2020

Optimizing Cell Culture Media for Combined Use for Ex Vivo Lung Bioengineering

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Abstract

The prevalence of lung diseases such as chronic obstructive pulmonary disease (COPD) is on the rise and with it the demand for donor lungs for transplantation in end-stage disease patients (Wagner et al., 2013). This demand continually exhausts the available donor lung supply, therefore, novel alternatives for lung transplantation are being investigated. One of these potential solutions is the creation of a bioengineered synthetic lung for use in transplantation. A successful methodology would result in an independence from the limited supply of donor lungs as well as a solution to immune rejection by the transplant recipient, as cells from the recipient could be used to cellularize the engineered lung and minimize rejection. A proposed protocol for the synthesis of bioengineered lungs involves the use of a lung scaffold, obtained by removing all cellular material from a pre-existing lung, leaving on the extracellular matrix proteins, and seeding it with patient cells to grow a new, viable lung (Uriarte et al., 2018). However, one problem that currently hinders progress is the ability to seed multiple cell lines on a single scaffold. Currently a single cell line can be seeded, however this is not representative of a completely functioning lung possessing over 40 types of cells. Therefore, this study focused on developing a preliminary protocol on how to co-seed multiple cell lines, traditionally cultured in different growth media, on a single scaffold. In this study, cell culture was conducted with human lung fibroblast cells (HLFs) and human bronchial epithelial cells (HBEs). The two cell lines were initially grown and observed in their own respective media to determine proportional growth rates. They were then seeded individually and as co-seeds in plates varying in media components and in cell counts. It was found that a co-seed of HLF and HBE cells is possible over a seven-day period in a media composed of 50% HBE and 50% HLF media. Growth in this trial followed an exponential trend, and the cells were interspersed indicating that they were cohabitating in a healthy manner.

Introduction

There are currently two viable solutions for end-stage lung diseases: lung transplants and long-term lung assist devices. However, these two treatment plans are not able to support the number of people requiring lung therapy in the US. Lung transplantation is significantly limited by the

lack of donors and the high complication potential brought on by the recipient's immune system. The median waiting time for a lung transplant in the US has been reported to be 4 months, while 34.7% of candidates have to wait over a year. Between 2010 and 2012, an average of 329 patients died while on the waiting list per year. (Valapour et al., 2014). Lung transplantations also have a high risk of rejection, with about 16% pf patients experiencing acute rejection within the first year post-transplant (Valapour et al., 2020). Lung assist devices, specifically extracorporeal membrane oxygenation devices, play a role in supporting those waiting for transplants, however, they require hospitalization and special care and are therefore not a cost efficient solution (Doufle et al., 2016). These shortcomings leave a serious gap in end-stage lung disease therapies, exacerbated by the fact that COPD is predicted to become the third leading cause of death by the year 2020 (Lopez, 2006).

Organ bioengineering has been investigated as a novel solution to the shortage of donor lungs. The goal of organ bioengineering is to create functional human organs with preserved structure which lack any foreign material that could be recognized and attacked by host immune systems (Tsuchiya et al., 2014). This will increase the number of available organs, since there will no longer be a reliance on matching donors, and theoretically decrease the post-transplant complications from host immune responses. Bio-printing organs has been suggested as a solution, but current manufacturing technology is unable to create the complex structures essential for lung function (Wagner et al., 2013). The process of decellularizing and recellularizing organs provides the opportunity to fulfill both aforementioned goals of bioengineering lungs, as the extracellular matrix structure can be preserved while all cellular components are removed. If the extracellular matrix of an organ was obtained and then recellularized using cells from the recipient, the host immune response would be circumvented. This strategy has been shown to regenerate other tissues such as blood vessels, heart and liver (Wertheim et al., 2012, Ott et al., 2008, Krawiec et al., 2012). Using previous methods and models, lung recellularization is within the realm of possibility in the near future.

Bioengineering lungs begins with a lung scaffold, and many different scaffolds have been experimented with. Human lungs are being investigated as the most logical lung scaffold available. For purposes of experimentation, animal scaffolds are being investigated as well. Avian lungs have been subjects of particular interest, due to their increased gas exchange efficiency resulting from being functional at a high altitude. Therefore, avian lungs could provide the most effective scaffolds for recellularized lungs (Wrenn et al., 2018). Once a scaffold has been obtained, it needs to be recellularized with the cell types necessary for lung function.

This study was conducted to address the issue of co-culturing the necessary cells of a functional lung. Currently there is no protocol for seeding more than one cell line on a single scaffold. To achieve a bioengineered lung ready for transplant, the 40+ cell types must be cultured together. The goal of this study was to address the hypotheses human bronchial epithelial (HBE) and human lung fibroblast (HLF) cells are amenable to co-culture and a combination of each respective media leads to an increase in proliferation and viability of both. cell types in co-culture

To investigate this hypothesis, cells were co-cultured in various concentrations with various medias. These investigations will gain insight into key steps in the biosynthetic lung creation progress, and allow for progression towards a completely bioengineered functional lung.

Materials & Methods

Methods for Determining Relative Growth Rates

Lines of both HBE and HLF cells were established in tissue culture flasks three days prior to beginning cell culture experimentation. HBE media contained 99% Keratinocyte-Serum Free medium, 1% streptomycin and penicillin solution, 0.005 mg/mL insulin and 500 ng/mL hydrocortisone supplement. HLF media contained 89% minimal essential media, 10% fetal bovine serum and 1% streptomycin and penicillin solution. Supplement included in Keratinocyte-Serum Free medium and minimal essential medium are listed in **Table 1.**

Table 1

Minimal Essential Media		Keratinocyte-Serum Free Media*	
Component	Concentration (mg/L)	Component	Concentration (mg/L)
Amino Acids		Bovine Pituitary Extract (BPE)	50.0
L-Arginine hydrochloride	126.0	rEGF (human recombinant)	0.005
L-Cystine 2HCl	31.0	$CaCl_2$	N/A
L-Glutamine	292.0	L-Glutamine	N/A
L-histidine hydrochloride-H2O	42.0	Phenol Red	N/A
L-Isoleucine	52.0		
L-Leucine	52.0		
L-Lysine hydrochloride	73.0		
L-Methionine	15.0		
L-Phenylalanine	32.0		
L-Threonine	48.0		
L-Tryptophan	10.0		
L-Tyrosine disodium salt dehydrate	52.0		
L-Valine	46.0		
Vitamins			
Choline chloride	1.0		
D-Calcium pantothenate	1.0		
Folic Acid	1.0		
Niacinamide	1.0		
Pyridoxal hydrochloride	1.0		

Riboflavin	0.1
Thiamine hydrochloride	1.0
i-Inositol	2.0
Inorganic Salts	
Calcium Chloride anhydride	200.0
Magnesium Sulfate anhydride	97.67
Potassium Chloride	400.0
Sodium Bicarbonate	2200.0
Sodium Chloride	6800.0
Sodium Phosphate monobasic	140.0
Other components	
D-Glucose (Dextrose)	1000.0
Phenol Red	10.0

MEM catalog reference # 11095, Keratinocyte-SFM catalog reference # 17005-042

**Some concentrations and additional components are reserved by the manufacturer*

HLF and HBE cells were plated in two 12-well tissue culture plates using the schematic outlined in **Figure 1**. The first plate consisted of exclusively HLF cells while the second consisted of exclusively HBE cells. Each cell line was plated in 2 mL total of their respective medias. Medium was added to each well and the cells were seeded directly in the center of each well. Cell were seeded in varying amounts in order to be able to determine the confluency at 7 days achieved by the different cell seed counts. Cell concentrations were in accordance with a cell count that was conducted prior to seeding utilizing a hemocytometer. After seeding, each plate was swirled to ensure complete homogenization of the wells. The plates were incubated for a total of eight days. Observations under a standard light microscope were made and recorded on day 3 and day 8 to assess confluency.

200	**200**	**400**	**400**
800	**800**	**1600**	**1600**
3200	**3200**	**6400**	**6400**

Figure 1: Cell seed counts per well in a 12-well plate culture.

Methods for Determining Cell Survivability in Presence of Foreign Media

Lines of both HBE and HLF cells were established in tissue culture flasks three days prior to beginning cell culture experimentation. HBE and HLF media components were identical to those mentioned previously.

HLF and HBE cells were plated in two 12-well plates as shown in **Figure 2**. An estimation of a cell seeding numbers that would result in near 90% confluency for both cell lines was conducted based on the above experiment. This estimation was used to seed the plates in this experiment. Each trial was done in duplicate. Media was added to each well preceding cell seeding, in correspondence to the ratios presented in **Figure 1**. For the combined media using part HLF and part HBE media, a stock media solution was first created. Cells were plated in the center of each well, and swirled to ensure equal dispersion. Seeding numbers (**Figure 3**) were in accordance with a cell count that was conducted with a hemocytometer prior to seeding. The plates were incubated for a total of seven days. Observations under a standard light microscope were made and recorded on day 2, 5 and day 7 to visually assess cell growth.

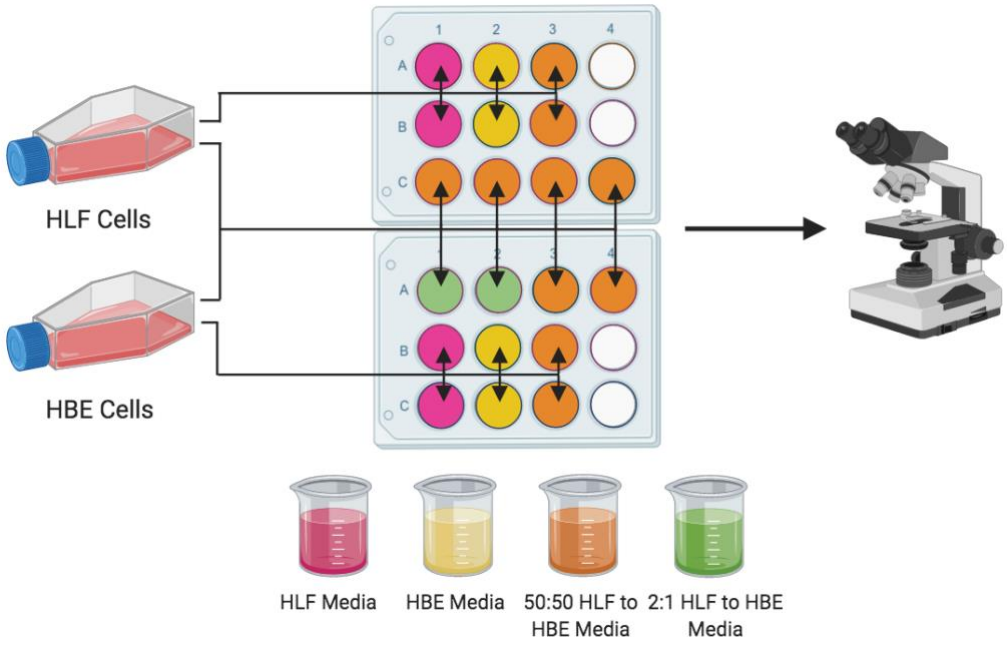


Figure 2: Schematic for *Methods for Determining Cell Survivability in Presence of Foreign Media* experiment. Cell lines were established in flasks and seeded in isolation and as co-seeds in vary media concoctions. Observations were conducted on days 2, 5 and 7. Schematic prepared with BioRender.

Cell Media								
	3200 HLF 100% HLF	3200 HLF 100% HLF	3200 HLF 100% HBE	3200 HLF 100% HBE	1000 HBE 2000 HLF 50:50	1000 HBE 2000 HLF 50:50	2000 HBE 1000 HLF 50:50	2000 HBE 1000 HLF 50:50
	2000 HBE 100% HLF	2000 HBE 100% HLF	2000 HBE 100% HBE	2000 HBE 100% HBE	1000 HBE 2000 HLF 1:2 HBE:HLF	1000 HBE 2000 HLF 1:2 HBE:HLF	2000 HBE 2000 HLF 50:50	2000 HBE 2000 HLF 50:50
	3200 HLF 50:50	3200 HLF 50:50	2000 HBE 50:50	2000 HBE 50:50				

Figure 3: Cell seed counts and media ratios for 12-well plate culture.

Methods for Obtaining Quantitative Data of Cell Growth

Seeding cell counts were determined by results found in the *Determining Relative Growth Rates* experiment. It was found that the wells seeded with 2000 HBE and 2000 HLF produced the healthiest and most balanced result, so this seed count was used for all future co-seeds. Additionally, the HBE cells in the experiment above were not as proliferative as expected even in their own media, so the seed count for HBEs in isolation was increased from 2000 to 3000.

Seed counts used are displayed in **Figure 4**. The plates were incubated for a total of seven days. Photos with an EVOS microscope were taken at 10X magnification and a PrestoBlue Cell Viability assay (Invitrogen) was performed on days 1, 3, 5 and 7 to test for cell viability and proliferation. This experiment was done in triplicate.

3200 HLF	3200 HLF	3200 HLF	3200 HLF
3000 HBE	3000 HBE	3000 HBE	3000 HBE
2000 HLF 2000 HBE	2000 HLF 2000 HBE	2000 HLF 2000 HBE	2000 HLF 2000 HBE

Figure 4: Cell seed counts per well in a 12-well plate culture.

Methods for Cell Growth Trend Analysis

The data gathered in the three biological trials in the experiment outlined above were used to create growth curves for each cell line in each different media. First, the four technical replicates in each trial were averaged to get a single data point for the fluorescence each day in each media. These values were then averaged across all three trials to produce one graph for each cell type representing the data gathered in all three biological replicates. Standard error was calculated between the three average values for each plot point found in each biological replicate.

Results

The primary goal of this study was to determine the relative growth rates of HLF and HBE cells lines, the survivability of each cell line in different medias and in co-culture, and gain quantitative data on cell growth in co-culture. Observational data was used to determine relative growth rates and the survivability of each cell line in different media. Photomicrographs and the PrestoBlue Cell Viability Assay were used to fulfill the third objective of gaining quantitative growth rate data.

The observed confluencies on the day 8 of the Relative Growth Rate experiment are displayed in **Figure 5**. An initial cell seeding count of 800 HBE cells resulted in 60% confluency, indicating that this cell count would cover roughly half of a well at the end of future seven day experiments. An initial cell seeding count of 1600 HLF cells resulted in 50%

confluency, indicating that this cell count would cover roughly half of a well at the end of future seven day experiments.

Seeded cells/well	HLF	HBE
200	3%	10%
400	10%	40%
800	30%	60%
1600	50%	80%
3200	90%	100%
6400	100%	100%

Figure 5: Confluency observations on day 8 of relative growth rate experiment. These numbers are strictly observations, and as such are not perfectly accurate. Reported numbers are a consensus from multiple observers.

The data collected via PrestoBlue Assay pertaining to HLF cells is displayed in **Figure 6**. Days 1, 3, 5, and 7 are plotted against relative fluorescence units. The green curve represents cells in combination media, the pink line represents cells in HLF media and the blue line represents cells in HBE media. HLFs performed similarly in both HLF media and combination media, producing an exponential growth curve. However, they did not perform well in HBE media, scarcely growing at all.

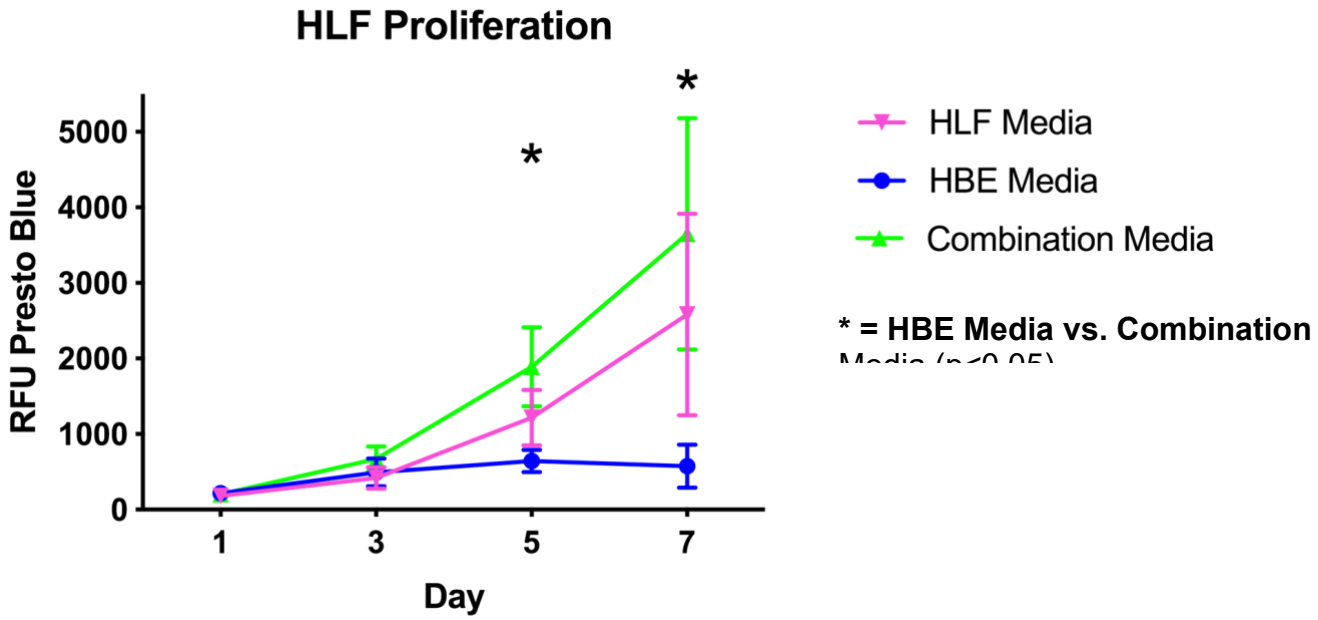


Figure 6: HLF growth curves in HBE, combination and HLF media. Plot points were determined by averaging respective values in 3 biological replicates. Relative fluorescence units were normalized to media without cells. A one-way ANOVA test was conducted on the data showing statistical significance between HBE and Combination media on days 5 and 7. Values are depicted as means ± standard error.

Images taken each day of HLF cells in each media are shown in **Figure 7**. Images are similar across all three trials. In HLF media, cells appear healthy and had an expected morphology. Growth appears to follow a standard, exponential trend. In HBE media, the cells looked unhealthy and appeared to be dying. As the experiment progressed, little to no growth was apparent in the images. In combination media, cells look relatively healthy with few apoptotic bodies, and had the expected morphology. They also appear to follow a similar growth trend as HLF cells in HLF media alone, with trials 2 and 3 exemplifying this the best.

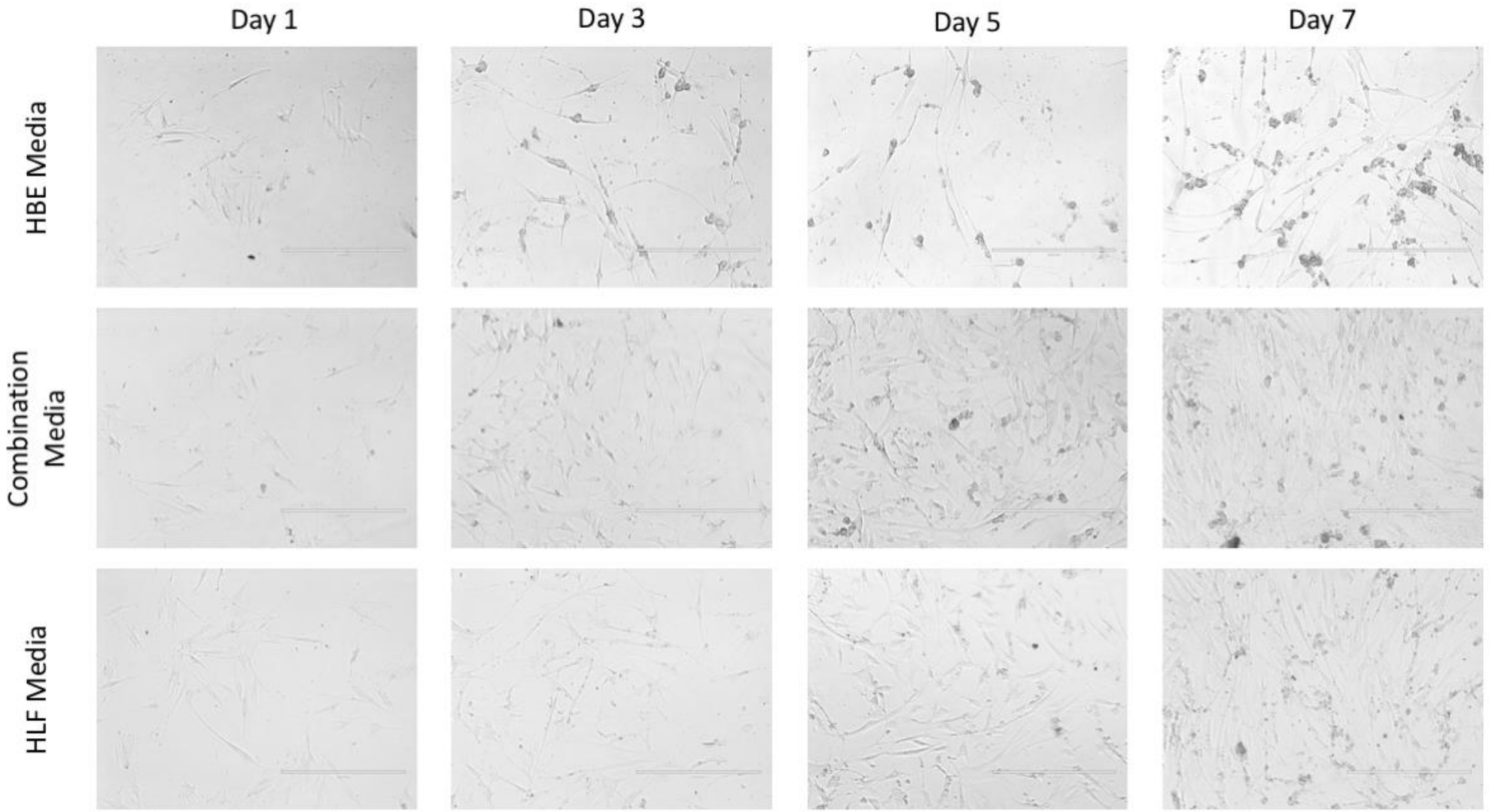


Figure 7: Photos taken of HLF cells at 10X magnification using an EVOS microscope on Day 1, 3, 5 and 7. Images are from trial 3 and are representative of three biological replicates.

The data collected via PrestoBlue Assay pertaining to HBE cells is displayed in **Figure 8**. Days 1, 3, 5, and 7 are plotted against relative fluorescence units. The green curve represents cells in combination media, the pink line represents cells in HLF media and the blue line represents cells in HBE media. HBEs performed the best in combination media, producing a strong exponential growth curve. An exponential growth curve was always produced by HBE media. In HLF media, HBE cells did not perform well, scarcely growing at all.

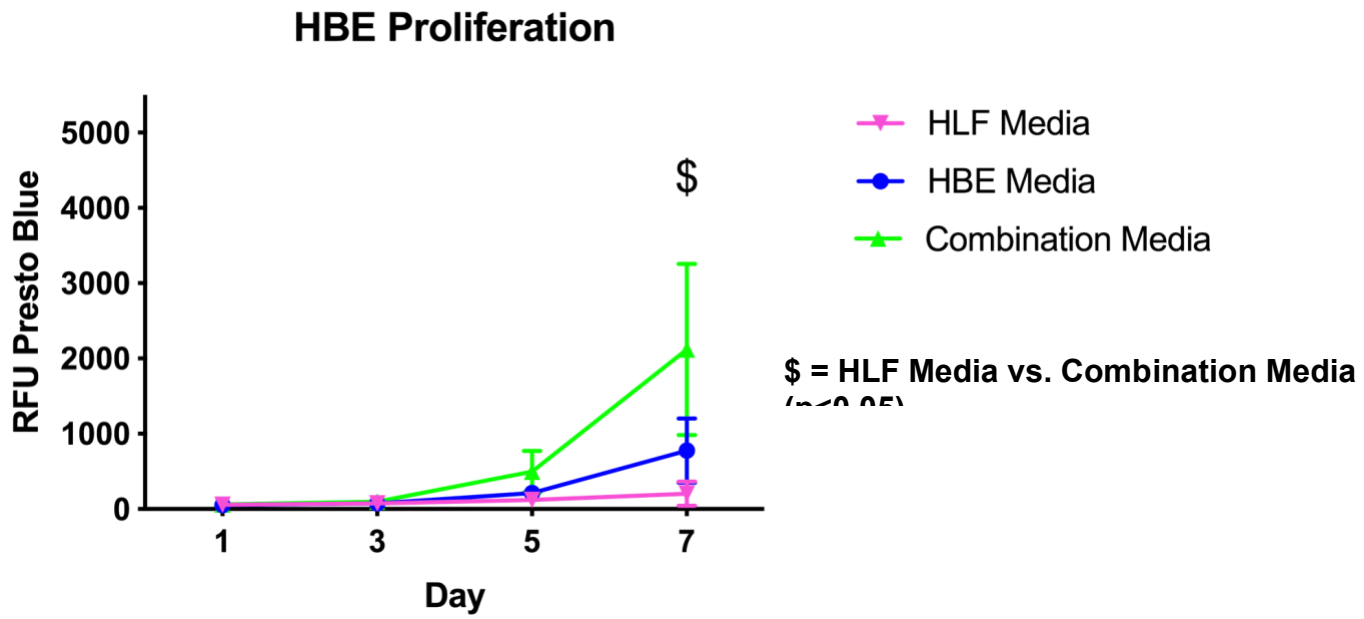


Figure 8: HBE growth curves in HBE, combination and HLF media. Plot points were determined by averaging respective values in 3 biological replicates. Relative fluorescence units were normalized to media without cells. A one-way ANOVA test was conducted on the data showing statistical significance between HLF and Combination media on day 7. Values are depicted as means ± standard error.

Images taken each day of HBE cells in each media are shown in **Figure 9**. Images are similar across all three trials. In HBE media, cells appear healthy, had an expected morphology, and appear to grow exponentially each day. In HLF media, cells looked very unhealthy and are sparsely populated. In combination media, cells look relatively healthy with few apoptotic bodies, had the expected morphology, and appeared to grow at an exponential rate, though reduced when compared to HBE media alone. This is contrary to the calculated growth curves seen in **Figure 8**, as combination media appears to produce the best growth. This suggests that the images taken of HBEs in combination media and HBE media are not representative of the entire well. Trial 1 exemplifies this the best.

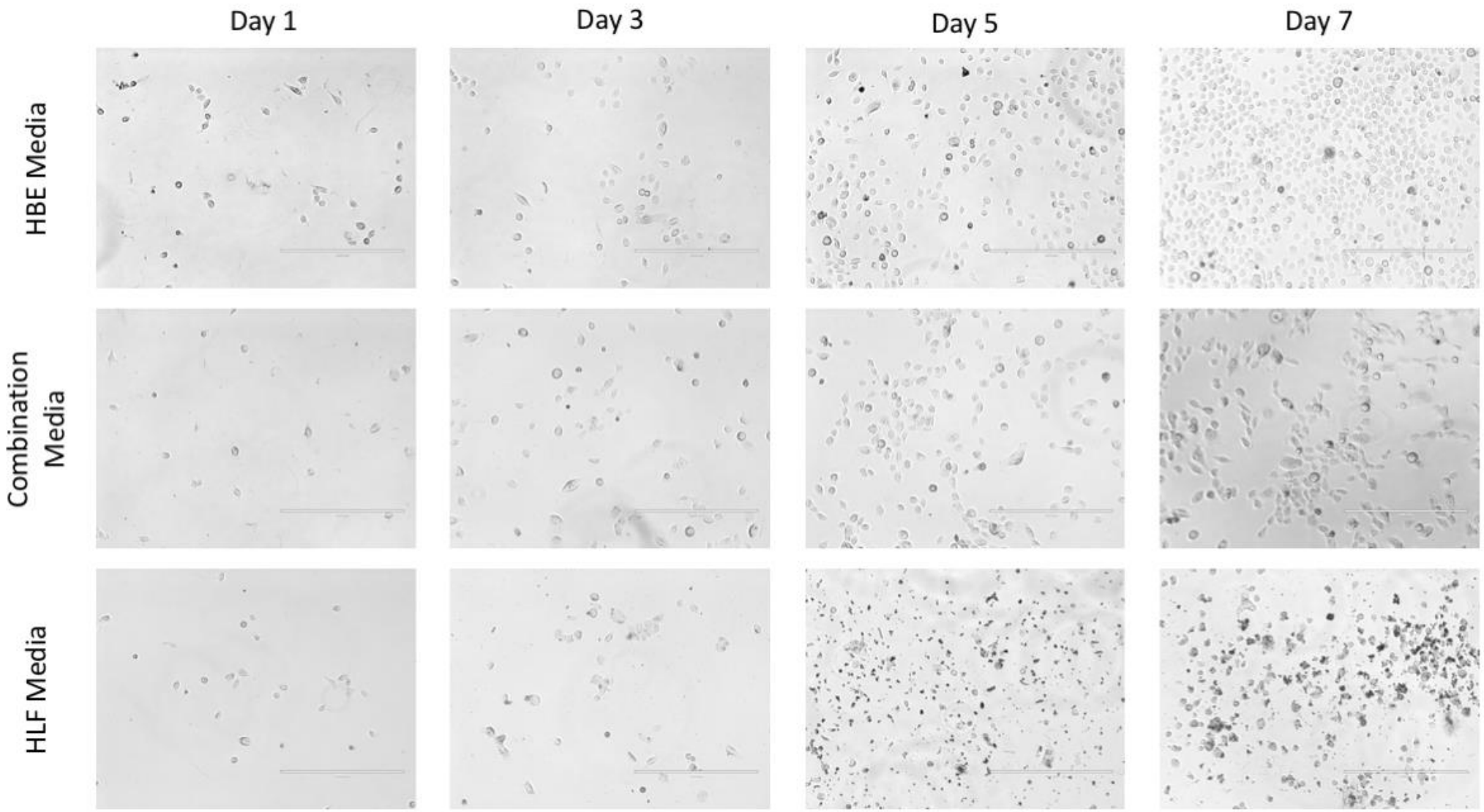


Figure 9: Photos taken of HBE cells at 10X magnification using an EVOS microscope on Day 1, 3, 5 and 7. Images are from trial 3 and are representative of three biological replicates.

The data collected via PrestoBlue Assay pertaining to wells containing combinations of cells is displayed in **Figure 10**. Days 1, 3, 5, and 7 are plotted against relative fluorescence units. The green curve represents cells in combination media, the pink line represents cells in HLF media and the blue line represents cells in HBE media. These cell combinations performed best in combination media, producing a strong exponential growth curve. They also produced an exponential growth curve in HLF media. However, they did not perform well in HBE media, scarcely growing at all.

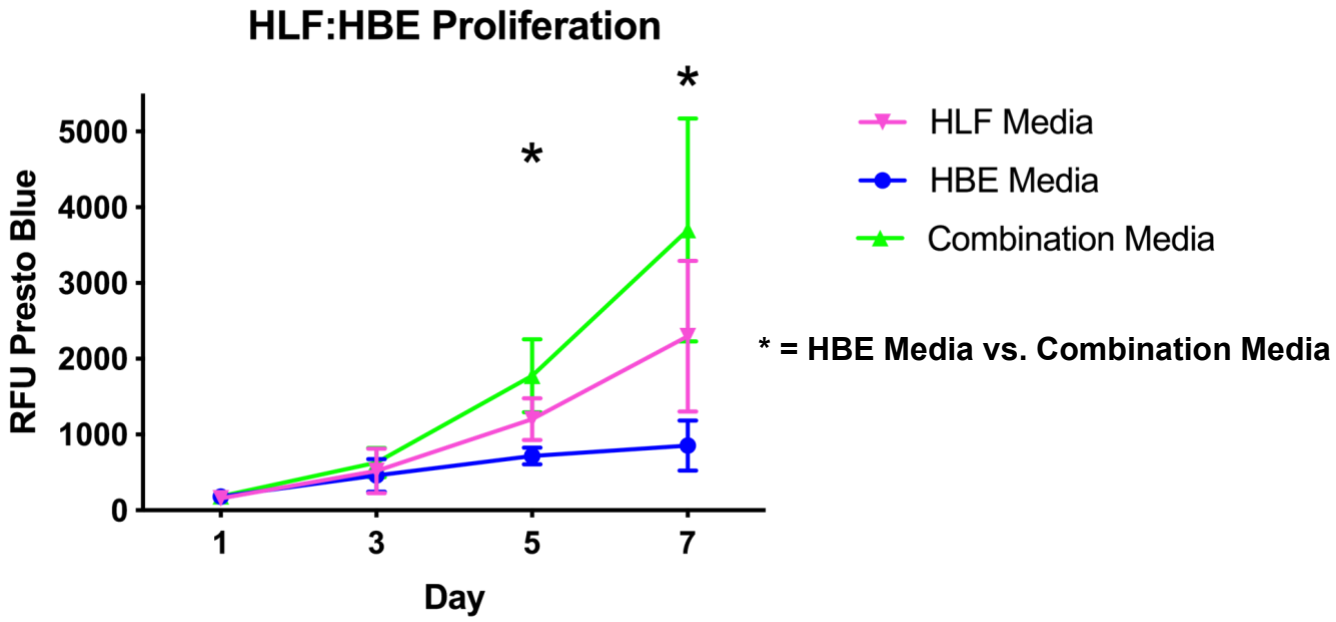
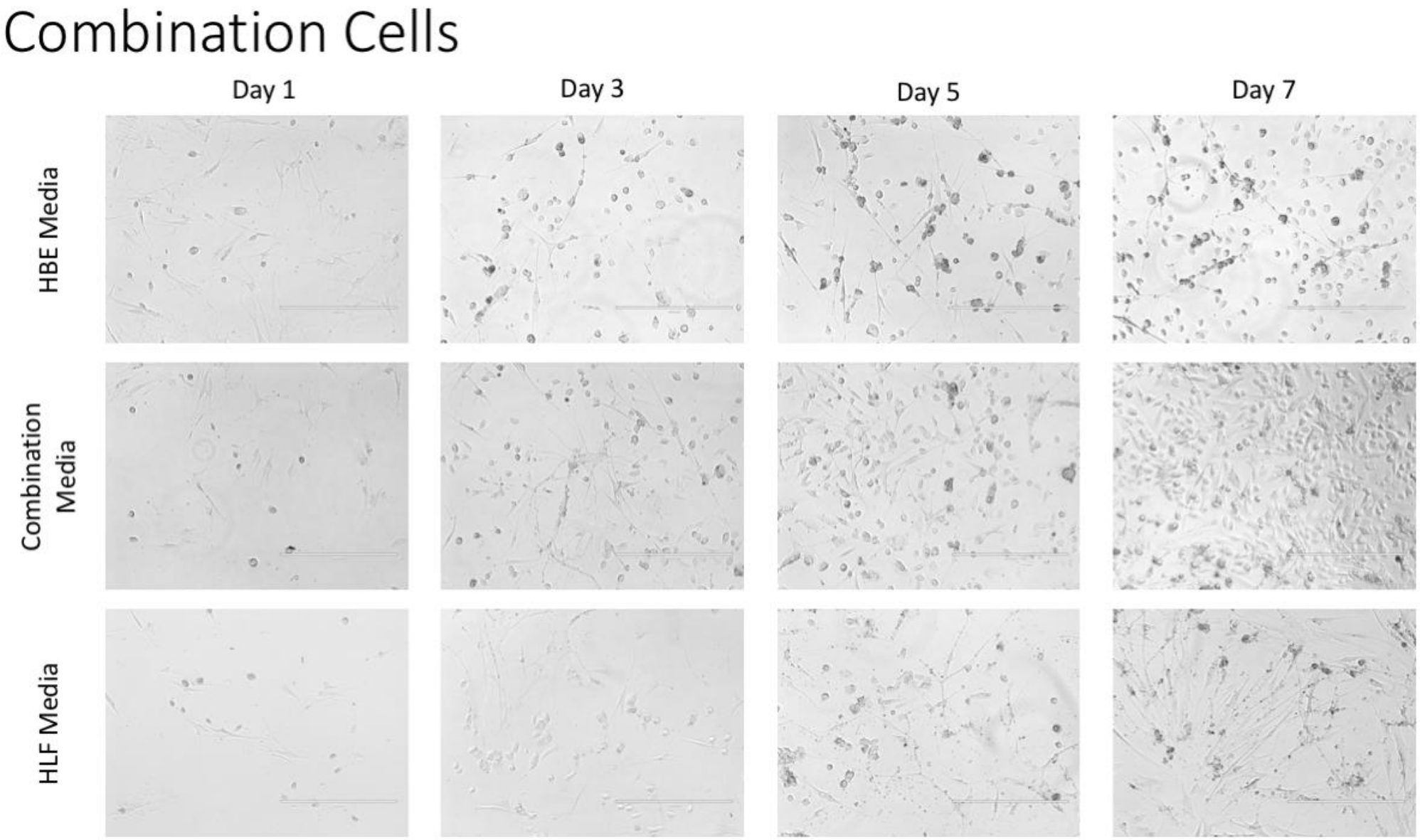


Figure 10: Combination cell growth curves in HBE, combination and HLF media. Plot points were determined by averaging respective plot point in 3 biological replicates. Relative fluorescence units were normalized to media without cells. A one-way ANOVA test was conducted on the data showing statistical significance between HBE and Combination media on days 5 and 7. Values are depicted as means ± standard error.

Images taken each day of combination cells in each media are shown in **Figure 11**. Images are similar across all three trials. In HLF media, both cell types appear to be present, healthy and interspersed. This is also true in combinational media, and cell populations appear to be denser than in HLF media alone. Images from trials 2 and 3 exemplify this the best. In HBE media, neither cell line has a large presence and the HLF cells appear unhealthy.



F**igure 11:** Photos taken of HLF and HBE combination cells at 10X magnification using an EVOS microscope on Day 1, 3, 5 and 7. Images are from trial 3 and are representative of three biological replicates.

Discussion

In the growth rate determination of this study, it was found that HBE cells grow much more rapidly than HLF cells, reaching confluency after 8 days with just 3200 initial cells, while HLF cells needed 6400 cells initially to reach confluency in the same timeframe. This indicated that future co-seed experiments should have a higher concentration of HLF cells seeded than HBE cells. A goal of future co-seed experiments was to have each cell line take up roughly half of a well. It was observed that an initial cell seeding concentration of 800 HBE cells resulted in 60% confluency, while an initial cell seeding concentration of 1600 HLF cells resulted in 50% confluency. Using these numbers, it was determined that future co-seeding experiments would seed 1000 HBE cells and 2000 HLF cells in 12-well plates.

The next stage of the study was assessing cell survivability in the presence of foreign medias. After taking photos on day seven, it found that cells could grow in the presence of the other cell's media, though they needed their own media to proliferate in a healthy manner. It was found that a 50:50 split of the two medias resulted in growth of both cells lines, based on morphology. The wells that produced the healthiest looking co-seed were the ones that had 2000 of both cells lines seeded initially. Therefore, co-seed cell counts for future experiments were changed to reflect this. Additionally, it was observed that HBE cells were not as proliferative expected, even in their own media. The HBE seed cell count was increased from 2000 to 3000 cells per well to help alleviate this issue.

When collecting quantitative data, it was found that HLF cells in isolation performed similarly in both HLF and 50:50 HLF and HBE media combinations, and performed poorly in HBE media. It can be inferred that HLF cells are not hindered by HBE media, as the presence of such media did not affect cell performance. However, it can also be inferred that the presence of HLF media is required and that HBE media does not supply the cell line with all the essential nutrients it needs to grow. HBE cells in isolation followed a similar trend, performing well in HBE and combinational media and poorly in HLF media. Again, it can be inferred that the presence of HLF media does not hinder the growth of HBE cells, however, HBE cells require components only found in their own media to grow successfully. The co-seeded trials performed best in combination media, well in HLF media and poorly in HBE media. Photographs taken of combination cells in combination media showed that based on morphology, both cells were present, healthy and interspersed. This suggests that the presence of HLF cells boosted HBE cells viability, even without its own media present. The literature supports this claim, as fibroblastic cells have been found to provide a feeder role producing a wide variety of ECM components, growth factors and other bioactive compounds to support the proliferation of the surrounding cells (Rajabalian et al., 2003). In this study, HLF cells were likely supporting the growth of the HBE cells by secreting these compounds allowing for steeper growth curves in combination cell trials. The fact that HBE cells within the combination cell trails grew in HLF media alone can be contributed to the support of HLF cells, minimizing the need for the HBE specific media.

An interesting observation can be noted in the photo of HBE cells in combination media on day 7. While overall morphology looks healthy, some cells express a slightly more elongated shape than HBE cells in HBE media. A possible explanation for this could be that those cells are experiencing epithelial-mesenchymal transition, which allows epithelial cells to assume the phenotype of a mesenchymal cell (Kalluri et al., 2009).

Conclusions

The co-seed results and inferences were based solely on morphology. To confirm that both cells indeed were healthy in combination media and HLF media, specific cell populations must be determined. Flow cytometry provides a method to determine such populations. I had planned to do this as the final part of my project. However, this was interrupted by the COVID-19 shut down. In future experiments, the contents of each well on day 7 should be fixed and run with a flow cytometry protocol targeting antibodies specific to each cell in order to determine relative cell densities of HLFs and HBEs.

There was a large margin of error between biological replicates when determining cell growth curves. All trials followed a common trend, but each had varying end population densities. In order to ensure accuracy, more trials should be conducted in order to decrease the standard error between each trial and gain further understanding on each conditions growth curve.

This experiment has shown that HLFs and HBEs can be successfully co-cultured in one media, and that co-culturing produces a possible synergistic effect between the two cell lines that is not present while culturing in isolation. To provide additional insight supporting the overall goal of optimizing conditions for lung recellularization, the project could be extended numerous ways. The apparent support of HBE cells by HLF cells could have recellularization order implications, and experiments could be run to determine if adding one line to an already established culture of the other would further optimize cell growth. Additionally, repeat trials could be conducted on hydrogels and lung 3D structures to investigate the role of growth substrate on cell growth curves. Media concoctions also need further optimization, and trials could be run with slightly altered ratios of combination media.

Acknowledgements

I would like to thank everyone in the UVM Microbiology and Molecular Genetics department and everyone in the Weiss Laboratory for their assistance as I was conducting my research, especially my thesis mentor Dr. Daniel Weiss. I would also like to thank Juan Jose Uriarte for his biomedical engineering expertise, and Evan Hoffman for his cell culture knowledge and continual support throughout the entirety of my project.

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