Wake Forest® School of Medicine

Institute for Regenerative Medicine

2018

Summer Scholar Program Research Day & Poster Symposium

AUGUST 8, 2018

Piedmont Triad Community Research Center,
Winston-Salem, NC

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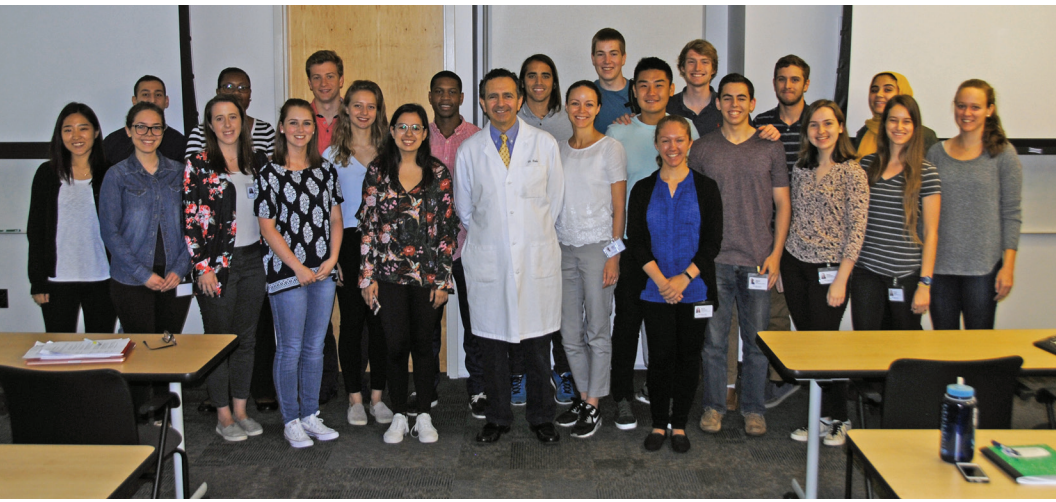

WFIRM 2018 Summer Scholars Program
Final Research Day & Poster Symposium



CELEBRATING MULTIDISCIPLINARY RESEARCH EXPERIENCES FOR UNDERGRADUATE SCHOLARS IN CHALLENGING AREAS OF REGENERATIVE MEDICINE

Wednesday, August 8, 2018

The Wake Forest Institute for Regenerative Medicine (WFIRM) is an international leader in translating scientific discovery into clinical therapies. Our mission is to improve patients' lives through regenerative medicine.
A significant challenge in this promising field is developing the next generation of engineers, scientists, clinicians and entrepreneurs cognizant of the challenges and approaches needed to solve regenerative medicine problems and design functional replacement tissues and organs. Here at WFIRM, inherently tied to our mission is the training of next generation experts to whom we will look toward to continue to advance and deliver upon the promise of this field and make a lasting impact on health conditions ranging from heart disease, diabetes, injury and aging.

Congratulations to our WFIRM Summer Scholars 2018

Today we invite you to join us in celebrating the graduation of 24 summer undergraduate scholars, who participated in our annual 10-week summer research program. May 30th marked the beginning of our 2018, 10-week, Summer Scholar Program. Today's Final Research Day with oral presentations and scientific poster session provides the opportunity for undergraduate researchers who join us from across the United States and globe, to present their summer research. We offer congratulations to our talented scholars, their faculty mentors and near-peer mentors comprised of teams of graduate, postdoctoral fellows and lab technicians. All providing important contributions and support. Much success is extended to our 2018 cohort of undergraduate scholars in their future educational and career pursuits. WFIRM is privileged to be a part of their intellectual and professional growth. We thank you all for joining us today and the support you have extended to these fine young adults.

Anthony Atala, MD
Director, Wake Forest Institute for Regenerative Medicine

Joan F. Schanck, MPA
Summer Scholars Program Director

WFIRM 2018 SUMMER SCHOLARS FINAL RESEARCH DAY

Wednesday, August 8, 2018

Oral Presentation Venue: Piedmont Triad Community Research Center (PTCRC)
115 S. Chestnut Street, Winston-Salem, NC 27101

Poster Session and Luncheon Venue: Wake Forest Institute for Regenerative Medicine,
391 Technology Way, Winston-Salem, NC 27101

SCHEDULE

7:45 am to 8:00 am	Summer Scholars Arrival at PTCRC: Photo Session
8:00 am to 8:30 am	Guest Arrivals: Registration w/coffee, biscuits and muffins at PTCRC
8:30 am to 8:45 am	Welcome and Overview Anthony Atala, MD, Director, WFIRM and Joan Schanck, MPA, Summer Scholars Program Director
8:45 am to 10:00 am	Summer Scholars' Presentations – Part I (Note: Group Q&A follows each session)

Session 1

1 Agne Nixon Washington State University	*BIOENGINEERING OF OVARIAN FOLLICLE-LIKE CONSTRUCTS*
2 Olivia Cornett Salem College	*IN VITRO DIFFERENTIATION OF HUMAN KLINEFELTER SPERMATOGONIAL STEM CELLS IN 3D TESTICULAR ORGANOID SYSTEM*
3 Nicole VanOstrand Rochester Institute of Technology	EVALUATING THE EFFECT OF ISCHEMIC STROKE ON THE BBB USING AN *IN VITRO* HUMAN BRAIN MODEL
4 Grahame Evans Duke University	*DEVELOPMENT AND ANALYSIS OF A LENTIVIRAL-LIKE CAS9 PARTICLE DELIVERY SYSTEM TO TREAT DUCHENNE MUSCULAR DYSTROPHY*

Session 2

5 Nancy Rutishauser Fordham College at Rose Hill	*DEVELOPMENT OF AN OXYGEN-RELEASING ANTIOXIDANT POLYMERIC SCAFFOLD FOR SUSTAINED OXYGEN DELIVERY*
6 Sean Muir Wake Forest University	*PORCINE KIDNEY ECM-DERIVED HYDROGEL AS A PHYSIOLOGICALLY RELEVANT SUBSTRATE FOR IN VITRO KIDNEY MODELING*
7 Jake Gray University of North Texas	*DEVELOPING SMALL MOLECULES BASED THERAPIES FOR INNER EAR SENSORY CELL REGENERATION AND HAIR FOLLICLE REGROWTH*
8 Grigory Manyak Case Western Reserve	*USING SMALL MOLECULES TO COMBAT OXIDATIVE STRESS*

Session 3

9 Erin Maloney University at Buffalo	*IMMERSION BIOPRINTING ORGANOID CONSTRUCTS IN MULTI-WELL PLATES FOR INCREASING THROUGHPUT OF 3D DRUG SCREENING*
10 Ugne Ziausyte Carnegie Mellon University	*USE OF 3D LIVER ORGANOIDS IN SCREENING NOVEL DRUG FOR TREATMENT OF ALCOHOL INDUCED LIVER FIBROSIS*
11 Andrew Rabah University of Michigan	*MOLECULAR METHODS TO DETERMINE SAFETY ASPECTS OF A CELL- AND GENE-BASED THERAPY FOR HEMOPHILIA A*

12 Victoria Kusztos Tufts University	*CYTOTOXIC T LYMPHOCYTES ARE SIGNIFICANTLY HIGHER IN COLONIC TISSUE FROM GI-SYMPTOMATIC CHILDREN WITH AUTISM*

10:00 am to 10:15 am Coffee Break

10:15 am to 11:20 am Summer Scholars' Presentations – Part II
(Note: Group Q&A follows each session)

Session 4

13 Boeun Hwang University of Illinois at Urbana-Champaign	*inCITE OPTICAL TISSUE CLEARING IS COMPATIBLE WITH POST PROCESSING STAINING AND ANALYSIS*
14 Robert Masi Washington and Lee University	*INCITE TISSUE CLEARING PRESERVES NATIVE STRUCTURES IN BLADDER AND INTESTINE*
15 Kate Singletary Davidson College	*OPTICAL CLEARING TECHNIQUES FOR IMAGING COMPOSITE TISSUES*
16 Amit Cudykier North Carolina State University	*FIBER OPTIC IMAGING OF COLORECTAL CANCER ORGANOIDS*

Session 5

17 Maryam Eugenia Elizondo Rice University	*RHEOLOGICAL CHARACTERIZATION OF BIOINKS FOR EXTRUSION 3D PRINTING*
18 Joseph Grech Michigan State University	*OPTIMIZATION OF dECM BASED BIOINK FOR 3D BIOPRINTING OF RENAL CONSTRUCT*
19 Zishuai Chou University of California, Berkeley	*DECELLULARIZED HUMAN SKIN-DERIVED ECM AS A SUPPLEMENT TO FIBRIN HYDROGEL FOR SKIN BIOPRINTING APPLICATIONS*

Session 6

20 Bianca Cordazzo Vargas Harvard University	*EFFECTS OF MICROGRAVITY ON NATURAL KILLER CELL ANTI-LEUKEMIC CYTOTOXICITY*
21 Julie Leonard-Duke Georgia Institute of Technology	*OPTIMIZING HUMAN AMNION EPITHELIAL CELL PROLIFERATION FOR CELL THERAPY*
22 Rayia Johnson Winston-Salem State University	*THE EFFECTS OF PULSED ELECTROMAGNETIC FIELDS ON THE GROWTH AND DIFFERENTITATION OF SKELETAL MUSCLE STEM CELLS*
23 Rawdah Elbahrawi University of Alabama at Birmingham	*LOCAL DELIVERY OF STEM CELL-RECRUITING FACTOR AND SKELETAL MUSCLE MATURATION FACTOR FOR IN SITU SKELETAL MUSCLE REGENERATION*

11:20 am to 11:35 pm Wrap-Up/Certificates of Completion
(**Note:** Walk to WFIRM for Poster Session & Luncheon)

11:45 pm to 12:30 pm Poster Session at WFIRM (2nd Floor Collaboration Area)

12:30 pm to 1:45 pm Lunch with Scholars at WFIRM (2nd Floor Collaboration Area)

1:45 pm to 2:30 pm Lab Tours at WFIRM
(**Note:** Guests sign-up during registration. Scholars assist with demos, overview of their work in lab at stations)

Introducing the 2018 WFIRM Summer Scholars

Summer Scholar	Primary Faculty Mentor(s)
Zishuai Chou University of California, Berkeley Bioengineering, Junior	**Anthony Atala, MD** Professor and Director of WFIRM
Bianca Cordazzo Vargas Harvard University Undeclared, Freshman	**Graca Almeida-Porada, MD, PhD** Professor and **Christopher Porada, PhD** Associate Professor
Olivia Cornett Salem College Chemistry, Senior	**Hooman Sadri-Ardekani, MD, PhD** Assistant Professor
Amit Cudykier NCSU/UNC Biomedical Engineering, Junior	**Shay Soker, PhD** Professor and CSO
Rawdah Elbahrawi University of Alabama at Birmingham Biomedical Engineering, Sophomore	**Ji Hyun Kim, PhD** Instructor
Maryam Eugenia Elizondo Rice University Bioengineering, Junior	**James Yoo, MD, PhD** Professor, Assoc. Director **Sang Jin Lee, PhD** Associate Professor
Grahame Evans Duke University Biology, Junior	**Baisong Lu, PhD** Assistant Professor
Jake Gray University of North Texas Biology, Senior	**John Jackson, PhD** Associate Professor
Joseph Grech Michigan State University Human Biology, Junior	**James Yoo, MD, PhD** Professor, Assoc. Director **Sang Jin Lee, PhD** Associate Professor
Boeun Hwang University of Illinois at Urbana-Champaign Bioengineering, Junior	**Frank Marini, PhD** Professor
Rayia Johnson Winston-Salem State University Exercise Physiology, Junior	**Tracy Criswell, PhD** Assistant Professor
Victoria Kusztos Tufts University Biology, Junior	**Steve J. Walker, PhD** Associate Professor
Julie Leonard-Duke Georgia Institute of Technology Biomedical Engineering, Junior	**Sean Murphy, PhD** Assistant Professor
Erin Maloney University at Buffalo Biomedical Engineering, Junior	**Aleks Skardal, PhD** Assistant Professor and **Thomas Shupe, PhD** Assistant Professor

Summer Scholar	Primary Faculty Mentor(s)
Grigory Manyak Case Western Reserve University Medical Anthropology, Pre-Medical, Freshman	**Anthony Atala, MD** Professor and Director of WFIRM
Robert Masi Washington and Lee University Biochemistry, Freshman	**Frank Marini, PhD** Professor
Christopher McCoy Winston-Salem State University Exercise Physiology, Sophomore	**Young Min Ju, PhD** Instructor
Sean Muir Wake Forest University Medicinal Chemistry, Sophomore	**Khalil Bitar, PhD** Professor and **Giuseppe Orlando, MD, PhD** Marie Curie Fellow and Assistant Professor
Agne Nixon Washington State University General Studies, Biological Sciences, Junior	**John Jackson, PhD** Associate Professor
Andrew Rabah University of Michigan Cell and Molecular Biology, Junior	**Graca Almeida-Porada, MD, PhD** Professor and **Christopher Porada, PhD** Associate Professor
Nancy Rutishauser Fordham College at Rose Hill Biological Sciences, Junior	**Emmanuel Opara, PhD** Professor
Kate Singletary Davidson College Psychology, Pre-medical	**Vijay Gorantla, MD, PhD** Associate Professor
Nicole VanOstrand Rochester Institute of Technology Biomedical Engineering, Sophomore	**Anthony Atala, MD** Professor and Director of WFIRM
Ugne Ziasyte Carnegie Mellon University Biological Sciences, Sophomore	**Colin Bishop, PhD** Professor

BIOENGINEERING OF OVARIAN FOLLICLE-LIKE CONSTRUCTS

*A. Nixon, S. Sivanandane, R. Sequeira, J. Jackson
*Summer Scholar, Wake Forest Institute for Regenerative Medicine

A significant subpopulation of reproductive age women suffer from premature ovarian failure (POF) due to primary ovarian pathologies and treatments for disease, resulting in devastating effects on fertility and quality of life. While recent advances in reproductive technologies show promise, many women with POF have no follicular reserve and limitations of current therapies indicate a need for alternatives. Oogenesis and folliculogenesis were thought to occur solely in the perinatal period, however a 2004 study found that oogonial stem cells (OSCs) are present in postnatal mice and human ovaries, suggesting potential for lifelong oogenesis.[1] Under this premise, novel methods of in vitro follicle maturation and hormone replacement therapy were developed utilizing rat models.[2,3] The objective of this study was to further these developments and utilize OSCs and Granulosa/Theca progenitor cells isolated from human ovarian tissue to bioengineer human follicle-like constructs and assess morphology and function in 2D and 3D cultures with the long term goal of oocyte maturation and hormone secretion.

Human ovaries were acquired via organ procurement organizations. Tissue digestion was followed by isolating DDX4 and IFITM3 positive OSCs, used to form the follicular construct core. The negative cells were differentiated with specialized media to form Granulosa Cells (GCs) and Theca Cells (TCs), the supporting layers of the construct. Follicle-like organoids were assembled in Aggrewell 400 plates with cells layered in successive 48 hour intervals. Each was designed with a core of 200 OSCs, middle layer of 500 GCs, and outer layer of 300 TCs. Organoids were separated into groups, subjecting identical organoids to basal media vs treatment media (FSH + LH) as well as 2D suspension culture vs 3D collagen embedded culture. A subset of organoids cultured in 2D suspension had various cell types pre-stained with different fluorescent cell trackers to assess cell migration and maintenance of follicular structure at different time points by epifluorescent and confocal imaging. Unlabeled organoids were assessed by live/dead assay to analyze viable cell distribution over time. Organoids embedded in collagen for 3D culture were assessed for altered structure/function using H&E and IHC staining and observation of GC and TC markers. Media was sampled at regular 48 hour intervals for ELISA assessment of hormone production. Additionally, 30,000-cell organoids were engineered containing varied concentrations of collagen, fibronectin, laminin, and poly-L-Ornithine to mimic a basement membrane-like structure. The surfaces were analyzed using Scanning Electron Microscopy (SEM).

GCs and TCs might not have been fully differentiated, as evidenced by the number of organoids migrating from wells and aggregating. An absence of hormones suggested by ELISA further supports this finding. Within most organoids cells remained in their respective layers, with OSCs located in the periphery of the construct. Moving forward, migration may be prevented by selecting a different organoid formation system. Both the 3 and 2-layer organoids remained intact in collagen and suspension, with a small number of cells migrating from the periphery. Conversely, 1-layer organoids dispersed, implying that supportive cells play a role in core structure maintenance. Within the organoids, connective tissue remained intact while the number of nucleated cells slightly decreased. Live/dead assay indicated that viability of the cells was maintained over time. Finally, SEM revealed that organoids engineered using laminin and fibronectin exhibited greater cell-to-cell communication and were denser. Further analyses of ECMs are recommended to optimize a basement membrane-like structure.

Acknowledgements: The summer scholar's research reported was supported by National Science Foundation.

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IN VITRO DIFFERENTIATION OF HUMAN KLINEFELTER SPERMATOGONIAL STEM CELLS IN 3D TESTICULAR ORGANOID SYSTEM

*O. Cornett, N. Pourhabibi Zarandi[1], Guillermo Galdon[1], S. Howards[2], S. Kogan[1,2], A. Atala[1,2], H. Sadri-Ardekani[1,2]
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Klinefelter Syndrome (KS) is the most common genetic disorder (1 in 600 new males born) causing infertility in males. A KS patient has 47 chromosomes with an additional X-chromosome in the sex chromosomes (47XXY), rather than the typical 46 XY. This disorder is characterized in majority of KS patients by hypogonadism and sterility after puberty. Recently our group has developed an *in vitro* 3-dimensional (3D) human testicular organoid (HTO) system from normal human testicular cells, which was able to produce testosterone and differentiate spermatogonia stem cells (SSC) to post-meiotic stage. The objective of this study is to use the same system to create 3D HTOs from *in vitro* propagated human KS testicular cells as a novel infertility treatment of KS patients.

Previously isolated, propagated and cryopreserved KS testicular cells in a 2-dimensional (2D) culture system were thawed, recovered and propagated in StemPro Complete media in 37°C for a week. After trypsinization, cells were incorporated into 3D HTOs using ultra low attachment round bottom plates with 10,000 cells per HTO for 48 hours. Then, the newly formed organoids were refreshed with differentiating media and kept in 34°C for 3 weeks. Over the period of 3 weeks, multiple assays were performed. To evaluate the viability of the HTOs, live/dead staining followed by confocal microscopy imaging was done. To estimate the cells activity, ATP assay was utilized. To assess the structure of the HTOs, at each time point they were fixed, embedded in paraffin blocks and stained for Hematoxylin & Eosin (H&E), Masson Trichrome and PGP9.5 (as a marker for undifferentiated spermatogonia). To assess the maintenance of the four major testicular cell types (Spermatogonia, Sertoli, Leydig an Peritubular cells) and tracking the spermatogonia differentiation, quantitative Reverse transcriptase PCR (qRT-PCR) and digital Reverse transcriptase PCR (dPCR) were performed using different cell type specific markers; ZBTB16 for undifferentiated, DAZL for differentiating, SYCP3 for meiotic and PRM1 and ACR for early and late post meiotic germ cells. To monitor the testicular somatic cells, expressions of STAR for Leydig cells, CYP19A1 for Sertoli cells and ACTA2 for peritubular cells were measured. Moreover, at each timepoint HTOs were collected and dissociated using GMP qualified collagenase (SERVA) to count the number of cells in each HTO.

Using the isolated cells from human KS testis, a well-defined spherical HTOs were formed after 48 hours with the average diameter of 448 µm. Live/dead assay showed stable and viable organoid at all timepoints. ATP activity of HTOs revealed a drop in first week of culture which stayed stable for the rest of the time. H&E staining showed that HTOs preserved their structure during the whole 3 weeks of study. PCR results confirmed the presence of all four major testicular cell types, and the ability of the system to differentiate spermatogonial cells to post meiotic stage. Dissociation of the HTOs revealed a decrease in the number of cells throughout the 3-week study, however most of the cells were viable (viability >90%) in all timepoints.

In summary, 3D HTO system was created from isolated and propagated human KS testicular cells in 2D culture. These organoids were able to maintain their viability and structure at least for 3 weeks in culture. In addition, SSCs were differentiated to post meiotic germ cells. This study is showing a promising future as a clinical application to address infertility in KS patients.

Acknowledgements:
This research effort was supported by Urology Care Foundation Research Scholar Award Program, American Urological Association (AUA) Southeastern Section; American Society of Andrology (ASA), and the Diversity Initiative/National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Diversity Funding. A sincere thank to Abinav Udaiyar for his assistance during the research process.

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EVALUATING THE EFFECT OF ISCHEMIC STROKE ON THE BBB USING AN *IN VITRO* HUMAN BRAIN MODEL

*N. VanOstrand, G. Nzou, J. Jackson, A. Atala
*Summer Scholar, Wake Forest Institute for Regenerative Medicine

Neurological disorders affect more than 100 million Americans and stroke is one of the more prevalent of these disorders. Strokes affect approximately 6.8 million people and cost the Unites States $109.6 billion on average every year. Strokes are more prevalent in the elderly than in younger people and as the life expectancy continues to rise, the number of strokes is expected to rise. By 2030, the number of people suffering from stroke is expected to reach 9.5 million. Better therapies are needed. Current therapies are not very effective as they must be given within a certain time after having a stroke and frequently those suffering from a stroke arrive in the hospital too late thereby missing the effective window for these therapies. Current models from which current therapies are developed do not closely mimic the microvasculature and blood brain barrier [BBB] of the human brain. Therefore, we have developed a spheroid model for stroke that includes all six major cell types in the human brain.

The spheroids were developed by mixing 20% human neurons, 15% human oligodendrocytes, 5% human microglia, 15% human astrocytes, 30% human brain microvasculature endothelial cells, and 15% human brain vascular pericytes in a hanging drop. On day 6, half of the spheroids developed were placed in the hypoxic chamber with 0.1% O_2 for 24 hours to model acute ischemic stroke while the other half were cultured under normal growth conditions. The spheroid's supernatants were used to run an ELISA to investigate the cytokine secretion and total protein extraction was performed to quantify and compare the protein expression under normoxia vs. hypoxia.

We established the inflammatory response in spheroids under hypoxic conditions by assessing pro-inflammatory cytokines (IL-8, IL-2, IL-1β, MCP-1, TNF-α, and IL-6) and anti-inflammatory cytokines (IL-13 and IL-4). We noted higher secretion of pro-inflammatory cytokines under hypoxic condition compared to normoxic condition. This upregulation of pro-inflammatory cytokines can be attributed to both endothelial and glial activation under hypoxic condition. Low anti-inflammatory cytokine secretion under hypoxic condition was unexpected since higher amounts of anti-inflammatory mediators should be present under these stress condition to counteract the effects of pro-inflammatory agents. Then we assessed the expression of the most prevalent proteins found in the basement membrane: fibronectin [FN] and laminin [LAMA1]. They play important roles in the maintenance of BBB integrity. FN was upregulated under hypoxic conditions. FN supports cell survival through oxygen supply by inducing angiogenesis. However, LAMA1 was downregulated under hypoxic condition spheroids and this helps to explain BBB dysregulation under hypoxia because LAMA1 maintains the integrity of the BBB.

Previous qualitative analysis showed a disruptive distribution of tight junctions under hypoxic conditions. Interestingly, quantitative evaluation of VE-Cadherin, Claudin-5, ZO-1, Occludin, and Beta Catenin showed that these tight junctions were upregulated in the hypoxic conditioned spheroids. This means that cells are producing more tight junction proteins to resolve the compromised BBB. However, the tight junction proteins may be internalized which explains the less tight junctions at the cell junctions under hypoxic condition shown previously.

In summary, the spheroid model described here may function as an *in vitro* model for stroke. Future work is geared towards looking at the effects of 2-Arachidonoylglycerol and Secoisolariciresinol Diglucoside as possible anti-inflammatory agents that could reverse the effect of a stroke by reducing reactive oxygen species and thereby restoring the tight junctions and normal expression of cytokines and proteins.

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4

DEVELOPMENT AND ANALYSIS OF A LENTIVIRAL-LIKE Cas9 PARTICLE DELIVERY SYSTEM TO TREAT DUCHENNE MUSCULAR DYSTROPHY

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Duchenne muscular dystrophy (DMD) is a potent muscle degeneration disease caused by mutations in the X-linked dystrophin gene. Most of these mutations cause disruption of the reading frame, leading to dystrophin production impairment and progressive muscle failure in affected tissue areas (1). One promising treatment option for DMD is exon skipping using the CRISPR/Cas9 gene editing system (2, 3). Utilizing the high selectivity of this bacterial system, paired with a novel lentiviral delivery method of the system's components *in vivo*, we performed exon deletion in human muscle cells from DMD patients to recover dystrophin production, theoretically producing partially deleted but functional dystrophin protein. We sought to evaluate this use case in the context of development and further optimization of our novel lentiviral-like particles to deliver CRISPR/Cas9 in a variety of cell lines. In this study, we used CRISPR/Cas9-mediated gene editing to delete exon 51, a strategy potentially able to treat roughly 14% of all DMD patients. We initially designed two sgRNA sequences on introns 50 and 51 to identify the optimal combination of sgRNAs for exon 51 deletion. We then used the best sequence combination to create lentiviruses to deliver two sgRNAs targeting sequences within introns 50 and 51. We verified exon 51 deletion efficiency by transducing 293T cells with our novel lentiviral Cas9 particle delivery system and another lentivirus to deliver two sgRNAs. Our PCR analysis showed efficient deletion of exon 51 with this strategy. To test the exon deletion efficiency of our system in more relevant cell lines, we transitioned to trans-differentiated muscle cells from fibroblasts of DMD patients. We are currently working on optimization of our detection method, specifically for use in muscle cell lines. We plan to use RT-PCR to detect exon deletion in trans-differentiated muscle cells. Also, we plan to use immunocytochemistry to examine recovery of dystrophin production in muscle cells from DMD patients. Although many groups are exploring the usage of CRISPR/Cas9 system to treat DMD by exon skipping (3), our Cas9 mRNA delivery strategy addresses the concerns of mutagenesis caused by long-term Cas9 expression.

Acknowledgements:
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DEVELOPMENT OF AN OXYGEN-RELEASING ANTIOXIDANT POLYMERIC SCAFFOLD FOR SUSTAINED OXYGEN DELIVERY

*N. Rutishauser, K. Enck, E. C. Opara
*Summer Scholar, Wake Forest Institute for Regenerative Medicine

Type 1 diabetes is an autoimmune disease in which the immune system attacks the insulin-producing β-cells in pancreatic islets. Management consists of regular insulin injections and blood glucose monitoring. Islet transplantation has been shown to free type 1 diabetes patients from exogenous insulin dependence[1]. One obstacle to routine clinical use is the hypoxic injury to isolated islets. β-cells have huge oxygen requirements, utilizing 10-12% of the blood flow to the pancreas while only making up 1-2% of the pancreas by mass [2]. During isolation and the transplantation period, islet cells are exposed to an extended period of hypoxia while waiting for angiogenesis. Revascularization begins around 2-4 days post-transplantation and is not complete until 10 days; islet function may be lost during this period[3].

One strategy to overcome this obstacle is the use of particulate oxygen-generating substances (POGS), like calcium peroxide (CPO), to supplement the oxygen supply. CPO reacts with water to produce oxygen. Hydrogen peroxide is formed as an intermediate, which can generate reactive oxygen species that cause oxidative stress on the cell. The addition of catalase speeds up the reaction, reducing the risk of radical formation but also causing rapid oxygen release. This can lead to hyperoxia, which may also negatively impact islets[4]. Therefore, a sustained oxygen delivery system that mimics normoxia is needed. This study aims to characterize a chemically modified CPO compound that will allow for both sustained oxygen generation and free radical scavenging.

Polyurethane antioxidant calcium peroxide (PUAO-CPO) incorporates a hydrophobic polymer to delay the access of water to CPO as well as the antioxidant ascorbic acid to scavenge radicals and reduce the risk of oxidative damage. PUAO-CPO was synthesized according to a modified version of the Parvaiz et. al protocol in which the final compound was lyophilized and cryomilled into a powder[5]. Oxygen generation was measured with a dissolved oxygen probe in a hypoxic chamber. The DPPH assay was used to measure antioxidant capacity and compared to trolox and ascorbic acid, two known antioxidants.

Oxygen generation lasted up to 2.5 days when using the 10% PUAO-CPO (percent CPO by weight) with the equivalent of 1mg CPO in 25 mL PBS. The 1%, 5%, and 10% PUAO-CPO all demonstrated a more consistent oxygen release over time compared to that of 1mg CPO with 1μL catalase. All compounds had a similar area under the curve, showing that the oxygen capacity was consistent between groups. All PUAO-CPO compounds demonstrated high antioxidant capacity with percent inhibition of the DPPH radical increasing significantly as the sample concentration increased. At 10mg/mL after 45 minutes, the PUAO-CPO compounds had an antioxidant capacity equivalent to 7.05-7.52mM trolox and 0.99-1.05mM ascorbic acid. The percent inhibition of the 1% and 5% PUAO-CPO was not significantly different at 45 minutes, however there was a small but significant difference between these two groups and the 10% PUAO-CPO. Even though the 10% PUAO-CPO had the least antioxidant, the weight needed to generate physiologic levels of oxygen was much lower than the other compounds, making it more relevant for co-encapsulation. It may be practical to synthesize PUAO-CPO at a higher percentage CPO in order to maximize oxygen release since PUAO appears to be a highly potent antioxidant at small concentrations. With promising data on oxygen generation and antioxidant capacity, future directions include co-encapsulating the PUAO-CPO with islets and observing cell viability and functionality over time.

Acknowledgements: This summer research position was made possible by the Henry H. and Dorothy Ray Henley Fund.

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Porcine Kidney ECM-derived Hydrogel as a Physiologically Relevant Substrate for *In Vitro* Kidney Modeling

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Drug development costs have surged to over 2.6 billion dollars in total. Once marketed, a drug has an 89.5% attrition rate primarily due to cumulative toxicity effects on either the liver or kidneys. Some drugs including non-steroidal anti-inflammatory drugs and aminoglycosides have been shown to cause acute kidney damage when administered within recommended dosages. Early detection of toxicity to these organs could be facilitated by "organ's-on-a-chip" serving as functional and anatomical surrogates for human organs. The complex nature of organ anatomy and the physiology of the nephron filtration systems could be mimicked *in vitro* if technology could provide a microenvironmental system relevant to the specific organ of interest. Hydrogels have characteristics relevant to substrate-based platforms that support "organ specific" microenvironments modified to enhance the viability and performance of cell delivery tools and platforms. Our goal was to construct a porcine kidney extracellular matrix (ECM)-derived hydrogel as a substrate for Madin-Darby Canine Kidney (MDCK) cells, which were seeded onto 3-dimensional scaffolds representing (1) collecting tubule, (2) collecting tubule and ureteric bud, and (3) distal convoluted tubule of a kidney. The ultimate goal was to create renal constructs that could be used to test drug toxicity *in vitro*.

Producing 3-Dimensional (3D) scaffolds for cell seeding. Three kidneys designs were modeled using auto computer aided instrumentation to represent the collecting tubule, the uretic bud, and distal convoluted tubule. The designs were printed using a fused deposition modeling 3D printer (MakerBot Replicator+, MakerBot Industries LLC. 11201). The 3D printed designs were imbedded in polydimethylsiloxane (PDMS) and coated with pluronic F-127 to create a viable channel platform for MDCK cells in hydrogel.[1]

Producing extracellular matrix (ECM) hydrogels. Kidney ECM-derived hydrogels were produced by decellularizing and solubilizing tissue of organs without destroying the fundamental ECM components: collagen, glycosaminoglycans, and elastin. We produced hydrogels using published decellularization methods that could be converted into a hydrogel following an optimized protocol specific to kidney ECM by varying time, temperature, and pH conditions. Specifically, fresh kidneys (n=4) were harvested from 25-30kg Yorkshire pigs. All kidneys were decellularized according to *Orlando et al*, cannulated and decellularized, concurrently.[2] The kidneys were perfused with water and heparin followed by 0.5% sodium dodecyl sulfate (SDS) and then perfused with 1X phosphate-buffered saline solution (PBS).

Kidneys were separated into two groups. Group 1 was sliced into 1cm by 1cm blocks, decellularized, lyophilized for 5-7 days, and fragmentized with Cryo Mill (SPEX SamplePrep 6870). Group 2 was sliced, delipidized, using a standard protocol of chloroform and methanol, lyophilized, and fragmentized with Cryo Mill. Both groups were re-solubilized to produce a homogenous mixture of ECM, which was reconstituted to produce a specific concentration of hydrogel. The kidney ECM-derived hydrogel was then combined with GelMA hydrogel in a 2:1 ratio to sustain gelation. Groups 1 and 2 hydrogels were placed in the above designs and seeded with MDCK cells to form tubules. Characterization of ECM included histological staining, DNA, collagen, GAGs, and elastin quantitation.

Our results showed that both Group 1 and Group 2 formed a hydrogel however, the hydrogel was not sustainable unless combined with GelMA. This is the first porcine kidney ECM-derived hydrogel produced that is less harsh to renal tissues than previous methods. The delipidized ECM-GelMA hydrogel was shown to be a viable cell substrate for MDCK cells.

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DEVELOPING SMALL MOLECULES BASED THERAPIES FOR INNER EAR SENSORY CELL REGENERATION AND HAIR FOLLICLE REGROWTH

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Introduction

Regenerative Medicine is now beginning to represent a valuable tool to cure several clinical conditions in both acute injuries and chronic diseases. Certain tissues, such as the sensory epithelia within the inner ear of adult mammals, lack the regenerative capacity to restore sensory cells once damaged, making subsequent impairments to the auditory or vestibular system permenant.[1,2] When follicular epithelial stem cells, which ultimately give rise to hair follicles, become dormant or die the resultant hair loss is also permanent.[3,] One promising approach to restore the regenerative capacity of such tissues is to reprogram dormant stem cells residing within that tissue. In situ molecular reprogramming via small molecules appears to have the potential to be further developed as drugs to stimulate patients' endogenous cells to repair and regenerate *in vivo*. The goal for this project was to test WFIRM developed small molecules to (1) induce the reprogramming of mouse cochlear cells, and (2) to induce early reactivation of anagen and follicular neogenesis in a telogenic mouse model.

Methods

HEI-OC1, mouse cochlear cells, were treated with four small molecules (W951, W108, W942, and W16) at multiple concentrations at different time points, and MTS assays were carried out to measure cell growth. Further, proliferation was measured using EdU incorporation assay. Cells were immunostained for reprogramming factors and were quantified using INCell Analyzer. For Hair Regrowth studies, in vivo experiment was carried out by Jareer K. et al. In brief, a small molecule, W108, was topically applied twice (2-day gap) on the shaved dorsal skin of C57BL/6J telogenic mice, and after 14 days the mice were observed for hair regrowth, euthanized, and the test skin from each mouse was collected, formalin fixed, and processed into paraffin blocks. Five-micron thin sections were H&E stained, observed for hair follicle generation and the number of hair follicles were quantified. Additionally, skin sections were immunostained with a proliferation marker, Ki67, to confirm induction of anagen.

Results and Conclusions

MTT assay results suggested that all the tested small molecules induced highest proliferation of HEI-OC1 cells within 48 hours of treatment at different concentrations. They showed potent effect even at 1nM concentration. Further, EdU incorporation quantitative assay further confirmed that these small molecules enhance HEI-OC1 proliferation. Further, immunostaining and IN Cell analysis of compound treated cells with reprogramming factors like SOX-2, C-Myc and OCT-4 showed quantitative increases in their expression compared to control. Our results provide first evidence of the proliferative capabilities and potency of these small molecules in a cochlear cell line.

Topical application of W108 to the shaved skin of telogenic mice appeared to induce the onset of the anagen phase of the hair growth cycle, as evident from the growth of hair within 14 days of compound treatment. Further histological analysis using H&E staining revealed W108 treated mice had a large number of dark pigmented and newly generated hair follicles compared to that of control mice. Immunofluorescent staining showed expression of proliferative marker Ki67 within the hair follicles of compound treated mice compared to untreated ones. These results show that W108 was able to regrow hair by activating dormant hair follicles in telogenic mice. This provides evidence that it may be further developed as a potential treatment for hair loss in humans.

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USING SMALL MOLECULES TO COMBAT OXIDATIVE STRESS

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Background: Redox imbalance within the cell that is caused by increased reactive oxygen species (ROS) levels leads to oxidative stress. Chronic oxidative stress is responsible for a plethora of diseases such as fibrosis, cancer, liver disease, and neurological disorders.[1] Previous studies have revealed a large number of detoxifying "phase II" proteins that are upregulated to counter the effects of oxidative stress; the expression of these proteins is collectively controlled by the transcription factor Nrf2.[1] While the Nrf2-driven oxidative stress-response system allows cells and tissues to counteract the destructive effects of oxidative stress, continued ROS overexpression can ultimately lead to the manifestation of disease.

Objective: The goal of this study was to characterize and validate small molecules that have been shown to induce the phase II protein heme oxygenase, and to investigate their ability to combat the damage caused by oxidative stress.

Methods: Human muscle progenitor cells (MPCs), human lung fibroblasts from patients with idiopathic pulmonary fibrosis (IPF), and hepatic stellate cells (HSCs) were cultured in 96-well plates and treated with small molecule compounds synthesized at WFIRM (W135, W141, and W145). HSCs were grown as spheroids in low-attachment round-bottom plates. To test protection against oxidative stress, cells were incubated in the presence of hydrogen peroxide (75–500 μM). To test protection against alcohol poisoning, HSCs were incubated with 50 mM ethanol in an evaporation-resistant chamber[2,3]. Toxicity assays were performed by treating cells with ethidium homodimer. For heme oxygenase immunofluorescence staining, cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, blocked with 5% bovine serum albumin, and immunostained with heme oxygenase antibody that was detected with a fluorescein-conjugated secondary antibody. All fluorescent cells were quantitated using the InCell Analyzer 2000 (GE), which measures both the percentage of dead cells stained with propidium homodimer as well as the intensity of heme oxygenase expression. RNAi was performed by treating cells in 96-well plates with 20 nM siRNA (Qiagen) and 1.7 μl/mL siLentFect lipid reagent (BioRad).

Results: Silencing of the Nrf2 pathway reduced compound-induced heme oxygenase activation, indicating that the compounds' activities were at least partially Nrf2-dependent. Treating MPCs that were poisoned with 450 nM H_2O_2 with W135, W141, and W145 decreased peroxide-induced cell death by 19.2%, 18.8%, and 15.0% respectively. Next, we tested disease models starting with IPF cells, where the three compounds were shown to decrease the proportion of peroxide-induced cell death by 25.8%, 17.1%, and 20.1% respectively; these rates were better than in normal lung fibroblasts (13.2%, 8.1%, and 16.9%, respectively). This is potentially because fibrotic lung cells have a higher baseline oxidative stress condition than their healthy counterparts, and may therefore react differently to the compounds. In the alcohol poisoning model[2,3] with HSC spheroids, the three compounds decreased ethanol-induced cell death by 27.8%, 20.9%, and 3.7% respectively. Lastly, we compared heme oxygenase activation by our compounds to its genetic activation via silencing the enzyme's transcriptional repressor Bach1, and found that both pharmacological (compound) and genetic (Bach1 silencing) activation of the protein were equivalent.

Conclusion: These small molecule compounds exhibit promising cytoprotective abilities in a variety of cell types *in vitro* through their induction of heme oxygenase. Our findings suggest that these compounds have the potential to protect against other oxidative and cytotoxic stressors in addition to the models already shown.

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IMMERSION BIOPRINTING ORGANOID CONSTRUCTS IN MULTI-WELL PLATES FOR INCREASING THROUGHPUT OF 3D DRUG SCREENING

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Introduction: The current drug development pipeline takes approximately fifteen years and $2.6 billion dollars to bring new drugs to market. To test the drug's efficacy before reaching human trials, tests are run on 2D cell culture models and animal models. However, these models are often not representative of the human body. 2D culture changes the morphology and physiology of cells, directly affecting the drug response, disease progression, and overall function of cells [1]. Similarly, animal models have a different anatomy and physiology than that of humans. One solution would be to use 3D organoids for drug screening. 3D organoids have been shown to be far more representative of key biomarkers in pathologies such as cancer [1]. Through incorporating 3D organoids, candidate drugs can be screened in human-based models before human trials, thereby increasing chances of success and reducing costs. One limitation in employing organoids in drug screening has been the difficulty in consistently creating large numbers of organoids in form factors compatible with high throughput screening (e.g. 96- and 384-well plates). We present a technique to bioprint human tissue organoids in 96-well plates to increase throughput of drug screening.

Objective: To develop a technique to consistently fabricate large numbers of human organoids in 96-well plates for increased throughput of drug screening.

Methods: We printed our cells using both the Allevi BioBots and CellInk INKREDIBLE printers. Two hydrogel bioinks comprised of natural extracellular matrix components were tested. Images of live/dead-stained were taken with a macro-confocal microscope to assess viability, while MTS assays were performed to quantify mitochondrial metabolism of the cells over time. HepG2, Caco2, and A549 cells were cultured using standard protocols.

Results: We have created a novel immersion printing technique to bioprint organoids directly into multi-well plates for increased-throughput drug screening. We printed our organoids into a 96-well plate that contains a support gelatin bath [2]. Once completed, the gelatin is removed, and the organoids are left in cell culture media. To demonstrate the flexibility of the approach, we printed with two different bioinks: a thiolated hyaluronic acid and methacrylated collagen hydrogel and the commercially available Hystem hydrogel. The Hystem gel was crosslinking midprint leading to problems during the printing process. Organoids were cultured for 7 days and a MTS assay and live/dead staining was taken at the day 1, 3, 5, and 7 time points demonstrating high viability immediately following bioprinting and over time in culture.

Conclusion: In conclusion, we present an easy, reproducible technique to increase throughput of drug screening which provides high viability and versatility with different bioinks and bioprinters. In the future, we plan to use this technique as a tool for personalized medicine. Specifically, we plan to take biopsies from cancer patients and create tumor organoids to test which chemotherapy cocktail would be most effective for that patient as demonstrated in the Mazzocchi *et al* paper "In vitro patient-derived 3D mesothelioma tumor organoids facilitate patient-centric therapeutic screening [3]."

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USE OF 3D LIVER ORGANOIDS IN SCREENING NOVEL DRUG FOR TREATMENT OF ALCOHOL INDUCED LIVER FIBROSIS

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Alcohol abuse is one of the main instigators of liver fibrosis. Excessive alcohol intake increases the permeability of the intestinal wall, releasing lipopolysaccharide (LPS) into the blood where it stimulates Kupffer cells through the CD14/Toll-like receptor-4. Stimulated Kupffer cells release inflammatory cytokines and reactive oxygen species (ROS), which in turn activate stellate cells leading to inflammatory dysregulation, production of excess collagens and finally apoptosis of hepatocytes (1). The most effective treatment is abstinence from alcohol consumption, but in severe cases the only curative treatment is liver transplant. Currently, therapies are being developed that are aimed at reversing late stage liver fibrosis through the use of drugs.

We have previously reported on the *in vitro* development of 3D multicellular human liver organoids which closely mimic the major liver functions seen *in vivo*. Further, by treating these organoids with LPS we have developed an in vitro disease model which closely resembles alcohol induced liver fibrosis (ALF) (2). The aim of this project was to use this new human fibrosis model to assess the effectiveness of a novel small molecule ML290 in the treatment of ALF. ML290, works as an agonist of the human relaxin receptor and increases production of the relaxin peptide without interfering with existing pathways. Although relaxin is known to produce anti-fibrotic effects, its use as a therapeutic drug is limited by its expense, and short half-life.

The hypothesis to be is that ML290 will be able to reduce fibrosis, as measured by a reduction in collagen and production after LPS stimulation in our hepatocyte organoid model of ALF.

The organoids were created by allowing the 4 major liver cell types (Kupffer cells, stellate cells, endothelial cells, and hepatocytes) to self-aggregate by gravity over 4 days in low adhesion 96 well plates or 256 well agarose micro-molds. For half – life analysis, 1uM ML290 and 1uM propranolol were incubated in medium or medium containing organoids. The concentration at defined intervals between 0 and 5 days was measured by mass spectrometry. Dose-response assays were performed by treating organoids with either media only, LPS only, or LPS and a titration of ML290 100mM – 1nM. These plates were left for four days, after which supernatant was removed and tested for pro-collagen concentration through ELISA. Collagen protein within the organoids was assessed through confocal whole mount immunofluorescence and staining of fixed sections. A fibrotic index was established by scanning the images using software that counted the number of DAPI stained nuclei (blue) and collagen staining (red) pixels. Finally, RNA extracted from organoid and the expression of approximately 84 fibrosis related genes was assessed by RT-qPCR.

The data suggested that the half – life of ML290 was almost 19.87 days and therefore stable enough for therapeutic use. Dose – response data showed a decrease in fibrosis with an increase in concentration of ML290, illustrating the efficacy of the molecule in reducing fibrosis in a dose – dependent manner. No apparent toxicity was observed at concentrations of 100 micromolar. Combined, the results suggest that ML290 can potentially be used as a drug to reverse liver fibrosis.

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Molecular Methods to Determine Safety Aspects of a Cell- and Gene-based Therapy for Hemophilia A

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Background: Hemophilia A [HA] is an X-linked congenital bleeding disorder caused by a defect in coagulation factor VIII [FVIII], resulting in a significant decrease in FVIII activity. The severe form of HA is characterized by spontaneous bleeding leading to chronic hematomas, hemarthrosis, hematuria, and intracranial hemorrhages. HA patients must undergo frequent prophylactic infusion of FVIII protein, which can cost (in the USA) $150,000–$300,000 per year (1). In addition, about 30% of people receiving protein replacement therapy develop neutralizing antibodies, or inhibitors, rendering the treatment ineffective (2). Furthermore, many of the world's hemophiliacs do not have access to protein replacement therapy, creating the need for the development of new long-term or corrective treatments. We have recently shown that pre-natal transplantation of human placental cells [PLC] transduced with a lentiviral vector encoding an expression/secretion-optimized variant of FVIII [mcoET3], referred to as PLC-mcoET3, resulted in curative plasma levels of FVIII following birth, in a large preclinical animal model. If successful, this approach could enable the birth of a corrected child who needs no further treatments, or at least the treatment could induce immunologic tolerance to FVIII protein, circumventing the immunological barriers associated with postnatal treatment (3).

Study Objectives: Although the stable insertion of mcoET3 in PLCs led to high levels of FVIII production by the transduced cells, the integration of transgenes into the host cell genome is not risk-free. The potential for proviral integration into active transcription units can result in insertional transformation of the transduced cells leading to unsafe clonal proliferation after transplantation. In order to better understand the safety profile of the PLC-mcoET3 therapeutic platform, we investigated the integration sites in PLC-mcoET3, as well as in 3 other PLC populations transduced with lentiviral vectors encoding other expression/secretion-optimized variants of FVIII. In addition, using molecular approaches, we also identified the locations of engraftment of the PLC-mcoET3 after fetal transplantation, and determined whether the mcoET3 was still being expressed by the transplanted cells.

Methods: In order to perform integration site analysis, PLCs transduced using three different codon-optimized lentiviral vectors, mcoET3, lcoET3, hsqET3, and a non-codon optimized ET3 were cultured and lysed for gDNA extraction. Each gDNA sample was digested with three different blunt-end restriction endonucleases (DraI, SspI and HpaI). An adaptor molecule was then ligated to the ends of the gDNA fragments, and a long-range nested PCR reaction was performed using primers specific to the 3' LTR of the lentiviral vector, and to the adaptor molecule. Secondary PCR products were then directly cloned and sequenced. To determine sites of PLC-mcoET3 engraftment, RT-qPCR was performed on RNA isolated from tissues of two lambs who received prenatal treatment, using primers designed to be specific to either mcoET3 or human FVIII. Standard curve analysis was performed to enable quantification of expression of ET3 in engrafted tissues.

Results and Conclusions: Analysis of mRNA transcripts from liver and spleen tissue of two prenatally transplanted lambs revealed engraftment and expression of mcoET3 and human FVIII in PLCs. For the same tissues, levels of mcoET3 FVIII mRNA transcripts were higher than those of human FVIII, demonstrating that the transgene had not been silenced and was effectively producing mcoET3. Multiple sites of integration were identified in PLC-mcoET3 and PLC-ET3. Detailed integration site analyses of PLC-lcoET3 and PLC-hsqET3 are underway, but data thus far support the safety of this approach to HA treatment.

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CYTOTOXIC T LYMPHOCYTES ARE SIGNIFICANTLY HIGHER IN COLONIC TISSUE FROM GI-SYMPTOMATIC CHILDREN WITH AUTISM

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Gastrointestinal (GI) problems are more common in children with autism spectrum disorder (ASD) than in typically developing (TD) children[1]. Moreover, many children with ASD present with GI symptoms suggestive of an inflammatory bowel disease-like (IBD-like) condition however, following investigative ileocolonoscopy with biopsy, conventional histology with hematoxylin and eosin (H&E) staining often does not reveal any marked abnormalities. Using immunohistochemistry, an earlier study reported an increase in $CD8^+$ density and intraepithelial lymphocyte numbers in children with ASD and GI symptoms that was disproportionate to the inflammation seen on routine histologic evaluation, indicating a distinct lymphocytic colitis[2]. The goal of this study was to investigate this finding in another case/control cohort by comparing levels of CD8, a cytotoxic T cell and gut inflammation marker, in colon samples from GI-symptomatic children with ASD and TD children.

Biopsies from the right colon were obtained via colonoscopy from children with ASD and TD children who presented with GI symptoms suggestive of an IBD-like disease. These tissues were embedded in paraffin blocks, and we first prepared slides and performed conventional H&E staining. This was followed by immunohistochemistry with a primary CD8 antibody from a mouse host (dilution 1:750) and a secondary goat anti-mouse antibody (1:1000) with an emission wavelength of 594 nm. Tissues were permeabilized with 0.2% Triton-X100 in PBS and antigen retrieval was performed with 0.01 M citrate buffer at pH 6. Dako protein block and TrueBlack Autofluorescence Quencher were used to reduce nonspecific staining and autofluorescence. Tissues stained with secondary antibody only were the negative control and the positive control was a human spleen sample. Density of cells with CD8, average intensity of CD8 fluorescence per area, and total intensity of CD8 fluorescence per area was assessed semi-quantitatively following one second exposure with fluorescent microscopy.

As predicted, H&E staining showed unremarkable colonic mucosa with no diagnostic abnormalities in both the ASD and TD group. Immunohistochemical analysis using a CD8-specific antibody resulted in a mean total intensity of fluorescence in cells with CD8 that was significantly greater in ASD than in TD samples ($p=0.0031$), and the total intensity per area was also significantly greater in samples from ASD versus TD children ($p=0.000753$). We observed a trend that children with ASD have a higher density of cells with CD8 than TD children, as well as a higher average intensity of CD8 fluorescence per area; however these differences did not reach statistical significance.

These findings revealed that, compared to GI-symptomatic TD children, children with ASD and GI symptoms have a marked increase in CD8 fluorescence intensity, suggesting a cytotoxic T lymphocyte infiltration that was not apparent on routine histologic examination. Further analysis of CD8 reactivity is required to determine the significance of the observed CD8 infiltration. Other inflammatory markers such as CD3 and CD4 should also be examined via immunohistochemical analysis.

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inCITE OPTICAL TISSUE CLEARING IS COMPATIBLE WITH POST PROCESSING STAINING AND ANALYSIS

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Recent advances in 3D microscopy have enabled imaging large tissue samples without sacrificing three-dimensional details. However, imaging quality of confocal and multiphoton microscopy deteriorates rapidly at imaging depth of several hundred micrometers as light scattering and light absorbing moieties in the tissue prevent light from focusing on the plane of interest [1]. Optical tissue clearing (OTC) allows a deeper imaging depth by removing light impeding components as well as by matching refractive indices (RI) of the tissue. Though several OTC techniques have been developed, many of these techniques require harsh chemicals or detergents that can damage, degrade and shrink tissues and impact the intactness of extracellular matrices (ECM), frequently unraveling and disorganizing the collagen matrix. We also noted that many of these techniques appear specialized for clearing of neural tissues and will damage non-neural tissues and surrounding ECM. Therefore, there is a need for a milder and gentler clearing technique that perfoms similar, but can be applied to other non-neural types of organs. Here, we investigate the ability to perform post-OTC processing of whole tissues using two different clearing techniques, one using alcohol dehydration followed by BABB refractive index matching (uDISCO), and the other (inCITE), a hybrid method which exposes the tissue to low concentration of delipidation followed by mild RI matching using a tunable RI media on a range of whole mouse organs.

uDISCO and inCITE clearing methods were each applied to a set of mouse whole organs. uDISCO was performed as previously described [2]. For the inCITE technique, a patent pending tissue clearing protocol, fixed and hydrogel embedded tissues were treated with a combination of mild detergents, and surfactants, in a solution termed the “lipid magnet” for 10-14 days, in a pressurized recycling perfusion setup followed by final clearing and RI matching. Untouched control and cleared organs were analyzed for light transmission, transparency, light scatter, and ability to image at depth. In order to assess the quality of tissue after optical clearing, thin sections of cleared whole organs were taken and stained with hematoxylin and eosin, picrosirius red and subjected to multiplxed/multispectral analysis to determine the impact of optical clearing on the post processing tissue quality and ability to detect IHC antigens.

Our findings suggest that as compared to uDISCO, which optically cleared all tissues in a few days, inCITE resulted in a similar transparency of organs; especially lung, small intestine, bladder, and muscle, however it took 14 days. Organs treated with uDISCO showed a 1/3 reduction in size due to the alcohol dehydration process, while the inCITE did not impact the organ size. Furthermore, we observed less architectural changes in tissues cleared with inCITE, as compared to uDISCO especially in terms of cell planar relationships, which are critical in understanding the structure-function interactions. Interestingly, both uDISCO and inCITE did not impact the compatibility with H&Es, picrosirius red, and immunofluorescent staining/antigen retrieval after the optical clearing processing, suggesting both technqiues are compatible with post-processing.

This study demonstrates that the inCITE technology enables clearing of various whole mouse organs and is compatible with post processing. This novel clearing method will be used to analyze tissue structures of large tissue samples at cellular and subcellular levels without the loss of three-dimensional information or severe morphological changes.

Acknowledgement: This research was made possible by the Douglas Jerome Bodner Fund for Research in Regenerative Medicine.
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INCITE TISSUE CLEARING PRESERVES NATIVE STRUCTURES IN BLADDER AND INTESTINE

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In regenerative medicine, it is vitally important to perform high resolution tissue imaging for structural and functional characterization. Recent advances in microscopy have afforded three dimensional imaging, overcoming the limitations of traditional histology to provide a more comprehensive examination of tissue. However, volumetric imaging is limited to a depth of roughly 1 mm due to the inherent light-scattering properties of tissue. This stems from differences in refractive indices between tissue components, especially at the boundaries of large molecules such as lipids, resulting in an opaque appearance. The emergence of optical tissue clearing (OTC) allows for whole-tissue microscopic imaging by removing light scattering moieties, thereby rendering tissue transparent. While recent OTC technologies have improved deep tissue imaging, there are limitations to broad implementation. Many methods utilize caustic chemicals which risk tissue shrinkage and structural damage, and OTC chemistries have largely been targeted towards neurological tissue without optimization for non-neural structures. Thus, there is need for procedural optimization and effective tissue-sparing chemistries before OTC can offer more comprehensive investigation of tissue.

The objective of this study was to compare the clearing efficacy and structural preservation of two recently developed OTC methods, uDISCO (ultimate Dimensional Imaging of Solid Cleared Organs) and InCITE (index matched Clear Imaging for Tissue Evaluation), when applied to bladder and small intestine, two organs with promising regenerative potential. uDISCO is an alcohol-based clearing technique, using gradient tert-butanol solutions for dehydration and benzyl alcohol-benzyl benzoate (BABB) for clearing and refractive index matching. InCITE is a hybrid clearing method, combining ECM-preserving hydrogel with mild pressure-assisted detergent-based delipidation and alcohol clearing with refractive index matching in 2,2'-thiodiethanol (TDE). Whole bladders and small intestines from fluorescently labeled transgenic mice were harvested and subjected to their respective clearing methods. Photographs were taken every other day qualitatively compare clearing efficacy. Quantitative measurements utilizing optical transmittance tests were used to assess the completeness of clearing, and assays were performed to determine preservation of protein content post-clearing. Following clearing, intact tissue sections were imaged using confocal and multiphoton microscopy to volumetrically visualize structure, ECM content, and imaging depth ability. Additionally, histological sections were taken from cleared and uncleared tissue and stained with hematoxylin and eosin, picrosirius red, and a panel of IHC antibodies. 2D multispectral images were acquired and processed using VisioPharm® software to quantitatively compare morphology, ECM preservation, and cellular organization for each clearing method.

We found that uDISCO and InCITE both achieve satisfactory tissue transparency within 14 days. However, InCITE-cleared tissue maintained original morphology and size, whereas uDISCO-treated samples exhibited considerable shrinkage. 3D microscopy revealed that native tissue architecture and behavior was better preserved with InCITE, whereas uDISCO tissue displayed slight damage and compacting. Quantitative analysis showed that InCITE better maintained ECM content and cellular arrangement as compared to uDISCO. Protein loss was also minimized with InCITE thanks to the structurally-sparing hydrogel. In summary, our results show that InCITE is a powerful tool for achieving tissue transparency with structural preservation, allowing for greater understanding of regenerative organ behavior.

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15

OPTICAL CLEARING TECHNIQUES FOR IMAGING COMPOSITE TISSUES

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Background: Optical tissue clearing (OTC) is a novel technique that removes light-scattering molecules in inherently translucent or opaque tissues, such as solid organs, rendering them transparent to light, while retaining 3-dimensional integrity in the cleared tissue (1). We have optimized OTC techniques for solid organs (eg. lung, heart, brain) to enable high quality imaging of structural, morphological, and compositional features in treated tissues compared to conventional histology (2). Here in, we evaluated for first time, the efficacy of our OTC techniques [uDISCO and index matched clear imaging for tissue evaluation (inCITE)] in clearing mouse facial flap (with eyeball) and mouse hind limb tissues that included skin (with hair/adnexa), skeletal muscle, vessels, nerve, fat, cartilage, bone, and bone marrow components.

Methods: Murine limb and face flap explants were obtained (n=5) following perfusion fixation with paraformaldehyde (PFA). Untreated controls (n=2) were compared to OTC treatment (n=3). Of treated samples, half were limbs and half were face flaps. Bone tissue in limbs was decalcified with formic acid and EDTA for 2-4 days. Following hydrogel fixation, samples were cleared with either uDISCO or inCITE techniques. All cleared samples were analyzed using histological and immunohistochemical staining/analysis for specific markers (skin, muscle, bone, nerve and vessel), and 3-dimensional confocal imaging was performed to compare cleared versus uncleared (stained/unstained) tissues treated with either technique.

Results: Analysis of the composite tissues revealed that in comparison to controls, cleared tissues allowed for higher quality staining and imaging because of enhanced tissue transparency. Both uDISCO and inCITE techniques successfully cleared limb and face flaps. uDISCO technique was fast and efficient in clearance of composite facial flaps and limbs. However, the harsher chemical process compromised structure and planar composition resulting in distorted imaging and immunohistochemical analysis versus control treatment. In comparison, the milder chemical process of the inCITE successfully cleared composite tissues with preserved microanatomic as well as 3-dimensional integrity due to the hydrogel matrix fixation. This allowed high quality imaging (compared to controls or uDISCO) and preserved sensitive cellular elements (including bone marrow) and molecular as well as morphological features (including tissue interfaces) of composite tissues.

Conclusion: We successfully confirmed the effectiveness of OTC techniques in composite tissues including osteo-myo-cutaneous and multiple germ-layer derived components. We established a bone decalcification protocol for limb tissues that retained integrity of surrounding tissues while rendering transparency for high quality imaging. Our protocol has potential applications in 3-dimensional imaging analysis of vascularized composite allografts such as face or limb transplants. Further optimization of OTC techniques could allow high quality imaging delineation of bone marrow engraftment, bone healing, nerve regeneration, immune cell infiltration, or graft vasculopathy (as in chronic rejection) in these transplants. This technique could hold promise in regenerative medicine applications to evaluate tissue interfaces of engineered composite tissue constructs to determine the in-vivo dynamics of healing, engraftment and vascularization.

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FIBER OPTIC IMAGING OF COLORECTAL CANCER ORGANOIDS

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Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer in the United States and the second deadliest cancer with over 50,000 deaths annually. Clinically relevant *in vitro* models are crucial for the development of more precise and effective novel therapeutics for CRC. Micro-engineered tumor technology (tumor organoids) is a 3-dimensional, hydrogel-based *in vitro* tumor model designed to better recapitulate the *in vivo* tumor microenvironment (stroma) [1].Utilizing a novel fiber-optic-based imaging system (FOBIS) capable of delivering precise laser excitation in real-time non-destructive manner[2], we constructed an image of CRC cells co-incubated with stromal cells in the tumor organoid model. The FOBIS technology can open the door for non-invasive analysis of cancer and stromal cells within a tumor-like tissue for future studies of cancer therapy and drug development applications.

3D tumor organoids were constructed by mixing CT26 -GFP murine CRC cells and murine mesenchymal stem cells (mMSC)-mCherry cells with hyaluronic acid and collagen, in 1:3 ratio, and subsequent cross-linking by ~1second of UV irradiation. Organoids were positioned over micro-imaging channels (MIC) embedded in PDMS for FOBIS analysis. Excitation laser light delivered from within the MIC was rastered across a region of interest (ROI) within the organoid to stimulate fluorescent emission at individual points that were captured by an electron multiplying CCD (EMCCD) camera positioned below the organoid. Each ROI was imaged once for CT26 -GFP signal and once for mMSC -mCherry signal. The individual points were then assembled into a matrix and processed using MATLAB to reconstruct a single image of the two different fluorescent signals.

Reconstructed images of the seeded tumor organoids were obtained and demonstrated single-cell resolution of up to 15µm. These reconstructed images were compared with conventional control images taken by CCD camera. Cell locations in the reconstructed images correlated with their locations in the control images supporting the notion that the FOB system is capable of reconstructing accurate images of the tumor organoid based on assembly of individual point excitations.

We demonstrated the FOBIS' capability to image 3D tumor organoid using individual point excitations, yielding images comparable in quality to standard CCD-based microscopy. The system was able to provide laser exaction in a precise and controlled manner to different ROIs in the tumor organoid. Future research can utilize the FOBIS to determine the effects of cancer therapy drugs on cancer and non-cancer cells of the tumor.

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RHEOLOGICAL CHARACTERIZATION OF BIOINKS FOR EXTRUSION 3D PRINTING

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Extrusion 3D printing is the most common additive manufacturing modality for rapid assembly of geometric constructs in a layer-by-layer fashion. This type of printing is useful for bioengineering as it allows for the three-dimensional fabrication of biological components for *in vivo* or *in vitro* study. While extrusion printing is possible for a variety of bioinks, the choice of materials depends on the ability of the material to be extruded in a uniform manner as well as retain its structural integrity after extrusion (1). These criteria for "printability" are related to their mechanical properties. Rheology, or the study of deformation and flow of materials, can serve as a method of determining a viscoelastic material's solid and liquid character, as well as its ability to withstand shear stresses in the printing nozzle. Rheology can describe these characteristics through the storage and loss moduli, which correspond to these solid and liquid qualities respectively. The ability to be extruded through a nozzle and react to these shear stresses correspond to the power law function and the material's shear thinning coefficients, K and n (2).
For an acceptable "printable" bioink, no single biomaterial has been shown to have extrusion uniformity, sufficient structural integrity, and the ability to be used with cells. Composite bioinks must be created with combinations of different biomaterials to achieve suitable "printability." This project aims to characterize the rheological properties of individual biomaterials for the application of creating composite bioinks that can be successfully utilized for bioprinting applications.

In this study, a list of hydrogels commonly used in extrusion printing was compiled for rheological analysis. A literature review was conducted to find a range of three hydrogel concentrations used for each gel. The TA Instruments Discovery Series Hybrid 2 Rheometer was used for analysis of the gels using a 40 mm diameter cone-plate geometry for uniform shear rates during testing at a 1° angle and 33 µm truncation gap. All gels were tested at 18°C with the exception of thermosetting agarose which was also tested at 22°C. The two tests conducted were a strain sweep from 0.02 to 1.0% strain, which measures the viscoelastic region of the material and yields the storage and loss moduli of bioinks, and a frequency sweep from 0.01 Hz to 100 Hz at 0.2% strain which measures polymer relaxation by frequency dependence and yields the complex viscosity of the material.

With increasing concentrations of the hydrogels, both storage and loss moduli increase for all gels tested. In the case of thermosetting gel, agarose, the storage and loss moduli also increase with increasing temperature. The shear thinning coefficients, calculated from the power law for non-Newtonian fluids, showed that all gels tested had n values of less than one, showing that all gels used previously in extrusion printing modality have some degree of shear thinning. However, it remains clear that some n values were more shear-thinning than others. It is possible these highly pseudoplastic gels could potentially be combined with highly structurally secure gels to create a more "printable" composite gel. In summary, this study rheologically characterized several common bioinks at multiple concentrations and temperatures. In the future, this database can be used as the basis for creating composite bioinks.

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OPTIMIZATION OF dECM BASED BIOINK FOR 3D BIOPRINTING OF RENAL CONSTRUCT

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Kidney diseases such as End Stage Renal Disease (ESRD) and Chronic Kidney Disease (CKD) remain a formidable health problem in the United States, claiming around 50,000 lives a year[1]. Current medical therapies include kidney dialysis, which is non-curative and costly, and kidney transplantation, which is curative, however, risks organ rejection and there exists an organ shortage[1]. A potential alternative treatment approach to kidney disease is to utilize the tissue engineering approach of 3D bioprinting to form a structure that ultimately restores the function of the kidney in human patients. The major prerequisite for the 3D bioprinting process is to form a suitable bioink capable of supporting cell growth, maturation, and functions.

It has been suggested that the use of decellularized extracellular matrix (dECM) hydrogels is optimal to synthetic hydrogels when 3D bioprinting cells, as dECM bioinks are better able to provide a microenvironment with crucial cues for cell engraftment[2]. Prior to my being at WFIRM, Dr. Lee's laboratory created a dECM bioink composed of the whole kidney and found it optimal to GelMA bioinks for kidney cell proliferation and functionality. That said, the objective of this study was to create a printable dECM bioink that is optimal to the whole kidney dECM bioink by analyzing the ECM components and functionality of dECM bioinks composed of the renal cortex, the renal medulla, and the whole kidney, respectively. We hypothesize that a cortical dECM bioink will provide the most crucial cues for cell growth, maturation, and functions; and, thus, optimal to the other groups.

Our dECM bioink formulation process started with perfusing four porcine kidneys to remove all cellular components and debris. After decellularization, the kidneys were dissected to separate the renal cortex from the renal medulla. Samples were then enzymatically digested and methacrylated to create a crosslinkable photosensitive bioink. Qualitative histological stains including H&E, Masson's Trichrome, Alcian Blue/Sirius Red, Collagen IV and Laminin, in addition to collagen and sGAG quantitative assays, were completed throughout the bioink formulation process. Bioinks were printed first, without cells, to determine their printing properties. Subsequently, bioinks were seeded with human kidney cells, extruded using 'Integrated Tissue and Organ Printing System' (ITOP), and crosslinked using UV light. Printed constructs were incubated at 37°C for post-print evaluation.

Complete removal of cellular components of kidneys was confirmed with H&E staining, presence of collagen in the dECM was confirmed with Masson's Trichrome and Alcian Blue/Sirius Red staining, and presence of sGAGs in the dECM was confirmed with Alcian Blue/Sirius Red staining. Additionally, immunohistochemistry staining was utilized to confirm the presence of laminin and collagen IV in the dECM. Collagen and sGAG quantitative assays indicated that both proteins were preserved is similar amounts throughout the decellularization, digestion, and methacrylation processes. Printability tests showed that both the medullary and cortical dECM bioinks have good printability. Post printing evaluation of the bioprinted constructs by cell viability staining indicated survival of human kidney cells seeded in the dECM bioinks of both the cortex and the medulla one hour after printing.

In conclusion, the bioink formulation process utilized in this study is successful in preserving vital extracellular components and can be used for 3D bioprinting of kidney constructs. Forthcoming functionality results will be useful in assessing the optimal dECM bioink of the three groups.

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19

DECELLULARIZED HUMAN SKIN-DERIVED ECM AS A SUPPLEMENT TO FIBRIN HYDROGEL FOR SKIN BIOPRINTING APPLICATIONS

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Background: Burn injuries represent a significant clinical burden in the United States, with 1.1 million injuries annually requiring medical attention[1]. For patients with full-thickness burns, the healing process proves particularly challenging with success rates below 50%[2]. The standard of care for burn injuries includes autologous skin grafting, which requires sufficient amounts of harvest sites and can be scarce in patients with severe wounds. Bioprinting has been proposed as a complementary method for *in vitro* fabrication of full-thickness skin with multiple cell types organized into biomimetic layers. Fibrin hydrogel is one candidate for skin bioprinting, however, its post printing mechanical strength is limited. It has been shown that the addition of collagen significantly increases fibrin hydrogel mechanical strength[3]. While collagen is the major component of human skin extracellular matrix [ECM], other ECM proteins may foster additional cellular attachment and activity[4]. Thus, we propose human skin-derived ECM as a potential supplement to fibrin hydrogel.
Objective: Evaluate the efficiency of decellularized human skin-derived ECM supplement in improving fibrin hydrogel's mechanical strength and biological performance.
Methods: Human skin was decellularized and solubilized into an ECM solution. The efficiency of decellularization was evaluated through scanning electron microscopy [SEM] and histology. Solubilized ECM gelation potential was observed independently and compared against fibrin hydrogel and Fibrin-ECM gel [FEG]. The surface structure of the hydrogels was visualized by SEM. Rheological properties were evaluated at varying temperatures and ECM concentrations to optimize printability. To access biological performance, fibroblasts isolated from human skin were cultured in FEG. Cell proliferation and viability, as well as cell-laden construct mechanical strength and structural integrity, were evaluated.
Results: SEM and histology confirmed successful skin decellularization. Solubilized ECM gelled at a pH 3, with dissolution once neutralized to pH 7. ECM, Fibrin hydrogel, and FEG were similar structurally on SEM, with the maintenance of fibrin crosslinking in FEG. All hydrogel compositions displayed shear thinning properties and their strength and viscosity increased at higher concentration, but they became weaker and less viscous with increased temperature. Alamar Blue proliferation assay revealed that higher ECM concentration resulted in a decreased rate of cell proliferation on 2D culture, but an increased rate on 3D culture. Cell viability within all cell-laden constructs decreased over 15 days, but viability was better maintained in FEG. All cell-laden constructs exhibited a decrease in mechanical strength over 15 days, but FEG displayed higher storage modulus than fibrin hydrogel at all time points. Histological assessment of the constructs illustrated improved maintenance of structural integrity in the FEG construct.
Conclusions: Decellularization and solubilization of human skin yielded ECM solution, a potential supplement to fibrin hydrogel for skin bioprinting. The ECM solution was unable to be used independently as a bioink, as gelation only occurred at pH 3, an unsuitable environment for cells. 1% ECM was selected as an optimal concentration for FEG based on its enhanced mechanical strength and structural stability over time, maintenance of cell viability, and improved cell proliferation compared with fibrin hydrogel. Further work must be done to characterize ECM solution protein composition and toxicity, evaluate printability, and assess cellular activity with associated ECM remodeling within engineered skin constructs.
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EFFECTS OF MICROGRAVITY ON NATURAL KILLER CELL ANTI-LEUKEMIC CYTOTOXICITY

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One of NASA's objectives is to send humans to Mars in the early 2030s[1]. These future missions outside of Low Earth Orbit (LEO) will place astronauts outside the protection of the Earth's magnetosphere, exposing them to significant doses of galactic cosmic radiation (GCR) and solar energetic particles (SEP). In addition, astronauts will be subjected to conditions of microgravity (μG) for extended periods of time, which could add to the deleterious health effects of radiation. Prior work in our lab showed that mice repopulated with human hematopoietic stem/progenitor cells (HSC) that had been exposed to mission-relevant doses of space radiation developed human T-cell acute lymphoblastic leukemia (T-ALL)[2]. In the present studies, we focused on defining the effects of μG on natural killer (NK) cells, which are effector lymphocytes of the innate immune system that play a critical role in tumor surveillance. As such, alterations in their number and/or function would be predicted to exacerbate cancer risks arising from exposure to GCR/SEP radiation. Determining how μG affects NK cells is thus of crucial importance to better understand the potential risks associated with future missions and to design effective countermeasures.

To test the hypothesis that μG could impair the anti-leukemic cytotoxicity of NK cells, we used established cell lines of human NK cells (NK-92MI) and human leukemic cells (K-562). NK-92MI cells were first cultured for 24 hours in normal gravity (1G) or in simulated μG, modeled by placing the cells within Synthecon High Aspect Rotation Vessels (HARVs) attached to a Rotary Cell Culture System (RCCS), and their ability to lyse K-562 cells was then assessed using a non-radioactive cytotoxicity assay based upon activity of lactate dehydrogenase (LDH), an enzyme released upon cell lysis. NK-92 MI cells and K-562 cells were cultured both individually and together at a 10:1 effector: target ratio to measure the cytotoxicity of NK-92MI cells against K-562 cells. Results obtained in 1G and μG were analyzed and compared. Our findings to-date have demonstrated a substantial decrease in NK-92MI cytotoxicity against K-562 cells when the effector cells were cultured in μG as opposed to 1G. These findings thus support our hypothesis that conditions of μG may add to astronaut cancer risk, and highlights the need for further studies to define the mechanism(s) responsible.

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OPTIMIZING HUMAN AMNION EPITHELIAL CELL PROLIFERATION FOR CELL THERAPY

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Introduction: Inflammatory lung diseases are characterized by a cycle of increased inflammation, which leads to fibrosis in the lungs and a long-term decrease in lung function. Human Amnion Epithelial Cells (hAECs) are a promising cell therapy treatment option as they can be isolated from the placenta after birth and have the immunomodulatory properties ideal for treating these diseases. The biggest hinderance to using hAECs in large-scale treatment is that one donor may only provide enough cells for one treatment and hAECs are extremely difficult to expand in culture. We hypothesized that conditional reprogramming will cause hAECs proliferate to a greater extent. We evaluated whether the conditionally reprogrammed cells maintained their immunomodulatory effects with and without priming with an inflammatory cocktail. We hypothesized that priming with tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) will enhance the immunomodulatory properties of hAECs.

Materials and Methods: Human Amnion Epithelial Cells that had previously been cryopreserved were thawed and plated on T25 collagen coated flasks (Corning) with either serum-free media (DMEM/F12 10%FBS) or conditional reprogramming media (proprietary). Cells were permitted to grow to 80% confluency before they were passaged or cryopreserved. Cells to be used for the lymphocyte proliferation assay were split into two groups, primed and unprimed. The primed group was exposed to TNFα and IFNγ for 24 hours. Next, we performed a lymphocyte proliferation assay evaluating four groups of hAECs for their ability to suppress proliferation: 1) unprimed serum-free media hAECs, 2) primed serum-free media hAECs, 3) unprimed conditional reprogramming media hAECs, 4) primed conditional reprogramming media hAECs.

Results and Discussion: The two medias were compared for cell proliferation, attachment rate and population doubling rate. Differences were evident in the attachment rate when the media was cleared 72 hours after initial seeding. In addition, when assessing the proliferation of the cells over time the population doubling time varied between the two medias. These differences indicate that the conditional reprogramming media does influence the overall expansion process in hAECs. The effects of the media on lymphocyte proliferation when unstimulated and stimulated by an inflammatory cocktail were evaluated through a lymphocyte proliferation assay. The stimulation was found to have an impact on the amount the lymphocytes replicated. Additionally, the variations between the assays performed with the two medias were recorded. It can be concluded that both inflammatory stimulation as well as expansion media has an effect on the immunomodulatory characteristics of hAECs.

Conclusions: The results of this study indicate that expanding hAECs in conditional reprogramming media influences their proliferation and attachment rate when compared to standard growth media. In addition, priming with inflammatory factors results in notable differences in the proliferation of lymphocytes as does the media the primed cells were expanded in.

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THE EFFECTS OF PULSED ELECTROMAGNETIC FIELDS ON THE GROWTH AND DIFFERENTITATION OF SKELETAL MUSCLE STEM CELLS

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Background: Skeletal muscle accounts for approximately 40% of the average adult body mass and musculoskeletal disorders are a primary cause of disability in the military and civilian populations. Although, therapeutic options for the treatment of mild and moderate skeletal muscle injury exist, there are no therapies that 100 percent effective for larger traumatic injuries. Pulsed electromagnetic field (PEMF) therapy has been approved for clinical use for the treatment of bone fractures, tendonitis and cancer (Markov 2007) and has been used to reduce inflammation, edema and pain. Furthermore, PEMF has been shown to have direct effects on the growth and differentiation of neurons, osteoblasts, chondrocytes and embryonic stem cell lines (Qinlong Ma, 2016; Nagarajan Selvamurgan, 2017; Dinesh Parate, 2017). The mechanism of PEMF effects is still unclear, but is thought to be dependent on changes in ion changes within cell membranes.

The **objective** of this study was to determine if PEMF may prove an effective therapy for skeletal muscle injuries. In these initial studies, we wanted to determine if PEMF had any effect on the growth and differentiation of C2C12 skeletal muscle stem cell line. In a second experiment we examined the effect of PEMF on the activation of the NFκB inflammatory pathway. We **hypothesized** that PEMF treatment would increase the proliferation and/or differentiation of C2C12 cells, and to decrease activation of the inflammatory NFκB pathway.

Methods: Our study consisted of three experiments to determine the effects of PEMF on C2C12 cells. PEMF was induced using Helm Holtz coils to generate the magnetic fields. We controlled the time of exposure, intensity and number of pulses in this experiment. (1) To determine proliferation, C2C12 cells were grown in growth media and treated with or without PEMF for 4 days (15Hz, 2mT for 30 minutes/day). Growth curves were generated using an Incucyte machine (Sartorius). (2) To determine the effects of PEMF on cell differentiation, we grew C2C12 cells in differentiation media and treated with or without PEMF for ten days. Incucte was used to generate growth curves and cells were fixed and stained with a MHC (Myosin heavy Chain) antibody. (3) C2C12 cells that expressed a red fluorescently labeled NFκB responsive reporter gene were grown in growth media prior to treatment with various increasing amounts of the inflammatory protein TNFα (0, 0.1, 1, 10 ng/ml). After treatment with TNFα, cells were exposed to PEMF with cells receiving no PEMF treatment as control. The Incucyte was used to determine activation of the NFκB promoter.

Results: PEMF treatment showed no significant effect on the proliferation and differentiation of C2C12 cells. However, PEMF treatment decreased NFkB reporter activity after exposure of C2C12 cells to a high dosage of TNFα (10ng/ml).

Conclusions: This data suggests that PEMF may alter inflammatory pathways in skeletal muscle stem cells. Since this was a pilot experiment, these experiments will need to be repeated to confirm these results.

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LOCAL DELIVERY OF STEM CELL-RECRUITING FACTOR AND SKELETAL MUSCLE MATURATION FACTOR FOR IN SITU SKELETAL MUSCLE REGENERATION

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Volumetric muscle loss is a result of loss of skeletal muscle either through traumatic incidents or surgical incidents that leads to functional impairment. Ultimately, with the loss of the skeletal muscle the muscle's ability to regenerate or ultimately heal is lost as well. About 4.5 million annually undergo surgical procedures in the United States alone. Current treatment methods include functional free muscle transfer. However, a functional free muscle transfers is often limited by unavailability of suitable host tissue and poor grafting efficacy which prevents functional restoration of muscle mass.

Currently, bioengineered skeletal muscle tissue using culture-expanded cells can be a promising solution to achieve functional recovery of volumetric muscle injuries; however, this approach involves extensive cell expansion steps that requires a large amount of time and energy. In addition, there is also the risk of the cells death during/post implantation. In situ tissue regeneration strategy of recruitment of host endogenous stem/stromal cells to the site of injury is seen as a viable alternative.

In this study, we utilized the in-situ tissue regeneration strategy to treat skeletal muscle injuries. The objective of this study is to develop a scaffolding system for efficient delivery of biological factors for in situ skeletal muscle regeneration. 3D bioprinting is utilized in order to fabricate a skeletal muscle construct that mimics native skeletal muscle tissue organization and that allows for the delivery of biological cues. To recruit host endogenous stem/progenitor cells into the construct and their skeletal muscle differentiation, stromal cell-derived factors (SDF) and insulin-like growth factors (IGF) were incorporated into a fibrin-based bio-ink. SDF is responsible for the recruitment of stem cells and IGF is responsible for the skeletal muscle differentiation of the recruited cells. The release kinetics of SDF and IGF were investigated through ELISA, a quantitative assay. The results showed that the SDF and IGF were released from 3D bioprinted skeletal muscle constructs up to two weeks in vitro. Our results demonstrate that the 3D bioprinted scaffolding system is feasible to deliver IGF and SDF which ultimately may contribute to functional restoration in volumetric muscle injury in vivo.

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Targeted Angiogenesis To Control Neovascularization Using a Drug-Eluting Scaffold

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There are about 1.8 million Americans living with amputations. There are many reasons for amputations but the most common reason is due to the narrowing of the arteries, called peripheral artery disease[1]. As a result of this, the affected tissue begins to die. Fortunately therapeutic angiogenesis could possibly regenerate damaged or lost tissues in ways previously considered impossible, and may prevent unnecessary amputations. Vascularization of engineered tissues are critical for success upon the implantation. All engineered tissues require a vascular network to provide the nutrients and oxygen needed for survival in *vivo* except for the cornea and cartilage[2]. The challenge with these engineered tissues are that the vascular ingrowth of these tissues take weeks to occur naturally this causes tissues to become starved of the essential nutrients needed, which in return leads to death of the tissue. This study tested the effectiveness of using a heparin-conjugated scaffold chemically bound with a growth factor, specifically Vascular Endothelial Growth factor or VEGf. It was hypothesized that the heparinized scaffold will control the release of VEGf to increase the density and growth speed of the vascular network.

Initially 10mL of toluidine blue was added to a known amount of heparin solution that was cross-linked using EDC/NHS in a MES buffer solution with pH of 5.5 and activated for 30 minutes. The heparin solution was then incubated at 37 degrees Celsius for 4 hours with gentle shaking. This solution was diluted and measured at an absorbance of 530 nm to create a calculation curve to determine unknown amounts of conjugated heparin. New samples were made using the cross-linked heparin solution to optimize conjugation time, variables were 1, 2, 4, 12 and 24 hours. Samples were then washed 3 times with 5 mL of DI water centrifuging at 1500rpm for 5 minutes after each wash. Supernatant was discarded and heparin was then stained with 2 mL of toluidine blue and incubated for 4 hours. Precipitate was then washed 2 times using 5mL of HCl solution, centrifuged at 3500 rpm for 10 minutes with each wash. Supernatant was discarded and finally 5mL of 80% ethanol/.01 M NaOh mixture (4/1) solution was added to dissolve precipitate and absorbance was measured at 530 nm. After time dependent study heparin concentration dependent experiments were performed. Samples underwent same protocol of time dependent studies using optimal the optimal conjugation time and applied to different heparin concentration amounts, variables were 1mg/mL, 5mg/mL and 10mg/mL performed and measured at an absorbance of 530nm to find optimal conditions for the conjugation of heparin. Assay of model protein lysozyme was also conjugated with heparin to test loading efficiency and to be tested in delivery system

Analysis of the time dependent conjugation study showed 4 hours of conjugation time provided the most conjugated heparin compared to the other time variables. Analysis of the concentration dependent sudy shows that 1 mg/mL contained the most conjugated heparin. Finally the graph of our model protein lysozyme was linear with r squared value at 94% but our hypothesis was refuted when measuring at absorbance of 285 showed a doubling in value as compared to stock solution, when we expected to see a decrease. For future studies our VEGf delivery system will need to be further analyzed and tested for functionality.

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COCHLEA-ON-A-CHIP MICROFLUIDICS SYSTEM FOR DRUG SCREENING

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Introduction

Experimental in vitro systems that accurately model the physiological realities of tissues or cells in vivo are vital for drug screenings. Three-dimensional cultures and microfluidics systems are two developments that provide improvements upon the traditional two-dimensional in vitro cultures. 3D cultures better replicate the in vivo function of certain cell types than 2D cultures, while microfluidics systems increase the accuracy of in vitro models by simulating the vasculature, and even allowing for the involvement of multiple tissue/cell type interactions within the same model system.[1]

Sensory hair cells within the inner ear are responsible for our sense of hearing and balance. The loss of these sensory cells can result in varying degrees of deafness and impairments to balance.[2,3] Unlike many non-mammalian animals, the sensory epithelia within the inner ears of adult mammals lacks the capacity to regenerate lost or damaged hair cells, making any subsequent auditory or vestibular impairments permanent.[2,3] The enduring nature of these impairments makes the development of systems that more accurately model the physiological responses of the inner ear sensory epithelium crucial. Using a 3D cochlear model within a microfluidics system during a drug screening could provide a more accurate model for how inner ear cells may react in response to various drugs, or to the by-products produced by the drug's interaction with other tissue types. This would increase the chances of identifying potentially ototoxic side effects in prospective pharmaceutical drugs. Here we began the development of a three-dimensional cochlear microfluidics model for the screening of ototoxic drugs.

Methods

HEI-OC1 mouse cochlear cell spheroids were characterized by immunostaining and tested for viability with live-dead assays up two weeks after initial seeding. Within low-attachment round-bottom well plates, spheroids were treated with the known ototoxic drugs cisplatin and gentamicin, and live-dead assayed after 24 and 48 hours. Additionally, other spheroids were transferred into a microfluidics chip system. The spheroids were fixed within the microfluidics chamber with a photocurable hyaluronic acid-based gel and a media flow of 9 ul/min was generated through Teflon tubing via a peristaltic pump. Cisplatin and gentamicin were added to the media reservoirs of the system, and caspase and live-dead assays were run at 24 and 48 hour time points to examine the extent and mechanism of cell death.

Results and Conclusion

Immunostaining of spheroids revealed markers specific for both supporting cells and hair cells (sox2 and prestin respectively) were abundantly present, and live-dead staining showed minimal cell death up to two weeks of spheroid culture. These 3D cultured spheroids showed dose dependent cytotoxicity towards cisplatin and gentamicin. Spheroids within the microfluidics system also showed an increase in cell death compared to control. Cell death observed in spheroids treated with 50uM and 100uM cisplatin after 24 and 48 hours respectively corresponded to increases in caspase activity, an apoptosis marker. Spheroids treated with gentamicin showed a moderate increase in cell death compared to control as well. These pilot experiments provide evidence to suggest that HEI-OC1 cells could be used for creating a reliable in vitro 3D cochlear cell model for use in ototoxic drug screenings, and their applicability for use in microfluidic system experiments.

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