A novel bioreactor for the perfusion of electrospun fibre scaffold supporting 3D engineered tissue production – Application of human skeletal satellite muscle cells and human embryonic stem cells differentiated towards neural cells

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Introduction

Electrospinning allows for the production of scaffolds mimicking that of in vivo extracellular matrices. With the additional flexibility of altering topography, porosity, protein coating and polymer composition it provides a suitable platform for 3D culture systems. While the scaffold provides excellent support for growing and differentiating cells, the cell density required for production of tissue-like constructs will eventually be limited by insufficient distribution of oxygen and nutrients.

To overcome this problem we have designed and built a bioreactor system that allows the scaffold to continuously be perfused by an orthogonal flow of medium. Furthermore, this system is fully automated with live monitoring of pH, oxygen and temperature allowing for a robust culture with low maintenance.

Our goal in our studies is to evaluate this system with regards to proliferation and differentiation of skeletal muscle cells as well as embryonic stem cells to dense tissue-like constructs.

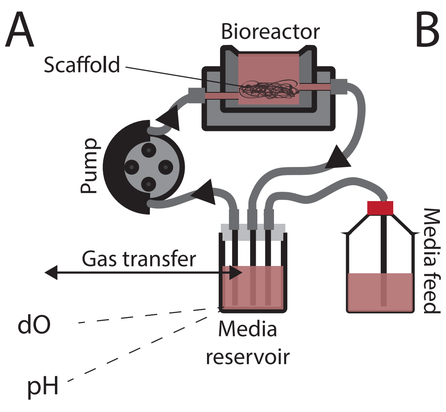
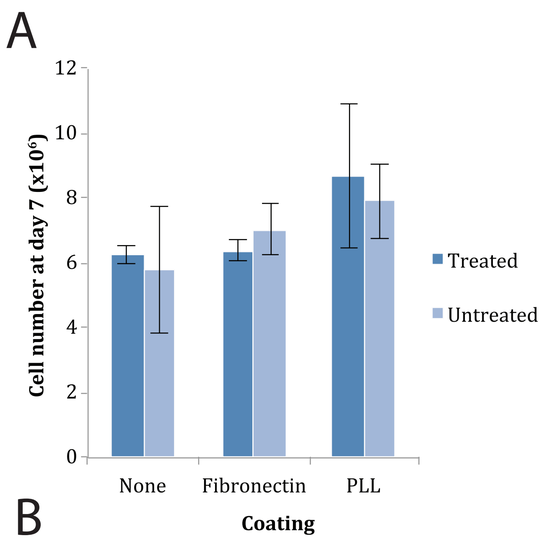


Figure 1 - Overview of the mini-bioreactor.
A) Schematic of the bioreactor system. B)Cultivation of skeletal muscle cells in four mini-bioreactors in parallel.

Bioreactor cultivation overview

4 or 8 bioreactors equipped with polycaprolactone (PCL) fibre scaffolds (up to 3 mm thick) were cultured in parallel. The cells were inoculated in the scaffold, cultured overnight in static conditions and then transferred to the bioreactor where the medium recirculation was operated at flow rates between 0,032cm/min to 0,239cm/min. The glucose consumption and lactate accumulation were monitored during the perfusion culture as an indicator of cell growth. At the end of the culture, the following analyses were performed: DNA quantification after papaine digestion, scaffold staining by calceine to analyze the distribution of living cells, immunocytochemistry and qPCR.

Results



Optimisation of the proliferation of SkMCs in the mini bioreactors

Human skeletal muscle cells (SkMCs) were seeded on polycaprolactone (PCL) scaffolds of 3 mm thickness within bioreactor inserts. The scaffolds were cultured in static incubator conditions for 16-24 hours before being transferred into the bioreactors where they were cultured for a further 6 days. The growth medium (12 ml/bioreactor) was circulated at flow rate of 0.75 ml/min and a half medium change was performed every day after a 3-day batch process.

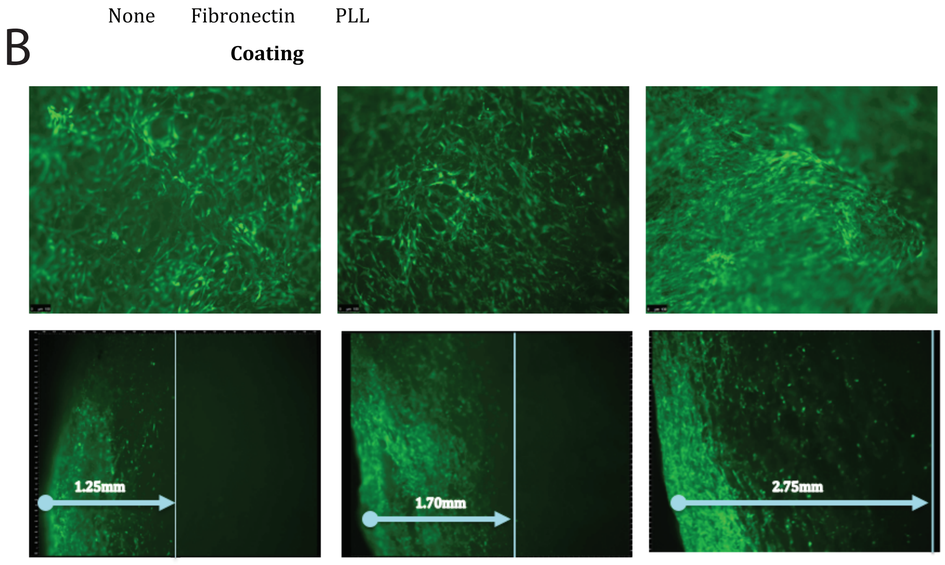


Figure 2 - Expansion of SkMCs on 3 mm thick ENF PCL scaffolds and the effect of plasma treatment and fibronectin and PLL coating after 7 days of culture.

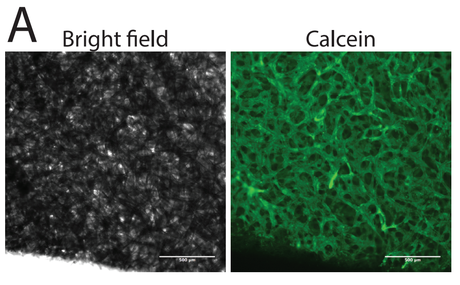
(A) Average number of cells quantified per scaffold by measurement of DNA (n=3). PLL coating resulted in a significant cell increase compared to the uncoated scaffolds (marked with an asterisk), while the effect of plasma-treatment and fibronectin coating were not significant in comparison to untreated and uncoated controls. (B) Images of live skeletal muscle cells (stained green using Calcein AM dye) on the scaffold surfaces and in-depth penetration. Uniform cell distribution and cell spreading were observed on the top surfaces of all scaffolds examined, and this was independent of the coating while the in-depth penetration of cells into the scaffolds was found to be dependent on the type of coating. The arrows depict the vertical infiltration distance of cells into the scaffolds with the round end indicating the top surface where the cell suspension was deposited.

Cultivation of embryonic stem cells under perfusion

Initially the bioreactor system was tested using embryonic stem cells confirm if it was a suitable platform for culturing and differentiating cells. To create a sufficient microenvironment for the stem cells PCL scaffolds of 200µm was coated in a stepwise manner with the extracellular protein laminin-521 along with the cell-cell adhesion protein E-cadherin-fc. To these scaffolds cells were seeded a density of 1.5x106 cells/scaffold. The cells were then cultured for 1 day in static conditions followed by 3 days in bioreactors under a 0,032cm/min perfusion rate. After the cultivation the scaffolds were initially analysed using calcein staining to investigate the viability of the cells. After the 4-day cultivation the cells had grown to a dense layer covering the entire scaffold (figure 1a). Further investigation of the pluripotency markers Oct3/4 and Nanog using antibody staining revealed a clear expression, indicating that the cells could proliferate without spontaneous differentiation.

Differentiation of embryonic stem cells to neural cells in perfusion bioreactors

To further investigate whether these bioreactors also could support the differentiation of embryonic stem cells, a 15-day protocol based on dual SMAD inhibition was tested. The protocol utilizes three inhibitors; IWP-2, LDN193189 and SB431542 to direct the stem cells towards a neural lineage. Similar to the stem cell expansion, cells were cultured under a 0,032cm/min flow rate with a daily media exchange rate of 50%. Cells were harvested at day 5, day 10 and day 15. As seen in figure 4a cells at day 15 were healthy and covered the entire scaffold as indicated by the calcein stain (fig 4a). Staining of markers Tuj1 and Pax6 clarified the differentiation in to a neural liniage (fig 4b). Further analysis of gene expression of the forebrain marker FoxG1 showed a clear increase over the 15 days (fig 4c). Pax6 on the other hand had an expression peak at day 5, with a drop towards day 10 and 15 (figure 4d), this hits well with its description as an early neural progenitor marker. Finally the cell number was estimated to be 23x106 cells at day 15, which within these scaffolds is a density of 370x106 cells/cm3 (fig 4e).



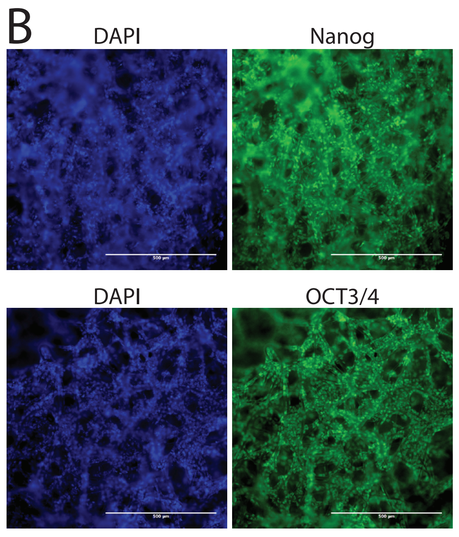


Figure 3 – Cultivation of embryonic stem cells in perfusion bioreactors.
(A) Bright field image and calcein stain of cells after the 4-day cultivation. (B) Staining of harvested cells using anti-Oct3/4 and anti-Nanog antibodies, counterstain with DAPI. Scale bars 500µm.

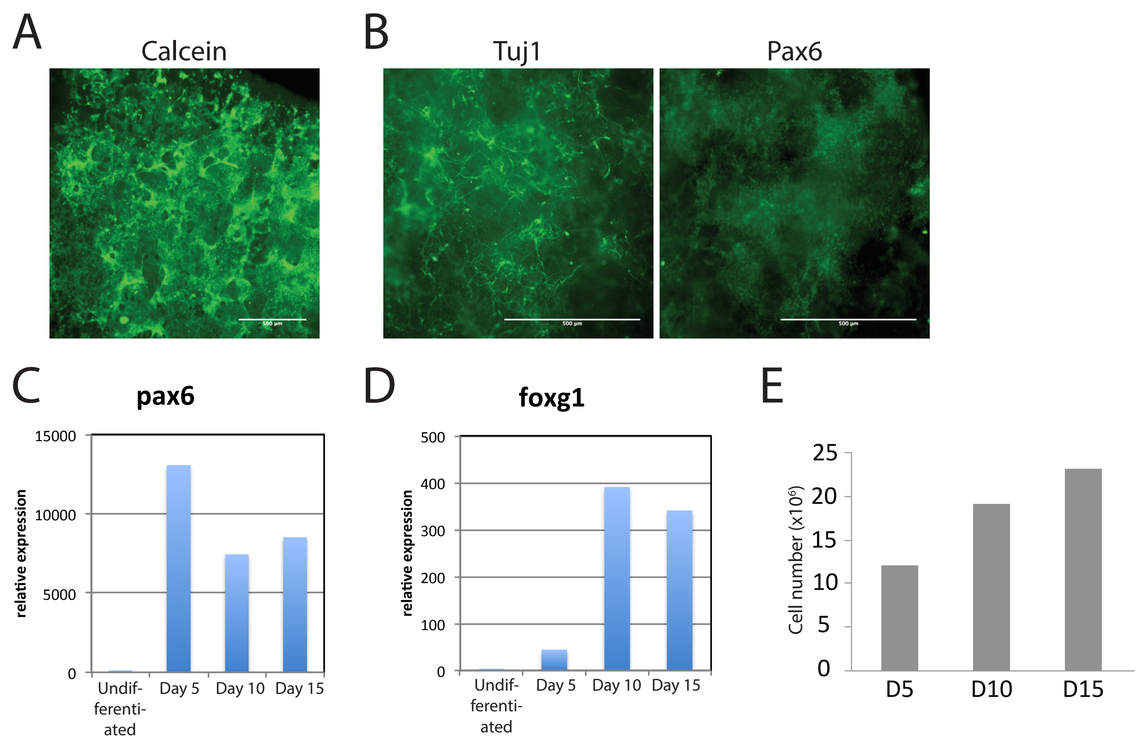


Figure 4 – Differentiation of embryonic stem cells towards neural lineage

(A) Calcein stain of cells harvested on day 15. (B) Antibody stain of markers Tuj1 and Pax6. (C) Gene expression of Pax6 at day 5, 10 and 15 relative to undifferentiated embryonic stem cells. (D) Gene expression of Tuj1 at day 5, 10 and 15 relative to undifferentiated embryonic stem cells. (E) Cell amounts quantified using DNA quantification. Scale bars 500µm.

Conclusions & perspectives

This bioreactor system provided an excellent way to culture both embryonic stem cells and skeletal muscle cells. Furthermore we show that differentiation of the embryonic stem cells was possible and yielded a highly dense culture of neural cells in only 15 days. As we could obtain these densities it is be possible to culture the cells longer to obtain tissue like structures in the 3D scaffold environment, these could useful for a range of studies like developmental biology, drug testing, regenerative medicine etc.

As this system is fully automated and easy to use, long-term cultures and differentiations can be executed with ease.

Acknowledgements

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