Dec 15;146(1):1–8.

29. Horbelt D, Boergermann JH, Chaikuad A, Alfano I, Williams E, Lukonin I, et al. Small molecules dorsomorphin and LDN-193189 inhibit myostatin/GDF8 signaling and promote functional myoblast differentiation. J Biol Chem. 2015 Feb 6;290(6):3390–404.