Investigations into Serum Components Which Support Proliferation in Culture: The Crucial Role of Albumin-Associated Lipids

Oliver Newsom

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Reading Committee:

Lucas Sullivan, Chair

Patrick Paddison

Akhila Rajan

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Abstract

Investigations into Serum Components Which Support Proliferation in Culture: The Crucial Role of Albumin-Associated Lipids

Oliver Newsom

Chair of the Supervisory Committee:
Lucas Sullivan
Department of Human Biology

Serum is a ubiquitous additive to culture media despite its complex and heterogeneous composition, which can affect experimental reproducibility. Development of a defined serum-free culture medium would improve experimental consistency; however, the environmental requirements for proliferation which are provided by serum remain unclear. To determine how serum supports growth of cells in culture, I used a combination of live-cell imaging and liquid chromatography-mass spectrometry (LC-MS) to investigate serum components that enable sustained proliferation in culture. Importantly, the primary serum components that enable proliferation were identified as trace metals and serum lipids. Moreover, despite access to a broad range of lipid species, albumin-bound lipids were selectively depleted from media during cell proliferation, and a combination of these lipid classes with essential soluble serum components was sufficient to support cell proliferation in the absence of serum. Utilizing the serum-free media as a tool to investigate variables which influence consumption, I found that lipid scavenging occurs through a mass action mechanism, independent of FA structure and non-competitive with other albumin-associated lipid classes. The critical role of lipid scavenging in sustaining cell proliferation in culture was further supported by the finding that impairments to lipid synthesis had minimal effects on proliferation, while disruption of exogenous FA utilization effectively reduced cell proliferation in culture. These results advance our knowledge of serum components that support proliferation in culture, particularly the role of environmental lipids, and provide a serum-free culture medium that may be applied to various cell lines.

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List of Acronyms

Acronym	
ACAC	Acetyl-CoA Carboxylase
ACS	Acyl-CoA Synthetase
ACSL	Acyl-CoA Synthetase Long-chain Family Member
ACSL1	Acyl-CoA Synthetase Long-chain Family Member 1
ACSL3	Acyl-CoA Synthetase Long-chain Family Member 3
ACSL4	Acyl-CoA Synthetase Long-chain Family Member 4
ACSL5	Acyl-CoA Synthetase Long-chain Family Member 5
ACSL6	Acyl-CoA Synthetase Long-chain Family Member 6
ACSBG	Acyl-CoA Synthetase Bubblegum Family Member
B5 (PANTOTHENATE)	D-Pantothenate
BSA	Bovine Serum Albumin
BMIPP	123I-beta-methyl-p-iodophenyl Pentadecanoic Acid
BODIPY	A type of fluorescent dye used to label fatty acids
CD36	Cluster of Differentiation 36
CHOP	C/EBP Homologous Protein
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats associated Cas9
DMEM	Dulbecco's Modified Eagle's Medium
ESCs	Embryonic Stem Cells
FA	Fatty Acid
FASN	Fatty Acid Synthase
FAT	Fatty Acid Translocase
FATP	Fatty acid transport protein
FBS	Fetal Bovine Serum
GSK	Abbreviation for GSK2194069
GPx	Glutathione Peroxidase
GPx4	Glutathione Peroxidase 4
HDL	High-Density Lipoprotein
H1299_NucRFP	H1299: human non-small cell lung cancer cell line; NucRFP: Nuclear Red Fluorescent Protein
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLM	Human Plasma Like Media
IDL	Intermediate-Density Lipoprotein
ITS	Insulin, Transferrin, and Selenium

Acronym	
iPSCs	Induced Pluripotent Stem Cells
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LC-MS	Liquid Chromatography-Mass Spectrometry
LPC	Lyso-Phosphatidylcholine
LPE	Lyso-Phosphatidylethanolamine
MFSD2a	Major Facilitator Superfamily Domain-Containing Protein 2a
MeOH	Methanol
MUFA	Monounsaturated Fatty Acids
PBS	Phosphate Buffered Saline
PLA2	Phospholipase A2
PUFA	Polyunsaturated Fatty Acids
SCD1	Stearoyl-CoA Desaturase 1
SREBP	Sterol Regulatory Element-Binding Protein
SOD	Superoxide Dismutase
SPT	Serine Palmitoyltransferase
SL	Sphingolipids
SM	Sphingomyelin
TAG	Triacylglycerides
Triacsin C	A specific inhibitor of the acyl-CoA synthetase long-chain (ACSL) family
UPR	Unfolded Protein Response
U-13C glucose	Uniformly labeled Carbon-13 glucose
VLDL	Very-Low Density Lipoprotein

Foreword

The development and use of cell culture has been indispensable to the advancement of the science of medicine. Cell culture aims to model cell behavior and phenotypes that occur in vivo, within a controlled system that can be more easily manipulated to investigate cell physiology. As culture methodologies and technologies improve, the influence of the cellular environment on cell behavior has become increasingly appreciated. Thus, while cell culture has significantly advanced our understanding of many biological phenotypes and has been critical to development of biological manufacturing, many cell culture applications still rely on antiquated resources like the use of animal serum for sustained cell growth. In particular, fetal bovine serum (FBS) is the most common additive, containing a high abundance of growth factors, nutrients, and a heterogeneous mix biologically active components that are incompletely defined. Moreover, natural animal products exhibit inherent compositional variability. In addition to masking cellular phenotypes through undefined serum components, this variability also hinders experimental consistency and reproducibility. Thus, there has been a reemergence of efforts to replace FBS in cell culture with a chemically defined medium which supports proliferation across a broad range of cell lines. Importantly, development of such a media requires understanding of the essential components in FBS that support sustained growth in culture. Thus, as detailed in this thesis, my research aimed to identify the essential serum components that support proliferation, with an emphasis on serum-derived lipids. Moreover, I systematically tested variables involved in exogenous lipid utilization to

determine the role of serum lipids in supporting cell proliferation and to elucidate the requirement of environmental lipids in culture media. These findings have implications for the development of serum-free culture media. Additionally, understanding the essential role of scavenged lipids and the patterns of lipid scavenging suggest novel therapeutic approaches to target these pathways in cancer and that targeting exogenous lipid utilization may be more efficacious than inhibiting de novo lipid synthesis directly.

1. Chapter 1

Section 1.13, titled "Environment influences gene dependency and cell phenotypes." contains Figure from a manuscript published in Cell Metabolism [1]. The authors and their affiliations are listed below:

Authors: Oliver J. Newsom[1], Lucas B. Sullivan[1*]

Affiliations: [1]Human Biology Division, Fred Hutchinson Cancer Center, Seattle, WA, 98109, USA. *Correspondence to: lucas@fredhutch.org.

All conceptualization, writing, and editing were performed by me and Dr. Lucas Sullivan

1.1. Overview of cell culture in biomedical research

1.1.1. Origins of cell culture

Cell culture is a mainstay in modern scientific research and its development revolutionized the fields of science and medicine. The origins of cell culture date back to the late 19th century, as biologists began exploring methods to study cell biology outside of the body with greater control [2]. In 1882 Sydney Ringer developed Ringer's solution- one of the first saline solutions- containing a simple mixture of sodium, potassium, calcium, and magnesium. Ringer's solution could maintain the contractility of frog heart explants for physiologic studies outside the body [3]. Shortly thereafter, in 1885, the embryologist William Roux successfully maintained sections of the medulla from a chicken embryo in a warm saline solution for days [2]. The early successes of Ringer and Roux were important for motivating others to explore methods to cultivate cells outside the body.

Building upon these foundations, in 1907 Ross Harrison, who is considered by many to be the grandfather of modern cell culture, developed the hanging drop method to cultivate frog embryonic nervous tissue. In these experiments neurons derived from the medullary tube and ectoderm from frog embryos were cultured in a coagulated lymph solution collected from the frog lymph sac [4]. Over multiple days and weeks, Harrison observed nerve cell projections growing from the cells in the clotted media, providing important evidence supporting the neuron doctrine hypothesis, which argues that the nervous system is made of discrete cells rather than a fused network [5], [6]. These findings further demonstrated the utility and applications of ex vivo cultivation of cells for study.

Shortly thereafter, Alexis Carrel and his colleague Montrose Burrows further refined culture methods to permit the cultivation of chicken fibroblast explants [7]. This work ultimately led to a series of studies which resulted in multiple important advances in cell culture. First, they concluded that blood serum was superior to lymph solution in cultivation of warm-blooded animal cells [8]. Secondly, they determined that repeated washes and regular media changes improved the health of cultures and extended their lifespan [9], [10], [11]. Lastly, they invented a prototype of the modern cell culture flask, which provided an alternative to the hanging drop method and enabled effective 2D expansion of cells in vitro [11]. In addition to the improvement of aseptic techniques, these innovations enabled Carrel and Burrows to maintain various adult and embryonic tissues- including those from dogs, cats, humans, and rats- in culture outside the body. Carrel, who was eventually awarded the Nobel Prize in Physiology or Medicine for his work on vascular surgery and transplantation, also demonstrated that cells in culture undergo a wound-healing process similar to that of cells in vivo. Together, these early experiments expanded the use of cell culture in science and medicine.

Although the foundational work in cell culture development is primarily attributed to Harrison, Carrel, and Burrows, it was later acknowledged that Margaret Reed also made important contributions to the field. Indeed, in 1908 Reed successfully cultivated guinea pig bone marrow tissue in a tube of nutrient-rich agar, representing the first in vitro cultivation of mammalian cells [2]. Similar to Carrel and Burrows, Reed continued to improve cell culture methodologies by exploring the requirements for cells in culture. She found that a modified Locke's salt solution containing glucose, amino acids, and 15%

chicken bouillon was more effective for cell growth than a balanced saline solution alone, and that glucose was essential for maintaining cell viability [12]. These experiments also represent some of the first attempts to find alternatives to serum use in media in an effort to develop a more standardized solution for the cultivation of cells[13].

Together, these studies were foundational in the improvement of tissue culture and the development of modern techniques used today. They demonstrated that cells from various organisms could be cultivated in vitro when maintained in the appropriate media conditions. Indeed, Carrel and Burrows' discovery that serum was effective in supporting proliferation across many cell types was influential in establishing serum as a component of modern culture media. Reed's efforts to find critical culture media requirements were among the first attempts to reduce or eliminate the use of animal-derived components in media.

1.1.2. **The development and expansion of cell lines**

Early cell culture experiments were reliant on primary cell lines, which were obtained directly from core biopsies, fine-needle aspirates, tissue resections, or autopsy specimens for ex vivo cultivation. Importantly, these primary cell lines lasted for days to weeks before proliferation eventually slowed and stopped altogether and collection of new tissue was required. However, in 1943 Wilton R. Earle successfully established the first immortalized cell line from mouse fibroblasts, termed L-cells, which could be maintained in culture indefinitely [14]. Soon thereafter, George O. Gey developed a cervical cancer cell line that was maintained in culture for over a year in a mix of chicken plasma, bovine embryo extract, and human placental cord serum [15]. This cell line, which became known as HeLa cells, represented the first immortalized human cell line and in addition to L-cells,

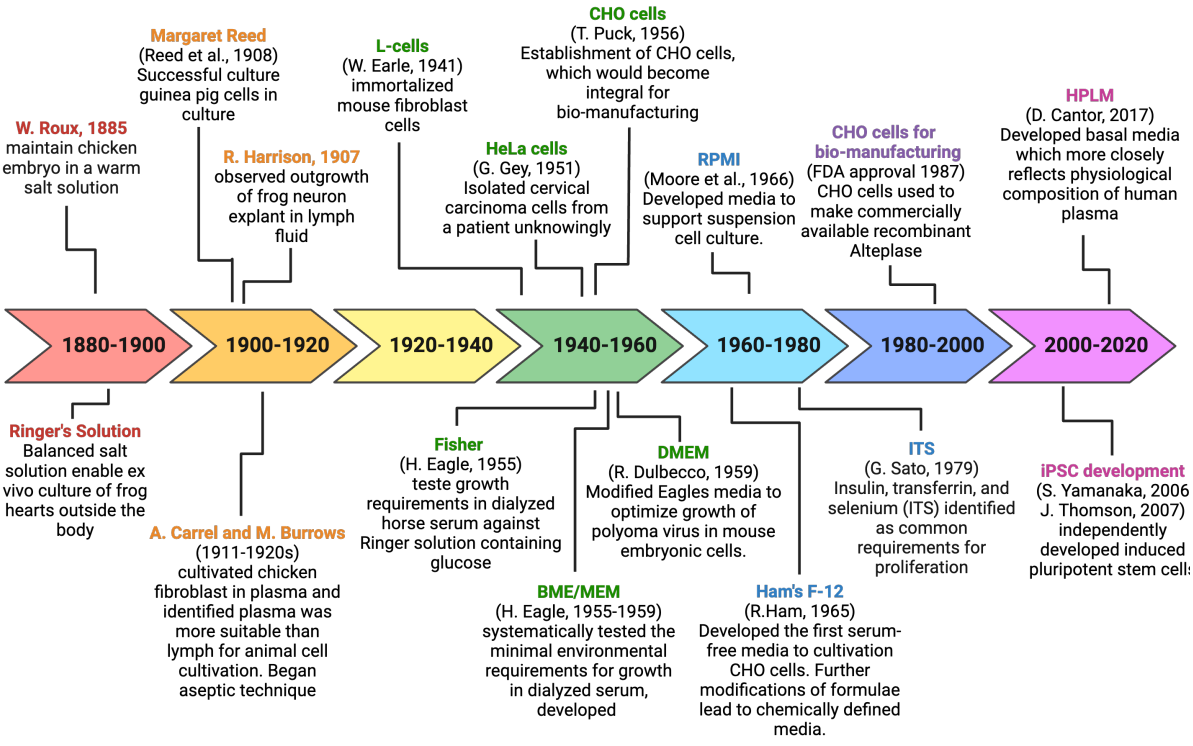

Figure 1.1: History of cell culture and culture media. Timeline of important advancements and milestones in the development of culture media and establishment of cell lines.

revolutionized modern cell culture by enabling widespread access to immortalized cell lines for studying cell biology.

Despite the enormous scientific contribution of HeLa cells, they were collected unknowingly from Henrietta Lacks, a cancer patient at Johns Hopkins, raising questions about patient privacy, ownership, and informed consent in science and medicine [16]. Over the years, scientists increasingly recognize the responsibility of ethically conducting research. Nonetheless, HeLa cells have been indispensable for major scientific advances, including vaccine development, exploration of cancer metabolism, drug development, and the study of radiation's effects on human cells [17], [18], [19], [20], [21], [22] .

The continued establishment of immortalized cell lines further spurred scientific advances as their application to science and medicine grew. Notably, the establishment of Chinese Hamster Ovary (CHO) cells by Theodore Puck in 1957 was another important milestone in cell culture [23]. CHO cells are rare in their capacity to survive in either adherent or suspension cultures and their ability to produce substantial amounts of protein have made them valuable for biomanufacturing purposes [24], [25]. Moreover, since few zoonotic pathogens share hamsters and humans as a vector, CHO cells pose minimal risk for disease transmission to humans, making them well suited for production of biologics destined for human use and consumption [26]. Importantly, as will be discussed later, the use of CHO cells for biologics production requires unique culture media conditions that lack animal products to further minimize the risk of pathogen transfer to humans [27].

Most lab cultivated cell lines grow as adherent cells in two-dimensional (2D) monolayers on the bottom of culture flasks or dishes. However, in the mid-1970s an acute lymphoblastic leukemia (ALL) cell line derived from a 14-year-old boy was established, termed Jurkat cells [28]. Unlike adherent cells, Jurkat cells grow exclusively in suspension, and their widespread use has been instrumental in advancing our understanding normal T-cell biology, leading to critical discoveries in T-cell receptor physiology [29], [30]. Additionally, the relative ease of suspension cell maintenance also serves as a useful system for high throughput applications like genetic screens [31]. Indeed, both Jurkat and Hela cells exemplify the utility of immortalized cancer cell lines for understanding transformed cell biology and have additionally led to improved understanding of non-transformed cells.

As the scientific fields continued to progress, and the applications of cell culture broadened, so did the efforts to model different developmental and disease processes more accurately using cell culture. In 2006, Shinya Yamanaka demonstrated that adult mouse cells in vitro could be reprogrammed back into a pluripotent state with only four transcription factors, termed Yamanaka factors, generating induced pluripotent stem cells (iPSCs) [32]. Only a year later James Thomson- who was also responsible for the derivation of human embryonic stem cells (ESCs)- independently produced human iPSCs from adult fibroblast [33], [34]. Together, these discoveries helped advance personalized medicine, regenerative medicine, and disease modeling, further broadening the applications of cell culture technologies [34]. Collectively, the development of the various cell lines and

refinement to cell culture techniques has been paramount in advancing our understanding of human health and disease, ultimately leading to significant improvements in medicine.

1.1.3. Environment influences gene dependency and cell phenotypes.

One of the primary observations that has emerged from studying cells in culture and in vivo is how environmental composition affects cells health and proliferation [35], [36], [37]. In vivo, the local environment of the tissue shapes the nutrient composition and availability to cells. The field of cancer research has been paramount to understanding the interplay between environmental composition and cell phenotype, particularly through studies investigating the effects of the tumor microenvironment on cancer cell fitness [38], [39]. As methodologies to study cell physiology improve, the role the extracellular environment in determining cell behavior and gene essentialities has begun to emerge. For instance, when nutrients are limited, cells must rely on endogenous synthesis pathways to compensate for environmental scarcity; conversely, when the microenvironment is rich in nutrients, cells are less reliant on endogenous biosynthetic pathways. Understanding how the cellular environment affects cellular behavior is critical for optimizing media formulations that support proliferation of specific cell lines. Moreover, understanding the in vivo environment can improve media formulations to better model those cellular behaviors and phenotypes in culture.

Advances in cellular and molecular analyses have uncovered that environmental nutrient composition can buffer endogenous metabolic impairments, and conversely, that exogenous nutrient availability can determine gene essentiality [39]. For example, genetic

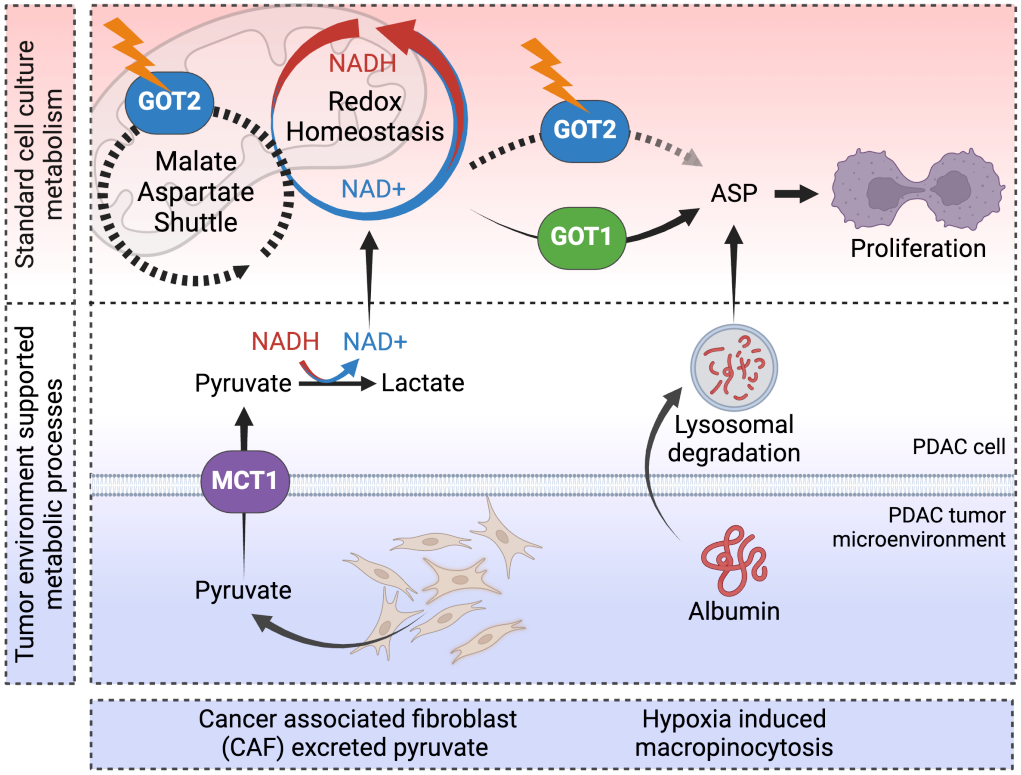

Figure 1.2: Environmental nutrients buffer endogenous metabolism. GOT2 loss stalls the malate aspartate shuttle leading to an imbalance in NAD+/NADH homeostasis and thereby impairing endogenous synthesis of aspartate (ASP) by GOT1 or GOT2 in PDAC cells. PDAC interactions with the tumor microenvironment can bypass the metabolic limitations of GOT2 deficiency that otherwise occur in standard cell culture conditions. Cancer associated fibroblasts (CAFs) can secrete pyruvate which, when scavenged through MCT1 and converted to lactate, can restore the NAD+/NADH ratio in the absence of the malate aspartate shuttle activity in GOT2-deficient PDAC cells, allowing for ASP synthesis through GOT1. Additionally, hypoxia-induced macropinocytosis and lysosomal degradation of extracellular proteins provides a direct source of aspartate to bypass the effects of aspartate synthesis disruption. In both cases, the tumor environment supports aspartate acquisition pathways to enable PDAC tumor cell proliferation in the absence of GOT2 function.

disruption of glutamic-oxaloacetic transaminase 2 (GOT2)- one of the two enzymes responsible for aspartate synthesis- results in stalled proliferation by disrupting the malate-aspartate shuttle and impairing redox balance (Figure 1.2) [1]. Environmental factors, such as pyruvate and/or the presence of albumin, enables cells to circumvent

intrinsic metabolic limitations by (1) restoring the NAD+NADH ratio, or (2) providing cells with a direct source of aspartate from catabolized albumin.

Other studies showed that cells grown in culture media containing physiologically relevant amounts of uric acid exhibit decreased sensitivity to 5-floururacil (5-FU). The mechanism of resistance was identified as uric acid-mediated inhibition of uridine 5' monophosphate synthase (UMPS), an enzyme essential for intracellular activation of 5-FU [40]. These findings, observed in vitro, may explain why patients treated with 5-FU for cancer therapy require larger doses than predicted for cytotoxic effects. Together, these studies exemplify the importance of the cellular environment composition, showing how it can dictate gene essentiality and cellular behavior. Furthermore, these studies underscore that understanding environmental composition is crucial for interpreting experimental results both in vivo and in culture.

Rewiring of cell metabolism is a hallmarks of the cancer and is key to malignant transformation, sustaining proliferation rates, and enabling invasion and metastasis to distant sites within the body [41]. Metabolic alterations are influenced by local nutrient availability, which is dictated in vivo by the tumor microenvironment and vascular infiltration into the tumor [42], [43]. In contrast, the environmental composition in culture is determined by culture media formulations and other bioactive factors found in serum or other biological additives supplemented in media [44]. However, the undefined and heterogeneous nature of these supplements can mask the relative importance of specific media components to cell phenotypes in culture. Additionally, serum components that may not be present in the tumor microenvironment can buffer cell metabolism, making it

challenging to determine the precise metabolic requirements of cancer cells [45].

Collectively, these observations highlight the central role of exogenous nutrient composition in shaping cellular phenotypes, gene essentiality, and response to both metabolic and pharmacologic perturbations. By uncovering how cell-intrinsic and -extrinsic factors enable cells to circumvent these perturbations, researchers can improve experimental design to better reflect physiological conditions. Additionally, these observations demonstrate culture media formulations significantly influence cell behavior and phenotypes, including the heterogeneous mix of bioactive molecules provided in serum. Ultimately, refining our knowledge of environmental requirements- particularly through the development of chemically defined culture media that support proliferation across a broad range of cells can improve both the reproducibility of in vitro research and reveal required/ancillary environmental components supporting cell growth and potentially be used to improve the effectiveness of cancer therapies in vivo.

1.2. Development of culture media

Natural Medias	
Coagulant/clots, Tissue extracts, Biological fluids	Clotted plasma, blood, or lymph Various tissue extracts, including embryos, liver, spleen, pituitary Plasma, serum, lymph, amniotic fluid, and pleural fluid.
Synthetic Media	
Serum-Containing	Human, bovine, porcine, or other serum is used as a media additive
Serum-Free	Crude serum protein extracts are used as serum supplements
Chemically Defined	No undefined components, including biological fluids/extracts

Table 1.1: Categories of cell culture media and components/additives included

Before the development of immortalized cell lines, cell culture relied on primary cell lines collected from fresh tissue that could be cultivated in vitro for weeks to months [2]. Once immortalized cell lines became commonplace in research, investigators sought to develop standardized media formulations optimized for sustained proliferation. Much of the early work investigating media requirements for proliferating cells was performed in L-cells and HeLa cells [46], [47].

The primary goal of culture media is to support robust growth in vitro while approximating the physiological environment cells experience in vivo, enabling investigations of cell behavior in a controlled and accessible system. In general, culture media are divided into two categories- natural and synthetic- which are discussed further below.

1.2.1. Natural media

Natural culture media were among the first medias used to grow cells, relying entirely on products derived from biological sources such as coagulated clots, tissues extract, or biological fluids (Table 1) [2], [48]. These medias are considered undefined, as their make-up is complex, heterogenous, and many of the components are unknown. While this complexity can sometimes more closely mimic the in vivo environment, it also introduces uncontrolled variability that effects experimental consistency and reproducibility.

1.2.2. Synthetic media

In contrast to natural media, synthetic media are formulated using a defined mix of organic and inorganic compounds [2]. These medias can be further divided into serum-supplemented, serum-free, or chemically defined medias (Table 1):

- Serum-supplemented media is composed of a basal media and includes a small percentage of serum to enhance proliferation, typically ranging from 5-20%. This is the most commonly used synthetic media category because it supports a broad range of cell lines that require serum for sustained growth.
- Serum-free media, by definition, does not contain serum. However, serum-free medias may still include crude protein extracts, albumin, and serum globulin to support growth.

- Chemically defined media are the most defined category of culture medias and exclude any undefined components but may include purified recombinant proteins.

Although more highly defined medias improve experimental consistency and reproducibility, they lack the multitude of bioactive compounds- both known and unknown- found in biological sources. As a result, they are less useful for a broad range of cells. Consequently, no serum free or chemically defined media can currently support culture across most cell lines.

1.2.3. Development of basal media

The foundational efforts to determine the minimal nutritional requirements for cells in culture largely began in the 1950s. It was recognized that despite the required addition of serum to culture media for most cell lines, the complex mix of metabolites, growth factors, and biologically active components made investigation of individual factors challenging. One of the first systematic approaches to investigate the minimal required components for growth was performed by Albert Fischer, who dialyzed horse serum against Ringer's solution containing glucose [49]. This process removes low molecular weight components smaller than 10kDa, including amino acids, metals, and other small soluble serum components, while retaining larger proteins and insoluble components. Chick embryo myoblast cultures grown in dialyzed serum failed to maintain proliferation, ultimately leading to the induction of apoptosis, underscoring the essential role of low molecular weight serum factors. Further investigations uncovered specific amino acids, particularly

cysteine, as essential for myoblast survival [50]. Moreover, while addition of downstream cysteine fates into the media, such as glutathione improved proliferation, Fisher concluded exogenous amino acids fulfill an indispensable role in supporting growth in culture.

Building upon these findings Harry Eagle conducted seminal work to determine the minimum essential media requirements for culture of L-cells and HeLa cells in culture[46], [47]. He observed that certain non-essential amino acids were conditionally essential in culture, reflecting tissue specific metabolism and biosynthetic pathways that are not universally utilized in all cell lines. Eagles work resulted in the development of Basal medium Eagle (BME), which includes 13 amino acids and eight vitamins [46], [47]. Because of its simple composition, BME is not suitable for culturing of many cell lines. Therefore, Minimum Essential Medium (MEM) was developed by modifying BME to include higher concentrations of specific amino acids, six inorganic salts, eight water soluble vitamins, and dialyzed serum [51].

The utility of MEM in supporting a variety of cell lines provided a foundational media which was further modified to improve the growth of select cells lines. Among the many modifications of MEM, Renato Dulbecco developed Dulbecco's Modified Eagle's Medium (DMEM), which significantly increased the concentration of amino acids and vitamins relative to BME and proved especially suitable for studying the plaque forming ability of polyoma virus in mouse embryonic cells [52]. Over time, DMEM has been further modified to include glycine, serine, iron, and pyruvate. Moreover, for rapidly proliferating cells, such

as cancer cells in culture, DMEM modifications often include an increase in the concentration of glucose to 25mM to support the increased metabolic demands.

During this period, the establishment of lymphocytes in culture led to the development of RPMI at the Roswell Park Memorial Institute (RPMI) [53]. RPMI contains additional amino acids lacking in DMEM, such as alanine. Importantly, DMEM and RPMI are not equally effective at supporting growth of cell lines, exemplifying differences in exogenous nutritional requirements exhibited by cells for proliferation [54], [55].

As cell culture methodologies expanded into the commercial and biomanufacturing settings, the reliance on serum addition into culture media posed unique limitations and risks, including potential contamination with zoonotic pathogens. In response, Richard Ham [56] pioneered the formulation of F-10 and subsequently F-12 media. Notably, Ham's F-12 was the first serum-free culture medium that successfully enabled the cultivation of CHO cells by replacing serum with albumin and fetuin, two serum proteins capable of binding a variety metabolites and minerals respectively. Additionally, this media formulation was the first to introduced trace metals like copper and zinc. Nonetheless, most other cell lines still required the addition of serum in this media for optimal growth.

As the push for a fully chemically defined media grew, the formulation of Ham's F-12 was further modified to replace the use of albumin and fetuin with linoleic acid and putrescine, and further adjusted the concentrations of amino acids and vitamins [2]. This formulation was the first truly chemically defined medium and helped pave the way for large-scale biomanufacturing using CHO cells without animal products.

Other serum-free medias have been developed to meet the growth requirements of embryonic stem cells and iPSCs [57]. Typically, these formulations use DMEM or a combination of DMEM and F-12 as a basal media, supplemented with various growth factors, cytokines, and small molecules to maintain cell pluripotency. These different media formulations highlight that many of the components identified early in the exploration of nutritional requirements for proliferation continue to guide the evolution of culture media today.

A recent addition to the various culture media, is the development of human plasma like media (HPLM) in 2017 [40]. HPLM contains many components found in other common basal medias, while its metabolite concentrations have been adjusted to more closely reflect those measured in human plasma. Importantly, the HPLM formulation includes over 60 polar metabolites found in human plasma- like creatine, taurine, and uric acid- to better replicate the in vivo environment [40], [58]. Similar approaches have also been used in the generation of other medias more closely resembling physiological nutrient levels, which have all been found to alter sensitivities to various metabolic and signaling pathways [37], [59], [60], [61].

1.3. Basal media composition

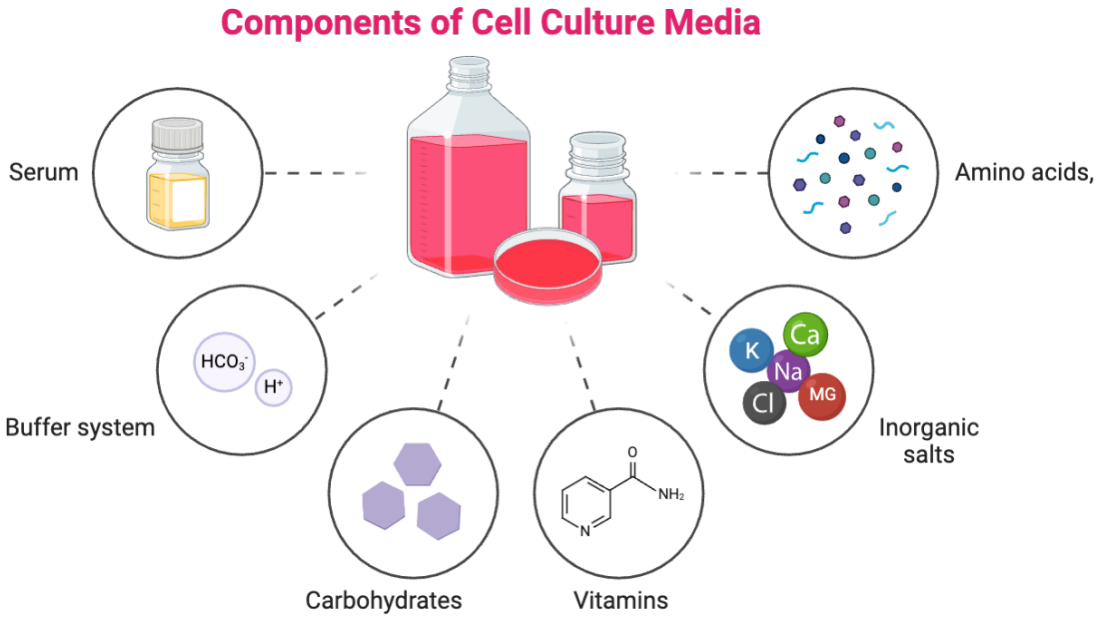

Figure1.3: The primary components included in synthetic cell culture medias. All synthetic culture medias are formulated using a mix of amino acids, inorganic salts, vitamins, carbohydrates, some buffer system, and most include the addition of serum. The concentrations of these different components and addition of other miscellaneous constituents, differentiates these medias from each other.

Basal media serve as the base nutrient source for cultured cells, providing many of the required nutrients for proliferation (Figure 1.3). The purpose of basal media is to provide these nutrients at sufficient levels to maximize cell proliferation in culture, which may not necessarily directly reflect physiologically relevant concentrations. Although many different formulations of culture media exist, RPMI, DMEM, Ham's F-12, and more recently HPLM have emerged as the primary basal medias of choice for mammalian cell culture [62].

Each formulation shares many similar constituents- including carbohydrates, amino acids, vitamins, minerals, salts, and a buffering agent [63]. However, slight

compositional variations of these media can significantly influence cell growth, reflecting the distinct environmental requirements of different cell types. In most cases, researchers supplement basal media with a serum additive, which delivers a complex and heterogeneous mix of growth factors and biologically active molecules that are otherwise absent in basal media.

1.3.1. Carbohydrates

Carbohydrates are the primary energy source in culture media, with glucose being the most commonly added sugar (Figure 1.3). Other monosaccharides like fructose, galactose, and lactose can be included in specialized media formulation[64]. Once glucose enters the cell, it is rapidly oxidized via glycolysis and the tricarboxylic acid (TCA) cycle, generating ATP for various cellular functions [65]. In addition to energy production, glucose derived carbons can be utilized as substrates for biosynthetic pathways, such as alanine and fatty acid biosynthesis. Thus, inclusion of carbohydrates has been an essential component of culture media since the beginning of tissue culture, and limiting carbohydrates impairs cell growth and viability [66].

Cancer cells in culture and in vivo exhibit the Warburg effect, characterized by high glucose uptake and conversion to lactate despite adequate oxygen supply [65]. Indeed, the increased glycolytic activity of cancer cells underlies the principle of the ^{18}Fluorodeoxyglucose PET scan, where metabolically active tumor cells accumulate the isotope tracer and enable visualization of tumors with imaging [67]. Thus, to accommodate the elevated glycolytic activity in culture, media intended for rapidly proliferating

transformed cell lines often include elevated glucose concentrations, typically between 10 to 25 mM [68]. These glucose levels are in contrast to normal blood glucose concentrations, which range from 4-6 mM, underscoring the excessive glucose concentrations provided in standard culture conditions [69]. One reason for the providing excess glucose in culture media is that without glucose replenishment with vascular profusion, cell consumption would rapidly deplete glucose and lower concentrations below physiological concentration and impair cell proliferation.

1.3.2. Buffering system

Maintaining the environmental pH is essential for proper enzyme function, making the buffering component of media a key aspect of its formulation (Figure 1.3). Most media formulations use bicarbonate as a buffer, which is ideal for maintaining a pH between 7.2 and 7.4 in standard incubator conditions that contain 5% CO_2 [48]. Bicarbonate is particularly relevant because it is one of the primary buffering components in blood, produced endogenously by carbonic anhydrases [70]. Although physiologic bicarbonate concentrations typically range between 22 to 32 mM- and most culture medias contain similar levels- DMEM has is approximately 44mM [62].

While bicarbonate remains the most common buffer system in culture media, several alternative buffers, such as HEPES, help maintain the pH independent of CO_2 levels. Additional chemical buffers like MOPS, MES, and BES can be used, although they may interact with various media components and can result in metal chelation [71].

1.3.3. inorganic salts

inorganic salts are another component of cell culture media, where they provide both ionic and osmotic balance, making them an indispensable inclusion to media formulations (Table 1.2). Environmental inorganic salts are required for proper cell functions, including maintenance of membrane potential via the Na/K pump and the crucial role of magnesium in stabilizing transition states during chemical reactions [72], [73]. The presence of these salts also facilitates utilization and uptake of other environmental components, such as the sodium dependent metabolite transporters, including amino acids and some lipid classes [74], [75]. Calcium in particular is a potent signaling molecule and can drive protein conformational changes that direct many cellular functions [76]. Although culture media salt concentrations generally reflect normal ranges of human plasma, the formulations for RPMI contains decreased calcium and magnesium salt concentrations. RPMI, for example, contains 0.42 mM Ca^{2+} and 0.04 mM Mg^{2+}, while the normal range in plasma is 1.11-1.32 mM Ca^{2+} and 0.62-0.99 mM Mg^{2+} [62].

Inorganic Salts			
DMEM	**RPMI**	**Ham's-F12**	**HPLM**
Calcium chloride	Calcium nitrate	Calcium chloride	Ammonium chloride
Ferric nitrate	Magnesium sulfate	Cupric sulfate	Calcium chloride
Magnesium sulfate	Potassium chloride	Ferrous sulfate	Calcium nitrate
Potassium chloride	Sodium Bicarbonate	Magnesium chloride	Magnesium chloride
Sodium Bicarbonate	Sodium chloride	Potassium chloride	Magnesium sulfate
Sodium chloride	Sodium Phosphate	Sodium Bicarbonate	Potassium chloride
Sodium Phosphate		Sodium chloride	Potassium phosphate
		Sodium phosphate	Sodium bicarbonate
		Zinc sulfate	Sodium chloride
			Sodium phosphate dibasic

Table 1.2: Inorganic salts included in DMEM, RPMI, Ham's F-12, and HPLM formulations.

1.3.4. Amino acids

Amino Acids			
DMEM	**RPMI**	**Ham's-F12**	**HPLM**
L-Arginine	L-Arginine	L-Alanine	L-Alanine
L-Cystine	L-Asparagine	L-Arginine	L-Arginine
L-Glutamine	L-Aspartate	L-Asparagine	L-Asparagine
Glycine	L-Cystine	L-Aspartate	L-Aspartate
L-Histidine	L-Glutamate	L-Cystine	L-Cystine
L-Isoleucine	L-Glutamine	L-Glutamate	L-Glutamate
L-Leucine	Glycine	L-Glutamine	L-Glutamine
L-Lysine	L-Histidine	Glycine	Glycine
L-Methionine	L-Isoleucine	L-Histidine	L-Histidine
L-Phenylalanine	L-Leucine	L-Isoleucine	L-Isoleucine
L-Serine	L-Lysine	L-Leucine	L-Leucine
L-Threonine	L-Methionine	L-Lysine	L-Lysine
L-Tryptophan	L-Phenylalanine	L-Methionine	L-Methionine
L-Tyrosine	L-Proline	L-Phenylalanine	L-Phenylalanine
L-Valine	L-Serine	L-Proline	L-Proline
	L-Threonine	L-Serine	L-Serine
	L-Tryptophan	L-Threonine	L-Threonine
	L-Tyrosine	L-Tryptophan	L-Tryptophan
	L-Valine	L-Tyrosine	L-Tyrosine
		L-Valine	L-Valine

Table 1.3 : Amino acids included in DMEM, RPMI, Ham's F-12, and HPLM formulations.

Similar to glucose, amino acids have been included in culture media since the early days of tissue culture and are critical components of media formulations (Table 1.3). Amino acids are required substrates for protein synthesis and support numerous metabolic pathways. The differentiation between essential and non-essential amino acids has been recognized for nearly a century. Essential amino acids must be obtained through the diet because human cells are unable to synthesize them de novo [77]. Interestingly, some non-essential amino acids are still required in culture media, as not all tissues

express the synthesis machinery required for amino acid production, thus amino acids like alanine, asparagine, aspartate, glutamine, glycine, proline, and serine are often included and/or supplemented into culture media to enhance cell proliferation [78], [79].

In addition to their role as building blocks, some amino acids, like glutamine, serve anaplerotic functions, providing both carbons and nitrogen to replenish metabolic intermediates in key pathways like the TCA cycle [60]. Early studies by Fischer, found impaired cell proliferation and viability when amino acids were removed from media via dialysis [50]. Building upon these findings Harry Eagle systematically identified the specific amino acids required in the extracellular environment to support optimal cell growth [46], [47]. More recent studies have shown deprivation of specific amino acids, such as asparagine and glutamine, can impair proliferation. This finding is the basis for therapeutic strategies involving asparaginase and glutaminase inhibitors to limit tumor growth in cancer, underscoring the role of environmental amino acids for cell proliferation [80], [81], [82].

The concentrations of amino acids in the blood can vary depending on many factors, including diet, lifestyle, age, and between different amino acids [83], [84], [85], [86]. Nonetheless, most culture media aim to approximate these physiologically relevant levels. Glutamine is often supplemented at higher concentrations to meet the significant metabolic demands of rapidly dividing cells over several days without resulting in full depletion.

1.3.5. Vitamins

Vitamins serve as cofactors in numerous biochemical reactions that underlie cell proliferation and function. The included vitamins in media formulations are primarily water soluble B vitamins (Table 1.4) For example, thiamine (B1), riboflavin (B2), and niacinamide (B3) participate in energy metabolism, while folic acid (B9) is involved in one carbon metabolism and required for nucleotide synthesis [87]. Thus, these vitamins are all constituents of RPMI, DMEM, Hams F-12 and HPLM. Biotin (B7), which is occasionally included in some basal media, supports carboxylation reactions, while other added vitamins contribute to redox balance and gene regulation [87].

Although vitamins are integral to normal animal physiology, not all are universally needed at the cellular level and specific cell lines exhibit unique requirements. For example, cells dependent upon lipid synthesis for proliferation require the addition of biotin in the media, whereas the presence of exogenous lipid sources can attenuate the need for biotin inclusion [88].

Vitamins			
DMEM	**RPMI**	**Ham's-F12**	**HPLM**
Thiamine (B1)	Riboflavin (B2)	Thiamine (B1)	Thiamine (B1)
Riboflavin (B2)	Thiamine (B1)	Riboflavin (B2)	Riboflavin (B2)
Niacinamide (B3)	Niacinamide (B3)	Niacinamide (B3)	Niacinamide (B3)
D-Pantothenate (B5)	D- pantothenate (B5)	D- pantothenate (B5)	D- pantothenate (B5)
Pyridoxine (B6)	Pyridoxine (B6)	Pyridoxine (B6)	Pyridoxine (B6)
Pyridoxal (B6)	Biotin (B7)	Biotin (B7)	Biotin (B7)
Folic acid (B9)	Folic acid (B9)	Folic acid (B9)	Folic acid (B9)
Choline chloride	Cobalamin (B12)	Cobalamin (B12)	Cobalamin (B12)
Myo-inositol	p-Aminobenzoic acid	Choline chloride	p-Aminobenzoic acid
	Choline chloride	i-inositol	Choline chloride
	Myo-inositol		i-inositol

Table 1.4 : Vitamins included in DMEM, RPMI, Ham's F-12, and HPLM formulations.

1.4. Role of serum in cell culture

Serum is the acellular aqueous phase of blood that remains after coagulation, containing a complex mix of proteins, electrolytes, hormones and other biologically active substances and has long been recognized as a key component to culture media [89]. The serum proteome is estimated to contain roughly 1,800 proteins and over 4,000 metabolites, underscoring the many bioactive serum factors that can potentially influence cell proliferation [90], [91], [92]. Historically, a variety of serum types- ranging from rabbit and chicken to horse and bovine- have been used in culture and are typically supplemented at 5-20% in media. Importantly, serum composition is inherently variable, influenced by factors such as species, age, and diet.

Today, fetal bovine serum (FBS) is the most commonly used serum supplement in modern cell culture. Importantly, FBS was shown to support robust proliferation and karyotype maintenance in cell lines from diverse sources, and evidence indicated that serum from fetal fluid exerted stronger growth promoting effects than serum from adult animals. Its widespread adoption in the 1950s, following observations that FBS provides high levels of growth factors and sufficient nutrient composition, while containing lower concentrations of complement proteins and antibodies compared to other types of serum [2], [23]. Individual serum components which have been shown to influence growth are discussed further below.

1.4.1. Growth factors

Historically, the growth-promoting effects of FBS were largely attributed to the high levels of growth factors present in fetal fluids. Growth factors are signaling molecules which promote cell division and differentiation and can be proteins, peptides, or lipid-based structures [93]. Most surface receptors for growth factors exhibit tyrosine kinase activity upon binding to their ligands and initiate downstream signal transduction cascades [94]. Alternatively, lipid-based structures, typically in the form of steroids, bind intracellular receptors which then translocate to the nucleus to initiate their downstream effects.

The role of specific serum derived hormones has been explored and shown to promote cell proliferation. Indeed, insulin, epidermal growth factor (EGF), and insulin like growth factor (IGF-1), result in the activation of the PI3K/AKT pathway, leading to increased cell survival, cell motility, and inhibition of apoptosis [94]. Other hormones, such as androgens and estrogens can also exert potential growth promoting effects via direct transcriptional changes, particularly in cell lines exhibiting increased expression of their target receptors, such as Her2+ breast cancer cell lines or androgen dependent prostate cancer cells [95], [96], [97], [98]. Thus, the presence and composition of growth factors, particularly in a fetal fluid rich in these factors, underscore a key reason for the broad utility of serum use in cell culture, and the motivation for finding a suitable alternative that may more closely reflect the growth factors and hormones cells are exposed to in an adult human.

1.4.2. Serum proteins

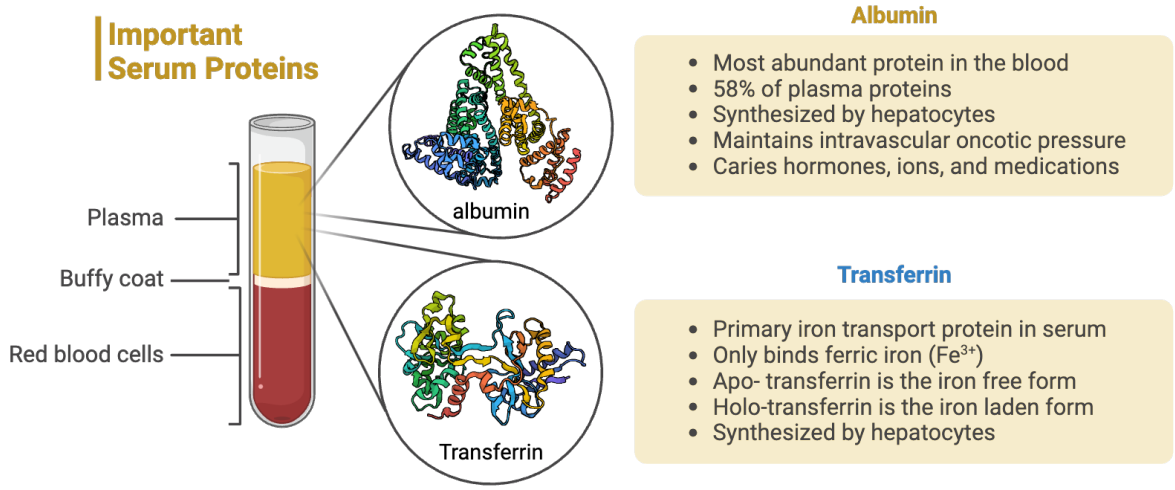

Figure 1.4: Albumin and transferrin are two critical serum components. Albumin is a large carrier protein which has many different ligands. Transferrin is the primary iron carrier molecule in the serum. Both albumin and transferrin shown to support cell proliferation in vitro and have been included in serum free media formulations.

Serum contains numerous proteins that play essential roles both in the body and in culture (Figure 1.4) [99]. Albumin, the most abundant serum protein, maintains oncotic pressure in the vasculature, binds a variety of ligands, and serves as a major source of free thiols via a single unpaired cysteine residue. Human serum albumin (HSA) is approximately 66k Da and consists of three homologous helical domains, each subdivided into A and B subdomains, containing 6 and 4 helices respectively [100]. Albumin binds a broad spectrum of endogenous ligands, including FAs, bilirubin, and hemin, in addition to exogenous molecules such as warfarin and ibuprofen [101]. Consequently, drugs with high albumin binding affinity may require increased dosages to achieve an effective concentration in vivo and in cell culture.

Bovine serum albumin (BSA) and HSA share roughly 76% sequence identity and display similar properties. In cell culture, albumin is a major source of metabolites for the

cell due to its carrying capacity of hydrophobic compounds found in serum, particularly FA and other FA containing lipids important for cell metabolism [102]. In addition to the ligands it carries, albumin can also serve as a source of amino acids through endocytosis and lysosomal degradation. For instance, pancreatic ductal carcinoma (PDAC) cells, which reside in desmoplastic nutrient scarce conditions in vivo, rely on consumption of exogenous protein sources, and require the addition of FA-free albumin in culture media for optimal growth [1], [103]. While the exact mechanism of cell surface interaction with albumin remains unclear, gp60 has been proposed as an albumin receptor that can trigger caveolae endocytosis into the cell [104], [105]. Alternatively, PDAC cells have also been observed to uptake bulk extracellular material including albumin and catabolize it to liberate the amino acids and support cell growth through a process known as micropinocytosis [106].

Other serum proteins are also vital to cell culture, including the transferrin family of proteins, which serves as the primary iron carrier in the blood (Figure 1.4) [107]. These proteins are approximately ~80 kDa, but can vary in size depending upon isoforms included in the mature protein and exist in two form: apo-transferrin (iron-free) and holo-transferrin (iron-laden) [107]. Because free iron can readily generate reactive oxygen species (ROS), transferrin chelates ferric (Fe^{3+}) iron and additionally prevents oxidative damage [108]. Studies have demonstrated the essential role of iron homeostasis in culture and revealed transferrin addition is important for serum free culture systems [109], [110].

Under physiological conditions, there is little free iron in circulation due to the sufficient abundancy of transferrin to bind excess iron. The ratio of iron to transferrin is described by the total iron binding capacity (TIBC), and when the ratio of iron to transferrin

is less than one, nearly all iron is sequestered bound to transferrin [111]. Cells uptake transferrin via a transferrin receptor that binds two transferrin molecules and then undergoes receptor-mediated endocytosis [112]. Endosomal acidification breaks the iron-transferrin interactions, allowing iron to bind ferritin, the major intracellular iron storage protein, and apo-transferrin is recycled back to the cell surface and exported [113].

Extracellularly, transferrin can only bind Fe^{3+} iron, whereas ferrous (Fe^{2+}) iron does not bind. other serum components, like ceruloplasmin and citrate, can oxidize Fe^{2+} to Fe^{3+}, enabling transferrin binding [114], [115]. Although citrate catalyzed iron oxidation results in ROS production, ceruloplasmin mediated iron oxidation produces water. Thus, the concentrations of citrate and iron should be considered in culture media, as they may result in increased oxidative stress in vitro.

1.4.3. Trace elements

Although not the most abundant component of serum, trace metals have important functions in cellular processes. While the significance of serum iron and iron carrier proteins are discussed above, most basal media formulations lack elements such as copper and zinc, making serum-derived sources critical. Zinc is an essential co-factor for proper enzyme function and an integral component of zinc finger proteins, which are responsible for DNA-protein interactions [116]. Moreover, both zinc and especially copper are important for essential metabolic enzymes and antioxidant proteins, including super oxide dismutase (SOD) [117]. While typically present in low micromolar to nanomolar

concentrations, these metals are vital for cellular homeostasis, and their imbalance can induce oxidative stress and apoptosis.

Beyond metals, other trace elements in serum also play critical roles in cell culture. For instance, selenium has been studied extensively for its role in proliferation-in vitro- and effect on health- in vivo. Although overexposure to selenium can be toxic, selenium is integrated into the proteome via selenoproteins, with the modified amino acids-selenocysteine and selenomethionine [118]. Notably, selenoproteins are central to the antioxidant response pathways. For example, glutathione peroxidase (GPx), thioredoxin reductase, and selenoprotein P are critical to the cellular antioxidant response [118], [119]. Indeed, GPx4, is critical to quenching lipid peroxides which can otherwise propagate through cellular membranes resulting in ferroptosis and media compositions containing selenium can modify cell line function through its impact on ferroptosis suppression [37].

Because selenium is highly reactive and is involved in redox reactions, selenium concentration in media may also lead to unintended toxicity. Although not a typical component of basal media, selenium is provided through serum addition and can be added as a media supplement. Indeed, selenium has been explored as an exogenous culture additive in various forms, including selenium (Se), selenium Dioxide (SeO_2), and selenite (SeO_3), as well as organic forms of selenium, which have been conjugated to other organic moieties [120], [121].

1.4.4. Serum lipids

In addition to the many soluble factors provided by serum in media, lipids are another critical component that support cell growth. Lipids are hydrophobic molecules characterized as poorly soluble in aqueous solutions that play essential roles in membrane structure, energy production and storage, signaling functions, and can regulate protein activity via post-translational modifications [122].

The environmental lipidome can significantly affect cellular fitness and viability. Studies utilizing lipid depleted serum, either through charcoal stripping or chemical extraction, have reported impaired cell proliferation and increased sensitivity to metabolic perturbations [123], [124]. Conversely, the addition of lipids to culture media can be protective and improve cell growth under conditions of metabolic stress, such as redox imbalance and hypoxia [125], [126].

Historically dialyzed serum has been used to systematically investigate serum components essential for cell growth. However, because lipids are bound to large macromolecular lipoproteins or serum albumin, they are not removed by dialysis. Techniques such as charcoal stripping or chemical extraction of serum lipids have exhibited mixed efficacy in their removal [123], [127]. Therefore, studies systematically exploring the role of serum lipids have been challenging, and different approaches are required to characterize how cells adapt to fluctuating lipid availability and the role of particular lipids in cell growth.

1.4.4.1. Fatty acids

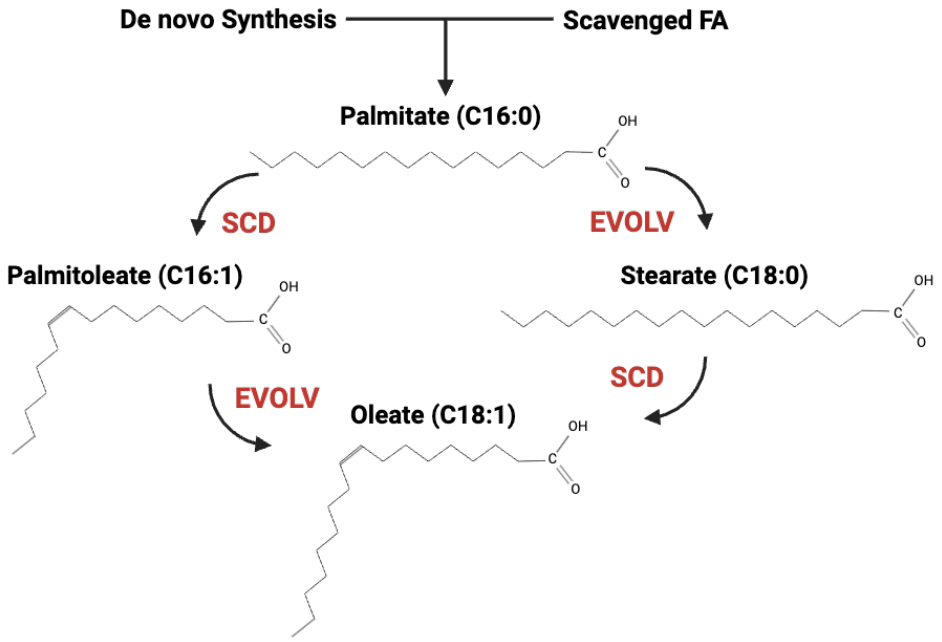

Figure 1.5: De novo synthesis and scavenging contribute to the FA pool and are modified by lipid metabolic enzymes. are the most basic lipid metabolite used in cellular membranes. FA can be de novo synthesized via endogenous metabolic pathways, where the first product it palmitate. Palmitate can then undergo through a series of metabolic reactions which can elongate the FA and/or desaturate it, producing the various saturated and mono-unsaturated FA found in the lipidome. Serum derived FA also enter the lipid metabolic network and are modified by the same enzymes as endogenously produced FA. Stearoyl CoA Desaturase (SCD), elongase enzymes (EVOLV).

Fatty acids (FA) are aliphatic molecules with a terminal carboxylic acid group and are a primary lipid class from which other complex lipids are derived. Although short chain FAs can contribute to energy metabolism, long chain FAs (14-22 carbons) containing up to 6 double bonds are the primary structural components of membrane lipids [128] . The hydrophobic nature of FA chains drive lipid bilayer formation via electrostatic interactions and hydrophobic shielding. Importantly, variations in chain length and degree of saturation are primary determinants of membrane fluidity [129]. Longer, saturated chains increased

Van Der Waals forces and reduce fluidity, while double bonds cause kinks in the FA structure that disrupt these interactions and lead to increased membrane fluidity [129]. Both exogenous scavenging and de novo synthesis FA contribute to the cellular lipid pool [130], [131], [132].

De novo FA synthesis typically begins with glucose-derived acetyl-CoA, although other metabolites such as glutamine and acetate can also feed into the acetyl-CoA pool [133], [134], [135]. Acetyl-CoA is subsequently carboxylated by acetyl-CoA carboxylase (ACAC) to produce malonyl-CoA. Fatty acid synthase (FASN) uses malonyl-CoA to catalyze the serial condensation of an acetyl group to the growing acyl chain, releasing CO_2 in the process [136]. The first product of de novo FA synthesis is palmitate (C16:0), which can then be elongated by a family of elongases (ELOVL) or desaturated (primarily by stearoyl-CoA desaturase, SCD1) to produce the various FAs in the cellular lipidome (Figure 1.5) [137], [138], [139]. Importantly, exogenous FAs scavenged from the serum can also enter lipid metabolic pathways, and be modified via elongation and desaturation, highlighting the plasticity of lipid metabolism, and the difficulty in isolating it to a single exogenous source [139].

Although cells can synthesize both saturated and monounsaturated FAs, particularly in the case of cancer cells which increase expression of lipogenic enzymes, human cells lack the desaturases required to produce polyunsaturated FAs (PUFA) (Figure 1.6) [140]. PUFA are key signaling molecules and are especially involved in inflammatory signaling. For instance, the omega-6 PUFA, arachidonic acid (20:4), is converted by cyclooxygenase (COX) isoenzymes into pro-inflammatory prostaglandins [140], [141]. In

contrast, the omega-3 PUFAs, docosahexaenoic acid (C22:6) and eicosapentaenoic acid (20:5), exhibit antagonist signaling functions and are considered anti-inflammatory, producing resolvins and dampening inflammatory pathways [142], [143].

The FA composition of animal sera commonly used in cell culture can vary widely. For example, FA profiling of FBS reveals low levels of linoleic acid (C18:2) compared to other animal sera. This indicates, the serum lipidome can fluctuate, impacting studies into lipid metabolism and lipid- signaling [144]. Moreover, lot-to-lot variations within a particular species of animal serum also hinders experimental consistency, and demonstrates the importance of standardizing serum lipid composition [145].

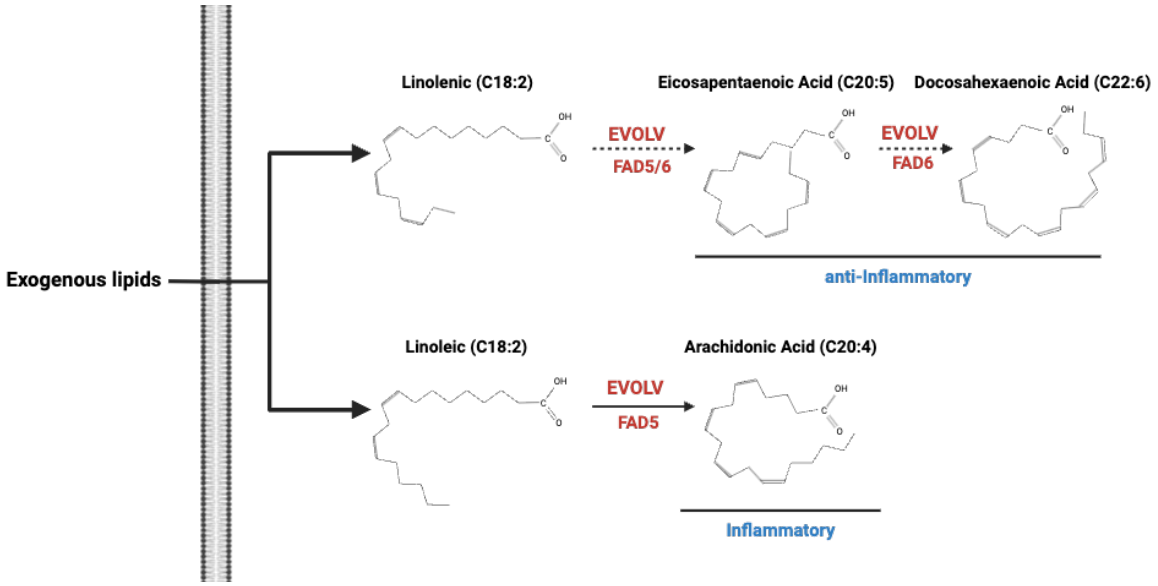

Figure 1.6: Schematic of poly-unsaturated FA metabolism. PUFAs are dietarily essential and must be scavenged from the environment. The desaturase enzymes responsible for double bonds at sites located in linolenic and linoleic acid are not expressed in humans. Thus, dietarily sourced PUFA are scavenged from the environment and then can be modified be intracellular enzymes including various elongase family of enzymes (EVLOL) and two other desaturase enzyme, fatty acid desaturase 5/6 (FAD5/6).

1.4.4.2. Phosphoglycerolipids

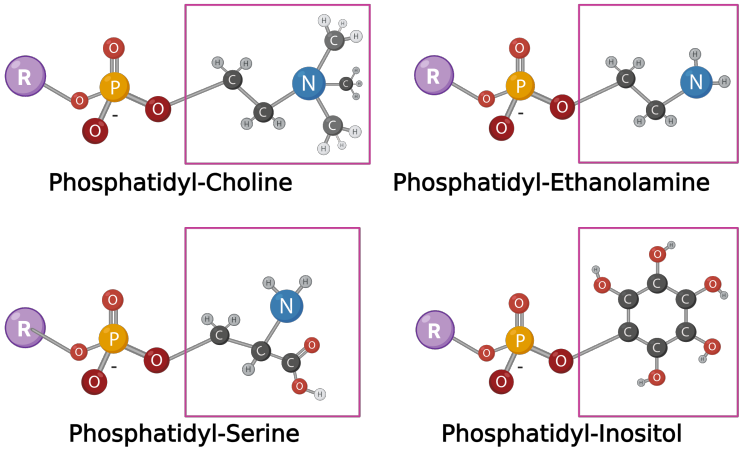

Figure 1.7: Phospholipid polar head group structure. The phosphate polar head group is covalently attached at the sn-3 position of the glycerol backbone which can contain either 1 or 2 FA conjugated via an ester bond at the sn-1 or sn-2 position. The most common polar head groups are phosphatidylcholine (PC), phosphatidylethanolamine (PE), while other important phosphatidylserines (PS) and phosphatidylinositol (PI) are less abundant.

Phosphoglycerolipids are a major class of membrane lipids composed of a glycerol backbone, and one or two FA attached via an ester linkage at the sn-1 and sn-2 positions, and a polar head group connected via a phosphodiester bond in the sn-3 position. Typically, sn-1 position on the glycerol backbone is conjugated to the more saturated FA, while the sn-2 position is occupied by a more unsaturated FA [146]. The polar head group of phosphoglycerolipids can contain various different polar head groups and most commonly include, choline, ethanolamine, serine, or inositol (Figure 1.7) [147].The variety of polar head groups and FA chain structures give rise to the diverse repertoire of phospholipids in the mammalian lipidome.

These lipids are the predominant structural component of cellular membranes, forming bilayers in which the polar head group is oriented towards extracellular and intracellular

aqueous phase, and the hydrophobic fatty acid tails are oriented inwards [129]. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant phosphoglycerolipids in cellular membranes, providing essential structural support [148]. Phosphatidylserine (PS) and Phosphatidylinositol (PI), though less abundant, fulfill key additional functions. PS carries a negative charge and predominantly found on the cytosolic leaflet of the membrane; however, during apoptosis it translocates to the extracellular leaflet, signaling phagocytic cells to engulf the dying cell [149]. Importantly, the hydroxyls on the inositol polar head group of PI can contain different combinations of phosphorylations which are provide potent signaling functions for the cell [150]. Additionally, PI phosphorylations signals which membrane the lipid resides in, helping cellular proteins localize to specific membranes. In serum PC and PE are the most abundant phosphoglycerolipids, with PS and PI present at lower levels.

Lyso-phosphoglycerolipids, such as lyso-PC (LPC), are generated when phospholipase A_2 (PLA_2) cleaves a FA chain from a phosphoglycerolipid, producing a free fatty acid and a phosphoglycerolipids containing only a single FA chain [151]. While lyso-lipids are intermediates of metabolic pathways, LPC molecules have also been found to be more than simple metabolic intermediates and have been shown to exhibit signaling functions, particularly during oxidative stress and inflammatory responses [152]. Changes to serum LPC concentration have also been associated with various disease states, including serum concentration increases in cardiovascular disease and diabetes, while Alzheimer's disease is associated with decreases in LPC concentrations [153], [154], [155]. Additionally, hypoxia is reported to both increase the production of LPC in the serum and

support cell metabolism during hypoxia [126], [156]. In addition to supplying cellular lipids for cells proliferation, LPC can be re-condensed with activated FA by acyl-CoA:lysophosphotdylcholine aceyltransferase (LPCAT), regenerating PC for membrane lipids [157].

1.4.4.3. Neutral lipids

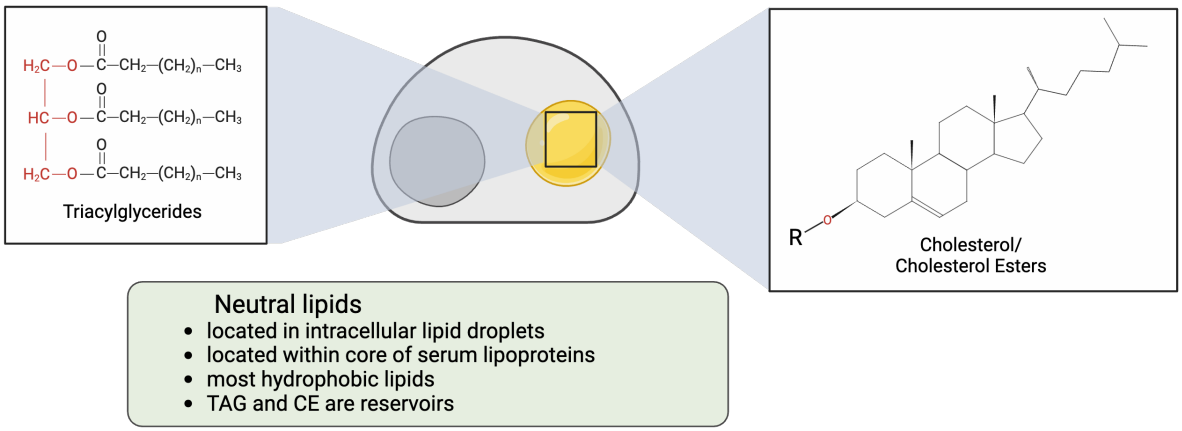

Figure 1.8: Triglyceride and cholesterol ester structure. Neutral lipids are non-charge lipid molecules which include triacylglycerides (TAG) and cholesterol esters (CE). TAG and CE are stored intracellularly in lipid droplet stores, while they are carried in serum within lipoprotein cores. CE are formed from the condensation of cholesterol for a FA chain on the carbon 3 position of cholesterol. FA side chain (R).

Cholesterol esters (CE) and triacylglycerides (TAG) are neutral lipids that are typically sequestered in the core of lipid droplets and lipoprotein, where they are shielded from the surrounding aqueous environment, due to their hydrophobic nature (Figure 1.8) [158]. Structurally similar to phosphoglycerolipids, TAGs are composed of a glycerol backbone, but rather than containing a polar head group, all the glycerol hydroxyl groups are occupied by FAs. TAGs primarily function as reservoir for FAs, providing an energy source during nutrient scarcity or metabolic stresses like hypoxia, and help to maintain the proper balance FA within the cellular lipidome [159], [160].

CE are unique lipid classes that consist of a cholesterol molecule esterified to a FA at its free hydroxyl group on the carbon 3 position and is the main form of cholesterol transported in serum. FA esterification neutralizes the hydroxyl charge, resulting in a more hydrophobic molecule. Upon hydrolysis via cholesterol ester hydrolases (CEH), free

cholesterol is released and incorporated into cellular membranes [161], [162]. In membranes, cholesterol is often associated with sphingolipids, promoting tighter packing and enabling the formation of lipid rafts, membrane microdomains with low membrane fluidity that are critical for membrane signaling and transport [163], [164].

CEs are generated through the enzymatic transfer of a FA chain from PC molecules onto cholesterol via the action of lecithin cholesterol acyltransferase (LCAT), producing both a CE and an LPC molecule in the process. In vivo, CE is constantly broken down and reformed during the process of lipoprotein cycling between tissues and the liver [165].

1.4.4.4. Sphingolipids

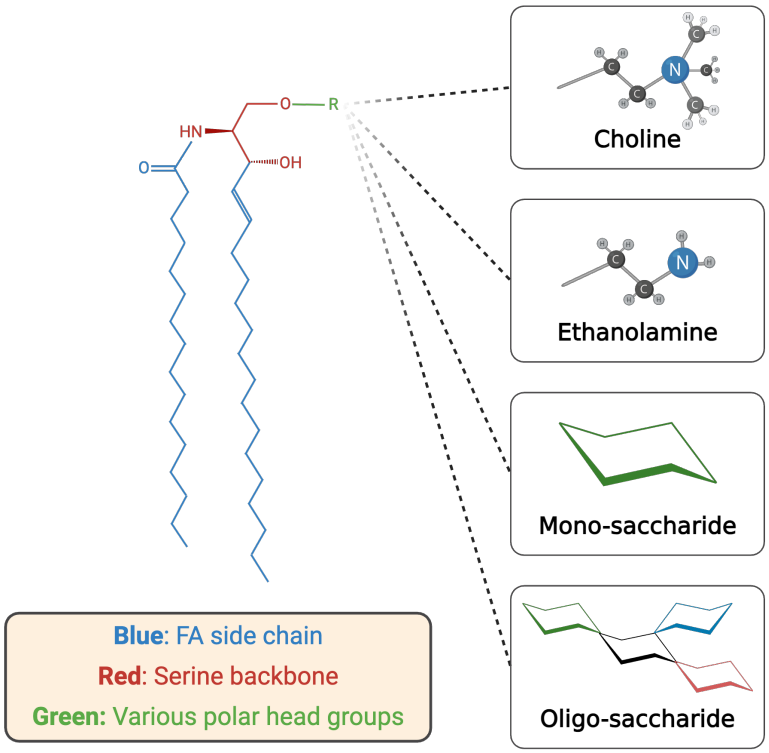

Figure 1.9: Sphingolipid structure. Sphingolipids are composed of serine backbone, while two FA chain and variable polar head groups. The most common polar head group is choline, and is termed sphingomyelin (SM). Glycosphingolipids drive structural diversity of sphingolipids and are important for signaling purposes.

Sphingolipids (SL) are a large class of unique polar lipids that, unlike glycerophospholipids, are built upon a serine backbone. The synthesis of SL begins in the ER with the condensation of FA-CoA with L-serine via the enzyme serine palmitoyltransferase (SPT) to produce sphingosine, where the preferred FA substrate is palmitoyl-CoA [166]. Sphingosine can undergo additional modifications, including condensation with other FAs, addition of various polar head groups, and glycosylation, all of which create a diverse repertoire of lipids (Figure 1.9).

The most abundant form of SL both in the serum and in the cell is sphingomyelin (SM), which consists of a choline polar head group ester linked to the serine backbone and

constitutes greater than 90% of the sphingolipid content of serum [167]. SM play a vital role to the formation of lipid rafts, where the free hydroxyl of the serine backbone interactions electrostatically with cholesterol, contributing to the tight membrane packing, required for membrane raft stabilization[168]. Therefore, SM are important for various cellular processes including signal transduction. Impairment of sphingolipid synthesis disrupts raft formation and can disrupt cellular signaling pathways.

In addition to their unique structural make up in the cellular lipidome, SL have various polar head groups which are influential in their cellular function. Like glycerophospholipids, choline is the most abundant polar head group, whereas ethanolamine is also commonly found, and certain sphingolipids can include various oligosaccharide polar head groups. The oligosaccharide polar head groups can be further modified with chemical groups such as sialic acid, giving increased functional diversity to this class of lipids[166]. These variations add to the functional diversity of this class of lipids, allowing them to serve as receptors and interact with various extracellular ligands.

The role SL play in cell metabolism is complex and varied, contributing to both structural and signaling functions. One of the primary roles of SL is to stabilize lipid rafts along with cholesterol, supporting membrane structure and organization [163]. Additionally, certain oligosaccharide containing lipids and of SL derivatives possess important signaling functions, including influencing apoptosis, cell proliferation, and stress response pathways [169], [170]. Interestingly, disruption to sphingolipid metabolism can affect lipid scavenging; either by impairing interactions with lipid carrier proteins, and/or interfering with membrane raft stability and membrane protein function [171]. The

diversity in SL structure- and importance in cellular signaling- makes it challenging to fully elucidate their role in cell biology.

1.5. Effects of environmental lipids on cell proliferation

Cell culture has been indispensable for studying the role of environmental lipids in supporting cell growth. The role exogenous lipids play in cell behavior is complex and influenced by multiple variables, including: the abundance of environmental lipids, the types of lipids present, and the metabolic state of the cell [124], [132]. The contribution of lipid scavenging and de novo lipid synthesis to cell growth in culture and in vivo settings remains unclear, as environmental lipids can be abundant in the microenvironment and many cancer cells additionally increase expression of lipogenic enzymes relative to parental tissue [130]. Historically, research exploring lipid metabolism has argued that exogenous lipids play a dominant role in cell proliferation. Conversely, recent studies exploring alterations to lipid metabolism in cancer have emphasized the role of lipid synthesis in providing cellular lipids for proliferation and have been proposed as a therapeutic target for the treatment of different cancers [130], [172].

In some cancer cell lines, free FAs have been shown to contribute to cell proliferation, serving as an important lipid source. Much of the foundational work exploring FA scavenging was done using Ehrlich ascites tumors (EAT) cells, a mouse mammary adenocarcinoma cell, which could be passaged by simple injection into the intraperitoneal space of mice, where they proliferation in the fluid-filled abdominal cavity known as ascites[173], [174], [175], [176], [177]. These experiments concluded that exogenous FAs

were a significant source of lipids for EAT cells, and both the environmental concentration and ratio of FA to BSA was a major determinant of uptake [178]. Further, FA uptake by EAT cells was found to be an energy independent process, minimally influenced by uptake of other metabolic substrates such as glucose. Together, these experiments argued that cells heavily rely on FA scavenging, particularly when environmental lipids are abundant.

Studies using other cell lines to explore lipid scavenging also showed environmental lipids are a significant contributor to the lipid pool during proliferation. Indeed, proliferating fibroblast and HeLa cells were shown to preferentially incorporate scavenged lipids into their membrane lipids during proliferation when grown in physiologically relevant concentrations [179]. Extracellular hydrolysis of TAG, producing free FA and glycerol, provides an extracellular source of FA for uptake in vitro. In this study, heat inactivation of serum attenuated TAG hydrolysis and resulted in FA depletion, which was attributed to lipolytic enzyme denaturation. Upon uptake, extracellular FAs entered lipid metabolic networks and could be traced into intracellular triglycerides and phospholipids [180]. Similarly, additional studies traced FAs derived from VLDL into the cellular the lipidome, and reduced de novo FA synthesis was observed when VLDL was supplemented in the media [181]. More recent examples using isotopically labeled palmitate found both non-transformed and transformed cells preferentially incorporated exogenous palmitate into membrane lipids during proliferation when supplied in physiologically relevant concentrations [179]. Additionally, studies comparing the effects of ketogenic diets and caloric restriction on tumors in mice demonstrated that, despite similar decreases circulating glucose and insulin, ketogenic diets maintained the lipid abundance in the

tumor microenvironment and were ineffective in reducing tumor growth, supporting a critical role for environmental lipids in supporting cancer cell proliferation [182]. Collectively, these studies highlight the metabolic contribution of environmental lipids in supporting cell proliferation, in both in vivo and in cell culture.

Cholesterol carried in lipoproteins also supports the growth of other cell lines. When the contribution of de novo cholesterol synthesis versus environmental scavenging was assessed in in vivo, the majority was found to be taken up from the environment, with 3% synthesized in EAT tumors [183]. The high degree of scavenged cholesterol was attributed to the elevated abundance of VLDL in the peritoneal fluid. Consistent with these observations, studies using the MBIII mouse lymphoblast strain, showed that triglycerides, phospholipids, and cholesterol were rapidly scavenged from the media [184],[180],[185].

1.5.1. Deleterious effect of exogenous lipids

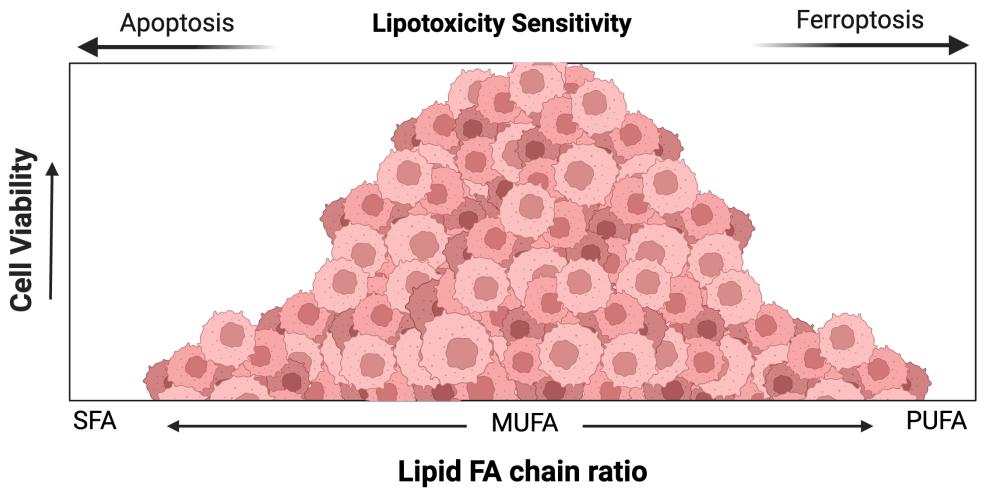

Figure 1.10: FA saturation ratio dictates sensitivity to lipotoxicity. The ratio of SFA:MUFA:PUFA determines the sensitivity to lipotoxicity. Overabundance of saturated FA in the cellular lipidome pushes cells towards apoptosis and reduced viability, whereas and overabundance of PUFA sensitize membrane lipid to lipidperoxide, resulting in ferroptosis.

Although scavenged lipids support proliferation, the environmental lipid composition can also have deleterious effects on cell fitness and viability. Cellular stress resulting from disruption to lipid homeostasis is referred to as lipotoxicity. It can occur when the saturation index (the relative abundance of SFA:MUFA:PUFA in cellular lipids) shifts too far towards one extreme, or when the rate of lipid uptake outpaces the cell's ability to incorporate these lipids into its lipidome or storage pools (Figure 1.10) [186].

Accumulation of saturated FAs (SFA) results in disruption to lipid homeostasis, culminating in lipotoxicity induced apoptosis. While the exact mechanism is not fully understood, evidence indicates that SFA lead to endoplasmic reticulum (ER) stress and initiation of the unfolded protein response (UPR) [187], [188]. The ER membrane is enriched for unsaturated FA providing increased membrane fluidity necessary for proper

protein folding [189]. As saturated FAs accumulate intracellularly, the ER membrane lipid remodeling results in decreased fluidity and impaired membrane folding, leading to CHOP activation [190], [191]. Prolonged UPR activation and ER stress resulting from saturated FA accumulation ultimately leads to induction of apoptosis.

Conversely, overabundance of PUFA dictate cellular sensitivity to ferroptotic cell death. Ferroptosis is the iron dependent cell death resulting from peroxidation of membrane lipids [192]. The process of ferroptosis involves the generation of hydroxyl radicals generated by Fenton reactions between Fe^{2+} and H_2O_2 [193]. ROS react with double bonds present in complex lipids, particularly membrane lipids enriched for PUFAs, to generate lipid peroxides. This oxidative stress can ultimately propagate across cellular membranes, even traveling from one cell to another across long distances (>5mm) [194]. Sensitivity to ferroptosis is influenced by both endogenous lipid metabolism pathways, as well as the environmental PUFA availability, where elevated PUFAs in the environmental sensitize cells to ferrotopic cell death[193].

The ratio of FA within the cellular lipidome determines sensitivity to both forms of lipotoxicity. Monounsaturated FAs (MUFA), particularly oleate (C18:1), are critical for protecting against lipotoxic stress [195], [196]. Indeed, oleate can displace PUFA from membrane lipids and protect against ferroptotic stress and help to maintain proper membrane dynamics. Together, these observations reveal the interplay between exogenous lipid composition and sensitivity to differing forms of lipotoxicity, and show environmental lipids are influential in both proliferation and maintaining cellular lipid homeostasis.

1.6. Lipid transport

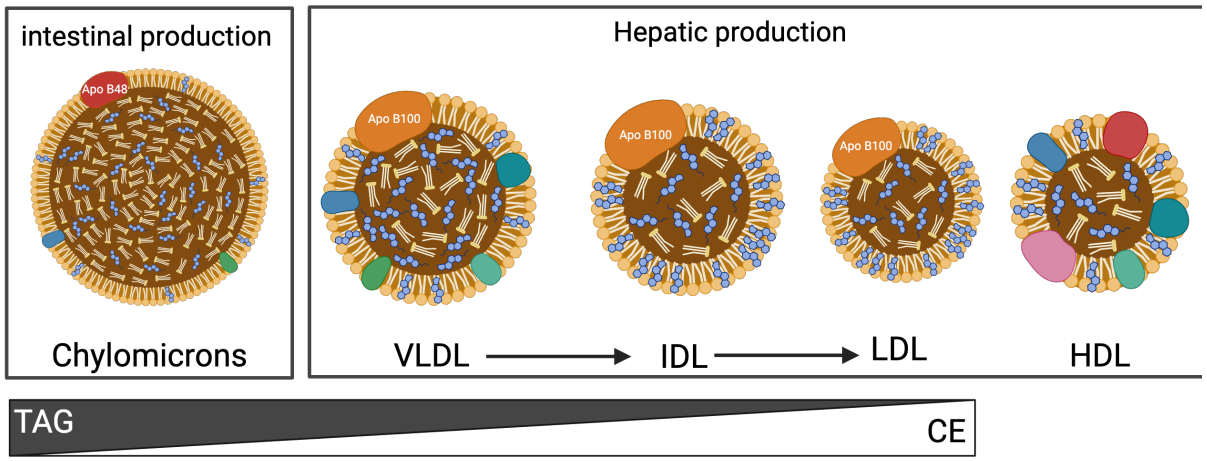

Figure1.11: The different forms of lipoproteins found in the serum. Intestinal enterocytes produce chylomicrons which are rich in TAG, while hepatocytes produce VLDL, which mature in the blood as metabolic enzymes act on the complexes, forming IDL and ultimately LDL, which have the highest ratio of CE to TAG. Hepatocytes also produce HDL, which are responsible for reverse transport of lipid back to the liver.

The hydrophobic nature of lipids makes them poorly soluble in aqueous solution and thus require specialized forms of transport. One of the primary mechanisms of lipid transport in serum involve lipoproteins. Lipoproteins are large lipid complexes packaged and secreted into the serum by hepatocytes and enterocytes [127]. Lipoproteins contain a mix of different lipid classes including, phosphoglycerolipids lipids, neutral lipids, and sphingolipids and their relative abundance changes with the maturity of the lipoprotein complex. Lipoproteins have an outer shell of phospholipids- primarily PC and PE, SM- and various proteins embedded in the membrane [197]. The lipid shell surrounds an inner core of neutral lipids, including TAG and CE, which are shielded from the aqueous solution [198], [199].

Chylomicrons are large lipoprotein complexes produced post prandially from enterocytes and are characterized by the presence of ApoB-48 protein on the lipoprotein

shell, with a large concentration of TAG in the particle core [127]. In contrast, hepatocytes are responsible for the production of all other lipoproteins in the body which contain the ApoB-100 protein, which is important for recognition and cellular uptake. The lipoproteins produced by hepatocytes are secreted as very-low density lipoproteins (VLDL) with a normal serum concentration of 30 mg/dl, containing roughly 70% TAG [200]. As lipoprotein are metabolized and mature in serum and lipids are scavenged by cells, the lipid profile of VLDL is altered, the ratio of CE to TAG increases, altering the size and density of the VLDL to intermediate-density lipoprotein (IDL), and ultimately into a low-density lipoprotein (LDL) (Figure 1.11) [113,114]. LDL is the terminal lipoprotein particle and contains the highest ratio of CE to TAG and are considered pro-atherogenic. High density lipoproteins (HDL) exhibit antagonist functions by exchanging lipids with other lipoproteins, particularly CE, and transporting lipids back to the liver ultimately for repackaging into VLDL or excretion through the bile.

In contrast to large lipoprotein complexes, lipids containing single FA chains, such as LPC, LPE, and free-FA are predominantly transported in serum bound to albumin [203]. Albumin contains roughly 7 binding sites for lipids and functions to increase solubility and delivery to cells [102]. Albumin-bound lipids can be produced either through cellular secretion from cells like adipocytes or derived intravascularly through metabolic lipolysis of lipids contained within lipoproteins via various serum enzymes.

1.6.1. Mechanisms of lipid scavenging

While exogenous lipids have been shown to impact cell proliferation and viability in culture, the mechanisms and proteins involved in the lipid scavenging pathway remain poorly defined. A handful of different proteins and protein families have been demonstrated to play a role in lipid uptake and utilization of exogenous lipids, but the precise mechanisms and role in lipid metabolism are still unclear (Figure 1.12). While Fatty acid transport proteins (FATP) have also been implicated in uptake of exogenous lipids, their reported involvement is similar to the Long-chain fatty acyl CoA synthetase family (ACSL) family of proteins discussed further below [204].

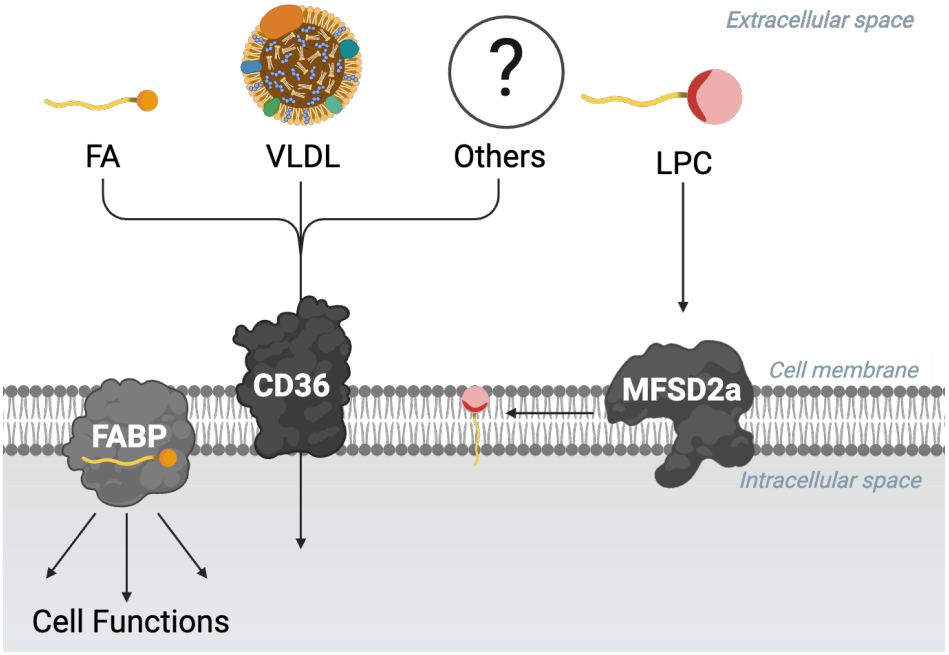

Figure 1.12: Schematic of reported lipid transport components. The major lipid transport proteins involved in uptake of FA and LPC. (1) MFSD2a is involve in the uptake of environmental LPC and is particularly associated with PUFA containing LPC across the blood brain barrier. (2) CD36 is a transmembrane glycoprotein reportedly responsible for binding and uptake of multiple ligands related to lipid metabolism, including FA and oxidized lipoproteins. (3) FABP is a family of proteins involved in intracellular trafficking of FA within the cell and have also been detected in extracellular fluid.

1.6.2. Major facilitator superfamily domain-containing protein 2a

Major Facilitator Superfamily Domain-Containing Protein 2a (MFSD2a) is a membrane transporter that has been demonstrated to facilitate the sodium dependent uptake of LPC [75]. In particular MFSD2a has been implicated in the uptake of LPC molecules containing docosahexaenoic acid (C22:6) across the blood brain barrier, although MFSD2a has also show transport capabilities of LPC molecules containing other FA like palmitate and oleate in vitro[205], [206]. Importantly, mutations in MFSD2a have been associated in impairment to nervous system development and microcephaly resulting from reduced PUFA in the central nervous system [207]. Interestingly, despite the benefit of LPC during metabolic stress and their contribution to the lipidome, MFSD2a is a reported tumor suppressor and expression impairs cancer growth [208], [209], [210]. Thus, the exact role of MFSD2a in LPC uptake and lipid scavenging outside of the setting of the blood brain barrier and development remains unclear.

1.6.3. CD36 fatty acid transporter

There is ongoing debate within the scientific field regarding whether FA uptake is an active protein mediated process or occurs passively [211], [212]. Among the proteins reported to be involved in FA uptake, CD36- also known as Fatty Acid Translocase (FAT)- is the most frequently referenced. It is a transmembrane glycoprotein from the scavenger receptor class B family, expressed on the surface of many cells [213]. CD36 is reported to be involved in many cellular functions, including lipid metabolism, immune response, inflammation, and signal transduction [214]. One proposed mechanism of CD36-mediated

FA uptake was recently demonstrated in adipocytes, where FA treatment triggered caveolae-dependent CD36 internalization. This process requires post-translational palmitoylation at Tyr91, and disrupting CD36 palmitoylation reduced FA uptake activity [215]. Consistently, blocking endocytosis or the lipid raft formation, where endocytosis occurs, with pharmacological inhibitors also reduced FA uptake, indicating a critical role of endocytosis in CD36 mediated FA uptake [213], [215].

While endocytic uptake of FA via CD36 is supported by studies in adipocytes, other evidence of CD36 involvement in FA uptake comes from genetic studies and FA tracers [216]. CD36 protein expression and mRNA expression correlates with exogenous FA uptake, while genetic disruption of CD36 impairs uptake of fluorescently labeled FAs, such as Fluorinated BODIPY FA-analogs. However, BODIPY FA analogs contain bulky charged moieties on the omega end of the FA, and it is not clear these reporters accurately model FA metabolism, particularly for investigating uptake of FA. Some studies suggest CD36 exhibits substrate specificity for MUFAs, which is suggested to be important in maintaining lipid homeostasis during cancer cell matrix detachment[215], [217]. However, cells lacking CD36 still maintain the ability to scavenge exogenous FAs, indicating additional cellular factors influence FA scavenging from the environment. Interestingly, inhibition of FASN upregulates CD36 in colorectal cancer cells, supporting a role in exogenous lipid acquisition [218].

In vivo, CD36 expression has been linked to metabolic disease, including insulin resistance, hyperlipidemia, and hypertension. An estimated 2-3% of certain populations exhibit CD36 deficiency, which is associated with persistent lipoprotein remnants in

circulation and reduced uptake of the FA-analog ^{123}I-beta-methyl-p-iodophenyl pentadecanoic acid (BMIPP) [219]. Indeed, beyond its role in FA uptake and similar to other scavenger B receptors in the same protein family, CD36 has also been shown to facilitate uptake of oxidized lipoproteins and phospholipids, indicating its role in lipid metabolism is complex and heterogeneous [220].

CD36 also promotes cancer cell progression and metastasis in certain tumor types, including breast, brain, and ovarian cancer [221], [222], [223]. While reported pharmacological inhibitors of CD36 like sulfo-N succinimidyl FA analogs have been shown to disrupt intracellular metabolism with many off target effects, targeting CD36 is being explored clinically with the development of anti-CD36 monoclonal antibodies, which have been shown in preclinical studies to reduce tumor growth in cancer cells with high CD36 expression [224], [225]. Combined, cell culture and in vivo studies demonstrate CD36 contributes to lipid metabolism and disease progression, but the exact role CD36 plays in FA uptake and ligand specificity remains unclear.

1.6.4. Fatty acid binding proteins

Fatty acid binding proteins (FABP) are a family of ubiquitously expressed, low molecular weight proteins, responsible for intracellular transport of FA. Twelve FABP isoforms have been identified and are named according to the tissues where they were first identified or where they are most highly expressed [226]. In tissues with high lipid metabolic demands, such as liver, adipose, and the heart, FABPs can constitute 1-5% of the cytosolic proteins [227]. FABPs share similar protein structure, characterized by a β-

barrel composed from of ten anti-parallel β-sheets, forming an inner ligand binding pocket that accommodates a single long chain FA at a time [228].

FABP facilitate the solubilization of hydrophobic FAs, allowing them to be shuttled within the aqueous cellular environment. FABPs are responsible for shuttling FA to various cellular organelles. For instance, FABP2 (intestinal FABP) is important for FA uptake from the intestines during digestion, and FABP3 (heart FABP) is involved in FA transport to the mitochondria for β-oxidation, particularly important for cardiomyocyte energy production [229], [230].

FABPs are also implicated in various disease states, including cancer. FABP4 (adipocyte FABP), has been shown to be overexpressed in breast, prostate, and colon cancers, and FABP5 (epidermal FABP) overexpression is associated with worse cancer prognosis[231], [232], [233]. Together, these observations indicate FABPs mediated lipid transport and utilization are influential cellular lipid metabolism and can impact cancer cell proliferation.

1.6.5. Low density lipoprotein receptor

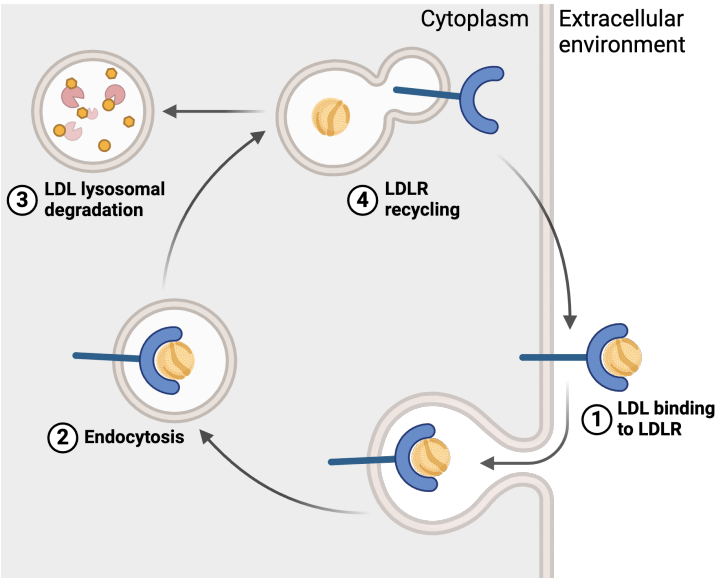

Figure 1.13: Schematic of LDLR function and lipoprotein uptake. Lipoproteins bind the LDLR receptor and trigger endocytosis of the receptor-lipoprotein complex. The lipoprotein is then degraded in the lysosome, liberating the lipoprotein lipids, and the LDLR receptor can return to the plasma membrane to bind additional lipoproteins.

The low-density lipoprotein receptor (LDLR) is an endocytic receptor family of proteins that is involved in lipoprotein uptake and arguably the best well characterized lipid transport protein. The LDLR ubiquitously expressed transmembrane proteins binds apoB-100 and apoE containing lipoproteins and is important for maintaining cholesterol homeostasis [234]. Thus, this family of proteins interacts primarily with hepatocyte produce lipoproteins, while chylomicrons produced in the intestines do not bind with LDLRs. Upon ligand binding, the LDLR dimerizes and is internalized by receptor mediated endocytosis via clathrin-coated pits (Figure 1.13) [235]. The LDLR can return to the plasma membrane via endocytic cycling or can be degraded by the lysosome [235]. Elevated LDLR expression is associated with cancer progression and metastasis in various cells, and uptake of lipoprotein can significantly contribute the lipidome [236]. In addition to

increased lipid uptake supporting elevated proliferation rates of cancer cells in vivo, It is suggested that elevated expression of the LDLR results in outcompeting local immune cells, effectively depriving them of nutrients in the microenvironment and reducing immune function [237].

1.6.6. Long-chain fatty acyl CoA synthetase family

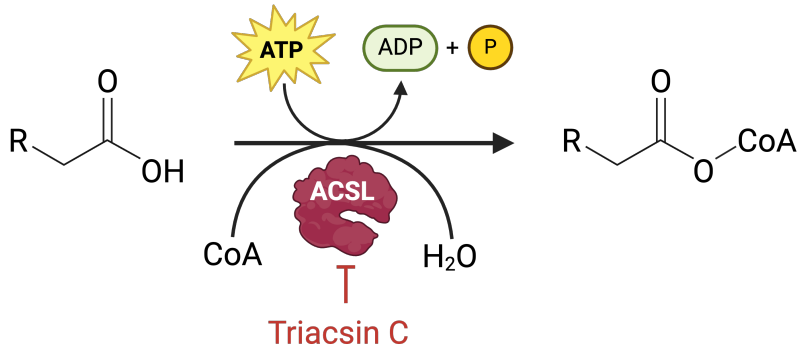

Figure 1.14: ACSL catalyze the ATP dependent condensation of FA with Coenzyme A. ACSL family are responsible for FA activation, producing a fatty acyl-CoA. Triacsin C is a pan inhibitor of ACSL family members and prevent FA activation.

Whether acquired from exogenous source or through endogenous lipolysis, FA must be activated via condensation with coenzyme A (CoA) to form fatty acyl-CoA esters (Figure 1.14) [238]. This reaction is catalyzed by multiple homologous proteins belonging to the ACSL, FATP, and bubblegum (ACSBG) subfamilies, which activate FAs with a leaving group which enables fatty acyl-CoA to enter into the lipid metabolic pathway. While FATP and ACSBG protein members have been shown to influence uptake of FA from the environment, the ACSL family and it's cells physiology roles have been the most well studied [204]. The ACSL family of proteins is comprised of 5 different isoforms (ACSL1, 3, 4, 5, and 6), the ACSL family influences critical intracellular lipid fates, including lipid

remodeling, and membrane synthesis [239]. Different isoforms exhibit different intracellular locations and substrate preferences. Their activity is critical to intracellular FA fates, including FA β-oxidation, lipid remodeling, and membrane lipid synthesis. Their activity is regulated by post- translational modifications, and ACSL expression is controlled transcriptionally by PPAR and SREBP [240].

Different ACSL isoforms are exhibit different tissue expression and subcellular localization. ACSL1 is widely expressed across metabolically active tissues like the liver, heart, adipose, and muscle and facilitate activations of both saturated and unsaturated FA in the mitochondria and the ER [241]. ACSL3 is primarily localized to the mitochondria, ER, and surface of lipid droplets, and reportedly exhibits preference for palmitate activation, but is also essential for utilization of exogenous monounsaturated FA [159]. Additionally, ACLS3 has been demonstrated to influence lipid droplet biogenesis and tumorigenesis[i] of clear cell renal cell carcinoma cells [242]. Studies investigating modifiers of ferroptosis revealed ACSL4 selectively activates PUFAs like arachidonic acid for entry into the lipidome [243].

Identified pharmacological inhibitors of ACSL family acidity have been useful in understand the role of ACSL in cell metabolism. Triacsin C is a pan-ACSL inhibitor, which has been used to investigate the consequences of FA inactivation [244], [245]. In some cell lines, triacsin C treatment resulted in impaired TAG synthesis, while PC synthesis was less affected [244]. In other systems, ACSL impairment resulted in FA accumulation within cells treated with VLDL [246]. Together, these findings indicated ACSL mediated FA-activation is crucial for utilization and incorporation into the cellular lipidome.

1.7. Drawbacks and controversy of FBS use in cell culture

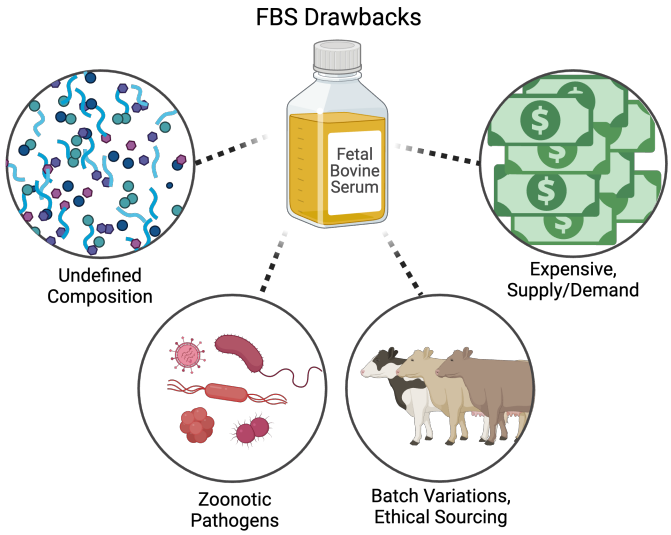

Figure 1.15: Diagram of drawback for FBS use. FBS is a complex and undefined mix, whose full composition is unknown, reducing experimental consistency and reproducibility. FBS can carry zoonotic pathogens which can infect cells in culture. As a biological product, FBS, exhibits batch to batch variations in its composition. The use of FBS is typically one of the largest expenses in cell culture and prices fluctuate with supply and demand.

While the use of FBS and animal serum is ubiquitous in cell culture for its growth promoting properties, it also presents multiple drawbacks (Figure 1.15). Since FBS is a biological product obtained from multiple animals, the composition of nutrients, proteins, and hormones vary [247]. Further, while there are standard processing steps, which include coagulation of blood, centrifugation to clarify the supernatant, and filtration to remove remaining cellular components, there remains a risk for microbial contamination for viruses, prions, or other pathogens. This variability and risk of culture contamination pose challenges in both research and biomanufacturing settings and can reduce experimental consistency and product safety [248], [249].

Ethical and regulatory concerns also undermine the value of FBS use in culture [250]. FBS is collected from pregnant cattle during meat processing, making it a byproduct of the meat industry. Although FBS collection and processing is restricted to government approved facilities across the world, many scientists and activists have pushed for a more humane alternative. Furthermore, some countries prohibit the slaughter of pregnant cattle, resulting in reduced opportunities for fetal serum collection.

Other issues with the demand for FBS in culture media relates to the supply and cost. Since FBS is a byproduct of the meat industry, its availability and price fluctuates with both supply and demand of meat [251]. Indeed, FBS is often one of the largest expenses in cell culture applications. Furthermore, with the rise of cultured meat and plant-based alternatives, the global supply of FBS may decline further, causing increased costs.

The issues and drawbacks of FBS addition in culture media has encouraged efforts to develop a serum-free media optimized for growth in culture [252]. A serum-free media alternative would standardize nutritional support for cells, reduce or eliminate animal-derived components, limit risk of culture contamination, and improve experimental reproducibility- currently hindered by the variability of animal serum. Defined media formulations are promising for reducing ethical concerns of FBS collection and mitigating the concerns of waning supply [247].

1.7.1. Serum alternatives to FBS

Various FBS alternatives have been explored to address the ethical and practical issues of FBS addition in culture media [253]. However, many of the proposed FBS substitutes, including adult animal serum, pituitary extracts, chick embryo extracts, bovine ocular fluid, plant extracts, and even earthworm coelomic fluid are also variable and chemically undefined in nature [247]. While these address some of the ethical concerns of FBS collection and use, they also carry their own drawbacks and the composition of these biological products do not necessarily support growth as well as serum. Other serum-free formulations containing recombinant hormones and other growth factors have also been investigated, yet not have proven to support proliferation across a broad range of cells as FBS [254]. Importantly, although commercially serum-replacement are available, they are typically proprietary, and their exact formulations undisclosed- in effect resulting in the similar issues as the undefined nature of FBS [255].

Efforts to understand and replace serum in cell culture involve systematically testing the requirement of different serum derived growth factors and nutrients that support proliferation in culture. Many such studies identified serum components, such as insulin, transferrin, and selenium (ITS), as general requirements for many cells in culture [256]. Given these uncertainties, many researchers continue to use ITS and ITS-related supplemented under serum-restricted, but not serum-free, conditions [109], [257]. Thus, commercial formulations of ITS were made and are often used in low serum applications. Some ITS formulations include other serum factors, which can aid proliferation in low serum conditions- such as linoleic acid (C18:2), BSA, or trace metals to enhance growth

further. Indeed, ITS remains particularly important in biopharmaceutical production, where minimizing or omitting serum entirely is desirable for consistency and safety.

1.8. Gaps in current understanding

Despite progress in understanding some of the environmental dependencies required for proliferation fulfilled by serum in media, FBS remains a mainstay in most culture systems. Its heterogeneous mix of bioactive compounds influence cellular phenotypes, yet which of these are required for cell growth and which are merely beneficial is not fully understood [258]. This uncertainty is exacerbated by the complex and undefined makeup of serum, underscoring a gap in our knowledge of the exact role in supporting proliferation. Indeed, most high-throughput genetic screens used to identify essential genes under various conditions routinely use serum-containing media, potentially obscuring results [58]. Undefined serum components can mask or modulate key genetic or metabolic dependencies, particularly in the case of the abundant growth factors present in the fetal serum.

While there have been some successful efforts to develop serum-free media formulations tailored to select cell types, no single alternative has proven sufficient for supporting the diverse array of cell lines used in research [259]. This limitation therefore motivates the need for further research into cell type-specific media requirements, particular the role of lipids and other underexplore serum components. Addressing these gaps in knowledge will be critical in moving beyond the reliance of FBS use in culture towards a more defined, ethically sourced, and reproducible culture practices [259].

1.9. Thesis focus

The central aim of this thesis is to explore the critical role of serum in supporting cancer cell proliferation in culture, particularly the role of serum lipids. Because serum can exert powerful- yet incompletely characterized- effects on cell proliferation and metabolism, this work aims to explore how serum lipids contribute to cancer cell proliferation. While the role of select serum constituents are known to affect various aspects of cell biology, the role which serum lipids support cell proliferation and viability in culture remains insufficiently understood.

1.9.1. Central hypothesis:

The guiding hypothesis is that serum lipids play pivotal functions for sustaining cell proliferation. By systematically dissecting the contributions of these lipids, this thesis will explore how exogenous lipid availability drives growth in culture.

1.9.2. Importance of understanding lipid contribution to optimize cell culture

Insights gained into the function of serum lipids in culture have significant implications beyond basic research. In particular, understanding lipid dependencies in culture can inform the biology of cells in vivo and reveal potential strategies to target serum lipid utilization for cancer treatment [260]. Beyond cancer research, investigating how lipids affect cell proliferation and fitness may offer broader benefits for

biomanufacturing purposes, where media optimization is a primary determinant of product yield and quality, and aid in the development of a chemically defined media that mitigates drawbacks from serum use [261].

2. Chapter 2

This chapter is from a manuscript titled, "**Defined media reveals essential roles of lipid scavenging to support cell proliferation**".

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The authors and their affiliations are listed below:

Authors: Oliver J. Newsom[1], Lucas B. Sullivan[1*]

Affiliations: [1]Human Biology Division, Fred Hutchinson Cancer Center, Seattle, WA, 98109, USA. *Correspondence to: lucas@fredhutch.org.

2.1. Abstract

Fetal bovine serum (FBS) is a ubiquitous yet undefined additive in cell culture media, and its poorly defined functional contributions have hindered efforts to develop an alternative to serum supplementation in media. Here, using live-cell imaging and liquid chromatography-mass spectrometry, we show that serum provides consumed factors, particularly metals and albumin-bound lipids, that sustain cell proliferation in culture. Moreover, supplementing basal media with ITS, containing necessary metals, and albumin-associated lipids can replace FBS in media and enabling sensitive quantification of lipid consumption in a defined serum-free system. We observe that fatty acids (FA) are consumed through a mass-action mechanism, with minimal competition from other lipid

classes, and that pharmacologic disruption of FA activation reduces uptake and impairs cell proliferation. Together, this work identifies metabolic contributions of serum in cell culture and provides a platform for building cell culture systems that sustain proliferation without the undefined and heterogenous components of FBS.

2.2. Introduction

Since the origins of cell culture, animal serum has been an essential additive for supporting robust and sustained growth of most cell lines [2], [7]. Among the various serum additives used in culture media, fetal bovine serum (FBS) is the most widely used in biomedical research, primarily attributed for its roles in providing a complex mix of growth factors, lipids, and other nutrients [2], [25]. However, despite the ubiquitous use of FBS, its fundamentally undefined nature poses a challenge for scientific understanding, since the many biologically active components cannot be readily uncoupled for study using traditional culture media formulations. In addition, the inherent batch-to-batch variability of FBS can influence experimental outcomes and thus contribute to reproducibility issues [45], [145]. Aside from its biological influence, FBS also has the potential to introduce zoonotic pathogens into cell culture systems, resulting in safety concerns, particularly in the context of biomanufacturing [248]. Finally, it is unclear whether fetal bovine fluid is relevant for studying post-natal human disease, as its composition may inappropriately influence biological phenotypes and inadequately model the in vivo environment. These issues therefore highlight the need to develop serum-free alternatives for cell culture.

A major hurdle to excluding FBS from cell culture media is that serum components required to sustain cell proliferation remain poorly defined. For instance, although growth factors are a well-known constituent of FBS, the extent to which specific serum-derived hormones drive cell proliferation remains uncertain [262]. In the case of cancer cell culture, the requirement for fetal serum rich in growth factors is questionable, as a hallmark of cancer is constitutive activation of growth factor signaling pathways in the absence of exogenous cues [263]. Moreover, fetal growth factors may impair our understanding of oncogenic signaling in cancer by masking phenotypic effects of alterations to cancer-associated growth factor pathways. Alternatively, FBS may support cancer cell proliferation by providing small molecule nutrients, including lipids, soluble metabolites, vitamins, and trace metals. Indeed, several studies have found that modifying the relative proportion of these molecules in media through various filtering, extraction, and formulation strategies can influence genetic and metabolic dependencies of proliferating cancer cells [58], [61], [264], [265]. The potential nutritional contributions of FBS are also implied when considering that some biologically important vitamins (e.g. biotin and cobalamin) and metals (e.g. iron, zinc, and copper) are absent in many commonly used cell culture media formulations [58], [62]. Indeed, culture media additives that support proliferation in serum restricted conditions often include trace metals or the metal carrier protein transferrin [2], [56]. Nonetheless, the development of a serum-free media that enables robust proliferation across a broad range of cell lines remains a challenge since the role of serum remains unclear. Collectively, these issues underscore the need for a minimal media capable of supporting cell proliferation without the addition of serum, which would facilitate

investigations into the environmental requirements for proliferation, including the metabolic and signaling factors that are otherwise inextricably fulfilled by the numerous serum components.

To explore environmental dependencies of serum for cell proliferation in culture, we combined live cell imaging with liquid chromatography-mass spectrometry (LC-MS) to investigate the role of FBS components— particularly serum lipids—in supporting cancer cell proliferation. Despite the broad range of lipid species in FBS, our findings reveal that cells selectively consume albumin-bound lipid classes during proliferation. Furthermore, combining these lipid classes with cell culture additives that provide micronutrients is sufficient to function as an effective FBS replacement, enabling the uncoupling of metabolic variables that are normally provided by FBS. Our results underscore the critical role of lipids in supporting cell growth and provide a platform to investigate lipid metabolism and other contributions of serum in a defined environment.

2.3. Materials and methods

2.3.1. Reagents

Unlabeled oleic acid (O1008) and palmitic acid (P0500) used in proliferation experiments were purchased from Sigma. U-13C oleic acid (CLM-460), U-13C palmitic acid (CLM-409-0.1), and d11-arachidonic acid (10006758) isotope standards were obtained from Cambridge Isotopes. 5-13C oleic acid (9004089), 4-13C palmitic acid (30550), LPC 18:1 (20959), and LPC 16:0 (10172) used in consumption assays were purchased from Cayman

Chemical. 18:1 LPE (846725P), LPE 16:0 (856705P), LPC 18:1-d7 (791643), LPE 18:1-d7 (791644), and lipids used to generate calibration curves SPLASH® LIPIDOMIX® Mass Spec Standard (330707), LightSPLASH™ LIPIDOMIX® Quantitative MS Primary Standard (330732), were purchased from Avanti Polar Lipids. The chemical inhibitors used in this study GSK2194069 (SML1259) and triacsin C (2472) was purchased from Sigma and Tocris, respectively. FBS lots used for serum lipid comparison were purchased from Cytiva (SH30396.03; Lot No: AH30469640, and AK30775909), Gibco (A52567-01; Lot No: M3009140RP), and Corning (35-077-CV; Lot No:22023001).

2.3.2. Cell culture

Cell lines were acquired from ATCC (143B, H1299, HCT116, HT1080, A549, Jurkat), JCRB Cell Bank (OCUG1), or as a gift from Dr. Supriya Saha, Fred Hutch (CCLP1, SSP25). H1299 NucRFP cells were previously generated, as described in [266]. Cell identities were confirmed by satellite tandem repeat profiling and cells were tested and found to be free from mycoplasma (MycoProbe, R&D Systems). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, 50–003-PB) supplemented with 3.7 g/L sodium bicarbonate (Sigma-Aldrich, S6297), 10% FBS (Cytiva, SH30396.03) and 1% penicillin-streptomycin solution (Sigma, P4333). Cells were incubated in a humidified incubator at 37 °C with 5% CO_2.

2.3.3. Reagent preparations

Numerous cell culture additives containing Insulin-Transferrin-Selenium (ITS) are currently available, often containing supplements beyond its namesake additives, resulting in heterogeneous ITS formulation offerings that are sometimes referred to as "ITS-plus." The ITS mixture used here was that of Insulin-Transferrin-Selenium Solution Plus (100X), Animal Free (GenDEPOT, CA202), which was used as purchased or made in-house by assembling its components (or subsets thereof, as described) into a 100x stock solution containing 0.5 g/L recombinant insulin (Sigma I9278), 0.6 g/L recombinant transferrin (Sigma, T8158), 0.67 mg/L of sodium selenite (Sigma, 214485), 10 mg/L ethanolamine (Sigma, E0135), 2.04 mg/L ferric citrate (Sigma, F3388), 863 μg/L of zinc sulfate (Sigma, 83265) and 1.6 μg/L of copper(II) sulfate (Sigma, C8027) in water. These solutions were used as a spike-in supplement at 1% into culture media. Lipid mixes were made by dissolving lyophilized lipids in 100% MeOH to a final concentration of 100 mM and lipid solutions were aliquoted and stored in glass vials at -20 °C in glass. Lipid solutions were then diluted to a final concentration of 200 μM in DMEM media containing 50 μM Bovine Serum Albumin Fraction V, heat shock, fatty acid free (Sigma, 3117057001) and placed in a flask and shaken at 37 ° C for 1 hour. 200 μM lipid solutions were then diluted in DMEM to the final working concentration. Unless otherwise noted, lipid mixes were combined at a 95:5 molar-ratio of oleate:palmitate FA chains for both FA mix and LPC mix, while LPE lipid mix was a 60:40 molar-ratio of oleate:palmitate FA chains.

2.3.4. Incucyte/proliferation experiments

H1299 NucRFP cells were trypsinized (Corning, 25-051-CI), resuspended in media, counted (Beckman Coulter Counter Multisizer 4 or Nexcelom Auto T4 Cellometer), and seeded overnight onto 24-well dishes (Thermo, 142475) with an initial seeding density of 5,000 cells/well. After overnight incubation, wells were washed 3 times in phosphate-buffered-saline (PBS) and 1 ml of treatment media was added. Experiments were conducted in DMEM (Corning 50-013-PB) without pyruvate, supplemented with 3.7 g/L sodium bicarbonate (Sigma, S5761) and 1% penicillin-streptomycin solution (Fisher, 15-140-163) and 1 mM sodium pyruvate (Sigma-Aldrich, P8574) and supplemented with the indicated treatments. Plates were imaged in real time using the IncuCyte S3, at 20X magnification with a 400 ms exposure for the red channel and set to capture images every 6 hours. For standard proliferation assays, initial and final cell counts were collected to calculate proliferation rates.

2.3.5. Consumption experiments

Cells were seeded on 6-well dishes (Corning, 087721B) in DMEM containing 10% FBS. The following day, the wells were washed 3 times in PBS before swapping cells into 2 ml of the indicated media. Plates were placed back in the incubator for 1 hour to allow for the cells and media to equilibrate. For consumption experiments in 10% FBS/DMEM, media was collected at the indicated time and cells were counted. For consumption experiments in the serum replacement media, cell counts were collected from a parallel plate at time 0 and 100 μl of media was collected at 2-hour intervals for 6 hours. At the final time point, cell

counts were collected from the well to calculate cell hours and proliferation rates during the assay.

2.3.6. Lipidomic sample preparations

Media samples were extracted using a single-phase extraction protocol, where 30 μl of media was combined with a 270 μl of 2:1 ratio of ethyl acetate (Thermo, 022912.K2):2-propanol (Sigma, 34863) containing isotopically labeled lipid standards in glass conical vials (Microsolv technology, 9512S-0CV-T-RSD), resulting in a final extraction solvent of 6:3:1 ethyl acetate: 2-propanol: water. Samples were vortexed for 10 minutes and centrifuged at max speed to pellet debris. 200 μl of supernatant was transferred to a clean glass vial before drying down using a refrigerated vacuum concentrator (Fisher, 10269602). Once dry, lipids were resuspended in 100ul of 65:30:5 acetonitrile (Sigma, 34851): 2-propanol: water and transferred to glass autosampler (Fisher, 03-452-330) vials for mass spectrometry analysis. Mass spectrometry runs were analyzed using Skyline software for metabolomics analysis [267]. Semi-targeted lipidomics analysis of serum lipids was normalized to Avanti SPLASH deuterated standard for each class, whereas targeted consumption assays included standards for each individual lipid.

2.3.7. Isotope tracing

H1299 cells were seeded in a 6-well dish at an initial density of $2x10^5$ cells per well. The following day, cells were washed twice with PBS and swapped to DMEM without glucose, glutamine, pyruvate, or phenol red (Sigma, D5030) supplemented with 10% dialyzed FBS (Sigma, F0392), 1% penicillin-streptomycin, 25 mM U-^{13}C glucose (Cambridge Isotopes

Laboratory, CLM-1396), and 4 mM ^{12}C glutamine (Sigma, G5792). GSK2194069 treated cells were supplemented with 200 nM of GSK and all plates were placed back in incubator for 24 hours before extraction.

2.3.8. Complex lipid analysis

A 2 μL injection of sample (held at 10°C in the autosampler) was made onto a hypersil gold column (1.0x150mm with a 1x10mm guard, at 50°C), and was eluted (from 32% to 97% "B" over a 36-minute total run time) using a multi-step gradient. The composition of the mobile phases used consists of "A" = water:acetonitrile at 60:40 v/v with formic acid at 0.1% and 10mM ammonium formate, and "B" = acetonitrile:IPA at 10:90 v/v containing 10mM ammonium formate with formic acid at 0.1%. Eluted analytes were analyzed with a Q-Exactive HF-X at 120k resolution in the MS1 mode in both positive & negative polarities in the same run, with ddMS2 performed on the top 15 precursors at 30k resolution with stepped NCE energies of 25 and 30 respectively. Identification of lipids was made by accurate mass comparison to an in-house database of standards and validated by comparison of the MS2 spectra to predicted fragmentation patterns. Prior to data collection, the performance of the instrument and chromatographic system were evaluated for retention time consistency and signal intensity using an injection of 1,2-distearoyl-sn-glycero-3-phosphocholine (PC 18:0) solubilized in 50:50 (v/v) mobile phase A and B. Additionally, instrument performance and mass calibration are evaluated throughout the sample run by a daily injection of Lipidomix SPLASH mix (Avanti Polar Lipids) containing 14 deuterium-labeled lipid species.

2.3.9. Fatty acid analysis

Post extraction, fatty acid samples were re-suspended in acetonitrile/IPA/water 65:30:5 v/v/v, and a 2 μL injection was made onto a Kinetex C8 core-shell column (2.1 x 100 mm, 2.6 μm, and attached 2.1 mm i.d. SecurityGuard Ultra C8 guard column). The autosampler is held at 10°C, while the column is kept at 40° C through the run. The mobile phase composition consisted of "A" = water with acetic acid (at 0.1%) mixed with 5% (by volume) of mobile phase "B", where "B" = acetonitrile/methanol/0.1% acetic acid (80:15:5 v/v/v). A semi-isocratic gradient is used to elute the analytes off the column, beginning at 20% for 0-1 minute, followed by a rapid increase of "B" to 66%, which is then held for 6.5 minutes, followed by a further increase of "B" to 100%. Finally, a short wash phase is followed by a re-equilibration phase of 7 minutes at starting conditions (20%), with total injection-to-injection times of 22 minutes. Eluted analytes were detected with the Q-Exactive HF-X in MS1 mode over a mass range of 210-600 m/z using negative polarity. Analytes were identified by accurate mass comparison to an in-house database of standards. Prior to analysis and throughout the sample series, instrument performance is evaluated using a mixture (diluted 1:4 using 50:50 mobile phase "A" and "B") containing 10 monounsaturated fatty acids at 10 ng/μL each (Cayman Chemical). Mass calibration, retention times and peak intensities were monitored to ensure consistent performance. To compensate for contamination from background saturated and mono-unsaturated FAs, multiple volumes of media were extracted to generate a dose response curve, allowing for the calculation of serum-attributable FA content.

2.3.10. Quantification and statistical analysis

All graphs and statistical analyses were performed in GraphPad Prism 9.0. Technical replicates, defined as parallel biological samples independently treated, collected, and analyzed during the same experiment, are shown. Experiments were verified with independent repetitions showing qualitatively similar results. Details pertaining to all statistical tests can be found in the Figure legends.

2.4. Results

2.4.1. Serum provides essential consumable factors to support cell proliferation

FBS is a key additive to culture media for supporting cell proliferation, yet the mechanism by which serum-restriction impairs cell proliferation remains incompletely understood. Cell proliferation measurements typically utilize initial and end-point cell counts to calculate proliferation rates while assuming a constant doubling time throughout the experiment, which is unlikely to be a reasonable assumption in all settings. For instance, we propose two hypothetical models of proliferation defects that can result in the same final cell count: (1) A rate-limitation phenotype, where restriction of growth factors results in a slowed but constant proliferation rate, or (2) a depletion phenotype, where proliferation rate remains stable initially but decreases once essential serum factors are exhausted (Fig S1A). Given that these two models reflect different biological mechanisms, we hypothesized that understanding the kinetics of proliferation defects during FBS restriction may inform how FBS supports cell proliferation.

To investigate the contribution of FBS to proliferation, we expressed nuclear-localized red fluorescent protein in H1299 non-small cell lung cancer cells (H1299 NucRFP) and used live-cell imaging to track nuclei counts in real-time as a readout of cell proliferation in DMEM media with various FBS concentrations. As expected, FBS was required for sustained cell proliferation, and final cell counts were proportional to media FBS concentration (Fig 1A, left). Interestingly, when comparing the kinetics of cell population growth over time, initial increases in cell counts were similar across all FBS concentrations, however the rate of cell population growth slowed over time proportional to FBS concentrations (Fig 1A, right). When we converted changes in cell count to time-resolved proliferation rates, the kinetics closely matched those predicted for a depletion phenotype (Fig 1B, Fig S1A). Notably, upon slowing, the rate of proliferation decay was comparable in each case of FBS limitation, with the primary difference being that the onset of proliferation defects occurred later in conditions with higher FBS concentrations (Fig 1B). These findings therefore support the hypothesis that FBS restriction impairs cell proliferation by limiting consumable components necessary for sustained proliferation.

Since basal media lacks numerous components of FBS that could support proliferation, we next tested whether supplementation with a defined additive containing insulin, transferrin, selenium, ethanolamine, and biologically relevant metals (ITS, see methods), a mixture shown to shown to support cell proliferation in serum restricted conditions, could similarly restore cell proliferation to these cells during FBS limitation [109], [110]. Indeed, ITS was sufficient to fully restore the proliferation rate kinetics of

H1299 NucRFP cells cultured in 0.5% FBS to match that of cells cultured in 10% FBS (Fig 1C). We next investigated which component(s) of the ITS mixture mediated the proliferation restoration in low FBS conditions and observed that transferrin and metals are the dominant contributors to H1299 NucRFP proliferation upon serum-restriction (Fig S1B). Consistent with the limitation of these micronutrients in low serum conditions, titrating down the concentration of ITS in 0.5% FBS restored the depletion phenotype (Fig S1C). These data therefore indicate that a primary limitation in serum restricted conditions is the depletion of trace metals that are not otherwise provided in the basal media. We next tested whether adding ITS could replace FBS altogether. Although ITS improved the overall number of doublings compared to DMEM alone, these cells still exhibited a decaying proliferation rate indicative of a delayed depletion phenotype (Fig 1D). This suggests that while ITS is beneficial under reduced FBS conditions, presumably by solving the first order deficiency for metals, it cannot replace FBS entirely, as it lacks an additional essential serum component that is required to sustain cell proliferation.

We next explored how other factors in FBS may contribute to cell proliferation of ITS treated cells in basal media. Serum-derived lipids serve as a metabolic resource that are utilized during cell proliferation which can be conditionally essential during metabolic stress [124], [126], [179], [264]. Thus, we tested the impact of lipid supplementation by adding a fatty acid (FA) mix composed of palmitate and oleate, the two most abundant saturated and mono-unsaturated FA species in serum, conjugated to bovine serum albumin (BSA) [128], [268]. Like ITS, FA mix improved overall cell proliferation relative to basal media alone, but cell proliferation rate eventually decayed over time (Fig 1E).

Importantly, the co-supplementation of ITS with FA mix in basal media prevented cell proliferation rate decay and was sufficient to maintain exponential growth in the absence of FBS, albeit with a modest rate limitation phenotype compared to cells cultured in 10% FBS (Fig 1E, Fig S1D). We also investigated if this combination could support the proliferation of a diverse group of cancer cell lines, including cells deriving from non-small cell lung cancer (A549), osteosarcoma (143B), cholangiocarcinoma (CCLP-1), and colon cancer (HCT116). Measuring proliferation rates by cell count, we observed that the combination of ITS and FA mix was sufficient to support serum-free cell proliferation in each cell line (Fig 1F). While the addition of FBS to culture media still adds convenience for various cell culture functions, including inactivating trypsin while splitting adherent cells and providing attachment factors that encourage cells to adhere to culture dishes, we found that this FBS replacement formulation can maintain robust cell proliferation in Jurkat suspension cells for greater than four weeks (Fig S2). These findings therefore highlight that the requirement for serum can be largely obviated for cancer cell culture experiments by providing a source of metals and fatty acids, which are otherwise absent from basal media (Fig S1D).

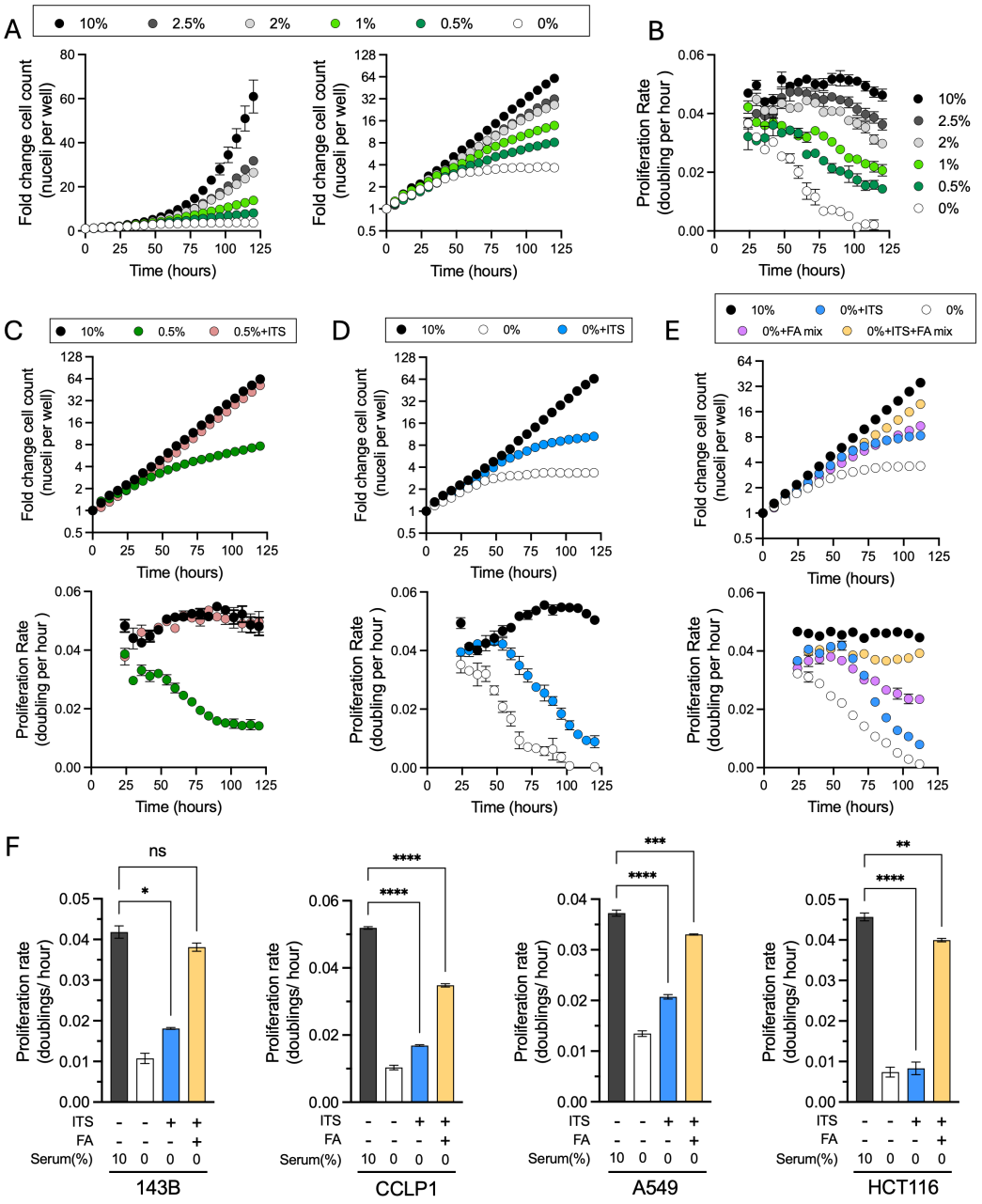

Figure 1. Effects of serum limitation on cancer cell proliferation rates.

(A) Fold change in H1299 NucRFP cell counts over time when cultured with varying concentrations of FBS. Data are shown on a linear scale (left) and a log2 scale (right). **(B)** Moving average of the proliferation rate (calculated from four adjacent time points) for cell nuclei counts measured in (A). **(C)** H1299 NucRFP cells grown in 10% FBS or 0.5% FBS treated with vehicle or ITS. Fold change in cell counts (top) and moving average of proliferation rate (bottom) **(D)** H1299 NucRFP cells cultured in 10% FBS or in DMEM alone, treated with or without ITS. Fold change in cell counts (top) and moving average of proliferation rate (bottom) **(E)** H1299 NucRFP cells cultured in 10% FBS or in DMEM alone and treated with either a FA mix (100 µM), ITS, or both. Fold change in cell counts (top) and moving average of proliferation rate (bottom) **(F)** Proliferation rates of A549, CCLP1, HCT116, and 143B cells grown in 10% FBS compared to DMEM supplemented with either ITS or a combination of ITS and FA mix, measured by initial/final cell counts. Error bars indicate mean ± SEM (n=3). Abbreviations: ITS, insulin–transferrin–selenium mix that also contains ethanolamine and trace metals; FA, fatty acid mix. Statistical significance was assessed using one-way ANOVA Brown-Forsythe and Welch ANOVA tests (F). ns = not significant, *p < 0.05, ****p < 0.0001.

2.4.2. FBS lipidome and its consumption by proliferating cells

While our data demonstrate that exogenous FAs can fulfill a major role of FBS of providing lipids to support growth in culture, FBS is a heterogeneous mixture that contains a wide repertoire of lipid species and so it remains unclear which serum lipids are normally consumed by cultured cells. Because multiple lipid species may redundantly support proliferation, we next examined the lipid composition of FBS and measured which lipids are consumed by proliferating cells. The serum lipidome includes diverse lipid species which vary in the number of FA chains each lipid contains, lipid head group, as well as the length and saturation of the hydrocarbon chains [269]. To characterize the major lipid classes in FBS and their variability across different FBS lots, we used semi-targeted quantitative LC-MS to profile lipid metabolites in four different FBS lots (Fig 2A). Consistent with prior reports, the most abundant lipid classes included neutral lipids, where sterol esters (SE) were much more abundant than triacylglycerols (TAG) [270]. Additionally, polar lipids like sphingomyelin (SM), and glycerophospholipids, including phosphatidylethanolamine (PE), phosphatidylcholine (PC), and their precursor lysolipids, lyso-PC (LPC) and lyso-PE (LPE) were also detected in all FBS samples. Importantly, while the detected lipid classes were consistent across FBS lots, the relative and total abundance of lipid classes varied between different lots of FBS. These findings therefore demonstrate the inherent variability of using serum in cell culture settings and highlight the need to explore the roles of FBS-derived lipids in a more controlled system.

We next sought to determine which serum lipids are consumed by proliferating H1299 NucRFP cells. To focus on the lipids present in FBS at biologically relevant concentrations

for proliferation, we ranked the detected lipids from most abundant to least and excluded those that constituted the bottom 1% of the total available FBS lipid pool, narrowing the analysis from 563 species to 163 lipids spread across eight classes, varying in FA chain length and degree of saturation (Fig 2B-C, S3). Among these, SEs were the most abundant lipid class, which were quantified at 3.22 +/- 0.098 mM, while TAGs were the least abundant lipid class, at 16.73 +/-0.67 μM. PEs, the most abundant glycerophospholipid detected, and PCs were present at 381.93 +/-8.24 μM and 164.78 +/- 2.16 μM, respectively; while SMs were detected at 49.36 +/- 1.02 μM. The lipid precursors, LPC and LPE, were detected at lower concentrations of 108.69 +/- 1.36 μM and 23.65 +/- 0.74 μM. Although background from unlabeled saturated and monounsaturated FAs is a known challenge in lipidomics that affects accurate quantification, we quantified FAs at 113.40+/-7.03 μM, representing only a small fraction of the total serum lipid pool despite sufficiency in supporting proliferation in the serum-substitute media.

To identify which FBS-derived lipids are consumed from the media during proliferation, we collected spent media from cells cultured in 10% FBS containing media at multiple time points and profiled the change in the media lipidome over time. Notably, only a subset of available lipids showed meaningful depletion relative to their starting concentration. Specifically, the lipid precursors containing only a single fatty acid, which include FA, LPC, and LPE were selectively consumed, with > 80% depletion in most species relative to the starting amount over 96 hours (Fig 2D). In contrast, the complex lipids including SE, TAG, PC, PE, and SM exhibited minimal change with <5% depletion in most species during the same period. Notably, the pattern of media lipid consumption was closely associated with

the mode of transport in serum. Indeed, highly consumed lipid species including FA, LPC, and LPE, are primarily transported bound to BSA, whereas SE, TAG, PC, PE, and SM are carried in lipoprotein complexes [102], [151], [270], [271], [272]. To determine whether selective albumin-associated lipid consumption is specific to H1299 NucRFP cells or represents a broader phenotype of proliferating cancer cells, we profiled media lipids during growth across multiple cell lines. Notably, depletion patterns were broadly similar across all cell lines tested, with cells exhibiting selective depletion of albumin-bound lipids and minimal consumption of lipoprotein-associated lipids during proliferation (Fig S4). Altogether, the preference for albumin-bound lipids during proliferation across various cells suggests that these lipids fulfill a general metabolic requirement for cancer cell proliferation in culture.

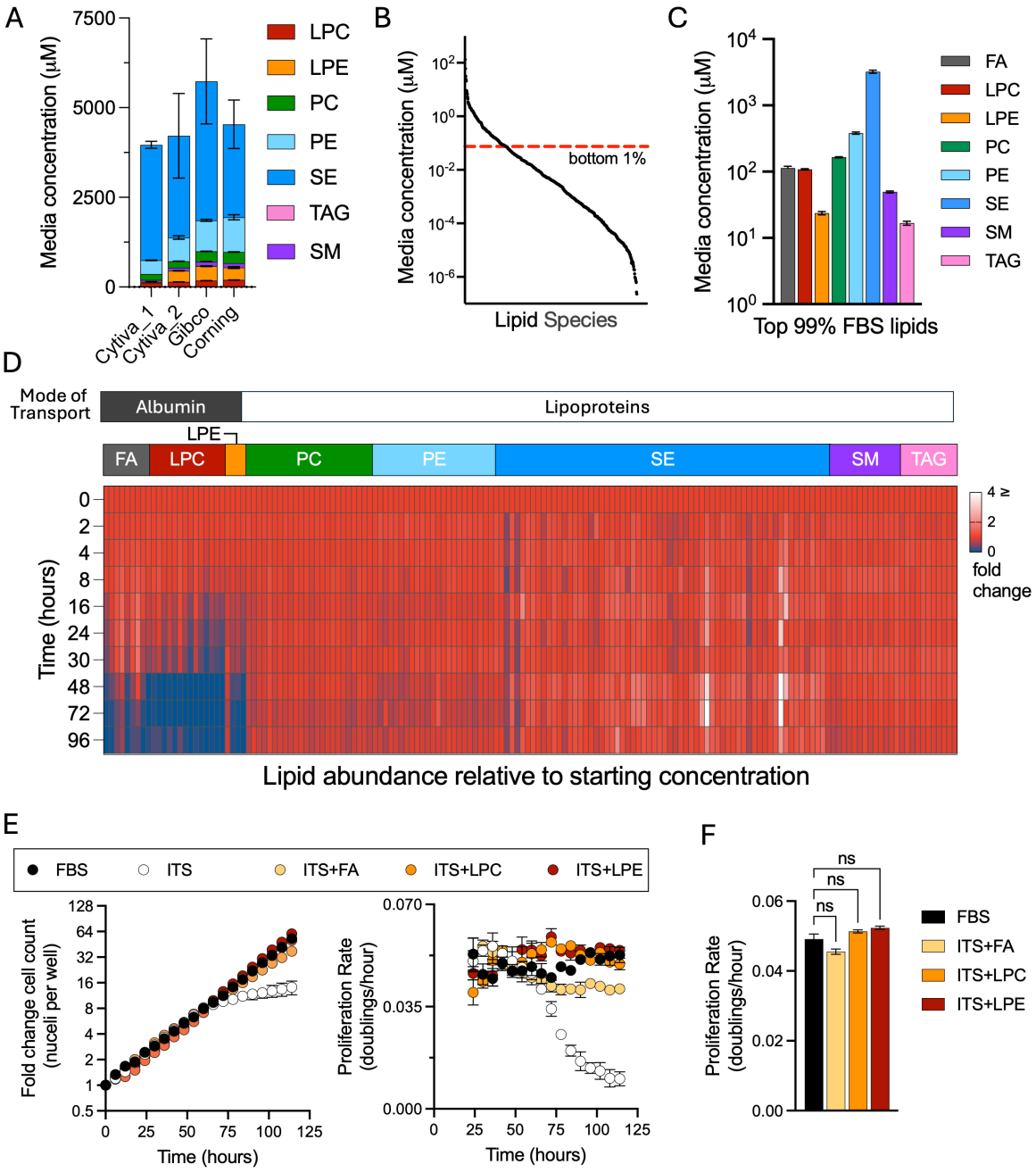

Figure 2. Quantification of lipid depletion during cell proliferation.

(A) Comparison of lipid profiles across different lots of commercially available FBS. **(B)** All detected lipids ranked from most abundant to least abundant, where lipids which fall below the red line indicate the bottom 1% of total lipids in FBS. **(C)** FBS lipid concentration in 100% FBS used in the remaining experiments. **(D)** Relative concentrations of the top-most abundant lipids normalized to initial concentrations during cell proliferation. **(E)** Proliferation of H1299 cells cultured in either 10% FBS or DMEM supplemented with ITS plus one of the following: LPC, LPE, or FA mix. Fold change in cell counts (left) and moving average of proliferation rate (right). **(F)** Average proliferation rate of cells over the entire assay. Error bars represent mean ± SEM (n = 3). Abbreviations: SE, sterol esters; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingolipids; TAG, triacylglycerols; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; FA, fatty acids. Statistical significance was assessed using one-way ANOVA Brown-Forsythe and Welch ANOVA tests (F). ns = not significant.

2.4.3. Consumed lipid species redundantly fulfill the lipid requirements of serum

Our data indicate that FAs can fulfill an essential function of FBS in supporting cell proliferation, yet in the context of FBS-containing media, FAs are consumed alongside LPC and LPE lipid species. We thus sought to determine whether these albumin-associated lipid classes were redundant or distinct in their ability to support cell proliferation. We therefore cultured H1299 NucRFP in serum-free media containing ITS, with or without co-supplementation of individual albumin-associated lipid classes and tracked their effect on proliferation kinetics (Fig 2E). Notably, like the combination of ITS and FA mix, both the LPC and LPE lipid mixes were equally sufficient to fulfill the lipid demand for cell proliferation and prevent the depletion phenotype of cells cultured in the absence of serum. Indeed, the average proliferation rate was not statistically different between the cells grown in 10% FBS and any of these serum-free medias (Fig 2F). These data therefore indicate that albumin-associated lipid classes can redundantly fulfill the lipid requirement for cell proliferation.

2.4.4. Lipid consumption dynamics are a function of environmental availability

The mechanisms and variables that dictate lipid consumption remain poorly understood. We used our serum-free media formulation as a tool to further explore the factors that influence lipid consumption of proliferating cells, using serum-free media containing FAs and/or LPCs as a lipid source. We first cultured cells across a dilution series of FA or LPC mixes and quantified consumption rates. Similar to 10% FBS, cells grown in serum-free media readily consume exogenous FAs and LPCs (Fig 3 A-B). Notably, the consumption rate for both FA and LPC increased proportionally with media concentration,

indicating that lipid consumption is predominantly a function of concentration and cells can scavenge lipids at increased rates when they are more abundant in the environment.

Our data indicate that lipid consumption rates within a lipid class are primarily concentration dependent, but it is unclear if consumption of one class influences consumption of other classes. We next sought to explore how the availability of FA-containing molecules, specifically FAs and LPCs, impacted the consumption of each other. We cultured cells across a dilution series containing an equimolar concentration of FAs and LPCs and compared the consumption rates to those measured when cells were grown in media containing a single lipid class (Fig 3 C-D). Surprisingly, despite sharing BSA as a carrier molecule, FA and LPC uptake was unaffected by the presence of the other lipid class, further supporting non-competitive, concentration-dependent lipid consumption dynamics for both lipid classes. To further explore lipid intrinsic variables affecting lipid consumption, we tested if consumption was selective for FAs based upon chain structure. We thus cultured H1299 nucRFP cells in a fixed concentration of total FAs while varying the molar ratio of palmitate to oleate and quantified consumption of each lipid individually. Interestingly, consumption of each FA followed a similar trend as other uptake variables - showing that, independent of FA chain length or saturation, FA uptake was instead primarily dependent on environmental concentration (Fig 3E). Together, these findings indicate lipid scavenging of FAs and LPCs occurs through a class-independent, mass-action transport mechanism which is unaffected by the FA structure.

Lipids bound to albumin are in dynamic binding equilibrium, and unbound free-FAs are considered the primary form scavenged by cells [273]. Thus, the availability of lipids for

uptake is suggested to be influenced not only by the concentration of lipids in the environment, but also the concentration of albumin, which compete for FA binding with the cell [177]. We therefore examined how BSA concentration affects FA consumption by culturing cells in media containing a fixed concentration of FA with varying amounts of BSA in the media (Fig 3F). Consistent with previous reports, the consumption of FA from the media scaled proportionally with the FA:BSA ratio, where a higher FA:BSA ratio was associated with increased FA consumption, despite lower BSA available to cells [273]. These data are consistent with a model in which, as the FA binding sites on BSA approach saturation, the concentration of free-FA increases, leading to proportionally higher lipid consumption. Thus, higher FA:BSA ratios drive increased lipid consumption, supporting the finding that available FA concentration primarily governs consumption rates. Altogether, these data provide insight into the factors governing lipid consumption by proliferating cells and demonstrate the utility of a defined serum substitute media to isolate the variables governing nutrient uptake.

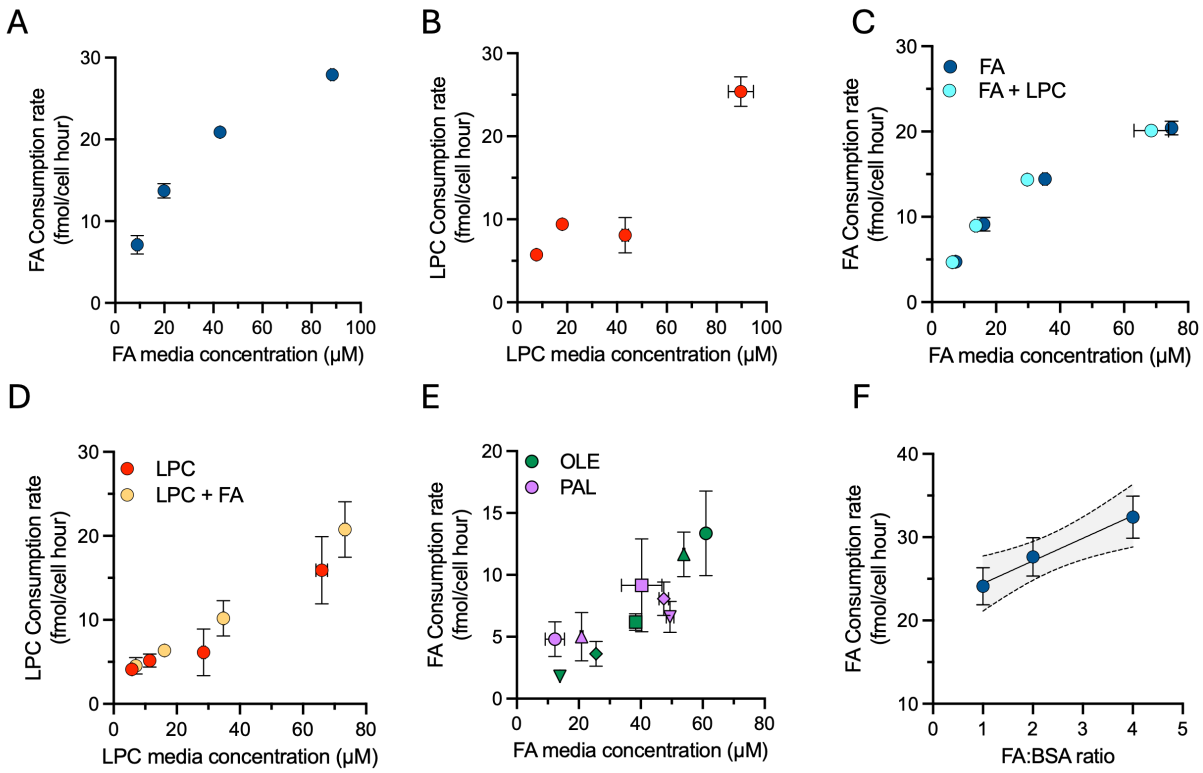

Figure 3. Exploration of lipid scavenging kinetics in serum-free systems.
(A–B) Consumption rate of total FA (A) and LPC (B) by cells cultured in serum-free media across a concentration series of either lipid mix. **(C–D)** Consumption rates of FA (C) or LPC (D) by cells cultured in serum-free media containing an equimolar mixture of FA and LPC, compared to cells grown in media containing only a serum-free media containing a single lipid class of either FA (C) or LPC (D). **(E)** Consumption rates of palmitate and oleate in cells grown in a fixed concentration of total FA concentration with different molar ratios of oleate-to-palmitate (indicated by matching shapes: ○ 4:1 △ 2:1 □ 1:1 ◇ 1:2 ▽ 1:4). **(F)** Consumption rate of FA in cells cultured at a fixed concentration of FA with varying concentrations of BSA. Error bars represent mean ± SEM (n = 3). Abbreviations: OLE, oleate; PAL, palmitate.

2.4.5. Lipid scavenging is required for cancer cell proliferation

Metabolic demand is typically a major determinant of nutrient consumption, yet our data indicate that lipid consumption is driven by environmental concentration [274]. Thus, we next sought to explore whether perturbations to cellular metabolism in FA synthesis or FA activation influenced lipid consumption. We used GSK2194069 (GSK), a fatty acid synthase (FASN) inhibitor, or triacsin C (TriC), a pan inhibitor of the acyl-CoA long-chain (ACSL) family of proteins, which catalyze the condensation of FAs with coenzyme A (Fig 4A) [275], [276]. Using isotope tracing, we confirmed that GSK was effective in preventing *de novo* palmitate synthesis, as evidenced by the loss of isotope incorporation from U-^{13}C glucose into palmitate (16:0), the first product of FASN-mediated FA synthesis (Fig 4B). GSK treatment also blocked higher order labeling species of the downstream FA oleate (18:1), but did not abolish the amount of oleate with the M+2 isotopologue (Fig 4B). These data demonstrate on-target inhibition of FASN, as cells maintained the capability to elongate/desaturate unlabeled upstream scavenged FAs to produce oleate. Interestingly, despite substantially impairing FASN activity, GSK had no effect on the cell proliferation of H1299 nucRFP cells cultured in FBS containing media, indicating that *de novo* fatty acid synthesis is not required in these conditions (Fig 4C).

FASN impairment had no effect on cell proliferation in standard culture conditions, prompting us to investigate how disruption to lipid metabolism influences FA sourcing. We thus cultured cells across a dilution series of FAs in the presence of GSK or TriC, and quantified FA consumption in the media. Surprisingly, inhibiting FASN-dependent lipid synthesis had no measurable impact on FA consumption, in line with our earlier observation

that lipid uptake is primarily determined by lipid availability (Fig 4D). In contrast, TriC treatment resulted in near complete loss of FA consumption from the media at all concentrations, consistent with an essential role of ACSL-dependent FA activation for entry of scavenged FAs into metabolic networks. These data therefore indicate that FA consumption occurs independent of perturbations to endogenous FA synthesis and, in contrast, can be directly blocked by disruption of proteins involved in the scavenging pathway.

As we previously demonstrated that lipid scavenging is essential for proliferation, we next tested the consequence of GSK and/or TriC treatment on proliferation kinetics. Consistent with the dominant role of lipid scavenging in supporting cell proliferation, treatment with GSK had no effect on the proliferation kinetics of H1299 NucRFP cells, while TriC treatment impaired cell proliferation (Fig 4E). Notably, when lipid uptake capacity was constrained by TriC treatment, GSK co-treatment now caused a modest additional antiproliferative effect. These data indicate that, while fatty acid synthesis capacity appears unable to meet lipid synthesis demands on its own, it can modestly contribute to proliferation when lipid uptake is constrained. Finally, to assess whether the relationship between lipid synthesis and scavenging is conserved across various cell lines, we tested the effects of GSK and/or Tri C on 143B, CCLP1, A549, HCT116, and HT1080 cells grown in the serum-free media by cell counts (Fig 4F). As with H1299 cells, all cell lines showed sensitivity to TriC treatment and were insensitive to GSK treatment alone. Interestingly, however, there were heterogeneous responses to the combination of TriC and GSK, with some cell lines showing additional proliferation defects upon GSK co-treatment (H1299,

143B, CCLP1) while others were unaffected (A549, HCT116, HT1080), presumably reflecting cell intrinsic differences in lipid synthesis capacity (Fig 4E-F). Collectively, these findings demonstrate that lipid scavenging is essential for proliferation and that *de novo* lipid synthesis is dispensable in the presence of exogenous lipid sources.

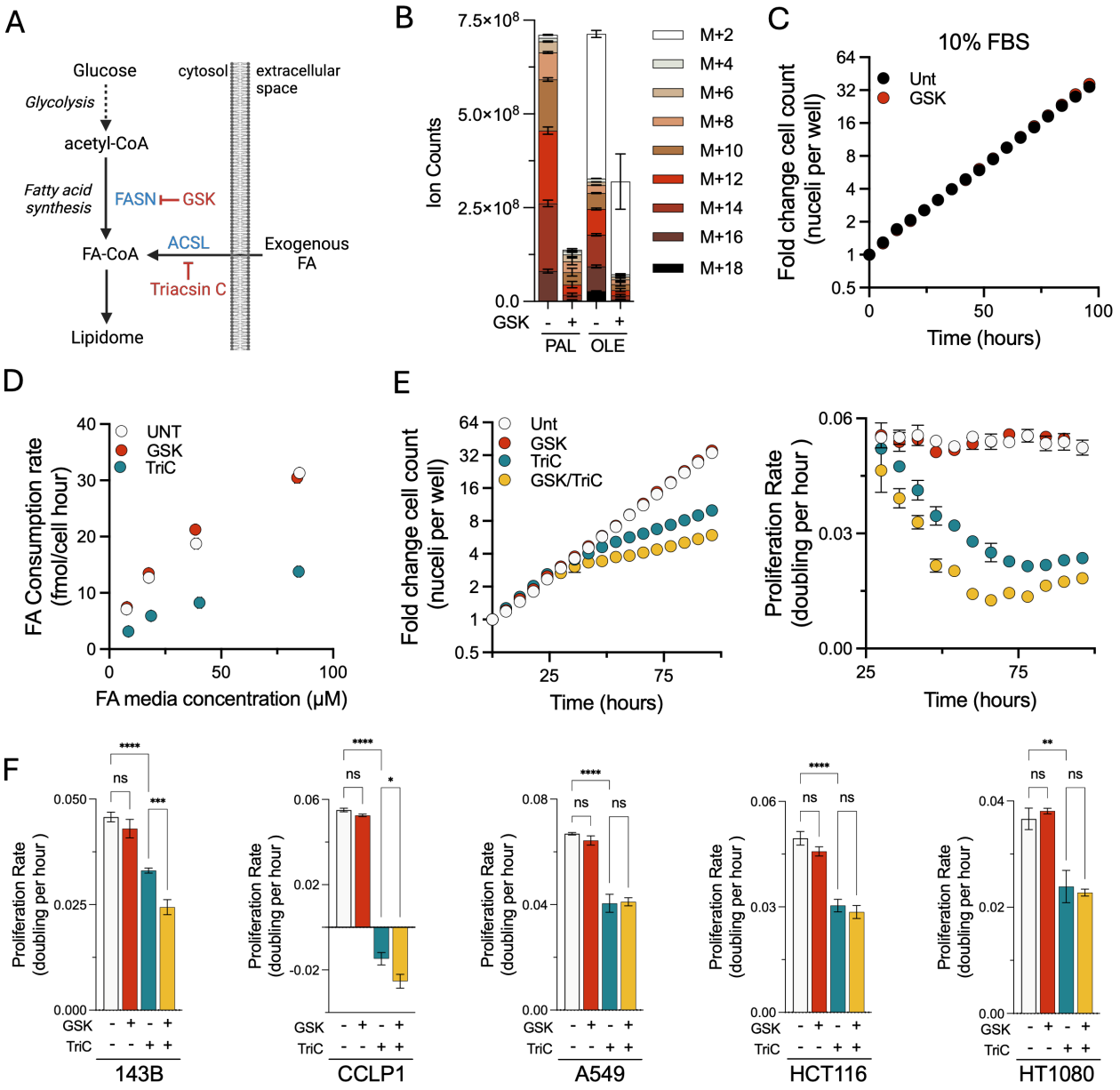

Figure 4. Disruption of fatty acid activation limits lipid scavenging.

(A) Schematic of FA sources into the cellular lipidome with steps inhibited by GSK and triacsin C. **(B)** Isotopologue distribution of palmitate (C16:0) and oleate (C18:1) in cells cultured in U-13C labeled glucose with or without GSK (200 nM). **(C)** H1299 NucRFP cells grown in 10% FBS containing media treated with or without GSK. **(D)** Consumption rate of FA by cells cultured across a FA dilution series treated with either vehicle, GSK, or triacsin C (4 μM). **(E)** H1299 NucRFP cells grown in serum-free media containing FA treated with either GSK, triacsin C, or both. Fold change in cell counts (top) and moving average of proliferation rate (bottom). **(F)** Average proliferation rate of A549, CCLP1, HCT116, 143B, and HT1080 cells grown in FA containing serum-free media treated with GSK or triacsin. Abbreviations: PAL, palmitate; OLE, oleate. Error bars represent mean ± SEM (n = 3). Statistical significance was assessed using one-way ANOVA Brown-Forsythe and Welch ANOVA tests (F). ns = not significant, *p < 0.05, ****p < 0.0001.

2.5. Discussion

FBS, a ubiquitous additive in culture media, supports proliferation across numerous cell types; however, the specific serum components needed for growth are not well defined. Here, we combined live-cell imaging with LC-MS to show that metals and albumin-associated lipids in serum are crucial factors for sustaining cancer cell proliferation in culture. We observed that albumin-bound lipids, such as FA, LPC, and LPE, were rapidly consumed from the media, while lipoprotein-transported lipids were minimally depleted during proliferation. Furthermore, we found that a defined mixture of insulin, transferrin, selenium, ethanolamine and various trace metals was an effective serum replacement (predominantly for providing metals) when combined with albumin-associated lipids, providing a tool to investigate lipid consumption in a controlled lipid environment. Using this defined media, our results support a model whereby albumin-associated lipid consumption occurs in a mass-action manner independent of FA structure, with minimal competition with other lipid classes. This uptake did not correspond to lipid demand, as inhibiting *de novo* lipid synthesis had minimal effects on lipid scavenging, however direct disruption of components in the scavenging pathway blocked lipid uptake and impaired cell proliferation.

Our results contribute to the development of serum-free tissue culture media, which could have benefits for research beyond the cost savings and ethical considerations of omitting FBS [255]. For instance, recent data has shown that many genetic dependencies are influenced by the nutrient composition in the microenvironment; therefore serum-free conditions provide an opportunity to uncouple the numerous metabolic variables inextricably supplied by FBS [58], [277]. Although it could be argued that the molecular

complexity of FBS more closely models *in vivo* conditions, the composition and abundance of bioactive molecules may also be inappropriate for modelling the cellular microenvironment experienced in an adult human and thus may mask important phenomena. In addition, our work is in agreement with other related studies in finding that FBS lots exhibit substantial variation in their metabolomic profiles, particularly lipids, which also significantly differs from human serum [45], [128]. This work is thus a step towards defined media conditions, which may facilitate future studies using genetic screens in cells grown in defined media to uncover which metabolic and signaling pathways are buffered by serum constituents, thereby potentially identifying additional serum factors that support proliferation in other contexts.

One of the major findings from using a serum-free media formulation to explore lipid consumption is that FA scavenging in proliferating cells is primarily driven by the concentration of exogenous lipids rather than by selective uptake based upon lipid structure. Indeed, treatment of cells with increased levels of FAs is associated with several effects on cell physiology including driving lipid droplet formation, causing lipotoxicity when excess saturated FAs accumulate intracellularly, and promoting ferroptosis when excess polyunsaturated FAs are enriched in membrane lipids [186], [192], [278], [279]. The vulnerability to these outcomes based on lipid environment thus supports our conclusion that FA consumption rate is primarily determined by exogenous availability rather than metabolic need, making cells susceptible to the abundance and composition of environmental lipids. This results therefore underscore the potential utility of nutritional

approaches that may modify the tumor lipid microenvironment to promote cancer cell lipid states that may be favorable for therapeutic targeting.

While proteins involved in FA and LPC transport (e.g. CD36 and MFSD2a) have been implicated to exhibit substrate preference based upon FA structure, it is unclear if this leads to relevant differences in substrate consumption during cell proliferation [205], [217]. Consistent with the minimal selectivity of scavenging based on fatty acyl structure, screens aimed at identifying lipotoxicity modifiers have predominantly uncovered factors that regulate intracellular lipid homeostasis, such as those involved in lipid droplet formation and phospholipid remodeling, rather than lipid transporters [195], [196]. Moreover, because lipid consumption is proportional to exogenous concentrations, interpretations of selective lipid uptake may instead reflect differences in initial abundance and lipid stability.

Despite the increase in lipogenic enzyme expression commonly observed in cancer cells, our data underscore an indispensable role of exogenous lipids in fulfilling the lipid requirement for proliferation [280], [281]. Indeed, we observed exogenous lipid sources sustained serum-free cultivation of cells, and our results are consistent with the minimal effects of disruption to *de novo* lipid synthesis on cell proliferation when exogenous lipids are available [282]. The critical nature of lipid scavenging is further supported by the modest clinical efficacy of FASN inhibitors as a monotherapies for cancer treatment, as lipid-rich microenvironments *in vivo* may enable efficient lipid scavenging that diminishes the importance of *de novo* lipid synthesis [182], [283], [284]. Indeed, cells cultured in physiologically relevant concentrations of lipids, which is approximately an order of magnitude greater than what is present in media containing 10% FBS, have been shown to

preferentially incorporate scavenged lipids into cellular membranes during proliferation [128], [179]. Further, we identify the ACSL family of proteins as crucial mediators of exogenous FA scavenging through metabolic trapping. Indeed, disruption of FA activation with triacsin C abolishes FA scavenging, consistent with the essentiality of FA condensation with coenzyme A for entry into the cellular lipidome [204]. Importantly, previous studies using genetic and pharmacological perturbations to ACSL family members indicate these proteins are crucial in FA incorporation into neutral lipids and phospholipids; our data additionally directly implicates this family of proteins in the FA scavenging pathway [244]. Interestingly, we find that only when FA scavenging was constrained could FA synthesis detectably contribute to cell proliferation, albeit heterogeneously across cell lines. Together, these findings suggest that targeting lipid scavenging could be a more effective therapeutic approach to target the increased lipid demand of proliferating cancer cells, though any such strategy would have to overcome the redundancy of alternate albumin-bound lipid sources.

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3. Chapter 3

3.1. Conclusions

Our study explored the role of serum in supporting cell proliferation by combining live-cell imaging with LC-MS to measure the depletion of lipids overtime. Our major findings were that adding serum to the media primarily provided factors consumed during growth in culture, rather than functioning predominantly through supporting growth signaling cascades (i.e. by fetal growth factors). Furthermore, only select serum-derived lipids were consumed during cancer cell proliferation, particularly those bound to albumin, and their consumption was driven by exogenous concentrations. By contrast, lipoprotein-transported lipids were less utilized. Importantly, we also found that combining an ITS solution- which also contained metals- with albumin-bound lipid classes was sufficient to replace serum in the media, enabling quantitative experiments that explored factors influencing lipid consumption. In the context of previous studies, our results also highlight the importance of exogenous lipids in supporting cell proliferation.

3.1.1. Unanswered questions

Despite progress in identifying the necessary media components for serum-free cultivation of cells, a broadly applicable serum-free medium has not yet been identified. Our findings indicate that albumin-bound lipids are critical for in vitro proliferation, however it is unclear that cells in vivo exhibit the same selectivity in uptake. Moreover, the concentration of lipoproteins in FBS are significantly lower than in adult human serum and

includes only LDL and HDL lipid particles [285]. Thus, lipoproteins may play are larger role in supporting cell proliferation in vivo, and lipid consumption should be explored further in other contexts to determine if the same selectivity is exhibited by cells in vivo.

The role of lipid scavenging for tumor growth in vivo remains unclear. Cancer cells often occupy nutrient-scare niches due to poor vascularization, which may limit environmental lipid delivery in the tumor microenvironment [46,47]. Slower proliferation rates observed in vivo settings and the potential increased reliance on de novo lipid synthesis due to nutrient scarcity, may influence the importance of lipid scavenging. Genetic disruption to scavenging components followed by in vivo tumorigenesis and tumor growth studies would help uncover which scavenging pathways are utilized in vivo.

Beyond the role of lipids, serum-free media conditions provide the opportunity to explore additional environmental requirements masked by the complex and undefined composition of serum. Much like the early experiments of Fischer and Eagle- who systematically assessed the contribution of individual metabolites with dialyzed serum- or serum-free system enables investigations of how specific nutrients support growth [46], [47], [50]. Use of serum-free medias for these purposes could similarly improve our understanding of lipid uptake and offer a platform to uncover additional cellular dependencies that can be exploited for therapeutic benefits.

While our work and other related studies demonstrate that defined media components can replace the requirement for serum, a universal serum replacement has not yet been identified [109], [253]. This motivates exploration into other environmental and nutritional requirements for cell growth of other cell lines.

3.2. Specific future research directions

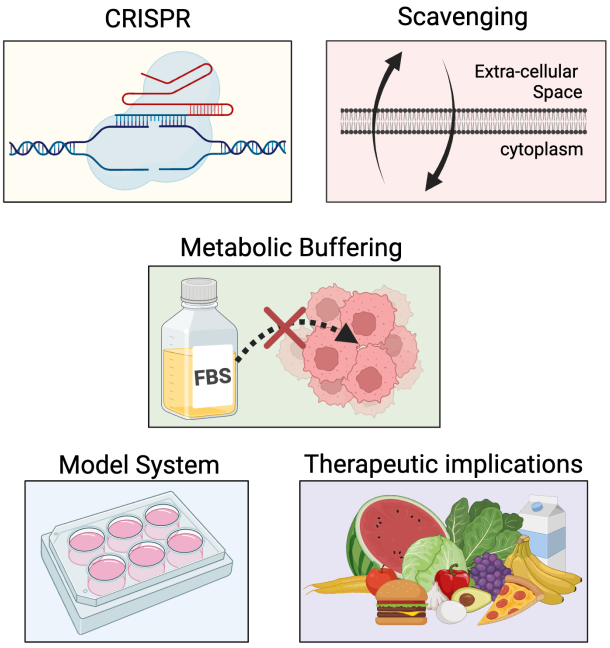

Figure 3.1: Schematic of future directions. (1) These findings indicate the serum free media can support extended cell growth and can be used in genetic screens to identify genes involved in lipid uptake. (2) This system can additionally be used identify other components which are scavenged from the media. (3) This media can be used to test cell behavior in the absence of metabolic buffering derived from serum. (4) The serum free media may be an effective model for other cell lines and across various cells to enable proliferation in the absence of serum. (5) Findings from our consumption data indicate dietary modification and changing serum lipids have therapeutic applications to target cellular lipidome.

3.2.1. CRISPR-Cas9 screens

Our work reveals a useful serum-free culture system, which provides a versatile tool for studying lipid metabolism and function in a controlled environment (Figure 1.14). Indeed, one challenge to studying lipid metabolism is the inability to uncouple and isolate specific lipid classes due to the heterogeneous makeup of serum and presence of multiple lipid classes. Our formulation enables inclusion of select lipid species, such as individual

PUFAs, without confounding and/or antagonistic interactions from other lipids. This provides an opportunity for more precise analyses of lipid signaling, metabolism, and overall cellular fitness in response to specific lipids in the environment.

An important next step is to clarify the machinery which controls lipid uptake and utilization. Forward CRISPR/Cas9 genetic screens in our serum-free system may identify essential proteins and pathways involved in this process. For instance, a screen that compares cell proliferation in media containing FA or LPC relative to media containing 10% serum could uncover proteins involved for uptake and/or incorporation of each lipid class. Additionally, conditions that include FA chains of different length or degrees of saturation can reveal enzymes that exhibit substrate selectivity in utilization and metabolism. Importantly, refining the lipid environment to a single class or species has the potential to identify new components of lipid scavenging machinery and clarify the roles of CD36, MFSD2a, and ACSL family members in the uptake of exogenous lipids, while minimizing environmental lipid redundancy. Indeed, typical screens include the addition of 10% serum, which provide multiple lipid classes we identified as sufficient to support proliferation and therefore lipid scavenging components may be masked by lipid redundancy in the media [58].

As proper signal detection in such screens requires multiple population doublings, it's important that the media supports sustained growth and serial passaging of cells. Importantly, we show that our serum-free media is sufficient to sustain long-term robust proliferation of Jurkat suspension cells, which do not require enzymatic dissociation from

culture plates with trypsin, or the presence of serum derived anti-trypsin during passaging and would be a suitable cell line for such a genetic knockout screen.

Beyond lipid metabolism, the serum-free media would be useful in exploring additional pathways masked by the presence of serum components. Comparing growth in serum-free media to serum-containing media would provide insight into metabolic dependencies that are otherwise buffered by serum factors. For instance, genetic disruption to nucleotide synthesis may have blunted effects in serum-containing media because nucleotides and related metabolites in serum can buffer metabolic impairments [58]. In contrast, serum-free conditions are entirely dependent on de novo synthesis pathways in the absence of exogenous nucleotide sources. Therefore, identifying the growth requirement differences between serum-free and serum-containing conditions provides a tool to identify environmental metabolic and signaling factors supporting cellular fitness. The serum-free culture media enables investigates into lipid-scavenging pathways and other metabolic requirements, which will improve our understanding of how environmental composition supports cell metabolism and proliferation.

3.2.2. Optimization of serum-free media

The serum free media described here provides a minimal media which can be further modified to optimize growth of other cells in serum free media. Including additional serum factors required for growth of cell lines, such as inclusion of androgens for culture of androgen dependent cancer cells may require the addition of testosterone to culture

media to support proliferation and can broaden the utility of this serum-free media recipe [286], [287]. Indeed, while a universal serum-free media may not be practical, identifying the minimal requirements for different cell lines can provide a base media for further modification, similar to advancement which followed after the development of MEM. Thus, the media formulation described here can act as a base serum free media that can be further modified to support growth of other cell lines.

Although this study primarily focused on FA and LPC, this media is a base which can be further adjusted to meet the demands of more cell lines to make a universal culture media which supplies cells with the required nutrients for growth. Additionally, the media composition described here uses albumin as a carrier protein, and identification of alternative lipid carrier molecules would result in both a defined and animal free media composition.

Improved serum free culture medias are also attractive in the field of biomanufacturing. While a media formulation for CHO cell growth in chemically defined media has been previously determined, the development of the culture meat industry is also striving to identify optimal media formulations for the growth of various cell lines[89], [288], [289]. Like biomanufacturing for therapeutic purposes, culture meat needs to exclude the use of animal products which can introduce zoonotic pathogens. For example, prion diseases derived from ingestion of infected meat products can lead to the development of Creutzfeldt disease[290].

Additionally, traditional cell passaging uses enzymatic dissociation from plates for serial subculturing, with serum inactivating the enzymes during passaging. Optimization of

serum free systems may require novel methods to passage cells that maintain seeding viability.

Since the development of immortalized cell lines, researchers have been working to identify the components required for proliferation and have continued to identify the serum components required for proliferation. This work indicated exogenous lipid sources is a crucial component which has gained relatively less attention.

3.2.3. Therapeutic implications

Beyond its utility in culture, our observations using serum-free media indicate cells non-selectively scavenge FA-containing lipids from the environment, which has implications for cancer therapy. In support of our findings of non-specific uptake, other studies have reported that the lipid prolife of cell reflects the composition of the environmental lipidome [144], [182]. Together, this suggests that modifying the serum lipidome in vivo, either through a dietary or pharmacologic approach can directly influence the composition of the cellular lipidome [291].

When considered in the context of lipotoxicity, the consumption patterns observed in this work indicate therapeutic strategies which alter the serum lipidome can influence cellular sensitivity to lipotoxicity. For instance, increasing dietary intake of PUFA containing lipids, such as fish oils that are enriched for omega 3-FA, may increase PUFA incorporation into the cellular lipidome and disrupt the FA balance within the cell and potentially sensitizing cells to ferroptotic stress. Furthermore, cells may be additionally pushed towards this form of lipotoxicity via pharmacologic inhibitors such as sulfasalazine, an FDA

approved medications demonstrated to sensitize cancer cells to ferroptosis by disrupting the cellular antioxidant capabilities [292], [293]. Alternatively, others have shown tumors in mice fed high-fat diets- rich in saturated FAs- and treated with FA desaturase inhibitors exhibit decreased tumor growth due to disrupted lipid homeostasis and saturated lipotoxicity[182]. Importantly, these results additionally imply the serum lipidome can buffer the cellular lipidome during endogenous lipid perturbations[294]. In the broader picture, our results indicate that dietary lipids and serum lipid composition must be considered when targeting lipid metabolism in human health and disease.

4. References

All Figures were made using Biorender and Graph pad.

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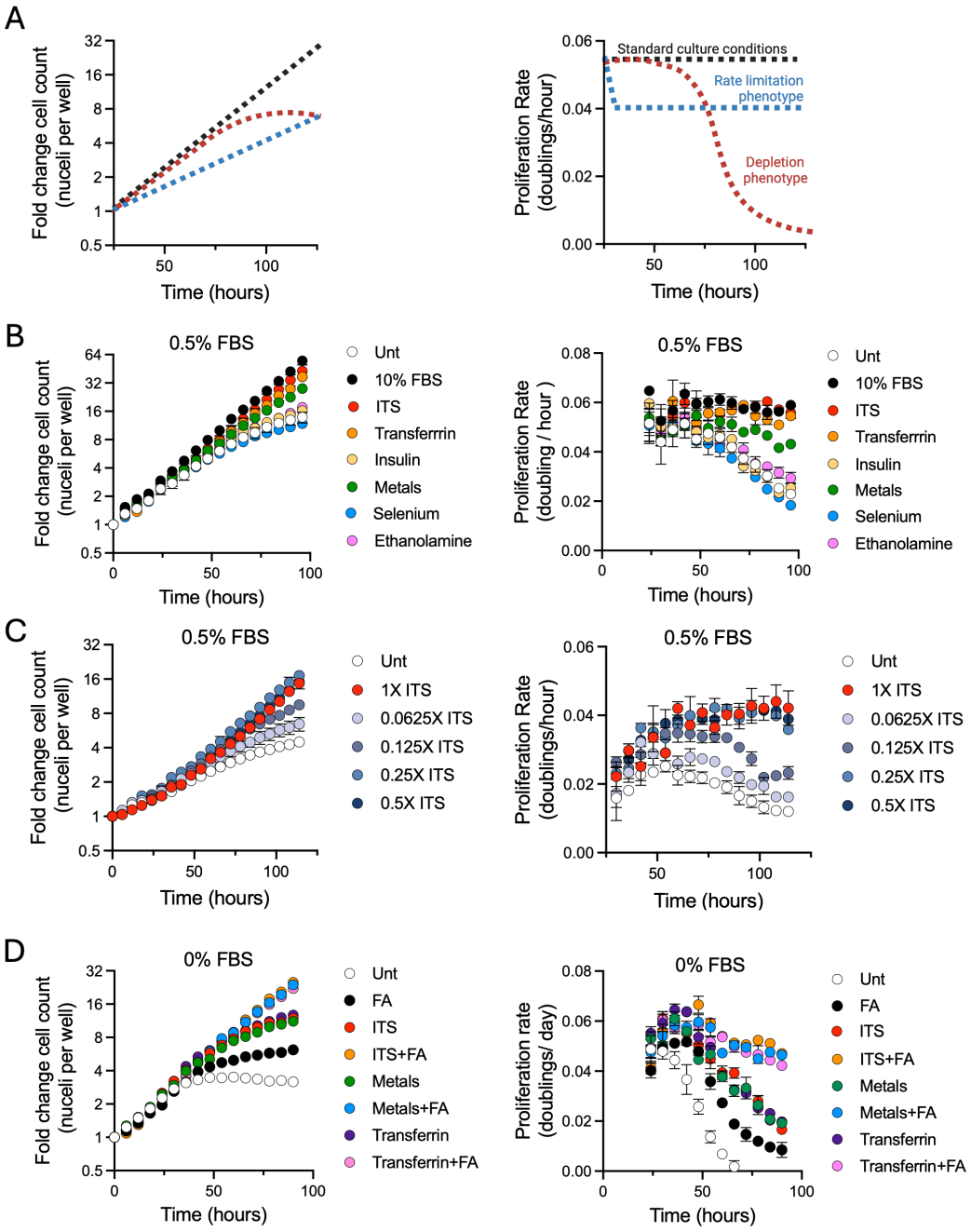
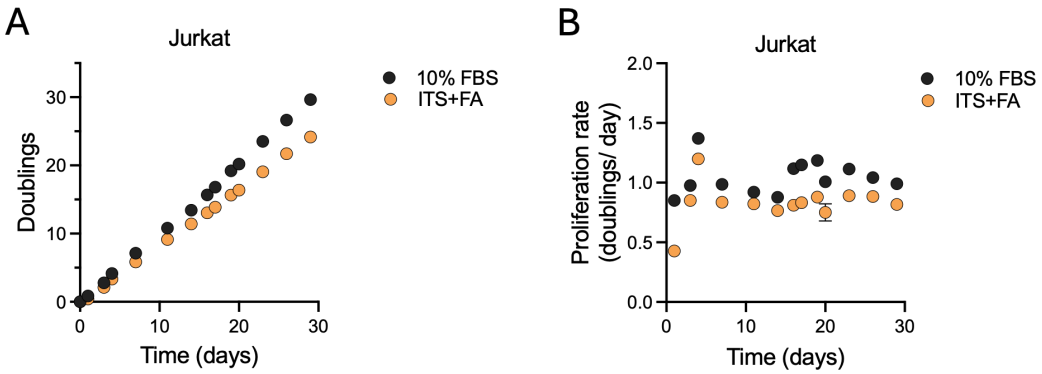
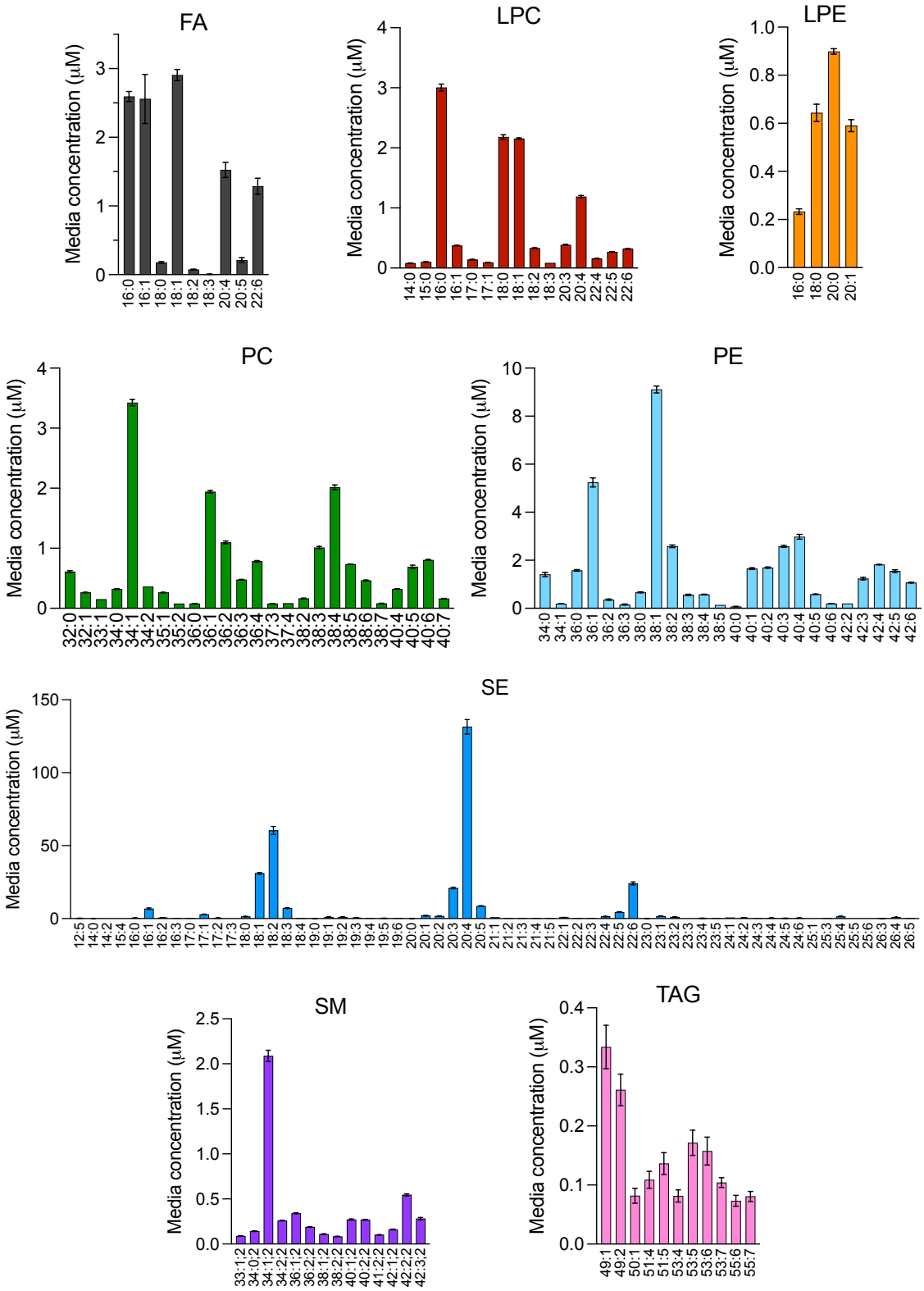
5. Supplemental Figures



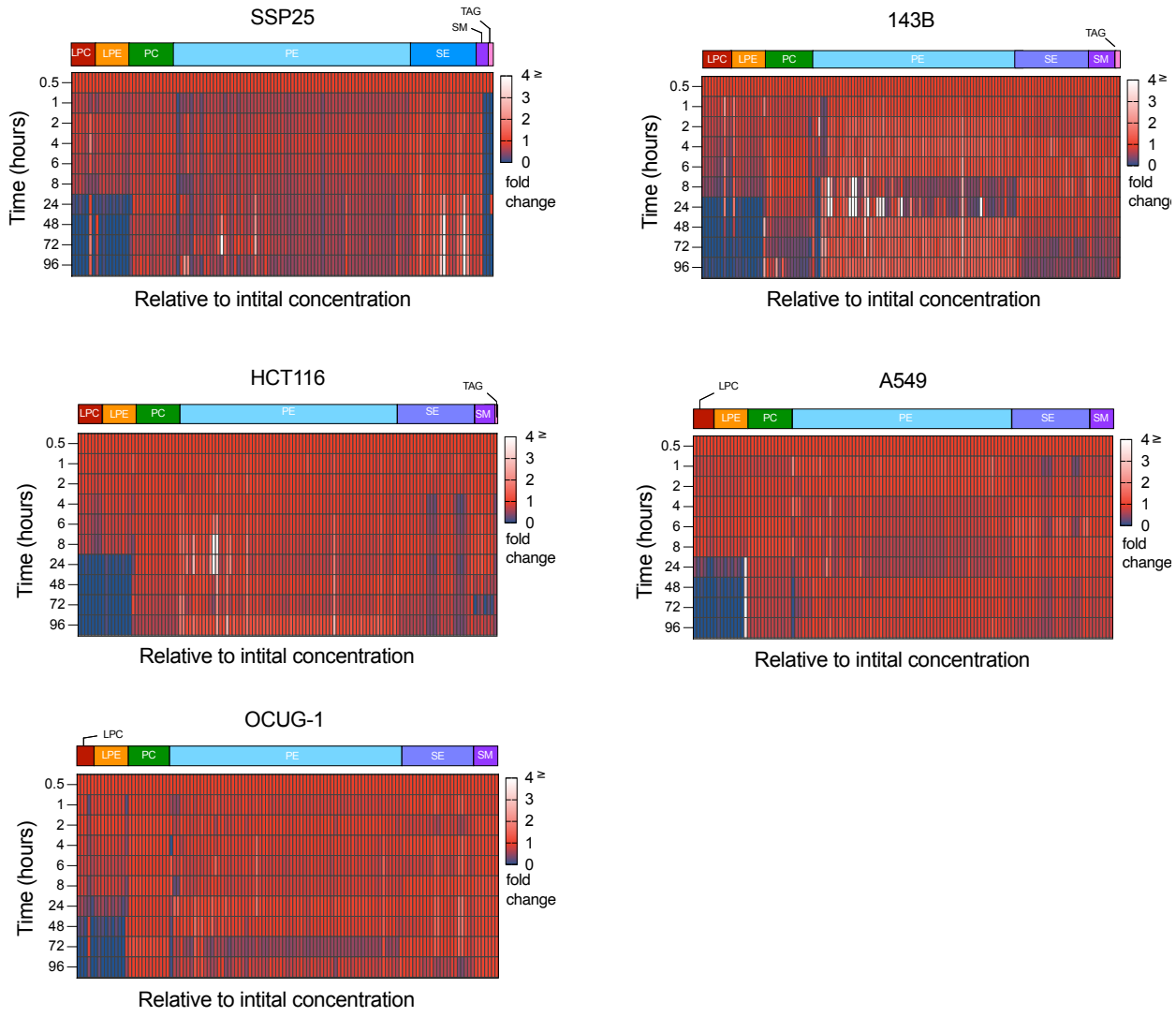
Supplementary Figure 1. Serum-restriction results in a depletion phenotype.
(A) Predicted changes to cell growth kinetics upon two theoretical modes of proliferation inhibition. Fold change in cell counts (left) and moving average of proliferation rate (right). The blue dotted line reflects a rate limitation phenotype (consistently slowed) compared to the red dotted line which reflects a depletion phenotype (progressive loss of proliferation over time), which can appear identical if only counting initial and final timepoints. **(B)** Growth kinetics of cells cultured in 0.5% FBS with individual components of the ITS mix. Fold change in cell counts (left) and moving average of proliferation rate (right). **(C)** Growth kinetics of cells cultured in 0.5% FBS supplemented with a decreasing titration of the complete ITS mix. Fold change in cell counts (left) and moving average of proliferation rate (right). **(D)** Growth kinetics of cells cultured in DMEM supplemented with and without FA, and either ITS, transferrin, or trace metals. Fold change in cell counts (left) and moving average of proliferation rate (right). Error bars represent mean ± SEM (n = 3).



Supplementary Figure 2: Serum free media supports sustained Jurkat cell growth.
(A-B) Jurkat cell proliferation in serum-free media relative to culture media containing 10% FBS. Population doublings (A) and proliferation rate (B). Error bars represent mean ± SEM (n=3).



Supplementary Figure 3. Lipid profiles of FBS.
Lipid species that constitute the top 99% of the total lipid pool detected in 10% FBS containing media. The x-axis indicates the number of carbons and the number of double bonds present in each lipid species. Error bars represent mean ± SEM (n = 3). Abbreviations: SE, sterol esters; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingolipids; TAG, triacylglycerols; LPC, lysophosphatidylcholine; LPE,



Supplementary Figure 4. Lipid consumption patterns are conserved across cell lines. Media lipid depletion from 10% FBS containing media for cholangiocarcinoma (SSP25), osteosarcoma (143B), non–small cell lung cancer (A549), colon cancer (HCT116), and gallbladder carcinoma (OCUG-1).