The Creation of an Animal Component-Free Medium for Mammalian Cells in 2D and 3D Microenvironments

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Abstract

In modern biotechnology, the ability for researchers to grow cells in laboratory settings (*in vitro*) allows them to harness the power of cell culture science to study diseases, develop new technologies, and better understand biological processes. Cell culture medium is the vehicle that supplies cells *in vitro* with the nutrients necessary for maintaining normal cell growth, metabolism, and function. Culture medium often contains added supplements from animal sources that provide a complex mixture of hormones, growth factors, lipids, and other components required for many mammalian cells to survive outside of their native environments. Specifically, fetal bovine serum (FBS) is the most widely used serum supplement for mammalian cell culture, yet its limitations and drawbacks have prompted a search for alternative formulations. The disadvantages of using animal-based supplements such as FBS are highlighted when investigations into their manufacture demonstrate unethical means of harvesting, and analyses of different samples show inconsistencies. While alternative serum-free (SF) options exist, they are often cell-type specific, may contain residual animal-derived components, and are usually developed for non-universal conditions. There is continual progress being made in this field, yet it needs to be built upon by fabricating SF formulations that can grow multiple cell types while remaining fully animal component-free (ACF). This thesis addresses the need for an ACF substitute for FBS in mammalian cell culture by focusing on the development of a medium formulation through empirical experimentation. By identifying essential components for cell growth and function from the literature, various concentrations of these components were systematically tested to evaluate their impact on cell viability and morphology in different types of microenvironments. Diverse microenvironments for cell cultures can be carried out depending on the application, including traditional two-dimensional (2D) growth on Petri dishes, but also three-dimensional (3D) culture on complex scaffolds or in suspension vessels. To explore the universal nature of the medium formulation developed in our work, three distinct investigations were undertaken. In our first investigation, we assess the effectiveness of the optimized formulation on cells grown in 2D conditions on Petri dishes (Chapter 2). In our second investigation, we slightly modify the formulation to cater to another distinct cell type which grows in suspension cultures. Here, we examine our formulation's effectiveness as the suspensions are cultivated in spinning flasks (Chapter 3). In our third investigation, we assess the efficacy of the formulation in supporting cell growth for the same cell types from our first investigation, but cultured on plant-derived 3D scaffolds, as opposed to flat, 2D Petri dishes (Chapter 4). The results demonstrate that the formulated medium can support robust cell growth and preservation of morphology across diverse cell types, comparable to the performance of an FBS-based medium. While further research is needed to extend these findings to other cell types and applications, this work highlights the importance of specific media components in mammalian cell culture and provides a framework for SF medium development in multiple microenvironments. A review on the fundamentals of cell culture, mechanisms of cell growth *in vitro*, medium components, serum alternatives, 2D vs 3D microenvironments, and their applications serves as the introductory chapter to this thesis (Chapter 1).

Statement of Originality

As far as the author is aware, this collection of work represents original research and the investigations outlined in this thesis are formatted as scientific manuscripts. The work presented in this thesis is the author's own original work, produced by themselves, unless otherwise indicated. The work was carried out under the guidance of Dr. Andrew E. Pelling at the University of Ottawa in the Center for Interdisciplinary Nanophysics Laboratories. The initial chapter presents an extensive introduction which serves to supply the reader with an elementary understanding of the relevant subject matter (Chapter 1). Subsequently, manuscripts which outline the original experimental research performed over the course of this degree are presented (Chapters 2-4), which are currently being formatted for potential publication in select scientific journals. To close the thesis, the final chapter summarizes and discusses any future applications and prospective channels arising from this comprehensive investigation (Chapter 5).

Contributions

Statement of contributions

I, Nicolette B. Mogilever, was the predominant contributor to the work enclosed in this thesis. All included experiments were constructed, executed, and analyzed by myself, with the guidance of Dr. Andrew E. Pelling. The presented work includes long-term cell culture, scaffold fabrication, preparation for and execution of confocal, phase, and light microscopy, transcriptomic profiling, image and data analysis, and statistical analyses. In certain instances, collaborative efforts with other researchers were necessary. Dr. Andrew E. Pelling edited all manuscripts and advised on their execution. Marie-Hélène Godin Pagé, M.Sc., aided in executing the mRNA and cDNA extraction protocols as well as qPCR analysis. Master's students Rose Boudria and Anjolaoluwatikiitan Solola (a visiting summer student from Cornell University) helped maintain some cell cultures.

Conferences and workshops

- Mogilever, N. B. Third prize at the Young Scientist Event. (2021). The suitability for public consumption of meat analogues. Case study at the *Science and Technology for Meat Analogues Conference*. (Wageningen University, Netherlands).
- Mogilever, N. B. Participation in discussions with students, scientists, researchers, companies, and activists. (2022). The future of cultured meat and other cell-cultured foods. *New Harvest Conference*. (New York, USA).

Other contributions

- Campuzano, S., Mogilever, N. B., & Pelling, A. E. (2020). Decellularized plant-based scaffolds for guided alignment of myoblast cells. *BioRxiv*, 2020-02.
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Abbreviations

- 4',6-diamidino-2-phenylindole (DAPI)
- Animal component-free (ACF)
- A one-way analysis of variance (ANOVA)
- American Type Culture Collection (ATCC)
- Complementary deoxyribonucleic acid (cDNA)
- Confocal laser scanning microscopy (CLSM)
- Cyclin-dependent kinase (CDK)
- Deoxyribonucleic acid (DNA)
- Dimethylsulfoxide (DMSO)
- Dulbecco's modified eagle medium (DMEM)
- Embryonic stem cells (ESCs)
- Epidermal growth factor (EGF)
- Extracellular matrix (ECM)
- Fetal bovine serum (FBS)
- Figure (Fig.)
- Filamentous actin (F-actin)
- Fibroblast growth factor 2 (FGF2)
- Ham's F-12 (HF12)
- Henrietta Lacks cells (HeLa)
- Human foreskin fibroblasts (HFF)
- Induced pluripotent stem cells (iPSCs)
- Insulin-Transferrin-Selenium (ITS)
- Machine learning (ML)
- Minimum essential medium alpha (αMEM)
- Mesenchymal stem cells (MSCs)
- Madin-Darby canine kidney (MDCK)
- Messenger ribonucleic acid (mRNA)
- Mechanistic target of rapamycin (mTOR)
- Paraformaldehyde (PFA)
- Penicillin/Streptomycin (P/S)
- Phosphate buffered saline (PBS)
- Propidium iodide (PI)
- Quantitative polymerase chain reaction (qPCR)
- Reverse transcription quantitative polymerase chain reaction (RT^2-qPCR)
- Revolutions per minute (rpm)
- Roswell Park Memorial Institute (RPMI/RPMI-1640) media
- Serum-free (SF)
- Serum-free media/medium (SFM)
- Sodium dodecyl sulfate (SDS)
- Standard error of the mean (SEM)
- Three-dimensional (3D)
- Tissue culture polystyrene (TCPS)
- Transforming growth factor beta 1 (TGFβ)
- Triton X-100 (TX)
- Two-dimensional (2D)

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Chapter 1 | Introduction

1.1 Foreword

The use of cell culture in scientific exploration has been pivotal to major biological developments for human society.[1] The practice of animal cell culture involves isolating cells, tissues, and/or organs and maintaining their vitality in an artificial setting that mimics natural conditions.[2,3] This method is known as *in vitro* cell culture, opposite from *in vivo* conditions where cells are studied within their natural biological context, often within living organisms.[4] Both *in vivo* and *in vitro* experimentation methods have their own strengths and limitations. *In vivo* studies can offer insights into real-life responses but can be more challenging to conduct due to complexities associated with animal models.[5] *In vitro* studies can offer precise control over experimental variables but do not fully replicate the complex interactions found within living organisms.[5] Regardless, both methodologies have allowed for tremendous advancements in biology since they are the techniques used by scientists to study cellular processes, model disease mechanisms, discover new drugs, personalize medicine, and produce recombinant proteins, antibodies, gene therapies, and tissue-engineered constructs for many applications.[6-10]

As these advancements have unfolded, *in vitro* methodologies continue to serve as the cornerstone for elucidating cellular behaviour and paving the way for transformative breakthroughs in biological research. The success of *in vitro* cell cultures relies extensively on cell culture media – the "cell food" that provides all the necessary nutrients, growth factors, vitamins, and other essential components that cells need to survive and multiply when they are not in their native environment anymore.[11] The media that is used in mammalian cell cultures is often tailored to the specific cell type of study and can be supplemented with a variety of components so that cell function and proliferation are preserved.[12] Despite this, there is a lot of overlap among the types of culture media utilized across industrial and research settings, with animal serum being one of the most used supplements as it naturally contains many of the factors that mammalian cells need to be viable in an artificial setting.[11] While animal serum, particularly fetal bovine serum (FBS), has long been a staple supplement in cell culture media to support cell viability and growth, its use raises concerns regarding ethical considerations, batch variability, high costs, and risk of transmitting infectious agents.[13] Consequently, there is a growing necessity to explore alternative strategies that can effectively replace FBS while maintaining optimal cell culture conditions. In this thesis, I aim to address these concerns by investigating alternative supplements to FBS for specific cell types cultured in both two-dimensional (2D) and three-dimensional (3D) conditions, with the goal of identifying substitutes that can sustain cell growth while minimizing ethical and variability concerns.

The objective of this first chapter is to introduce key concepts in cell culture and its implications relevant to the research presented in this thesis. It provides an overview of the historical development of culture medium, the general mechanisms governing cell proliferation, and explores how various components of media could influence cellular growth and behaviour. Additionally, it delves into the differences between 2D and 3D cell growth methodologies to

better understand the later chapters of this thesis. Furthermore, current alternatives to traditional culture media such as serum-free formulations and their potential applications are described.

1.2 History of Cell Culture Media

In 1882, what is said to be the simplest form of a cell culture medium was developed for the purpose of keeping frog hearts beating after their removal.[14,15] This medium, named Ringer's solution after the scientist that created it, contained a salt solution formulated to resemble that of bodily fluids. This development jumpstarted the creation of other solutions that contained components like inorganic salts and sometimes glucose. Over time, new balanced salt solutions for a variety of applications were developed by other scientists, such as with Locke's solution, Tyrode's solution, Krebs-Ringer solution, Gey's solution, Earle's solution, and Hanks' solution.[16-21] Although these solutions could keep cells and tissues viable outside of the body, they could do so for a few days at most. It was only years later, in 1907, that a biologist by the name of Ross G. Harrison was credited with the first animal tissue culture. By removing nervous tissue from a frog and cultivating it in frog lymph fluid, he observed the growth of nerve fibers after a few weeks.[22] In 1910, Montrose T. Burrows, a cancer researcher and surgeon, began studying under Harrison, later to discover that lymph fluid was not sufficient to grow most cells outside of their native environment. Instead, he chose blood plasma as a candidate for culture medium and successfully grew chicken embryonic stem cells (ESCs) in chicken plasma,[23] and later other mammalian cell types.[24] During this time, Burrows' mentor, Alexis Carrell, experimented with embryonic extracts, blood plasma, and lymph fluid with some success,[25] but this led to a new scientific inquiry that would still be a salient question in the modern day – what is inside these animal-derived fluids that actually causes cells to proliferate and survive?

As researchers grappled with the question of the specific components within animal extracts that are responsible for cell maintenance *in vitro*, a shift towards creating precisely defined media formulations began to emerge. Between the 1910s and the 1950s, scientists Margaret and Warren Lewis experimented with modified salt solutions enriched with amino acids, glucose, and other additives to enhance cell cultivation.[26] With different researchers experimenting, it was eventually found that glucose was crucial for cell survival, while partially hydrolyzed proteins and amino acids promoted cell growth.[27] However, efforts to identify specific growth-promoting substances were inconclusive. Other scientists, Vogelaar and Erlichman, demonstrated the effectiveness of hormones like insulin and thyroxine mixed with Ringer's solution, glucose, plasma, and some other amino acids in cultivating human fibroblasts for extended periods.[28] Many subsequent attempts were made to further enhance media composition, where eventually vitamins A, B1, B2, and C were added,[29] yet achieving fully defined media without any animal-derived components remained a challenge.

In this era, when healthy cells were taken from organisms to be grown *in vitro*, it was extremely rare for them to continue growing in an unlimited capacity, even with the right culture medium. In the 1940s, cell culture scientists would source cells directly from animals at the beginning of each experiment as there was no way to keep cells alive after a certain number of cell divisions.

During this time, an important innovation was made by harnessing the power of cancerous cells – unrestrained division. Wilton R. Earle used carcinogens to force mouse fibroblasts to proliferate indefinitely, creating the first immortalized cell line.[30] Later in 1951, George O. Gey and his colleagues harvested uterine cervical cancer cells from their patient Henrietta Lacks to create the first infinitely proliferating human cell line (named HeLa).[31] Although this was a monumental discovery to the field of cell culture, its important to mention that this was an unethical practice of exploitation, as Henrietta Lacks, a Black woman that was being treated for her cancer at one of the only hospitals that legally provided medical care to Black people in America at the time, never consented to this procedure, violating her right to bodily autonomy, and not herself nor her family was ever compensated.[32] The case of Henrietta Lacks underscores the long-standing inequities in medical treatment and research faced by marginalized communities, particularly Black folks.[33-35] Despite the profound injustice surrounding the origin of HeLa cells, their establishment fundamentally changed the landscape of cell culture research. With readily available and standardized cell lines, researchers no longer needed to sample cells from animals at the beginning of each experiment. This development facilitated more consistent and reproducible experimental conditions and consequently, researchers worldwide could now study the effects of culture media on cells with greater precision, leading to rapid advancements in the field.

Following these advancements, there were 2 major strategies for discovering the components that would be crucial to maintaining cells in vitro. The first strategy was to use minimal amounts of dialyzed animal serum (serum that has been processed to remove low molecular weight components in order to reduce the concentration of certain factors that could interfere with assays or cellular processes[36]) and slowly add other proteins and factors to it, to see how cells would behave.[11] The second strategy was to only mix together definitive components without any serum to then observe cell behaviour. Fischer was a pioneer in the first method and by extracting low molecular weight fractions from sera, he uncovered that amino acids are a key component in the survival of cells in culture.[37,38] Later on in 1955, by expanding upon Fischer's method, Harry Eagle found an ideal solution that was necessary for growing mouse L cells and HeLa cells.[39,40] The medium coined Eagle's minimum essential medium (MEM) contained glucose, 13 amino acids, eight water-soluble vitamins, six inorganic salts, and a minimum amount of dialyzed serum.[41] Based on the cell type in question, other researchers modified Eagle's MEM, leading to advancements in media for carcinosarcoma cells and lymphocytes.[42-44]

During this period, other researchers were studying how including no proteins and no serum at all could lead to successful cultivation of mammalian cell types in vitro. They were able to create some formulations (e.g. Medium 199, CMRL1066, NCTC109, MB 752/1) that contained a minimum of 40 components each to grow mouse L cells or chick embryo-derived cells for more than 3 weeks.[45-47] These formulations included things like glucose, amino acids, iron, vitamins, inorganic salts, coenzymes, cholesterol, purine bases, and pyruvate.[48] Although many of these studies did not use animal extracts, other researchers reported the need for supplementing Medium 199 with serum (from adult bovines) for cell proliferation.[39] This finding was in line with the other studies which commonly implemented biological fluids and extracts like sera from various sources, yet the occasional successful cultivation of specific cell types was still

poorly understood.[49] These biological extracts ranged from various animal-based plasma or serum derivatives to milk, embryo extracts, and amniotic, lymph, and spinal fluids.[22,23,25,27,50,51] In the 1950s, researchers discovered that serum from fetal bovines was superior in supporting the growth of cultured cells, mainly because of its higher content of growth factors and lower presence of antibodies that might interfere with cell growth.[52,53] Fetal bovine serum (FBS) was first introduced in 1958 only to help stimulate cell growth but as it became a more commonly used supplement, its superior properties in maintaining cells in vitro were uncovered, leading to its widespread adoption.[54,55]

Still, this was not enough to standardize protocols for defined mammalian cell culture so Richard G. Ham thought to better mimic *in vivo* conditions, whole proteins needed to be added to the medium. Through experimentation, he created Ham's F-10 medium which contained albumin and fetuin in addition to the major components mentioned above, in order to grow Chinese hamster ovary (CHO) cells.[56] It was further modified to create Ham's F-12 medium by adding some fatty acids, since the F-10 version was not successful in growing more than one cell type.[57] Ham's group researched media throughout the 1960s and 1970s, where animal proteins were newly being processed into peptides that could be used for supplements in culture media. For example, although insulin was discovered in the 1920s, it was not industrially processed to be used as a supplement for culture media until the 1960s.[58,59] Around the same time, growth factors were discovered and although their supplement to serum-free media increased cell growth, this was often still less effective than using serum itself.[60-63] Concurrently, the commercial availability of FBS increased and it became a standard component for almost all animal cell culture media, but because it was still unclear why the slurry of components within serum actually maintained cells in culture, many researchers were still experimenting with defined components that could potentially outperform serum in the maintenance of cells *in vitro*. Through further experimentation, Ham's group showed that a trace element, called selenite, was essential to serum-free culture of some human cell types.[64] At the same time, another research group led by Gordon H. Sato demonstrated that several hormones in combination with selenite, transferrin, and some growth factors were necessary.[65] Sato's and Murakami's group also found that ethanolamine, an important component for the formation of cellular membranes, is essential for hybridoma cultivation.[66] From these findings, many groups experimented with these components in serum-free formulations for a variety of cell types, leading to a plethora of newly commercialized supplements.[49,66-69] Due to the integration of FBS into cell culture protocols alongside basal media formulations (like the ones mentioned above), increased commercial production and availability of FBS made it easier for laboratories around the world to access high-quality serum.

In the 1980s, a larger push towards defining media formulations was seen – that is, developing cell culture media with precisely known components and without FBS or minimal animal-derived ingredients. This was due to a new revolutionary technology at the time that began to transform biotechnology and medicine – recombinant DNA technology. By inserting DNA encoding a specific protein into the genome of a host cell (like E. coli, yeast, or some animal cells), the cell can then produce that protein.[70] Such proteins are crucial for various applications, including the development of new medications, vaccines, and diagnostics.[71] As this technology

was advancing, researchers increasingly used animal cells as hosts to produce complex proteins that are more compatible with human use. However, the process of producing these proteins in animal cells brought about a new challenge: the need to optimize the production process to be as efficient and clean as possible. One significant hurdle was the traditional use of FBS in the culture medium because while FBS nurtures cell growth, it also contains a mix of undefined biological substances that can interfere with the purification of the desired recombinant proteins.[13] Impurities from FBS make it difficult to isolate the target protein without contamination.[72] To enhance the efficiency of biopharmaceutical production, researchers began experimenting to optimize previous formulations, employing techniques such as monitoring the changes in the concentrations of medium components and by-products in the culture. This approach helped to fine-tune the conditions needed for maximal production of the target proteins while ensuring the process remained as streamlined and contaminant-free as possible. Through these initiatives along with modifications to host cells, the production yield per cell has risen almost tenfold between 1986 and 2004.[72]

For an in-depth timeline of all the relevant technical innovations in the development of cell and tissue culture, see *Table 1.1* in *Freshney's Culture of animal cells*.[73] In the modern day, researchers around the world use modified versions of the mediums described previously, thanks to the efforts of the last century. Depending on the cell type and application, different basal mediums are used (i.e., Ham's F-12, Eagle's MEM, Dulbecco's MEM, etc.) along with supplements like growth factors, hormones, animal sera (most commonly FBS), and other elements. Despite the advancements in formulating cell culture media that cater to specific research needs and applications, the quest for an ideal, universal culture medium persists. Currently, no fully defined, animal component-free (ACF) medium exists that can universally support the growth and functionality of all mammalian cell lines as effectively as FBS. Many efforts are underway to develop modern SFM and these endeavors will be detailed later in *Section 1.5*. Prior to this, a deeper understanding of the molecular mechanisms underlying cell growth and the specific roles of various culture media components will be explained to provide valuable insights into why these elements are essential for maintaining viable and functional cells *in vitro*.

1.3 Mechanisms of Cell Growth and Nutritional Requirements in Culture

Animal cells are attuned to a complex array of nutritional and environmental stimuli that orchestrate their proliferation and functionality. In general, animal cells *in vivo* thrive under specific conditions that comprehensively balance factors such as the availability of nutrients, components that stimulate or dampen growth, and interactions with surrounding support structures. More specifically, the native 3D environment cells reside in includes a matrix made up of collagen and various structural proteins woven with protein-sugar molecules that help bind water (proteoglycans), chemical signals from nearby cells, and mechanical forces from everything that surrounds them.[74] For mammalian cell cultures *in vitro*, there is a deviation from these natural conditions. Although the fundamental macromolecular (e.g. glucose, amino acids, lipids) requirements for survival remain essential, cell behaviour is slightly altered

because of the artificial culture medium that changes the exact stimuli the cells receive.[11,75-77] Additionally, cells *in vitro* are often grown on 2D, flat substrates that are void of the typical complex extracellular matrix (ECM) found in vivo, which causes changes in their spatial arrangement and as a result their cellular function.[78,79] Before delving into the intricate workings of these complex intracellular changes, we will first explore the basic necessities of cells and their typical growth. Following this, we'll examine how these fundamentals shift when cells are cultured *in vitro*, and identify which components are crucial for maintaining proliferation and function.

1.3.1 Cellular Proliferation

Cell growth refers to a net increase in size and mass, which for any given organ system typically occurs in three parts: hyperplasia (increase in cell number), hyperplasia with hypertrophy (increase in cell size), and/or hypertrophy alone.[80] Hyperplasia represents a critical developmental stage and occurs in different organs for varying periods, with some organs undergoing cellular replacement throughout their lifespan.[81] Malnutrition during hyperplasia results in decreased cell numbers, irreversible by later nutrient intake, while malnutrition after this period results in decreased cell size, which can generally be reversed.[80,82] In cell culture, one basic objective is usually to achieve an increase in cell number without a net change in individual cell size (i.e. hyperplasia without hypertrophy). This process is often referred to as cellular "growth" and also as multiplication or proliferation. The concept of cellular multiplication is critical in understanding how cells respond to nutritional inputs. The increase in cell number is particularly important during developmental stages and results from an increase in the rate of the cell cycle – the series of events that take place leading to replication which involve distinct phases.[83-85] Moreover, the transition from hyperplasia to hypertrophy, where cell size increases, indicates a different nutritional need that is often linked to protein synthesis and increased cellular architecture components.[86]

Cell growth demands a significant amount of energy since nutrients must be converted into macromolecules (carbohydrates, proteins, lipids), and this process is quickly halted when conditions are unfavorable. Autophagy, the process that involves degrading and recycling cellular components, acts as a counterbalancing mechanism to respond to stressors and for managing cellular quality control.[87] When faced with nutrient scarcity, DNA damage, excessive oxidation, viral infections, or other stressors, cells swiftly shut down the ATP-consuming activities associated with growth.[86] This rapid shutdown allows cells to conserve their resources or redirect them to address the stressor. This balanced program between cell growth and autophagy is regulated by many intracellular signalling cascades that involve a variety of proteins. One of the most important pathways in understanding this regulation is the mTOR signaling pathway.[88] mTOR is a protein which forms the core of two distinct complexes: mTORC1 and mTORC2, which regulate different aspects of cell function.[89] (Fig. 1.1) mTORC1 is mainly involved in the regulation of metabolism and cell growth in response to changes in nutrients, growth factors, and available energy.[90,91] This protein complex promotes lipid biosynthesis, protein synthesis, and inhibits autophagy. mTORC2 is predominantly involved in managing cell survival, cytoskeletal organization, and metabolism via activating Akt signaling (another

intracellular signaling pathway that goes beyond the scope of this thesis).[90,92] Cells in culture are often subjected to stress conditions such as nutrient depletion, hypoxia, or oxidative stress. Understanding mTOR's role in autophagy allows for the design of interventions that can help cells cope with these stresses, enhancing cell survival and maintenance.[93,94]

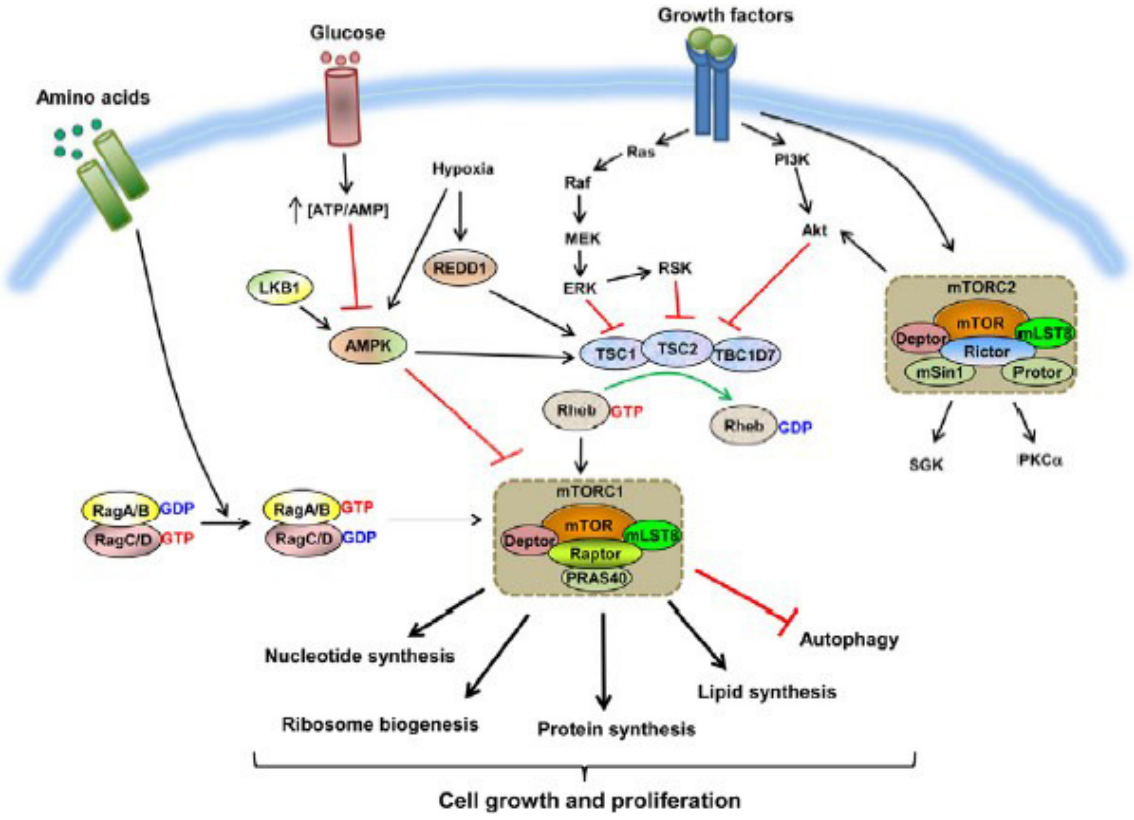

Figure 1.1: The mTOR signaling pathway and its function for cell growth and proliferation. The illustration shows the principle signaling pathways that govern mTORC1 and mTORC2, as well as the composition of each mTOR complex. Various signals from extracellular amino acids and growth factors, cellular energy levels, and stressors converge on mTORC1 through the depicted mechanisms. When mTORC1 is activated, it significantly promotes cell growth by enhancing anabolic processes like lipid, nucleotide, and protein synthesis as well as ribosome biogenesis. In contrast, mTORC2 is primarily regulated by growth factors and unlike mTORC1 does not respond to nutrient- or stress-related upstream signals. *This figure is reproduced from Kim et al.'s 2013 review.*[95]

Cell growth is an intricate process influenced by a myriad of signaling pathways. These pathways integrate various environmental signals, such as growth factors and cell density, to regulate proliferation.[96] Intracellular elements like cyclins and tumor suppressor proteins manage the cell cycle's progression, with disruptions in these pathways often leading to diseases such as cancer.[97] Tumor suppressors like p53 and Rb serve as crucial cell cycle checkpoints, halting the cycle in response to damage.[98] While the mTOR pathway is a key player in this complex

network, many other pathways, such as those activated by growth factors (which will be discussed in *Section 1.3.3*), also contribute to changes in the cell cycle by initiating cascades that involve cyclins and cyclin-dependent kinases (CDKs). Although understanding these mechanisms is vital for therapeutic strategies against uncontrolled cell growth, this thesis will focus on the extracellular factors affecting cell cycle progression, particularly in the context of culture media's relevance to cell growth.

1.3.2 Cellular Differentiation

While almost all cells in one organism contain the same genome, they exhibit different phenotypes and can perform a variety of functions. These distinct cell types, each defined by unique gene expression patterns, are formed during development and subsequently maintained in a stable state.[99] This process of specialization is known as cellular differentiation and can involve various regulatory mechanisms. During the process of differentiation, cells undergo changes in gene expression, morphology, and functional characteristics, resulting in the emergence of specialized cell types which ensures that each cell type can carry out its specific functions effectively.[100-102] Many signalling pathways are crucial in orchestrating differentiation and the outcomes are also linked to changes in metabolism.[103] (Fig. 1.2) This process is influenced by the cellular microenvironment, including the presence of differentiation-inducing factors (like growth factors and transcription factors) and the physical properties of the substrate.[104-106] The external environment significantly influences cellular differentiation via nutrient availability, oxygen levels, mechanical forces, and cell-cell interactions that can play crucial roles in determining cell fate.[107-109] For example, nutrient-rich conditions and appropriate growth factors can promote differentiation, while nutrient deprivation or hypoxia can induce stress responses that affect differentiation outcomes.[110-112] Additionally, the ECM provides structural support and biochemical signals that guide cellular behavior and differentiation.[113,114]

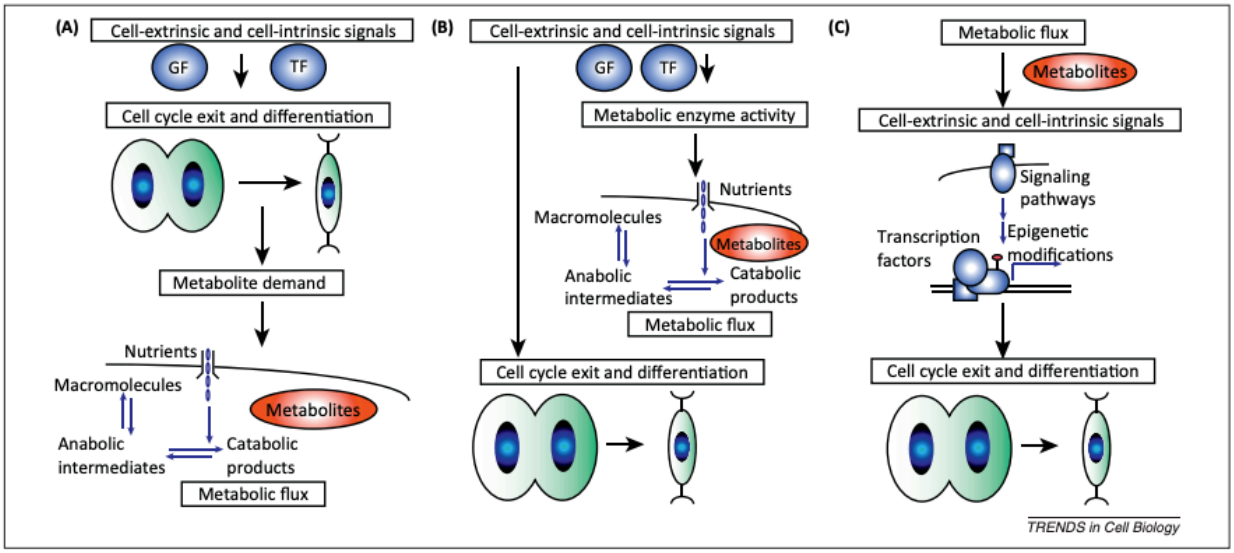

Figure 1.2: Metabolic alterations play a crucial role in cell differentiation.

(A) Growth factors (GFs) and transcription factors (TFs) signal cells to cease proliferation and begin differentiating. As cells exit the cell cycle, their metabolic needs shift (highlighted in red), such as by decreasing the incorporation rates of nucleotides and amino acids into macromolecules. These shifts can redirect metabolic flux, the rate that substrates are converted through metabolic pathways. **(B)** Cellular signals (GFs, TFs) cause cells to differentiate and modulate the activity of metabolic enzymes. This coordinated regulation leads to a reconfiguration of metabolic pathways to meet the new demands of differentiated cells, such as lowering the synthesis rates of nucleotides and amino acids. **(C)** Adjustments in metabolic flux result in altered concentrations of certain metabolites. These metabolites can then regulate signaling proteins, which in turn affect cell differentiation. *This figure is reproduced from Agathocleous et al.'s 2013 review.*[103]

A significant pathway related to proliferation, differentiation, and metabolism is the PI3K/Akt/mTOR pathway, which promotes anabolic processes and is activated by certain growth factors.[115-117] This pathway enhances glucose uptake and glycolysis by regulating glucose transporters and glycolytic enzymes, and it also stimulates fatty acid and protein synthesis to meet the demands of cellular proliferation.[118] Additionally, it plays a key role in determining the fate of stem and progenitor cells.[119] The interplay between metabolic pathways and signaling networks is vital for both proliferation and differentiation, with aerobic glycolysis serving multiple functions beyond just supporting biosynthesis and energy supply. By integrating signaling pathways and metabolic changes, cells can efficiently transition from a proliferative state to a differentiated one, ensuring that their metabolic needs align with their new functions. In cell culture, achieving a balance between proliferation and differentiation depends on the desired outcome and is essential for generating functional tissues and studying developmental processes.

1.3.3 Cell Metabolism and Nutrient Requirements

Metabolic function is a key determinant of cell growth rate and must be regulated in tandem with the cell cycle. For the purpose of this thesis, this section focuses on the metabolic requirements of normal, healthy cells that closely mirror those of their *in vivo* counterparts. Thus, this explanation of cell metabolism and nutrient needs is not comprehensive and mainly focuses on results obtained from *in vitro* cell cultures. Although a rigorous analysis of nutrient requirements for normal mammalian cells in a fully defined medium is incomplete, existing data from cellular nutrition and whole-animal nutritional experiments help explain cellular demands in culture.

Nutritional growth requirements for cultured cells include nutrients, hormones, and growth factors, and can vary based on environmental conditions such as temperature, pH, osmolarity, and substrate (the material upon which cells grow *in vitro*). A nutrient can be defined as a chemical substance utilized by cells for structural components, biosynthesis, or energy metabolism.[120] *In vivo* nutrition relies on the body's homeostatic systems, such as the liver and kidneys, to regulate extracellular concentrations of nutrients and waste products. In contrast, cells *in vitro* lack organs, thus lacking these regulatory systems and cannot be "fed" simply by introducing a daily allowance of essential nutrients.[120] Studying cellular nutrition is complicated

because of the immense diversity in nutrient requirements which vary not only between species but also with the developmental stage of the animal.[121-123] Regardless, cells still require macromolecules to carry out their functions, such as proteins for enzymatic activities and structural integrity, lipids for membrane formation and energy storage, nucleic acids for genetic information and regulation, and carbohydrates for energy supply and cellular signaling (Fig. 1.3). These macromolecules can be synthesized or converted from the components present in the culture medium, often in forms that are readily absorbable and at concentrations that mimic physiological conditions to ensure proper cellular function and growth.[13,120,124,125] In this section, we will explore each essential growth requirement for cellular metabolism and nutrition in culture and why they are crucial. In the following *Section 1.3.4*, the contents of standard animal serum-supplemented culture medium will be discussed and related to the demands of cell cultures presented here. It is important to note that the microenvironment and the substrate in which cells are cultured significantly impacts their behavior, metabolism, and nutritional requirements, yet this will be covered in the following portion of this thesis, in *Section 1.4*.

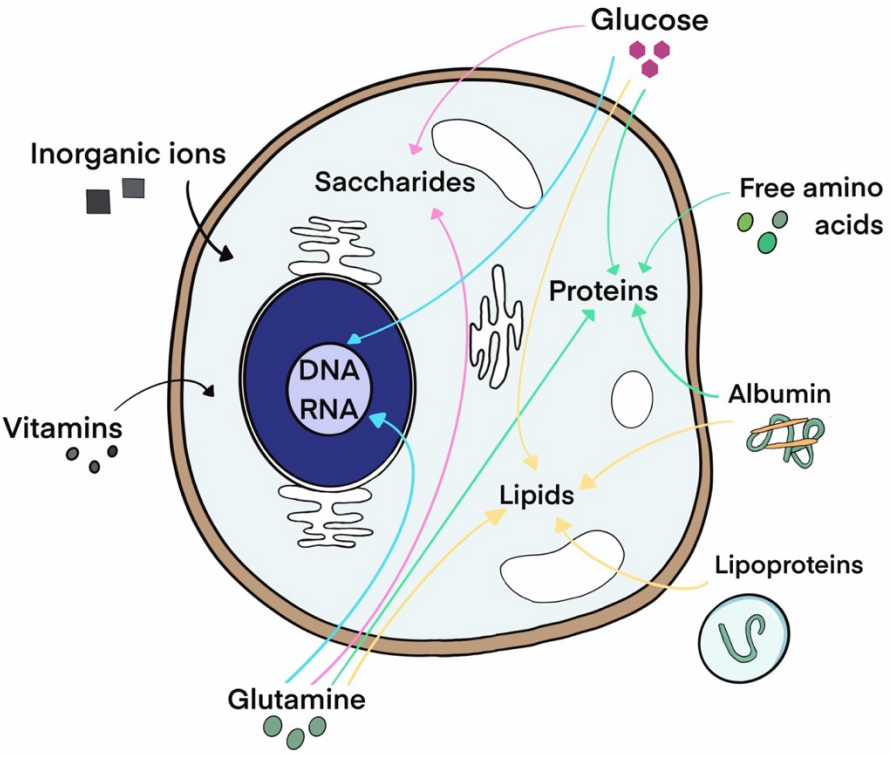

Figure 1.3: The primary roles of key nutrients found in mammalian blood in the formation of cellular macromolecules.

The contents on the inside of the cell (DNA/RNA, saccharides, proteins, and lipids) are the building blocks that are essential to cell maintenance and proliferation. The contents on the outside of the cell (inorganic ions, vitamins, glutamine, lipoproteins, albumin, free amino acids, and glucose) are required to make the building blocks intracellularly. Glucose and glutamine are vital for the intracellular synthesis of nucleic acids (DNA and RNA). Saccharides primarily originate from glucose, with nitrogen components provided by glutamine. Protein synthesis utilizes amino acids that are either imported directly in their free form or produced through the breakdown of

extracellular proteins like albumin. Most lipids, which are transported by lipoproteins and albumins, are not always essential for mammalian cells and can be synthesized from glucose and glutamine. The unlabelled cellular structures in this diagram provide context to the general organelles found within the animal cell. Nutrients are not drawn to scale. *Adapted from Palm et al., Nature, 2017, Fig. 1A.*[126]

Amino Acids: Nearly every function carried out in a cell is mediated by proteins, making the production of proteins essential for cellular life.[127] Amino acids, the building blocks of proteins, are thus crucial for cells in culture.[128] They are required for the synthesis of structural proteins that maintain cell shape and integrity, as well as for enzymes that catalyze metabolic reactions.[129] Amino acids also play a role in the synthesis of signaling molecules and hormones that regulate various cellular processes, including growth, differentiation, and response to environmental stimuli.[130] Additionally, certain amino acids serve as precursors for nucleotides and other bioactive molecules necessary for DNA and RNA synthesis.[131-133] Without a sufficient supply of amino acids, cells in culture would be unable to maintain protein synthesis, leading to impaired growth, reduced metabolic activity, and eventual cell death. Essential amino acids are defined as ones that cannot be produced on their own and thus must be obtained through the diet.[134] Non-essential amino acids can be synthesized by cells but may still need to be supplemented in culture to support optimal growth.[135] Intact animals have complex systems that allow for nutrient sharing/recycling, metabolic interactions, and homeostatic mechanisms between tissues and organs.[136-138] These complexities are not present in cell cultures, which is why cells *in vitro* often require both essential and non-essential amino acids to be present. Therefore, the design of cell culture media trends toward including all 20 amino acids involved in protein synthesis, with a balance optimized for specific cell types.[139] Providing an adequate and balanced mixture of amino acids in the culture medium is vital to support the diverse and essential functions that proteins perform within the cell.

Carbohydrates: The primary energy source for cells in culture are carbohydrates, crucial for carrying out various cellular functions. The most common carbohydrate utilized in cell culture medium is glucose, which cells metabolize through glycolysis and oxidative phosphorylation to generate ATP, the energy currency of the cell.[140] This energy supports a wide range of cellular activities, including biosynthesis, cell division, and maintenance of cellular homeostasis.[141] Additionally, glucose is a key precursor for the pentose phosphate pathway, which provides ribose for nucleotide synthesis and NADPH for reductive biosynthesis and antioxidant defense.[142] Pyruvate, a key intermediate in glucose metabolism, is often included in culture media as an additional energy source. Pyruvate can be further metabolized in the mitochondria through the citric acid cycle, enhancing ATP production, or it can serve as an antioxidant to neutralize reactive oxygen species, protecting cells from oxidative stress.[143] Beyond glucose, other carbohydrates such as galactose and fructose can also be used in specific cell culture applications, though less frequently.[144] These alternative sugars may be included in media formulations to study metabolic pathways or to tailor the nutrient environment to the needs of particular cell types. Animal cells rapidly metabolize glucose and glutamine *in vitro*, leading to their quick depletion and the accumulation of lactate and ammonium ions, which can inhibit cell growth, and as a result culture medium does have to be replaced quite frequently.[145]

Overall, the availability of carbohydrates, including glucose and pyruvate, is vital for sustaining cellular energy levels and supporting the anabolic and catabolic processes necessary for cell viability and proliferation *in vitro*.

Lipids: From acting as structural components of cell membranes, providing energy storage, and serving as key players in signal transduction and biosynthetic pathways, lipids are crucial to the survival of mammalian cells.[146] Phospholipids, cholesterol, and glycolipids form the lipid bilayer of the cell membrane which maintains cell integrity and fluidity, while fatty acids supply energy through oxidation, supporting cellular activities.[147,148] Additionally, other lipid-derived molecules are integral to intracellular signaling, influencing processes such as growth, differentiation, and apoptosis.[149] Commonly used lipids in culture media include fatty acids like palmitic acid and oleic acid, cholesterol, and various phospholipids, which are often provided through lipid-rich supplements.[150] However, the requirement for added lipids in cell culture media is debated. In previous cell culture studies where supplementation of lipids was deemed unnecessary, animal-based sera were often used, which inherently contain lipoproteins and free fatty acids.[151] Research has indicated that *de novo* (from scratch) synthesis of fatty acids and cholesterol is inhibited when adequate supplies of lipids are available in the culture medium, which can lead to lipid accumulation in cells.[152,153] Overall, the inclusion of lipids in culture media in some capacity is essential for replicating physiological conditions and ensuring optimal cell growth and function, depending on the cell type.[154]

Vitamins: Vitamins are essential to mammalian cells in culture because they play crucial roles in various biochemical processes necessary for cell growth, function, and survival. Key vitamins often included in culture media are B-vitamins (such as B1, B2, B3, B5, B6, B7, B9, and B12), and sometimes vitamin C (ascorbic acid).[155] B-vitamins function as coenzymes in metabolic pathways, facilitating energy production, nucleotide synthesis, and the metabolism of amino acids and fatty acids.[156] For instance, vitamin B1 (thiamine) is vital for carbohydrate metabolism,[157] while vitamin B12 (cobalamin) is essential for DNA synthesis.[158] Vitamin C serves as an antioxidant and is crucial for collagen synthesis, aiding in maintaining the extracellular matrix.[159,160] Certain vitamins are often not included in culture media due to their instability or the assumption that cells can synthesize or recycle them.[155] When these vitamins are not included in the medium, cells may rely on their metabolic pathways to synthesize or regenerate them, assuming they have the necessary precursors and enzymes.[126] Alternatively, if the culture medium lacks these vitamins and the cells cannot synthesize them, supplementation with serum or specific vitamin-rich additives may be necessary to ensure optimal cell growth and function. In some cases, the absence of certain vitamins may not significantly impact cell culture if the specific cell type does not have high requirements for those vitamins or can compensate through other metabolic mechanisms.[126] Overall, while vitamins are critical for many cellular functions, their inclusion in culture media is tailored to the specific needs and capabilities of the cultured cells.

Major Ions and Trace Elements: Major ions, such as sodium, potassium, calcium, magnesium, chloride, and phosphate, are essential for maintaining osmotic balance, membrane potential, and pH stability, which are critical for cellular homeostasis.[161] Sodium and potassium ions, for

instance, are key players in the function of the sodium-potassium pump, which is required for maintaining membrane potential and driving various transport processes.[162] Calcium ions are integral to signal transduction pathways, muscle contraction (relevant for muscle-related cell types), and enzyme activity regulation.[163] Magnesium acts as a cofactor for numerous enzymatic reactions, including DNA and RNA synthesis, while phosphate is crucial for energy storage and transfer through ATP.[164] Trace elements, including zinc, iron, copper, manganese, and selenium, although required in smaller amounts, are equally important.[165] They serve as cofactors for enzymes involved in redox reactions, DNA synthesis, and repair, as well as cellular respiration.[166] Iron, for example, is a key component of hemoproteins, like cytochromes, playing a pivotal role in oxygen transport and cellular respiration. Iron also helps regulate oxidative stress by balancing the generation and neutralization of reactive oxygen species.[167,168] Zinc is involved in DNA synthesis and repair, as well as immune function.[169] The precise regulation and supplementation of these ions and trace elements in culture medium are essential to replicate the *in vivo* environment, ensuring that cells can carry out their metabolic functions, grow, and proliferate effectively yet levels of each element must be carefully controlled to maintain osmotic balance and membrane potential.[170]

Hormones and Growth Factors: In cell culture, hormones and growth factors play essential roles in regulating cellular processes vital for cell health, growth, differentiation, and function.[49] Insulin is one of the most critical hormones used in vitro, as it facilitates glucose uptake, promoting energy production and anabolic processes.[171] Insulin activates key signaling pathways, such as the PI3K/Akt pathway, which are crucial for protein synthesis and cell growth, maintaining the metabolic and proliferative states of cultured cells.[172] In some cultured cell lines, thyroid hormones, such as thyroxine (T4) and triiodothyronine (T3), regulate metabolism, growth, and development, making them important for maintaining the overall physiological balance.[173,174] Hydrocortisone, a glucocorticoid, is often included in culture media to modulate inflammatory responses and enhance the survival and function of specific cell types, like fibroblasts and epithelial cells, by mimicking the anti-inflammatory and immunosuppressive effects observed *in vivo*.[175-178] Growth factors are equally crucial in cell culture, influencing various aspects of cellular behavior and development. Epidermal growth factor (EGF) is vital for cell proliferation and differentiation, binding to its receptor EGFR and activating downstream signaling pathways that drive cell cycle progression and mitosis.[179-181] Fibroblast growth factor (FGF) plays a significant role in angiogenesis, wound healing, and embryonic development, supporting the proliferation and differentiation of a wide range of cell types.[182-184] Transforming growth factor-beta (TGF-β) is involved in regulating cell growth, differentiation, and apoptosis, as well as maintaining extracellular matrix production and immune responses.[185-189] These growth factors, along with other important factors such as platelet-derived growth factor (PDGF) and insulin-like growth factors (IGFs), create a microenvironment that closely mimics physiological conditions, ensuring that cells in culture can perform their natural functions and respond to experimental conditions in a controlled manner.[185,190,191] It is important to note that the specific requirements for these hormones and growth factors can vary depending on the cell type, and not all cells will necessarily require every hormone and growth factor listed above.

1.3.4 Understanding Traditional Culture Media

Having established a foundational understanding of the nutrient and growth requirements for cells in culture, we now turn our attention to the components that underpin the growth of typical cell cultures, elucidating why standard media formulations are effective. The classic cell culture medium consists of a basal medium, supplemented with animal-derived components. Basal media, devoid of animal products on their own, essentially serve as a nutrient-rich slurry, with their contents typically derived from plant extracts, synthetic sources, or purified chemicals.[192,193] These media are formulated to supply essential nutrients, such as amino acids, vitamins, inorganic salts, glucose, and buffering agents, necessary for basic cellular functions and metabolism.[194] Despite their advantages, basal media alone are insufficient for optimal cell proliferation as they lack the complex mix of growth factors, hormones, and other bioactive molecules present in serum.[11] Over time, it has become evident that nearly every cell type has unique requirements for medium supplements, making a universal cell and tissue culture medium impractical. Different cell types possess distinct receptors critical for survival, growth, and differentiation, and this diversity necessitates the use of specific basal media (with variations in nutrient content) tailored for each cell type.[195,196] Consequently, developments have created a variety of basal media formulations including Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI-1640), and Minimal Essential Medium (MEM), which differ in terms of nutrient concentrations and the inclusion/exclusion of certain elements. For a comprehensive understanding and comparison of the most commonly used basal mediums and their features designed for specific cell types, see Yao et al.'s 2017 review.[11]

Serum, a crucial additive to basal medium, is derived from the blood of animals, and is a complex mixture of nutrients and proteins that supports cell growth in culture.[11,193] Sera from various animals, such as horses, pigs, chicken, sheep, and goats can be used in cell culture,[197] but fetal bovine serum (FBS) is the most commonly used due to its high concentration of growth factors and low levels of antibodies, which promote optimal cell growth and minimize immunological interference.[198] However, the use of serum comes with significant drawbacks. Firstly, the manner by which serum is harvested has raised ethical concerns as there is potential suffering of the fetus during the collection phase, and the pregnant cow must be sacrificed to do so.[199,200] Additionally, serum composition can vary significantly between batches (due to the diet and environment of the pregnant animal), leading to inconsistencies in experimental results.[201,202] Despite modern technology, various batches of FBS from a single supplier have been found to exhibit wide variations in component concentrations, leading to significant differences in cell proliferation between lots.[200,203] Moreover, the use of animal products for the fabrication of new medicinal products is strongly unadvised due to the high risk for contamination during the harvesting procedure with viruses, mycoplasma, and other pathogens.[204-206] The disadvantages of using animal sera do not stop here. The presence of undefined components in serum makes it difficult to pinpoint specific factors responsible for observed cellular behaviors, and the unsteady supply of FBS (due to beef and dairy prices, feed costs, and outbreak of diseases) continually causes increases in its cost from year to year.[207-208] Despite the drawbacks associated with using animal serum, their benefits remain clear in their

universal ability to support cell growth and proliferation, thanks to the rich mix of growth factors, hormones, and attachment molecules they provide.

Specifically, FBS contains a complex mix of components like hormones, growth factors, proteins (such as albumin and transferrin), lipids, vitamins, minerals, and attachment factors.[209] The proteins, lipids, and hormones in FBS contribute to membrane stability, intracellular signaling pathways, and proper growth and proliferation.[13,210] Attachment factors, such as fibronectin and vitronectin, are crucial for the adhesion of cells to the culture substrate, which promote proper construction of the rest of the ECM *in vitro*.[211-213] Even though we can begin to understand why cells grow with animal serum, it remains challenging to pinpoint the exact requirements for each cell type because FBS contains approximately 1,800 proteins and over 4,000 metabolites, and the precise contributions of various hormones, nutrients, growth factors, and other components in serum to cell proliferation are still not well understood.[210,214-216] There have been many efforts to characterize the most important components found within FBS pertaining to cellular growth (Table 1.1), yet their concentration and exact mechanisms of action are obscure. Given its composition and the analysis of the nutrient requirements of proliferation, differentiation, and metabolism highlighted in *Section 1.3.3*, it is logically consistent that FBS is highly effective in supporting mammalian cells in culture. The rich array of growth-promoting substances mimics the natural environment found in animal blood, which inherently supports tissue growth and development.

Category	**Components**
Serum proteins	Albumin, Antithrombin-III, Fibronectin, Globulins, Hemoglobin beta fetal chain, Kininogen, Laminin, Plasminogen, Serum spreading factor, a1-Antitrypsin, α2-HS-glycoprotein, α2-Macroglobulin, β2-Glycoprotein
Transport proteins	Apolipoprotein, Transcortin, Transferrin, α1-Lipoprotein, β1-Lipoprotein
Enzymes	Alanine aminotransferase, Alkaline phosphatase, Aspartate aminotransferase, cGMP-specific 3’,5’-cyclic phosphodiesterase, Lactate dehydrogenase, Lactoperoxidase, Phosphokinase, Prothrombinase, Transaminase, γ-Glutamyl transferase
Hormones	Adrenocorticotropic hormone, Corticosteroids, Follicle-stimulating hormone, Glucagon, Growth hormone (bovine), Insulin, Luteinizing hormone, Parathyroid hormone, Pituitary glandotropic factors, Prostaglandins, Prolactin, Testosterone, Thyroid hormones, Thyroxine, Vasopressin
Growth factors and cytokines	Basic fibroblast growth factor (bFGF), Endothelial cell growth factor (ECGF), Epidermal growth factor (EGF), Fibroblast growth factor (FGF), Glial growth factor, Insulin-like growth factor (IGF) (e.g., IGF-binding protein 2, IGF-binding protein 4, IGF- II), Interferons, Interleukins, Nerve

	growth factor (NGF), Platelet-derived growth factor (PDGF), Prepro-insulin-like growth factor I, Transforming growth factor (TGF) (e.g., TGFβ1)
Fatty acids and lipids	Cholesterol, Ethanolamine, Free and protein-bound fatty acids, Phosphatidylethanolamine, Phospholipids, Triglycerides
Carbohydrates	Fructose, Galactose, Glucose, Glycolytic metabolites, Mannose, Ribose
Nonprotein/nitrogens	Amino acids, Creatinine, Polyamines, Purines/pyrimidines, Urea, Uric acid
Vitamins	Retinol/retinoic acid (vitamin A), Ascorbic acid (vitamin C), Vitamin B-Group: Biotin, Cobalamin, Folic acid, Niacinamide/nicotinic acid, Pantothenic acid, Pyridoxine/pyridoxalphosphate, Riboflavin, Thiamine, α-Tocopherol (vitamin E)
Minerals	Ca, Cl, Cr, Cu, F, Fe, I, K, Mn, Mo, Na, Ni, Se, Sn, Zn
Inorganic compounds	Alkaline phosphate, Phosphate
Others	Bilirubin, CO, CO_2

Table 1.1: Identified components of fetal bovine serum relevant to cell growth or maintenance. The relationship between fetal bovine serum components and cell growth, proliferation, maturation, and differentiation is still under scientific inquiry. Although precisely identifying the ingredients and contents remains challenging, numerous studies have investigated the growth factors, hormones, proteins, cofactors, and minerals that influence cell culture. This table provides concrete examples of components found within FBS as evidenced by different biological assays. *This table is reproduced from Lee et al.'s 2022 study,[201] with modifications from several other studies.[208-210,217]*

For effective cell growth and maintenance of human or animal cells *in vitro*—whether in primary cultures or continuous cell lines—specific culture conditions must be established. These conditions should closely resemble the physiological environment *in vivo*, taking into account factors such as temperature, pH, osmolarity, and oxygen supply.[218] It is important to note that the experimental goals, research context, and cell culture conditions will affect the criteria for defining 'healthy' cells *in vitro*, which may be defined as the ability to maintain a stable phenotype and viability while demonstrating normal cellular functions without any signs of stress. The integration of basal media, supplemented with either serum or specific animal-derived components, along with maintaining the culture system at physiological temperature (37°C), ensures that cells receive the necessary nutrients, growth factors, and attachment signals required for optimal proliferation and function, closely mimicking the *in vivo* conditions.

1.4 Cell Culture in 2D and 3D Environments

Cells *in vivo* are part of complex tissues with three-dimensional (3D) architectures, interacting with the extracellular matrix (ECM) and neighboring cells. *In vitro*, cells are typically grown on two-dimensional (2D) surfaces, which differ from their *in vivo* counterparts due to the lack of 3D geometry and systemic homeostatic regulation.[219] Since the early 1900s, flat 2D cultures have been the standard yet research published within the last 20 years suggests that 2D models misrepresent the intricate cellular interactions and physiological responses observed *in vivo*.[220,221] To address these limitations, researchers have developed various 3D culture systems, including suspension cultures in bioreactors, scaffolding systems, and organoids.[219,222] These systems offer more physiologically relevant environments that better mimic *in vivo* conditions. In this thesis, we explore the use of serum-free media for culturing mammalian cells in both 2D (Chapter 2) and 3D (Chapters 3 and 4) environments. This section delves into the differences in cell behavior observed in 2D versus 3D environments, focusing on how these conditions might affect cellular responses, morphology, and functionality. Herein, these two culturing approaches are defined and the advantages and limitations of each are explored.

1.4.1 Cell Adhesion and Mechanotransduction in 2D

In culture, cells adhere to the substrate and each other through interactions with the ECM and cell surface receptors. The cytoskeleton, composed of actin filaments, microtubules, and intermediate filaments, regulates cell shape, motility, signaling, division, and provides structural support. Cells can sense extracellular mechanical inputs through mechanotransduction, a process where mechanical signals are converted into biochemical responses.[221] They do so by interacting with their environment via testing its elasticity through anchoring and pulling actions. These actions rely partly on myosin-driven contractility and transcellular adhesions, which involve creating protein complexes (called focal adhesions) with integrins, cadherins, and other adhesion molecules to transmit forces to the surrounding substrates.[223] (Fig. 1.4) Normal tissue cells not only exert these forces but also react to the sensed resistance through cytoskeletal organization and other cellular processes.[223,224] This response occurs regardless of whether the resistance comes from the natural tissue matrix, a synthetic substrate, or a neighboring cell. This ability to sense and respond to mechanical stimuli affects various aspects of growth, differentiation, and metabolism.[225] In tissue engineering, creating artificial environments that guide cells through various phases over time is essential. However, the changes cells induce in their surroundings complicate this process. The continuous interaction between cells and their matrices, involving mechanosensing, signal transduction, cell responses, and matrix remodeling, makes it challenging to determine how cells decide to grow, differentiate, or undergo apoptosis.[225]

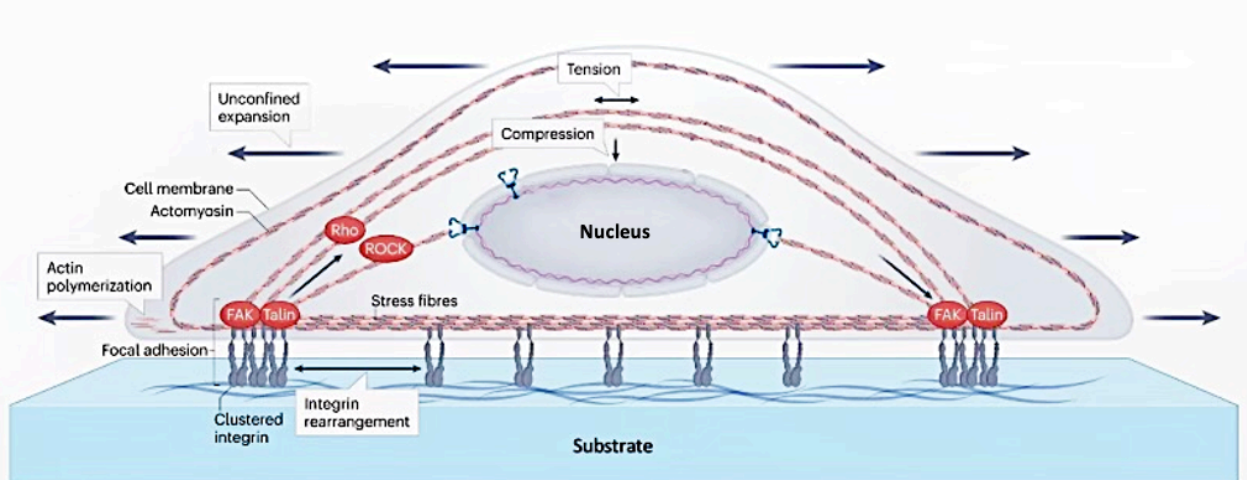

Figure 1.4: Mechanism of Cellular Response to Substrate Stiffness in 2D Environments.
Cells detect the stiffness of their substrate by applying contractile forces on 2D surfaces through stress fibers and focal adhesions. This process activates proteins like focal adhesion kinase (FAK), talin, Rho, and ROCK at the adhesion sites. This promotes the maturation of adhesions and the formation and contractility of stress fibers. These forces are then transmitted to the nucleus, altering nuclear envelope tension and opening nuclear pores. This change facilitates the entry of transcription regulators into the nucleus, ultimately influencing the cell's phenotype. Additionally, in a 2D environment, cells can spread out laterally without any mechanical constraints. *This figure is reproduced and modified slightly from Saraswathibhatla et al.'s 2023 study.*[226]

In 2D cultures, cells are restricted to a flat surface, which limits their ability to form natural cell-cell and cell-ECM interactions. This lack of three-dimensionality leads to altered cytoskeletal organization and function, which can significantly impact cellular behavior and experimental outcomes.[226] (Fig. 1.4) Attachment in a 2D environment changes the cell's shape and geometry, causing the cell to flatten.[227,228] This flattening affects the internal cytoskeleton and nuclear shape, which subsequently alters gene and protein expression.[229,230] For instance, in drug discovery and disease modeling, the flat nature of 2D cultures often results in responses that do not accurately reflect *in vivo* conditions, leading to discrepancies in the efficacy and safety profiles of potential therapeutics.[231] Additionally, cancer research studies are negatively affected when experiments are carried out in 2D environments, as they do not mimic the natural architecture of tissues or tumours.[232] Due to the limitations of 2D cultures, 3D cultures have gained popularity, driven by advancements in 3D culture techniques that better replicate *in vivo* environments and provide more accurate models for studying cellular behavior and function.

1.4.2 Suspension Cultures and Bioreactors

A suspension culture is a method of growing cells in a liquid medium where the cells are not attached to a solid surface, unlike in a Petri dish. Cells are freely suspended in the nutrient-rich liquid, allowing them to grow and proliferate while being gently agitated to ensure even distribution of nutrients and gases.[233] This type of 3D culture is especially useful for cells that

naturally exist in suspension, such as certain blood cells, and is commonly used in large-scale production of cells and cellular products like antibodies, vaccines, and recombinant proteins.[234,235] Bioreactors, initially associated with industrial processes like fermentation, were first used in tissue engineering for simply mixing the medium of Petri dishes. Over time, bioreactors have advanced to support the *in vitro* creation of tissues like skin, tendons, blood vessels, cartilage, and bone, and to serve as models for studying cell function and tissue development.[236] As bioreactors have become more sophisticated, they now offer semi-automated, closely monitored, and tightly controlled environments for cell and tissue culture.[237] These advanced systems enable precise control of physicochemical parameters, making it easier to study the effects of biological, chemical, or physical cues on cell functions in a 3D context.[237,238] Many types of bioreactor systems exist (e.g. rotating wall vessels, direct perfusion systems, hollow fibres, spinner flasks, mechanical force systems)[237] but to maintain relevance to this thesis, only one will be described. Spinner flasks are one type of vessel used for suspension cultures that contains a rotating paddle or impeller to keep the cells in constant motion.[238] This agitation prevents cells from settling at the bottom, promotes even distribution of nutrients and oxygen, and mimics the dynamic environment of a living organism. The spinner flask is often used in research and industry where a higher volume of cell culture is needed, as it allows for efficient cell growth and scalability compared to traditional Petri dishes.[239-241]

Suspension cultures and 3D bioreactor systems offer distinct advantages over traditional 2D cultures. Both allow for high-density cell growth and are scalable for industrial applications, which is essential in biotechnology and pharmaceutical research.[233,238] However, maintaining consistent cell viability and function across large volumes can be challenging. They can be costly and technically demanding, requiring specialized equipment and expertise and often, scaling up bioreactor systems can lead to variability in cell behavior and product quality.[236,242,243] Due to the complexity of maintaining adequate nutrient and oxygen distribution and effectively eliminating waste products throughout the entire culture, issues such as nutrient starvation and metabolite toxicity can cause cells to form spheroids with hypoxic centers containing necrotic cells.[244] Despite these challenges, bioreactors remain crucial in modern cell culture, as their ability to provide more physiologically relevant environments and support large-scale production makes them invaluable in many areas of research and industry.

1.4.3 Other Modalities for 3D culture

Three-dimensional culture models can be categorized into several types: studies involving whole animals and organotypic explant cultures (tissue samples taken from organisms, including embryos), cell spheroids (clusters of cells that grow in a spherical shape), microcarrier cultures (cells grown on tiny beads), and tissue-engineered models (artificially created tissues). While some 3D culture models do not need scaffolds, the use of scaffolds (structures that support cell growth) has greatly increased in the past ten years.[238] Whole animals and organotypic explants are used in research to obtain precise tissue-specific information by studying cells within their natural environment, and is often used in studies of neural physiology.[245,246] While the tissue architecture is maintained in these types of 3D cultures, sustaining specimen integrity and deep imaging of samples can be difficult.[247]

Cellular spheroids are simple 3D models that form when adherent cells naturally clump together. These spheroids can be made from various cell types using methods like hanging drops, rotating cultures, or concave plates.[247-249] They do not need scaffolds and can easily be observed using light, fluorescence, and confocal microscopy. Because of these features, spheroids are often used to study solid tumor growth and spread, and in numerous therapeutic research applications, such as high-throughput screening for drugs.[250]

As the size and complexity of a 3D cell model increase, porous scaffolds become essential to support cells, aid in cell adhesion, help with nutrient and gas exchange, and prevent issues like cell death due to poor nutrient flow.[247,251,252] Scaffolds have different purposes based on their intended use. For clinical applications, biodegradable scaffolds that the body can replace with native tissue are ideal.[252] These scaffolds must support cell growth and differentiation, match the defect size, and degrade without causing toxicity or immune reactions.[238] On the other hand, scaffolds may be used as 3D models to study tissue biology or for drug and cosmetic testing.[253] Here, replicating the natural tissue structure and enabling detailed imaging of cell functions are key. Materials for scaffolds include metals, glasses, polymers, ceramics, and components derived from animals and plants (of which will be described further in Chapter 4).[252,254-257] Hydrogels, which are water-based gels that provide a supportive environment for cells and can be hybridized with other materials mentioned above, are also commonly used.[258,259] A crucial requirement for all scaffolds in culture is to (in some capacity) mimic the ECM so that adherent cell types can begin to attach and proliferate *in vitro*.[260] Adjusting the surface chemistry of these materials can enhance or reduce protein adsorption and cell attachment, and many studies have been conducted to explore the optimization of these properties.[261-264] Scaffolds and hydrogels can provide more realistic environments for cell growth due to their 3D nature, but they can also be complex to design and fabricate, and are still not exact replicas of natural tissues.

1.4.4 Conclusions in 2D and 3D Cultures

3D cell culturing methods offer promising potential for scientific breakthroughs that have been difficult to achieve with traditional 2D cultures. Despite their advantages, 3D cultures present challenges such as higher costs and they are still imperfect in replicating natural cell microenvironments.[265] One significant hurdle is fluorescence microscopy, which in 3D cultures requires capturing a series of images at different depths (z-stack), unlike the simpler single-layer imaging in 2D cultures.[266] Even so, one survey found that two-thirds of respondents (i.e., scientists and researchers) plan to switch from 2D to 3D cell cultures, with many having already made the transition.[267] As more researchers adopt 3D cultures, it is expected that new methodologies will emerge to address current limitations.

Biomaterials and the other 3D culturing systems mentioned above are often incorporated into cultures in tissue engineering to mimic the natural 3D microenvironment's biochemical and biophysical properties. Various natural and synthetic biomaterials are used to develop scaffolds that support cell growth in conditions that closely resemble *in vivo* environments. However, choosing the appropriate biomaterial for specific cell types can be challenging, as different

materials can cause variability, be less biocompatible than their counterparts, and often contain animal-derivatives which introduce ethical concerns and potential contamination risks.[268]

Although 2D culturing methods are simpler and more widely practiced, they can be less applicable than 3D methods. Mechanotransduction is altered in both 2D and 3D environments, but is more changed in 2D environments, affecting various aspects of cell behavior, including growth, differentiation, and response to stimuli.[221,269] 3D cell cultures provide deeper insights into cell adhesion and migration mechanisms, as their 2D counterparts exhibit differences in gene expression.[270-272] For example, fibroblasts on a 2D substrate differ in shape and adhesion protein distribution compared to those cultured in 3D collagen.[273-275] Developing 3D culture systems involves managing numerous factors such as medium composition, biomaterials, mechanical stress, oxygen content, pH, and medium perfusion, all of which influence the quality and quantity of cultured cells.[247] Issues like cell agglomeration and nutrient/waste diffusion must also be addressed to standardize culture methods and prevent heterogeneous cell populations, necrosis, and inhibited cell proliferation.[269,276] Moreover, there is a significant need to produce large quantities of high-quality cells for biomedical applications. Allogenic cell therapy for clinical trials requires billions to trillions of cells, necessitating substantial advancements in media, culturing devices, and techniques.[269] Future work in this field is likely to focus on integrating 2D and 3D culture methods to leverage the strengths of both approaches. This could involve developing hybrid systems that combine the simplicity of 2D cultures with the physiological relevance of 3D cultures. Additionally, advances in medium formulation will be crucial for optimizing cell growth and function in both 2D and 3D environments. By tailoring media formulations to the specific needs of cells in different culture conditions, researchers can improve experimental outcomes and advance our understanding of cell biology in multi-dimensional microenvironments.

1.5 Efforts in Serum Alternative Fabrication

1.5.1 How to Develop Serum-Free Media

Through understanding the nutrient requirements of cells in culture, why they grow, and how 2D and 3D environments affect them, we can appreciate why FBS is often used to maintain cell cultures. Nevertheless, serum-free media (SFM) developments have been on the rise since the 1960s and continue to be a focal point of research and innovation in the modern day. Before diving into the details of these advancements, we will first define the different types of serum-free alternatives and review the numerous studies that have recommended methods for their creation. With countless studies exploring alternatives to FBS, it is crucial to define these alternatives clearly, as they vary significantly—some still contain animal components, while others do not (Table 1.2). Even without serum, media containing animal derivatives can pose ethical concerns, batch variability, and the risk of contamination.[11,277]

Term	Definition	Supplement Example
Serum-based	Media containing animal or human serum.	• FBS • Human serum
Serum-free	Media containing neither serum nor unprocessed plasma but may contain discrete proteins or bulk protein fractions, which may or may not be animal-derived.	• Animal/human-derived supplements (platelet lysates, serum fractions) • Animal/plant hydrolysates • A mixture of human/animal-derived or recombinant proteins, hormones, growth factors, and lipids
Xeno-free	Media containing only human-derived supplements (e.g., no animal serum) so that the finished product is considered to be human sourced.	• Human serum or plasma • Human-derived supplements (e.g. human platelet lysates)
Animal component-free	Media containing neither animal- nor human-derived components. The final product is not in any way derived, manufactured with or otherwise exposed to any animal- or human-derived components during manufacturing operations.	• Plant hydrolysates • A mixture of recombinant materials (proteins, hormones, cytokines, and/or growth factors) that are expressed and processed from qualified cell lines
Chemically-defined	Media containing no proteins, growth factors, hydrolysates, or components of unknown composition. These media contain only components whose chemical compositions and structures are known and have all of their chemical species specified.	Recombinant materials such as proteins, hormones, cytokines, and/or growth factors
Protein-free	Media containing no proteins but often containing hydrolyzed proteins and small peptides with low molecular weight.	• Hydrolyzed proteins • Small peptides such as insulin

Table 1.2: Classification and definitions of cell culture medium types.
Serum-free media are defined as no sera present but with additives like proteins sourced from animals or humans. Media that contain only human derivatives are defined as xeno-free. Animal component-free media do not contain any human- or animal-derived components. Chemically-defined media consist of recombinant proteins and synthetic ingredients. Protein-free media lack full proteins but may contain peptides and hydrolyzed proteins. *Table is reproduced from Karnieli et al.'s 2017 review.*[278]

Early efforts to cultivate cells in serum-free, hormone-supplemented media aimed to elucidate the function of serum in cell culture. Researchers tried to identify and replace all physiologically relevant serum components to support cell proliferation, but these attempts were initially met with limited success.[13] The pioneering work of Hayashi and Sato in 1976 marked a

breakthrough, as they replaced serum by adding specific hormones that maintained growth and initiated differentiation in certain cells, leading to the creation of an effective SFM.[49,65,279] In the last 20 years, many components have been identified to maintain distinct cell types in SFM and the variety of cell-specific media continue to expand. There are numerous reviews on developing SFM,[11,13,278,280-292] many of which share overlapping conclusions, which will be described here.

To develop a new SFM that is generalizable across various cell types, a common starting point is a 50:50 mixture of two basal mediums: DMEM and Ham's F-12. This combination integrates DMEM's high amino acid content with the enriched nutrients of Ham's F-12.[49,293] The basal medium should also include an essential supplement known as ITS (insulin, transferrin, and selenium). Insulin has been a vital component to cell culture and is the most commonly used hormone additive to SFM.[171] Transferrin is crucial for iron transport into cells,[294] while selenium, a trace element, protects cells against oxidative stress through its role in selenoproteins.[295]

Some cell types can survive in basal media alone[120,296] but most require additional supplements to thrive. Among these supplements, albumin plays a crucial role in acting as a carrier protein, transporting and stabilizing various molecules such as lipids, hormones, and vitamins, which are vital for cellular functions.[297] Additionally, albumin helps to maintain osmotic pressure and pH balance in the culture environment, providing stable and supportive conditions.[298] Recently, various recombinant forms of albumin have been introduced commercially. These recombinant albumins are more likely to meet regulatory standards and can serve as animal component-free ancillary products for cell therapy and regenerative medicine. Another component that is often added to SFM is glutamine, an amino acid that serves as a crucial building block for proteins and is vital for cell growth and division.[299] In the context of cell culture, glutamine provides a necessary source of energy for rapidly dividing cells and supports the synthesis of nucleotides and other essential molecules.[300] Despite its benefits, glutamine can be unstable in solution and can produce toxic ammonia, so commercialized, more stable alternatives like GLUTAMAX™ are recommended as additives.[301] Nowadays, some commercially available basal media like DMEM contain Glutamax already, speaking to its popularity in the serum-free culture industry.

The addition of specific hormones and growth factors will be highly cell type specific, and since their functions were discussed in *Section 1.3.3*, we will not delve into their details here. Briefly, hormones, which are physiological constituents in blood and present in serum, were among the first supplements that were experimented with SFM development. As mentioned, insulin is critical in all serum-free formulations, while other commonly added hormones include glucocorticoids, triiodothyronine, and cAMP-elevating agents.[13] Growth factors, crucial for increasing cell proliferation and stimulating specific cell functions, are typically added to basal media, although they should be carefully considered based on the cell type in question. The most commonly used growth factors for SFM development include fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF).[278] Protease inhibitors, substances that help protect cells by preventing the breakdown of proteins, such as α1-antitrypsin and α2-macroglobulin, are not essential to SFM,

yet can be considered in their development.[302] The specific needs for key growth-promoting elements also differ based on factors such as the intended application, type of culture substrate, the culture system (i.e. 2D vs 3D), and relevant regulatory standards.

Researchers should consider incorporating extracellular matrix components to facilitate cell attachment, as animal-sera typically provide the necessary attachment factors required by adherent cell types in culture.[303] Without FBS, cells may struggle to adhere to the culture surface, which can impede their growth and viability.[304] To address this, various additives and coatings can be used to enhance cell attachment such as fibronectin, collagen, laminin, and vitronectin.[213,305] These proteins mimic the natural ECM, providing binding sites that cells can adhere to, thus supporting their growth and function. Additionally, culture substrates can be pre-coated with several materials to improve cell adhesion. Common coatings include: synthetic polymers (e.g. Poly-L-lysine, Poly-D-lysine) that enhance the electrostatic interaction between the cell surface and the culture substrate, promoting better attachment[306]; Matrigel, a gelatinous protein mixture that resembles the complex extracellular environment found in many tissues[307,308]; collagen, which provides a natural scaffold that supports cell adhesion and growth[309]; gelatin, a denatured form of collagen[310]; or, synthetic/plant-based peptides that mimic specific adhesive motifs found in natural ECM proteins.[278]

To validate SFM, a series of controlled experiments can be conducted to compare cell growth, viability, and function against a standard serum-containing medium. This involves assessing cell proliferation rates, morphological characteristics, and specific markers of cell health and differentiation through techniques such as flow cytometry, microscopy, and biochemical assays. Additionally, performing stress tests to evaluate the medium's ability to support cells under various conditions, such as detection of nutrient depletion or targeting and measuring markers of oxidative stress, can provide further validation.[11] Reproducibility is key, so repeating experiments across multiple cell lines and different iterations of media will help confirm the consistency and reliability of the developed formulation. Existing media formulations can be found through literature surveys or online databases, which, despite limitations, provide valuable resources for further development and optimization.[13,208,311]

1.5.2 Current Serum-Free Media Formulations

Having outlined the principles and methodologies for developing SFM formulations, we now turn our attention to reviewing the existing formulations that have shown great promise. The most effective and innovative SFMs currently available will be highlighted, providing insights into their components, applications, and the specific cell types they support. By examining these formulations, we can identify the best practices for future development but despite the identification of many alternatives to FBS, including biological fluids, industrial by-products, and chemical combinations, no single serum-free or chemically-defined medium can replace FBS for all cell types currently. Over the past sixty years, alternatives such as pituitary extracts, chick embryo extracts, bovine milk fractions, and plant-based proteins have been investigated.[210] While these alternatives show promise, they often still contain animal-derived components,

which come with their own challenges. Here, we will examine pre-existing studies that developed SFM which are chemically-defined and predominantly animal component-free (ACF). Numerous chemically defined or semi-defined SFM products are now available commercially for cell culture applications. It is important to note that medium formulations are often not fully disclosed. As a result, despite being marketed as chemically-defined, the true chemical composition and concentration of many commercial products can be unclear.[278] One example of an infamous commercially-available SFM is the Essential 8™, developed initially for induced pluripotent stem cells (iPSCs), which gained popularity due to its minimalistic formulation consisting of only eight essential components.[312] There have been other efforts to create SFM for different cell types used in regenerative medicine, including human mesenchymal stem cells (hMSCs), iPSCs, and T cells,[284,312,313] and in cultivated meat, including bovine satellite cells and bovine myoblasts.[278,315-319] Some optimized SFMs have shown superior growth-promoting capabilities compared to traditional serum-containing counterparts.[320-322] Other cell lines like CHO and HEK293 cells have fostered countless serum-free studies, due to their unique capabilities.[323] CHO cells are notable for their ability to grow without FBS, which facilitated the production of the first therapeutic protein, human plasminogen activator, in 1986. CHO cells continue to be preferred for recombinant protein manufacturing because the ease of serum-free conditions enhance production safety and reduce processing variability. Reports approximate that around 80% of monoclonal antibodies approved between 2014 and 2018 were produced using CHO cells, which is why over 22 CHO-focused FBS-free media formulations exist.[324,325] Similarly, the HEK293 cell line, which thrives in FBS-free medium and has a high transfection efficiency, is widely used for therapeutic protein production.[326]

Other cell lines have also been the focus of developing SFM, reflecting the broad scope and potential of these formulations. For instance, Vero cells, a key cell line in vaccine (e.g. inactivated polio, rabies) production, have successfully adapted to serum-free conditions.[287,327-329] Additionally, MDCK cells, which are crucial for influenza vaccine production, have been effectively cultured in SFM that enhance the vaccine production yield.[330-332] Other noteworthy examples include SFM for hybridoma cells used for monoclonal antibody production for diagnostic and therapeutic applications,[66,333,334] and for neuronal cell lines which has enabled more accurate modeling of neural development and neurodegenerative diseases, as well as the testing of neuroprotective drugs.[68,335-337] For example, the SH-SY5Y neuroblastoma cell line has been successfully cultured in SFM, supporting studies on Parkinson's disease and other neurological disorders.[338-340] Hematopoietic stem cells (HSCs) used in blood and bone marrow research represent another significant success story. SFM formulations have been developed to support the growth and differentiation of HSCs, which are critical for understanding hematopoiesis and developing treatments for blood disorders.[341-343] Finally, countless SFM have been developed for cell lines used in fundamental research with variations in their components, such as for myoblasts,[344-348] fibroblasts,[349-352] osteoblasts,[353-356] and various epithelial cell lines.[357-361] These formulations are tailored to support the specific growth and differentiation needs of each cell type. For instance, myoblasts, which are precursor muscle cells, often require media supplemented with specific growth factors like IGF or FGF, which promote their proliferation and differentiation into mature muscle cells.[363-364] Fibroblasts, essential for connective tissue research, thrive in media containing components that support their collagen

synthesis and extracellular matrix formation.[365] Osteoblasts, which are bone-forming cells, benefit from media enriched with calcium and phosphate to facilitate mineralization.[366-368] Epithelial cells, critical for studies on barrier functions and tissue regeneration, require media that maintain their polarity and tight junction integrity.[369,370] These tailored formulations enable researchers to investigate cellular behaviors and functions with greater precision and consistency, advancing our understanding of basic biological processes. The variation in the components often implemented for each cell type and their concentrations are reviewed in Table 1.3.

Hormone, Growth Factor, or Binding Protein	**Concentration**	**Responsive Cell Types/Lines**
Insulin	0.1 – 10 μg/ml	All cell lines
Glucagon	0.05 – 5 μg/ml	**Human:** colon carcinoma, lung epidermoid carcinoma **Rat:** liver parenchyma, hepatocyte
Follicle Stimulating Hormone	0.05 – 0.5 μg/ml	**Human:** ovarian cells **Rat:** ovarian cells **Mouse:** sertoli **Porcine:** granulosa
Growth Hormone	0.05 – 0.5 μg/ml	**Human:** B cells **Rat:** osteoblasts **Mouse:** sertoli **Chicken:** chondrocytes
Epidermal Growth Factor (EGF)	1 – 100 ng/ml	**Human:** Hela, breast cancer lines, colon carcinoma, human lung epidermoid carcinoma, pancreatic carcinoma **Mouse:** sertoli, embryonic fibroblast, myoblasts **Porcine:** MSC
Fibroblast Growth Factor (FGF)	1 – 100 ng/ml	**Human:** Hela, mammary/breast epithelium, MSC, hematopoietic stem cells, keratinocytes **Mouse:** myoblasts, fibroblasts **Rat:** microglia, fibroblasts **Hamster:** kidney fibroblast **Canine:** MDCK **Porcine:** pluripotent stem cells **Bovine:** satellite cells
Transforming Growth Factor beta (TGFβ)	0.5 – 100 ng/ml	**Human:** articular chondrocytes, MSC, melanocytes **Mouse:** hepatocytes, myoblasts **Rat:** hepatocytes, kidney cells, microglia **Bovine:** chondrocytes

Insulin-like Growth Factor (IGF)	10 – 100 ng/ml	**Human:** embryonic stem cells **Rat:** osteoblasts **Porcine:** adipose tissue **Rabbit:** chondrocytes **Hamster:** CHO
Triiodothyronine	1 – 100 pM	**Human:** mammary epithelium, lung epidermoid carcinoma, colon carcinoma, skin fibroblast **Rat:** pituitary epithelium **Porcine:** adipose tissue **Bovine:** chondrocytes **Canine:** MDCK
Hydrocortisone	10 – 100 nM	**Human:** MSC, Hela, mammary epithelium **Rat:** myoblast **Canine:** MDCK
Transferrin	0.5 – 100 μg/ml	Most cell lines
Albumin	0.5 – 5 mg/ml	**Human:** blastocyst, pluripotent stem cells, umbilical vein endothelial cells, fibroblasts, keratinocytes, satellite cells, **Mouse:** embryonic fibroblast, lung fibroblast, mammary epithelium, myoblast **Rat:** glia, myoblast, satellite cells, **Hamster:** CHO **Bovine:** myoblast **Chicken:** myoblast
Attachment Factors (e.g. fibronectin, vitronectin, laminin)	5 – 100 μg/ml	All adherent cell lines may benefit

Table 1.3: Stimulatory factors for cell lines in serum-free media.
Cell lines listed are ones that have used the corresponding component to maintain high (>80%) viability in a serum-free study previously. This is not a fully comprehensive list. For the cell lines listed, the concentrations given are the effective range commonly used in modern SFM. Some specific requirements of less commonly used nutrients or substrate modifications are not listed. *This table is a modern and adapted take on Sato et al.'s table from 1980.*[49]

Alternative methods for developing SFM include using microbial fermentation to synthesize recombinant growth factors and utilizing non-animal origin extracts.[371] Microbial fermentation, also known as precision fermentation, has long been employed in biotechnology to produce commercial quantities of molecules, such as pharmaceutical insulin. By producing the most crucial molecules to cell culture found in FBS through this method, it's possible to replace serum with precisely defined media tailored for certain cell lines.[372] Another promising approach involves using extracts from non-animal sources, which are often cheaper and simpler to produce.[373] These extracts come from fungi, soybeans, bacteria, or microalgae, many of which

are already used in food production.[374-378] Despite their enticing costs and straightforward means of production, the variability in extract compositions can affect the final product, and their use in bioreactors is not yet fully established.[372,379,380]

Continuous innovation is essential to address the unique requirements of different cell types and applications. With new formulations regularly emerging, the landscape of SFM is ever-expanding. A valuable resource for researchers is the FBS-free database, which provides information on media from commercial sources or described in the scientific literature for a variety of cell types.[381] This database is instrumental in facilitating the transition to serum-free systems by offering access to a wide range of validated and experimental media formulations. The examples discussed illustrate the diverse applications and potentials of SFM across various cell lines and research domains. However, to drive further innovation and refinement, integrating computational approaches will be essential to help optimize formulations and address the complex requirements of different cell types more efficiently.[382]

1.5.3 Challenges in Serum-free Development

Serum-free media offer several advantages over serum-containing media, including reduced risk of contamination, more consistent results, and better control over the cellular environment. One significant advantage of SFM is the ability to tailor the composition to the specific requirements of different cell types but also to induce differentiation or maintain cells in a particular state, providing greater experimental control. Even so, SFM development faces several significant challenges and limitations. One primary issue is the need for optimization across different cell types, which can be a lengthy and costly process since the medium requirements for different cell types may not overlap in their contents. For example, satellite cells from diverse animal species have unique requirements and respond differently to specific factors used in serum-free environments.[292,383,384] This necessitates the development of tailored formulations for each cell type, which can be resource-intensive.[323] Another significant drawback is the suboptimal cell proliferation rates occasionally achieved with SFM compared to traditional serum-containing media.[315,316,376,379,380] Although some cell lines like CHO and HEK293 cells have exhibited better proliferation rates in modern optimized serum-free formulations, it is often the case that SFMs developed for other cell lines do not yet match the efficiency of serum-based ones in promoting cell growth. Moreover, the cost of producing chemically-defined media is substantially higher due to the need for recombinant growth factors which are expensive because of complexities in their synthesis and purification.[385,386] This economic barrier is a significant obstacle to the widespread adoption of SFM, especially for large-scale applications.

Regulatory and sourcing challenges further complicate the development and implementation of SFM. Developing acceptable substitutes for the various functions of serum involves identifying and sourcing reliable, certified components, which can be difficult given the variability in quality and supply of biological materials.[278] For instance, human serum albumin, transferrin, or platelet-derived lysate, while potential substitutes, face restrictions in sourcing and quality assurance due to regional regulations.[387] Moreover, the transition to xeno-free or chemically-

defined media introduces additional complexities, as these media become increasingly cell type- and process-specific, requiring extensive testing and validation to ensure efficacy across different applications.[388]

Another drawback to SFM development is the challenge in validating and defining what constitutes a truly healthy cell. There are numerous metrics and methodologies to assess cell health, leading to variability in standards and outcomes. Furthermore, using FBS-grown cells as a control is logical in certain contexts, but it has limitations. FBS does not exactly replicate *in vivo* conditions, meaning comparisons are often made to a standard that exists primarily because it has been the most effective since the inception of cell culture. This reliance on FBS as a benchmark may obscure more accurate assessments of cell health and function in serum-free environments.

Beyond the scientific and technical challenges of developing SFM, the ethical and environmental implications of continuing to use animal-derived products like FBS are significant. The harvesting of FBS raises serious ethical concerns, as it involves the collection of serum from fetal calves often with no anesthetization, contributing to the broader ethical issues associated with industrial animal agriculture.[198] The environmental impact of animal agriculture cannot be overstated; it is a major driver of deforestation, greenhouse gas emissions, water pollution, and biodiversity loss.[389-391] By developing and adopting SFM, we can help mitigate these environmental damages and promote more sustainable and responsible scientific practices. Transitioning to serum-free alternatives is not only a scientific necessity but also a moral and environmental imperative, aligning research with global efforts to reduce our ecological footprint and advance more humane approaches to biotechnology.

In summary, optimization for diverse cell types, suboptimal proliferation rates, high production costs, environmental impacts, and regulatory and sourcing challenges all contribute to the complexity of this endeavor. Despite these obstacles, continued research and innovation are essential to overcome these barriers, enabling the broader use of SFM in various scientific and industrial applications. From commercial to open-source, research-driven serum-free formulations, there is a clear variety in the types of alternatives to FBS-based cultures. Different cell types thrive in SFM, while others do not. The universality in culturing many types of animal cells lies in the large number of components found in animal sera, but the dependence on animal-derived products remains a significant concern. This issue has prompted pleas from scientists to seek alternative solutions due to ethical considerations, potential for contamination, and the variability associated with animal-based materials.[198] Regardless, this is an ongoing endeavor, and there are pros and cons to both animal-based and animal-free culture, necessitating continued investigation.

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Chapter 2 | The development and validation of an animal component-free media for select cell types in 2D conditions

§ Motivation

There is a growing demand for sustainable and cost-effective practices in biomedical research. Traditional cell culture methods often rely on animal-derived components (such as fetal bovine serum (FBS)), which can pose ethical, reproducible, and economic challenges. The use of FBS not only raises concerns about animal welfare, but also introduces variability and potential contaminants that can affect experimental reproducibility and data interpretation. Developments in serum-free media are rising, yet no standard exists in quantitatively assessing their effectiveness for mammalian cells in culture. Additionally, their specificity poses a drawback in culturing different types of cells and new formulations are needed to support a broader range of cell lines under various experimental conditions. With the rise of advancing biotechnologies and therapeutic developments, newly characterized, multi-use serum alternatives are vital to push these industries forward.

§ Hypothesis and Objectives

The goal of this study was to create a fully ACF media to grow 4 commonly used cell lines in research, without the use of animal serum. A secondary goal of this study was to quantitatively characterize the differences in morphology and gene expression of the studied cell types in the ACF media and compare them to the typical effects of FBS in mammalian cell culture. We hypothesized that the ACF media would support the growth and maintenance of the selected cell lines similarly to FBS-containing media, as the components we chose in the formulation were proven to be useful in previous serum-free studies. Additionally, we hypothesized that although the ACF media would foster proliferation, that distinct morphological characteristics would be observed with altered gene expression profiles related to stress response and extracellular matrix production.

2.1 Abstract

Fetal Bovine Serum (FBS) is one of the most commonly used media supplement for the maintenance of mammalian cell types, yet the expensive costs, ethical concerns, and lot-to-lot variation have provoked a clear need for a serum that is standardized and derived from non-animal sources. Several serum-free formulations have been developed in the past, however they are often cell type specific, contain animal-derived components, and lack long-term culture validation. In this study, we developed a novel animal component-free (ACF) medium and

investigated its effectiveness on four commonly used mammalian cell lines via long-term (up to 90 days) morphological, transcriptomic, and proliferative analyses. Cells cultured in our ACF medium exhibited comparable cellular morphologies and equal or greater growth rates compared to cells cultured with FBS. Additionally, differentially expressed genes between the FBS-grown and ACF-grown groups were predominantly associated with functions linked to proliferation and cell attachment. The findings from this study indicate that this medium is a suitable replacement to FBS-containing medium for several common cell lines.

2.2 Introduction

Cell culture systems, pivotal for studying physiological, biological, and pharmacological activities at the cellular level, have significantly advanced biology with broad applications ranging from drug evaluation and tissue engineering to vaccine production and cultivated meats.[1-4] Cell culture medium, the vehicle that supports cell viability, is essential to any cell culture *in vitro*.[5,6] The proteins, vitamins, minerals, hormones, growth factors, and other key nutrients that are responsible for providing this support to mammalian cells must be supplied by the medium they are grown in.[7,8] The major supplier for these essential components has been commonly derived from animal sera, with fetal bovine serum (FBS) being the 'gold standard' supplement since its introduction to cell culture scientists in 1958.[9,10] Other mammalian-derived sera from chicken, sheep, pig, turkey, and horse blood can also be used in certain protocols or for specific cell types,[11] but FBS continues to be the most widely used supplement. A by-product of the animal agriculture industry, its production varies based on changes in the public's beef consumption, outbreaks of bovine-related diseases, and changes in crop prices for herd feed.[12] The high demand for meat products has led to large cattle inventories and therefore it is unsurprising that FBS can be relatively easily sourced and distributed to scientists worldwide.[12,13] Although practical, FBS carries several scientific and ethical concerns and ideally a more sophisticated, controllable and reproducible alternative should be developed.[14,15]

The high degree of lot-to-lot variability in FBS can be one significant cause of irreproducibility in research findings from lab to lab around the world.[14-17] This has a detrimental impact on academic science and on the commercialization of biotechnologies aimed at producing consumer products, cell-based therapeutics, and medical assays, and it is not fully understood how each component contributes to either proliferation or differentiation, at what capacities, and in differing cell types.[18-20] Chemically-defined, serum-free media (SFM) alternatives pose fewer ethical concerns, eliminate the potential of biological contaminants, create more controlled culture conditions, have more stable costs and could be less expensive altogether.[14,21,22] Many literature reviews on SFM development methodologies and essential components have been conducted.[14,15,18-20] Commercially available and open-source serum supplements have been produced previously with over 100 different serum-free formulations to date, however they are typically optimized for the growth of specific cell types,[13,15] for example chondrocytes,[25-29] myoblasts,[30-33] satellite cells,[34-37] HeLa cells,[38-40] Vero cells,[41-45] and a variety of fibroblastic lines.[46-51] It is important to note that although there is some overlap in the components used by these groups, the implemented concentrations and ingredients vary

between most of the developed formulations and the quantitative assessment methods used to validate each formulation differ greatly from one another, making comparisons difficult.

These alternative media additives provide a strong starting point for the development of a more generalized serum replacement. The goal of the research presented here is to develop and validate a general-use, well-characterized, ACF serum supplement to address the obstacles described above, for several different adherent cell lines. The ACF medium we developed was validated by looking at proliferation and morphological analyses as well as transcriptomics in long-term culture. Herein we demonstrate the creation of a synthetic and recombinant component-based medium that is validated for the NIH 3T3 fibroblast, C2C12 myoblast, MC3T3 pre-osteoblast, and HFF human foreskin fibroblast cell lines, which can be utilized, and independently validated by scientists worldwide who depend on mammalian cell culture processes. The experiments carried out here provide the cell culture community with verification of a functional serum replacement and create a modern starting point for non-computational serum-free fabrication.

2.3 Results

2.3.1 Serum-free media creation

A multi-step methodology was carried out in order to investigate various combinations of supplements for cell culture media. We examined their interactions and fine-tuned their concentrations to create a SFM formulation suitable for supporting the growth of NIH 3T3 fibroblasts, and later made minor adjustments of the formulation for 3 commonly used cell lines in research. To do so, the following steps were carried out:

1. By assessing the studies and reviews that investigated serum-free media formulations, essential components to adherent mammalian cell culture of widely used cell lines were identified.[14-51] From these reviews and primary research articles, a database was created to summarize any components that were used in successful serum-free culture as well as their concentrations. By adapting the recommendations from van der Valk's 2010 review, the essential components needed to begin formulating SFM are presented (Fig. 2.0). We examined the components which overlapped studies most and then chose the ones that could be made ACF to start in our first formulation. Initially, this consisted of a strongly recommended basal medium (DMEM/F-12), 2 hormones (insulin, prostaglandin E2), 2 carrier proteins (transferrin, albumin), 1 lipid derivative (ethanolamine), and 3 growth factors (epidermal growth factor (EGF), fibroblast growth factor (FGF2), transforming growth factor beta (TGFβ).

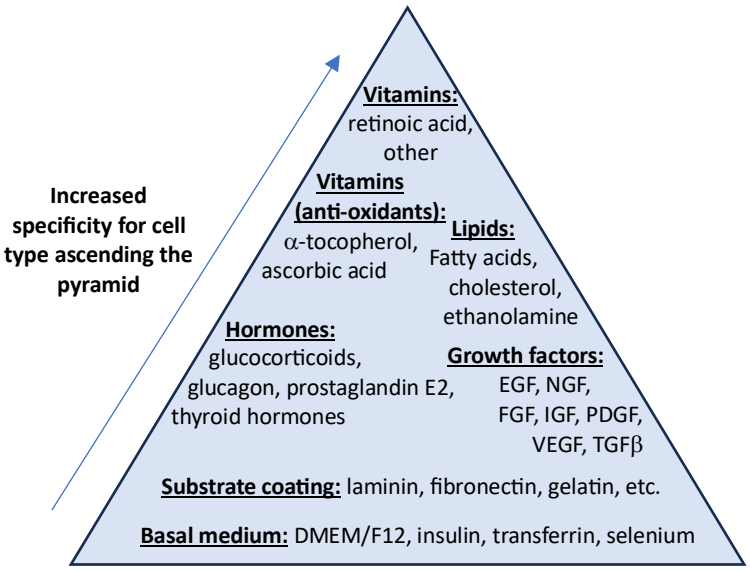

Figure 2.0: Schematic modular approach for the development of serum-free media.
Start the formulation at the base of the pyramid and move upwards to increase specificity for the desired cell line. Each heading provides examples of the types of components that can be added but is not a comprehensive list. *Adapted from van der Valk, 2010.*[23]

2. With this initial formulation, NIH 3T3s were sequentially adapted from serum-containing medium to serum-free medium over a 10-day period *(see Methods Section 2.5.3).* Next, cell viability using trypan blue exclusion was measured after seven days of culture. Although this initial formulation allowed for high viability (>90%) after seven days, the morphology of the cells as shown using phase contrast microscopy exhibited much more elongated, spindle-like morphology as compared to the cells grown in FBS-containing medium. Thus, further optimization of the components was required.
3. The concentrations of four components were first chosen to be modified including the 3 growth factors (EGF, FGF2, TGFβ) and 1 hormone (Prostaglandin E2, PGE2). The reason that these factors were chosen to be modified initially while keeping the others constant was because the other components showed much more consistency across serum-free media studies both in their implementation and concentration, while these growth factors and hormone were implemented quite differently across successful serum-free research. In the past, matrices for culture media components have been used to identify the most significant factors that influence a certain outcome.[147,148] In this study, we modified this approach by setting up a matrix that allowed us to screen for different combinations of concentrations of these four chosen media components. We implemented this using a non-statistical approach with 2 desired outcomes: a) maintaining high viability (>90%) at the seventh day of culture, and b) exhibiting a similar morphological pattern via phase contrast microscopy as compared to cells grown in FBS-containing media. Table 2.1 summarizes all concentrations and outcomes found in this optimization study.

Medium type	*Component*				*Cell viability*		*Morphology*
	A (TGFβ) **+ = 5ng/mL** **- = 1ng/mL** **-- = 0 ng/mL**	**B (FGF2)** **++ = 50ng/mL** **+ = 25ng/mL** **- = 1ng/mL** **-- = 0ng/mL**	**C (EGF)** **++ = 25 ng/mL** **+ = 10ng/mL** **- = 1ng/mL** **-- = 0ng/mL**	**D (PGE2)** **+ = 10ng/mL** **- = 5ng/mL** **-- = 0ng/mL**	**Survived to Day 1 of 0% FBS**	**Survived to Week 1 of 0% FBS**	**Similar interspacing and elongation to FBS-grown NIH 3T3 cells**
1	+	++	++	+	✓	✓	×
2	+	++	-	+	✓	✓	×
3	+	++	--	+	✓	×	×
4	+	+	--	+	✓	×	×
5	+	-	-	+	✓	✓	×
6	-	-	++	+	✓	✓	×
7	-	-	+	+	✓	✓	×
8	-	-	-	+	✓	✓	×
9	-	-	-	-	✓	✓	×
10	-	-	-	--	✓	✓	×
11	-	--	-	+	×	×	×
12	--	-	+	+	✓	✓	✓
13	--	-	+	--	✓	✓	✓
14	--	-	-	+	✓	✓	×
15	--	-	-	-	✓	✓	×
16	--	-	-	--	✓	✓	×
17	--	--	-	+	×	×	×
18	--	--	--	+	×	×	×

Table 2.1: Optimization matrix for final ACF medium formulation.
Components A, B, C, and D were chosen based off the literature suggesting that these growth factors and hormones may be related to more volatile changes in cell growth. Their concentrations designated as (++) represent the high end of the given range of concentrations based off the preliminary medium formulation. Their concentrations are designated as (-) represent the low end of the given range of concentrations based off pre-existing literature. Their concentrations designated as (--) represent a concentration of zero. Each of the 18 media formulations were allowed to proliferate for at least 7 days since any exposure to FBS, if possible.

4. From this matrix, we found several combinations that allowed for our 2 desired outcomes. Next, to create simplicity and minimize costs downstream, we chose the formulation that used the minimal concentration of growth factors but still fostered the same outcomes, since growth factors are often responsible for most of the high serum-free media costs.

5. Finally, this formulation was used to quantitatively assess long-term proliferation in NIH 3T3 fibroblasts and HFF human foreskin fibroblasts. This final formulation did not have optimal success with the other 2 cell lines used in this study (C2C12 myoblasts, MC3T3 osteoblasts) as their proliferation rates were lower and morphologies too dissimilar from their FBS-grown counter parts. As a result, two media components were altered for these cell lines. Firstly, the basal medium was changed to their native basal medium that is most commonly used for their culture – DMEM for C2C12 and αMEM for MC3T3 cells. And lastly, after similar optimization steps described above, the concentration of FGF2 increased from 1 ng/mL to 30 ng/mL for both cell lines, TGFβ was kept as is, and PGE2 was eliminated altogether. Separate from the medium formulation, a substrate coating was added to increase cell adhesion in all cell lines – the details of this finding and execution are detailed in the Methods (*Section 2.5.4*). The final medium formulation used for all cell types is summarized in Table 2.2. This final iteration of the ACF formulation is the medium used in the treatment groups in the following presented results.

Component	**Final Working Concentration**	**Company, Catalogue #**
Albumin, recombinant expressed in rice	1 mg/mL	Sigma, A9731
Animal origin-free ITS (100X) • Insulin, recombinant derived from yeast • Transferrin, recombinant derived from rice • Selenium, synthetic	Animal origin-free ITS (5X) • 50 ug/mL • 27.5 ug/mL • 25 ng/mL	Sigma, SCM055
Ethanolamine, synthetic	1.5 mg/mL	Sigma, E0135
Fibroblast growth factor 2, recombinant expressed in E. coli	1 ng/mL (NIH/3T3, HFF) 30 ng/mL (C2C12, MC3T3)	Sigma, SRP4038
Epidermal growth factor, recombinant expressed in E. coli	10 ng/mL	Sigma, SRP3196
Transforming growth factor beta, recombinant expressed in E.	5 ng/mL (C2C12, MC3T3)	Sigma, GF346

Table 2.2: Final optimized formulation for animal component-free medium.
Final recipe for optimized medium formulation used in this study. These components were added in supplement to the same basal medium as used in the FBS-grown control groups (*see Methods for basal medium description, Section 2.5.3*) with 1% Penicillin and Streptomycin.

2.3.2 ACF medium supports fibroblast proliferation and viability

After sequential media adjustment, NIH 3T3s were inoculated onto tissue culture plates and cultured for 7 days. At days 1, 4 and 7 cell concentration was quantified in an automated cell counter (Fig. 2.1A). On days 1 and 4 there was no difference in cell concentration (P=0.4468 and P=0.0776, respectively), however on the seventh day of culture, the ACF medium resulted in a significantly lower cell concentration compared to the FBS medium group (P=0.0362). Furthermore, Annexin-V/PI staining revealed that both ACF and FBS groups were able to maintain high cell viability (>80%) at each time point (Fig. 2.1B). Phase contrast images reveal significantly lower cell concentration in ACF vs FBS media on the seventh day of culture (Fig. 2.1C-D). Although qualitative, it was observed that cells in ACF medium tended to easily shear off the culture plates indicating that in the absence of FBS, cells were poorly adhered to the culture plate. Therefore, we conducted a series of experiments which explored the use of animal and plant-based surface coatings to solve this problem *(detailed in the Methods, Section 2.5.4*).

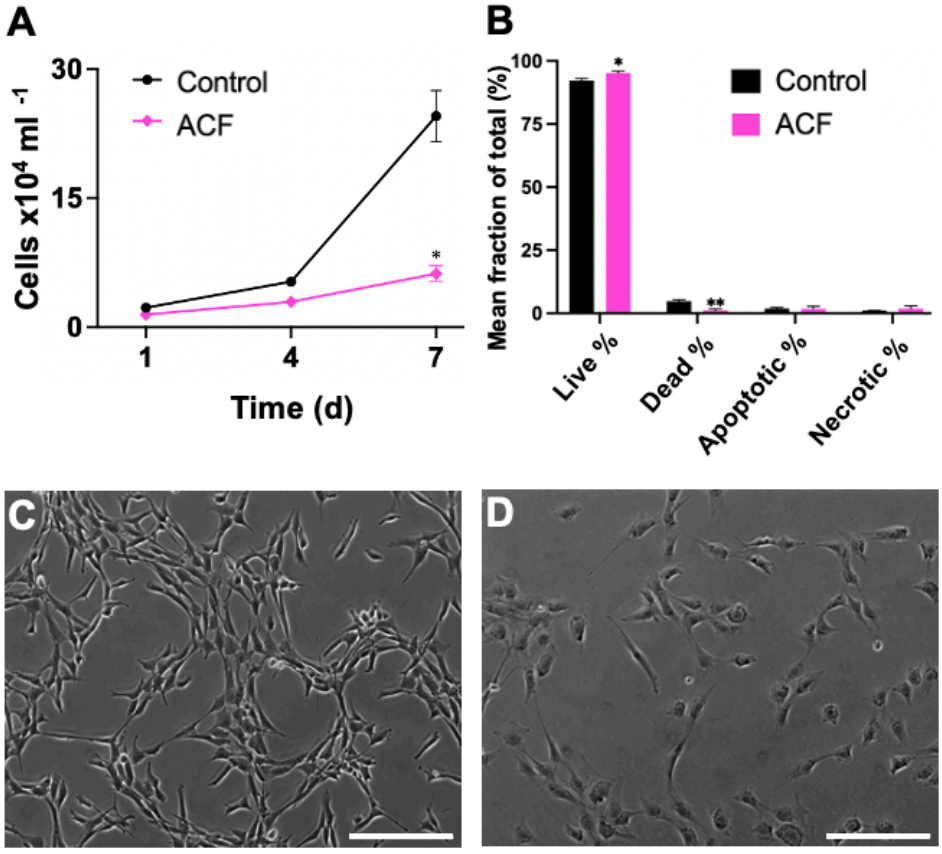

Figure 2.1: Short-term (<7 day) cell viability and morphology of NIH 3T3 fibroblasts.
(A) Mean concentration of cells (x10^4 per ml) after 1, 4, and 7 days since initial seeding. **(B)** Mean live, dead, early apoptotic, and necrotic cell populations represented as a percent of the total cell concentration, measured by Annexin-V/PI staining. N=3 for each time point and medium condition. Each biological replicate is the mean of 3 technical replicates. Data are presented as

mean ± SEM where *P<0.033, **P<0.002, ***P<0.001, as compared to the control medium. Most representative phase contrast images. Scale bars, 200μm. **(C)** Control medium (10% FBS). **(D)** ACF medium (no coating).

We included gelatin in our analyses as it is a widely used animal-derived coating that is known to increase adhesion in cell cultures.[52-56] We also chose a plant-based surface coating to use in our analyses as the goal of this work was to maintain the fully ACF quality of the culture system. Among the array of plant-based coatings applicable to adhesion in cell culture, soy stands out as a particularly promising candidate due to its unique combination of adhesive proteins, polysaccharides, and bioactive compounds.[57-60] Specifically, soy-derived proteins, such as soy protein isolate (SPI), have been recognized for their ability to form thin films on surfaces and promote cell adhesion.[61-63] These proteins possess adhesive domains that can interact with cell surface receptors, facilitating attachment to the substrate.[64,65] Furthermore, soy isoflavones, such as genistein and daidzein, have been reported to modulate cellular signaling pathways involved in adhesion and cytoskeletal organization.[66-68] These compounds can activate integrin-mediated signaling cascades, leading to enhanced focal adhesion formation and strengthening of cell-substrate interactions.[68,69] Thus we hypothesized that the application of a soy-derived coating to cell culture dishes could provide an adhesive microenvironment conducive to fibroblast attachment and spreading, similar to the effect that gelatin is widely used for. As described in the Methods *(Section 2.5.4)*, we developed a methodology to create a surface coating for Petri dishes from soy protein which was then used to support the adhesion of fibroblasts in ACF medium.

Cells were then cultured either on gelatin-coated dishes (ACF-gelatin) or on soy-coated dishes (ACF-soy). Cells grown in ACF-soy and ACF-gelatin showed similar total cell concentrations to the FBS-based group at days 1, 4, and 7. On the seventh day of culture, total cell concentrations in coated groups (ACF-gelatin and ACF-soy) were insignificantly changed from the FBS group (P=0.0850, P=0.0504, respectively) (Fig. 2.2A). All ACF media groups were able to proliferate (viability > 94%) by the seventh day of culture as determined by Annexin-V/PI staining (Fig. 2.2B). In order to view the morphology of the cells at these time points, phase contrast images were obtained. Fibroblasts grown in ACF-gelatin (Fig. 2.2E) and ACF-soy (Fig. 2.2F) showed similar densities (>75%) to the FBS group (Fig. 2.2C), consistent with the proliferation data. ACF-soy grown cells tended to grow in distinct clusters, similar to the morphology exhibited by the FBS group. Such recognizable clustering was not apparent in ACF-gelatin and the non-coated ACF groups.

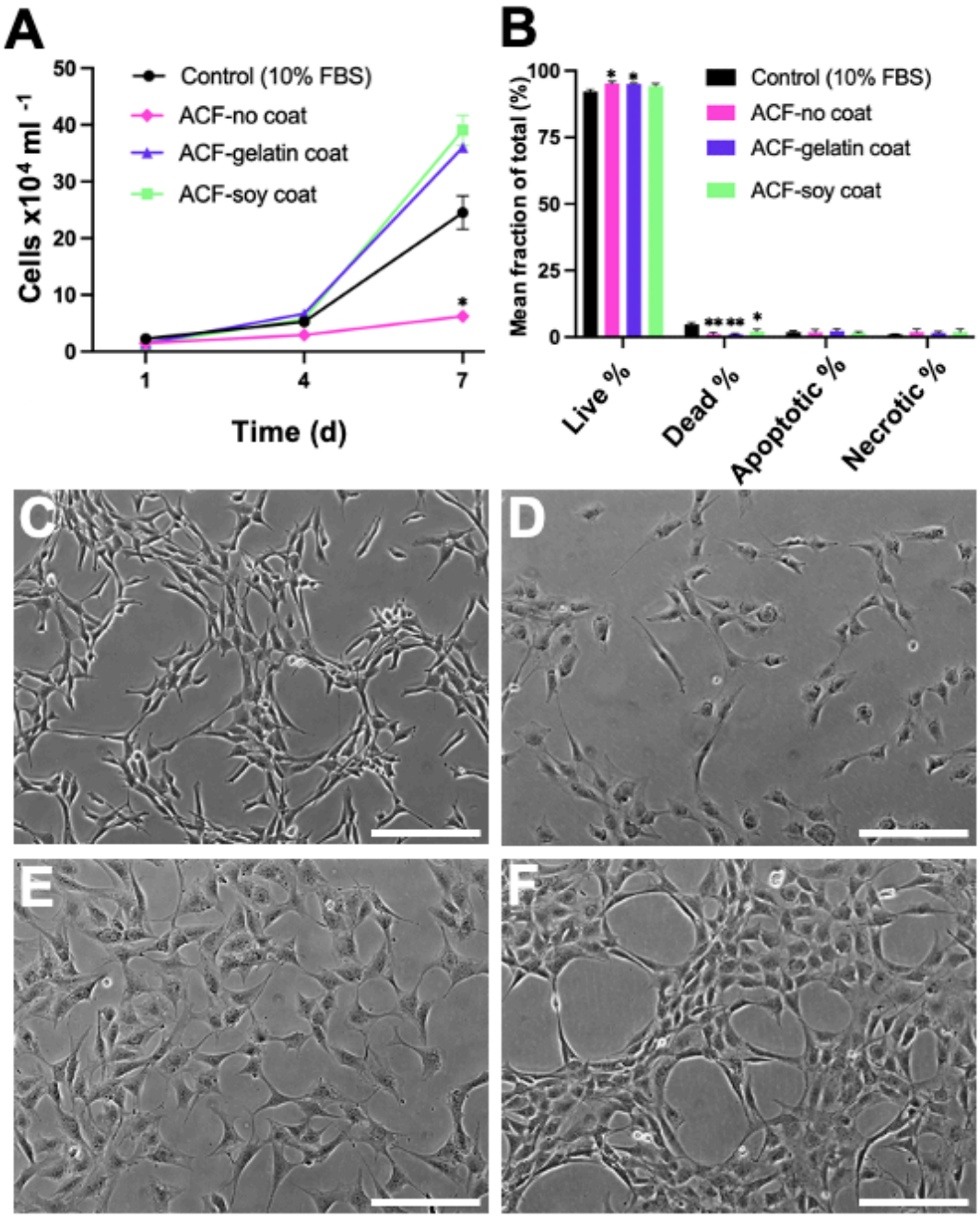

Figure 2.2: Short-term (<7 day) cell viability and morphology of NIH 3T3 fibroblasts in all ACF media conditions.

(A) Mean concentration of cells (x10^4 per ml). **(B)** Mean percentage of live, dead, early apoptotic, and necrotic cells as measured by Annexin-V/PI staining. N=3 for each time point and media condition. Each biological replicate is the mean of 3 technical replicates. Data are presented as mean ± SEM where *P<0.033, **P<0.002, ***P<0.001, as compared to the control medium. **(C-F)** Most representative phase contrast images. Scale bars, 200μm. **(C)** Control medium (10% FBS). **(D)** ACF medium (no coating). **(E)** ACF medium (gelatin-coated). **(F)** ACF medium (soy-coated).

To better understand the long-term proliferative capabilities of the ACF medium on fibroblasts, we continued to culture the cells for up to 90 days. Every 30 days, assays for cell concentration and viability were performed, along with phase contrast microscopy. After 90 days in culture, all ACF conditions showed similar total cell concentrations to the FBS group at days 30 and 60 since full medium adjustment (Fig. 2.3A). On the 90th day of culture, ACF-soy grown cells showed a significantly increased total cell concentration as compared to the FBS group (P=0.0040), while the ACF-gelatin and non-coated ACF groups showed insignificant changes in total cell concentration (P=0.0713, P=0.9854, respectively) (Fig. 2.3A). As shown by Annexin-V/PI staining, all ACF media groups were still viable after 90 days of culture (>95%) (Fig. 2.3B). Fibroblasts grown in ACF-gelatin medium showed a significantly increased live cell percentage as compared to the FBS group (P=0.004), while non-coated ACF and ACF-soy groups demonstrated an unchanged live cell percentage as compared to the FBS group. Phase contrast imaging demonstrates similar morphologies of ACF-soy cells to serum-grown cells, with non-coated and gelatin-coated ACF groups more elongated and less spread out (Fig. 2.3C-F).

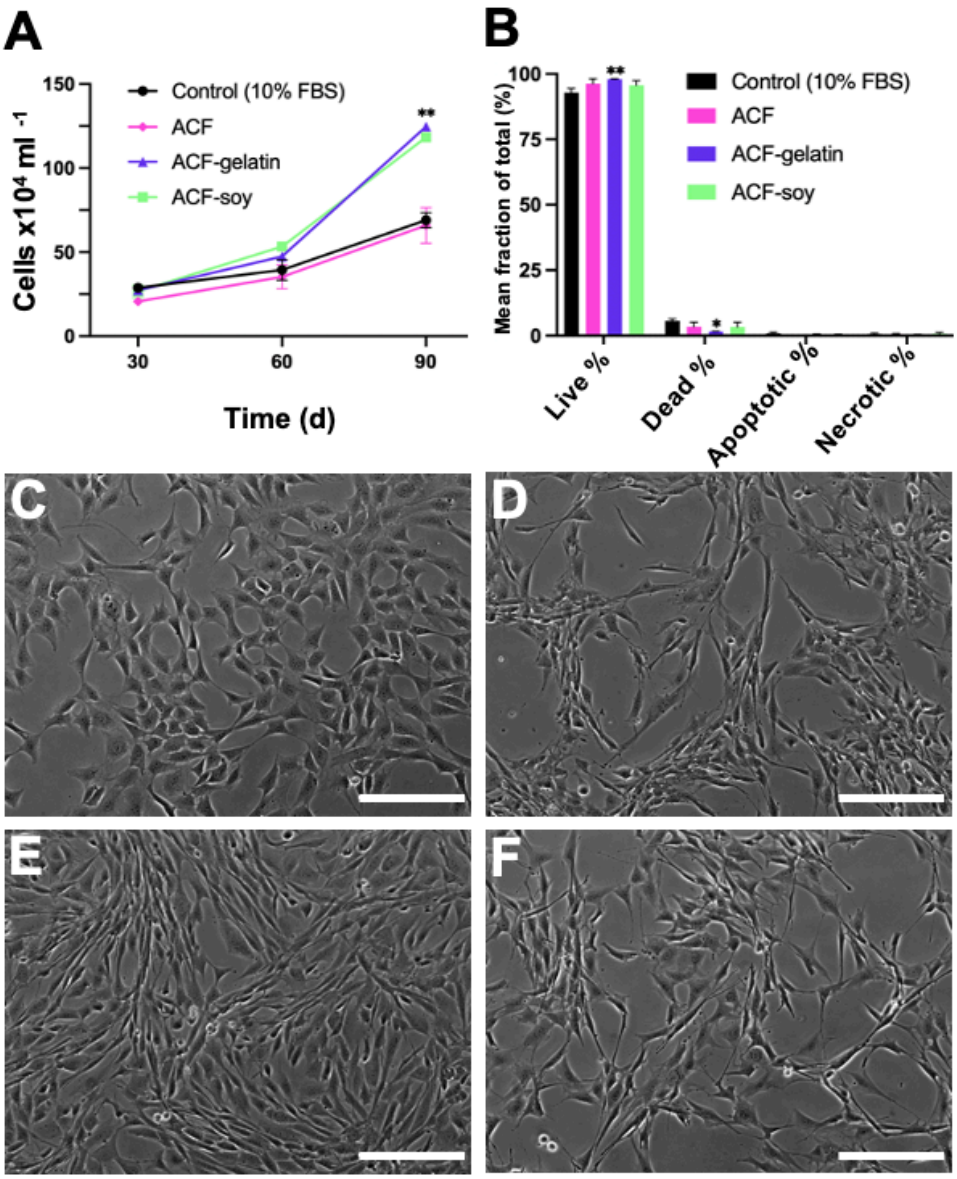

Figure 2.3: Long-term (30-90 day) cell viability and morphology of NIH 3T3 fibroblasts.
(**A)** Mean concentration of cells (x10^4 per ml). **(B)** Mean percentage of live, dead, early apoptotic, and necrotic cells as measured by Annexin-V/PI staining. N=3 for each time point and media condition. Each biological replicate is the mean of 3 technical replicates. Data are presented as mean ± SEM where *P<0.033, **P<0.002, ***P<0.001, as compared to the control medium. **(C-F)** Most representative phase contrast images. Scale bars, 200μm. **(C)** Control medium (10% FBS). **(D)** ACF medium (no coating). **(E)** ACF medium (gelatin-coated). **(F)** ACF medium (soy-coated).

2.3.3 Morphological differences of fibroblasts are minimal with ACF media

A major goal of this study was to not only create a serum-free alternative to FBS that can foster viable cells for short- and long-term periods but also that can allow typical cell lines used in research to behave similarly to those grown with FBS-based medium. When transitioning adherent cells from serum-containing to SFM, notable changes in cellular morphology are often observed.[70-73] Adherent cells cultured in SFM may exhibit alterations in their shape, size, and overall appearance compared to those cultured in serum-supplemented conditions.[70-74] These changes can be attributed to the absence of growth factors, hormones, and other serum components that play crucial roles in cell signaling and regulation of cellular processes.[75-77] Typically, cells cultured in SFM may display a more flattened or elongated morphology, with reduced cell-to-cell contact and altered cytoskeletal organization.[78-80] This morphological shift is often accompanied by changes in cellular behavior, such as decreased proliferation rates, alterations in gene expression profiles, and modifications in cell adhesion properties.[81-84] As a result, a morphological analysis on NIH 3T3s was performed to better understand how the ACF medium impacts cell size and shape as compared to cells grown in FBS-supplemented medium. The selection of nuclear and cell body areas as quantitative metrics that reflect morphology was driven by their relevance to fundamental cellular processes, including proliferation, differentiation, and migration, which are known to be influenced by changes in nutrient availability and signaling cues present in the culture medium.[85-88] This analytical strategy enabled us to discern subtle but significant differences in cellular morphology across distinct treatment groups, providing valuable insights into the cellular adaptations elicited by serum-free culture conditions.

Morphological analysis was performed via confocal laser scanning microscopy (CLSM) and fluorescent staining to analyze nuclear and cell body morphology for each culture condition. Cells in all groups demonstrated a typical fibroblastic morphology, with similar levels of interspaced, flattened cells (Fig. 2.4A-D). Image quantification allowed us to measure nuclear and cell body areas as well as their ratios (Fig. 2.4E-G). Similar mean nuclear areas of NIH 3T3s were found in the FBS-based medium (118.4±1.3μm) and in ACF-soy medium (114.5±1.1μm). These nuclear areas were found to be on average 24% smaller in uncoated ACF medium (90.2±1.0μm) and on average 30% larger in ACF-gelatin medium (152.7±1.6μm). Similar mean cell body areas of the cells were found in the FBS group (876.7±15.6μm) and in ACF-soy medium (854.1±17.5μm). The cell body areas were found to be on average 54% smaller in uncoated ACF medium (400.8±7.3μm) and on average 29% larger in ACF-gelatin medium (1129.7±20.4μm). Correspondingly, nuclear to cell area (N:C) ratios were calculated. Uncoated ACF medium

demonstrated significantly different N:C ratios than the FBS group (P=0.0001), where no significant differences in N:C ratios obtained from the ACF-gelatin (P=0.9999) and ACF soy (P=0.9985) groups were found.

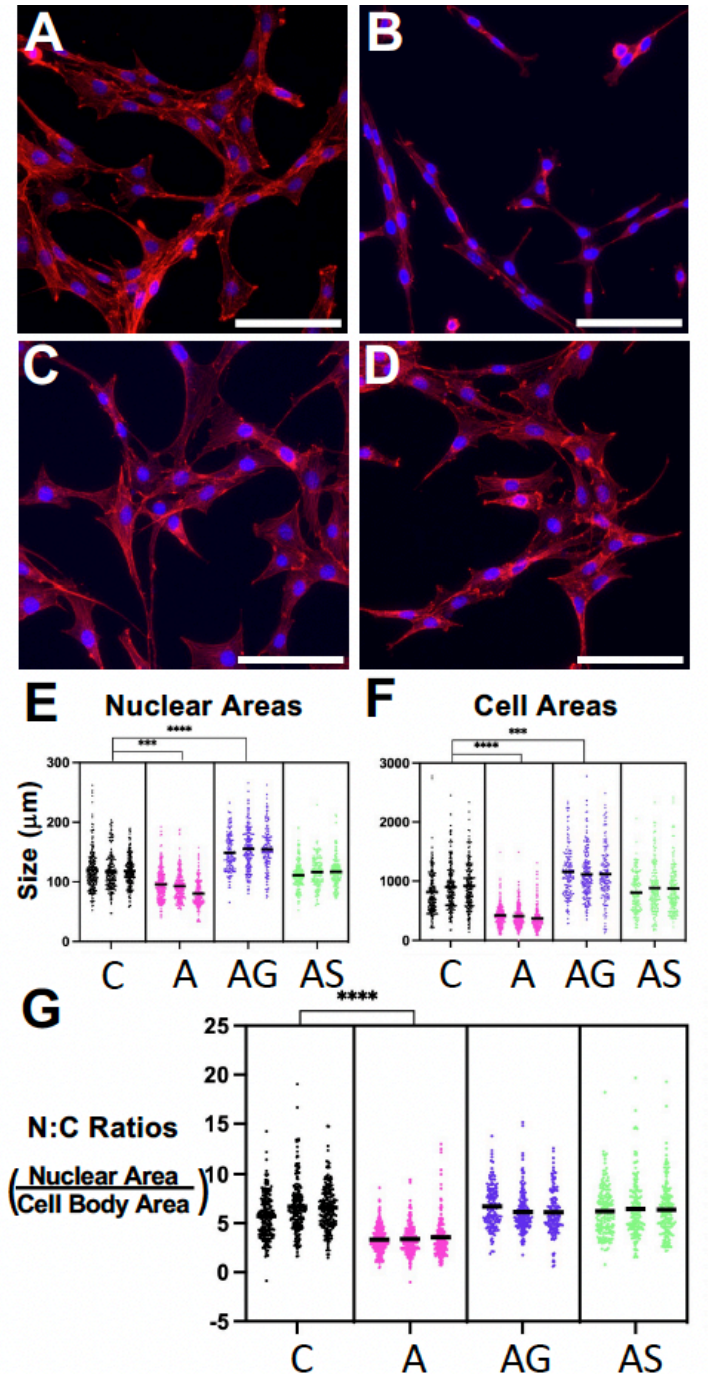

Figure 2.4: Morphological quantification of NIH 3T3 fibroblasts via cell sizing.
(A-D) Representative confocal laser scanning microscopy images quantified after 7 days since initial seeding. Nuclei stained with DAPI (blue) and F-actin with Alexa Fluor™ 647-Phalloidin (red).

Scale bars, 100um. **(A)** Control medium (10% FBS). **(B**) ACF medium (no coating). **(C)** ACF medium (gelatin-coated). **(D)** ACF medium (soy-coated). **(E-G)** Beeswarm plots with nucleus & cell body areas as well as nucleus to cell body (N:C) ratios across media types (C=control, A=ACF no coating, AG=ACF-gelatin, AS=ACF-soy). Each point represents 1 cell. N=3 for each medium condition with 150+ technical replicates per biological replicate. Centered horizontal black lines represent the mean for each replicate. *P<0.033, **P<0.002, ***P<0.0002, ****P<0.0001 as compared to the control medium.

2.3.4 Expression levels of select cell cycle and ECM genes are unchanged in ACF media

To explore any mRNA-level differences across media groups, RT-qPCR was used to examine a panel of commonly expressed genes important to cell cycle regulation, apoptosis, and extracellular matrix (ECM) and adhesion on NIH 3T3 fibroblasts grown in ACF media with different coatings, as compared to growth in FBS-containing medium. In total, 84 genes were assessed in a custom-made plate using Qiagen's RT2 Profiler PCR Arrays. The full panel, their abbreviations, and fold changes with P-values can be found in the Appendix, Table A.1. The selection of genes for the RT² Profiler PCR Array was driven by the overarching goal of understanding the fundamental molecular mechanisms underlying fibroblast function and cellular health. By integrating genes from Qiagen's pre-made panels on ECM and adhesion, apoptosis, and cell cycle regulation, our custom panel aimed to comprehensively assess the molecular landscape governing fibroblast behavior. Fibroblasts orchestrate tissue integrity and function through their dynamic interplay with the ECM, their capacity to modulate cell-cell and cell-matrix interactions, and their pivotal roles in proliferation, survival, and response to environmental cues.[89,90] Thus, by interrogating genes associated with ECM/adhesion, apoptosis, and cell cycle regulation, our analysis aimed to provide comprehensive insights into the molecular underpinnings of normal fibroblast function, with implications for tissue homeostasis, repair processes, and potential therapeutic interventions.

After 30 days in culture, mRNA was harvested from all ACF media groups and the FBS group, where all steps necessary for RT-qPCR were carried out. During analysis, ACF media groups were compared to the FBS group, where genes with a >2-fold difference, a P-value of <0.05, and a C_T value cut off of 35 were deemed significantly changed from the FBS group, as represented in the clustergram (Fig. 2.5A). Volcano plots generated from this data exhibit these results for all genes studied (Fig. 2.5B-D). Of all genes in the panel, 54 genes for non-coated ACF group, 40 genes for ACF-gelatin group, and 39 genes for the ACF-soy group were found to be insignificantly different than the FBS group, represented by the black points in each volcano plot. To better understand some of the differences among the ACF media groups and why they may have caused NIH 3T3s to behave slightly differently from one another, we examined the significantly changed genes by categorizing them into functional groups - apoptosis (Fig. 2.5E), cell cycle (Fig. 2.5F), and ECM/adhesion (Fig. 2.5G). 7 genes relating to apoptosis were differentially expressed across all groups. Among these, 3 genes (BNIP3L, XIAP, AKT1) were downregulated (between 2- and 4-fold) across all groups. DAPK1 was up-regulated across all groups (between 5- and 6-fold) and IL10 was heavily up-regulated in gelatin (28.21-fold) and soy (12.12-fold) groups, with no change in expression in the non-coated group. 11 genes relating to cell cycle (CCNA2, SMC1A, CDC6,

ATR, RBL1, BIRC5, CDKN3, CDC7, WEE1, AURKA, CHEK1) were down-regulated to a similar extent across all groups (between 2- and 5-fold). 8 genes relating to ECM/adhesion were differentially expressed. Up regulation of LAMA2 (ECM component) was present in all groups, although it showed highest expression in the non-coated group (13.86-fold) as compared to gelatin (2.26-fold) and soy (7.00-fold) groups. Consistent down-regulation of ITGA5 (integrin sub-unit) and CCN2 (matricellular protein) was present in all groups (between 3- and 4-fold). Other down-regulated genes showed varied expression across non-coated, gelatin, and soy groups: FN1 (-2.2-fold, -8.18-fold, -3.24-fold, respectively), SELP (-9.96-fold, -4.09-fold, -4.86-fold, respectively), FBLN1(-14.32-fold, -2.45-fold, -7.69-fold, respectively), and TGFBI (-4.5-fold, -14.51-fold, -7.47-fold, respectively). Finally, among all differentially expressed genes relating to ECM/adhesion, only COL5A1 showed down-regulation among gelatin (-5.62-fold, P=0.0005) and soy (-3.61-fold, P=0.0007) groups, with non-coated groups remaining unchanged.

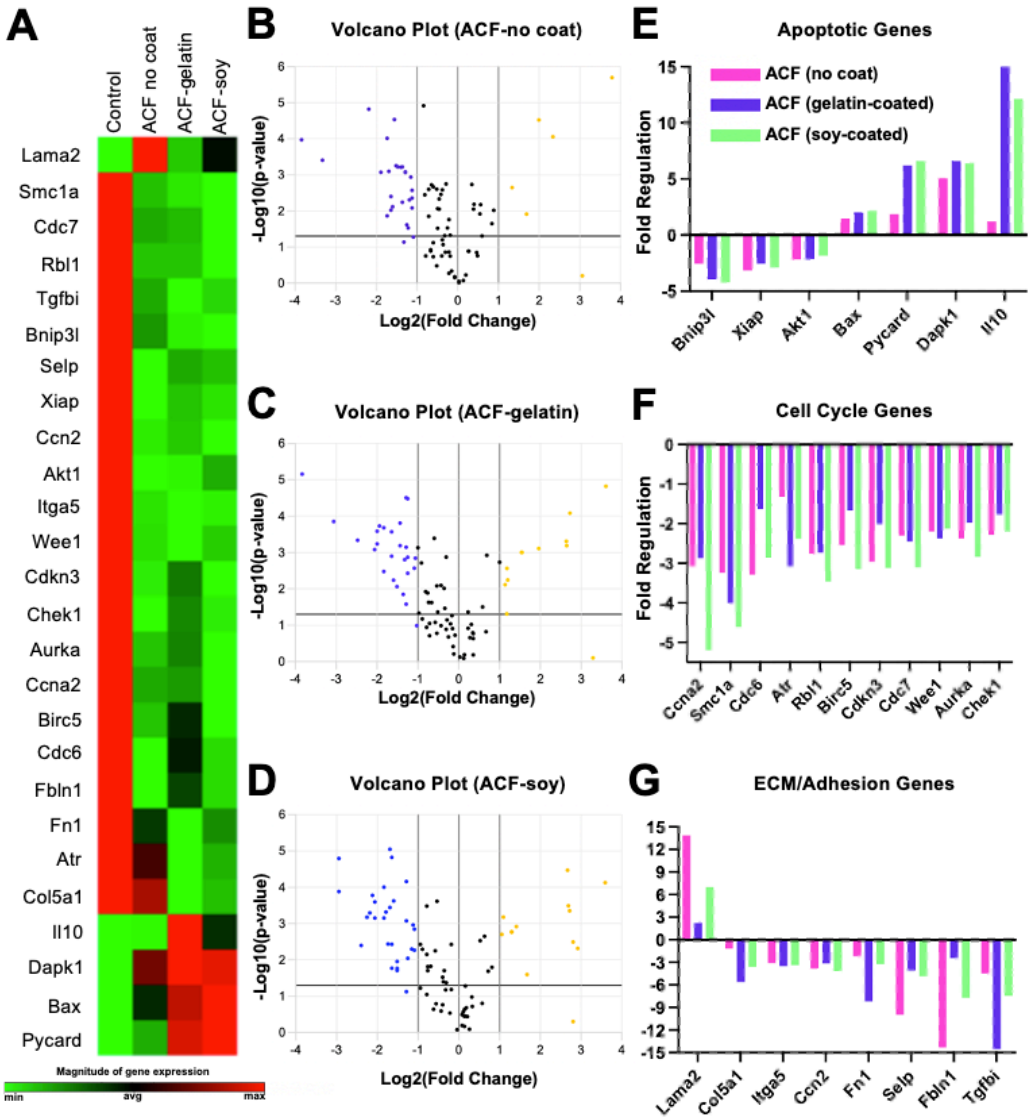

Figure 2.5: Differentially expressed genes between each media condition and the control through RT² profiler PCR array analysis.

(**A)** Clustergram of significantly altered genes (compared to the control group) from custom-designed gene panel across all groups. Each condition was analyzed in triplicate. Red represents maximum expression, green represents minimal expression, and black represents average levels of expression. **(B-D)** Volcano plots for each media condition. In these scatter plots, yellow points on the right side of the vertical line represent up-regulated gene expression as compared to the control group. Blue points on the left of the vertical line represent down-regulated gene expression as compared to the control group. Black points within the vertical lines represent unchanged gene expression, where fold change <2. Any points above the horizontal line at y=1.3 represent differentially expressed genes with a significant P-value <0.05. **(E-G)** Fold regulation of significantly differentially expressed genes across media conditions, grouped by function. The significant threshold for these genes was set as log2(fold change) > 2. This is compared with the control (FBS-based) group, for which fold regulation for each gene is normalized to 1. Significance values were defined at $P<0.05$.

2.3.5 ACF medium can support the growth of several adherent model cell lines

Although the ACF medium in this study was used to test its proliferative capacity on NIH 3T3 fibroblasts, we also selected other adherent cell lines to test the medium's universal growth capabilities. In this set of experiments, C2C12 mouse myoblasts, HFF human fibroblasts, and MC3T3 mouse pre-osteoblasts were grown in both ACF-soy and FBS mediums and cultured for 30 days. It is important to note that the media formulations used for different cell types were slightly altered to meet each cell line's base needs. HFF fibroblasts were cultured in the medium identical to that used for NIH 3T3s, but for C2C12 and MC3T3 cell lines, a minor change in the basal medium, concentration of FGF2, and addition of TGFβ was needed for optimized serum-free growth. On the 30th day of culture, mean cell concentrations were computed where each group showed a significantly increased total cell concentration in the ACF-soy group as compared to the FBS group (Fig. 2.6A). All cell types in ACF-soy medium were able to viably proliferate (C2C12s and MC3T3s: viability >94%, HFFs: viability >86%) as determined by Annexin-V/PI staining, with no significant changes in viability groups (Fig. 2.6B). In order to survey the confluency and general morphology of the cells at these time points, phase contrast images were obtained (Fig. 2.6C-H). All cell types grown in ACF-soy showed similar densities (>75%) to the FBS-based group. It is evident that the morphology (distinct clustering, cell elongation, specific characteristics) of each cell type was conserved when qualitatively comparing each image to its corresponding FBS-based control.

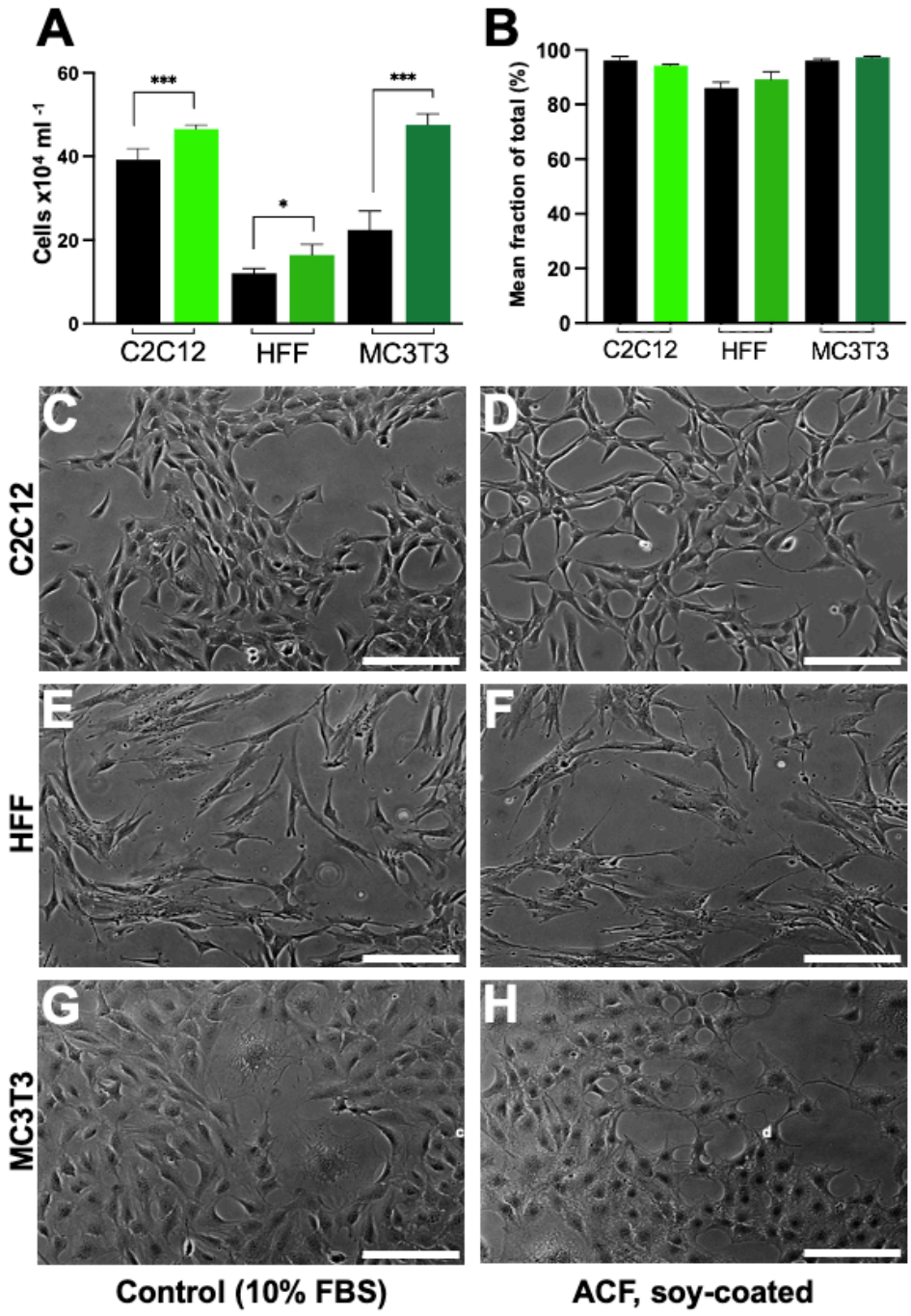

Figure 2.6: Cell concentration, viability, and morphology of 3 cell lines after 30 days.
(A) Mean concentration of total cells (x10^4 per ml). **(B)** Mean percentage of live cells as measured by Annexin-V/PI staining. N=3 for each cell line and each media condition. Each biological replicate is the mean of 3 technical replicates. Data are presented as mean ± SEM where *P<0.033, **P<0.002, ***P<0.001, as compared to the control (FBS-based) medium. **(C-H)** Most representative phase contrast images. Scale bars, 200μm. Each row presents images for one cell line. **(C,E,G)** Control medium (10% FBS). **(D,F,H)** ACF medium (soy-coated).

2.4 Discussion

Alternatives to fetal bovine serum have been continually emerging over the last few decades, reflecting a sustained effort to develop more controlled and defined culture conditions.[91-93] Previously fabricated SFM supplements serve as a robust foundation for creating a more universally applicable serum substitute but despite these commendable advancements, the growing reliance on FBS in various sectors, including industry, pharmaceutical manufacturing, cell culture, and research, underscores the need for a precisely regulated, well-defined serum-free alternative capable of accommodating diverse cell lines.[94,95] The research conducted in this study involves the thorough evaluation of an ACF culture system's potential as a replacement for FBS. It encompasses an examination of overall cell viability and proliferation capacity for 4 commonly used adherent cell lines in research, as well as morphological and transcriptomic analyses for one model cell line, NIH 3T3 fibroblasts. A major point of this work is to not only validate a new ACF medium via a variety of techniques, but also to provide the cell culture community with a formulation that can be used on more than one cell line.

In our study, various concentrations of growth factors and proteins that are typical of SFM development were combined and initially tested on fibroblasts. By conducting short-term (<7 day) qualitative and quantitative analyses, we were able to identify an ideal cocktail of components to be used in further experiments. We defined the 'optimal' formulation as one that was able to preserve cell viability and morphology for at least 7 days compared to the FBS condition and which contained a minimal number of components at the lowest effective concentrations. This optimization process was accomplished through a matrix which allowed us to test different concentrations of synergistic components (*Table 2.1*). Interestingly, our final formulation did not include transforming growth factor beta (TGFβ) and prostaglandin E2 (PGE2), which other SFM studies have often recommended and/or implemented themselves.[23,96-99] TGFβ is typically involved in regulating cell growth, proliferation, and differentiation,[100,101] while PGE2 is known for its role in inflammation and cell signalling.[102] Our data suggested that their inclusion altered cell morphology and did not significantly enhance cell viability under our specific conditions. This indicates that the requirements for maintaining NIH 3T3 fibroblasts may differ from other cell types or conditions in other SFM studies. Although PGE2 was excluded for all cell lines, TGFβ was still included in the growth of C2C12 myoblast and MC3T3 pre-osteoblast lines. Moreover, concentration dependencies played a crucial role in our findings. For example, epidermal growth factor (EGF) exhibited a dose-dependent effect where higher concentrations led to a more cobble-stone morphology as compared to the control. The minimal effective concentrations of components were critical not only for cost-effectiveness but also for mimicking typical morphologies of FBS-grown cells.

Cell adhesion in serum-free cultures has always been a factor of importance since FBS is the source of cell-adhesion promoting factors (e.g. vitronectin, fibronectin) to the culture system.[23,92,103] Thus, its absence often poses a problem for SFM development in adherent cell lines.[32,104] Although the adaptation of cells to suspension cultures or pre-coating substrate vessels with factors such as vitronectin or gelatin have been commonly employed solutions to such a problem, these solutions can be undesirable due to excess costs and their animal-

derived nature. Significant research has been conducted employing media that incorporate plant-derived hydrolysates, as evidenced in prior studies.[44,105-113] These plant-based hydrolysates are often preferred over animal tissue-derived counterparts due to safety, ethical, and cost considerations. Soy protein hydrolysates have demonstrated a substantial capacity to enhance cell growth and the production of recombinant proteins in cell cultures, and have been integrated into SFMs previously.[106-113] By assessing nuclear and cell body areas, we sought to capture the variations in morphology that may reflect underlying cellular responses to the altered culture environment. When using gelatin or soy protein hydrolysate as a substrate coating and then culturing the cell lines of focus in this study, the ACF medium showed enhanced growth rates in both short-term (<7 day) and long-term (90+ day) conditions. When fibroblasts were grown in the ACF medium without any substrate coating, nuclear to cell body ratios were significantly smaller than the FBS group. Without changing any components of the ACF medium but instead coating the cell culture substrates with either gelatin or soy protein hydrolysate solutions, nuclear to cell body ratios were rescued and found to be insignificantly different as compared to the FBS-grown groups. These findings suggest that soy protein hydrolysate is a crucial component to maintaining the morphology of cells in this culture system, and that it may be a suitable plant-based substitute to gelatin coating in certain cell culture applications. Unlike previous studies that utilized combinations of FBS with soy protein hydrolysates, our approach eliminates all animal-derived components from the culture system, addressing both ethical concerns and cost-effectiveness more comprehensively. This complete removal of animal components reduces the risk of variability and contamination associated with animal-derived materials. Furthermore, by comparing the nuclear to cell body ratios under different substrate coatings, our findings highlight the essential role of soy protein hydrolysate in maintaining cell morphology and function. This contrasts with previous methodologies that often relied on animal-derived coatings, thus underscoring the novelty and practicality of our approach in creating a completely animal-free culture system.

In addition to proliferative and morphological forms of validation, we assessed the transcriptomic effects of this ACF medium on NIH 3T3 fibroblasts. The majority of genes relating to cell cycle, apoptosis, and ECM/adhesion remained unchanged, yet differentially expressed genes in the three serum-free groups, each with different coatings (non-coated, gelatin, and soy) revealed intricate molecular adaptations governing cell behavior. The complex processes of cell adhesion have a direct impact on the interactions between cells and their surrounding ECM, thereby influencing critical events like cell proliferation, differentiation, and tissue formation.[114,115] A shared response across all groups, regardless of coating type, was the upregulation of Laminin alpha 2 (LAMA2), a fundamental component of the basement membrane, crucial for cell adhesion, differentiation, and migration.[116] This upregulation present in all ACF groups indicates a concerted effort to fortify adhesion in serum-free conditions. Notably, the non-coated group exhibited the highest upregulation of Lama2 (13.86-fold), suggesting a compensatory effort to establish robust cell adhesion in the absence of FBS or any substrate coating. Gelatin is known to enhance cell adhesion via RGD motifs, a repeat amino acid sequence that is recognized by integrin receptors on cell surfaces, which when bound promote adhesion and other signalling cascades.[53,54] We speculate that RGD motifs present in Lunascin, a protein found within soy, may be replicating gelatin's adhesive effects in culture

which could also explain its compensatory effect in rescuing nuclear to cell body area ratios. However, it is also plausible that the soy protein hydrolysate may simply have altered the surface charge of the substrate, thereby promoting better adhesion through electrostatic interactions. Studies have shown that surface charge can significantly influence cell adhesion by affecting protein adsorption and integrin binding.[117-119] This change in surface charge could enhance cell attachment and spreading without necessarily involving specific adhesive motifs. The downregulation of Collagen Type V Alpha 1 (Col5a1) in the gelatin (-5.62) and soy-coated groups (-3.61) supports this notion. Collagen V is integral to the ECM, contributing to collagen fibril assembly.[120] The reduced expression of Col5a1 may indicate that cells in these coated conditions did not need to synthesize as much collagen, likely due to the external adhesive cues provided by the coatings, which facilitated cell attachment and maintained cell morphology.

Furthermore, the downregulation of fibronectin (FN1), transforming growth factor beta induced (TGFBI), cellular communication network factor 2 (CCN2), fibulin-1 (FBLN1), and integrin alpha 5 (ITGA5) in all groups signifies a collective deviation in cell-ECM interactions. This group of genes are all crucial components of the ECM that facilitate cell adhesion by interacting with integrins and other matrix proteins, promoting stability within the tissue microenvironment.[121-124] This downregulation, particularly pronounced in the non-coated group, implies a shift in cell-ECM interactions, potentially influencing cell attachment and migration capabilities. These findings correlate to the observation that cells in SFM tend to be less adherent, likely due to the lack of attachment factors that are typically present in FBS, typically like fibronectin.[23,125,126]

Expanding our investigation to genes related to the cell cycle, a consistent downregulation of Cdc7, Cdkn3, Chek1, and Wee1 across all groups implies a collective suppression of cell cycle regulatory mechanisms in serum-free conditions. Cdc7 and Chek1 are crucial for DNA replication and repair, while Cdkn3 and Wee1 are involved in checkpoint control.[127-130] This downregulation likely reflects an adaptive response to the serum-free environment, where a slower cell cycle may help conserve resources and ensure cell survival under nutrient-limited conditions. The upregulation of Death-Associated Protein Kinase 1 (DAPK1) in all ACF groups indicates an increased sensitivity to apoptotic signals. DAPK1 is a pro-apoptotic kinase involved in mediating cell death in response to various stress signals.[131] This heightened apoptotic potential may have served as a protective mechanism, ensuring that damaged cells were efficiently removed, thus maintaining the overall health of the culture. Finally, the upregulation of PYCARD and Interleukin-10 (IL10) specifically in the gelatin and soy-coated groups reveals an interesting modulation of inflammatory responses. PYCARD is associated with inflammasome activation, leading to the production of pro-inflammatory cytokines,[132] while IL10 is an anti-inflammatory cytokine that helps regulate immune responses and prevent excessive inflammation.[133] While cells grown in vitro in FBS are considered the standard for cell culture, they do not replicate the native in vivo environment. The observed changes in the balance of inflammatory and immune responses in serum-free conditions are not necessarily negative; rather, they reflect adaptations to a different culture environment. These adaptations result in similar outcomes in terms of morphology and cell viability, suggesting that neither condition is inherently better, but each supports cell health through different mechanisms.

While this study provides a promising resource for researchers and serves as a valuable foundation for ongoing media development, it's evident that further optimization of media, focusing on cost-effectiveness and application to more cell lines, is necessary. The current approach in this work involved a step-by-step exploration of individual media components to identify suitable combinations for adherent cell lines. The interactions between these components can be complex and highly interdependent as they work in synergy to provide the necessary conditions for cells to grow and function properly. An in-depth understanding of the interplay between culture media components can be found in Yao and Asayama's 2017 review.[134] Given the intricate interaction of nutrients, growth factors, hormones, buffering agents, and trace elements within cell cultures, it's improbable that our soy-based ACF medium is already in its optimal form. There's a possibility that certain modifications of our medium (e.g. without coating, with gelatin coating, or higher concentrations of soy coating) might provide advantages in untested cell lines or in 3D culture modalities (e.g. suspension cultures). Computational methods for media development are better equipped to address multifactorial challenges like this and should be utilized to advance serum-free media for various applications.[135,136]

The research presented in this study introduces a completely animal component-free, soy-based medium to serve as a suitable replacement to FBS for NIH 3T3 cell culture in addition to other select cell lines (MC3T3, C2C12, HFF). However, achieving the universal nature of mammalian cell culture maintenance that FBS possesses, with the goal of preserving simplicity in the formulation, resemblance to typical cellular morphologies and behaviours, and cost-effectiveness, demands further refinement. Future endeavors to undertake these challenges might involve computational approaches using multifactorial analysis, machine learning algorithms, and other high throughput methods to better understand signalling pathways and the synergistic effects of different components in cell culture. Regardless, our work here has provided the cell culture community with new knowledge. The cellular proliferation rates in the soy-based medium proved to be at least as favorable, if not better than that in the conventional FBS-based medium. Morphological attributes of cells grown in the soy-based medium were matched to the FBS group and the majority of 84 genes studied by RT-qPCR remained unchanged. Our results not only offer definitive support for using soy protein hydrolysate as a supplement to serum-free culture but also contribute to transparent methods in serum-free media development. These findings have significant implications for refining cell culture methodologies, enhancing reproducibility, and tailoring culture conditions for diverse experimental settings.

2.5 Methods

2.5.1 Media formulation

We conducted a multi-step approach to explore different combinations of additives for cell culture media. We analyzed how these components interacted and adjusted their

concentrations to develop a SFM recipe capable of supporting NIH 3T3 fibroblast growth. The optimization process is discussed in full in the Results (*Section 2.3.1*).

2.5.2 Cell lines and culture conditions

Murine embryonic fibroblast NIH 3T3 cells (ATCC-CRL-1658), murine MC 3T3 E1 Subclone 4 pre-osteoblast cells (ATCC CRL-2593), murine C2C12 myoblast cells (ATCC CRL-1772), and human foreskin fibroblast HFF cells (ATCC-SCRC-1041) were purchased from ATCC. For routine cell maintenance, cells were cultured at 37°C in 5% CO2, passaged using 0.05% trypsin-EDTA (Cytiva, SH3023602), and counted using trypan blue with an image-based cell counter (Countess™ II FL, Invitrogen). Initially, NIH 3T3s and HFFs were maintained in proliferation medium consisting of 1:1 DMEM/Ham's F-12 (Sigma, 51445C) supplemented with 10% (v/v) FBS (Wisent, cat. 098150, lot. 185729) and 1% (v/v) penicillin/streptomycin (P/S; 100 U/mL penicillin, 100 mg/ml streptomycin; Cytiva, SV30010). The same protocol was used for the maintenance of MC3T3 cells and C2C12 cells but with different basal mediums - MEM Alpha 1X (Gibco, A1049001) and DMEM (Sigma, D0822), respectively.

To maintain consistency, the medium used to culture the FBS-grown groups contains the same basal medium but is supplemented with 10% FBS (Wisent, cat. 098150, lot. 185729). It is also important to note that this one lot of FBS has been secured for the entire duration of this study, to minimize variability across FBS-grown samples.

Cells were maintained in 10cm culture dishes at a seeding density of $8.8x10^3$ cells/cm^2 and sub-cultured every 2 days at around 85% confluence. After 2 passages in this serum-containing medium, cells were either kept in this same medium, designated to be controls grown in serum-containing medium, or went on to undergo the serum adaptation process, designated to eventually become the treatment groups grown in 100% serum-free medium.

2.5.3 Adaptation and serum-free medium

Serum adaptation was completed through five sequential passages over ten days, to diminish any stress the serum switch may have caused on the cells of the treatment groups. This adaptation process was repeated every round of media optimization before other validation. The cells in the FBS groups did not undergo the serum adaptation process, although they were sub-cultured every 48 hours in serum-based medium during this time in order to mature to the same passage number as the treatment groups. All FBS groups and all treatment (ACF) groups were individually cultured in triplicate in 10cm culture dishes at seeding densities of $8.8x10^3$ cells/cm^2. All sub-culture procedures are identical to those explained above, other than the animal component-free (ACF) medium used in the adaptation process for the treatment groups. Cells of treatment groups were initially cultured in 100% FBS-based medium and after 48 hours were then sub-cultured in medium consisting of 80% FBS-based medium and 20% ACF medium. Every 48 hours, as the cells reached around 85% confluency, they were sub-cultured to sequentially reduced serum amounts, with 60% FBS-based medium and 40% ACF medium, followed by 40% FBS-based medium and 60% ACF medium, 20% FBS-based medium and 80%

ACF medium, and finally 100% ACF medium. In the following sections, day 0 for each treated group is defined as 1 passage post seeding cells in 100% ACF medium.

Treatment groups of NIH 3T3s were adapted and routinely cultured in a variety of in-house developed ACF culture media formulations. These formulations are composed of DMEM/Ham's F-12 (Sigma, 51445C) with 1% (v/v) P/S as its base, to match the FBS group's medium base. They are supplemented with a variety of hormones, carrier proteins, lipids, minerals, and growth factors. The final iteration of the ACF medium's components and its corresponding concentrations can be found in Table 2.2. Basal media is the foundational liquid medium that provides essential nutrients, pH buffers, and other necessary components to support the growth of cells in vitro.[91] It is often supplemented with antibiotics, animal sera, and/or growth factors to create a specific environment tailored to the needs of the cells being cultured. The reason that DMEM/F-12 medium was chosen as the basal medium is because when traditional mediums (i.e. DMEM, MEM, Ham's F-12) were used in serum-free cultures, their ability to maintain growth was often inadequate, yet a 1:1 ratio of DMEM and Ham's F-12 exhibited better performance when used for serum-free culture of certain cell types.[137,138] As a result, DMEM/F-12 basal media has been recommended by a variety of studies aiming to create serum-free media formulations.[14,18,19] In this study, this basal medium combined with ACF supplements was ideal for growing NIH 3T3 fibroblasts and HFF human fibroblasts, but C2C12 myoblasts and MC3T3 pre-osteoblasts were grown in other basal mediums that are typically recommended for their growth in standard protocols (DMEM and αMEM, respectively). In the FBS groups for these cell lines, the same basal medium is used as in the ACF-supplemented groups.

2.5.4 Animal- and plant-based substrate coatings

SFM alternatives often incorporate supplementary additives to facilitate cell adhesion, compensating for the absence of components typically provided by FBS, thereby ensuring optimal cell growth and viability.[52,139-141] Such additives are often animal-derived and include extracellular matrix components such as fibronectins, vitronectins, laminins, and collagen.[52,141-143] A notable approach in achieving animal-free conditions has involved incorporating plant-based components as viable alternatives.[91,144] These components commonly include extracts from wheat, soy, rice, chickpea, pea, and other protein-rich botanical sources and have shown to enhance cell proliferation and improve recombinant protein production in some mammalian cell lines.[44,105,106,145] Initially, a gelatin coating on the substrate was used to increase cell adhesion and to observe if it would have an effect on the poor attachment seen in the preliminary SFM we created. Through researching the literature, soy protein hydrolysate was identified as a substitute to gelatin coating in this context. Renowned for its rich nutritional profile and diverse bioactive compounds, soybean stands out as a promising candidate to promote cell viability, proliferation, and aiding in adhesion.[107,146] Soy protein hydrolysates generated through diverse methods have outperformed other plant-based hydrolysates in enhancing cell growth in a diverse set of cell lines and culture applications.[44,106-113] Via further validation, soy as a coating was deemed an essential piece to the success of our final ACF media. Gelatin coatings were produced by dissolving gelatin (Sigma, G9136) in DI H2O at a

concentration of 1g/L. Soy coatings were produced by dissolving soy protein hydrolysate (Sigma, S1674) in DI H2O at a concentration of 4g/L. Gelatin and soy solutions were autoclaved once to sterilize. 24 hours before cell seeding, 10cm dishes were each coated with 8mL of soy or gelatin solutions. Right before cell seeding, solutions were aspirated from each dish and corresponding media was carefully added to not disrupt the particles adsorbed to the surface.

2.5.5 Proliferation rates and viability

The passage number for all groups and their replicates in these studies was P8 at day 0. Day 0 of short- and long-term counts is designated as 1 passage after the treatment groups were placed in 100% ACF medium, post-10-day serum adaptation process. At this point, FBS and ACF groups were sub-cultured with their corresponding media and inoculated with seeding densities pertaining to either short-, or long-term counts, described in detail in the following sections. For each timepoint and each group (FBS or ACF), 3 biological replicates were analyzed. 3 technical replicate values were obtained for each biological replicate in every condition.

All FBS and ACF groups were inoculated with a seeding density of $1x10^4$ cells/ml per culture plate on day 0. All conditions were monitored for this short-term growth period, with readings taken after 1, 4, and 7 days of culture. At each time point, cells were trypsinized and centrifuged at 1000 rpm (97g) for 3 minutes. After supernatant removal, cell pellets were re-suspended in 2 ml of PBS before being treated with the Annexin V-FITC Apoptosis/Staining Detection Kit (Abcam, ab14085). Briefly, for each sample, 1000 ml of 1 x binding buffer was added to the cell pellet after centrifugation and PBS removal. After re-suspension in binding buffer, Annexin V-FITC (5 ml) and PI (5 ml) were added to 100 ml of cell suspension. After 15 minutes of incubation at room temperature in the dark, 400 ml of 1 x binding buffer was added, resulting in a final volume of 500 ml for each sample. Keeping samples on ice in the dark, all samples were counted using the Countess™ II FL cell counter equipped with fluorescence filters (Ex 482/25, Em 524/24; EVOS Light Cube for GFP; Invitrogen, AMEP4951) and (Ex 531/40, Em 593/40; EVOS Light Cube for RFP; Invitrogen, AMEP4952). To avoid false recognition of debris or evident noise by the cell counter, an arbitrary size threshold level was set (size of 2–30 mm) along with the corresponding fluorescence intensity (relative intensity of 40–255) for each assay. Each threshold level was confirmed by reference to past studies of NIH 3T3 cell diameter and by examination of the real-time detection image of each cell using the counter.

For long-term growth studies (90-day – NIH 3T3; or 30-day – other cell types), all FBS and ACF groups were inoculated with a seeding density of $5x10^4$ cells/ml per culture plate on day 0. All groups were monitored for a period of 90+ days or 30 days in triplicate. During this period, subcultures for each dish were performed every 72 hours, where cell viability was quantified using trypan blue and the automated cell counter. Live cell counts were recorded for each replicate and then inoculated at a concentration of $5x10^4$ cells/ml in new dishes at each subculture. Cell viability was quantified at 30, 60, and 90 days of culture (shown in this study), and 120, 150, 180, and 210 days of culture (data not shown) via the Annexin V-FITC Apoptosis/Staining Detection Kit in the same manner as described above.

2.5.6 Fluorescent staining and microscopy

At day 0, cells for each condition were seeded in 35mm dishes at a concentration of $1x10^4$ cells/mL. After 7 days, cells were washed three times with 1 x PBS. The samples were fixed in 4% paraformaldahyde (PFA) for 10 min and then washed with PBS three times. Following the final PBS wash, cell permeabilization was achieved via addition of room temperature 0.02% TritonX-100 for 3 minutes. F-actin was stained using 1:200 Alexa Fluor™ 546 Phalloidin (Invitrogen, A22283). Nuclei were visualized using 1:500 DAPI (Invitrogen, D1306). Phalloidin and DAPI were added together in PBS at their respective concentrations and then incubated for 1 hour in the dark at room temperature. Following 3 washes with PBS, Vectashield mounting medium was added to each sample. A round glass coverslip was carefully placed on the surface of the dish, and samples were visualized by confocal laser scanning microscopy through the coverslip by inversion of the 35mm dish on an AFM insert. Confocal imaging was performed on an A1R high speed laser scanning confocal system on a TiE inverted optical microscope platform (Nikon, Canada) with appropriate laser lines and filter sets.

Image processing was executed via ImageJ open access software using a semi-automatic method. Briefly, DAPI (blue) and Phalloidin (red) channels were split. Next, Z-stacked 3D volume images were prepared for each channel. For the DAPI channels, a threshold was set, converting the colored max intensity projection to a binary image. Next, the Analyze Particles plugin was used to measure each nucleus' area, perimeter, circularity, roundness, aspect ratio, and other measures. If nuclei in the binary image were slightly warped and not fully picked up by the thresholding algorithm, that nucleus was excluded from data analysis. For the Phalloidin channels, each cell body was manually selected using the polygon selection tool and then measured within the software. The same measurements obtained from the nuclei were acquired. DAPI channels and Phalloidin channels were set side by side when performing the manual analysis on the cell bodies to ensure the order in which the cell bodies were counted would match the corresponding nucleus. This allowed for proper computation of each nucleus area to cell body area (N:C) ratios. Cell bodies of the Phalloidin channels were excluded if they were found on the bounds of the image or if they were obscured by other cells to accurately compute their area. 30 images were analyzed for each media group with included cells as follows: FBS group (619 cells included in analysis), ACF no coat group (671 cells included in analysis), ACF gelatin group (477 cells included in analysis), and ACF soy group (493 cells included in analysis). Beeswarm plots were created in GraphPad Prism, version 10.2.3 for MacOsx (GraphPad Software, La Jolla, CA, USA).

Transmitted light images were acquired on an inverted TiE microscope (Nikon, Canada) with phase contrast optics. Images were analyzed using ImageJ open access software (http://rsbweb.nih.gov/ij/). Brightness and contrast adjustments were the only manipulations performed to images.

2.5.7 Gene expression and analysis

For NIH 3T3s, 30 days after sequential serum adjustment, gene expression analysis was performed for each biological replicate in both FBS and ACF groups. Each plate of cells was trypsinized, centrifuged at 1000 rpm (97g) for 3 minutes, and re-suspended in 1 mL of appropriate medium. Next, the cell suspension was centrifuged, washed with PBS, and centrifuged once again. Supernatant was collected, leaving only the cell pellet. Purification of total RNA was performed using the RNeasy Mini Kit (Qiagen, 74104) for animal cells using spin technology. cDNA was reverse transcribed from 2 μg total RNA from each sample, using the Qiagen RT2 First Strand Kit (Qiagen, 330404) following the manufacturer's protocols. qPCR was performed using a custom-made RT2 profiler PCR array (Qiagen, 330171) following the manufacturer's protocols. A full list of the gene panel can be found in Table 3.3. Reactions were performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) and fold changes in gene expression were calculated via the $\Delta\Delta$CT method. Normalization of the raw data was performed with species-specific housekeeping genes B2m and Gusb using the geometric mean as the normalization factor. During qPCR analysis, CT cut-off value used was 35. The primers chosen in the customized plate included 32 primers for cell cycle genes, 25 for apoptotic genes, 27 for extracellular matrix and adhesion genes, and 5 for housekeeping genes. This gene expression panel was carried out in each of the following groups, each with 3 biological replicates: control (FBS) group, treatment group 1 (ACF medium without coating), treatment group 2 (ACF medium with gelatin coating), and treatment group 3 (ACF medium with soy coating),

2.5.8 Statistical analysis

A two-way analysis of variance (ANOVA) was applied to assess the changes in cell viability across media groups and across time. Using post-hoc tests (Dunnet's multiple comparisons test), the specific differences between media groups at each time point were explored. The mean of all the biological replicates for each treated group was compared to the mean of the replicates for the FBS group. A one-way ANOVA using Dunnet's method for multiple comparisons was applied to analyse the nuclear to cell area ratios across media groups. Differences were considered statistically significant with *P<0.033, **P<0.002, ***P<0.001. Statistical analyses for RT-qPCR were provided by Qiagen's online analysis software where P<0.05 was considered statistically significant. Captions for figures and tables describing experimental results state the number of biological replicates (N) as mean ± SEM, unless otherwise stated. The results were analyzed using GraphPad Prism, version 10.2.3 for MacOsx (GraphPad Software, La Jolla, CA, USA).

2.6 References

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Chapter 3 | Characterizing Jurkat T cell viability and metabolism in spinner flasks using 100% animal component-free media

§ Motivation

Suspension cultures are pivotal in fields like immunotherapy, vaccine production, and recombinant protein manufacturing. These applications often demand large-scale cell culture systems that can sustain high cell densities and ensure consistent cell growth and productivity. Spinner flasks serve as an intermediate step between small-scale laboratory experiments and full-scale bioreactor operations, providing a dynamic environment that supports cell growth in suspension through continuous agitation and nutrient exchange. There is a critical need to develop and validate serum-free media for suspension cell lines in these conditions, as standard animal-based media pose numerous disadvantages. By demonstrating that suspension cells can thrive in serum-free conditions within these systems, we aim to pave the way for more efficient and cost-effective production methods that are vital for commercial and clinical applications. This transition from serum-based to serum-free media in scalable systems aligns with the industry's push towards more sustainable, ethical, and reproducible manufacturing practices.

§ Hypothesis and Objectives

The goal of this study was to modify our previously developed ACF media to grow Jurkat T cells, a common model for lymphocyte research, without the use of animal serum. A secondary goal of this study was to perform the cell culture in three-dimensional conditions, in spinner flasks, which provide an analogous environment to bioreactor culture at a smaller scale. We hypothesized that the ACF media that was previously developed would need to be modified in order to accommodate the needs for this cell line. More specifically, we postulated that most factors related to cell adhesion would need to be removed, as the nature of this cell line is to grow in suspension without the need for adhesive elements in the culture.

3.1 Abstract

In recent years, dynamic, three-dimensional (3D) cell cultures have become increasingly utilized in large-scale production for applications such as drug, antibody, and vaccine development, tissue engineering, stem cell culture, and even cultured meat. Although these modalities, which commonly use vessels like bioreactors, are continuously being expanded upon and implemented into novel biological advancements, industrial demands continue to push for more optimized systems. Many cell types, including stem cells and lymphocytes, have been successfully grown in three-dimensional cultures, yet production volumes, harvesting procedures, oxygen exchange, and formulation of non-animal media supplement remain as barriers to achieving cost-effective, ethical, and consistent cultures at larger scales. In this study, we cultivate Jurkat T cells in spinner flasks using a modified version of a previously tested animal

component-free (ACF) medium and compare cell viability and morphology to cells expanded in an FBS-based control. Under continuous spinning over a two-week period, the Jurkat cells grown in this ACF medium retain high viability (>97%), and their morphology is preserved. We establish a simple yet effective culture protocol for maintaining Jurkat cells in non-animal derived dynamic cell suspension, with the goal of providing the serum-free culture community with further documentation of proof for successful animal-free cell culture.

3.2 Introduction

For several decades, Fetal Bovine Serum (FBS) has been used as the most common serum-supplement for the in vitro cell culture of eukaryotic cells around the world.[1] Presently, FBS is a staple in cell and tissue culture studies and is required for fundamental experiments in cell biology to uncover new knowledge in cell therapies, tissue engineering, modelling of diseases, pharmaceutical manufacturing, diagnostic tools, and more.[2,3] Despite its widespread use in these industries, animal-based sera can pose many drawbacks, especially in large-scale manufacturing, with their high costs[4,5] potential mycoplasma or viral contamination,[6,7] and lot-to-lot variability that could alter batch consistency.[8,9]

Advances in chemically-defined and serum-free media for cultures grown on two-dimensional (2D) adherent surfaces have been on the rise in recent years.[10-15] However, employing 2D adherent cell lines in large-scale operations by industries that rely on FBS for cell maintenance can be challenging.[16-18] The management of numerous adherent culture vessels such as flasks, roller bottles, or multi-stacked setups, involve tasks like coating vessels, passing cells, and extensive harvesting.[19-23] These time-consuming and laborious factors are some of the reasons which limit practical scalability. To upscale cell production efficiently, a more tunable manufacturing process is preferred.[24-26]

Dynamic suspension culture systems involve continuous motion or agitation of the culture medium, often through regular shaking, stirring, or perfusion.[27-30] They offer numerous benefits, such as controllability, scalability, and easier harvesting and cell feeding options. Dynamic, as opposed to static 2D cultures, can have better and more uniform oxygen diffusion, clearance of metabolites, and distribution of nutrients.[31-34] Furthermore, when suspension culture systems are integrated into a bioreactor, they can become even more conducive to automation. Many groups have successfully shown that a variety of cell types can be maintained in suspension cultures, using serum-free media.[35-41] The cell types used in these studies include pluripotent stem cells, embryonic stem cells, mesenchymal stem cells, vero cells, Chinese hamster ovary cells, and MDCK cells, and their applications vary greatly.

One of the most common suspension cell lines, Jurkat T cells, are often used as models in cell therapy,[42,43] anti-cancer drug discovery[44-46] and other cell-based applications.[47-49] In previous studies, Jurkat T cells have been grown in a wide range of modalities; in suspension in spinner flasks and bioreactors, in microcapsules in bioreactors,[47,50] in static conditions on regular culture dishes, and even in 2D with reduced serum conditions.[51] Several studies have cultured serum-free Jurkat cells[42,51-57] but to the best of our knowledge, no group has specifically sought out to

find a replacement to FBS-supplemented medium for this cell line, while focusing their research objectives on the validation of that chemically-defined medium. In our previous study, we developed an animal component-free (ACF) medium specifically designed for select adherent cell types, validating its effectiveness through cell viability, morphological, and transcriptomic analyses (*Chapter 2*). This research highlighted the potential of our medium to reduce dependency on animal-derived components while maintaining high cell viability and functionality. In the present study, we modify our previous ACF medium to be used in dynamic suspension culture for Jurkat T cells, in order to help advance the other efforts of serum-free media development over the last decade. Herein, we characterize cell viability, metabolism, and morphology of Jurkat cells grown in spinner flasks over a two-week period. With this analysis of serum-free dynamic suspension culture, we aim to contribute to the knowledge and implementation of non-animal derived components into 3D culture environments for future research endeavours.

3.3 Methods

3.3.1 Cell line and static cultures

Human leukemia T lymphoblasts, Jurkat T cells (ATCC-TIB-152) were received as a gift from the 2021 CAN-RGX team at the University of Toronto. Cells were cultured at 37°C in 5% CO2, in proliferation medium consisting of RPMI-1640 (Sigma, A10491-01) supplemented with 10% (v/v) FBS (Wisent, cat. 098150, lot. 185729) and 1% (v/v) penicillin/streptomycin (P/S; 100 U/mL penicillin, 100 mg/ml streptomycin; Cytiva, SV30010). For routine static culture, cells were maintained in Petri dishes (100mm diameter) with a density of $2x10^6$ cells/mL via counting using trypan blue with an automated image-based cell counter (Countess™ II FL, Invitrogen).

3.3.2 Adaptation to ACF medium

The ACF medium used in this study was modified upon a formulation used for adherent cell types in a previous study (*Chapter 2, Section 2.5*). Table 2.1 describes in detail the initial process for medium optimization, and a revised version of this procedure is described here. Briefly, the literature was scanned to assess commonly used components of serum-free media tailored to Jurkat cells, summarized in Table 3.1.

Study	**Basal medium**	**Supplements**	**Modality**	**Viability**
Youn et al. (2017) Cytoprotective Encapsulation of Individual Jurkat T Cells within Durable TiO2 Shells for T-Cell Therapy.[42]	RPMI-1640	B-27 serum free supplement GlutaMAX	Static, encapsulated	28% viability after 14 days
Audiffred et al. (2010) Characterization and applications	RPMI-1640 or	N/A	Static	No quantitative measurements

of serum-free induced adhesion in jurkat suspension cells.[51]	Opti-MEM			of viability. Adherence assays indicate majority cells viable after 24 hours.
De Leo. (2009) Adhesion of Jurkat cells in defined media and subtrates.[52]	RPMI-1640	N/A	Static	No quantitative measurements of viability. Adherence assays indicate majority cells viable after 24 hours.
Xu et al. (1995) Effect of lysophospholipids on signaling in the human Jurkat T cell line.[53]	RPMI-1640	Lysophosphatidic acid, or Lysophosphatidyl serine, or Sphingosyl-phosphorylcholine	Static	Similar thymidine incorporation as controls after 24 hours, at low concentrations of additives.
Purvis et al. (1995) HIV type 1 Tat protein induces apoptosis and death in Jurkat cells.[54]	RPMI-1640	N/A Jurkats are Tat transfected	Static	"Cells show prolonged survival". Data not shown.
Caricchio et al. (2002) Fas, ceramide serum withdrawal induce apoptosis via a common pathway in a type II Jurkat cell line.[55]	RPMI-1640	N/A	Static	No measures of viability of cells alone.
Whitford et al. (2010) Animal Component Free T-Cell Culture. In Cells and Culture.[56]	CDM4T medium	Insulin-like Growth Factor 1 Exact components unclear	Dynamic, bio-reactor	Viability decreased to ~25 to ~50% after 12 days of culture, depending on the bioreactor condition.

Table 3.1: Review of serum-free culture of Jurkat cells in different conditions.
Only 0% animal-sera conditions are included from each study. Studies which looked at serum-reduced conditions only are not included. Results from each studies' viability testing are

presented in the final column. All studies used antibiotics in their formulations as additives but are not mentioned in this table.

This summary demonstrated that the RPMI-1640 medium was widely used in serum-free Jurkat cell culture and thus, this was the basal medium that was chosen for the base of the SFM formulation. Further, it was unclear exactly which components could be ideal to fortify this medium for Jurkat cell culture since the summary of the literature showed no consistent additives among research groups. As a result, other literature on serum-free culture was examined, but for human and murine lymphocytes in general, not specifically for Jurkats alone (Table 3.2).

Study	**Basal media**	**Cell type**	**Supplements**
Causey et al. (1994). A serum-free medium for human primary T lymphocyte culture.[58]	N/A	Primary human lymphocytes	1:1 mixture of commercially available serum-free media AIM V (Gibco, undisclosed contents) and EX-CELL 300 (undisclosed contents)
Kaldjian et al. (1992). Enhancement of lymphocyte proliferation assays by use of serum-free medium.[59]	N/A	Primary murine lymphocytes	AIM V (Gibco, undisclosed contents)
Hsia et al. (1979). Mixed lymphocyte reactions in serum-free medium.[60]	Medium 199	Primary human lymphocytes	2mM glutamine 0.5% bovine serum albumin
Rumley et al. (1984) Effects of different protein supplements on mitogen responses of human peripheral blood lymphocytes.[61]	RPMI-1640	Primary human lymphocytes	1% human serum albumin or 1% bovine serum albumin
Herzberg et al. (1987). T cell growth without serum.[62]	N/A	T cells	Transferrin Interleukin-2
Blaehr et al. (1988) Mitogen-induced lymphocyte transformation in four different serum-free media.[63]	RPMI-1640	Primary human lymphocytes	2mM glutamine One of the following commerically produced serum substitutes: SF-X (Costar) or FEB-100 (Biochrom)

Yssel et al. (1984). Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones.[64]	Iscove's modified DMEM	Primary human lymphocytes	5 ug/ml insulin 35 ug/ml transferrin 0.02 mM ethanolamine 2.5 ug/mL bovine serum albumin 1 ug/ml palmitic acid 1 ug/ml linoleic acid 1 ug/ml oleic acid
Hashizume et al. (1983). Identification of lactoferrin as an essential growth factor for human lymphocytic cell lines in serum-free medium.[65]	1:1 DMEM/Ham's F-12	Human lymphocytic cell lines: Bri 7, AL-60, RPMI 8226, NL, HYON, Jijoye (P-2003), CCRF-SB, CCRF-HSB-2 and CCRF-CEM. Mouse lymphocytic cell lines: NS-1 MPC 11, P3U1 and X63.6.5.3	15 mM HEPES 1.2 g/L $NaHCO_3$ 25-35 ug/ml lactoferrin, or 625-1400 ug/ml transferrin
Needleman et al. (1981). Human lymphocyte transformation induced by mitogens and antigens in a serum-free tissue culture system.[66]	RPMI-1640	Primary human lymphocytes	2 mM glutamine 10 mM HEPES 1 mM $MgSO_4$ 5-10 ug/ml phytohemagglutinin 5-10 ug/ml concanavalin A 7.5-15 ug/ml pokeweed mitogen

Table 3.2: Review of serum-free media contents for successful culture of lymphocytes in different conditions.

Basal media and added supplements are presented where identifiable. Each study included exhibited similar proliferation and viability to controls containing FBS. All studies used antibiotics in their formulations as additives but are not mentioned in this table.

The most commonly used additives in these studies included glutamine, insulin, transferrin, albumin, and fatty acids, which provided us with a promising starting point. Interestingly, these components overlapped with our previous ACF medium study *(Chapter 2)* and thus, they were chosen to initially culture Jurkat cells at the same concentrations from the previous study. Trypan blue exclusion results showed ~50% Jurkat cell viability after 7 days in culture and thus

we followed serum-free culture recommendations to increase insulin and transferrin concentrations to potentially increase viability.[67,68] By increasing the insulin, transferrin and selenium concentrations by 5X as those in the original recipe, cell viability increased significantly. The exact concentration and each component used further in this study are explained in Table 3.3.

Component	Final Working Concentration	Company, Catalogue #
Albumin, recombinant expressed in rice	1 mg/mL	Sigma, A9731
Ethanolamine, synthetic	1.5 mg/mL	Sigma, E0135
Animal origin-free ITS (100X) Insulin, recombinant derived from yeast Transferrin, recombinant derived from rice Sodium selenite, synthetic	*Animal origin-free ITS (5X)* Insulin: 50 ug/mL Transferrin: 27.5 ug/mL Sodium selenite: 25 ng/mL	Sigma, SCM055

Table 3.3: Animal component-free medium components for Jurkat T cells.
These components were added in supplement to the same basal medium as used in the control, RPMI-1640 with 1% P/S. This is the medium used to grow Jurkat cells in all ACF experiments across the study, other than the negative control. It is modeled after the formulation process described in *Chapter 2, Section 2.5*.

After 2 passages in the serum-containing medium post-thawing, Jurkat cells were either kept in this same medium, designated to be controls grown in serum-containing medium, or went on to undergo the serum adaptation process, designated to eventually become the animal component-free (ACF) group grown in 100% serum-free medium. Serum adaptation was carried out through a series of five consecutive passages spanning over a period of ten days. This process aimed to alleviate any potential stress induced by the switch in serum composition on the cells within the treatment group. After splitting the cells grown in the control medium, they were transferred to a medium composed of 80% FBS-based medium and 20% ACF medium. Every 48 hours, they underwent sub-culturing with decreasing proportions of serum, eventually transitioning to 20% FBS-based medium with 80% ACF medium, and finally, 100% ACF medium on the tenth day. The same process was performed for the negative control group grown in RPMI-1640 medium with no additives.

3.3.3 Dynamic culture in spinner flasks

After serum adaptation, Jurkat cells in both the control and ACF media groups were inoculated into separate disposable spinner flasks (Corning, CLS3152), with a seeding density of 5×10^4 cells/mL and cultured for a period of 14 days. To allow for proper aeration and prevent overflow during agitation, 125 mL spinner flasks were handled using a final media volume of 50mL. Spinners were maintained at 37°C in 5% CO_2 on a magnetic controller (Thermo Scientific™ Cimarec™ Biosystem 40B) with continuous stirring at a speed of 45 rpm. To allow for proper gas exchange, each sidearm screw cap was loosened by one full counter-clockwise turn during the

culturing procedure. Every 3 days, the total content of each spinner was centrifuged and the spent media was aspirated. The cell pellet was re-suspended and placed back into the corresponding spinner flask to a final volume of 50 mL, containing only fresh media. Samples of cell cultures were taken on days 1,3,5,7,9,11,13, and 14 for further evaluation described in the following sections. 3 spinner flasks for each media condition (N=3) underwent experimentation.

3.3.4 Cell viability and metabolic assays

Every 48 hours, starting at day 1 of culture, 2 mL samples were taken in triplicate from each medium condition. After pelleting by centrifugation, samples were re-suspended in 4 mL of complete medium. Next, trypan blue solution 0.4% (Gibco, 15250061) was added to the samples at a 1:1 ratio. Automated cell counting was performed using the Countess™ II FL cell counter with 3 technical replicates per biological replicate.

To provide a second measure for cell viability, Calcein-AM/PI staining was performed at the same time points as executed for trypan blue exclusion. Briefly, 2 mL samples were taken from each spinner flask every 48 hours. Samples were pelleted by centrifugation and then washed with PBS three times. Next, 5 ug/mL of Calcein-AM (Sigma-Aldrich, 206700) and 5 ug/mL of Propidium Iodide (Invitrogen, P3566) in PBS was used to re-suspend cell pellets. After incubation in the dark for 1 hour at 37ºC, samples were again washed with PBS twice. Prior to counting, samples were re-suspended in 4 mL of PBS. Finally, counting was performed using the Countess™ II FL cell counter equipped with fluorescence filters (Ex 482/25, Em 524/24; EVOS Light Cube for GFP; Invitrogen, AMEP4951), (Ex 531/40, Em 593/40; EVOS Light Cube for RFP; Invitrogen, AMEP4952). An arbitrary size threshold level was set (size of 5–25 nm) to avert false recognition of debris or noise, for each assay. The size thresholding level was determined by reference to past studies of Jurkat cell diameter and by analyzing the real-time detection image of individual cells using the counter.

The Alamar blue dye can be used as a direct measure of cell proliferation via metabolic activity. Every 48 hours, 2 mL samples were taken from each spinner flask (N=3 per condition) and after pelleting by centrifugation and washing with PBS, cells were re-suspended in fresh and complete RPMI-1640 medium with 10% (v/v) Alamar blue (Invitrogen, A50100). Next, 100uL per well was plated onto a 96-well plate (N=8 technical replicates per biological replicate) and incubated at 37ºC for 4 hours in the dark. Finally, plates were assessed via absorbance reading in a plate spectrophotometer (Epoch 2, BioTek, Winooski, VT, USA) using absorption peaks at 570nm and 600nm. A negative control was implemented where 10% Alamar blue was added to complete RPMI-1640 medium but with no cells present. Separately, a positive control was implemented by adding ascorbic acid (5%) to the same contents as the negative control, reducing 100% of the Alamar blue dye. To calculate the OD values obtained by the plate reader, the following equation was used:

% Reduction of Alamar blue = $[(E_{oxi600} \times A_{570}) - (E_{oxi570} \times A_{600})] / [(E_{red570} \times C_{600}) - (E_{red600} \times C_{570})] \times 100$

$= [(117216 \times A_{570}) - (80586 \times A_{600})] / [(155677 \times C_{600}) - (14652x\ C_{570})] \times 100$

Where:

- E is the molar extinction coefficient of oxidized or reduced Alamar blue reagent at 570 or 600nm
- C is the mean absorbance of the negative control wells at 570 or 600nm
- A is the mean absorbance of the treated condition wells at 570 or 600nm

3.3.5 Microscopy and fluorescent staining

After 1, 7, and 14 days of culture in spinner flasks, 2mL samples were taken in triplicate for each condition and placed in 35mm Petri dishes. Transmitted light images were acquired on an inverted TiE microscope (Nikon, Canada) with phase contrast optics at 10X magnification. Images were analyzed using ImageJ open access software (http://rsbweb.nih.gov/ij/). Brightness and contrast adjustments were the only manipulations performed to images.

At 7 and 14 days of culture in spinner flasks, samples were centrifuged and washed three times with 1 x PBS. Samples were fixed in 4% PFA for 10 min and then washed with PBS three times. Next, cell permeabilization was performed via addition of 0.02% TritonX-100 for 3 minutes. F-actin was stained using 1:200 Alexa Fluor™ 546 Phalloidin (Invitrogen, A22283). Nuclei were visualized using 1:500 DAPI (Invitrogen, D1306). Phalloidin and DAPI were added together in PBS at their respective concentrations and then incubated for 1 hour in the dark at room temperature. Following 3 washes with PBS, 50 μL from each sample was carefully added to a glass cover slip. After allowing the cells to settle onto the surface of the coverslip, samples were visualized by confocal laser scanning microscopy. Confocal imaging was performed on an A1R high speed laser scanning confocal system on a TiE inverted optical microscope platform (Nikon, Canada) with appropriate laser lines and filter sets at 40X magnification. Image processing was executed via ImageJ open access software. Briefly, DAPI (blue) and Phalloidin (red) channels were split. Next, Z-stacked 3D volume images were prepared for each channel and scale bars were added.

3.3.6 Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyze cell viability across media groups, using Tukey's multiple comparisons test for the Trypan blue exclusion assay. To explore the differences of Alamar blue reduction between media groups at each time point, a two-way ANOVA was implemented using Šidák's multiple comparisons test. The mean of all the biological replicates for the ACF group was compared to the mean of the replicates for the control group. Captions for figures describing experimental results state the number of biological replicates (N) as mean ± SEM, unless otherwise stated. The results were analyzed using GraphPad Prism, version 10.2.3 for MacOsx (GraphPad Software, La Jolla, CA, USA).

3.4 Results

3.4.1 ACF medium supports T cell viability and metabolism

After subsequent medium adjustment, Jurkat cells were cultured in suspension in spinner flasks for 14 days. Initially, cell viability was measured across control (FBS-based), negative control (basal medium only), and ACF media groups using Trypan blue exclusion via an automated cell counter, providing readings of total cell concentration, and viable cell percentage (Fig. 3.1). The negative control was included to better understand the contribution of basal medium (RPMI-1640) on cell growth alone, where the viable cell percentage after 24 hours significantly decreased ($40.3 \pm 1.2\%$, $^{**}P < 0.001$), and any viable cell growth was slowed to a halt by the fifth day of culture (Fig. 3.1A-B). Jurkat cells grown in the ACF medium condition were able to proliferate (mean viability across time, $97.5 \pm 1.0\%$), with no significant change in growth rate or viability as compared to the control group (mean viability across time, $97.8 \pm 0.7\%$) (Fig. 3.1B). The confluency and general morphology of these cells was observed during this time period using phase contrast imaging. After 1 day in spinner flask culture, samples from both media conditions were visualized where both groups show similar low confluency with distinct rounded morphology of Jurkat cells (Fig. 3.1C-D). After two weeks of growth, both groups exhibited increased confluency (>90%), while preserving the typical rounded morphology along with some cell clustering and debris accumulation, characteristic of Jurkat cells at higher concentrations (Fig. 3.1E-F). No significant differences across media conditions was observed.

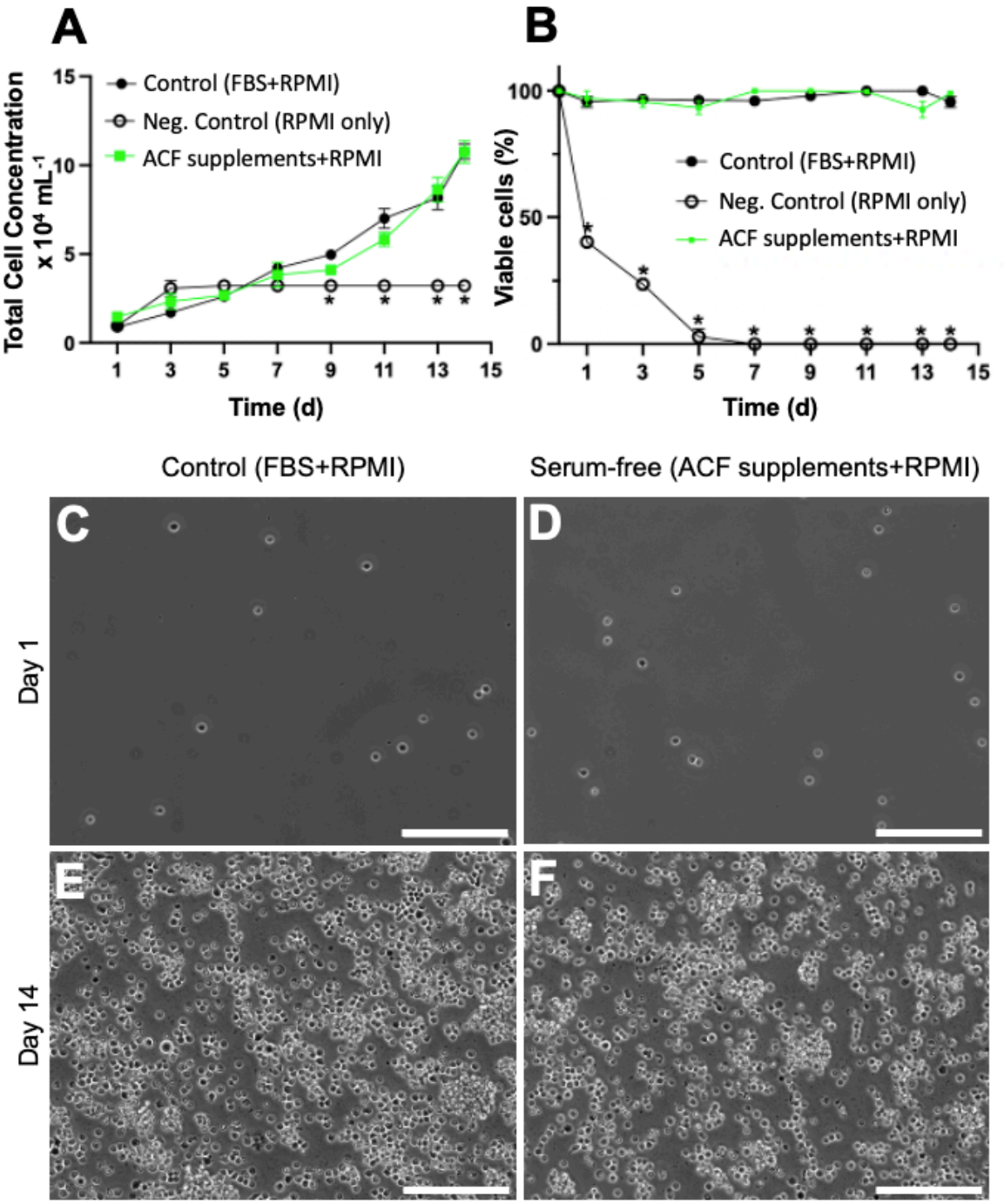

Figure 3.1: Cell viability analysis of dynamic culture of Jurkat cells.

(A) Mean concentration of total number of cells (x10^4 per mL) as measured by Trypan blue exclusion via automated cell counting. **(B)** Percentage of viable cells at each time point. N=3 for each time point and each medium condition. Each biological replicate is the mean of 3 technical replicates. Data are presented as mean ± SEM where *P < 0.01. **(C-F)** Most representative phase contrast images, 10X. Scale bars, 200μm. **(C,E)** Control medium (10% FBS) after 1 and 14 days, respectively. **(D,F)** ACF medium after 1 and 14 days, respectively.

To better document the proliferative capability of the ACF medium developed for this study, the Alamar Blue assay was used to assess the amount of metabolically active cells temporally. Jurkat cells exhibited similar levels of reduction of resazurin (oxidized component of Alamar blue which indicates increased metabolic activity) across media groups for each time point (Fig. 3.2). In comparing the control and ACF media conditions across the 14-day time period using a two-way ANOVA, it was found that the type of medium used had no significant effect on the metabolic activity over time.

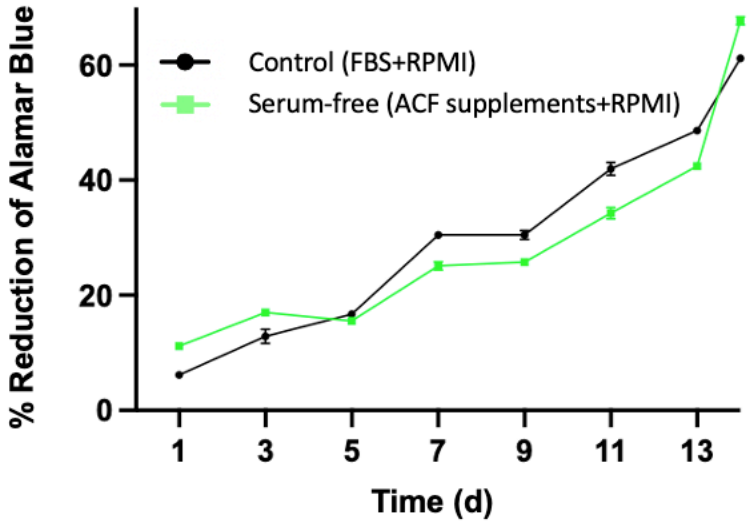

Figure 3.2: Indirect metabolic activity of Jurkat cells in dynamic culture over 2 weeks.
Time course of mitochondrial activity via percent reduction of Alamar blue dye. Each time point was separately measured. N=3 for each time point and each media condition. Each biological replicate is the mean of 8 technical replicates. Data is expressed as mean ± SEM where *P < 0.01.

To visualize viability, Calcein-AM and propidium iodide (PI) staining was implemented at 7 and 14 days of culture (Fig. 3.3). Viable cells stained by Calcein-AM appear green under fluorescent imaging, while dead cells stained by PI appear red. Observation of the stained cells revealed notable differences in cell viability between the two time points. At one week, both conditions displayed a similar staining pattern, with a large portion of living cells and a proportionally miniscule amount of dead cells (Fig. 3.3A-B). After two weeks, the overall cell density appeared to be much higher, with the percentage of dead cells remaining low (Fig. 3.3C-F). Visual examination of the cells under both media conditions did not reveal any significant changes in cellular morphology between the two time points. Cells appeared round or oval-shaped with a homogeneous distribution, consistent with typical Jurkat T cell morphology. No apparent signs of cell aggregation, debris accumulation, or abnormal cellular structures were observed.

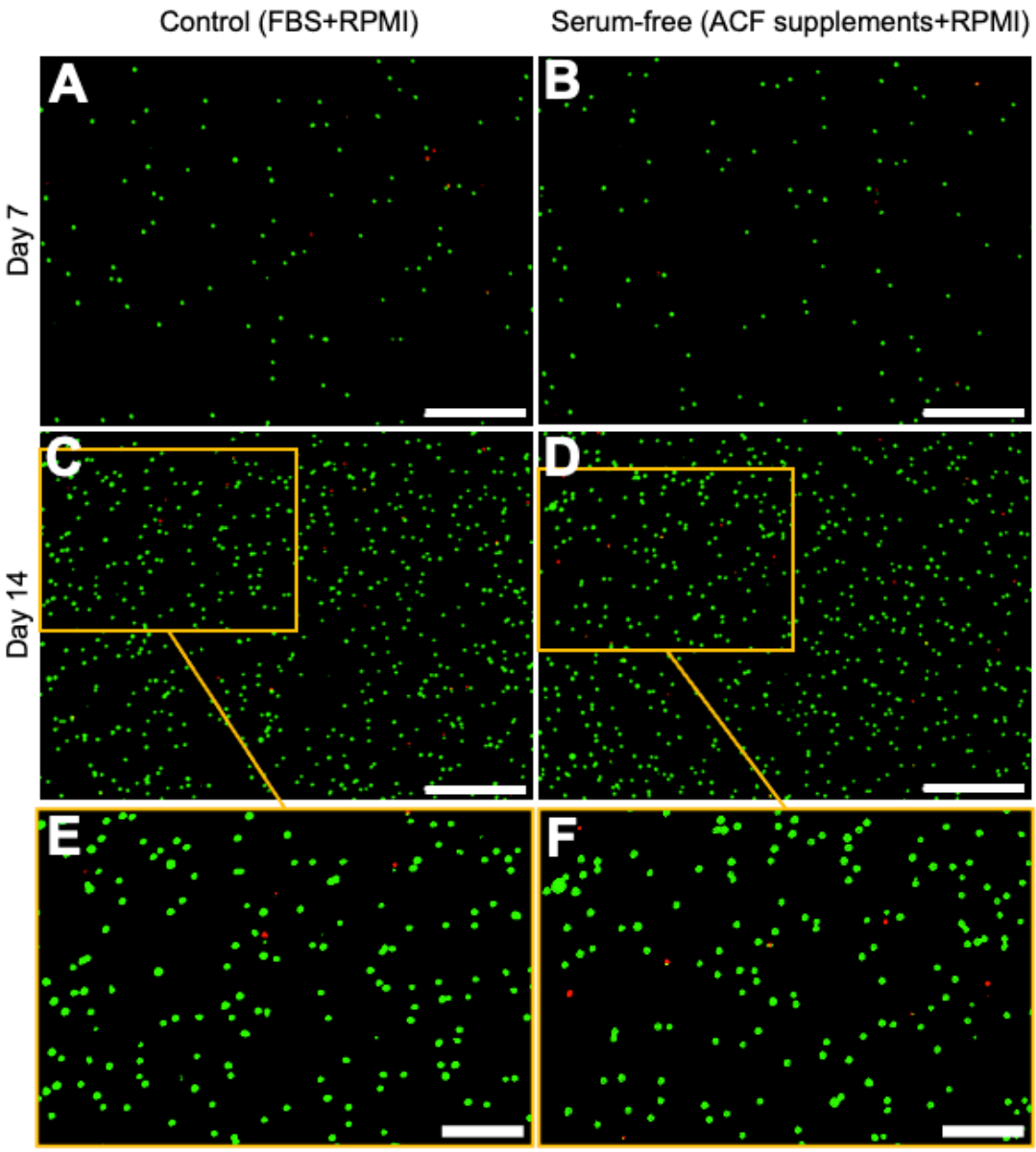

Figure 3.3: Most representative fluorescent images of live/dead staining.
Live (Calcein-AM, green) and dead (Propidium Iodide, red) cells visualized by CLSM. **(A-D)** Scale bars, 200µm. **(E-F)** Scale bars, 75µm. **(A,C,E)** Control medium (10% FBS) after 1 and 14 days, respectively. **(B,D,F)** ACF medium after 1 and 14 days, respectively

3.4.2 Jurkat cell morphology is preserved in dynamic ACF culture

To investigate changes in cellular morphology and cytoskeletal organization over time, Jurkat cell nuclei were stained with DAPI and F-actin was stained with Phalloidin at 7 and 14 days (Fig. 3.4). After 7 days, microscopic examination of stained cells revealed distinct morphological features consistent with early-stage growth (Fig. 3.4A-B). Typical Jurkat cells exhibited a rounded morphology, as indicated in previous studies that cultured them in FBS-containing medium.[69] In both conditions, well-defined nuclei with typical actin organization (minimal, rounded) were observed.

Cells cultured for 14 days displayed a substantial increase in cell density (Fig. 3.4C-D). Both conditions exhibited a similar pattern of cellular distribution and morphology, with cells appearing more tightly packed into clusters, compared to the 7-day time point. While most cells retained their rounded morphology, some cells appeared larger, possibly indicating multinucleation or increased nuclear size. However, there were no apparent changes in the organization or distribution of actin filaments. Notably, there were no visually significant differences observed in general morphology between the two conditions at either time point.

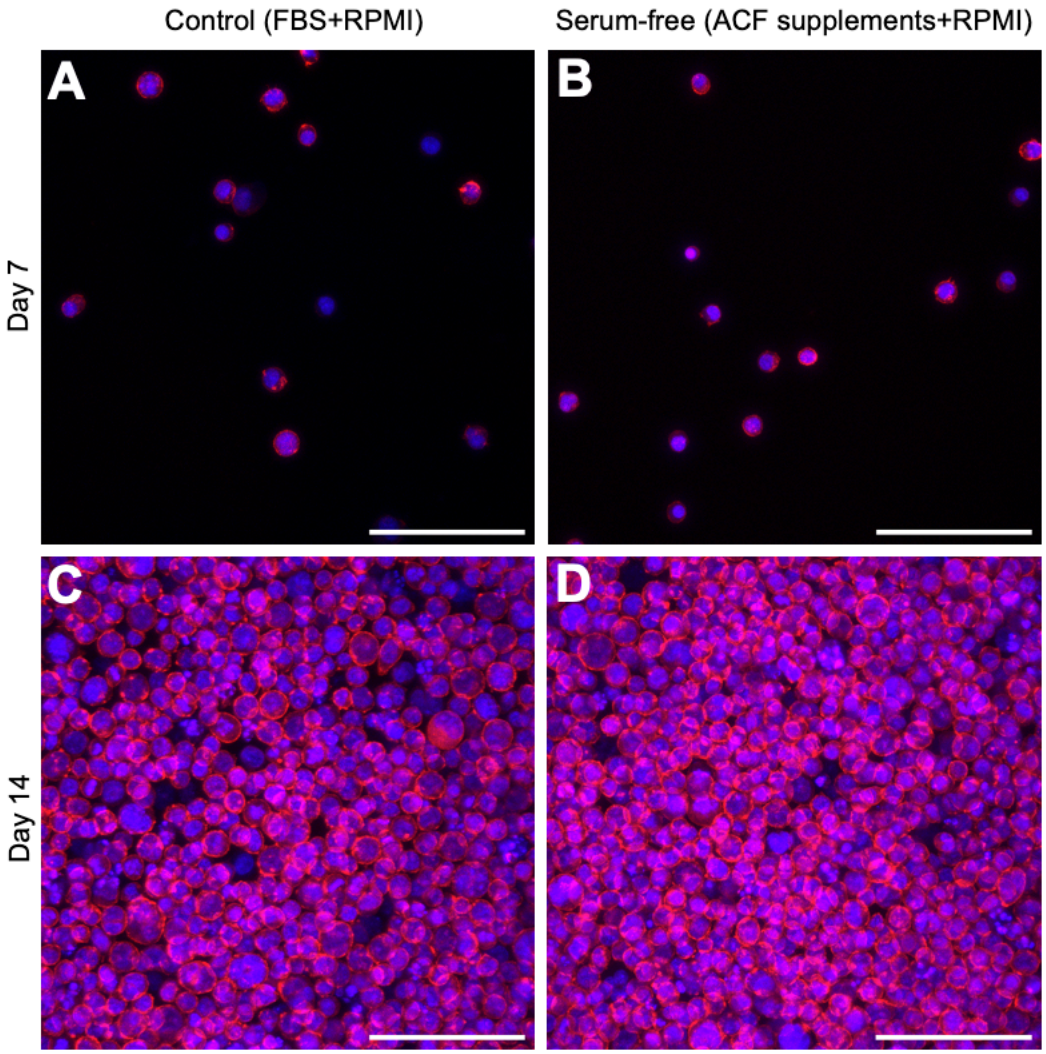

Figure 3.4: Nuclear and F-actin organization of Jurkat cells over time across.
(A-D) Representative confocal laser scanning microscopy (CLSM) images quantified after 7 and 14 days since initial seeding. Nuclei stained with DAPI (blue) and F-actin with Alexa Fluor™ 546-Phalloidin (red). Scale bars, 100μm. Magnification, 40X. **(A,C)** Control medium (10% FBS) after 1 and 14 days, respectively. **(B,D)** ACF medium after 1 and 14 days, respectively.

3.5 Discussion

The immortalized leukemic T-cell line, Jurkats, are very popular in studies representing immune cells for a wide range of applications.[49,70] Using lymphocytes in vitro as models for drug discovery, immunotherapy, and studies of cell encapsulation are increasingly requiring more tunable manners of production.[42-49] To create even greater control over the culture environment, implementing chemically-defined medium can facilitate regulatory compliance by allowing for better batch consistency and documentation of cell-based products.[67,71] The reduced risk of viral, mycoplasma, and prion contamination of animal-free culture is also a plus where product purity is crucial in biopharmaceutical production[1]. Moreover, SFM formulations can be tailored to specific cell types, optimizing production and cost effectiveness.

Jurkat cells can be grown in static suspension but are also often cultured in dynamic conditions – in agitated flasks, spinner flasks, and large bioreactor set ups. Yet, to the best of our knowledge, no team has actively aimed to discover a substitute for FBS-supplemented medium for this cell line in long-term dynamic cultures. In Table 3.1, a review of all studies that used SFM to culture Jurkat cells for other applications are summarized. Most commonly, RPMI-1640 or Opti-MEM were used as basal media, with a couple of additives that vary vastly from study to study. Additionally, only one study that implemented dynamic culture in bioreactors was identified – all other recognized studies employed static cultures. In large-scale manufacturing, dynamic suspension cultures are often preferable to static ones, as they can be easily scaled up to larger volumes.[72,73] Additionally, they do not require the labour of detaching adherent cells during passaging, create a more homogenous environment for cell growth, can have better nutrient and gas exchange, and can more closely mimic conditions in vivo.[37,74-76]

Previously, we developed a more complex ACF medium for several adherent cell lines that successfully preserved proliferation for NIH 3T3 fibroblasts for over 210 days, as well as C2C12 myoblasts, MC3T3 pre-osteoblasts, and HFF human foreskin fibroblasts for 30 days in culture *(Chapter 2)*. Our optimization process began by changing the basal medium of the former medium to RPMI-1640, typical of Jurkat culture. Additionally, we modified the ACF supplements from the previous study (which was designed to culture adherent cells in 2D) by removing transforming growth factor beta (TGFβ), fibroblast growth factor (FGF2), and epidermal growth factor (EGF) while keeping the other components: albumin, ethanolamine, insulin, transferrin, selenium. With insulin, transferrin, and selenium at 5X the concentration, we were able to successfully grow Jurkat T cells in spinner flasks over 14 days. Jurkat T cells, being suspension cells, do not require adherence to a surface or the production of extracellular matrix (ECM) components to maintain their growth and proliferation. This is in contrast to adherent cells, which rely heavily on the presence of specific growth factors to support ECM production and cell attachment, critical for their survival and proliferation in a 2D culture environment.[77] Adherent cells require these factors to synthesize and organize proteins such as collagen, fibronectin, and laminin, which constitute the ECM and provide structural support and signaling cues essential for their function.[78] In suspension culture, the need for ECM components is bypassed as cells grow in a three-dimensional environment without attachment requirements.

Consequently, growth factors like TGFβ, FGF2, and EGF, which are primarily involved in promoting cell adhesion, migration, and ECM production, become less critical.[79-81] For instance, TGFβ is known to induce ECM protein expression and deposition, while FGF2 and EGF stimulate cellular processes that enhance ECM interactions and stability.[82-84] By removing these factors from our medium formulation, we optimized it for Jurkat T cells, which do not depend on ECM-related signals for their growth in suspension. The increased concentrations of insulin, transferrin, and selenium likely compensated for the metabolic and proliferative needs of the cells. Insulin can act as a growth promoter by stimulating glucose uptake and metabolism, transferrin ensures adequate iron supply critical for cellular processes, and selenium is essential for antioxidant defense and maintaining redox balance.[85-89] Thus, the absence of ECM-dependent growth factors in our ACF medium formulation did not impede Jurkat T cell growth, as these cells inherently do not rely on ECM production and attachment, unlike adherent cell types.

As a result of this formulation process, we successfully established a fully ACF, robust, and dynamic suspension culture system for Jurkat T cell maintenance, while preserving typical cell proliferation rates and morphologies. Cell viability assays showed that the viable cell density in the ACF medium group was similar to the control group containing FBS after 14 days of growth in spinner flasks with continuous medium exchange. By incorporating a variety of viability detection techniques, we were able to confirm the robustness of this medium in preserving proliferation over time. Trypan blue exclusion with automated cell counting, visualization of live/dead cells with Calcein-AM/PI, and Alamar blue assays all showed consistent results where both FBS-based and ACF mediums fostered increased growth of Jurkat cells until the 14th day of culture, with proportionally minimal amounts of dead cells. Although metabolic activity of cultures was not measured directly, Alamar blue results provide an indirect measure of metabolism because enzymes from cellular mitochondria reduce its non-fluorescent oxidized components and a colorimetric change then occurs where the reduced form becomes pink and fluorescent.[90] Taken together, these results indicate that the ACF medium was able to preserve regular growth and metabolism in Jurkat cells, whereas some SFM studies of the same cell line have demonstrated weaker growth rates across time compared to controls.[42,56] Cultures without any FBS or other additives and only basal medium show almost 100% cell death by the fifth day of culture, consistent with previous studies that elucidated the same results.[42]

The imaging studies we implemented using phase contrast and CLSM reveal consistent cellular distribution and morphology across media types and time points. Although cells appear to cluster and tightly pack together after two weeks of growth compared to earlier time points, no significant differences in the distribution of actin filaments was observed.

Interestingly, there have been reports of SFM instigating cell adhesion in Jurkat cells.[51,52] SFM can be tunable to different properties, which may be advantageous to future researchers that use this cell line for different applications. The basal medium used in these studies that focused on cell adhesion, Opti-MEM, contains insulin, transferrin, and other additives. The ACF medium used in our study also contains insulin and transferrin, but with other additives *(see Section 3.3.2 and Table 3.3 for details on medium formulation and optimization*). It is hard to say how

the choice of medium caused Jurkat cells to remain in suspension or to begin to adhere, as it is possible that each medium component works in a dose-dependent manner and/or synergistically with other components. As the specific concentrations and exact additives of Opti-MEM remain proprietary, it is difficult to make the correct assumption. Other studies which developed SFM formulations for primary lymphocytes commonly use glutamine, insulin, albumin, and different types of fatty acids as supplements.[60-66] Glutamine is a crucial amino acid in cell culture media, serving as a primary carbon and nitrogen source for biosynthesis of nucleotides and amino acids which supports cell growth and proliferation.[91] For lymphocytes, including Jurkat T cells, glutamine metabolism is particularly important as these cells have high proliferative rates and metabolic demands.[92] Moreover, insulin facilitates glucose uptake and metabolism, promoting cell survival and proliferation.[93] Albumin, often used as a carrier protein, binds and stabilizes various bioactive molecules, including hormones, vitamins, and fatty acids, thereby improving their availability to cells.[94] Additionally, albumin helps to maintain osmotic pressure and pH balance in the culture medium.[95] Albumin has been used in countless SFM studies for its aforementioned properties and continues to be a staple in serum-free development.[11-13,60,61,64,96,97] Finally, fatty acids are vital components of cell membranes and play roles in energy production and signaling pathways.[98] The inclusion of different types of fatty acids such as linoleic acid or palmitic acid can support membrane fluidity and integrity, which is critical for rapidly dividing cells like lymphocytes, and they have been implemented in SFM formulations previously.[53,64,99]

Speculatively, the combination of these components in SFM might mimic the complex milieu provided by FBS, preserving cell proliferation and function through multiple pathways. For instance, insulin and albumin could work together to enhance nutrient uptake and stabilize metabolic conditions, while glutamine and fatty acids provide essential building blocks and energy sources. Although we do not include any fatty acids in our ACF medium, we do include ethanolamine which may help support cell membrane integrity in cell cultures by serving as a precursor for certain phospholipids.[100] The specific ratios and interactions between these components could create a conducive environment for Jurkat T-cell growth and maintenance, potentially explaining why our ACF medium formulation was successful in supporting Jurkat cultures in dynamic suspension. Further studies could explore the dose-dependent effects of these additives and their synergistic interactions to optimize SFM formulations for various cell types, including primary lymphocytes and other immune cells.

In summary, this study demonstrates that RPMI-1640 based medium with several ACF additives can act as a promising SFM for culturing Jurkat T cells in dynamic suspension culture within spinner flasks. The work presented here aims to address some of the concerns of industries that rely on serum-free suspension culture. However, future research should aim to expand these findings to other suspension cell lines, further optimize culture conditions for long-term functionality, and explore cost-effective strategies in various biomedical and biotechnological applications. Overall, this work lays a foundation for building serum-free culture technologies, and offers potential benefits in terms of scalability, reproducibility, and ethical considerations for cell-based research and bioprocessing industries.

3.6 References

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Chapter 4 | Validating an animal component-free media for select cell types cultured on apple-derived scaffolds

§ Motivation

There is a rising demand to enhance 3D culture systems with the use of biocompatible scaffolds for advancing regenerative medicine and new biotechnologies. Current 3D scaffolding methods for cell cultures often depend on animal-derived media for providing nutrients, which limit their applicability, and pose potential contamination and reproducibility challenges. By developing a plant-based scaffolding system in conjunction with a serum-free media, we aim to create a more effective and versatile platform that can be expanded on for tissue engineering. The integration of natural scaffolds with ACF media could revolutionize cell culture by providing an animal-free environment for creating complex tissue structures, ultimately contributing to the development of innovative treatments and technologies in regenerative medicine and beyond.

§ Hypothesis and Objectives

The goal of this study was to expand upon our previously optimized ACF media to develop an advanced 3D culture system using apple-derived scaffolds in serum-free conditions. We hypothesized that our ACF media formulation, combined with soy-coated scaffolds, would facilitate cell adhesion, proliferation, and maintenance at levels comparable to traditional FBS-based media. Moreover, we aimed to characterize any differences in uncoated and soy-coated scaffolds in order to test soy protein's capacity for initiating adhesion in cell cultures.

4.1 Abstract

The use of plant-based scaffolds for mammalian cell culture has emerged as a promising avenue in tissue engineering and three-dimensional (3D) cell culture applications. Previous studies have demonstrated the feasibility of growing mammalian cells on animal-plant hybrid scaffolds, showcasing their potential for various biomedical applications. Herein, we present a fully animal component-free (ACF) medium formulation, previously validated for mammalian cell growth in 2D environments, that is capable of supporting cell growth on soy-coated apple scaffolds. In this study, we cultivate NIH 3T3 fibroblasts, C2C12 myoblasts, and MC3T3-E1 osteoblasts on decellularized apple scaffolds in serum-free medium over a 14-day period. Through cell viability assays and fluorescence microscopy, all cell types maintain typical morphology and cell growth as compared to the control groups, cultured with FBS-containing medium. Cells cultured in ACF medium grown on uncoated apple scaffolds exhibit slower growth rates. This integrated approach builds upon the repertoire of available scaffolds for tissue engineering and underscores the versatility of this ACF medium in supporting diverse cell culture environments. Our findings not only provide insights into the synergistic interaction between plant-derived

scaffolds and serum-free culture but also lay the groundwork for the development of novel strategies in regenerative medicine and cellular agriculture.

4.2 Introduction

The convergence of biology and materials science has fueled ground-breaking advancements in tissue engineering and regenerative medicine.[1] Among these innovations, the development of three-dimensional (3D) microenvironments for in vitro cell culture has gained momentum in recent years. This is mainly due to the innate limitations of two-dimensional (2D) culture substrates like glass or tissue culture polystyrene (TCPS), since they lack the layered architecture needed to stimulate complex signals in native, 3D in vivo environments.[2,3] Many 3D scaffolds for cell culture have been developed from both naturally-derived and synthetic sources, and each deliver a wide range of applications.[4-8] Several groups have been successful in harnessing the scaffolding abilities of plant-derived cellulose for mammalian cell culture. Decellularized plants such as apple, carrot, mushroom, spinach, leek, celery, potato, tobacco leaf, broccoli, and parsley have been proven capable of fostering growth in varying capacities for myoblasts,[9-16] fibroblasts,[9,17,18] mesenchymal stem cells (MSCs),[19-22] endothelial cells,[19,23] pre-osteoblasts,[24-26] smooth muscle cells,[19,27-29] breast cancer cells,[30] induced pluripotent stem cells (iPSCs),[31,32] neuroblastomas,[33] adipose tissue,[26,29,34] and satellite cells.[16,35,36]

Preceding studies from our group have leveraged the biocompatibility of apple-derived scaffolds to facilitate cellular adhesion and proliferation in a variety of cell types. More specifically, successful long-term (12-week) viability and vascularization of fibroblasts, myoblasts, pre-osteoblasts and epithelial cells was demonstrated by our group previously.[9-11,24,25] The favorable outcomes seen in this culture system can be explained by the nutrients supplied by culture medium containing fetal bovine serum (FBS) along with the porous interior structure of apple hypanthium tissue which contains openings that allow for nutrient and gas exchange, vital to 3D cell culture.[37,38]

In parallel, the quest for animal component-free (ACF) cell culture media has gained traction, driven by concerns regarding xenogeneic contamination, batch variability, and ethical considerations associated with traditional serum supplements such as FBS.[39,40] Building upon this necessity, we have previously formulated a fully ACF medium, devoid of any animal serum, to sustain mammalian cell growth. Previous validation of this medium in 2D culture systems on Petri dishes *(Chapter 2)* and 3D suspension culture in spinner flasks *(Chapter 3)* has highlighted its efficacy and compatibility with various cell types, laying a solid groundwork for further exploration.

Herein, we bridge these two realms of inquiry by combining our ACF medium with the cultivation of mouse fibroblasts, myoblasts, and osteoblasts on apple scaffolds. By extending the application of our medium formulation to 3D culture environments, we aim to validate its efficacy and robustness in supporting cellular growth and function within the intricate architecture of plant-derived scaffolds. This integrated approach not only enhances our understanding of the interplay between biomaterials and cell culture media but also holds

profound implications for the development of advanced tissue engineering strategies where animal-free systems are desired. Previous studies have cultured select mammalian cell lines on scaffolds without any serum, yet to the best of our knowledge, none have done so on plant-derived cellulose scaffolds with fully ACF media formulations.[41-48]

The major goal of this research is to elucidate the synergistic potential of apple-derived scaffolds and our previously developed ACF medium in facilitating mammalian cell culture in 3D environments. Often in serum-free studies, cell adhesion is a significant challenge, as the absence of serum, which naturally contains adhesion-promoting elements, removes the necessary extracellular matrix (ECM) proteins that may instigate the initial attachment of cells in culture.[49,50] In studies involving cell culture on scaffolds, this issue is particularly pronounced. Many plant-derived proteins have been used as promising candidates for increased cell adhesion.[51-56] In particular soybean is evidenced to support cell adhesion and proliferation, likely due to its biocompatibility, natural abundance, and presence of attachment-promoting amino acid sequences (i.e. RGD, Arg-Gly-Asp).[57-70] Thus, we employ soy protein's benefits in this study to enhance the ACF culture system's capacity to maintain mammalian cells on plant-derived scaffolds. Through cell viability assays using metabolic activity and fluorescent imaging revealing morphological structure, we demonstrate that cells cultured on soy-coated apple scaffolds in ACF medium are maintained to a similar degree as in standard medium. With the validation of this medium in a 3D culture environment, we aim to add to the innovative applications of regenerative medicine, cultivated food products, and beyond.

4.3 Methods

4.3.1 Scaffold Production

The scaffold preparation protocol is based upon previous studies from our research group.[9-11,13,24,25] Briefly, McIntosh Red apples were cut into 1mm sections (as measured by a Vernier caliper), using a mandolin slicer. Scaffolds were created using a 6mm biopsy punch to carve 6mm disks from the sliced hypanthium tissue. Decellularization was performed by submerging in 0.5% SDS solution and shaking at 100 rpm for 48 hours. To remove any leftover surfactant, scaffolds were washed with dH_2O three times and then incubated at room temperature in 100mM $CaCl_2$ for 24 hours. To remove salt residue, scaffolds were washed three times with dH_2O. To sterilize, scaffolds were incubated with 70% ethanol for 24 hours at room temperature. Finally, scaffolds were washed with sterile dH_2O five times and kept in fresh sterile dH_2O until one day before scaffold seeding.

4.3.2 Cell lines and Culture Conditions

Murine embryonic fibroblast NIH 3T3 cells (ATCC-CRL-1658), murine C2C12 myoblast cells (ATCC CRL-1772), and murine MC3T3-E1 Subclone 4 osteoblast cells (ATCC CRL-2593) were purchased from ATCC. Cells were cultured in 10 cm dishes at 37°C in 5% CO_2, passaged using 0.05% trypsin-EDTA (Cytiva, SH3023602), and counted using trypan blue to maintain ~85% confluency at each passage for routine cell maintenance. NIH 3T3s were maintained in proliferation medium

consisting of 1:1 DMEM/Ham's F-12 (Sigma, 51445C) supplemented with 10% (v/v) FBS (Wisent, cat. 098150, lot. 185729) and 1% (v/v) P/S (100 U/mL penicillin, 100 mg/ml streptomycin; Cytiva, SV30010). Different basal mediums were used to culture the other cell lines while using the same protocol: MC3T3-E1 - MEM Alpha 1X (Gibco, A1049001) and C2C12 - DMEM (Sigma, D0822).

The ACF medium group underwent a 10-day sequential serum adaptation process before being cultured in 100% ACF medium. Following two passages in the serum-containing medium, cells were split into two media groups: control medium (containing FBS) and animal component-free medium (ACF, no supplements of animal origin). The growth media for the control groups consisted of basal media [DMEM/Ham's F-12 (for NIH 3T3) or DMEM (for C2C12) or MEM alpha (for MC3T3)] with 1% (v/v) Penicillin/Streptomycin (P/S) as its base, and supplemented solely with 10% FBS (Wisent, cat. 098150, lot. 185729). A single batch of FBS has been acquired for the research conducted over the entire thesis, aiming to reduce fluctuations among control samples. The ACF culture medium contains the exact same base as the control (basal medium for specific cell type + 1% P/S). It is supplemented with a variety of hormones, carrier proteins, lipids, minerals, and growth factors. The formulation is identical to that of our previous study with its final contents in Table 4.1. For a full description on the optimization and formulation testing process, see *Chapter 2, Section 2.5.3.*

Component	**Final Working Concentration**	**Company, Catalogue #**
Albumin, recombinant expressed in rice	1 mg/mL	Sigma, A9731
Ethanolamine, synthetic	1.5 mg/mL	Sigma, E0135
Animal origin-free ITS (100X) • Insulin, recombinant derived from yeast • Transferrin, recombinant derived from rice • Selenium, synthetic	Animal origin-free ITS (5X) • 50 ug/mL • 27.5 ug/mL • 25 ng/mL	Sigma, SCM055
Fibroblast growth factor 2, recombinant expressed in E. coli	1 ng/mL (NIH 3T3) 30 ng/mL (C2C12, MC3T3)	Sigma, SRP4038
Epidermal growth factor, recombinant expressed in E. coli	10 ng/mL	Sigma, SRP3196
Transforming growth factor beta, recombinant expressed in E. coli	5 ng/mL (C2C12, MC3T3)	Sigma, GF346

Table 4.1: Animal component-free media components for scaffolding study.

These components were added in supplement to the same basal medium as used in the corresponding control for each cell type. Basal mediums differed for each cell line. Some

components listed here varied for cell type as listed in brackets. Otherwise, all supplements remained constant across cell lines.

4.3.3 Scaffold Coating and Seeding

One day before cell seeding, apple scaffolds were placed in soy solution to be used for ACF soy-coated conditions, or they remained in PBS for control and non-coated ACF media conditions. Soy coatings were produced by dissolving soy protein hydrolysate (Sigma, S1674) in DI H2O at a concentration of 4g/L. 6 hours before cell seeding, each apple scaffold was placed in media (either control medium or ACF medium) and kept at 37°C in 5% CO_2. At the time of seeding, cells grown in 10cm Petri dishes (as described and adjusted above) were counted using an automated image-based cell counter (Countess™ II FL, Invitrogen) after trypsinization, centrifugation, and re-suspension in corresponding medium. Each apple scaffold was placed in a 24-well plate and inoculated with cells in a 10 μL droplet, at different concentrations depending on the type of experiment. To allow for proper attachment, media were added to each well to submerge the scaffold only after four hours since the initial inoculation. Scaffolds that were prepared for NIH 3T3 viability assays were seeded at a density of $1.5x10^6$ cells/mL and scaffolds that were prepared for staining and imaging were seeded at a density of $1x10^6$ cells/mL. Scaffolds that were prepared for C2C12 and MC3T3 staining were seeded with $5x10^5$ cells/mL. For a period of two weeks for each condition, media were removed and exchanged for fresh media every 3 days.

4.3.4 Cell Viability

Cell proliferation for NIH 3T3s was measured via the Alamar blue assay. After one, four, and seven days of culture, media were removed from the wells surrounding each cell-covered scaffold (N=24 per medium condition per day). Scaffolds were washed twice with PBS, placed in 96-well plates, and then covered in complete basal medium with 10% (v/v) Alamar blue (Invitrogen, A50100). In the dark, plates were incubated at 37ºC in 5% CO_2. Finally, absorbance was read in a plate spectrophotometer (Epoch 2, BioTek, Winooski, VT, USA) using absorption peaks at 570nm and 600nm. A negative control was implemented where 10% Alamar blue was added to complete basal medium but with no scaffolds or cells present.

4.3.5 Staining and Microscopy

Calcein-AM/Propidium Iodide (PI) staining for NIH 3T3s was performed after 7 and 14 days of culture to assess live/dead cell ratios on scaffolds over time. Briefly, scaffolds (N=3 per condition per time point) were washed with PBS three times. Next, 2.5 μg/mL of Calcein-AM (Sigma-Aldrich, 206700) and 5 μg/mL of Propidium Iodide (Invitrogen, P3566) in PBS was used to submerge the scaffolds in the dark for 1 hour at 37ºC. Samples were again washed with PBS three times before visualization using Confocal Laser Scanning Microscopy (CLSM) on an A1R high speed laser scanning confocal system on a TiE inverted optical microscope platform (Nikon, Canada). Images were taken at 10X magnification with appropriate filter sets and lasers. ImageJ (Fiji) open access software was used to process images where Calcein-AM (green) and Propidium Iodide (red) channels were split and Z-stacked volume images were prepared.

The same staining and imaging protocol was employed for all cell lines in this study. To prepare nuclei, actin, and cellulose-stained scaffolds, samples were washed with PBS three times and fixed with 10% formalin for 10 minutes at room temperature. Next, 0.1% TritonX-100 was added to permeabilize cells for 5 minutes. Following three PBS washes, scaffolds were blocked with 25% BSA at room temperature for 30 minutes. Next, scaffolds were incubated with 1:500 DAPI (Fisher, D1306) and 1:200 Alexa Fluor™ 488 Phalloidin (Invitrogen, A22283) in 25% BSA in the dark for one hour. Then, cellulose was stained using 0.008% Congo Red (Sigma) for 15 minutes. After three washes with PBS, Vectashield mounting medium was added to each sample. By placing each sample on a glass coverslip, CLSM was performed on an A1R high speed laser scanning confocal system on a TiE inverted optical microscope platform (Nikon, Canada) with appropriate laser lines and filter sets at 10X magnification. Using ImageJ, DAPI (blue), Phalloidin (green) and Congo red (red) were split and then Z-stacked 3D volume images were prepared with scale bars. No other image manipulations were performed other than altering certain brightness and contrast settings.

4.3.6 Statistical analysis

The statistical analysis performed for Alamar blue reduction between media groups at each time point was characterized using a two-way ANOVA with Šidák's multiple comparisons test. The mean of all the biological replicates for the ACF non-coated and soy groups were compared to the mean of the replicates for the control group. The number of biological replicates in the captions for figures describe the number of biological replicates (N) as mean ± SEM, unless otherwise stated. The results were analyzed using GraphPad Prism, version 10.2.3 for MacOsx (GraphPad Software, La Jolla, CA, USA).

4.4 Results

4.4.1 Metabolic activity and viability of NIH 3T3 cells is preserved in soy-coated scaffolds

NIH 3T3 cells were cultured on apple-derived scaffolds under three different media conditions: control medium containing 10% FBS, ACF medium without scaffold coating, and ACF medium with a soy-coated scaffold. Alamar Blue assays conducted over a two-week period revealed that scaffolds coated with soy and grown in ACF medium exhibited no significant difference in metabolic activity compared to the control containing FBS (P=0.78) (Fig. 4.1A). However, scaffolds without any coating, grown in ACF medium, showed a significantly decreased metabolic rate by the end of the 14-day period (P=0.03), indicating slower proliferation rates as compared to the other media conditions.

Cell viability was assessed using Calcein-AM and PI staining at days 7 and 14 of culture. On day 7, no significant differences were observed in cell viability across the three media conditions (Fig. 4.1B-D). The soy-coated ACF group and the control group exhibited similar levels of green fluorescence (live cells), along with some red fluorescence (dead cells), demonstrating similar cell viability in these groups after two weeks (Fig. 4.1.E-F). However, by day 14, a marked

decrease in the green fluorescence signal was observed in the non-coated ACF group, indicating a reduced number of living cells (Fig. 4.1G).

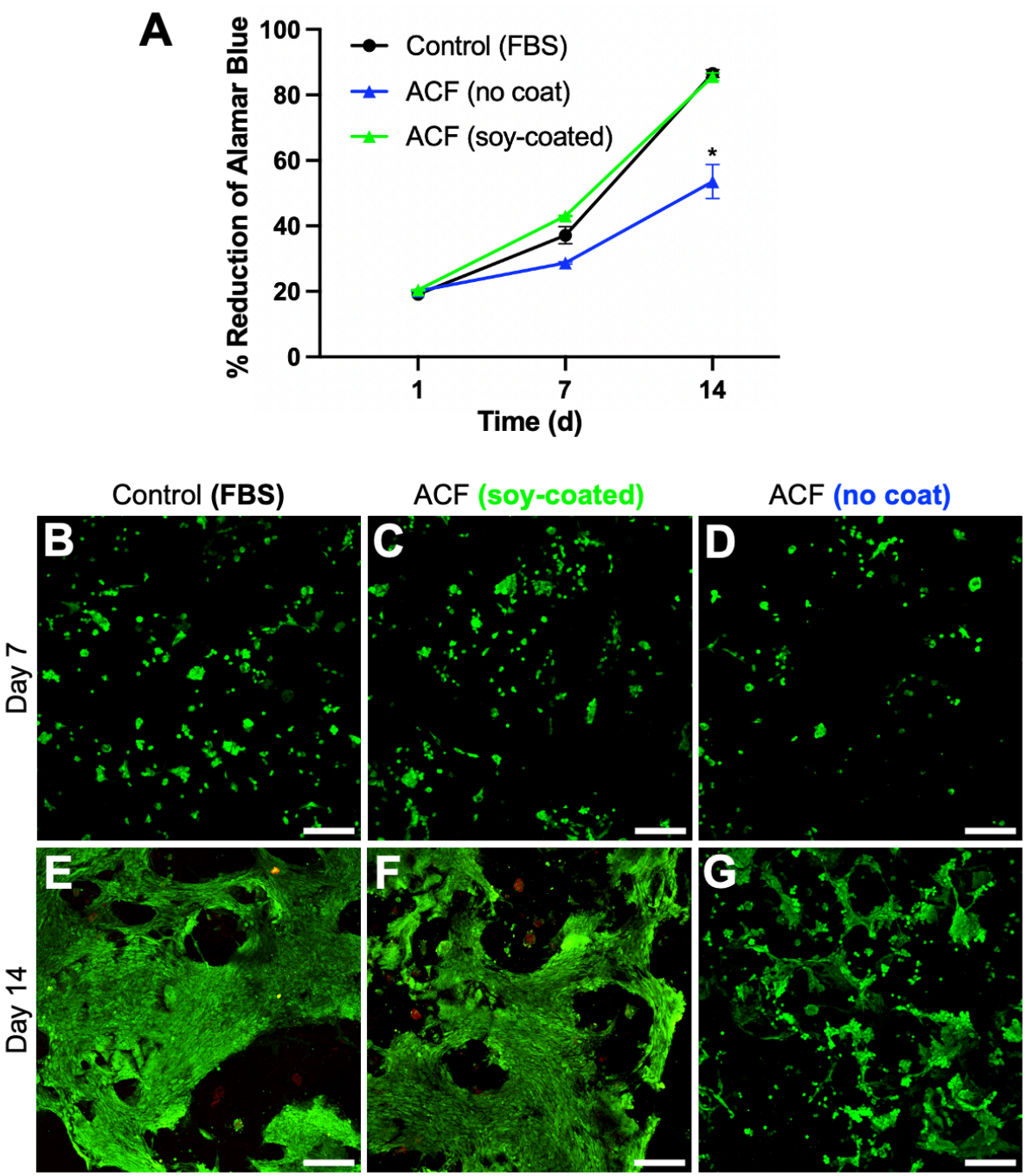

Figure 4.1: Cell growth and viability of NIH 3T3s over 2 weeks in various media and scaffolding conditions.

(A) Mean percent reduction of Alamar Blue solution over time with Control (black), ACF (blue), and ACF with soy coating (green) conditions. Experiments were conducted in triplicate for each condition, with 8 technical replicates per biological replicate. Each time point was separately measured and is expressed as mean ± SEM where *P<0.05. **(B-G)** Cells treated with Calcein-AM/PI

after 7 **(B-D)** and 14 **(E-G)** days. Calcein-AM stained viable cells (green) while PI stained dead cells (red). Media and scaffold conditions are organized into columns. Scale bars, 200μm.

4.4.2 Morphology is maintained in soy-coated scaffolds across cell lines

NIH 3T3 cells stained with DAPI (nuclei), Phalloidin (F-actin), and Congo red (cellulose) were imaged by CLSM after 7 and 14 days of culture. After seven days of culture, no significant changes in cell quantity and morphology were visible across media conditions (Fig. 4.2A-C). Despite this, by the 14th day of culture fewer cells were observed on the scaffolds in the non-coated ACF group, as indicated by the lower intensity of DAPI and phalloidin signals (Fig. 4.2D). Conversely, the soy-coated ACF group and the control group showed similar cell densities and distribution across both time points, with robust actin filament staining and uniform nuclear staining, indicating healthy cell growth and scaffold integration (Fig. 4.2E-F).

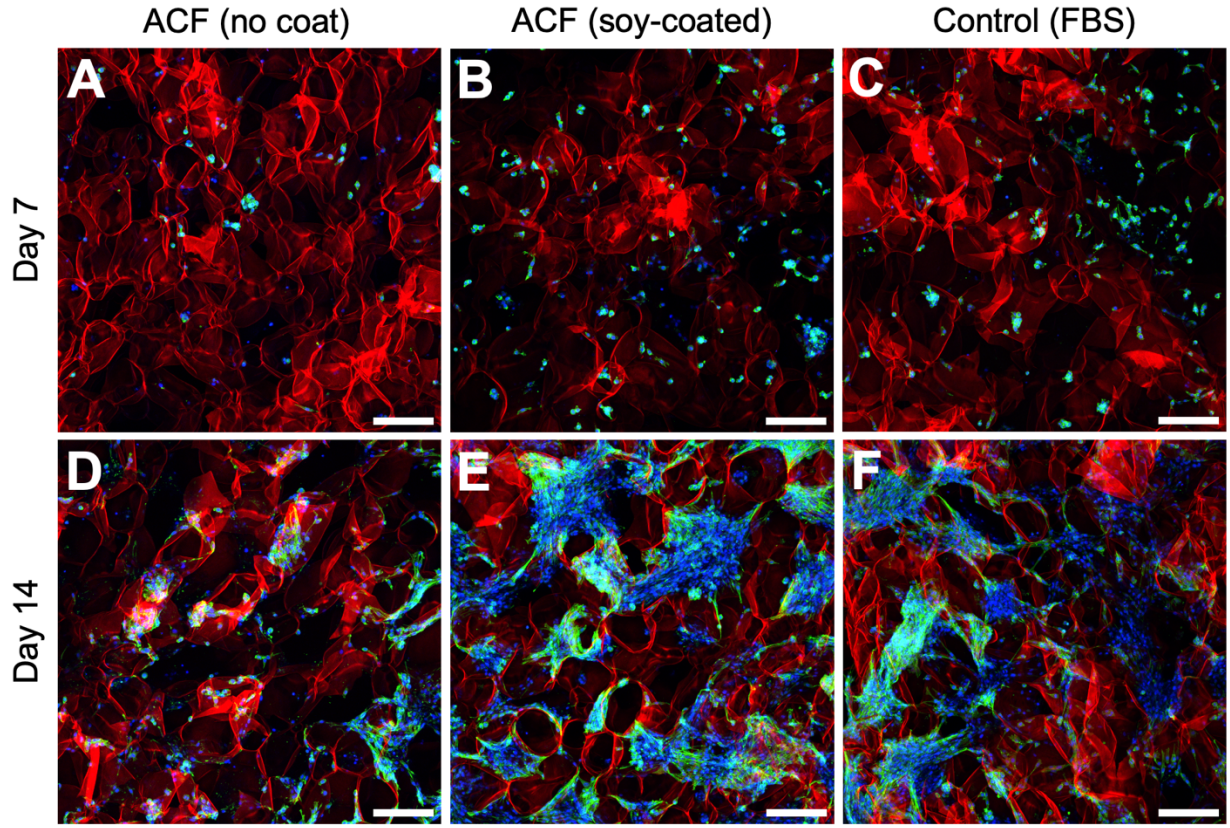

Figure 4.2: Fixed and stained NIH 3T3 cells cultured on 3D cellulose scaffolds in various media conditions.
Specific fluorescent staining using confocal laser scanning microscopy reveals the cellulose structure of the apple (red, Congo Red), actin filament (green, Phalloidin) and nuclei (blue, DAPI). Cells were cultured in these scaffolds for 7 **(A-C)** or 14 **(D-F)** days prior to staining and imaging. Confocal volumes were acquired and projected in the XY plane. Media conditions are organized into columns. Scale bars, 200μm.

To further validate the effectiveness of the ACF soy-coated scaffolds, similar confocal imaging and staining protocols were applied to C2C12 myoblasts and MC3T3 pre-osteoblasts. These cell types were cultured in a slightly altered ACF medium formulation described in detail in Table 4.1, with 2 media conditions per cell type: uncoated scaffold in control medium (FBS) and soy-coated scaffold in ACF medium. After 14 days of culture, CLSM revealed comparable cell densities and morphologies for each cell type in both media conditions (Fig. 4.3). C2C12 myoblasts exhibited typical spindle-shaped morphology, with alignment following the native cellulose structure (Fig. 4.3A-B). MC3T3 pre-osteoblasts showed uniform, polygonal monolayers with a high degree of cell spreading, in both cases (Fig. 4.3C-D).

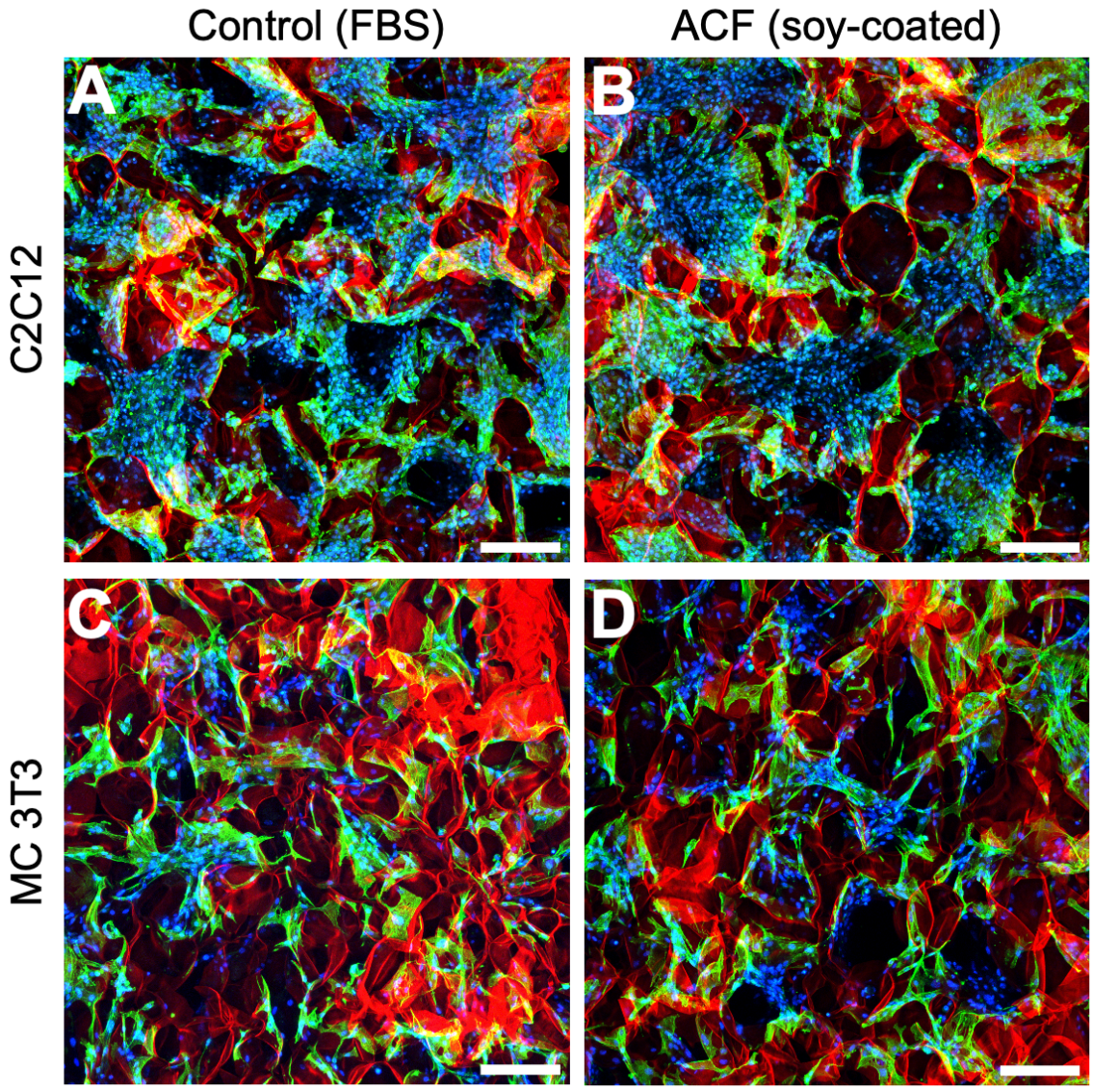

Figure 4.3: Fluorescent images of C2C12 and MC3T3 cells cultured on apple scaffolds. Fluorescent staining via confocal laser scanning microscopy reveals the cellulose structure of the apple (red, Congo Red), actin filament (green, Phalloidin) and nuclei (blue, DAPI). **(A-B)** C2C12

myoblasts cultured with FBS-containing medium **(A)** and ACF medium with soy coating **(B)**. **(C-D)** MC3T3 pre-osteoblasts cultured with FBS-containing medium **(C)** and ACF medium with soy coating **(D)**. Cells were cultured in these scaffolds for 14 days prior to staining and imaging. Confocal volumes were acquired and projected in the XY plane. Scale bars, 200um.

4.5 Discussion

In this study, we evaluated the effectiveness of a previously validated ACF medium formulation *(Chapter 2)* for the growth of NIH 3T3 cells, C2C12 myoblasts, and MC3T3 pre-osteoblasts on apple-derived scaffolds. From our previous study, we highlighted the potential of this ACF medium for adherent cell lines in a two-dimensional (2D) setting, as they were cultured on a flat substrate. Here, we demonstrate the expanded capabilities of this formulation in a more three-dimensional (3D) setting, emphasizing the importance of developing media formulations that can support cell proliferation in both environments. On a flat, 2D substrate, cells are spread in a manner by which only one side is exposed to extracellular matrix (ECM) interactions, whereas ECM interactions may be present on multiple facets of cells on more complex 3D substrates.[71,72] This difference in environmental stimulus has shown to alter signaling pathways, thereby changing gene expression profiles of the same cell type grown in 2D versus 3D conditions.[73,74] The neighbouring cells and surrounding ECM can have a profound impact on cell behaviour because when cell attachment is limited to a single plane, the complexity of signals received by cells is restricted.[74-76] In 3D cultures, multidirectional mechanical signals create a more intricate network of biochemical cues which in turn affect processes like proliferation, differentiation, and migration.[78-83] Interestingly, the medium we developed for 2D culture was capable of effectively maintaining the same cell types over a 14-day period in a 3D setting on apple-derived scaffolds, without any alteration. With serum-free media (SFM) development, the ability to maintain consistent proliferation across different substrates and dimensionalities without adjusting media composition is essential for consistency, standardization, and scalability.[84,85] A single, adaptable medium reduces the need for developing and optimizing multiple specialized formulations for different stages of production in applications where SFM are required.

The effectiveness of our ACF medium in both 2D and 3D settings may be attributed to its content of essential nutrients and growth factors that support cellular function. It has been shown that signaling cascades vary between 2D and 3D cultures, with 3D ones exhibiting more relevant signaling mechanisms to native microenvironments.[76-83,86] For instance, based on changes in local compression or tension (which can vary between 2D and 3D environments),[87-91] cultured cells can enter the growth phase or instead initiate apoptosis to the same chemical stimuli.[92-95] In our study, the same chemical stimuli (i.e. the mix of growth factors, hormones, and other proteins of our formulation) allowed for high viability (>90%) in both 2D and 3D modalities across cell types. Therefore, we speculate that the mixture of albumin, insulin, transferrin, selenium, ethanolamine, FGF2, EGF, and, depending on the cell type, TGFβ, all contribute to an environment that is similar enough to that of FBS that it allows for successful cell maintenance. This speculation is supported by various studies that have demonstrated the efficacy of these components in a variety of SFM formulations previously.[40,84,96-126] For instance, Ham and McKeehan (1979) developed a SFM containing insulin, transferrin, and selenium that

supported the growth of numerous mammalian cell lines, and these components have been successfully implemented by most SFM research since then, highlighting their necessity in mammalian culture.[127] Modern-day studies have demonstrated that a combination of recombinant albumin, insulin, transferrin, and selenium as a base can support bovine,[128,129] pig,[130,131] and sheep[132] embryo development, as well as the growth of human mesenchymal stem cells,[133-135] oocytes,[136,137] chondrocytes,[111-115] myoblasts,[118,126,138] satellite cells,[120,124] Vero cells,[99,102,103] and a variety of fibroblastic lines.[105,108,139] This is further confirmed by commercially available products like Essential 8™, which includes FGF2 and TGFβ in addition to most of the other components mentioned above. Other commercially available serum-free formulations often include at least some if not most of these elements to maintain various cell types.[140,141] The reason that albumin, insulin, transferrin, selenium, ethanolamine, FGF2, EGF, and TGFβ were chosen in our study is due to the successful research described above. For each of these components, we created a broad range of concentrations based on the reported values in the literature. We initiated our optimization process by selecting concentrations that were at the midpoint of these ranges. Through iterative rounds of testing and fine-tuning, we identified optimal concentrations for supporting the proliferation and maintenance of fibroblasts. Specifically, fibroblasts thrived with moderate levels of FGF2 and no TGFβ. When we transitioned to optimizing the medium for myoblasts and osteoblasts, we observed distinct differences in their requirements. Myoblasts and osteoblasts demanded higher concentrations of FGF2 to support their growth and could not survive without the inclusion of TGFβ. The full detailed rationale for this final formulation can be found in *Chapter 2, Section 2.3.1.* These observations underscore the importance of cell-type-specific optimization in medium formulation. By understanding the specific needs of different cell types, researchers can create more effective and targeted culture conditions. Future studies should continue to explore and refine these formulations, considering the unique requirements of different cell types.

Our results show that the soy-coated scaffolds in ACF medium significantly enhance cell proliferation on 3D plant-derived scaffolds, achieving growth rates comparable to FBS-containing medium. This finding was also discovered in our previous study where soy protein hydrolysate was used to coat Petri dishes before culture *(Chapter 2).* The interaction between soy protein and the apple-derived cellulose scaffolds may create an optimal bridge for cell attachment. The soy protein coating on the cellulose scaffold can potentially interact with the cellulose fibers, enhancing the scaffold's bioactivity.[142] This may be due to the presence of RGD (arginine-glycine-aspartic acid) motifs in lunasin, a soy peptide known for its cell adhesion properties.[143] Additionally, the mere presence of peptides from soy protein can provide sites for cellular attachment and may also support sustained cell growth and function by maintaining a conducive microenvironment.[144] Transcriptomic data from our previous study revealed that while most genes related to cell cycle, apoptosis, and ECM/adhesion remained unchanged, specific differentially expressed genes indicated complex molecular adaptations *(see Section 2.3.4).* Notably, the upregulation of Laminin alpha 2 (LAMA2) across serum-free conditions suggested enhanced adhesion efforts. Downregulation of ECM-related genes like Collagen Type V Alpha 1 (Col5a1) in soy-coated groups supports the hypothesis that external adhesive cues from soy protein reduce the need for ECM synthesis. These transcriptomic insights underscore

the potential of soy coatings to mimic serum's adhesive properties and may explain the comparable cell proliferation and maintenance to FBS-grown cells seen in this study.

These results highlight the potential of our ACF medium as a viable alternative for 3D cell culture systems, which are increasingly used in tissue engineering and regenerative medicine. Future work should focus on further optimizing the ACF medium formulation and scaffold coatings to enhance cell differentiation and tissue-specific functions. Investigating the molecular mechanisms underlying the interaction between soy peptides and cellulose scaffolds could provide deeper insights into how these components contribute to improved cell attachment and proliferation.

Advancing the ACF medium and natural scaffolds further would involve several key steps. First, it would require refining the ACF formulation to better mimic the complex nutritional and signaling environments provided by FBS, potentially through the inclusion of synthetic or plant-derived growth factors and adhesion molecules and through computational methodologies. Additionally, improving the physical and biochemical properties of natural scaffolds, such as their porosity, mechanical strength, and bioactivity, could enhance their ability to support tissue-specific cell functions. The major contribution of this work is the development of more ethical and sustainable cell culture practices, reducing reliance on animal-derived products like FBS. This is particularly significant in the context of producing cultured meats, developing tissue grafts for medical applications, and creating more reliable in vitro models for various applications. Ultimately, the integration of natural scaffolds with ACF media formulations hold promise for advancing regenerative medicine, cultivated food products, and beyond, paving the way for more ethical, cost-effective, and high-quality solutions in these fields.

4.6 References

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Chapter 5 | Conclusions

5.1 Future Directions

Over the past few decades, significant progress has been made in the development of serum-free media (SFM) formulations, with the potential to revolutionize cell culture techniques by eliminating the need for animal-derived components like FBS. FBS has long been a staple in cell culture due to its rich composition of growth factors, hormones, and nutrients that support a wide range of cell types. However, ethical concerns, batch variability, and supply constraints have driven the search for viable alternatives.[1] This thesis has made substantial contributions to this field through the creation and validation of a novel SFM that supports the growth of various cell types, including fibroblasts, myoblasts, pre-osteoblasts, and lymphoblasts, both in 2D and 3D cultures. Our findings demonstrate that the animal component-free (ACF) formulation we developed matches, and in some cases surpasses, the performance of FBS-containing medium in terms of cell viability, mRNA expression, and morphological characteristics.

The significance of this work lies not only in the creation of an effective ACF medium but also in its broader implications for the field of cell culture. Existing studies have made strides in developing SFMs, but many are limited in scope, often focusing on a single cell type or application. For example, research that has tackled the animal-derived media problem within cultivated meats, such as work by Kolkmann et al. (2020), primarily addressed the needs of bovine myoblasts, while Messmer et al. (2022) and Stout et al. (2022) developed a SFM specifically for bovine satellite cells.[2-4] In contrast, our formulation is versatile, supporting a diverse array of cell types across different culture conditions. This positions our work as a significant advancement, providing a more universal solution that can be adapted for various biomedical applications.

The first paper established the efficacy of the ACF medium in promoting cell growth in 2D (on Petri dishes), showing promising results across all tested adherent cell types. These results were comparable to those observed in the studies by Chaturvedi et al. (2015), who developed a SFM for mouse myoblasts, but our medium demonstrated broader applicability, extending to fibroblasts and pre-osteoblasts. Our medium formulation overlaps with theirs for 5 out of 9 components, but with differing concentrations and with fully animal-derived components.[5] The second paper extended these findings to lymphoblasts cultured in spinner flasks with a modified version of the ACF medium, confirming that the formulation supports robust cell growth under dynamic, 3D conditions. This aspect of our research aligns with the work of Youn et al. (2017), who explored lymphoblasts in 3D culture systems using SFM, yet our results suggest superior performance in maintaining cell viability and proliferation over extended culture periods.[6] The third paper further validated our medium by demonstrating its effectiveness in supporting cell growth on soy-coated apple-derived scaffolds, a more complex 3D culture system using the same cell types as in the first study. This approach not only provides a novel application of the developed SFM but also opens the door to more sustainable and ethical tissue engineering practices.

Despite these promising results, transitioning from FBS-containing medium to SFM in large-scale bioprocessing poses several challenges.[7-9] One significant hurdle is the need for extensive validation and optimization to ensure consistent performance across various cell lines and culture conditions. Currently, the global demand for FBS is estimated to exceed 800,000 liters per year, primarily driven by the biopharmaceutical industry and research institutions.[10] With prices ranging from $300 to $1,000 per liter, the economic and ethical implications of reducing reliance on FBS are substantial.[11] However, we do not fully understand the exact requirements for cell growth for each cell line, and the precise reasons why animal sera work so effectively to cultivate cells remain unclear. This is partly due to the complex and undefined composition of FBS, which includes thousands of components in varying concentrations. Moreover, we lack perfect measures for assessing functionality and cell "health," making it hard to ensure that cells grown in SFM are equivalent to those cultivated with FBS. Additionally, the absence of animal components in cell culture media can affect the regulatory approval process for cell-based therapies, necessitating comprehensive safety and efficacy studies to meet stringent regulatory requirements.[12] Addressing these knowledge gaps and developing robust methods for evaluating cell viability and functionality will be crucial for the successful adoption of SFM in bioprocessing and therapeutic applications.

To address these challenges, future research should focus on optimizing and expanding the application of the ACF medium developed in this thesis, along with other successful SFMs that are either available commercially or through open-source research. One avenue is to refine the formulation to support a wider range of cell types, particularly those that are more challenging to culture, such as primary cells and induced pluripotent stem cells (iPSCs). This could involve identifying growth factors that are easier to source and more cost-effective, potentially lowering the overall cost of SFM. Companies like Future Fields are presently working on these challenges. This Canadian company in particular is producing recombinant proteins using fruit flies to address the economic and ethical issues typically associated with standard recombinant protein production. Additionally, emerging technologies, such as the production of self-sufficient modified cells capable of generating cultured tissue without the need for exogenous growth hormones, offer exciting potential to further reduce costs and improve the efficiency of SFM-based culture systems in the production of cultured meat and other biotechnological applications.[13] Further to those obstacles, investigations into the long-term effects of SFM on cell behavior and genetic stability are essential to ensure the reliability, functionality, and safety of this approach for various biomedical applications. For instance, studies could explore the epigenetic changes that may occur in cells cultured in SFM over extended periods, which could have implications for their use in regenerative medicine.

Future studies should also explore the integration of our ACF medium with advanced 3D culture systems, including microcarriers and bioreactors, aiming to replicate the in vivo environment more closely. Tan et al. (2015) mentions that SFM requirements differ between microcarrier and monolayer cell cultures, and that optimization is required for larger scale microcarrier cultures.[14] Such work can be combined with advancements in bioreactor technology to fine-tune parameters for SFM in large-scale applications. By systematically optimizing factors like agitation speed, oxygen transfer rates, and nutrient supply, researchers can create more reliable

and scalable systems that closely mimic the physiological conditions found in vivo. Additionally, future research could involve developing co-culture systems that mimic tissue complexity and investigating the interactions between different cell types within these systems. Recent advancements in 3D culture systems, such as organ-on-a-chip technologies, offer promising platforms for enhancing tissue engineering applications by providing more physiologically relevant conditions.[15,16] The incorporation of microfluidic devices could allow for more precise control over the culture environment, leading to improved tissue models and potentially reducing the reliance on animal models in preclinical testing.

Another promising avenue for future research involves leveraging computational modeling and machine learning to identify the most ideal SFM formulations for different cell types. Machine learning algorithms can analyze vast datasets of cell culture conditions and conclusions to detect patterns and predict optimal media compositions. By integrating AI with high-throughput screening techniques, researchers can rapidly test numerous media components and experimental outcomes. Such work is already being pioneered by Cosenza et al., whose group is using a truncated genetic algorithm to store and model prior knowledge to predict optimal media conditions.[17,18] This approach could be expanded to include a broader range of cell types and culture conditions, potentially accelerating the development of more effective SFMs. Recently, the Alberta Machine Intelligence Institute (AMII) has partnered with New Harvest (a research organization that is dedicated to the field of cellular agriculture) to create a fellowship centered around combining the power of artificial intelligence and machine learning to solve common problems within the field such as designing and optimizing serum-free media formulations, establishing cell lines, analyzing microscopy images, and optimization bioprocesses involved in large-scale production.[19] An overview of a machine learning approach for analyzing biological data is presented in Figure 5.1.

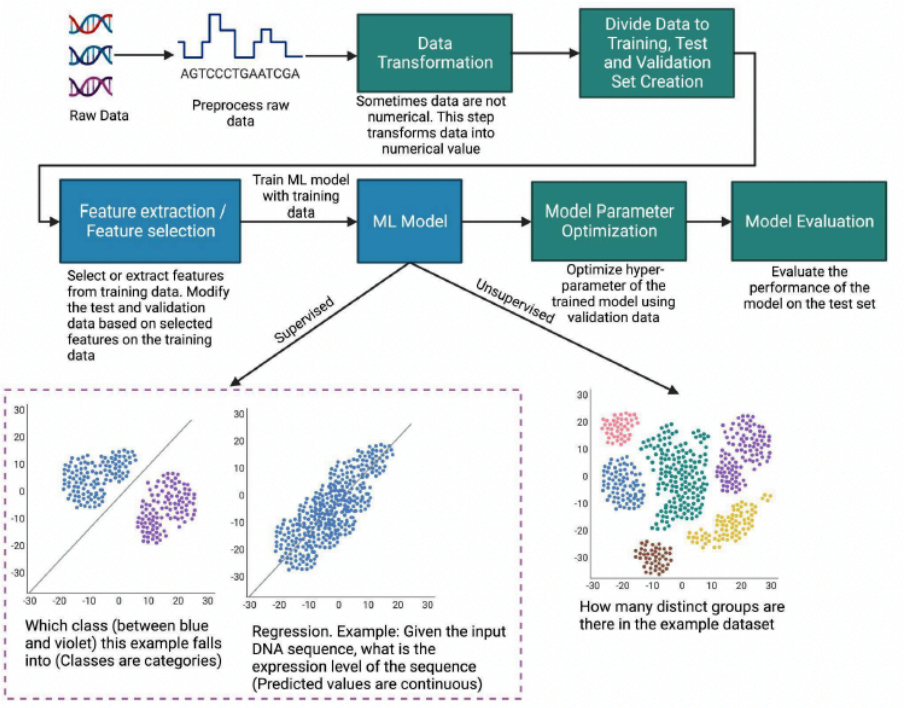

Figure 5.1: Machine learning workflow for biological data analysis.
Overview of a machine learning (ML) approach for analyzing biological data. Raw biological data, such as DNA sequences, are first processed and converted into a numerical format. This data is then divided into three sets: training, validation, and testing. Important features are selected to focus on the most relevant biological information. The ML model is trained to recognize patterns or make predictions using the training data, and its parameters are fine-tuned based on the validation data to improve accuracy. The model's ability to make accurate predictions is then evaluated using the test data. The figure illustrates different ML tasks: supervised learning for predicting outcomes or classifying data into categories, and unsupervised learning for discovering natural groupings within the data. *This figure is reproduced from Todhunter et al.'s 2024 review.*[19]

Other cutting-edge technologies, such as CRISPR for precise genetic modifications, in tandem with high-resolution imaging techniques, can be used in future studies to provide deeper insights into the molecular mechanisms underlying cell growth and differentiation in serum-free conditions.[20] These technologies can help elucidate how the absence of animal serum influences cellular pathways and gene expression, thereby guiding the development of more effective and tailored SFM formulations. For instance, CRISPR could be used to knock out specific genes to study their role in cell survival and proliferation in SFM, offering insights that could lead to the creation of more targeted media formulations.

In conclusion, this thesis has laid a solid foundation for the development of ACF media that can potentially replace FBS for select cell types. The continued refinement and expansion of this technology holds great promise for advancing cell culture practices, reducing reliance on animal products, and ultimately contributing to more ethical and sustainable biomedical research. By addressing the challenges and harnessing recent technological advancements, the field can move towards more consistent and regulatory-compliant cell culture practices that better replicate the in vivo environment, improve the predictability and efficacy of cell-based therapies, and optimize the use of SFM in cellular agriculture and other industries that require animal-free culture solutions. The potential synergies between our SFM and emerging technologies, such as organ-on-a-chip and AI-driven media design, could lead to transformative advancements in cell culture and tissue engineering, paving the way for more reliable and scalable applications in both research and industry.

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Appendix

Gene Name Abbreviation	Gene Name	NCBI Gene ID	Fold change (non-coated ACF), p-value, special note	Fold change (ACF-gelatin), p-value, special note	Fold change (ACF-soy), p-value, special note
Abl1	c-abl oncogene 1, non-receptor tyrosine kinase	11350	0.47 P=0.0529	0.69 P=0.1665	0.58 P=0.0896
Cdc6	cell division cycle 6	23834	0.30 P=0.0136	0.61 P=0.0890	0.35 P=0.0194
Cdkn2b	cyclin-dependent kinase inhibitor 2B	12579	1.20 P=0.1310 B	2.92 P=0.0010 B	2.46 P=0.0017 B
Rad17	RAD17 checkpoint clamp loader component	19356	1.10 P=0.8512	1.59 P=0.1514	1.49 P=0.1583
Aifm1	apoptosis-inducing factor, mitochondrion-associated 1	26926	0.63 P=0.1190	0.54 P=0.0666	0.52 P=0.0595
Casp7	caspase 7	12369	0.95 P=0.7077	2.27 P=0.0027	1.76 P=0.0159
Mcl1	myeloid cell leukemia sequence 1	17210	0.46 P=0.0082	0.84 P=0.2096	0.59 P=0.0196
Xiap	X-linked inhibitor of apoptosis	11798	0.32 P=0.0095	0.39 P=0.0143	0.35 P=0.0111
Ctnnb1	catenin (cadherin associated protein), beta 1	12387	0.71 P=0.0302	0.7 P=0.0429	0.62 P=0.0150
Itgb4	integrin beta 4	192897	0.78 P=0.0859	1.5 P=0.0316	1.25 P=0.1048
Thbs3	thrombospondin 3	21827	0.38 P=0.0062	0.32 P=0.0003	0.33 P=0.0002
Hsp90ab1	Melanocortin 1 receptor		0.41 P=0.0008	0.53 P=0.0020	0.46 P=0.0011
Atr	ataxia telangiectasia and Rad3 related	245000	0.76 P=0.1776	0.33 P=0.0058	0.42 P=0.0011
Cdc7	cell division cycle 7	12545	0.43 P=0.0297	0.41 P=0.0263	0.32 P=0.0170

Cdkn3	cyclin-dependent kinase inhibitor 3	72391	0.34 P=0.00003	0.5 P=0.0007	0.32 0.00002
Rb1	retinoblastoma 1	19645	1.02 P=0.9724	0.74 P=0.1028	0.86 P=0.2926
Akt1	thymoma viral proto-oncogene 1	11651	0.46 P=0.0027	0.47 P=0.0027	0.54 P=0.0051
Casp8	caspase 8	12370	0.88 P=0.0470	0.9 P=0.1244	0.76 P=0.0298
Naip1	NLR family, apoptosis inhibitory protein 1	17940	1.2 P=0.1310 B	2.92 P=0.0010 B	2.46 P=0.0017 B
Adamts5	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)	23794	1.46 P=0.0122	0.79 P=0.0342	1.55 P=0.0022
Fbln1	fibulin 1	14114	0.07 P=0.0001	0.41 P=0.0007	0.13 P=0.0001
Lama1	laminin, alpha 1	16772	1.20 P=0.1310 B	2.92 P=0.0010 B	3.2 P=0.0252
Tgfbi	transforming growth factor, beta induced	21810	0.22 P=0.00002	0.07 P=0.00001	0.13 P=0.00002
MGDC	Mouse genomic DNA control	N/A	N/A	N/A	N/A
Aurka	aurora kinase A	20878	0.42 P=0.0242	0.51 P=0.0464	0.35 P=0.0172
Cdk1	cyclin-dependent kinase 1	12534	8.34 P=0.6282	9.79 P=0.7914	7.00 P=0.04964
Chek1	checkpoint kinase 1	12649	0.44 P=0.0049	0.57 P=0.0118	0.45 P=0.0050
Rbl1	retinoblastoma-like 1 (p107)	19650	0.36 P=0.0006	0.37 P=0.0006	0.29 P=0.0005
Bad	BCL2-associated agonist of cell death	12015	0.7 P=0.0025	0.66 P=0.0004	0.69 P=0.0002
Dapk1	death associated protein kinase 1	69635	5.06 P=0.0001	6.62 P=0.0001	6.37 P=0.00003

Nfkb1	nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105	18033	0.61 P=0.0025	0.74 P=0.0080	0.58 P=0.0003
Ccn2	connective tissue growth factor	14219	0.27 P=0.0008	0.32 P=0.0013	0.24 P=0.0007
Fn1	fibronectin 1	14268	0.45 P=0.0011	0.12 P=0.0001	0.31 P=0.0004
Lama2	laminin, alpha 2	16773	13.86 P=0.000002	2.26 P=0.0490	7.00 P=0.0032
Vcan	versican	13003	0.73 P=0.0089	0.28 P=0.0002	0.67 P=0.0056
RTC	Reverse Transcription Control	N/A	N/A	N/A	N/A
Bcl2	B cell leukemia/lymphoma 2	12043	1.51 P=0.0492	1.10 P=0.6465	1.65 P=0.0205
Cdk2	cyclin-dependent kinase 2	12566	0.40 P=0.0724	0.49 P=0.1027	0.41 P=0.0750
Ddit3	DNA-damage inducible transcript 3	13198	1.38 P=0.1427	2.20 P=0.0076	2.08 P=0.0020
Shc1	src homology 2 domain-containing transforming protein C1	20416	0.71 P=0.0086	0.87 P=0.0911	0.80 P=0.0666
Bax	BCL2-associated X protein	12028	1.48 P=0.0067	2.00 P=0.0911	2.14 P=0.067
Diablo	diablo, IAP-binding mitochondrial protein	66593	0.64 P=0.0019	0.69 P=0.0082	0.78 P=0.0197
Nol3	nucleolar protein 3 (apoptosis repressor with CARD domain)	78688	1.15 P=0.5931 A	1.28 P=0.2333 A	1.10 P=0.6835 A
Cdh1	cadherin 1	12550	1.40 P=0.1760 A	2.92 P=0.0010 B	2.46 P=0.0017 B
Itga2	integrin alpha 2	16398	1.2 P=0.1310 B	3.90 P=0.0008	2.46 P=0.0017 B

Lamb2	laminin, beta 2	16779	0.84 P=0.0295	0.91 P=0.3842	0.77 P=0.0398
Vtn	vitronectin	22370	0.86 P=0.5341 A	1.09 P=0.8141 A	0.97 P=0.8387 A
RTC	Reverse Transcription Control	N/A	N/A	N/A	N/A
Birc5	baculoviral IAP repeat-containing 5	11799	0.39 P=0.0057	0.60 P=0.0234	0.32 P=0.0037
Cdk6	cyclin-dependent kinase 6	12571	0.95 P=0.6706	1.27 P=0.1614	1.16 P=0.3650
Dst	dystonin	13518	1.79 P=0.225	2.30 P=0.0057	2.67 P=0.0012
Skp2	S-phase kinase-associated protein 2 (p45)	27401	0.57 P=0.0036	0.48 P=0.0014	0.47 P=0.0014
Bcl2l1	BCL2-like 1	12048	0.90 P=0.4653	1.28 P=0.1705	1.26 P=0.1930
Fas	nan	nan	2.52 P=0.0022	12.18 P=0.00002	7.59 P=0.0048
Pycard	PYD and CARD domain containing	66824	1.85 P=0.0096	6.21 P=0.0005	6.60 P=0.0004
Col1a1	collagen, type I, alpha 1	12842	1.31 P=0.0088	0.82 P=0.0709	1.10 P=0.2318
Itga4	integrin alpha 4	16401	1.20 P=0.1310 B	2.92 P=0.0010 B	2.46 P=0.0017 B
Lamc1	laminin, gamma 1	226519	0.77 P=0.0635	0.40 P=0.0016	0.52 P=0.0038
Actb	actin, beta	11461	0.30 P=0.00001	0.37 P=0.0002	0.28 P=0.0001
RTC	Reverse Transcription Control	N/A	N/A	N/A	N/A
Casp3	caspase 3	12367	1.02 P=0.9093	1.24 P=0.0958	1.14 P=0.2070
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	12575	0.70 P=0.3321	1.28 P=0.6869	1.20 P=0.8115

Itgb1	integrin beta 1 (fibronectin receptor beta)	16412	0.66 P=0.1863	0.58 P=0.1139	0.74 P=0.2562
Smc1a	structural maintenance of chromosomes 1A	24061	0.31 P=0.0008	0.25 P=0.0006	0.22 P=0.0005
Bid	BH3 interacting domain death agonist	12122	0.66 P=0.0759	0.67 P=0.0834	0.76 P=0.1716
Igf1r	insulin-like growth factor I receptor	16001	0.61 P=0.0140	0.63 P=0.0237	0.59 P=0.0153
Ripk1	receptor (TNFRSF)-interacting serine-threonine kinase 1	19766	0.80 P=0.0483	0.59 P=0.0129	0.58 P=0.0070
Col4a1	collagen, type IV, alpha 1	12826	1.32 P=0.0065	0.94 P=0.1874	1.06 P=0.3228
Itga5	integrin alpha 5 (fibronectin receptor alpha)	16402	0.32 P=0.0040	0.28 P=0.0033	0.30 P=0.0036
Selp	selectin, platelet	20344	0.10 P=0.0004	0.24 P=0.0008	0.21 P=0.0007
B2m	beta-2 microglobulin	12010	1.27 P=0.0018	0.89 P=0.0544	1.12 P=0.0525
PPC	Positive PCR Control	N/A	N/A	N/A	N/A
Ccna2	cyclin A2	12428	0.33 P=0.0077	0.35 P=0087	0.19 P=0.0040
Cdkn1b	cyclin-dependent kinase inhibitor 1B	12576	0.58 P=0.1778	0.61 P=0.1952	0.65 P=0.2294
Mcm2	minichromosome maintenance complex component 2	17216	0.35 P=0.0006	0.26 P=0.0002	0.23 P=0.0002
Trp53	transformation related protein 53	22059	0.86 P=0.0173	1.02 P=0.7390	1.08 P=0.2704
Birc2	baculoviral IAP repeat-containing 2	11797	0.66 P=0.0021	1.61 P=0.0012	1.44 P=0.0030
Il10	interleukin 10	16153	1.20 P=0.1310 B	28.21 P=0.0067	12.12 0.0001
Traf2	TNF receptor-associated factor 2	22030	0.88 P=0.1782	1.17 P=0.1209	1.10 P=0.3597

Col5a1	collagen, type V, alpha 1	12831	0.86 P=0.1383	0.18 P=0.0005	0.28 P=0.0007
Itgav	integrin alpha V	16410	0.81 P=0.1390	0.81 P=0.1297	1.12 P=0.3565
Sgce	sarcoglycan, epsilon	20392	3.23 P=0.0122	2.92 P=0.0010 B	2.46 P=0.0017 B
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	14433	0.56 P=0.00001	0.41 P=0.00003	0.31 P=0.00001
PPC	Positive PCR Control	N/A	N/A	N/A	N/A
Ccne1	cyclin E1	12447	1.02 P=0.8925	1.21 P=0.2207	1.06 P=0.6413
Cdkn2a	cyclin-dependent kinase inhibitor 2A	12578	1.20 P=0.1310 B	2.92 P=0.0010 B	2.46 P=0.0017 B
Notch2	nan	nan	0.63 P=0.1289	0.92 P=0.6166	0.68 P=0.1613
Wee1	WEE 1 homolog 1 (S. pombe)	22390	0.46 P=0.0044	0.42 P=0.0037	0.47 P=0.0054
Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	12177	0.39 P=0.0006	0.25 P=0.0003	0.24 P=0.0003
Mapk1	mitogen-activated protein kinase 1	26413	0.73 P=0.0046	0.78 P=0.0096	0.81 P=0.0426
Trp53bp2	transformation related protein 53 binding protein 2	209456	0.77 P=0.0026	0.81 P=0.0013	0.88 P=0.0038
Ctnna1	catenin (cadherin associated protein), alpha 1	12385	0.41 P=0.0009	0.42 P=0.0014	0.41 P=0.0008
Itgb3	integrin beta 3 binding protein (beta3-endonexin)	67733	3.98 P=0.00003	6.26 P=0.0006	6.47 P=0.0003
Sparc	secreted acidic cysteine rich glycoprotein	20692	0.82 P=0.0018	0.42 P=0.0003	0.41 P=0.00007
Gusb	glucuronidase, beta	110006	0.75 P=0.0035	1.17 P=0.0433	0.88 P=0.0656
PPC	Positive PCR Control	N/A	N/A	N/A	N/A

Table A.1: Gene panel used in RT2 PCR Profiler Array.

Entire panel was purchased in a kit from Qiagen. Fold-Change ($2^{-\Delta\Delta C_T}$) is the normalized gene expression ($2^{-\Delta C_T}$) in the Test Sample (ACF conditions) divided by the normalized gene expression ($2^{-\Delta C_T}$) in the Control Sample (FBS-grown cells). Special notes can be given either *A* or *B*. *A* signifies that the gene's average threshold cycle is relatively high (>30), so its relative expression level is low in both control and test samples, and the p-value for the fold change is either unavailable or quite high (P>0.05). *B* means that this gene's average threshold cycle is either not determined or greater than the defined cut-off (C_T>35), meaning that the expression was undetected and therefore results are un-interpretable.