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Tethered growth factors on biocompatible scaffolds improve stemness of cultured rat and human neural stem cells and growth of oligodendrocyte progenitors

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ABSTRACT

Currently, there is no widely accepted technique to efficiently and reproducibly grow stem and progenitor cells *in vitro*. Stem cells require contact with extracellular matrices as well as signals from growth factors to proliferate and to retain their stemness. We have developed a novel tissue culture platform (StemTrix cultureware) that transforms standard tissue culture plasticware into a multi-functional chitosan-based scaffold that supports the expansion of neural stem cells. The StemTrix scaffold is comprised of chitosan with immobilized heparin which in turn tethers heparin-binding growth factors. The scaffold is also coated with an adhesive ECM protein. Here we demonstrate that fibronectin or the RGD peptide contained in fibronectin are equally effective in promoting the adhesion, viability and growth of rat and human neural stem cells. When FGF-2 and heparin-binding EGF are tethered to the StemTrix cultureware neural stem cells grow ~3 times faster and remain in a more primitive state as determined by both Western Blot and gene expression analyses. Another important feature of this new culture platform is that the NSCs remain in a primitive and proliferative state for 4 days without refreshing the culture medium or providing new growth factors, which represents a 20-fold extension of FGF-2's biological activity vs when it is freely soluble in the medium. To test the utility of this scaffold for propagating other types of stem cells and progenitors we tethered platelet-derived growth factor (PDGF) and FGF-2 alone and in combination to the scaffold and tested the efficacy of this platform to maintain primary oligodendrocyte progenitors or the CG-4 cell line in a primitive state. Oligodendrocyte progenitors plated onto this multifunctional film proliferated for at least 3 days without providing soluble growth factors while inhibiting the expression of the differentiation marker myelin-basic protein. Oligodendrocyte progenitors proliferated 3 times more rapidly than cells maintained on fibronectin-coated culture substrates in culture medium supplemented with soluble FGF-2 and PDGF. Finally, we show that StemTrix cultureware can be produced using clinical grade components, providing users with a fully defined platform suitable for clinical use that maintains stem cells or progenitors in a more uniform and primitive state.

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1. Introduction

1.1. Stem cells and progenitors are difficult to propagate and maintain in culture

There is currently no universal platform to efficiently and reproducibly grow stem and progenitor cells (SPCs) *in vitro*. SPCs are commonly used in research to model injury and disease, examine drug and pharmaceutical effects, investigate tissue repair mechanisms for preclinical studies and for determining underlying genetic causes of disease. For instance, human neurons, which are virtually impossible to obtain or isolate by traditional methods, can be easily studied *in vitro* through advances in stem cell technology that enable them to be produced by differentiating

Abbreviations: BLBP, brain lipid binding protein; BSA, bovine serum albumin; CHG, chitosan-heparin-genipin; DMEM, Dulbecco's modified Eagle's Medium; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; hbEGF, heparin binding epidermal growth factor; FGF-2, fibroblast growth factor-2 Fnfn; GFAP, glial fibrillary associated protein; HBS, HEPES buffered salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hESC, human embryonic stem cell; HGF, hepatocyte growth factor; hNSC, human neural stem cell; IKVAV, Isoleucine-Lysine-Valine-Alanine-Valine; IOD, Integrated Optical Density; MBP, myelin basic protein; NSC, neural stem cell; OPC, oligodendrocyte progenitor; PDGF, platelet derived growth factor; RGC, radial glial cell; RGD, Arginine-glycine-Aspartic Acid peptide sequence; RGM, radial glial cell basal media; SPC, stem progenitor cell; VEGF, vascular endothelial growth factor.

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pluripotential stem cells into neural stem cells (NSCs). The demand for SPCs use in clinical applications is consistently increasing but they must be expanded in culture before *in vivo* use to produce sufficient numbers of cells for clinical efficacy. However, maintaining SPCs for research or clinical applications is difficult, time-consuming and costly. Traditional cell culture techniques use polystyrene tissue culture plates coated with an adhesion peptide or a growth matrix like Matrigel® for cell attachment. Because the growth factors used have short half-lives, SPC cultures often require daily medium changes to replenish growth factors that become denatured or depleted. Due to small margins of error, this methodology often fails to maintain the SPCs in a homogenous, proliferative and undifferentiated state. For example, fibroblast growth factor-2 (FGF-2), which is an essential survival factor and mitogen for NSCs, has been reported to have a half-life of 24 h at 32 °C [1]. This time is reduced to less than 5 h under standard culture and physiological conditions, i.e. 37 °C [2,3]. On the other hand, its stability increases when affixed to heparan sulfate proteoglycans [4]. Factors such as FGF-2 are necessary to maintain many types of SPCs in a primitive and proliferative state. However, frequent media changes result in oscillations in growth factor concentrations that decrease proliferation, increase heterogeneity and increase the risk of contamination. Furthermore, this constant replenishment of media and growth factors is both expensive and tedious.

SPCs also require contact with extracellular matrices (ECM) to proliferate and to retain the cardinal characteristics of stem cells (stemness), which can be defined as slowly dividing, self-renewing, undifferentiated, multipotent cells that express specific antigenic markers. The ECM is a scaffold that provides cells with structural and functional support. It is comprised of interconnected proteins and proteoglycans that creates an adhesive framework. Attachment to the individual components of this matrix transduces mechanical signals that regulate both basic and complex cellular processes. The proteins and proteoglycans that comprise the ECM bind to a number of cell surface receptors that affect proliferation, migration, differentiation, survival and other functions [5–8].

Here we describe enhancements to a scaffold that we formulated several years ago. We introduce modifications to address both the growth factor and ECM requirements to provide a reliable, low maintenance and affordable two-dimensional platform for SPC growth. Our novel scaffold is comprised of natural and inexpensive biomaterials that combine the activation of integrin signaling pathways and long-term stabilization of growth factors to enhance the propagation, stemness and homogeneity of SPCs.

2. Materials and methods

2.1. Chitosan scaffold

2.1.1. Chitosan scaffold mixture

Crustacean-based chitosan powder (low molecular weight ~50 kDa, with ~75–85% deacetylation (DDA) was purchased from Sigma-Aldrich (Cat #448869) and ultrapure mushroom-based chitosan was purchased from Synolyne (Belgium). Both were made into 3% (w/v) chitosan solutions. Each chitosan solution was mechanically stirred at 500 rpm while adding acetic acid to 3% (v/v). The mixture was covered and stirred for 30 min at 500 rpm before speed was increased to 1000 rpm for 2 h to overnight at room temperature. The resulting solution was collected and centrifuged at 4000 rpm for 10 min to remove impurities. All scaffolds were produced with crustacean-based chitosan unless otherwise stated.

2.1.2. Chitosan coating films for tissue culture vessels

The chitosan solution was pre-warmed to 45 °C and then pipetted onto warmed tissue culture plates or dishes of various dimensions, insuring the absence of bubbles or gaps in coating. Any excess solution was removed leaving a thin film coating. The coating was allowed to dry for at least 24 h at room temperature under clean conditions. The chitosan coatings were neutralized using 0.5 M NaOH for 10 min and then rinsed 4 times with distilled water. The coating was sterilized in 70% ethanol, followed by 4 rinses with sterile distilled water. For added substrate cell adhesion, research grade heparin sodium salt from porcine intestinal mucosa (5 mg/mL; 10X stock) purchased from Sigma-Aldrich (St Louis, MO) or clinical grade heparin purchased from Sagent Pharma (Schaumburg, IL) was added to a crosslinker Genipin (100 mg/mL; 1000X stock) that was pre-dissolved in DMSO and purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Both were further dissolved in HBS (50 mM HEPES Balanced Salt Solution dissolved in 0.9% NaCl solution pH 7.5) immediately prior to adding to solidified chitosan coating film. The heparin-genipin was allowed to adhere to the chitosan overnight at room temperature. Then all vessels were gently washed 4 times in HBS followed by 2 times in double distilled water (pH 7.5). Plates were allowed to air dry and could be stored for later use at room temperature. For cell adhesion, the chitosan-heparin-genipin (CHG) coating were subsequently adsorbed with solutions diluted to equivalent molarities of 45 mM with either human FN (FN) (stock 10 μg/mL) purchased from BD BioSciences (Franklin Lakes, NJ), Gly-Arg-Gly-Asp-Ser (RGD) and Cys-Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg (IKVAV), purchased from Sigma-Aldrich (Cat #sG-4391 and C-6171), were prepared in distilled water, covered and left overnight at room temperature. For quicker adhesion, double the concentration was placed on the cultureware and placed in a 37 °C incubator for 4 h. The plates were rinsed 1 time with water and inoculated with different concentrations of recombinant human FGF-2, human heparin-binding epidermal growth factor (hbEGF), human platelet derived growth factor-AA (PDGF-AA) or human PDGF-AB purchased from Peprotech (Rocky Hill, NJ) or R&D systems, (Minneapolis, MN) dissolved in 1 μg/mL BSA solution for 2 h at room temperature. Finally, the tissue cultures vessels were gently rinsed once and seeded with desired cell density. For controls, standard stem cell growth conditions were employed, which included FN coated tissue culture plates and media supplemented with growth factors daily.

2.2. Cell culture

2.2.1. RG3.6 cell line

RG3.6 is an immortalized cell line obtained by introducing *v-myc* to radial glial cells from embryonic cortex day 13.5 of green fluorescent protein positive (GFP^+) rats (donated by Dr. Martin Grumet, Rutgers, New Brunswick) [9]. RG3.6 cells were grown as adherent monolayers on coated tissue culture dishes in radial glial medium (RGM) containing equal parts of Dulbecco's modified Eagle's Medium (DMEM) and F12 medium supplemented with B27, 50 μg/mL gentamicin and 50 μg/mL apo-transferrin. The stock media was furthered supplemented to 10 ng/mL FGF-2 in 1 ng/ml heparan sulphate at final concentration. Ten percent of the medium was changed every day and replaced with equal volume of a 10X FGF-2 containing media (100 ng/mL) to maintain a constant supply of growth factors.

2.2.2. Human neural stem cells

Human Neural Stem Cells purchased from Sigma-Aldrich (St Louis, MO) were derived from H9 (WA09) human embryonic stem

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