2022

Growing Meat on Plants: Using intermediate CBD-RGD fusion proteins to improve bovine satellite cell attachment on cellulose-based scaffolds

Julian Cohen

Growing Meat on Plants:
Using intermediate CBD-RGD fusion proteins to improve bovine satellite cell attachment on cellulose-based scaffolds

A Thesis Presented

by

Julian Mentis Cohen

To the Keck Science Department

of

Claremont McKenna, Scripps, and Pitzer Colleges

In Partial Fulfillment of

The Degree of Bachelor of Arts

Senior Thesis in Biophysics

April 25th, 2022

Abstract

Cellular agriculture is an emerging technology aiming to replace existing methods for animal agriculture with tissue engineering and cell culture-based technologies. Cultured meat falls within this purview, using a biomimetic approach to recreate animal muscle tissue through tissue engineering. In the attempt to diminish the necessity of animal-derived materials within this process, plant-based scaffolds can be used as a substrate upon which stem cells are cultured. Due to the unfavorable environment of cellulose for mammalian cell-surface proteins, the approach was taken of coating cellulose nanofiber films with a fusion protein composed of a cellulose binding domain (CBD) protein and the cell-adhesion peptide motif RGD, upon which bovine satellite cells were then cultured. Using this protein as an intermediate upon which each component can bind, our results indicate statistically-significant enhancement of cell attachment within this system when using an FBS-containing media formulation.

Table of Contents

Introduction

Cultured Meat

As we look ahead to the next decade, substantial efforts must be made to address the rising challenges presented by our world's climate and public health crises. Confronting these challenges force us to explore the myriad ways in which food systems are both part of the problem, while also holding the promise and possibility of being part of the solution. It is with this view that we consider the role of conventional meat production - and its effects on the environment, public health, and social relations - and how a transition to novel forms of meat production offered by cellular agriculture might mitigate or reverse these effects.

One area into which cellular agriculture can make inroads is on the front of environmental impact. Indeed, livestock contributions to the Earth's volume of the three major greenhouse gases is staggering: it is responsible for 9% of carbon dioxide, 39% of methane, and 65% of nitrous oxide emissions (FAO, 2006). Conventional agriculture also requires huge amounts of land, some of which is used for animal grazing and the vast majority of which is used for growing monocrop corn and soybeans for animals feed, often leading to deforestation and destruction of ecological landscapes (Hecht, 1993). Prospective lifecycle analyses, however, conclude that switching from conventional meat production to that of cultured meat might reduce land usage by 99%, water usage by 90%, and energy consumption by 40% (Tuomisto and de Mattos, 2011). More recent life cycle analyses, it should be noted, suggest that cultured meat production could require smaller quantities of agricultural inputs and land than livestock, but which could come at the expense of more intensive energy use (Mattick et al, 2015).

There are also serious concerns with conventional meat production from a public health perspective. Cardiovascular disease, diabetes, and colorectal cancer have long been associated with the consumption of red meat (Wolk, 2017). Meat production is also responsible for development and transmission of many foodborne illnesses, such as Salmonella, Campylobacter, and E. coli, vectors responsible for millions of medical illnesses a year in the United States (CDC, 2012). Across livestock operations around the world, there is the twin problem of widespread antibiotic administration to farm animals, while farms simultaneously create conditions that are fertile to harboring potentially pathogenic microbes (animal proximity, genetic homogeneity, and interaction with fecal matter) which, given the former, are more likely to develop antibiotic resistance (Mathew et al, 2007). The livestock industry also drives a great deal of human-animal interaction. In addition to the daily interaction that ranch and slaughterhouse workers have with farmed animals (and the outsized risk these people face for contracting avian and swine influenzas), increasing demand for meat production has led to the further encroachment onto lands previously-uninhabited by humans and the increased associated human interaction with potential pathogens in said environments (Greger, 2007; Slingenbergh, Gilbert, de Balogh, & Wint, 2004). Cellular agriculture, by contrast, offers flexibility in potential nutritional composition (Stout et al, 2020), and will reduce the need to interact regularly with farmed and wild animals alike.

Conventional meat production also necessitates the slaughter of animals. For many, this killing of sentient beings is immoral, and should be avoided as much as possible. While some animals raised for slaughter live relatively longer and more conformable lives, the vast majority of meat production on a global scale takes place on Concentrated Animal Feeding Operations, or

CAFO's (Dickson-Hoyl & Reenberg, 2009). Animals living in these conditions often do not have adequate space for movement, defecation, or the capacity for socialization with members of their own family. The average age for slaughter of a cow raised for meat is somewhere in the range of 4 to 5 years, as opposed to the 20-year lifespans they usually have (De Vries & Marcondes, 2020).

Additionally, it is worth noting that conventional meat production is notoriously inefficient from an energetic point of view. For instance, cattle have a bioconversion rate of only 15% (Egbert & Borders, 2006). From the point of view of maximizing caloric output for agricultural input, this is highly inefficient and ought to be optimized through alternative means. Finally, while it has become more common in recent years for food producers and distributors in western countries to cater towards vegan and vegetarian diets, as the global population increases - and standards of living in developing countries increase along with it – demand for meat is likely to substantially increase (Post, 2006). Indeed, this trend has been played out over the last two decades, with global meat consumption increasing by 58% between 1998 and 2018, a result of population increase, changing consumer preferences, and income growth (Whitnall and Pitts, 2019).

It is thus imperative that alternatives to this system are explored. One such possibility for this can be found at the nexus of tissue engineering and food science: cellular agriculture. Cellular agriculture is an alternative agricultural process that uses cell culture to produce food and fiber that would otherwise be produced through conventional agricultural means (Rubio et al, 2019). Within this burgeoning field is the development of technologies necessary for creating cell-cultured, or "cultured" meat.

How to get there: Tissue Engineering

The field of cultured meat seeks to recreate the processes by which meat is usually made. Instead of producing edible muscle and fat tissue in animal bodies, cultured meat aims to produce the same tissues and products *ex vivo*. To produce these biomimetic products, it is important to understand the composition of muscle and fat tissue, and the processes by which they usually develop.

Mammals, such as cows and pigs, contain three distinct kinds of muscle tissue: skeletal muscle, smooth muscle, and cardiac muscle**.** Skeletal muscle tissue is both what mammals use to move as well as what we usually consume when eating mammalian meat. It is comprised predominantly of three main components: muscle cells, intramuscular fat, and extracellular matrix proteins, the organization of which determines the tissue's characteristic features. (Dickson-Hoyle & Reenberg, 2009)**.**

Muscle tissue is made up of bundles of muscle fibers. Muscle fibers are long, multinucleated muscle cells there are in turn comprised of bundles of myofibrils. Myofibrils contain contractile units known as sarcomeres, which use the proteins myosin, actin, troponin, and tropomyosin as a means to create the power stroke of contraction (Dickson-Hoyle & Reenberg, 2009)**.** The other main component in muscle tissue is the extracellular matrix (ECM) that attaches to, stabilizes, encloses, and separates muscle fibers (Marieb 2007). The ECM is a complex meshwork consisting of collagens, glycoproteins, proteoglycans, and elastin (Takala and Virtanen, 2000; Halper and Kjaer, 2014), providing three-dimensional structure and support

to the tissue, as well as stimulating integrin-dependent cell signaling pathways that enable integral cellular functions (Takala and Virtanen, 2000; Halper and Kjaer, 2014).

To mimic the structure of muscle tissue for cultured meat, the generative capacity of stem cells can be leveraged to recreate these hierarchical structures. Stem cells are extremely versatile, making them natural building blocks from which to build *in-vitro* tissue. This is because stem cells perform two crucial biological functions: they can divide with ease and high frequency, and they are able to differentiate into many distinct cell types. While there are several different varieties of stem cells, the two most general groups are embryonic stem cells and somatic stem cells.

Embryonic stem cells are present during development in utero. After the creation of a zygote and the development of a blastula, embryonic stem cells aggregate into clusters known as germ layers. There are three germ layers - the endoderm, mesoderm, and ectoderm - each composed of embryonic stem cells that have differentiated substantially enough to exit the stage of pluripotency (the ability to differentiate into any cell type in the body), and into multipotency (the ability to differentiate into a narrower subset of cell types). Each germ layer accounts for the development of different organ systems and tissue types that will eventually make up the body of a mammal. It is the mesoderm from which skeletal muscle tissue is created.

Satellite cells, by contrast, are the somatic stem cells which are responsible for regenerating skeletal muscle tissue. Satellite cells are triggered into asymmetrical division following tissue damage, upon which they will divide into both differentiated muscle cells as well as greater numbers of satellite cells. Asymmetrical division enables the body to immediately address the injury using the former cell type, while taking advantage of the latter's high replicative ability to retain future regenerative capacity.

The process of differentiation is triggered by a combination of different biochemical and biophysical signals in and around the cellular environment. These signals activate the expression of genes necessary for differentiation into and functionality as their new cell type. During embryonic development, for example, molecular families that induce the creation of different germ layers that determine cell fate include fibroblast growth factors (FGFs), the Wnt family, the superfamily of transforming growth factors—β(TGFβ), and bone morphogenic proteins (BMP) (Zakrzewsk et al, 2019). The Mesoderm-derived structures that appear as a result then generate the body's first muscle fibers. Subsequent waves additional fibers are generated along these template fibers (Bentzinger et al, 2012).

Prior to injury, satellite cells reside in the basal lamina of muscle fibers and are characterized in their quiescent state by the expression of the Pax7 gene, an important factor for self-renewal in this cell type and the primary marker of satellite cell identity (Olguin et al, 2004). The microenvironment surrounding satellite cells which has the right mix factors (e.g., extracellular proteins, signaling molecules, etc.) to maintain their quiescent state is called the "satellite cell niche" (Bentzinger et al, 2012).

Once there is an injury to muscle tissue, however, damaged fibers release growth factors such as TNF-alpha, HGF, and FGF, which activate satellite cell signaling pathways necessary for entry into the cell cycle (Almeida et al, 2016). Following the proliferation phase that this triggers, a subgroup of these satellite cells will start down their path towards their eventual fate as muscle cells. Using extracellular matrix protein scaffolding as a template for regeneration of muscle tissue, environmental signals induce a transition away from Notch signaling, which expands the progenitor pool of adult skeletal muscle upon injury, and toward canonical Wnt3a signaling necessary for efficient myoblast differentiation and muscle regeneration (Brack et al.

2008). This leads to the differentiation of myoblasts and their fusion into myotubes, which in turn fuse with each other or to a previous fiber to repair the damaged muscle tissue (Almeida et al, 2016). As satellite cell make their journey down the path toward their fate as mature muscle cells, different genes are expressed at varied levels. For example, fusion competent cells express Myod, while early differentiated cells express Myogenin. Satellite cells which have entered the last stages of differentiation into skeletal muscle will express Myosin Heavy Chain, a structure integral to contraction (Brack et al, 2008).

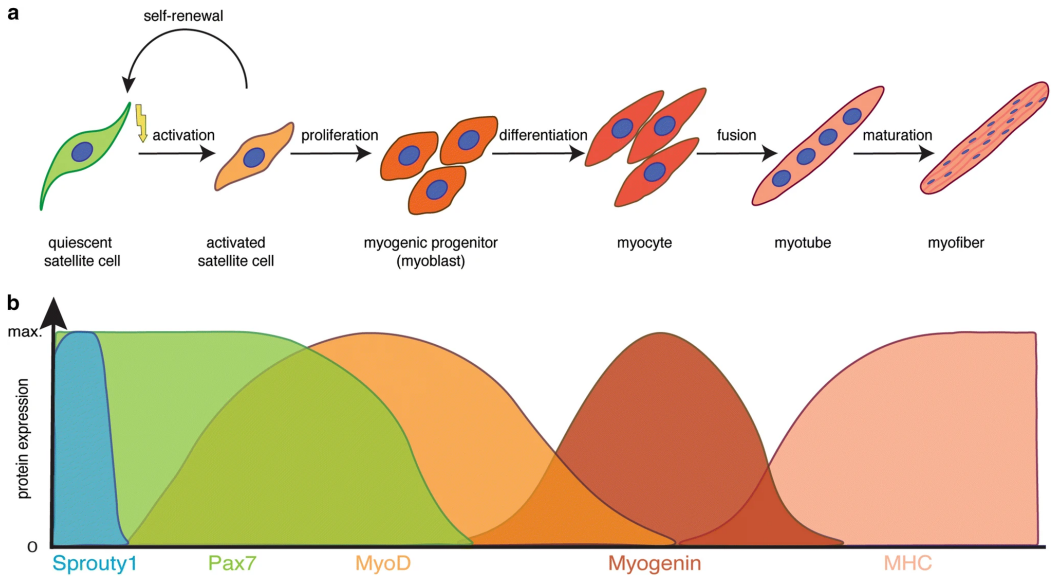


Figure 1: Progression of myogenic stem cells into mature muscle fibers, along with expression of key myogenic genes. **(a)** Satellite cells are activated and start to proliferate, thereby generating myogenic progenitor cells. Upon differentiation, myogenic progenitor cells differentiate into myocytes, which fuse to form myotubes and mature to become myofibers, the contractile unit of skeletal muscle. **(b)** Expression of key genes regulating myogenic cell fate. Figure taken from Schmidt et al, 2019.

In the attempt to harness the biological machinery needed for tissue development, tissue engineers recreate these processes by using the same major components: stem cells, extracellular scaffolding, and signaling molecules to direct cell fates (Ikada, 2006). Specific choices for these components will depend on the organ of interest; regrowing brain matter for a mouse will call for different materials, cell types, and media additives than those used for growing a set of capillaries in a caterpillar. Choice of cell types can vary based on organism, degree of genetic manipulation, and role in development. For this last consideration, tissue engineers often choose between somatic stem cells (such as satellite cells) and embryonic cells with greater pluripotency. Since the early 2000's, there have also been the use of induced pluripotent stem cells, or iPSC's, which utilize somatic cells (such as fibroblasts) that have been reprogrammed back into states of greater pluripotency (Yamanaka & Takahashi, 2006). For scaffolding, ECM proteins like collagen are often are used, and cast into molds for preferred geometry or formed into microcarriers (Post, 2012). Media types vary across cell type and tissue specifics, but are broadly comprised of the necessary sugars, fats, nutrients, vitamins, amino acids, and growth factors needed to induce proliferative and differentiated phases in the cell populations.

For tissue engineers trying to culture bovine skeletal muscle tissue, the cell type of choice for many is the bovine satellite cell (BSC), owing to its ability to differentiate easily (Post, 2012). The choice in scaffolding materials range, with cellulose, alginate, and mycelium having

been explored (Seah et al, 2022). To promote satellite cell proliferation, DMEM media supplemented with fetal bovine serum (FBS) is often used, though serum-free alternatives are also in development (Stout et al, 2021). In serum-containing systems, reduction of serum protein concentration in media is a tactic used to trigger differentiation. Another strategy often used in conjunction with this is culturing satellite cells to the point of confluency, so that cell-to-cell interaction will induce differentiation (Stout et al, 2020). The stress and strain generated from cell attachment to anchored scaffolding structures has also been shown to induce differentiation, thus designating mechanical stimulation as an important factor in muscle tissue development (Post, 2012).

As we look to the future, large-scale bioreactors will be needed for large-scale production of cultured meat (Datar et al, 2009). Recent advancements have been made in the use of bioprinting technologies (Kang et al, 2021) and perfusion-based bioreactors (Specht et al, 2018) as ways to vascularize and construct more complex three-dimensional cultured tissues, though this is an area where more research is needed.

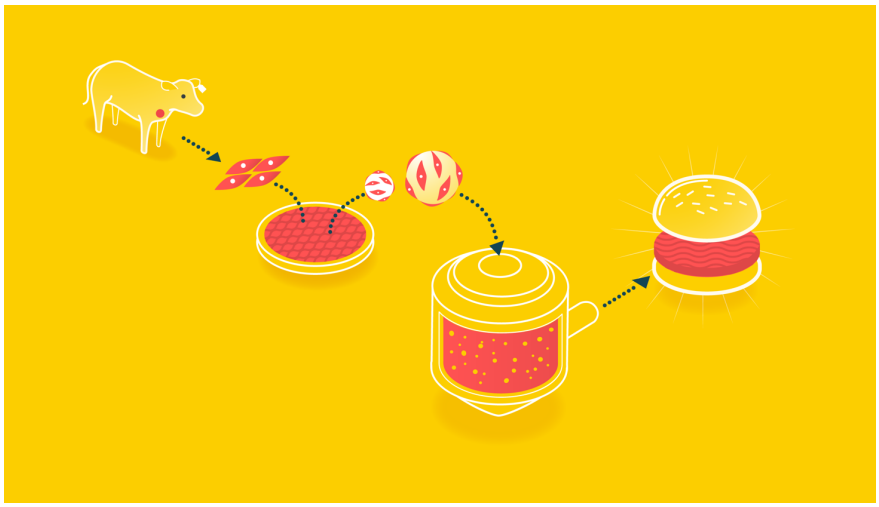


Figure 2: Generalized workflow shown for production of cultured meat, showing biopsy from animal, proliferation of cells in culture, attachment to scaffold, differentiation into muscle tissue, and harvesting for consumption. Illustration by Nick Counter for New Harvest // CC BY-NC-SA 4.0.

Project Overview

A substantial barrier to scaling up cultured meat production is the difficulty presented by the use of animal-free scaffolds. While extracellular matrix (ECM) proteins such as collagen and fibrin contain optimal proteins for cell adhesion, such materials are either animal-derived or costly to produce recombinantly (Wang et al, 2017). For this reason, scaffold materials sourced from cellulose-based agricultural waste present a great opportunity to both upcycle otherwise-unusable waste within the food system while offering a low-cost, animal-free material. However, cellulose-based scaffolds are bio-inert, and therefore offer a sub-optimal substrate for cell attachment, proliferation, and differentiation (Mayer, 2003).

While a great deal of effort has gone into the development of chemically modified cellulose-based scaffolds for improved cell adhesion (Courtenay, 2017), such methods have their limitations due to the frequent addition of animal-derived or costly recombinant proteins.

Instead, one promising option is the use of a fusion protein comprised of the integrin-binding motif Arg-Gly-Asp (RGD) and a cellulose binding domain (CBD) to coat cellulose surfaces in order to increase the adhesion of bovine satellite cells (BSC) to cellulose. This fusion protein would act as an intermediate between the cells and the scaffold, with the RGD binding to integrins on the cell membrane and the CBD binding to the cellulose scaffold. If this approach proves effective, such information could lay crucial groundwork for the future possibility of engineering bovine satellite cells to produce this fusion protein themselves, cutting costs and possibly serving as an economically feasible path to scaling up cultured meat production.

Indeed, an essential component of tissue engineering is the presence of a material for cells to attach to. These materials, often referred to as scaffolds or extracellular matrices (ECM), provide structural support to encourage multicellular organization, as well as stimulating adhesion-mediated mechanical signals that cells require to undergo necessary biological processes (Khalili & Ahmad, 2015). In the context of biomedical engineering, scaffolds are usually comprised of synthetic or natural polymers (Obrien, 2011). Scaffolds for cultured meat production, however, have the added design constraints of edibility, texture, and animal-free sourcing. While a great deal of mammalian tissue utilizes collagen as an effective ECM (Du Lullo et al, 2002) its sourcing is both ethically and financially problematic, given the necessity of either animal slaughter or expensive recombinant production (Wang et al, 2017).

Thus, it might be promising to look across biological kingdoms and consider the building blocks of a vast array of plant life: cellulose. Nanocellulose has been shown to be a promising biomaterial for medical applications of tissue engineering including blood vessels, bone, liver, cartilage and adipose tissue (Bacakova et al, 2019). Other studies have explored the concept of cellulose in the form of decellularized plants (e.g., spinach, apples) for in-vitro muscle cell culture (Campuzano & Pelling, 2019. Gershlak et al, 2017. Modulevsky et al, 2014). Despite the many advantages of cellulose as a scaffold and biomaterial, one disadvantage to using cellulose is its unfavorable environment for cell attachment and adhesion - a crucial point given that robust cell attachment is important for inducing strong cellular differentiation into mature muscle and adipose tissues present in meat (Mayer, 2003). For this reason, one promising strategy could be the introduction of a fusion CBD-RGD protein to act as an intermediate structure, mediating the attachment between a cellulose-based scaffold and integrins on bovine satellite cells (BSC).

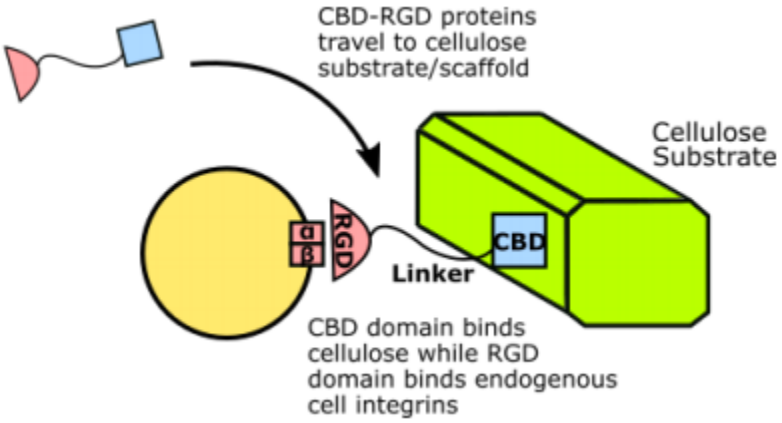


Figure 3: Fusion proteins containing cellulose binding domains and RGD binding motifs (CBD-RGD), added exogenously in media, to enable BSC attachment onto cellulose substrates. CBD-RGD proteins bind to cellulose on one end with its CBD domain and to cells on the other end with its RGD domain.

Cellulolytic enzymes found in numerous organisms are comprised of a catalytic domain and a cellulose binding domain (CBD). While many CBD's exist, that of the fungus *Trichoderma koningii* has been previously incorporated into CBD-RGD fusion proteins (Chen et al, 2002), and was therefore used in this project. RGD peptide motifs are a key target for cellular integrins, and are involved in cell adhesion to many different extracellular matrix proteins (Ruoslahti, 1996). These proteins have extensive precedent in tissue engineering for a range of applications (Want et al, 2013. Jeschke et al, 2002), and therefore make a promising counterpart for CBD-RGD fusion protein.

Such CBD-RGD fusion proteins have previously been used for a number of tissue systems, including in the attempted promotion of chrondogenis in adipose stem cells (Chang et al, 2009), and investigations into improved clinical osteogenesis (Visser et al, 2013). Hsu et al investigated its effects on promoting cellular adhesion to biomedical polyurethane in a variety of cell types, reporting that the effect on cellular adhesion correlated with the amount of CBD-RGD physically adsorbed on the material surface (Hsu et al, 2004). Their findings demonstrated that a small amount of protein (20 µl of 0.1 mg/ml protein coating on a 15 mm slide) displayed a maximal effect in promoting cellular adhesion. In this work, I investigated the impact that this fusion protein could have on bovine satellite cells (BSCs) and studied its impact on cell attachment, growth, and differentiation on cellulose-based scaffolds.

Materials and Methods

CNF Films - Construction

Two-dimensional biofilms were constructed from soluble cellulose nanofiber (CNF). 3% CNF solution (Cellulose Lab, 6 Beechwood Cres, E3B 2S8 Fredericton, New Brunswick, Canada) was diluted with deionized water (DIW) to a final concentration of 1% CNF solution. 1% CNF solution was then cast into 100mm culture dishes and spread for complete covering. CNF-containing culture dishes were left to dry in a chemical fume hood. Depending on the environmental conditions, drying times varied from 24 hours to 48 hours. Dried films (Figure 4) were then cut into 10mm-diameter circle-shaped samples using biopsy punches. CNF samples were then sterilized in the autoclave at 121° C for with a 15-minute dry heating cycle and 15-minute drying cycle.

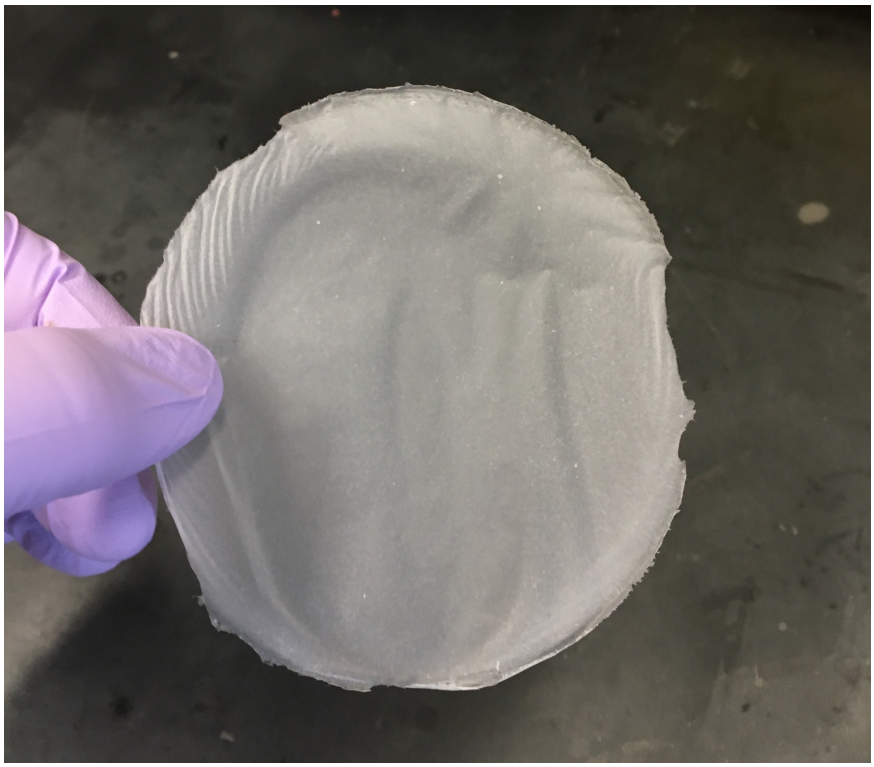


Figure 4: Cellulose Nanofiber (CNF) film after casting, drying, and removal from culture dish.

CBD-RGD Fusion Protein - Peptide Reconstitution

The amino acid sequence used for the CBD-RGD fusion protein is as follows: PTQHWGQCGGIGYSGPTVCASGTTCQVLNPYYSQCLPTTPTGRGDSAS. Synthesis, purification, and quality control analysis of the fusion protein was performed by Alan Scientific, with HPLC-grade purity of 96.48%. Protein was provided in powdered form, and was

reconstituted in acetonitrile and deionized water (1 mg peptide dissolved into 0.33 mL ACN, with 0.67 mL H_20 subsequently added). Protein stock solution was then made (0.4 mg/ml) (in 200 mM Tris–HCl (pH = 7.4), 100 mM NaCl, 20 mM $CaCl_2$), sterilized with a syringe filter and stored in 1 mL aliquots in a freezer at –20° C. Protein stock solution was warmed to room temperature before use.

CBD-RGD Fusion Protein - Film Coating

To prepare protein coating, thawed protein stock solution was diluted in Tris-HCl buffer to 0.04 mg/mL. For all experiments, cellulose nanofiber samples were placed into wells of a low-attachment 24-well plate. For each CNF sample, protein coating was achieved by dispensing 500 μL of 0.04 mg/mL solution into each sample-containing well, and left to adsorb for 1 hour. After one hour, remaining solution was aspirated from sample-containing wells, and samples were washed with 500 μL PBS to remove any non-adherent fusion protein.

General Culture

Cell Sourcing

Bovine satellite cells (BSC) were isolated from a biopsy sample of a Simmental calf from Tufts Veterinary School [Grafton, MA]. Isolated cells were kept in vials containing one million cells each, frozen in fetal-bovine serum supplemented with 10% DMSO and stored in liquid nitrogen. DMSO-containing frozen cell suspensions were thawed and dispensed into fetal bovine serum (FBS)-containing growth media (DMEM, 20% FBS, 1 ng/mL FGF-2, and 1% antibiotic) to neutralize the cytotoxic effects of DMSO. Cell suspension was then placed in a centrifuge and spun down for 5 minutes at 300 RCF at room temperature, with DMSO and media-containing supernatant subsequently aspirated off. Cell pellet was then resuspended in FBS-containing growth medium and 500,000 cells were seeded into each laminin-coated T-175 cell culture flask used (0.25 ug/cm2 laminin of iMatrix 511-silk) and cultured in incubators at 37° C.

Routine cell passaging was performed as follows: Flasks were aspirated, washed with PBS, and aspirated again to remove media. 3 mL of trypsin was then added to each flask, followed by incubation for 5 minutes at 37° Celsius. Trypsinized cells were then removed from flasks and added into conical tubes containing 7 mL of FBS-containing media to neutralize the cytotoxic effects of trypsin. Cell suspension was then centrifuged at 300 RCF for 5 minutes to separate the cell pellet from the media supernatant, after which the remaining trypsin-containing media was aspirated. The cell pellet was then resuspended in 10 mL of FBS-containing media. Cells were counted using NucleoCounter® NC-200™ (Chemometec). After cells were counted, 500,000 cells were seeded into each new laminin-coated T-175 cell culture flask and cultured in incubators at 37° C.

Cell Seeding onto CNF Films and Subsequent Culture

To seed CNF films, BSCs were passaged as above and seeded at a density of 20,000 cells/cm^2 for proliferation experiments or 100,000 cells/cm^2 for differentiation experiments. This was done in a 24-well low-attachment plate, with 3 replicates of each treatment: Without fusion protein in FBS media and with fusion protein in FBS media, and without fusion protein in

serum-free media and with fusion protein in serum-free media. B8 media was used as the serum-free option during seeding, and was changed to Beefy-9 media during the next subsequent feeding. B8 media contents was as follows: 1 L DMEM, 200 mg L-ascorbic acid 2-phosphate, 20 mg Insulin, 20 mg Transferrin, 20 μg Sodium selenite, 40 μg FGF2, 100 ng NRG1, and 100 ng TGFb3. Beefy-9 media used contained the same contents, with 800 μg recombinant Albumin added. FBS media as described previously was used for the serum-containing option. Media was replaced every two days for the duration of culture.

Cell Attachment Experiments

Live-Dead Stain

To qualitatively determine whether coating CNF scaffolds with CBD-RGD fusion protein had an effect on bovine satellite cell attachment, a live-dead stain and subsequent microscopy imaging was performed. The LIVE/DEAD® Viability/Cytotoxicity Kit (ThermoFischer Scientific) discriminates live from dead cells by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. The Live-Dead stain was as follows: The light in the biosafety cabinet was turned off due to the assay's photosensitivity, after which media was aspirated from each well. Each sample was then washed with PBS to remove any residual unbound cells, and aspirated out. Live-Dead stain and the appropriate media type was then added to each sample at a 1:1 ratio. The well plate was then covered with tinfoil to avoid light exposure, and left to sit at room temperature for 30 minutes. After 30 minutes, tinfoil was removed and samples were extracted to image on the Keyence microscope. Measurements were taken on days 1 and 4 after seeding, with assays done on separate plates designated for each respective day.

Presto Blue Assay

To quantitatively determine whether coating CNF scaffolds with CBD-RGD fusion protein had an effect on bovine satellite cell attachment, a Presto Blue assay was performed. When added to cells, the PrestoBlue® reagent (ThermoFischer Scientific) is modified by the reducing environment of the viable cell and turns red in color, becoming highly fluorescent. This color change is then detected using fluorescence or absorbance measurements. Assay was done on low-attachment plates with a n = 3 for each treatment group: without protein in FBS media and with protein in FBS media, and without protein in serum-free media and with protein in serum-free media. Due to the assay's photosensitivity, lights in the room and biosafety cabinet were turned off. Media was aspirated from each sample-containing well, and each sample was then washed with PBS to remove any residual unbound cells, and aspirated out. A 9:1 mixture of the respective media type to Presto Blue solution was then added to each well, in addition to wells without samples to control for differences among fluorescence levels between media types (n = 3 for controls of each media type). The plate was then covered with tinfoil to protect against light exposure, and left to incubate for 1 hour at 37º C. After 1 hour, solution from each sample was transitioned into wells in a 96-well plate, with two technical replicates done for each biological replicate. The 96-well plate was then placed in a microplate reader to detect

fluorescence levels for each sample, with excitation at 560 nm and emission at 590 nm. Measurement were taken, using the same 24-well plates, on days 1 and 4 after seeding.

Cell Proliferation Experiments

To assess whether the CBD-RGD protein had any effect on the proliferative capacities of BSC's cultured on CNF films, a growth curve was conducted. To achieve this, the Presto Blue assay was performed on days 1, 4, 7, and 10 after seeding, using the same protocols as described for the cell attachment experiments.

Cell Differentiation Experiments

To assess whether the CBD-RGD protein had any effect on the ability of BSC's to differentiate into muscle fibers, cells were cultured in their varying treatment groups and immunofluorescent staining was performed on said cultures. Because of limited success of cultures growth within serum-free media conditions, differentiation experiments were limited to serum-containing cultures, using protein-coated CNF films and non-coated CNF films for culture. Samples were each seeded with 100,000 cells/cm^2, and media was changed every two days until day 7 after seeding, at which point one final media change was performed. Culture conditions were otherwise identical to those described above. Cultures on different plates were incubated for 7 and 14 days, respectively, following this final media change to simulate starvation conditions known to induce differentiation. Upon the completion of each of these time periods, immunofluorescent staining was performed to determine morphological markers characteristic of muscle formation. Staining was performed as follows: cells were fixed with 4% paraformaldehyde (ThermoFisher #AAJ61899AK) for 30 minutes, washed in PBS, permeabilized for 15 minutes using 0.5% Triton X (Sigma #T8787) in PBS, blocked for 45 min using 5% goat serum (ThermoFisher #16210064) in PBS with 0.05% sodium azide (Sigma #S2002), and washed with PBS containing 0.1% Tween-20 (Sigma #P1379). Primary MHC antibodies (Developmental studies hybridoma bank #MF-20, Iowa City, IA, USA) were diluted to 4 μg/mL in blocking solution containing 1:100 Phalloidin 594 (ThermoFisher #A12381) and added to differentiated cells. Primary antibodies were incubated overnight at 4º C. The following day, cells were washed with PBS + Tween-20, incubated with secondary antibodies for MHC (ThermoFisher #A-11001, 1:1000) for 1 hour at room temperature, washed with PBS + tween-20, and mounted with Fluoroshield mounting medium with DAPI (Abcam #ab104139, Cambridge, UK) before imaging.

Results

Cell Attachment Experiments

FBS System

Cell attachment experiments were first performed on cultures containing FBS media. Microscopy images reveal increased cell adhesion on day 1 after seeding. This trend continued through day 4 (Figure 5 – A). Results indicate that the CBD-RGD protein improves initial cell adhesion, and that the favorable cell-material interaction which is facilitated by the fusion protein has long-term efficacy throughout culture. A Presto Blue assay was also performed to indirectly measure cell numbers through fluorescence measurements of metabolized resazurin (Figure 5 – B). To detect if there were a significant difference in cell attachment between the two sample groups cultured using FBS-containing media, a standard t-test was performed on this data. Assay was performed on three biological replicates (n = 3) for each treatment group, each biological group having two technical replicates. Results show a significant increase in cell adhesion on day 1 ($p < 0.001$) for cells seeded onto fusion-protein coated films, which supports the conclusions from the Live-Dead stains. After 4 days of culture, cell attachment to the protein-coated films was likewise increased compared with uncoated films ($p < 0.0001$). Taken together, these results demonstrate clear evidence supporting the hypothesis that in a system using FBS-containing media, coating cellulose nanofiber films with a CBD-RGD fusion protein enhances bovine satellite cell attachment to the substrate.

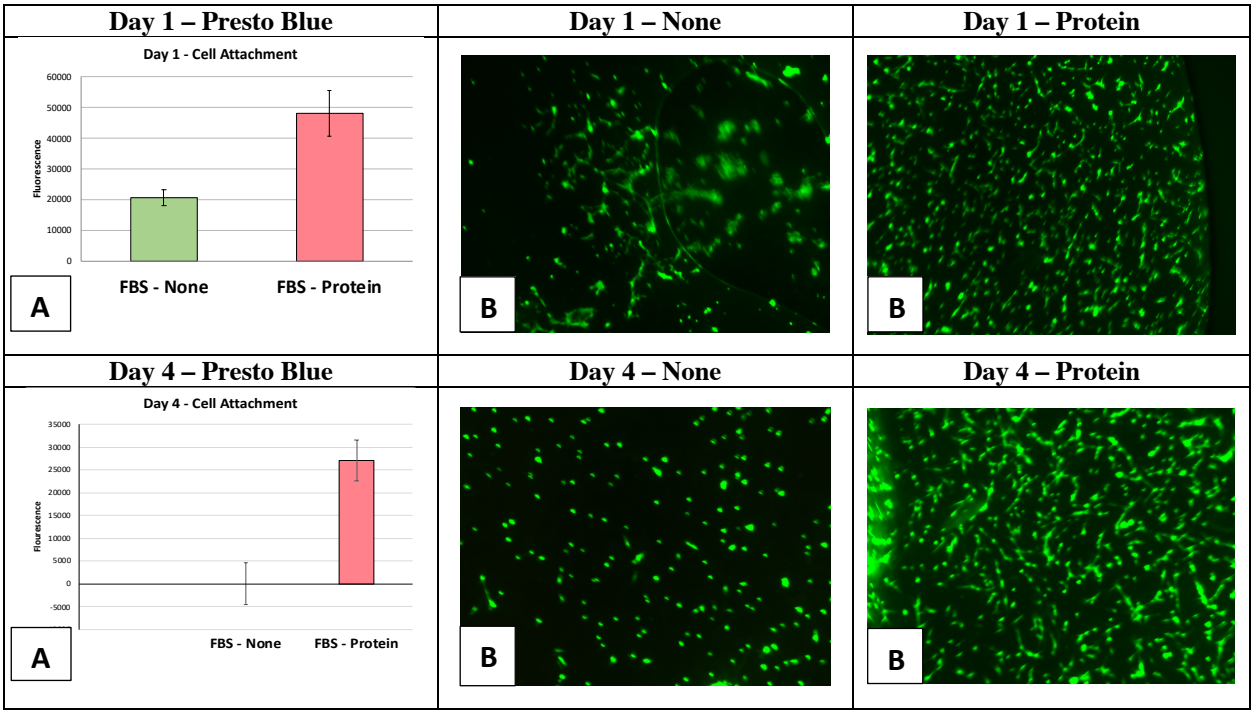


Figure 6: Cell attachment experiments performed on FBS-containing cultures. Data from presto blue assays performed on days 1 and 4 after seeding (A). Microscopy images taken following Live-Dead stain for viable cells are shown on days 1 and 4 after seeding (B). Fluorescent dots represent live cells. "None" indicates treatment without fusion protein, while "protein" indicates treatment with fusion protein.

Serum-Free System

Cell attachment experiments were also performed on cultures containing serum-free media. Microscopy images reveal no clear differences between treatments one day after seeding, nor at 4 days after seeding (Figure 6 – A). To detect if there were a significant difference in cell attachment between the two sample groups cultured using serum-free media, a standard t-test was performed on data from the fluorescence levels of metabolized resazurin obtained using the Presto Blue assay (Figure 6 - B). This was performed on three biological replicates (n = 3) for each treatment group, each biological group having two technical replicates when performing the assay. Results show no significant difference between treatments groups one day after seeding ($p = 0.627894$). While no significant difference was seen between uncoated and coated samples on day 4 ($p = 0.055$), there was a strong trend towards improved outcomes with the coating. Taken together, these results show that for a system using serum-free media (B8 and Beefy-9), coating cellulose nanofiber films with a CBD-RGD fusion protein has little to no effect on bovine satellite cell attachment to the substrate immediately after seeding, though it is possible that cell attachment might be improved over time.

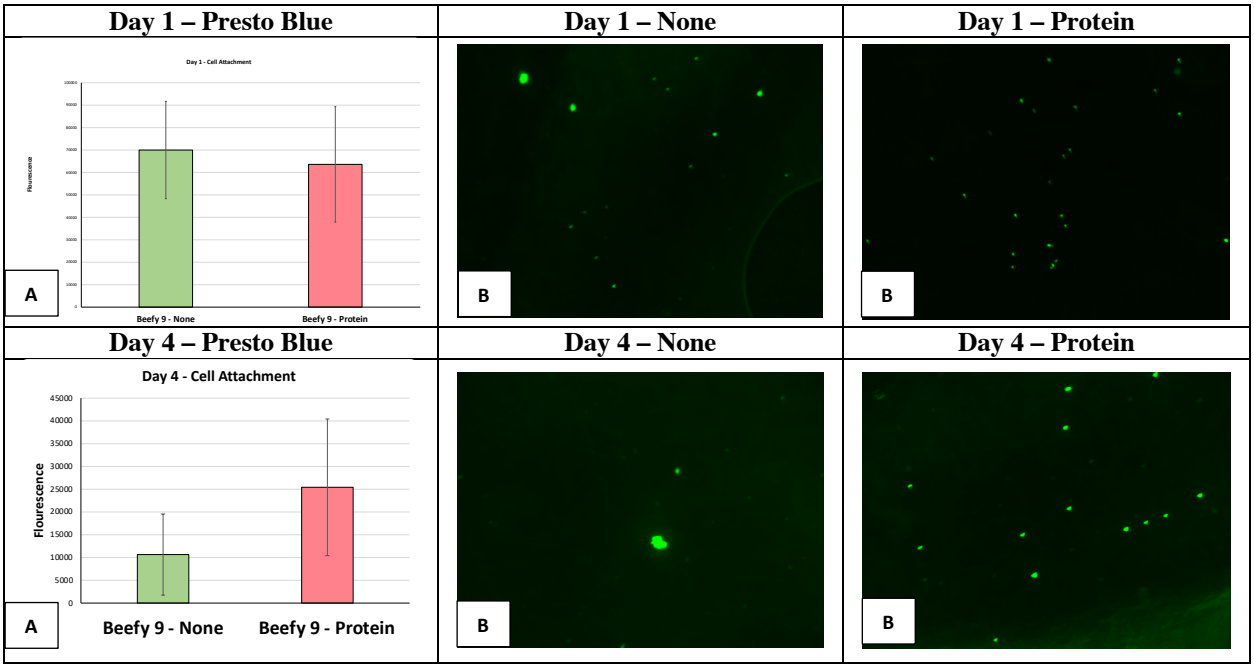


Figure 6: Cell attachment experiments performed on serum-free cultures. Data from presto blue assays performed on days 1 and 4 after seeding (A). Microscopy images taken following Live-Dead stain for viable cells are shown on days 1 and 4 after seeding (B). Fluorescent dots represent live cells. "None" indicates treatment without fusion protein, while "protein" indicates treatment with fusion protein.

Cell Proliferation Experiments

FBS System

To assess the CBD-RGD fusion protein's effect on cell proliferation within an FBS media-based system, the Presto Blue assay was performed on days 1, 4, 7, and 10 after seeding for cells growing on a protein-coated CNF film and cells growing on a non-treated CNF film, respectively. This was performed on three biological replicates (n = 3) for each treatment group, each biological group having two technical replicates when performing the assay. Cells grown on protein-coated CNF films consistently outperformed those grown on CNF without the fusion protein (Figure 7), with a statistically significant difference between the treatment groups on day 1 ($p = 0.003109$), day 4 ($p = 0.000956$), day 7 ($p < .0001$), and day 10 ($p = 0.001254$).

While there was significant difference in relative cell number between the two treatments on each day after seeding, this does not tell the whole story. Indeed, fusion protein-treated cells exhibited higher initial attachment and greater proliferation over the first 4 days than their non-protein-treated counterparts, but their cell numbers began to decline thereafter, indicating cell death. This might have been owed into cell detachment from the surface, as samples were washed to remove non-adherent cells at the outset of every Presto Blue assay. This could point to the possibility of CBD-RGD protein inactivity over time, resulting in protein detachment from the substrate and subsequent cell detachment as a result. That being said, non-protein-treated cells also exhibited statistically significant cell death by day 10, though at a different rate from the protein-treated group.

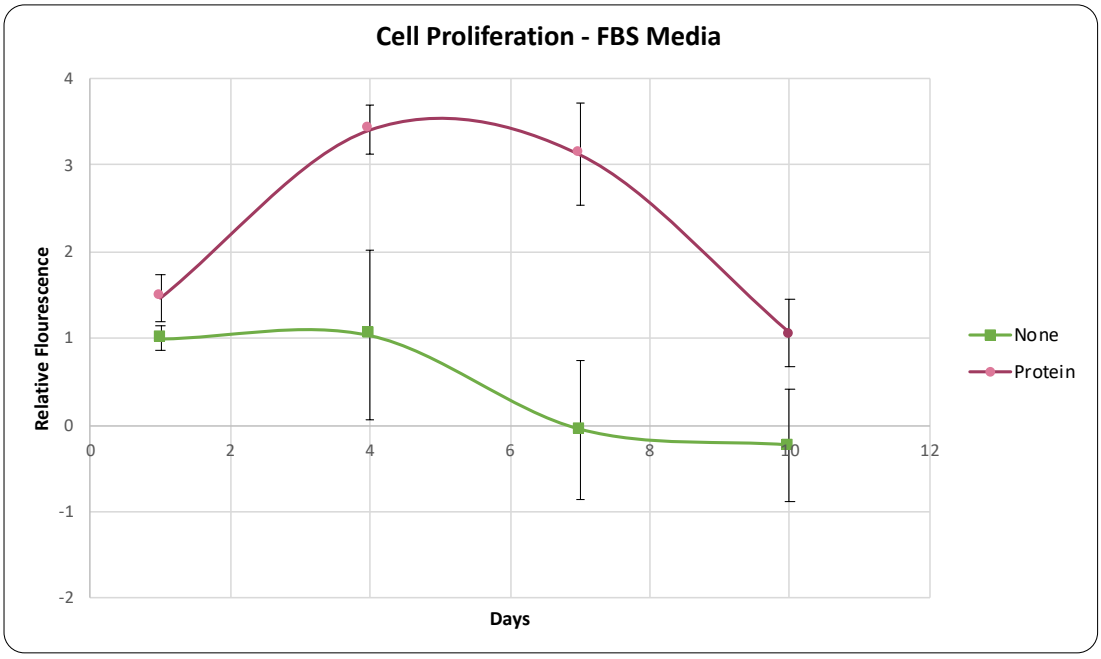


Figure 7: All data points were normalized to Day 1 fluorescence levels for cultures without protein. "None" signifies cultures without fusion protein, and "Protein" indicates cultures with fusion protein.

Serum-Free System

To assess the CBD-RGD fusion protein's effect on cell proliferation within a serum-free media-based system, the Presto Blue assay was performed on days 1, 4, 7, and 10 after seeding for cells growing on a protein-coated CNF film and cells growing on a non-treated CNF film, respectively (Figure 8). This was performed on three biological replicates (n = 3) for each treatment group, each biological group having two technical replicates when performing the assay. Cells grown on protein-coated CNF films showed no statistically significant difference from those grown on CNF without the fusion protein, a trend held for each day on which a measurement was made. The t-test comparing these two groups demonstrated two-tailed p-values and for each day as followed. Day 1: $p = 0.906059$, Day 4: $p = 0.837885$, Day 7: $p = 0.476314$, Day 10: $p = 0.206779$.

In each treatment, both groups exhibited perceived but statistically insignificant growth, as well as perceived but statistically insignificant cell death. They appeared to do so at very similar rates, pointing to the conclusion that the CBD-RGD fusion protein did not have any effect on cell growth within this system.

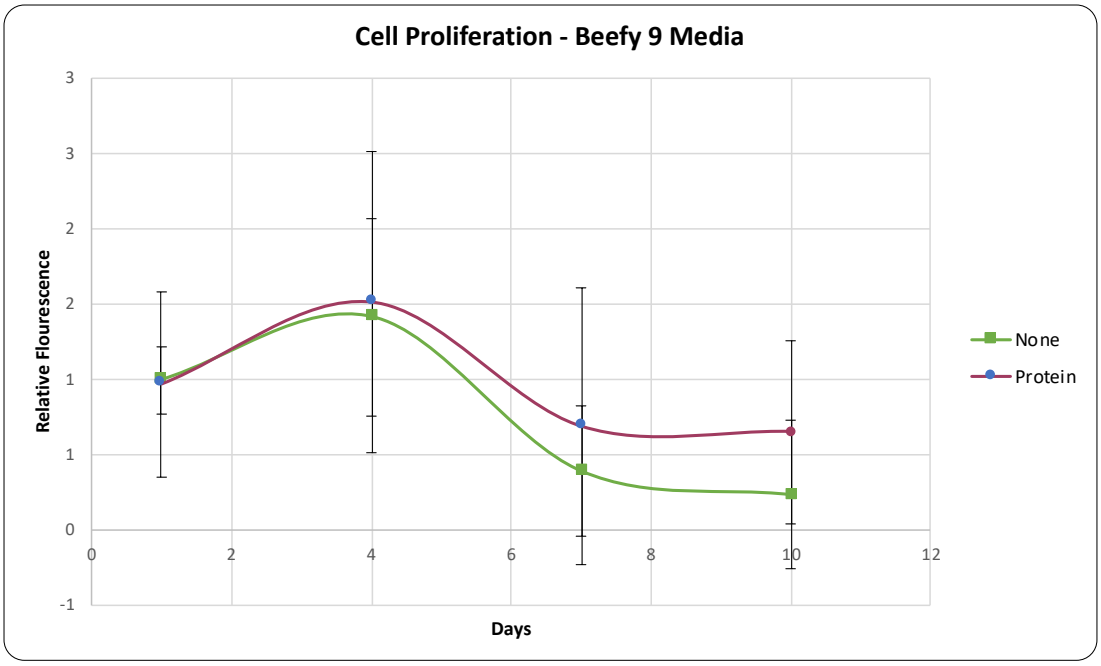


Figure 8: All data points were normalized to Day 1 fluorescence levels for cultures without protein. "None" signifies cultures without fusion protein, and "Protein" indicates cultures with fusion protein.

Muscle Formation Experiments

Given serum-free BSC cultures' low attachment levels, slow growth rate, and relative inability to achieve confluency within this system, while also taking into account the importance of confluency for inducing differentiation, experiments to elucidate the cells' ability to differentiate into muscle tissue were limited to cell culture systems containing FBS-based media. Microscopy images taken after immunofluorescent staining for myosin heavy chain (green), actin (red), and cell nuclei (blue) reveal no clear differences between cultures using the CBD-RGD fusion protein and cultures without it. Cultures in each treatment group failed to reach confluency and exhibited minimal differentiation. Microscope images for cultures without fusion protein on day 7 did reveal some multinucleated cells, though their sphere-like shapes indicate possible coiling of the fibers from detachment from the substrate on one end of the fiber. Day 7 images of cultures with the fusion protein also show limited differentiation (Figure 9), with sporadic formation of multinucleated, striated cells. Day 14 images of cultures without protein again show limited cell numbers and limited differentiation, with again the possible sporadic formation of coiled muscle fiber. Day 14 images (Figure 10) of cultures using fusion protein also show limited cell numbers and associated differentiation, with sporadic formation of possible multinucleated muscle fibers with greater degrees of actin and myosin heavy chain-striation. Such results should be viewed with a degree of skepticism, and indicate a need for further research on the matter. Like in the growth curve, it is possible that initially-applied fusion protein coats might have become unbound, necessitating the need for reapplication of fusion protein solution over the course of long-term culture.

Day 7

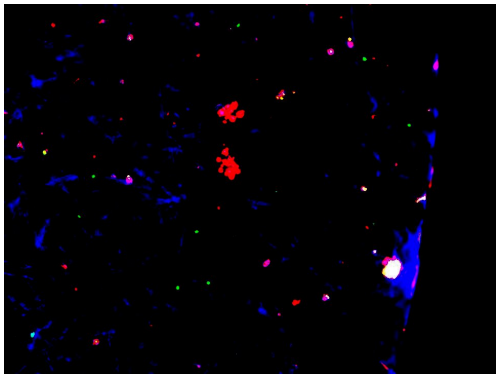
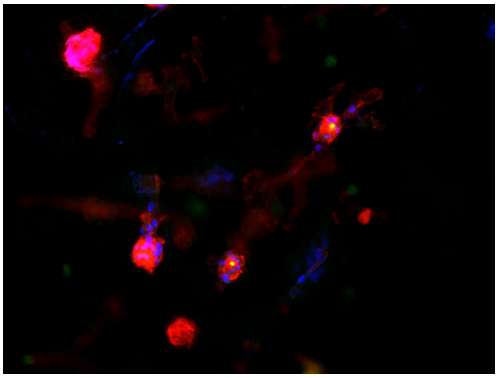
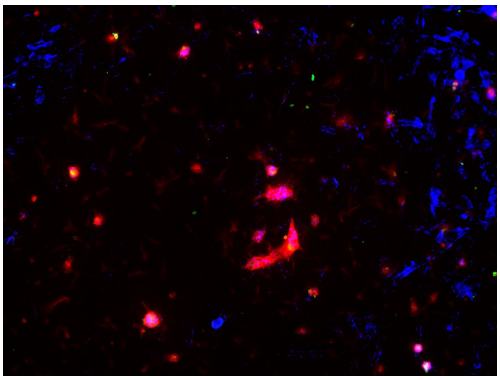
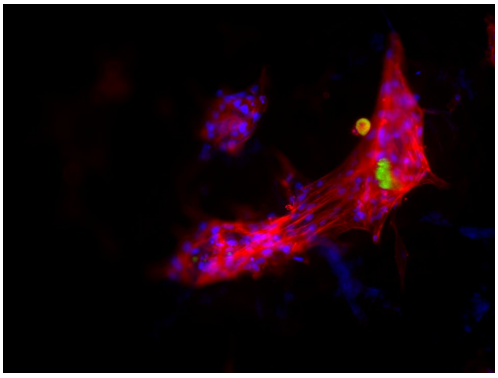
4x magnitude - None	20x magnitude - None
	
4x magnitude - Protein	**20x magnitude - Protein**
	

Figure 9: Immunofluorescent staining to elucidate cell fate on the differentiation path to muscle fiber formation within a FBS media-containing system. Microscopy images taken on day 7 after inducing differentiation. Green = Myosin Heavy chain, Red = actin, Blue = nuclei. "None" indicates treatment without fusion protein, and "Protein" indicates treatment with fusion protein.

Day 14

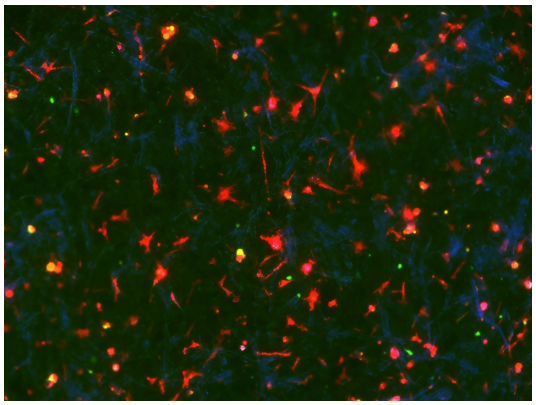
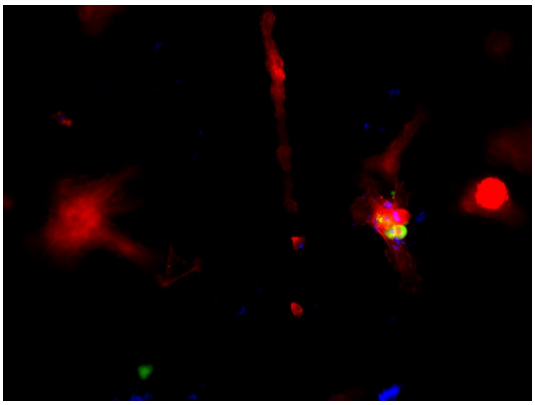
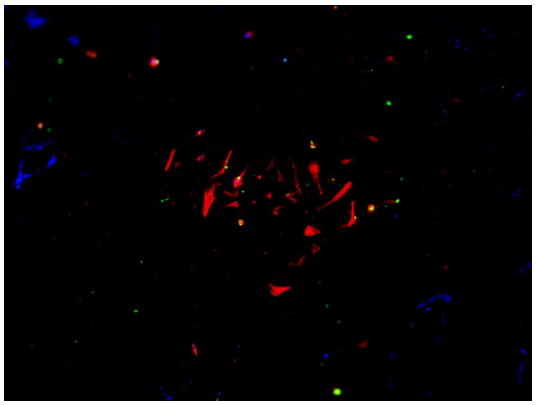
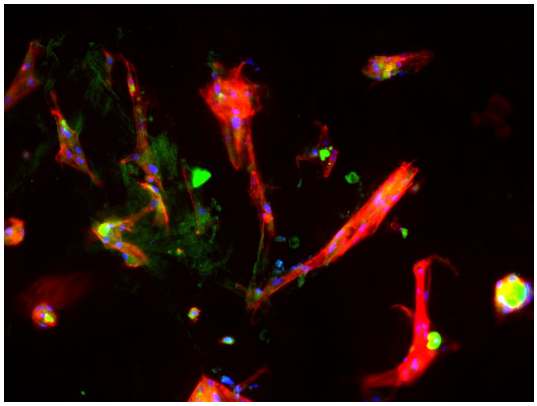
4x magnitude - None	20x magnitude - None
	
4x magnitude - Protein	**20x magnitude - Protein**
	

Figure 10: Immunofluorescent staining to elucidate cell fate on the differentiation path to muscle fiber formation within a FBS media-containing system. Microscopy images taken on day 14 after inducing differentiation. Green = Myosin Heavy chain, Red = actin, Blue = nuclei. "None" indicates treatment without fusion protein, and "Protein" indicates treatment with fusion protein.

Discussion

With the goal of removing animal-derived materials from cultured meat production systems, we sought to tackle two problems simultaneously: creating greater biocompatibility between plant-based scaffolds and mammalian cell types, and reducing the reliance on fetal bovine serum within media formulations. With regard to accomplishing the former, we attempted to harness the CBD-RGD fusion protein's dual compatibility in binding to both cellulose and mammalian cell surface integrins to enhance bovine satellite's attachment to cellulose nanofiber scaffolds. Due to cellulose's natural abundance, edibility, and capacity for being upcycled from agricultural waste, progress on this front would help enable tissue engineering of cultured meat's transition away from the use of animal-derived or expensive recombinantly-produced collagen as a scaffold.

On this front progress was made, though with still a number of questions yet unresolved. Indeed, cell attachment experiments with FBS media-containing cultures demonstrated a statistically-significant enhancement of BSC attachment to CNF scaffolds when the CBD-RGD fusion protein was applied. Growth over a relatively short period in FBS-containing systems was likewise enhanced, though cell populations declined over time in both protein-coated and non-coated cultures. This latter phenomenon points to the possibility of the fusion protein's decreased activity over time, perhaps through unbinding or degradation. Future research would be well-served to determine the protein's binding dynamics over time to both cellulose and cells as a way determine the limiting factors within the system and to analyze whether the reapplication of protein over the course of culture might be necessary. Such research may also improve proliferation and differentiation of BSC's into muscle fibers, an objective that must be accomplished if such a system is seriously considered for use in cultured meat production.

Interestingly, the enhancement of cell attachment seen in FBS-containing cultures was not replicated in their serum-free counterparts. This was surprising, and was in many ways counter-intuitive. This is because FBS contains a number of proteins that also enhance cell attachment to the extracellular matrix (Johnson, 2013). This fact led us to the hypothesis that FBS-containing media might mask any possible effect that the fusion protein might have on promoting cell attachment, and that a possible enhancing effect might be more pronounced in a serum-free system devoid of FBS proteins. The oppositive, of course, was observed, begging a number of questions. It might be possible that there is a component of FBS that enhances the CBD-RGD protein's binding capacity, such as an enzyme changing the fusion protein's conformation to accomplish this. It is also possible that there is some synergistic effect created by the presence of FBS proteins and the fusion protein, where one enhances the effect of the other. Such questions ought to guide future research into the matter, particularly as development serum-free media alternatives continues.

If this method of improving the biocompatibility of mammalian cell types cultured onto plant-based scaffolds is further validated and optimized, one can envision a future in which a number of different paths that R&D might take. One such direction could be investigating whether the CBD-RGD fusion protein might have a similar effect when other cell types relevant to cellular agriculture, such as avian, porcine, or fish cell types. Another obvious direction would be to determine whether these effects translate from two-dimensional culture (using CNF films) to three-dimensional culture, a step integral to the creation of any large-scale muscle and fat tissue. Finally, genetic engineering of cells to endogenously express and secrete the fusion

protein might present a possible way of integrating the protein into the system without incurring additional supply chain costs that would come from sourcing from recombinant production.

In conclusion, this study aimed to explore the effect of a CBD-RGD fusion protein in enhancing bovine satellite cell attachment to cellulose-based scaffolds. While results show a promising starting point, it is clear that further research is needed. If successful, this research direction could enable the use of plant-based scaffolds, and help with the realization of the potential offered by cultured meat technologies.

Acknowledgements

I'd like to thank Andrew Stout, John Yuen, Sophie Letcher, Ning Xiang, Michael Saad, and Natalie Rubio for their scientific input, mentorship, and encouragement over the course of this project. I'd also like to thank David Kaplan for offering me a place in his lab, and the wisdom and kindness he has shown to me throughout. This project was enabled by the generous financial support of New Harvest, an organization that has given me the support and inspiration to become part of the alternative protein movement and the cellular agriculture community. Finally, I'd like to thank Emily Wiley for her encouragement and support in turning this research project into a thesis that I can be proud of.

Literature Cited

Almeida, C. F., Fernandes, S. A., Ribeiro Junior, A. F., Keith Okamoto, O., & Vainzof, M. (2016). Muscle satellite cells: exploring the basic biology to rule them. *Stem cells international*, *2016*.

Bacakova, L. et al. Versatile Application of Nanocellulose: From Industry to Skin Tissue Engineering and Wound Healing. Nanomaterials 9, 164 (2019).

Bentzinger, C. F., Wang, Y. X., & Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor perspectives in biology*, *4*(2), a008342.

Brack AS, Conboy IM, Conboy MJ, Shen J, Rando TA. 2008. A temporal switch from Notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. Cell Stem Cell 2: 50–59.

Brack, A. S., & Rando, T. A. (2012). Tissue-specific stem cells: lessons from the skeletal muscle satellite cell. *Cell stem cell*, *10*(5), 504-514.

Campuzano, S. & Pelling, A. E. Scaffolds for 3D Cell Culture and Cellular Agriculture Applications Derived From Non-animal Sources. Front. Sustain. Food Syst. 3, 38 (2019).

CDC (2012). CDC estimates of foodborne illness in the United States. http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html Accessed: Jan 20th, 2012.

Chang, J. C., Hsu, S. H., & Chen, D. C. (2009). The promotion of chondrogenesis in adipose-derived adult stem cells by an RGD-chimeric protein in 3D alginate culture. *Biomaterials*, *30*(31), 6265-6275.

Chen DC, Hu NT, Chen YJ, Hseu TH. Chimeric proteins with a cellulose binding domain. [US patent 6407208B1]; 2002: 6–18.

Courtenay, J. C. et al. Surface modified cellulose scaffolds for tissue engineering. Cellulose 24, 253–267 (2017).

Datar, I., & Betti, M. (2010). Possibilities for an in vitro meat production system. *Innovative Food Science & Emerging Technologies*, *11*(1), 13-22.

De Vries, A., & Marcondes, M. I. (2020). Overview of factors affecting productive lifespan of dairy cows. *Animal*, *14*(S1), s155-s164.

Di Lullo, Gloria A.; Sweeney, Shawn M.; Körkkö, Jarmo; Ala-Kokko, Leena & San Antonio, James D. (2002). "Mapping the Ligand-binding Sites and Disease-associated Mutations on the Most Abundant Protein in the Human, Type I Collagen". *J. Biol. Chem.* **277** (6): 4223–4231. doi:10.1074/jbc.M110709200. PMID 11704682.

Dickson-Hoyle, S., & Reenberg, A. (2009). The shrinking globe: Globalisation of food systems and the changing geographies of livestock production. *Geografisk Tidsskrift-Danish Journal of Geography*, *109*(1), 105-112.

Egbert, R., & Borders, C. (2006). Achieving success with meat analogs. Food Technology, 5 (Jan).

FAO (2006). Livestock's long shadow— Environmental issues and options. : FAO publications.

Fergal J. O'Brien, Biomaterials & scaffolds for tissue engineering. Materials Today, Volume 14, Issue 3, 2011, Pages 88-95. ISSN 1369-7021.

Gershlak, J. R. et al. Crossing kingdoms: Using decellularized plants as perfusable tissue engineering scaffolds. Biomaterials 125, 13–22 (2017).

Greger, M. (2007). The human/animal interface: Emergence and resurgence of zoonotic infectious diseases. Critical Reviews in Microbiology, 33(4), 243–299

H. C. Olguin and B. B. Olwin, "Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a poten- tial mechanism for self-renewal," Developmental Biology,vol. 275, no. 2, pp. 375–388, 2004.

Halper, J., and Kjaer, M. (2014). "Basic components of connective tissues and extracellular matrix: elastin, fibrillin, fibulins, fibrinogen, fibronectin, laminin, tenascins and thrombospondins," in Progress in Heritable Soft Connective Tissue Diseases, ed. J. Halper, (Dordrecht: Springer), 31–47. doi: 10.1007/978-94-007- 7893-1_3.

Hecht, S. B. (1993). The logic of livestock and deforestation in Amazonia. *Bioscience*, *43*(10), 687-695.

Hsu, S. H., Chu, W. P., Lin, Y. S., Chiang, Y. L., Chen, D. C., & Tsai, C. L. (2004). The effect of an RGD-containing fusion protein CBD-RGD in promoting cellular adhesion. *Journal of biotechnology*, *111*(2), 143-154.

Ikada, Y. (2006). Challenges in tissue engineering. *Journal of the Royal Society Interface*, *3*(10), 589-601.

Jeschke, B., Meyer, J., Jonczyk, A., Kessler, H., Adamietz, P., Meenen, N. M., ... & Nies, B. (2002). RGD-peptides for tissue engineering of articular cartilage. *Biomaterials*, *23*(16), 3455-3463.

Johnson M. Fetal bovine serum. Mater Methods 2013;3: 175.

Kang, D. H., Louis, F., Liu, H., Shimoda, H., Nishiyama, Y., Nozawa, H., ... & Matsusaki, M. (2021). Engineered whole cut meat-like tissue by the assembly of cell fibers using tendon-gel integrated bioprinting. *Nature communications*, *12*(1), 1-12.

Key, T. J., Davey, G. K., & Appleby, P. N. (1999). Health benefits of a vegetarian diet. Proceedings of the Nutrition Society, 58(2), 271–275.

Khalili, A. A., & Ahmad, M. R. (2015). A Review of Cell Adhesion Studies for Biomedical and Biological Applications. *International journal of molecular sciences*, *16*(8), 18149–18184. doi:10.3390/ijms160818149

Larsson, S. C., & Wolk, A. (2006). Meat consumption and risk of colorectal cancer: A meta-analysis of prospective studies. International Journal of Cancer. Journal International

Marieb, Elaine Nicpon; Hoehn, Katja (2007). *Human anatomy & physiology*. Pearson Education. p. 133. ISBN 978-0-321-37294-9.

Mathew, A. G., Cissell, R., & Liamthong, S. (2007). Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. *Foodborne pathogens and disease*, *4*(2), 115-133.

Mattick, C. S., Landis, A. E., Allenby, B. R., & Genovese, N. J. (2015). Anticipatory life cycle analysis of in vitro biomass cultivation for cultured meat production in the United States. *Environmental science & technology*, *49*(19), 11941-11949.

Mayer, U. Integrins: Redundant or important players in skeletal muscle? Journal of Biological Chemistry (2003). doi:10.1074/jbc.R200022200.

Mayer, U. Integrins: Redundant or important players in skeletal muscle? Journal of Biological Chemistry (2003). doi:10.1074/jbc.R200022200

Modulevsky, D. J., Lefebvre, C., Haase, K., Al-Rekabi, Z. & Pelling, A. E. Apple Derived Cellulose Scaffolds for 3D Mammalian Cell Culture. PLoS One 9, e97835 (2014).

Pette, D., & Staron, R. S. (1997). Mammalian skeletal muscle fiber type transitions. *International review of cytology*, *170*, 143-223.

Pimentel, D., & Pimentel, M. (2003). Sustainability of meat-based and plant-based diets and the environment. The American Journal of Clinical Nutrition, 78(3 Suppl), 660S–663S.

Post, M. J. (2012). Cultured meat from stem cells: Challenges and prospects. *Meat science*, *92*(3), 297-301.

Rubio, N., Datar, I., Stachura, D., Kaplan, D., & Krueger, K. (2019). Cell-based fish: a novel approach to seafood production and an opportunity for cellular agriculture. *Frontiers in Sustainable Food Systems*, *3*, 43.

Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. *Annual review of cell and developmental biology*, *12*(1), 697-715.

Schmidt, M., Schüler, S. C., Hüttner, S. S., von Eyss, B., & von Maltzahn, J. (2019). Adult stem cells at work: regenerating skeletal muscle. *Cellular and Molecular Life Sciences*, *76*(13), 2559-2570.

Seah, J. S. H., Singh, S., Tan, L. P., & Choudhury, D. (2022). Scaffolds for the manufacture of cultured meat. *Critical Reviews in Biotechnology*, *42*(2), 311-323.

Slingenbergh, J., Gilbert, M., de Balogh, K., & Wint, W. (2004). Ecological sources of zoonotic diseases. Revue Scientifique et Technique (International Office of Epizootics), 23(2), 17.

Specht, E. A., Welch, D. R., Clayton, E. M. R., & Lagally, C. D. (2018). Opportunities for applying biomedical production and manufacturing methods to the development of the clean meat industry. *Biochemical Engineering Journal*, *132*, 161-168.

Stout, A. J., Mirliani, A. B., Soule-Albridge, E. L., Cohen, J. M., & Kaplan, D. L. (2020). Engineering carotenoid production in mammalian cells for nutritionally enhanced cell-cultured foods. *Metabolic engineering*, *62*, 126-137.

Stout, A. J., Mirliani, A. B., White, E. C., Yuen, J. S., & Kaplan, D. L. (2021). Simple and effective serum-free medium for sustained expansion of bovine satellite cells for cell cultured meat. *bioRxiv*.

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *cell*, *126*(4), 663-676.

Takala, T. E., and Virtanen, P. (2000). Biochemical composition of muscle extracellular matrix: the effect of loading. Scand. J. Med. Sci. Sports 10, 321–325. doi: 10.1034/j.1600-0838.2000.010006321.x.

Tuomisto, H. L., & de Mattos, M. J. (2011). Environmental impacts of cultured meat production. Environmental Science & Technology, 45(14), 6117–6123.

Visser, R., Arrabal, P. M., Santos-Ruiz, L., Fernandez-Barranco, R., Becerra, J., & Cifuentes, M. (2013). A collagen-targeted biomimetic RGD peptide to promote osteogenesis. Tissue Engineering Part A, 20(1-2), 34-44.

Wang, F., Li, Y., Shen, Y., Wang, A., Wang, S., & Xie, T. (2013). The functions and applications of RGD in tumor therapy and tissue engineering. International journal of molecular sciences, 14(7), 13447-13462.

Wang, Tianyi & Lew, Jie & Jayaraman, Premkumar & Poh, Chueh Loo & Win Naing, May. (2017). Production of Recombinant Collagen: State of the Art and Challenges. Engineering Biology. 1. 10.1049/enb.2017.0003.

Wang, Tianyi & Lew, Jie & Jayaraman, Premkumar & Poh, Chueh Loo & Win Naing, May. (2017). Production of Recombinant Collagen: State of the Art and Challenges. Engineering Biology. 1. 10.1049/enb.2017.0003.

Whitnall, T., & Pitts, N. (2019). Global trends in meat consumption. Agricultural Commodities, 9(1), 96-99.

Wolk, A., 2017. Potential health hazards of eating red meat. J. Intern. Med. 281, 106–122. https://doi.org/10.1111/joim.12543.

Zakrzewski, W., Dobrzyński, M., Szymonowicz, M., & Rybak, Z. (2019). Stem cells: past, present, and future. *Stem cell research & therapy*, *10*(1), 1-22.