

Engineering Mammalian Muscle Satellite Cells to Produce Viral Antigens

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## Abstract

Utilizing a transposon-based genetic engineering system, transgenic hemagglutinin (from Influenza B) and spike glycoprotein antigen (from SARS-COV-2) were individually inserted into both primary bovine muscle satellite cells and C2C12 murine myoblasts. Expression of the antigens after engineering was verified via immunofluorescent staining, implying successful expression and sufficient structural formation to present antibody-binding epitopes. Cell identities and differentiation capacities were maintained after engineering as shown by immunofluorescence staining for PAX7, a muscle stem cell marker, and MHC, a late-stage muscle differentiation marker. Though ELISA quantification of the hemagglutinin antigen within the cells was attempted, along with a challenge to the antigen stability via simulated cooking, insufficient data was gathered from these tests to draw any statistical conclusions. Ultimately, the results of this study provide initial evidence of the potential of developing edible vaccines using cell-cultivated meat technology.

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Abstract .....	ii
Acknowledgments .....	iii
Table of Contents .....	iv
List of Tables and Figures .....	vi
1. Introduction .....	1
1.1 Vaccines .....	1
1.2 Modes of Vaccine Delivery .....	2
1.3 Oral Vaccines .....	4
1.4 Advantages of Oral Vaccination.....	5
1.5 Low Prevalence of Oral Vaccines .....	7
1.6 Edible Organisms as Vaccines .....	11
1.7 Cultivated Meat as Vaccines.....	11
2. Objectives .....	13
3. Materials and Methods .....	14
3.1 Plasmid design and assembly.....	14
3.2 Primary bovine satellite cell culture.....	15
3.3 C2C12 cell culture .....	15
3.4 Transfection and selection .....	15
3.5 Immunofluorescence staining .....	17
3.6 ELISA quantification of antigens with lysis and cooking .....	18
4. Results .....	20
4.1 Plasmid assembly .....	20
4.2. Transfections .....	20

4.3 Immunostaining for antigen presence .....	23
4.4 Immunostaining for cell identity and maturity .....	23
4.5 ELISA quantification of hemagglutinin .....	24
5. Discussion .....	29
5.1 Plasmid construction .....	29
5.2 Transposon-based engineering of BSCs and C2C12s.....	30
5.3 Immunofluorescent labeling of engineered proteins .....	31
5.4 Confirming cell identity and differentiation capacity .....	32
5.5 ELISA quantification of antigen expression with a stability challenge .....	33
5.6 Challenges during this project .....	33
5.7 Regulatory and Consumer Acceptance .....	36
6. Future work .....	38
6.1 ELISA quantification of antigen levels .....	38
6.2 Further evaluate the results generated by this study .....	38
6.3 Testing for the stability of the antigens as an oral therapeutic .....	39
6.4 Determine the bioavailability of the recombinant antigens after digestion .....	39
6.5 Testing for immune stimulation .....	40
6.6 Increasing antigen expression and alternative engineering .....	40
6.7 Peptide engineering for increased stability .....	41
6.8 Animal study challenge .....	41
7. References .....	42

List of Tables and Figures

Table 1. Comparing the advantages and disadvantages of vaccines delivery methods ..... 3

Table 2. Overview of the current FDA approved oral vaccines in the United States ..... 6

Table 3. Biological hurdles of oral vaccine delivery from administration to immune response ... 10

Figure 1. An overview of the experimental process described in this this study. A) Viral antigen gene sequences were acquired and optimized for mammalian expression. Gene cassettes assembled with a cytomegalovirus (CMV) promotor to simultaneously express puromycin resistance genes (PuroR) and have bi-cistronic expression of either hemagglutinin (Influenza B) or spike glycoprotein (SARS-COV-2) and green fluorescent protein (eGFP) were incorporated into plasmids containing Sleeping Beauty inverted repeat transposable elements. B) Cultured bovine satellite cells and C2C12 murine myoblasts were transfected to incorporate the cassette via transposon mediated engineering. C) Engineered cultures were collected, cooked, and lysed, then measured by sandwich ELISA for quantification of the transgenic antigen ..... 19

Figure 2. Electrophoresis gel results from fragment amplification prior to assembly. PSB = Sleeping Beauty plasmid; Hema = hemagglutinin gene; Spike = spike glycoprotein gene. The first number in “X.X” denotes the initial gene sample purified from an *E.coli* carrier, the second number denotes the PCR reaction replicate; “Hema1.2” was the first purification of the Hema gene and the second replicate amplified from that purification. A) Hema gene and PSB backbone amplified for Gibson assembly. B) Spike and PSB backbone amplified for Gibson assembly. C) Spike gene and alternative PSB backbone amplified for Golden Gate assembly ..... 21

Figure 3. Green fluorescent protein reporter gene fluorescence in live cultures of engineered primary bovine satellite cells (BSCs) and C2C12 murine myoblasts. A) Representative photos of

engineered cultures expanded and stored to cryobank. B) Representative images of cultures with low cell counts or strange morphology that were present in replicate wells after transfection and not stored or used for further experiments. Scale bars = 500  $\mu\text{m}$  unless otherwise noted ..... 22

Figure 4. Immunostaining for antigen expression (red) in bovine satellite cells (BSCs) and C2C12 murine myoblasts engineered with the CMV-Hema-GP (A) or CMV-Spike-GP (B) insert seven days after initiating differentiation. The cultures were counterstained with DAPI, a nuclei stain (blue) and exhibit endogenous fluorescence from green fluorescent protein expression (green). Scale bars=100 $\mu\text{m}$  unless otherwise noted ..... 25

Figure 5. Immunostaining for antigen expression (red) in bovine satellite cells (BSCs) with the CMV-Hema-GP insert seven days after initiating differentiation. The cultures were counterstained with DAPI, a nuclei stain (blue) and exhibit endogenous fluorescence from green fluorescent protein expression (green). Morphology of this culture does not match replicate cultures of engineered BSCs at this stage of growth. Morphology shown more closely matches expected shape of fibroblasts in culture. Due to these stains, this set of cells was not used for further experiments. Scale bars=100 $\mu\text{m}$  ..... 26

Figure 6. Bovine satellite cells (BSCs) and C2C12 murine myoblast cultures engineered with CMV-Hema-GP or CMV-Spike-GP inserts stained for the presence of Pax7 (red), a muscle precursor protein marker, and counterstained with DAPI (blue), a nuclei stain. Stains co-localize on the nuclei as expected, appearing magenta at the overlap of signal. Scale bars=500 $\mu\text{m}$  unless otherwise noted ..... 27

Figure 7. Bovine satellite cells (BSCs) and C2C12 murine myoblast cultures engineered with CMV-Hema-GP or CMV-Spike-GP inserts stained for the presence of MHC (red), a marker of

mature muscle myotubes and counterstained with DAPI (blue), a nuclei stain. Scale bars=  
500µm ..... 28

## 1. Introduction

### 1.1. Vaccines

Vaccines have been a vital tool in protecting human health since the late 1700s when Dr. Edward Jenner showed that cowpox infection provided protection against the much deadlier smallpox disease<sup>1</sup>. Today, many vaccines are based on small non-replicative protein subunits of a specific virus or bacteria called antigens. These antigens are typically surface presenting proteins native to a pathogen. When a pathogen invades a host, antibodies produced within the host body have a chance to bind to these antigenic proteins, and upon doing so, trigger an immune response in which immune cells learn to recognize and destroy the pathogen<sup>2-6</sup>. The immune system can then remember this infection, and should the same pathogen be encountered again, react to stop the infection before it can cause harm<sup>1,7</sup>. In many cases, disease occurs before the immune system can react to the pathogen, and protection is only provided against re-infection. By proactively exposing the immune system to the antigens for a specific pathogen without the chance of infection, vaccines take advantage of this process, and prime the immune response to destroy that pathogen before disease is caused<sup>8,9</sup>. While the usage and coverage of vaccinations have been expanding for centuries, human diseases continue to change and emerge over time<sup>10</sup>. This has been particularly apparent with the late-2019 emergence of the SARS-COV-2 virus (COVID-19), which became a global pandemic before effective vaccines were developed based on the spike glycoprotein antigen<sup>6</sup>. The influenza virus, responsible for the flu illness, has had effective vaccines available for decades based on the influenza hemagglutinin antigen, however, these vaccines must be continually updated and re-administered on an annual basis to protect against ever-changing strains<sup>11</sup>.

## 1.2 Modes of Vaccine Delivery

Vaccines can be administered in a variety of ways depending on the format of the vaccine. Injection by needle is the most common route of administration for vaccines and includes intramuscular, subcutaneous, and intradermal targets. Vaccines can also be delivered via intranasal spray and oral administration<sup>12</sup>, and are considered mucosal vaccines<sup>6</sup>. Each of these methods have their advantages and disadvantages in terms of production, storage, stability, and efficacy<sup>9,13-16</sup> (Table 1). Injected vaccines are generally very effective and provide long lasting immunity<sup>1,12,13</sup>, but carry a risk of non-compliance due to needle aversion<sup>17,18</sup>, as well as having the potential for unwanted side effects such as lingering pain and discomfort at the injection site<sup>19</sup>. Injected vaccines also only trigger the systemic immune response<sup>20</sup>. Intranasal vaccines are typically administered as a spray and take advantage of the mucous membrane in the nasal cavity to allow uptake of the antigens. These vaccines are easy to administer, and they can trigger a systemic as well as a mucosal immune response. However, nasal vaccines have shown a lower efficacy and longevity compared to their injectable counterparts<sup>9,16</sup>. Oral vaccines, usually in tablet or liquid form, share the ease of administration of nasal, as well as triggering a similar mucosal and systemic immune response<sup>14,21,22</sup>. Generally, oral vaccines have a lower cost of production and distribution compared with injection-based vaccines as well<sup>14,21,22</sup>. The most significant challenge for oral vaccines is the need to remain stable in the gastrointestinal tract, an environment of wide variation in pH and proteolytic enzymes<sup>9,23</sup>, though methods to preserve the stability of these vaccines have been developed.

**Table 1**  
**Comparing the advantages and disadvantages of vaccines delivery methods**

Delivery Method	Typical Formats	Common Advantages	Common Disadvantages	Ref
Injection	•Needle	<ul style="list-style-type: none"> <li>• Bypasses the mucosal membrane and can directly interact with the immune system</li> <li>• Strongly effective at preventing disease</li> <li>• Long lasting immune protection</li> </ul>	<ul style="list-style-type: none"> <li>• Needle aversion lowers compliance</li> <li>• Only triggers the systemic immune response</li> <li>• Pain and discomfort at the injection site</li> <li>• Stringent storage requirements are necessary to preserve stability</li> </ul>	8,9,15,19,20
Oral	•Tablet •Liquid	<ul style="list-style-type: none"> <li>• Both systemic and mucosal immune responses are activated, providing more broad immune protection throughout the body</li> <li>• Less stringent storage requirements compared to many injection-based formulations</li> </ul>	<ul style="list-style-type: none"> <li>• Requires additives to prevent degradation of the vaccine in the gastrointestinal tract</li> <li>• Higher dosages are needed to allow sufficient uptake to provide effective immune protection</li> <li>• More difficult to include effective adjuvants</li> </ul>	9,14,16,20–24
Nasal	•Spray	<ul style="list-style-type: none"> <li>• Both systemic and mucosal immune responses are activated, providing more broad immune protection throughout the body</li> <li>• Can avoid allergic reactions to components commonly found in injectables</li> </ul>	<ul style="list-style-type: none"> <li>• Lower efficacy and duration of immune protection</li> </ul>	9,13,16,20

### 1.3 Oral Vaccines

There are currently only four diseases for which there are oral vaccines authorized by the Food and Drug Administration (FDA) for use in the US<sup>25</sup> (Table 2). These include vaccines against Adenovirus Type 4 and Type 7, Cholera (a disease caused by bacterium *Vibrio cholerae*), Rotavirus, and Typhoid (a disease caused by bacterium *Salmonella* serotype Typhi). The Adenovirus Type 4 and Type 7 vaccine is only currently available to US military personnel and is administered as two tablets, one containing each type of the virus. These tablets are enteric coated, allowing them to remain unaffected by the acidic environment of the stomach, but will readily dissolve in the more alkaline environment of the intestines, releasing the live virus within the intestinal tract<sup>26</sup>. The oral cholera vaccine is administered in the US under the tradename VAXCHORA. This vaccine is based on a live-attenuated bacterium engineered to remove the *ctxA* gene responsible for synthesis of the cholera toxin<sup>27</sup>. Packets of lyophilized bacterium and a sodium bicarbonate buffer are dissolved in water and swallowed. The bicarbonate buffer protects the virus from the acidic environment in the stomach until it reaches the intestinal tract. For rotavirus, there are two oral vaccines approved in the US for use on infants only. The first of these, RotaTeq, is administered as a liquid containing live human-bovine reassortant rotaviruses exhibiting antigens of five human rotavirus types in a solution of multiple pH buffers<sup>28</sup>. The second, ROTARIX, consists of a lyophilized attenuated rotavirus strain combined with a liquid diluent containing disodium adipate, also a pH buffer<sup>29</sup>. The oral typhoid vaccine available in the US is distributed under trade name Vivotif and is comprised of a live-attenuated *Salmonella typhi* strain lyophilized in capsules containing various pH buffers and stabilizers with an enteric coating to protect the contents from the stomach environment<sup>30</sup>. For all these vaccines, a major component of the formulation is intended to protect the contents from the acidic stomach environment and is

achieved by utilizing a low-pH resistant coating and/or pH buffers. Low pH environments damage the proteins of these microbes and can adversely affect the capacity of antibodies to bind to the epitope sites of these proteins, limiting the immune recognition of antigens and consequently preventing immunity<sup>31-33</sup>. The protection of these proteins specifically and the live microbes generally until they reach the point of uptake is crucial for the effectiveness of these oral vaccines.

#### 1.4. Advantages of Oral Vaccination

While currently limited in use, oral vaccinations hold great promise due to specific advantages over injection-based vaccines, including stimulating a mucosal immune response, simplified production, self-administration, stability in distribution and storage, and compliance<sup>14,34,35</sup>. Injectable vaccines mostly trigger the systemic immune response dominated by Immunoglobulin G (IgG) antibodies local to blood and many bodily fluids, whereas it has been shown that, in addition to a systemic immune response, oral vaccines absorb into the mucosal epithelium of the intestines, triggering a mucosal immune response dominated by Immunoglobulin A (IgA) antibodies<sup>7,20</sup>. The lymphocytes triggered by the vaccine to produce IgA in the intestinal lining may then relocate to remote mucosal effector sites in the colon, the respiratory tract, the reproductive tract, as well as salivary and mammary glands<sup>5,6,20</sup>, bringing greater immune protection to these regions of the body<sup>6,20</sup>. Oral vaccines also have the advantage of not involving injections. Whereas injectable vaccines require trained personnel to administer the vaccines in a controlled environment, oral vaccines can be distributed for self-administration<sup>21,36</sup>.

**Table 2****Overview of the current FDA approved oral vaccines in the United States**

<b>Vaccine</b>	<b>Target Pathogen</b>	<b>Disease</b>	<b>Format</b>	<b>Protective Additives</b>	<b>Ref</b>
Adenovirus Type 4 and Type 7 vaccine	<i>Adenovirus</i> Type 4 and Type 7	<ul style="list-style-type: none"> <li>• Febrile acute respiratory disease</li> </ul>	<ul style="list-style-type: none"> <li>• Lyophilized live virus in 2 tablets, one for each type</li> </ul>	<ul style="list-style-type: none"> <li>• pH sensitive enteric coating</li> </ul>	26
VAXCHORA	<i>Vibrio cholerae</i> serogroup O1 (bacterium)	<ul style="list-style-type: none"> <li>• Cholera</li> </ul>	<ul style="list-style-type: none"> <li>• Lyophilized live-attenuated bacteria reconstituted in water with the <i>ctxA</i> gene responsible for synthesis of cholera toxin engineered out</li> </ul>	<ul style="list-style-type: none"> <li>• Sodium bicarbonate pH buffer added during reconstitution of lyophilized bacteria</li> </ul>	27
RotaTeq	<i>Rotavirus</i> types G1, G2, G3, G4, G9	<ul style="list-style-type: none"> <li>• Rotavirus gastroenteritis</li> </ul>	<ul style="list-style-type: none"> <li>• Liquid solution containing live human-bovine reassortant rotaviruses exhibiting antigens of 5 different human rotavirus types</li> </ul>	<ul style="list-style-type: none"> <li>• Sucrose, sodium citrate, sodium phosphate monobasic monohydrate, sodium hydroxide</li> </ul>	28
ROTARIX	<i>Rotavirus</i> types G1, G3, G4, G9	<ul style="list-style-type: none"> <li>• Rotavirus gastroenteritis</li> </ul>	<ul style="list-style-type: none"> <li>• Lyophilized live-attenuated virus reconstituted in liquid diluent (liquid formulation also distributed)</li> </ul>	<ul style="list-style-type: none"> <li>• Diluent contains calcium carbonate as a pH buffer</li> </ul>	29
Vivotif	<i>Salmonella</i> serotype typhi (bacterium)	<ul style="list-style-type: none"> <li>• Typhoid fever</li> </ul>	<ul style="list-style-type: none"> <li>• Lyophilized live-attenuated strain <i>Salmonella typhi</i> Ty21a in capsules</li> </ul>	<ul style="list-style-type: none"> <li>• pH sensitive enteric coating</li> <li>• Various pH buffers and stabilizers</li> </ul>	30

This would reduce or eliminate some of the costs of administering an injectable vaccine incurred by providers and by extension their patients, including the costs to train healthcare personnel, provide protective equipment, maintain a sterile environment for administration, and pay staff for the labor of administration<sup>34,36</sup>. Injections also contribute to vaccine hesitancy, due in part to needle aversion in both adults and children<sup>17</sup>, as well as the time burden of attending an appointment at a healthcare provider<sup>18</sup>. Vaccination compliance may be improved by oral self-administration if injections by a healthcare provider are unnecessary.

### 1.5 Low Prevalence of Oral Vaccines

Despite the advantages of oral vaccine delivery, most vaccines are still formulated to be administered by injection. Though the advantages of oral delivery described above point towards this route of administration being a more therapeutically beneficial method of vaccination, the dearth of available oral vaccines seem to be primarily driven by preconceptions around the efficacy of oral vaccines and questions pertaining to the adequacy of the immune response triggered by oral delivery<sup>37</sup>. This has generally resulted in oral vaccines only being utilized for special populations, such as military use for adenovirus and infant use for rotavirus. Yet there are several valid scientific and logistical drawbacks compared to injectable vaccines that have limited the pursuit of oral vaccines development (Table 3). Stability in the digestive environment remains one of the main concerns. The low pH, wide pH variance, and proteolytic enzyme activity in the gastric fluids can degrade and denature the antigenic proteins used in the vaccines, rendering them unable to confer immunity<sup>21,22,31</sup>. While the FDA approved oral vaccines described here have found solutions by utilizing resistant coatings and buffers to protect against this environment, the process necessary to optimize these measures would not be necessary for injectables. There is also the consideration of efficacy in terms of dosage. The environment of the human digestive tract can vary widely

between individuals and is impacted by time of day and recency of meals<sup>38,39</sup>. Despite the protective measures that have been developed to improve the stability of the antigens in this environment, this variability must be accounted for when determining the optimal concentration of antigen necessary in a standard dose to provide sufficient immune protection for all consumers<sup>9,16</sup>. Efficacy is also impacted by the limited time of action available after administering the oral vaccine as it passes through the gastrointestinal tract, with the critical point of uptake occurring in the small intestine potentially only lasting a few hours<sup>38,39</sup>. These considerations can increase the complexity of evaluation necessary to determine the necessary effective dosage prior to commercial distribution.

Most injectable vaccines are administered with an adjuvant, an additive meant to actively trigger an immune reaction at the site of the injection, thereby directing immune cells to the area and increasing their contact with the antigenic proteins<sup>1,9</sup>. For use in oral delivery, these adjuvants would need to survive the same gastrointestinal environment as the antigens before uptake, and would also need to be limited in the immune response they provoke before reaching the point of uptake to prevent premature immune activation. Uptake itself is a concern for oral vaccines as well. Injectables by their nature will be processed and metabolized by the body as they are administered past the mucosal membrane into tissues<sup>9,12</sup>. Oral vaccines must be absorbed through the mucosal membrane of the intestines to interact with the immune system. This typically occurs at Peyer's patches, regions of the intestinal lining which contain microfold cells that sample antigens from the intestinal lumen to present to immune cells as a natural defense against pathogens that enter the gastrointestinal tract<sup>3,40</sup>. While this function can be taken advantage of for the purposes of administering vaccines orally, the capacity of these microfold cells to uptake a

given antigen must be considered, as well as the capacity for the antigens taken up to then bind to antibodies presented by B lymphocytes<sup>3,5,20</sup>.

**Table 3****Biological hurdles of oral vaccine delivery from administration to immune response**

Stage	Duration	Challenge	Impact	Ref
Stage 1: Oral cavity	Seconds to minutes	<ul style="list-style-type: none"> <li>Salivary lactoperoxidase</li> </ul>	<ul style="list-style-type: none"> <li>Produces oxidized metabolites that have a significant bactericidal effect. This enzyme would primarily affect live bacteria-based vaccines, but could also lead to some antigen protein degradation</li> </ul>	38,41
		<ul style="list-style-type: none"> <li>Esophageal lysozyme</li> </ul>	<ul style="list-style-type: none"> <li>Breaks polysaccharides in the cell wall of bacteria, causing the cells to lyse. This enzyme would primarily affect live bacteria-based vaccines</li> </ul>	39,42
Stage 2: Stomach digestion	1-2 hours	<ul style="list-style-type: none"> <li>Hydrochloric acid</li> </ul>	<ul style="list-style-type: none"> <li>Hydrochloric acid released into the stomach environment drops the pH of the fluids there to ~2, which causes denaturation of most proteins, including antigens</li> </ul>	31,32,38
		<ul style="list-style-type: none"> <li>Pepsin</li> </ul>	<ul style="list-style-type: none"> <li>Pepsin is a proteolytic enzyme that can degrade antigenic proteins. It is the most critical enzyme to consider for oral vaccine formulation due to its efficiency of protein cleavage</li> </ul>	38,43,44
Stage 3: Pre-uptake intestinal tract digestion	1-5 hours	<ul style="list-style-type: none"> <li>Trypsin (with carboxypeptidase and chymotrypsin)</li> </ul>	<ul style="list-style-type: none"> <li>Trypsin, a proteolytic enzyme released by the pancreas into the intestinal lumen, works with two other proteolytic enzymes, carboxypeptidase and chymotrypsin, to degrade and breakdown proteins</li> </ul>	39,44
Stage 4: Uptake at the mucous membrane of the small intestine	-	<ul style="list-style-type: none"> <li>Antigen endocytosis by microfold cells in Peyer's patches</li> </ul>	<ul style="list-style-type: none"> <li>Antigens must bind to receptor sites on microfold cells to be taken up via endocytosis. Only site where antigens in the intestinal lumen will be presented to immune cells, so a lack of binding will prevent immune protection.</li> </ul>	3,40
Stage 5: Immune response	-	<ul style="list-style-type: none"> <li>Antibody binding and activation of B lymphocytes</li> </ul>	<ul style="list-style-type: none"> <li>Microfold cells present antigens they uptake to B lymphocytes, immune cells that express surface bound antibodies. The antigens must be able to bind to these antibodies to trigger further antibody production by the B cells. B lymphocytes also present antigens to other immune cells such as T lymphocytes for further immune responses which also require adequate receptor binding to trigger the response</li> </ul>	2,3,40

## 1.6. Edible Organisms as Vaccines

One promising method for oral vaccine production being explored is using recombinant genetic techniques to express viral antigens in edible organisms<sup>22,45</sup>, including staple agricultural plants, algae, yeasts, insect-cells and lactic acid bacteria<sup>22</sup>. These organisms endogenously produce the antigens within their cells and preliminary studies have shown that they can provide immune protection when eaten<sup>22</sup>. A typical component of conventional vaccine production is the processing and purification of recombinant antigens<sup>46</sup>, however, these studies indicate that the use of these edible organisms for vaccine production would make these steps unnecessary and could lead to faster manufacturing and lower production costs<sup>22,35</sup>. This method also holds promise for reducing the complications involved with distribution of conventional vaccines. Many injectable vaccines require storage at very low temperatures to prevent degradation<sup>15,36</sup>. This has required the use of costly cold-chain distribution networks for vaccines, where storage conditions must be carefully planned at every step from manufacturing to administration<sup>36</sup>. However, some recombinant organism-based edible vaccines have shown promise to maintain antigen stability within the cells and tissues with minimal temperature preservation, with one seed-based vaccine study showing stability that could extend for years at ambient temperatures<sup>24,35</sup>, adding to the potential cost savings this type of vaccine may provide.

## 1.7 Cultivated Meat as Vaccines

One source of recombinant viral antigens that has not been previously explored is the use of transgenic mammalian cells to produce edible muscle tissue containing recombinant viral antigens. Mammalian cells, including bovine skeletal muscle cells, have the potential to be a good platform for vaccine production as they are already being explored as a source of cultivated meat<sup>47</sup>. Cultivated meat production involves *in vitro* culturing of food-relevant cells (e.g., muscle and fat)

sourced from animals that include conventional livestock, aquatic animals, and even insects<sup>48,49</sup>. These cells are expanded at large scale and then formatted into an edible product to provide a cell-based alternative to conventionally produced meat<sup>48,50,51</sup>. Recent advancements in the field of *in vitro* cell-culture have made it possible for cultivated meat to be seriously considered as consumer products<sup>47,50</sup>. Cultivated meat products are already available to consumers in certain regions of the world, with numerous companies well on their way to having consumer cultivated meat products available in the US<sup>52</sup>. Cultivated meat has numerous potential advantages over animal-based meats including avoidance of the ethical concerns around raising animals for slaughter, reduced land use requirements, and improved food safety<sup>47,51</sup>. A recent study has shown it is possible to customize the nutritional profiles of cultivated meats using recombinant genetic techniques<sup>53,54</sup> to engineer bovine satellite cells (BSCs) to produce carotenoids<sup>47</sup>, antioxidant compounds that are typically found in plants and have shown some capacity to reduce potential negative health effects of eating red meat<sup>55</sup>. In the present study, these same genetic engineering techniques were used make BSCs as well as C2C12 murine myoblasts endogenously express viral antigen proteins, with the potential to serve as an edible meat-based vaccine. Inevitable advancements in cultivated meat production that drive down costs and increase production and availability could also apply to meat-based vaccines, potentially improving vaccine access globally.

## 2. Objectives

### Specific Aim

The specific aim of the research described here was to genetically engineer cultured muscle stem cells to endogenously produce viral antigens. This aim was intended to explore whether engineered cultivated meat could serve as an effective edible vaccine delivery mechanism.

*Objective 1 – Construct a vector plasmid containing a viral antigen gene flanked by Sleeping Beauty transposon elements*

The gene sequences of hemagglutinin from the Influenza B virus and spike glycoprotein from SARS-COV-2 were planned to be incorporated into a *Sleeping Beauty* plasmid backbone by Gibson assembly and standard molecular cloning techniques. The antigen cassettes would include a promoter for constitutive mammalian expression, puromycin resistance genes for selection, and a green fluorescent protein (GFP) reporter for assessing gene expression.

*Objective 2 – Engineer muscle stem cells with the assembled transposable plasmid construct to express a viral antigen*

Using standard transfection techniques, the antigen gene cassette would be introduced into muscle stem cells along with another plasmid holding the *Sleeping Beauty* transposase gene. Transposase expressed from the plasmid in the muscle stem cells would insert the antigen cassette into the genomic DNA of the cells.

*Objective 3 – Assess the yield of the recombinant antigens and challenge their stability*

Engineered muscle cells would be exposed to heat to evaluate the stability of the produced antigens if cooked; raw “tartar” cells will also be evaluated. Antigen presence and yield would be assessed by immunostaining and protein quantification assays, comparing results for raw cells to cooked to measure stability.

### 3. Materials and Methods

#### 3.1. Plasmid design and assembly

The amino acid sequences for the Influenza B virus antigen hemagglutinin and the COV-SARS-2 antigen spike glycoprotein S1 subunit were obtained from UniProt (accession numbers A0A3Q9T421 and P0DTC2, respectively) and optimized for expression in *Bos taurus* in codon optimization software (IDT, Coralville, IA). All procedures in this research tested hemagglutinin or the spike glycoprotein independently. Plasmid constructs containing an ampicillin resistance gene and either the optimized hemagglutinin gene or the optimized spike glycoprotein gene were ordered from ThermoFisher's GeneArt gene synthesis service (ThermoFisher Scientific, Waltham, MA, USA). A *Sleeping Beauty* transposon vector backbone pSB-CMV-GP, containing *Sleeping Beauty* inverted repeats, a puromycin resistance gene, and a green fluorescent protein (GFP) gene under a cytomegalovirus (CMV) promoter was constructed for prior work<sup>47</sup>. Final Sleeping Beauty transposon vectors pSB-CMV-Hema-GP (hemagglutinin) and pSB-CMV-Spike-GP (spike glycoprotein) were constructed via Gibson assembly (SGI-DNA #GA1100, San Diego, CA, USA) or Golden gate assembly (New England Biolabs, NEB, #E1602, Ipswich, MA, USA) for bicistronic expression of the antigen gene sequence and GFP under the CMV promoter (Fig. 1A), utilizing Q5 high-fidelity polymerase (NEB #M0492S) for polymerase chain reactions that were verified by 1% agarose gel-electrophoresis. All ordered and constructed plasmids were maintained in 10-beta high efficiency competent *E.coli* (NEB #C3019) using standard molecular cloning techniques, verified with Sanger sequencing (Genewiz, Cambridge, MA, USA) and purified via GeneJet miniprep (ThermoFisher #K0503).

### 3.2. Primary bovine satellite cell culture

Primary bovine satellite cells (BSCs) were previously isolated, characterized, and cryopreserved<sup>47</sup>. The BSCs were expanded on tissue culture plastic using standard techniques, maintained in growth media comprised of Dulbecco's Modified Eagle Medium (DMEM) Glutamax (ThermoFisher #10566024) containing 20% fetal bovine serum (FBS; ThermoFisher #26140079), 1 ng/mL FGF-2 (ThermoFisher #13256-029), and 1% antibiotic-antimycotic (ThermoFisher #15240062). During seeding, 0.25  $\mu\text{g}/\text{cm}^2$  iMatrix recombinant laminin-511 (Iwai North America #N892021, San Carlos, CA, USA). was added to the BSC growth media. Cells were cultured to 80% confluence at 37°C in 5% CO<sub>2</sub>, with media changes every 2-3 days and were subsequently passaged with 0.25% trypsin-EDTA (ThermoFisher #25200056) or were cryopreserved in FBS supplemented with 10% dimethyl sulfoxide (DMSO, Sigma Aldrich #D2650, St. Louis, MO, USA). Cells were counted in suspension using either a standard hemocytometer, or a NC-200 automated cell counter (Chemometec, Allerod, Denmark). Myogenic differentiation was promoted by not changing the medium for 5-7 days to achieve serum starvation<sup>47</sup>.

### 3.3. C2C12 cell culture

C2C12 murine myoblasts (ATCC #CRL-1772, Manassas, VA, USA) were maintained on tissue-culture plastic in media comprised of DMEM Glutamax containing 10% FBS and 1% antibiotic/antimycotic. Cells were passaged, cryopreserved, and differentiated as described here for the BSC cultures, excluding the laminin supplement during seeding.

### 3.4. Transfection and selection

Transfection of BSCs and C2C12s was achieved using either a Lipofectamine 3000 kit (ThermoFisher #L3000008) or a Nucleofector 4D electroporation device (Lonza, Cologne,

Germany). For the Lipofectamine kit, BSCs and C2C12s were seeded into 6-well tissue culture plates and grown to ~85% confluency. The cultures were then washed with phosphate-buffered saline (PBS) and incubated in OptiMEM media (ThermoFisher #31985062) containing the Lipofectamine 3000 reagents, the *Sleeping Beauty* antigen plasmid construct, and pCMV(CAT)T7-SB100, a plasmid containing the *Sleeping Beauty* transposase<sup>31</sup> that was a gift from Zsuzsanna Izsvak<sup>53</sup> (Fig. 1B). After 6 hours of incubation at 37°C, growth media respective to either cell type was added in a 1:1 ratio with OptiMEM to provide a minimal amount of serum. After 24 hours, the media was fully changed to the appropriate growth medium. For transfections with the Nucleofector 4D device, BSCs and C2C12s were seeded into T75 tissue culture flasks and grown to ~85% confluency. The cells were then washed with PBS and detached with trypsin, centrifuged to remove the supernatant, and resuspended in growth media. A manufacturer recommended protocol optimized for C2C12 transfection was followed utilizing the SE reagent kit for the 4D device (Lonza #V4XC-1032). This protocol used the same plasmid constructs as described for the Lipofectamine protocol. Cells transfected in the 4D device were then plated in T25 tissue culture flasks. For both transfection techniques, a subset of cells was transfected with the plasmid CMV-GFP-Pur constructed for prior work<sup>47</sup> containing only the GFP expression gene and puromycin resistance gene, to serve as a control. 48 hours after transfection, the media was removed from the cultures and replaced with growth media containing 0.25ug/ml puromycin (ThermoFisher #A1113803) to select for transfected cells. The cultures were then incubated in this selective media for 1 week, changing the media every 2-3 days. Positive transformation was verified by imaging live cultures to check for GFP expression. Stably transformed BSCs and C2C12 cultures were expanded to 80% confluence then cryopreserved for use in all subsequent experiments.

### 3.5. Immunofluorescence staining

To confirm the expression of the antigen proteins as well as the identity and maturation state of the cells, cultures of the transfected cells were established from cryopreserved samples and maintained in 24 well tissue culture plates. When the cultures had achieved ~90% confluency, media changes were halted to induce differentiation. After 5 days of differentiation, cultures were stained using standard immunofluorescence protocols as previously described<sup>22</sup>. Briefly, cultures were fixed in 4% paraformaldehyde (PFA) (ThermoFisher #J61899.AP), washed with PBS, then permeabilized using 0.5% Triton X (Sigma #X100). After a wash with PBS containing 0.1% Tween-20 (Sigma #9005-64-5), the cultures were then blocked with PBS containing 5% goat serum (ThermoFisher #16210072) and 0.05% sodium azide (Sigma #16210064). After another wash with PBS containing 0.1% Tween-20, cultures were exposed to primary antibodies in blocking buffer. To stain for the antigens, anti-hemagglutinin mouse antibodies (Novus Biologicals #NBP3-11657, Centennial, CO, USA) or anti-spike glycoprotein rabbit antibodies (ThermoFisher # MA5-36247) were added to their respective cultures at an antibody to buffer ratio of 1:500. Subsets of each culture were alternatively stained with either Myosin heavy chain (MHC) mouse antibodies (Developmental Studies Hybridoma Bank #MF-20, Iowa City, IA, USA) or Paired Box 7 (Pax7) rabbit antibodies (ThermoFisher #PA5-68506), both diluted in blocking solution to 1:500. The cultures were then incubated overnight at 4°C. After incubation, the fixed tissues were washed with PBS containing 0.1% Tween-20, then soaked in blocking buffer. The cultures were then exposed to buffer containing a DAPI nuclear stain (Abcam #ab228549, Waltham, MA, USA) at 1:1000 and, respective to the host of the primary stain, either Goat-anti-rabbit 594 antibodies (ThermoFisher #A-11012) or Goat-anti-mouse 594 antibodies (ThermoFisher #A-11032) at 1:500 ratio. After a final wash with PBS, the stained cultures were

stored in PBS and imaged with a fluorescence microscope, and the resulting images were processed using ImageJ software.<sup>56</sup>

### 3.6. ELISA quantification of antigens with lysis and cooking

Cultures of the cells engineered to express hemagglutinin (spike protein engineered cells were not evaluated) as well as the GFP control cells were expanded and differentiated as described above. They were then collected with trypsin and transferred to conical tubes containing PBS, then centrifuged to pellet the cells. The supernatants were removed and the pellets were resuspended in PBS to a concentration of 250,000 cells/mL. The cell solutions were then aliquoted to microfuge tubes. One subset of the cells were lysed with RIPA lysis buffer (ThermoFisher #89900) using the manufacturer recommended protocol. Following lysis, a subset of the lysed and un-lysed cell aliquot tubes were then exposed to 100°C in a heat block for 10 minutes to simulate cooking, while the remaining samples were kept “raw”. The hemagglutinin concentration in these solutions was then measured by colorimetric sandwich ELISA (Fig. 1C) using a commercial hemagglutinin antibody pair (Novus Biological #NBP3-11657) following standard ELISA protocols or by a Hemagglutinin ELISA kit (SINO Biological # KIT11053, Wayne, PA, USA).

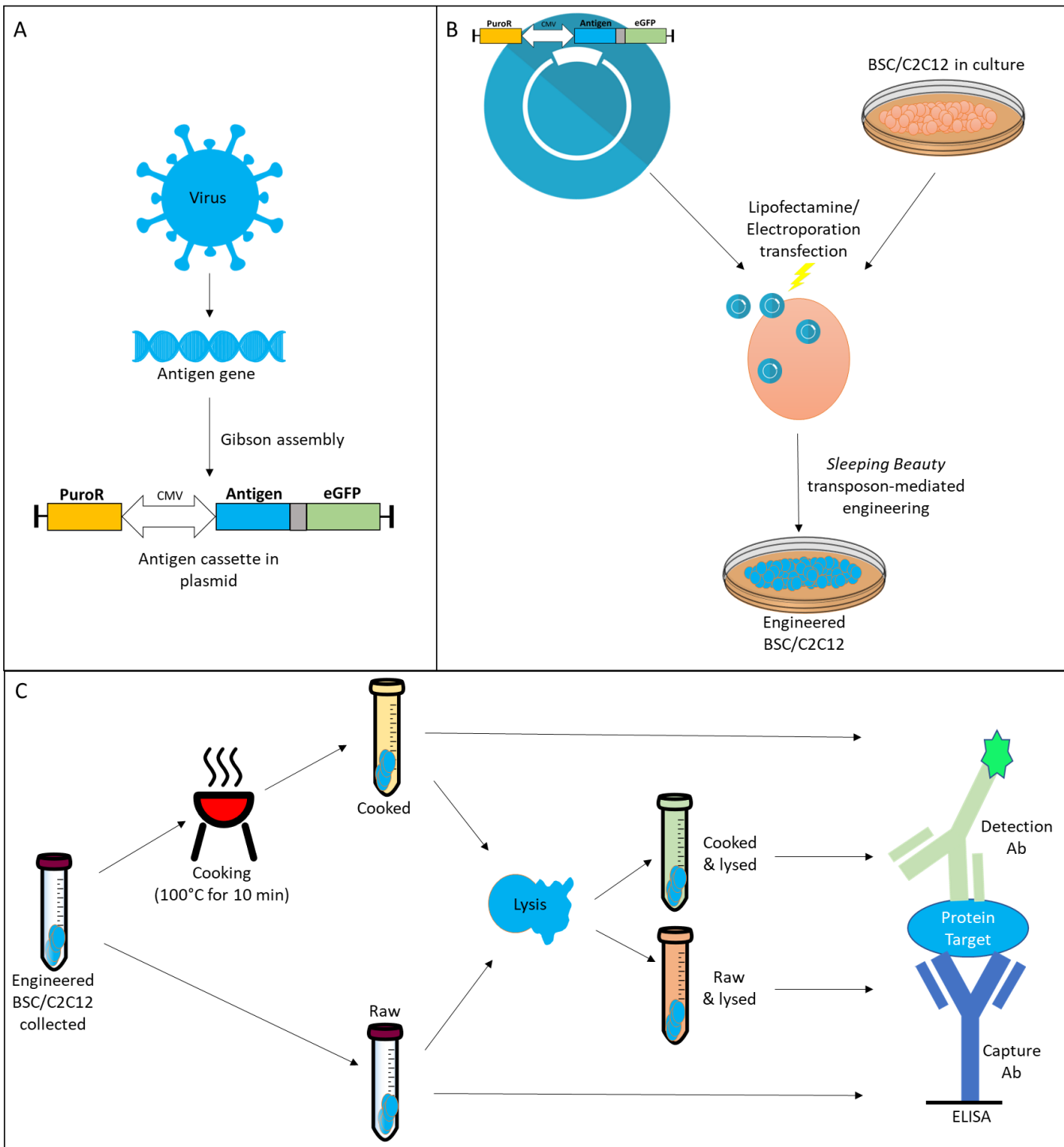


Figure 1. An overview of the experimental process described in this study. A) Viral antigen gene sequences were acquired and optimized for mammalian expression. Gene cassettes assembled with a cytomegalovirus (CMV) promoter to simultaneously express puromycin resistance genes (PuroR) and have bi-cistronic expression of either hemagglutinin (Influenza B) or spike glycoprotein (SARS-COV-2) and green fluorescent protein (eGFP) were incorporated into plasmids containing *Sleeping Beauty* inverted repeat transposable elements. B) Cultured bovine satellite cells and C2C12 murine myoblasts were transfected to incorporate the cassette via transposon mediated engineering. C) Engineered cultures were collected, cooked, and lysed, then measured by sandwich ELISA for quantification of the transgenic antigen.

## 4. Results

### 4.1 Plasmid assembly

Sequences amplified by PCR for use in the construction of the CMV-Hema-GP plasmid by Gibson assembly, as well the CMV-Spike-GP plasmid by Gibson assembly and Golden Gate assembly were successfully verified by gel electrophoresis with probes for the backbone plasmid amplification and the antigen genes (Fig. 2). After transformation of the assembled plasmids into competent *E.coli*, Sanger sequencing of colonies with probes for the overlap regions between the backbone and the antigen gene were used to select colonies containing successfully assembled plasmids. From this, optimal colonies were expanded and stored for later propagation of the plasmids.

### 4.2. Transfections

Transfections of BSCs and C2C12s was successful for both the CMV-Hema-GP insert and the CMV-Spike-GP insert, as well as for the control insert CMV-GFP-GP, as verified by survival of the cells post-transfection in the selective puromycin containing media, as well as via fluorescence imaging of the live cultures (Fig. 3A). GFP expression was not observable in the imaging for the BSCs engineered with the CMV-Spike-GP insert, and higher exposure times were necessary to observe fluorescence in the C2C12s engineered with the CMV-Spike-GP insert, despite the survival of the cultures in the selective media. This suggests reduced gene expression of the spike glycoprotein, perhaps due to cellular difficulties in transcribing or translating this sequence. For each transfection, multiple replicate samples were engineered in parallel, and some replicates did not produce cultures that had the expected morphology or cell survival and were not utilized for further experiments (Fig. 3B).

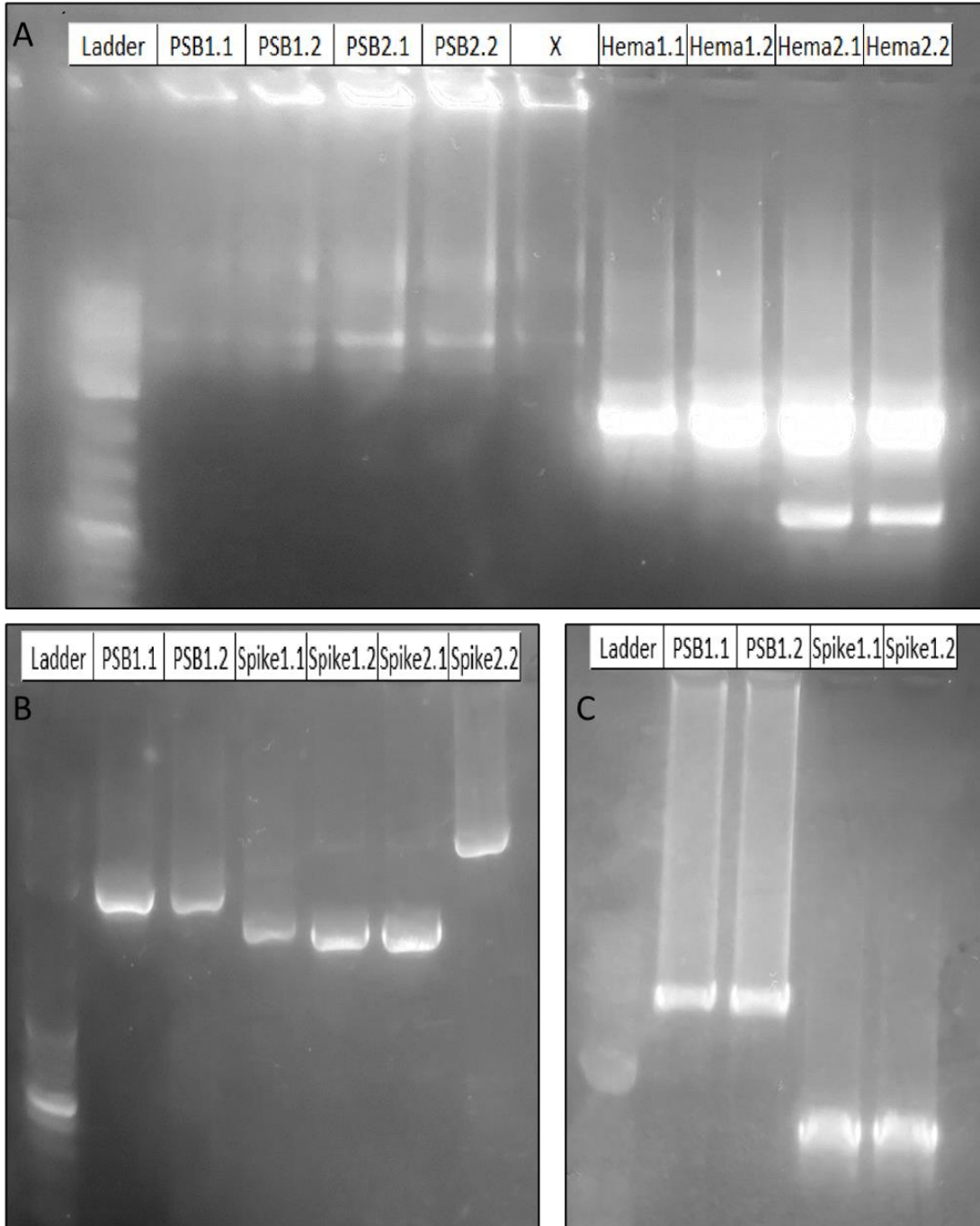


Figure 2. Electrophoresis gel results from fragment amplification prior to assembly. PSB = *Sleeping Beauty* plasmid; Hema = hemagglutinin gene; Spike = spike glycoprotein gene. The first number in “X.X” denotes the initial gene sample purified from an *E.coli* carrier, the second number denotes the PCR reaction replicate; “Hema1.2” was the first purification of the Hema gene and the second replicate amplified from that purification. A) Hema gene and PSB backbone amplified for Gibson assembly. B) Spike and PSB backbone amplified for Gibson assembly. C) Spike gene and alternative PSB backbone amplified for Golden Gate assembly.

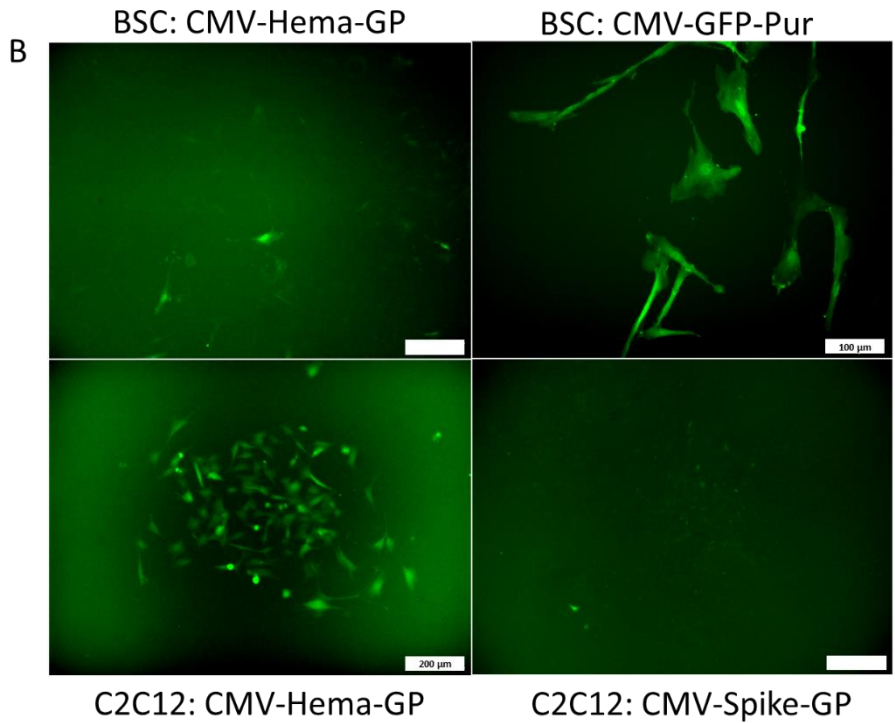
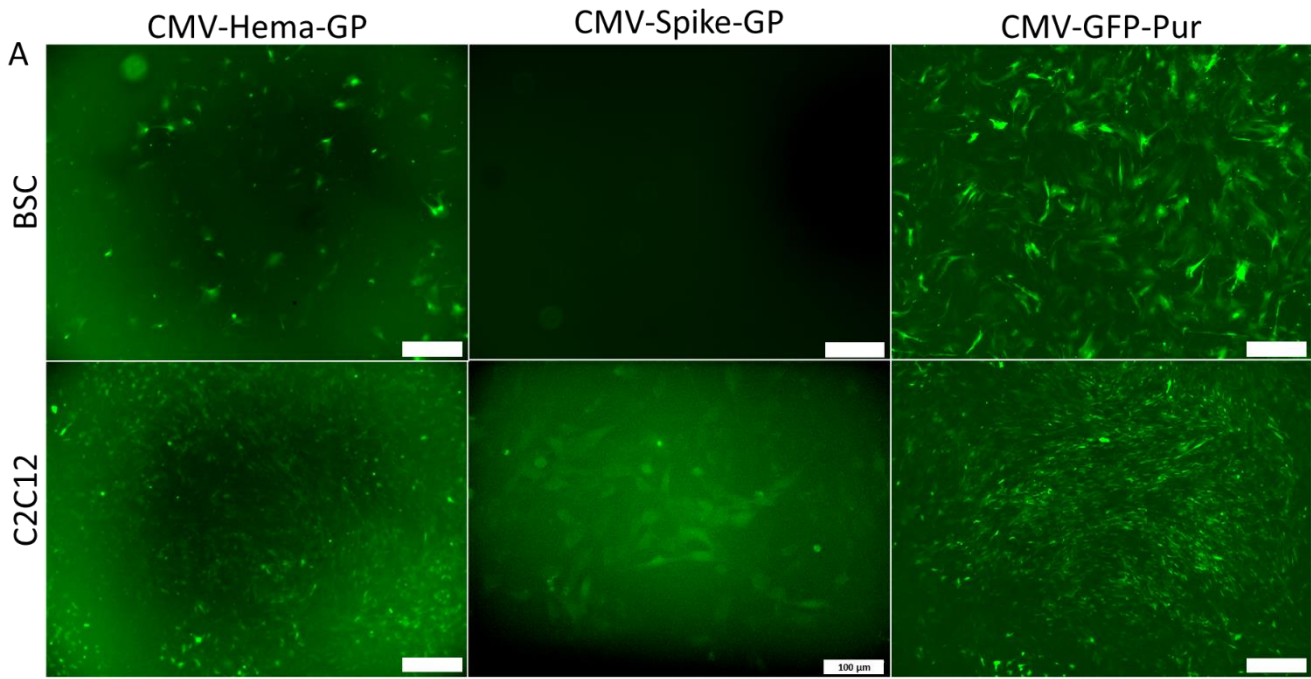


Figure 3. Green fluorescent protein reporter gene fluorescence in live cultures of engineered primary bovine satellite cells (BSCs) and C2C12 murine myoblasts. A) Representative photos of engineered cultures expanded and stored to cryobank. B) Representative images of cultures with low cell counts or strange morphology that were present in replicate wells after transfection and not stored or used for further experiments. Scale bars = 500  $\mu\text{m}$  unless otherwise noted.

#### 4.3. Immunostaining for antigen presence

Immunostaining results indicated the presence of the hemagglutinin protein in both BSCs and C2C12s (Fig. 4A) engineered with the CMV-Hema-GP insert. Spike glycoprotein expression was also seen in the BSCs and C2C12s engineered with the CMV-Spike-GP insert (Fig. 4B). No signal for either hemagglutinin or spike glycoprotein was present in the CMV-GFP-Pur control cultures, as expected. Low expression of GFP was seen in both BSCs and C2C12s with the CMV-Spike-GP insert as was seen in the transfection confirmation GFP imaging. In one subset of the BSCs cultures engineered with the CMV-Hema-GP insert, an unexpected morphology was seen after differentiation (Fig. 5). These cells have morphology more akin to that expected for fibroblasts, and though clearly engineered to express hemagglutinin, this sample was not used for further experimentation as fibroblasts were not the target cell type for this study.

#### 4.4. Immunostaining for cell identity and maturity

For all engineered cultures, Pax7 expression localized to the nuclei was detected during proliferation via immunostaining, a confirmation of the muscle precursor identity of the cells in culture (Fig. 6). For the C2C12 cultures with the CMV-Spike-GP insert, Pax7 staining is present in the nuclei of the cells in culture, but also shows some non-specific binding in what may be spontaneously forming myotubes in the culture based on appearance. The reason for this was not determined in this study. MHC expression was detected in both BSC and C2C12 cultures engineered with the CMV-Hema-GP insert, but only the C2C12 cultures containing the CMV-Spike-GP insert, and not the BSCs, showed observable expression of MHC via immunostaining (Fig. 7).

#### 4.5. ELISA quantification of hemagglutinin

No reliable ELISA results were obtained due to assay failures as determined by the R-squared values of the standard curves being below an acceptable threshold.

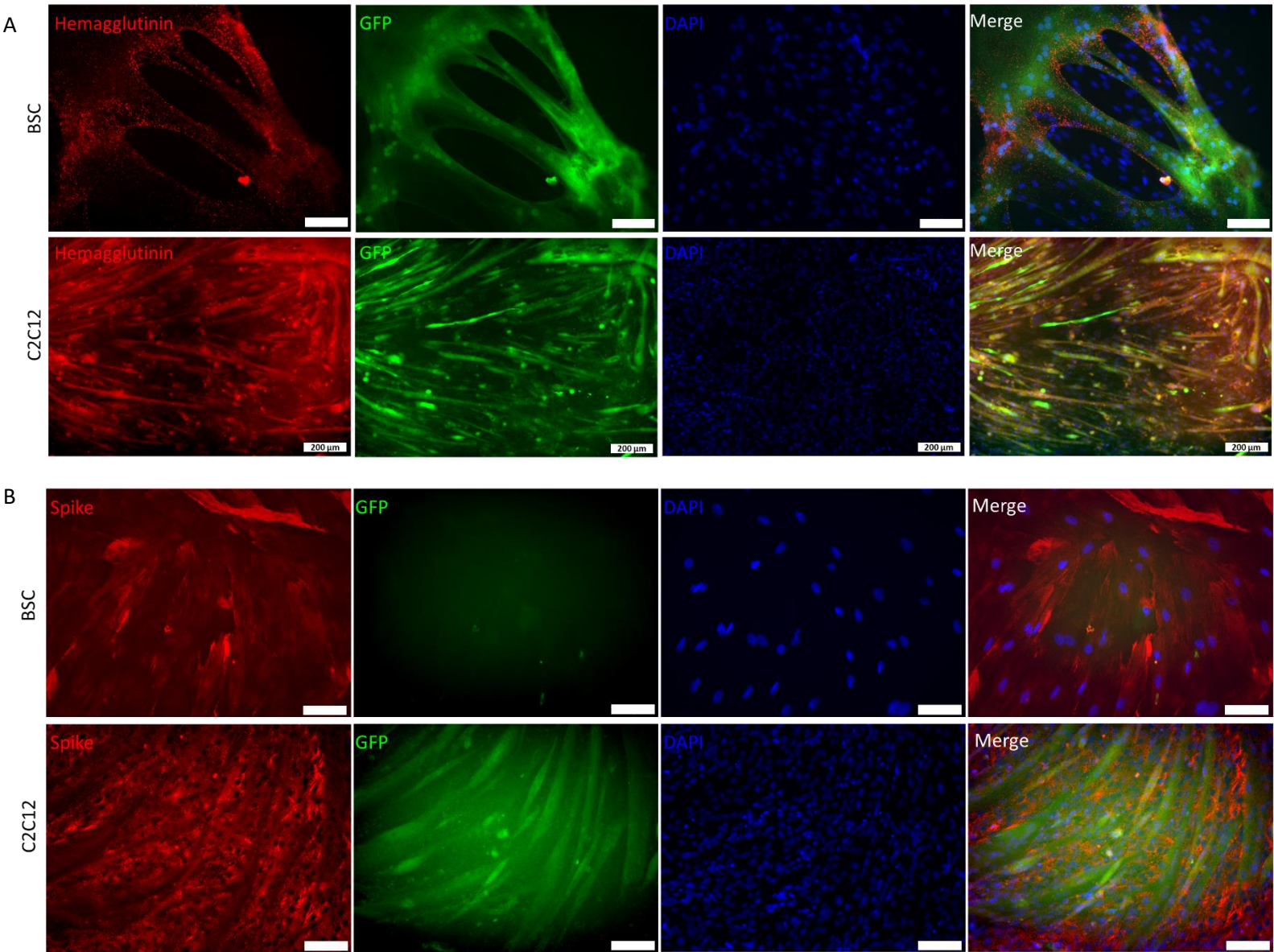


Figure 4. Immunostaining for antigen expression (red) in bovine satellite cells (BSCs) and C2C12 murine myoblasts engineered with the CMV-Hema-GP (A) or CMV-Spike-GP (B) insert seven days after initiating differentiation. The cultures were counterstained with DAPI, a nuclei stain (blue) and exhibit endogenous fluorescence from green fluorescent protein expression (green). Scale bars=100μm unless otherwise noted.

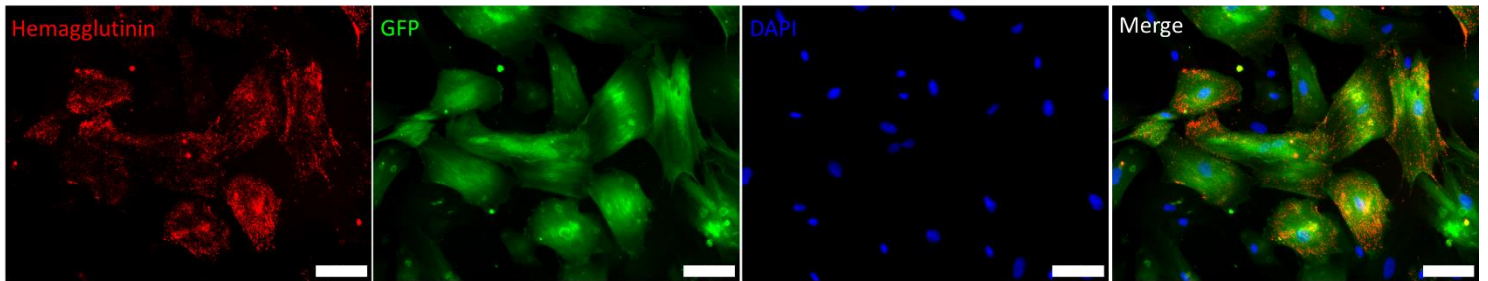


Figure 5. Immunostaining for antigen expression (red) in bovine satellite cells (BSCs) with the CMV-Hema-GP insert seven days after initiating differentiation. The cultures were counterstained with DAPI, a nuclei stain (blue) and exhibit endogenous fluorescence from green fluorescent protein expression (green). Morphology of this culture does not match replicate cultures of engineered BSCs at this stage of growth. Morphology shown more closely matches expected shape of fibroblasts in culture. Due to these stains, this set of cells was not used for further experiments. Scale bars=100 $\mu$ m

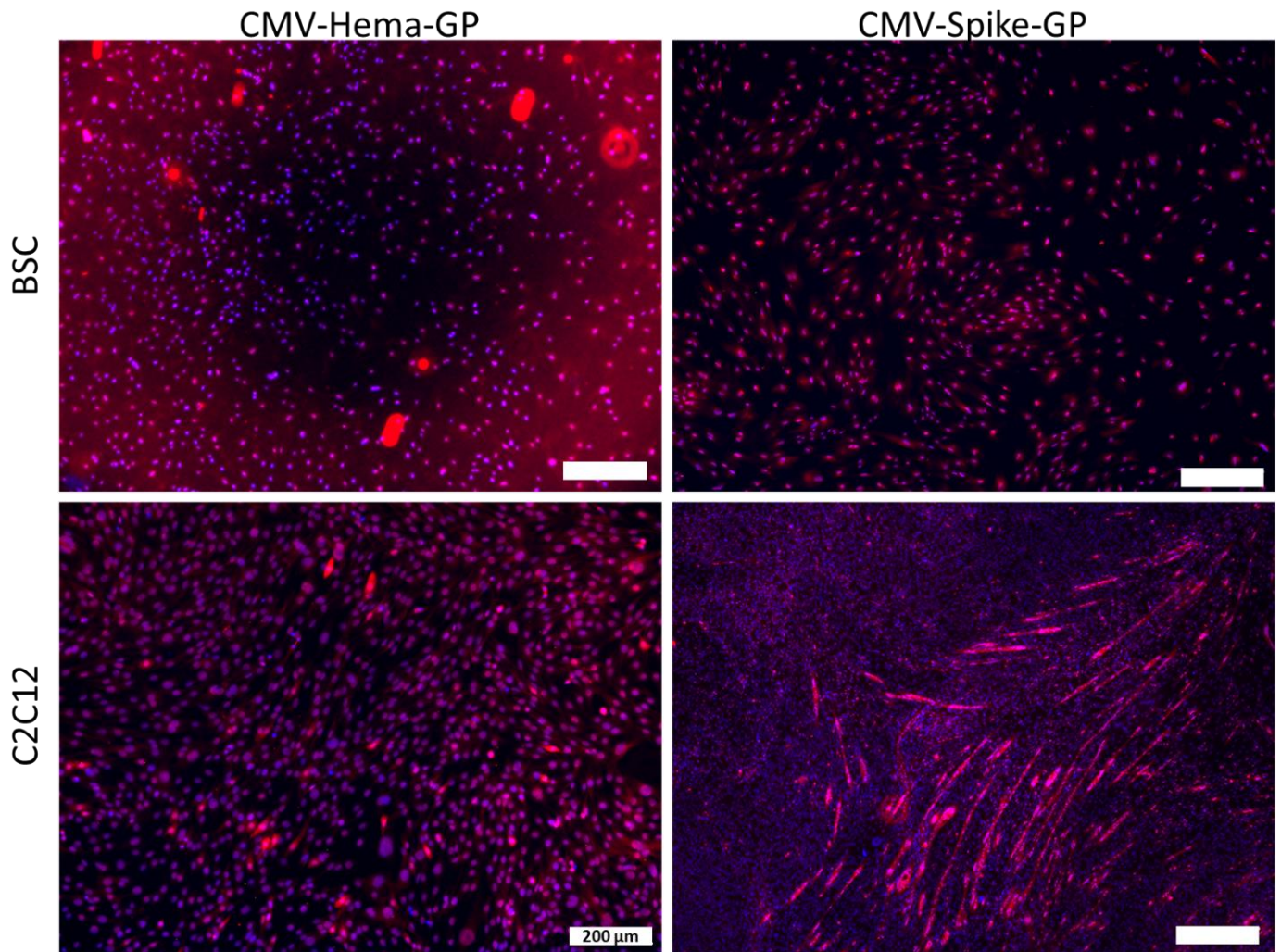


Figure 6. Bovine satellite cells (BSCs) and C2C12 murine myoblast cultures engineered with CMV-Hema-GP or CMV-Spike-GP inserts stained for the presence of Pax7 (red), a muscle precursor protein marker, and counterstained with DAPI (blue), a nuclei stain. Stains co-localize on the nuclei as expected, appearing magenta at the overlap of signal. Scale bars=500 $\mu$ m unless otherwise noted

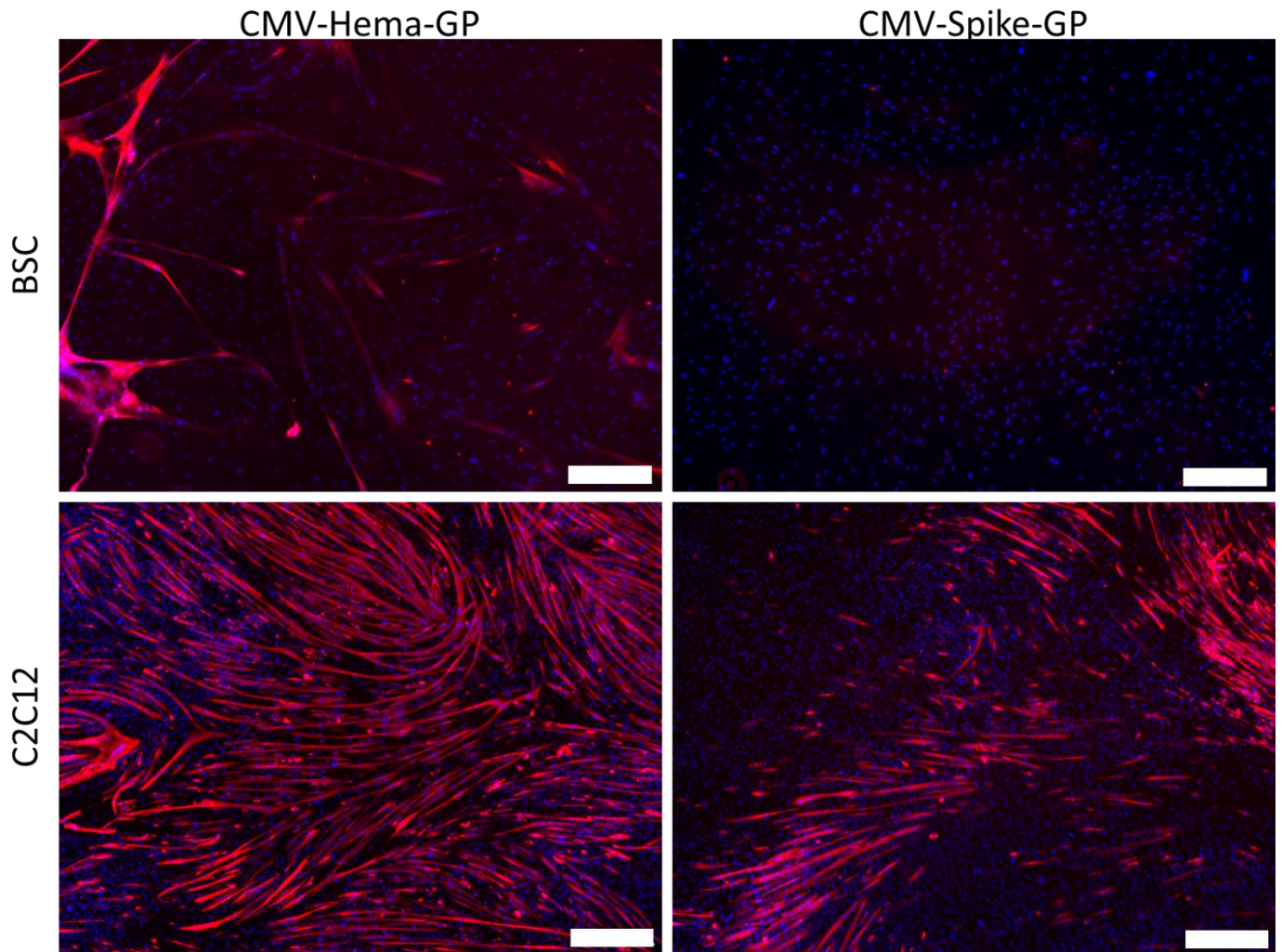


Figure 7. Bovine satellite cells (BSCs) and C2C12 murine myoblast cultures engineered with CMV-Hema-GP or CMV-Spike-GP inserts stained for the presence of MHC (red), a marker of mature muscle myotubes and counterstained with DAPI (blue), a nuclei stain. Scale bars=500 $\mu$ m

## 5. Discussion

This study intended to show that mammalian muscle cells could be engineered to express viral antigens with the necessary conformation to bind to antigen-specific antibodies. It also intended to measure the antigen expression by these engineered cells. In pursuing these objectives, successes were achieved in genetic engineering, however the quantification of the engineering results remains elusive.

### 5.1 Plasmid construction

To address the first objective of this study to construct the necessary plasmids containing the antigen engineered cassettes, PCR gel results allowed the selection of the best amplified components prior to the assembly. For the hemagglutinin cassette, PCR products “PSB 2.2”, the *Sleeping Beauty* backbone plasmid segment, and “Hema 1.1”, the hemagglutinin gene containing fragment, were chosen for assembly (Fig 1A). For the initial spike protein plasmid assembly attempt, PCR products “PSB1.1” and “Spike1.2” (Fig 1B) were chosen. A second attempt to assemble the spike plasmid also used PCR products from its respective amplification labeled “PSB1.1” and “Spike 1.2” (Fig 1C). Successful assembly as evidenced by Sanger sequencing was achieved from these selected amplified solutions.

Implications: In showing that these viral genes can be assembled into plasmids utilizing the assembly methods described here, the results imply that the genes for other viral antigenic proteins could be similarly assembled into transposable cassettes using this method. Multiple antigens could likely be incorporated into the same assembly providing multivalent coverage for strains of a single virus or even coverage of multiple different virus types in a single transgenic culture.

## 5.2. Transposon-based engineering of BSCs and C2C12s

Prior research showed that the *Sleeping Beauty* transposon system was capable of engineering BSCs to express carotenoids<sup>47</sup>, to provide added nutritional benefits to cultivated meat. As such, this system was chosen as the platform to engineer both primary bovine muscle stem cells and immortalized mouse muscle stem cells to express therapeutic compounds; one of two viral antigens. For both the Influenza B hemagglutinin antigen as well as the SARS-COV-2 spike glycoprotein antigen, this system was successfully able to insert cassettes containing the genes for these proteins into the genome of both stem cell types, as shown by the expression of the reporter GFP and survival in the selective media, achieving the goal of the second objective of this project.

Implications: The successful incorporation of multiple viral antigenic proteins into two different species of mammalian muscle cells is an important finding. This implies that the success of this engineering method is not specific to antigen identity, nor the origin species of the cells. This study also showed that the nature of the cells, be they primary or an immortalized line, did not impact the capacity for successful engineering and expression. These findings show that this method has the potential to be adapted for other antigen proteins and provide protection against a wide variety of pathogens, as well as be utilized across a broader range of cell lines and primary cultures from many other species than were used in this study. As production of cultivated meat from a broader range of species is optimized over time, more efficient and cost-effective bulk production may be developed for species other than the two used in this study, and this could occur with primary cell lines or immortalized lines. The engineering results shown here do not suggest these differences are a limit for viral antigen production.

### 5.3. Immunofluorescent labeling of engineered proteins

An important consideration for this project was whether viral proteins expressed in mammalian cells would achieve the conformational folding necessary to be effective as an antigen. This relies on the proteins having the proper formation needed for antibody binding, an essential component of the immune response needed to confer immune protection. Immunofluorescent staining relies on the binding of primary antibodies specific to a given protein, therefore the successful use of antibody staining for the antigens in these engineered cells implies that these transgenic proteins are not only expressed and present in both cell types, but that they are at least as conformationally structured as is necessary to bind with antibodies and would potentially have the capacity to trigger an immune response if used as a therapeutic.

Implications: Mechanisms of protein expression vary widely in such disparate organisms as mammals and viruses. Accurate expression requires that the amino acid chain is not only fully translated, but also folded into the necessary three-dimensional structure. Antibodies rely on being able to bind to epitope sites, regions of proteins that are only present with proper folding. By using commercial antibodies with binding specificity to the native viral proteins and showing that they can also bind to the transgenic viral proteins produced by the mammalian cells, especially after amino-acid optimization for expression in mammal cells, it can be posited that antibodies in the human immune system have the potential to also bind to these proteins and subsequently trigger an immune response that would identify and provide protection against the native pathogen. Though the epitope binding that occurred in the staining described here may not match the binding sites typically targeted by conventional vaccination, further study may elucidate the validity of this claim.

#### 5.4 Confirming cell identity and differentiation capacity

Given that the long-term concept for these cells is to serve as an oral therapeutic via cultivated meat, it is important that the engineered cells analyzed in this study have the presumed identity (i.e., muscle cells), and retain the capacity to differentiate into mature muscle tissue. Cell identity of the engineered cultures was confirmed by staining for the presence of Pax7, a protein involved in myogenic regulation of muscle stem cells. The cells also retained their differentiation capacity, as evidenced by the presence of MHC, a protein expressed in mature muscle myotubes, in staining of cultures that had been induced to differentiate by serum starvation. The lack of observable MHC in the BSC cultures engineered with the CMV-Spike-GP may indicate that the engineering impacted the capacity of these cells to mature and could require re-engineering in the hope of the insert occurring at a less impactful site in the BSC genome. It could alternatively indicate that the production of the spike glycoprotein slows the overall maturation rate of the cultures and that these cells would still mature, but at a longer time scale than the bounds of the work done in this study. Further, efforts to achieve enough cell doublings to have a sufficient cell bank for the needed experiments may have led to cell senescence, limiting the differentiation potential of the cells. Ultimately, more experiments would be needed to evaluate this further.

Implications: Cultures of muscle stem cells can often contain populations of fibroblasts which, if present, could be engineered in the same manner as the muscle cells, allowing them to survive selection and grow in subsequent cultures. As the intended use for the engineered cells in this project is conceptualized to be a meat-based vaccine, ensuring the cultures are the expected cell type is important, and the muscle cell specific Pax7 antibody binding shown here validates that for the cultures in this study, though some cultures did show some morphology that suggested fibroblast presence, however upon discovery were not used for any further experiments. It will

also likely be important as a consumer product to produce structured muscle tissue rather than stem cell mass to truly be considered meat. The engineering process used for this study involves random insertions of genetic material into the source cell genome, and this could potentially disrupt the capacity for the cells to properly differentiate. The presence of MHC as shown by staining in all but the spike expressing BSCs indicates that the other cultures retain the ability to differentiate and were not impacted in this way by the engineering, but that this remains an important consideration in further study of this concept.

### 5.5 ELISA quantification of antigen expression with a stability challenge

Therapeutics generally have an optimal effective dosage necessary to confer their therapeutic effects, with the quantity of hemagglutinin used in a typical intramuscular flu shot being around  $15\mu\text{g}$ <sup>57</sup>. As such, measuring the antigen concentration produced in engineered cell tissue is an important consideration, as the volume of oral therapeutic meat needed to attain a therapeutically relevant dose of the antigen would need to be reasonable for consumption. This could be affected by the preparation method of the meat prior to this consumption, hence the need to test a real-world use case in which the meat would be cooked prior to this consumption. However, ELISA measurements of the hemagglutinin levels both before and after cooking of the cells containing the CMV-Hema-GP insert were not able to produce reliable results at the time of writing due to assay failures. This remains an important point which will need to be determined for this concept to move forward.

### 5.6 Challenges during this project

After selecting a representative sample of transformed colonies for sequencing, initial Sanger sequencing attempts for all plasmid constructs were not able to validate successful

assembly. It is likely that the colonies selected contained backbone plasmids that had re-circularized to themselves, carrying antibiotic resistance that allowed the resulting *E.coli* colonies to survive, but not the antigen gene insert of interest. However, subsequent transformation experiments with the same assembly products provided at least one colony per attempt that did show valid assembly via sequencing results and were used for the proceeding steps in this study. In further attempts to perform these assembly methods with these types of fragments, the sequencing of a higher number of transformed colonies may provide a better chance of getting a success earlier in the process.

Transfection of muscle cell cultures to express the spike protein was greatly prolonged by experimental failures and the proceeding troubleshooting steps taken. Engineering of the hemagglutinin expressing cells proceeded without issue, with all attempts to transfect the cells providing cultures that robustly expressed GFP after puromycin selection, and these cultures were used throughout the rest of the project. However, initial engineering efforts with the spike protein cassette resulted in death of the muscle cell cultures after puromycin selection. The initial theory was that the transfection with Lipofectamine had failed, so a second transfection attempt was made, which again resulted in the same die-off of the cultures after switching to selective media. The protocol was then changed to utilize electroporation-based transfection. However, this too resulted in the same die off after selection. Troubleshooting further back in the process, the spike protein containing plasmid was re-assembled using Golden Gate assembly rather than the Gibson assembly protocol that had been performed for all prior assembly up to that point. After sequencing the resulting plasmid to show proper incorporation of the spike protein gene with this alternative assembly method, transfection experiments proceeded only to result in the same die off at the selection stage. At this point, the underlying *Sleeping Beauty* plasmid backbone came into

question. Due to all the experiments failing to survive the puromycin condition, the spike cassette containing plasmid from the Golden Gate assembly was sequenced for the puromycin gene and results showed that it was not intact. This was not expected, as the same source for the backbone plasmid had been used for the hemagglutinin engineering without issue. As a result, a fresh sample of purified backbone plasmid was isolated from a different stock of the *E.coli* in which the backbone plasmid was propagated. The spike containing plasmid was then assembled and sequenced for both the spike gene incorporation and for the puromycin gene. Results from this showed both were present and transfections proceeded, successfully producing the spike protein expressing muscle cells used in the rest of the project. The issue occurring at the earliest stages of the process, along with the successes of the hemagglutinin work using the same material unfortunately obscured the problem, leading to troubleshooting backwards though the process trying to pinpoint the issue. Experimental troubleshooting for similar endeavors may benefit from more thorough validation of the materials being utilized at each stage to prevent similar occurrences.

Attempts to probe for hemagglutinin concentration by ELISA were unable to provide the quantitative data desired due to a failure to produce valid standard curves. Initial attempts were performed using a hemagglutinin antibody pair and self-prepared reagents, as well as self-coating 96 well assay plates with the primary hemagglutinin capture antibody. After multiple attempts failed to produce valid standard curves, a hemagglutinin-specific ELISA kit was acquired, and the experiments repeated. With the kit containing pre-prepared reagents and an assay plate pre-coated with the capture antibody, it was expected that any user error involved in the failures with the antibody-pair procedure would not influence the results and allow usable data to be gathered. However, multiple attempts with this kit also failed to provide valid standard curves to which data

could be fit. User error remains a likely culprit for these failures and perhaps a more practiced hand with this assay protocol would be better able to get results from this method, however time and material constraints limited further attempts at the time of writing.

## 5.7 Regulatory and Consumer Acceptance

Regulatory challenges may prove to be a larger hurdle than the scientific challenges in the development of a consumer ready edible vaccine based on cultivated meat. Despite the prevalence of genetically engineered plant food products available in the US, at the time of writing, only two transgenic animal lines are approved for consumption in the US, an Atlantic Salmon engineered for faster growth and a pig engineered to remove an allergen<sup>58</sup>. Currently, there are no cultivated meat products approved for sale in the US<sup>59</sup>, and regulatory approval of therapeutics is notoriously difficult<sup>60</sup>. A transgenic cultured-beef product containing therapeutic compounds would need to overcome not just one, but all these regulatory hurdles, and would have to gain acceptance by not only the FDA, but also the USDA<sup>59</sup>. Yet there are promising signs for regulatory acceptance of cultivated meat products in the US, with recent announcements from the FDA that, after pre-market consultation, there are no further questions concerning the safety of products from California-based cultivated meat companies Upside Foods and GOOD Meat Inc<sup>61,62</sup>. As the cultivated meat industry leads the push for regulatory approval of these products, they will open the door for a meat-based vaccine and may provide valuable lessons through their efforts.

Consumer acceptance may be as significant a hurdle for these vaccines as regulatory approval in the US. A review of global studies over the past decade asking whether participants would try a cultivated meat product varied in response from as low as 5-11%, up to over 63%<sup>63</sup>. Even at 63% willingness, a great deal of hesitancy is implied for the average consumer, and these studies did not even consider GMO cultivated meat, with GMO food independently having its own

consumer acceptance issues<sup>64</sup>. Add to this the inevitable marketing concerns of therapeutics purposefully incorporated into these edible products, and the problem may be even further exacerbated. Yet as the cultivated meat industry becomes more established and can point to official safety approval of their products, along with a rise in consumer familiarity with these products as they come to market, favorable views of an edible cultivated meat vaccine may rise in tandem.

## **6. Future work**

In the near term there are multiple additional avenues of research that could be explored using the cells engineered in this project. ELISA quantification of antigen levels needs to be further explored for both the hemagglutinin expressing cells and the spike protein expressing cells. Antigen stability, bioavailability experiments, and expression optimization would also be beneficial for the progress of this concept. Longer term, a more fully developed vaccine would benefit from testing with disease challenges in animal models.

### **6.1 ELISA quantification of antigen levels**

Quantification of the antigen levels produced by the cells remains one of the most critical pieces of information that should be determined for the engineered lines generated by this study. Additional attempts with commercial kits to generate this data should prove fruitful, as well as an exploration of alternate antibodies for capture and tagging of the antigen proteins. Therapeutic dosage will be an important parameter for the use of these cells as a commercial vaccine, with hemagglutinin injectable vaccines holding ~15ug of antigen per strain in each dose. This would likely need to be higher for an oral vaccine as a lower proportion of the antigen will be expected to make contact with immune cells after digestion compared to an injectable formulation.

### **6.2 Further evaluate the results generated by this study**

The results described here generated additional points of interest that would be useful to explore. The lack of GFP expression in the BSCs engineered with the CMV-Spike-GP insert, despite survival of this culture in the selective agent and validation of expression of the antigen, could be explored further. This could include experiments with a longer culture time to determine if these engineered cells require longer to differentiate, staining for earlier markers of

differentiation such as MyoD or myogenin, and sequencing of the engineered cells to try and determine the insert site and any relevant genes at that region that may have been impacted.

The staining study showed a wide variety of antigen locations within the cells across the engineered inserts and cell types, so a more in-depth exploration of this may help determine how these proteins are being packed, aggregated, and/or excreted by the various engineered lines. This could be useful for determining how protected and accessible the antigens may be within the structure of the cell.

### 6.3 Testing for stability of the antigens as an oral therapeutic

Given that the long-term intent of these cells is to be used as an edible vaccine, further testing of the impact of cooking, as well as an exploration of the impact of digestion on the antigen proteins could be explored. To test for the ability of the expressed antigens to remain stable after passing through the digestive tract to the small intestine, a simulated in vitro gastric fluid digestion could be performed as previously described<sup>43</sup> by exposing engineered cells to HCl and pepsin to simulate gastric digestion, then a pancreatin-bile solution to simulate intestinal digestion. ELISA quantification of antigen levels before and after the digestion could indicate the impact of this challenge.

### 6.4 Determine the bioavailability of the recombinant antigens after digestion

Were the antigen to remain stable after cooking and digestion, it would still need to bypass the intestinal mucous membrane and subsequently trigger an immune response. To explore this capacity, the antigens could be challenged to bypass a model of the intestinal mucous membrane consisting of Caco-2 intestinal cells (ATCC #HTB-37) cultured to confluency in a Transwell plate system (Corning #3398, Corning, NY, USA)<sup>33</sup>. Antigen solutions could be dosed on one side of the membrane, then the supernatant on the other side could be measured via ELISA for the

presence of the antigen. Immunostaining of the Caco-2 cells after this process would also provide information as to whether the cells uptake the antigen to any degree.

#### 6.5 Testing for immune stimulation

One of the most pivotal aspects of the recombinant antigens that will need to be determined is the capacity to trigger an immune response to confer immunity. This could be evaluated by a modified antibodies from lymphocyte supernatants (ALS) assay performed as previously described<sup>65</sup>, by exposing unfractionated peripheral blood mononuclear cells (PBMCs) in culture to the produced antigens and testing for antibody production via protein assays. B lymphocytes in the PBMC population present antibodies, and upon binding with an antigen, these cells produce additional antibodies which could be measured by this assay.

#### 6.6 Increasing antigen expression and alternative engineering

Antigen expression levels will have a substantial impact on resources required to produce pharmacologically relevant quantities of antigen via the proposed methods. To increase this expression, cultures of these engineered cells could be grown in the presence of higher concentrations of puromycin to select for cells expressing the inserted genes at higher levels, with the assumption that higher expression of the puromycin resistance gene confers survivability at higher concentrations and that the higher expression of this gene will be directly related to the expression level of the antigen gene. Alternative methods of engineering to increase the copy number of the inserted cassette, as well as using engineering techniques such as CRISPR for directed genome integration to safe harbor locations within the host genome, could both aid in increasing expression levels of the antigen genes. In addition, an exploration of alternative promoters to the CMV used in this study may reveal promoters that provide more efficient

expression of a given antigen gene as well as promoters that may be more optimal for a given species.

#### 6.7 Peptide engineering for increased stability

The stability of a protein in a given environment is highly influenced by the bonds holding the three-dimensional structure of the protein. Small changes in the amino-acid sequence of proteins have been shown to provide better stability of this structure under certain environmental challenges<sup>66</sup>. By applying these techniques via altering the inserted antigen gene prior to cell transfection, modified antigens could be expressed that could have better low-pH and high heat stability, and potentially also have more resistance to proteolytic activity as well, reducing the need or volume of protective additives necessary in the formulation of a final vaccine product.

#### 6.8 Animal study challenge

After more thorough development, cultured meat vaccines will need to move beyond *in vitro* analysis to *in vivo* drug trials in animal models. This could be in the form of a mouse study in which the engineered meat could be fed to the mice both with and without an infection challenge, measuring metabolism by the host as well as the parameters of the intended immune protection. These tests would help determine effective dosages, efficacy, longevity, and vaccine stability. The impact of protective additives, as well as immune stimulating adjuvants, could also be explored in the complex *in vivo* environment, which may reveal aspects that could not be captured by *in vitro* experiments. Results from studies like this would direct the further development of this concept towards the goal of an edible cultivated meat vaccine.

## 7. References

1. O'Hagan, D. T. & Valiante, N. M. Recent advances in the discovery and delivery of vaccine adjuvants. *Nat Rev Drug Discov* **2**, 727–735 (2003).
2. Heath, W. R., Kato, Y., Steiner, T. M. & Caminschi, I. Antigen presentation by dendritic cells for B cell activation. *Curr Opin Immunol* **58**, 44–52 (2019).
3. Jung, C., Hugot, J.-P. & Barreau, F. Peyer's Patches: The Immune Sensors of the Intestine. *Int J Inflam* **2010**, 1–12 (2010).
4. Kumar, B. V., Connors, T. J. & Farber, D. L. Human T Cell Development, Localization, and Function throughout Life. *Immunity Reviews* **48**, 202–213 (2018).
5. Renegar, K. B., Small, P. A., Boykins, L. G. & Wright, P. F. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. *J Immunol* **173**, 1978–1986 (2004).
6. Smith, N. *et al.* Distinct systemic and mucosal immune responses during acute SARS-CoV-2 infection. *Nature Immunology* *2021 22:11* **22**, 1428–1439 (2021).
7. de Smet, R., Allais, L. & Cuvelier, C. A. Recent advances in oral vaccine development: Yeast-derived  $\beta$ -glucan particles. *Hum Vaccin Immunother* **10**, 1309 (2014).
8. Vaillant, A. A. J. & Grella, M. J. *Vaccine (Vaccination)*. *StatPearls* (StatPearls Publishing, 2022).
9. Zhang, L., Wang, W. & Wang, S. Effect of vaccine administration modality on immunogenicity and efficacy. *Expert Rev Vaccines* **14**, 1509–1523 (2015).
10. United States Centers for Disease Control and Prevention. How Flu Viruses Can Change: Drift and Shift. Available at: <https://www.cdc.gov/flu/about/viruses/change.htm>. (2021). (Accessed: 11th November 2021)
11. United States Centers for Disease Control and Prevention. How Influenza (Flu) Vaccines Are Made. Available at: <https://www.cdc.gov/flu/prevent/how-fluvaccine-made.htm>. (2021). (Accessed: 11th November 2021)
12. United States Centers for Disease Control and Prevention. Vaccine Administration Route and Site. Available at: <https://www.cdc.gov/vaccines/hcp/admin/administer-vaccines.html>. (2021). (Accessed: 11th November 2021)
13. Kim, J. & Jesus, O. De. *Medication Routes of Administration*. *StatPearls* (StatPearls Publishing, 2023).
14. Criscuolo, E. *et al.* Alternative Methods of Vaccine Delivery: An Overview of Edible and Intradermal Vaccines. *J Immunol Res* **2019**, (2019).

15. Thielmann, A., Puth, M. T., Kersting, C., Porz, J. & Weltermann, B. Vaccine cold chain in general practices: A prospective study in 75 refrigerators (Keep Cool study). *PLoS One* **14**, (2019).
16. Kar, S. *et al.* Oral and intranasal vaccines against SARS-CoV-2: Current progress, prospects, advantages, and challenges. *Immun Inflamm Dis* **10**, (2022).
17. Taddio, A. *et al.* Survey of the prevalence of immunization non-compliance due to needle fears in children and adults. *Vaccine* **30**, 4807–4812 (2012).
18. Larson, H. J., Jarrett, C., Eckersberger, E., Smith, D. M. D. & Paterson, P. Understanding vaccine hesitancy around vaccines and vaccination from a global perspective: A systematic review of published literature, 2007–2012. *Vaccine* **32**, 2150–2159 (2014).
19. Shah, V. *et al.* Pharmacological and Combined Interventions to Reduce Vaccine Injection Pain in Children and Adults: Systematic Review and Meta-Analysis. *Clin J Pain* **31**, S38 (2015).
20. Holmgren, J. & Czerkinsky, C. Mucosal immunity and vaccines. *Nature Medicine* **11**:4 **11**, S45–S53 (2005).
21. Vela Ramirez, J. E., Sharpe, L. A. & Peppas, N. A. Current state and challenges in developing oral vaccines. *Adv Drug Deliv Rev* **114**, 116 (2017).
22. Kurup, V. M. & Thomas, J. Edible Vaccines: Promises and Challenges. *Mol Biotechnol* **62**, 79 (2020).
23. Tashima, T. Delivery of Orally Administered Digestible Antibodies Using Nanoparticles. *Int J Mol Sci* **22**, (2021).
24. Takaiwa, F. Seed-based oral vaccines as allergen-specific immunotherapies. *Hum Vaccin* **7**, 357–366 (2011).
25. United States Food and Drug Administration. Vaccines Licensed for Use in the United States. Available at: <https://www.fda.gov/vaccines-blood-biologics/vaccines/vaccines-licensed-use-united-states>. (2021). (Accessed: 11th November 2021)
26. Barr Labs Inc. HIGHLIGHTS OF PRESCRIBING INFORMATION - Adenovirus Type 4 and Type 7. Available at: [https://www.amlc.army.mil/Portals/73/Documents/Adenovirus% 20Package% 20Insert.pdf?ver=2020-03-04-141425-743](https://www.amlc.army.mil/Portals/73/Documents/Adenovirus%20Package%20Insert.pdf?ver=2020-03-04-141425-743). (2020). (Accessed: 10th April 2023)
27. Emergent Travel Health Inc. HIGHLIGHTS OF PRESCRIBING INFORMATION - VAXCHORA. Available at: <https://www.fda.gov/media/128415/download>. (2022). (Accessed: 10th April 2023)
28. Merck Sharp & Dohme Corp. Package Insert - RotaTeq. Available at: <https://www.fda.gov/media/75718/download>. (2006). (Accessed: 10th April 2023)

29. GlaxoSmithKline Biologicals. Package Insert and Patient Information - ROTARIX. Available at: <https://www.fda.gov/media/163009/download>. (2008). (Accessed: 10th April 2023)
30. Crucell Switzerland LTD. Vivotif Package Insert USA. Available at: <https://www.fda.gov/media/75988/download>. (2013). (Accessed: 10th April 2023)
31. Lund, P. A. *et al.* Understanding How Microorganisms Respond to Acid pH Is Central to Their Control and Successful Exploitation. *Front Microbiol* **11**, 2233 (2020).
32. Beasley, D. E., Koltz, A. M., Lambert, J. E., Fierer, N. & Dunn, R. R. The Evolution of Stomach Acidity and Its Relevance to the Human Microbiome. *PLoS One* **10**, (2015).
33. Hubatsch, I., Ragnarsson, E. G. E. & Artursson, P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nature Protocols* **2007** 2:9 **2**, 2111–2119 (2007).
34. Amin, K., Rae, M., Artiga, S., Young, G. & Ramirez, G. Where do Americans get vaccines and how much does it cost to administer them? - Peterson-KFF Health System Tracker. <https://www.healthsystemtracker.org/chart-collection/where-do-americans-get-vaccines-and-how-much-does-it-cost-to-administer-them/#item-start>.
35. Waheed, M. T. *et al.* Need of cost-effective vaccines in developing countries: What plant biotechnology can offer? *Springerplus* **5**, 1–9 (2016).
36. Griffiths, U. *et al.* Costs of delivering COVID-19 vaccine in 92 AMC countries. Updated estimates from COVAX Working Group on delivery costs. (2021) Available at: <https://www.who.int/publications/i/item/10665337553>. (Accessed: 11th November 2021)
37. New, R. R. C. Formulation technologies for oral vaccines. *Clin Exp Immunol* **198**, 153 (2019).
38. Livovsky, D. M., Pribic, T. & Azpiroz, F. Food, Eating, and the Gastrointestinal Tract. *Nutrients* **12**, (2020).
39. Sensoy, I. A review on the food digestion in the digestive tract and the used in vitro models. *Curr Res Food Sci* **4**, 308 (2021).
40. Gullberg, E. & Söderholm, J. D. Peyer's patches and M cells as potential sites of the inflammatory onset in Crohn's disease. *Ann N Y Acad Sci* **1072**, 218–232 (2006).
41. Magacz, M., Kędziora, K., Sapa, J. & Krzyściak, W. The Significance of Lactoperoxidase System in Oral Health: Application and Efficacy in Oral Hygiene Products. *Int J Mol Sci* **20**, 1443 (2019).
42. Ferraboschi, P., Ciceri, S. & Grisenti, P. Applications of Lysozyme, an Innate Immune Defense Factor, as an Alternative Antibiotic. *Antibiotics* **10**, (2021).

43. Yun, S., Habicht, J. P., Miller, D. D. & Glahn, R. P. An in vitro digestion/Caco-2 cell culture system accurately predicts the effects of ascorbic acid and polyphenolic compounds on iron bioavailability in humans. *J Nutr* **134**, 2717–2721 (2004).
44. Fu, Z., Akula, S., Thorpe, M. & Hellman, L. Marked difference in efficiency of the digestive enzymes pepsin, trypsin, chymotrypsin, and pancreatic elastase to cleave tightly folded proteins. *Biol Chem* **402**, 861–867 (2021).
45. Nascimento, I. P. & Leite, L. C. C. Recombinant vaccines and the development of new vaccine strategies. *Brazilian Journal of Medical and Biological Research* **45**, 1102 (2012).
46. Zhao, M. *et al.* Affinity chromatography for vaccines manufacturing: Finally ready for prime time? *Vaccine* **37**, 5491–5503 (2019).
47. Stout, A. J., Mirliani, A. B., Soule-Albridge, E. L., Cohen, J. M. & Kaplan, D. L. Engineering carotenoid production in mammalian cells for nutritionally enhanced cell-cultured foods. *Metab Eng* **62**, 126–137 (2020).
48. Rubio, N. R., Xiang, N. & Kaplan, D. L. Plant-based and cell-based approaches to meat production. *Nat Commun* **11**, (2020).
49. Rubio, N. R., McCartney, N. E., Trimmer, B. A. & Kaplan, D. L. Biofabrication with insect cells. *Trends Entomol* **16**, (2020).
50. Post, M. J. *et al.* Scientific, sustainability and regulatory challenges of cultured meat. *Nature Food* 2020 1:7 **1**, 403–415 (2020).
51. Mattick, C. S., Landis, A. E., Allenby, B. R. & Genovese, N. J. Anticipatory Life Cycle Analysis of In Vitro Biomass Cultivation for Cultured Meat Production in the United States. *Environ Sci Technol* **49**, 11941–11949 (2015).
52. Saigol, L. & Keown, C. Is cell-based meat the next big thing? Here are 5 companies leading the revolution. MarketWatch. Available at: <https://www.marketwatch.com/story/is-cell-based-meat-the-next-big-thing-here-are-5-companies-leading-the-revolution-2020-10-06>. (2020). (Accessed: 11th November 2021)
53. Mátés, L. *et al.* Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. *Nature Genetics* 2009 41:6 **41**, 753–761 (2009).
54. Aronovich, E. L., McIvor, R. S. & Hackett, P. B. The Sleeping Beauty transposon system: a non-viral vector for gene therapy. *Hum Mol Genet* **20**, R14 (2011).
55. Falowo, A. B., Fayemi, P. O. & Muchenje, V. Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. *Food Res Int* **64**, 171–181 (2014).
56. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 2012 9:7 **9**, 671–675 (2012).

57. United States Food and Drug Administration. Sanofi Pasteur 450/477 Fluzone® Quadrivalent. Available at: <https://www.fda.gov/media/119856/download>. (2021). (Accessed: 11th November 2021)
58. United States Food and Drug Administration. Intentional Genomic Alterations (IGAs) in Animals. Available at: <https://www.fda.gov/animal-veterinary/biotechnology-products-cvm-animals-and-animal-food/intentional-genomic-alterations-igas-animals> (Accessed: 10th April 2023)
59. United States Food and Drug Administration. Human Food Made with Cultured Animal Cells. Available at: <https://www.fda.gov/food/food-ingredients-packaging/human-food-made-cultured-animal-cells> (Accessed: 10th April 2023)
60. Forum on Neuroscience and Nervous System Disorders; Board on Health Sciences Policy; Institute of Medicine. *Improving and Accelerating Therapeutic Development for Nervous System Disorders: Workshop Summary*. Washington (DC): National Academies Press (US); 2014 Feb 6. 2, Drug Development Challenges. Available at: <https://www.ncbi.nlm.nih.gov/books/NBK195047/> (Accessed 10<sup>th</sup> April 2023)
61. United States Food and Drug Administration. FDA Completes First Pre-Market Consultation for Human Food Made Using Animal Cell Culture Technology. Available at: <https://www.fda.gov/food/cfsan-constituent-updates/fda-completes-first-pre-market-consultation-human-food-made-using-animal-cell-culture-technology>. (Accessed 10<sup>th</sup> April 2023)
62. United States Food and Drug Administration. FDA Completes Second Pre-Market Consultation for Human Food Made Using Animal Cell Culture Technology. Available at: <https://www.fda.gov/food/cfsan-constituent-updates/fda-completes-second-pre-market-consultation-human-food-made-using-animal-cell-culture-technology>. (Accessed 10<sup>th</sup> April 2023)
63. Bryant, C. & Barnett, J. Consumer acceptance of cultured meat: A systematic review. *Meat Sci* **143**, 8–17 (2018).
64. Sendhil, R. *et al.* Genetically modified foods: bibliometric analysis on consumer perception and preference. *GM Crops Food* **13**, 65 (2022).
65. Kirkpatrick, B. D. *et al.* Comparison of the Antibodies in Lymphocyte Supernatant and Antibody-Secreting Cell Assays for Measuring Intestinal Mucosal Immune Response to a Novel Oral Typhoid Vaccine (M01ZH09). *Clin Diagn Lab Immunol* **12**, 1127 (2005).
66. Teufl, M., Zajc, C. U. & Traxlmayr, M. W. Engineering Strategies to Overcome the Stability-Function Trade-Off in Proteins. *ACS Synth Biol* **11**, 1030–1039 (2022).