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STUDIES UTILIZING DECELLULARIZED HUMAN AMNIOTIC MEMBRANE AND SW-620 CELLS AS A PRELUDE TO 3D *IN VITRO* CULTURES

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STUDIES UTILIZING DECELLULARIZED HUMAN AMNIOTIC MEMBRANE AND SW-620 CELLS AS A PRELUDE TO 3D *IN VITRO* CULTURES

A THESIS APPROVED FOR THE
STEPHENSON SCHOOL OF BIOMEDICAL ENGINEERING

BY

_____
Dr. Vassilios Sikavitsas, Chair

_____
Dr. Edgar O'Rear

_____
Dr. Roger Harrison

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Abstract

The prevalence of cancer in modern society has increased from 14.1 million new diagnoses and 8.2 million deaths in 2012 to an estimated 21.7 million new diagnoses and 13 million cancer-related deaths projected in 2030 [1]. The development of novel methods for 3D *in vitro* tumor growth can help to understand better the progression of the disease and eventually provide new therapeutic approaches, especially at the personalized medicine level. Human amniotic membrane is a promising biological scaffold for the growth of cancer cells and ultimately tumorigenesis. The membrane is antimicrobial and contains important extracellular matrix components for cancer cell growth. SW-620 colon cancer cells were seeded on the surface of the decellularized human amniotic membrane in the presented studies. SW-620 colon cancer cells were shown to proliferate on decellularized amniotic membrane in a shortened seeding and culturing two day procedure. A five day seeding and culturing on the decellularized membrane procedure resulted in a three day proliferative period (days 0 through 3) with a 60% seeding efficiency on day 0 (555,000 initially seeded cells). The number of cells plateaued from days 3 to 5. These results provide a promising proof-of-concept in 3D *in vitro* monolayer culture. Future work is necessary to improve the use of human amniotic membrane as a 3D *in vitro* scaffold through layering or surface modifications. Comparison of the genotypic expression between the *in vivo*, 3D *in vitro* amniotic membrane and 2D culture models using PCR is also necessary to determine if the cells are behaving like cells in physiological conditions.

1. Introduction

Impact of Cancer

Cancer is a rapidly-evolving, devastating disease that is projected to be diagnosed in 39.6% of men and women in the United States at some point in their lives [2]. In 2016, the projections for new cancer cases in the United States alone was 1.7 million with roughly 600,000 people perishing from the disease. On the global scale, there were an estimated 14.8 million new cases and 8.2 million cancer-related deaths in 2012 [3]. Cancer has a significant impact on global health and is the second highest cause of death in the world.

The complexity of cancer, which comprises many different diseases, requires extensive research to understand its progression and to improve therapeutic treatments. In the following sections, methods of 2D and 3D cell culture and animal models for study of cancer proliferation and metastasis are discussed. An overview of colon cancer and the characteristics of the amniotic membrane is also included.

Introduction to Metastasis

Primary cancer removal is often followed by reappearance of the tumor in the same or an alternative site via metastasis. Metastasis is actually the predominant cause of cancer-related fatalities. The exact mechanisms of metastasis and cause of cancer are still only conjecture, which increases the need for cancer-based research.

Animal Models

Currently, animal models are used to study tumor growth and other aspects of cancer biology. The predominant animal model used for cancer research is the mouse [4]. Specifically, nude mice are used to study the growth of human cancers. Zebrafish and rats are also used as animal models for cancer research. Mice and other animal models are important to develop studies for general cancer research and preclinical research. Animal models represent an *in vivo* system with the cancer behaving similarly to the native environment. The limitation of animal models is that even though they are a living system, they do not perfectly represent how the cancer develops and progresses in the human body. In addition, nude mice have a compromised immune system. Apart from the biological differences, tumor size is an additional factor where animal models and human patients differ [5]. The maximum size that a tumor can reach in a mouse model is much smaller than what would be found in human tumors. This can impact the overall growth and metabolic state of the tumor and lead to different responses when testing drug efficacy.

Human Clinical Models

Human clinical models are also used in modern cancer research, especially when studying chemotherapy efficacy on cancer *in vivo*. The largest issues with using humans, especially as a preliminary model to study cancer, are finding patients who qualify and are willing to participate in the trial. When drugs are fast-tracked into human clinical trials, long term effects and other side effects are unknown and may detrimentally impact the patient. Human tumors are the most accurate in terms of the actual growth and development of the

cancer since the cancer lines being studied are originally derived from humans but can be surrounded by ethical obstacles for finding trial participants.

Personalized medicine is currently evolving to include cancer patients with highly diversified tumor characteristics that are otherwise categorized in general groups in order to develop therapeutic approaches. Readily available primary cancer cells from biopsies make *in vitro* models, which can be used to understand better the response of cancer cells to a variety of chemotherapeutic drugs, a potentially powerful tool in rapid screening of anti-cancer agents. Cancer treatments formulated in *in vitro* models will spare the patient from unnecessary harm due to aggressive treatment options and observations can be made as to which drugs work on a patients' specific cancer. Individualized treatment plans are under development and are being explored through immunotherapies and targeted therapies and can be expanded through the application of 3D *in vitro* cancer growth models. 2D and 3D models still need to be refined in order to reflect *in vivo* conditions and be fully manipulated *in vitro*.

In Vitro **Models**

Modern cancer research has utilized both 2D (monolayer culture on tissue culture plates) and 3D *in vitro* models. The current focus of these models is tumor growth, cell migration, cell adhesion, maintenance of *in vivo*-like morphology mimicking the characteristics of gene expression, metabolic state, cell-cell interactions of the native tumor microenvironment [6]. 3D *in vitro* models refer to cellular constructs that extend beyond a simple monolayer. They can be generated using exclusively cellular aggregates that can grow into tumor

spheroids. Alternative approaches include the use of hydrogels to entrap cancer cells and allow them to grow in an environment containing components of their native extracellular matrix and growth factors [7]. Synthetic and natural scaffolds have also been used as they provide extended surface area available for cancer cell attachment and growth. Microfluidic networks have also been tested in cancer research and they constitute a special category between 2D and 3D models [8].

2D Static *in vitro* Culture

2D *in vitro* static cell culture involves a culture environment devoid of flow and monolayer growth. Generally, static culture is performed in a petri dish, flask or well plate and media is supplemented to the container [7]. Static culture is beneficial to culture studies since a vast majority of cells can be cultured in this way and is widely used due to ease of use. The major weakness of 2D monolayer culture is the inability to recreate the 3D nature of actual tumors and studies have shown that the lack of three dimensionality compromises the phenotypic characteristics of many cancer cell types. Another weakness of 2D *in vitro* cell culture is that there are no extracellular matrix components present which limits the extent of which *in vitro* cancer cells can resemble *in vivo* cellular growth.

2D *In Vitro* Culture on Inserts

One example of a 2D modeling environment is culture on inserts. Culturing on inserts is useful when trying to understand angiogenesis, transport, cell-cell interactions and invasion [9]. The main premise of cell culture inserts is

that they consist of a thin, porous membrane that allows for the media to pass through the membrane [10]. This allows both the lower and upper surface of the cells to be exposed to the same nutrients. The membrane can consist of a synthetic material of a known porosity. The porosity is meant to mimic *in vivo* conditions due to the fact that surfaces in the body are generally porous to some degree and allow for extracellular cell-cell signaling. Some of the common materials used for the membrane are polycarbonate, polyethylene terephthalate, cellulose esters and polytetrafluoroethylene [9],[11]. These inserts are also used to grow co-cultures of different cell types using the same environment. Cells can be grown on top of the porous membrane and below the membrane on the bottom of the well plate. This allows for cell communication as well as controlled co-culturing. One of the main limitations of culturing on cell inserts is that the shape of the insert does not resemble the shape of a tumor *in vivo*. The main growth progression is in a monolayer which is not how tumors progress *in vivo*. Culture using cell inserts is a better method of 2D *in vitro* culture when compared to traditional growth in flasks due to the ability to maintain a co-culture and allow for cell-cell communication, which both mimic *in vivo* conditions.

3D *In Vitro* Hydrogel Models

One 3D culture method is imbedding cells in natural or synthetic materials in a hydrogel. For embedding, cells can either be seeded onto a porous surface or entrapped in a hydrogel. Hydrogels can be formed from many different materials, both synthetic and natural, and are used to mimic the natural

extracellular matrix microenvironment. Some examples of synthetic hydrogel materials include: polyethylene glycol, poly(hydroxymethyl methacrylate), polyvinyl alcohol, and polycaprolactone [7]. Natural materials include: alginate, chitosan, hyaluronan, dextran, collagen and fibrin. Matrigel is another example of a naturally-based material. Matrigel primarily contains collagen IV, laminin and enactin and was originally derived from Englebreth-Holm-Swarm tumors in mice [12]. One potential limitation of a 3D hydrogel tumor model is that cell adhesions are not part of the entrapment process. If there is a need to study cell attachment, then a hydrogel would be lacking due to the fact that the cells are physically trapped and are not required to adhere through their own processes.

Special precautions are needed when cross-linking is required in hydrogel formation. Some chemical cross-linking can be toxic to cells and UV or light based cross-linking can also be harmful to the cells [13]. Despite this, hydrogels can be tuned to a specific desired environment in terms of the material and directional growth for a 3D cell culture.

3D *In Vitro* Synthetic Scaffolds

Synthetic scaffolds are also used as a platform for 3D *in vitro* cancer models. These scaffolds are considered to be a solid scaffold and can be created through a variety of methods. This includes 3D printing, electro-spinning and other methods to create pores [14]. Some examples of materials that are used to form synthetic scaffolds include poly(lactic) acid, poly(glycolic acid) and poly(lactic-co-glycolic acid). These specific materials are biodegradable which could have mixed results in 3D tumor growth. The positive of biodegradability

is that the cancer cells will be able to proliferate and take over the structure previously occupied by the scaffold material. On the contrary, the scaffold may break down before the tumor architecture is established and bi-products of degradation may impact the growth of the cancer and possibly make the culture less representative of *in vivo* conditions. The benefits of synthetic scaffolds as a base of cancer cell growth is that they can be reproduced, especially when using a 3D printed scaffold. The same template can be used to create more scaffolds with specific parameters and porosity. A limitation of using synthetic scaffolds is that the scaffold can influence the directionality of cellular growth and result in deviations from *in vivo*-like growth patterns. They also do not contain extracellular matrix components and are considered inert in terms of interactions with the cells. Research in our lab (Cortes/McKernan/Sikavitsas, unpublished) demonstrated a lack of adhesion and growth of cancer cells seeded on 3D printed poly(lactic acid) scaffolds as well as electro-spun poly(lactic acid) scaffolds. The cancer cells were not able to bind to either of these scaffolds in a 48-hour period even when adhesion peptides (RGD) were incorporated on the poly(lactic acid) surface.

3D *In Vitro* Natural Tissue Models

Scaffolds derived from natural tissues also demonstrate promise as a 3D culture platform. Especially for human-derived tissues, the propensity to support cellular growth and proliferation is evident [7]. They contain an extensive network of extracellular matrix components that promote cell binding. Specific examples of natural-derived tissues used in current research include the small

intestine submucosa (SIS), human umbilical vein (HUV) and amniotic membrane to name a few [15]. Natural materials can be decellularized before seeding with new cells since the presence of the preexisting cells if often undesirable with the exception of cases where co-cultures of cancer cells are explored. The decellularization process results in some damage to the native architecture of the extracellular matrix; however, cells can still favorably adhere to the decellularized tissues using the extracellular matrix components which still closely resemble the native state. Specifically, the human amniotic membrane is a thin material that seems to resemble a 2D environment as opposed to a 3D environment. This can be remedied by layering membranes on top of each other to increase the scaffold thickness. Cells can be seeded between the layers to also improve cell growth in three dimensions. Significant limitations include individual variation as well as reliable sourcing. Natural materials, especially of human nature, can be difficult to source. Individual variation is a challenge and variability in sample size is almost impossible to eliminate.

Co-culture Using Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) have been thought to impact the growth of tumors *in vivo* through cellular communication. The impact of MSCs on tumor growth is questionable due to the fact that many studies have demonstrated that they increase growth and many others have demonstrated that they inhibit growth [16]. Stem cells can promote tumor growth by means of differentiating into pericytes or endothelial cells involved in angiogenesis. The

secretion of VEGF by the MSCs also helps to promote angiogenesis within the tumor. Tumor cells can communicate with MSCs and other cells within the tumor by use of extracellular vesicles [17]. This communication method is two-way with both the MSCs and tumor cells able to produce extracellular vesicles. The vesicles contain non-coding RNA (ncRNA) which influences cancer initiation, progression and pre-metastatic niche formation. MSCs are also involved in immune suppression of B and T lymphocytes and natural killer cells [16]. This allows for the tumor to continue with growth and proliferation with only limited immune resistance. It is also believed that MSCs can influence and control the epithelial-to-mesenchymal transition (EMT) which leads to increase metastasis and invasiveness. The inhibition/increase discrepancy may be partially due to the timing of MSC introduction into the tumor. Some studies demonstrate that introducing MSCs to established tumors inhibits the growth of the tumor. Other studies demonstrated that the addition of MSCs with cancer cells improved tumor formation. Introducing MSCs with cancer cells at the beginning of growth may be useful to promote angiogenesis and tumor proliferation in 3D *in vitro* models.

3D *In Vitro* Tumor Spheroids

Currently, 3D cancer models have focused on several different materials and experimental designs. Some examples include culturing multicellular aggregates (spheroids), hydrogels, synthetic scaffolds and seeding cells on natural tissue.[6]. Maintaining the health of cancer spheroids over time is a challenge. As the spheroid expands in the radial direction due to cell

proliferation, the spheroid becomes more tightly packed with cells in the interior experiencing hypoxia [18]. The transport limitations of spheroids result in a rapidly expanding mass due to direct contact with the media and nutrients and a necrotic center that has little to no access to vital nutrients. One benefit of the spheroid is the natural form that it takes as it grows. Spheroids are aggregated so that the tumor-like form already exists as the cells proliferate. A downfall to a tumor spheroid model is that in the purest form, there is not existing extracellular matrix. Apart from this, the tumor size is limited in the radial direction due to the transport limitations that occur without angiogenesis.

Goal of Study

The main point of this study is to determine the growth and proliferation of cancer cells seeded on a decellularized human amniotic membrane. The amniotic membrane was specifically chosen for this application due to accessible sourcing as well as being human-derived. The cancer line that has been used is the SW-620 colon cancer cell line. The results of this aim will be developing preliminary model for future work involving surface modification and 3D cancer cell growth using the amniotic membrane.

Colon Cancer Overview/Motivation

Colon cancer is the third most common type of cancer found throughout the world for both sexes [19]. There are more than a million new cases each year, with roughly half of those cases being fatal. In this study, the SW-620 colorectal adenocarcinoma cell line was chosen due to the resemblance of the

amniotic membrane to the mucosa of the colon. This line was also chosen due to its rapid proliferation rate.

Colon Cancer Staging and Treatment

Colon cancer is classified using a staging system. Stage 0 refers to carcinoma *in situ*, or a grouping of abnormal cells located in the intraepithelial or lamina propria of the colon [20]. Stage 0 is the lowest stage classification for colon cancer. The worst and most progressed stage of colon cancer is stage 4. Stage 4 is uniquely characterized by metastasis to either 1 or multiple other organs. The primary tumor can be in any classification and the lymphatic system has been invaded to some extent.

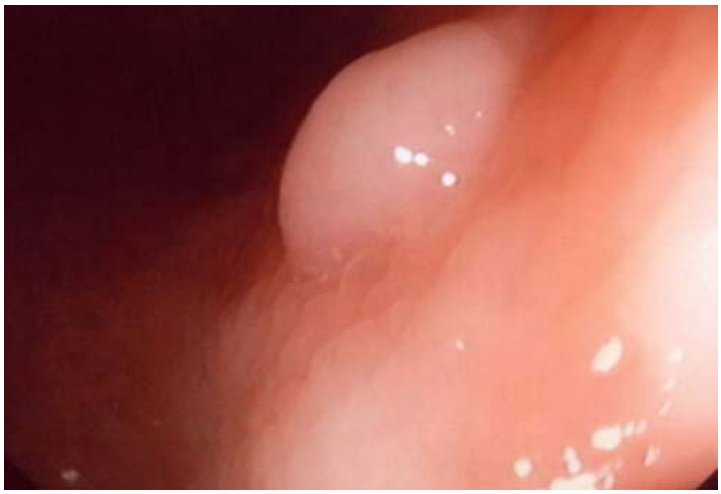


Figure 1: Colon cancer polyp [21]

The tumor initially appears as a small polyp (Figure 1) which can either be benign or malignant [21]. Polyps can be discovered through a routine colonoscopy performed by a physician. Some benign tumors have the ability to become malignant so surgical removal of abnormal polyps is advised. If not surgically removed, the polyps can grow in size to cause physical discomfort

along with other gastrointestinal abnormalities. Typically, colon cancer survival has not improved despite advances in chemotherapies and radiation-based treatments. The survival rate ranges from 90% in the initial stage 0 to stage 2 detection on a 5-year basis [22],[23]. The rate drops to as low as 10% for 5-year survival when detected at stage 4 metastasis. Tumor excision through surgery allows for this high rate of 5-year survival for the lower stage patients [22]. Stage 4 colorectal cancers are generally treated with chemotherapy. Drugs that are currently on the market consist of 5-fluorouracil, leucovorin and several others. Some drugs are now utilizing targeted approaches using monoclonal antibodies. These drugs specifically target the epidermal growth factor receptor (EGFR) to slow or stop the rate of metastasis. Others are able to inhibit growth and development pathways needed for cancer metastasis. Bevacizumab targets the VEGF system when used in combination with other chemotherapy drugs [24]. Targeting the VEGF pathway is important to limit tumor growth. VEGF is responsible for controlling angiogenesis in healthy cells and is upregulated in cancer cells [25]. Limiting the development of new blood vessels in cancerous tumors ensures that the cells on the interior of the tumor do not receive proper nutrients. Tumors rely on a network of blood vessels for nutrient transport and are not sustainable through nutrient diffusion processes alone.

Colon Cancer Metastasis Mechanisms

Current mechanisms for metastasis in cancer cells include the influence of cancer stem cells (CSCs) [22]. It is not known if these cells mutate from healthy sources of stem cells or if cancer cells have the ability to dedifferentiate

into CSCs. Regardless of source, CSCs are responsible for the tumorigenic and metastatic properties of cancer cells. In epithelial cell cancers, cells undergo epithelial-to-mesenchymal transition and obtain properties similar to stem cells [22],[23]. These properties include infinite proliferation, self-renewal and apoptotic resistance. EMT is the critical step in order for cancer cells to expand from a primary grouping of abnormal cells to a metastatic phenotype with the ability to travel to distant locations in the body. EMT is also characterized by increased drug resistance and the down regulation in production of E-cadherin. The loss of E-cadherin allows the cells to separate in attachment to neighboring cells and migrate throughout the body. This process occurs relatively quickly in colorectal cancers and contributes to the aggressiveness and poor prognosis of metastatic stage 4 cancer. The metastatic colon cancer cells invade the basement membrane and are able to invade both the lymphatic and circulatory system. This allows for them to traverse the lumen of the vessels (intravasation) and exit the lumen (extravasation) to reach other organs and produce new tumors.

Overview of the Amniotic Membrane

The amniotic membrane, or amnion, is a thin, extraembryonic membrane of the placenta that functions as an immune barrier to the fetus [26]. The chorionic membrane, or chorion, is found on the outside of the amniotic membrane and is also part of the placenta. The amniotic membrane is composed of several different components including: fibronectin, elastin, nidogen, collagen I, III-VI, elastin and hyaluronic acid. These components are generally found as part of the extracellular matrix of many cells. The amniotic membrane is

compatible with many different ECM components and ideal as a tissue engineering scaffold due to its low immunogenicity. The DNA of the membrane epithelial cells contain Fas L, TNF and TRAIL which are all genes that induce apoptosis. These genes can cause leukocytes to commence apoptosis which reduces the effectiveness of an immune response against the amniotic membrane. The amnion is also antiangiogenic and is avascular in nature. The amniotic membrane is antimicrobial due to its production of β-defensins which kill invading microbes. The anti-inflammatory properties derive from the suppression of cytokines that cause inflammation as well as IL-1α and IL-1β expression. The cells of the amniotic membrane also produce secretory leukocyte proteinase inhibitor and elafin which also contribute to the anti-inflammatory properties. The combination of these factors make the amniotic membrane an ideal choice as a scaffold material for tissue engineering. The membrane also maintains its mechanical strength and shape even after excision from the placenta and chorion and after the decellularization process. This enhances the stability as a scaffold material. The membrane is also semipermeable which could assist nutrient diffusion or cellular penetration. Literature states that its thickness ranges from 70-180 μm in thickness which could be plausible for cells to traverse or permeate [26]. Stacking of the amniotic membrane has also been utilized with cell seeding to create an improved 3D *in vitro* scaffold (Mathilde/Nollert, thesis).

2. Materials

Cell Line

SW-620

The SW-620 cell line is derived from a 51 year old male Caucasian patient [27]. The cells were harvested from the lymph node (metastatic site) after the original colorectal adenocarcinoma spread. The characterization of disease progression was Duke's type C. The cell line is considered to be adherent epithelial cells but these specifically have already gone through metastasis. These cells were purchased in September 2016 from American Type Culture Collection (ATCC) and delivered in a frozen state. They were cultured and expanded to grow the cell stock when they arrived in November 2016. Extra cells were frozen and stored in liquid nitrogen until needed for culture. To date, these cells have only been cultured for DNA quantification and for seeding on the amniotic membrane.

Amniotic Membrane

Amniotic membranes used in this thesis were sourced from Norman Regional Hospital in Norman, OK. These amniotic membranes were 5 days old when donated at no charge. Specifically, the membranes in this thesis were processed by Julian Arrizabalaga in October 2016 and decellularized by him.

3. Methods

Cell Culture

SW-620

The SW-620 cells were cultured in accordance to the guidelines set in place by the ATCC with some slight modifications [27]. Cells taken from cryopreservation in liquid nitrogen were thawed in a water bath and added to a Falcon tube, supplemented with Leibovitz's L-15 media (Life Technologies ™). This Falcon tube was then centrifuged for 5 minutes at 1100 rpm. The supernatant was pipetted out of the Falcon tube and the concentrated cells were re-suspended with L-15 media to a concentration of 1 million cells/mL. The supernatant was removed in this process in order to remove any DMSO which is toxic to cells in general [28]. In each T-75 flask, 1 mL of cell and media mixture was added. Each flask was supplemented with another 9 mL of L-15 media to a final flask volume of 10 mL.

For general culture and expansion, the cells were rinsed with 3 mL of PBS per flask the day after plating. The PBS was suctioned out with a pipette and 10 mL of L-15 media were added to the flask. After this step, media was changed every 3 days (2 days between each media change). Media changes were continued until cells reached 85-90% confluent on the bottom of the flask. The splitting procedure is documented in the section following the culture of PC3 cells. SW-620 cells were split using 1:4 to 1:10 ratio of original flasks to new flasks needed.

SW-620 cells were prepared for cryopreservation by splitting the cells and re-suspending the cells using cryogenic media. 1 mL of the cell media mixture with a concentration of 1 million cells per 1 mL was placed into each 1 mL cryogenic tube. Tubes were placed in a slow, controlled freezing container and placed in a -80°F freezer overnight. The next day, tubes were taken and placed in cryogenic storage tanks for long-term storage.

Splitting

Splitting a cell culture, or subculturing, refers to removing the cells from the original flask and moving them into new flasks to expand their growth. There are several important factors to determine the correct time to split a cell culture [28]. Confluence, or the cell monolayer density on the bottom of a flask or culture vessel, is a prime indicator of when to split. This can be visually determined using a light microscope and is characterized by high cell density and little to no areas of space without cells. When cells reach confluence, they need to be split within a 24 hour period or else there is a risk of apoptosis and longer recovery when the flasks are subcultured. Timing is also an indicator of a subculture requirement. Yield and growth are consistent as long as the seeding density is maintained and should occur over a set time period. Each cell line has different timing requirements and multiplication times. Confluence and timing are the most used methods of determining when a cell culture should be split within this lab and this thesis.

Splitting requires several different materials and is based on a standardized protocol within the Sikavitsas lab with minor adjustments. Media,

trypsin and PBS must first be brought to temperature in a 37°C water bath. The specific media used is the same media used for culture of a particular cell line. Once they are up to temperature, cells are placed under the Bio-Hood and the existing media is suctioned out of the flask. 3 mL of Dulbecco's phosphate buffered saline (PBS, Life Technologies™) is added to each culture flask and gently flowed over the bottom of the flask. This step removes any existing debris or serum that can inhibit the action of trypsin [28]. The PBS is then suctioned out of each flask and 2 mL of trypsin is added to each flask. This is gently rinsed over the bottom of the flask to ensure contact with the entire cell monolayer. Flasks are then incubated at 37°C and the designated CO_2 setting for 5 minutes. After this incubation period, fresh media (8 mL/flask) is added to the trypsin and used to rinse the monolayer and suspend cells in the media mixture. This rinse is repeated by suctioning and flowing the media along the bottom of the flask. After roughly 15 rinses using the same media, the media, trypsin and cell suspension is taken out of the flask and placed in a Falcon tube for centrifugation. If the total cell/media/trypsin volume is under 15 mL, a 15 mL Falcon tube is used for centrifugation. If the total cell/media/trypsin volume is under 50 mL and greater than 15 mL, a 50 mL Falcon tube is used for centrifugation. More Falcon tubes can be used if the total volume exceeds 50 mL.

A small, well-mixed sample (<1 mL) is taken from the Falcon tube in order to be counted. Cells were counted using a hemocytometer. 20 µL of the well-mixed cell/media sample was combined with 20 µL of trypan blue and

mixed. A glass cover slip was placed on top of the hemocytometer and 10 μL of the trypan blue/media mixture was pipetted into each side of the hemocytometer. Cells were counted on both sides of the hemocytometer. Live cells appeared as bright, white circles under the microscope. The specific counting protocol is found in Appendix 11.

The Falcon tube is spun at a speed of 1100 rpm for 5 minutes until a cell pellet forms at the bottom of the tube. All supernatant is suctioned out using a sterile glass pipette, leaving a small amount of media directly around the cell pellet. Cancer cells have a tendency to not pack as tightly as other cells and can result in cell loss if the small amount of media is not maintained around the pellet at this step. The cells are resuspended to a concentration of 10^6 cells/mL of fresh media and pipetted so that 1 mL of cell resuspension is placed in each new flask. This is supplemented with 9 mL of fresh media so that each flask contains a total volume of 10 mL. The splitting ratio differs between cell lines and is discussed for each cell line in the cell culture section of this thesis.

Decellularization of Amniotic Membranes

This protocol is the current protocol for amniotic membrane decellularization in Dr. Nollert's lab and was performed by Julien Arrizabalaga to prepare all amniotic membranes used in this thesis. The decellularization protocol is found in Appendix 12.

The first steps of the decellularization protocol involved removing the amniotic membrane from the chorionic membrane and rest of the placenta. This is performed by blunt dissection by hand. Once the amniotic membrane is

isolated, the umbilical cord is removed for other projects and the rest of the placenta is discarded as biohazard. The membrane is placed in a beaker of distilled water to removed blood and swell the spongy layer. The membrane is placed on a flat tray with the stromal side facing upwards. The spongy layer on top of the stromal side is removed by hand to not destroy the actual membrane. Using a 3D printed rectangle template (6.5 cm by 9.5 cm), the membrane was cut into smaller rectangles using a scalpel. A small cut was made at the bottom left hand corner of each rectangle in order to differentiate the top from the bottom. The membranes are placed in a 250 mL bottle and placed in the -86°C freezer for two hours. At the end of the two hour freeze, they are placed in the water bath at 37°C for 15 minutes and frozen/thawed again. After the second thaw, membranes are rinsed with distilled water and transferred to a 500 mL bottle filled with 400 mL of distilled water and 0.03% (w/v) sodium dodecyl sulfate. These bottles are then placed on an orbital shaker for 12-24 hours at 100 rpm. Membranes are washed in new bottles of 400 mL of distilled water and transferred to new bottles at 5, 15 and 40 minutes and 1, 6 and 24 hours. In order to remove residual DNA, membranes are incubated with 20-50 μg/mL DNase in a Tris buffer solution on an orbital shaker for 2 hours at 100 rpm. Membranes are then rinsed with distilled water. To sterilize the membranes, they are placed in a solution of 0.2% (v/v) peracetic acid and 4% ethanol in distilled water for 2 hours on an orbital shaker at 100 rpm. The bottles are wrapped in aluminum foil to maintain sterilization activity of the peracetic acid. Under the Bio-Hood, the membranes are washed in sterile distilled water at 100 rpm for 10, 20 and 30

minutes. They are washed again for 24 hours in distilled water at the same orbital shaker conditions. After the final distilled water wash, membranes are transferred into sterile PBS and the pH is recorded right after transfer and after 24 hours and should be in the biological pH range (7.2 to 7.4). The membranes are transferred into a final sterile PBS solution with antibiotics and stored in the 4°C refrigerator until needed.

Static Membrane Growth

Decellularized amniotic membranes were obtained from Julian Arrizabalaga and Jin Liu from Dr. Nollert's research lab. The decellularization protocol was performed by Julien and sterile, decellularized membranes were placed in PBS for storage in the 4°C refrigerator. These membranes were placed in the PBS in October 2016.

Membranes were prepared the day before cell seeding for both cell growth studies. Stainless steel A4 washers (also provided by Julien and Dr. Nollert's lab) were sterilized in batches of six in the anprolene sterilizer for 12 hours. Tools were also sterilized in the same batch as the washers. Specific tools included a pair of curved hemostats, a scalpel handle, a pair of scissors, a large forceps, beakers and a large petri dish. Membranes were taken from the sterile PBS and placed on the large petri dish using a large forceps. This was done one at a time to avoid crowding or contamination. The cut corner of each membrane was oriented so that the cut corner was on the bottom left side of the membrane. This ensures that cells are seeded on the correct side of the membrane. A 3D printed square template was placed on the membrane and the scalpel equipped

with a sterile blade was used to cut out small squares of membrane. The dimension of this template and the approximate dimensions of the membrane was 2.5 cm X 2.5 cm. The total surface area of the cut membrane was 6.25 cm^2. The measured thickness of the amniotic membrane (n=1) was 86.6 +/- 6.1 μm in the laboratory. This value was measured by taking ten different measurements on various parts of a decellularized membrane using an iGaging® micrometer. The micrometer measures to an accuracy of +/- 4 μm. Each square was transferred into a sterile, non-tissue culture 6-well plate with care taken to ensure that the correct side was facing upwards. A washer was carefully placed on top of the flattened, square membrane in each well plate (Figure 2). The washer thickness, outer diameter and inner diameter dimensions were 0.2, 3.4 and 2 cm, respectively. The resulting exposed membrane surface area for cell seeding was 3.14 cm^2. The washer is not biologically reactive with the membrane or cells and is used to keep the membrane flat and stable on the bottom of the well plate.

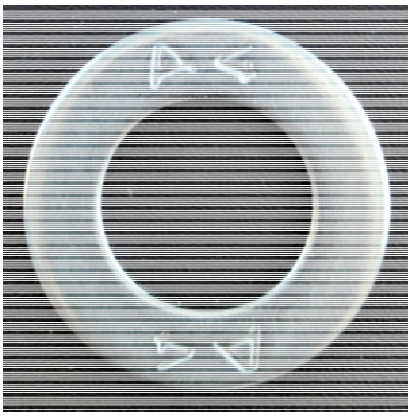


Figure 2: Stainless steel washer

This process was continued until all well plates were filled with membrane squares and washers. Each well was supplemented with 5 mL of L-

15 media. All well plates were placed in the incubator at 37°C and atmospheric CO_2 overnight to bring to temperature.



Figure 3: Representative 6-well plates with membranes

On the day of seeding, the cell flasks were split according to the splitting protocol. Cells were counted using the hemocytometer and trypan blue staining. Each cell-seeded membrane was seeded with 500,000-1,000,000 cells per 0.15 mL. Control membranes were not seeded with cells and were supplemented with L-15 media. The first seeding was done with the initial concentration of cells in the volume of 0.15 mL placed directly on the membrane in the inner circle of the washer. All well plates were then incubated in the 37°C and atmospheric CO_2 incubator for an hour. After the hour, membranes were reseeded by taking the supernatant from the sides of the washer and pipetting it back onto the center of the membrane. At this time, media was placed in the water bath to bring up to 37°C. Well plates were incubated for another hour and reseeded at the end of that hour. In total, there were 3 seeding periods and 2 hours of incubation. After the last seeding period, 5 mL of L-15 media was pipetted carefully and slowly onto the top of each washer. By placing media onto the washer, cells were less likely to be washed off due to the media while still filling the well with media. The media flowed off of the washer and over the amniotic membranes. The day

0 sample was taken at this point. All other well plates were placed back into the incubator until samples needed to be taken.

For the static membrane growth experiments, the SW-620 cell line was used. This explains why L-15 media was used for all static membrane experiments. Sample sets were taken in triplicate. The sampling process requires a pair of hemostats or forceps and a pair of scissors. The washers are removed from the wells and placed on a petri dish. One at a time, the amniotic membrane is lifted from the well using the hemostats and cut into small pieces using the scissors. The small pieces are placed into a small sample tube filled with 1 mL of nanopure water. Once all the samples are placed in their respective collection tubes, they are sonified for 5 seconds each. 200 µL of trypsin is added to each collection tube under the Bio-Hood. They are then incubated for 1 hour in the 37°C/atmospheric CO_2 incubator. Following the incubation step, samples are sonified for 10 seconds each and placed in the -20°F freezer until the freeze/thaw cycles can be completed.

Overview of the Five Day Membrane Study

Media changes were performed on day 2 (before the sample) and on day 4 (after the day 4 sample and before the day 5 sample). This would ensure that cells are favorably adhering to the membrane and can withstand a media change. The SW-620 cells were still used for the study. A total of 555,000 cells suspended in 0.15 mL of media was seeded onto each cell-seeded, experimental membrane. No fixed samples were taken during the trial in order to save resources and only focus on growth in terms of DNA content present per membrane.

Sampling: Two Day Membrane Study

Day 0 samples were taken immediately following the third seeding period. A total of 3 DNA assay samples were taken and processed according to the DNA assay preparation protocol per day for membranes seeded with cells. Two DNA assay samples were taken on both day 0 and day 2 for the membranes without cells. On all sample days, 3 membrane samples were taken and fixed for the membranes seeded with cells. A fixed sample was taken on day 0 and day 2 for membranes not seeded with cells. The samples were fixed by gentle submerging into PBS 3 times and then placed in fixing solution (10% formalin in PBS (v/v)) for 15 minutes. After the 15 minute period expired, samples were placed in a labelled collection tube filled with 1 mL of 70% ethanol and stored in the refrigerator.

Sampling: Five Day Membrane Study

For each day of sampling, three samples were taken and processed according to the DNA assay protocol for each experimental group. For example, on day 0, there were three samples taken for the control (no cell) group and three samples take for the experimental (SW-620) group. Each sample was analyzed in triplicate during the DNA assay and averaged together to get the final value. This was different from the first experiement in that each day sample had a corresponding control sample for accuracy.

Method of Analysis

DNA Assay

Preparing the sample for analysis differs slightly when analyzing a tissue material versus a synthetic scaffold. For the amniotic membrane scaffolds, samples to be used for DNA assay were taken according to the lab protocols found in the Sikavitsas lab. This specific protocol can be found in Appendix 6. Each membrane scaffold was taken from the well and cut into small pieces using scissors. The small pieces were placed in a small sample tube in 1 mL of nanopure water. Each sample was then sonified for 5 seconds. After sonication, 200 µL of trypsin was added to each sample tube. The samples were then incubated at 37°C and atmospheric air conditions for 1 hour. The reason that they were incubated in atmospheric air was because the SW-620 cells are cultured in that condition. Following the hour of incubation, samples were sonified again for 10 seconds each and placed in the -20°C freezer.

The samples being assayed were taken out of the freezer and allowed to thaw at room temperature. During this period, the DNA standards were prepared using a serial dilution method. Standard 4 was prepared to a concentration of 3 µg/mL by combining 30 µL of DNA standard and 970 µL of nanopure water. For consistency, the serial dilution was performed in the same manner for each assay needed in this thesis but it could be calculated to make different volumes of each standard by using the $C_1*V_1=C_2*V_2$ equation for dilutions. To ensure proper concentrations, each standard was vortexed for a few seconds before proceeding to the next standard. To make standard 3, 700 µL of standard 4 was added to 1.4 mL of nanopure water. Standard 2 was prepared by adding 1 mL of

standard 3 to 2.8 mL of nanopure water. Finally, standard 1 was prepared by adding 2 mL of standard 2 to 4 mL of nanopure water. The final concentrations of standards 1, 2 and 3 were 0.1, 0.3 and 1 μg/mL respectively. Standard 0 simply consists of pure nanopure water.

The buffer solution was prepared according to the "DNA Assay" protocol sheets found within the lab. Appendix 4 includes the protocol for preparing the buffer solution as well as preparing the well plates for assay. The amount of buffer varied with the amount of samples being analyzed. The volume amount of 20X TE buffer solution and amount of nanopure needed to dilute to a 1X buffer was listed on the assay sheet. Once the buffer was mixed, half of the volume was transferred to another container. The secondary container was wrapped in foil to protect from the light and set aside to be mixed with PicoGreen later on.

A DNA assay was preformed using an Invitrogen™ Quant-iT™ PicoGreen™ dsDNA Assay Kit [29]. Opaque 96-well plates were prepared and the layout of the well plate was drawn in the lab notebook to ensure accuracy and precision. Initially, 107 μL of buffer solution (the half not wrapped in foil) was pipetted into each occupied well. This includes wells occupied by the standards. Before adding samples or standards to the wells, each was vortexed gently to ensure homogeneity. Once mixed, 43 μL of sample or standard was added to the designated wells. Each sample/standard was analyzed in triplicate. The assay protocol sheets also included the amount of PicoGreen needed for the number of samples. The designated volume of PicoGreen was added to the

buffer half wrapped in the foil. This set was performed in the dark since PicoGreen is light sensitive and premature exposure to light skews the results of the assay. Once the PicoGreen buffer solution was vortexed, 150 μL was added to each occupied well. The plate is covered for 5 minutes with foil to set before running through the spectrophotometer/fluorometer.

Population Doubling Rate

The population doubling rate was calculated using the results of the DNA assay and confirmed using the microscope images in Figure 5. The cell number determined from the assay was multiplied by two to determine the doubled population. The amount of time to reach population doubling was then interpolated using the assay results (values with cell numbers between the doubled day 0 value) and the calculated doubled population for day 0.

Statistical Analysis

Statistical significance was determined using the T-test Excel built-in function. A star above the bars in the graph signifies a p-value of less than 0.05. Error bars represent the standard deviation for each sample.

4. Results

Static Amniotic Membrane Culture

Experiment #1: Two Day Culture

The first experiment was a true proof-of-concept trial to determine if the SW-620 cells were viable when seeded on the decellularized amniotic membranes. The seeding concentration per membrane was 10^6 cells/membrane. The 10^6 cells were contained in 0.15 mL of media which was seeded onto each membrane.

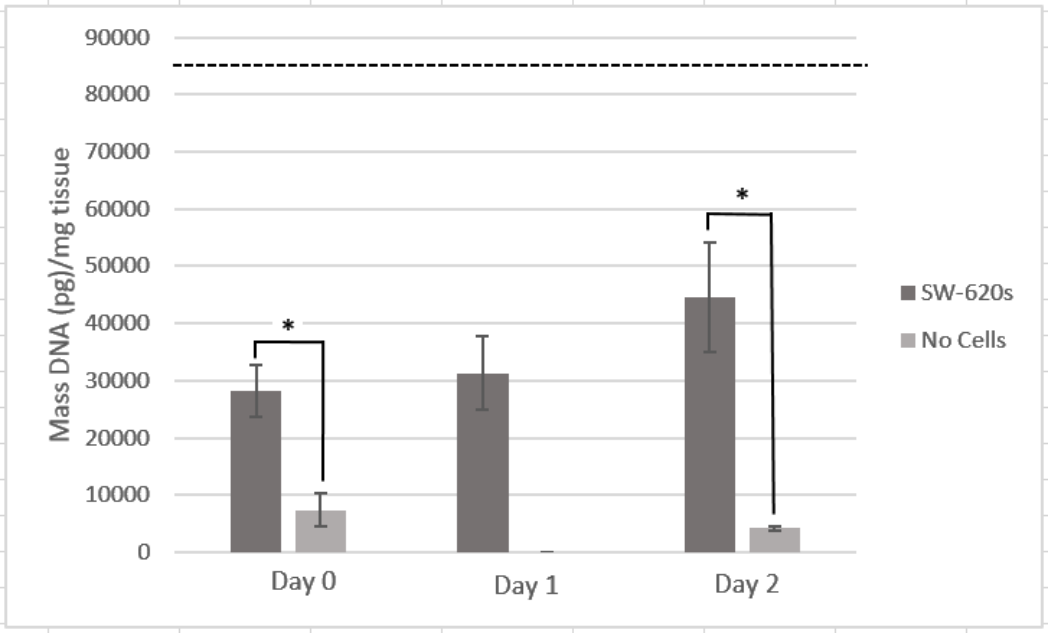


Figure 4: Two day static membrane experiment with SW-620s; dashed line represents the initial number of cells seeded

The results of the DNA assay for the day 0 controls, the day 2 controls and the days 0-2 cell-seeded samples are depicted in Figure 4. A substantial number of cells were lost and did not adhere from the initial seeding to the time of the day 0 sample collection. The values did begin to increase after the initial drop between seeding and day 0.

Experiment #2: Five Day Culture

The pilot study was repeated and expanded from two days to five days. Images were also taken for a visual representation of the cell proliferation on the membrane. Figure 5 tracks the progress of SW-620 cell growth from before the day 1 sample up until right before the day 5 sample. The same wells (day 5, middle no cells and day 5, middle cells) were imaged over the course of the entire experiment for consistency.

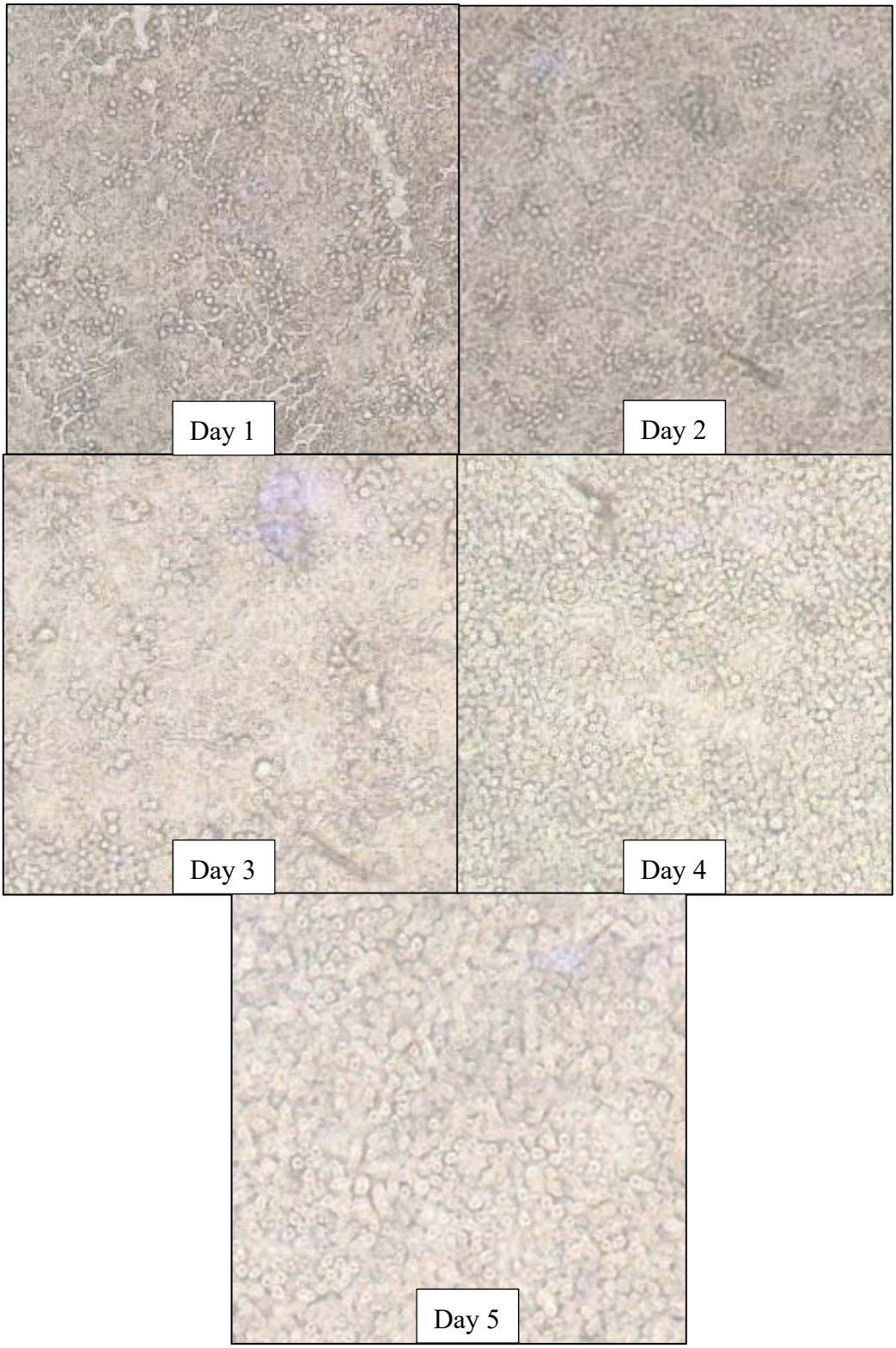


Figure 5: Days 1-5 static membrane growth seeded with SW-620 cells



Figure 6: Days 1-5 static membrane controls without cells

Figure 6 shows the images taken of the control membranes without any cells. No growth appears on the surface and no cross contamination of cells ended up on the control membranes.

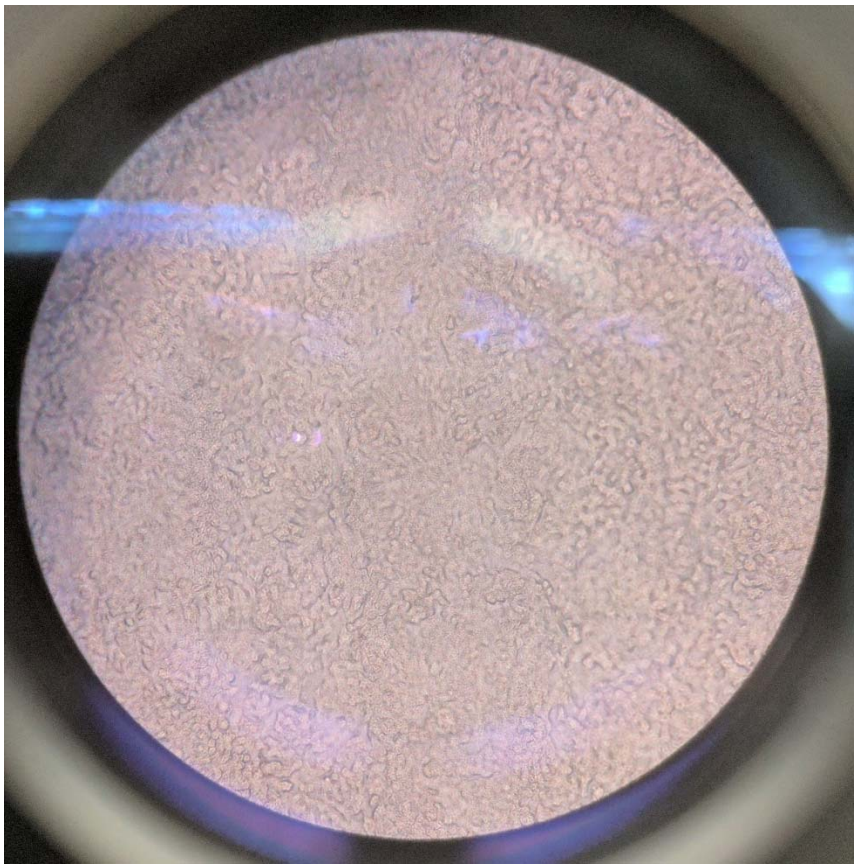


Figure 7: Day 5 amniotic membrane without cells

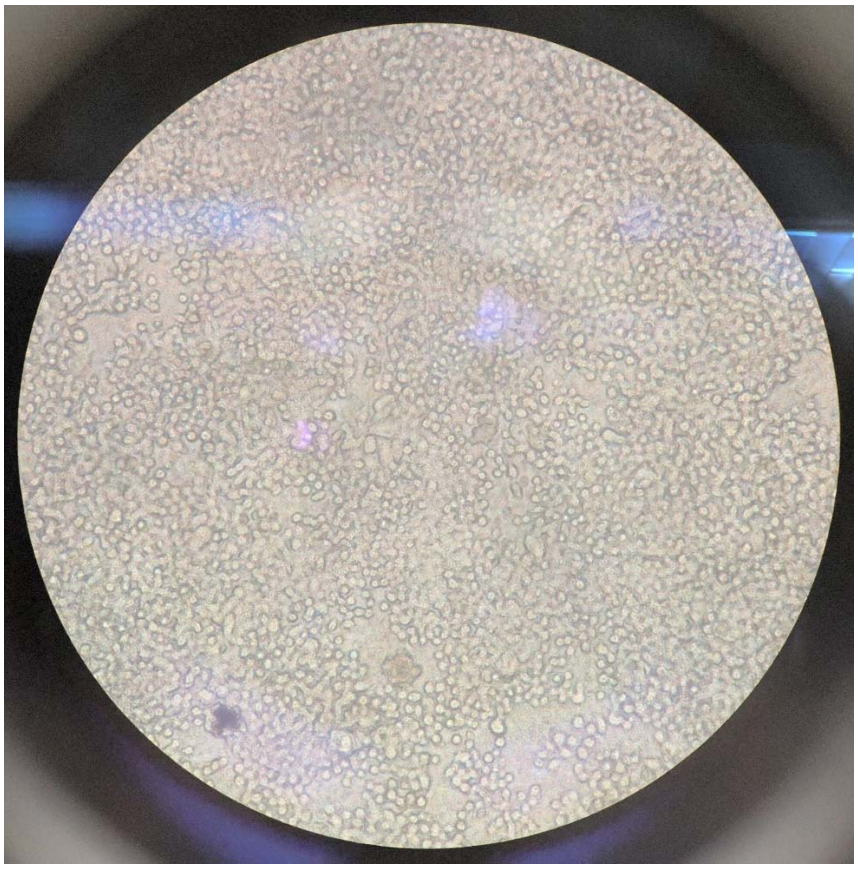


Figure 8: Day 5 amniotic membrane seeded with SW-620 cells

Figures 7 and 8 are higher magnification images (using the 20X setting on the light microscope) of day 5 right before samples were taken. The surface architecture of the membrane can be observed on the membrane without cells. The membrane with cells shows about a 70-80% confluency of cells on the surface. Cells can be seen throughout the entire surface of the membrane.

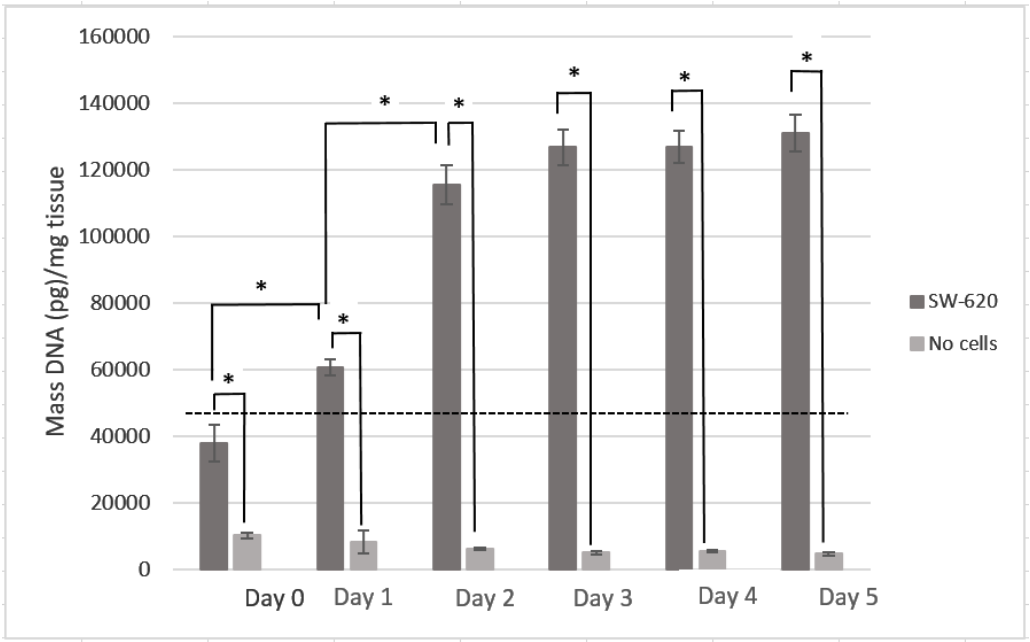


Figure 9: Five day static membrane experiment with SW-620s; dashed line represents the initial number of cells seeded

There were 555,000 SW-620 cells initially seeded onto the membrane with an observed seeding efficiency of 60% on day 0 as observed in Figure 9. There was a significant increase in the mass of DNA per mg of tissue between days 0 and 1 as well as days 1 and 2. The DNA mass per mg of tissue values plateaued from days 3 to 5, with no significant increase or decrease over these days. There was a 300% increase in the mass value of DNA per mg of tissue comparing the value for day 0 to day 5. The population doubling rate of the SW-620 cancer cell seeded on the amniotic membrane was estimated to be between 1 and 2 days.

5. Discussion

Static Amniotic Membrane Culture

The main goal of this study was to determine the seeding efficiency and proliferation of SW-620 cells seeded and cultured on the surface of decellularized human amniotic membrane. The amniotic membrane cancer cell cultures are critical proof-of-concept experiments (Figures 4 and 9) that will potentially lead to more extensive investigation of the use of the human amniotic membrane as a 3D *in vitro* cancer model. The experiments presented include visual images of the membrane (Figures 5-8) and are able to confirm the proliferation of SW-620 cancer cells on the surface of the membrane. From the top down view, cells can be viewed forming a monolayer on the surface of the membrane. Media changes that often pose cell detachment if cell adhesions are weak, did not seem to impact the adhesion or growth of the cancer cells on the surface of the amniotic membrane. Increasing the extent of the culture period can help to determine if cells are able to eventually organize into a small tumor on the human amniotic membrane.

Colon cancer tumors begin as small polyps on the surface of the lining of the colon. This is a flat, long environment since the mucosa of the colon extends along the circumference and length of the colon. The human amniotic membrane can physiologically resemble this flat environment. The possibility of seeding healthy colon endothelial or mucosal cells on the membrane first and then introducing the colon cancer cells may even be able to accelerate the tumorigenesis process and allow the exploration of colon cancer cells with

healthy cells residing in that microenvironment and may be critical in tumor formation or metastasis.

Figures 5 and 6 depict images of the decellularized amniotic membrane over the five day culture period. Figure 5 represents the membranes that were seeded with SW-620 cells and Figure 6 shows the membranes that were not seeded with cells. Cells appear as rounded shapes with slightly darker nuclei. Cells can be seen proliferating in a monolayer in Figure 5. The images of the membranes that were not seeded with cells only display the surface architecture of the membrane and do not contain any rounded cell shapes. This signifies that there was no cross contamination of cells to the control membranes. Figures 7 and 8 provide a view of the amniotic membrane before the day 5 sample was taken (on day 5) zoomed at 20X magnification. Figure 7 depicts the control membrane and Figure 8 depicts the membranes seeded with SW-620 cells. Cells in Figure 8 can be differentiated from the surface architecture of the membrane in Figure 7 in that the cells appear as consistently small and rounded figures. The surface of the membrane appears irregular in shape and not small, round shapes. The cell monolayer in Figure 8 is nearly confluent, which is a promising result in the proliferation of SW-620 cancer cells on the amniotic membrane.

One metric that can be used to compare the growth of the SW-620 cells *in vitro* on the amniotic membrane to *in vitro* 2D flask cultures and *in vivo* tumor growth is the population doubling rate. The doubling rate is most accurate when measured before the culture reaches high levels of saturation. This is done to limit the amount of impact that cell crowding has on the proliferation rate.

Using the DNA assay results and images taken through the light microscope of the amniotic membrane, the population doubling rate can be estimated. For the *in vitro* amniotic membrane culture, the population doubling rate was approximately measured to be between 1 and 2 days (30 hours, Figure 9). Using Appendix 14 (2D culture growth of SW-620 cells) and literature cultures, the population doubling rate for 2D flask culture is 26 hours [30]. A study growing SW-620 tumors in Rowett athymic rats determined that the doubling rate *in vivo* was 5.5 days [31]. The value for the *in vitro* membrane study falls in between the physiological doubling rate than 2D flask doubling rate. This could suggest that culturing cells on the thin amniotic membrane results in a semi-3D environment, but more experiments are required to strengthen the validity of this observation as discussed in the future aims section.

6. Conclusion

SW-620 cancer cells were able to be seeded and proliferate onto the decellularized human amniotic membrane in *in vitro* culture studies. The cancer cells were able to form a nearly confluent monolayer on the surface of the amniotic membrane. SW-620 cells seeded on the amniotic membrane had a population doubling rate in between the doubling rate of an *in vivo* rat tumor model than the 2D flask culture. There are still unanswered questions regarding this study, including cell penetration and gene expression as well as improving the three dimensionality of the amniotic membrane. Cell penetration can be quantified through histology, gene expression can be determined and compared to *in vivo* conditions using PCR and the three dimensionality can be improved by layering the membrane and seeding cells between the layers. Though these questions need to be addressed further, the preliminary results regarding the monolayer growth of the SW-620 cells on the decellularized amniotic membrane were promising.

7. Future Aims

There is more work needed in order to fully understand how these cells bind to the membrane and grow in longer time periods. Histology, successful fluorescent staining and Live/Dead assay would be useful to understand if there is any cellular penetration occurring through the membrane as well as cell viability. In order to have a viable 3D model, there must be cellular penetration and in turn, tumorigenesis. Staining and histology would be necessary to see the cellular penetration by repeating the five day study. PCR would also be useful to quantifying the cellular activity of the SW-620 cells growing in a monolayer versus a 3D *in vitro* model versus an *in vivo* animal model. Cells generally express different genes depending on their environment. Confirming that these cells are able to act like *in vivo* cells is necessary before moving forward with amniotic membrane as a scaffold and a clinically relevant model solution. According to literature, there are several genes that can be used to assess colon cancer behavior using RT-PCR. The housekeeping gene that can be used was glyceraldehyde-3-phosphate dehydrogenase (GADPH) [32]. There are 15 relevant genes that can be examined. These genes are as follows: monocarboxylate transporter (MCT), human glutamate decarboxylase (GAD67), human serine proteinase inhibitor (P19), human glutathione transferase M3 (GSTM3), human subunit C of V-ATPase (vat C), *homo sapiens* mRNA for translocation protein-1, human cyclin D (cyclin D1), human ataxia-telangiectasia locus protein (ATM) gene, *homo sapiens* apoptosis-related protein TFAR15 (TFAR15), human CO-029, human surface antigen (ESA), *homo*

sapiens mRNA for ASM-like phosphodiesterase 3b, human interferon regulatory factor (Humirf5) and *homo sapiens* mRNA for IFN-inducible γ2 protein. This is not a complete list of genes that can be monitored for the SW-620 cell line but can be used as a starting point for RT-PCR monitoring.

Repeating the five day study with PC-3 cells, MDA-MB-231 cells and other cancer cell lines would also be necessary to determine if the successful seeding and proliferation was cell-specific or can be repeated for different cell lines. The morphology of both of those lines is different than that of the SW-620s and their reaction to the amniotic membrane needs to be documented. Additionally, repeating the experiments with natural (non-decellularized) membrane could be used as a control measure. This would determine if there is any significant changes between the decellularized and non-decellularized membrane and any interactions between the existing cells and cancer cells and could be explored further.

Since the amniotic membrane is very thin in nature, surface modifications can be made to the membrane in order to expand into more sophisticated 3D models. Models involving larger scale 3D spaces resembling *in vivo* tumors require a larger scaffold capable of supporting cell growth in all three dimensions as opposed to only a monolayer. The amniotic membrane scaffold can be stacked in order to provide a larger scaffold. This scaffold can be seeded with cells in between the layers to promote cell penetration through the membrane to form an *in vitro* tumor. The type and extent of modifications depends on the results from the attachment of the other cancer lines to the

decellularized, unmodified membrane. Testing the other lines first will determine if modifications are necessary or if the cells bind to the amniotic membrane. This will also ensure that the seeding results determined using the SW-620 cells are not only cell-line specific and can be repeated with different cell lines. Further research is necessary to determine the adhesion mechanisms of cancer cells to each other and to other tissues.

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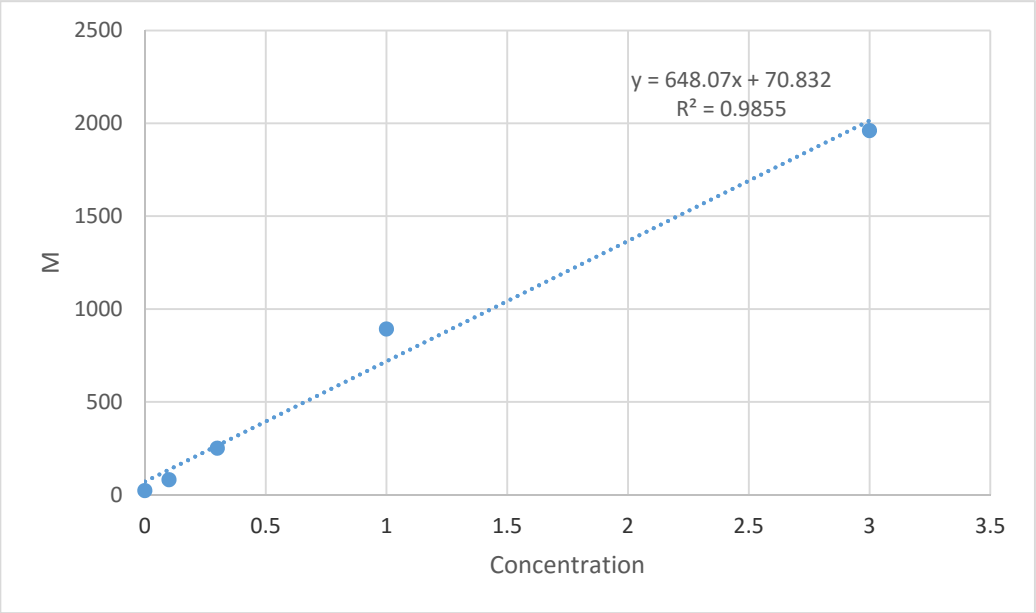
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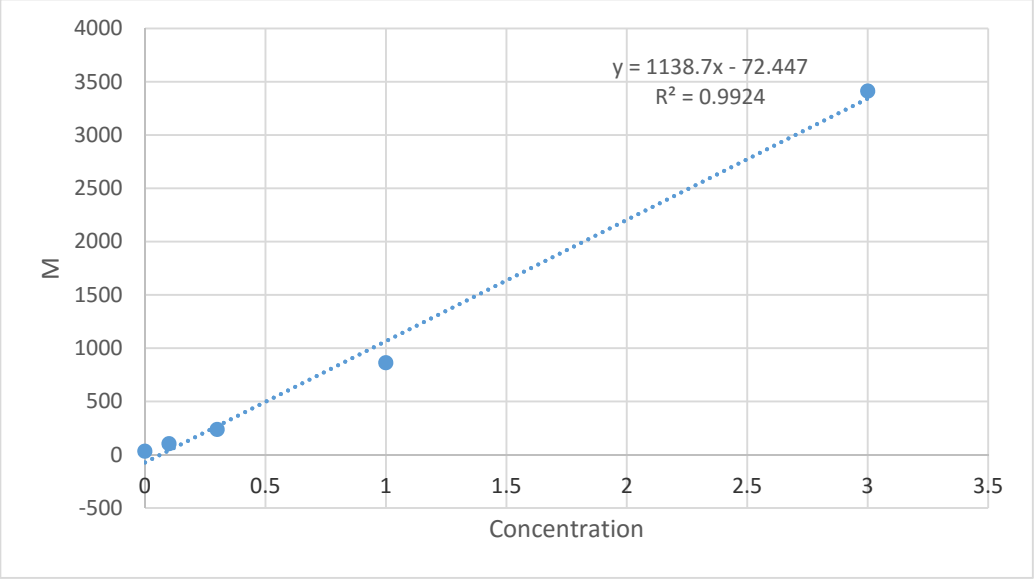
[36] W. S. Inc. "What is Nanopure Water," https://www.apswater.com/article/334/What-is-Nanopure-Water.

9. Appendices

Appendix 1: Standard curve for SW-620 DNA quantification

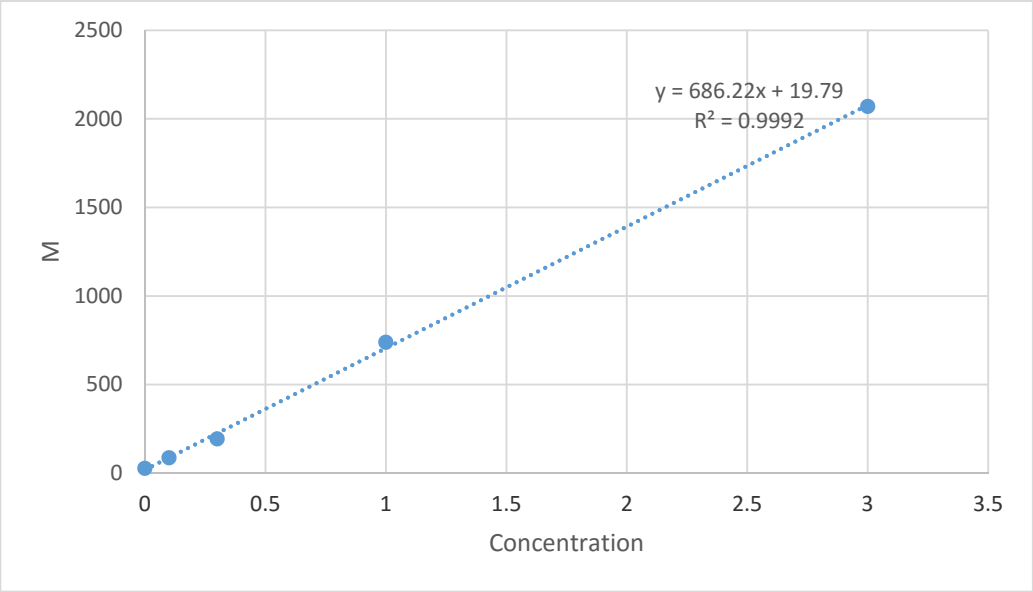


Appendix 2: Standard curve for two day membrane experiment

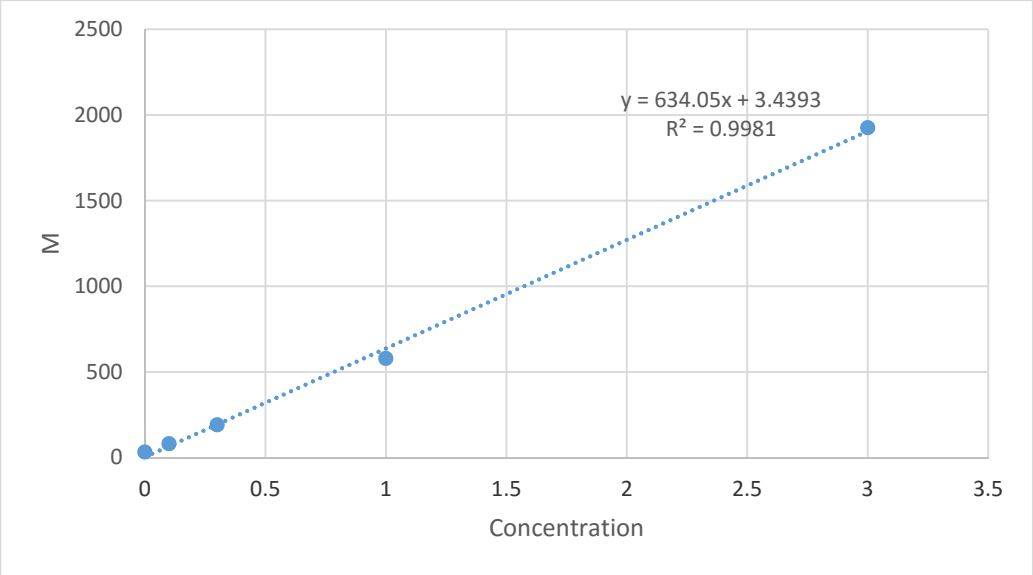


Appendix 3: Standard curves for five day membrane experiment

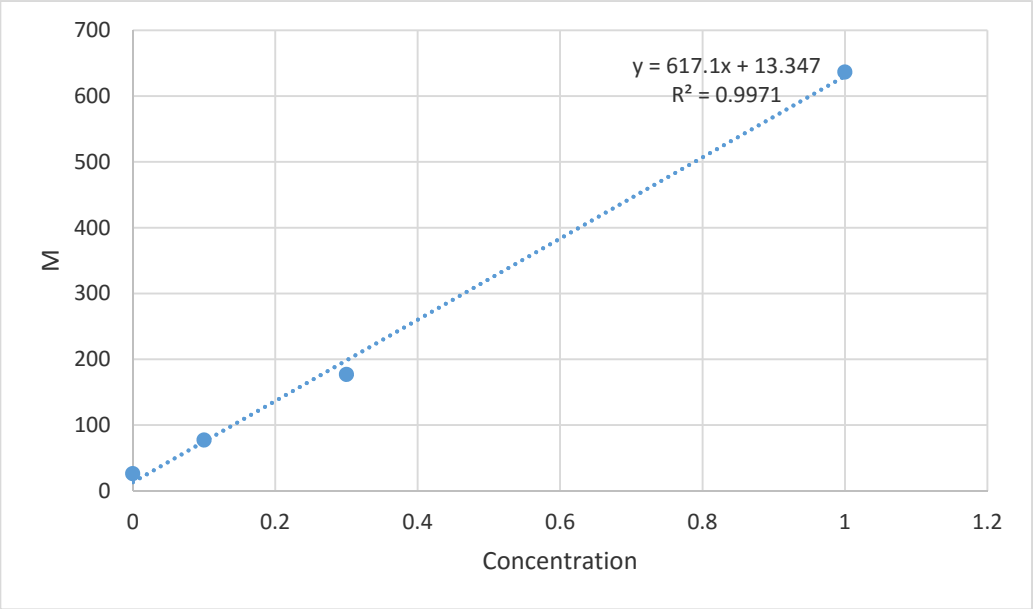
Day 0 and 1



Day 2 and 3



Day 4 and 5



Appendix 4: DNA Assay Protocol

DNA Assay

(PicoGreen dsDNA Quantitation Assay)

NOTES:

- PicoGreen dye is light sensitive. When storing or thawing avoid exposure to light.
- Keep fully prepared well plates in the dark until you are ready to put the plate in the microplate reader
- DNA standards made with DI water break down quickly and are only good for 2 days
- Plate reader does not accurately read outside wells of 96 well plates. Use only the 60 interior wells of the plate. (**X** in plate layout indicates wells not to be used)

MATERIALS:

1. PicoGreen dye (Quant-iT PicoGreen dsDNA reagent, P7581, Invitrogen (-20 °C))
2. 20x TE buffer solution (200 mM Tris-HCl, 20 mM EDTA, pH 7.5 (4 °C))
3. 100 µg/mL λ DNA standard solution (-20 °C)
4. 100 mL beaker
5. Centrifuge tubes
6. Falcon tubes (15 mL)
7. Falcon tube (50 mL)
8. Opaque flat bottom 96 well plate

PROTOCOL:

1. Fill 100 mL beaker with DI water
2. Prepare DNA standards from 100 µg/mL λ DNA standard solution in centrifuge tubes as follows:

Volume of standard used (µL)	Volume DI water (µL)	Standard concentration obtained (µg/mL)
0	1000	0
1	999	0.1
3	997	0.3
10	990	1
30	970	3

3. Design well-plate layout
   a. Each sample should be assayed in triplicate (**X** indicates wells not to be used)

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	X	X	X	X	X	X	X	X	X	X	X
B	X	S-1	S-2	S-3	S-4	S-5						X
C	X	S-1	S-2	S-3	S-4	S-5						X
D	X	S-1	S-2	S-3	S-4	S-5						X
E	X	ST-1	ST-2	ST-3	ST-4	ST-5						X
F	X	ST-1	ST-2	ST-3	ST-4	ST-5						X
G	X	ST-1	ST-2	ST-3	ST-4	ST-5						X
H	X	X	X	X	X	X	X	X	X	X	X	X

4. Determine the total volume of buffer solution need
   a. V_{buffer} (µL) = ((# of Samples + # of standards)*3+3)*300 µL
   b. $V_{20xTEbuff}$ (µL) = V_{buffer} (µL) /20
   c. V_{DIH2O} (µL) = V_{buffer} (µL) – $V_{20xTEbuff}$ (µL)
5. Pipette calculated volumes of 20x TE buffer solution and DI water into falcon tube
6. Cap falcon tube and vortex to mix
7. Evenly divide total volume of buffer into two 15 mL falcon tubes

DNA Assay Continued

8. Pipette in every pre-assigned well 107 µL of 1x TE buffer
9. In every pre-assigned well, pipette 43 µL of the designated standard or sample
10. Determine volume of PicoGreen dye needed
    a. Vol total buffer (µL) /400 = Vol of PicoGreen (µL)
11. Remove PicoGreen dye from freezer and thaw
12. Pipette calculated amount of PicoGreen dye into 15 mL falcon tube containing unused half of 1x TE buffer
13. Cap falcon tube and vortex to mix
14. Pipette 150 µL of dye solution into each occupied well
15. Incubate plate in the dark at room temp (25 °C) for at least 5 min
16. Measure fluorescence of wells at 480 nm excitation and 520 nm emission

Appendix 5: Cell Splitting Protocol

Splitting Cell Cultures

NOTES:

- Make sure to label flasks with:
  1. Cell type
  2. Passage
  3. Date
  4. Your name (can be initials)
- When removing media from pelleted cells do not let vacuum pipette go past the top of the bend of the cone of the falcon tube. Going past the bend can result in loss of cells.

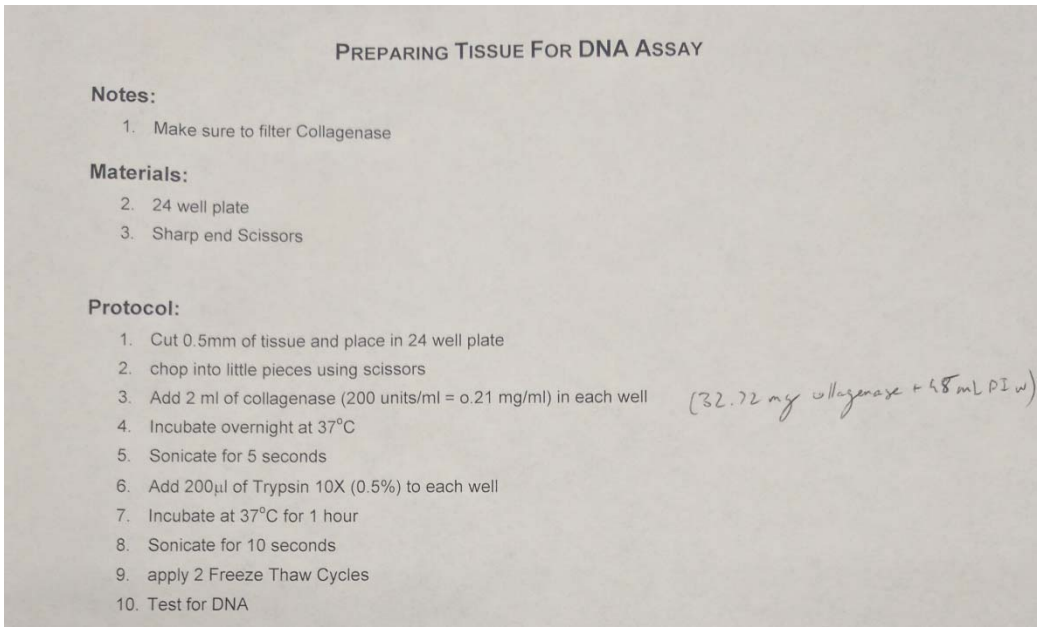
MATERIALS:

1. Phosphate buffered saline (PBS)
2. α Minimal essential media (αMEM)
3. Trypsin (25200, Invitrogen)
4. 15 mL falcon tubes
5. Glass pipettes
6. Disposable pipettes

PROTOCOL:

1. Carefully remove old media from culture flask using a glass pipette and vacuum
2. Rinse cells with 3-5 mL of PBS
3. Remove PBS with a glass pipette and vacuum
4. Pipette 1 mL of Trypsin into flask
5. Close flask and place in incubator for 5-10 min (monitor with microscope to detect when detachment has taken place)
6. Remove flask from incubator and tap flask against counter to dislodge any cells that have not been fully detached by Trypsin
7. Rinse cells from bottom of flask with 9 mL of fresh αMEM
8. Remove cell suspension from flask and place in 15 mL falcon tube
9. Make sure cells are evenly distributed throughout media then take small sample for counting
10. Count cells
11. Centrifuge cell suspension at ~~2,000~~ 1100 RPM for 5 min using centrifuge in D204
12. Carefully remove media from pellet using a glass pipette and vacuum
13. Suspend cells with fresh media at 375,000 to 1 million cells per 1 mL of media (The cell concentration depends on cell type and desired culture time)
14. Pipette resuspended cells into new sterile flasks at 1 mL per flask
15. Supplement flasks with 9-10 mL of fresh αMEM to bring the flasks total volume to 10-11 mL
16. Close flasks and label
17. Place flasks in incubator

Appendix 6: Tissue Preparation for a DNA assay



PREPARING TISSUE FOR DNA ASSAY

Notes:

1. Make sure to filter Collagenase

Materials:

2. 24 well plate
3. Sharp end Scissors

Protocol:

1. Cut 0.5mm of tissue and place in 24 well plate
2. chop into little pieces using scissors
3. Add 2 ml of collagenase (200 units/ml = o.21 mg/ml) in each well (32.72 mg collagenase + 48mL PIW)
4. Incubate overnight at 37°C
5. Sonicate for 5 seconds
6. Add 200µl of Trypsin 10X (0.5%) to each well
7. Incubate at 37°C for 1 hour
8. Sonicate for 10 seconds
9. apply 2 Freeze Thaw Cycles
10. Test for DNA

Appendix 7: PBS Preparation

Phosphate Buffered Saline Preparation

(For Cell Culture)

NOTES:

- Should make 1 L of PBS containing 1% antibiotic that can be used for cell culture
- Make sure to label PBS bottles with:
  1. Type of PBS (for cell culture or not for cell culture)
  2. Your initials
  3. Date PBS was made
- Antibiotic should be in aliquots located in the freezer (-20 °C)
- When thawing antibiotic in water bath do not leave in more than 15 min. If left in water bath too long contents will precipitate and adhere to falcon tube.
- Use cell culture tested sodium hydroxide, and hydrochloric acid.
- Rinse off pH probe before placing it in or removing it from its storage buffer.

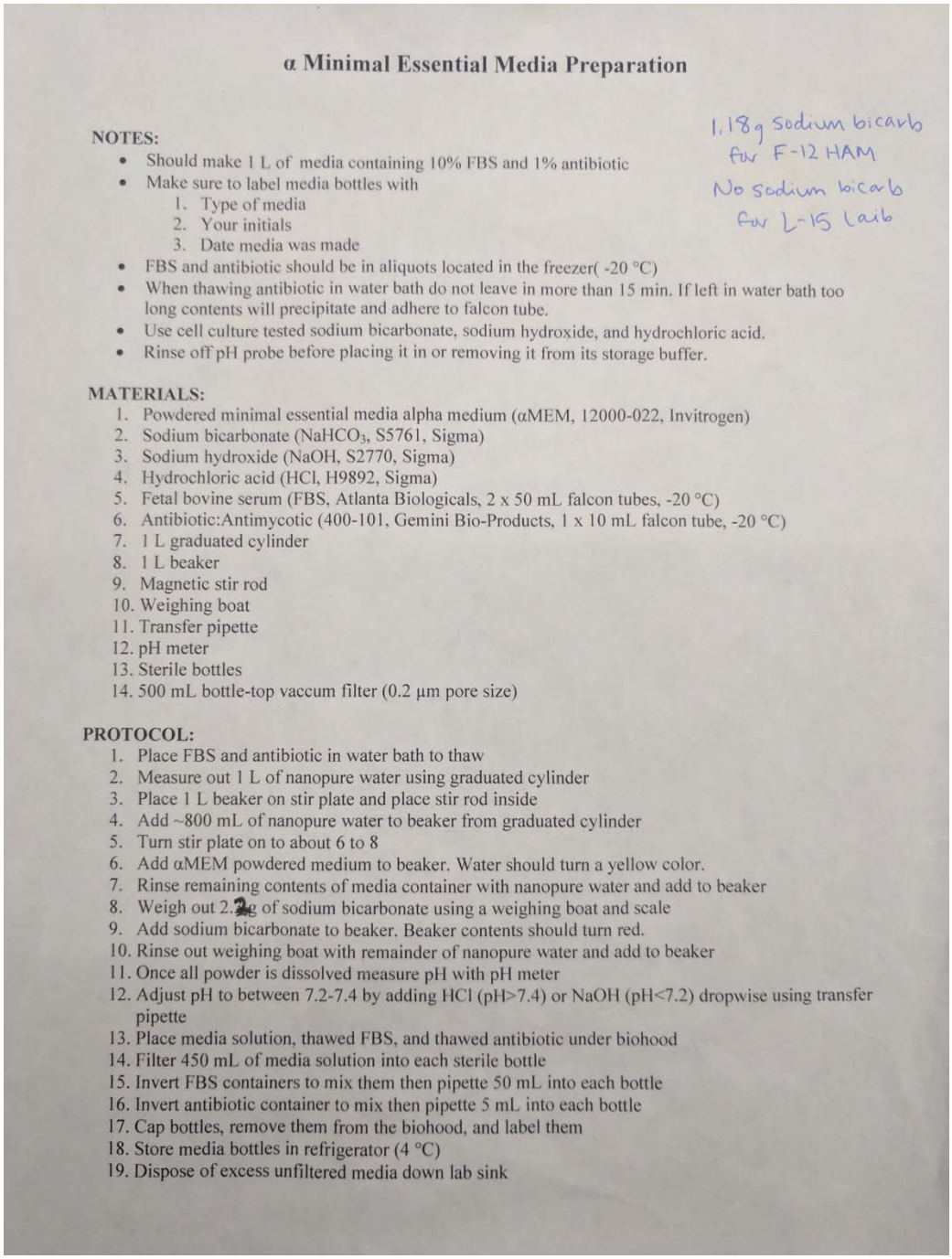
MATERIALS:

1. Powdered Dulbecco's phosphate buffered saline (PBS, D5652, Sigma)
2. Sodium hydroxide (NaOH, S2770, Sigma)
3. Hydrochloric acid (HCl, H9892, Sigma)
4. Antibiotic:Antimycotic (400-101, Gemini Bio-Products, 1 x 10 mL falcon tube)
5. 1 L graduated cylinder
6. 1 L beaker
7. Magnetic stir rod
8. Transfer pipette
9. pH meter
10. Sterile bottles
11. 500 mL bottle-top vaccum filter (0.2 µm pore size)

PROTOCOL:

1. Place antibiotic in water bath to thaw
2. Measure out 1 L of nanopure water using graduated cylinder
3. Place 1 L beaker on stir plate and place stir rod inside
4. Add ~800 mL of nanopure water to beaker from graduated cylinder
5. Turn stir plate on to about 6 to 8
6. Add PBS powder to beaker
7. Rinse remaining contents of PBS powder container with nanopure water and add to beaker
8. Add remainder of nanopure water to beaker
9. Once all powder is dissolved measure pH with pH meter
10. Adjust pH to between 7.2-7.4 by adding HCl (pH>7.4) or NaOH (pH<7.2) dropwise using transfer pipette
11. Place PBS solution and thawed antibiotic under biohood
12. Filter 500 mL of media solution into each sterile bottle
13. Invert antibiotic container to mix then pipette 5 mL into each bottle
14. Cap bottles, remove them from the biohood, and label them
15. Store PBS bottles in refrigerator (4 °C)

Appendix 8: Media Preparation



α Minimal Essential Media Preparation

1.18g Sodium bicarb for F-12 HAM
No sodium bicarb for L-15 Laib

NOTES:

- Should make 1 L of media containing 10% FBS and 1% antibiotic
- Make sure to label media bottles with
  1. Type of media
  2. Your initials
  3. Date media was made
- FBS and antibiotic should be in aliquots located in the freezer(-20 °C)
- When thawing antibiotic in water bath do not leave in more than 15 min. If left in water bath too long contents will precipitate and adhere to falcon tube.
- Use cell culture tested sodium bicarbonate, sodium hydroxide, and hydrochloric acid.
- Rinse off pH probe before placing it in or removing it from its storage buffer.

MATERIALS:

1. Powdered minimal essential media alpha medium (αMEM, 12000-022, Invitrogen)
2. Sodium bicarbonate ($NaHCO_3$, S5761, Sigma)
3. Sodium hydroxide (NaOH, S2770, Sigma)
4. Hydrochloric acid (HCl, H9892, Sigma)
5. Fetal bovine serum (FBS, Atlanta Biologicals, 2 x 50 mL falcon tubes, -20 °C)
6. Antibiotic:Antimycotic (400-101, Gemini Bio-Products, 1 x 10 mL falcon tube, -20 °C)
7. 1 L graduated cylinder
8. 1 L beaker
9. Magnetic stir rod
10. Weighing boat
11. Transfer pipette
12. pH meter
13. Sterile bottles
14. 500 mL bottle-top vaccum filter (0.2 µm pore size)

PROTOCOL:

1. Place FBS and antibiotic in water bath to thaw
2. Measure out 1 L of nanopure water using graduated cylinder
3. Place 1 L beaker on stir plate and place stir rod inside
4. Add ~800 mL of nanopure water to beaker from graduated cylinder
5. Turn stir plate on to about 6 to 8
6. Add αMEM powdered medium to beaker. Water should turn a yellow color.
7. Rinse remaining contents of media container with nanopure water and add to beaker
8. Weigh out 2.2g of sodium bicarbonate using a weighing boat and scale
9. Add sodium bicarbonate to beaker. Beaker contents should turn red.
10. Rinse out weighing boat with remainder of nanopure water and add to beaker
11. Once all powder is dissolved measure pH with pH meter
12. Adjust pH to between 7.2-7.4 by adding HCl (pH>7.4) or NaOH (pH<7.2) dropwise using transfer pipette
13. Place media solution, thawed FBS, and thawed antibiotic under biohood
14. Filter 450 mL of media solution into each sterile bottle
15. Invert FBS containers to mix them then pipette 50 mL into each bottle
16. Invert antibiotic container to mix then pipette 5 mL into each bottle
17. Cap bottles, remove them from the biohood, and label them
18. Store media bottles in refrigerator (4 °C)
19. Dispose of excess unfiltered media down lab sink

Appendix 9: Cryogenically Freezing Cells

Cryogenically Freezing Cells

NOTES:

- Make sure Mr. Freeze is thawed out before use. If Mr. Freeze is found in -80 °C freezer place at room temperature for a day to thaw.
- Make sure to label vials with:
    1. Description of Contents (i.e. cell type, passage, amount of cells)
    2. Date
    3. Your Name (can be initials)
    4. Professors Initials
- When removing media from pelleted cells do not let vacuum pipette go past the top of the bend of the cone of the falcon tube. Going past the bend can result in loss of cells.

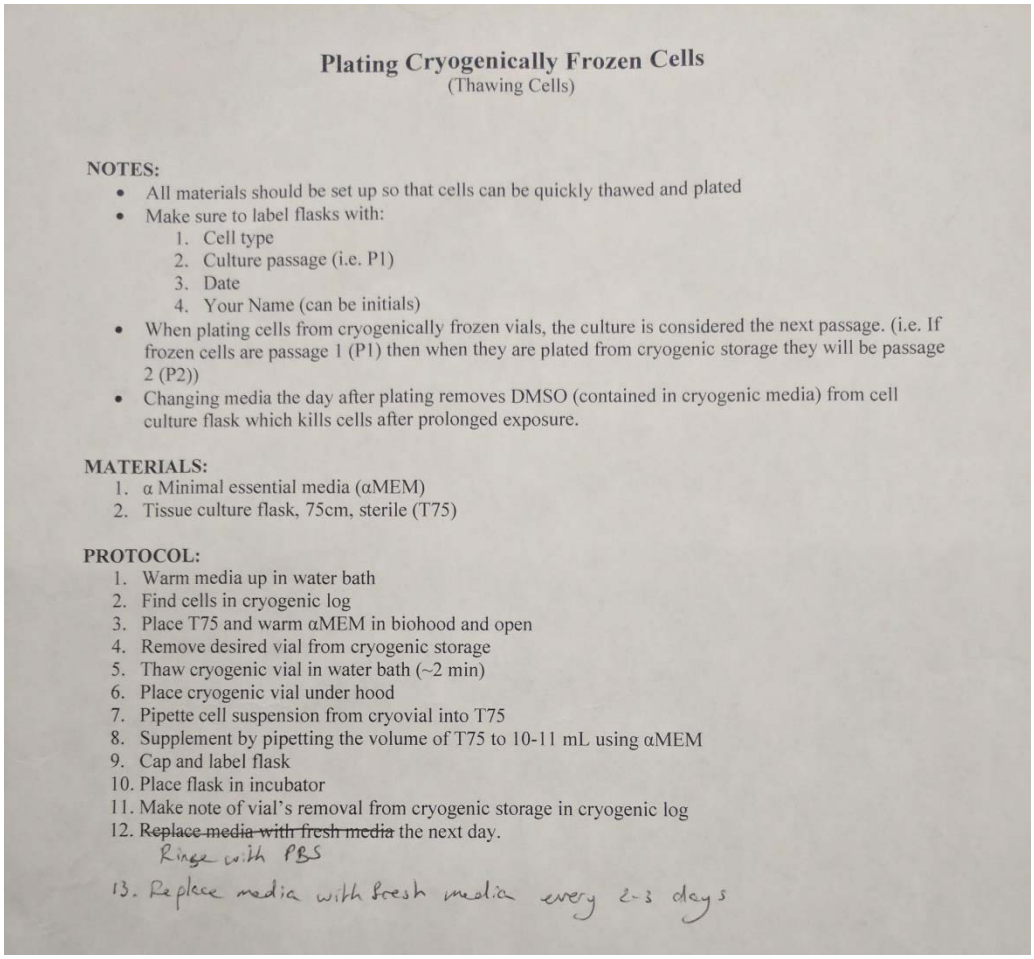
MATERIALS:

1. Dimethylsulfoxide (DMSO)
2. Fetal bovine serum (FBS, Atlanta Biologicals)
3. Phosphate Buffered Saline (PBS)
4. α Minimal essential media (αMEM)
5. Trypsin (Invitrogen)
6. 15 mL falcon tubes
7. Glass pipettes
8. Disposable pipettes
9. Cryogenic vials
10. Mr. Freeze

PROTOCOL:

1. Create cryogenic media composed of 10% DMSO in FBS
2. Carefully remove old media from culture flask using a glass pipette and vacuum
3. Rinse cells with 3-5 mL of PBS
4. Remove PBS with a glass pipette and vacuum
5. Pipette 1 mL of Trypsin into flask
6. Close flask and place in incubator for 5-10min (monitor with microscope to detect when detachment has taken place)
7. Remove flask from incubator and tap flask against counter to dislodge any cells that have not been fully detached by Trypsin
8. Rinse cells from bottom of flask with 9 mL of fresh αMEM
9. Remove cell suspension from flask and place in 15mL falcon tube
10. Make sure cells are evenly distributed throughout media then take small sample for counting
11. Count cells
12. Centrifuge cell suspension at 2000 RPM for 5 min using centrifuge in D204
13. Carefully remove media from pellet using a glass pipette and vacuum
14. Suspend cells in cryogenic media at 1-2 million cells per 1 mL of media
15. Pipette 1 mL of suspended cells per cryogenic vial
16. Close vials and label
17. Place vials in Mr. Freeze evenly starting in center and moving outward
18. Close Mr. Freeze and place in -80 °C Freezer (located in Dr. Harrison's lab, D201) for ~12 hours (or overnight) to allow vials to freeze gradually
19. Transfer vials to liquid nitrogen cryogenic storage (located in D211)
20. Create entry for the deposit of cells in cryogenic log

Appendix 10: Plating Cells from Cryopreservation



Plating Cryogenically Frozen Cells
(Thawing Cells)

NOTES:

- All materials should be set up so that cells can be quickly thawed and plated
- Make sure to label flasks with:
  1. Cell type
  2. Culture passage (i.e. P1)
  3. Date
  4. Your Name (can be initials)
- When plating cells from cryogenically frozen vials, the culture is considered the next passage. (i.e. If frozen cells are passage 1 (P1) then when they are plated from cryogenic storage they will be passage 2 (P2))
- Changing media the day after plating removes DMSO (contained in cryogenic media) from cell culture flask which kills cells after prolonged exposure.

MATERIALS:

1. α Minimal essential media (αMEM)
2. Tissue culture flask, 75cm, sterile (T75)

PROTOCOL:

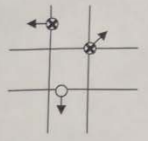
1. Warm media up in water bath
2. Find cells in cryogenic log
3. Place T75 and warm αMEM in biohood and open
4. Remove desired vial from cryogenic storage
5. Thaw cryogenic vial in water bath (~2 min)
6. Place cryogenic vial under hood
7. Pipette cell suspension from cryovial into T75
8. Supplement by pipetting the volume of T75 to 10-11 mL using αMEM
9. Cap and label flask
10. Place flask in incubator
11. Make note of vial's removal from cryogenic storage in cryogenic log
12. ~~Replace media with fresh media~~ the next day.
    Rinse with PBS
13. Replace media with fresh media every 2-3 days

Appendix 11: Counting Cells with the Hemocytometer

Counting Cells Using Hemocytometer

NOTES:

- Counts are usually performed on ~100 µL samples taken after cells are lifted from plates but before centrifugation.
- Cell counts are usually performed outside of the biohood.
- Trypan blue stains dead cells a dark blue color. When counting cells only count cells that are alive indicated by a white translucence.
- When counting cells. Cells that fall on borders move to the nearest outer section for counting. Cells on inside border intersections and outside section borders are not counted.

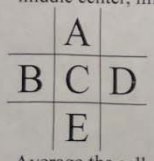


MATERIALS:

1. Centrifuge tubes
2. Trypan blue
3. Hemocytometer
4. 2-20 µL pipettor
5. 0.5-10 µL pipette tips
6. 1-200 µL pipette tips

PROTOCOL:

1. Take ~100 µL sample from well mixed cell suspension and place in centrifuge tube.
2. Make sure suspension sample is mixed well then pipette 20 µL of suspension sample and 20 µL of trypan blue into a clean centrifuge tube.
3. Use heated breath to form condensation on hemocytometer cover slip and place over grid sections of hemocytometer base.
4. Make sure trypan blue/cell suspension is well mixed then carefully pipette 10 µL of trypan blue/cell suspension mixture to each side of hemocytometer using the filling troughs on each side. (Make sure there are no air bubbles on either side. If there is the hemocytometer needs to be washed and refilled)
5. Count the cells in the 5 heavy grid sections on both sides. (See picture, top center, middle left, middle center, middle right, bottom center)



6. Average the cell counts for each side of hemocytometer then add them together.
7. Calculate the number of cells in the suspension
   a. (A+B+C+D+E)/5 = ave
   b. (ave_1 +ave_2) * 10 * amount of suspension media(µL) = # of cells
   c. Example: # of cells suspended in 10 mL of media if cell count is 45
      i. 45 *10 * 10,000 µL = 4.5 million cells

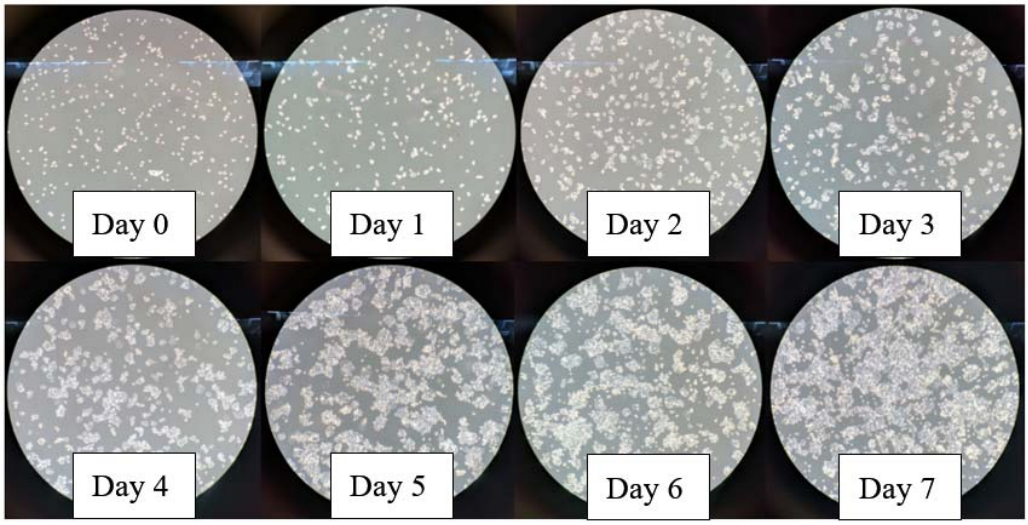
Appendix 12: Decellularization of the Amniotic Membrane

3.1 AMNIOTIC MEMBRANE DECELLULARIZATION PROTOCOL

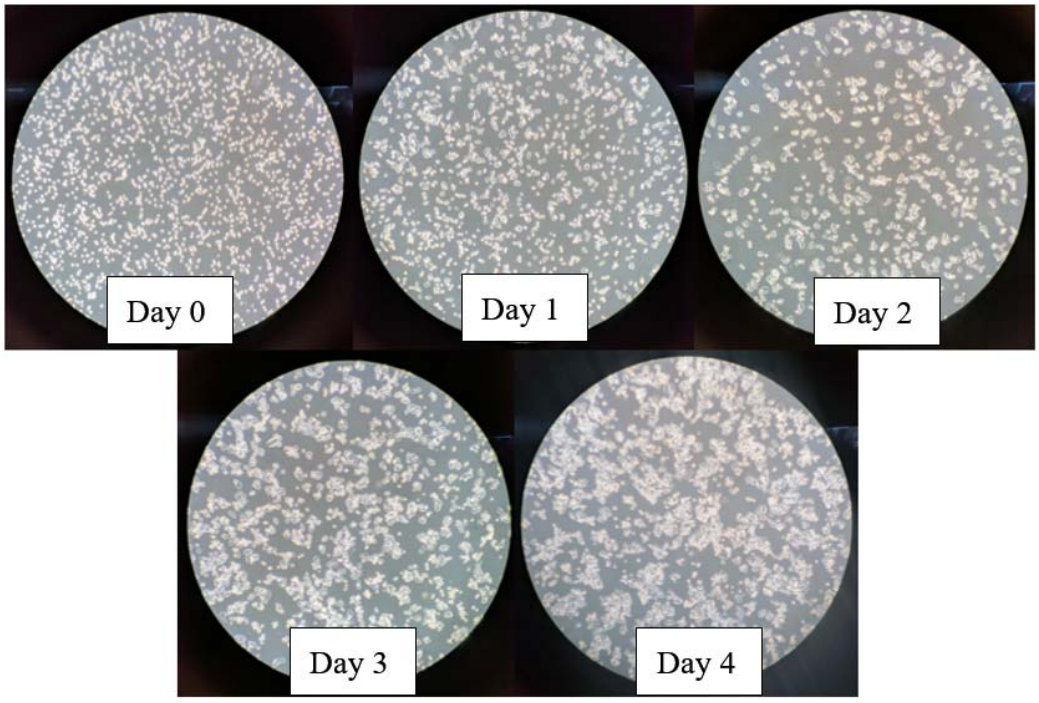
PROCEDURE:

1. Physically separate amniotic membrane from chorionic membrane with blunt dissection
2. Rinse membrane in a beaker of distilled (DI) water and agitate for several minutes to remove blood and facilitate swelling of the spongy layer
3. Remove the membrane from the beaker and lay flat with the stromal side facing-up (stromal side can be identified by its thick, loosely-connected collagen surface)
4. Using a mechanical scraper, gently remove the nonhomogeneous spongy layer in a sweeping motion while being careful not to damage the underlying basement membrane.
5. Keeping the membrane flat, use a scalpel to cut several rectangular sections of about 8 X 15 cm. Discard any tissue with holes or tears.
6. Rinse membranes in DI water again to rid unwanted blood clots then place each membrane into separate bottles
7. Place the membranes into empty 250 mL bottles and freeze at -86°C for about two hours, then allow them to thaw in a water bath or incubator at 37°C for 15 minutes. Repeat this process.
8. Briefly rinse the membranes in DI water, and then transfer to a 500 mL bottle with 400 mL DI water and 0.03% (w/v) sodium dodecyl sulfate (SDS). Place on an orbital shaker to agitate at 100rpm for 12-24 hours.
9. Wash the membranes by transferring them to new bottles of 400mL DI water on an orbital shaker at 100 rpm (making changes every 5, 15 and 40 minutes, and 1, 6 and 24 hours) to remove residual chemicals
10. Incubate membranes with 20-50µg/mL DNase in a Tris buffer solution (50mM Tris-HCl, pH 7.5, 10mM $MgCl_2$) at 37°C for 2 hours on an orbital shaker at 100rpm to remove remaining DNA.
11. Rinse again in DI water
12. Finally, subject membranes to a mixture of 0.2% (v/v) peracetic acid (PAA) and 4% ethanol in DI water for 2 hours on an orbital shaker at 100rpm to ensure that they are fully sterilized (wrap in aluminum foil to maintain activity)
    MEMBRANE IS NOW STERILE. MAKE ALL REMAINING TRANSFERS UNDER BIOHOOD
13. Perform several washes in STERILE DI water on shaker at 100rpm (10min, 20min, 30min)
14. Perform another STERILE DI water wash for 24 hours on shaker at 100rpm
15. Place membrane in STERILE PBS solution for 24 hours on shaker at 100rpm. Record pH after moving membrane to PBS, then record pH of PBS after 24 hours (should be 7.2<pH<7.4)
16. Place final membranes into STERILE PBS solution with antibiotics and store in 4°C fridge
    Label bottles with your name and dates of 1. when obtained, 2. when stored

Appendix 13: SW-620 Culture in Flask (directly from cryopreservation)



Appendix 14: SW-620 Cells Culture in Flask (progression after splitting)



Appendix 15: Glossary

Glossary

Amniotic membrane: Innermost, thin membrane of the placenta

Cryopreservation: The state of freezing cells in liquid nitrogen at -196°C

F-12k HAM: Media used to culture PC-3 cells

Gleason score: A score quantified by a pathologist to classify prostate cancer

In situ: In a localized state or condition [33]

In vitro: Occurs in a laboratory vessel outside of the body [34]

In vivo: Made to occur within a living organism [35]

L-15: Media used to culture SW-620 and MDA-MB-231 cells

MDA-MB-231: Breast cancer cell line obtained from ATCC

Nanopure water: Water with 18.2 megaohm ionic purity with negligible bacteria [36]

PC-3: Prostate cancer cell line obtained from ATCC

Quant-iT™ PicoGreen™ dsDNA Assay Kit: DNA assay kit used to determine concentration of DNA in a sample using absorbance

RPMI-1640: Media used to culture PC-3 cells

Seeding: Adding cells to a material or flask at a specific concentration and volume

Sonication: Sound energy is applied to agitate particles in a sample

Splitting: The action of lifting cells from a flask and moving them to new flasks for culture expansion

Static culture: Culture conditions with no flow regime

SW-620: Colon cancer cell line obtained from ATCC

Tumorigenesis: Process of generating new tumors

Appendix 16: Abbreviations

Abbreviations

ATCC: American Type Culture Collection

ATM: Human ataxia-telangiectasia locus protein

CO_2: Carbon dioxide

CSC: Cancer stem cell

DMEM: Dulbecco's minimal essential medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EGFR: Epidermal growth factor receptor

EMT: Epithelial-to-mesenchymal transition

ER: Estrogen receptor

ESA: Human surface antigen

GAD67: Human glutamate decarboxylase

GAPDH: Glyceraldehyde-3-phostphate dehydrogenase

GSTM3: Human glutathione transferase M3

Humirf5: Human interferon regulatory factor 5

MCT: Monocarboxylate transporter

MSC: Mesenchymal stem cell

ncRNA: Non-coding RNA

P19: Human serine proteinase inhibitor

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PLLA: Poly-L-lactic acid

PR: Progesterone receptor

PSA: Prostate-specific antigen

RNA: Ribonucleic acid

TFAR15: *Homo sapiens* apoptosis-related protein TFAR15

Vat C: Human subunit C of V-ATPase