CMBES/SCGB

2016 CMBEC39 Conference
Calgary AB
May 24–27, 2016

MICROCARRIER SCREENING FOR SKIN-DERIVED PRECURSOR SCHWANN CELL CULTURE IN STIRRED TANK BIOREACTORS

Kimberly Bowal[1*] Tylor Walsh[2,3,*], Rajiv Midha[4], Michael Kallos[1,2,3]

[1] *Chemical and Petroleum Engineering, Schulich School of Engineering, University of Calgary*
[2] *Pharmaceutical Production Research Facility (PPRF), Schulich School of Engineering, University of Calgary*
[3] *Biomedical Engineering Graduate Program, Schulich School of Engineering, University of Calgary*
[4] *Hotchkiss Brain Institute and Department of Clinical Neurosciences, Cumming School of Medicine, University of Calgary*
**These authors contributed equally to this work*

INTRODUCTION

The Peripheral Nervous System (PNS) contains glial cells called Schwann cells (SCs). Schwann cells can either be myelinating, which myelinate large-diameter axons, or non-myelinating which support small diameter sensory axons [1]. In addition to their myelinating capabilities, SCs have a regenerative capability allowing for functional repair of the peripheral nervous system following an injury. Although the PNS is believed to possess some regenerative potential, after most injuries the adult mammalian nervous system has poor functional recovery since it is unable to complete significant endogenous repair (Sun and He, 2010). To enhance the regeneration capability of nerves, cellular therapy is a promising approach.

SCs have become the leading candidate for cellular therapy and regeneration of injured peripheral nerves due to their regenerative capabilities [1]. They are typically harvested from nerve tissue but they can also be differentiated from stem cells such as embryonic stem cells (ESCs), bone marrow mesenchymal stem cells (BM-MSCs), and skin derived precursor cells (SKPs) [2]–[4]. SKPs are a self-renewing multipotent somatic cell derived from skin tissue which under specific culture conditions can be differentiated into Schwann cells (SKP-SCs) [4]. These SKP-SCs offer a great alternative to nerve SCs as they are easily obtainable from the patient's skin allowing for autologous therapy. Recent reports show that the SKP-SCs show great potential for improving axonal regeneration and behavioral recover after peripheral nerve damage [5]. The development of methods of expanding large quantities of cells is crucial for cellular therapy. Current expansion protocols to grow the SKP-SCs focus around static cultures in petri dishes, well plates, or flasks. However, this method is not adequate for the large quantities of cells needed to develop a treatment. Recently, to address this problem, we have been able to grow the SKP-SCs in suspension bioreactors on microcarriers [6]. Microcarrier-bioreactor cultures offer a number of benefits over traditional static culture by increasing the surface area to volume ratio, as well as adding process control, flexible operation, homo-generous culture conditions, on-line sampling, and the ability to harvest in-situ.

Microcarriers are small particles that allow adherent cells, such as SKP-SCs, to grow in suspension bioreactors. It is important that the microcarriers promote cell attachment and proliferation. To try and accommodate the many different cell types, there are a number of commercially available microcarriers with many different properties such as material of construction, surface charge, size, surface area, coating, and porosity. Because of this, the selection of the appropriate microcarrier is a critical step of microcarrier/bioreactor culture.

There have been limited studies published comparing the growth of SKP-SCs on different microcarriers in suspension, and therefore an

investigation into commercially available microcarriers that support the rapid, reproducible, and controlled expansion of SKP-SCs in stirred tank bioreactors is required.

MATERIALS AND METHODS

SKP-SC Static Culture expansion

Rat SKP-SCs were obtained from Dr. Jeff Biernaskie's laboratory (Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary). Skin derived precursors were generated and subsequently differentiated into Schwann cells as previously described [4]. All SKP-SCs used were line 3, passages 7-9. Frozen SKP-SCs were stored in the vapour phase of liquid nitrogen, thawed at 37°C, and re-suspended in SKP-SC medium, which consisted of a 3:1 ratio of Mulbecco's Modified Eagle Medium (DMEM) and F-12 + GlutaMax Nutrient Mix. This mixture was then supplemented with 1% N-2 supplement, 1% penicillin/streptomycin, 50 ng/mL recombinant human neuregulin-1-1β, 5μM forskolin, and 1% fetal bovine serum (FBS).

Static (monolayer) expansion of rat SKP-SCs involved seeding 10 cm culture dishes at a density of 5000 cells/cm^2 and then placed in a humidified CO_2 controlled incubator at 37°C and 5% CO_2. Cells were passaged upon reaching confluency (day 4 of culture). Passaging involved washing the SKP-SCs with DMEM followed by an incubation step for 5 min with TrypLE Express to dissociate the cells from the surface of the dish. The cell suspension was then centrifuged at 1000 rpm for 5 min at room temperature, the supernatant discarded and remaining pellet re-suspended in SKP-SC medium and counted using the trypan blue exclusion method. The SKP-SCs were then either re-plated or inoculated on the microcarriers.

Microcarrier culture

All microcarrier cultures were performed in either 125mL spinner flasks or 6-well plates designed to avoid attachment of cells to the plate or vessel surface. This was achieved in low attachment 6-well plates, while the spinner flasks were coated in Sigmacote. Sigmacote was applied to the spinner flask wall and impeller surface area and aspirated. Vessels were left to dry for 24 hours then washed with phosphate buffered saline (PBS) followed by double distilled water and autoclaved prior to use.

All microcarriers used in the comparison were prepared according to the manufacturer's instruction. For all microcarrier cultures, after initial hydration and sterilization by autoclaving, the microcarriers were inoculated into the bioreactors or plates in SKP-SC medium for at least 8 hours in a humidified incubator at 37°C and 5% CO_2 in air to allow for the conditioning of the microcarriers. After this period, the SKP-SCs were added with fresh medium to 60% of total volume, for 24 hours, to aid in attachment to the microcarriers. Cells were inoculated at 4 cells/microcarrier which is 4444 cells/cm^2. After the 24 h inoculation period, the remaining volume of medium was added for a total of 125 mL. Microcarrier sampling was done by taking 0.5 mL and staining it with 0.5% crystal violet in methanol for pictures. Growth curves were conducted by taking 3 mL samples and incubating them in 0.1% crystal violet and 0.1 M citric acid, for at least 1 hour, to lyse the cells releasing the nuclei to be counted.

RESULTS

Cell Attachment

Four commercially available microcarriers (Plastic Plus, ProNectin F, Hillex II, and Cytodex 3) were initially screened in low attachment 6-well plates to identify the best microcarriers for attachment. The cells were inoculated into either serum free SKP-SC medium or SKP-SC medium contain 1% FBS. Attachment after 1, 2, 4.5, and 18.5 hours of incubation was assessed visually through the use of photomicrography (data not shown) and quantitatively (fig. 1) using the crystal violet nuclei counting method to determine the cell density on the microcarriers. After 1 hour, differences in attachment were already noticeable. After the full inoculation period of 18.5 hours, Cytodex 3 and Hillex II had the best attachment in both serum containing and serum free conditions. Serum free cultures seemed to have better attachment for all microcarriers with the exception of Cytodex 3. Because of the high attachment rate of Cytodex

3 and Hillex II, these two microcarriers were used to test the expansion of the SKP-SCs in suspension bioreactors.

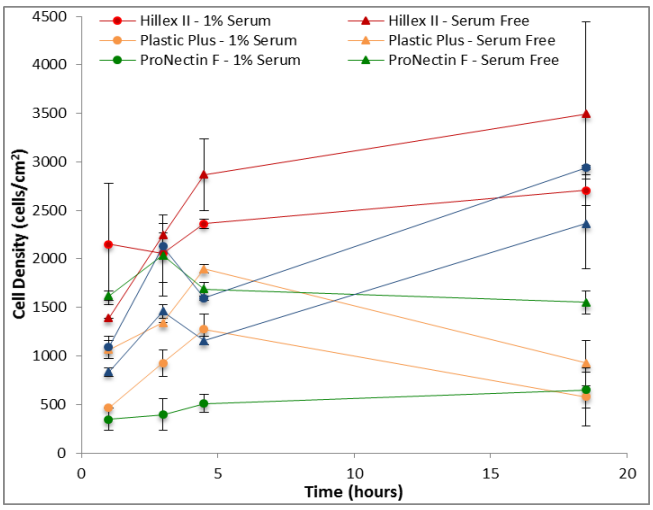

Figure 1: Cell density plot of attachment of SKP-SCs on selected microcarriers in well-plate culture

Cell Growth in Bioreactors

The studies conducted in the low attachment well plates provided an indication as to the performance of the microcarriers, so it was important to conduct spinner flask experiments for microcarrier comparison, as this would be more representative of how the microcarriers would affect the growth in practice. The two best microcarriers in terms of attachment (Cytodex 3 and Helix II) were used to culture SKP-SCs in 125 mL bioreactors to observe the growth kinetics on each microcarrier. Cells were inoculated at 4444 cells/cm^2 in either serum containing or serum free SKP-SC medium. Cell density on microcarriers over the culture period was assessed by photomicrography (fig. 2) and using the crystal violet nuclei counting method (fig 3).

SKP-SC growth was found to be significantly greater on the Cytodex 3 microcarriers than Hillex II microcarriers for the SKP-SC medium containing 1% serum. Cytodex 3 microcarriers in serum free medium did not support the growth of SKP-SCs and resulted in the lowest cell density of the conditions tested. Cell growth was similar on the Hillex II microcarriers (in serum free and serum containing conditions) and Cytodex 3 (in 1% serum) from days 1-4. However, by day 6 of culture the cell density was found to be significantly higher on Cytodex 3 than the Hillex II when considering the maximum densities for each. When evaluating the maximum densities, there was significant difference between serum free and serum containing for both Cytodex 3 and Hillex II microcarriers. There were also significant differences between the cell densities on Cytodex 3 and Hillex II in both conditions.

The bioreactor cultures were also compared to the traditional static expansion methods. It can be seen that the peak cell density of the SKP-SCs in static occurs at approximately the same time as the Hillex II (1% serum) and Cytodex 3 (1% serum) cultures. The maximum cell density in the static is higher than the peak densities found for the Hillex II and Cytodex 3 cultures in serum-free medium, but lower than the other two conditions.

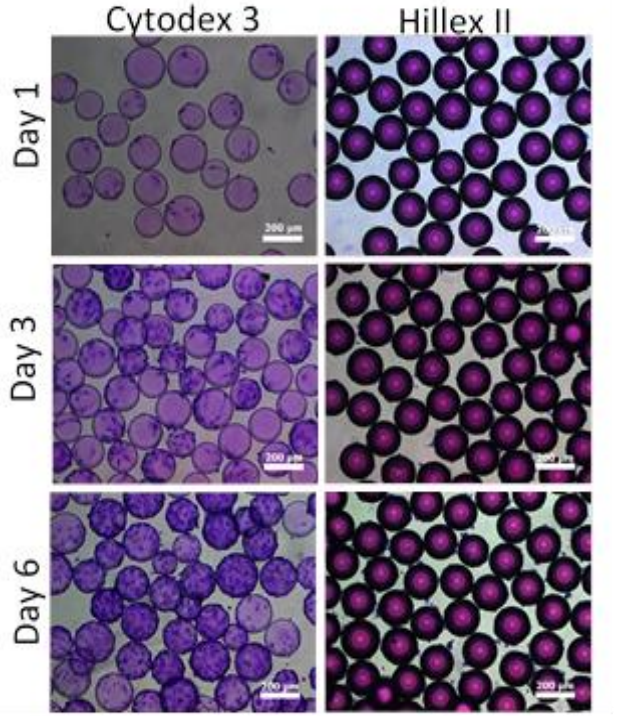

Figure 2: Photomicrographs of bioreactor growth of SKP-SCs on selected microcarriers at best conditions. Scale bars represent 200 µm.

It was also observed in all experiments that the Hillex II microcarriers changed colour (clear to red) within a few hours of being inoculated in medium, regardless of the serum condition. Since the media also changed colour (from red

to clear), it is believed that the Hillex II microcarriers absorbed the phenol red contained in the SKP-SC medium. This was not observed in any of the other microcarriers.

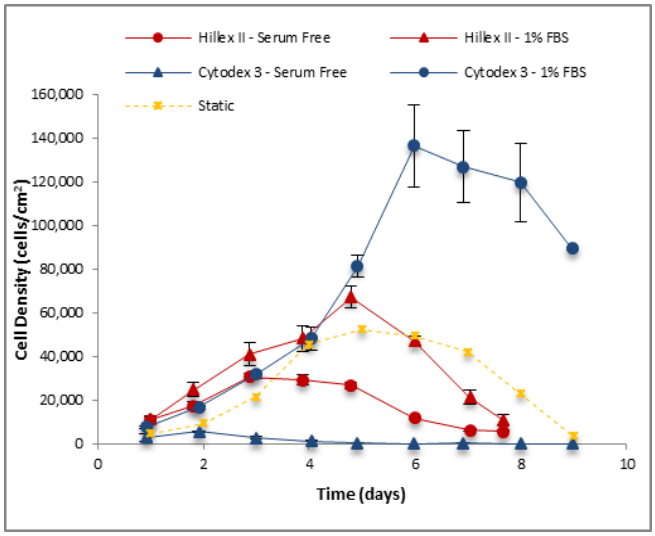

Figure 3: Cell density plot of growth of SKP-SCs in static and in biroreactors on Hillex II and Cytodex 3 microcarriers.

DISCUSSION

The selection of the most appropriate microcarrier is a critical step of any microcarrier based cell therapy process. There is very little data on the attachment and growth of SKP-SCs on commercially available microcarriers, and screening of these microcarriers was needed.

The greatest attachment density was reached within the 18.5 h inoculation period on the Hillex II microcarriers in the serum free SKP-SC medium with the second highest attachment being Cytodex 3 in 1% serum. The other two microcarriers (Plastic Plus, and Pronectin F) have poor attachment and were not used passed the screening phase. Even though the Hillex II microcarriers had better initial attachment compared to the Cytodex 3, the SKP-SCs expanded to higher densities on the Cytodex 3 over the 9-day culture period. One reason we think the growth on the Hillex II was so poor was that when they absorbed the phenol red, they may have also absorbed some of the nutrients thus limiting the cell growth. One other factor that could attribute to the difference in growth kinetics is that the Cytodex 3 microcarriers have a collagen coating, which may lead to their higher maximum cell density compared to Hillex II. In both cases, the bioreactors with serum containing medium had higher cell densities than the static with serum containing medium.

CONCLUSIONS

Finding the most appropriate microcarrier for SKP-SC expansion is a critical component in process development. This step needs to be done early in the research to avoid costly changes later. Findings from this investigation suggest that to culture SKP-SCs in serum containing medium, it is best to use Cytodex 3 microcarriers, but in serum free medium it is best to Hillex II. These are very promising results as they demonstrate that these cells can be readily grown in suspension bioreactors on microcarriers. Therefore these cells have a great potential to be scaled up and used in a clinical process to treat nerve injuries.

ACKNOWLEDGEMENTS

This study was supported by a CRIO Project grant from Alberta Innovates-Health Solutions (AIHS). The authors would like to thank Jeff Biernaskie for providing cells for this study.

REFERENCES

[1] H. A. Kim, T. Mindos, and D. B. Parkinson, “Plastic Fantastic : Schwann Cells and Repair of the Peripheral Nervous System,” *Stem Cells Transl. Med.*, no. 2, pp. 553–557, 2013.

[2] J. W. McDonald, X. Z. Liu, Y. Qu, S. Liu, S. K. Mickey, D. Turetsky, D. I. Gottlieb, and D. W. Choi, “Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord.,” *Nat. Med.*, vol. 5, no. 12, pp. 1410–1412, 1999.

[3] M. C. O. Rodrigues, A. A. Rodrigues, L. E. Glover, J. Voltarelli, and C. V Borlongan, “Peripheral nerve repair with cultured schwann cells: getting closer to the clinics.,” *ScientificWorldJournal.*, vol. 2012, p. 413091, Jan. 2012.

[4] J. a Biernaskie, I. a McKenzie, J. G. Toma, and F. D. Miller, “Isolation of skin-derived precursors (SKPs) and differentiation and enrichment of their Schwann cell progeny.,” *Nat. Protoc.*, vol. 1, no. 6, pp. 2803–12, Jan. 2006.

[5] H. T. Khuong, R. Kumar, F. Senjaya, J. Grochmal, A. Ivanovic, A. Shakhbazau, J. Forden, A. Webb, J. Biernaskie, and R. Midha, “Skin derived precursor Schwann cells improve behavioral recovery for acute and delayed nerve repair.,” *Exp. Neurol.*, vol. 254, pp. 168–79, Apr. 2014.

[6] A. Shakhbazau, L. Mirfeizi, T. Walsh, H. M. Wobma, R. Kumar, B. Singh, M. S. Kallos, and R. Midha, “Inter-microcarrier transfer and phenotypic stability of stem cell-derived Schwann cells in stirred suspension bioreactor culture,” *Biotechnol. Bioeng.*, vol. 9999, no. xxx, p. n/a–n/a, 2015.