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The influence of extracellular matrix composition on the differentiation of neuronal subtypes in tissue engineered innervated intestinal smooth muscle sheets

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Abstract

Differentiation of enteric neural stem cells into several appropriate neural phenotypes is crucial while considering transplantation as a cellular therapy to treat enteric neuropathies. We describe the formation of tissue engineered innervated sheets, where intestinal smooth muscle and enteric neuronal progenitor cells are brought into close association in extracellular matrix (ECM) based microenvironments. Uniaxial alignment of constituent smooth muscle cells was achieved by substrate microtopography. The smooth muscle component of the tissue engineered sheets maintained a contractile phenotype irrespective of the ECM composition, and generated equivalent contractions in response to potassium chloride stimulation, similar to native intestinal tissue. We provided enteric neuronal progenitor cells with permissive ECM-based compositional and viscoelastic cues to generate excitatory and inhibitory neuronal subtypes. In the presence of the smooth muscle cells, the enteric neuronal progenitor cells differentiated to functionally innervate the smooth muscle. The differentiation of specific neuronal subtypes was influenced by the ECM microenvironment, namely combinations of collagen-I, collagen-IV, laminin and/or heparan sulfate. The physiology of differentiated neurons within tissue engineered sheets was evaluated. Sheets with composite collagen and laminin had the most similar patterns of Acetylcholine-induced contraction to native intestinal tissue, corresponding to an increased protein expression of choline acetyltransferase. An enriched nitrergic neuronal population, evidenced by an increased expression of neuronal nitric oxide synthase, was obtained in tissue engineered sheets that included collagen-IV. These sheets had a significantly increased magnitude of electrical field stimulated relaxation, sensitive maximally to nitric oxide synthase inhibition. Tissue engineered sheets containing laminin and/or heparan sulfate had a balanced expression of contractile and relaxant motor neurons. Our studies demonstrated that neuronal subtype was modulated by

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varying ECM composition. This observation could be utilized to derive enriched populations of specific enteric neurons in vitro prior to transplantation.

Keywords

Neural stem cell; neural tissue engineering; extracellular matrix; adult stem cell; enteric nervous system

1. Introduction

An uninterrupted enteric nervous system with the preservation of myenteric ganglia is required for intestinal motility and function [1, 2]. Motor neurons of the myenteric ganglia pre-dominantly express acetylcholine/tachykinins (excitatory) or nitric oxide/inhibitory peptides/purines (inhibitory) to mediate smooth muscle contraction and relaxation [3, 4]. Partial, selective or total loss of neurons is reported in several disorders including, but not limited to, Hirschsprung's disease, achalasia, and inflammation [5-8]. Neural-crest derived enteric neuronal progenitor cells have been isolated from adult mammalian guts, including ganglionic bowel of patients with Hirschsprung's disease [9-12]. These cells have the ability to differentiate into neuronal and glial phenotypes [13-15]. However, there is little information and understanding of microenvironment driven differentiation and limited studies describing subsequent functional behavior of these differentiated neurons in vitro [10, 11].

The ECM has been long known to provide permissive and non-permissive environmental cues for migration and differentiation of neuronal progenitor cells [16]. Collagen IV, laminin and heparan sulfate proteoglycans play an important role in guiding the migration of neural-crest derived cells and formation of the enteric nervous system [17-19]. Moreover, myenteric ganglia are surrounded by an ECM composed of type IV Collagen, laminin and a heparan sulfate proteoglycan [20].

Type I fibrillar collagen has favorable biological properties and has been widely used in neural tissue engineering [21, 22]. Collagen IV has been demonstrated to promote neurite outgrowth in neuroepithelial progenitor cells and in sympathetic peripheral neurons in vitro [23, 24]. Collagen IV aids the colonization of the embryonic gut and modulates selective neurotrophic signaling [20, 25]. The IKVAV peptide in laminin interacts with a neurally acquired receptor on post-migratory neural crest stem cells promoting their differentiation [26]. In the gut, heparan sulfate proteoglycan is required for GDNF signaling [27]. Glycosaminoglycan – growth factor interactions additionally stabilize and increase local bioavailability of growth factors, thus inducing neurite outgrowth [28]. The ECM can provide a background upon which cell-cell and cell-matrix signaling can work to regulate phenotypes of differentiating enteric neuronal progenitor cells.

In previous studies using 2D culture substrata, we demonstrated that varying ECM composition of 2D culture substrata influenced neuroglial differentiation of adult enteric neuronal progenitor cells [29]. The number of neurons, neurite lengths and preliminary neuronal network formation were all enhanced in culture substrata that contained collagen

IV, laminin and heparan sulfate. The previous studies, however, were carried out on 2D coated glass coverslips. Moreover, we examined neuronal and glial differentiation, without documenting the phenotype or functionality of the differentiated neurons. In the present studies, we utilize tissue engineering as a tool to provide enteric neuronal progenitor cells with 3D viscoelastic ECM microenvironments. Given that the cues for differentiation arising from a contractile phenotype of smooth muscle cells would be constant, we hypothesized that ECM composition could differentially regulate the generation of specific neuronal subsets. The objective of the current undertaking was to determine the presence of differentiated motor neurons obtained and investigate their ability in mediating smooth muscle contraction/relaxation.

We utilize a previously described tissue engineered model of intestinal longitudinal smooth muscle sheets, where uniaxial alignment of a smooth muscle monolayer was facilitated by the use of substrate microtopography [30]. We innervated these tissue engineered sheets using enteric neuronal progenitor cells embedded with hydrogels of varying ECM composition. Differentiated neuronal composition (cholinergic, nitrergic, peptidergic) within tissue engineered sheets was evaluated. Functional neuronal physiology mediating smooth muscle contraction/relaxation was also evaluated in the tissue engineered sheets using real-time force generation measurements.

2. Materials and Methods

2.1 Materials

All tissue culture reagents (including media, supplements, and natural mouse laminin) were purchased from Invitrogen (Carlsbad, CA). Rat tail type I collagen and mouse collagen type IV were purchased from BD Biosciences (Bedford, MA). Heparan sulfate was purchased from Celsus (Cincinnati, OH). Growth factors were purchased from Stemgent (Cambridge, MA). All primary antibodies were purchased from Abcam (Cambridge, MA), unless specified otherwise.

2.2 Isolation and primary culture from adult rabbit GI tissues

Enteric neuronal progenitor cells were isolated from jejunal biopsies of adult New Zealand White rabbits using a collagenase/dispase digestion technique and cultured in neuronal growth media, as described previously [29]. Cells aggregated to form enteric neurospheres. Longitudinal smooth muscle cells were isolated from the adult rabbit sigmoid colon as described previously [30]. Isolated longitudinal smooth muscle cells were expanded in culture until confluency.

2.3 Composition and characterization of ECM hydrogels

ECM hydrogels were made with the following components:

- i. Collagen I gels (800-1600µg/ml);
- ii. Collagen I (800µg/ml) and Collagen IV (200µg/ml) gels composite gels;
- iii. Collagen I and Collagen IV with laminin (5-10µg/ml);

iv. Collagen I and Collagen IV with laminin and heparan sulfate (10-20 μ g/ml).

Other components of the gel included: 1% fetal calf serum, 0.1X antibiotics in Dulbecco's modified Eagle's medium. 0.1N Sodium hydroxide was used to adjust pH to ~7.4 for gelation.

2.3.1 Rheological characterization of ECM hydrogels—Oscillatory rheometry (ATS RheoSystems) was used to measure viscoelastic moduli of ECM gels. 20mm parallel base plates were used to perform a stress sweep of the sample at 1Hz. ECM gels were allowed to gel in situ between the parallel plates at 37°C. The viscoelastic modulus was obtained from a linear region of the stress-strain curve, at strains lower than 10%, within the sensitivity ranges for torque and strain of the rheometer. 3-5 individually manufactured ECM gels were measured to determine an average viscoelastic modulus. Compositions that resulted in a matrix viscoelasticity within the range of 150-300Pa were utilized for further experimentation, so as not to let stiffness be a variable in influencing neuroglial differentiation.

2.3.2 Characterization of ultrastructure of ECM hydrogels—Sample preparation of ECM hydrogels for scanning electron microscopy was adapted from Stuart et al. [31]. Gels were dehydrated through graded ethanol (10% to 100%). Hydrogels were dried at critical point using carbon dioxide exchange. The resulting dehydrated ECM discs were mounted onto metallic stubs with conducting carbon tape, sputter coated with gold, and visualized using an AMRAY 1910 Field Emission Scanning Electron Microscope. Constant working distance and magnification were maintained to image all samples. NIH Image J was used to measure and compare fiber diameters. Porosity was determined using Image J from micrographs obtained from at least three-independent samples of dehydrated ECM gels.

2.4 Tissue engineering innervated intestinal smooth muscle sheets

The tissue engineering process was adapted from Raghavan *et al.* [30]. Briefly, 500,000 longitudinal smooth muscle cells were aligned uniaxially for 4 days on 35mm diameter circular Sylgard molds containing wavy microtopographies. Enteric neurospheres were treated with Accutase to obtain single cell suspensions. 200,000 cells were resuspended in the appropriate ECM solution and overlaid on the aligned smooth muscle monolayer. Upon gelation, neuronal differentiation medium (neurobasal-A) was added, supplemented with B27 and 1% fetal bovine serum. Differentiation medium was exchanged every second day. Enteric neuronal progenitor cells were allowed to differentiate within the hydrogel for a period of 10 days. Smooth muscle cells compacted the ECM hydrogel over the next 10 days, forming ~1cm long innervated smooth muscle sheets, anchored between silk sutures. Phase microscopy was used to image neuronal differentiation at the edge of the tissue engineered sheets.

2.5 Biochemical characterization of neuroglial composition in tissue engineered sheets

At day 10, tissue engineered sheets were harvested in radioimmunoprecipitation buffer to isolate protein. Protein concentration was estimated spectrophotometrically using the Bradford assay. 20 μ g of protein from each sample was resolved electrophoretically and

transferred to polyvinylidene difluoride membranes. Membranes were blotted with antibodies for neuronal β III Tubulin, neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), and Smoothelin. β -Actin was used to confirm equal loading. HRP-conjugated secondary antibodies were used to visualize proteins using enhanced chemiluminescence.

2.6 Immunohistochemical characterization of neuron composition in tissue engineered sheets

Tissue engineered sheets were fixed in 4% formaldehyde and washed extensively in glycine buffer. Immunohistochemical staining was performed following previously established protocols utilized for staining differentiated neurons within bioengineered tissues [32]. Sheets were blocked with 10% horse serum and permeabilized in 0.15% Triton-X for 45 minutes. Permeabilized sheets were incubated with primary antibodies directed against Vasoactive Intestinal Peptide (VIP), ChAT and nNOS for 60 minutes at room temperature. Following antibody incubation, sheets were washed three times with phosphate buffered saline, pH 7.4. Tissue engineered sheets were incubated with appropriate fluorophore conjugated secondary antibodies for 45 minutes, washed in phosphate buffered saline and imaged using an inverted fluorescence microscopy (Nikon Ti-E, Japan). For a negative control, incubation with the primary antibody was skipped, and only fluorophore conjugated secondary antibodies were used to visualize background fluorescence.

2.7 Measurement of physiological function in innervated tissue engineered sheets

Myogenic and neuronal functionality were assessed using real-time force generation as previously described [30, 33]. 4-5 individual tissue engineered sheets for each ECM composition were tested. Tissue engineered sheets were anchored between a stationary pin and measuring pin of a force transducer (Harvard Apparatus, Holliston MA) at 0% stretch. The organ bath maintained temperature at 37°C. An additional 10% stretch was applied using a vernier control. Tissues were immersed in 4ml of medium, which was exchanged at the end of every experiment following a brief wash with fresh medium. Peak contraction or maximal relaxation was quantified following pharmacological or electrical stimuli, and compared between tissue engineered sheets with varying ECM compositions.

Before each treatment, tissues were washed in fresh warm medium and allowed to equilibrate to a baseline. The following stimuli were used independently to assess physiological functionality of the tissue engineered sheets: **1)** 60mM Potassium chloride to assess electromechanical coupling integrity of the smooth muscle; **2)** 1 μ M Acetylcholine (contractile agonist); **3)** Electrical field stimulation (5Hz, 0.5ms, 40V) applied using parallel plate platinum electrodes. Preincubation with neuronal blocker, tetrodotoxin (TTX) was used to dissect myogenic and neuronal components of contraction/relaxation. Pre-incubation with specific inhibitors were used to identify functional neuronal subtypes: **1)** nNOS-blocker N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME; 300 μ M); and **2)** VIP-receptor antagonist [D-p-Cl-Phe6, Leu17]-Vasoactive Intestinal Peptide (VIP-Ra; 2 μ M). Following stimulation and subsequent contraction/relaxation and recovery, tissues were washed with fresh medium, and allowed to re-establish a baseline before the next treatment. Equilibrated baseline was arbitrarily set to zero, to measure contraction/relaxation due to a stimulus.

2.8 Data Analysis and statistical methods

Densitometry on western blots was performed using BioRad Quantity One (Hercules, CA). Raw data was acquired from the force transducer at 1000 samples/second. Second order Savitsky-Golay smoothing was applied to data using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA). Area under the curve (AUC) was measured from the time of addition of pharmacological agonist/electrical field to the end of the contraction/relaxation response. Extent of inhibition by pharmacological inhibitors was calculated by expressing the AUC of contraction/relaxation in the presence of the inhibitor as a percentage of the AUC in the absence of the inhibitor. One way ANOVAs with Tukey post-tests were used to compare means using GraphPad Prism. $p < 0.05$ was considered significant. Physiological evaluation and densitometry was carried out between 3-5 tissue engineered sheets within each experimental set; all values are expressed as mean $\pm$ SEM.

3. Results

3.1 Ultrastructure and viscoelastic properties of ECM hydrogels

All compositions of ECM hydrogels gelled at 37°C within 30 minutes. Scanning electron micrographs revealed a fibrous structure in type I Collagen gels (Fig 1A). The fibers were randomly oriented, with diameters averaging at $478.3 \pm 19.31\mu\text{m}$. With the addition of type IV Collagen, network-like structures were observed (Fig 1B). Cables of fibers within the networked structures were thicker, with average diameters of $714.8 \pm 36.67\mu\text{m}$. Addition of laminin to the hydrogels did not alter the ultrastructure or the networked suprastructure (Fig 1C). With the addition of heparan sulfate, the fibers within the networked structures were pulled more tightly together and cabled (Fig 1D). The dehydrated ECM gels displayed a porous appearance, with average porosity ranging from 40.77% - 43.95% (Table 1).

Viscoelastic moduli were measured in hydrated ECM gels using oscillatory rheometry. Type I Collagen gels had increasing viscoelastic moduli with increasing collagen concentration ranging from $72.6 \pm 4.86\text{Pa}$ (800 $\mu\text{g}/\text{ml}$) to 182.3 ± 2.6 (1600 $\mu\text{g}/\text{ml}$) to $424 \pm 2\text{ Pa}$ (3200 $\mu\text{g}/\text{ml}$). The addition of 200 $\mu\text{g}/\text{ml}$ collagen IV to 800 $\mu\text{g}/\text{ml}$ collagen I increased the modulus of the gels to $236 \pm 13.53\text{ Pa}$. The addition of laminin had no effect on viscoelastic moduli (compare $236 \pm 13.53\text{ Pa}$ to $220.7 \pm 16.27\text{ Pa}$). 10 $\mu\text{g}/\text{ml}$ of heparan sulfate caused an increase in the modulus of ECM hydrogels ($287 \pm 20.11\text{ Pa}$, $p < 0.05$). Table 1 summarizes that the final ECM gels evaluated had viscoelastic moduli ranging from 182Pa to 287Pa.

3.2 Neuronal differentiation in engineered innervated intestinal smooth muscle sheets

Uniaxially aligned smooth muscle cells compacted overlaying ECM hydrogels over 10 days in culture as described before [30]. The resultant tissue engineered sheets were ~1cm long, and a few cell layers thick. In the presence of smooth muscle, the enteric neuronal progenitor cells differentiated within the ECM hydrogel. Neuronal differentiation was identified morphologically by microscopic examination at day 10, demonstrating similar differentiation profiles expressed by enteric neuronal progenitor cells, both in vitro [29] and in tissue engineered constructs [32]. Several differentiated neurons were observed in tissue engineered sheets, regardless of the ECM composition (Fig 2). Arrows in the figures indicate numerous instances of neuronal clustering and preliminary neuronal networking.

3.3 Neuronal composition in engineered innervated intestinal smooth muscle sheets

Immunoblotting was used to assess neuronal composition within tissue engineered innervated intestinal smooth muscle sheets. Blotting for β -actin demonstrated that equal amounts of protein were assayed. Representative blots for each protein are shown, indicating the approximate molecular weight at which they appear on the gels (Fig 3E). Contractile phenotype of constituent smooth muscle was demonstrated by the similar expression of smoothelin, within the tissue engineered sheets (Fig 3B). The expression of Smoothelin was constant, regardless of the ECM composition of the sheets, indicating that the constituent smooth muscle cells maintained a contractile phenotype.

3.3.1 Neuronal differentiation—Pan neuronal marker β III Tubulin expression was similar amongst all tissue engineered sheets, despite the ECM composition (Fig 3A). This suggested that irrespective of the ECM composition, neuronal differentiation of enteric neurospheres proceeded similarly in the presence of smooth muscle cells. β III Tubulin expression ranged from 21.65 ± 1.43 AU – 28.98 ± 0.85 AU. β III Tubulin expression was similar amongst various ECM gel compositions (ns; Fig 3A), indicating similar neuronal differentiation.

3.3.2 Cholinergic neurons—Choline acetyltransferase (ChAT) expression was used to detect the presence of cholinergic neurons (Fig 3C). Collagen I (33.73 ± 1.13 AU) and collagen I/IV/laminin (28.82 ± 1.21 AU) sheets had a significantly elevated expression of ChAT compared to sheets with composite collagen and/or heparan sulfate. Immunoblotting demonstrated an enriched cholinergic neuron population in tissue engineered sheets manufactured with collagen I only or composite collagen I/IV with laminin. The presence of cholinergic neurons was additionally confirmed using immunohistochemistry (Fig 4E-H).

3.3.4 Nitroergic inhibitory motor neurons—Neuronal nitric oxide synthase (nNOS) expression was used to detect the presence of inhibitory nitroergic motor neurons (Fig 3D). Sheets with collagen IV (with or without laminin/heparan sulfate) had a significantly higher nNOS expression ranging from 26.37 ± 1.29 AU – 28.15 ± 2.69 AU. Conversely to ChAT, collagen I sheets had minimal nNOS expression (11.33 ± 2.85 AU). Presence of nNOS was additionally confirmed using immunohistochemistry (Fig 4I-L).

3.3.4 VIP-ergic inhibitory motor neurons—Vasoactive intestinal peptide (VIP) motor neurons were identified using immunohistochemistry. VIP neurons were abundant, with increased immunofluorescence in composite hydrogels with laminin and heparan sulfate (Fig 4A-D).

3.4 Agonist-induced contractility of tissue engineered innervated smooth muscle sheets

3.4.1: Potassium Chloride-induced contraction—Electromechanical coupling integrity of constituent smooth muscle cells was first evaluated using potassium chloride (KCl). KCl treatment elicited rapid contractions that were sustained for ~5 minutes (Fig 5). Peak maximal contraction in response to KCl was similar between the different tissue engineered sheets (Fig 5A-D), ranging from 279.5 ± 4.79 μ N to 296.5 ± 6.26 μ N. This correlated with the equivalent expression of contractile smooth muscle marker, Smoothelin,

indicating that the constituent smooth muscle cells within the tissue engineered sheets maintained a contractile phenotype regardless of ECM composition. Furthermore, KCl-induced contractions in tissue engineered sheets were similar to native rabbit intestinal tissues (Fig 5E) in time course, but slightly reduced in magnitude. Peak KCl-induced contractions in native tissue averaged $373.5 \pm 10.63 \mu\text{N}$. KCl-induced contraction was unaffected by pre-treatment with neuronal blocker TTX (red traces; Fig 5), indicating myogenic electromechanical coupling integrity. Figure 5F demonstrates that the area under the curve of contraction was similar in all tissue engineered sheets, and was significantly higher in native rabbit intestinal tissues. Although reduced in magnitude compared to native tissue, KCl-induced contractions were similar among the different tissue engineered sheets, indicating a robust contractile smooth muscle phenotype unaffected by the ECM composition.

3.4.2: Acetylcholine-induced contraction—Exogenous addition of $1 \mu\text{M}$

Acetylcholine (ACh) was used to simulate agonist-induced contraction. All tissue engineered sheets contracted in response to ACh, and sustained contractions up to ~ 5 minutes post stimulation with ACh (Fig 6A-D). Tissue engineered sheets with composite collagen I/IV with laminin had a significantly elevated peak maximal ACh-induced contraction (Fig 6C; $232.9 \pm 8.167 \mu\text{N}$), as well as an elevated area under the curve of contraction ($47606 \pm 2054 \text{ AU}$). Magnitude of ACh-induced contraction was still significantly lower compared to contraction in native tissue ($342.6 \pm 3.15 \mu\text{N}$; $70448 \pm 5876 \text{ AU}$). However, the time course of contraction was very similar to native tissue in tissue engineered sheets containing laminin, reaching maximal contraction within a minute of agonist stimulation. Collagen I sheets also had an elevated ACh-induced contraction (Fig 6A; $238.9 \pm 13.72 \mu\text{N}$; $42668 \pm 2172 \text{ AU}$) corresponding to the elevated ChAT protein expression. However, the kinetics of contraction did not match native tissue.

In order to estimate the smooth muscle (myogenic) component of ACh-induced contraction, neurotoxin TTX was used as a pretreatment (red traces, Fig 6). Area under the curves of contraction was compared with and without TTX pre-treatment in order to estimate %inhibition (Fig 6F). %Inhibition of ACh-induced contraction in the presence of TTX was highest in two ECM conditions: i) collagen I sheets ($72.77 \pm 2.45\%$); and ii) collagen I/IV/laminin sheets ($60.58 \pm 1.66\%$). These values of %inhibition were similar to that observed in native tissue ($72.73 \pm 3.66\%$) upon TTX-pretreatment. This increased neuronal contribution to ACh-induced contraction also correlated with the elevated protein expression of ChAT in collagen I and composite collagen I/IV/laminin sheets (Fig 3C). TTX-pretreatment inhibited ACh-induced contraction to a significantly lower extent in collagen I/IV $\pm$ heparan sulfate sheets, ranging from $48.36 \pm 4.36 \%$ (Heparan sulfate) to $50.31 \pm 4.22\%$ (collagen IV; Fig 6F).

3.5 Relaxation in engineered innervated sheets in response to electrical field stimulation

Electrical field stimulation (EFS) at 5Hz, 0.5ms was used to stimulate neurons within the tissue engineered sheets to produce relaxation of smooth muscle (Fig 7). The extent of relaxation was quantified as area under the curve of relaxation. Extent of relaxation significantly varied amongst the tissue engineered sheets with varying ECM compositions.

Sheets bioengineered with collagen IV, which displayed elevated nNOS expression, had higher relaxation compared to sheets bioengineered with collagen I only (compare 109693 ± 8465 AU in collagen I/IV sheets to 23142 ± 4921 in collagen I sheets). Sheets containing laminin and/or heparan sulfate also had significantly elevated relaxation compared to collagen I sheets (68395 - 69025 AU). In response to EFS, native tissues relaxed generating 101550 ± 11279 AU. Tissue engineered sheets with collagen IV and/or laminin and/or heparan sulfate additionally had a time course of relaxation most similar to native tissue. Maximal relaxation was achieved within 2 minutes of EFS, and a subsequent recovery of basal force was complete within 10 minutes. Upon pre-treatment with TTX, EFS-induced relaxation was inhibited entirely (red traces, Fig 7).

3.5.1 Inhibition of nitric oxide synthase—In order to identify the presence and functionality of nitrergic neurons, an inhibitor of nitric oxide synthase (L-NAME) was used (green traces, Fig 8). %inhibition was determined by comparing areas under the curves of maximal relaxation with and without the L-NAME pre-treatment. %inhibition with L-NAME treatment was the lowest in collagen I sheets ($33.37 \pm 8.37\%$; green trace, Fig 8A). This corresponded to the low protein expression of nNOS in collagen I sheets compared to sheets containing collagen IV (Fig 3D). In contrast, the inhibition of nNOS activity attenuated relaxation upto $61.71 \pm 2.82\%$ (green trace, Fig 8B) in collagen I/IV sheets. In sheets containing laminin and heparan sulfate, %inhibition with L-NAME varied between $62.28 \pm 2.75\%$ (laminin, Fig 8C) to $57.16 \pm 1.91\%$ (heparan sulfate). This inhibition is significantly elevated compared to collagen I sheets, corresponding to the increased expression of nNOS observed in the collagen I/collagen IV sheets (Fig 3C). Native tissues had a higher %inhibition with L-NAME ($78.02 \pm 2.85\%$).

3.5.2: Inhibition of the VIP-receptor—The functionality of VIP-ergic neurons was assessed using a VIP receptor antagonist (VIP-Ra). Pre-treatment with VIP-Ra inhibited maximal relaxation in all tissue engineered sheets to varying extents ranging from $55.55 \pm 3.92\%$ - $65.92 \pm 5.38\%$ (pink traces, Fig 9). Inhibition of EFS-induced relaxation indicated the presence of differentiated VIP-ergic neurons in tissue engineered sheets.

4. Discussion

Neural stem cell transplantation is a promising therapeutic approach to repopulate neurons within enteric ganglia. A complete loss of neurons is reported in HSCR, and a partial loss of selective neuronal subtypes is documented in achalasia and stenosis [7, 8, 34]. Several groups have injected enteric neuronal progenitor cells into experimental models of aganglioneurosis, demonstrating the feasibility of transplantation [9, 12, 35]. However, there is inadequate focus on differentiation of progenitor cells into mature neuronal subtypes, and subsequent assessment of functionality. Here, we describe a method to bias differentiation of enteric neuronal progenitor cells in vitro, prior to transplantation.

The ECM microenvironment, consisting of collagens, laminin and proteoglycans, not only acts as a structural framework for cells, but also plays an active role in aiding neurotrophic signaling [16, 36]. For these studies, we chose to evaluate four ECM components (collagen I, collagen IV, laminin and heparan sulfate), three of which are known to be present in adult

myenteric ganglia [37]. Collagen IV has been documented to be favorable for neurite outgrowth and neuronal differentiation [23, 24, 38]. Laminin has long been known for its neurite promoting activity, in central, peripheral, and enteric neurons [26, 29, 39]. The role of the heparan sulfate proteoglycan in neuronal differentiation is also well documented, both developmentally and in regenerative medicine applications [40-42]. Fibrillar Collagen I was used additionally in these studies for ease of gelation and incorporation of other ECM components within a 3D hydrogel.

Apart from composition, substrate elasticity has been demonstrated to affect the differentiation of adult neural stem cells, with neuronal differentiation reported between 100-500Pa [43, 44]. ECM hydrogel compositions were adjusted in order to maintain their viscoelastic modulus within the range suitable for neuronal differentiation (Table 1). Structural architecture was verified using scanning electron microscopy, wherein the addition of collagen IV demonstrated the presence of network structures, similar to self-assembled collagen IV in the mammalian basement membrane [45]. The addition of laminin did not alter the ultrastructure, because it was expected to coat collagen fibers evenly. Additionally, laminin was also not expected to alter the stiffness/viscoelasticity of the gels, given the manner of its interaction with the collagens [46-48]. The glycosaminoglycan chains of heparan sulfate are documented to cross link between laminin and collagen IV, thereby pulling fibers into a more compact structure, and slightly increasing the viscoelasticity of ECM gels [45, 49].

4.1 Smooth muscle cells within tissue engineered sheets drive the differentiation of enteric neuronal progenitor cells

Tissue engineered sheets provided a good modality to assess variability of differentiated neurons due to ECM composition as well as the functionality of differentiated neurons. The proximity to smooth muscle promoted the differentiation of enteric neuronal progenitor cells extensively. In vitro differentiation of neural stem cells in the presence of gut-derived factors has been demonstrated previously by us and others [32, 33, 50]. Neurotrophic factors (NT-3, Neurturin, GDNF) and morphogens (BMP-2/4) capable of driving enteric neuronal progenitor cell proliferation and differentiation have been demonstrated to arise from the smooth muscle and mesenchyme of the developing and adult gut [51-53]. Recently, the postnatal bowel was demonstrated to support the differentiation of enteric neuronal progenitor cells, strengthening the fact that cues for differentiation can be derived from the postnatal gut [54, 55]. Hence, it was expected that smooth muscle cells within the tissue engineered sheets would drive the differentiation of enteric neuronal progenitor cells. We evaluated all tissue engineered sheets to ensure that the constituent smooth muscle cells demonstrated a contractile phenotype expressing Smoothelin (Fig 3B). Smoothelin expression has been previously demonstrated to be essential for contractility of smooth muscle [56]. In line with the equivalent expression of smoothelin, myogenic electromechanical coupling integrity was also equivalent in the tissue engineered sheets (Fig 5A-D). Similar patterns of contractions were observed in tissue engineered sheets in response to KCl, regardless of ECM composition.

4.2 ECM modulates differential neuronal subtypes while supporting overall smooth muscle-driven neuronal differentiation

In the presence of the smooth muscle, enteric neuronal progenitor cells differentiated, and expressed similar amounts of pan-neuronal marker β III Tubulin (Figure 3A), suggesting that smooth muscle derived factors and substrate viscoelasticity were suitable for overall neuronal differentiation. However, on closer examination of neural subtypes, there was a differential expression of excitatory and inhibitory markers within tissue engineered sheets with varying ECM compositions (Fig 3).

Sheets containing laminin had a balanced expression of both ChAT and nNOS. Kinetics of Ach-induced contraction in laminin sheets was most similar to native tissue, indicating the presence of an increased viable cholinergic neuronal component in composite collagen/laminin sheets. Furthermore, attenuation of EFS-induced relaxation by L-NAME (~62%) was also kinetically similar to native tissue (~78%), indicating the presence of a nitrergic neuronal component.

Collagen I, in the absence of any other matrix components, was the ECM of choice when an enriched cholinergic neuronal population was required, with a significantly diminished nitrergic neuronal population (Fig 3C-D). While these sheets demonstrated a robust TTX-sensitive Ach-induced contraction commensurate with the heightened ChAT protein expression, relaxation in response to an electrical field was diminished. Furthermore, there was minimal attenuation of relaxation upon the inhibition of nNOS, correlating with the low nNOS expression.

Composite Collagen I/IV sheets had an enhanced nNOS protein expression, with an associated increase in EFS induced relaxation (Fig 7B). AUC of relaxation in composite Collagen I/IV sheets was comparable to native intestinal tissue. However, both ChAT expression and contraction was lower in composite collagen sheets.

4.3 ECM is a framework upon which smooth muscle derived factors regulate differentiation of neural subtypes

Overall, the studies in this paper contribute towards understanding the impact of environmental and molecular mechanisms associated with enteric neural subset activation. Here we demonstrate a critical role of collagen I and collagen IV containing ECM environments in promoting excitatory and inhibitory motor neurons, respectively. The ECM microenvironment plays a role in modulating neurotrophic as well as morphogenetic signaling. Morphogenetic signaling via the BMP family expressed in fetal gut is important for the phenotypic diversity of enteric ganglia, including nitrergic and VIP-ergic neuron differentiation [57] [58] [59, 60]. Collagen IV is documented to modulate BMP signaling, and heparan sulfate modulates GDNF signaling in the gut [25, 27]. Immunoreactivity of neurotrophic factor, NT-3, has been observed in ganglia and in the ECM molecules surrounding them, suggesting a role for a Collagen IV-based ECM to modulate NT-3 signaling [61]. The constituent smooth muscle phenotype in tissue engineered sheets was contractile, expressing smoothelin, and generating contractions and relaxations approaching ~60% of those generated by native intestinal tissue. Differentiation cues arising from the

constituent smooth muscle cells drove enteric neuronal differentiation. Furthermore, it is likely that the ECM could act as a framework for smooth muscle-derived factors, enhancing or inhibiting their effects, resulting in the generation of differential neuronal phenotypes.

5. Conclusions

The studies in this paper used tissue engineering as a tool to evaluate the effect of ECM composition on the differentiation of adult enteric neuronal progenitor cells into mature neuronal subtypes. Furthermore, our studies indicated that different neuronal subtypes emerged within microenvironments that varied in their ECM composition. A combination of biochemical, immunohistochemical and physiological analyses revealed that several functional differentiated neuronal subtypes were present in tissue engineered intestinal sheets, capable of mediating smooth muscle contraction/relaxation. Neuronal populations varied from being highly cholinergic (collagen I), highly nitrergic (collagen IV), or balanced between the two (laminin and/or heparan sulfate). Additionally, peptidergic neurons with VIP immunoreactivity were also detected. Our studies indicate that neuronal differentiation was modulated by varying the composition of ECM microenvironments. Enriched populations of differentiated neurons can be derived within transplantable tissue engineered sheets, using ECM microenvironments. ECM microenvironments may also facilitate adequate trophic support and phenotype maintenance of differentiated neurons.

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Figure 1. Scanning electron micrographs of dehydrated ECM gels

Images were obtained at constant magnification and constant working distance. (A) Collagen I fibers were randomly oriented, and presented a dense fibrous structure; (B) Composite Collagen I/IV sheets demonstrated evidence of the formation of network-like structures; (C) There was no difference in ultrastructure with the addition of laminin; (D) Evidence of cabling and cross-linking was observed with the addition of heparan sulfate. Average porosity was determined and summarized in Table 1. Viscoelastic modulus was calculated using oscillatory rheometry of ECM gels in their hydrated state, and tabulated in Table 1. Scale bar 10 μ m.

Figure 2. Neuronal differentiation within tissue engineered sheets

Phase contrast micrographs were obtained at the edge of the tissue engineered sheets.

Evidence of neuronal differentiation and initiation of network formation was observed in all ECM compositions at day 10. The arrows indicate instances of preliminary neuronal networking. Scale bar 200µm.

Figure 3. Immunoblot analysis of tissue engineered longitudinal sheets

Sheets were assessed for expression of neuronal differentiation, constituent smooth muscle phenotype, and excitatory and inhibitory neural markers. Densitometry was used to quantify band intensities, to quantify and compare expression. (A) Neuronal β III Tubulin expression was similar amongst all four matrices suggesting that all ECM compositions supported neuronal differentiation; (B) Constituent smooth muscle within the tissue engineered sheets maintained contractile phenotype, demonstrated by similar Smoothelin expression; (C) Choline acetyltransferase (ChAT) expression was significantly ($*p < 0.05$) elevated in Col I and Col I/IV/Laminin sheets compared to Col I/IV and Col I/IV/Lam/HS sheets; (D) Neuronal nitric oxide synthase (nNOS) expression was significantly lower ($**p < 0.001$) in Col I sheets compared to elevated levels in all tissue engineered sheets containing Col4 with or without laminin and/or heparan sulfate. (E) Representative immunoblots are provided along with β Actin, demonstrating equal loading.

Figure 4. Immunofluorescence for differentiated neurons within tissue engineered sheets
 Differentiated neurons within tissue engineered sheets were stained with markers for vasoactive intestinal peptide (VIP; red), choline acetyltransferase (ChAT; red) or neuronal nitric oxide synthase (nNOS; green). (A-D) Numerous differentiated VIP-ergic neurons were present in tissue engineered sheets. (E-H) Differentiated excitatory cholinergic neurons expressing ChAT were present within tissue engineered sheets; (I-L) Differentiated inhibitory nitroergic neurons expressing nNOS were present within tissue engineered sheets. Scale bar 100 μ m.

Figure 5. Potassium chloride induced contraction of tissue engineered sheets

60mM Potassium chloride (KCl) was used to examine the electromechanical coupling integrity of the constituent smooth muscle cells within the tissue engineered sheets. The black traces indicate the contraction in response to the addition of KCl. The red traces indicate the addition of KCl in the presence of a neuronal blocker, TTX. Pre-treatment with TTX did not inhibit KCl-induced contraction. The ECM composition of the tissue engineered sheets did not affect smooth muscle contraction, evidenced by similar contractile patterns in response to KCl stimulation. A robust and immediate contraction was observed upon addition of KCl (indicated by the arrows) in all tissue engineered sheets (A-D), similar to native rabbit intestinal tissue (E). Peak contraction in response to KCl ranged between 279.5μN and 296.5μN in tissue engineered sheets, and averaged at 373.3 ± 10.63 in native tissue. (F) The area under the curve of KCl-induced contraction was quantified to demonstrate no significant (ns) difference in contraction in tissue engineered sheets, with a slightly elevated ($*p < 0.05$) magnitude in native tissue.

Figure 6. Acetylcholine induced contraction

Addition of 1μM Acetylcholine (ACh; arrow) resulted in contraction of tissue engineered sheets, as well as native tissue. Red traces demonstrate ACh treatment in the presence of neuronal blocker, TTX. (A-E) Representative tracings of ACh-induced contraction in tissue engineered sheets and native tissue. Magnitude of ACh-induced contraction varied between tissue engineered sheets. Comparison of the area under the curve of contraction demonstrated that tissue engineered sheets approached 31.5% (Col I) - 67.6% (Laminin) of contraction observed in native tissue. In the presence of TTX, magnitude of ACh-induced contraction was attenuated. Quantification of inhibition (F) revealed that the degree of inhibition with TTX varied amongst the tissue engineered sheets with different ECM compositions. Highest inhibition was observed in Col I ($72.77 \pm 2.5\%$) and Col I/IV/Laminin ($60.58 \pm 1.7\%$) sheets, indicating an elevated presence of cholinergic neurons contributing to ACh-induced contraction. Significantly lower inhibition ($*p < 0.05$; $48.36 \pm 4.3\%$ - $50.31 \pm 4.2\%$) was seen in Col I/IV and Col I/IV/Lam/Heparan sulfate sheets. TTX pre-treatment attenuated ACh-induced contraction by $72.73 \pm 3.7\%$ in native tissue.

Figure 7. Electrical Field Stimulation induced relaxation in tissue engineered sheets

Electrical Field stimulation (EFS; shaded gray area) was used to stimulate relaxation in tissue engineered sheets (A-D) and native tissue (E). Red traces indicate TTX pre-treatment. EFS induced relaxation was significantly attenuated by TTX-pretreatment ($90.9 \pm 2.41\%$ - $94.41 \pm 0.93\%$), indicating that differentiated neurons within the tissue engineered sheets were capable of evoking smooth muscle relaxation. The magnitude of relaxation varied amongst the tissue engineered sheets (summarized in Table 3). (F) Quantification of the area under the curve of relaxation indicated that Col I sheets had a significantly low ($***p < 0.001$; 23142 ± 4921 AU) magnitude of relaxation. Relaxation in Col I/IV sheets (109693 ± 8465 AU) were similar to those observed in native tissue (101550 ± 11279 AU) in response to the electrical field, indicating the presence of elevated levels of inhibitory motor neurons capable of mediating relaxation. Relaxation was higher in Col I/IV/Laminin (69025 ± 7154 AU) and Col I/IV/Lam/Heparan sulfate (68395 ± 8228 AU) sheets, when compared to Col I, also indicating a similar increase in the presence of inhibitory motor neurons.

Figure 8. Inhibition of relaxation with L-NAME

The functionality of nitrergic neurons was studied by inhibiting EFS-induced relaxation with L-NAME, a non-metabolizable substrate for nNOS. The green traces indicate EFS in the presence of L-NAME. Pretreatment with L-NAME attenuated EFS-induced relaxation in all tissue engineered sheets (A-D) and native tissue (E). (F) Quantification of the area under the curve for relaxation indicated that the extent of L-NAME inhibition varied amongst the tissue engineered sheets. Col I sheets had a significantly lower %inhibition with L-NAME ($*p < 0.05$; $33.4 \pm 8.4\%$) corresponding to the lowest immunoblot expression of nNOS. The degree of L-NAME inhibition was higher in tissue engineered sheets containing Col I/IV and/or laminin and/or heparan sulfate (57.16% - 62.28%), corresponding to the higher immunoblot expression of nNOS. Attenuation of relaxation in the presence of L-NAME was $78 \pm 2.9\%$ in native tissue.

Figure 9. Inhibition of relaxation with VIP-Ra

The functionality of VIP-ergic neurons was studied by inhibiting EFS-induced relaxation with VIP-Ra, a VIP-receptor antagonist. Purple traces indicate EFS in the presence of VIP-Ra. In the presence of VIP-Ra, EFS-induced relaxation was attenuated in tissue engineered sheets (A-D) and in native tissue (E), indicating the presence of functional VIP-ergic neurons capable of mediating smooth muscle relaxation upon electrical field stimulation. Area under the curve of relaxation was quantified, to calculate the %inhibition of relaxation in the presence of VIP-Ra (F). Extent of VIP-Ra induced inhibition of relaxation varied from $56.55 \pm 3.12\%$ - $63.11 \pm 3.2\%$ in tissue engineered sheets, and averaged at $73.32 \pm 3.23\%$ in native tissue.

Table 1

ECM composition	Viscoelastic modulus (Pa)	Porosity (%)
Coll	182.3 ± 2.6	43.95 ± 0.47
Coll/Coll4	236 ± 13.5	43.95 ± 0.79
Coll/4Laminin	220.7 ± 16.27	42.28 ± 0.51
Coll/4Laminin/Heparan sulfate	287 ± 20.11	40.77 ± 0.29

Table 2

ECM/Force	Force (μ N)	Area under the Curve (AU)	Inhibition of contraction by TTX (%AUC)
Collagen I	238.9 $\pm$ 13.72*	42668 $\pm$ 2172*	72.77 $\pm$ 2.47
Collagen I/IV	170.5 $\pm$ 7.56	22201 $\pm$ 2507	50.31 $\pm$ 4.23
Collagen I/IV/Laminin	232.9 $\pm$ 8.17*	46409 $\pm$ 2361*	60.58 $\pm$ 1.68
Collagen I/IV/Laminin/Heparan sulfate	185.1 $\pm$ 10.55	37447 $\pm$ 2534	48.36 $\pm$ 4.36
Native intestinal tissue	342.6 $\pm$ 3.15**	70448 $\pm$ 5876**	72.73 $\pm$ 3.66

Table 3

ECM/Force	Force (μ N)	Area under the Curve (AU)	Inhibition of relaxation by TTX (%AUC)	Inhibition of relaxation by L-NAME (%AUC)	Inhibition of relaxation by VIP-Ra (%AUC)
Collagen I	-146.9 ± 10.63	23142 ± 4921	90.99 ± 2.41	33.37 ± 8.37	56.55 ± 3.12
Collagen I/IV	-389.7 ± 12.19	109693 ± 8465	94.41 ± 0.93	61.71 ± 2.82	63.11 ± 3.2
Collagen I/IV/Laminin	-250.8 ± 32.02	69025 ± 7154	92.09 ± 1.24	62.28 ± 2.75	60.96 ± 5.19
Collagen I/IV/Laminin/Heparan sulfate	-239.5 ± 20.28	68395 ± 8228	92.78 ± 0.93	57.16 ± 1.91	61.92 ± 4.77
Native intestinal tissue	-355.8 ± 17.47	101550 ± 11279	93.05 ± 0.71	78.02 ± 2.85	73.32 ± 3.23