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A cell bank paradigm for preclinical evaluation of an analogous cellular product for an allogeneic cell therapy

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Abstract

Toward the translation of allogeneic cell therapy products, cell banks are needed not only to manufacture the final human product but also during the preclinical evaluation of an animal-based analogous cellular product (ACP). These cell banks need to be established at both the master cell bank (MCB) level and the working cell bank (WCB) level. Inasmuch as most of the development of cell therapy products is at academic centers, it is imperative that academic researchers understand how to establish MCBs and WCBs within an academic environment. To illustrate this process, using articular cartilage as the model, a cell bank for an ACP was developed (MCBs at passage 2, WCBs at passage 5) to produce self-assembled neocartilage for preclinical evaluation (constructs at passage 7). The cell bank system is estimated to be able to produce between 160 000 and 400 000 constructs for each of the six MCBs. Overall, the ACP cell bank yielded constructs that are analogous to the intended human product, which is critical toward conducting preclinical evaluations of the ACP for inclusion in an Investigational New Drug application to the FDA.

1. Introduction

While cell therapy products can be used to treat a wide variety of indications, to date, only 32 products categorized as cellular and gene therapy (CGT) products have been approved by the Food and Drug Administration [1]. The lack of approved CGT products can be partially attributed to the challenging regulatory environment and lack of precedent within the field, as recently reviewed [2]. Products that rely upon living cells for production have proven to be difficult to translate due to challenges in manufacturing, reproducibility, storage, funding, lack of infrastructure, and regulatory ambiguity.

In the case of cell therapy products that use an allogeneic cell source (accounting for 13 of the 32 currently approved CGT products [1]), cell banks can be used to manufacture reproducible batches of the product at large scales [3]. Historically, cell banks have been utilized to aid in the manufacturing process of medical-grade biopharmaceuticals and vaccines such as rabies vaccine, polio vaccine, lymphoblastoid interferon, recombinant tPA, and the immunosuppressant drug OKT3 [4]. As novel allogeneic cell therapy products are developed and translated, the use of cell banks to manufacture cell therapies will likely grow in prevalence. However, currently, much of the research that leads to novel CGT products is conducted at academic universities [5], where there is a lack of infrastructure to manufacture clinical-grade cell therapy products. In the university setting, researchers often conduct basic science for publication purposes. While basic science may be preclinical in nature it is not always applicable for inclusion in an FDA submission. To conduct preclinical evaluations that may be included in an FDA Investigational New Drug (IND) submission the studies must be of a certain caliber, and the requirements are often unclear to academic researchers. One such challenge is that when an allogeneic cell therapy is in development, a cell bank must be established at the ‘Master Cell Bank’ (MCB) [6] level and ‘Working Cell Bank’ (WCB) [6] level for both the final human clinical product and the preclinical evaluation of the product. To facilitate the
translation of allogeneic cell therapy products more effectively, the methods for establishing a cell bank system for IND-enabling studies should be further defined within the literature.

The FDA defines analogous cellular products (ACPs) as ‘cellular products derived from the animal species used for testing that are analogs of the ultimate clinical product in phenotype and biologic activity’ [7]. While the safety and activity of certain cell-based products may be successfully assessed through the transplantation of human cell-based products into an immunosuppressed animal model, preclinical evaluation may also require the development of an ACP. For example, the FDA’s ‘Guidance for Industry: Preparation of IDEs and INDs for Products Intended to Repair or Replace Knee Cartilage’ specifies preclinical evaluation in large animal models [8], which are generally not immunosuppressed. Accordingly, Matrix-induced Autologous Chondrocyte Implantation (MACI) described the use of ACPs in their released pharmacology/toxicology review with rabbit and horse studies conducted using species-specific cells rather than human cells [9]. Despite this precedent, in general, more guidance is needed on how ACPs should be manufactured, qualified, and used in animal studies. In addition to having analogous phenotype and biologic activity, analogy between the human product and ACP would include a parallel manufacturing structure. Therefore, in the case of allogeneic products where a cell bank would be used to manufacture the final human product, a cell bank system should be employed for the production of ACPs as well.

The FDA has described a cell bank as a ‘collection of cells of uniform composition derived from a single source’ [6]. Cell banks may be used at different stages of the translational vector as illustrated in figure 1(A). A primary objective of a cell bank is to ensure an ‘adequate supply of equivalent cells’ for the life of the product [3]. Figure 1(B) illustrates a cell bank in a two-tiered system as described by the FDA [3, 6, 10]. Cells are isolated from a source and cryopreserved at a low passage in a ‘Master Cell Bank’ (MCB) [6] in a uniform collection of ampoules. The MCB is subjected to quality control (QC), such as screening for microbiological agents (e.g. sterility, endotoxin, mycoplasma, and adventitious agents), confirming cell identity, assessing purity, and validating the functional output (i.e. potency) of the cell population [11]. An ampoule of the MCB can be further expanded through a defined number of passages and aliquoted into a secondary collection of uniform ampoules known as a ‘Working Cell Bank’ (WCB) [3, 10]. Provided the MCB has been thoroughly characterized, WCB QC can be more limited in nature, requiring that the cells be checked for contaminants that may have been introduced during culture, using sterility and in vitro adventitious agent testing [3, 6, 10]. Cell growth kinetics as quantified by population doubling level or passage level of cell banks and end-of-production cells should be defined [6]. The FDA does not provide guidance on the limit to the extent to which cells can be expanded and manufacturing information on current cellular and gene therapy products is difficult to obtain. However, there is precedent for using highly passaged cells as long as tumorigenicity and chromosomal aberrations are not present. For example, a banked chicken cell line described in US Patent 5672485 has been accepted beyond passage 160 for the production of food-grade meat products [12, 13]. A two-tiered cell bank system that utilizes passaged cells allows for the long-term preservation of a consistent, comprehensively characterized cell source to enable the generation of numerous product batches with high uniformity [14].

Often, there is a substantial gap in manufacturing requirements between academic research and clinical-grade cell therapy production. However, since many CGT products are developed in the academic setting [5], there is a need to streamline the process of translating academic discoveries to the clinic. In an academic setting, cell banking practices are often small-scale and constrained by the resources available (e.g. monetary, personnel, and temporal). Clinical-grade cell lines usually are neither feasible nor necessary for basic science. For example, costly adventitious agent testing typically is unnecessary for cells intended to be used in basic research only and not for implantation. Thus, the contrast between traditional academic cell storage and clinical-grade quality cell banks can be substantial, but bridging this gap by the development of an intermediary cell bank solution for ACPs can facilitate the translation of academic research. Because ACPs should mimic the intended human product to the fullest extent possible, when designing an ACP cell bank, analogous parameters and methods should be used to the intended human cell bank (e.g. passage number, bioactive factors utilized, standard operating procedures). If structured and documented appropriately, an ACP cell bank may be utilized for both basic research and preclinical evaluations of a potential product, which could aid in transitioning cell therapies from academic research through the translational vector.

Biologic variability poses a significant challenge to the translation of biologic research [15, 16]. Due to concerns about biologic variability, basic research usually requires the use of multiple donors to ensure that findings are applicable to the population at large rather than individual donors. Pooled populations of cells have been used to increase experimental output while obtaining a representative cellular response [17]. However, pooled donor populations are not accepted for manufacturing human cellular products [18]. Considering the safety of an implant, using cells from a single donor reduces the risk of disease transfer and immune response. Moreover, for
A biologic product manufacturing, it may be desirable to identify a highly functional donor (i.e. cells that have exceptional functional properties for a particular application) to obtain the highest clinical efficacy of the product, further motivating cell banking at the single donor level. Therefore, there is a fundamental difference between the pooled donor cell banks often used in academic research versus the single donor cell banks used in manufacturing therapeutics.

The objective of this work is to describe a paradigm for developing an ACP cell bank including both MCBs and WCBs for conducting preclinical evaluation of a product toward the submission of an IND application. This study was motivated by prior work in the temporomandibular joint, which demonstrated that self-assembled neocartilage is tolerated immunologically (i.e. rare evidence of macrophage immunoreactivity and no evidence of multinucleated giant cells, neutrophils, eosinophils, or capsule formation) and improves the functional repair (i.e. 4.4 times more complete defect closure and 3.4-fold stiffer repair tissue) of the temporomandibular joint disc [19]. The ACP cell bank developed in this study is intended to generate self-assembled neocartilage for examination in the Yucatan minipig, toward addressing the indication of repairing articular cartilage defects. This study harnessed recent advances in the use of growth factors and aggregate culture to rejuvenate the chondrogenic phenotype of highly passaged chondrocytes [20] to establish a cell bank.

Figure 1. (A). Cell banks used throughout the translational vector. (B). The cell bank system described by the FDA. Cell banks generally consist of a two-tiered system that includes a master cell bank (MCB) and working cell bank (WCB) that are both subjected to quality control (QC). (C). The analogous cellular product (ACP) cell bank developed for generating self-assembled neocartilage. Six minipig (MP) donors were used, and MCBs were generated from each donor at passage 2. MCBs were then validated, and donors were selected for inclusion in a pooled WCB and single donor WCB at passage 5.
system for cartilage tissue engineering applications. Specifically, we have demonstrated that chondrocytes passaged up to P11 can be used to generate self-assembled neocartilage with functional properties on par with low passage chondrocytes [20]. MCBs were established at passage 2, WCBs were established at passage 5, and constructs that are analogous to the intended human product (i.e. human neocartilage from extensively passaged juvenile costal chondrocytes) were generated at passage 7. The concept of identifying a highly functional donor for a specific indication (i.e. articular cartilage repair) was demonstrated. In addition, a cell bank consisting of pooled donors was generated to reduce biological variability during basic research and development. Altogether, the development of an ACP cell bank for IND-enabling studies will aid in the translation of self-assembled neocartilage implants by using analogous manufacturing techniques to the intended human product.

2. Results

2.1. Structure of the ACP cell bank

A cell bank as described by the FDA [3, 6] is depicted in figure 1(B), and the ACP cell bank generated in this study is depicted in figure 1(C). While the ACP cell bank uses the same general framework as cell banks described by the FDA, for this study, cells from multiple donors are assessed and selected for inclusion in (1) single donor and (2) pooled donor WCBs. Specifically, cells were isolated from six juvenile minipig donors (5–7 months old, three male, three female) and expanded to generate MCBs derived from each donor. We estimate that the juvenile cells used in this study correspond to approximately 10–12 years of age in the human [21]. It has been reported that in the human, chondrocytes isolated from donors under 13 years of age had greater proteoglycan production and collagen type II and type IX gene expression than adult donors [22]. Therefore, selection criteria of the minipig donors mimic those of the human donors. The MCBs were used to generate constructs and evaluated in terms of morphology, histology, biochemistry, and mechanical properties. The flow chart for selecting MCB donors to be used in WCB production is illustrated in figure 2. All MCB donors that generated constructs that had an acceptable morphology were included in the pooled WCB. A highly functional donor was selected using a functionality index, as described previously [23–25]. The functionality index is a weighted average of the primary release criteria for the intended human product (i.e. aggregate modulus and Young’s modulus) where a functionality index value closer to 1 is considered more similar to native tissue. The functionality index equation is given below

\[
\text{Functionality index} = \frac{1}{2} \left[ \left(1 - \frac{E_{\text{Eng}}^C - E_{\text{Nat}}^C}{E_{\text{Nat}}^C}\right) + \left(1 - \frac{E_{\text{Eng}}^T - E_{\text{Nat}}^T}{E_{\text{Nat}}^T}\right) \right]
\] (1)

In this equation, \(E_{\text{Nat}}^C\) is the aggregate modulus of native tissue, \(E_{\text{Eng}}^C\) is the aggregate modulus of engineered tissue, \(E_{\text{Nat}}^T\) is the tensile Young’s modulus of native tissue, \(E_{\text{Eng}}^T\) is the tensile Young’s modulus of engineered tissue. The MCB that yielded cells with the functionality index closest to 1 was used to generate a single donor WCB. The FDA requires specific testing during the development of a biologic product including viability, sterility, mycoplasma,
adventitious agents, purity via endotoxin, potency, and primary release criteria. While previous studies have used a functionality index to describe both biochemical and mechanical properties of neocartilage [23–25], the functionality index used here is intended to combine only the quantitative primary release criteria (i.e. mechanical properties) into a single value. This overall structure is parallel to the intended human cell bank to be used in clinical trials and product manufacturing.

2.2. Validation of MCBs
As shown in figure 3, all constructs displayed a round flat morphology, without evidence of notable curling, folding, or balling that can manifest in high passage constructs [20]. All quantitative data are listed in

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Figure 3. Validation of master cell banks. (A) Gross morphology, hematoxylin and eosin (H&E) staining, picrosirius red staining, and safranin-O staining of each group. (B) Tensile characteristics and (C) compressive characteristics of constructs. Different uppercase letters indicate statistically significant differences among groups ($p < 0.05$, $n = 5–7$ per group). Absence of letters or same letters indicate no significant differences. 'MP' denotes minipig, 'UTS' denotes ultimate tensile strength.
ascending donor from minipigs 1 through 6 unless otherwise stated, and minipig donors are abbreviated as MP (e.g. MP1 represents minipig donor 1). The thicknesses of the constructs were 1.01 ± 0.18 mm, 0.42 ± 0.03 mm, 0.60 ± 0.07 mm, 0.89 ± 0.07 mm, 0.72 ± 0.10 mm, and 0.81 ± 0.10 mm. Diameters of the constructs were 6.20 ± 0.14 mm, 5.32 ± 0.08 mm, 6.33 ± 0.24 mm, 6.36 ± 0.11 mm, 6.47 ± 0.38 mm, and 6.17 ± 0.25 mm. Histologically, the constructs generated from MP2 had cells that appeared to be smaller than those from the other donors. Moreover, lighter hematoxylin and eosin (H&E), safranin-O, and picrosirius red staining was observed on MP2 constructs, and lacunae were not evident. MP1 MP3, MP4, MP5, and MP6 had relatively consistent histological staining.

The biochemistry results are summarized in table 1. Notably, constructs from MP2 had a significantly higher percent DNA to dry weight than the constructs from MP1 (p = 0.003), MP3 (p = 0.01), MP4 (p = 0.02), and MP5 (p = 0.02). The percent glycosaminoglycan (GAG) per wet weight from MP2 constructs was significantly lower than other donors being only 3.1 ± 1.7%, while others ranged from 5.3% to 8.2%.

The functional mechanical properties of constructs from each donor are shown in figure 2. No statistically significant differences were observed in terms of Young’s modulus and ultimate tensile stress (UTS). Specifically, Young’s modulus for constructs generated from each donor was 0.9 ± 0.6 MPa, 1.0 ± 0.4 MPa, 2.3 ± 1.3 MPa, 1.7 ± 0.9 MPa, 1.6 ± 1.1 MPa, and 0.8 ± 0.2 MPa. UTS was 0.3 ± 0.1 MPa, 0.7 ± 0.6 MPa, 0.5 ± 0.3 MPa, 0.4 ± 0.2 MPa, 0.4 ± 0.2 MPa, and 0.4 ± 0.1 MPa, respectively, but no significant differences were detected. Strain-at-failure was 0.4 ± 0.2, 1.0 ± 0.8, 0.3 ± 0.2, 0.3 ± 0.2, 0.4 ± 0.2, and 0.6 ± 0.2 with MP2 being significantly higher than MP3 (p = 0.03). In terms of compressive properties, the aggregate modulus values of the constructs generated were 173 ± 53 kPa, 42 ± 19 kPa, 368 ± 138 kPa, 225 ± 20 kPa, 224 ± 134 kPa, and 281 ± 46 kPa. MP2 constructs had significantly lower aggregate modulus values than all constructs from donors except for MP1 (p < 0.0001 MP3, p = 0.01 MP4, p = 0.01 MP5, p = 0.001 MP6). Additionally, MP3 had a significantly higher aggregate modulus value than MP1 (p = 0.01). Shear modulus values were 86 ± 27 kPa, 19 ± 9 kPa, 147 ± 38 kPa, 108 ± 20 kPa, 101 ± 56, and 127 ± 32 kPa. The shear modulus of constructs from MP2 was significantly lower than constructs from all other donors. No significant differences were observed in permeability, and values were 32 ± 26 10−15 m² s−1 N−1, 41 ± 40 10−15 m² s−1 N−1, 41 ± 33 10−15 m² s−1 N−1, 30 ± 23 10−15 m² s−1 N−1, 28 ± 18 10−15 m² s−1 N−1, and 19 ± 15 10−15 m² s−1 N−1.

2.3. Sex-related differences
While the objective of this study was not to assess sex as a biologic variable (SABV), post hoc analysis of the data was used to determine the number of donors that would be required to detect sex-related differences. Post hoc power analysis of the aggregate modulus data found that an n = 128 would be needed to detect a difference between the male and female donors with a power of 0.8 and an alpha of 0.05 (male mean = 194 kPa, standard deviation = 164 kPa; female mean = 243 kPa, standard deviation = 32 kPa). The same calculation with UTS values yielded an n = 38 (male mean = 0.49 MPa, standard deviation = 0.24 MPa; female mean = 0.36 MPa, standard deviation = 0.01 MPa). Power analysis of Young’s modulus values indicated that an n = 2.28 million would be needed to detect sex-related differences (male mean = 1.37 MPa, standard deviation = 0.78 MPa; female mean = 1.37 MPa, standard deviation = 0.47 MPa). Other parameters yielded similarly high n values to detect sex-related differences.

2.4. Donor selection results
Based on the intended indication of the human product (i.e. articular cartilage repair), release criteria for the ACP will be (1) morphology of the construct and (2) mechanical properties of the construct. First, for a cell source to be validated for a given application, it must be able to produce the desired morphology (i.e. flat constructs that can be implanted into a cartilage defect). Cells from all MCBs produced constructs with the desired flat morphology and, thus, were included in the pooled working cell bank. For the mechanical criterion, a highly functional donor for the single donor working cell bank was identified by evaluating the weighted average of functional release criteria (i.e. aggregate modulus and Young’s modulus) compared to native tissue. Using equation (1) and adult minipig condyle cartilage values of 450 kPa aggregate modulus and 13.3 MPa Young’s modulus, the functionality index of MP1 was 0.22, MP2 was 0.08, MP3 was 0.49, MP4 was 0.31, MP5 was 0.31, and MP6 was 0.34. While these values can be further enhanced (i.e. brought closer to 1) through the application of bioactive factors, this value represents how closely constructs generated from each donor mimic native articular cartilage before additional stimuli are applied. Comparing these values, MP3 had the highest functionality index value and was selected as the donor with the highest inherent functional output. Therefore, MP3 was used to generate the single donor cell bank.

2.5. Validation of WCBs
WCBs were validated both with and without the use of bioactive factors. Bioactive factors were used to
Table 1. Phase 1 biochemistry results. Different uppercase letters indicate statistically significant differences among groups ($p < 0.05$, $n = 6$ per group). Absence of letters or same letters indicate no significant differences.

<table>
<thead>
<tr>
<th>Master Cell Bank Donor</th>
<th>Sex</th>
<th>Water Content (%)</th>
<th>DNA/Wet Weight (%)</th>
<th>DNA/Dry Weight (%)</th>
<th>GAG/Wet Weight (%)</th>
<th>GAG/Dry Weight (%)</th>
<th>GAG/DNA ($\mu g/\mu g$)</th>
<th>Collagen/Wet Weight (%)</th>
<th>Collagen/Dry Weight (%)</th>
<th>Collagen/DNA ($\mu g/\mu g$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>86 ± 2 A,B</td>
<td>0.01 ± 0.01 B</td>
<td>0.04 ± 0.04 B</td>
<td>5.3 ± 0.4 C</td>
<td>17.4 ± 3.4</td>
<td>555 ± 262 A</td>
<td>0.9 ± 0.2</td>
<td>2.8 ± 0.5 B</td>
<td>92 ± 50 A</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>87 ± 1 A</td>
<td>0.04 ± 0.02 A,B</td>
<td>0.33 ± 0.27 A</td>
<td>3.1 ± 1.7 D</td>
<td>28.1 ± 20.8</td>
<td>115 ± 99 C</td>
<td>0.8 ± 0.4</td>
<td>6.6 ± 3.7 A</td>
<td>25 ± 14 B</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>82 ± 1 C</td>
<td>0.03 ± 0.02 A,B</td>
<td>0.08 ± 0.05 B</td>
<td>8.2 ± 0.9 A</td>
<td>24.6 ± 7.1</td>
<td>415 ± 231 A,B</td>
<td>1.0 ± 0.2</td>
<td>2.9 ± 0.5 B</td>
<td>51 ± 36 A,B</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>85 ± 1 B,C</td>
<td>0.03 ± 0.01 A,B</td>
<td>0.09 ± 0.05 B</td>
<td>5.9 ± 0.8 B,C</td>
<td>18.7 ± 8.8</td>
<td>235 ± 84 B,C</td>
<td>0.9 ± 0.5</td>
<td>2.5 ± 1.1 B</td>
<td>34 ± 20 B</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>82 ± 1 C</td>
<td>0.04 ± 0.01 A,B</td>
<td>0.09 ± 0.02 B</td>
<td>7.1 ± 0.5 A,B</td>
<td>17.6 ± 2.7</td>
<td>203 ± 27 B,C</td>
<td>0.9 ± 0.1</td>
<td>2.3 ± 0.2 B</td>
<td>27 ± 5 B</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>83 ± 2 C</td>
<td>0.04 ± 0.01 A</td>
<td>0.18 ± 0.08 A,B</td>
<td>6.9 ± 0.6 A,B,C</td>
<td>26.4 ± 7.9</td>
<td>159 ± 23 B,C</td>
<td>0.6 ± 0.4</td>
<td>2.1 ± 1.3 B</td>
<td>15 ± 1 B</td>
</tr>
</tbody>
</table>
enhance the functional properties through a regimen of TGF-β1, chondroitinase-ABC (C-ABC), and lysyl oxidase like 2 (TCL) stimulation, as previously described [26]. The constructs generated from both the single donor and pooled donor WCBs were flat in morphology. The single donor WCB produced constructs that were thicker and larger in diameter than constructs generated from the pooled WCB. Dimensions of the constructs generated were 0.38 ± 0.03 mm, 0.44 ± 0.07 mm, 0.27 ± 0.04 mm, and 0.19 ± 0.02 mm in thickness and 5.54 ± 0.09 mm, 5.43 ± 0.08 mm, 4.84 ± 0.10 mm, and 4.43 ± 0.09 mm in diameter; data presented in the following order: single donor unstimulated, single donor TCL, pooled donor unstimulated, pooled donor TCL.

Values obtained from the biochemical assays are included in table 2. Two-factor analysis of variance (ANOVA) was used to analyze data with cell bank (i.e. single vs. pooled) and treatment (unstimulated or TCL) as factors. The effect of cell bank and the effect of TCL were both significant factors with regard to hydration (p = 0.01 and p = 0.02, respectively). Using the same statistical method on other biochemical data, DNA per dry weight was also significantly affected by both factors (p = 0.001 and p = 0.005 for the cell bank and TCL factors, respectively). However, DNA per wet weight only was significantly impacted by the effect of TCL (p = 0.0001). GAG content was significantly affected by cell bank when normalized by either wet weight (p = 0.0002) or dry weight (p = 0.01). When normalized to DNA, GAG was significantly affected by TCL only (p = 0.0001). Collagen content was significantly affected by both cell bank (p < 0.0001) and TCL (p < 0.0001) when normalized to wet weight but only TCL when normalized to dry weight (p = 0.0005). When normalized to DNA, collagen was significantly affected by both cell bank (p = 0.006) and TCL (p = 0.002).

Mechanical properties of the constructs (figure 4) were also influenced by cell bank and application of TCL treatment. The effect of TCL significantly affected Young’s modulus (p = 0.01). UTS was significantly affected by both TCL (p = 0.0001) and cell bank (p = 0.003). However, neither factor significantly affected strain-at-failure. In terms of compression data, the effect of cell bank significantly affected aggregate modulus (p < 0.0001), shear modulus (p < 0.0001), and permeability (p = 0.01).

Based on adult minipig condyle cartilage properties of 450 kPa aggregate modulus and 13.3 MPa Young’s modulus, the functionality index for the groups were 0.23 for unstimulated single donor, 0.35 for TCL single donor, 0.16 for unstimulated pooled donors, and 0.18 for TCL pooled donors. While these functionality index values indicate that additional bioactive factors or stimuli (e.g. fluid-induced shear [27]) may be needed to bring values closer to 1 for knee articular cartilage repair, the functional properties of the constructs are similar to articular cartilage in other joints. For example, for the cervical facet joint, with an aggregate modulus of 243 kPa and Young’s modulus of 6.7 MPa in the minipig [28], the constructs achieve a functionality index of 0.42, 0.67, 0.31, 0.35 for single unstimulated, single TCL, pooled unstimulated, and pooled TCL, respectively. We have previously demonstrated that an implant with a functionality index of 0.42 was able to regenerate the temporomandibular joint disc [19]. Therefore, the functionality index values observed in the current study indicate that the constructs may be implantable for the indication of facet repair but may need further enhancement for the indication of knee repair.

2.6. Resources expended and potential construct yield of cell bank system
To achieve a cell bank of the magnitude described here, a notable resource expenditure was required. An estimated 700 person-hours and 1500 flasks were needed to establish the MCBs and WCBs alone, without accounting for cell bank validation steps. The cumulative continuous cell culture time to establish the six MCBs was approximately 6 months. Establishing the two WCBs required an additional 3 months of continuous culture. Cell bank validation steps for MCBs and WCBs accounted for an additional 5 months of continuous culture. Overall, the resource requirements were notably higher than in standard academic studies.

Though a large number of resources were required to generate this cell bank system, the cell bank is expected to result in multiple studies toward the translation of self-assembled neocartilage. Specifically, between 42 and 104 ampoules were generated per MCB at passage 2 with a concentration of 15 million cells per vial, depending on cell yield from each donor. Each ampoule is capable of generating a WCB of an estimated 64 vials at passage 5 assuming a four-fold expansion factor. Each vial within the WCB could generate approximately 60 constructs at passage 7 assuming a four-fold expansion factor, 50% yield out of aggregate rejuvenation [20], and 2 million cells per construct. With these assumptions, we estimate that the MCBs produced in this study could yield between 160 000 and 400 000 5 mm diameter constructs at passage 7. Therefore, the cell banks described here have long-term usability for both basic science and preclinical evaluation of the ACP.

3. Discussion
The objective of this study was to develop a cell bank for an analogous animal product (i.e. analogous cellular product or ACP) to support the translation of a potential allogeneic cell therapy from the academic
Table 2. Phase 2 biochemistry results. Different uppercase letters indicate statistically significant differences among groups ($p < 0.05$, $n = 7$ per group). Absence of letters or same letters indicate no significant differences. ‘TCL’ denotes treated with TGF-$eta_1$, C-ABC, and lysyl oxidase like 2.

<table>
<thead>
<tr>
<th>Working Cell Bank</th>
<th>Treatment</th>
<th>Water Content (%)</th>
<th>DNA/Wet Weight (%)</th>
<th>DNA/Dry Weight (%)</th>
<th>GAG/Wet Weight (%)</th>
<th>GAG/Dry Weight (%)</th>
<th>GAG/DNA (µg/µg)</th>
<th>Collagen/Wet Weight (%)</th>
<th>Collagen/Dry Weight (%)</th>
<th>Collagen/DNA (µg/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>Unstimulated</td>
<td>87 ± 2 A</td>
<td>0.03 ± 0.03 B</td>
<td>0.12 ± 0.08 B</td>
<td>3.4 ± 0.7 B</td>
<td>12.8 ± 2.3 B</td>
<td>179 ± 103 A</td>
<td>1.2 ± 0.2 B</td>
<td>4.6 ± 1.2 C</td>
<td>61 ± 33 A</td>
</tr>
<tr>
<td>Single</td>
<td>TCL</td>
<td>81 ± 4 B</td>
<td>0.07 ± 0.02 A,B</td>
<td>0.27 ± 0.11 B</td>
<td>5.7 ± 1.8 A</td>
<td>21.9 ± 8.4 A,B</td>
<td>82 ± 17 A,B</td>
<td>2.6 ± 0.5 A</td>
<td>10.2 ± 3.4 A,B</td>
<td>39 ± 8 A,B</td>
</tr>
<tr>
<td>Pooled</td>
<td>Unstimulated</td>
<td>88 ± 4 A</td>
<td>0.03 ± 0.03 B</td>
<td>0.39 ± 0.51 B</td>
<td>3.4 ± 1.1 B,C</td>
<td>38.7 ± 16.2 A</td>
<td>204 ± 128 A</td>
<td>0.7 ± 0.2 B</td>
<td>7.5 ± 2.0 A,B</td>
<td>43 ± 24 A</td>
</tr>
<tr>
<td>Pooled</td>
<td>TCL</td>
<td>87 ± 4 A</td>
<td>0.09 ± 0.03 A</td>
<td>1.26 ± 0.68 A</td>
<td>1.6 ± 1.2 C</td>
<td>23.8 ± 19.3 A,B</td>
<td>20 ± 17 B</td>
<td>0.9 ± 0.3 B</td>
<td>11.9 ± 5.2 A</td>
<td>10 ± 2 B</td>
</tr>
</tbody>
</table>
Figure 4. Validation of working cell banks. (A) Gross morphology, hematoxylin and eosin (H&E) staining, picrosirius red staining, and safranin-O staining of each group. (B) Tensile characteristics and (C) compressive characteristics of constructs. Different uppercase letters indicate statistically significant differences among groups ($p < 0.05$, $n = 5–7$ per group). Absence of letters or same letters indicate no significant differences. 'MP' denotes minipig, 'UTS' denotes ultimate tensile strength, 'Unstim' denotes unstimulated, 'TCL' denotes treated with TGF-$eta$1, C-ABC, and lysyl oxidase like 2.

setting to clinical trials. An ACP composed of animal cells may be required for IND-enabling studies to demonstrate the safety and activity of a product in a non-immunosuppressed animal model. According to the FDA, the ACP should mimic the characteristics and manufacturing process used to generate the final human cellular product [7]. To illustrate this process, an ACP cell bank for self-assembled neocartilage was developed, which may be used as a model to guide future cell therapy development. Chondrocytes were passaged using previously developed conservative chondrogenic passaging methods [20] and used to generate an ACP cell bank. MCBs were frozen at passage 2, and WCBs were frozen at passage 5.
MCB cells were screened to identify a highly functional donor with superior functional output to be utilized in the single donor WCB, which is analogous to the intended human product. Concurrently, all donors that generated morphologically appropriate constructs were included in a pooled WCB that could be used for basic research. This ACP cell bank structure supports translational efforts in advancing a cell therapy product from academic research to clinical trials.

Clinically used allogeneic cell therapy products are derived from a single human donor to decrease the risk of disease transmission and immune rejection. The FDA has released guidance for making donor-eligibility determinations for human donors [29], but the same criteria have not been outlined for animal-derived ACPs during preclinical evaluation. Many of the diseases that are screened for, as described in the FDA’s Guidance for Industry on Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products [29], are irrelevant in animal screening. Therefore, in the current study, selecting a donor based on functionality was emphasized, because donor eligibility requirements, such as testing for human diseases, are not applicable to the porcine model. In this study, we demonstrate that different Yucatan minipig donors yielded cells with significantly different functional outputs, and the MP3 donor produced constructs with superior mechanical properties as indicated by the functionality index. Encouragingly, although the highly functional donor was selected based on constructs at passage 3, the superior functional output observed from MP3 was maintained through passage 7, indicating that the high functional properties of the donor cells may be maintained through cell expansion. Specifically, during phase 2 of this study where constructs from WCBs were generated, single donor constructs from MP3 had superior mechanical properties compared to pooled donor constructs (i.e. 2.9-times higher aggregate modulus, 1.4-times higher Young’s modulus). This suggests that, using the passing and rejuvenation techniques described in this study, screening for functional output at a lower passage can give insight into how the cells will behave at high passage.

The functionality index used to select a highly functional donor and to assess the quality of the final product may need to be modified on an indication-specific basis. In the current study, a functionality index was used to aggregate the functional properties that would be used as release criteria for articular cartilage repair. Since both compressive and tensile characteristics are critical to the function of the intended human product, both compressive aggregate modulus and tensile Young’s modulus values are factored into the functionality index. Other articular cartilage sources may have different target native tissue values. For example, as described in the results section, when tuned to the facet joint indication with an aggregate modulus of 243 kPa and a Young’s modulus of 6.7 MPa [28], the functionality index reaches up to 0.67 for the single donor stimulated with TCL treatment. However, the release criteria included within the functionality index may need to be modified for different indications. We have previously described how cartilage exists within a fibrocartilage spectrum [30]. Fibrocartilages, such as the temporomandibular joint disc, which functions primarily in tension, may place greater emphasis on the tensile characteristics of the construct and may not include the aggregate modulus within the functionality index calculation. Interestingly, during the first phase of this study, MP2 was demonstrated to have a lower aggregate modulus value but a higher UTS than any other donor. Thus, the functional properties observed in cells generated from MP2 could position it to be a highly functional donor for fibrocartilage applications. Therefore, a functionality index can be developed for different indications with different release criteria. This concept could be applied to the donor selection process for other cell therapy products in development.

While the ACP manufacturing process should mimic the intended human product to the greatest extent possible, typically there are areas of notable difference between an ACP and the equivalent human product. Good Laboratory Practice (GLP) is a quality system in place for nonclinical animal studies and Good Manufacturing Practice (GMP) is a quality system in place for product manufacturing. While the human product must be developed under GMP, there is limited availability of GMP facilities that allow animal cells because of cross-contamination issues. As recently reviewed, many of the currently approved cellular and gene therapy products did not fully comply with GLP- or GMP-level manufacturing practices during their preclinical animal studies [2]. This challenge is exacerbated by the fact that much of the research on cellular and gene therapy products is currently conducted at academic institutions rather than large pharmaceutical companies with their own GMP facilities. As recently described, 15 of the 16 companies that have developed approved cellular and gene therapy products (excluding hematopoietic stem cell cord blood products) were associated with academic institutions [5]. Some academic institutions may have clean rooms or GMP facilities, however, such facilities are not appropriate for manufacturing of an ACP. Animal cells may need to be cultured in non-GMP conditions. However, preclinical evaluations of cell therapy products must be conducted under GLP conditions or provide a ‘statement of the reason for the noncompliance’ and provide an explanation of ‘whether the deviation(s) impacted study outcome’ [31]. Actions that can be taken to
aid in this requirement include the development of a GLP-like quality management system, detailed standard operating protocols, in-depth record keeping on the work conducted and on the maintenance of the equipment and facilities, and third-party oversight of the studies (quality assurance).

Another key difference between ACPs and the final human product is the microbiological testing requirements. Standard safety tests that are run on human cell banks include sterility, endotoxin, mycoplasma, and adventitious agents [10]. However, there are challenges in replicating these tests in an ACP cell bank. For example, sterility testing, (i.e. bacterial and fungal testing) is a common requirement for human MCBs and WCBs [10]. GMP manufacturing for human products is typically done under extremely clean conditions and avoids the use of antibiotics during manufacturing. If animal cells used in ACPs are grown outside of GMP facilities with clean rooms, they would likely require the use of antibiotics during long-term cell culture to avoid bacterial and fungal contamination. The use of antibiotics creates the potential of masking the sterility testing outcome (i.e. yielding a false negative). Therefore, the validity and utility of sterility testing for ACPs is a concern. Adventitious agent testing is another example of how the testing requirements between the ACP and human product can differ. The standard set of adventitious agent tests applied to human cells (i.e. CMV, HIV-1 & 2, HTLV-1 & 2, EBV, B19, HBV, and HCV testing [10]) cannot be applied to animal cells, because the viruses tested are applicable only to humans, though species-specific testing may be considered (e.g. screening for porcine adventitious agents). Given the differences in ACP and human cell bank testing capabilities, researchers should consider which assays may be scientifically appropriate for their specific ACP and whether or not the assay would mimic the intended human product’s manufacturing process.

Other important aspects of this work include addressing sex as a biologic variable (SABV), developing a donor selection algorithm, and generating a pooled cell bank for basic research. Notably, while we did not specifically examine sex as a factor within this study, post hoc power analyses of the data demonstrated that very high n numbers (e.g. over 2000 000 in the case of Young’s modulus) would be needed to detect sex-related differences. Therefore, SABV within the context of generating self-assembled neocartilage is not a primary concern when performing the donor selection process. We established an algorithm to identify a highly functional donor that uses both qualitative and quantitative criteria, using an analogous methodology to the donor screening process for manufacturing the intended human product. Additionally, we developed a method for pooling cells to produce a well characterized WCB that can be used long term to carry out basic science studies, which reduces concerns of biologic variability within future research. Future studies can further build upon these concepts as cartilage repair therapies are developed to treat different indications (e.g. repair of the temporomandibular disc, meniscus, and interphalangeal joints).

In conclusion, this was the first effort to generate a cell bank for the production of ACPs to support the preclinical evaluation of a cell therapy product. Cells from six donors were used to generate six separate MCBs, which were then screened for potency via the assessment of functional properties as release criteria, in an analogous manner to the human product. A highly functional donor was selected, and the cells from the selected donor were able to generate constructs with higher functional properties compared to a pooled cell population. While the methods used here were tuned to articular cartilage repair, these methods can be modified to different cartilage repair indications by modifying the functionality index for a given application. More broadly, the methods described here represent the first effort to describe how an ACP cell bank may be applied toward translational research for cell therapy products. By structuring an ACP cell bank in the same manner as the cell bank that will be used for the human cell therapy product, researchers may lay the foundation for the successful translation of their research from an academic setting to clinical use.

4. Materials and methods

4.1. Isolation and expansion of costal chondrocytes

Costal cartilage was obtained from 5–8 month old Yucatan minipigs (three male and three female) within 1 d of the animals being culled for purposes unrelated to this study (S&S Farms, Romona, CA). Muscle, fat, connective tissues, and perichondrium were removed from all ribs to expose the costal cartilage. Cartilage was excised from the ribs and minced into 1 mm³ cubes, placed in washing medium consisting of Dulbecco’s modified Eagle’s medium with high glucose/GlutaMAX™ (Life Technologies, Grand Island, NY) and 1% penicillin–streptomycin–fungizone (P/S/F; Lonza, Basel, Switzerland). The tissue was then digested in 0.4% pronase (Sigma-Aldrich, St. Louis, MO) and 3% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) in washing medium on an orbital shaker at 50 RPM for 1 h at 37 °C. The pronase solution was then replaced with collagenase type II ( Worthington, Lakewood, NJ) at a concentration of 630 active units/ml in washing medium and placed on an orbital shaker at 50 RPM for 18 h at 37 °C. After digestion, the isolated chondrocytes were passed through a 70 µm strainer to remove any undigested cartilage, rinsed with washing medium, and treated with ammonium–chloride–potassium lysing buffer for 4 min at 37 °C to lyse red blood cells, as previously described.
[32]. Afterwards, cells were rinsed twice more in washing medium and centrifuged at 400 × g for 5 min between each wash step to pellet the cells. Cells were then plated at a density of 2.5 million per T225 in chemically defined chondrogenic culture medium (CHG; Dulbecco’s modified Eagle’s medium with high glucose/GlutaMAX, 1% P/S/F, 1% nonessential amino acids (Life Technologies, Grand Island, NY), 1% ITS+ Premix (BD Biosciences), 50 µg ml⁻¹ ascorbate-2-phosphate (Sigma-Aldrich, St. Louis, MO), 40 µg ml⁻¹ L-proline (Sigma-Aldrich, St. Louis, MO), 100 µg ml⁻¹ sodium pyruvate (Sigma-Aldrich, St. Louis, MO), and 100 mM dexamethasone (Sigma-Aldrich, St. Louis, MO)), supplemented with 2% fetal bovine serum, 1 ng ml⁻¹ TGF-β1 (PeproTech, Rocky Hills, NJ), 10 ng ml⁻¹ platelet-derived growth factor (PeproTech, Rocky Hills, NJ), and 5 ng ml⁻¹ basic fibroblast growth factor (PeproTech, Rocky Hills, NJ). The cells were cultured until confluent (~11 d) changing the media every 3–4 d. Cells were then lifted via 0.05% trypsin–EDTA followed by incubation in 0.2% w/v collagenase supplemented with 3% FBS in DMEM with 1% PSF and pipetted up and down every 15 min until the aggregates were fully dissociated. Agarose wells were prepared 2 d before use using 2% agarose. Agarose wells were formed that were 5 mm in diameter, using a negative mold [33]. CHG was used to rinse the wells three times over the course of 2 d before seeding to remove any PBS. Cells were seeded at a density of 2 million cells per construct in 100 µl of CHG medium (or CHG + TGF-β1 in the TCL group in Phase 2). After 4 h, an additional 400 µl was added to the wells. Medium was changed every day, and constructs were unconfined from their agarose wells on day 2. For the TCL-treated groups described in phase 2, the regimen was as follows: TGF-β1 was applied at 10 ng ml⁻¹ from seeding through day 28, c-ABC (Sigma-Aldrich, St. Louis, MO) was applied at 2 Units per ml for 4 h in day 7, and LOXL2 (Signal Chem, Richmond, Canada) was applied at 0.15 µg ml⁻¹ from days 7–21 with 0.146 µg ml⁻¹ hydroxylysine (Sigma-Aldrich, St. Louis, MO) and 1.6 µg ml⁻¹ copper sulfate (Sigma-Aldrich, St. Louis, MO), as previously described [34]. Constructs were cultured for 4 weeks, at which time they were tested. Images were taken of each construct, and then the constructs were partitioned for assays.

4.2. MCB and WCB generation

Cells from one minipig at a time were thawed, seeded, and passaged, as described above until passage 2. At that point, they were frozen in identical ampoules at a concentration of 30 million cells per 1 ml freezing medium. The ampoules from each MCB were used to make constructs (as described below), and the constructs were subjected to quality control (e.g. mechanical testing, biochemistry, and histology, as described below).

All donors that had an appropriate morphology (figure 2) were included in the pooled WCB. The donor that yielded constructs with the highest functional properties, as identified by the functional index, was used to generate the single donor WCB. Briefly, the functionality index (equation (1)), as described in previous studies [23–25], was used to generate a weighted average of the primary functional outputs of the construct including aggregate modulus and tensile Young’s modulus. Cell passaging and freezing for the cells of the WCBs were performed in the same way as for the MCBs. For the WCBs, cells were frozen at a concentration of 15 million cells per 1 ml freezing medium at passage 5.

4.3. Construct generation

Constructs from MCBs were generated at P3 and constructs from WCBs were generated at P7. Cells were expanded from the MCBs or WCBs, lifted, and seeded into aggregate rejuvenation [20]. Aggregate rejuvenation was conducted by seeding cells into agarose covered 25 × 100 mm dia. petri dishes at a concentration of 750 000 cells ml⁻¹ in CHG supplemented with 10 ng ml⁻¹ TGF-β1, 100 ng ml⁻¹ growth differentiation factor 5 (GDF-5), and 100 ng ml⁻¹ bone morphogenetic protein 2 (BMP-2). The petri dishes were placed in an incubator on an orbital shaker at a speed of 50 RPM for 24 h. The cells were then left in static culture for the remainder of the 14 d incubation time; the medium was changed every 3–4 d. Aggregates were digested with a 45 min incubation in 0.05% trypsin-EDTA followed by incubation in 0.2% w/v collagenase supplemented with 3% FBS in DMEM with 1% PSF and pipetted up and down every 15 min until the aggregates were fully dissociated.

Agarose wells were prepared 2 d before use containing 2% agarose. Agarose wells were formed that were 5 mm in diameter, using a negative mold [33]. CHG was used to rinse the wells three times over the course of 2 d before seeding to remove any PBS. Cells were seeded at a density of 2 million cells per construct in 100 µl of CHG medium (or CHG + TGF-β1 in the TCL group in Phase 2). After 4 h, an additional 400 µl was added to the wells. Medium was changed every day, and constructs were unconfined from their agarose wells on day 2. For the TCL-treated groups described in phase 2, the regimen was as follows: TGF-β1 was applied at 10 ng ml⁻¹ from seeding through day 28, c-ABC (Sigma-Aldrich, St. Louis, MO) was applied at 2 Units per ml for 4 h in day 7, and LOXL2 (Signal Chem, Richmond, Canada) was applied at 0.15 µg ml⁻¹ from days 7–21 with 0.146 µg ml⁻¹ hydroxylysine (Sigma-Aldrich, St. Louis, MO) and 1.6 µg ml⁻¹ copper sulfate (Sigma-Aldrich, St. Louis, MO), as previously described [34]. Constructs were cultured for 4 weeks, at which time they were tested. Images were taken of each construct, and then the constructs were partitioned for assays.

4.4. Histology

Histology samples were fixed in 10% neutral-buffered formalin. Samples were processed, embedded, and sectioned at a thickness of 6 µm. Slides were then stained using hematoxylin and eosin, safranin-O, and picrosirius red, as previously described.

4.5. Biochemistry

Samples that were isolated for biochemistry were weighed before and after lyophilization to obtain wet and dry weights. DNA was quantified using a PicoGreen™ assay [35], and GAG was quantified through a dimethylmethane blue (DMMB) kit (Biocolor, Carrickfergus, UK), as per manufacturers’ protocols. A modified hydroxyproline assay was used to assess collagen content, as previously described [36].
4.6. Mechanical testing
Compressive testing was carried out through the use of creep indentation testing. A 3 mm diameter punch of neocartilage tissue was glued to the base of a creep indentation apparatus and loaded using a 1.0 mm diameter porous tip under constant load, as previously described [37]. Aggregate modulus, shear modulus, and permeability were calculated using finite element optimization to fit the creep curves to a linear biphasic model [38]. Tensile properties of the constructs were assessed by uniaxial tensile testing. Neocartilage sections were cut into a dog bone conformation and pulled at a rate of 1% strain per second until failure, as previously described [39]. A custom MATLAB (MathWorks) code was used to calculate Young’s modulus, UTS, strain-at-failure, toughness, and resilience using a linear elastic model.

4.7. Statistical analysis
All statistical analyses were conducted using Prism 9 (GraphPad, Boston, MA) except for power analyses, which were conducted in SAS (SAS Institute Inc., Cary, NC). Phase 1 (i.e. MCB characterization) data were analyzed using a one-way ANOVA with Tukey’s post hoc and \( \alpha = 0.05 \) (\( n = 5–7 \) per group). Phase 2 (i.e. WCB characterization) data were analyzed using a two-way analysis of variance with Tukey’s post hoc and \( \alpha = 0.05 \) (\( n = 5–7 \) per group).

Data availability statement
All data that support the findings of this study are included within the article (and any supplementary files).

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