Effect of Brain-Derived Extracellular Matrix on Neuronal Stem Cell Differentiation

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Introduction and Background

Traditional methods investigating the molecular mechanisms of neurodegenerative disease entail the use of two-dimensional cell cultures or animal models. Combined, they have been efficient methods for the basic study of disease pathology, pharmacological development, and for the testing of treatments. However, traditional 2-D culture for disease models lack the ability to recapitulate normal physiological conditions with extreme accuracy. Novel approaches to the study of the molecular processes of disease entail the fabrication of tissue-specific protein-derived hydrogels for three-dimensional culturing of cells. Many such structures have been produced using substrates extracted from animal or human tissue, specifically extracellular matrix (ECM) components, granting a more biomimetic representation of the human physiological conditions. Animal-derived, commercially available products (Matrigel), have been successfully applied to the development of three-dimensional neuronal culture models and the study of neurodegenerative diseases [1, 2]. In addition, human mammary tissue-derived hydrogels are regularly applied in our laboratory to the study of breast cancer dynamics. With the notion that the cellular microenvironment has direct impacts on cell fate, and the goal to produce an accurate model to study the mechanisms characteristic of neurodegenerative disease, we report a novel method for the production of three-dimensional hydrogels derived from porcine brain tissue [3]. Here we investigate our novel method adapted from existing protocols to determine the effects that a more accurate *in vivo* like environment has on neural stem cells derived from induced pluripotent stem cells [4-6]. We demonstrated the efficiency of our protocol in the removal of cells and preservation of extracellular matrix components of the native tissue, and we evaluated the suitability of the final product as a substrate for neural stem cells and neuron cultures.

Generation of Porcine Brain-Derived Hydrogels

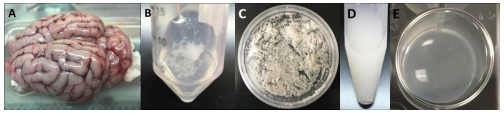


Figure 1. Fresh brain tissue was extracted from pig heads in our laboratory **(A)** and washed with sterile water. The tissue was cut and decellularized for 24-48 hours in 2% N-Lauryl Sarcosine (NLS). The detergent allows removal of all the cells from the tissue, while maintaining intact extracellular matrix proteins. The product was washed 10 times with sterile-water to ensure complete removal of NLS, and subsequently washed in Isopropyl alcohol (IPA) for 24-48 hours to remove any lipid component **(B)**. Residual IPA was removed with 10 sterile water washes. The decellularized tissue (extracellular proteins) was lyophilized to ensure complete removal of fluids **(C)**. Successively the product was then mechanically homogenized, and ground using LN2. The brain-derived product was enzymatically digested in a 1mg/ml pepsin solution in 0.1M HCl **(D)**. The acidic product was then dialyzed using 6-8kDa dialysis tubing and sterile PBS solution at 7.4 pH to elevate the pH to neutral status. The products obtained from this protocol are self-gelling when kept above 4-8°C. **(E)** Hydrogels are prepared by placing a set volume of product in the appropriate container and incubated at 37°C for 2 hours. The substrate is ready for application for 2D and 3D cell cultures.

Neural Stem Cell survival, proliferation and differentiation on 2D brain-derived substrates

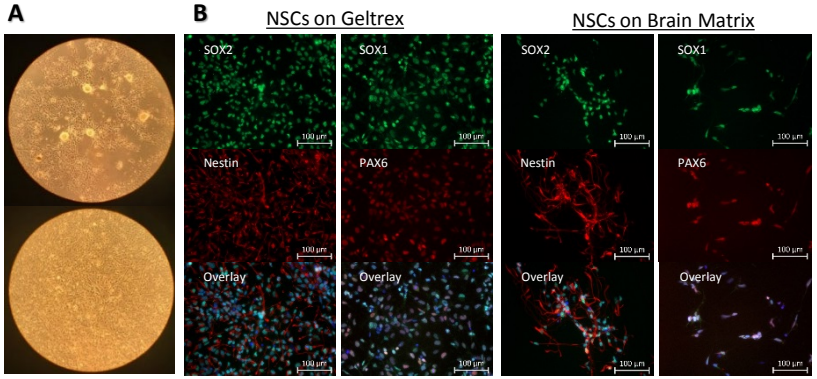
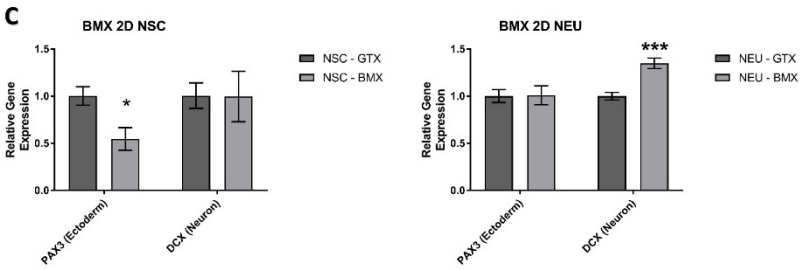


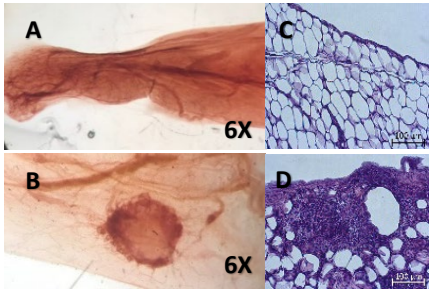
Figure 2. The brain matrix-derived substrate was tested for two-dimensional culture of neural stem cells and their differentiation into neurons. Cell culture plates were first coated with 0.1% PEI solution for 2hr and rinsed three times with sterile water. The brain matrix product (BMX) was diluted to a concentration of 0.5mg/mL in cold DMEM/F12, and used to coat the plates. After overnight incubation at 37°C, the coated plates were washed three times with sterile water. Neural stem cells (NSC) were seeded onto the coated plates and cultured following established culture methods [7]. Survival and proliferation of NSCs on the brain-derived substrate was evaluated via phase microscopy **(A – top)**, and compared to NSCs seeded on Geltrex® coated plates **(A – bottom)**. The identity of the cells was qualitatively evaluated through immunocytochemistry **(B)**. Cells were fixed, permeabilized and labeled with primary and secondary antibodies. The cells were labeled with Nestin, PAX6, SOX1 and SOX2 markers. The first two are NSC markers, while the latter are indicative of pluripotency. It is possible to observe that all the markers were expressed in NSCs cultured both on Geltrex® and on BMX, indicating that the brain derived substrate allows for the maintenance of NSCs identity in two-dimensional cultures.

The ability of NSCs to undergo spontaneous or chemically induced differentiation into neurons was evaluated through Quantitative Real Time Polymerase Chain Reaction (qRT-PCR). At day 17 from plating, NSCs grown on Geltrex® and on BMX were tested for expression of PAX3 and DCX genes **(C – left)**. The first is an NSC marker, while the second is a early neuronal marker. Significantly decreased levels of PAX3 were observed in NSCs grown on BMX, while DCX levels were comparable between the two substrates. Differentiation into neurons was chemically induced culturing NSCs in medium supplemented with Retinoic Acid and Purmorphamine [8] [9] [10]. PAX3 and DCX expression was evaluated after 7 days of differentiation **(C – right)**, and while PAX3 expression was comparable between the substrates, DCX expression was significantly increased in NSCs differentiated on the BMX substrate.



Neural Stem Cells Survival and Proliferation in Live Mice

Figure 4. The porcine brain matrix-derived substrate was tested for its ability to promote the *in vivo* survival and proliferation of stem cells. Neural stem cells were injected in the mammary glands of live mice, both in absence or presence of hydrogels, and were retrieved after 10 weeks. In absence of the brain-derived hydrogels, no growth was observed in any of the samples retrieved **(A)**. However, the majority of the samples containing the brain-derived substrate presented ingrowths **(B)**. Upon Hematoxylin and Eosin staining, presence of nucleic acids was reported in the ingrowths **(D)**, while not in the mammary gland **(C)**, suggesting the ability of our product to promote cell survival and proliferation.



Conclusion

With the project proposed we fabricated a porcine brain matrix-derived substrate ideal for two- and three-dimensional culture of neural stem cells and neurons. We demonstrated the ability of the brain derived substrate to promote survival, proliferation and differentiation of neural stem cells in vitro, and its biocompatibility in vivo. Future aims entail further characterization of the product, and its adoption in the study of asymmetrical division of neural stem cells.

Funding

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