COMPLEXATION OF LIPIDS WITH CYCLODEXTRIN CARRIERS FOR FULLY DEFINED SUPPLEMENTATION OF CELL CULTURE

by

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DEDICATION

First and foremost, I thank God, and my Lord Jesus Christ for giving me the ability, guidance, and stamina to do this work.

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GLOSSARY

ALA	Alpha Linolenic Acid
AP	Alkaline phosphatase
APC	Adenomatous polyposis coli
AscPT	Ascorbyl Palmitate
Allogenic	From the same organism
Autologous	From a different organism
BSA	Bovine Serum Albumin
DHA	Docosahexanoic Acid
DMEM	Dulbecco"s Modified Eagle Medium
EPA	Eicosahexanoic Acid
ESC	Embryonic Stem Cell
FBS	Fetal bovine serum
GSK-3	Glycogen Synthase Kinase 3
HFF	Human Foreskin Fibroblasts
hESC	Human embryonic stem cell
H9	Human embryonic stem cells (9)
iPSC	Induced pluripotent stem cell
MBCD	Methyl beta cyclodextrin
µg	Microgram
µl	Microliter

GLOSSARY – CONTINUED

µL	Micromol
ng	Nanogram
OA	Oleic Acid
PA	Palmitoleic Acid
PBS	Phosphate buffered Saline
PT	Palmitoleic Acid

ABSTRACT

Induced Pluripotent Stem Cells (iPSCs) hold great promise for revolutionizing medicine and research. Scientists are currently able to reprogram adult cells of almost any type to a genetically "open" state, pluripotency, wherein they lose the characteristics of their original cell type, and revert to a state that can reproduce indefinitely, and can be differentiated to many different cell types. IPSCs are currently grown in "chemically defined" media that contains no animal derived components. This media eliminates animal and human sera because these tend to be quite variable and confound the reprogramming process, but the chemically defined media in use has almost no lipid content. The central goal of this project was to develop methods for chemically defined addition of lipids to cell culture media. The methods developed promise to be an advance in stem cell and general cell culture methodology, providing more reproducible experimental results, and supporting cells in culture with optimized lipid contents.

In order to facilitate the addition of lipids to cell culture media without animal serum or serum albumin, complexation of individual lipids with a methyl β-cyclodextrin starch was accomplished without addition of other molecules or oxidation of delicate lipids at a 1:1 stoichiometry. The lipid/MBCD complexes are soluble in aqueous media, and can be added individually or as a mixture to cell cultures.

Application of complexed lipids to stem cells and fibroblasts revealed significant differences in lipid responses. Supplementation of human fibroblasts with a mixture of complexed lipids and other elements caused a 60% increase in proliferation over a 10 day period. Supplementation of stem cells with complexed lipids significantly increased proliferation, without reduction of pluripotency. Differences in lipid responses were also found between iPSC and embryonic stem cells, that may help elucidate differences between genetic or metabolic states which may point the way for more effective reprogramming of adult cells to pluripotency.

INTRODUCTION

The Importance of Induced Pluripotent Stem Cells

Stem cells, defined as undifferentiated "master" cells, have the ability to self-renew indefinitely. They are pluripotent, meaning they have the capacity to differentiate into cell types from all three major germ layers. These unique qualities hold great potential for advancement in cell research and medicine. In addition to the exciting potential for personalized medicine and therapies, these cells can also replace cancer cells in drug testing, more closely modeling normal cell response and human disease states (Zuba-Surma et al. 2012).

Until recently, embryonic stem cells (ESCs) were the only pluripotent cells available. ESCs are harvested from either the inner cell mass or epiblast of embryos (Thompson and Williams 2018). While ESCs remain the gold standard for pluripotency, they pose severe limitations for therapeutic application. First among these is host immune response, which can result in rejection of implanted cells. Every cell in a body carries antigen molecules on its surface. The structures of these antigens communicate to the immune system if the cell is foreign, causing an immune response (Choo 2007) and, if the cell is detected as foreign, the immune system attacks it. Transplantation of cells or organs that do not adequately match the antigen type of the recipient can cause the patient to reject the transplant. This can result in patient death.

Secondly, implantation of cells from a donor or embryo introduces the possibility of transmitting genetic disease from the donor. Mutations that may have gone unnoticed

in the donor may be compounded with mutations in the recipient, causing novel and unforeseen problems. Finally, ethical concerns about the death of the embryo from which cells were harvested has given rise to a political backlash, resulting in a ban on harvest of human embryos in the U.S in 2001 (Murugan 2009). Existing human ESC lines were not banned and these lines continue in research use. ESCs are an important standard for pluripotency, but iPSCs offer much greater potential for therapy and disease.

Use of Stem Cells for Drug Research

Cancer cells, the only cells other than stem cells that proliferate indefinitely, are used for most drug research (Zuba-Surma et al. 2012), which creates at least one major problem. Cancer cells favor aerobic glycolysis for energy production, instead of oxidative phosphorylation, which most normal cells use for energy generation. Reliance on aerobic glycolysis, called the Warburg effect, nearly eliminates mitochondrial activity, leaving mitochondrial toxicity largely unreported in drug trials using cancer cells (Dykens and Will 2007, Marthiens et al. 2010). Oxidative phosphorylation occurs exclusively in the mitochondria, and is a much more efficient method of ATP production than glycolysis. Oxidative phosphorylation tends to produce Reactive Oxygen Species (ROS), which can be useful for signaling in small amounts, but are toxic and even deadly in large amounts (Auten and Davis 2009). Glycolysis supports lipogenesis, which supports synthesis of lipids necessary for assembly of new membranes in highly proliferative cells, but produces lactic acid as a waste product, which can be toxic to surrounding cells in high concentrations (Freund and Croughan 2018). Embryonic stem cells (ES) have very low numbers of mitochondria, as they have not developed yet, but

iPSCs have normal numbers of mitochondria, so are able to utilize oxidative phosphorylation. Use of iPSCs instead of cancer cells for drug testing could eliminate errors in pharmaceutical testing that have resulted in unforeseen side effects such as those from the popular fluoroquinolone family of antibiotics (Marchant 2018).

Another research model in wide use today is animal cell lines. hiPSC's can replace these animal models, which do not adequately mimic human cell responses due to interspecies differences (McGonigle and Ruggeri 2014). These differences include the ability of mouse cells to produce vitamin C, while human cells cannot, and differences in the cytochrome P450 system of enzymes, which metabolize 75 % of drugs (Uhi 2015). Human iPSCs have the potential to be used to more closely model human disease states for more specific and effective drug testing (Medvedev et al. 2010). The media environment may also need to be altered to mimic some disease states, and nutrients may actually become part of treatments for disease, as we more fully understand their roles in cell health and behavior.

History of iPSCs

In 2006, Shinya Yamanaka reported the successful reprogramming of murine fibroblasts to pluripotency by retrovirally introducing four genetic factors, Oct4, Sox2, c-Myc and Klf4, to primary cells (Takahashi and Yamanaka 2006). These cells are called induced pluripotent stem cells (iPSCs). Successful factor introduction was measured by resistance to the antibiotic Geneticin418 (G418). Resistance to G418 was imbued to cells by transfection of a resistance gene within the gene for Fbx15. Fbx15 (FBXO15 in humans) is a protein that is expressed when Oct4 and Sox 2 are transcribed. Fbx15

normally degrades KIF1 Binding Protein (KBP), a mitochondrial transport protein. When Fbx15 is expressed, mitochondrial proteins are not transported from the nucleus, and a developmental bottleneck ensues, preventing production of mitochondria, causing low mitochondrial activity and low production of reactive oxygen species (ROS) (Donato et al. 2017). KBP effects, and their dependence on the activity of the core pluripotency factors Oct4 and Sox2, promoted the use of the expression of Fbx15 as the first marker of successful reprogramming of fibroblasts into iPSCs. While cells in this initial study were considered pluripotent, their ability to form tumors that contained cells from all three cellular germ lines was not reliable. Additionally, these first iPSCs did not generate live offspring when implanted into murine blastocysts, suggesting that they were not fully pluripotent (Takahashi and Yamanaka 2006).

In 2007, the same four transcription factors were used to induce human fibroblasts to pluripotency (Takahashi et al. 2007). This was a breakthrough and promised to provide solutions to the major problems associated with the use of stem cells in medicine. When used for therapy on the donor, iPSCs do not carry the immune or potential genetic issues of ESCs, and have opened a plethora of possibilities for research and therapy. In the 2007 human cell study, a mouse promoter for retroviruses, Slc7a1 was introduced to the human fibroblasts before reprogramming to increase efficiency of transfection. Cells were grown in media supplemented with 10% fetal bovine serum (FBS) and grown on a feeder layer comprised of mouse embryonic fibroblasts (Takahashi et al. 2007). Since this initial experiment, many variations have been tested, including variations of transcription factors, low oxygen conditions, and a variety of nutrients and small

molecules in the media (Ma et al. 2017). A major advance has been the move to more defined media, which involves elimination of animal tissues and extracts, such as feeder layers and sera (Chen et al. 2011).

Reprogramming and culture of human induced pluripotent stem cells (hiPSCs) is currently one of the most investigated methodologies in biomedical research. The potential of hiPSCs for use in research and therapy is enormous (Compagnucci and Bertini 2017, Liu et al. 2017, Omole and Fakoya 2018), but its effective application is hindered by issues of safety and reproducibility. The Food and Drug Administration (FDA) has further defined these issues as deficits of homogeneity, efficiency, and differentiability (Knoepfler 2012). The chemically defined lipid supplements described in this work may help to overcome two of the three hurdles identified by the FDA: homogeneity and efficiency. The final hurdle, differentiability, must be addressed, as well, to produce a more reliable and efficient iPSC methodology. If conditions for reprogramming and maintenance of hiPSCs are improved, differentiability may also be improved, as the roots of differentiation problems are thought to result from incomplete reprogramming (Ohi et al. 2011). This problem may be better understood by an illustration of the cellular machinery involved in reprogramming and cell fate decisions.

Reprogramming Mechanisms

Reprogramming mechanisms are not completely understood, but the process has been broken down into three stages (Samavarchi-Tehrani et al. 2010). In the initiation stage, genes associated with somatic traits are down regulated, and chromatin remodeling

begins. This requires silencing by methylation of somatic genes, and acetylation of histone proteins in the chromatin, making more genes accessible for transcription. Secondly, in the maturation stage, genes associated with the pluripotent cell-type are expressed, requiring demethylation and expression of silenced genes. Finally, the cells stabilize in a pluripotent state through methylation of reprogramming genes, further methylation of somatic genes, and chromatin remodeling through acetylation of histones, reflecting the new epigenetic state. The desired result is that cells become morphologically and epigenetically homogenous and form colonies (Polo et al. 2012).

While molecular mechanics of some protein interactions involved in reprogramming are documented, there are multiple diverse and somewhat stochastic paths available to many proteins. Lingering problems with low efficiency and variable quality of iPSCs strongly suggest that there are mechanisms operating in cells that effectively attenuate the signals and mechanisms that are activated by the reprogramming process (Ohi et al. 2011, Ori Bar-Nur 2011). These forces exert themselves through metabolic (Zhang et al. 2016, Shyh-Chang 2017), inflammatory (Säemann et al. 2000, King and Goodell 2011), oxidative (Yanes et al. 2010, Lin et al. 2018) and other signaling pathways (Huang et al. 2015, Tejada-Romero et al. 2015). These pathways appear to be effected by thousands of nutrients (Kogteva and Bezuglov 1998) and small molecules (Zhang 2010, Zhang et al. 2012, Zhang and Wu 2013, Bar-Nur et al. 2014). Although we are aware of some of the effectors, they continue to confound the reprogramming of iPSC's, resulting in low efficiency, and unreliable genotypes that make their therapeutic use unreliable (Yoshihara et al. 2017).

Protocols for reprogramming somatic cells to pluripotency consist of transfecting cells with one or more transcription “factors”, and then maintaining those cells in some form of media, often the same growth media used for the source cells, while transcription is activated. Transcription is known to be effected by the presence of nutrients such as ascorbic acid (Yin et al. 2013, Cimmino et al. 2018) and sodium butyrate (Zhang, 2013) and proteins such as β-catenin and e-cadherin (Huang et al. 2015), as well as the products of several cellular signaling pathways such as TGFβ, Wnt and IGF (Watabe 2003, Dalton 2013, Tejada-Romero et al. 2015). RNA produced may or may not be efficiently translated into protein (Kwak and Lis 2013), and functional proteins expressed may perform diverse functions including, but not limited to: further activation of genes (Wohrle et al. 2007), regulation of cellular activities by signaling (Hlavacek and Faeder 2009) and acting as substrates and cofactors for cellular activities (Hlavacek and Faeder 2009, Vallerie and Hotamisligil 2010). The diverse activities of proteins expressed during reprogramming may also cause regulatory or metabolic cellular responses unrelated to their primary or known functions, such as concentration based activation of regulatory mechanisms (Valenta et al. 2012, Krauss 2014). These complex and interdependent mechanisms are not fully understood. Here we present a discussion of current transfection methods, transcription and expression mechanisms, most commonly used factors and their activities. We also present the complexities of one central pluripotency-related pathway, the Wnt pathway that illustrates, in part, the delicacy and complexity of the processes involved in reprogramming cells to pluripotency.

Transfection

The process of reprogramming is initiated by the introduction of specific nucleic acids into cells. These nucleic acids are commonly called factors, but they are actually genes that code for the transcription factor (TF) proteins after which they are named (Kim and Eberwine 2010). Once produced, TFs can control gene expression by creating a "cascade" effect, wherein the presence of small amounts of one protein trigger the production of larger amounts of a second, which may trigger production of even larger amounts of a third, and so on (Clancy 2008, He et al. 2013). Conversely, the expression of some proteins triggers the expression of other proteins that feedback to regulate the expression of the original protein, or effect the function of cellular pathways that attenuate downstream functions (Chen et al. 2008). While we are aware of primary functions of the proteins expressed, these secondary effects are largely unknown.

To date, approximately 1600 human TFs have been catalogued and matched with binding motifs (Fulton, 2009, Vaquerizas, 2009, Wingender, 2015). These genes may be inserted into cells in several ways. Methods in current use include: infection with viruses (O'Keefe 2013), coating DNA with lipids (lipofection) enabling it to enter through the membrane of the cell (Felgner et al. 1987), and administration of electrical force (electroporation) to briefly cause holes in the cell membrane through which genes may be inserted (Hahn and Scanlan 2010). Once inside the cell, transport proteins recognize genetic material and facilitate its entry into the nucleus (Bai et al. 2017), where genes may be inserted into the genome, or transiently expressed (Coffin 1997). In the process of reprogramming, the delivered genes code for transcription factors that

stimulate the expression of proteins that effect the changes necessary to render the cells pluripotent (Clancy 2008). Efficiency of entry into the nucleus, and whether the genes are inserted into the genome are dependent both on the method used to transfect cells, and the type of DNA used (Kim and Eberwine 2010).

Viral Transfection. Gene delivery is most commonly accomplished using viruses. Viruses naturally deliver genetic material into cells, forcing cells to use their replicating machinery to express viral genes (Coffin 1997). Retroviruses have the ability to make DNA from their RNA genomes using the reverse transcriptase enzyme (RT). This cDNA can then be inserted into the host cell genome, causing an inheritable change which allows for permanent expression of a gene construct, such as small interfering RNA (siRNA) or proteins that fluoresce when certain conditions are met, called reporters (Barker and Planelles 2003). The term Retrovirus refers to simple retroviruses, while the term lentivirus refers to “complex” retroviruses (Shao and Wu 2010). The main differences between retro- and lenti-viruses are their abilities to access the genome, and the presence of some additional genes in lentiviruses. Retro viruses can only access the nucleus during mitosis, when the nuclear membrane disassembles briefly during division, while lentiviral DNA can be transported across the intact nuclear membrane (O'Keefe, 2013). Both types of virus are appropriate to use, depending on applications and cell types.

Viral particles deliver protein containers, called cores, into the cellular cytoplasm upon infection. These cores contain RNA, reverse transcriptase (RT) and integrase enzymes (Warnock et al. 2011). Once inside, RT begins to produce copies of

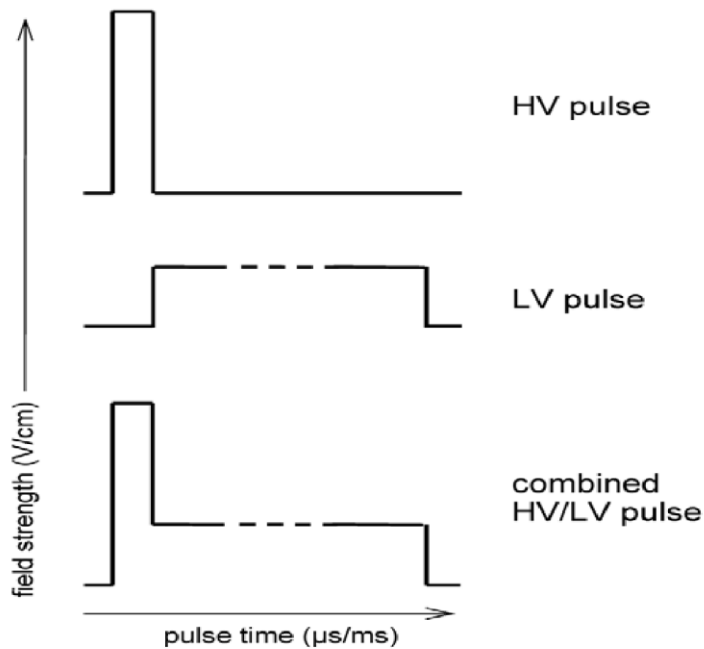
the RNA sequences, and produces DNA from these replicates. The integrase enzyme facilitates the introduction of the viral DNA into the host cell genome (Escors 2012). Integrase processes the 3' ends of the viral DNA leaving a cytosine-adenine (CA) tail. CA tails can then be attached to 5' phosphorylated ends of a double-stranded cut made in the host cell genome (Craigie et al. 1990). Integrase then repairs the gaps in the DNA, creating five base-pair repeats around the inserted gene sequence (Mizuuchi 1992).

Naturally occurring viruses force cells to replicate viral protein indefinitely once introduced to cells, causing pathology and even cell death. To prevent this, modern transfection viruses have been engineered by replacing the genes encoding for viral replication with the genes for desired proteins. This produces “replication defective” vectors that can efficiently deliver the genes of interest without the undesirable effects of viral infection (Coffin 1997). Non-integrating viruses have also been engineered by mutations in the integrase gene, and by mutations in the long terminal repeat sequences that contain the CA sequence necessary for integration (Banasik 2010). These methods reduce integration exponentially, but do not eliminate it entirely (Kantor 2011). Replication-defective viral vectors can usually hold inserts of up to 10 kilobases (kb) (Shao 2010), making it possible to deliver multiple genes simultaneously (O'Keefe, 2013). These improvements make non-integrating viruses a popular method of reprogramming. Nonetheless, viral vectors are not ideal for therapeutic applications because insertion into the recipient genome remains possible, and can cause defective protein synthesis, oncogenesis, and other aberrations (Baum 2003).

Lipofection. Introduction of cationic chaperones to facilitate DNA passage through the cellular membrane began with the use of calcium phosphate (Graham 1973) and DEAE-dextran (Pari 2004), and continues with use of polybrene to enhance viral transfection (Davis, 2002). These techniques have been successful on many cell lines, but efficiency is generally low, and there have been issues with toxicity. Today, cationic facilitators are generally used to enhance the efficiency of other methods (Felgner et al. 1987). Use of lipids to facilitate transfer, called lipofection, is also most commonly used with plasmid DNA, but with greater efficiency and lower toxicity than early cationic methods. DNA is spontaneously encapsulated in a liposome at very high efficiency. The liposome is easily fused to the lipid membranes of cells in culture, and its DNA contents are delivered into the cytoplasm (Felgner et al. 1987). Cellular transport and transcription then proceed as mentioned above.

Electroporation. Electroporation was first described in 1982 using fibroblasts (Wong and Neumann 1982). DNA was first caused to adsorb to the cell membrane using 20mM magnesium chloride and then 5–10 kV/cm electrical charge was applied with a duration of 5–10 μs. Results were comparable to calcium phosphate and DEAE-dextran methods in use at the time. Cell membranes are unable to pass electrical current except through ion channels. Subjecting cell membranes to a high-voltage electric field results in temporary breakdown and the formation of "pores" that are large enough to allow molecules like DNA to enter or leave the cell (Potter and Heller 2018). The membrane quickly closes the pores once the electrical pulse is over, and the cell's natural tendency to retain DNA then maintains the transfection. Today, electroporation methods have

been optimized for cell lines and applications (Chu et al. 1987, Wolf et al. 1994). An advancement in electroporation, called nucleofection, was developed in 2003 by the Amaxa Company (Maasho et al. 2004). This method combined a brief high voltage pulse with a lower voltage pulse of greater duration (Stroh et al. 2010) (figure 1), and was found to deliver DNA approximately 50% more efficiently, with cell viabilities of 82-86% (Distler et al. 2005).



Stroh et. al. 2010

Figure 1. Mechanics of nucleofection. The high voltage pulse (HV) is 5kV for milliseconds. The low voltage pulse is 1.5kV for a range of milliseconds. Pulse duration varies depending on cell type.

Nucleofection has developed to a highly reliable method that uses premade, disposable nucleofection vials, nucleofection solution, and preset programs for different cells lines. Its efficiency and high cell viability, along with its ability to deliver non-viral DNA directly into the nucleus (Lakshmipathy et al. 2004) makes it a method of choice

for delivery of genetic factors in iPSC reprogramming and differentiation (Lakshmipathy et al. 2003).

Transcription and Translation

Transcription factors are proteins involved in regulating gene expression. A typical TF has multiple functional domains, not only for recognizing and binding to the appropriate DNA sequence, but also for interactions with other TFs, with coactivator proteins, with RNA polymerase II, with chromatin remodeling complexes, and with small noncoding RNAs (Roeder 1996).

Epigenetic structures must sometimes be overcome in order to make it possible for TFs to bind to DNA. In eukaryotes, DNA is stored in tightly wound packages called nucleosomes. Genes associated with a nucleosome are not available for transcription by most TFs, because they are wound around positively charged proteins called histones (Marino-Ramirez et al. 2005).

Acetylation of histone proteins and methylation of DNA are key regulators of the accessibility of DNA for transcription. Modifications that can change the chromatin structure and silence or activate genes include phosphorylation, acetylation, methylation, ubiquitination, sumoylation, glycosylation and biotinylation. Of these mechanisms, methylation and acetylation have been the most studied in the area of reprogramming and differentiation (Konsoula, 2012). These two mechanisms accomplish the restructuring of nucleosomal DNA, silencing of somatic genes in early reprogramming, activation of pluripotency genes in mid reprogramming, and stabilization of the pluripotent state in late reprogramming. Roles of other mechanisms have not been well studied.

Methylation of DNA is a primary mechanism of silencing of genes, and DNA methylation patterns are important epigenetic markers. Methyltransferase enzymes place methyl groups on promoters of certain genes to block their activation. A major methyltransferase, DNA methyltransferase 1, transfers methyl groups to cytosine residues in cytosine and guanine rich areas of DNA known as CpG islands (Bhattacharya et al. 1999). These areas contain 60% of known promoters in the mammalian genome, and this methylation silences transcription directly by interfering with the binding of TFs and indirectly by favoring the formation of repressive chromatin by methyl DNA-binding proteins (Godini et al. 2018). Methylation can be singular, or multiple methyl groups may be placed in a single promoter region. The location of the methylation is a factor in whether the gene is silenced or just inhibited. Recently, it has been reported that methylation does not inhibit binding of all TFs, in fact some preferentially bind methylated promoters (Kribelbauer et al. 2017). These aspects of methylation mechanisms, many of which are not understood, confer differing levels and types of gene inhibition (Godini et al. 2018). Methylation is a central function of cellular fate decision, and must be activated in early reprogramming to silence somatic genes. Little is known about the mechanisms of reprogramming that cause methylation of somatic genes, except that reprogramming factors cause down regulation of methytransferase enzymes and transcription factor binding can cause demethylation. Methylation and demethylation are continual processes, and are regulated by many pathways and transcription factors (Ivanov, 2016, Rodriguez, 2013, Yin, 2013).

Acetylation is accomplished by histone acetyl transferase (HAT) enzymes. These enzymes can add negatively charged acetyl groups to positively charged lysine residues on histones, decreasing the charge differential between the DNA backbone and the histone, and causing the DNA to unwind from histone proteins (Godini et al. 2018). Histone deacetylases (HDAC) remove acetyl groups, stabilizing the epigenetic "settings" of the cell (Konsoula and Barile 2012). Regulation of expression of HATs or HDACs is one way that chromatin remodeling can be regulated. Regulation of expression involves methylation and demethylation, as well as TF binding and these are known effects of some of the most common reprogramming factors (see transcription factors), but we also know that acetyl-CoA, a central metabolite produced by multiple metabolic pathways including glucose metabolism, fatty acid β-oxidation, and amino acid degradation, is the source of acetyl groups for the process (Choudhary et al. 2014). Recently, it has been shown that endogenous lipids are a preferred source of acetyl groups for chromatin remodeling (McDonnell et. al 2016). This further suggests that nutrients and metabolic mechanisms play an important part in reprogramming.

Demethylation enables TFs to bind to and promote pluripotency gene transcription in reprograming. Demethylation is accomplished in two ways: passively and actively. Passive demethylation is accomplished by interfering with the Dnmt1 gene, which codes for DNA methyltransferase 1 (DNMT1). When the DNMT1 enzyme is not present, methylation of newly formed DNA does not happen, so newly formed cells do not carry the methylation patterns of the parent cell, leaving them open to new epigenetic changes (He et al. 2017). Active demethylation is done by excision of the methylated

cytosine, and replacing it with an unmethylated cytosine (Jost et al. 1995). This is done by as yet unknown DNA polymerase and ligase enzymes. Two known promoters of active demethylation are growth arrest and DNA-damage-inducible protein 45 alpha (GADD45a), a small acidic nuclear protein induced by stress (Barreto et al. 2007), and ascorbic acid (Yin et al. 2013). Interestingly, ascorbic acid is used extensively in current reprogramming media, and is shown to increase reprogramming efficiency (Yin et al. 2013, Cimmino et al. 2018). It is unknown exactly how much of reprogramming demethylation is active and how much is passive. Novel demethylation mechanisms may be involved that have not yet been elucidated.

A class of TFs called "pioneer" factors can overcome epigenetic barriers, which usually prevent the cell from losing its somatic traits by sequestering genes that are not relevant to the adult cell type (Zaret and Carroll 2011). They do this by directly binding to DNA in nucleosomes, and recruiting other TFs and HDACs to remodel the structure to allow further transcription. Pioneer factors can also overcome methylation silencing by binding to areas with concentrations of cytosine and guanine, and inhibiting methylation (Konsoula 2012). These processes make more DNA available for transcription, creating a plethora of possibilities including pluripotency. Oct4 and SOX2, well-known reprogramming factors, are pioneer TFs.

Transcription is initiated when TFs bind to promoter and enhancer sequences on DNA, and recruit RNA polymerases (RNAPs). Eukaryotes have three types of RNAP: RNAP I synthesizes ribosomal RNA (rRNA), RNAP II synthesizes messenger RNA (mRNA), and RNAP III synthesizes transfer RNA (tRNA) (Cooper 2000). We

will focus here on RNAP II, since it is responsible for most human protein transcription. All eukaryotic TFs contain a binding site for RNAP II. The transcription process is initiated by general transcription factors (GTFs), which are ubiquitous in all cells, and TATA box binding protein (TBP). Binding of TBP to the TATA gene sequence introduces a ~90° bend in the DNA at the promoter sequence (Roeder 1996). General transcription factors TFIIA and TFIIB are then recruited, followed by the recruitment of RNAPII/TFIIF. This positions the 5' end of the template DNA in the cleft of RNAP II (He et al. 2013). General transcription factors TFIIE and TFIIH are then recruited to complete the pre-initiation complex. DNA helicase enzymatic proteins move ahead of RNAP II, separating and unwinding the DNA using the energy released during the process of binding, hydrolysis and release of ATP. This creates a transcription "bubble" (Wang et al. 1992), allowing nucleotides to be accessed by RNAP II, and TFIIH then threads downstream DNA into the active site of RNAP II in an ATP-dependent manner (figure 2). RNAP II then begins forming a complementary strand of mRNA from the exposed template strand of the DNA (figure 2) (Lehninger 2000). When the nascent mRNA is extended to 20–60 nucleotides, RNAP II pauses. This is a regulatory mechanism, and more TFs are necessary to release RNAP II into the "productive elongation" stage (Kwak and Lis 2013).

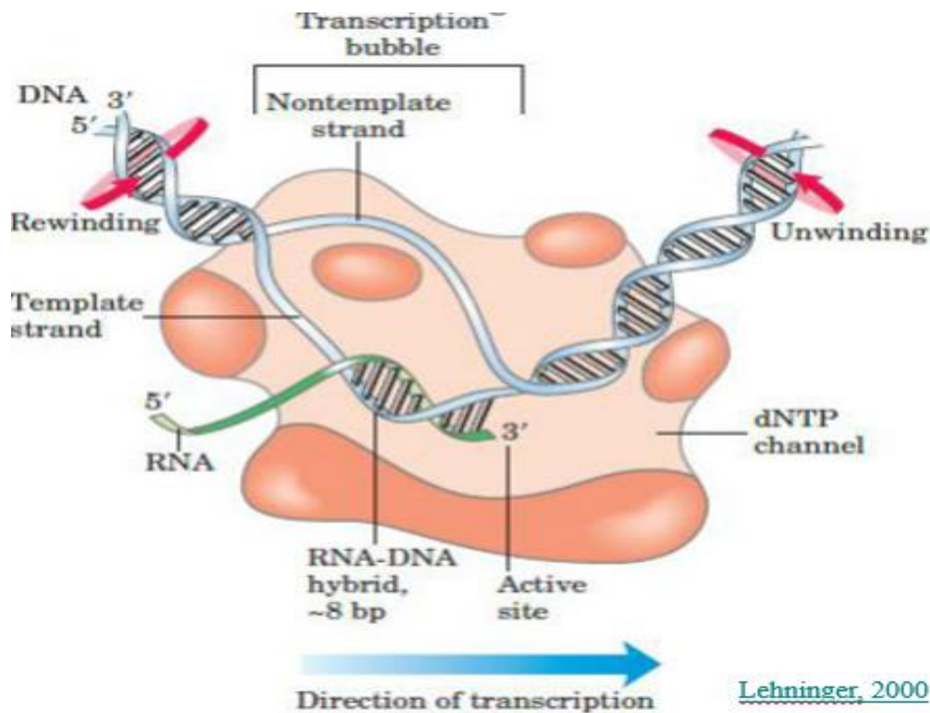


Figure 2. Transcription Model. RNA polymerase (brown shape) stabilizes unwound DNA, and produces a copy of a specific gene, proceeding until reaching a "stop" codon.

Productive elongation is known to be stimulated by a number of other transcription factors, including general transcription factors, TFIID, TFIIF and TFIIS, mentioned earlier. Interestingly, two TFs associated with pluripotency, cMyc and NF-κB, are known to attenuate polymerase pausing (Kwak and Lis 2013), possibly accounting for their positive effects on reprogramming.

Once pausing is overcome, transcription continues until a stop codon TAA, TAG, or TGA is encountered. mRNA is then released and can be translated into a chain of amino acids.

Translation requires another large protein, a ribosome, which coordinates the attachment of amino acids according to the three base pair code. Enzymes called aminoacyl tRNA synthetases, recognize the anticodon loop, and chemically links transfer

RNA (tRNA) to an amino acid specific to that anticodon. tRNAs carrying specific amino acids are recruited and bound to mRNA by ribosomes at the "anticodon loop". This tRNA will then be accepted by the ribosome when its anticodon corresponds to the codon being synthesized, and its amino acid will be added to the growing peptide chain (Lehninger 2000). Once a polypeptide is synthesized, numerous other proteins, nutrients and molecules are often needed to facilitate proper folding and modify it to produce a functional protein (Knorre et al. 2009). The process of transcription and translation is referred to as protein expression.

Reprogramming Transcription Factors

The now famous Yamanaka transcription factors are genes that encode the proteins Octamer Binding Protein3/4 (OCT4), Sex-determining region Y box 2 (Sox2), Kruppel like factor 4 (KLF4), and c-MYC, a member of the MYC family of oncogenes (Takahashi and Yamanaka 2006). Another set of successful reprogramming factors, described by James Thompson in 2007, replaced KLF4 and c-MYC with genes coding for Lin28, an RNA binding protein, and NANOG, a member of the homeobox protein family that is active in early embryonic development (Yu et al. 2007). OCT4, SOX2 and KLF4 are believed to work in concert by co-occupying the promoter and enhancer regions of a large set of genes most often expressed in embryonic stem cells (Plath et al. 2017). These are often referred to as pluripotency genes. In contrast, solitary binding of these factors in ES cells is generally associated with transcriptional repression (Kim et al. 2008). Additionally, OCT4, SOX2 and KLF4 have been shown to collaborate with stage-specific TFs to direct both somatic promoter inactivation and pluripotency promoter

activation to coordinate reprogramming(Chronis, Fiziev et al. 2017).and this may explain how OCT4, SOX2 and KLF4 are able to silence somatic gene expression early in the course of reprogramming, and activate pluripotency genes later (Kim et al. 2008). c-MYC, targets cell cycle and metabolism related genes (Chen et al. 2008), and is not necessary for reprogramming to pluripotency, but acts to improve efficiency of reprogramming(Rand, Sutou et al. 2018) (Rand et al 2018) LIN28 is known to regulate cell proliferation, often in concert with SOX2 by regulating mRNAs that are important for both growth and translation (Cimadamore et al. 2013). NANOG, while not known to be essential for reprogramming, is known to prevent differentiation (Hyslop et al. 2005). Numerous other TFs and over 6000 genes are known to be implicated in the reprogramming process and it remains unclear exactly how transcription factors cause cells to revert to a pluripotent state. Here we present known activities of the six most commonly used pluripotency factors.

OCT4. OCT4 protein is considered a master regulator of pluripotency. It is also a "pioneer" TF, meaning it is able to effect transcription of genes that are bound up in nucleosomes, and therefore unavailable for transcription by most TFs (Zaret and Carroll 2011). OCT4 achieves this by recruiting the chromatin remodelling factor BRG1 to support not only its own binding but also make DNA more available for binding of the other factors necessary for reprogramming, including SOX2 and NANOG (King 2017). Oct4 also regulates the cell cycle, stimulating progression of cells from the G1 phase of the cell cycle, to the S phase, wherein the genes are completely duplicated in preparation for division (Lee et al. 2010). These functions make OCT4 an essential factor for

initiation of reprogramming. The effects of OCT4 are not that simple, however, as cooperation with SOX2, NANOG, and KLF4, amongst many others, has been shown to promote transcription of numerous genes necessary for pluripotency, supporting changes like the mesenchymal to epithelial shift that occurs in early reprogramming of fibroblasts (Li et al. 2010), and promotion of numerous other genes (Kim et al. 2008). When bound alone to promoters, OCT4 represses expression of somatic cell specific genes, preventing differentiation (Kim et al. 2008). Oct4 has also been found to interact with the protein β-catenin in the nucleus, regulating several cascades of transcription and cellular signaling, discussed later. In a study of over 6600 promoters, OCT4 was found to bind with 783, with close proximity to transcription start sites, so it is clear that the activities of this essential pluripotency factor are not fully understood (Kim, 2008).

SOX2. SOX2 is closely associated with OCT 4, and both are often found cooperatively binding promoters. SOX2 is also a pioneer transcription factor is categorized as a transcriptional activator, and has been shown to promote expression of other pluripotency proteins, NANOG and Lin28 (Narayan et al. 2017). SOX2 is known to bind to approximately 819 other promoters located close to transcription start sites (Kim et al. 2008). OCT4 and SOX2 form a complex that often co-occupies target genes. Other proteins found to bind in concert with OCT4/SOX2 include SMAD3, a product of the transforming growth factor beta (TGFβ) signaling pathway, which is required for hESC maintenance, but inhibits reprogramming (Chen et al. 2011, Dalton 2013). While all the mechanisms of SOX2 are not known, its association with OCT4 is known to

activate pluripotent genes while repressing lineage-specific ones, depending on the presence of other effectors (Boyer et al. 2005, Chen et al. 2008).

KLF4. KLF4 is known to facilitate cell cycle arrest at the Gap1 phase, preventing progression to the DNA synthesis phase of the cell cycle. This function does not appear to support reprogramming, but cells are responsive to extracellular signaling during the G1 phase (Hindley and Philpott 2013), and in concert with the other factors, this may be beneficial in the early stage of reprogramming, causing a pause for epigenetic reprogramming to be effected. KLF4's role in the activity of the original 4 pluripotency factors in reprogramming initiation may have been to attenuate OCT4 and c-MYC activity, which strongly promotes transition to the S phase, or suppression of p53 protein, which also arrests cell cycle progression at G1 and inhibits NANOG expression, but this is unknown. In the maturation stage, KLF4 cooperates with OCT-4 and SOX-2 to activate ESC-specific genes, such as NANOG (Schmidt and Plath 2012). While OCT4 and SOX2 are known to coordinate with KLF4, KLF4 binds to approximately twice as many sites genome-wide as either of the other factors, suggesting numerous independent roles for KLF4 (Kim et al. 2008). KLF has been successfully replaced in reprogramming by several other transcription factors, including LIN28 and NANOG, discussed below.

c-MYC. Cellular MYC, or c-MYC, is one of three known MYC proteins. Myc proteins are transcriptions factors that promote proliferation by stimulating transcription and enhancing elongation of active genes by releasing RNAP from transcriptional pause, and stimulating its reload onto promoters (Rahl et al. 2010).

Overexpression of c-MYC is known to lead to oncogenesis, but it also stimulates mitochondrial fission, and upregulates glycolysis, both important aspects of early pluripotency (Prieto et al. 2018). C-MYC targets are different from those of OCT, SOX2 and KLF4, coding for transcription factors that enhance transcription of active genes, but are not essential for the epigenetic changes made during reprogramming (Nakagawa et al. 2008). Given these activities, it is not surprising that sustained expression of c-MYC past the initiation stage of reprogramming promotes differentiation (Nie et al. 2012).

LIN28. LIN28 is an RNA binding protein that is best known for promoting pluripotency via regulation of a number of microRNA's, including the let-7 family, a family of mRNAs known to block reprogramming, inhibit proliferation and promote differentiation (Roush and Slack 2008). LIN28 represses let-7g, improving cell proliferation, and is active in regulation of the other 11 human let-7 genes. LIN28 also interacts with many mRNA's that promote cell growth and survival (Peng et al. 2011). Effects of LIN28 are similar to those of c-MYC, and like c-MYC, LIN28 enhances reprogramming efficiency, but is not essential to the reprogramming process.

NANOG. NANOG is a transcription factor that is expressed in the late stage of reprogramming, and is thought to function as a stabilizer of the pluripotent state (Hyslop et al. 2005). It has been found to be essential to the progression of cells from the de-differentiated state to full pluripotency (Silva et al. 2009). NANOG is not considered essential to reprogramming because its expression is one of the results of OCT4 and SOX2 in late reprogramming. Many of the stabilizing effects of NANOG are attributed to its promotion of the expression of REX1 protein, which stimulates expression of

cyclins B1 and B2, and by inducing mitochondrial fission in concert with cyclinB/CDK1 (Son et al. 2013). In addition to the well-established roles of cyclins in cell cycle regulation, cyclin B1 is essential for chromatin condensation, which is a necessary condition for mitosis (Gong and Ferrell 2010). Cyclin B2 is localized in the Golgi apparatus, and participates in Golgi disassembly during mitosis.

It is clear that effects of pluripotency transcription factors are complex and mediated by numerous other factors. Additionally, they are not the only primary regulators of cell behavior. External signals (Tejada-Romero 2015, Cimmino, 2018, Galli 2011), metabolites (Zhang 2013, Lin 2018), selected small molecules (Bar-Nur 2014, Ma 2017), and reactive oxygen species (Lees 2017, Khacho 2016, Rasmussen 2018) have also been found to play important roles in protein expression, and specifically in cell fate decisions like pluripotency and differentiation (Kwak and Lis 2013, Ma et al. 2017).

Differentiation

While differentiation is not a subject of this work, a discussion of some aspects may illuminate further the need for specific, chemically defined conditions for cells that are used for reprogramming and eventual differentiation. Differentiation mechanisms vary greatly depending on what cell type is desired. Most protocols involve use of specific culture conditions to cause differentiation to the desired state. Here are a few examples of differentiation protocols.

To differentiate stem cells to the hepatic lineage, a 20 day protocol consisting of media supplementation for 5 days with 100ng/mL Activin A, and culturing in 20% O_2

conditions, followed by 5 days with 20ng/mL bone marrow protein 4 (BMP4) and 10ng/mL fibroblast growth factor 2 (FGF2) in 4% O_2 conditions (Vallier, Alexander et al. 2005). This is followed by 5 days with 20ng/mL hepatocyte growth factor (HGF) in 4% O_2 conditions, and finally 5 days in specialized hepatocyte growth medium supplemented with oncostatin M, a cytokine of the leukemia inhibitory factor (LIF) family (Vallier, Alexander et al. 2005).

Differentiation of iPSCs to the skeletal muscle lineage begins with plating iPSCs on matrigel™, a culture plate coating commonly used for maintenance of stem cells. The reasoning behind this is that matrigel contains laminin, collagen IV, heparan sulfate proteoglycans and entactin/nidogen, all known to induce differentiation to the myogenic lineage. Cells are maintained on matrigel in "commitment medium" consisting of 10% FBS and 100µM mercaptoethanol, a reducing agent. This produces a myogenic precursor cell that is still highly proliferative, and cell populations are expanded in "proliferation media" with 10% FBS and 50µM mercaptoethanol, with 5ng/mL FGF, additional amino acids, 0.5µM oleic acid and 0.5µM linoleic acid, along with additional iron. Cells are then transfected with MYO-D, a master differentiation factor for the myogenic lineage (Pownall et al. 2002), and placed in "differentiation media" supplemented with 2% horse serum, along with a number of nutrients known to aid in reprogramming, including retinol and ascorbic acid 2-phosphate (Hore et al. 2016), hormones such as corticosterone and progesterone, and some lipids and lipid related nutrients including linoleic and linolenic acid, lipoic acid and alpha tocopherol (vitamin E) (Burridge et al. 2014).

Differentiation to the neural lineage has been efficiently accomplished using only media cues. Cells are initially placed in serum free media supplemented with TGFβ inhibitor and Noggin, a protein known to inhibit BMP ligands of the TGFβ pathway (Hirsinger et al. 1997). Upon day 5 of differentiation, the TGFβ inhibitor is removed and increasing amounts of neuron specific media supplemented with insulin, transferrin and progesterone (Bottenstein 1985) is added at each feeding (every 2 days) maintaining concentrations of Noggin (Chambers et al. 2009). This protocol induces motor neurons in 19 days. Differentiation to other types of neurons, such as dopaminergic neurons, has proven more difficult.

Notably, these three representative protocols rely heavily on media contents, modulating function of the Wnt (Lian et al. 2012), BMP and TGFβ pathways (Kattman et al. 2011), and stimulating cells with hormones and nutrients to promote differentiation.

Efficient differentiation is the final obstacle to therapeutically applicable iPSCs, but differentiation efficiencies are variable, and 50-80% differentiation is considered highly efficient (Burridge et al. 2014, Maguire et al. 2017, Gu 2018). Differentiation is assessed by presence of certain receptors and markers on the cell, and this may not be a complete picture of the cellular epigenetic state, as illustrated above. The term naïve has been coined to refer to iPSCs that express the markers of pluripotency, and do not have residual epigenetic markers (Bates and Silva 2017). New protocols are continually being developed for differentiation of stem cells to specific lineages, and increasingly the focus has been placed on media supplements as active

components of the differentiation decision (Sachs et al. 2003, Chen et al. 2011, Inge et al. 2011, Lin et al. 2018).

Application of Stem Cells to Therapy

Application of stem cells to therapy is a goal of current research and clinical trials. It is accepted that transplantation of a patient's own cells (allogenic) is much safer than use of stem cells from a donor (autologous), but results of trials have been disappointing, and risks remain high (Marks et al. 2017). Allogenic transplants eliminate immune rejection issues, but may cause relapse of the disease state, especially when applied to genetically related disease. Use of autologous hematopoietic stem cells to replenish blood cells after chemotherapy is common, but requires immune suppression, and has a reported non-relapse mortality rate of 20-30% (Wang et al. 2019). Other applications have also remained problematic. In one case, a patient was treated with injections of allogeneic stem cells from different sources to reduce the effects of a stroke (Berkowitz et al. 2016). The treatment led to the development of a brain tumor, which led to paraplegia. Another case involved autologous stem cell treatment for macular degeneration using stem cells derived from adipose tissue. Three people were given the treatment, and all experienced worsening vision. Two became blind (Kuriyan et al. 2017). Currently, allogenic stem cell therapy is used most commonly for ocular and hematological applications (Martin 2017). iPSCs are, however, being used very effectively to model disease states in vitro, making it possible to learn more about the mechanisms of disease (Zuba-Surma et al. 2012, Rami et al. 2017). This modelling is done by reprogramming cells from diseased patients to pluripotency, differentiating them

to cell lineages that are not practical for sampling, such as neurons, and modeling the disease state in vitro. Modeling of disease states, and analysis of cellular responses to stimuli such as nutrients, small molecules, and transfection can be a powerful tool for design and testing of novel treatments, including stem cell transplants, and can definitely be a valuable resource for continued research to improve iPSC quality and reliability.

The Wnt Pathway

The Wnt pathway is a central regulator of cell fate determination, cell migration, cell polarity, neural patterning and organogenesis (Komiya 2008), and has emerged as a central pathway of cell reprogramming (Rasmussen et al. 2018). The Wnt receptor is a transmembrane protein called Frizzled (Baarsma et al. 2013). Adjacent to the Wnt receptor is the co-receptor, low-density lipoprotein receptor-related protein 5/6 (LRP5/6), which is activated by binding of a palmitoleic or palmitic acid. Binding of Wnt protein to Frizzled, accompanied by binding of a palmitoyl moiety to LRP5/6 recruits the Disheveled and Axin proteins to the inner surface of the cell membrane, disabling a protein complex called the β-catenin degradation complex and activating "canonical" Wnt signaling (Galli et. al 2011). The binding of Wnt proteins to the Frizzled receptor without lipid binding to the LRP 5/6 co-receptor does not activate the canonical pathway, but instead activates the non-canonical Planar Cell Polarity and Ca^{2+} pathways (Gao and Chen 2010, Baarsma et al. 2013). This lipid dependent differential activation is definitive to the effect of Wnt signaling. Without specific lipids, canonical signaling is not possible.

The canonical Wnt pathway supports pluripotency and cell survival, while the non-canonical pathways are essential for programmed cell death, cytoskeletal rearrangement, differentiation, and embryonic development, among other functions (Gao and Chen 2010). Interference with non-canonical Wnt activity can cause cells with genetic or phenotypic flaws to continue to proliferate, leading to disease and even cancer (Zanconato et. al. 2016, Holland et al. 2013) Therefore, it follows that encouraging canonical Wnt signaling promoting pluripotency, and minimizing non-canonical pathway activity while leaving its vital activities intact, may be a promising path for stem cell health and homogeneity.

Canonical Wnt Signaling

Canonical Wnt activation is accomplished by binding of a palmitoylated Wnt protein to the Frizzled receptor, along with binding of the palmitoyl moiety to the LRP5/6 receptor (figure 3). The cytoplasmic tail of LRP binds to Axin in a Wnt- and phosphorylation-dependent manner, and the cytoplasmic portion of Frizzled recruits disheveled (Galli and Burrus 2011). This paired binding activates the canonical Wnt pathway (He 2008, Nusse and Varmus 2012).

The initial steps of canonical Wnt activation are the binding of Axin to the cytosolic tail of LRP5/6, and the binding of disheveled to the cytosolic side of Frizzled. Axin binding causes disassembly of the β-catenin degradation complex of proteins, which includes Glycogen Synthase Kinase 3 (GSK3), Casein kinase 1 (CK1), Protein phosphatase 2A (PP2A) and Adenomatous Polyposis Coli (APC), Yes Associated protein

(YAP), and Transcriptional co-Activator with PDZ-binding motif (TAZ) (figure 3) (Azzolin 2014, Huang et al. 2015).

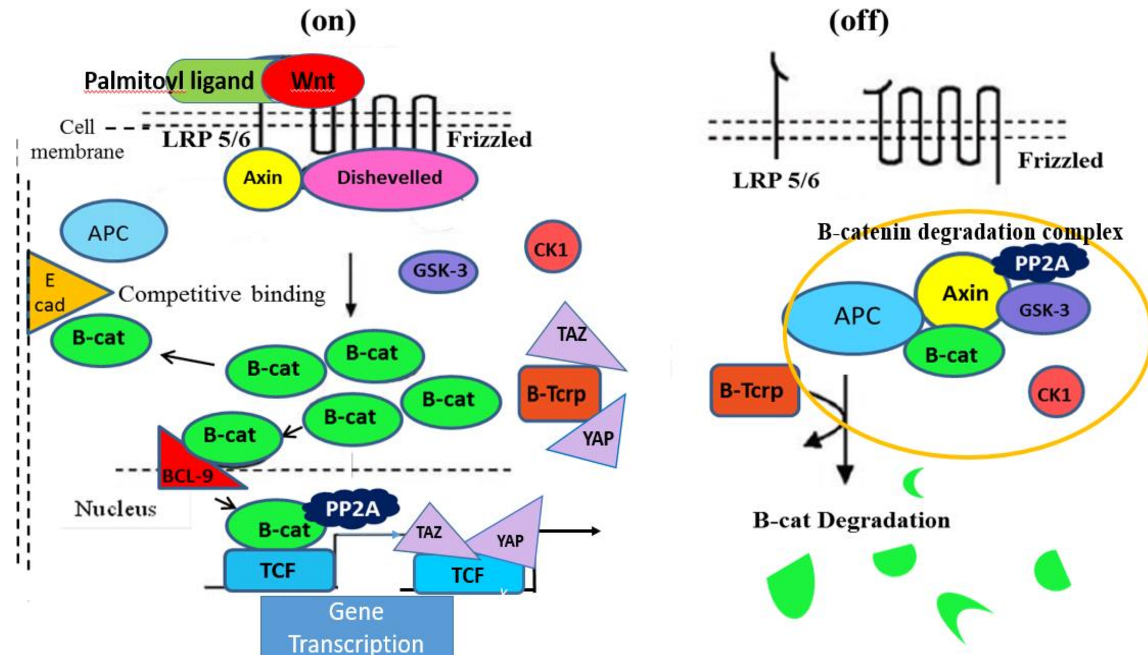


Figure 3. A Model of Canonical Wnt Signaling. PA: palmitoleic acid. APC: Adenomatous Polyposis Coli, B-cat: Beta Catenin, E Cad: E-cadherin, GSK-3: Glycogen Synthase Kinase 3, CK1: Casein kinase 1, YAP: Yes Associated Protein, TAZ: Transcriptional coactivator with PDZ-binding motif, β-Trcp: β-transducin repeat containing protein, TCF: T-cell factor

Each of these proteins play important roles in downstream Wnt related activities, but the most important effect of complex deactivation by canonical Wnt signalling is that the β-catenin protein is no longer degraded by ubiquitination, so its concentration builds up in the cytoplasm. This triggers transport to the nucleus, facilitated by the B-cell Lymphoma 9 (BCL-9) protein.

In the nucleus, β-catenin interacts with the T-cell factor/Lymphoid enhancer factor (TCF/LEF) group of transcriptional mediators. This binding is mediated by PP2A from the degradation complex, and results in expression of genes supporting

pluripotency, proliferation and cell survival, such as c-Myc and Cyclin D (Wohrle et al. 2007).

Components of the β-catenin degradation complex have been found to be active in other cellular mechanisms when canonical Wnt signaling is activated. There is disagreement about whether the β-catenin degradation complex is dismantled upon recruitment of the Axin scaffold protein to the membrane, but there is abundant evidence that the proteins of the complex are active in other ways during canonical Wnt activation (Cohen and Frame 2001, Schittek and Sinnberg 2014, Ye 2015). This includes PP2A modulation of the activities that are initiated by the nuclear activities of β-catenin, APC adhesion functions and CK1 activities. Known roles of these proteins are too numerous to catalog here, but a brief overview of known roles that are relevant to reprogramming and differentiation may illuminate the benefits of maintaining these proteins in functional form during culture, reprogramming and maintenance of iPSCs, instead of silencing, which is often done to improve reprogramming efficiency.

The central role of B catenin

β-catenin is a multifunctional protein and interacts with many other proteins including cadherins, Adenomatous Polyposis Coli (APC), BCL-9, and the LEF/TCF domain.

Interaction with β-catenin is essential for the function of cadherins in cell adhesion by establishing a link to the actin cytoskeleton (Marthiens et al. 2010). Two cadherins, E-cadherin and N-cadherin are relevant in this context. N-cadherin has been found to have no effect on stem cell proliferation and maintenance (Kiel 2009). E-

cadherin is a membrane-associated protein, which binds α-catenin, β-catenin, and plakoglobin (γ-catenin). B-catenin forms a direct link between E-cadherin and α-catenin, and α-catenin interacts with and signals the actin cytoskeleton. E-cadherin is also a regulator of cell cycle and cell fate, through its signaling function (Redmer et al. 2011). Canonical Wnt signaling down-regulates expression of E-cadherin, minimizing β-catenin binding at the membrane, and potentially inhibiting cell-cell adhesion (Berx et al. 2008). Adhesion, and the signaling it induces are essential for colony formation: an important secondary step in stem cell reprogramming (Redmer et al. 2011).

B-catenin binding with APC is essential for spindle orientation in the centrosome during cell division (Marthiens et al. 2010). Lack of APC connections on both ends of the chromosomal spindle leads to asymmetric orientation during cell division, resulting in cells that are not identical (Marthiens et al. 2010). Asymmetric division is the way stem cell populations provide cells for healing and growth, producing one differentiating cell and one stem cell, and this is also source of spontaneous differentiation in cell culture.

APC and E-cadherin compete for binding to β-catenin, making availability of β-catenin even more important during reprogramming of somatic cells to pluripotency. β-catenin must be available both for symmetric spindle orientation, and then for cell-cell adhesion (Valenta et al. 2012).

When β-catenin is plentiful in the cytoplasm, it is also available for binding with the BCL9 protein, which shuttles it into the nucleus (Brembeck 2004), where the other primary function of β-catenin can be accomplished. In the nucleus, β-catenin associates

with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. These factors repress gene transcription in the absence of β-catenin. When activated by β-catenin, they modulate and facilitate the activity of β-catenin by recruiting it to various promoters and enhancers in DNA, causing the transcription of target genes (Valenta et al. 2012). Other factors also effect activation of DNA transcription by β-catenin, including some that attenuate canonical Wnt signaling (Baarsma et al. 2013). These observations further illustrate the complex balance and feedback mechanisms of the Wnt pathway.

A 12-armadillo repeat array of β-catenin (figure 4) is the main mediator of transcription factor interactions (Bienz and Clevers 2003).

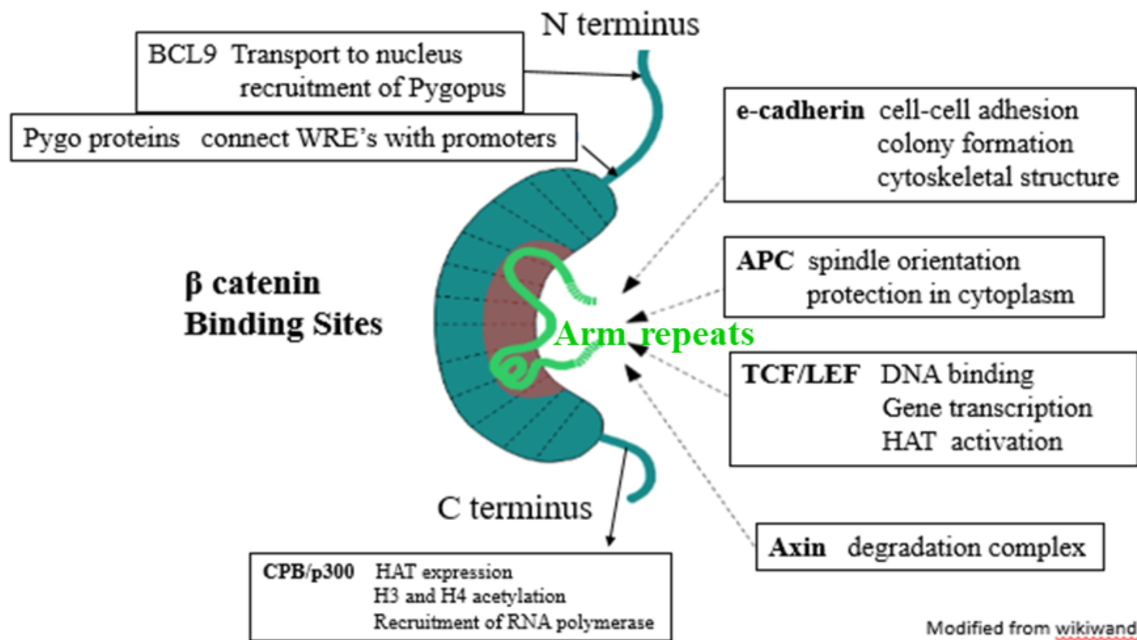


Figure 4. Known Binding Sites of β-catenin. Central are the armadillo (ARM) repeats, which bind to numerous partners. Notable binding partners in Wnt activation are e cadherin, APC, the TCF/LEF domain, and Axin. CPB and p300 proteins bind at the C-terminus, and BCL9 binds at the N terminus, recruiting pygopus (pygo) proteins and translocating β-catenin to the nucleus (https://en.wikipedia.org/wiki/Beta-catenin)

Domains in the amino and carboxyl tails also activate and facilitate Wnt target transcription by promoting expression and activation of Histone Acetyltransferases (HAT), recruiting RNA polymerase and connecting Wnt Response Elements (WRE) to promoters (Valenta et al. 2012).

Thus another layer of complexity is added to the signaling and protein reactions involved in Wnt signaling.

Proteins of the β-catenin Degradation Complex

GSK3 is the most often silenced protein of the β-catenin degradation complex. Silencing of GSK3 cause the dephosphorylation of Axin, APC and β-catenin in canonically Wnt activated cells (Cohen and Frame 2001). E-cadherin can then interact with the freed β-catenin resulting in cell-cell adhesion and APC is functionalized tto interact with β-catenin for chromosomal spindle orientation in the centrosome (Baarsma et al. 2013). Both of these functions are essential for stem cell maintenance in the pluripotent state (Marthiens et al. 2010). GSK3 also activates the extremely important nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which promotes cell survival. Furthermore, GSK3 inhibits the cell cycle progression activators cyclin D, cyclin E and MYC, activates p53 related apoptosis pathways (Maurer 2014), and is important in phosphorylating β-catenin for degradation. While these roles seem to counteract efficient reprogramming, they are important for elimination of excess β-catenin, and for programmed death of cells with genetic anomalies (Marthiens et al. 2010). The intricate crosstalk between pathways that culminates in the overall effect of

GSK3 is not well understood, but it is clear that silencing of this protein, while enhancing pluripotency, would leave several essential cellular functions inactive.

CK1 is another kinase with numerous functions. Relevant to stem cells, CK1 plays a role in spindle formation and cell cycle progression, and blocks apoptosis (Schittek and Sinnberg 2014). CK1 phosphorylates β-catenin in the destruction complex, priming the protein for phosphorylation by GSK3. CK1 also phosphorylates Axin, as part of its binding to the cytosolic tail of the LRP5/6 receptor, in the initial stages of canonical Wnt signaling (Baarsma et al. 2013).

PP2A is a phosphatase, and as such it is able to reverse the phosphorylation dependent activation of GSK3 and CK1 (Thompson and Williams 2018). PP2A activates GSK3 by dephosphorylating it at Ser9. Additionally, PP2A subunits are essential for interaction of β-catenin with E-cadherin at the cell membrane, resulting in cell to cell adhesion. In its dimerized state, PP2A stabilizes β-catenin by dephosphorylating it. PP2A also stabilizes the c-Myc protein, which is a strong activator of stem cell self-renewal (Thompson and Williams 2018). C-Myc is one of the original Yamanaka factors used to induce pluripotency, and its transcription is promoted by Wnt activity.

APC protects β-catenin in the cytoplasm when the degradation complex is intact, and is also an essential component of adherens junctions in the nucleus, which position chromosomes during mitosis (Marthiens et al. 2010).

Two recently elucidated components of the β-catenin degradation complex, the Yes-associated protein (YAP) and the Transcriptional coactivator with PDZ-binding motif (TAZ), enhance association of the β-transducin repeat containing protein (β-Trcp)

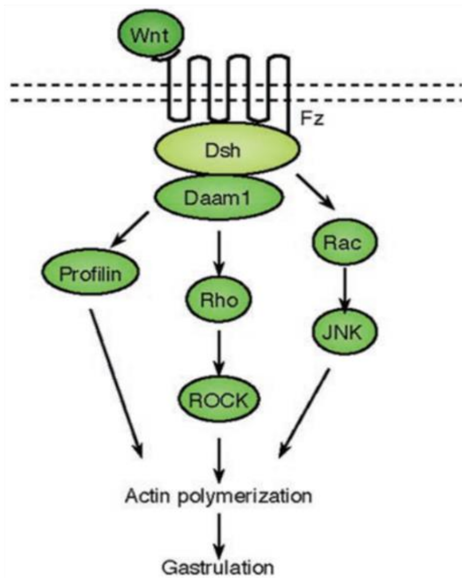
with the β-catenin destruction complex, when canonical Wnt signaling is off. Association with the destruction complex disrupts the function of β-Trcp, protecting β-catenin from degradation (Azzolin 2014). Activation of Wnt releases YAP and TAZ to locate to the nucleus, where they interact with the TCF/LEF domain, activating some of the same proliferation promoting genes as β-catenin, such as c-myc and survivin (Zanconato et al. 2016). YAP and TAZ activity is also modulated by the HIPPO pathway, which is signaled by spatial stresses such as stiffness of matrix, shear stress, and proximity of other cells (Aragona et al. 2013). Hippo activation causes phosphorylation of Large Tumor Suppressor Kinase (LATS), which in turn phosphorylates and inactivates YAP and TAZ, downregulating expression of the genes they promote.

The cross regulation of the Hippo and Wnt pathways, and the complex interactions of their components are not well understood. The Insulin signaling pathway, Transforming Growth Factor β (TGFβ)/Activin/Nodal pathway, Fgf2 pathway and the Notch pathway have also been shown to modulate the effects of Wnt pathway activation (Dalton 2013). This complex network of interactions insures that the specific needs of a particular cellular situation can be met, while avoiding aberrant growth or other deleterious behavior (Aragona et al. 2013). It is clear that silencing any single component of this complex system would risk aberrant cell behavior, such as oncogenesis, by suspending feedback mechanisms. For this reason, a nutrient based system to activate canonical Wnt signaling, without disabling the proteins involved, promises to support desired cellular activities without sacrificing other essential functions of these proteins, leaving regulatory and complementary crosstalk intact.

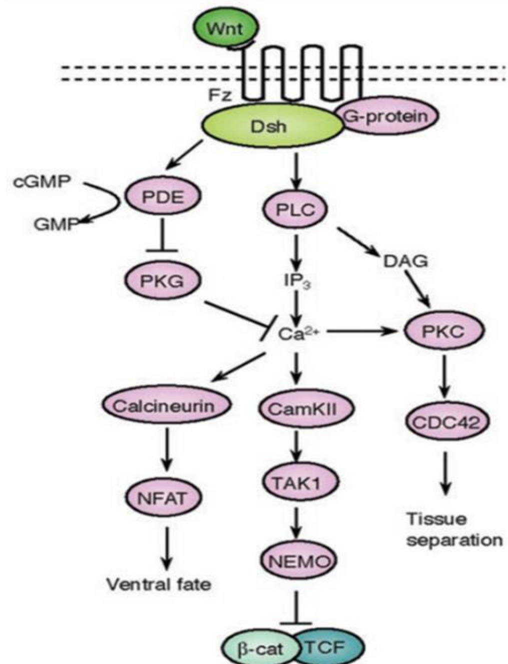
Non-canonical Wnt Signaling

The non-canonical Wnt pathways have been shown to be stimulated by Wnt binding to the Frizzled receptor in the absence of the palmitoyl adduct to the LRP5/6 co-receptor (He 2008). In non-canonical Wnt signaling, the disheveled protein initiates activation of two pathways important in development, the planar cell polarity pathway (PCP) and the calcium signaling cascade (Ca^{2+}). Like the canonical pathway, activation is mediated by many other signals, shaping the cellular response according to the presence of other ligands and inputs (figure 5).

Planar Cell Polarity Pathway



Ca^{2+} Signal Transduction Cascade



Komiya and Habbas Organogenesis, 2008

Figure 5. Non Canonical Wnt Signaling. Note that the PCP pathway stimulates Rho associated protein kinase (ROCK) and the Ca^{2+} cascade results in cessation of βcatenin/TCF target gene expression and differentiation.

When PCP is activated, the small GTPases Rho and Rac are also activated. These factors then go on to stimulate production of Rho-associated protein kinase (ROCK), which induces cytoskeletal reorganization, and often results in "blebbing" and cell death in stem cells (Schmandke et al. 2007).

ROCK inhibitors are commonly used in iPSC culture, to reduce apoptosis when seeding from cryopreservation (Park 2015). The PCP pathway is essential for the symmetric development around a central plane of an embryo and Actin cytoskeletal reorganization. PCP also controls gastrulation, the initiation of embryonic development. This suggests the PCP pathway is active in differentiation, which must be avoided during maintenance, and reversed for successful reprogramming.

A poorly understood mechanism of the PCP is the stimulation of Jun N-terminal Kinase (JNK). JNK is a member of the mitogen activated protein kinase family of proteins, which are central to the process of mitosis (Zhang and Liu 2002). JNK participates in stem cell mitosis, and activates apoptosis where appropriate. JNK responds to many molecular cues, so down regulation of non-canonical Wnt does not necessarily abrogate its essential functions (Dhanasekaran and Reddy 2008). In fact, JNK response depends on the mode of its activation, and has been shown to be necessary for stem cell proliferation in some circumstances. JNK pathways are also activated by infection, oxidative stress, DNA damage, and inflammation, providing a necessary repair or the apoptotic response for damaged or genetically impaired cells (Tejada-Romero et al. 2015). Interestingly, excess of free fatty acids or sugars can also activate JNK apoptotic pathways *in vivo* which can act through inactivation of insulin receptors (Vallerie and

Hotamisligil 2010). This nutrient effect indicates again that media contents can be very important in preventing excessive JNK related apoptosis, while silencing of this pathway may be deleterious to the quality and homogeneity of the cell population (Tejada-Romero et al. 2015).

The Ca^{2+} pathway is triggered by binding of Wnt to the Frizzled receptor, without lipid binding to the LRP5/6 receptor. This causes release of Ca^{2+} from the endoplasmic reticulum, resulting in Ca^{2+} cascades (De 2011). These cascades cause muscle contraction, mitochondrial swelling, expression of inflammatory proteins, and apoptosis (Sassone-Corsi 2012). The Ca^{2+} cascade stimulated by non-canonical Wnt also activates Wnt5a production, and participates in degradation of β-catenin.

Non-canonical Wnt pathways respond to multiple signals in addition to the binding of a Wnt to the frizzled receptor, and are regulated by enzymes and crosstalk from several sources (Aragona et al. 2013, Huang et al. 2015, Lees et al. 2017). These complex interactions, stimulated by nutrients and cytokines available to cells, modulate non-canonical Wnt responses and provide signals for many essential cellular functions (Kahn 2011) including healing and metabolism.

The downregulation of non-canonical Wnt signaling, which occurs with stimulation of canonical Wnt signaling, may eliminate or reduce the need for other interventions like ROCK inhibitors and gene silencing, while maintaining minimal intrusion into the natural functions of non-canonical Wnt pathways, and their effects on the interdependent networks of other pathways and mechanisms.

Canonical and Non-Canonical Wnt Pathway Interaction

LRP5/6 binding to a lipid has been shown to be essential for the activation of the canonical Wnt pathway. Wnt binding to Frizzled without the lipid modification that binds the LRP5/6 site may activate the non-canonical planar cell polarity (PCP) pathway, resulting in Rock activation, and the Ca2+ signal transduction cascade related to the JNK pathway. Disruption of the balanced interaction between E-cadherin, APC, and the catenins has been found to cause epithelial–mesenchymal transitions (EMTs) (Wang 2013). These transitions may be part of development, causing cells to migrate normally or part of oncogenic behaviors, causing cells to invade neighboring tissues (Redmer et al. 2011).

The competitive relationship between β-catenin/E-cadherin and β-catenin/APC binding effects whether the spindle is oriented to cause identical or differential daughter cell production (APC binding) or cell-cell adhesion (E-cadherin binding). Adhesion also directly effects the activation of the Hippo pathway, which interprets adhesion signals to determine cell density and stress, responding with release of YAP and TAZ, which act to slow proliferation (Aragona et al. 2013). Many target genes of Wnt signaling influence cadherin adhesion, and many downstream proteins directly impact E-cadherin activity (Huang et al. 2015). E-cadherin activity, combined with other inputs, such as Hippo response and non-canonical Wnt activity, contribute to the proper orchestration of cell adhesion (Azzolin 2014). Wnt and Hippo signals, in concert with their mutual regulation and the participation of non-canonical JNK and ROCK apoptosis controls are all essential to healthy cell fate decisions (Aragona et al. 2013). Providing cells with the lipid ligands

necessary for Wnt pathway stimulation without silencing of essential related proteins, and without the confounding factors introduced by sera, may provide a better foundation for reprogramming and maintenance of iPSCs using nutrient stimulation, and may also be crucial for avoiding oncogenesis (Pruszynska et al. 2018).

<u>Fatty Acid Effects on Wnt Activity</u>

Differential lipid modification of specific cysteine and serine residues of Wnt proteins has been shown to be essential in differential activation of canonical or non-canonical Wnt signaling pathways (Bryja et al. 2009, Galli and Burrus 2011). Mass spectrometry studies have shown that fatty acylation of Wnt protein utilizes fully saturated palmitate (C16:0) at cysteine 77 (S-palmitoylation) and monounsaturated palmitoleic acid (C16:1) at serine 209 (O-palmitoylation) (He 2008, Nile and Hannoush 2016). This supports the idea that differential and desirable activation of the Wnt pathways may be achieved by selectively making these lipids available in culture media. Modification of both the cysteine and the serine residues are required for normal signaling, but palmitoylation of the serine residue, has been shown to be more critical than cysteine palmitoylation in ß-catenin dependent signaling (Galli and Burrus 2011), suggesting that the canonical Wnt pathway is selectively activated by the palmitoleic acid ligand. Palmitate is thought to be essential in Wnt protein synthesis, transport, and secretion (Coudreuse and Korswagen 2007), but, high levels of palmitate have been closely associated with cell death due to JNK activation (Dhanasekaran and Reddy 2008) and production of Reactive Oxygen species (ROS), indicating it may be more conducive to the activation of the non-canonical Wnt pathway (Egnatchik et al. 2014). Both

palmitic and palmitoleic acids were tested in this work, as the distinctions are still unclear, and multiple undiscovered roles may exist for each of these fatty acids.

Effects of Wnt Activation on Stem Cells

In summary, stem cell maintenance requires stimulation of proliferation and pluripotency genes, absence of differentiation signals, production of identical offspring cells, and quality control mechanisms to maintain genetic integrity and pluripotency (Zhang et al. 2012, Dalton 2013, Shyh-Chang 2017).

Canonical Wnt signaling supports the reprogramming and pluripotency of cells by activation of histone acetyltransferases, stimulation of proliferation and cell cycle genes, and provision of β catenin for cell-cell adhesion and spindle orientation in the centrosome (Wohrle et al. 2007, Huang et al. 2015).

Histone acetylation is the way DNA is “unraveled” from histone proteins, and demethylation of DNA makes genes epigenetically silenced by methylation available for transcription in reprogrammied cells. In contrast, differentiation involves deacetylation of histones, which causes DNA to wrap around them, making a nucleosome, and rendering the DNA unavailable for transcription. When an acetyl group attaches to a lysine residue on the surface of a histone, the positive charge on the lysine is neutralized, causing the DNA that is wrapped around it to “unravel” (Nakayasu et al. 2017). This opens up binding sites that were not available for transcription when the DNA was tightly “wrapped” around the histone. Canonical β-catenin transcription stimulates expression of histone acetyltransferase proteins, stimulating the “unravelling” of DNA, and the process of reprogramming. Fully unraveled DNA, if possible, would be ideal, but it is unclear

whether any but the very first few cells of a blastocyst actually have this conformation (Wu et al. 2016). The DNA in iPSCs as we know them today have been "unraveled" to differing degrees depending on the source cells, and the methods and media used to reprogram and maintain them (Newman and Cooper 2010, Ivanov et al. 2016). The epigenetic state of iPSCs is not equivalent to that of embryonic stem cells, retaining some histone structure, and methyl markers from the original cell type (Halevy and Urbach 2014).

Wnt target genes also express proteins essential for mitochondrial reproduction and proteins that induce S-phase transition such as cyclin D1, cyclin D2 and c-Myc, and promote transcription of key pluripotency factors OCT4, NANOG and SOX2 (Wohrle et al. 2007, Rasmussen et al. 2018). Through these and other effects, stimulation of canonical Wnt maintains ESC's in a more naïve state, meaning the DNA is more "unraveled".

β-catenin is essential for cell to cell adhesion, and for adherens junctions in the centrosome. Interactions between β-catenin, E-cadherin and Axin anchor stem cells to a support cell in a stromal niche and provide adhesion to neighbors in an epithelial niche (Berx et al. 2008).

Placement of adherens junctions is believed to effect cell fate by determining the orientation of the spindle just prior to division (Marthiens et al. 2010). This process is dependent on Adenomatous Polyposis Coli (APC), which is a part of the β-Catenin degradation complex, and is liberated from this complex for relocation to the membrane by stimulation of the canonical Wnt pathway (Figure 3). Symmetric division produces

two identical daughter cells, while asymmetric division produces two different cells, at least one of which will be primed to differentiate after division (figure 6).

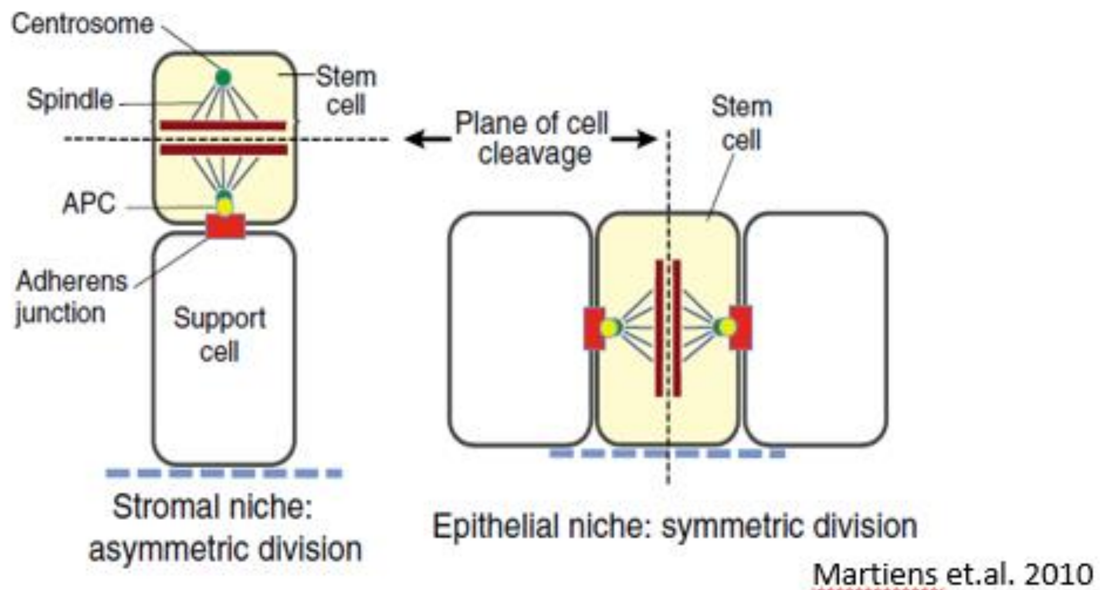


Figure 6. Spindle Orientation in Stem Cell Division. Adenomatous Polyposis Coli (APC) is essential to symmetric division of cells, producing identical daughter cells. Production of identical daughter cells is important for promotion of homogenous populations. APC is liberated when canonical Wnt is activated.

APC protein is recruited to adherens junctions in a β-catenin dependent manner, positioning the chromosomal spindle just prior to cell division (Komiya 2008, Marthiens et al. 2010). When canonical Wnt is activated, both APC and β-catenin are plentiful, making symmetrical spindle orientation much more likely. This leads to fewer differentiating cells in culture (Ye 2015).

The fate of β-catenin is partially regulated by tyrosine phosphorylation, which induces BCL-9 binding and nuclear transport, and disrupts E-cadherin binding, which is essential for cell-cell adhesion (Inge et al. 2011). E-cadherin expression is promoted by several other proteins, including AP2 and c-Myc, depending on signals from other

receptors and pathways (Berx et al. 2008). This balance and crosstalk further underlines the importance of stimulating the Wnt pathway in a biologically relevant and unobtrusive way, as can be accomplished with lipid and nutrient supplementation, to avoid crossing the fine line to oncogenic or other aberrant development.

Obstacles to Therapeutically Applicable iPSCs

Reprogramming Efficiency

Reprogramming yield of iPSCs is measured by number of colonies produced from a given number of primary cells. Published colony yields from transfection of Yamanaka's four factors range from 0.056% to 0.19% in fibroblasts (Medvedev et al. 2010). Attempts to improve this ratio have used numerous combinations of cloning factors, several small molecules (Zhang et al. 2012, Ma et al. 2017) and gene silencing. Some results claim up to 56% yields using small molecules and nutrients, including inhibitors of glycogen synthase kinase β (GSK3-β) (Bar-Nur et al. 2014, Omole and Fakoya 2018). GSK3 inhibition eliminates β-catenin degradation, and so allows β-catenin related activities, but does not otherwise modulate Wnt pathway activities such as non-canonical activities, sequestering of Axin to the cytoskeleton or release of CK1, PP2A, APC, YAP, and TAZ proteins from the degradation complex (figures 3 and 5).

More recently, use of mRNA for reprogramming has shown promise with yields as high as 90% (Kogut et al. 2018), but these experiments used RNA with the Myc transactivation domain fused to the Oct 4 promoter. This type of genetic modification may result in permanent and unpredictable changes in the genome. Additionally, media

conditions in these experiments included animal derived supplements (Wang et al. 2017). These complex additives contain unknown components, and have been shown to be the source of variation in cultured cell experiments.

Primary cells used to generate hiPSCs must be harvested from volunteers or patients, so to minimize the amount of tissue and time required for reprogramming, increased reprogramming efficiency is of the utmost importance in iPSC applicability for research and therapy. Also of concern is the efficiency of other modifications that may be made to hiPSCs during research or therapy, such as gene correction. Once a population of hiPSCs is established, it may be used for tissue generation (Li et al. 2017), including organ generation (De Vos and Assou 2017), for modeling of healthy or diseased states in research (Elitt et al. 2018), or for application to genetic therapy, via gene correction (Gross et al. 2012). In all cases, the level of pluripotency, and the viability of the stem cell population are of utmost importance. If residual histone coiling and methylation markers of the source cell genotype remain, there is potential for the source cell type to reemerge. Cells used for research must reliably respond to experimental variables in the manner of the *in vivo* cell type they represent, or incorrect experimental results will ensue. Cells used for gene therapy must maintain their genotype and phenotype through a process of genetic editing, and emerge competent to carry the genetic correction to their progeny, without reversion to their former cell type.

The first step in the process of reprogramming is to choose a method of introduction of reprogramming factors. There are a variety of methods, with varying efficiencies and safety factors (Turinetto et al. 2017, Omole and Fakoya 2018) as

diagrammed in Figure 7. In this work, it was important to introduce reprogramming factors without a viral vector, as viral vectors tend to insert into the host genome and stay there. Viral insertions introduce unpredictable effects, as these inserts may cause the cell to die, perturb protein synthesis, or lead to oncogenesis (Brouwer et al. 2016, Turinetto et al. 2017). To avoid the use of viral vectors, nucleofection, an advanced form of electroporation, is often used to introduce non-viral reprogramming factors (figure 7). A form of DNA called minicircle™ DNA may also be used to carry transcription factor genes. The minicircle is a compact vector that can be loaded with DNA, and does not contain bacterial or viral DNA that can be inserted into the genome.

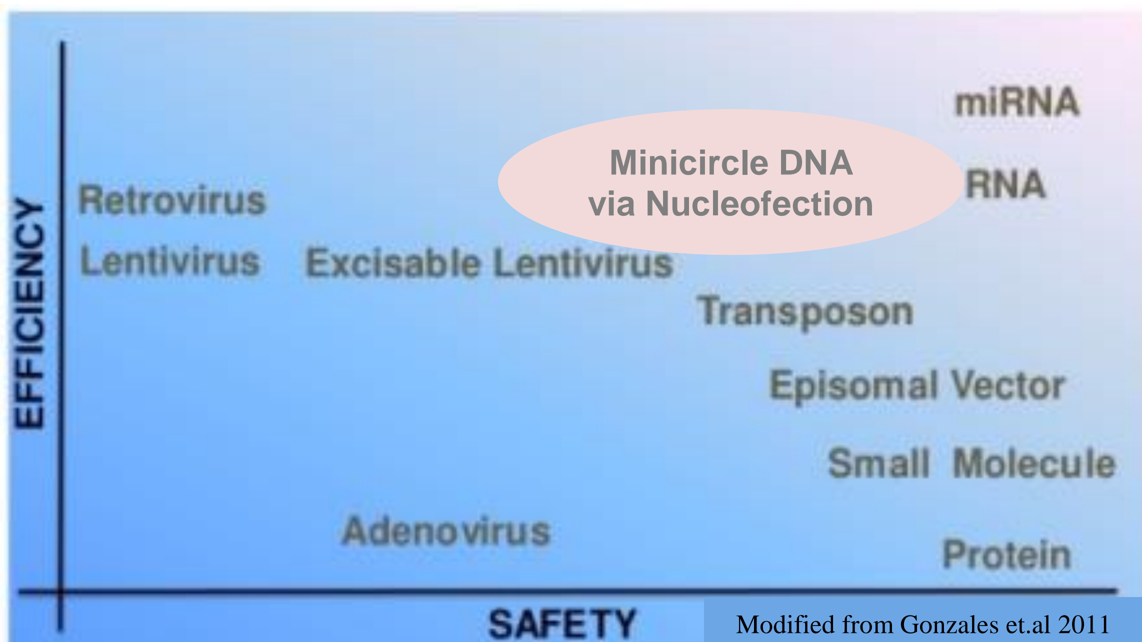


Figure 7. Relative Efficiency and Safety of Reprogramming Methods. Safety refers to the risks associated with unintended genetic insertion, oncogenesis, and cell death due to the method.

The use of minicircles eliminates the possibility of DNA insertions, improving safety for potential transplant recipients, and eliminating the possibility of genetic

mutations in iPSC lines that may be used for research (Jia et al. 2010). The minicircle method also eliminates problems associated with use of monocystronic methods, in which each factor is on its own plasmid, leading to uneven application, such as mRNA (González 2011). Minicircle DNA applied via nucleofection, as used in this work, offers 80% transfection efficiency without insertions.

Use of mRNA is considered the most safe and efficient method of gene transfection, but in a multi-factor application, such as reprogramming to pluripotency, it is important that each cell receives at least one copy of each of the factors. This is impossible to achieve when multiple factors are delivered independently. With the minicircle DNA format, all four factors are carried in each minicircle, ensuring even distribution of all four factors. This minicircle, polycystronic format is highly efficient and transgene free (Jia et al. 2010). Reprogramming efficiency remains low, even with this system, underlining the need for more optimization of the reprogramming process. Polycistronic mRNA is currently being tested, and holds great promise for virus free, safe and efficient reprogramming (Castro et al. 2018).

Homogeneity

As the interdependence between nutrient driven cell signaling and gene expression is explored, the importance of homogeneity of iPSC populations becomes more apparent. Cells that are not fully reprogrammed may signal others in unpredictable ways (Hlavacek and Faeder 2009, Dalton 2013). This was illustrated in 2010 by a large meta-analysis of the genetic homogeneity of human pluripotent stem cells (Newman and Cooper 2010). A significant correlation between expression signatures of 15 genes

commonly expressed by embryonic and induced stem cells were analyzed. Significant variation in expression levels of these genes was found between hiPSCs of the same cell line origin, that were cultured in different labs (Newman and Cooper 2010). Lab-specific expression differences were greater than gene expression differences between iPSCs and ESCs. This variability was attributed to stochastic environmental differences, further underlining the weakness of current techniques in providing homogeneously reprogrammed and cultured iPSCs, and also illustrating the importance of environmental and nutrient effects on iPSC homogeneity. Activation of a strong pluripotency regulation pathway, like the canonical Wnt pathway (Rasmussen et al. 2018), along with use of standardized, chemically defined culture conditions, may help overcome some of these variations.

Canonical Wnt activation inhibits glycogen synthase kinase 3-β (GSK3-β) activity without inactivating it completely (Maurer 2014). This provides β-catenin, PP2A, YAP and TAZ to the nucleus, stimulating expression of pluripotency genes. Canonical Wnt stimulation also provides Adenomatous Polyposis Coli (APC) from the β-catenin degradation complex to adherens junctions in the nucleus, and stimulates expression of e-cadherin proteins needed for cell adhesion and signalling (Ye 2015). These adhesion proteins allow spindle alignment to occur symmetrically (APC), deactivate the planar cell polarity pathway (non-canonical Wnt) which supports differentiation and apoptosis (E-cadherin) (Gao and Chen 2010), and provide a strong transcription signal (β-catenin) that may help overcome stochastic variation in reprogramming (He 2008 et al. 2013). Culture of iPSCs, or any cell type, in chemically

defined conditions, may also provide a more homogeneous population by preventing highly variable animal cytokine content from effecting cell fate decision, metabolism, or viability. Close inspection of components of many "defined" cell culture products almost always reveals some animal sourcing. On the other hand, use of synthetic or recombinant matrices, chemically defined media, and xeno free reprogramming methods have proven very effective for stem cell culture, and may be good replacements for general cell culture with controlled addition of beneficial lipids (Chen et al. 2011).

Differentiability

Differentiation, defined as the process through which a cell undergoes changes in gene expression to become a more specific type, is both a problem to be avoided, in long-term culture of iPSCs, and a goal in cases where the differentiated cells are desired. Initially, the purpose is to reprogram and then maintain cells in the pluripotent state. During cell expansion and maintenance, the goal is to prevent differentiation, and encourage self-renewal. During maintenance, cell populations may be purified, genetically altered, or used for drug or genetic research. The pluripotent state allows cells to continually proliferate, providing sufficient numbers of cells for transfection and selection of cells that properly received transfection, to populate a matrix for organ development, or to support other types of research. Differentiation to a target cell type requires precisely controlled conditions, as described above, and fully pluripotent cells to be successful (Hagbard et al. 2018). Fully pluripotent cells require complete reprogramming. This is accomplished by acetylation of the histone proteins in the chromatin to expose DNA and demethylation of cytosine residues in the exposed DNA,

as described above. Reprogramming causes overexpression of proteins that support histone acetylation and DNA demethylation, but this is a process that takes weeks. Somatic cell genotype continues to recede when cells are cultured in chemically defined conditions. Although mechanisms for this have not been elucidated, this suggests that culture in chemically defined conditions may also support increasing efficiency in both reprogramming and differentiation (Beers et al. 2012, Hagbard et al. 2018). The diminishing of somatic cell properties during long term iPSC culture, also suggests that many iPSCs may not be fully reprogrammed by current methods.

While iPSCs currently available possess traits of pluripotency, as defined by presence of specific protein markers like SSEA4, OCT4, SOX2, Tra 1-80 and others, many studies have shown that current reprogramming techniques produce iPSCs with residual epigenetic barriers to pluripotency maintenance and differentiation (Doi 2010, Ori Bar-Nur 2011, Miyagi-Shiohira and Nakashima 2018). Inconsistent DNA methylation, changes in the number of gene copies, and impaired gene function have all been found in iPSCs (Ivanov et al. 2016, Tesarova et al. 2016). These problems are caused both by reprogramming methods that are not fully effective, and by culture conditions that do not fully support reprogramming. Limitations of both reprogramming methods and culture conditions therefore, cause heterogeneity of iPSC populations, that can result in spontaneous differentiation in culture, and difficulty in precisely and permanently differentiating to a desired cell type.

Development of more *in vivo*-like culture “niches” for reprogramming iPSCs, providing a genome more thoroughly stripped of epigenetic marks on which to perform

differentiation, may be supplemented by similarly designed "niches" to support differentiation. There is evidence that certain nutrients that support targeted signaling pathways like Canonical Wnt will also be important to the differentiation process (Huang et al. 2015, Knobloch et al. 2017). Some of these nutrients are lipids, and some may be modulated by lipids. Lipid roles in these mechanisms may be more fully elucidated by chemically defined lipid supplementation experiments.

Chemically Defined Culture

As methods of stem cell culture developed, a media called TeSR emerged in which embryonic stem cells (ESC) could be cultured (Chen et al. 2011, Hagbard et al. 2018). TeSR has 18 components, in addition to the 52 components that make up its DMEM/F12 base (Chen et. al. 2011). The TeSR formulation was developed around the use of Bovine Serum Albumin (BSA). Continuing difficulties relating to maintenance of pluripotency, or spontaneous differentiation (Chen et al. 2011), necessitated a new look at stem cell culture media. Initially, it was discovered that Human Embryonic Stem Cells (hESC's) benefitted from the use of human serum albumin and human sourced matrix proteins to provide the lipids and growth factors essential for cell survival without spontaneous differentiation (Chen et al. 2011). As iPSCs became the stem cells of choice, elimination of all tissue components and sera from the cultures, to form chemically defined conditions, became the goal for reliable and reproducible stem cell culture (Omole and Fakoya 2018).

Stem cells require a protein matrix or coating to adhere to the culture plate. Initially, a “feeder” layer of mouse embryonic fibroblast cells was used (Hagbard et al. 2018). Matrigel^{TM}, a coating derived from mouse tumors, has been widely used in stem cell culture (Hagbard et al. 2018). While Matrigel^{TM} has been proven to support stem cells in a pluripotent state, it is variable by batch, much like FBS (Hughes et al. 2011). Additionally, animal sourced cells and matrices carry unknown amounts and types of cytokines, and may carry viral particles capable of infecting humans, and other bioactive molecules that can trigger immune responses in a transplant recipient, or remain in the cultured cell line (Desai et al. 2015). On the other hand, a matrix that does not provide attachment signals to cells undermines the ability of the population to remain pluripotent and homogenous (Hagbard et al. 2018), as adhesion and its signaling response is an important part of the pluripotent fate decision. Synthetic matrices are certainly fully defined, but they lack this signaling ability. Laminin, a protein abundant in the basement membranes on which many cells grow *in vivo*, can be produced in a recombinant form, eliminating the issues associated with variable biological sources, and still providing the adhesion feedback necessary. Coating the culture plate with a recombinant form of a naturally occurring adhesion protein is a fully defined way to provide stem cells with a functional surface on which to grow. Currently, laminin, fibronectin, or vitronectin are the most common matrices used in fully defined stem cell culture (Miyazaki et al. 2017).

Fibroblasts, the most facile source cell for reprogramming, are normally cultured in FBS containing media. Dermal fibroblasts, as cultured in this work, do not require a matrix to adhere, but as they become stem cells, they acquire the need for a

matrix. This is because in the pluripotent state, signaling via cell to cell adhesion, as discussed above, is an important mechanism, providing cues *in vivo* that differentiation or proliferation is necessary, as in wound healing, where signaling molecules released from distressed cells signal nearby progenitor cells to detach, mobilize to the wound site, and differentiate to produce new tissue (Zvia 2010). In vivo, stem cells often adhere to certain cell types when quiescent, and become mobilized when detached (Marthiens et al. 2010). Once transfection of pluripotency genes is effected, both somatic and pluripotent states must be supported simultaneously, while the cells undergo the reprogramming process. This reprogramming process takes one to several weeks, and source cells must continue to proliferate while the process of reprogramming is completed. This is currently accomplished by the changing of media ingredients during reprogramming, as set out by different protocols that differ according to source cell type. Clearly use of animal sera, containing unpredictable amounts and types of nutrients and proteins (Hagbard et al. 2018, Omole and Fakoya 2018), is not desirable during this process. Addition of specific lipids and trace elements to induce desired signaling and to support source cells like fibroblasts, without the addition of animal sera or serum albumin, may greatly improve the reprogramming process. This calls for addition of the lipids and trace elements necessary for source cells from FBS, in a chemically defined and specific manner. Variation from chemically defined standards for culture conditions during reprogramming introduces components that can potentially contain molecules that may have signaling properties, significantly effecting cells (Zhang 2010).

<u>The Importance of Media</u>

Numerous studies have produced insights into nutrient effects on stem cell health and proliferation *in vitro* (Säemann et al. 2000, Chung 2010, Bar-Nur et al. 2014). These studies seem to indicate that improvements in culture media may be a key to optimization of both homogeneity and efficiency. Mimicking the *in vivo* cell "niche" is possible only through optimization of media ingredients, matrices, and incubation variables. The media environment appears to be the most dynamic and complex variable in this process (Chen et al. 2011). Chemicals and nutrients in the media provide signals to cells, and the media is a conduit for intercellular signaling (Freshney 2010). Modification of secreted proteins, such as the Wnt proteins discussed later, are often essential to the reception of signals between cells. Receptors such as Tra 180 and Tra 1-61, used to identify stem cells, are "decorated" with sugars and lipids from the environment or media to modulate their activities (Natunen et al. 2011). Stem cells in particular have been shown to modify their behavior according to their sensing of proximal stem cells, and do not easily differentiate unless a "quorum" of cell density is sensed *in vivo* (Zvia 2010). Additionally, a myriad of intricate chemical and nutrient interactions activate hundreds of diverse receptors to influence cell fate and behavior decisions (Hlavacek and Faeder 2009).

<u>Media and reprogramming</u>

Reprogramming signals cause DNA to "uncoil" from histones and support the removal of epigenetic methylation markers that "deactivate" genes that do not correspond to the adult cell type (Rodriguez et al. 2013). These two functions enable cells to adopt a

pluripotent "stem" phenotype. Signaling pathways that control the removal of epigenetic marks contain self-regulating mechanisms, and competing signaling cascades are activated by the chemical environment produced by changes in methylation and acetylation (Wohrle et al. 2007). Nutrients like ascorbic acid, and some lipids have been identified as potent cofactors and signaling molecules in the reprogramming process (McDonnell et al. 2016, Sharma et al. 2018). This indicates that it is very important to control the media environment during reprogramming to produce a clean signaling and nutrient niche. Elimination of unknown and variable components in the media will support full and efficient reprogramming and maintenance of iPSCs.

Inherent in the details of the effects of media on reprogramming are some key elements that may also improve differentiability, as it is logical to assume a more completely reprogrammed stem cell will have a more accessible genome when prompted to differentiate. A more fully reprogrammed cell, with minimal residual epigenetic DNA histone coiling or methylation, will also be less likely to spontaneously revert to source cell traits, as the epigenetic pattern will not be predisposed to a particular fate. This tendency to revert to source cell phenotype is called epigenetic memory recall, and has plagued transplantation studies (Doi 2010, Ohi et al. 2011, Miyagi-Shiohira and Nakashima 2018). Careful control of the nutrient environment may improve results of long-term in vitro culture by reducing or eliminating spontaneous differentiation and other phenotypical and metabolic shifts.

Development of specific media supplements for reprogramming and maintenance of iPSCs can make these cells more reliable for research and therapy and

help to move the field one step closer to realization of their great promise. To this end, research continues to optimize iPSC systems using gene silencing, small molecule additives, nutrients and other media components, matrix proteins, and oxygen and temperature conditions (González 2011). Nevertheless, reprogramming efficiency remains low in general, with reported efficiencies over the wide range of 0.00002 - ~ 1% (Wang et al. 2017) and iPSC gene methylation profiles are variable between labs, and do not mimic that of ESCs as closely as desired (Ivanov et al. 2016, Miyagi-Shiohira and Nakashima 2018).

Chemically Defined Media

In 2011, Guokai Chen, James Thompson, and coworkers systematically evaluated each of the 18 components of TeSR medium, and eliminated all but 8, resulting in Essential 8 culture medium (E8) (Chen et al. 2011). This medium does not contain BSA or any type of serum albumin. E8 is the first fully chemically defined medium, and led to a system that includes use of recombinant proteins like vitronectin for attachment layers, and non-enzymatic passaging using EDTA instead of trypsin (Beers et al. 2012, Garitaonandia et al. 2015) as used in this work.

E8 contains 150nM linoleic acid as the only lipid content (https://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-formulations/dme-f12.html). While linoleic acid is the only "essential" fatty acid, the catabolism and anabolism required to produce other fatty acids from it requires specialized fatty acid membrane transporters (FAT's) and enzymes which are located predominantly in muscle and liver tissue. Additionally, production of necessary lipids

from one lipid moiety is energetically costly, and exogenous uptake of lipids has been shown to be necessary for both fibroblasts and stem cells (Folmes et al. 2013). In the same study, inhibition of fatty acid uptake inhibited cellular proliferation. Additionally, fatty acid oxidation, the process by which cells break down fatty acids, upregulates oxidative phosphorylation and down-regulates protein kinase C, which has been shown to support self-renewal (Lin et al. 2018). Oxidative phosphorylation for energy production is a more efficient alternative to the Warburg effect, and is stimulated by lipid availability. All this evidence suggests that lipids, and especially specific fatty acids, may play a dynamic part in the process of reprogramming and maintenance of pluripotency.

Essential 8 medium encourages the proliferation of pluripotent cells, while allowing differentiated cells to die, assumably due to lack of nutrients and growth factors specifically necessary for differentiated cells, producing excellent results in long-term culture (Chen et al. 2011). However, during reprogramming source cells retain their original nutrient requirements for proliferation and health during the process of converting to the pluripotent state. This suggests that stepwise changing of media ingredients, from those ideal for fibroblast growth to those ideal for stem cell growth, would be optimal during reprogramming. Hence the need to develop chemically defined lipid supplements for culture of HFF and other source cells, to reduce the presence of unknown growth factors, nutrients and cytokines that can interfere in the reprogramming process.

Fibroblasts are normally cultured using 10% FBS containing media. Blood cells also require serum addition (Gross et. al. 2012). Therefore, we have embarked on strategies for addition of lipids in a chemically defined and specific manner appropriate for use with the E8 system in each stage of somatic cell reprogramming and stem cell maintenance.

The process of developing a multi stage system was begun in 2012 with the production of Essential 6 media (Chen et al. 2011). E6 is E8 without transforming growth factor β (TGFβ) or fibroblast growth factor (FGFβ), providing flexibility for addition of growth factors during the different stages of reprogramming (Beers et al. 2012). We discuss the further development of such a flexible system using lipids and fatty acids.

Fatty Acid Deficiency

While E8 and the fully defined culture method it supports have been an important step forward in culture of iPSCs, the problems of low reprogramming efficiency, stem cell homogeneity and differentiability still linger. Lack of lipids in media has been found to induce de novo lipid synthesis, reducing mitochondrial respiration and altering cell behavior, often supporting oncogenesis (Lamaziere et al. 2013, Lin et al. 2018). The membrane of every cell is composed of phospholipids, comprised of fatty acids joined by a glycerol molecule and topped by a polar phosphate head, so these structural materials are essential for proliferating cells. Specific fatty acids, like palmitoleic and palmitic acids discussed above, have also been found to be important in signaling and protein modifications that are important to the cell fate decision and many aspects of cell health

(Graber et al. 1994, McDonnell et al. 2016). A central signaling pathway, the Wnt pathway, is dependent on specific fatty acids for signaling and to coordinate cell fate decisions (Galli and Burrus 2011, Nile and Hannoush 2016). Low levels of lipids in fully defined culture media favors a metabolic change away from fat burning in oxidative phosphorylation to support lipogenesis. Oxidative phosphorylation is the preferred method of energy production in most mammalian cells, and is prevalent in naïve stem cells in vivo (Shyh-Chang 2017). In light of these considerations, chemically defined addition of fatty acids to iPSC culture media promises to enhance iPSC production by providing the energy, structural and signaling tools that iPSCs need to efficiently and fully reprogram to pluripotency and proliferate efficiently.

Fatty Acids in Cell Culture

In mammals, only two fatty acids, linoleic (18:2ω6) and linolenic (18:3 ω3) acids, have been found to be essential (Di Pasquale 2009). Essential means the organism cannot manufacture these lipids, so they must be provided through diet. It must be noted that these fatty acids are essential to the mammalian organism, but not necessarily to any specific cell line in culture. In intact organisms, cellular systems process nutrients, which are delivered via vehicles like serum albumin to specific cell types, which use what they require, but may also be effected by the presence of other nutrients and molecules through signaling pathways. In culture, cells are not supported by diverse types of cells and organs that serve to produce metabolites, and delivery systems to transport them. Each cell line must be given everything it needs in the culture niche.

Most cells are capable of manufacturing a wide range of lipids, although capabilities and requirements vary greatly between cell types (Freshney 2010). In culture, it seems optimal to provide the specific lipids and fatty acids required for the needs of each cell type while avoiding others, as it has been found that fatty acids are not only structural molecules and energy sources for cells, but also can be potent signaling molecules (Papackova and Cahova 2015) and post translational modifiers of vital proteins (Nile and Hannoush 2016). Fatty acids can act reversibly at distinct locations to modify cell signals, and can be powerful mediators of cell behavior (Glatz and Luiken 2015). Specific fatty acids can also modify the activity of ion channels, lipases, kinases, G-proteins, and other components of cellular response mechanisms (Sumida and Nunez 1994, Resh 2013).

In culture of stem cells, lipid contents must be carefully controlled, as the presence of some types of lipids can stimulate differentiation. A cocktail of lipids is used to differentiate neurons (Bieberich 2012) and two phospholipids have been shown to cause differentiation of iPSCs to the cardiac lineage (Sharma et al. 2018), but specific fatty acids can also support and even stimulate reprogramming through signaling pathways (Zhang and Wu 2013, Shyh-Chang 2017, Lin et al. 2018). Exogenous lipids provide a preferred carbon source for histone acetylation (McDonnell et al. 2016) and stimulate and modulate the Wnt pathway (Holland et al. 2013). These are some aspects of lipid effects that have been observed and there are likely many more.

Problems with use of Serum and Serum Albumin

Serum Albumin is the most abundant protein in plasma and occurs at a concentration near 45 mg/mL. The serum albumin protein is made up of 585 amino acids and contains 30 alpha helices connected by random coils (Kragh-Hansen et al. 2002). This secondary structure folds to make three domains of 10 helices each, resulting in seven binding sites (figure 8).

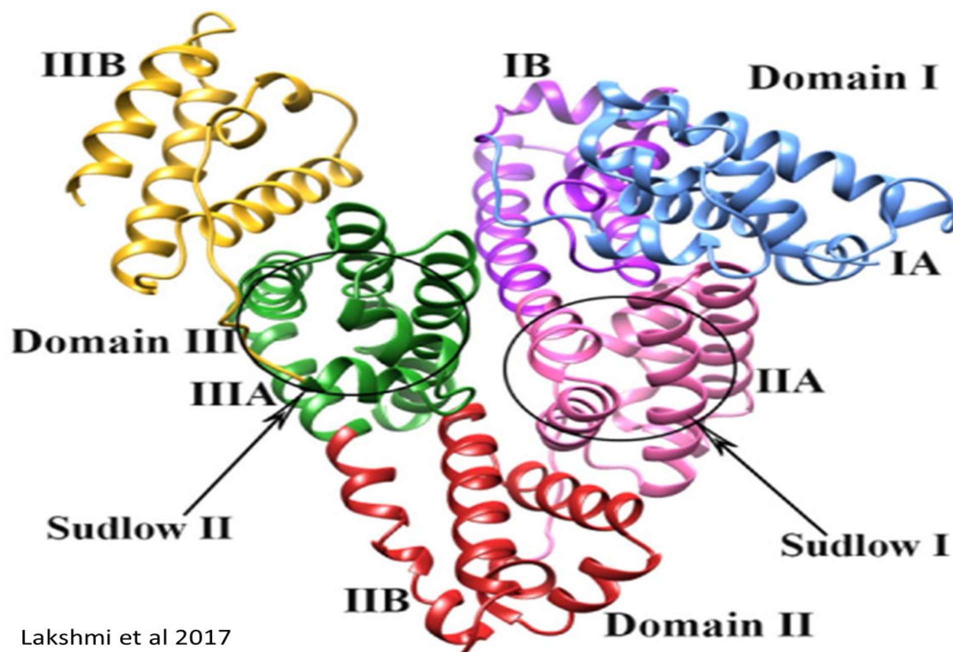


Figure 8. Structure of Human Serum Albumin with Binding Sites labeled I - IIIb. Fatty acids are normally bound at domains I and III

Under normal physiological conditions, serum albumin carries only two lipids at a time on average (Kragh-Hansen et al. 2002). Domains I and III contain the most favorable fatty acid binding sites (Curry et al. 1998). This leaves a majority of sites available for binding cytokines, glycoproteins, metals, drugs, hormones, toxins and other metabolites (Lakshmi et al. 2017). Changes in lipid binding allosterically effect binding

affinity at other sites by rearrangement of domains I and III, relative to domain II (Fasano et al. 2005).

With the capacity to carry at least seven different compounds, serum albumins are rather unpredictable in their binding behaviors and quite variable in types of materials carried. *In vivo*, serum albumins are active carriers of proteins and cytokines as well as lipids, and this variable cargo can include potent factors effecting cellular health and behavior. The difference between the needs of a cell type being cultured and the collective needs of an entire organism are enormous (Saeidnia 2015). Serum and serum albumin carry numerous proteins and growth factors that may not be appropriate for a given individual cell type, as well as nutrients of general importance like lipids and trace elements (Fasano et al. 2005). When sera are used in cell culture media, this wide range of proteins and factors are available for interaction with cells in culture, causing unintended variability in experimental results by providing signaling molecules and nutrients to cells that are consistent with the general needs of the donor organism, while potentially lacking content specific for the cultured cell type. Because content of serum and serum albumin vary so greatly, the amounts and types of cytokines in FBS and human serum (HS) cannot be reliably published. Averages can still illustrate the differences between human and bovine serum cytokine content (Table 1). These differences are compounded by the specificity of the needs of single cells lines. Additionally, human serum has the same potential issues of contamination and variability, in spite of the fact that it is commonly used in commercially prepared

"defined" and "xeno free" media. Chemically defined media is superior in this regard, but is still lacking in lipids.

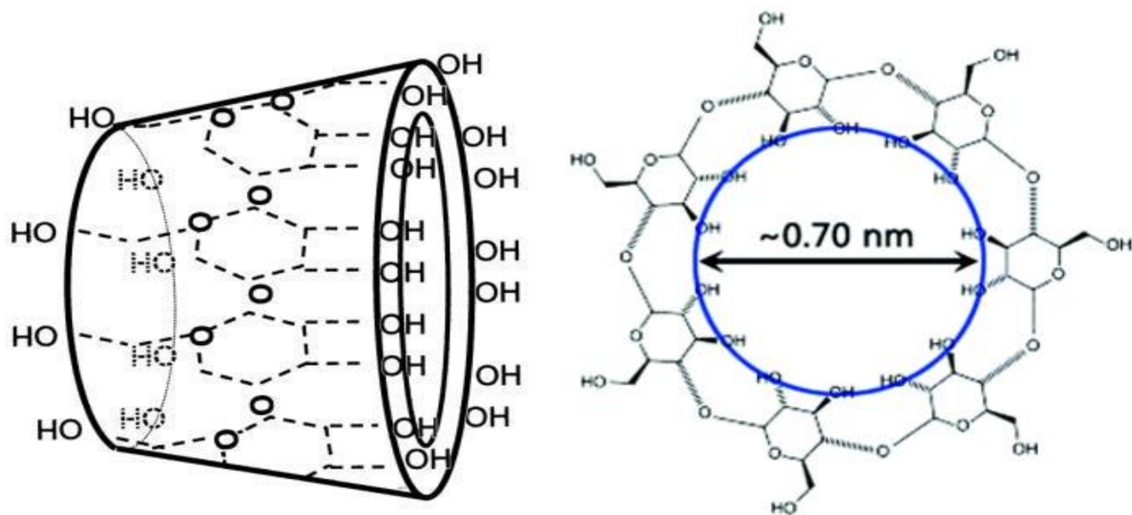
Growth factor	**Human Serum**	**Bovine Serum**
Insulin like Growth Factor 1	76 ng/mL	111 ng/mL
Transforming Growth Factor β1	47 ng/mL	12.6 ng/mL
Platelet Derived Growth Factor	5 ng/m:	1-100 ng/mL
Epithelial Growth Factor	1.5 ng/mL	1-100 ng/mL
Hepatocyte Growth Factor	0.80 ng/mL	undetected
Vascular Endothelial Growth Factor	0.063 ng/mL	1-100 ng/mL
Fibroblast Growth Factor	0.0019 ng/mL	37.3 ng/mL

Table 1. Average Growth Factor Contents in Human and Bovine Serum. Amounts are extremely variable. Human serum data (Rauch, et al. 2011) Bovine serum data (Freshney, 2010)

The problems associated with use of serums and serum albumins would seem to preclude their use in a process like reprogramming, where clear signals must be given to cells (Folmes et al. 2013, Yao et al. 2016). Use of serum in general cell culture has long been in question (Van der Valk 2010). To accomplish delivery of specific lipids without the use of serum or serum albumin, it was necessary to find a way to deliver hydrophobic lipids to cells in a controlled fashion, avoiding fatty acid micelle formation and without the use of animal protein carriers. The complexation protocol developed in this work may facilitate that goal.

<u>Cyclodextrins: Chemically Defined Carriers for Fatty Acids</u>

Cyclodextrins are cyclic oligomeric starches composed of α-1-4-linked glucose units. Cyclodextrins are barrel shaped, with a hydrophobic interior and hydrophilic exterior (figure 9). They are relatively widely used in food, cosmetics, and drugs (Del Valle 2004). Cyclodextrins are named using Greek letters, based on the number of glucose units (α = 6, β = 7, γ = 8).



https://en.wikipedia.org/wiki/Cyclodextrin

Figure 9. Structure of Methyl beta cyclodextrin. A cyclic oligsaccharide with a barrel type structure whose interior is hydrophobic and exterior is hydrophilic.

The number of glucose units determines the size of the hydrophobic “barrel”. 2-hydroxylpropyl-β-cyclodextrin (HP-β-CD), a hydroxyalkyl derivative, is an alternative to α-, β- and γ-cyclodextrin, with increased water solubility (Pessine et al. 2012).

Many different cyclodextrins (CD) have been used to improve solubility of hydrophobic drugs in aqueous solutions, such as culture media or blood (Sharma and Baldi 2016). Toxicity of CDs has been extensively studied in animals and humans, and they have all been found to be non-toxic at a daily dose below 600mg/kg body weight

(Antisperger 1996). In an Ames Test assay (up to 1000 μg/plate) and an *in vivo* micronucleus test (up to 5000 mg/kg/day) there was no evidence that cyclodextrins are mutagenic or toxic *in vitro* (Leroylechat et al. 1994).

The lipophilic interiors of CD barrels provide a non-polar environment within which a variety of non-polar molecules of appropriate sizes will tend to form inclusion complexes in aqueous solution (Loftsson and Brewster 2012). The driving force of complex formation is a classic hydrophobic effect (Tanford 1973). Water molecules are displaced by the hydrophobic guest molecules (figure 10), releasing water molecules to the aqueous exterior solvent, increasing entropy and resulting in a more stable lower free-energy state (Szetjli 1998). No covalent bonds are formed in the complexes, and the complex remains dynamic, allowing for dissociation under favorable circumstances, such as contact with a cell membrane (Greenbergofrath et al. 1993). MBCD in particular has been shown to effectively deliver cholesterol to cell membranes (Brunaldi et al. 2010).

chemistryworld.com

Figure 10. Complexation of a hydrophobic molecule (rotoxane) with cyclodextrin (green). The hydrophobic portion of the guest molecule (blue) is inserted into the cyclodextrin “barrel” resulting in a soluble, reversible complex.

CDs have been widely used for drug delivery for decades. MBCD has also been used to control excess cholesterol in humans by complexing with it *in vivo*, and enhancing its elimination (Sanchez et al. 2011). While studies have been done on cyclodextrin complexation with linoleic, oleic, palmitic acids (Szente et al. 1993), and cholesterol (Sanchez et al. 2011), complexation with other fatty acids of interest has not been comprehensively studied. Fatty acid/CD complexation methods and stoichiometry of complexes were not well established before this work.

Cyclodextrins Used in This Work

Methyl Beta Cyclodextrin (MBCD), used in this work, has been shown to maintain a low concentration of unbound (free) fatty acids in aqueous solution, and to readily dissociate with fatty acids at the lipid membrane (Brunaldi et al. 2010). FA (soap) micelles are toxic to cells but the cyclodextrins are nontoxic (Leroylechat et al. 1994). This makes them promising carriers for delivery of fatty acids to stem cells in fully defined media.

CD complexation has also been shown to protect unsaturated fatty acids from oxidation (Kim 2000). This is due to the localization of double bonds within the CD barrel, limiting its exposure to oxidative molecules. This is very advantageous for cell culture applications, since oxidation damages polyunsaturated fatty acids and quells many of their desirable functions (Borsonelo and Galduroz 2008).

Studies thus far have primarily used an excess of CD (up to 12 CD:1 FA) to study delivery and uptake of hydrophobic molecules by CD (Skoulika et al. 1999, Bojinova 2003). In this work, we have sought to establish an optimal stoichiometry for

complexation to minimize the presence of free/uncomplexed CD, maximizing fatty acid availability, and minimizing unintended complexation with nutrients in media, which happens when uncomplexed CD concentrations are greater than 700µM.

Other Lipid Effects on Pluripotency and Reprogramming

The Warburg Effect

The Warburg Effect is the reliance of cells on aerobic glycolysis instead of oxidative phosphorylation (ox/phos) for ATP production. This metabolic change was first found in cancer cells by Otto Warburg and has recently been found to be present in stem cells (Riester et al. 2018). Under normal conditions, cells break down glucose through glycolysis, and the resulting pyruvate is oxidized in the mitochondria through ox/phos, producing large quantities of adenosine triphosphate (ATP), the central molecule of cellular energy. Anaerobic glycolysis is utilized under low oxygen conditions. The switch to glycolysis in aerobic conditions, even in the presence of fully functional mitochondria, virtually eliminates mitochondrial ox/phos activity, reduces production of Reactive Oxygen Species (ROS), and supports lipid neogenesis, however it is a much less efficient method for energy production, and produces large amounts of lactic acid (Lees et al. 2017).

It is not known whether cells adopt aerobic glycolysis because of low oxygen conditions in the stem cell niche or the developing embryo, or if the switch may be a necessary condition for pluripotency (Abdel-Haleem et al. 2017). Mitochondria are not plentiful in embryonic cells, and that has been suggested as a reason for the prevalent use

of aerobic glycolysis in ESCs (Lees et al. 2017). Production of necessary building blocks and metabolites for rapid proliferation is also a suggested reason for Warburg metabolism (Wang et al. 2017). Chief among these necessary building blocks are members of the lipid family, as they are necessary for production of cell membranes.

Supplementation of media with lipids may attenuate the switch to aerobic glycolysis, which may either aid or inhibit reprogramming. The difference may lie in which specific lipids are available, with results dependent on signaling effects, or on the use of lipids for energy.

Histone Acetylation

Histone acetylation is a central mechanism of reprogramming, as it causes the unravelling of DNA from the histone protein, making genes available for transcription. Recently, lipids have been found to be a major carbon source for histone acetylation, donating 90% of the carbons found in remodeled chromatin (McDonnell et al. 2016). This indicates that lipid supplementation of stem cell media may substantially support the rapid proliferation that remains a hallmark of pluripotency.

Fatty Acid Uptake and Oxidation

The process of taking up fatty acids from the intercellular space, and the β-oxidation process that cells use to breakdown lipids for energy enhances proliferation of neural stem cells (Brasaemle 2006) and increases pluripotency of iPSCs by inhibiting protein kinase C (Fillmore et al. 2015, Knobloch et al. 2017, Wang et al. 2017, Lin et al. 2018). These findings differ between cell type, and conditions differ considerably

between studies, but they certainly indicate an active role for lipids in cell fate and maintenance. A study using palmitate found that cells preferred to take up fatty acids from their environment, rather than synthesizing them for use in membranes (Yao et al. 2016). The application of specific lipids in fully defined conditions using the MBCD carrier system we have developed, could illuminate the effects of specific lipids on different cell types in different states of pluripotency, and quiescence. Application of fatty acids in the presence of serum, serum albumin, or live feeder cells cannot be expected to result in reliable information, due to the presence of variable amounts of other lipids and bioactive molecules, and the additional variable of feeder cell metabolism. Thus we sought to develop chemically defined individual lipid supplements and mixtures, that could be optimized for any cell type being used in reprogramming.

Research Goals

The complex interactions of cellular metabolic pathways, signal responses, and genetic regulation in stem cell reprogramming and maintenance are not fully understood. The Wnt pathway, discussed above, is only one of the pathways that impact cell health and behavior. The complexity illustrated by our discussion of the Wnt pathway is assumably repeated in many other mechanisms that have not been discussed here.

Effects of the interplay between metabolism and gene transcription can be observed in the cell behaviors and phenotypes that result. To accomplish this, it is essential to know exactly what molecules are available to a cell population if results are to be reproducible and analysis is to be effective. This, and the problems with use of sera, gave rise to the goal of developing optimized, chemically defined lipid supplements

for cell lines used in research and therapy. This work sought first to discover a tool for supplementation of stem cell culture media with lipids in the absence of serum and serum albumin. This lead to the development of lipid supplements to replace sera in fibroblast cell culture. Further, the goal was to develop several fully defined lipid and nutrient mixtures that would effectively support cells through the process of reprogramming to pluripotency, and to aid in the maintenance of pluripotency.

The initial task was the complexation of lipids with MBCD, which enabled the addition of single lipids and combinations of lipids to cell media. This facilitated the analysis of the effects of each lipid on different cell lines. Results of initial supplementation experiments indicate that this new approach may be a valuable tool in research to elucidate cellular pathway interactions, and their interplay with lipids. Importantly, it may also expedite development of fully defined supplements for any cell line, and for each step of reprogramming. The fundamental goal tha has emerged is optimization of fully defined lipid supplements to enhance research by minimizing variability and unrecognized interactions in stem cell culture. This will also potentiate research into the addition of lipids to optimally support many different cell lines *in vitro*, and development of specialized chemically defined nutrient mixtures to support reprogramming, differentiation, implantation, and other aspects of iPSC utilization.

MATERIALS AND METHODS

Cell Culture

Cells used in this study were fully characterized Cy_2 induced pluripotent stem cells (Cy_2iPSC) from blood, a generous gift from Dr. Nasir Malik of the NIH, H9 ESCs, a gift from Dr. Renee Reijo-Pera of MSU, and HFF cells, a gift from Dr. Sandra Halonen of MSU (Appendix A). Reprogramming experiments were performed using low passage HFF1 (ATCC cat#SCRC-1041, lot#3003912).

Original aliquots of cells were expanded and cryopreserved in Bambanker^{TM} cryopreservation medium (FUJIFILM Wako, Cat#302-14681). These stocks were used for all Cy_2iPSC applications, with passage numbers noted. H9 and iPSC stem cells were grown in Essential 8 media (Life Technologies, cat#A1517001), using recombinant human Vitronectin (Thermofisher, cat#A14700) or Laminin 511(Gibco Cat#A29249, lot#1958446) as a matrix, and passaged by dissociation using Versene^{TM} EDTA/trypsin dissociation reagent (Thermofisher, cat#15040066), unless counting was necessary, in which case, cells were dissociated to single cell suspension using Accutase^{TM} (Fisher, cat#NC9839010). HFF cells were grown in high glucose DMEM with pyruvate and glutamate (Thermofisher cat#10569044), supplemented with 10% FBS (Thermofisher cat#10437028) unless lipid supplements were used, and Pen-Strep antibiotic (Life Technologies cat#15140-122).

Lipid supplementation experiments were performed in tissue culture treated 12 well plates, with E8 media. This was supplemented with lipids and in some cases other

nutrients as noted. iPSCs were grown on vitronectin coated plates, and plated at 70,000cells/well for the proliferation experiments. H9 ESCs were plated at 68,000 cells/well for proliferation wxperiments. At day four, cells were dissociated with Accutase (Sigma Aldrich cat#A6964, lot#SLBX5621) for 5 minutes, resulting in a single cell suspension, and counted using a hemocytometer. HFF cells were seeded on tissue culture treated 12 well plates at a density of 100,000 cells/well, and dissociated using TrypLE™ Express (Gibco ref# 12605, lot#1951057) for 5-7 minutes, resulting in a single cell suspension, and counted using a hemocytometer.

Reprogramming was performed using minicircle DNA (ABM Good cat#G389) containing all four Thompson factors: Oct4, Sox2, NANOG and Lin28. DNA was delivered by nucleofection using an Amaxa Nucleofector™ II unit, set on program U-023, and using the Amaxa Human Dermal Fibroblast Kit (Lonza, cat#VAPD-1001).

After nucleofection, cells were plated in Essential 6 media (Life Technologies, cat#A1516401), which is E8 without the growth factors TGFβ and bFGF. Hydrocortisone (Sigma Aldrich cat# H0888-1G) was added to initial reprogramming media at a concentration of 1μM. Some reprogramming media was supplemented with Wnt3A protein (R&D Systems (cat#5036-WN-010/CF), sodium butyrate (Sigma cat#B5887 lot#091M5302V), and cyclodextrin complexed lipids as noted (Table 2). Controls included use of the FGM Bulletkit (Lonza Cat#CC3132, lot#0000699478), with no additional supplementation, DMEM 1X + Glutamax (Gibco cat#10569-010, lot#1967493) supplemented with 10% FBS (Gibco Cat#26140-079, lot#1998657) and

Penicillin/Streptomycin (Gibco Cat#15140122 lot#1902420). E6 and FA media was not supplemented with antibiotic in the first five days of reprogramming.

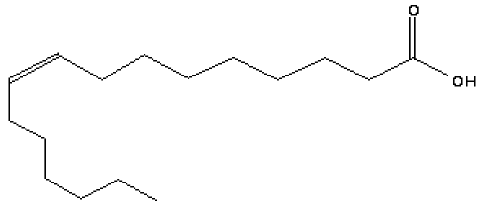
Media	100µM NaB	1µM Wnt3A	1µM HC	10µM PA	1mM Asc PT	1mM Asc Acid	1mM Octanoate	Other FA
E6	R2	R1	R1				R1	
FGM								
FBS								
FA1	R2	R1	R1	R1 R2	R1 R2		R1	R1 R2
FA2 (no PT)	R2	R1	R1	R1 R2		R1 R2	R1	R1 R2
FA3 (no PA)	R2	R1	R1		R1 R2		R1	R1 R2

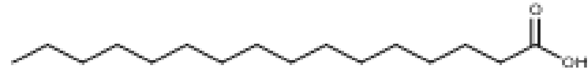
Table 2. Contents of Reprogramming Media. Other FA=1µM α Linolenic acid, 10µM Cholesterol Sulfate, 10nM DHA, and 1µM Oleic acid. R1= Days 1-5 after nucleofection, R2=Days 5-25. FGM= FGM Bulletkit used as directed. FBS=HFF growth medium (DMEM+ 10%FBS).

Five days after nucleofection, media was changed to Essential 6 media (Life Technologies cat# A1516401) without hydrocortisone, but with sodium butyrate (NaB) added as noted. Colonies were counted visually, at 20X magnification on a Leica DMIL microscope, and cryopreserved or prepared for analysis when numbers were adequate.

Fatty Acid Complexation

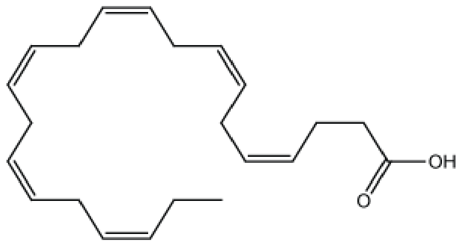
Eight fatty acids and cholesterol, were tested for their effects on cells. These included Docosahexanoic acid (DHA), eicosapentanoic acid (EPA), oleic acid (OA), palmitoleic acid (PA), α linolenic acid (ALA) and cholesterol (Cayman Chemical cat#s 90310, 10009871, 15106, 90110, 90210, 90260, and 3121) and octanoic acid (MP Biomedicals Cat#100391) (figure 11).

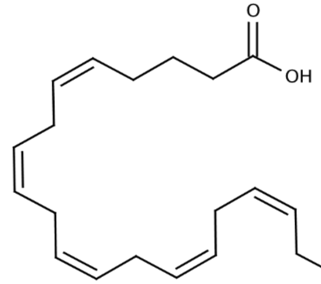




Palmitoleic acid C16:1 ω7

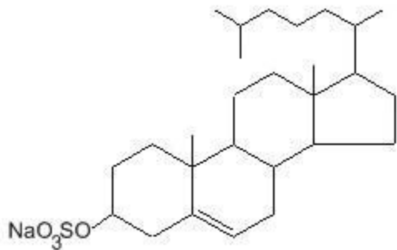
Palmitic acid C16:0

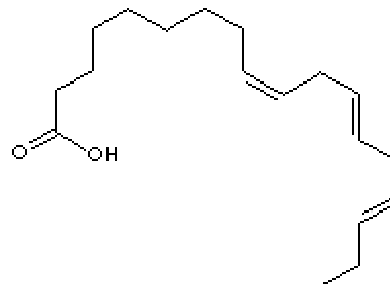




Docosahexanoic acid C22:6 ω3

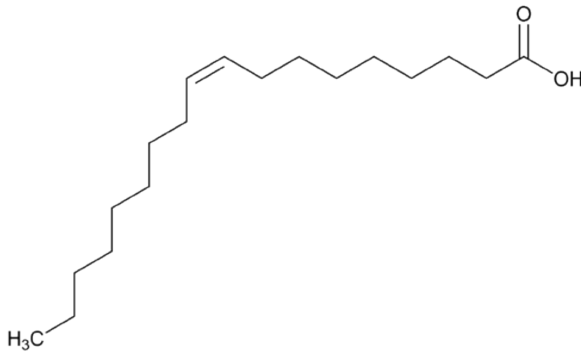
Eicosahexanoic acid C20:5 ω3

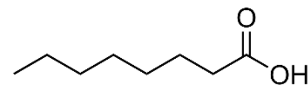




Cholesterol Sulfate C27:1

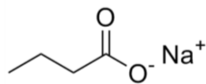
α Linolenic acid C18:3 ω3





Oleic Acid C18:1 ω9

Octanoic acid C8



(sodium) Butyrate (C4)

Figure 11. Structures of lipids used in this work.

Three forms of cyclodextrin, hydroxypropyl beta cyclodextrin (Cayman Chemical cat#16169 lot#0469751-16), methyl β-cyclodextrin (MBCD)(Fisher cat#AC377110250), and γ cyclodextrin (TCI Chemical cat#C0869) were initially tried as complex partners for lipids. MBCD produced the best stoichiometry with all lipids except palmitic acid, and complexes readily lyophilized to form a crystalline powder. Hydroxypropyl cyclodextrin produced a waxy product after lyophilization, and required 2:1 stoichiometry to complex with DHA and palmitoleic acid.and γ cyclodextrin did not solubilize all lipids at 1:1 stoichiometry. MBCD was thus chosen for lipid complexation, and was diluted in sterile deionized water for solvation of lipids. Complexation was done in a Branston 2510 sonicating water bath, in septa vials in which lipids were provided by the vendors when possible, using argon gas flushing for delicate unsaturated lipids.

Where necessary, initial solutions were transferred to 10mL sterile septa vials (Allergy Laboratories cat#SEV1020S, lot #SEV2101217), using Hamilton syringes to avoid lipid adhesion to transfer vessels, and original vials were rinsed with a dilute (0.02g/mL) solution of MBCD, to improve transfer efficiency.

Pure fatty acids and cholesterol sulfate were provided in 1mL septa vials. DHA was dissolved in ethanol, palmitoleic acid was packaged as a neat oil, and Cholesterol sulfate was a lyophilized powder. In order to avoid oxidation, MBCD in sterile deionized water at a concentration of 0.4g/mL (300µM) or 0.2g/mL (150µm) was added dropwise via syringe directly through the septa of the vials containing lipids. Vials were then placed in a sonicating water bath, and sonicated for 15 minutes to one hour. Temperatures were controlled in the sonicating water bath, starting at 25°C and rising to

a maximum of 55°C in the case of oleic acid. With the exception of palmitate, all lipids were complexed at 1:1 stoichiometry to achieve fully solvency, as evidenced by visual examination of solution turbidity (Table 3).

Complexation was initially determined by solutions turning clear, with no residual material floating or adhering to the sides of the container.

Lipid	Form as shipped	Amount (mg)	Molecular weight	Amount (µmoles)	Condition	MBCD (mL)	M L
Palmitoleic	In EtOH	100	393.06	254.41	2	2.64	0.1
Cholesterol sulfate	powder	25	466.72	53.56	1	0.356	0.1
α Linolenic	powder	50	278.43	179.58	1	1.21	0.1
Oleic	In EtOH	500	282.47	1770.09	2	11.9	0.1
Oleate (caprylic)	Neat Oil	91*	282.47	322.15	1	4.24	0.1
DHA	powder	50	328.48	152.21	2	1.01	0.1
EPA	powder	50	302.45	165.31	2	1.0861	0.1

Table 3. Protocol Components of MBCD Complexation with Relevant Lipids. In all cases, MBCD solution was added dropwise through the septa while sonicating. Conditions code: 1=Sonicate at RT, 2= flush with argon, sonicate at RT under argon allowing temperature to increase to 45°C, 3=Sonicate using water temp of 40°C to start, continue until clear, or 55°C. All solutions were stirred overnight at RT in the dark. *100µL neat oil

MBCD:lipid complexation was confirmed by spectrophotometry (see analytical methods). Palmitic acid (Sigma cat#P0500 lot#SLBQ8866V), the largest saturated fatty acid used, required over 10:1 CD:lipid stoichiometry for complexation with all cyclodextrins. Resulting palmitate solutions contained cyclodextrin aggregates, and were

toxic to cells. This problem was overcome by using 6-O-Palmitoyl L-Ascorbic acid (Selleck Chemicals cat#S2532), a molecule made by condensation of ascorbic acid and palmitic acid (Humeau et al. 1995). The resultant molecule is soluble in aqueous media up to 13mM (Swern 1949), and can be added directly into media at experimental concentrations (less than 10mM), making complexation unnecessary (figure 12).

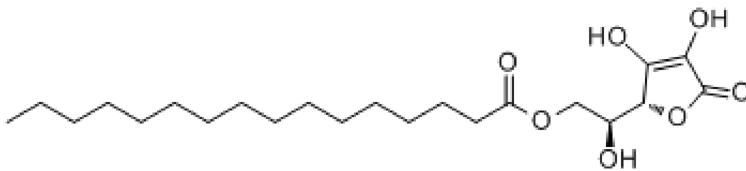


Figure 12. 6-O-Palmitoyl L Ascorbic Acid. Soluble to 13mM in aqueous solution.

Molecular Biology

Redox sensitive green fluorescent proteins (roGFP) were a gift of Dr. Tobias Dick of The University of Heidelberg, Heidelberg Germany. They consisted of glutaredoxin-1 (Grx1)–reduction-oxidation sensitive GFP2 (Grx1-roGFP2), Grx1-roGFP2 connected to a mitochondrial targeting sequence (mito Grx1-roGPF2), roGFP2 connected to oxysterol-binding protein 1 (Orp1) (roGFP2-Orp1), and roGFP2-Orp1 with a mitochondrial targeting sequence (mito-roGFP2-Orp). The proteins encoded in these plasmids fluoresce differently when glutathione or ORP1 peroxidase are oxidized or reduced, in cellular redox reactions. Since some of the GFP's contain a mitochondrial targeting sequence, this makes it possible to see and measure levels of oxidative stress, and its location in the mitochondria or cytoplasm of the cell.

It was originally decided that the genes should be placed in pLPCX lentiviral vectors. Plasmids were expanded in Top 10 competent E. coli, harvested using the Qiagen miniprep system and purified using a10% agar gel electrophoresis purification. Primers were designed to cut the genes of interest from the pLPCX retroviral vectors (Table 4). Resulting sequences were expanded using PCR, purified, and concentrations verified using nanodrop spectrophotometry.

Plasmid	Primers
pLPCX GRX1ro-GFP2	5' CACCATGGCTCAAGAGTTTGTGAAC 3' 25bp 48%GC MT 56°C (FWD) 5' TTACTTGTACAGCTCGTCCAT 3' 21bp 43%GC MT 55°C (REV)
pLPCX mito GRX1ro- GFP2	5' CACCATGGCCTCCACTCGTGTCC 3' 23bp 65%GC MT 62°C (FWD) 5'TACTTGTACAGCTCGTCCATGCC 3' 23bp 52%GC MT 60°C (REV)
pLPCX ro-GFP2-Orp1	5' CACCATGGTGAGCAAGGGCGAG 3' 22bp 64%GC MT 60°C (FWD) 5' CCCATCGATCTATTCCACCTCTTTCAA 3' 27bp 44%GC MT 56°C (REV)
pLPCX mito ro-GFP2- Orp1	5' CACCATGGCCTCCACTCGTGTCC 3' 23bp 65%GC MT 62°C (FWD) 5' CCCATCGATCTATTCCACCTCTTTCAA 3' 27bp 44%GC MT 56°C (REV)

Table 4. Primers Designed for Expansion of Target Sequences. Sequences were expanded using PCR, and cloned into pENTR vectors for repackaging in lentiviral vectors.

Samples of the resulting DNA were submitted for sequencing (Sequetech) to determine if they were correct and intact (Appendix C). The genes were then packaged into pENTR D TOPO entry vectors designed for use with the Gateway™ packaging system. Once this was done, virus was made using LentiX packaging single shots (Clontech cat#631275) and HEK 293XT cells. Virus was added to Cy_2iPSC cells cultured in E8. After 24 hours, the infected cells were selected using puromycin. Colonies that correctly received the roGFP insert were expanded for analysis.

Unfortunately, the functional genes ORP1 and GRX, and the mitochondrial targeting sequence (mito) were not annotated in the literature. These sequences were located, and primers were redesigned to include these essential portions of the gene, including Orp1, and mitochondrial targeting sequences (figures 13-16).

Figure 13. Glutathione Sensitive Cytosolic GFP. As annotated in the literature (left) and with located GRX sequence (right). Primers in purple (right). Fragment length 1135 bp

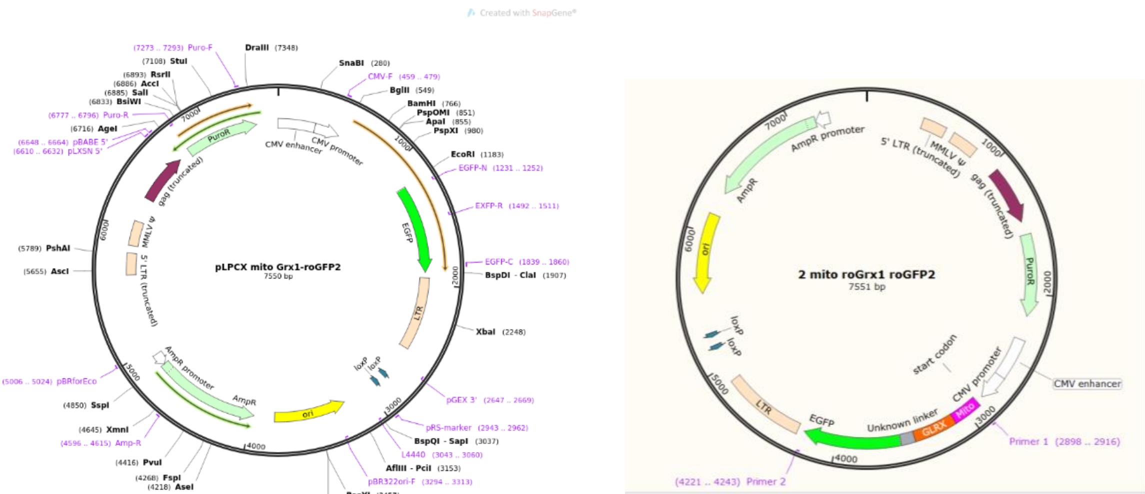


Figure 14. Glutathione Sensitive Mitochondrial GFP. As annotated in the literature (left) and with located GRX and mitochondrial locator sequences (right). Primers in purple (right). Fragment length 1349 bp

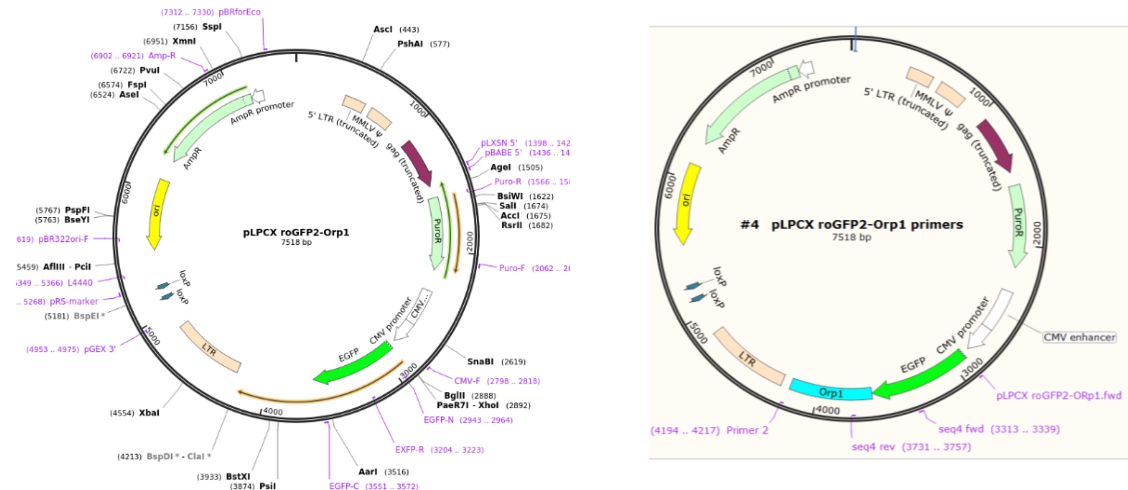


Figure 15. Peroxidase Sensitive Cytosolic GFP. As annotated in the literature (left) and with located Orp1 sequence (right). Primers in purple (right). Fragment length 1322 bp

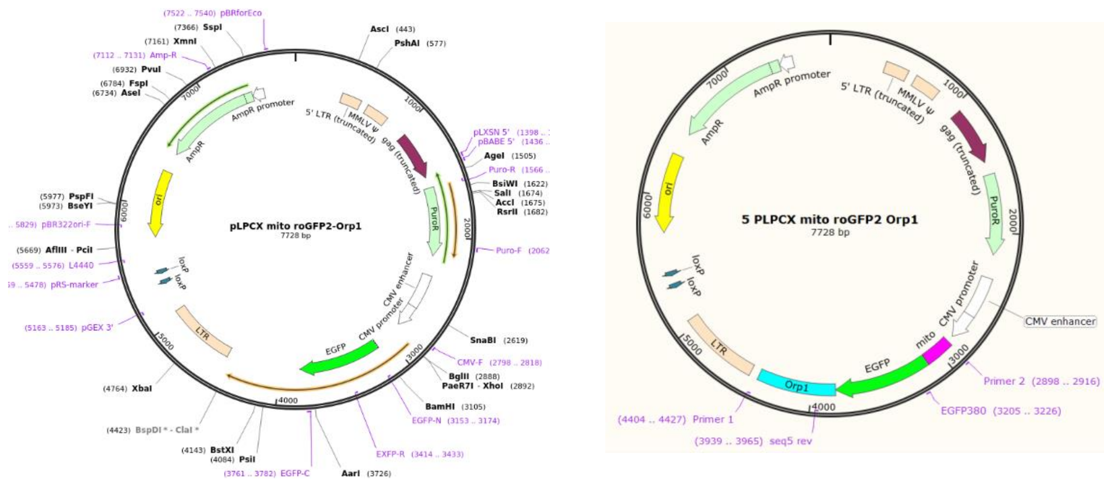


Figure 16. Peroxidase Sensitive Mitochondrial GFP. As annotated in the literature (left) and with located Orp1 and mitochondrial locator sequences (right). Primers in purple (right). Fragment length 1533 bp

Sequences resulting from newly designed primers were much longer than those carrying only the GFP portion. They were submitted for sequencing, using primers designed to analyze overlapping parts from each end, since sequencing accuracy declines sharply when sequence length is greater than 700 bp.

Results were good for all but the mitochondrial ORP1 sequence, which showed some uncertainty within the gene as annotated by N notation, meaning results are not sure which base pair occupies that position (Appendix C). The mitochondrial Orp1indicator is the largest sequence, exceeding even the overlapping sequence technique employed to accommodate the lengthy roGFPs, so that may explain the unknown or varying N positions. A sequence comparison was used to assure the sequences were correctly excised and expanded. Gene fragments were inserted into pENTR vectors and packaged in lentiviral vectors.

Resulting virus was concentrated by filtration and resuspended at 10X in PBS. Viral titers were then added to Cy_2iPSCs at 60-70% confluency in E8 media and allowed to infect cells with the roGFP sequences. Cells were selected using Puromycin (Gibco cat#A1113803), since the lentiviral envelope contained resistance to this antibiotic.

Repeated trials returned little or no functional protein in cells, so pLPCX plasmids containing the roGFP genes were directly packaged using the RetroX packaging system (Clontech cat#631530). This method required no cloning, as the pLPCX vector is an entry vector for the RetroX packaging system. Yields were much higher, and virus was acquired by packaging using HEK GP2 293 packaging cells. Resulting virus was concentrated using the RetroX Concentrator (Clontech cat#631455) and stored at -80 for later use in oxidative stress experiments.

Analytical Methods

To corroborate successful complexation, a phenolphthalein assay was employed to quantify the amount of uncomplexed MBCD present in complexed solutions. MBCD/FA complexes were diluted in 20mM Na_2CO_3 to a concentration of 2mM, and adjusted to pH of 10.5 for optimum phenolphthalein absorption. A 0.2mM phenolphthalein solution was prepared in Na_2CO_3 and also adjusted to pH 10.5. A Standard curve was prepared using phenolphthalein at pH 10.5 diluted with pH 10.5 Na_2CO_3, and samples were prepared by mixing 0.5mL of MBCD/FA solutions and 0.5mL of phenolphthalein solution. To determine phenolphthalein concentration when mixed with pH 10.5 water alone, a base measurement was run using 0.5mL Na_2CO_3 and

0.5mL 0.2mM phenolphthalein solution. Measurements were then taken using 0.5mL 2mM MBCD/FA solutions, and 0.5mL 0.2mM phenolphthalein, making the concentrations 1mM MBCD/FA and 0.1mM phenolpthalein. Spectrophotometric measurements were made on a Cary 60 spectrophotometer at 555nm, measured λmax of phenolphthalein. Measurements were made in triplicate and the results were compared to the standard curve of phenolphthalein concentration that had been prepared (figure 17).

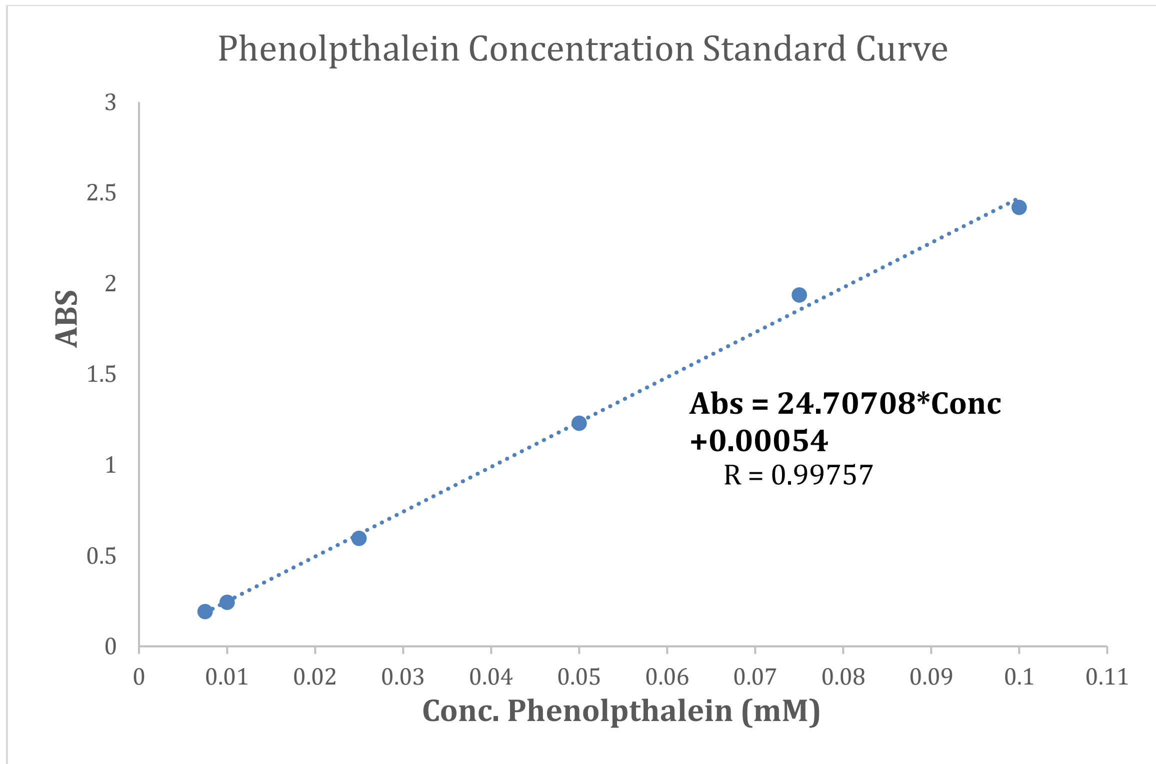


Figure 17. Standard Curve of Known Concentrations of Phenolphthalein at pH 10.5. All measurement were done in triplicate, and averages plotted (Appendix B).

RESULTS

Fatty Acid Complexation

Addition of fatty acids to cell culture media in a fully defined manner requires use of a carrier to replace albumin. The carrier must proffer solubility on the fatty acids maintaining appropriate levels in free form without inhibiting their delivery to the cell membrane, or altering their activities. This implies molecular coordination, or non-covalent complexation of lipids with a carrier, as the best route. Docosahexanoic acid (DHA), eicosapentanoic acid (EPA), oleic acid (OA), palmitoleic acid (PA), α linolenic acid (ALA) and cholesterol were successfully complexed with MBCD at 1:1 stoichiometry. Palmitic acid (PT), the largest saturated fatty acid used, required 10:1 stoichiometry, so was not satisfactorily complexed. A condensation of ascorbic acid and palmitate, 6-O-Ascorbyl L-Palmitate, which is somewhat water soluble, was used instead to carry palmitate in culture media, and did not require additional modification.

Palmitic acid (PT) required more than 10 molar equivalents of MBCD to become soluble. At this concentration, aggregates of cyclodextrin were noted in media, possibly making the palmitic acid unavailable, and potentially causing the complexation of MBCD with other nutrients in the media. Both of these possibilities are unfavorable. Ascorbyl palmitate was found to be soluble at the concentrations used in this work. Ascorbyl palmitate provides both palmitate, which may take part in Wnt processing, and extra ascorbic acid, which is known to be necessary for DNA demethylation, and has antioxidative effects. Both of these activities are likely to be favorable.

Once complexation with MBCD appeared to be accomplished, it was necessary to establish that complexes had actually formed. To do this, phenolphthalein was used in a spectrophotometric assay. Phenolphthalein is highly colored at high pH, easily occupies the barrel of MBCD in a 1:1 stoichiometry, and is colorless when complexed (Skoulika et al. 1999). Phenolpthalein is easily displaced from MBCD by more hydrophobic molecules such as lipids. It follows that a phenolphthalein solution of known concentration would have a reduction in absorbance reflecting the molarity of uncomplexed cyclodextrin in solution. It was found that less than 7% of MBCD in the complexed solutions was free, proving that at least 93% were complexed with lipids (Table 5).

Complexation Analysis				
Expected Concentrations:	MBCD:FA	1mM		
	Phenolpthalein	100µM		
	Free Phenolpthalein conc. (µM)	Standard Deviation (+/-) µM	Free MBCD conc. (µM) (base - complexes)	Free CD % (FreeMBCD/MBCD: FA)./100
Base (no MBCD/FA)	96.1	0.250	0.00	0
DHA	60.3	0.034	35.80	3.58
Palmitoleic Acid	22.3	0.004	73.80	7.38
Cholesterol	95.6	0.259	0.50	0.05
Oleic Acid	35.7	0.026	60.40	6.04
a Linoleic Acid	28.5	0.006	67.60	6.76

Table 5. Results of Spectrophotometric Analysis of MBCD/FA Complex Solutions. Phenolphthalein concentrations when mixed with MBCD/FA solutions were subtracted from (base) concentration of Phenolphthalein without added MBCD/FA. The difference is due to complexation of phenolphthalein with free MBCD at 1:1 stoichiometry. The equation of the line was Abs = 24.70708C +0.00054 R=0.99757

The small amount of free MBCD may be attributed to the non-covalent nature of the complexation, allowing for equilibrium with small amounts of free lipid in solution.

Free CD concentrations were assumed to reflect free lipid concentrations, and these concentrations were then compared to critical micelle concentrations of the lipids used, to ensure levels of free lipid were actually within reasonable limits. With the exception of oleic acid, which was the most difficult to complex, all lipids concentrations were at or beneath concentrations that would cause soapy micelles to form (Table 6). These results indicated that complexation was complete, and that MBCD was delivering low free fatty acid concentrations for cells as desired.

Lipid	**Estimated Free Lipid Concentration in MBCD**	**Critical Micelle Concentration from literature**
DHA	36μM	100μM
Palmitoleic Acid	74μM	150μM
Cholesterol Sulfate	0.5μM	0.5μM
Oleic Acid	60μM	6μM
A Linolenic acid	68μM	150μM

Table 6. Comparison of Assumed Free Lipid Concentrations and Corresponding Reported Critical Micelle Concentrations.

Supplement Effects on Stem Cells

Initial experiments were carried out on fully characterized iPSCs from blood, provided by NIH (Cy_2iPSCs). These cells were plated and fatty acids complexed with cyclodextrin were added. Cyclodextrin alone was also added to determine if it had any

part in observed effects. It was determined that cyclodextrin was not toxic at the concentrations used, and proliferation was not significantly different from control cells. Several lipids did improve proliferation of cultured Cy_2iPSCs. Serial dilution was then performed using the lipids that showed promise, revealing the most effective concentrations (Table 7). Surprisingly, the essential fatty acid linolenic acid (18:2 ω3) (ALA) and ω-3 eicosaopentanoic acid (20:5 ω3)(EPA) showed results similar to control. Docosahexaenoic Acid (22:6 ω3) (DHA), Palmitoleic Acid (16:1ω7), and Cholesterol, a 27 carbon lipid, were selected for further study.

Cells were cultured for 52 days with fatty acid supplementation, including 10 passages using EDTA based dissociation solution, and continued to proliferate without appreciable differentiation as measured by visual examination of cell morphology. The most effective concentrations were 7.5nM DHA, 750nM Cholesterol, and 7.5µM Palmitoleic acid.

Fatty Acid Concentration	Control E8 only	Eicosapentanoic Acid (EPA)	Docosahexanoic Acid (DHA)	Alpha Linolenic Acid (ALA)	Cholesterol	Palmitoleic Acid
7.5 µM	AVG: 9.56×10^5	0	0	9.21×10^5	1.35×10^6 141%	1.42×10^6 149%
750 nM	AVG: 9.56×10^5	1.13×10^6 118%	7.84×10^5	1.05×10^6 110%	1.50×10^6 157%	1.16×10^6 121%
75 nM	AVG: 9.56×10^5	1.11×10^6 116%	1.13×10^6 118%	8.11×10^5	1.42×10^6 149%	1.21×10^6 121%
7.5 nM	AVG: 9.56×10^5	1.11×10^6 116%	1.37×10^6 143%	9.49×10^5	$9.66\ 10^5$	8.80×10^5

Table 7. Results of Lipid Serial Dilution on Cy2iPSCs. Effects of individual lipids on iPSC counts after 4 days supplementation. Cells were evenly seeded in 6 well plates at a seeding of 300,000 cells/well.

Identical serial dilutions were performed on H9 embryonic stem cells and Cy_2iPSCs. Each sample consisted of four wells, evenly seeded at 68,000 cells/well and 70,000 cells/well respectively, and cells were counted at day four post seeding.

Results of serial dilutions with ESC and iPSC revealed large differences in proliferative responses to ALA, AscPT, DHA and ascorbic acid (figure 18).

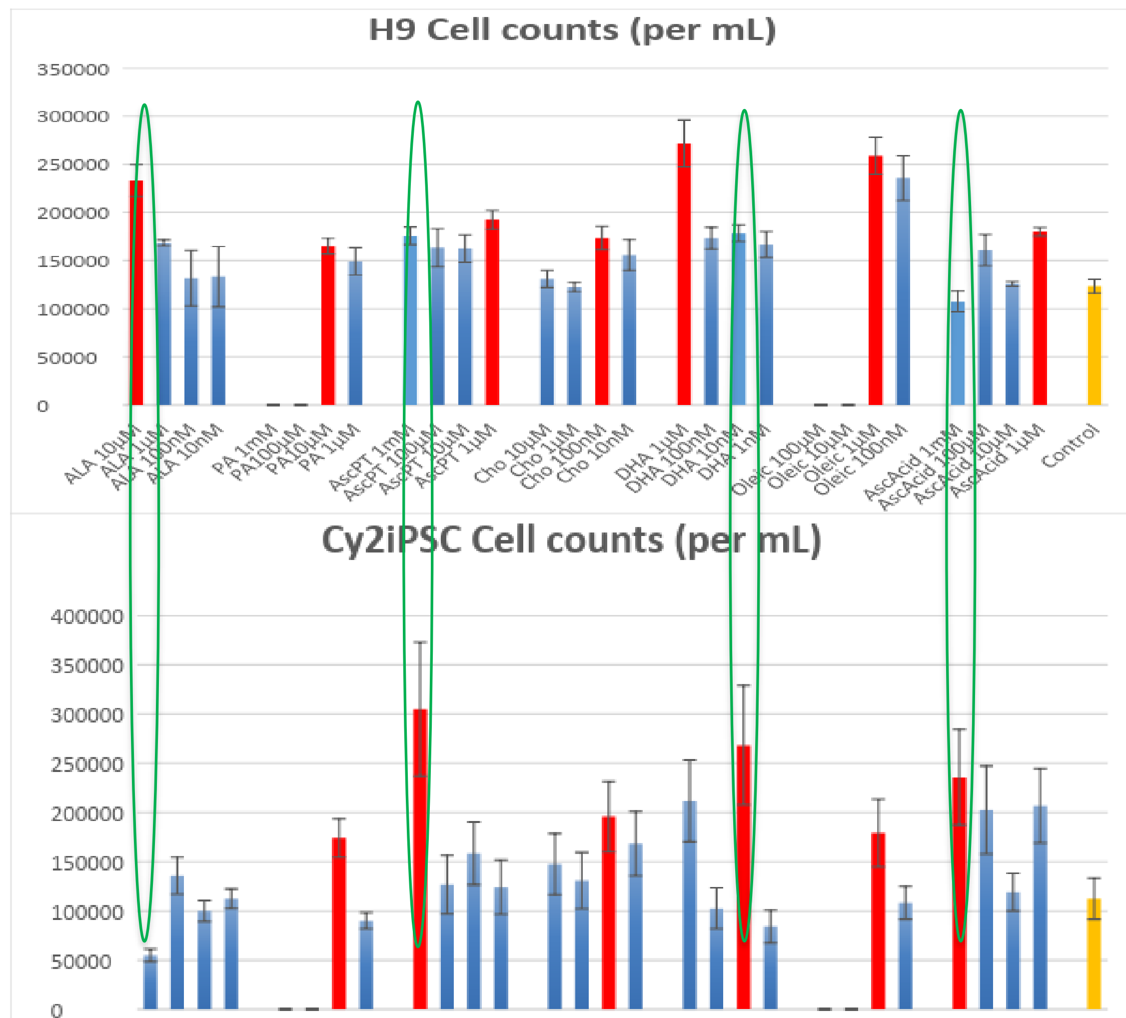


Figure 18. Identical Serial Dilutions of Lipids on iPSC and ESC. Addition of single lipids shows distinct differences in response to ALA, AscPT, DHA and Asc Acid. Green ovals indicate corresponding lipid concentrations that provided significantly different responses between stem cell types.

These differences correspond with other studies comparing iPSCs with ESCs and finding differences, including that ESC's are more fully demethylated by approximately 20% (Bock et al. 2011, Omole and Fakoya 2018), probably because embryonic stem cells have never been methylated, having never been differentiated.

Explanation of these responses requires a review of some of the nutrient effects discussed earlier. Ascorbic acid is known to be essential in DNA demethylation (Young et al. 2015, Cimmino et al. 2018), so the proliferative response to both ascorbic acid and ascorbyl palmitate by iPSCs seems to indicate that they may be in need of more demethylation.

Another aspect of these differences is palmitate. Palmitate is one of the lipids utilized by the Wnt pathway to produce, excrete, and receive Wnt proteins. The strong proliferative response to 100 times more AscPT may be due to the palmitate, the ascorbic acid, or a combination of both.

To deconvolute the effects of Wnt related lipids palmitate and palmitoleic acid, and the relationship with ascorbic acid, another cell proliferation experiment was performed using each lipid and ascorbic acid alone in E8 media, compared to three mixtures: $Cy_2$1 containing no AscPT, $Cy_2$2, containing no PA, and $Cy_2$3, containing 10μM concentrations of both PA and AscPT, plus ascorbic acid (figure 19). The results indicated that PA and PT both increase proliferation greatly alone, but mixtures with other fatty acids significantly diminish that effect, in spite of their concentrations being the same.

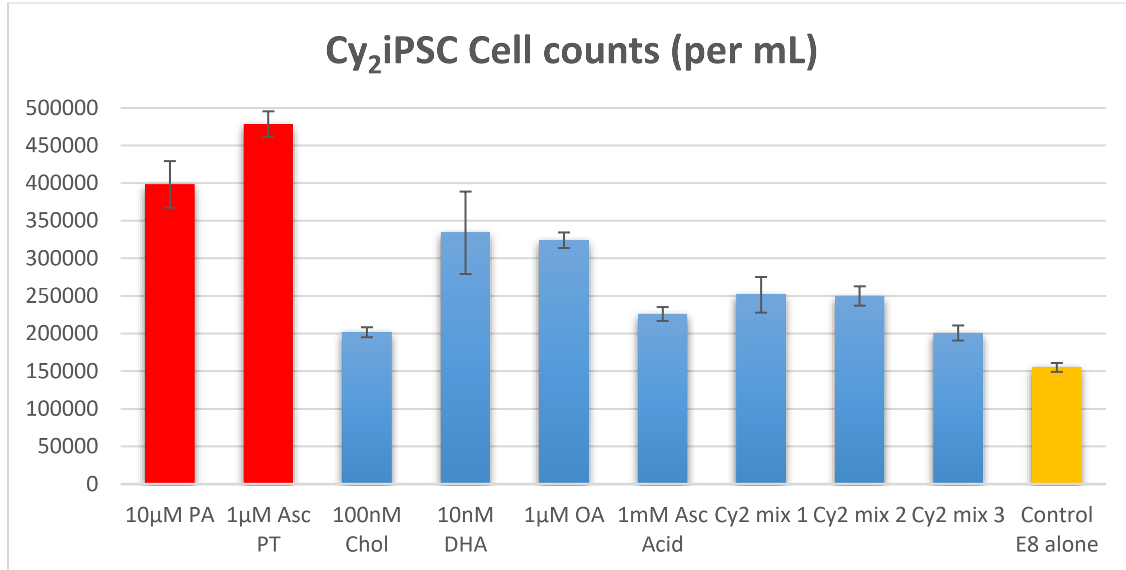


Figure 19. Cell Proliferation using Lipid Mixes. PA and Asc PT increased proliferation by close to 3 fold, while mixes containing them did not. Ascorbyl PT increased proliferation 2 fold over ascorbic acid alone. Cells were evenly seeded in 6 well plates at a density of 300,000cells/well, and counted 4 days post seeding.

Additionally, ascorbic acid alone increased proliferation only half as effectively as Asc PT. Interestingly, palmitate itself has been found to cause cell death under some conditions by its pro-oxidative nature (Oh et al. 2018). Palmitate has also been credited with increasing mitochondrial respiration (Egnatchik et al. 2014), which produces reactive oxygen species. Negative effects of palmitate may have been countered in this instance by the antioxidative effects of ascorbic acid.

Lipid effects on pluripotency were measured on the same cells using antibody staining for major markers Oct4, Sox2, Tra 1-60 and Tra 1-81, and differentiation marker SSEA1. Fluorescence intensity results showed that Oct4 and Sox2 were upregulated in all supplemented cells compared to E8 control (figure 20, appendix D). The negative indicator of pluripotency, SSEA1, showed no appreciable differentiation in any of the

samples. Tra 1-60 and Tra 1-81 indicators seem to be somewhat more expressed in the control sample compared to samples grown in media containing lipids.

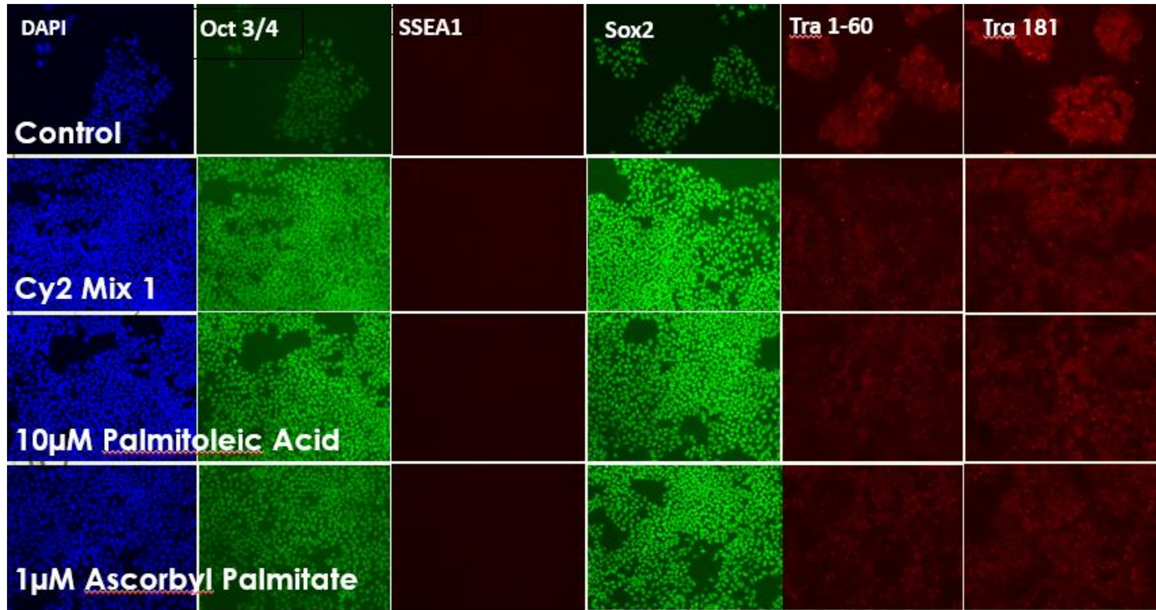


Figure 20. Staining of iPSCs after lipid supplementation. Cells were fixed and stained for pluripotency and differentiation proteins. Fluorescence intensity indicates levels of protein expression.

This seems to indicate some pluripotency genes are more active in the control group. The reason behind this may lie in the type of receptor Tra 1-81 and Tra 1-60 bind to, as these are primarily embryonic stem markers, or it may be explained by further analysis of lipid effects on pluripotency and pluripotency markers.

A preliminary experiment was also run to see if lipids might eliminate the need for ROCK inhibitor when initially thawed from cryopreservation. Four vials of frozen stock were thawed and plated using the ROCK inhibitor Thiazovivin, or a mix of all four fatty acids. Thiazovivin is a 2,4-disubstituted thiazole which has been shown to dramatically increase cell attachment and adhesion mediated integrin signaling by

regulating E-Cadherin mediated cell to cell signaling, and inhibiting the ROCK pathway (Park 2015).

Since the ROCK pathway is a part of the non-canonical Wnt pathway, which we hope to down regulate with the addition of fatty acids, and E-Cadherin is part of the canonical pathway, it seemed that a comparison of cell survival upon thawing from cryopreservation might indicate whether the fatty acids were likely to be effecting the Wnt pathway. The fatty acid mixture seemed to improve adhesion and cell survival significantly better than Thiazovivin, as measured by visual examination of colonies at day 3 post seeding. This result suggested that this mixture of fatty acids may down regulate the ROCK pathway, and stimulate E-cadherin related attachment. Although the mechanism for this is unclear, it is significant that the ROCK pathway is part of the non-canonical Wnt cascade, while E-cadherin adhesion is dependent on several components of the canonical Wnt pathway, including β catenin and APC. APC is a component of the β-catenin degradation complex, which is freed by canonical Wnt activation. These results provide some support for the theory that fatty acid availability effects Wnt pathway activity under the conditions of these experiments. Since lipids are also necessary for energy metabolism and structure, and their effects are naturally regulated and mediated by cells *in vivo*, lipid supplementation may be a healthier and more effective supplement than ROCK inhibitors to provide support for cells at seeding (figures 21-23).

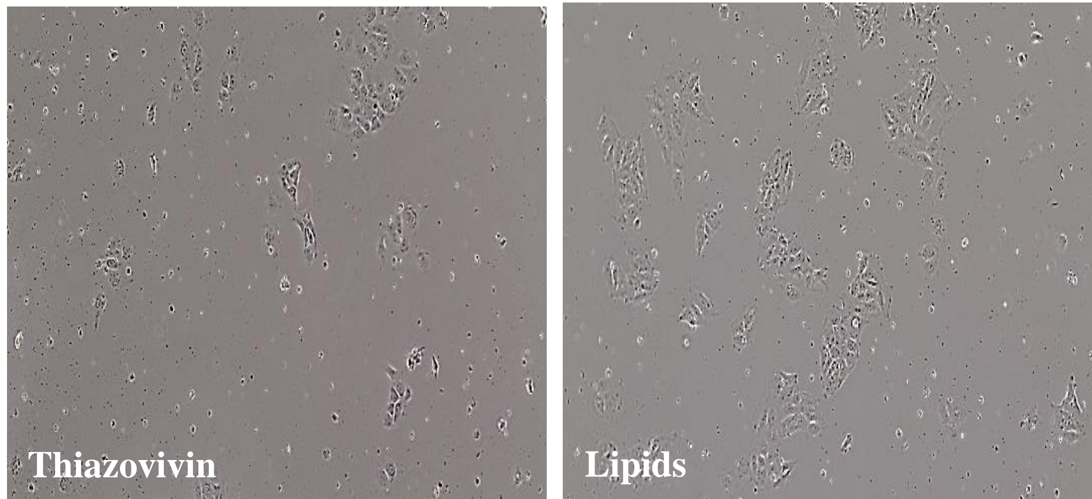


Figure 21. Thiazovivin and Lipid Addition to Passage 18 Cy_2iPSCs. Comparative effect on attachment after seeding from cryostorage

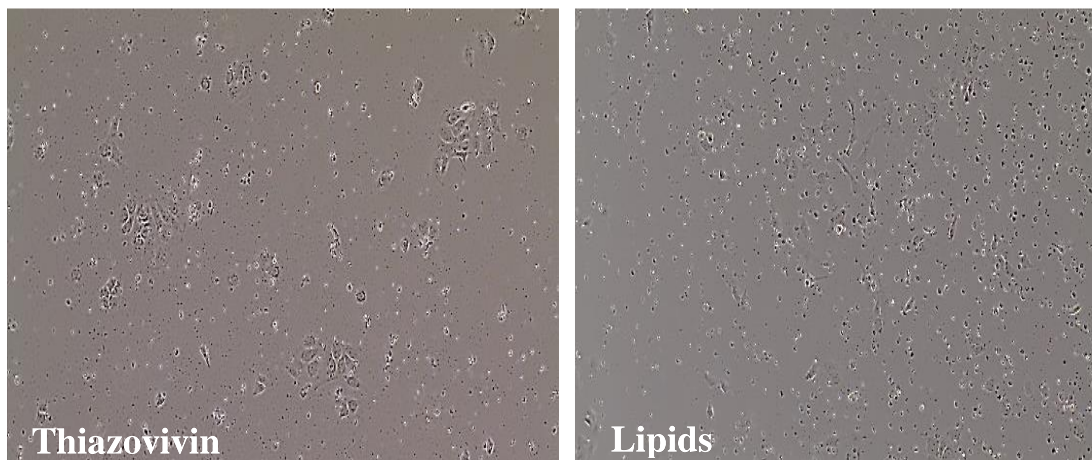


Figure 22. Thiazovivin and Lipid Addition to Passage 22 Cy_2iPSCs. Comparative effect on attachment after seeding from cryostorage

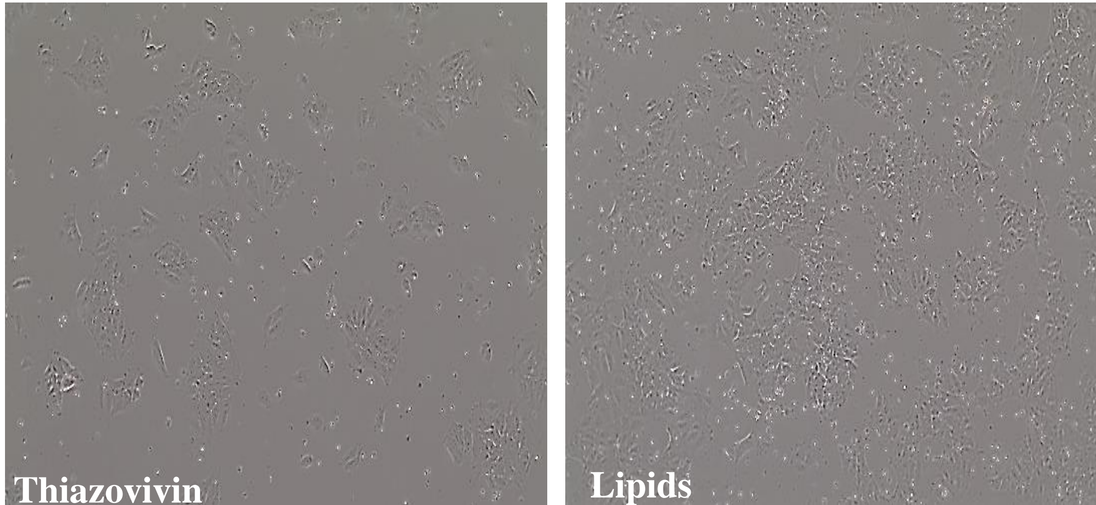


Figure 23. Thiazovivin and Lipid Addition to Passage 25 Cy_2iPSCs. Comparative effect on attachment after seeding from cryostorage
Lipids

Figures 21-23. Cryopreserved Cy_2iPSCs from various passages were thawed and seeded. Thiazovivin was used to aid attachment and survival in some wells, and was replaced with a lipid supplement in other wells. Cells attached and survived noticeably better with the addition of fatty acids.

Supplement Effects on Human Fibroblasts

Our early work on fibroblasts illustrated that human fibroblasts were more proliferative (figure 24), and were larger (figure 25) when heat treated human serum was added to the media, than when supplemented with FBS, which is also heat treated to inactivate the immune complement.

Human serum is much richer in fatty acids than FBS. From this we surmised that the type of fatty acid content in media might have a beneficial effect on cell health and proliferation in culture, although other factors may be involved. This observation motivated the use of distinct fatty acid mixtures and concentrations to assess their effects on the proliferation and self-renewal of pluripotent stem cells.

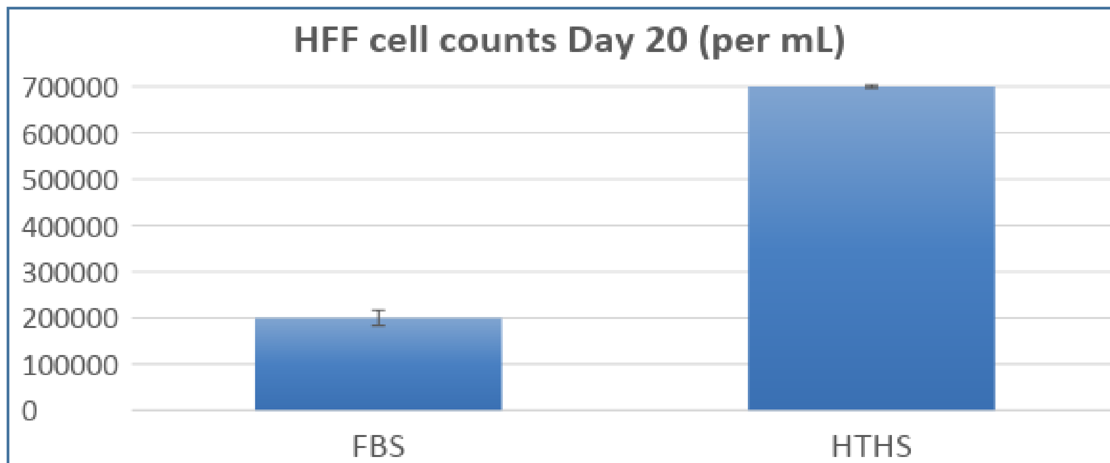


Figure 24. HFF Cell Counts per mL 5 Days after Passage 3, comparing human serum (HTHS) supplemented cell growth to that of FBS. Cells were seeded in 6 well plates at $6x10^4$ cells/well and passaged 1:5 at confluency. N=3

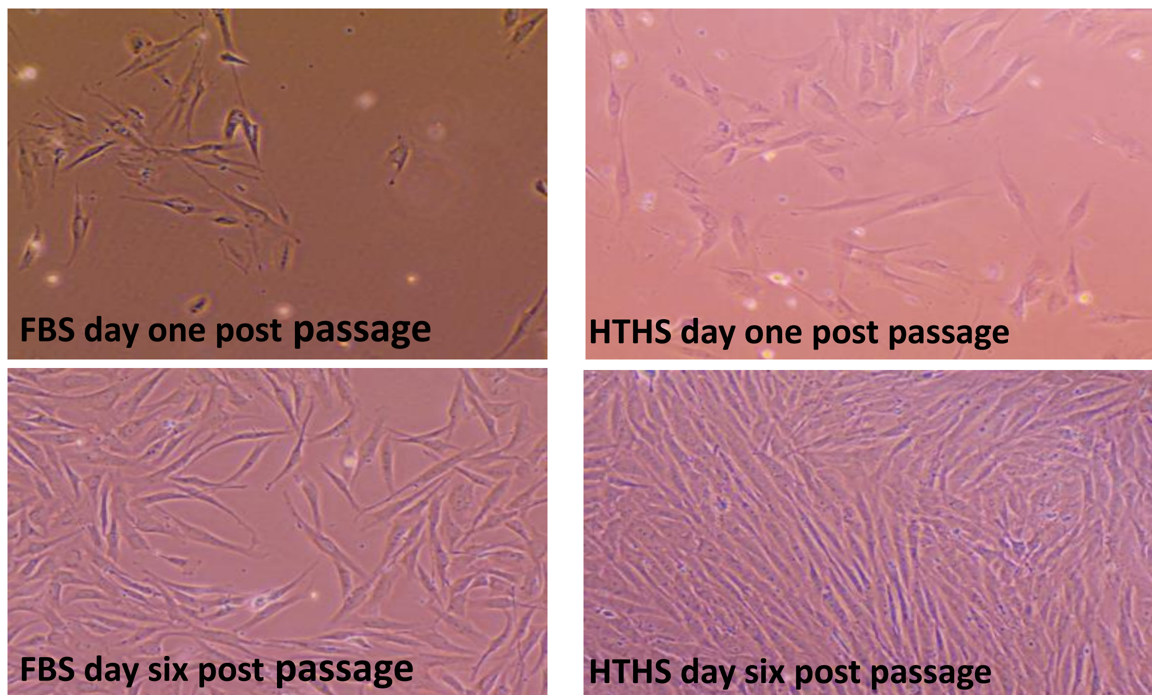


Figure 25. Adult Fibroblast Cells Exposed to Heat Treated Human Serum (HTHS), and Commercial Fetal Bovine Serum (FBS). Both serum additives were used at 10% concentration in DMEM complete medium with antibiotic/antimitotic.

Human serum is commonly used in the culture of HFF for iPSC generation (Hagbard et al. 2018), but cytokine, prion and other contaminating cargo can be an issue

with human serum as well. Fibroblasts are also the most commonly used cells in iPSC generation, so development of a fully defined lipid supplement to replace FBS in the culture of HFF may also be desirable for enhancement of reprogramming efficiency.

To determine what lipid contents are favorable for HFF growth, a serial dilution was performed, with cells evenly seeded at densities noted in corresponding figures, and counted at day four after seeding (figure 26).

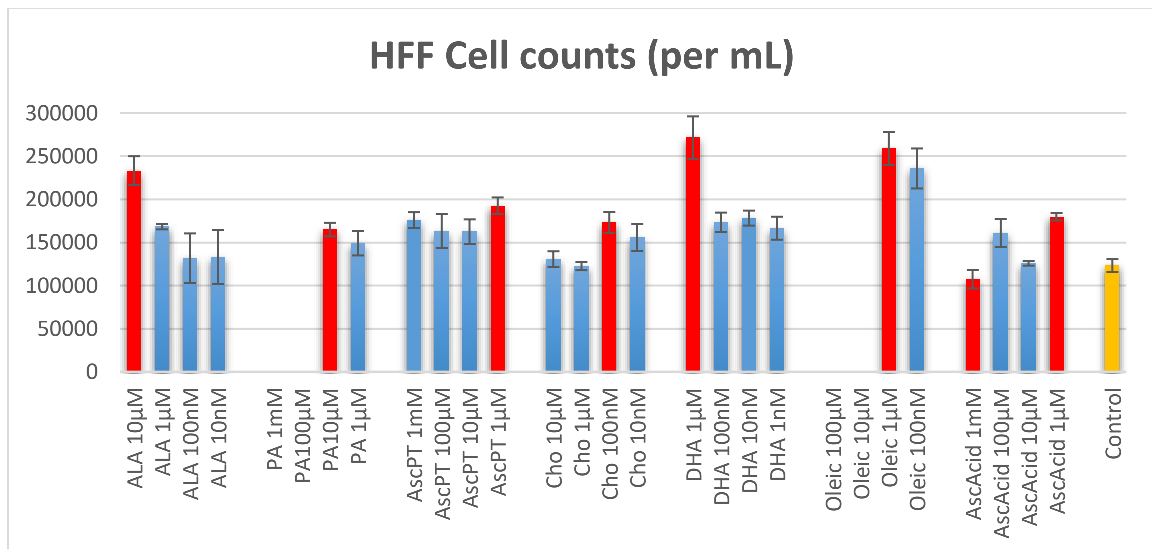


Figure 26. HFF Serial Dilution of Lipids. Cells were seeded at density of 80,000 cells/well in 12 well plates. No FBS was added to lipid supplemented media. No bar means cell death. N=3

From serial dilution results, target concentrations of lipids were supplemented with Trace elements A or B, insulin and holotransferrin, respectively, as these are also carried by serum, and are necessary for many cell types (Freshney, 2010). Cells were again seeded evenly in 12 well plates, and individual lipids were used, supplemented as noted. Results showed that a mixture of all six lipids tested was significantly more effective than any one lipid alone, unlike the results from stem cell trials (figure 27).

Trace element mixture A was more effective in supporting cell growth, and insulin and holotransferrin were not effective in promotion of proliferation in the short term of the experiment. Significantly, the cells given the mixture of six lipids proliferated almost 60% more than control cells given FBS.

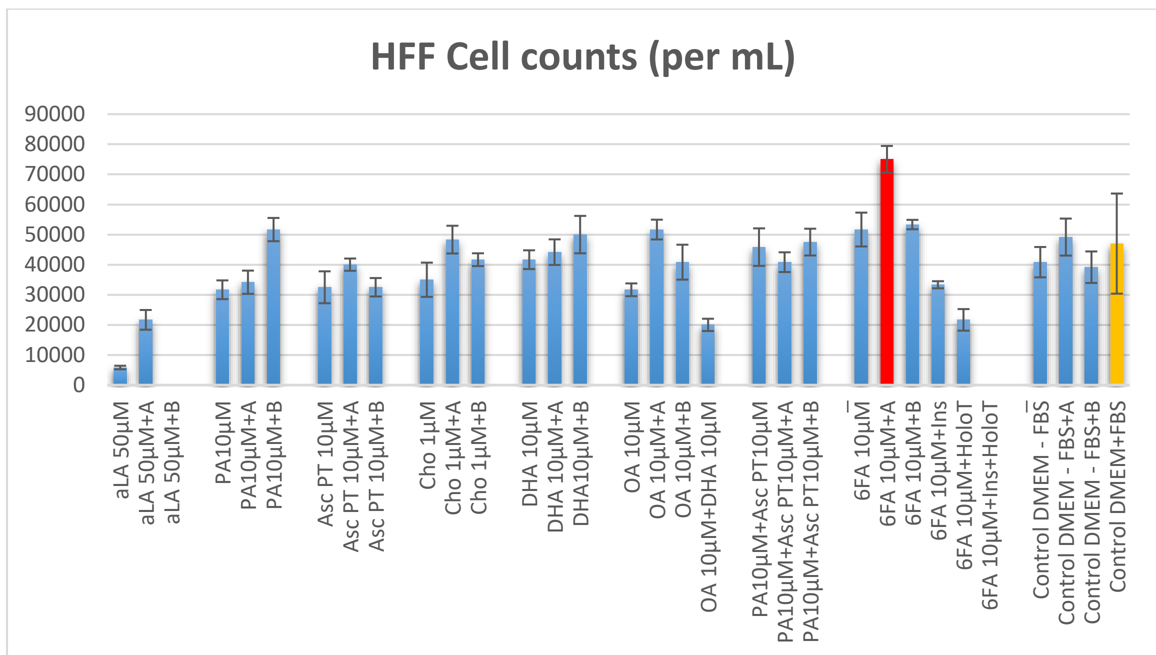


Figure 27. Proliferation of HFF with Supplementation of Different Lipids, Trace Elements, Insulin and Holotransferrin. N=3 for all conditions.

There was also a striking difference in standard deviations in this experiment (figure 26), indicating less variability with chemically defined lipids over FBS.

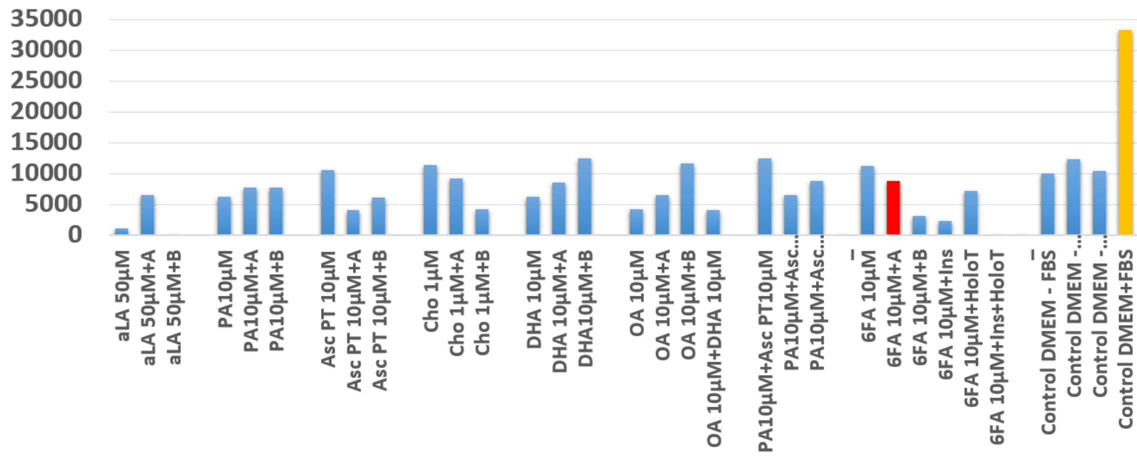


Figure 28. Standard Deviations of Proliferation Data from Supplementation of HFF Cells.

DISCUSSION

Importance and Application of Defined Lipid Supplements

As the field of iPSC research has grown, understanding of the cellular pathways and mechanisms involved in cell fate decision has been central to progress toward reliable and robust iPSC production, maintenance and differentiation. The content of the growth media has been re-evaluated and chemically defined conditions have been established to be optimal for production of genetically stable, fully pluripotent iPSCs. Nonetheless, use of animal cell feeder layers, animal and human sera, and complex proteins like serum albumin continues, even in media dubbed "defined" or "xeno free" (Desai et al. 2015). Culture of cell lines other than stem cells is still dominated by use of FBS. Problems and variability associated with use of these products, and notably FBS, are well documented, but an effective chemically defined lipid vehicle has not previously been developed.

In this work, the foundation has been placed for truly chemically defined lipid supplementation. Complexation of lipids with MBCD at 1:1 stoichiometry has been shown to be possible, and supplementation of cells with the complexed lipids has been shown to be effective, and provocative. Clearly, the precise and defined application of lipids this technology provides may pave the way for less variable results in cell studies, more effective use of lipids as signaling molecules, and potential replacement of FBS as a cell media supplement.

It was initially assumed that the essential fatty acids linoleic acid (LA 18:2 Δ 9,12) and its ω-3 counterpart alpha linolenic acid (ALA 18:3 Δ 9,12,15) would be primary parts of any effective lipid supplement, as cells cannot synthesize them, but can use them to produce many other lipids. ALA was tested along with the long chain omega three fatty acids, docosahexaenoic acid (DHA 22:6 Δ 3,6,9,12,15,18) and eicosopentanoic acid (EPA 20:5 Δ 5,8,11,14,17), which are the most bioactive components of fish oil. Omega three fatty acids were chosen due to their antioxidant properties, and their many positive biomedical properties *in vivo* (Stillwell and Wassall 2003). ALA proved to be have little effect on proliferation in the cells tested. Although the implications of this are muddied by the presence of small amounts of LA in the E8 base, this result seems to support the idea that cell types tested cannot easily synthesize lipids from LA, or that cells prefer to take some lipids up from their surroundings (exogenous uptake). If the cells tested could easily synthesize other needed lipids from the linoleic or linolenic fatty acids, it seems that the addition of other lipids would not be so much more effective at promoting cell proliferation.

DHA and EPA, while both antioxidant ω3 fatty acids, had very different effects on cell proliferation. Clearly DHA was the more effective lipid for iPSCs, from a proliferation standpoint. This may be due to the fact that cells can synthesize EPA from DHA, but most cells cannot synthesize DHA from EPA. This effect may be cell type specific, depending on which enzymes are expressed. More studies on a variety of cell lines are necessary to begin to determine optimum lipids for each cell type and application.

Two fatty acids that are associated with activation of the Wnt pathway, PT and PA, were investigated specifically because of their impact on Wnt pathway activation (Coudreuse and Korswagen 2007, Galli and Burrus 2011). PT and PA acid play important and different roles in Wnt production, secretion and activation (He 2008, Komiya 2008, Galli and Burrus 2011). Because PT was not successfully complexed, AscPT was used instead. This introduced an interesting dynamic, since PT is known to stimulate mitochondrial respiration, producing ROS, and causing cell death at some concentrations (Lees et al. 2017). Ascorbic acid, however, is a powerful antioxidant, and the combination of ascorbate and PT was nontoxic to cells, and even outperformed palmitoleic acid for stimulating proliferation when applied to some cell types. Measurement of the effects of these additions on Wnt pathway activities are yet to be analyzed. This is expected be an important step in the process of developing optimal lipid supplements to support stem cell reprogramming, maintenance and differentiation.

Octanoic acid was chosen as a reprogramming additive due to its distinction as a preferred acetyl group donor for histone acetylation (McDonnell et al. 2016). Reprogramming experiments have begun, but results are not yet available. The small fatty acid, butyrate, had also been identified as a reprogramming enhancer (Gaoyang 2010). This may be due to butyrate's inhibitive effects on the histone deacetylase enzyme, and its upregulation and stabilization of the micro RNA cluster 302/367(miR302/367) (Zhang 2013). The miR302/367 cluster is also a target of Oct4 and Sox2, two of the four main pluripotency factors used to stimulate reprogramming. Butyrate has also been found to be protective against hypoxic and hypoglycemic insults

and oxidative stress (Kang et al. 2014, Newman and Verdin 2014). These two lipids may also be useful in maintenance culture for certain cell types. More experimentation is being done on their effects on stem cell maintenance and on other cell types.

Both cholesterol and DHA are essential for construction of many membranes (Stillwell and Wassall 2003, Goluszko and Nowicki 2005) and both exhibited positive effects on stem cell proliferation, but no single lipid alone significantly increased HFF proliferation compared to supplementation with FBS. A mixture of six lipids, along with trace elements, was effective in replacing FBS in fibroblast culture for about 10 days. This is long enough to be useful for reprogramming, but more work is necessary if FBS is to be replaced in general fibroblast culture, as it was noted that cell numbers were reduced in longer term culture with the lipid mixture.

Differences in Cellular Lipid Requirements

Research in the interest of developing a fully defined media for HFF cells led to the discovery that the most abundant fatty acid in FBS is oleic acid, so oleic acid was also complexed and tried on three cell types: H9, iPSC, and HFF cells. Interestingly, the optimum oleic acid concentration for HFF (10µM) was toxic to stem cells, while 1µM OA significantly increased stem cell proliferation. While the mixture of lipids used in initial trials was very effective, later application of this mixture in larger dishes with lower cell density lacked the same proliferative result. Addition of growth factors is likely to be needed to effect the same proliferative success in larger applications and in long term culture of HFF.

Proliferation is an initial criteria for success of lipid supplements. Lipid supplementation promises to be protective against oxidative stress, which can be desirable in some differentiation protocols, or undesirable, as in maintenance cell viability in long term culture. Redox sensitive GFPs have been expanded and are ready to implement in cells to analyze the oxidative stress effects of supplementation with different lipids. Plasmids coding for redox sensitive GFPs can be virally transfected into cells, and enable them to report through fluorescence signals, the oxidative state of some important redox variables in the cell, including glutathione. Cells expressing these proteins may help to more clearly define the role and mechanisms of oxidative signaling in cell health and behavior.

As experimentation continues, reprogramming results will be used to inform further studies of lipid supplementation for stem cell applications. Important in this is the analysis of effects of lipid supplementation on the Wnt pathways. Wnt responses will be analyzed using rtPCR to determine relative amounts of downstream Wnt proteins expressed under different nutrient conditions.

Many questions remain, and additional optimization studies must be performed to establish use of fully defined lipid additives, but it seems likely that implementation of the tools developed in this project will greatly improve the results of stem cell culture for research, reprogramming of adult cells to pluripotency, maintenance of stem cells in culture, differentiation of stem cells to target cell lines, and possibly even use of lipids as therapies.

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APPENDICES

APPENDIX A

CELL SOURCE INFORMATION

Cell line name	CY2 [Human iPSC]
Synonyms	ND50020
Accession	CVCL_1E79
Resource Identification Initiative	To cite this cell line use: CY2 [Human iPSC] (RRID:CVCL_1E79)
Comments	From: NIH Center for Regenerative Medicine (CRM); Bethesda; USA.
Species of origin	Homo sapiens (Human) (NCBI Taxonomy: 9606)
Sex of cell	Male
Age at sampling	Age unspecified
Category	Induced pluripotent stem cell
Web pages	https://www.nimhgenetics.org/stem_cells/crm_lines.php
Cross-references	
Cell line collections	NHCDR; ND50020
Other	Wikidata; Q54817088

nih crm ipsc catalog

Catalog of induced Pluripotent Stem Cell (iPSC) from NIH CRM available for distribution through the NIMH Stem Cell Resource

Name or Designation	Description	iPSC Reprogramming Method	Starting Material	Source	Availability
Control lines:					
NCRM-1	iPSC Control	Episomal Plasmid	CD 34+ Cord Blood	NIH CRM Lonza Contract	Current
NCRM-2	iPSC Control	Episomal Plasmid	CD 34+ Cord Blood	NIH CRM Lonza Contract	Current
NCRM-3	iPSC Control	Episomal Plasmid	CD 34+ Cord Blood	NIH CRM Lonza Contract	Current
NCRM-4	iPSC Control	Episomal Plasmid	CD 34+ Cord Blood	NIH CRM Lonza Contract	Current
NCRM-5	iPSC Control	Episomal Plasmid	CD 34+ Cord Blood	NIH CRM Lonza Contract	Current
NCRM-6	iPSC Control	Episomal Plasmid	CD 34+ Cord Blood	NIH CRM Lonza Contract	Current
ND1.4	iPSC Control	Episomal Plasmid	Fibroblast (ATCC)	University of Wisconsin	Current
ND2.0	iPSC Control	Episomal Plasmid	Fibroblast (ATCC)	University of Wisconsin	Current
CY2	iPSC Control	Episomal Plasmid	Blood	NIH CRM CDI Contract	Current
1201C1	iPSC Control	Episomal Plasmid	Peripheral blood	Dr. Shinya Yamanaka (CiRA, Kyoto University)	Feb 2014
1205D1	iPSC Control	Episomal Plasmid	Peripheral blood	Dr. Shinya Yamanaka (CiRA, Kyoto University)	Feb 2014
1231A3	iPSC Control	Episomal Plasmid	Peripheral blood	Dr. Shinya Yamanaka (CiRA, Kyoto University)	Feb 2014

Cell line name	HFF-1		
Synonyms	HFF1		
Accession	CVCL_3285		
Resource Identification Initiative	To cite this cell line use: HFF-1 (RRID:CVCL_3285)		
Species of origin	Homo sapiens (Human) (NCBI Taxonomy: 9606)		
Hierarchy	Children:		
	CVCL_C253 (ATCC-DYR0100)	CVCL_X499 (ATCC-DYS0100)	CVCL_DQ54 (epiHFF1-B1)
	CVCL_F285 (HFF-1 IRR)	CVCL_F286 (HFF-1 MITC)	CVCL_C648 (HIPS #1)
	CVCL_C649 (HIPS #11)	CVCL_C650 (HIPS #14)	CVCL_C651 (HIPS #3)
	CVCL_C652 (HIPS #7)	CVCL_C653 (HIPS #8)	CVCL_C654 (HIPS #9)
	CVCL_DQ55 (vHFF1-iPS4)		
Sex of cell	Male		
Age at sampling	<1M		
Category	Finite cell line		
STR profile	**Source(s):** ATCC		
	Markers:		
	Amelogenin	X,Y	
	CSF1PO	7,11,12,13	
	D5S818	11	
	D7S820	9,10,12	
	D13S317	11,13	
	D16S539	10,12	
	TH01	7,9,9.3	
	TPOX	8,11	
	vWA	14,15,17,19	
Publications	PubMed=18415935; DOI=10.1387/ijdb.082590le Eiselleova L., Peterkova I., Neradil J., Slaninova I., Hampl A., Dvorak P. Comparative study of mouse and human feeder cells for human embryonic stem cells. Int. J. Dev. Biol. 52:353-363(2008)		
Cross-references			
Cell line collections	ATCC; SCRC-1041 BCRJ; 0275		
Cell line databases/resources	Lonza; 1293		
Ontologies	BTO; BTO:0004784 CLO; CLO_0003730		
Biological sample resources	BioSample; SAMN03471935 eagle-i; shared.eagle-i.net/i/0000014e-e033-4ab4-e23c-e57880000000		
Other	Wikidata; Q54883255		

APPENDIX B

SPECTROPHOTOMETRIC ANALYSIS OF COMPLEXATION

Concentration Analysis Report

Report time	8/2/2018 4:05:22 PM
Method	C:\Users\f89m731\f89m731.MCN
Batch name	C:\Users\f89m731\FA complexation Assay 8-6.BCN
Application	Concentration 5.0.0.999
Operator	

Instrument Settings

Instrument	Cary 60
Instrument version no.	2.00
Wavelength (nm)	553.0
Ordinate Mode	Abs
Ave Time (sec)	0.1000
Replicates	3
Standard/Sample averaging	OFF
Weight and volume corrections	OFF
Fit type	Linear
Min R^2	0.95000
Concentration units	mmol/L

Zero Report

Read	Abs (553.0 nm)
Zero	0.0419

Concentration Analysis Report

Report time	8/2/2018 4:15:41 PM
Method	C:\Users\f89m731\f89m731.MCN
Batch name	C:\Users\f89m731\FA complexation Assay 8-6.BCN
Application	Concentration 5.0.0.999
Operator	

Instrument Settings

Instrument	Cary 60
Instrument version no.	2.00
Wavelength (nm)	553.0
Ordinate Mode	Abs
Ave Time (sec)	0.1000
Replicates	3
Standard/Sample averaging	OFF
Weight and volume corrections	OFF
Fit type	Linear
Min R^2	0.95000
Concentration units	mmol/L

Calibration

Collection time 8/2/2018 4:06:01 PM

Standard	Concentration F mmol/L	Mean	SD	%RSD	Readings
Std 1					0.1919
					0.1915
					0.1911
	0.0075	0.1915	0.00040	0.20888	
Std 2					0.2416
					0.2416
					0.2415
	0.0100	0.2416	0.00006	0.02390	
Std 3					0.5956
					0.5947
					0.5948
	0.0250	0.5950	0.00049	0.08290	
Std 4					1.2283
					1.2300
					1.2303
	0.0500	1.2295	0.00108	0.08772	
Std 5					1.9389
					1.9330
					1.9380
	0.0750	1.9366	0.00318	0.16413	
Std 6					2.4144
					2.4217
					2.4182
	0.1000	2.4181	0.00365	0.15099	

Calibration eqn	Abs = 24.70708*Conc +0.00054
Correlation Coefficient	0.99757

Calibration time 8/2/2018 4:15:42 PM

Analysis

Collection time 8/2/2018 4:17:09 PM

Sample	Concentration mmol/L	F	Mean	SD	%RSD	Readings
DHA		R				1.4901
		R				1.4892
		R				1.4909
	0.0603		1.4901	0.00085	0.05708	
Palmitoleic Acid		R				0.5528
		R				0.5527
		R				0.5526
	0.0223		0.5527	0.00010	0.01809	
Cholesterol		R				2.3579
		R				2.3618
		R				2.3704
	0.0956		2.3634	0.00640	0.27061	
Oleic Acid		R				0.8841
		R				0.8830
		R				0.8835
	0.0357		0.8835	0.00055	0.06234	
a Linolenic Acid		R				0.7039
		R				0.7037
		R				0.7040
	0.0285		0.7039	0.00015	0.02170	
NaCo3		R				2.3802
		R				2.3778
		R				2.3685
	0.0961		2.3755	0.00618	0.26015	

Results Flags Legend

U = Uncalibrated
N = Not used in calibration
O = Overrange
R = Repeat reading

APPENDIX C

SEQUENCING DATA OF ROGFP CONSTRUCTS

Highlighted N reads mean the software could not identify the base pair. This is normal at the beginning and end of the sequence, but large sections of N reads inside the sequence indicates abnormal variability of sequences in that area. Potentially problematic areas are highlighted

Grx1-roGFP2 1135bp

fwd
NNNNCNANATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATT
GATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTCCGC
GGCCGCCCCCTTCACCATGGCTCAAGAGTTTGTGAACTGCAAAATCCAGCCT
GGGAAGGTGGTTGTGTTCATCAAGCCCACCTGCCCGTACTGCAGGAGGGCCC
AAGAGATCCTCAGTCAATTGCCCATCAAACAAGGGCTTCTGGAATTTGTCGA
TATCACAGCCACCAACCACACTAACGAGATTCAAGATTATTTGCAACAGCTC
ACGGGAGCAAGAACGGTGCCTCGAGTCTTTATTGGTAAAGATTGTATAGGCG
GATGCAGTGATCTAGTCTCTTTGCAACAGAGTGGGGAACTGCTGACGCGGCT
AAAGCAGATTGGAGCTCTGCAGACTAGTGGTGGTTCAGGTGGTGGTGGTTCA
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GGAGGATCAGGAGGAGAATTCGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
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AGTTCATCTCCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACC
ACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGC
AGCACGACTTCTTCAAGTCCGCCATGCCCGAANGCTACGTCCAGGAGCGCAC
CATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTC
GAGGGCGACACCCTGGNGAACCGCATCGAGCTGNNNNCATCGACTTCANNA
GGACGGCACATCCTNGGCACAAGCTGGAGTACACTACACTGCNACACNNCTA
TATCATNNNNNAN

rev

NNNNNNNNNNTAGGGGNNATCAGCNGGNNNNCAAATAATAGGATTTTTATT
TTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATAAGCAATGCTTTCTTA
TAATGCCAACTTTGTACAAGAAAGCTGGGTCGGCGCGCCCACCCTTTACTTG
TACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCA
GGACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGAGCAGGT
GCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTG
TTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCG
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CGTTGTGGCAGTTGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCC
TCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTC
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TTCAGGGTCAGCTTGCCGTANGTGGCATCGCCCTCGCCCTCGCCGGACACGCT
GAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACC
CCGGTGAACAGCTCCTCGCCCTTGCTCACGAATTCTCCTCCTGATCCTCCTCC
TCCTGATCCTCCCTCCTCCTGATCCTCCTCCACCTGAACCACCANCCACCTGA
ACCACCACCACCNNAACNCCACTANTCTGCAGAGCTNCGATCTGCTTTANCN
NCNN

mito Grx1-roGFP2 1349 bp

fwd

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CTCGCCTGGCCTCCCAGATGGCTGCTTCCGCCAAGGTTGCCCGCCCTGCTGTC
CGCGTTGCTCAGGTCAGCAAGCGCACCATCCAGACTGGCTCCCCCCTCCAGA
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GCAACAGCTCACGGGAGCAAGAACGGTGCCTCGAGTCTTTATTGGTAAAGAT
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TGGTGGTTCAGGTGGTGGTGGTTCAGGTGGAGGAGGATCAGGAGGAGGAGG
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GTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGC
CACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAG
CTGACCCTGAAGTTCATCTCCACCACCGGCAAGCTGCCCGTGCCCTGGNCCA
CCCTCGTGACCACCCTGACCNACGGCGTGCANNGCTTTCAGCCGCTACCCCG
ACNCATNANCN

rev

NNNNNNNNNTATAGGGGNNATCNGCTGGANGGCAAATAATGNTTTTTATTTT
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AGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGA
CCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGAGCAGGTGCTC
AGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCT
GCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGAT
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TGTGGCAGTTGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCC
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GCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTGCTT
CATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCAGGGTGGTC
ACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGGAGATGAACTTC
AGGGTCAGCTTGCCGTANGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGA
ACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCC
GGTGAACAGCTCCTCGCCCTTGCTCACGAATTCTCCTCCTGATCCTCCTCCTC
CTGATCCTTCCCTCCTCCNGATCCTCCTCCACCTGAANCACCACCACCTGAAC
CACCACCACNTGAACCACNCTAGTCNGCAGAGCTNCAATCNGCTTTANNNCG
NCAG

roGFP2-Orp1 1322 bp

fwd

NNNNNNCANATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAAT
TGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTCCG
CGGCCGCCCCCTTCACCATGGCCTCCACTCGTGTCCTCGCCTCTCGCCTGGCC
TCCCAGATGGCTGCTTCCGCCAAGGTTGCCCGCCCTGCTGTCCGCGTTGCTCA
GGTCAGCAAGCGCACCATCCAGACTGGCTCCCCCCTCCAGACCCTCAAGCGC
ACCCAGATGACCTCCATCGTCAACGCCACCACCCGCCAGGCTTTCCAGAAGC
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GTGTCCGGCGAGGGCGAGGGCGATGTCACCTACGGCAAGCTGACCCTGAAGT
TCATCTCCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACC
CTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGC
ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCAT
CTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAG
GGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAG
GACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACTGCCACAAC
GTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGA
TCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCA
GAACACCCCCATCGGCGACGGCCCCGNGCTGCTGCCCGANANCACTACCTGA
GCACCTGCTCCGCCCNGANCAAAGACCCCNNGAGAAGCGCGATCACATNNN
CNNNNGGANN

rev

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mito roGFP2-Orp1 1500bp

fwd

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Rev

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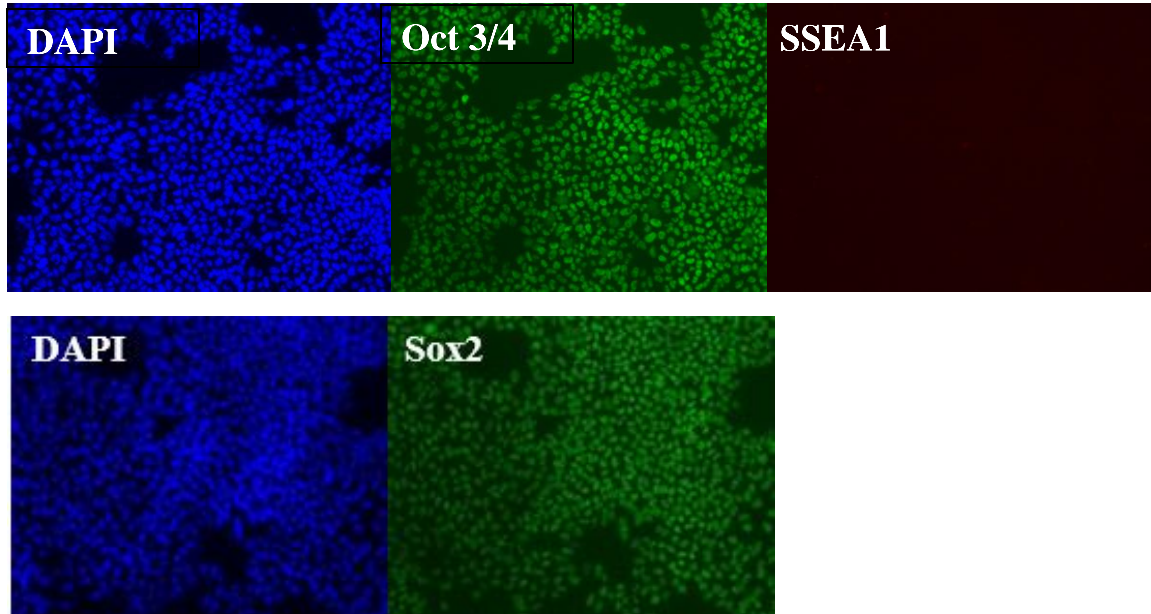
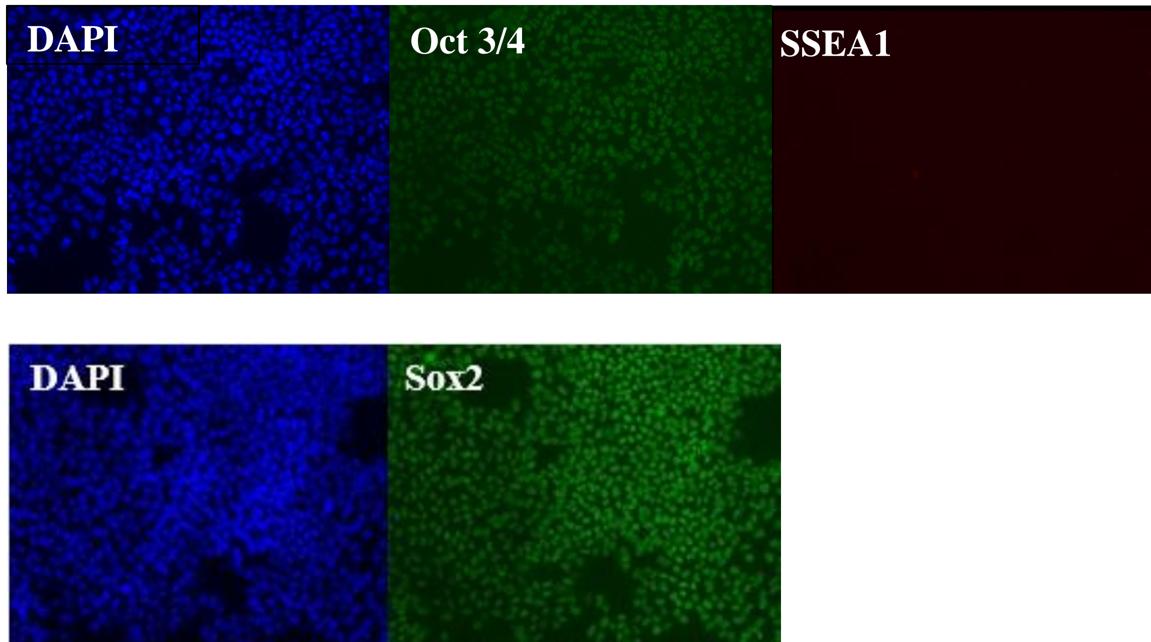
APPENDIX D

FLUORESCENT ANALYSIS OF PLURIPOTENCY MARKER EXPRESSION

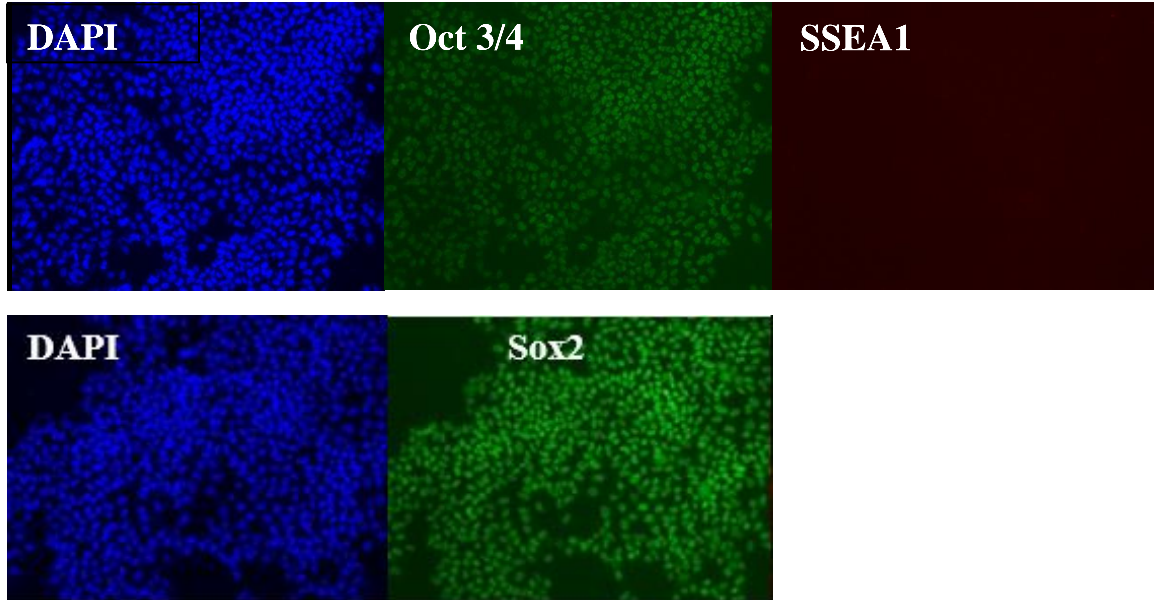
10 µM Palmitoleic Acid



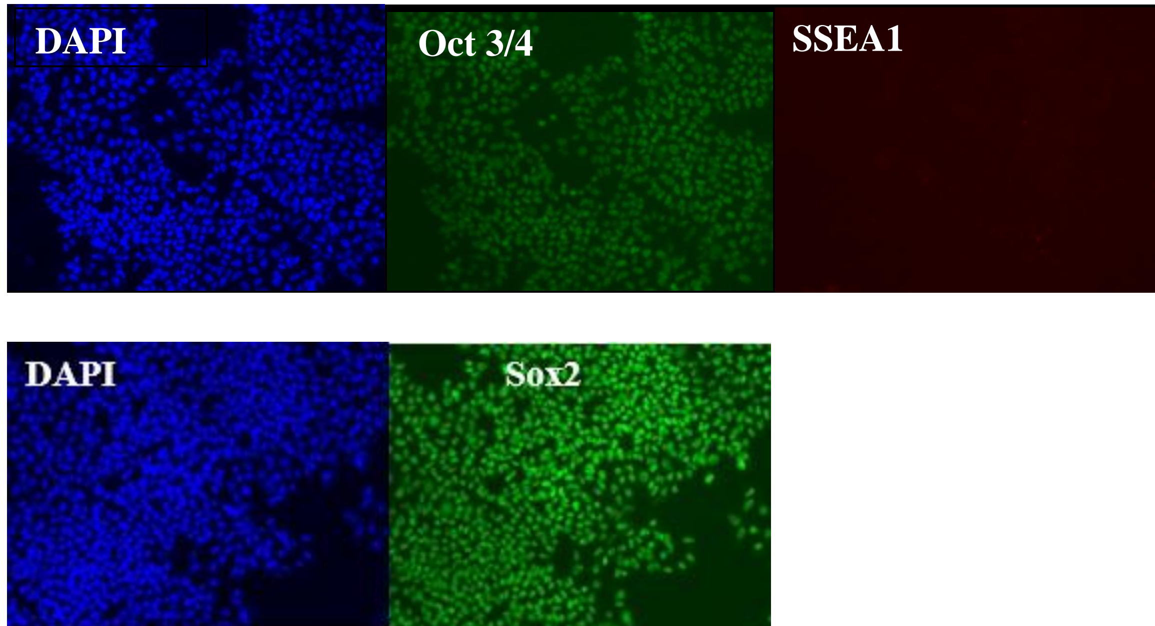
1 µM Ascorbyl Palmitate



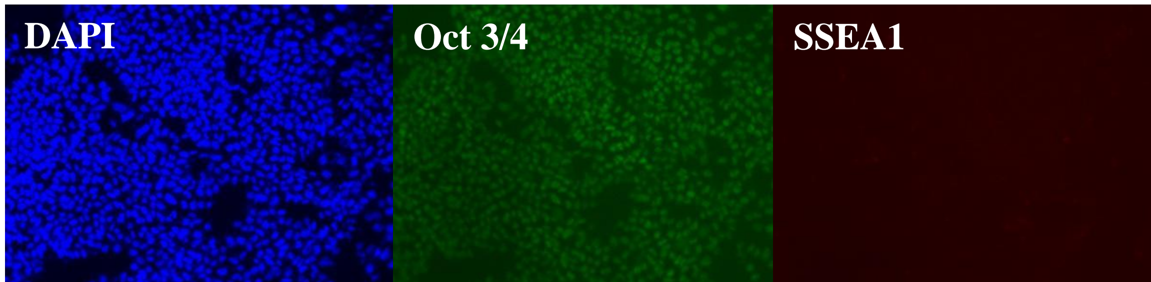
100nM Cholesterol Sulfate



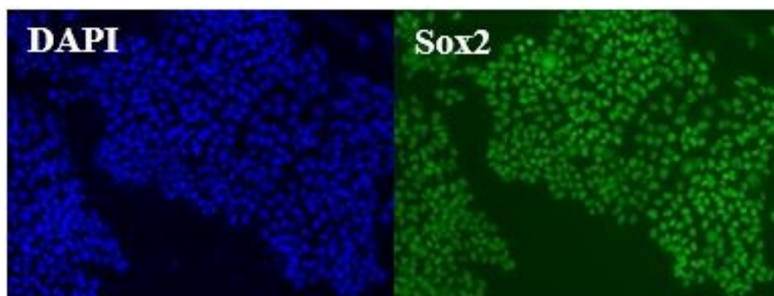
10nM DHA



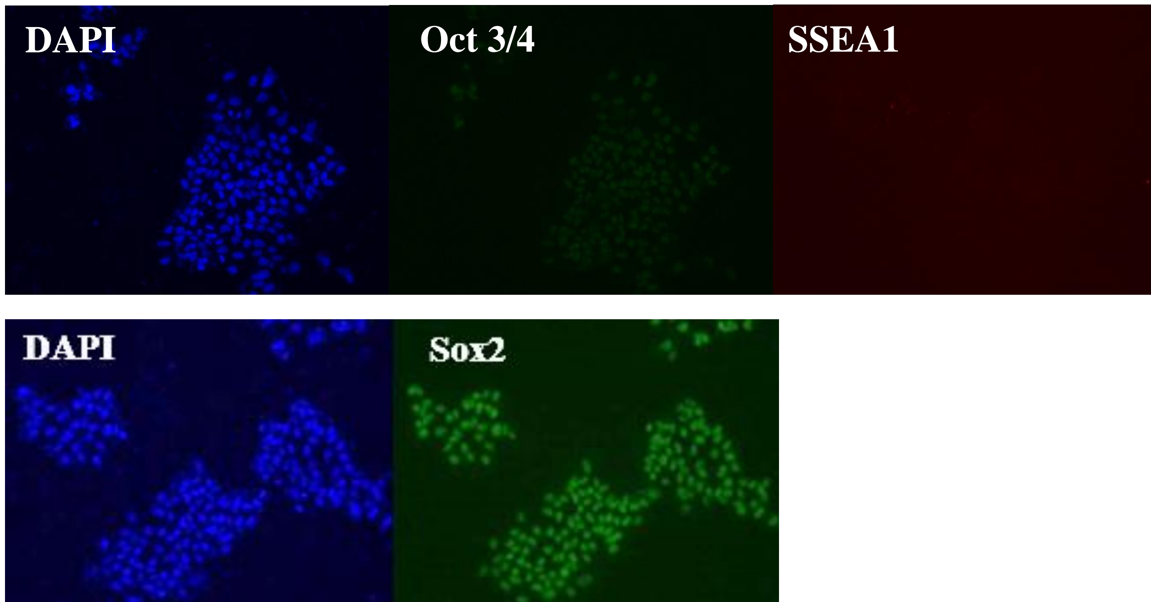
1μM Oleic Acid



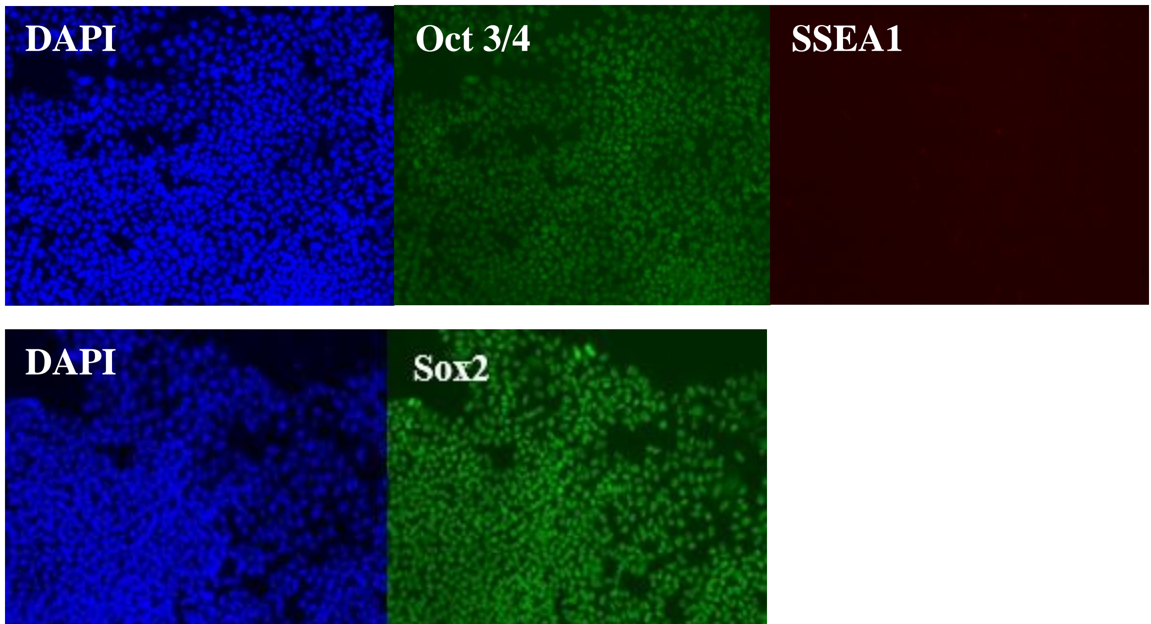
1mM Ascorbic Acid



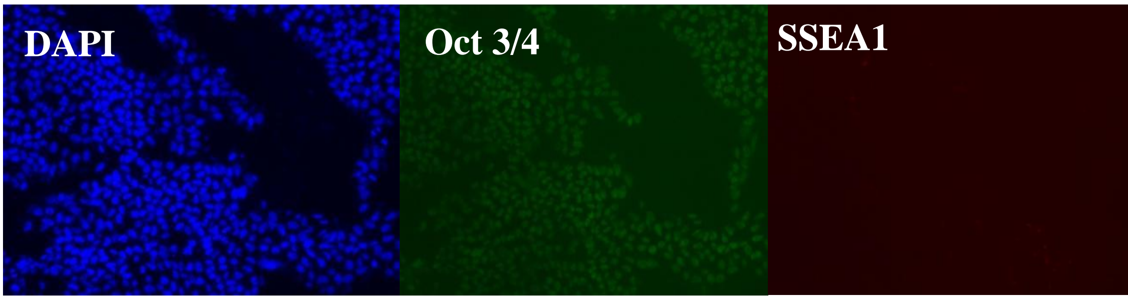
Control (E8 alone)

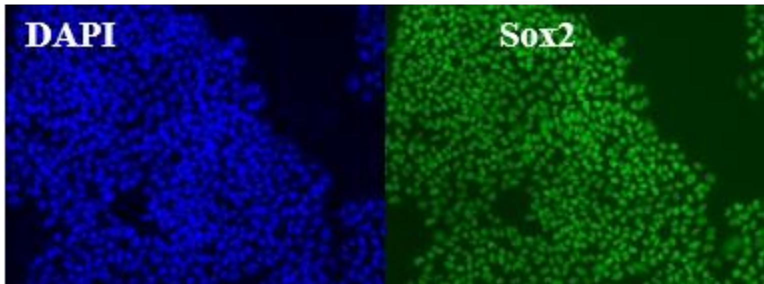


Cy2 Mix 1



Cy2 Mix 2





Cy2 Mix 3