A MICROFLUIDIC CAPTURE AND RELEASE METHOD FOR ISOLATION INTESTINAL PROGENITOR AND STEM CELLS FROM NATIVE RAT TISSUE ENABLING ADVANCES IN VASCULOGENIC CO-CULTURES.

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Not all who wander are lost.

-J.R.R Tolkien
ABSTRACT

Stem cells, multi-potent cells with capabilities to differentiate into many different cellular and tissue specific subtypes, are an active area of biological research. Stem cells reside within the human body in a variety different locations ranging from blood derived to tissue specific stem cells including cornea, skin, and intestinal. Movements towards intestinal stem cells, residing in the crypt niche, have demonstrated self-renewing capabilities in vitro. The identification of tissue specific cells, such as intestinal stem cells, have lead to therapeutic discoveries and advanced tissue engineering to give the ability to cultivate whole organs. While the identification of these stem cells has been made, an isolation procedure which does not disrupt the stem cells native state is a much needed technology. A new isolation technology could provide a translational bridge to advance research in understanding native tissue derived stem cells for cellular based therapeutics.

As a means to isolate intestinal stem cells, conventional methods rely on genetic hybridization techniques which employ a viral vector to modify host genetic code. The genetic technique works by changing the genetic code of the stem cell of interest to express a fluorescent protein. In turn, the tissue digestate can be sorted against the fluorescent protein using an instrument known as fluorescence activated cell sorter (FACS). The instrument has some significant caveats in use such as: extremely low isolation efficiency and low throughput, which impacts viability and overall culture plating efficiency.
This isolation approach is limited to hybridization techniques following a genetic model not indicative of a native stem cell. By contrast, this dissertation describes a novel microfluidic cell capture and release platform to enrich for rare tissue specific stem and progenitor cells within native conditions without the need of a FACS instrument. The platform incorporates the use of a microfluidic post array coupled with an alginate-PEG moiety containing a bound capture protein which allows for selective capture and release of target cells with a simple chelation step. Illustrated in chapter 3, incorporates anti-CD133 into the hydrogel for selective intestinal progenitor cell enrichment where as chapter 4 demonstrates stem cell isolation implementing anti-GPR49 as the capture protein. This approach has lead to a novel cell separation prototype to isolate intestinal stem cells from native tissue digestate without the need for conventional genetic hybridization techniques. In addition, the approach provides a greater throughput (35,000 cells/min) and higher viability (93%) in comparison to the state of the art.

Concurrent with the developed cell isolation platform, an investigation into vasculogenic co-cultures with crypts derived from native intestinal digestate to determine migrational responses was conducted. The motivation is to bridge a tag-free cell separation platform for native tissue digest to perform functional co-culture vascularization assays for intestinal tissue engineering. Rat aortic smooth muscle cells, a migratory vascular cell line, were co-cultured with cells derived from an enriched rat crypt suspension. The forming intestinal stem and progenitor cells clusters, enteroid and enterospheres respectively, elicited a migratory response to the vascular cell line. Quantification of a chemo-attractant, VEGF, demonstrated that the exogenous source is localized to the forming intestinal clusters within the co-culture. Additionally, confocal
microscopy aided in determining directionality of the migratory vascular cell line and identification of cell types via immuno-histochemical staining. These new insights into the intestinal enteroid and enterosphere induced vascular migration may allow for a better understanding in developmental cues and disease states as a result of over-vascularization.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ ix
LIST OF TABLES ........................................................................................................... x
1.0 INTRODUCTION..................................................................................................... 1

2.0 CRITICAL LITERATURE REVIEW ...................................................................... 6
  2.1 Microfluidic Enrichment Techniques .................................................................... 6
    2.1.1 Adhesion-Based Separations ........................................................................... 8
    2.1.2 Capture and Release ....................................................................................... 14
  2.2 Discovery and isolation of multi-potent intestinal cells ....................................... 17
  2.3 Intestinal Stem and Progenitor Cell Cultures ....................................................... 20
  2.4 Intestinal Co-cultures ......................................................................................... 24
  2.5 Intestinal Angiogenic and Vascularization Systems .......................................... 28
  2.6 Summary ........................................................................................................... 34

3.0 LABEL-FREE ISOLATION AND CULTURE OF INTESTINAL PROGENITOR
   CELLS FROM NATIVE RAT TISSUE USING MICROFLUIDIC DEVICES ......... 36
  3.1 Methods For Tag-Free Isolation of Intestinal Progenitor Cells ......................... 36
    3.1.1 Neonatal Rat Septicemians .......................................................................... 37
    3.1.2 Intestinal Tissue Digestion .......................................................................... 37
    3.1.3 Microfluidic Cell Isolation Device Fabrication ........................................... 38
    3.1.4 Intestinal Progenitor Cell Isolation Experiments ....................................... 39
    3.1.5 Flow Cytometry of Intestinal Digestate ...................................................... 39
    3.1.6 Culture of Isolated CD133+ Cells .................................................................. 39
3.1.7 Cultured Cell Analysis

3.1.8 On-chip Staining of CD133+ Cells

3.1.9 Statistics and Data Analysis

3.2 Results and Discussion for Intestinal Progenitor Microfluidic Enrichment

3.2.1 Microfluidic Isolation of Intestinal Progenitor Cells

3.2.2 Characterization of Digested Intestinal Tissue

3.2.3 Culture of Isolated Progenitor Cells

4.0 TAG-FREE ENRICHMENT OF LGR5+ STEM CELLS FROM WILD-TYPE RAT INTESTINE

4.1 Methods for an Optimized Microfluidic Enrichment Platform and Enteroid Promoting Culture System

4.1.1 Tissue Harvest and Digestion

4.1.2 Cell Capture Coating Materials

4.1.3 Validation of Capture Antibody

4.1.4 Microfluidic Device Preparation, Cell Capture, and Cell Release

4.1.5 Flow Cytometry of Intestinal Stem Cells

4.1.6 Culture of Enriched Cell Populations

4.1.7 Immuno-histochemical Staining of Organoid Structures

4.2 Results and Discussion for Selective Microfluidic Enrichment of Lgr5+ from Native Rat Intestine Producing Viable Enteroid Cultures

4.2.1 Microfluidic Enrichment of Lgr5+ Intestinal Stem Cells

4.2.2 Three-Dimensional Culture of Enriched Cells and Single Cell Derived Enteroids

4.2.3 Immuno-staining of Enriched and Un-enriched Structures in Matrigel

5.0 INTESTINAL ORGANOGENESIS ELICITS VASCULOGENESIS IN CO-CULTURE
5.1 Methods for a Novel Vasculogenic Co-culture System..............................72
  5.1.1 RASMC Culture.................................................................73
  5.1.2 Tissue Disaggregation..........................................................73
  5.1.3 Co-Cultures of Crypt Digestate and RASMCs.................................74
  5.1.4 Culture Medium VEGF ELISA Analysis........................................75
  5.1.5 Confocal Microscopy and Immuno-histochemical Staining of Co-cultures.75
  5.1.6 Statistical Analysis...................................................................76
5.2 Intestinal Crypt and rASMC Vasculogenic Co-Cultures.............................76
  5.2.1 Quantifying VEGF Response in RASMC and Crypt Digestate
       Co-Cultures...............................................................................80
  5.2.2 VEGF excess in crypt digestate and uptake inhibition via PF-4............81
  5.2.3 RASMC migration towards intestinal enterospheres via confocal
       microscopy..............................................................................83
6.0 CONCLUSIONS..................................................................................90
  6.1 Characterization of Intestinal Tissue Digestate Gives Insight into Influencing
       Ancillary Cell Types allowing for a Rational Design of an Intestinal Progenitor
       Cell Isolation Platform.................................................................92
  6.2 Optimization of Hydrogel Moiety through Reaction pH and Passivation
       Increased Intestinal Stem Cell Purity Giving Viable Enteroids In Situ........94
  6.3 Intestinal Organoids, Derived from Crypt Digestate, Secrete Exogenous VEGF
       to Promote Vascularization..........................................................97
7.0 RECOMMENDATIONS........................................................................100
8.0 NOMENCLATURE............................................................................104
9.0 REFERENCES....................................................................................106
LIST OF FIGURES

Figure 2.1 Soft-lithography method for fabricating rapid-prototype PDMS microfluidics devices. SU-8, photoresist, is spun onto a silicon wafer and exposed to UV light via a photo mask to obtain desired features. Adapted from Xia and Whitesides [1]………………………………………………8

Figure 2.2 Illustration of silane-GMBS chemistry used to functionalized microfluidic channels with a protein of interest. Channels are exposed to oxygen plasma and reacted with silane followed by a bi-functional dimmer (GMBS). Glycine is used as a simple protein but chemistry can react with any free amine terminus Figure adopted from Shilver Lake [2]…………..9

Figure 2.3 Optimization of a spiral geometry to ensure even distribution of cells in channel. The spiral geometry eliminates dead zones within the channels, allowing for consistent shear stresses for negative selection cell enrichment. Adapted from Green et al. [3]………………………………11

Figure 2.4 (a) A schematic diagram for the fabrication of the nanopatterned microfluidic device. (b) A photograph of the microfluidic device illustrated the respective patterned channels; (c) PUA flat surface, (d) 400 nm pillars, (e) 400 nm perpendicular lines, and (f) 400 nm parallel lines. [4]………………………………………………………………………...13

Figure 2.5 NIPPAAm, a thermo-responsive polymer, can be functionalized with antibodies. (A) General isolation procedure using a micropipette and a colorimetric temperature reference in the center channel. (C-F) Functionalized surfaces using biotinylated antibody and biotin binding protein. Selectively isolated CD4 positive cells and releases by temperature reduction at 32 °C. Figure adopted from [5]……………….15

Figure 2.6 Magnetic separation approach using tunable electromagnetic fields. Blood, containing target cells with superparamagnetic beads attached, and buffer stream are flowed into the devices. The electromagnetic fields displace target cells out of the blood streams into the buffer stream, enriching select cell types. Adopted from Plouffe et al. [6]………………………………16

Figure 2.7 Stem cell zone (bottom) contains CBCs that reside intercalated between paneth cells. +4 LRC are a quiescent cell type that lies above the paneth cell line, which falls within the +4 LRC model. Adopted from Barker et al. [7]…………………………………………………………………18
Figure 2.8 Populations within the intestinal crypt under the overlay of the stem cell zone and +4 LRC model. This figure illustrates transit-amplifying progenitor, crypt based columnar, and +4 LRC cells with their respective markers CD133, Lgr5, and BMI-1. Adopted Montgomery et al. [8].....19

Figure 2.9 Intestinal signaling within the crypt zone. Cascades and growth factors are necessary to trigger the differentiation cues. It is unknown what signaling occurs between CBCs and +4 LRCs. Adopted from David et al [9].....21

Figure 2.10 Bright field images of forming intestinal progenitor and stem cells in vitro. Intestinal progenitor cell clusters, enterospheres, (A) form short-lived cysts and remain round throughout the duration. Conversely, intestinal stem cells form self-renewing clusters, enteroids, (B) which form clear lumen and budding projections. [10].................................22

Figure 2.11 Day progression of FACS-sorted single-Lgr5 cells from a transgenic mouse model. The organoid forms a central lumen followed by hyperplasia after day 5. Adopted from Sato et al. [11]............................23

Figure 2.12 Lgr5 culture optimization using Wnt3a cofactor allowing for increased plating efficiency. Paneth cell dependence is illustrated by sorting Lgr5-paneth cell doublets and comparing to Lgr5-Lgr5 doubles in the presence/absence of Wnt3a. Adopted from Sato et al. [12].......................25

Figure 2.13 Bright field images of crypt derived enteroids in culture. Enteroids were short-lived (A) due to the lack of necessary growth factors such as: EGF and Noggin. Enteroids were seeded onto a monolayer of myofibroblasts (B) without the EGF and Noggin present and proliferated past day 2 in culture. [13]......................................................27

Figure 2.14 Over-expression of VEGF-A increases the severity of the colitis within the DSS treated models, where as VEGFR-1 decreases severity. (A) Weight loss measurements over time for DSS treated models injected with a adenovirus vector for VEGF-A, sVEGFR-1, or control virus. (B) Colitis score was quantified for the respective virus induced models. [14].............29

Figure 2.15 VEGF-A165 pathway affecting SMC migration. VEGF-A165 secreted from a breast cancer tumor cells in turn binding to VEGFR-1 and NRP-1 complex. VEGFR-1/NRP-1 complex initiate Akt cascade causing a migratory gene response of the SMC towards exogenous VEGF-A165 source. [15]..................................................31
Figure 2.16 Effect of VEGF-A\(_{165}\) on VEGFR-1/Flt-1, VEGFR-2/Flk1, and NRP-1 mRNA and protein expression in hAOSMC. (A) Western blots of VEGFR-1 receptor in smooth muscle and endothelial migratory cell lines with \(\beta\)-actin control. VEGF-A\(_{165}\) addition of hAOSMC to show VEGFR-1 expression. (B) Blots highlighting AOSMC and HUVEC VEGFR-2 expression in the presence of VEGF-A\(_{165}\). (C) NRP-1 expression in the presence of VEGF-A\(_{165}\) within hAOSMC and mRNA electrophoresis to further confirm. [15].

Figure 3.1 Microfluidic cell isolation device (a) containing an array of pillars (b). The pillars are coated with an alginate hydrogel functionalized with antibodies against CD133. Arrows indicating captured CD133+ cells (b) and outlined square box illustrating the area of the magnified section (c) of the stained capture chip. Scale bars, 40\(\mu\)m.

Figure 3.2 Purity comparison of injected cells from tissue digestate and released (isolated) CD133+ cells from microfluidic isolation; * denotes p < 0.005 and percent viability of the respective injected and released populations as determined by a live/dead assay ( **p > 0.01). n = 3.

Figure 3.3 Age progression culture of the injected heterogeneous suspension using the proposed culture conditions. Epithelial sheets, illustrated by the green asterisk (*) and cyst like structures (white arrows) were noticed at day 2. Expansion of these cysts structures (white arrows) and dissociation of cluster units (red arrows) were abundant at Day 4 (b). As the culture progressed to day 6 (c), many of the cyst structures dissociated (red arrows) and epithelial sheets (*) were retained. Scale bar, 25\(\mu\)m.

Figure 3.4 Day progression of post-released CD133 suspension into the proposed culture conditions. Red asterics (*) indicated points of reference to accurately track the enterosphere formation overtime. Early cluster onset is noticed at day 2 (a) while a distinct lumen-like morphology is noticed at day 4 (b). Apical expansion, indicated by the arrow, was demonstrated in day 7 (c); however, day 9 (d) demonstrated a dissociation (arrows) and expulsion effect on the enteroid. Scale bar, 50\(\mu\)m.

Figure 4.1 Fluorescence micrographs of an individual intestinal crypt from un-enriched tissue digestate suspension. The overall cell content in the crypt is seen in (a) and within this crypt three cells are observed to stain for GPR49/Lgr5. The merged image (c) illustrates the location of the positive population in relation to other cell types. Scale bar 50 \(\mu\)m.
Figure 4.2 Antibody binding specificity for injected intestinal digestate into a microfluidic post array. (A) Hexagonal post array off set by one 40µm to increase the amount of cellular collisions to the projections. A typical sample of a intestinal digestate population (D) before being injected into the microfluidics device. The suspension was stained with anti-GPR49/Lgr5, illustrated in red (B), and counterstained with a DAPI nuclear stain (C) while in the presence of a blocking solution. Scale bar, 50µm.

Figure 4.3 Optimization of antibody functionalized alginate allowed for improved capture efficiency and purity yields. The samples and formulations were divided into five protocols (a), each varying one variable. Mixing and incubation times are measured in minutes. The purity of the Lgr5+ enrichment device outputs was determined for each protocol and compared against the injected cell population (b). Representative flow cytometry data for the un-enriched digested tissue injected cell population (c) and enriched population (d). Each histogram was gated from the electron volume vs. side scatter regime to mitigate noise, and each gate was propagated through each sample. *p < 0.0005, **p < 0.001, ***p >0.05; n = 3.

Figure 4.4 Three-dimensional culture of Lgr5+ enriched cells compared to un-enriched cells in the absence of Wnt3. The un-enriched enterosphere culture (a-c) yielded different morphology at similar time points compared to the enriched population (d-f). Growth characteristics of an organoid body at Day 2 (d); projections highlighted by arrows at Day 3 (e), and small lumen formation, illustrated by dashed ring, with surrounding secreted apoptotic cells, at Day 4 (f). Scale bars, 100 µm.

Figure 4.5 Enriched and un-enriched structures in the presence of Lgr5 basal media constituents and Wnt3 following 3 days in culture. The majority of structures formed in the un-enriched culture (a,c) were cyst-like and harbored apoptotic cells, highlighted with red arrows. Lgr5+ enriched cells (b,d) exhibited similar morphology and lumen formation (indicated with dashed black ring) as in the Wnt3-absent study (Fig. 3e) at Day 3. Scale bars 100 µm (a,b) and 50 µm (c,d).
**Figure 4.6** Confocal micrographs of an enterosphere formed from un-enriched cells. These images represent a single z-stack. For imaging, this and other structures were extracted from matrigel after 4 days in culture. Dashed ellipses indicate regions of CD24 and GPR49/Lgr5 expression (A and B, respectively). CD24 expression is localized apically in the central domain and in varying levels of intensity (a). GPR49/Lgr5 is localized at regions of CD24 expression, but is only faintly discernible (white arrows highlight regions of highest expression; b). In the above enterosphere, apoptotic cells are discernible within the central lumen (c), while the overall morphology of the cyst-like enterosphere is that of a flat-disk, as illustrated in Fig. 8a. Scale bar, 25 μm

**Figure 4.7** Fluorescence confocal micrographs (from a single z-stack) of a representative enteroid formed by Lgr5+ enriched cells showing staining for CD24 (a), Lgr5 (b), and DAPI (merged image, c). This enteroid is a spherical structure with a hollow (luminal) interior, as reflected in the cartoon shown in Fig 8b. Co-localization of CD24 and Lgr5 expression is indicated by the white dashed circle in a-c. Scale bar, 10 μm

**Figure 4.8** Fluorescent confocal micrograph illustrating an GPR49 enriched intestinal stem cell via the proposed microfluidic separation. The enteroid was stained with a DAPI nuclear stain (a) and GPR49 specific for Lgr5 (c). Hollow domain is illustrated by the transverse image of the z-stack (b). Scale bar, 50 μm

**Figure 4.9** Illustrations of structures formed by un-enriched (A) and Lgr5+ enriched cells (B) in three-dimensional culture. The flattened disc morphology with apoptotic cells enclosed in the middle (a) is representative of the micrographs shown in Figs 5c and 6c. The hollow sphere morphology (b) generated by Lgr5+ enriched cells is representative of micrographs in Figs. 5d and 7c

**Figure 5.1** Vasculogenic co-culture containing small intestinal crypt digestate coupled with RASMCs. Co-cultures were sustained up to 6 days in where enterospheres or enteroids formed and a vasculogenic response was observed (A). Throughout the time course study, RASMC tubules within proximity to the intestinal cluster were quantified (D). In quantifying tubules, a 10μm zone from edge of cluster was drawn and tubules were counted within the dashed boundaries, as illustrated in Day 1 (B) and Day 4 (C) in culture. Scale bar 50 μm; *p > 0.05, **p < 0.05; n=3
Figure 5.2 Conditioned medium enables elongation of RASMC clusters in homogenous suspensions. Resulting images of RASMC culture medium conditioned experiment (A-F) against native culture (G-L). Cultures were stained with DAPI (A,D,G,J) and Phlannoidin-488 (B,E,H,K) to aid in illustrating the cell nuclei and actin filaments, respectively. Under the conditioned set, RASMC clusters form elongated arms (B,E) conversely within the native culture RASMC clusters remain unified (H,K). Scale bar 50µm.

Figure 5.3 ELISA assays specific towards VEGF were preformed with co-culture medium. Time course and medium extractions were preformed at Day 2 (A), Day 4 (B), and Day 6. ELISA data (C) was normalized against basal medium control (grey) and compared to medium within the vasculargenic co-culture (patterned). Scale bar 50µm; *p > 0.01, **p < 0.005, ***p < 0.005; n=3.

Figure 5.4 VEGF ELISA assays were performed with enteroid and enterosphere medium. Culture contained intestinal digestate only and eventual forming enteroid and enterosphere units. Intestinal digestate cultures were stained with DAPI and Phlannodin to aid in illustrated morphology. Numerous enteroid and enterospheres formed in culture (A-C) and many of the structures exhibited a hollow central domain (E-F). Time course and medium extractions were preformed at Day 2, Day 4 and Day 6. ELISA data (G) was normalized against basal medium control (grey) and compared to medium within the intestinal digestate culture (patterned). Scale bar 50µm; *p < 0.01, **p < 0.005, ***p < 0.005; n=3.

Figure 5.5 ELISA assays specific towards VEGF were preformed. Co-culture contained intestinal digestate and RASMC supplemented with proposed medium with the addition of PF-4. Time course and medium extractions were preformed at Day 2 (A), Day 4 (B), and Day 6. ELISA data (C) was normalized against basal medium control containing PF-4 (grey) and compared to co-culture PF-4 medium (patterned). Scale bar 50µm; *p < 0.005, **p > 0.01, ***p > 0.01; n=3.

Figure 5.6 Confocal images of RASMC and intestinal digestate co-culture within basal medium taken at day 2. DAPI nuclear stain (A) indicated surrounding populations and proximities of nuclei. EpCAM stain, illustrated in green, (B) indicated epithelial structures within the culture. A well-known progenitor marker, CD133, highlighted enterospheres within culture. Phlannoidin exposed actin filaments and striations of the RASMC and enterospheres (D). Asterics (*) indicates migrating RASMC towards the enterospheres where the arrow indicates a abundant actin expression.
Merged channel (E) exemplifies morphology and expression patterns of the RASMC and enterospheres. Scale bar 50µm…………………84

Figure 5.7  Vasculogenesis is exhibited in 3- dimensions and is specific towards developing intestinal organoids. Confocal image Z-projection of RASMC and intestinal digestate co-culture at day 2 in basal medium. DAPI (A) illustrates nuclear aggregation (enterosphere) and surrounding RASMC. Highlighting the epithelial structure, EpCAM (B), illustrated the enterosphere morphology and was further confirmed of its enterospheres nature via CD133 (C). Merged channel (D) indicated overall morphology and expression patterns. Phlannoidin highlighted actin filliments with EpCAM (E) and migratory patterns observed in the z-axis perspective. White asteric (*) highlights origin of RASMC culture layer extending to enterospheres (F) from the observed x-axis perspective. In the y-axis, red asteric illustrates a RASMC projection above the monolayer (G). Each asteric corresponding to the z-axis perspective (E). Scale bar 50µm…..86

Figure 5.8  PF-4 inhibites migration of RASMC towards the intestinal organoid. Confocal images of RASMC and intestinal digestate co-culture within inhibitor PF4 medium taken at day 4. DAPI (A) and Actin stain (B), confirmed the presence of the intestinal organoid and surrounding RASMC. Actin stain of the RASMC aids in discerning between migratory or prolifertive responses (B). Expression patterns are appreciated within the merged channel (C) highlighting nuclei directionality in addition to actin filament alignment . Scale bar 50µm………………………………87
LIST OF TABLES

Table 3.1  Intestinal digestate characterization insight into select tissue specific cell types and subtypes. The cell types and subtypes were labeled with their respective antagonist derived from an identical solution to the injected suspension. Populations were counted via flow cytometry and gated for positive populations. \(n=3\)………………………………………………………………………45
1.0 INTRODUCTION

Intestinal diseases and disorders are prevalent amongst multiple age groups around the globe. Disorders such as Crohn’s and Celiac disease are diagnosed 500,000 times a year with little understanding in origin and progression [16]. Intestinal cancers affect a world-wide population, and treatment options are limited to the scrutiny within the gastroenterology community on where these cancers originate. Efficacies of treatments are dependent upon where the cancer resides within the intestinal system and origin [17]. Until recent discoveries, the intestinal stem cell location within the intestinal crypts had been under much debate [7-9,18-28]. The discovery of self renewing niches within the intestinal crypt population have been widely studied and accepted within the gastroenterology community [11]. Recent advances in intestinal stem cell models have constrained the intestinal stem cells to actively cycling (Crypt Based Columnar) and quiescent (+4 Label Retaining Cells). In lieu of these discovered cell types, groups have investigated tumor-initiated and non-initiated cell types allowing for malignant polyp origin and symptomatic disease studies [29]. The discovery of ligand expression trends within the intestinal stem cell zone allows for isolation of progenitor and stem cell populations alike. Therefore, the newly identified markers will facilitate in isolation techniques to be developed allowing for further studies in disease and cancer origins in homogeneous cultures.

Origins of disease and disorders contained in the intestinal stem typically stems within the stem cell zone. The stem cell zone is comprised of multi-potent subtypes such as transit-amplifying cells, crypt based columnar (CBC), and +4 label retaining cells
(LRC). Recent discoveries have shed light on receptor expression and led to methods of identifying CBCs and transit-amplifying cells with immunoglobulin such as Leucine-coupled g-protein (Lgr5) and CD133 [8,27,30-33]. The newly identified markers facilitate isolation of these multi-potent cell subtypes and studies of their self-renewing capabilities. Until recently, there was a lack of a specific antagonist (antibody) to bind Lgr5 in the cell extracellular membrane [7,30,34]. However, recent immunological advances have lead to a commercial anti-Lgr5 antibody to bind intestinal stem cells [35,36]. These isolated cell types, intestinal progenitor and stem cells, are able to be sustained in culture forming self-renewing bodies known as enterospheres and enteroids, respectively [37-41]. Current culture and isolation methods will be outlined in detail within Section 2 of this dissertation. In light of these new culture methods, advances in understanding exogenous responses within culture have been of interest to the gastroenterology community [9,13]. Studying co-culture responses are necessary, enabling the discovery developmental cues and influences of extraneous cell types [12,42-50]. Recent interests have been towards gaining understanding to the aforementioned disease types to better treat symptomatic patients. To Investigators have been correlating the migration of vasculature and over-vascularization to developmental disorders, intestinal cancer, and inflammation responses [14,51-53].

Recent advances within intestinal culture systems have enabled self-sustaining organoid formation through supplemented cytokines. The discovery dependent cytokines, that are necessary to sustain an in vitro intestinal stem cell culture system, have been made possible through defined co-culture systems. The discovered cytokines and growth factors have enabled novel methods in culturing rare and unstable cell types [11,54]. For
example, the intestinal cell type responsible for maintaining bacterial homeostasis (Paneth cells) has recently been found to secrete a necessary growth factor, Wnt3, allowing for an increase in intestinal stem cell sustainability [12,55]. Recent studies have investigated vascular endothelial growth factor (VEGF) within intestinal systems to shed light on its effect within disease states such as: Crohn’s disease and IBS [51]. Hybridized mouse models, expressing Crohn’s pathogenesis, have shown increased vasculature within intestinal jejunum due to extraneous VEGF [14]. VEGF and common symbiotic intestinal bacteria, E. coli, have demonstrated an angiogenic response in vitro with immortalized intestinal stem and cancer cell lines [56]. Angiogenesis and vasculogenic responses will be described in more detail in Chapter 6 of this dissertation. Researchers have been probing into new avenues for use of native tissue types due to the intrinsic caveats with using immortalized cell lines and cells derived from hybridized mice models [57]. With this being said, there is a significant need to develop co-cultures and investigate disease pathogenesis from native tissue thus moving away from conventional label intensive isolation techniques.

Isolation techniques for obtaining pure populations of certain types of cells are essential for studies in biology; studying specific populations, allowing for growth, genomic, and proteomic investigations. Where the current conventions, fluorescent activated cell sorting (FACS) and magnet activated cell sorting (MACS), in cell isolation fall short are applications in tissue engineering. The state of the art systems illustrated allow the end user to obtain a 99% purity population from a heterogeneous sample. Intestinal stem and progenitor cells are typically isolated in this fashion. The pure samples are further cultured downstream, allowing for morphological, genomic, and
proteomic assessments. The cell sorting technology has its shortcomings and limitations in different regimes. MACS depend on the use of a permanent magnet with a strong magnetic potential. Magnets of this caliber rely on rare earth metals which generate a significant expense to the user. On the other hand, FACS analysis uses fluorescent labels that can be localized intra or extra-cellular domain. The ability to couple multiple fluorescent labels in different regions adds multiple dimensions of analysis to the investigator. Current cancer researchers utilize FACS isolation technique to model expression and regulation responses allowing for therapeutics to be developed. However, FACS instruments are extremely expensive to acquire and require extensive training. Furthermore, the machine has technical limitations in where it is constrained to process low sample volumes. The presented constraints have been hindering the gastroenterology and oncology community due to technological limitations [18]. The limitations of FACs are constrained to the separation time which could be upwards of 2 hours to sort against one marker. Time-sensitive cells, e.g stem cells, are difficult to isolate via FACs leading to low viability for the enriched population. Advances in the field of microfluidics allows for higher sample processes, ability to multiplex, and increased throughput capabilities.

Coupling a microfluidic cell separation and affinity capture would provide a novel platform in isolating multi-potent intestinal cells. Eluting the multi-potent cells in an enriched population while retaining viability will enable investigators to culture these cells from wild-type sources. In turn, available techniques for enriching multi-potent intestinal cells can be used in pharmaceuticals diffusion models, creating an accurate in vitro model using in vivo subtypes. In addition, the eluted cell types will be relatively homogeneous giving the end-user the ability for investigative studies into co-culture
systems and exogenous growth factors. Thus, a combination of microfluidics and a functionalized alginate hydrogel will provide the foundation in the capture and release method within a microfluidics platform tailored towards cell separation. The eluted cells will be cultured to form self-sustaining organoid and stained to determine localized expression establishing a similar comparison to conventional isolation techniques. The provided platform will accelerate field of tissue engineering and personalized medicine allowing for a more accurate representation of native tissue vs. the conventional techniques. With respect to co-cultures, investigations within vascularization will give insight into the developmental intestine resulting inflammation responses and disease states of wild-type specimens. This describes a novel microfluidics approach for intestinal progenitor and stem cell isolation that facilitates the isolation of viable and functional cells directly from wild-type tissue. In addition, this dissertation investigates in the co-culture of enterosphere, enteroid, and aortic smooth muscle to give insight into vasculogenic affects.
2.0 CRITICAL LITERATURE REVIEW

Cell isolation or purification is typically the first step in a broad range of biological studies, including genomic, proteomic, and phenotypic analysis [7]. In recent years, advances in separation techniques and technologies have resulted in isolated cell purities of 99% [58]. In the field of microfluidics, cell-separation techniques can be miniaturized allowing for low sample volume, high throughput, and low cost [6,59-73]. The review presented here is a current overview of microfluidics-based cell-separation techniques, illustrating the parameters, benefits, and shortcomings in the context of analyzing intestinal tissue digestate. Furthermore, this review aims to identify isolation markers for intestinal multipotent cell types, and their relation to current isolation techniques and methods for culturing. In addition, this section will also illustrate the shortcomings of current intestinal multipotent cell isolation and motivate for the need for a better microfluidics isolation method while retaining functional characteristics.

2.1 Microfluidic stem cell separation techniques

The microfluidics community has developed many different cell separation techniques to obtain a pure target population from a heterogeneous suspension. These approaches have intrinsic limitations and advantages [3,5,62,71,74-76]. Microfluidic stem cell isolation utilizes carefully designed chemistries and geometries to enrich subtypes based on morphology, phenotype, and expression trends. Integrating microfluidics in applications to cell separation enables high throughput capabilities, ability to multiplex, and reproducibility [71,77,78]. Laminar flow contributes to high reproducibility within microfluidics channels due to its low Reynolds number.
Microfluidic chips are fabricated in a variety of different ways depending on the need of the end user and its scale. On the academic scale, soft lithography is the most widely utilized fabrication technique. In this method, SU-8 photoresist is spun on a silicon wafer for a desired feature height within the micron scale. The spun photoresist is exposed to ultraviolet (UV) light via a transparent geometry of the feature mask, cross linking the exposed SU-8 and creating the desired features, as illustrated in Figure 2.1. The wafer is developed using SU-8 developer, which dissolves non-exposed regions of the photo resist allowing for a negative mold to form. The patterned mold can be used for rapid prototyping of devices by using poly(dimethylsiloxane) (PDMS), an elastomeric polymer. PDMS is poured over the developed features, and cured by heating after exposure to vacuum. Inlets and outlets are made through punchin with a non-beveled syringe, and the PDMS device is bonded to a glass slide after treatment with oxygen plasma, as seen in Figure 2.1.
Figure 2.1 Soft-lithography method for fabricating rapid-prototype PDMS microfluidics devices. SU-8, photoresist, is spun onto a silicon wafer and exposed to UV light via a photo mask to obtain desired features. Adapted from Xia and Whitesides [1].

Within the microfluidics community, this soft lithography technique is commonly used as a cost-effective approach to developing these devices with micron-scale features on a small production scale [1,75]. In general, microfluidic devices can be fabricated with different geometries, integrated with pumps, electrical and optical interfaces, allowing for cell quantification and separation. Furthermore, microfluidic chips can be integrated with each other allowing for versatility in a one-pass cell separation system [59,62]. Microfluidic techniques have key advantages in allowing for rapid prototyping, and when coupled with adhesive surface chemistry and specialized geometries, they can be tailored to cell separation.

2.1.1 Adhesion-Based Separations

In negative selection microfluidics channels are with proteins or antibodies that selectively bind to non-target cells within a heterogeneous suspension. Such microfluidic
channels are staged in series to deplete extraneous cell types, thus enriching a cell type of interest.

A widely accepted method for binding amine-functional ligands to a glass or silicone surface is silane chemistry [2]. As seen in Figure 2.2, free hydroxyl groups are generated from oxygen plasma treatment of glass substrates. The free hydroxyl sites are then reacted with mercapto-siloxane (silane), and a cyclic dimer known as N-(gamma-maleimidobutyryloxy) succinimide (GMBS). The GMBS reactive sites further react with the amine terminus of the ligand, in our case a protein or antibody used to target extraneous cell types.

![Chemical reaction diagram]

**Figure 2.2** Illustration of silane-GMBS chemistry used to functionalized microfluidic channels with a protein of interest. Channels are exposed to oxygen plasma and reacted with silane followed by a bi-functional dimmer (GMBS). Glycine is used as a simple protein but chemistry can react with any free amine terminus. Figure adapted from Shilver and Lake [2].

Recent experiments testing the affects of shear stress on cellular antibody-protein-ligand interactions have yielded methods in isolating cell subtypes with similar marker
expression [79]. However, advancement of this method is limited to the lack of understanding of the thresholds within these cellular ligand coupling. Methods for quantifying maximum shear-stress tolerances for binding interactions have been developed in microfluidics platforms [80]. Hele-Shaw devices have been fabricated using specially designed microfluidic device geometries, allowing for measurement of the effects of a range of different shear stresses on binding interactions within a single device (Figure 2.3) [3,81]. Measurement of shear response enables construction of a working design space for cell capture devices, utilizing appropriate magnitudes of shear stress to minimize nonspecific cell binding.

Combining knowledge of shear stress tolerances of cellular ligand interactions with surface modification has allowed researchers to develop geometries for enrichment of cells via depletion of unwanted cells [62]. Plouffe et al. [82] developed such a depletion platform with serpentine channel geometry. This approach was further optimized by Green et al. [3], who developed a spiral geometry, which limits dead space (Figure 2.3).
Figure 2.3 Optimization of a spiral geometry to ensure even distribution of cells in channel. The spiral geometry eliminates dead zones within the channels, allowing for consistent shear stresses for negative selection cell enrichment. Adapted from Green et al. [3].

Using this procedure, it was shown that a depletion of endothelial cells, smooth muscle cells, and fibroblasts while retaining an enriched population adipose derived stem cells. The stem cell population eluted from the spiral-staged system had a purity of 46% through a one-pass approach, while retaining high viability. This system demonstrated a unique and successful approach in cell enrichment, but the enrichment of subpopulations from tissue digestate is challenging using this technique owing to the adhesive nature of the derived cells leading to nonspecific binding of multiple cell types.

There are several challenges associated with implementing this technique with heterogeneous suspensions. Enrichment or depletion of multiple cell subtypes requires characterization of antibodies and proteins specific to each of those cell types [62]. Regarding experimentation with intestinal tissue, cell subpopulations are vast and many exhibit markers that are co-expressed in several subtypes [83],[30],[84]. Optimization for
a tissue system would require extensive studies of potential capture ligands. Despite these disadvantages and arduous undertakings, negative selection is useful in scenarios in which stem cells do not have a known marker localized in the extracellular domain [62],[82]. Furthermore, negative selection can be used in tandem with a positive selection approach to deplete an abundant cell population. However, in the case of intestinal progenitor and stem cell populations, antibodies have been developed against unique ligands allowing for selective capture [36],[31].

Recent advances in adhesion based cell separation have been providing new avenues of label-free cell isolation for similar cell subpopulations. Incorporating the knowledge of cell spreading and adherence, Kwon et al. [4] developed a unique method in isolating two different types of cancer cell lines. Herein, the group describes a unique microfluidics nano-patterning consisting of pillars, perpendicular lines, and parallel lines (Figure 2.4). Breast cancer carcinoma lines, MCF10A and MCF-7, were used as the model cell lines and further tested under a variety of shear stresses. Once the cells were flown into the microfluidic channel, varying durations of cell adhering to the respective geometries were performed. It was found that MCF10A and MCF-7 exhibited different adherent characteristics in situ within the microfluidics channel. Exposure to different rates of shear were investigated providing a foundation for a separation mechanism.
Figure 2.4 (a) A schematic diagram for the fabrication of the nanopatterned microfluidic device. (b) A photograph of the microfluidic device illustrated the respective patterned channels; (c) PUA flat surface, (d) 400 nm pillars, (e) 400 nm perpendicular lines, and (f) 400 nm parallel lines. [4]

The results of this study found MCF10A are not as adherent to glass substrates within 1 hr incubations; conversely, MCF-7 cells were able to be sheared off of polyurethane acrylate (PUA) at low shear rates at similar incubations. With this given information, the group deduced that a separation mechanism of shear rate and substrate can enable selective cell separation. However, the proposed separation design has some significant limitations when applied to a complex suspension such as intestinal digestate. This technique is limited to samples containing two cell types whereas tissue digestates contain > 12 cell subtypes [85,86].
2.1.2 Capture and Release

Positive selection depends on the notion that the cell population of interest has a known ligand to which an antibody or protein can be selectively attached. However, a major challenge in this approach is retrieval of the captured cells following separation.

Thermo-responsive polymers have been used in a variety of different applications [5]. In recent studies, (poly(N-isopropylacrylamide), (PNIPAAm) was used as the capture and release method, where the capture medium was sensitive to changes in temperature. As seen in Figure 2.5, PNIPAAm is coated and polymerized at 37 °C onto the surface of a microfluidic channel. The coating is further reacted with neutravidin, generating a binding site for a biotinylated antibody. In this study, anti-CD4 and anti-CD34 were covalently bound in separate channels to the PNIPAAm, allowing for selective capture of mononuclear and stem cells from whole blood, respectively. The device was then cooled to below 32 °C thus releasing the polymer-bound neutravidin, and releasing cells attached to the antibody-neutravidin complex. It was reported that this approach had high specificity (89%) and significant release efficiency (59%) [5]. However, this approach did not give rise to pure populations of either mononucleaus or stem cells.

The shortcomings of this technique is due to the polymetric properties of this platform leading to complex expensive chemistries and its adhesive nature. NIPPAAm non-specifically binds proteins, which in turn can lead to nonspecific binding of non-target cell types. Affinity captures and release using the NIPPAAm technique presents significant challenges in isolation of cells from intestinal tissue, owing to the adhesive nature of epithelial cells generating non-specific binding affects. Advances in magnetic capture and release within microfluidics have been further refined allowing for more
efficient yields in purity.

**Figure 2.5** NIPPAAm, a thermo-responsive polymer, can be functionalized with antibodies. (A) General isolation procedure using a micropipette and a colorimetric temperature reference in the center channel. (C-F) Functionalized surfaces using biotinylated antibody and biotin binding protein. Selectively isolated CD4 positive cells and releases by temperature reduction at 32 °C. Figure adapted from Gurkan et al. [5].

The approach to magnetic cell separation can be constrained to two different phenomena, permanent and electromagnet. Each phenomenon presents intrinsic advantages and challenges associated with incorporation into a microfluidics system. A magnetic moment sufficient enough to displace a cell requires attachment of superparamagnetic beads [6]. Beads are reacted with sulfo-NHS-EDC chemistry with carboxylic acid functionalized superparamagnetic beads. The chemistry gives rise to the ability of an antibody to form a stable amide bond with EDC. The antibody-functionalized superparamagnetic beads allows for selective targeting of cells, which can then be separated from a heterogeneous suspension via a magnetic field in appropriate
Numerous investigators have refined dynamic magnetic cell separation within microfluidics [87]. Xia et al. implemented a permanent magnetic approach under flow conditions [87]. *E. coli* were tagged with magnetic beads, as described above. The resulting separation efficiency of *E. coli* in saline was observed to be 89%, but decreased to 56% in a spiked blood sample [87]. The decrease in separation efficiency in the presence of blood was due, in part, to the iron ion within heme rings found in erythrocytes [87]. In Figure 2.6, Plouffe et al. developed a sheath-flow microfluidics system allowing for lateral displacement of target cells into a buffered solution stream [6].

**Figure 2.6** Magnetic separation approach using tunable electromagnetic fields. Blood, containing target cells with superparamagnetic beads attached, and buffer stream are flowed into the devices. The electromagnetic fields displace target cells out of the blood streams into the buffer stream, enriching select cell types. Adapted from Plouffe *et al.* [6].
The tunability allows for optimizations according to receptor densities and diameters of the target cells, enabling enrichment with purities greater than 90% [64]. Magnetic cell separation presents many advantages, especially for cell isolations from whole blood. However, the adhesiveness of epithelial cells can generate a coagulation affect, which can lead to non-target cell elution [88].

2.2 **Discovery and isolation of multipotent intestinal cells**

Intestinal topography consists of finger-like projections, commonly known as villi and invaginations named crypts. Cells below the crypt zone, contain multipotent stem cells, which contribute to the various subtypes, contained within the intestinal niche. The intestinal stem cells asymmetrically divide to form secretory, absorptive progenitor cells, or Paneth cells within the transit-amplifying region. The secretory and absorptive progenitor cells will terminally differentiate into enterocytes, goblet, or enteroendocrine cells [7]. Transit-amplifying cells, residing just above the stem cell zone, exhibit a known marker targeting the prom-1 receptor known as anti-CD133 [8,31,89]. Intestinal cells exhibiting anti-CD133 have been well characterized and studied in pure populations via FACS separation [23]. However, localization of intestinal stem cells and identification of single stem cells have been controversial topics within the gastroenterology community.
The localization of intestinal stem cells has been described in two different models (Figure 2.7): the +4 model and the stem cell zone model [7,8,18,90]. Figure 6 illustrates the positioning of +4 label retaining cells (LRC) residing above paneth cells at the base of the crypts, whereas the stem zone model contains crypt based columnar cells (CBCs) intercalated between paneth cells. Until recently, the +4 model was more widely accepted. However, with recent discoveries a new model was formed that incorporates element of both of the above models, as illustrated in Figure 2.8 [8]. The functions of the +4 LRC and CBCs are stem-like exhibiting quiescence and active cycling, respectively [91]. Researchers have only recently identified an extra cell maker for CBCs, a gene for an orphan receptor known as leucine coupled g-protein (Lgr5) [30,34].
Figure 2.8 Populations within the intestinal crypt under the overlay of the stem cell zone and +4 LRC model. This figure illustrates transit-amplifying progenitor, crypt based columnar, and +4 LRC cells with their respective markers CD133, Lgr5, and BMI-1. Adapted from Montgomery et al. [8].

Lgr5 is an orphan receptor that codes for the Wnt pathway [9]. Barker et al. isolated and identified the gene that codes for this unique receptor, which was only present at the base of the crypt. Confirming the presence of the Lgr5 receptor was reinforced using a transgenic mouse model. The technique genetically knocked-in alleles, where over expressing Lgr5 coupled the GFP crossing EGFP-IRES-creERT2 with Cre activated Rosa26-lacZ reporter [11,12]. Localizations were confined to the base of the crypt intercalated between paneth cells reinforcing the stem cell zone model. Coupling CBCs with over expressed GFP-Lgr5 gives rise to an isolation method via FACS.

Currently, investigators rely on expensive instrumentation to sort cells such as magnetic activated cell sorting (MACS) and FACS. Each separation method, FACS or MACS, presents its own intrinsic advantages and shortcomings. FACS is an extremely expensive instrument ($500,000) enriches cell populations to high purity but has low-
throughput compared to pre-plating techniques and negatively impacts cell viability, whereas MACS can achieve high throughput and efficiency but low purity is its short-coming. Studies of CBCs are typically performed with transgenic mouse models designed to overexpress GFP-transfected CBCs. Translation of this technique for tissue engineering applications is challenging owing to the requirement of significant genetic modification in order to be able to isolate CBCs. Until recently, a commercially available antibody that specifically recognizes Lgr5 receptor had not been available [30,34]. However, starting in 2010, anti-GPCR GPR49 has been produced and it specifically adheres to the Lgr5 receptor, allowing for cell separation without the use of transgenic mouse models [35]. Isolation of intestinal progenitor and stem cells are important for several biological applications, but developing a controlled culture method in which these cells proliferate and differentiate is crucial.

2.3 Intestinal Stem and Progenitor Cell Cultures

Multipotent stem cell culture requires a variety of different constituents in order to maintain an intestinal in vivo system. Growth factors specific to intestinal systems trigger signaling cascades which induce differentiation, proliferation, quiescence, and anoikis. Regulating these pathways is challenging, but investigators have been able to form single-cell CBCs into self-sustaining organoids [19,83,91-94]. Insights into the signaling cascade of CBCs and +4 LRCs have allowed pathways to be constrained to triggering/inhibiting Notch, Wnt, P13K, and BMP cascades. In Figure 2.9, gap junctions and intracellular communications allow for growth factors to promote terminally differentiated states. The Wnt pathway gives rise to secretory progenitor cells from CBCs and further differentiates into goblet cells with KIF-4 addition, and into enteroendocrine
cells in the presence Ngn-3. The Notch pathway triggers absorptive progenitor cell formation from CBCs, and further differentiates into enterocytes. The signaling cascade between +4 LRCs and CBCs is unknown, but it is believed that +4 LRCs remain quiescent and give rise to actively cycling CBCs [9].

Figure 2.9 Intestinal signaling within the crypt zone. Cascades and growth factors are necessary to trigger the differentiation cues. It is unknown what signaling occurs between CBCs and +4 LRCs. Adapted from David et al [9].

Culture of homogenous suspension of isolated intestinal progenitor (CD133+) and stem cells (Lgr5) yield distinct morphologies in vitro. Progenitor cell forming clusters, named enterospheres (Figure 2.10) are fairly short lived (3-13 days) in culture [10]. Enterospheres remain cyst-like and harbor apoptotic cells within in central domain. These clusters further expand, eventually becoming unstable and undergoes dissociation. Conversely, intestinal stem cells (Lgr5+) perform much differently than the progenitor cell population forming structures known as enteroids [10,32,95]. Enteroids are long-lived clusters which show distinct lumen formation coupled with budding projections for newly formed villi. Throughout this dissertation, intestinal progenitor and stem cell
clusters will be referred as enterospheres and enteroids, respectively.

**Figure 2.10** Bright field images of forming intestinal progenitor and stem cells in vitro. Intestinal progenitor cell clusters, enterospheres, (A) form short-lived cysts and remain round throughout the duration. Conversely, intestinal stem cells form self-renewing clusters, enteroids, (B) which form clear lumen and budding projections [10].

Identification and isolation of CBCs from small intestines facilitated the development of a culture system for sustaining in vivo signaling [96]. Self-sustaining enteroids derived from high-expressing Lgr5 CBCs were formed by the addition of endothelial growth factor (EGF), rspondin-1, and noggin. EGF binds to TGF-α receptors promoting a wound healing response [11]. Rspondin-1 triggers the Wnt pathway and allows for cells to differentiate into their respective progenitor subtypes as stated before [29,97,98]. Noggin facilitates in regulating the BMP pathway, giving rise to terminally differentiated cells from their respective progenitors. Viability of CBCs and progenitor cells is highly dependent on intracellular communications, and a disaggregation will result in programmed cell death or anoikis. An anoikis cascade can be inhibited with the addition of a rho-associated kinase inhibitor (ROCK) [99]. Intestinal cells, and more importantly stem cells, require a scaffold for growth due to the topographical cues these
cells need [94,100].

Investigators have successfully demonstrated growth of single-cell derived CBCs into enteroids with noticeable lumen present. As seen in Figure 2.11, growth of the CBC-derived enteroids initially forms a central lumen. As the cells begin to differentiate and migrate through the lumen, hyperplasia occurs where the morphological “buds” begin to form. The buds eventually form in vitro villi and release apoptotic enterocytes at the apex of each of the villi. Cellular phenotypes were assessed via immunohistochemical staining, and it was observed that all known cell types (goblet, enteroendocrine, enterocytes, and paneth cells) were present [11]. Further investigation into single-cell derived organoids revealed necessary signaling between paneth cell and CBC interactions via co-cultures.

**Figure 2.11** Day progression of FACS-sorted single-Lgr5 cells from a transgenic mouse model. The organoid forms a central lumen followed by hyperplasia after day 5. Adapted from Sato *et al.* [11]
2.4 Intestinal Co-cultures

Intestinal stem cells require a vast amount of growth factors and constituents to promote their multipotency and viability. Multiple groups have shown that the plating efficiency of pure CBCs that yield self-renewing organoids is ~ 6% [11]. The low plating efficiency is indicative of a missing growth factor or triggered signaling pathway promoting organoid formation. Within the crypt domain, interacted CBCs between paneth cells, suggested a dependent signaling niche inducing its active cycling capabilities [12]. Recent discoveries with Sox9 expression in the intestinal system have given leads into critical signaling between paneth cells and neighboring CBCs.

Gracz et. al investigated cultures of over expressed sox9+ paneth cells and their relation to anti-CD24 [38]. In the study, transgenic mice were designed to over express the Sox9 gene. Together with GFP transfection, Sox9 expression was characterized as high, medium, low, and sub low, and the cells sorted via FACS. Under similar conditions as previously reported by the same group [38], organoid growth was observed in conditions when Sox9 expression was low and CD24 signals were within the medium range. Furthermore, gene expression analysis indicated that Sox9 and CD24 expression correlated with Lgr5 expression. Specifically, high Sox9 expression was observed to correlate with lower Lgr5 expression and vice versa. With these correlations, investigators probed the interactions between high-expression Sox9 paneth cells and CBCs.
Figure 2.12 Lgr5 culture optimization using Wnt3a cofactor allowing for increased plating efficiency. Paneth cell dependence is illustrated by sorting Lgr5-paneth cell doublets and comparing to Lgr5-Lgr5 doubles in the presence/absence of Wnt3a. Adapted from Sato et al. [12].

Recent work by Sato et al has described increased plating efficiency with the co-culture experiments of paneth cells and CBCs [12]. As seen in Figure 2.12, co-culture with a 50/50 mixture of paneth cells and CBCs yielded different plating efficiencies in comparison to single/doublet Lgr5 and Lgr5/Paneth cells respectively. In order to develop a similar plating efficiency as a paneth/lgr5 doublet for an lgr5 singlet recombinant Wnt3a was needed to artificially stimulate the Wnt cascade. Growth in the presence of Wnt3a yielded a cyst-like morphology at similar time points in comparison to singlet Lgr5 cells. Wnt3a improves the efficacy of respondin-1, which gives rise to the increased plating efficiency [12]. The importance of this co-factor is demonstrated by
addition of porcupine inhibitor, which selectively deactivates the Wnt pathway, and eventually collapses the enteroid units [12]. However, recent investigations have shown that in vivo surrounding cell subtypes will provide necessary growth factors to sustain active cycling stem cells.

As mentioned, co-cultures are an essential tool in discovering necessary growth factors needed for cell maintenance and support. Investigations of the influences of non-intestinal cell types on intestinal cells are starting to be reported. Until recently, it was believed that only adjacent cell types secreting necessary growth factors influenced formation of enteroids in vitro. However, work from Laher et al. demonstrated an influence to enteroids multipotency from intestinal sub-epithelial myofibrolasts (ISEMFs) [13]. As illustrated in Figure 2.13, intestinal crypts without the presence of growth factors formed short-lived enteroids in culture. These bodies were only sustained for approximately 4 days before rapid dissociation (Figure 2.13A). Conversely, the crypt structures (Figure 2.13B) seeded onto a layer of ISEMFS in the absence of the necessary growth factors. It was found that the crypt structures formed long-lived enteroids structures similar to what was demonstrated by Sato et.al.
Figure 2.13 Bright field images of crypt derived enteroids in culture. Enteroids were short-lived (A) due to the lack of necessary growth factors such as: EGF and Noggin. Enteroids were seeded onto a monolayer of myofibroblasts (B) without the EGF and Noggin present and proliferated past day 2 in culture. Adapted from Lahar et al. [13]

One shortcoming of the above study is the lack of quantitative evidence for these secreted growth factors. The group does demonstrate the gene responses within ISEMFs indicating that there is in fact a gene that codes for secretion of factors such as FGF and Wnt. An appreciable insight would have been seen if conventional ELISA assays were performed to quantitate the exogenous growth factors. With this given data, one could gain insight on the amount of growth factors needed to sustain the enteroids culture system and translate these correlations to an in vivo model.
2.5 **Intestinal Angiogenic and Vascularization Systems**

Vascularization is a significant challenge within the field of tissue engineering. As researchers investigate further to develop *in vivo* like systems derived from tissue there is a clinical and research need to make systems self-sustaining via nutrient supply. One of the essential components of retaining tissue homeostasis *ex vivo* is vascular matrix allowing for delivery of nutrients and oxygen. Developmental vascularization models are sparse within the literature due to the lack of culture models and robust angiogenesis-promoting scaffolds. Recent studies have used immortalized cell lines embedded in extracellular matrix (ECM) based scaffolds and performed studies of endothelial cells (EC) and smooth muscle cell (SMC) recruitment to form neovascularized complexes [15,45,101-105]. The shortcomings of current cultures produce and in-growth of these vascular cells *in vitro* thus generate a lack of oxygen and states of ischemia [106]. The lack of robust culture methods to study vascular dependent disease states, especially within intestinal systems, is prevalent.

Intestinal disease pathogenesis is highly dependent on vascular response *in vivo* [14,16,51,107-109]. Within the intestinal system, it has been shown that many inflammatory states have direct correlation to vascular responses [14,51]. The primary constituent of these responses is a migratory based cytokine known as vascular endothelial growth factor (VEGF) [51]. VEGF influence has been studies in intestinal disease states such as irritable bowel syndrome (IBD), Chron’s disease (CD), and ulcerative colitis (UC [14]). In addition, VEGF has been found to be a critical role in angiogenesis of gastrointestinal carcinomas and directly linked to metastatic evolution [110].
Recent work by Scaldaferri et al. illustrated a UC disease state coupled with VEGF response. As demonstrated in Figure 2.14, mouse samples were genetically modified using a plasmid to overexpressed VEGF-A and sVEGFR-1 with an adenovirus vector. The control population was transfected with an inert plasmid. The role of weight loss in each group was examined, Figure 2.14A. VEGF-A knock-in mice experienced a significant weight loss over the 8 day study and yielded a 100% mortality rate. Conversely, sVEGFR-1 exhibited weight loss but ended at approximately 85%. Histological images indicated more vascular structures in the VEGF-A group whereas...
animals from the VEGFR-1 group exhibited much fewer structures than the control. The results indicated a significant colitis response within the VEGF-A group that yielded cysts within histological image Figure 2.14. While Scaladaferri et al. did determine that the disease mechanism of UC is in fact VEGF-A mediated, this study did not examine the concentration threshold of VEGF-A associated with the response. Furthermore, the group did not implement a negative control using a VEGF inhibitor such as platlet factor 4 (PF-4) [111].

As mentioned, intestinal stem and progenitor cells rely on Wnt signaling. Proliferative enteroids and enterospheres cultures require endogenous promoters such as R-spondin. It has been shown by Kazanskaya et al. that in developmental system, such as mouse embryos, r-spondin required cell subtypes generate a signal cascade causing a secretion of VEGF [54]. Within the study, Rsps3 (R-spondin type of protein) soaked beads were placed in culture with chicken chroidinic membrane and a strong proliferative response with intense angiogenesis was noticed. These cells were compared with human umbilical vein endothelial cells (HUVECs) and similar responses were observed in the presence of Rspo2 [112]. The postulated mechanism of action within cell types outside of endothelial subtypes is that Wnt dependent cell types uptake endogenous R-spondin, which catalyzes secretion of VEGF to promote angiogenesis. This deduction provides insight into a developmental mechanism for vascularizing structures. With respect to intestinal multipotent systems, R-spondin is a necessary co-factor to sustain these complex culture systems and signaling pathways within the stem and progenitor cell populations can lead to VEGF secretion thus causing migration of aortic smooth muscle and endothelial cells.
Subsets of VEGF have been known to cause different angiogenic responses in \textit{in vivo} and \textit{in vitro} within co-culture systems. More specifically and pertaining to intestinal systems, VEGF-A plays a critical role in inflammatory [14]. There is a significant lack of literature coupling VEGF-A with co-culture of intestinal multipotent and migratory cells. In addition, most angiogenic studies are solely pursued using endothelial cell types where there is a clear lack of supporting cell types such as smooth muscle cells. Smooth muscles cells (SMC) play an integral part within angiogenesis and vascular formation. SMCs reinforce vascular walls and migrate in inflammatory conditions.

![Figure 2.15](image)

**Figure 2.15** VEGF-A$_{165}$ pathway affecting SMC migration. VEGF-A$_{165}$ secreted from a breast cancer tumor cells in turn binding to VEGFR-1 and NRP-1 complex. VEGFR-1/NRP-1 complex initiate Akt cascade causing a migratory gene response of the SMC towards exogenous VEGF-A$_{165}$ source. Adapted from Banerjee et al. [15]

VEGF-A$_{165}$ mechanism and its effect on SMC was described by Banerjee \textit{et al} (Figure 2.15). Within their study, smooth muscle and endothelial cell lines including human
aortic smooth muscle cells (AOSMC) and human umbilical vascular endothelial cells (HUVECs) were characterized using conventional western blot techniques. The resulting western blots demonstrated the presence of a class of VEGF receptor denoted as VEGFR-1 Figure 2.16A. The group confirmed the receptor expression was dependent on VEGF-A_{165} through additional western blots and established efficacy upper-bound thresholds of 50ng/mL Figure 2.16B. VEGFR-2 was not present within AOSMC lines but was prevalent within endothelial cells, HUVECs. With this given information, the group was certain that VEGFR-1 was mutually exclusive to endothelial and smooth muscle cells and not with VEGFR-2. These conclusion led to the investigation of NRP-1/VEGFR-1 complex and migration with correlation to migration via β-actin formation. The NRP-1 to actin expression ratio increased in the presence of VEGF-A_{165} indicating a migrational response within AOSMC, Figure 2.16C.
Figure 2.16 Effect of VEGF-A\textsubscript{165} on VEGFR-1/Flt-1, VEGFR-2/Flk1, and NRP-1 mRNA and protein expression in hAOSMC. (A) Western blots of VEGFR-1 receptor in smooth muscle and endothelial migratory cell lines with β-actin control. VEGF-A\textsubscript{165} addition of hAOSMC to show VEGFR-1 expression. (B) Blots highlighting AOSMC and HUVEC VEGFR-2 expression in the presence of VEGF-A\textsubscript{165}. (C) NRP-1 expression in the presence of VEGF-A\textsubscript{165} within hAOSMC and mRNA electrophoresis to further confirm. Adapted from Banerjee et al. [15]

While the evidence of AOSMC response was noteworthy, studies of threshold levels would have added considerably more insights. Specifically, correlation of VEGF-A\textsubscript{165} concentration with NRP-1/VEGFR-1 complex concentration may have revealed threshold levels of VEGF-A\textsubscript{165} needed to generate a migration response. In addition, co-culture of HUVECs and AOSMCs would have given insight into the specificity of VEGF-A\textsubscript{165} to AOSMCs. Moreover, inhibition studies with AOSMCs would have given
more value to these studies to service as a negative control, these inhibitors could have included platelet factor 4 (PF-4) [111,113,114].

2.5 Summary

In summary, the above critical literature review illustrates the need for a selective capture and release device for isolation intestinal progenitors and stem cells from native rat tissue. Current purification methodologies for intestinal multipotent cell types rely on in situ hybridized animal models coupled with FACs analysis which is an unattractive method for tissue engineering, motivating rational design of a new technique.

Secondly, the above evaluation provides a foundation for complex intestinal stem and progenitor cells culture conditions. The review demonstrates what factors are needed to support a self-renewing enteroid and enterosphere in vitro. Tailoring culture methods to each in order to form self-renewing organoids requires embedding each population into a scaffold with the addition of the growth factors as described by Sato et. al Through optimization of culture conditions will aid in increasing organoid forming colonies and robust culture conditions of the purified cell types. Increase in plating efficiency of intestinal stem cells by the addition of Wnt3a is beneficial for growth and characterization.

Lastly, associations between intestinal disease and vascular processes described in the literature motivate more fundamental studies of the interactions between intestinal stem and progenitor cells and SMCs. The current literature lacks co-culture systems that include enterosphere and enteroid units coupled with vascular cell lines and insight into exogenous chemokines originating from multi-potent cell types. Without these systems,
there will be shortcomings in understanding the developmental pathways and inflammatory processes that generate vascular migration responses.
3.0 LABEL-FREE ISOLATION AND CULTURE OF INTESTINAL PROGENITOR CELLS FROM NATIVE RAT TISSUE USING MICROFLUIDIC DEVICES

The multipotent and regenerative capabilities of intestinal stem and progenitor cells are active areas of research. However, the nature and scope of these studies is limited by the difficulties associated in isolating these cells with sufficient retention of viability and function. This article describes how microfluidic devices coated with an antibody-functionalized hydrogel are capable of isolating CD133+ intestinal progenitor cells through positive selection without the need for any pre-processing labeling and with little impact on cell viability. In addition, the released and eluted progenitor cells form viable cluster units exhibiting in situ morphology consistent with literature. The significance of this work lies in the demonstration of a new isolation method for these fragile cells which can potentially facilitate in-depth studies of their function and their utilization in intestinal tissue engineering.

3.1 Methods For Tag-Free Isolation of Intestinal Progenitor Cells

There were 5 objectives within this section to have a successful isolation and culture of the intestinal progenitor cells. The first was to assess the optimal digestion and dispersion of the intestinal tissue, followed by characterization of the digestate via flow cytometry. The second objective was to optimize the flow conditions within the microfluidic device containing the formulated hydrogel. Thirdly, a confirmation of CD133 positive cells was in fact adhered within the device. In the fourth objective, an assessment of purity of injected and release of CD133 via flow cytometry was needed.
Finally, optimizing culture conditions to form enterosphere units in situ against the unenriched injected population. Overall, the methods created a platform to enriched intestinal progenitor cells (CD133+) for enterosphere forming cultures.

3.1.1 Neonatal Rat Specimens

Male and female neonatal Lewis rats were used and harbored in room temperature conditions with a 12 h light/dark cycle. For the progenitor cell isolation studies, neonatal rats between the ages of 2 to 5 days were utilized and sacrificed via decapitation. All studies and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Northeastern University.

3.1.2 Intestinal Tissue Digestion

Laprotonomy was performed on the sacrificed animals and small intestine tissue was extracted and stored in 1× Hank’s buffered salt solution (HBSS; Fisher Scientific). Intestines were split laterally to expose the epithelium and were fragmented into 1 mm samples. The digestion protocol employed to extract the cells was a modified version of that reported by Evans et. al. [115]. Briefly, intestine fragments were rinsed 5 times with HBSS in a 25 cm² culture flask and decanted. Next, 20 mL of a 300 U/mL collagenase (Sigma) and 0.1 mg/mL dispase II (Stem Cell Technologies) solution in HBSS was added to the culture flask. The contents were agitated using an orbital shaker at 110 cycles/min for 45 min at room temperature. The digested sample was further agitated via aggressive pipetting for 10 min and a series of two gravitational sedimentations were performed at 60 s intervals with extraction of supernatant. The sequential supernatant was centrifuged at 220 × g for 5 min at 25 °C. The pellet was resuspended in serum-free 1× Dubecco’s modified Eagle’s medium (DMEM) and centrifuged under the same conditions. The
pellet was then suspended in DMEM and the sample was strained through a 40 µm mesh. For microfluidic cell isolation, the strained sample was aliquoted and placed in 1 mL syringes (BD Biosciences).

3.1.3 Microfluidic Cell Isolation Device Fabrication

Microfluidic devices were fabricated using traditional soft lithography [1,116] at the George J. Kostas Nanoscale Technology and Manufacturing Research Center at Northeastern University. The physical dimensions and design of the devices were identical to those of devices described by Hatch et al.[117] These devices consist of polydimethylsiloxane (PDMS) patterned with 100 µm-diameter pillars bonded to glass slides (Fig. 3.1a,b).

The functional component of these devices is a hydrogel coating synthesized in situ that contains capture antibodies against CD133. This synthesis of this hydrogel was originally described by Hatch et al [117]. Briefly, the following components were combined: 45 mg alginic acid (Sigma), 22.5 4-arm amine polyethylene glycol (PEG; Creative PEGWorks, and 0.04 mg anti-CD133 (Miltenyi Biotec) and mixed for 1 h in MES buffer (2-(N-morpholino) ethanesulfonic acid; Pierce). Next, 14.8 mg sulfo-NHS (Sulfo-N-Hydroxysuccinimide; Sigma) and 4.8 mg N′-ethylcarbodiimide hydrochloride (EDC) (Sigma) were added to the primary reaction mixture and allowed to react for 30 min. The functionalized alginate solution was injected into microfluidic pillar array devices containing a saturated solution (1 g/mL) of Calcium Chloride in dionized water. The adsorbed alginate was coverted into a hydrogel coating by first flowing MES buffer and then 100 mM CaCl₂ solution in MES buffer following a final MES buffer rinse, all at a flow rate of 10 µL/min through each device.
3.1.4 Intestinal Progenitor Cell Isolation Experiments

Cell suspensions prepared as described above were injected into the cell isolation devices at a flow rate of 3 µL/min. A total suspension volume of 0.1 mL was injected into each device. Following the injection phase, each device was flushed with 0.1 mL of MES at a flow rate of 3 µL/min. Captured cells were eluted out of the devices by flowing through 0.1 mL of a saturated solution of ethylenediaminetetraacetic acid (EDTA) at a flow rate of 10 µL/min.

3.1.5 Flow Cytometry of Intestinal Digestate

Flow cytometry analysis of both the starting (i.e. tissue digestate) cell suspensions and the isolated cells was performed using a Beckman Coulter Quanta SC flow cytometer. The following conjugated fluorescent primary antibodies were utilized: anti-mouse CD271-FITC (Abcam), anti-human CD133-PE (Miltenyi Biotec), anti-rat CD68-PE (ABD Serotec), anti-human CD34-FITC (e-Bioscience), and anti-human CD31-PE (e-Bioscience). Mouse anti-human CD26 (Abcam) was conjugated with goat anti-mouse FITC IgG (Abcam). All primary and secondary antibodies were normalized, diluted 1:50, and resuspended in calcium/phenol red free HBSS. All data and gating was analyzed via FlowJo™ with a control overlay.

3.1.6 Culture of Isolated CD133+ Cells

Cells were eluted from each microfluidic isolation device into 1.5 mL microcentrifuge tubes containing 50 µL of Matrigel™ pre-polymer (BD Biosciences) on ice. Control populations were run in parallel to the enrichment process. The control suspensions contained unsorted intestinal digestate and stored in serum-free DMEM for the duration of the enrichment process. Both cell suspensions were embedded into the
Matrigel via pipetting to facilitate mixing. 150 μL of the suspension was plated on tissue-grade 24-well plates (BD Biosciences). As per previous protocols by Sato et. al. [11] 100 ng/mL Noggin (Peprotech), 25 ng/mL rat endothelial growth factor (EGF; Peprotech), and 500 ng/ml r-spondin-1 (R&D Systems) were supplemented to a its respective concentration in 500 μL of advanced DMEM/F-12 culture medium (Invitrogen,) containing 0.7 μL of 10 μM (ROCK (Y-27632); Stem Cell Technologies) [34,118-120]. The Matrigel was polymerized at 37 °C for 30 min after which the liquid remnants were decanted. The cultures were segregated into the injected digestate and the enriched CD133 suspension while being cultured under the proposed conditions above. The cells were cultured for up to 9 days at 37 °C with 5% CO2. Culture medium was refreshed every 4 days and growth factors were supplemented every 2 days.

3.1.7 Cultured Cell Analysis

Cell viability was evaluated utilizing a Live/Dead™ (Invitrogen) kit and counting using a hemacytometer. The viability of cells in the tissue digestate as well as the isolated suspension were tested utilizing the manufacturer’s recommended protocol.

3.1.8 On-chip Staining of CD133+ Cells

Injected cells were stained with mouse-anti-human CD133-PE diluted 1:50 in MES buffer. 0.1 mL of the fluorescent antibody solution was injected at the cellular injection rate of 3 μL/min and rinsed with MES buffer at the same injection rate. Images were obtained using a Nikon Eclipse TE2000 fluorescence microscope with a phycoerythrin (PE) filter with an excitation/emission wavelengths of 476 nm/578 nm and analyzed using Nikon Elements Advanced Research software.
3.1.9 Statistics and Data Analysis

Each of the experiments including characterization, viability, and purity data were repeated 3 times. Uncertainty was evaluated in the form of standard error which is the quotient of the standard deviation and the square root of the number of trials. Student t-test calculations for the purity data set were performed from a one-tail distribution with equal variance. For viability measurements, a student t-test was calculated as a two-tail distribution with unequal variance. T-tests were carried out using Kaleidagraph software. $p < 0.01$ was considered statistically significant.

3.2 Results and Discussion for Intestinal Progenitor Microfluidic Enrichment

The results in presented detail in the resulting characterized intestinal tissue digestate and encompassed a purification mechanism for intestinal progenitor cells from native rat intestine. The resulting digestate characterization highlighted multiple intestinal cell subtypes including extraneous populations located outside of the crypt niche. Microfluidic design coupled with a dissolvable alginate matrix functionalized with anti-CD133 provided a foundation for tag-free enrichment of selected progenitor cell populations. Confirmation of selective capture of said cells were probed utilizing on-chip fluorescent staining. An appreciable 5-fold enrichment of CD133 positive cells were obtained and the eluted cells maintained viability that formed enterospheres. As described in chapter 2, there exists a need for native isolation method for intestinal stem cells which does not rely on hybridized models.
3.2.1 Microfluidic Isolation of Intestinal Progenitor Cells

The microfluidic cell isolation device used in this study is shown in Fig. 3.1a. The pillar-based architecture of the channel provides a large surface area for cell attachment. Intestinal cell digestate was injected into these devices at rates ranging from 3-10 µL/min, however higher flow rates did not provide satisfactory capture of the targeted CD133+ progenitor cells; hence all experiments described herein were carried out using injection flow rates of 3 µL/min. To confirm that adhered cells were indeed CD133+, staining was carried out following the cell suspension injection (and rinse) step and the results are shown in Fig. 3.1c. Target cells are clearly adhered within the devices where some are attached to the posts. This image also indicates the presence of cell aggregates. These can either be present within the injected suspension or be formed when flowing cells encounter and bind to the captured cells. In either case, such aggregates may contain non-target cells.
Figure 3.1 Microfluidic cell isolation device (a) containing an array of pillars (b). The pillars are coated with an alginate hydrogel functionalized with antibodies against CD133. Arrows indicating captured CD133+ cells (b) and outlined square box illustrating the area of the magnified section (c) of the stained capture chip. Scale bars, 40µm.

Flow cytometry was utilized to characterize both the injected and eluted cell populations. Both populations were analyzed by utilizing anti-CD133-PE and the data were gated using a control overlay. Gating for positive populations was possible with a
control overlay, which contained a tissue digestate sample without any labeling antibodies. Particle diameter gating was used to include cell sizes between 8 and 18 µm and side scatter was implemented to minimize counting of debris (data not shown). Collagen and cell debris made up the majority of any extraneous particulates.

Figure 3.2 Purity comparison of injected cells from tissue digestate and released (isolated) CD133+ cells from microfluidic isolation; * denotes p < 0.005 and percent viability of the respective injected and released populations as determined by a live/dead assay ( **p > 0.01). n = 3.

The microfluidic cell isolation process resulted in a 5-fold enrichment of intestinal progenitor cells, as shown in Fig. 2 (p < 0.005). Note that digestate suspension was created using serum-free DMEM rather than supplemented culture medium as serum components were found to adversely affect target cell capture by suppression of antibody-antigen interactions (results not shown). The 2-step cell isolation process
consisting of capture followed by chelator-driven release did impact cell viability, although not to a significant extent \((p > 0.01)\) (Figure. 3.2)

3.2.2 Characterization of Digested Intestinal Tissue

The tissue digestate was characterized via flow cytometry to obtain an understanding of the relative numbers and types of cells being injected into the microfluidic cell isolation devices. Surface marker labeling was employed to identify cell types including enterocytes, glial cells, hematopoietic stem cells, endothelial cells, and intestinal macrophages. A control plot containing no antibody tags was overlaid onto an experimental plot to determine the appropriate gate for every particular cell type based on its shift from the control (data not shown). Another goal of this analysis was to assess the effectiveness of anti-CD133 as the capture molecule for intestinal progenitor cells, given that CD133 is expressed by endothelial, hematopoietic, and glial cells [121-123].

Table 3.1 Intestinal digestate characterization insight into select tissue specific cell types and subtypes. The cell types and subtypes were labeled with their respective antagonist derived from an identical solution to the injected suspension. Populations were counted via flow cytometry and gated for positive populations. \(n=3\)

<table>
<thead>
<tr>
<th>Target Population</th>
<th>Antibody</th>
<th>Percent Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterocyte</td>
<td>CD26</td>
<td>21.40 ± 5.50</td>
</tr>
<tr>
<td>Endothelial</td>
<td>CD31</td>
<td>11.75 ± 4.53</td>
</tr>
<tr>
<td>Hemapoetic stem cells</td>
<td>CD34</td>
<td>1.74 ± 1.12</td>
</tr>
<tr>
<td>Intestinal Macrophage</td>
<td>CD68</td>
<td>8.76 ± 6.76</td>
</tr>
<tr>
<td>Glial</td>
<td>CD271</td>
<td>4.74 ± 1.45</td>
</tr>
</tbody>
</table>

The major and minor constituent cell types of the intestinal tissue digestate are listed in Table 3.1. The most abundant cells were the intestinal progenitor cells, which
comprised 26% of the total cell content. We define these cells as CD133+ cells that do not express other markers associated with endothelial, hematopoietic, and glial cells. The relative abundances of enterocytes and intestinal progenitor cells observed are consistent with tissue compositions reported by Dekaney et al [23]. The high abundance of progenitor cells is attributable to the neonatal age of the rats [115]. CD26+ enterocytes (intestinal absorptive cells residing primarily within the villous) were the next most abundant cells, representing approximately 21% of the sample. The other cell types were relatively low in abundance and the most abundant cell type among these cells were CD271+ glial cells, an autonomic nervous system cell type that aids in digestions, represented approximately 4.7% of the total cell population [123]. CD133 expression in neural cells is relatively low,[124] thus decreasing the likelihood of capture of these cells by anti-CD133 coated surfaces. CD31+ endothelial cells [121] and CD34+ hematopoietic stem cells [122] were relatively low in concentration representing roughly 11% and 1.7%, respectively. At certain development stages, these cell types typically express CD133 [125] however the extent of such co-expression is typically very low [126]. Lastly, CD68+ macrophages were present only in extremely small numbers. The total characterized sample made up approximately 74% of the sample population; the remaining fraction is attributed to cellular/tissue debris, myofibroblasts, and smooth muscle cells [26,127].

3.2.3 Culture of Isolated Progenitor Cells

The culture of the injected and released intestinal suspensions produced significant variations in morphology and growth patterns. The injected population, containing the characterized digestate, was cultured in the same fashion as the release population.
Multiple different morphologies and cell types were observed while expanding within the wells. At day 2 (Fig 3.3a) two primary cell morphologies illustrated a cyst-like cluster in addition to planar mono-layers of cell sheets. The cell sheets were homeostatic determined by proliferative growth over the respective time points and its retained morphology(Fig. 3.3a-c), but the cyst-like bodies which exhibited a rounded morphology with quasi-lumen containing cells did not follow a similar retention. The cyst-bodies underwent a de-clustering and disassociated leading to eventual death of the once sustained cyst-body (Fig. 3.3b,c.). While not all the cyst-bodies dissociated at day 4 (Fig. 3.3b), as the day progression culture proceeded to later time points significant cyst-bodies remnants were noticed (Fig. 3.4c).
Figure 3.3 Age progression culture of the injected heterogeneous suspension using the proposed culture conditions. Epithelial sheets, illustrated by the green asterisk (*) and cyst like structures (white arrows) were noticed at day 2. Expansion of these cysts structures (white arrows) and dissociation of cluster units (red arrows) were abundant at Day 4 (b). As the culture progressed to day 6 (c), many of the cyst structures dissociated (red arrows) and epithelial sheets (*) were retained. Scale bar, 25μm.

The CD133 enriched population yielded a contrasting culture compared to the injected suspension. Enterosphere formation was noticed at day 2 (Fig. 3.4a) followed by a pseudo-lumen formation at day 4 (Fig. 3.4b). Within the central pseudo-lumen, cellular granularity was observed indicating these are apoptotic cells and were retained through the culture duration. The pseudo-lumen and adjoining cells produced an asymmetrical elongation affect (Fig. 3.4c) which is thought to be a budding phenomenon. However, the elongated segment eventually collapsed at day 9 (Fig. 3.4d) indicating this is a short-lived progenitor cluster or enterosphere. The short-lived nature of progenitor cell intestinal clusters (CD133) leads to eventual collapse of the entity entirely, but the enrichment
methods provide a stable culture to allow for longer study durations in comparison to an intestinal digestate suspension.

Figure 3.4 Day progression of post-released CD133 suspension into the proposed culture conditions. Red asterics (*) indicated points of reference to accurately track the enterosphere formation overtime. Early cluster onset is noticed at day 2 (a) while a distinct lumen-like morphology is noticed at day 4 (b). Apical expansion, indicated by the arrow, was demonstrated in in day 7 (c); however, day 9 (d) demonstrated a dissociation (arrows) and expulsion affect on the enteroid. Scale bar, 50µm

This chapter demonstrates the effectiveness of microfluidic cell isolation in obtaining enriched suspensions of intestinal progenitor cells from tissue digestate with retention of viability and reasonable speed (< 1 h). The ability to directly inject tissue digestate into the microfluidic cell isolation devices without any preprocessing labeling coupled with the relatively low residence time of 1.5 min$^{-1}$, enables significant viability retention
relative to FACS and MACS (both techniques typically require 2h minimum processing time). Such high viability isolation enables downstream applications such as organoid unit culture and co-cultures with other cell types. The ability to isolate individual intestinal progenitor cells rapidly and with reasonable throughput (~13,000 total cells per minute per device) and high viability also opens the possibility of seeding these cells into scaffolds that mimic intestinal topography [128]. Recent studies of intestinal stem cells have been carried out using labeling followed by FACS [11]. In addition to poor viability (6%, as reported by Sato et al. [11]) an obvious disadvantage of fluorescence-labeling (or any other type of labeling) is the inherent incompatibility with any implantable therapy [129].

Anti-CD133 was chosen as the capture antibody in this study based on its specificity toward early intestinal progenitor cells and its strong ligand-receptor interactions [31]. While CD133 is expressed by other cells in intestinal tissue, such as endothelial progenitor cells and hematopoietic stem cells, the low concentration of these cells in the intestinal tissue reduces the likelihood of their capture by the microfluidic isolation system. The synthetic chemistry of the capture/release hydrogel coating can, however, be readily extended to a different antibody.

Cultures of the injected and isolated cell populations provided some interesting insights in terms of the relationships between cell composition, morphology, and development. The composition of the culture medium for these experiments as designed to contain supplements known to support the formation of crypt-like morphologies [97,130,131]. The injected and enriched cultures yielded distinct morphologies and cell types. The injected suspension yielded multiple morphologies in addition to prolific
extraneous cell types. These extraneous populations have potential in influencing enterospheres and enteroids development due to the secreted soluble factors [13]. It is assumed that this is a plausible reason in why the cyst-like bodies within the injected population dissociated relatively quickly in comparison to the CD133 enriched culture. The enriched suspension formed clear enterospheres and similar culture patterns as seen in previously reported literature [123]. The culture enriched CD133+ demonstrated a sustained culture that was longer lived than the cyst-bodies contained within the un-enriched suspension. Sensitive intestinal organoid forming units, such as enterospheres and enteroids, assimilate extraneous cell types within its niche [12]. The assimilation affect can affect overall cluster viability thus the need of developing enrichment techniques to mitigate these phenomena.

The studies described above are representative of much broader and in-depth biological studies that could be performed to gain insights into the fundamental biology of intestinal stem and progenitor cells using microfluidic isolation techniques. The prelabel-free and relatively rapid nature of the microfluidic isolation process could potentially enable the advance from such studies into the development of cell-based therapeutics and tissue engineering of the intestine.
4.0 TAG-FREE ENRICHMENT OF LGR5+ STEM CELLS FROM WILD-TYPE RAT INTESTINE

The isolation and investigation of Lgr5+ intestinal stem cells has been limited by the low abundance of these cells (~2%) in tissue and challenges in isolating these cells with sufficient viability. Recent work with these cells has typically been carried out using transgenic mouse models designed to express green fluorescent protein (GFP)-containing Lgr5+ cells and isolating these cells by fluorescence-activated cell sorting (FACS). This chapter describes a tag-free method of isolating these cells from wild-type rat tissue and demonstrates the functional capabilities of these enriched cells in terms of enteroid-forming ability in three-dimensional culture. Using a microfluidic device containing a degradable hydrogel functionalized with antibodies against GPR49/Lgr5, the Lgr5+ content of digested neonatal rat small intestine was increased 24-fold to ~49% with full retention of viability. The Lgr5+ enriched cells formed characteristic enteroid structures following four days in culture with hollow sphere morphology in contrast to the un-enriched cells, which formed flattened disk structures containing apoptotic cells.

4.1 Methods for an Optimized Microfluidic Enrichment Platform and Enteroid Promoting Culture System

The four main elements of this aim are as follows. First, a digestion procedure needed to be developed to specifically isolate the intestinal crypts from the native rat tissue. Secondly, confirmation of the GRP49 marker specific towards intestinal stem cells was needed to illustrate binding specificity of the capture antibody. Third, optimization of the hydrogel was needed to increase capture efficiency of the target population. Finally,
culture of the enteroid forming units had to be performed in order to demonstrate the characteristic intestinal enteroid forming potential of the enriched Lgr5+ cells.

4.1.1 Tissue Harvest and Digestion
Crypt enrichment was performed as described by Sato et al. [11]. Small intestine was extracted, split laterally, and fragmented into 1 mm segments. Fragmented tissue was incubated in 2 mM EDTA at 4º C for 30 min. Tissue samples were separated from solution and placed in 20 mL of phosphate buffered saline (PBS, Gibco) for 10 min of agitation. The supernatant fluid was then collected and centrifuged at 150 \( \times g \) for three min; the pellet was collected, suspended in 10 mL of serum-free Dulbecco’s Modified Eagle’s Medium (DMEM, Cellgro) and centrifuged again at 150 \( \times g \). The pellet was suspended in 5 mL of serum-free DMEM solution and filtered through a 100 μm cell strainer. The solution was then filtered through 20 μm cell strainers into 1 mL microcentrifuge tubes.

4.1.2 Cell Capture Coating Materials
Antibody functionalized alginate synthesis was carried out using six different protocols but stoichiometric ratios of reagents remained constant throughout each scheme. In the first step, 1940 μL of 2-(N-morpholino)ethanesulfonic acid (MES; Thermo-Fisher), 0.04 mg anti-GPCR GPR49 (Lgr5 antagonist; Abcam), and 22.5 mg of 10 kD 4-arm poly(ethylene glycol) (PEG; Creative PEGWorks) were mixed for 30 min. The MES buffer pH was either held at 4.7 (protocol VI) or 6.0 (protocol II-V; using NaOH with a concentration of 1M). The antibody, buffer, and PEG solution were allowed to incubate for 60 min in protocol II. The other protocols, however, did not include such an incubation period; rather, other reagents were added immediately. Specifically, 13.8 mg
sulfo-NHS (Pierce), 4.8 mg EDC (Pierce), and 45 mg alginic acid (Thermo-Fisher) were added, allowed to mix for 60 min (protocols II-VI), and incubated for another 60 min in protocol II. The functionalized alginate associated with each protocol was injected into a 10 kD dialysis cassette (Thermo-Fisher) and suspended in MES buffer at the respective protocol pH for 48 hours to remove any EDC and sulfo-NHS not bound to alginic acid. Protocol conditions and incubation times are summarized within Fig. 4.3.

4.1.3 Validation of Capture Antibody

Unstrained intestinal digestate was fixed using 4% formaldehyde for 20 min, centrifuged at 500 × g for 5 min and resuspended in Lgr5 blocking buffer. Next, a 1:100 dilution of anti-GPR49/Lgr5 in PBS was added to the suspension, incubated over night at 4º C, centrifuged, and re suspended in blocking solution containing 1:100 goat anti-rabbit Alexafluor 568 which was allowed to incubate for 2 h. The suspension was mounted on a glass cover slip containing a DAPI nuclear stain and imaged using a Nikon Eclipse TE2000-U inverted microscope.

4.1.4 Microfluidic Device Preparation, Cell Capture, and Cell Release

Each device was filled with antibody-functionalized alginate and allowed to incubate for 60 min. To complete the process of hydrogel formation within the channels, the following reagents were flowed through each device in order: 100 µL of pH 6 MES buffer at 10 µL/min, 100 µL of 100 mM CaCl₂ at 10 µL/min, and 100 µL of 0.1% (protocol II-IV, VI) or 1.0% (protocol V) bovine serum albumin at 10 µL/min. A Harvard Apparatus PHD2000 syringe pump was used to control flow rates. Cell suspensions obtained following tissue digestion were agitated using a vortexer to disaggregate and disperse the cells, and 200 µL of this suspension was drawn into each 1
mL syringe used for injection into the microfluidic devices. Using these syringes, 100 µL of cell suspension was pumped through each device at a rate of 3 µL/min followed by 100 µL of pH 6 MES buffer at 3µL/min to rinse. Next, 100 µL of 50 mM EDTA solution was pumped through each device at 10 µL/min to release captured cells. For culture, cells were released into microcentrifuge tubes containing 50 µL of Matrigel (BD Bioscience) on ice.

4.1.5 Flow Cytometry of Intestinal Stem Cells

Flow cytometry analysis of both the starting (i.e. tissue digestate) cell suspensions and the Lgr5+ enriched cells was performed using a Beckman Coulter Quanta SC flow cytometer. Anti-GPCR GPR49 conjugated with goat anti-rabbit-RPE (Invitrogen) was used to quantify the Lgr5+ cell content in the un-enriched and enriched populations. Primary and secondary antibodies were diluted 1:50 in PBS.

4.1.6 Culture of Enriched Cell Populations

Suspensions with volumes of 100 µL containing approximately 600 Lgr5+ enriched cells/mL embedded in Matrigel were plated into individual wells of a 24-well plate and incubated for 10 min at 37ºC. Lgr5 basal medium was prepared with the following constituents (Invitrogen): 500 mL of advanced DMEM F-12, 5 mL N2 supplement, 10 mL B27 without vitamin A, 5 mL HEPES, 6.25 mL Glutamax. Each sample was rinsed with 350 µL of Lgr5 basal media to remove EDTA from the cell culture. Then, 17 µL of ROCK inhibitor (y-27632, Sigma-Aldrich) was added to 10 mL of Lgr5 culture medium. A 486 µL aliquot of this solution was added to each well along with growth factors at the following concentrations: 100 ng/mL of murine Noggin (Peprotech), 100 ng/mL of murine Wnt3 (Peprotech), 50 ng/mL of rat EGF (Peprotech), and 1 µg/mL of murine
Rspondin-1 (R&D Biosciences). After each growth factor was added, the well plate was moved into an incubator maintained at 37 °C, 5% CO₂. After two days of cell growth, the medium was removed and Lgr5 medium without ROCK inhibitor was added to each well. Growth factors were added at the following concentrations: 100 ng/mL of Noggin, 100 ng/mL of Wnt3, 50 ng/mL of EGF, and 500 ng/mL of Rspondin-1.

4.1.7 Immuno-histochemical Staining of Organoid Structures

Enriched organoids were fixed with 4% paraformaldehyde and rinsed with 2 mM glycine in PBS. A solution containing 6 U/mL of dispase (Stem Cell Technologies) in PBS was added and incubated for 1 h to release the organoids from Matrigel. Organoids were pipetted into 200 μL Lgr5 media blocking solution containing 3% BSA, 10% goat serum, 0.1% Triton X-100, 10 mM HEPES, and 10 mM glycine. Next, 1:50 dilutions of the staining antibodies, anti-GPCR GPR49 and anti-CD24 (Abcam), in blocking solution were added and incubated at 4°C overnight. Organoids were selectively mouth pipetted under an inverted microscope, out of solution and into 200 μL of blocking solution containing normalized concentrations of goat anti-mouse Alexafluor 488 (Invitrogen), goat anti-rabbit Alexafluor 568 (Invitrogen), and 0.5 μg/ml DAPI (Invitrogen) where they remained for 3 h. Negative controls followed similar procedure except for omission of primary antibodies. Finally, organoids were mounted on glass cover slides and confocal images were taken via a Perkin Elmer Ultraview VOX spinning disk confocal microscope.
4.2 Results and Discussion for Selective Microfluidic Enrichment of Lgr5+ from Native Rat Intestine Producing Viable Enteroid Cultures

Optimization of the proposed alginate binding chemistry including pH titration and passivation was performed due to the rarity in population of Lgr5+ cells. In light of the new discoveries in literature, a ligand to antibody validation was necessary to confirm specificity and attachment for the anti-GPR49/Lgr5 capture antibody. The resulting optimization yielded a 24 fold enrichment of intestinal stem cells from native rat tissue. The isolation procedure yielded self-renewing clusters, enteroids, which its native expression trends were confirmed via confocal microscopy.

4.2.1 Microfluidic Enrichment of Lgr5+ Intestinal Stem Cells

To determine the specificity of the capture antibody, anti-GPCR GPR49, immune-staining was performed on the un-enriched tissue digest suspension in the presence of a blocking buffer to mitigate non-specific binding. Figure 4.1 shows fluorescence micrographs of a representative crypt and Lgr5 expression is observed in two different areas. Fluorescence microscopy of single cells and small cell clusters Figure 4.2 reflected a consistent trend in that a small number of cells, relative to larger numbers of surrounding granular cells, stained positive for anti-GPR49/Lgr5.
Figure 4.1 Fluorescence micrographs of an individual intestinal crypt from un-enriched tissue digestate suspension. The overall cell content in the crypt is seen in (a) and within this crypt three cells are observed to stain for GPR49/Lgr5. The merged image (c) illustrates the location of the positive population in relation to other cell types. Scale bar 50 µm.

The antibody-functionalized hydrogel coatings employed in this work for Lgr5+ cell enrichment are compositionally similar to those originally designed and utilized by Hatch et al.[117] for endothelial progenitor cell isolation from whole blood. Given the difference in target cell type and source in the present work, optimization of several experimental parameters was carried out in order to increase the GPR49/Lgr5+ cell enrichment and purity. Specifically, four variables were examined: reaction pH (for the reaction involving functionalization of alginic acid with PEG and capture antibody), reaction time, passivation by BSA adsorption to suppress non-specific binding, and flow rate of digested tissue samples into the enrichment device. A summary of the conditions examined is shown in Figure 4.3A.
Figure 4.2 Antibody binding specificity for injected intestinal digestate into a microfluidic post array. (A) Hexagonal post array offset by one 40µm to increase the amount of cellular collisions to the projections. A typical sample of a intestinal digestate population (D) before being injected into the microfluidics device. The suspension was stained with anti-GPR49/Lgr5, illustrated in red (B), and counterstained with a DAPI nuclear stain (C) while in the presence of a blocking solution. Scale bar, 50µm

The effect of hydrogel formation reaction pH was investigated in order to improve interactions between the components involved, namely, the 4-arm PEG, EDC, and capture antibody. In comparing protocols III and VI, it is evident that a pH change from 4.7 to 6.0 had a significant overall effect on efficacy of the alginate hydrogel in capturing GPR49/Lgr5+ cells ($p < 0.001$; Figure 4.3A,B). Increasing the reaction pH deprotonates the carboxylic acid and amine reaction sites of PEG, EDC, antibody, and alginate, thus allowing an increase in reaction potentials of antibody-PEG and alginate-EDC conjugates
and driving the overall reaction to a greater degree of completion. Mixing and incubation times had no statistically significant effect on enrichment. Protocol III resulted in the greatest purity, with approximately 49% pure population of GPR49/Lgr5\(^+\) cells released; this formulation facilitated a 24-fold GPR49/Lgr5 enrichment relative to the un-enriched injected suspension (Figure 4.3B).

**Table a**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Injection Rate</th>
<th>Percent BSA (w/v)</th>
<th>pH</th>
<th>Alginate Formulation Step I (Mix/Incubation)</th>
<th>Alginate Formulation Step II (Mix/Incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-Injected</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>3μL/min</td>
<td>0.1%</td>
<td>6.0</td>
<td>29/60</td>
<td>29/60</td>
</tr>
<tr>
<td>III</td>
<td>3μL/min</td>
<td>0.1%</td>
<td>6.0</td>
<td>29/00</td>
<td>60/00</td>
</tr>
<tr>
<td>IV</td>
<td>5μL/min</td>
<td>0.1%</td>
<td>6.0</td>
<td>29/00</td>
<td>60/00</td>
</tr>
<tr>
<td>V</td>
<td>5μL/min</td>
<td>1.0%</td>
<td>6.0</td>
<td>29/00</td>
<td>60/00</td>
</tr>
<tr>
<td>VI</td>
<td>3μL/min</td>
<td>0.1%</td>
<td>4.7</td>
<td>29/00</td>
<td>60/00</td>
</tr>
</tbody>
</table>

**Figure 4.3** Optimization of antibody functionalized alginate allowed for improved capture efficiency and purity yields. The samples and formulations were divided into five protocols (a), each varying one variable. Mixing and incubation times are measured in minutes. The purity of the Lgr5+ enrichment device outputs was determined for each protocol and compared against the injected cell population (b). Representative flow cytometry data for the un-enriched digested tissue injected cell population (c) and enriched population (d). Each histogram was gated from the electron volume vs. side scatter regime to mitigate noise, and each gate was propagated through each sample. *\(p < 0.0005\), **\(p < 0.001\), ***\(p > 0.05\); \(n = 3\).
The adsorption of BSA following hydrogel formation within microfluidic devices reduced non-specific cell binding and the cell-cell binding within the devices, thereby allowing consistent fluid and cell flow throughout the devices. The concentration of BSA solution used for adsorption, varied in protocols IV and V, had little effect on purity (Figure 4.3A,B). The flow rate of the tissue digest sample into the enrichment devices was varied between 3 and 5 µL/min (protocols III and IV). At the higher flow rate, a significant drop in purity was observed, which suggests possible shear effects on the target cells at higher flow rates (Figure 4.3B). Note that the 49% value in Figure 4.3B represents an average of three values, the lowest of which was 42% and the highest of which was 52.5%. Each of these values is an average from three separate chips; the 61% purity value is from one device that generated a high level of purity.

The viability of the released cells, as measured using with calcein AM/ethidium bromide, was 84% with no statistically significant difference relative to the un-enriched suspension ($p > 0.05$).

### 4.2.2 Three-Dimensional Culture of Enriched Cells and Single Cell Derived Enteroids

Lgr5+ enriched cell suspensions were mixed with Matrigel for three-dimensional culture under conditions similar to those described by Sato et al.[11] Our culture technique for GPR49/Lgr5+ cells included growth factor constituents that were altered slightly relative to those utilized by other groups working with transgenic mouse models.[11] These constituents included rat endothelial growth factor (EGF) and murine rspondin-1. In addition, the Rock inhibitor, Y-26743, was used to improve culture stability and to prevent anoikis in the cell suspension [132]. Use of this inhibitor with the enriched cell population resulted in greater plating efficiency. However this inhibitor had little effect
on the culture of the un-enriched population (data not shown). Culture of enteroids from enriched single GPR49/Lgr5+ cells was carried out up to four days and compared against culture of the unenriched population (Figure 4.4). Growth was noticed at Day 2 and progressed into an expansion stage at Day 3, when areas of the lumen folded to produce projections (Figure 4.4F). Small lumen formation coupled with an increase of budding was observed at Day 4, illustrating clear differences compared to the nascent stage at Day 2 for the Lgr5+ enriched cells. The un-enriched population, by contrast, had a very different morphology (Figure 4.4A-C), with non-uniform lumen formation lacking projections characteristic of an enterosphere (progenitor) structure. For the Lgr5+ enriched culture, the plating and enteroid formation efficiency was similar in value (~6%) to that reported by Sato et al.[11].
Figure 4.4 Three-dimensional culture of Lgr5+ enriched cells compared to un-enriched cells in the absence of Wnt3. The un-enriched enterosphere culture (a-c) yielded different morphology at similar time points compared to the enriched population (d-f). Growth characteristics of an organoid body at Day 2 (d); projections highlighted by arrows at Day 3 (e), and small lumen formation, illustrated by dashed ring, with surrounding secreted apoptotic cells, at Day 4 (f). Scale bars, 100 µm.

A similar time course experiment was carried with Wnt3 added to the culture medium and the resulting comparison of Lgr5+ enriched and un-enriched cells in three-dimensional culture is shown in Figure 4.5. Under these conditions, multiple morphologies were apparent in the un-enriched culture, ranging from enterospheres with central lumen harboring apoptotic cells (Figure 4.5A,C) to epithelial sheets. A qualitative visual comparison at the Day 3 time point indicated that the addition of Wnt3 increased the number of enteroid forming units in the Lgr5+ enriched culture but had little effect on the un-enriched population. This effect can be observed by comparing Figure 4.4B vs. 4.5A and Figure 4.4E vs. 4.5B and was consistent throughout the volume of the three-dimensional cultures.
Figure 4.5 Enriched and un-enriched structures in the presence of Lgr5 basal media constituents and Wnt3 following 3 days in culture. The majority of structures formed in the un-enriched culture (a,c) were cyst-like and harbored apoptotic cells, highlighted with red arrows. Lgr5+ enriched cells (b,d) exhibited similar morphology and lumen formation (indicated with dashed black ring) as in the Wnt3-absent study (Fig. 3e) at Day 3. Scale bars 100 µm (a,b) and 50 µm (c,d).

5.2.3 Immunostaining of Enriched and Un-enriched Structures in Matrigel

Enriched and un-enriched structures were released from culture at Day 4 via exposure of Matrigel to dispase. Cultured structures were incubated with anti-GPCR GPR49 and anti-CD24, conjugated with Alexafluor 568 (red) and 488 (green), respectively, and DAPI. Confocal microscopy (Figures 4.6, 4.7, 4.8) facilitated determination of the
morphology and protein expression of these structures. All labeled structures exhibited minimal non-specific binding within the structures, but remnants of Matrigel occasionally generated fluorescent noise outside of the enterosphere and enteroids (Figure 4.7).

**Figure 4.6** Confocal micrographs of an enterosphere formed from un-enriched cells. These images represent a single z-stack. For imaging, this and other structures were extracted from matrigel after 4 days in culture. Dashed ellipses indicate regions of CD24 and GPR49/Lgr5 expression (A and B, respectively). CD24 expression is localized apically in the central domain and in varying levels of intensity (a). GPR49/Lgr5 is localized at regions of CD24 expression, but is only faintly discernible (white arrows highlight regions of highest expression; b). In the above enterosphere, apoptotic cells are discernible within the central lumen (c), while the overall morphology of the cyst-like enterosphere is that of a flat-disk, as illustrated in Fig. 8a. Scale bar, 25 μm.

The un-enriched culture (Figure 4.5) had a significant population of apoptotic cells illustrated by the DAPI nuclear stain (Figure 4.6C) exhibiting granular fragments localized in the central domain. This is consistent with the morphology shown in Figure 4.5C. Many of the enterospheres in the un-enriched culture did not exhibit any lumen folding for the culture duration and exhibited a bright CD24 signal in an elliptical pattern (Figure 4.6A). By contrast, the expression of GPR49/Lgr5 was faint (Figure 4.6A), and expression was limited to the apical region of the enterosphere. In other words,
GPR49/Lgr5 and CD24 were not uniformly co-expressed within the enterosphere (Figure 4.6A,B).

![Fluorescence confocal micrographs](image)

**Figure 4.7** Fluorescence confocal micrographs (from a single z-stack) of a representative enteroid formed by Lgr5+ enriched cells showing staining for CD24 (a), Lgr5 (b), and DAPI (merged image, c). This enteroid is a spherical structure with a hollow (luminal) interior, as reflected in the cartoon shown in Fig 8b. Co-localization of CD24 and Lgr5 expression is indicated by the white dashed circle in a-c. Scale bar, 10µm

The un-enriched structures exhibited flattened morphology containing cells within the central domain (Figure 4.5C, 4.5A) in contrast to the Lgr5+ enriched enteroids, which were spherical with hollow lumen (Figure 4.8B). In the enteroid shown in Figure 4.7, the white dashed circles highlight two adjacent cells located on the inner surface of the structure which co-express both CD24 (green) and GPR49/Lgr5 (red). The overall level of CD24 expression was lower in Lgr5+ enriched enteroids relative to their un-enriched counterparts (Figure 4.6A vs. 4.7A). Furthermore, the co-localized expression of both CD24 and GPR49/Lgr5 in the Lgr5+ enriched group was limited to the baso-lateral regions of the enteroid, consistent with known CD24 and GPR49/Lgr5 expression trends.[38]
Conventional methods of intestinal stem cell isolation have typically relied on transgenic mouse models and fluorescent label-based separation. Herein, we describe a separation method that does not require prior labeling of the target cells, coupled with the enrichment of Lgr5/GPR49 functional stem cells from wild-type animals. Until recently, the orphan receptor Lgr5 lacked an antibody to sufficiently bind to the domain of the extracellular receptor.[7] The availability of such an antibody enables isolation using our microfluidic technique, thus allowing for enrichment of Lgr5-expressing cells from

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**Figure 4.8** Fluorescent confocal micrograph illustrating an GPR49 enriched intestinal stem cell via the proposed microfluidic separation. The enteroid was stained with a DAPI nuclear stain (a) and GPR49 specific for Lgr5 (c). Hollow domain is illustrated by the transverse image of the z-stack (b). Scale bar, 50µm
native rat intestinal tissue with retention of viability, phenotypic identity, and growth potential. The rationale for implementing the use of neonatal rats was to have the highest starting concentration of Lgr5+ cells to aid in optimizing the proposed microfluidic system. Culture of these cells within Matrigel using an appropriate medium [12] was also performed, yielding organoids demonstrating CD24 expression coinciding with GPR49/Lgr5 expression in the central domain, consistent with the observations of other groups.[38]

**Figure 4.9** Illustrations of structures formed by un-enriched (A) and Lgr5+ enriched cells (B) in three-dimensional culture. The flattened disc morphology with apoptotic cells enclosed in the middle (a) is representative of the micrographs shown in Figs 5c and 6c. The hollow sphere morphology (b) generated by Lgr5+ enriched cells is representative of micrographs in Figs. 5d and 7c.

Our microfluidic cell enrichment technique involves one-pass flow of sample; tissue digestate is flowed through the microfluidic devices only once, and cells are recovered
after a wash step. This process provided a 24-fold enrichment of the GPR49/Lgr5+ cells in the tissue digestate to a concentration level of approximately 49%. This concentration level was the highest that could be attained after multiple rounds of optimization of the separation system and reflects the adhesive nature of the GPR49/Lgr5+ stem cells which are typically surrounded by one or more Paneth cells.[12] In other words, it is difficult to fully disperse the tissue digestate into individual cells. Nonetheless, efforts were made in the present work to increase dispersion, by for example immediately injecting cells into microfluidic devices after filtering post tissue digestion.

Optimization of the separation performance of our device was carried out by varying pH. Chemical interactions and stability between alginate, EDC, 4-arm PEG, anti-GPR49/Lgr5 were increased as the reaction pH became more basic (Fig. 4.3 A,B). Increasing the reaction pH deprotonates active sites in the 4-arm PEG which constrains antibody interactions to the 4 active sites,[133] thus inhibiting covalent binding to the alginate matrix, creating an adsorption effect. The use of a monoclonal antibody against GPR49/Lgr5, if available, will likely result in greater enrichment of the stem cells due to more specific binding.

Three-dimensional culture of the un-enriched and Lgr5+ enriched cell populations in Matrigel provided some interesting insight in terms of the relationships between enrichment level/cell composition, morphology, and the effect of a soluble factor, Wnt3. The un-enriched and Lgr5+ enriched samples were cultured in similar fashion as previously reported in literature.[11] To investigate morphological differences in the Lgr5+ enriched versus unenriched groups, the cultures were run in parallel and prepared under otherwise identical conditions. The un-enriched culture population had significant
morphological variations between structures within individual samples. Many of these culture structures remained in an enterospheric state harboring apoptotic cells (as exemplified in Figure 4.9 A-C). The un-enriched enterospheres were unchanged by the presence of Wnt3 and demonstrated similar morphologies as cultures without Wnt3. We postulate that un-enriched cultures may have contained doublets of Paneth-Lgr5\(^+\) cells, which sustain the necessary Wnt signaling, hence resulting in the null effect observed with versus without Wnt3.[12]

In the absence of Wnt3, the plating efficiency of the Lgr5\(^+\) enriched population was approximately 6\%, in close agreement with previously reported results.[11] In time course studies, cells in the Wnt3-deficient culture exhibited lumen formation and an eventual hyperplasic onset. These observations concur with the previously reported morphology of enteroids derived from individual Lgr5\(^+\) cells wherein secreted apoptotic cells surrounded the enriched enteroids, mimicking in vivo proliferation.[38] Exogenous Wnt3 mimics Paneth signaling facilitating the activation of R-spondin1 allowing for stabilization of single Lgr5 positive cells in vitro and thus enabling formation of enteroid units.[11] This phenomenon is consistent with the increase in plating efficiency to approximately \(\sim 30\%\) in the Lgr5\(^+\) enriched sample that we observed in the presence of Wnt3, similar to previous reports by Sato et al.[12]

Recent literature has shown that intestinal stem cell potential resides in two coded genes, Sox9 and Lgr5.[38] These genes are associated with the extracellular markers, which can be identified using antibodies against CD24 and GPR49/Lgr5 respectively.[38] To investigate the expression trends within the structures, immuno-histochemical analysis coupled with confocal microscopy was used. The un-enriched structures exhibit an
enterosphere morphology harboring apoptotic cells within the central domain. Within the structures formed by the un-enriched cells, high CD24 expression was localized to the apical membrane, while faint GPR49/Lgr5 expression was observed in the highest expressed regions of CD24 (Figure 4.6). These expression patterns are associated with enterospheres derived from transit-amplifying progenitor cells expressing prominin-1 (CD133). [31,38] Structures formed by Lgr5+ enriched cells had a strong degree of co-localization with regard to CD24 and GPR49/Lgr5 expression (Fig. 4.7). When compared with the un-enriched structures, the Lgr5+ enriched structures showed lower overall CD24 expression and higher GPR49/Lgr5 expression, consistent with enteroid structures derived from Lgr5+ intestinal stem cells.[38] The images in Fig. 5.8 also indicate a smaller enteroids size with a small hollow central lumen lacking the apoptotic cells seen in un-enriched populations (Figure 4.6). The morphology of the Lgr5+ enriched structures is similar to those previously reported in literature by Gracz et al. [38] using FACS-sorted cells and hence represents a functional validation of our microfluidic enrichment process.
5.0 INTESTINAL ORGANOGENESIS ELICITS VASCULOGENESIS IN CO-CULTURE

Vasculature within the intestine plays a crucial role in nutrient diffusion via villi and sustaining the intestinal niche. While there is some understanding of intestinal vascular development and disease states associated with over-vascularization in vivo including Crohn’s and Colitis, to our knowledge, there is a lack of studies of crypt-vascular co-cultures. Such studies would enable systematic studies of vasculogenic mechanisms in both normal and disease states. This chapter describes a novel co-culture system that incorporates a migratory vascular cell line, rat aortic smooth muscles cells (RASMC) along with intact crypts harvested from rat small intestine. Proliferative enteroids and enterospheres elicit a migratory response to the RASMCs via exogenous chemo-attractant, specifically VEGF. Quantification of VEGF through ELISA demonstrates a significant increase over time in VEGF levels in crypt digestate culture, decrease in co-culture, and increase when VEGF is inhibited by PF4. Stained co-cultures images via confocal microscopy demonstrate 3-dimensional migrational taxis to the organoid source.

5.1 Methods for a Novel Vasculogenic Co-culture System

The three essential elements of this aim are: (i) robust co-culture, (ii) cytokine quantification, and (iii) culture evaluation via microscopy. The objective of part (i) was to optimize the co-culture to promote enteroid and enterosphere formation while retaining the migrational capabilities of rat aortic smooth muscle cells (RASMC). The objective of part (ii) was to use conventional ELISA to quantify endogenous VEGF concentration within the co-culture medium at select time points against positive and negative controls.
Lastly, in part (iii), fluorescence microscopy was employed to stain actin filaments along with epithelial, and progenitor cell surface markers to characterize the co-cultures.

5.1.1 RASMC Culture

Rat aortic smooth muscle cells (RASMC) were cultured following the manufacturer’s (Lonza) recommended protocol. RASMCs were plated in 25cm² culture flasks in DMEM-F12 (Invitrogen) supplemented with 20% FBS (Hyclone) and 1% penicillin/streptomycin (P/S, Gibco). The cultured cells were grown to confluence and passaged with trypsin/EDTA. The maximum passage number of six was used to maintain migration capabilities of these cells [15]. RASMCs for co-culture experiments were grown to confluence in chamber slides (Thermo) layered with 120µL of Matrigel™ (BD) allowed to polymerize at 37°C for 20min.

5.1.2 Tissue Disaggregation

Intact intestinal crypt cells were obtained using the procedure described by Sato et. al [11]. Small intestine harvested from neonatal rats between 2-5 days of age was split laterally and segmented into < 1mm fragments. The small intestine fragments were rinsed five times in PBS (Gibco). Intestinal fragments were added to 25 mL 2 mM EDTA solution in PBS and incubated at 4°C for 30 min. The suspended fragments were rigorously agitated using a pipette tip for 10 min. Segments of disaggregated intestine were sequentially gravity-settled twice for 1 min intervals and the supernatant was extracted at each interval. The final fraction was centrifuged at 150 x g for 5 min and resuspended in 10 mL serum free DMEM (Gibco). The sample was centrifuged in the same conditions, as described previously, and resuspended in 5 mL DMEM. The
resulting heterogeneous mixture was sequentially passed through a 100 μm filter followed by a 20 μm cell strainer.

5.1.3 Co-Cultures of Crypt Digestate and RASMCs

Disaggregated intestinal suspension was aliquoted and embedded into Matrigel containing 10 μM jagged-1 (Jag-1) peptide (Peprotech) [11,134] at a concentration of approximately 10^4 cells/mL. Confluent RASMCs cultures within chamber slides were decanted and rinsed 2X with medium volume (500 μL) HEPES buffer (Invitrogen). Next, 80 μL of the intestinal cell-laden Matrigel suspension was added to each respective chamber slide containing confluent RASMCs. 500μL of advanced DMEM-F12 containing 10 mL N2 supplement (Invitrogen), 1% P/S, 5 mL B6 minus vitamin A (Invitrogen), and 10μM rho associated kinase inhibtor (Y-21746, Sigma). Growth factors: 50 ng/mL rat EGF (Peprotech), 1 μg/mL r-spondin-1 (R&D Systems), 100 ng/mL Wnt3 (Peprotech), and 100 ng/mL noggin-1 (Peprotech) were also added. In specified experimental groups, rat recombinant platelet factor 4 (PF4, Peprotech) and rat vascular endothelial growth factor (VEGF, Peprotech) were added in medium concentration to the respective experimental and control cultures. Cultures were sustained for 8 days, and growth factors and inhibitors were replenished to the culture medium every 48 hrs with a full medium exchange every 4 days. Control cultures consisted of Jag-1 supplemented Matrigel without intestinal cells placed onto the confluent RASMCs with the respective enteroid and enterosphere forming medium, as described above.
5.1.4 Culture Medium VEGF ELISA Analysis

ELISA measurements were carried out using a rat recombinant VEGF ELISA assay kit (R&D Biosystems) was implemented to determine the concentration of endogenous VEGF. Medium samples from the co-culture, crypt digestate (with no RASMC) culture, and the PF4 inhibited culture groups and respective controls were collected and strained with a 0.2 um syringe strainer at days 2, 4, and 6. The assay procedure was carried out as recommended by the ELISA kit manufacturer. Calibration graphs were generated via the set of VEGF standards provided and respective internal controls were implemented which were within range of the manufacturer’s boundary conditions. The VEGF concentration of medium from respective experimental and control populations was analyzed via a 96 well micro plate reader (Biotek Powerwave/XS). VEGF concentration of each sample (pg/mL) was calculated from the calibration curve post-subtraction of background. Experimental concentrations were normalized against the respective medium from culture controls e.g. cultures containing RASMCs and Jag-1 Matrigel without crypt digestate. Each sample set was run in triplicate.

5.1.5 Confocal Microscopy and Immuno-histochemical Staining of Co-cultures

Co-culture systems were rinsed two times medium volume (500 µL) of PBS, fixed with 4% paraformaldehyde (PFA), and rinse with 100 mM glycine (Invitrogen) in PBS. A blocking buffer consisting of DMEM-F12, 3% BSA (Sigma Aldrich), 1% goat serum (Invitrogen) 0.1% Titron X-100 (Sigma Aldrich), 100mM HEPES, and 100 mM glycine was used to mitigate non-specific binding of fluorescent probes. Primary antibodies mouse anti-human CD133-PE (Miltenyi Biotec) and goat anti-mouse EpCAM (Santa Cruz) were added at a 1:100 dilution. Actin filament staining was performed using
Phlannoidin-Alexfluor-647 (Invitrogen) and Phlannoidin-Alexfluor-488 (Invitrogen). Primary antibodies and actin stain were incubated overnight at 4°C within the co-cultures respective chamber slides. To visualize EpCAM, sheep anti-goat fluorescein (Vector Labs) was used as the secondary antibody at a 1:1000 concentration, incubated at 4°C for 3 hours. Nuclei were stained DAPI at a concentration of 0.5 µg/mL incubated for 5 minutes. Images were taken via a Perkin Elmer Ultraview VOX spinning disk confocal microscope.

5.1.6 Statistical Analysis

Unless otherwise indicated all experiments were carried out with \( n = 3 \) and error bars are reported as standard errors. A \( p \)-value from two-tailed unpaired Student’s \( t \) test less than 0.05 was considered statistically significant. All experiments were preformed independently, in triplicate.

5.2 Intestinal Crypt and rASMC Vasculogenic Co-Cultures

In order to determine the effects of enteroid and enterosphere on RASMCs contained within the same culture, a time progression study was performed. The extended studies provided morphological and qualitative RASMC migrational insight into long-lived enteroid and short-lived progenitor populations in addition. The co-culture duration of the crypt and RASMCs forming units was extended to day 6 to confirm stemness of enteroid units. In assessing media compatibilities it was found that the elicited migratory response of RASMCs was prominent at day 2 within the extended culture. RASMCs extended and migrated outward from their 2-D monolayer into a 3-D projection coming into contact with the enteroid units (Fig 5.1A). The stemness of the enteroid is clearly illustrated by its lumen folding and budding projections from its central domain. Efficacy
of culture medium and qualitative presence of VEGF concentration within crypt digestate cultures was determined via culture doping. The conditioned crypt digestate medium at day 2 was aliquoted and added to a homogenous RASMC monolayer. Cultures were stained with DAPI and actin to discern for morphological differences between the native and conditioned cultures. Native RASMC cultures exhibited a cluster formation which remained morphologically constrained to its group (Fig 5.2 G-L). Conversely, in the presence of the conditioned medium, sprouting of RASMCs from the centralized cluster became apparent indicating a response to the constituents within the organoid conditioned medium (Fig 5.2 A-F).

Figure 5.1 Vasculogenic co-culture containing small intestinal crypt digestate coupled with RASMCs. Co-cultures were sustained up to 6 days in where enterospheres or enteroids formed and a vasculogenic response was observed (A). Throughout the time course study, RASMC tubules within proximity to the intestinal cluster were quantified (D). In quantifying tubules, a10 µm zone from edge of cluster was drawn and tubules were counted within the dashed boundaries, as illustrated in Day 1 (B) and Day 4 (C) in culture. Scale bar 50µm; *p > 0.05, **p < 0.05; n=3.

Additional experiments examined time-points where the migratory response was the most prevalent in order to quantify RASMCs present within a specified boundary of individual intestinal organoids. The boundary was measured 10µm from the organoid
edge and RASMCs projections were quantified within the threshold demonstrated in the day 1 and day 4 representative images (Fig 5.1B, 5.1C), respectively. The numbers of relative RASMCs in proximity to the intestinal clusters were measured at days 1, 2, and 4. RASMCs in proximity to the intestinal clusters were present in abundance but statistically insignificant between day 1 and day 2 in culture. However, the comparison between day 2 and day 4 demonstrated a decrease in RASMCs presence relative to intestinal clusters alluding to a chemokine abundance at day 2 in culture.
**Figure 5.2** Conditioned medium enables elongation of RASMC clusters in homogenous suspensions. Resulting images of RASMC culture medium conditioned experiment (A-F) against native culture (G-L). Cultures were stained with DAPI (A,D,G,J) and Phlanoidin-488 (B,E,H,K) to aid in illustrating the cell nuclei and actin filaments, respectively. Under the conditioned set, RASMC clusters form elongated arms (B,E) conversely within the native culture RASMC clusters remain unified (H,K). Scale bar 50µm

5.2.1 Quantifying VEGF Response in RASMC and Crypt Digestate Co-Cultures

In determining the presence of a common chemo-attractant, VEGF, conventional VEGF ELISA assays were performed. The intestinal enteroid and enterosphere culture medium contains numerous growth factors to aid in stabilizing the self-renewing units. These growth factors and medium supplements have the potential to cross-react with the ELISA microwell plate. To verify possible cross-reactants, culture wells which parallel experimental co-cultures served as culture medium controls and contained Jag-1 Matrigel, intestinal cell culture medium, and a confluent monolayer of RASMCs. The control wells were spiked with essential growth factors under the same methodology in promoting enteroid units. The culture media from the co-culture and control were sampled from days 2, 4, and 6. Representative migratory images from day 2 and day 4 demonstrate a VEGF response in culture (Fig 5.3A, 5.3B), respectively. The ELISA assay yielded VEGF concentration from the co-culture media and the data was normalized against its respective control, illustrated in grey (Fig. 5.3C). VEGF concentration within crypt digestate and RASMCs co-culture media had no statistical difference at day 2; albeit, the subsequent days in culture yielded a decrease in
normalized VEGF concentration within the 4 and 6 days in culture. Confirmation of these VEGF trends via positive and negative control experiments confirmed the trends observed in the levels of endogenous VEGF.

**Figure 5.3** ELISA assays specific towards VEGF were performed with co-culture medium. Time course and medium extractions were performed at Day 2 (A), Day 4 (B), and Day 6. ELISA data (C) was normalized against basal medium control (grey) and compared to medium within the vasculargenic co-culture (patterned). Scale bar 50mm; *p > 0.01, **p < 0.005, ***p < 0.005; n=3.

### 5.2.2 VEGF excess in crypt digestate and uptake inhibition via PF-4

The confirmation of endogenous VEGF within the culture medium was further assessed via positive and negative controls. The positive control experiment involved a culture of crypt digestate in the intestinal enteroid forming medium. The crypt culture yielded *in situ* enteroid and enterosphere units initially forming at day 2 (Fig 5.4A) and organoid
expansion at day 4 (Fig 5.4B). Digestate culture was embedded within the required Jag-1-supplemented Matrigel and was seeded onto a monolayer of un-supplemented Matrigel without the presence of RASMCs. The ELISA assay yielded contrasting results in comparison to the experiments involving the RASMCs and crypt digestate co-culture (Fig. 5.4G). The normalized VEGF concentration of the crypt digestate has a statistically significant increase in exogenous VEGF presence against the respective control at day 2 in culture. In testing the effect of the exogenous VEGF in enteroid media, the day 2 crypt digestate medium was added to a confluent RASMCs monolayer. In this case, a decrease in VEGF levels was observed at Days 4 and 6.

**Figure 5.4** VEGF ELISA assays were performed with enteroid and enterosphere medium. Culture contained intestinal digestate only and eventual forming enteroid and enterosphere units. Intestinal digestate cultures were stained with DAPI and Phlannodin to aid in illustrated morphology. Numerous enteroid and enterospheres formed in culture (A-C) and many of the structures exhibited a hollow central domain (E-F). Time course and medium extractions were preformed at Day 2, Day 4 and Day 6. ELISA data (G) was normalized against basal medium control (grey) and compared to medium within the intestinal digestate culture (patterned). Scale bar 50µm; *p < 0.01, **p < 0.005, ***p < 0.005; n=3.

The study incorporating PF-4 provided further support for the postulate that the migratory response elicited in the RASMCs is due to the VEGF chemokine. A co-culture
of intestinal crypt digestate and RASMCs were seeded in similar fashion as described above. Enteroid promoting growth factors were added in addition to PF4 to inhibit VEGF. The migratory response was significantly diminished as seen in day 2 and 4 bright field co-culture images (Fig 5.5A, Fig 5.5B). Quantitative ELISA assays demonstrated a similar normalized VEGF response as seen in the crypt digestate culture where excess exogenous VEGF was present at day 2 (Fig 5.5C). However, day 4 and 6 of the negative control co-culture showed a statistical insignificant change in endogenous VEGF within media (Fig 5.5C).

![Figure 5.5](image)

**Figure 5.5** ELISA assays specific towards VEGF were performed. Co-culture contained intestinal digestate and RASMC supplemented with proposed medium with the addition of PF-4. Time course and medium extractions were preformed at Day 2 (A), Day 4 (B), and Day 6. ELISA data (C) was normalized against basal medium control containing PF-4 (grey) and compared to co-culture PF-4 medium (patterned). Scale bar 50mm; *p < 0.005, **p > 0.01, ***p > 0.01; n=3
6.2.3 RASMC migration towards intestinal enterospheres via confocal microscopy

Confocal microscopy coupled with immune-staining aided in demonstrating the phenotype of intestinal clusters, directionality of RASMCs, and defining epithelial structures. The co-cultures, whether PF-4 inhibited or uninhibited, were fixed at day 2 in culture. The co-culture was further stained with anti-CD133, anti-EpCAM, DAPI, and phlanoidin. Anti-CD133 aided in visualizing intestinal progenitor clusters or enterospheres where as anti-EpCAM confirmed that these clusters were epithelial. The phlanoidin specifically stains β-actin filaments within the cytoplasm, which are contained in nearly all cell types. Staining for these markers allows assessment of stemness, epithelial structure, and directionality.

Figure 5.6 Confocal images of RASMC and intestinal digestate co-culture within basal medium taken at day 2. DAPI nuclear stain (A) indicated surrounding
populations and proximities of nuclei. EpCAM stain, illustrated in green, (B) indicated epithelial structures within the culture. A well-known progenitor marker, CD133, highlighted enterospheres within culture. Phallloidin exposed actin filaments and striations of the RASMC and enterospheres (D). Asterics (*) indicates migrating RASMC towards the enterospheres where the arrow indicates a abundant actin expression. Merged channel (E) exemplifies morphology and expression patterns of the RASMC and enterospheres. Scale bar 50µm

Confocal images of the crypt digestate and RASMCs under uninhibited conditions demonstrated unique migrational response towards enterospheres. The progenitor and epithelial nature of the cluster was confirmed via CD133 (Fig 5.6C) and EpCAM (Fig 5.6B) staining. The CD133 expression was prominent amongst multiple populations and exhibited gradient patterns indicating degrees of progenitor-like states. Migrational patterns of the RASMCs were highlighted through the actin stain (Fig 5.6D). The directionality of RASMC migration was illustrated by the alignment of the actin filaments towards the enterospheres as denoted by asterisks in Fig. 5.6D. In addition, pre-migrational proliferation due to the over-expression of actin [135] in the direction of the enterosphere was observed, as indicated by the arrow in Fig. 5.6D. In addition to clear migration, encapsulation of enterospheres by RASMCs was noted in ancillary culture wells. The enterospheres were confirmed in a similar fashion as described with anti-CD133 (Fig 5.7C) and anti-EpCAM (Fig 5.7B). RASMCs circled and began to undergo encapsulation of the enterospheres as illustrated by the actin stain (Fig 5.7E). The confocal z-stack was analyzed and compressed into a z-projection to illustrate the 3-D nature of the culture (Fig 5.7E). The z-projection was dimensionally shifted to illustrated an x and y axis perspective (Fig 5.7F, Fig 5.7G). RASMCs migrated from the monolayer (Fig 5.7F) to the enterosphere and continued to migrate around the cluster to its end point
(Fig 5.7G) where the white and red asterisk denotes points of reference within its respective axis perspective.

Figure 5.7 Vasculogenesis is exhibited in 3-dimensional and is specific towards developing intestinal organoids. Confocal image Z-projection of RASMC and intestinal digestate co-culture at day 2 in basal medium. DAPI (A) illustrates nuclear aggregation (enterosphere) and surrounding RASMC. Highlighting the epithelial structure, EpCAM (B), illustrated the enterosphere morphology and was further confirmed of its enterospheres nature via CD133 (C). Merged channel (D) indicated overall morphology and expression patterns. Phlannoidin highlighted actin filliments with EpCAM (E) and migratory patterns observed in the z-axis perspective. White asteric (*) highlights origin of RASMC culture layer extending to enterospheres (F) from the observed x-axis perspective. In the y-axis, red asteric illustrates a RASMC projection above the monolayer (G). Each asteric corresponding to the z-axis perspective (E). Scale bar 50µm

Confocal micrographs of cultures containing the PF-4 inhibitor illustrated a significant decrease in RASMC migration. The PF-4 case was stained with DAPI and Phlannodin to aid in illustrating the nuclei and actin filaments, respectively (Fig 5.8A-C). Actin staining
revealed little migration of RASMCs in proximity to the intestinal cluster (Fig 5.8B).

RASMC In addition, the actin staining demonstrated a clear lumen of the intestinal cluster which could possess stem cell attributes indicating an enteroid.

Figure 5.8 PF-4 inhibits migration of RASMC towards the intestinal organoid. Confocal images of RASMC and intestinal digestate co-culture within inhibitor PF4 medium taken at day 4. DAPI (A) and Actin stain (B), confirmed the presence of the intestinal organoid and surrounding RASMC. Actin stain of the RASMC aids in discerning between migratory or proliferative responses (B). Expression patterns are appreciated within the merged channel (C) highlighting nuclei directionality in addition to actin filament alignment. Scale bar 50µm

This aim demonstrates a unique vasculogenic co-culture incorporating intestinal crypt digestate and RASMCs. The novelty of the technique resides in the optimized culture medium formulations to promote enteroids and enterosphere forming units while retaining RASMC migrational response. Exogenous factors of enteroids and enterosphere cultures has recently been studies and been showed to support extraneous intestinal populations. For instance, investigators have shown that paneth cells secrete Wnt3 providing a necessary growth factor to stabilize Lgr5+ intestinal stem cell derived enteroids [12,136]. In addition, co-cultures with intestinal crypt digestate coupled with non-epithelial cell subtypes such as myo-fibroblasts provided insight into other supporting exogenous growth factors such as Wnt3a and FGF [13,137]. Advances in
understanding intestinal disease pathogenesis, specifically Chron’s and IBD motivate the development of culture methods which incorporate self-sustained enteroids and enterospheres with migrational subtypes such as rASMCs.

RASMC migration was promoted by the presence of a gradient of VEGF constrained to the forming enteroids and enterospheres. Within the culture system, RASMC migration was predominantly observed between days 1 and 2 while proximal RASMC tapered off after day 4 in culture. Intuitively, as the intestine develops, active recruitment of vasculature is needed as crypt cells form budding villi projections mimicking in vivo development [138]. The bright-field images of the proximal vasculature were further confirmed via ELISA assays specific towards VEGF. ELISA results for the co-culture of RASMC and crypt digestate yielded little difference in VEGF concentration within the culture media at day 2. It was shown that the predominant migrational response was constrained to day 2, but VEGF was not present within the co-culture in that condition. The phenomena can be explained in the assimilative nature of the RASMC while migrating. As VEGF induces a chemotactic response from RASMCs, it is in turn up-taken by these cells thus producing a null effect. Implementing a positive and negative system aided in confirming the suggested phenomena producing statistical differences in VEGF levels in the culture medium. Culture of the crypt digestate provided evidence that the enteroids and/or enterospheres structures were the endogenous VEGF sources. In addition, inhibiting the up-take of VEGF using a competitive-binding protein, PF-4, reinforced the chemotactic dependence of RASMC while demonstrating an abundance of non-absorbed VEGF in the co-culture medium. While it is true that VEGF causes a chemotactic affect on endothelial cells and it is worth investigating similar
affects, this study’s scope was limited to that of rat derived crypt digestate and aortic smooth muscle cells due to the well-documented migrational dependence of this cell type on VEGF [139].

Confocal microscopy provided insight into the degrees of migration and phenotype of VEGF-originating intestinal clusters. RASMCs were strongly attracted to enterosphere clusters in cultures, suggesting that these clusters are the primary source of endogenous VEGF. Through confocal microscopy, the 3-D culture illustrated a migrational response along all 3 axes to such an effect that RASMCs migrated from their monolayer to an enterosphere residing above. In addition, actin filaments helped determine directionality of the RASMCs and demonstrated that VEGF origin was constrained to enterospheres units due to perpendicular migration towards the intestinal clusters. While the confocal images shed light onto migration directionality, it has been shown that ASMCs are not susceptible to all epitopes of VEGF.

Studies in the literature have examined VEGF-A in the context of its abundance in vivo intestinal systems [14]. ASMCs are susceptible to chemotactic effects of VEGF-A, particularly the VEGF-A165 isoform. VEGF-A165 has been shown to catalyze VEGFR1 and NRP-1 receptors to enhance SMC migration towards the chemotactic origin [15]. While studies have concluded that VEGF-A is a common inflammatory marker within the intestine, it could also serve as a developmental cue to promote neo-vasculator for forming villi [14,140,141]. The above study demonstrates a novel co-culture system in which enterospheres elicits a vasculogenic response to RASMCs. The system sheds light into originating species producing exogenous VEGF for this response and can potentially
give rise to new disease models to model disease processes and treatments for intestinal disorders such as Crohn’s and irritable bowel disease.
6.0 CONCLUSIONS

Investigations into intestinal stem cell biology have yielded great insight into developmental pathways and, in turn, correlating to disease states. Isolation of these intestinal stem and progenitor cells have been limited to using expensive separation mechanisms such as FACs coupled with non-native genetic hybridization methods. The genetic hybridization methods carry adverse affects, limiting compatibilities with certain assays and co-culture systems where a native system performs more accurately. Therefore, there is a significant need for the creation of a tag-free technology that does not rely on transfection techniques for cell isolation. This could contribute to understanding native systems for human translational studies and enable phenotypic sound development of co-cultures for insight into exogenous influences via supporting cell types.

The overall goal of this dissertation was to develop a robust microfluidic cell separation system with optimized capture and release platform to enable the enrichment of intestinal progenitor and stem cells from native rat tissue. However, intestinal stem cells are extremely rare and intestinal progenitor cells are more populated within the native intestine. In addition, epithelial cells are intrinsically sticky in nature which the platform and capture medium must couple dispersion features while maintaining specific capture affinity. To address these needs, microfluidics post array coupled with an alginic acid/PEG formulation combined dispersion of adherent cell types while selectively binding target populations from native digestate. The significance of this approach will provide a native isolation platform moving away from conventional transfection models giving rise to accurate co-culture models and functional assays. Moreover, yielded
culture optimization of crypt derived intestinal cells provided a foundation of co-culture studies and functional assays.

In this dissertation it was proposed that a device integrating a capture and release chemistry can be optimized to enrich intestinal stem (Lgr5+) and progenitor (CD133+) cells from native rat intestinal digestate while maintaining the ability to form its respective enteroid and enterosphere cluster units. In addition, the resulting culture optimization lead to the effective design of a novel co-culture system which allowed insight into vasculogenic cues in developing intestine. To achieve these objectives, three specific aims were pursued: (i) Characterize native rat intestinal digestate and optimize hydrogel chemistries for enrichment of intestinal stem and progenitor cells, (ii) culture and assess enrichment elutant against unenriched intestinal digestate, and (iii) develop and design intestinal digestate vasculogenic co-culture model and investigate influencing exogenous factors. Rat intestinal digestate cell types were characterized via flow cytometry; localized to the intestinal niche and ancillary cell types. The abundance of absorptive cells or enterocytes was predominant followed by progenitor cell populations. In addition, the characterization study alluded to a low presence of intestinal stem cells representing only ~2% of the total population. Aforementioned in chapter 2.0, negative selection is an attractive option for isolating rare cell types from model suspensions but this dissertation focus on a much more crude sample where the target population represents less than 5%. As mentioned in chapter 2.0, negative selection experiments were initiated via implementing a model intestinal system, but were ultimately discontinued due to significant cellular depletion, lack of translational robustness, and recent discoveries of unique markers for intestinal stem and progenitor cells [3,62]. The
means of a positive selection approach allowed for ease of use, viability retention, and robust system to isolate fragile progenitor and intestinal stem cells from tissue digestate consisting of greater than 10 cell types.


First, a rigorous characterization study of the intestinal digestate was performed highlighting key populations within the suspension and demonstrating specificity of the target intestinal progenitor cell population with anti-CD133. The characterization and on-chip staining are demonstrating selective adhesion of target intestinal progenitor cells to the dissolvable alginate-PEG moiety. In addition, the characterizations alluded to determining cross-reactivity of abundant extraneous cell subtypes such as enterocytes, myo-fibroblasts, and endothelial cells. The described cell types did not have any appreciable CD133 expression concluding that isolate CD133+ cells are intestinal progenitor cells. On-chip bright-field images of injected intestinal digestate allowed for a tailoring of micro-channel flow rates to mitigate fouling affects. An optimal flow rate of 3 µL/min was confirmed via flow cytometry data and fluorescent on-chip staining of CD133+ progenitor cells. With the given optimal flow conditions, an appreciable 4.5-fold enrichment of intestinal progenitor cells (CD133) from ~20% to 85% purity was achieved. The microfluidics enrichment method demonstrated little adverse affects in terms of cellular viability.
The ability to understand intestinal progenitor cells in a quasi-homogenous suspensions and further culture the tag-free enriched enterosphere forming units allows (i) culture optimization for the selected cell type and (ii) a fundamental understanding of partially differentiate progenitor cells into developmental cues giving rise to disease pathogenesis. Secondly, the selective microfluidic capture and release of adherent epithelial cells from complex tissue digestate allows for a valuable tool to the life sciences market thus enabling stem cell isolation from various mediums.

Intestinal progenitor cell culture robustness is demonstrated via colony forming potential. In demonstrating this affect, optimized growth constituents with a custom medium formulation was tailored towards progenitor cluster (enterospher) formation. Enterosphere culture tracking via in situ reference points illustrated lumen centralized apoptotic cells which expanded overtime. Concluding trends of the enriched progenitor cell enterosphere were confirmed at later time-points where enterospheres expelled its’ lumen contents; an indicative trend of short-lived enterospheres. Comparative studies illustrated the unenriched intestinal digestate suspension illustrated multiple morphologies present within the culture system ranging from enteroids (stem cell) to epithelial sheets. Within the context of this dissertation, both a microfluidic CD133+ enrichment platform and enterosphere morphological assessment played a critical role in isolating functional intestinal progenitor cells from complex native tissue digestate.
6.2 Optimization of Alginate/PEG Hydrogel through Reaction pH and passivation

**Increased Intestinal Stem Cell Purity Giving Viable Enteroids In Situ.**

As a build off the previous progenitor enrichment system, significant modifications to the chemical formulation and procedure was made due to the low abundance of intestinal stem within the native niche. Capture protein choice was essential in determining specificity towards the intestinal stem cells and its cross-reactivity with other niche subtypes. The capture protein implemented, anti-GPR49, was specific towards Lgr5 expression in the extracellular domain of mult-potent cell types. Confirmation of specificity was concluded via fluorescent microscopy of dispersed crypts indicated localized expression of regions known to have intestinal stem cells. To further confirm, dissociation of the isolated crypts illustrated single dispersed cells with distinct Lgr5 expression.

Similar microfluidics geometry was implemented to aid in the dispersion of the adherent epithelial cells to selectively capture to the hydrogel substrate. Tailoring of the hydrogel formulation was preformed to take in account the much lower starting population of Lgr5+ cells in the intestinal digestate. Under original formulation conditions of a reaction 4.7 pH, a similar 4.5 fold enrichment was seen which yielded ~18% purity of the target stem cells. Therefore, to improve the fold threshold a redesign of the alginate formulation and flow procedures were needed. As described in chapter 4.0, increasing the reaction pH to 6.0 further stabilized and prevented unwanted denaturing of the fragile antibody thus increasing self-life and efficacy. In addition, the increased reaction pH deprotonated free carboxylic acid and amino groups which forwarded the carbodiimide coupling thus increasing capture affinity. Incorporating a
pacification step via 0.1% BSA before injection aided in decreasing non-specific binding of the said target population but purity yields were not dependant on BSA concentration. Flow conditions were compared and an injection flow rate of 3 uL/min was chosen due to its decrease of shear affects in comparison to 5 uL/min. Here we can confirm that the reaction chemistry of the hydrogel is highly dependent on pH which yields higher purities at a pH 6.0 with the addition of a BSA passification step at 3 uL/min injection rate.

Following optimizing the microfluidic enrichment system to yield a purity of ~50% target Lgr5 positive cells, a culture study of the enriched suspension was investigated. Media formulations were customized for the enriched suspension to promote colony-forming units known as enteroids. Comparison cultures against unenriched populations were used to assess organoid morphology. As mentioned in section 4, the addition of a supporting cytokine Wnt3 was added to select cultures. While morphology was fairly consistent between the presence and absence of Wnt3, an appreciable increase in enteroid forming units was noticed in the enriched culture system which coincides with previously reported studies [142]. Enteroid units illustrated a hollow lumen absent of cells within the central domain. Conversely, many of the cluster units within the unenriched culture system contained an appreciable amount of cells constrained to its central lumen at early time points e.g. 24 hrs in situ. With the stark differences in morphology between the two systems, investigations within immuno-expression trends of the unenriched and the Lgr5 enriched organoids was preformed.

Crypt digestate cultures arise multiple different cluster forming units ranging from progenitor enterospheres, Paneth cell clusters, and stem cell enteroids. Primary means of differentiating the three organoid types is immune-expression and bright field
morphology assessment. As alluded to in chapter 4, unenriched clusters contained an abundance of cells contained within its central lumen at early culture time points; these indications are indicative of either progenitor (enterosphere) or Paneth cell clusters. Confirming that these clusters were of the presented nature, anti-CD24 (Paneth cell marker) coupled with a nuclear DAPI and Lgr5 stain assessed cellular presence and stemness of the cluster. The representative confocal image (Fig. 4.5) of the un-enriched cluster demonstrated a clear CD24 expression pattern coupled with a very low expression of Lgr5 where the nuclear stain highlighted numerous cells contained within the lumen. Transverse images of the unenriched cluster exhibited a non-spherical morphology. With the given expression patterns, one can conclude that the unenriched cultures contained Paneth cell clusters. With respect to the Lgr5 enriched system, enteroid morphology demonstrates an absence of cells within the central lumen at early culture time points and form spherical clusters with a hollow domain. Confocal microscopy highlighted these features via the nuclear DAPI stain and transverse images clearly illustrated the hollow lumen domain indicative of an enteroids. In addition, immune-expression showed a strong co-localization of CD24 and Lgr5 indicating the stemness of the cluster. Given the information presented in this dissertation, one can conclude that the optimized alginate chemistry increased enrichment capabilities of the target intestinal stem cell using a confirmed capture ligand. In addition, the released suspension formed viable enteroids units giving rise to expressions trends demonstrated by previous studies [11,12,37,143].
6.3 Intestinal Organoids, Derived from Crypt Digestate, Secrete Exogenous VEGF to Promote Vascularization

Optimized culture conditions to promote formation of enterospheres and enteroids within culture provided a significant foundation for investigations into influencing exogenous factors towards ancillary cell types. Vasculogenesis is an interesting phenomenon that plays to part in a variety of different instances including disease and developmental states. Nutrient transport through the intestine is highly dependent on the vasculature located within the villi. Intuitively, as the intestinal villi form in vivo a migrational cue must aid to promote vascular formation. In addition, vascular formation within disease states is highly dependent on VEGF secretion and response.

The co-culture system consisting of intestinal crypt digestate and RASMC were cultured with enteroids and enterosphere promoting medium. It was demonstrated from homogenous RASMC cultures with the proposed medium that these cells proliferated similarly to the manufacture’s medium. In addition, enteroids and enterospheres formed within the co-culture system indicating that influencing RASMC did not have an adverse affect in cluster formation. With this two criteria met, one can conclude the presented is an ideal co-culture system; with the evidence, that these two cell types can proliferate independently of each other allowing for native exogenous influences.

Bright-field images of the enteroids and enterosphere units co-culture with RASMC demonstrate clear migration of the RASMC towards the forming clusters. The question is what influencing chemo attractant is directing the RASMC. VEGF is the common chemo attractant associated with vasculogenesis and can directly influence RASMC migration. Investigations into the composition of the co-culture medium
involved a VEGF specific ELISA testing of multiple epitopes. The resulting data demonstrated a null effect against the respective control containing a homogenous population of RASMC. Conversely, VEGF presence within crypt digestate was appreciable over the culture time-course. Implementing a vasculogenic inhibitor, PF-4, showed little migration within the co-culture bright-field images and ELISA data of PF-4 conditioned culture medium demonstrated a similar trend to the crypt digestate culture. With the given trends, one can conclude that exogenous presence within the co-culture system is diminished due to the uptake of VEGF by RASMCs thus generating the null effect. In the inhibition case, PF-4 preferentially binds over competition VEGF to the VEGFR-1 receptor thus creating a presence of VEGF within the co-culture medium.

Further validation of RASMC migration was demonstrated via confocal microscopy. The four color stain highlighted epithelial and progenitor populations in addition to highlight actin filament alignment. The microscopy images showed clear 3-D migration of the RASMC towards enterosphere clusters. In the context of this dissertation, we can conclude that crypt digestate forming enteroids and enterospheres elicit a vasculogenic response to RASMC via a VEGF chemoattractant.

In conclusion, it was shown in this dissertation that the described cell separation device coupled with a dissolvable alginate/PEG capture protein moiety could serve a valuable tool for tag-free intestinal stem and progenitor cell isolation advancing tissue engineering[144-148]. In addition, the intestinal stem and progenitor cell form viable clusters and exhibit similar characteristics as presented in hybridized derived mouse models[11]. Native crypt derived clusters provided significant insight into vasculature migrational cues in a developmental context. The bridge between native cell isolation and
co-cultures will provide a new state of the art in a biological tool to advance tissue engineering [74, 85, 106, 149-154].
7.0 RECOMMENDATIONS

This dissertation presented the design and development of a tag-free microfluidic system yielding high purity for selective capture and release of intestinal progenitor and stem cells from native rat tissue which did not have a significant effect on cell viability. Culture procedures from the enriched samples established viable enterosphere and enteroid cluster formation akin to prior studies. In addition, this dissertation investigated the endogenous interactions of crypt digestate derived organoids and its migrational affects of rat aortic smooth muscle cells. The recommendations for future investigations, based on the presented results within this dissertation, are as follows:

1. Continue to investigate isolating multi-potent intestinal subtypes including quiescent cell niches such as +4 label retaining cells (+4 LRC).

2. Investigate applying the functionalize hydrogel to different separation mechanisms. Applications to a magnetic bead substrate could provide rapid separation of intestinal stem cells from native rat digestate.

3. Probe the VEGF exogenous source within the intestinal cell niche and determine the originated cell type.

**Recommendation 1: Isolating +4 label retaining cells from native rat digestate.** The first recommendation is a continuation in the work presented in section 4. To date, there has been very little investigations in isolating +4 LRCs from native tissue without the use of advance flow cytometry sorting methods. Recent extracellular markers have been discovered that selectively tag for quiescent +4 LRCs. With a proper antagonist, one can
functionalize the alginate-PEG hydrogel with a capture ligand that can selectively enrich for these rare +4 LRCs. In addition, these +4 LRCs have not been cultured in a homogenous population and it has yet to be determined whether this select subtype has self-renewing capabilities that can be sustained in vitro. Coupling the described microfluidic enrichment method with a novel capture protein specific for +4 LRCS will provide researchers functional insight of the cell type and lay the foundation for culture studies investigating its quiescent nature.

Recommendation 2: Synergize Magnetic separations with the dissolvable alginate hydrogel moiety.

The foundation of the presented dissertation relies, in part, to the unique capabilities of the hydrogel chemistry for selective cell capture and release which developed within our lab [66,117,155-157]. As described in sections 3 and 4, the hydrogel was used within microfluidic channels to selectively enrich for intestinal stem and progenitor cells from native intestinal digestate. Specifically in section 4, the hydrogel was optimized to provide better capture efficiency by increasing reaction pH and implementing a passive agent. With those results, it would be an interesting investigation to apply the optimized coating to a magnetic bead substrate. Magnetic micro-beads are a common tool used by life science researchers to rapidly isolate cells from complex solutions [158,159]. However, current limitations in magnetic micro-bead release capabilities, hinders isolation of sensitive stem cell subtypes [160]. In a paper published in 2008 by McGuckin et al., the investigators describe culturing embryonic-like stem cells from human umbilical cord blood using a magnetic separation approach [161]. The authors describe
their attempts in culturing the post-magnetic sorted CD34+ stem cells and observed apoptosis after seeding the cultures. In addition, the investigators mention a need of a viable method to release the magnetic microbeads from the post-sorted stem cells. Surprising, to the best of the authors knowledge, there is no literature sources where immuno-magnetic beads can be released by chelation without the need of His-tag NTA (nitrilotriacetic acid) chemistry [162]. Applications of the hydrogel in conjunction with magnetic beads would provide a viable release mechanism, which would serve as a viable tool for stem cell researchers.

**Recommendation 3: Discover endogenous VEGF source through selective enrichment of intestinal progenitor and stem cells coupled with genomic analysis.**

A third recommendation resides in continuing the investigation described in section 6. Researchers studying intestinal disease states such as Colitis and Crohn’s disease have found that inflammatory responses are a function of over-vascularization of the intestinal niche. In specific, Boqui et al implemented a transgenic mouse model over expressing vilVEGF1 and vilVEGF2 to study an affect against wild type [51]. Within their study, the group concluded that VEGF plays a significant role in development and disease states highlighting vasculature abnormalities and cyst formation via immuno-histochemical staining. While the breadth of the study demonstrated a correlation between inflammatory intestinal diseases and over vascularization, there was a significant lack of granularity in determining the root cause. In light of this dissertation, it has been shown that VEGF is naturally secreted by organoid derived crypt digestate illustrating there could be an originating cellular subtype that is native to the crypt niche. The recommendation
encompasses an experimental design that exploits this possibility, in which homogenous suspensions of intestinal stem cells and progenitor cells including +4 LRCs, Lgr5+, and CD133+ cells are rigorously characterized through genomic analysis. With the given analysis, one can probe into the subset of genetic markers including vilVEGF and vilVEGF2 determining the higher expression trends amongst the stem and progenitor types. Once identified, genetic sequences correlating to VEGF expression can be determined laying a significant foundation of knock-in/knock-out models to aid in studying developmental and disease states. Built genetic models can be implemented in the described co-culture system in section 6 of this dissertation. Overall, the proposed recommendation would determine the originating intestinal cell subtype thus advancing development in disease models for Colitis and Crohn’s leading to therapeutic breakthroughs.
8.0 NOMENCLATURE

\[ h = \text{Height} \]
\[ l = \text{Length} \]
\[ c = \text{Cell} \]
\[ m = \text{mass} \]
\[ dt = \text{Time} \]
\[ T = \text{Temperature} \]
\[ w = \text{Width} \]
\[ x = \text{Distance} \]
\[ a.u. = \text{arbitrary units} \]
\[ x = \text{vector in the x-direction} \]
\[ y = \text{vector in the y-direction} \]
\[ z = \text{vector in the z-direction} \]
\[ IgG = \text{immunoglobin} \]
\[ D = \text{Day} \]
\[ P = \text{Passage number} \]
\[ \text{Anti- Antibody} \]
\[ FITC = \text{Fluoracine (Emission 488nm)} \]
\[ PE = \text{R-Phycoerythrin (Emission 568nm)} \]
**Subscript**

Digestate = Intestinal cell suspension derived from tissue

Crypt = Location of intestinal stem and progenitor cells
9.0 REFERENCES


