Partial Chemical Reprogramming for Induced Cellular Rejuvenation

by

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Koç University

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ABSTRACT

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Aging is caused in part by epigenetic dysregulation, making cellular reprogramming a promising strategy to restore youthful function. Full induction of pluripotency resets cellular age but erases somatic identity, limiting therapeutic use. Partial reprogramming offers a compromise by transiently engaging reprogramming pathways to reverse hallmarks of aging while maintaining cellular identity. Chemical reprogramming has emerged as an alternative approach, using small molecules to remodel signaling and chromatin states in a more controllable and homogeneous manner than transcription factor induction.

Here we compare genetic and chemical reprogramming modalities for cellular rejuvenation and show that chemical cocktails can reproduce and, in some respects, surpass genetic partial reprogramming, reducing senescence and improving mitochondrial performance while avoiding induction of pluripotency. Chemical reprogramming led to a homogeneous early change in identity and pluripotency markers, while factor induction produced mixed subpopulations and prolonged treatment increased stress. Screening by systematic removal of cocktail components identified MLL-menin inhibition and chromatin regulators such as p300/CBP as necessary for senescence suppression. By contrast, RAR signaling was indispensable. Its removal not only elevated senescence but also diminished identity markers. These results establish chemical partial reprogramming as a promising alternative strategy for epigenetic rejuvenation. They highlight the balance between senescence reduction and lineage stability and set the stage for protocols that may translate into safer, tunable therapeutic approaches.

ÖZETÇE

Hücresel Gençleşme için Kısmi Kimyasal Yeniden Programlama

Ömer Can Ergül

Hücresel ve Moleküler Tıp, Yüksek Lisans

25 Ağustos, 2025

Yaşlanma kısmen epigenetik düzensizliklerden kaynaklanmakta ve bu durum hücresel yeniden programlamayı gençlik işlevlerinin geri kazandırılması için etkili bir strateji haline getirmektedir. Pluripotensinin tam indüksiyonu hücrelerin biyolojik yaşını sıfırlayabilse de somatik kimliği ortadan kaldırarak terapötik kullanımını sınırlandırmaktadır. Kısmi yeniden programlama, yeniden programlama yollarını geçici olarak devreye sokarak yaşlanma belirtilerini tersine çevirmeyi ve aynı anda hücresel kimliği korumayı mümkün kılan bir ara çözüm sunmaktadır. Kimyasal yeniden programlama ise küçük moleküller aracılığıyla sinyal ve kromatin düzenlemelerini transkripsiyon faktörü indüksiyonuna kıyasla daha kontrol edilebilir ve homojen bir şekilde yönlendiren alternatif bir yaklaşım olarak öne çıkmaktadır.

Bu çalışmada, hücresel gençleşme amacıyla genetik ve kimyasal yeniden programlama yöntemlerini karşılaştırdık ve kimyasal kokteyllerin genetik kısmi yeniden programlamayı yeniden üretebildiğini ve bazı açılardan aşabildiğini gösterdik. Bu etkiler arasında senesensin azaltılması ve mitokondriyal fonksiyonların iyileştirilmesi bulunurken, pluripotensi indüksiyonu gözlenmemiştir. Kimyasal yeniden programlama, hücresel kimlik ve pluripotensi belirteçlerinde homojen bir değişim oluştururken faktör indüksiyonu ise aynı pencerede heterojen alt popülasyonlar üretmiş ve daha uzun uygulamalarda stres artışı gözlemlenmiştir. Kokteyl bileşenlerinin sistematik olarak çıkarılmasıyla yapılan tarama, senesensin baskılanması için MLL–menin inhibisyonunun ve p300/CBP gibi kromatin düzenleyicilerinin gerekli olduğunu ortaya koymuştur. Buna karşılık RAR sinyallemesi vazgeçilmez bulunmuştur; bu yolun devre dışı bırakılması senesensi artırmış ve hücresel kimlik göstergelerini zayıflatmıştır. Bu bulgular, kimyasal kısmi yeniden programlamayı epigenetik gençleşme için güçlü bir alternatif olarak tanımlamakta, senesens kontrolü ile soy stabilitesi arasındaki dengeyi vurgulamakta ve daha güvenli, ayarlanabilir terapötik yaklaşımların geliştirilmesine temel oluşturmaktadır.

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ABBREVIATIONS

AAV	Adeno-associated virus
ATAC-seq	Assay for Transposase-Accessible Chromatin sequencing
ATF3	Activating Transcription Factor 3
bFGF	basic Fibroblast Growth Factor
cAMP	cyclic Adenosine Monophosphate
CBP	CREB-binding protein
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)
cDNA	complementary DNA
CiPSC	Chemically induced pluripotent stem cell
CpG	Cytosine-phosphate-Guanine dinucleotide
CR	Chemical reprogramming
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleoside triphosphates
DOT1L	Disruptor of telomeric silencing 1-like (H3K79 methyltransferase)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESCs	Embryonic stem cells
EZH2	Enhancer of Zeste Homolog 2
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GADD45A	Growth arrest and DNA damage-inducible alpha
HBSS	Hank's Balanced Salt Solution
HDAC	Histone deacetylase
HEK293T	Human embryonic kidney 293T cells
HGPS	Hutchinson-Gilford Progeria Syndrome
HP1	Heterochromatin protein 1
hTERT	Human telomerase reverse transcriptase
ICE	Inducible changes to the epigenome
iPSC	Induced pluripotent stem cell
ISG15	Interferon-stimulated gene 15
JNK	c-Jun N-terminal kinase
KSR	Kinase suppressor of Ras
LINE-1	Long interspersed nuclear element-1
LSD1	Lysine-specific demethylase 1
MDM2	Mouse double minute 2 homolog
MET	Mesenchymal-to-epithelial transition
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEI	Polyethylenimine
PUMA	p53 upregulated modulator of apoptosis
PSCs	Pluripotent stem cells
qPCR	Quantitative PCR
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RNase	Ribonuclease

ROCK	Rho-associated kinase
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SCNT	Somatic cell nuclear transfer
SESN1	Sestrin 1
SIRT6	Sirtuin 6
WT	Wild type
XEN	Extraembryonic endoderm cells

CHAPTER 1: INTRODUCTION

1.1 Biology of Aging

1.1.1 Defining biological aging

Biological aging can be defined as the progressive, time-dependent deterioration of an organism over time which leads to an increased susceptibility to death and disease. Unlike chronological age, biological age refers to the functional capacity of an organism. Biological age increases with decreased function which is generally caused by the accumulation of deleterious or deregulatory changes. In humans, advancing age is the strongest risk factor for cancer, cardiovascular disease, type II diabetes, and neurodegenerative disorders, all of which occur more often with age.

Chronological time measures years lived, but biological aging reflects the accumulation of molecular and cellular injury that increasingly exceeds the body's capacity to repair. Errors in DNA replication, metabolic byproducts, and external stress contribute to this damage. Aging does not progress uniformly across systems. Some tissues decline faster than others, and people vary in how quickly their bodies weaken. At the whole-organism level, the outcome is reduced resilience and impaired balance between stress and repair. Visible features include gray hair, loss of skin elasticity, reduced strength, slower healing, and a weaker immune system.

Classic definitions emphasize that aging is characterized by a "progressive loss of physiological integrity, leading to impaired function and increased vulnerability to death." (López-Otín et al., 2013). While chronological age cannot be altered, biological aging may be modifiable. That possibility has prompted interest in interventions aimed at slowing or reversing aspects of decline.

1.1.2 The hallmarks of aging

The hallmarks of aging were introduced as a framework to describe the causes and results of the aging process. These processes were divided into nine hallmark features that fulfill the criteria of (1) manifesting during aging, (2) its exacerbation accelerated aging and (3) amelioration slows aging. The original hallmarks consisted of genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intracellular communication. All together they represent the common features

of aging across organisms. Many of these hallmarks are interconnected and can be caused by or lead to other hallmarks. For example, DNA damage (genomic instability) can lead to cellular senescence, and these cells can then secrete pro inflammatory cytokines (altering intracellular communication). Building on this framework an updated consensus expanded the list to twelve hallmarks to include disabled macroautophagy, chronic inflammation, and dysbiosis (López-Otín et al., 2013, 2023).

These hallmarks are classified into three groups: (1) primary hallmarks, (2) antagonistic hallmarks and (3) integrative hallmarks. Primary hallmarks are characterized by being unequivocally negative. They typically lead to loss of biological information which leads to dysfunction and other hallmarks. Antagonistic hallmarks, in contrast have beneficial effect and can be detrimental depending on their intensity. Often at low levels they beget positive effects but when increased can become negative. Integrative hallmarks affect more complex high-level systems such as tissue homeostasis and arise as the consequence of primary and antagonistic hallmarks. Throughout the process of aging, damage involving the systems of the primary hallmarks is caused by regular biological processes and/or environmental triggers. Over time this damage accumulates and then can lead to the disruption of beneficial systems and lead to the antagonistic hallmarks. Finally when the cellular damage sustained by the primary and antagonistic hallmarks pass a certain threshold it begins to affect tissue level mechanisms and lead to the integrative hallmarks (López-Otín et al., 2013).

The hallmarks of aging are, currently the best attempt at providing a comprehensive definition for biological aging. The structured framework they provide links molecular damage to functional decline which can be used for quantifying and targeting the aging process.

1.1.3 Quantifying aging

A crucial challenge in aging research is how to measure aging. Chronological age alone is an imperfect predictor of health. Individuals of the same age can exhibit drastically different functional capacities and disease risks. Therefore, development of “biological age” metrics that better capture the “true” age of an organism or the amount of aging its experienced is of utmost importance to more effectively intervene with age related decline. One of the first formal attempts to predict lifespan was made by Benjamin Gompertz in 1825. From mortality records he created a model that showed that the risk

of death doubles every ~8 years after adulthood (Gompertz, 1825). This model, known as Gompertz law remains as a population level aging model that allows inferences to be made about likely remaining lifespan of an individual (Kirkwood, 2015). However, to target and treat aging individual assessment and more granular biomarkers are needed.

Studies have revealed multiple biomarkers of aging that correlate with functional decline. The biomarkers separate into two general categories: physiological and cellular. Because aging is fundamentally a multicellular process, its effects are often more evident at the systemic level making them easier to quantify. Changes in physiological markers such as blood pressure, cholesterol, inflammation and fitness metrics such as exercise tolerance, VO2 max lead to significant correlations with aging and thus can be used to infer biological age. In fact these markers can be combined into composite indices such as the frailty index which provide a strong correlation with mortality risk (Rockwood et al., 2017). Functional markers such as these are significantly more difficult to measure on the cellular levels especially in vivo. Instead changes in cellular markers that are correlated with the aging process are measured. Measurements targeting the individual hallmarks of aging can provide some relative quantification of aging. Genomic instability can be detected via elevated levels of γH2AX, a marker for double-strand breaks and genomic instability (Rogakou et al., 1998). Among the ways epigenetic alterations manifest are as heterochromatin erosion and global DNA hypomethylation (K. Wang et al., 2022). Loss of proteostasis leads to an increased number of protein aggregates and misfolded proteins (Hipp et al., 2019). Telomere attrition leads to telomere shortening which can be measured through telomere shortening assays (Rossiello et al., 2022). Cellular senescence increases to detrimental levels with age, quantified through senescence associated B-galactosidase (SA-β-gal) assays and assays involving cell cycle inhibitors (Di Micco et al., 2021). Mitochondrial dysfunction increases and leads to a measurable decrease in membrane potential and an increase in elevated reactive oxygen species (ROS) (Sun et al., 2016). All together these markers change with age and while none are individually definitive their combined assessment can provide insight into the relative “age” of a cell and can help interpret differences between samples, perturbations or interventions.

Aging clocks

Aging clocks have emerged as a tool to glean information about biological age through complex omics data. Training machine learning algorithms with data involving

the omics data of interest with an age reference allows them to extrapolate patterns and connections within the omics data which can then be used to quantify the age of cells/organisms through the same omics data alone. The most common age reference is chronological age however there are newer generation clocks that use metrics such as time to mortality, disease onset or phenotypic measures (Rutledge et al., 2022). The first such clock was a linear regression model trained on methylation levels at 88 age-associated CpG sites from saliva DNA, capable of predicting chronological age within ~5 years (Bocklandt et al., 2011). Later the concept of DNA methylation clocks were expanded to a multi-tissue, pan-age model that was applicable to both organismal and cellular aging establishing it as an applicable biomarker for aging. This clock was trained using 8000 samples from various DNA methylation datasets and selected 353 CpG sites from a potential 21,369 CpGs that had either a positive or negative correlation with age. When tested the clock showed a correlation of 0.96 with chronological age and a median absolute error of 3.6 years across various tissues. It detected an acceleration in aging in cancer datasets, averaging 36 years above expected chronological age (Horvath, 2013). DNA methylation data was a starting point for extrapolating aging information from omics data this the approach was later expanded to include transcriptomics (Peters et al., 2015; Fleischer et al., 2018), proteomics (Menni et al., 2015; Tanaka et al., 2018), metabolomics , and other types of large data like, glycomics (Krištić et al., 2014), microbiome composition (Galkin et al., 2020), and chromatin states (Morandini et al., 2024; de Lima Camillo et al., 2025).

Despite their utility there are certain limitations and shortcomings these clocks face. One major concern is that these clocks tend to be highly correlated with chronological age rather than attempting to capture the complexity of biological age. For clocks trained using chronological age, there is an implicit assumption that the individuals in the training set had a near one-to-one correspondence between chronological and biological age which is unlikely. Other common criticisms include the variability these clocks can observe across populations tissues and experimental conditions. For example a study found that 13 of 17 tested aging clocks exhibited significant oscillations in predicted age according to the time of day from the same samples (Koncevičius et al., 2024). While aging clocks are certainly able to capture some aspects of aging, for them to be considered definitive markers will require more testing and functional validations.

1.1.4 Accelerated aging models

Studying aging poses significant challenges, especially when attempting to connect physiological phenotypes to *in vitro* findings. Cellular models can lose, or attenuate age-related features once removed from their native tissue environment. Conversely conducting in vivo studies are often long, resource intensive and not always feasible. Accelerated aging models help address these limitations now by compressing aging-like changes into a shorter timeframe which allows for more rapid investigation of underlying mechanisms as well as experimental interventions. These models include naturally occurring human progeroid syndromes, caused by mutations in genes affecting processes such as DNA repair or nuclear lamina integrity, along with engineered animal strains and cell lines that either mimic these syndromes or produce entirely novel aging phenotypes not seen in humans. While valuable, such models generally recapitulate only certain aspects of aging, making them segmental rather than complete representations of the aging process (S. J. Mitchell et al., 2015).

Hutchinson-Gilford Progeria Syndrome

HGPS is commonly caused by a mutation in the LMNA gene (c.1824 C>T) that leads to a splicing mutant that leads to a truncated form called progerin. Progerin disrupts the structure of the nuclear lamina and induces a DNA damage response and epigenetic dysregulation (Batista et al., 2023). Physiologically patients with HGPS develop normally as infant but develop age-related symptoms such as alopecia, growth retardation, osteoporosis, atherosclerosis, and cardiovascular problems. The expected lifespan for a HGPS patient is 13-15 years (Merideth et al., 2008). HGPS also mirrors the cellular hallmarks of aging such as, elevated DNA damage, loss of heterochromatin and high levels of senescence providing a bridge between *in vitro* experiments and established in vivo aging data (Batista et al., 2023).

Engineered accelerated aging models

In addition to natural progeroid syndromes, engineered models exploit specific hallmarks of aging to induce a premature aging phenotype. Through genetic modifications that either mimic naturally occurring progeroid syndromes or inducing novel aging phenotypes not found in humans they can recapitulate some aspects of aging. Examples include telomere-deficient mice (Rudolph et al., 1999), mitochondrial DNA polymerase PolgA mutant mice (Trifunovic et al., 2004), and SIRT6 knockout mice

(Mostoslavsky et al., 2006). Another example is the inducible changes to the epigenome (ICE) mouse, which expresses I-PpoI endonuclease to make controlled double-strand breaks without mutagenesis that disrupt epigenetic integrity. These mice develop progressive aging phenotypes such as reduced tissue regeneration, cognitive decline, muscle weakness, and metabolic dysfunction (Yang et al., 2024). Together, such engineered systems serve as potential models for research while also elucidating the role of proteins and processes in aging.

1.2 Cellular Reprogramming

1.2.1 Cell fate determination in development

Cell types differ because of their epigenetic states, not their DNA sequence. The zygote divides to produce many cells that progressively diverge in potential and function. As it does so, cell potency narrows in a defined sequence. The zygote itself is totipotent and can generate an entire organism. It divides and specifies while doing so to produce pluripotent cells that can form all embryonic lineages, which then divide and specify to form multipotent cells capable of generating several related types. Finally, multipotent cells give rise to unipotent cells that produce one type, which they maintain for the rest of their existence. This restriction occurs through controlled changes in gene regulation rather than changes to the genome. These controlled changes, termed differentiation, lead to the ordered specification of cell identity (De Los Angeles et al., 2015).

Extrinsic cues and lineage-defining transcription factors activate tissue-specific programs while repressing alternatives. Stable patterns of DNA methylation, histone modification, chromatin accessibility, and three-dimensional genome architecture lock in these programs and are propagated through cell divisions. As a result, commitment is robust and largely one-way, preserving identity in tissues over time.

Waddington introduced “epigenetics” to describe how developmental processes link genotype to phenotype. He did so through the epigenetic landscape: a ball representing an undifferentiated cell at the top of a hill. As the ball rolls down the hill, it divides and enters branching valleys that deepen as specification proceeds. Eventually, the ball settles at the bottom in a valley that represents a stable cell identity (Waddington, 1942).

1.2.2 Somatic cell nuclear transfer and induced pluripotency

Differentiation brings with it a set of epigenetic changes, chromatin remodeling, DNA methylation, and transcription factor circuits that reinforce identity. These marks deepen the valleys and build barriers between them. Throughout much of the last century, differentiation was considered one-way. A cell that had reached maturity was thought unable to reverse direction.

That view shifted in 1962. John Gurdon demonstrated that nuclei from intestinal cells of tadpoles could direct development when placed into enucleated frog eggs. The host eggs produced normal tadpoles, showing that differentiation did not erase the genetic program (Gurdon, 1962). This work marked the birth of somatic cell nuclear transfer (SCNT). The concept was later proven in mammals. In 1997, Dolly the sheep was born after an adult mammary cell nucleus was introduced into an oocyte (Wilmut et al., 1997). SCNT has since been used across species and in humans to generate embryonic stem cells from patient nuclei. These studies demonstrated that cell identity rests on reversible epigenetic states rather than fixed genetic alterations. In SCNT, oocyte cytoplasm provides the reprogramming environment. DNA methylation is erased, paralleling events in fertilization, and embryonic transcription resumes as somatic programs are silenced.

A more accessible approach appeared in 2006. Shinya Yamanaka identified four transcription factors, OCT4, SOX2, KLF4, and c-MYC through screening of the factors present in the oocyte cytoplasm that were able to reprogram somatic nuclei. These factors, he called the Yamanaka factors could reprogram fibroblasts into induced pluripotent stem (iPS) cells. These iPSCs displayed properties of embryonic stem cells, including the ability to self-renew and form derivatives of all germ layers (Takahashi & Yamanaka, 2006). Induced pluripotency proceeds in stages. Initiation involves pioneer factor binding, mesenchymal-to-epithelial transition, and metabolic reconfiguration. Maturation brings partial activation of endogenous pluripotency genes, though exogenous factors remain required. Stabilization follows, with silencing of transgenes and establishment of self-sustaining networks. At that point, the cells meet functional definitions of pluripotency (David & Polo, 2014).

SCNT resets nuclei in a single step through oocyte cytoplasm, while iPSC induction progresses through extended factor expression. Both methods prove that differentiation is not final but epigenetically maintained. Reprogramming technologies

have practical impact. Patient-specific stem cells can be generated by nuclear transfer or factor-based induction. They offer models for disease study, drug testing, and possible sources for transplantation. This development established a foundation for systematic efforts to optimize reprogramming efficiency and extend applicability across diverse cell types

1.2.3 Barriers of cellular reprogramming

Converting a differentiated cell into a pluripotent or another fate is like trying to push it uphill along Waddington's landscape. Naturally, there are many barriers that resist this change. Cells invest in maintaining their identity via robust regulatory circuits and epigenetic locking, which serve as barriers to arbitrary conversion. Understanding these barriers is important both for improving reprogramming efficiency and for controlling partial reprogramming safely (Arabacı et al., 2021).

One major barrier is the chromatin state. Differentiated cells have silenced developmental genes through DNA methylation and histone modifications. During reprogramming, the OSKM factors must bind to target sites on DNA, but in somatic cells many of those target sites are embedded in heterochromatic regions. OCT4, SOX2, and KLF4 are pioneer factors that can engage nucleosomal DNA even in closed chromatin. Yet many OSKM target sites remain inaccessible until additional chromatin remodeling occurs. Early in reprogramming, a subset of cells show partial opening at pluripotency loci, allowing downstream factors to bind (Papp & Plath, 2013). Use of compounds like valproic acid, an HDAC inhibitor can enhance reprogramming efficiency by making chromatin more acetylated and accessible (Huangfu et al., 2008). Additionally, certain histone marks impede reprogramming, e.g. high levels of H3K9me3 in cells are associated with poor reprogramming. In fact, overexpression or chemical inhibition of H3K9 methyltransferases improves iPSC formation by removing that repressive barrier (Wei et al., 2017).

Another barrier is the need for a mesenchymal-to-epithelial transition (MET). Fibroblasts must become more epithelial-like early in reprogramming. This involves upregulating E-cadherin, downregulating snail, etc. If a cell cannot undergo MET, it stalls. TGF-β signaling maintains mesenchymal traits, so adding a TGF-β inhibitor (e.g. RepSox) to induce MET is known to boost reprogramming (Pei et al., 2019). The timing of this transition is critical; it typically occurs in the first week of reprogramming. So, the

need for MET acts as a barrier; only cells that successfully execute MET proceed to full iPSCs. A further barrier is senescence and DNA damage responses. Some cells, when pushed to express OSKM, experience proliferation stress or DNA replication stress and activate p53/p21-mediated senescence or apoptosis. It's known that deleting p53 greatly increases reprogramming efficiency, because p53 normally halts cells with DNA damage or oncogenic stress (Hong et al., 2009, p. 21). In reprogramming experiments, especially with older cells, a large fraction of starting cells might become senescent rather than pluripotent. Those senescent cells express p16^INK4a and other cyclin inhibitors which strongly suppress the cell cycling needed for reprogramming (Liu et al., 2019). Therefore, reprogramming favors cells that can evade senescence barriers. Indeed, cells with longer telomeres or higher endogenous resilience tend to reprogram better. Telomere length itself can be a barrier. During reprogramming telomerase is reactivated and telomeres are elongated, but if a cell's telomeres are critically short at the outset, it may senesce before completing reprogramming. Overexpression of telomerase (hTERT) can improve reprogramming of difficult cells, indicating telomere rejuvenation is one necessary step on the path (Ma et al., 2024).

Metabolic state constitutes another barrier: Differentiated somatic cells rely primarily on oxidative phosphorylation, whereas pluripotent stem cells use glycolysis. Reprogramming entails a metabolic shift to glycolysis, which is necessary for supporting the biosynthetic and epigenetic demands of the pluripotent state. If cells cannot adjust their metabolism they may fail to reprogram (Wu et al., 2016).

Cell cycle speed is another consideration. Faster cycling cells reprogram more readily, in part because each cell division provides opportunities to remodel chromatin and dilute out old epigenetic marks (Liu et al., 2019). For example, forcing cells into a quiescent state is detrimental to reprogramming. Conversely, providing growth factors or pathways that encourage cell proliferation helps overcome barriers. In fact one the Yamanaka factors, c-MYC is used for this very reason even though it acts independently of the other factors and isn't required for reprogramming (Singh & Dalton, 2009).

Cell identity itself provides resistance; some cell types are inherently easier to reprogram than others. Blood cells (B-cells) need additional factors or transformation to iPSCs, possibly because they have a very specialized chromatin and require turning off lineage-specifying transcription factors (Staerk et al., 2010). By contrast, adult stem or

progenitor cells are often easier to reprogram than fully mature cells. For example, neural stem cells can be reprogrammed with only OCT4. This implies that the further a cell is along the differentiation path, the more barriers it has accumulated (Kim et al., 2009). Hence, different cell types present different barriers; understanding those can guide factor selection.

In in vivo reprogramming, additional barriers come into play. The immune system can recognize and eliminate partially reprogrammed cells expressing embryonic antigens, and the microenvironment might not support cells that detach from their niche. Moreover, the body's tumor suppression mechanisms serve as a barrier, this is beneficial, as they prevent OSKM-expressing cells from forming teratomas in partial reprogramming protocols, but it also means fully "resetting" cells in vivo is strongly selected against (Melendez et al., 2022).

Finally, epigenetic memory can be a subtle barrier. Reprogrammed cells can retain residual DNA methylation marks from their cell of origin, which might bias them or require further passages to erase. For example, iPSCs derived from blood cells can carry methylation memory of blood-specific genes. Additional culture or specific factors may be needed to purge that memory completely (Scesa et al., 2021).

In conclusion, cellular reprogramming faces multiple layers of defense: epigenetic locking, cell cycle and metabolic requirements, stress responses, and the need to break out of a stable regulatory network. The very existence of these barriers indicates how fundamentally cells "want" to maintain their identity, which is normally good for organismal integrity. In the context of aging, these barriers also mean that attempting to rejuvenate cells by partial reprogramming requires an intricate balance. Enough perturbation to roll back epigenetic marks of aging, but not so much that cells lose their identity or activate powerful tumor suppressor responses. Researchers have identified exploitable barriers: adding chromatin-modifying chemicals, inhibiting p53, optimizing factor delivery and timing. Many of these barriers, once identified can be targeted by small molecules to not only streamline iPSC generation but even replace the Yamanaka factors altogether.

1.3 Chemical Reprogramming

Intrinsic barriers that lock a somatic cell into its identity can be exploited to change the epigenetic state of the cell. Chemical reprogramming is a strategy that targets those

barriers with small molecules, driving cells into a highly plastic, dedifferentiated state from which a new cell identity can be established. In contrast to transcription-factor-based methods where several lineage-specifying factors are introduced, chemical reprogramming uses combinations of compounds to modulate key signaling pathways and epigenetic regulators that maintain the somatic state.

1.3.1 Comparison of genetic and chemical reprogramming

Chemicals offer several advantages over genetic approaches for epigenetic manipulation. One advantage is tunability and reversibility. Small molecules act in a dose and time-dependent manner, allowing their effects to be adjusted by changing concentration or exposure duration. This makes it possible refine protocols by testing different combinations or dosages across diverse cell types and donor backgrounds. They are also able to modulate pathways transiently because they do not integrate into the genome. Chemical treatments avoid insertional mutagenesis and leave the genetic material intact. Chemicals also offer more uniform and safer application both in vivo and *in vitro*. Genetic methods rely on viral or plasmid delivery which can result in heterogeneous expression due to differences in copy number and integration sites. In contrast, small molecules are distributed through media and across tissues more equally. While doing so they commonly do not elicit an immune response.

Chemical manipulation is also more adaptable. Many of the molecules identified for pluripotency induction can be reapplied in other contexts. The same set of compounds that suppress fibroblast identity during reprogramming can be used for direct lineage conversions when combined with other chemical cocktails that signal differentiation cues. Transcription factor-based systems are more rigid. For example, OSKM is largely restricted to pluripotency induction.

Small molecules offer practical alternatives to transcription factors for epigenetic manipulation. They are inexpensive, stable, and straightforward to apply or withdraw from culture conditions. This supports scalability and facilitates translation into clinical settings. However, the benefits chemicals offer does come with certain challenges. A more precise understanding of the epigenome is required, as these methods do not simply exploit pre-existing transcriptional programs but rather rely on direct modulation of chromatin states and signaling pathways. Overall, chemicals provide a tunable, non-

integrative, and versatile alternative for cellular reprogramming and epigenetic manipulation.

1.3.2 Pathways involved in chemical reprogramming

Small molecules employed in reprogramming largely fall into two categories: epigenetic modifiers and signaling pathway modulators (J. Wang et al., 2023). The goal is to mimic the effect of introducing reprogramming transcription factors by instead altering the cell's own regulatory circuits through drugs.

One critical pathway is TGF-β signaling. TGF-β maintains cells in a mesenchymal, often more differentiated state, and inhibits the MET required for pluripotency (Katsuno & Derynck, 2021). Thus, chemical reprogramming cocktails invariably include a TGF-β pathway inhibitor. For example, RepSox (a molecule that inhibits TGF-β type I receptor) or SB431542 (another TGF-β inhibitor) are commonly used to promote MET, allowing fibroblastic cells to become epithelial-like and primed for pluripotency (Hou et al., 2013; Guan et al., 2022). In the all-chemical reprogramming of mouse cells, a TGF-β inhibitor was crucial in the early phase to initiate the transition (Hou et al., 2013).

Another target is the GSK3β kinase, which is part of the Wnt signaling pathway. Inhibition of GSK3β (e.g. CHIR99021) activates Wnt/β-catenin signaling, which is known to support pluripotency and self-renewal. CHIR99021 is present in many reprogramming and stem cell media because it helps maintain an active proliferative state and can induce expression of c-MYC target genes akin to what the Yamanaka factor c-MYC does (S. Ye et al., 2012). Moreover, GSK3 inhibition has the side effect of raising cellular β-catenin levels that can cooperate with OSK factors to activate key pluripotency genes (S. Ye et al., 2012).

Histone modifications are targeted by molecules like valproic acid (VPA), a broad HDAC inhibitor. VPA increases histone acetylation and thus relaxes chromatin globally, making it easier for other factors (endogenous or exogenous) to access gene promoters. The addition of VPA to Yamanaka factors was famously shown to boost human iPSC generation by >100-fold and being able to replacing MYC, highlighting how big a barrier chromatin compaction was (Huangfu et al., 2008). In fully chemical protocols, HDAC inhibitors or similar chromatin modulators (e.g. sodium butyrate) help erase somatic gene expression programs and reactivate silent pluripotency genes (Huangfu et al., 2008; Mali

et al., 2010). On the other side there are certain marks that must be reduced: BIX-01294 is a small molecule that inhibits G9a, a histone methyltransferase responsible for H3K9 methylation (a repressive mark). By inhibiting G9a, BIX-01294 lowers H3K9me2/3 levels and was able to replace OCT4 to reprogram cells to pluripotency (Shi et al., 2008). Another compound, 3-deazaneplanocin A (DZNep) was shown to deplete EZH2 and reduce H3K27 methylation, further breaking down epigenetic barriers (Wen et al., 2025).

DNA methylation modulators are also featured in chemical reprogramming protocols. RG108 or 5-azacytidine are DNMT inhibitors that can demethylate DNA and were historically some of the earliest tools to manipulate the epigenome. For example 5-azacytidine was used by Davis et al. in the 1980s to help convert fibroblasts to muscle by making the genome more responsive to MyoD (Davis et al., 1987). In some chemical reprogramming protocols DNMT inhibitors aid in erasing somatic methylation patterns, allowing expression of hitherto silenced pluripotency genes (Y. Wang et al., 2025).

Additional pathway targets include LSD1 (lysine-specific demethylase 1), which demethylates H3K4me1/2. Paradoxically, inhibiting LSD1 helps reprogramming. Tranylcypromine allowed reprogramming of mouse cells with a single gene, OCT4 (Li et al., 2011). LSD1 normally acts to repress gene activation by removing activating marks, so blocking it can keep promoters in a more permissive state. Tranycypromine was one of the seven chemicals that led to mouse chemically induced iPSCs and was a part of the human chemical reprogramming study as well (Guan et al., 2022; Hou et al., 2013).

cAMP signaling has also been a exploited target. Forskolin, an activator of adenylyl cyclase (raising cAMP), appears in some protocols as it can induce a more plastic state and synergize with other signals to promote cell cycling and morphologic changes (Insel & Ostrom, 2003).

Finally, chemicals who have targets that aren’t directly involved in somatic, or pluripotency related networks are used to increase reprogramming efficiency. Some examples include apoptosis and stress response inhibitors. As reprogramming is a stressful process and many cells undergo apoptosis. A small molecule Y-27632 (ROCK inhibitor) is often used when transitioning cells as it prevents dissociation-induced apoptosis (L. Zhang et al., 2011). Another stress related chemical is JNK kinase inhibitor JNK-IN-8 which is responsible for suppressing the reprogramming stress triggered

inflammation (T. Zhang et al., 2012). JNK-IN-8 was one the key chemicals to achieve human chemical reprogramming (Guan et al., 2022).

In summary, chemical cocktails work by coordinated pathway manipulation: block differentiation signals (TGF-β, GSK3), enhance pro-pluripotency signals (Wnt, cAMP), open up chromatin (HDAC, G9a inhibitors), and ensure cell survival/proliferation. Each chemical alone cannot reprogram a cell, but in combination, they push the cell through milestones similar to those achieved by OSKM factor expression (J. Wang et al., 2023). Chemical reprogramming therefore essentially reverse-engineers the cascades triggered by Yamanaka factors, demonstrating that the right extrinsic cues can converge to activate the endogenous gene regulatory network for pluripotency (Wen et al., 2025).

1.3.3 Chemical reprogramming protocols

Chemical reprogramming of murine cells

The first success of purely chemical reprogramming was achieved in mouse cells. Hou et al. reported inducing pluripotent stem cells from mouse fibroblasts using a cocktail of seven small molecules, without any transgenic factors. They called the resulting cells CiPSCs (chemically induced PSCs). The process was developed through trial and error by screening hundreds of candidates and combinations. The final cocktail, abbreviated as VC6TFZ, included: VPA (V), CHIR99021 (C), 616452 (6), Tranylcypromine (T), Forskolin (F), and DZnep (Z). The protocol consisted of three steps: an initial stage to loosen cell identity, an intermediate stage to activate the pluripotency network, and a maturation stage to stabilize the CiPSC colonies. The efficiency of reprogramming was low, 0.1-0.2% of cells became CiPSC colonies. Resulting in a lower effiency than traditional OSKM methods. Nonetheless the CiPSCs were bona fide pluripotent, expressing endogenous core pluripotency markers (OCT4, Nanog, etc.). They had demethylated pluripotency gene promoters, could differentiate into all three germ layers *in vitro*, and contributed to chimeric embryos. In other words, they were functionally similar to normal iPSCs.(Hou et al., 2013)

Key observations from the mouse chemical reprogramming: early on (within a week), the fibroblasts lost their spindle shape and underwent MET, forming tightly packed epithelial clusters, a sign of entering a more stem-like state. By 2-3 weeks, rare colonies emerged that resembled embryonic stem cell colonies. These were picked and

expanded. The process was very sensitive to timing and dosage of each component; omitting or mistiming a single molecule often aborted reprogramming, underscoring that each barrier must be lifted in the correct sequence. Interestingly, one of the compounds used was 616452 (RepSox), which inhibits TGF-βRI and was instrumental in inducing MET. Another, SP600125, a JNK pathway inhibitor, was used to suppress stress-induced apoptosis in intermediate stages (Hou et al., 2013).

Since the initial report, improvements have been made. Various chemical screens identified pathways and compounds that achieved higher efficiency or faster kinetics (Jin et al., 2023; Long et al., 2015; W. Wang et al., 2021). Additionally alternative starting cells were reprogrammed with chemical cocktails as well. Some cell types required additional chemicals whereas reprogramming was achieved with more minimal cocktails. For example, neural stem cells, being closer to pluripotency, were converted to ciPSCs with just 4-5 chemicals. They were able to omit several chemicals for reasons such as neural stem cells endogenously expressing SOX2 (J. Ye et al., 2016).

An interesting use of chemical reprogramming was to create not fully pluripotent cells but a partially reprogrammed intermediate state. Zhao et al. described inducing a stable partially reprogrammed state called XEN-like cells (extraembryonic endoderm-like) from fibroblasts using a related chemical approach (Zhao et al., 2015). These XEN-like cells were not pluripotent but were a steppingstone in the reprogramming trajectory. They report testing they were able to perform on this state allowed them to identify small molecules that increased reprogramming efficiencies 1000-fold greater than that of the previous protocol. These findings further demonstrate the precision chemical reprogramming provides for cell fate manipulation.

Overall, mouse cells provided the proof-of-principle for chemical reprogramming. The success revealed which pathways are absolutely required to be modulated. The same protocol was unable to reprogram human somatic cells. Nonetheless, it showed chemical reprogramming is possible but tends to be prolonged and stochastic as only a minority of cells manage to hit the required molecular events in sequence.

Chemical reprogramming of human cells

Translating chemical reprogramming to human cells has been more challenging. Human somatic cells are generally more resistant to reprogramming than mouse cells and

often require stronger or longer factor expression. Human cells are known to have increased epigenetic stability and reduced plasticity compared to their mouse counterparts. Adaptation of epigenetic manipulations from mice to humans take time. For example the adaptation of SCNT took a decade and a half to apply to humans after it was done in mice (Tachibana et al., 2013; Wakayama et al., 1998). To accommodate the difficulty experienced with human cells, special screens have been conducted to identify chemicals that boost human reprogramming. Small molecules were identified that could substitute for all transcription factors except OCT4; however, the translation of a fully chemical protocol took nearly a decade after the initial success in mouse systems (Zhu et al., 2010; Hou et al., 2013; Guan et al., 2022).

In 2022, Guan et al. reported the first full chemical induction of pluripotency in human somatic cells. They designed a stepwise approach in which early stages promoted cellular plasticity and downregulated the somatic program, followed by chemical combinations that established an endogenous pluripotency network. The protocol revealed distinct intermediate states, including an epithelial-like stage, a transient highly plastic state, and an extraembryonic endoderm (XEN)-like stage, before the eventual emergence of stable pluripotent colonies. These stages were characterized in detail using single-cell RNA sequencing and ATAC-seq, which confirmed early activation of regenerative and developmental regulators such as SALL4 and FOXA2. A key mechanistic finding was that inhibition of the JNK pathway was indispensable; without it, pro-inflammatory signaling remained active and blocked the formation of the early plastic intermediate necessary for reprogramming (Guan et al., 2022).

Following the 2022 study, refinements were reported that increased efficiency and shortened timelines. Liuyang et al. developed an optimized protocol by identifying “reprogramming boosters” that alleviated specific bottlenecks in the original process. The newer protocol reduced the reprogramming duration from roughly 50 days down to 3-4 weeks. They observed increases in efficiency some reaching up to 31% and consistent reprogramming across donor cell lines (Liuyang et al., 2023). More recently, Wang et al further improved the reprogramming protocol by identifying differences between cell lines with different efficiencies attempting to identify barriers in cell lines that were more refectory to the reprogramming process. They identified that histone modification related gene sets were higher in cells that had worse reprogramming efficiencies and through screening of a chemical library targeting histone modifiers they found KAT3A/KAT3B

inhibitor A-485 and KAT6A inhibitor as WM-8014 as enhancers. With the addition of boosters they discovered through library screening they achieved a protocol capable of generating ciPSCs in as little as 10 days (Y. Wang et al., 2025). Interestingly A-485 is known to impair OSKM reprogramming (Ebrahimi et al., 2019). Finding it to improve chemical reprogramming suggests that chemical reprogramming and genetic reprogramming may take entirely different paths to pluripotency. The fact that the Yamanaka factors aren't activated until cells acquired pluripotency supports this idea that the path of chemical reprogramming is distinct from the reprogramming induced by the pioneer transcription factors and that chemicals are able to regulate global gene expression not through the pathways the Yamanaka factors are involved in but rather through cell signaling pathways and epigenetic modifications.

Taken together, these studies establish that full chemical reprogramming of human somatic cells is possible. At the same time, the protocols remain long, complex, and variable across cell lines. A key priority is reproducibility; results must be independently replicated under standardized conditions before the method can be considered reliable. Beyond the technical challenges, recent work has demonstrated that human ciPSCs are capable of differentiating into functional cell types such as pancreatic islet cells, and that these derivatives can be transplanted safely into animal models and even patients, where they restore insulin production and normalize glycemic control (S. Wang et al., 2024). These findings indicate that ciPSCs are not only feasible to generate but can produce functional, clinically relevant derivatives with demonstrated safety in preclinical and early translational settings.

1.4 Partial Reprogramming

Pluripotent stem cells are restricted to the early embryo, where they generate all embryonic lineages before differentiating into progressively less potent cell types. Somatic cells, by contrast, are specialized and carry molecular hallmarks of chronological and biological aging. Complete reprogramming of adult somatic cells to pluripotency reverts them to an embryonic-like state, one that has not experienced any type of "aging. These observations raise the question of whether somatic cells can be partially reprogrammed to reverse features of aging without losing lineage identity. The principle is to expose cells to reprogramming factors (or chemical equivalents) transiently, long enough to induce epigenetic remodeling and restore youthful cellular function, but not long enough to erase cell identity and trigger full pluripotency. The possibility of such a

concept is support by observations that cells, during the early stages of iPSC induction, begin to exhibit youthful characteristics (e.g. improved mitochondrial function, longer telomeres, a drop in age-related DNA methylation) before they become pluripotent (Sarkar et al., 2020). If that early rejuvenation could be harnessed in situ, aging cells in tissues might regain youthful functionality.

1.4.1 Principles of partial reprogramming

The epigenetic alterations that, in part drive aging are shown to be reversible in many aspects. Somatic cell nuclear transfer (SCNT) is among the experiments that provided early proof of this reversibility. Dolly the sheep, the first large mammal to be cloned is known for developing osteoarthritis and ovine pulmonary adenocarcinoma leading to her premature death at 6.5 years (Giles & Knight, 2003). This led to suspicions that potentially Dolly was aging prematurely however, later studies on a cohort of sheep cloned from the same donor as Dolly showed normal health parameters and no signs of accelerated aging comparable to non-cloned controls (Sinclair et al., 2016). An analysis of published studies reviewing the health of cloned animals including cattle, sheep, pigs, and mice found that 77% had no health problems which is in line with natural populations (Cibelli et al., 2002). This demonstrates that reprogramming can fully reset epigenetic age and that such a reset does not inherently shorten lifespan. This supports the concept that age-related epigenetic changes are not fixed but can be erased and re-established without immediate diminishing returns. Although there is also the possibility that the clones with extensive epigenetic damage simply fail the SCNT experiment, further studies are required to better elucidate the dynamics of successful epigenetic resetting.

Partial reprogramming applies pluripotency factors for short intervals to induce rejuvenation while avoiding full dedifferentiation. The approach was first validated *in vivo* by Ocampo et al., who showed that cyclic expression of OSKM in a progeria mouse model extended lifespan by ~30% and improved multiple age-associated phenotypes without inducing teratomas or loss of tissue architecture (Ocampo et al., 2016). This established that transient exposure to reprogramming factors could reverse hallmarks of aging *in vivo* while preserving tissue identity. The rejuvenation observed in partial reprogramming can be conceptualized as a controlled erosion and re-stabilization of the epigenome. With aging, epigenetic fidelity erodes: DNA methylation patterns drift, chromatin accessibility becomes disorganized, and transcriptional networks lose precision. This results in compromised lineage stability and impaired functional

responses. Partial reprogramming can pull cells out of their terminal resting places in an eroded epigenome enough to partially erase aberrant marks and restore plasticity, but not enough to erase identity. To explain on the metaphorical epigenetic landscape: reprogramming can pull cells from their terminal resting places up the hill and allow them to roll back down to re-establish their somatic gene expression programs a more defined chromatin landscape. This “mini differentiation” event may be what we attribute the rejuvenation we observe to.

Considering partial reprogramming as potential therapeutic gives rise to several considerations that should be taken into account. First, the amount of reprogramming applied is critical, defined by either dose or timing. Prolonged exposure risks excessive identity loss, preventing cells from re-establishing their somatic state. Second, some degree of epigenetic erasure is required to reshape the epigenome, both by removing aberrant marks that accumulate with age and by restoring marks that are lost over time. Third, reprogramming should not interfere with essential cellular functions; it should avoid inducing stress responses or senescence and should enhance, rather than impair, mitochondrial activity. Finally, retention of somatic identity is indispensable. Without the ability of cells to return to their original differentiated state, the therapeutic value of partial reprogramming is abolished.

1.4.2 Key studies and outcomes

Early reports suggested that limited exposure to reprogramming factors might restore youthful features without erasing cellular identity. In senescent fibroblasts, short pulses of OSK or OSKM reset heterochromatin structure while avoiding full dedifferentiation (Manukyan & Singh, 2014). The first *in vivo* demonstration of partial reprogramming came from Ocampo et al., who used a progeroid mouse model. Cyclic OSKM induction (two days on, five days off) lowered markers of cellular and physiological aging and extended median lifespan by roughly 30%. No teratomas were formed, and cell identity was preserved. Short cycles also improved the state of late-passage murine and human cells in culture (Ocampo et al., 2016).Work that followed broadened the settings and delivery modes. In the eye, AAV-driven OSK restored youthful DNA methylation and transcript profiles, promoted axon regrowth after optic nerve injury, and improved vision in aged and glaucomatous mice, with effects requiring TET enzymes (Lu et al., 2020). In human fibroblasts and endothelial cells, transient mRNA delivery of OSKMLN reduced epigenetic age while maintaining lineage identity. It also

dampened inflammatory features in chondrocytes and improved regenerative capacity of old muscle stem cells (Sarkar et al., 2020). Cardiomyocyte-restricted OSKM pulses activated a fetal-like proliferative program and aided post-infarction repair. Controlled induction also supported skeletal muscle regeneration *in vivo* (Chen et al., 2021; C. Wang et al., 2021).

Transient expression of the Yamanaka factors lowered fibrosis and promoted wound repair (Doeser et al., 2018), boosted skeletal muscle repair without viral vectors (de Lázaro et al., 2019), and countered hippocampal aging with measurable memory gains (Rodríguez-Matellán et al., 2020). A single induction of OSKM early in life improved late-life survival of mice (Alle et al., 2022). Broader molecular rejuvenation was reported across multiple tissues during normal aging (Browder et al., 2022; Chondronasiou et al., 2022). Studies described improved liver plasticity (Hishida et al., 2022), mitigation of disc degeneration (Cheng et al., 2022), and restoration of youthful transcriptional programs after temporary identity suppression (Roux et al., 2022). Systemic late-life AAV-OSK extended remaining lifespan by 109% (Macip et al., 2024).

All together partial reprogramming evidence across human cell models and mouse tissues points to several shared features. Benefits require carefully limited exposure to avoid identity loss (Chen et al., 2021). Molecular data indicate partial resetting of epigenetic and transcriptional states linked to aging (Sarkar et al., 2020; Browder et al., 2022; Chondronasiou et al., 2022). Functional outcomes vary by tissue and depend on delivery method (Lu et al., 2020; Chen et al., 2021)

1.4.3 Safety considerations and biological limits

Although the literature supporting partial cellular reprogramming is growing it remains at an early stage of development as a potential therapy. Attempting to rejuvenate cells by repurposing the Yamanaka factors, originally optimized for complete dedifferentiation into induced pluripotent stem cells. Because this approach was developed for inducing pluripotency, it is not inherently configured for safe rejuvenation applications. Optimization, cognizant of safety concerns is therefore needed for anti-aging use, with precise control of reprogramming extent, timing, and factor delivery.

A primary safety concern is the risk of cancerous growth caused by the loss of cellular identity. Reprogramming factors activate embryonic programs and increase cellular plasticity. Sustained expression can drive somatic cells toward pluripotent or

progenitor-like states prone to malignant change. Early *in vivo* experiments with continuous OSKM produced teratomas in multiple organs (Abad et al., 2013). Teratoma formation traditionally serves as the classical assay for pluripotency, and its occurrence *in vivo* indicates that some cells crossed the threshold into an iPSC-like state. Unrestrained reprogramming also yields disorganized tissue growth and preneoplastic lesions. Consitutive OSKM expression in transgenic mice caused rapid mortality due to widespread loss of cellular identity in vital organs, particularly the intestinal epithelium and liver (Parras et al., 2023).

These organs underwent dysplasia and functional failure as cells reverted toward embryonic states. However, when reprogramming is applied in short inductions (2 days on, 5 days off), no early deaths or tumorigenesis were observed. Later studies have similarly found that transient or cyclic reprogramming can ameliorate aging hallmarks without inducing cancer or abnormal growth. For example, cyclic OSKM over 35 cycles increased lifespan by 33% without weight loss or illness in a progeroid mouse model (Ocampo et al., 2016). In wild-type aged mice, up to 7-10 months of cyclic OSKM induction produced no teratomas or obvious health detriments (Browder et al., 2022). These results suggest that control over factor expression can largely separate rejuvenation benefits from the cancer risk associated with full reprogramming. Nevertheless, even with cyclic protocols, an oncogenic risk remains.

Another concern during reprogramming is genomic instability. Cellular reprogramming involves widespread epigenetic remodeling and cell-cycle entry, which can introduce DNA damage and chromosomal stress. Full iPSC reprogramming is associated with replication stress and can yield cells with DNA strand breaks or copy-number variations (Laurent et al., 2011). Partially reprogrammed cells do not typically undergo the extensive cell divisions of iPSC derivation, so the genomic insult may be milder. However, repeated cycles of partial reprogramming could allow damage to accumulate over time. Each induction cycle may engage DNA-damage responses and transiently alter safeguards such as p53 while epigenetic marks are reset. Encouragingly, some short-term partial reprogramming experiments report a reduction in markers of genomic stress. A brief initiation-phase OSKM treatment was able to decrease DNA-damage foci (γH2AX) and improved DNA-methylation age in an Ercc1-deficient mouse model (Paine et al., 2024). This indicates that partial reprogramming can restore some aspects of genomic stability in aged cells. Nevertheless, introducing exogenous factors

and rapidly remodeling chromatin carries a potential to create new lesions. If viral vectors are used to deliver OSKM, insertional mutagenesis is an additional concern. Consequently, non-integrating delivery (such as mRNA or chemical cocktails) is being explored to avoid permanent genomic alterations.

In vivo reprogramming may also provoke immune responses. Introducing OSKM in an adult organism can trigger innate and adaptive immunity. Cells undergoing partial reprogramming could express embryonic antigens or stress signals that mark them for immune clearance. Reprogramming attempts can also activate a senescence-like program in a subset of cells as a protective barrier (Banito et al., 2009). Senescent cells secrete pro-inflammatory cytokines, thus cells entering senescence during partial reprogramming can lead to local inflammation. In addition, delivery platforms (e.g., viral capsids or repeated mRNA transfections) can trigger immune reactions. A robust immune response might eliminate partially reprogrammed cells or limit their function. Early mouse studies reported no obvious immune-mediated pathologies with transient OSKM expression (Ocampo et al., 2016), but systematic analyses are needed. It is plausible that immune surveillance contributed to the absence of teratomas in those experiments by clearing pluripotent cells before expansion. Further work on immune compatibility is therefore essential for clinical translation.

Perhaps the biggest challenge is to achieve rejuvenation without eroding normal cell identity and function. This is particularly difficult because the loss of cellular identity may be required for rejuvenation to occur. Pushing cells too far back along the developmental timeline can impair specialized functions and lead to organ failure, with the additional risk of malignant transformation. Excessive dedifferentiation produces immature cell states in tissues that require mature functionality. For example, in a heart-specific reprogramming study, 12 days of continuous OSKM induction caused cardiomyocytes to lose differentiated characteristics and many underwent apoptosis, resulting in compromised cardiac function (Chen et al., 2021). This demonstrates a narrow window between sufficient reprogramming to induce rejuvenation and over-reprogramming that harms tissue integrity. Exceeding this window led to fatal tissue failure in related models, underscoring the need to titrate the intervention (Parras et al., 2023). To address this, protocols are being refined to identify optimal protocols where rejuvenation is maximized but identity loss is minimized. There are distinct reprogramming phases: an initiation phase that triggers youthful gene expression, a

maturation phase that yields rejuvenation benefits, and a pluripotent phase beyond which cells lose identity (David & Polo, 2014). By terminating OSKM expression no later than the maturation phase, rejuvenation may be captured without crossing into an iPSC state (Gill et al., 2022). Practically, this has meant using shorter induction periods or lower factor doses. For instance, maturation-phase partial reprogramming protocols of approximately 10-14 days have been proposed, which yield greater rejuvenation than very brief inductions but stop short of complete dedifferentiation. Achieving this balance is organ dependent as different cell types have variable susceptibility to reprogramming. Highly regenerative tissues may dedifferentiate more readily and require more conservative dosing. Whereas tissues like muscle or brain might tolerate longer inductions. The challenge of tuning reprogramming intensity remains central. Conservative protocols may yield limited benefit, whereas aggressive ones risk pathology. Therefore, dose-response studies and functional assays are used to identify conditions that refresh epigenetic and transcriptional profiles while preserving phenotypic identity and tissue architecture.

Beyond these safety issues, there are biological limits to what partial reprogramming can achieve. Aging is multifactorial, and not all damage is reversible through epigenetic means. Partial reprogramming primarily resets epigenetic marks and can rejuvenate gene expression, improve cellular metabolism, and restore aspects of proteostasis (Sarkar et al., 2020). However, it cannot reverse all age-related outcomes. For example, it can’t correct accumulated genomic mutations. Large-scale structural changes in tissues are also not reversed. For example, insoluble protein aggregates such as amyloid plaques are not cleared by resetting epigenetic age. Some studies note that rejuvenated cells can show improved proteostasis, which may slow further accumulation, but existing aggregates and long-lived matrix crosslinks typically persist unless addressed directly (Paine et al., 2024; Sarkar et al., 2020). Epigenetic resetting is known to restore youthful cellular functions, it may be able to restore apoptotic competence as well, allowing highly damaged cells to undergo apoptosis or senescence and thereby be removed (Simpson et al., 2021). Nonetheless, large-scale tissue defects remain even when cells are partially rejuvenated. Thus, partial reprogramming addresses certain hallmarks of aging (epigenetic alterations, loss of proteostasis, mitochondrial dysfunction) but cannot directly fix others like accumulated mutations, extracellular aggregates, or cell

loss. Combination strategies will likely be required for more complete reversal or alternative strategies such as replacement must be explored.

Another long-term consideration is clonal selection and expansion. Reprogramming interventions may not affect every cell uniformly, some cells or clonal subsets could respond better and expand after treatment (Shakiba et al., 2019). This raises the possibility of clonal dominance, analogous to age-associated clonal hematopoiesis in blood. If partial reprogramming confers a survival or proliferative advantage to specific clones, tissue composition could shift over time. The risk may be amplified with repeated cycles or integrating gene delivery, since the subset of cells that stably acquire the reprogramming cassette could form a permanent lineage. Detecting such dynamics is difficult in short-term studies but is relevant for clinical translation. Lineage tracing and single-cell analyses will be important to verify that partial reprogramming benefits the targeted population broadly rather than selecting for a subset. At present, no significant clonal outgrowth has been reported in short-term mouse studies under transient OSKM regimens, but longer studies and genomic surveillance are needed.

Overall, partial reprogramming is an intervention to rejuvenate cells and tissues, but its safety and efficacy depend on careful optimization of timing, dosage, delivery, and monitoring.

1.4.4 Partial chemical reprogramming

Chemical partial reprogramming, using cocktails of epigenetic drugs to rewind cellular age without inducing pluripotency, is an attractive strategy for a therapeutic. It offers easier delivery and control compared to genetic methods. However, this is a nascent field with only a few proof-of-concept studies reported so far. Recent landmark papers have begun to explore chemical cocktails for cellular rejuvenation. Schoenfeldt et al. demonstrated that a short chemical treatment could rejuvenate aged human fibroblasts by improving key hallmarks of aging. The seven-chemical cocktail composed of RepSox, tranylcypromine, 3-deazaneplanocin A (DZNep), TTNPB, CHIR99021, forskolin, and valproic acid (originally used to induce iPSCs in mice) was able to reduce DNA damage marker γH2AX and restore youthful levels of H3K9me3 and H3K27me3 in old cells. They further optimized an even simpler two-molecule cocktail (2C), consisting of RepSox and tranylcypromine which more efficiently ameliorated cellular senescence, lowering senescence-associated β-galactosidase activity and the expression of p21/p53

and SASP factors, as well as reducing oxidative stress in the cells. Remarkably, administering this 2C regimen *in vivo* extended the lifespan and healthspan of C. elegans worms, demonstrating that chemical partial reprogramming can confer longevity benefits in a whole organism (Schoenfeldt et al., 2025).

Mitchell et al. also applied the 7C cocktail to fibroblasts from old mice, as well as the simplified two-factor version 2C (RepSox and tranylcypromine). They reported broad rejuvenation in these aged mouse cells. Partial chemical reprogramming with 7C or 2C upregulated mitochondrial oxidative phosphorylation pathways, reduced the accumulation of old-age metabolites, and lowered the cells' biological age as measured by both DNA methylation and transcriptomic clocks. Treated old fibroblasts showed functional improvements, including increased respiratory capacity and mitochondrial membrane potential, indicating a tangible reversal of age-related decline in vitro (W. Mitchell et al., 2024a).

Yang et al. screened for compounds that reverse cellular aging and identified multiple effective combinations. They also found that the seven-factor cocktail (7C) could rejuvenate human cells in vitro. Short-term treatment with such cocktails in replicatively aged human fibroblasts restored a youthful gene expression profile and significantly reversed their transcriptomic age, all while the cells retained their original identity as fibroblasts (Yang et al., 2023).

Crucially, none of the previous studies employed a human chemical reprogramming cocktail that had been developed and validated for induction of iPSCs from human cells. Instead, earlier work has largely focused on mouse systems or relied on chemical combinations optimized for murine reprogramming. As a result, while these studies provided valuable evidence that small molecules can partially reverse aging phenotypes, they did not directly address how a bona fide human reprogramming cocktail functions in human somatic cells.

The work presented in this thesis addresses this gap. By applying a human chemical reprogramming protocol to human fibroblasts under partial reprogramming conditions, we directly compare its rejuvenation effects to those produced by OSKM-based partial reprogramming. To our knowledge, no previous study has carried out such a side-by-side evaluation in human cells. This approach allows us to assess whether chemical reprogramming using a human-specific cocktail can approach the rejuvenation

outcomes of OSKM, and to define points of convergence and divergence between the two methods.

CHAPTER 2: MATERIALS AND METHODS

2.1 Human Fibroblast Isolation and Culture

Human adult skin fibroblasts were isolated from dermal biopsies obtained with written informed consent and Koç University Ethics Committee approval. Tissues were rinsed twice in PBS. Samples were cut into ~0.5-1 mm^3 pieces with a sterile scalpel and placed in 6-well plates under sterile coverslips with %15 FBS DMEM supplemented with 1% penicillin-streptomycin then incubated at 37 °C, 5% CO_2. Medium was changed at 24 h and every 2-3 days. Fibroblast outgrowth appeared by days 4-6. When the cells reached ~80% confluence, around day 10-14, cells were detached with 0.05% trypsin-EDTA, passaged 1:3-1:4, and expanded in %15 FBS DMEM supplemented with 1% penicillin-streptomycin and frozen for subsequent use.

2.2 Production of Viruses

HEK293T cells were seeded in 10cm dishes at 3.8×10^6 cells per dish in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. A mixture of packaging plasmid (psPAX2 for lentivirus, pUMVC for retrovirus) envelope plasmid (VSV-G), and transfer plasmid were used. Plasmid DNA and PEI were mixed at a 1:3 DNA:PEI mass ratio in serum-free DMEM and incubated 20-30 min at room temperature. After incubation the mixture was added dropwise to cells. After overnight incubation, medium was replaced with fresh DMEM + 10% FBS. Viral supernatants were collected at 48 h and 72 h, clarified at 3,000 × g for 10 min, and filtered through 0.45 μm PES membranes. For concentration, filtered supernatant was brought to 10% (w/v) PEG-8000 by adding a 50% PEG stock, incubated at 4 °C overnight, pelleted at 3,000 × g for 30 min, resuspended in ice-cold PBS, aliquoted, and stored at −80 °C.

2.3 Generation and Maintenance of Cell Lines

Young human dermal fibroblasts were infected with either retroviral pBABE-WT LMNA-GFP-Puro or pBABE-Progerin-GFP-Puro, or with lentiviral pCW57.1-TetO-WT LMNA-Puro-rtTA or pCW57.1-TetO-Progerin-Puro-rtTA. Puromycin selection was applied until a parallel non-infected control had no survivors. Selected cells were expanded in DMEM containing 10% FBS and cryopreserved for later experiments.

2.4 Molecular Cloning and Plasmid Preparation

For inducible progerin expression open reading frames from pBABE-LMNA-GFP-Puro (Addgene, #17662) and pBABE-Progerin-GFP-Puro (Addgene, #17663) were PCR-amplified with primers adding 5′ EcoRI and 3′ SalI sites to remove GFP and express untagged proteins. Amplicons were cloned into the doxycycline-inducible pCW57.1 backbone with EcoRI and SalI. Clones were screened and verified by Sanger sequencing.

For inducible reprogramming casettes inserts from pSIN4-E2F-O2S (Addgene, #21162) and pSIN4-CMV-K2M (Addgene, #21164) were PCR-amplified with primers adding 5′ NheI and 3′ SalI sites. PCR products were cloned into FuW-TetO-MCS (Addgene, #84008) with NheI and BamHI. Clones were screened and verified by Sanger sequencing.

2.5 Genetic Reprogramming

Fibroblasts were seeded at a density of 5×10^4 cells per well in 12-well plates or 2.5×10^4 cells per well in 24-well plates. Cells were transduced with either pSIN-E2F-O2S and pSIN-CMV-K2M lentiviruses or with FUW-TETO-O2S and FUW-TETO-K2M, together with FUW-M2-rtTA. Doxycycline (Dox) was added to induce factor expression. On day 6, cells were replated onto Geltrex-coated surfaces, and culture medium was switched to mTeSR. Medium was refreshed every two days. Reprogramming was considered complete upon emergence of colonies, at which point TRA-1-60 immunostaining was performed.

2.6 Genetic Partial Reprogramming

Fibroblasts were seeded at a density of 5×10^4 cells per well in 12-well plates or 2.5×10^4 cells per well in 24-well plates. Cells were transduced with lentiviruses carrying reprogramming factors (pSIN-E2F-O2S and pSIN-CMV-K2M, or FUW-TETO-O2S and FUW-TETO-K2M in combination with FUW-M2-rtTA). Following infection, cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (D10 medium).

For partial reprogramming, cells were not passaged during the induction period. Doxycycline was added to induce factor expression where applicable. Culture medium was replenished every two days. Reprogramming was terminated at the designated time points without colony formation, and cells were collected for downstream analysis.

2.7 Chemical Reprogramming

Chemical induction of human CiPS cells was performed in three stages. hADS cells and adult skin fibroblasts were seeded in high-glucose DMEM with 15% FBS 1.2-1.4 × 10^4 cells per well in 12-well plates, then switched the next day to Stage 1 medium and cultured at 5% O_2.

The standard Stage 1 medium was KnockOut DMEM with B27 2%, KSR 1%, GlutaMAX 1%, NEAA 1%, penicillin-streptomycin 1%, vitamin C 50 µg/ml, LiCl 5 mM, nicotinamide 1 mM, BMP4 40 ng/ml, and small molecules CHIR-99021 5 µM, 616452 10 µM, TTNPB 2 µM, SAG 0.5 µM, EPZ-5676 2 µM, DZNep 0.05 µM, ruxolitinib 1 µM, VTP50469 0.5 µM, AKT inhibitor 1 µM, JNK-IN-8 0.2 µM, SETD2-IN-1 0.2 µM, AM095 0.5 µM, WM-8014 1 µM, and A-485 0.5 µM. In a predefined variant used for optimization, Stage 1 and Stage 2 were prepared in KnockOut DMEM with 10% FBS and 10% KSR and without B27 or BMP4 was used where indicated. Cells reached confluence by day 8-10 in hASFs, at which point cultures were switched to Stage 2 and returned to 21% O_2.

The standard Stage 2 medium was KnockOut DMEM with B27 2%, GlutaMAX 1%, NEAA 1%, penicillin-streptomycin 1%, vitamin C 50 µg/ml, bFGF 200 ng/ml, and small molecules CHIR-99021 5 µM, 616452 10 µM, TTNPB 2 µM, SAG 0.5 µM, JNK-IN-8 0.5 µM, EPZ-5676 2 µM, DZNep 0.2 µM, ruxolitinib 1 µM, BIRB-796 2 µM, SGC-CBP30 2 µM, 5-iodotubercidin 0.5 µM, AKT inhibitor 0.2 µM, CX-4945 1 µM, all-trans retinoic acid 5 µM, and GSK-3685032 0.02 µM.

Stage 3 used KnockOut DMEM with B27 2%, GlutaMAX 1%, NEAA 1%, penicillin-streptomycin 1%, KSR 5%, vitamin C 50 µg/ml, heregulin-β1 20 ng/ml, and small molecules CHIR-99021 1 µM, Y-27632 10 µM, PD0325901 1 µM, and SB-590885 0.5 µM. For the first two to three days of Stage 3, the medium was supplemented with valproic acid 200 µM, tranylcypromine 10 µM, DZNep 0.2 µM, EPZ-5676 2 µM, and PY-60 10 µM, followed by valproic acid 200 µM and PY-60 2 µM for an additional two to three days. At the endpoint all cells were detached and stained with TRA-1-60 antibodies to quantify reprogramming efficiency through flow cytometry.

2.8 Chemical Partial Reprogramming

Partial chemical reprogramming of fibroblasts was performed using only the Stage 1 chemical induction conditions. Cells were seeded at 1.2–1.4 × 10^4 cells per well in 12-well plates in high-glucose DMEM supplemented with 15% FBS. After overnight attachment, cultures were switched to Stage 1 medium prepared in KnockOut DMEM containing 10% FBS, 10% KSR, GlutaMAX 1%, NEAA 1%, penicillin–streptomycin 1%, and vitamin C 50 µg/ml. Small molecules were added as follows: LiCl 5 mM, nicotinamide 1 mM, CHIR-99021 5 µM, 616452 10 µM, TTNPB 2 µM, SAG 0.5 µM, EPZ-5676 2 µM, DZNep 0.05 µM, ruxolitinib 1 µM, VTP50469 0.5 µM, AKT inhibitor 1 µM, JNK-IN-8 0.2 µM, SETD2-IN-1 0.2 µM, AM095 0.5 µM, WM-8014 1 µM, and A-485 0.5 µM. Cells were maintained under 5% O_2 during this treatment phase.

For rejuvenation experiments, cultures were returned to their standard growth medium (DMEM with 10% FBS) for a chase period to allow recovery and assessment of phenotypic outcomes.

2.9 Quantitative PCR (qPCR)

Total RNA was isolated using the MN NucleoSpin RNA kit per manufacturer instructions. For cDNA synthesis, 250 ng - 1 µg RNA was combined with 0.5 µL 10 mM dNTP , 1 µL 50 µM random hexamers and completed to 16.5 µL with nuclease free water. The samples were then heated at 65 °C for 5 min and chilled on ice. A reverse-transcription mix of 5 µL 5× First-Strand Buffer, 2 µL 0.1 M DTT, and 0.5 µL RNase inhibitor was added and incubated 10 min at room temperature. 1 µL MMLV-RT was then added and reactions were run at 37 °C for 60 min followed by inactivation at 70 °C for 15 min. cDNA was diluted to 100 µL with nuclease-free water. qPCR was performed on a LightCycler® 480 Instrument II using LightCycler® 480 SYBR Green I Master (Roche, cat. 04707516001) in 20 µL reactions: 10 µL master mix, 2 µL cDNA, 2 µL primer mix with each primer at 2.5 µM, and 6 µL nuclease-free water. Cycling: 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Relative expression was calculated by the ΔCt method, where Ct is the threshold cycle of the gene of interest and Cc is the threshold cycle of the selected housekeeping reference gene.

Gene Name	Forward Primer Sequence	Reverse Primer Sequence
h p16 / CDKN2A	CCCAACGCACCGAATAGTTA	ACCAGCGTGTCCAGGAAG
h p21 / CDKN1A	GTCACTGTCTTGTACCCTTGTG	CGGCGTTTGGAGTGGTAGAAA
h ATF3	TGCTCAGAGAAGTCGGAAGAA	TGGCACAAAGTTCATAGGGCA
h MMP13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
h LINE-1 TA-Family	CAAACACCGCATATTCTCACTCA	CTTCCTGTGTCCATGTGATCTCA
h BTG2	ACGGGAAGGGAACCGACAT	TGGTGTTTGTAGTGCTCTGTGA
h PROS1	TCTCAGAGGCAAACTTTTTGTCA	TTTTCAAAGACCTCCCTGGC
h MGST1	TCTGGACCCTGAACAGGAGG	TTGGTCTGGAATGGTGTTCC
h TAGLN2	GGACCGGAGGTGGACTG	GGCTGCGGGGAGAGAT
h IL6ST	AACAGCATCCAGTGTCACCT	TTTTCTGGAGGCAAGCCTGA
h ISG15	AGCCATGGGCTGGGA	CGATCTTCTGGGTGATCTGC
h DCN	CTTTACAACTCTCTTGCAAGGTTCC	TCTGTTGAAACGGTCCAGCC
h TMED9	GCGCGCTCTACTTTCACATC	TGCGTCCGGTAGTTTCCTATG
h SUN2	GAAGGCCCGTTTTGTTCAGT	CCCTGAAGAGAATCTGAAGGC
h FST	TGGAGTCACCTACTCCAGTG	TCACAGGACTTTGCTTTGATACA
h MAD2L2	AGCGCAGAGCCGGTAG	TTGCGTTTCTGGAAGATGCC
h NEFH	GAGGAGTGGTTCCGAGTGAG	GCCTCTCCAGTGAGTCCTTG
h SESN1	GCGTACACGGCCCCTTT	TAATGCCAAGTTCCTCGTCCT
h FYN	AGGCTTACGTGAAGGGAATTT	TAGTTGGGGATGGAGGTCAC
h TALDO1	GAGGAGGCGATTGCCTATGG	ATCAAAGGAGAGCCTTGCGT
h TERT	GGCACGGCTTTTGTTCAGAT	ACATGCGTGAAACCTGTACG
h COL1A1	GGCCCTCAAGGTTTCCAAGG	CACCCTGTGGTCCAACAACTC
h MDM2	TCCCAGCCTAGGTTTCAGAC	AACACGGAGCTTGAGAGGAA
h GADD45A	AACGACATCAACATCCTGC	AATGTGGATTNGTCACCAG
h PUMA	GACCTCAACGCACAGTACGAG	AGGAGTCCCATGATGAGATTGT

Table 2.1: List of qPCR primers used in this study.

2.10 Immunofluorescence Staining

Cells were seeded on 12-mm circular glass coverslips in 24-well plates. For imaging they were fixed in 4% paraformaldehyde for 30 minutes at room temperature then washed three times with PBS for 5 min on an orbital shaker. For blocking/permeabilization cells were incubated at room temperature for 60 minutes on an orbital shaker with 0.1% Triton X-100 and 5% goat serum in PBS. Primary antibodies were diluted in blocking buffer and incubated overnight at 4 °C. The next day cells were washed three times with PBS for 5 min. Secondary antibodies diluted in blocking buffer were applied for 1 h at room temperature in the dark. Cells were washed three times in

PBS for 5 min. Cover slips were mounted on microscope slides with VECTASHIELD® Antifade Mounting Medium with DAPI (H-1200-10) and imaged on a Leica DMi8 confocal microscope.

Antibody Name	Vendor	Catalog Number
Mouse anti-human γH2AX (D7T2V)	Cell Signaling	#80312
Rabbit anti-human H3K9me3 (D4W1U)	Cell Signaling	#13969
Rabbit anti-human H3K27me3 (C36B11)	Cell Signaling	#9733
Goat anti-rabbit Alexa fluor 594	Invitrogen	R37117
Goat anti-mouse Alexa fluor 594	Invitrogen	R37121

Table 2.2: Antibodies used for immunofluorescence staining.

2.11 Flow Cytometry

Cells were detached with 0.05% trypsin-EDTA and neutralized with DMEM supplemented with 10% FBS. 200,000 cells were pelleted at 1,000 × g for 5 min and washed once with 4% FBS in PBS (FACS buffer). Cells were resuspended in 100 µL staining buffer and incubated with 1 µL antibody (1:100) for 30 min on ice in the dark. Cells were washed 3× with staining buffer and resuspended in 300 µL FACS buffer. Samples with appropriate stained and unstained controls were acquired on a Beckman Coulter CytoFLEX flow cytometry device.

Antibody Name	Vendor	Catalog Number
PE anti-human TRA-1-60-R	BioLegend	330610
PE anti-human CD13	BioLegend	301704

Table 2.3: Antibodies used for flow cytometry staining.

2.12 Senescence-Associated β-Galactosidase Assays

For the plate-based senescence-associated β-galactosidase assay, cells were first washed twice with PBS and fixed for 5 min at room temperature using 2% formaldehyde and 0.2% glutaraldehyde in PBS. After fixation, cells were rinsed twice with PBS and then incubated with freshly prepared X-gal staining solution (40 mM citric acid/sodium phosphate buffer at pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, and 1 mg/ml X-gal in dimethylformamide) at 37 °C. Incubation was carried out for 14 h in a non–CO_2 incubator. Following staining, cells were washed twice with PBS and imaged. Afterwards cells were washed with methanol,

air-dried, and stored protected from light. SA-β-gal–positive cells were identified by the appearance of blue staining and quantified by bright-field microscopy.

For the flow-cytometry based assay, lysosomal pH was raised with bafilomycin A1 for 1 h at 37 °C. C12-FDG (5-dodecanoylaminofluorescein di-β-D-galactopyranoside) was added directly to the medium after 1 hour at a final concentration of 15 µM for 2 h at 37 °C. Cells were washed with PBS twice then detached using 0.05% trypsin-EDTA and inactivated with DMEM supplemented with 10% FBS. Cells were centrifuged at 1,000 × g for 5 min and resuspended in ice cold PBS and analyzed on a Beckman Coulter CytoFLEX flow cytometry device.

2.13 Mitochondrial ROS Measurement

Cells were washed twice with HBSS without Ca^{2+}/Mg^{2+}, then incubated with 5 µM MitoSOX Red (TargetMol) in HBSS for 30 min at 37 °C, 5% CO_2. After staining cells were washed twice with PBS and detached with Accutase. Cells were centrifuged at 1,000 × g for 5 min and resuspended in PBS. Samples were acquired on a Beckman Coulter CytoFLEX using the PE channel, and mitochondrial superoxide was quantified as median fluorescence intensity.

2.14 Cell Viability Assay

Cell viability and proliferation were measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Cat. No. G7570). Fibroblasts were seeded at a density of 1,500 cells per well in opaque, walled, clear-bottom 96-well plates. Cells were cultured in a Stage 1 base reprogramming medium consisting of DMEM supplemented with 10% fetal bovine serum, 10% knockout serum replacement, 1% non-essential amino acids, 1% penicillin-streptomycin, 1% GlutaMAX, 50 µg/mL L-ascorbic acid, and 5 mM lithium chloride, as well as 1 mM nicotinamide.

Test compounds were diluted to their Stage 1 working concentrations in this base medium, while control wells received an equivalent concentration of DMSO vehicle. Cell viability was assayed at 24-hour intervals. At each time point, CellTiter-Glo reagent was added directly to the culture wells, and luminescence was measured using a plate reader (BioTek Synergy). Raw luminescence values were background-subtracted and normalized to the DMSO control for each time point.

CHAPTER 3: RESULTS

3.1 Identification of Differentially Expressed Genes with Aging

A dataset from Fleischer and colleagues was used to study how gene expression changes with age. It contains fibroblasts from 133 people, ranging from one year old up to ninety-four. Because the samples cover such a wide span, age could be treated as a continuous variable instead of split into groups. Expression values for each gene were compared with donor age, and genes that rose or fell across the age range were noted. Many of the changes matched what has been described before in fibroblast aging, such as shifts in matrix genes, stress pathways, and metabolism. From this first step, the top one hundred positive and top one hundred negative correlations were kept. These lists were then checked against three online resources that track genes connected with aging. Genes that appeared in both our list and one of these resources were marked as stronger candidates, since they came up in more than one place.

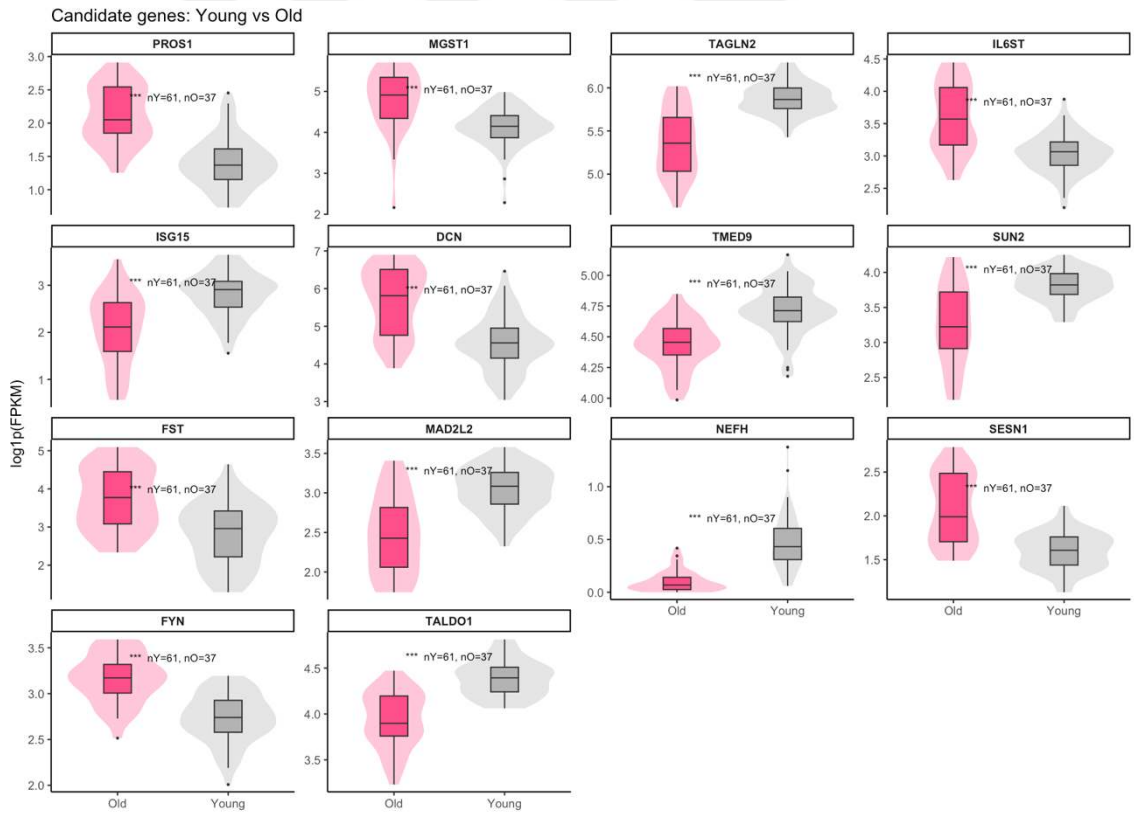

Figure 3.1: Expression levels of candidate genes between young and old groups. Young was considered < 35 and old was considered > 70.

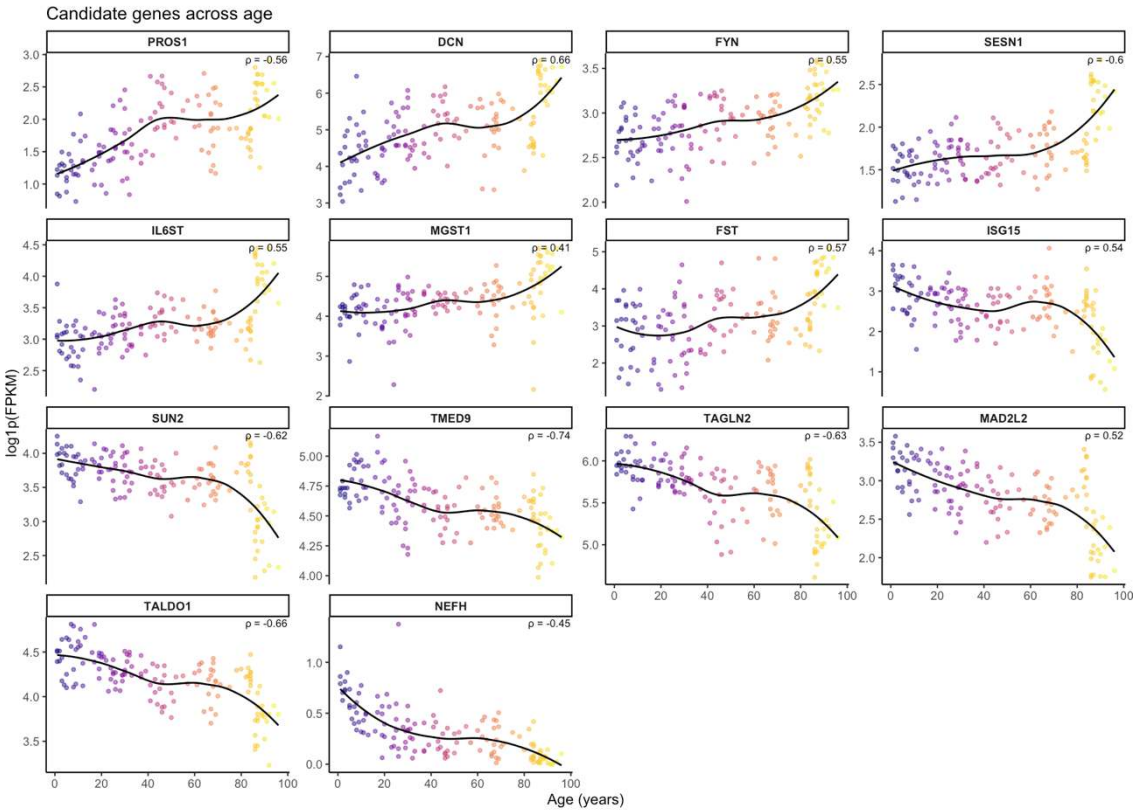

Figure 3.2: Expression levels of candidate genes across ages.

3.2 Characterization of Human Fibroblast Lines

To establish a baseline for assessing rejuvenation, young and old human fibroblasts were compared across multiple markers. Starting from passage 5, we noted the number of population doublings to track proliferative potential (Figure 3.3). Fibroblasts from older donors showed a slower accumulation of doublings than those from younger donors. The difference became more noticeable as the cultures progressed, reflecting the limited growth capacity typical of aged cells.

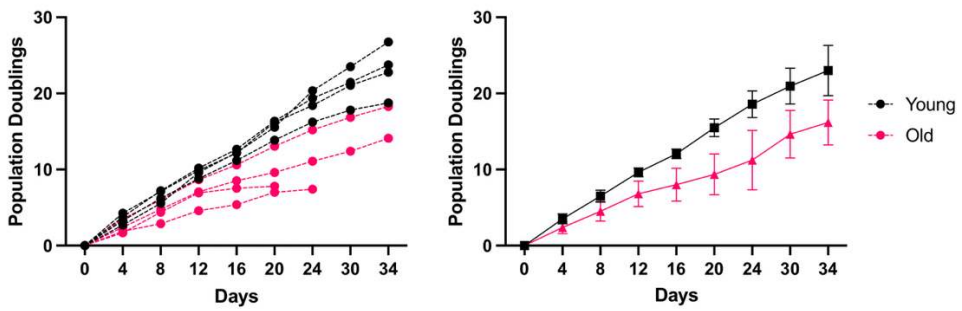

Figure 3.3: Population doubling graphs of young and old human fibroblasts individually (left) and aggregated (right).

A qPCR panel was designed based on the age-associated genes identified in Section 3.1 and genes commonly tested in the literature. Several genes showed significant differences in expression between young and old lines, most notably ATF3, BTG2, MMP13, ISG15 and SUN2 (Figure 3.4). By contrast, others such as p16 and p21 did not show a clear difference. Interestingly some of the genes showed the opposite pattern of what was expected according to the dataset (e.g. ISG15 and MAD2L2 are expected to be higher in young cells according to the dataset but we observe them to be significantly higher in old cells).

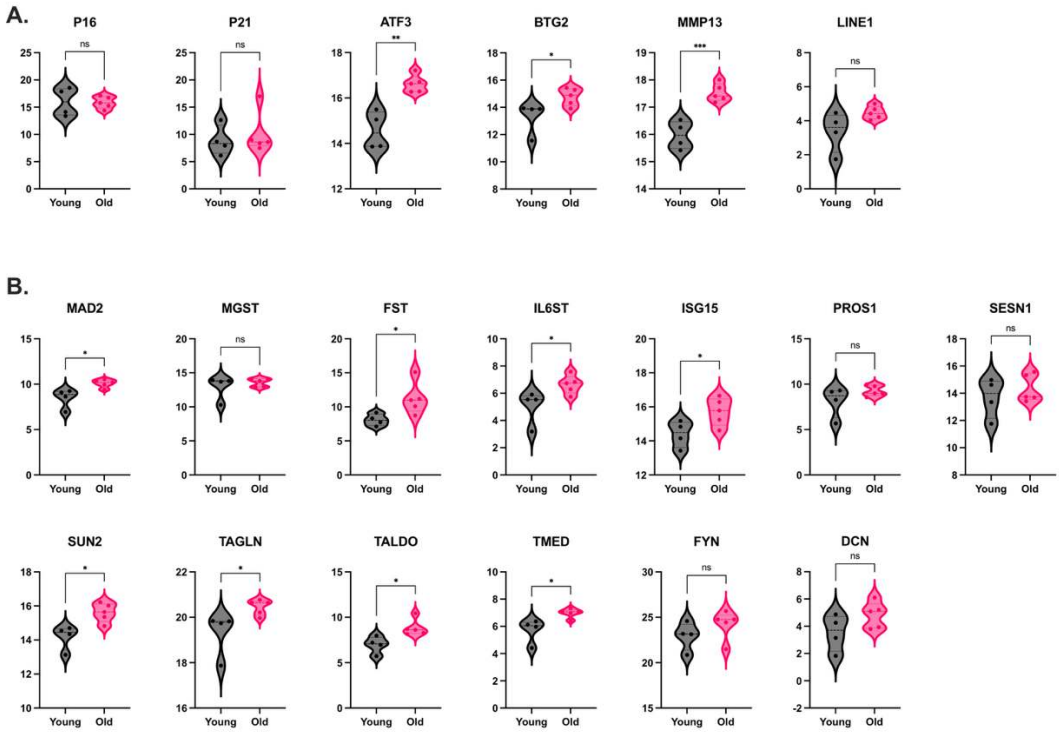

Figure 3.4: qPCR analysis of age-associated gene expression in young and old fibroblasts. (A) Common stress related genes from the literature. (B) Genes identified in RNA-seq analysis.

We next tested senescence status using β-galactosidase staining across all donor lines. Surprisingly, there was no consistent increase in staining in old fibroblasts compared with young ones (Figure 3.5).

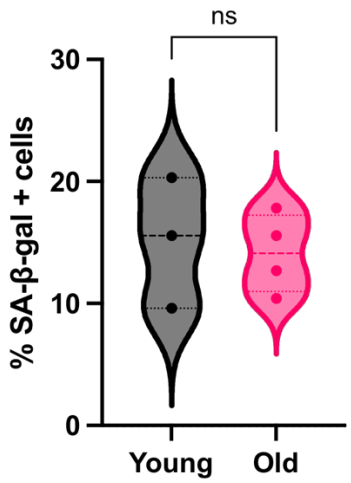

Figure 3.5: Senescence-associated β-galactosidase activity in young and old fibroblasts.

Reprogramming efficiency was then assessed by TRA-1-60 staining after OSKM induction (Figure 3.6). No major differences in colony formation or staining intensity were observed between young and old fibroblasts. Overall, the transcriptomic readout provided the clearest discrimination, while senescence and reprogramming efficiency did not differ substantially.

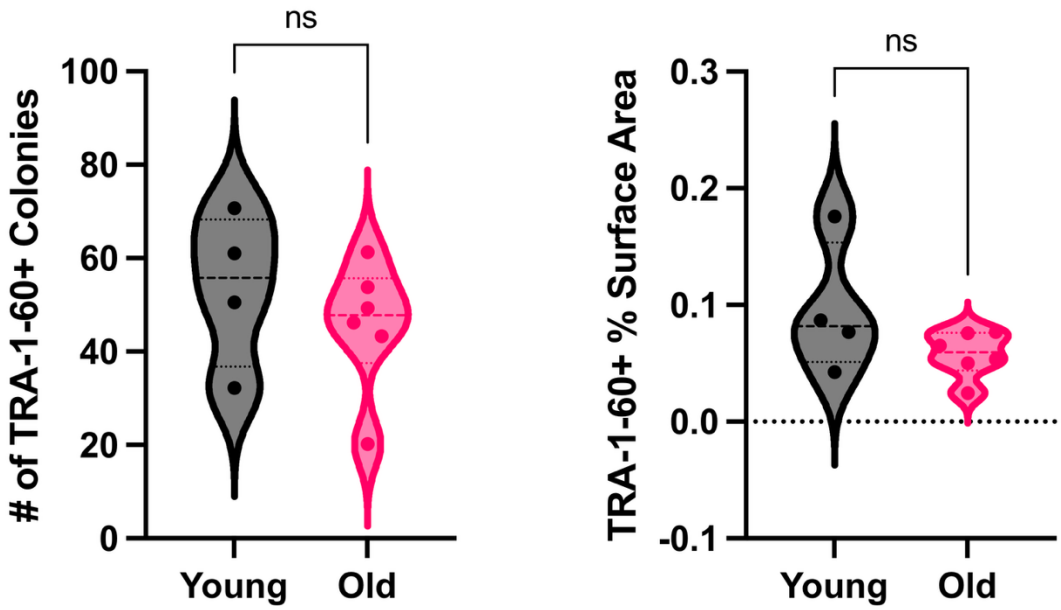

Figure 3.6: Reprogramming efficiency of human fibroblast lines under OSKM induction.

These findings suggested that our primary fibroblast set was not sufficient on its own as a robust aging model.

3.3 Characterization of HGPS Model Lines

To generate a stronger model of accelerated aging, we turned to Hutchinson-Gilford Progeria Syndrome (HGPS). The disease is caused by a dominant-negative mutation in LMNA that produces the mutant protein progerin. Four young fibroblast donor lines were transduced with retroviruses carrying either wild-type LMNA-GFP or progerin-GFP. Following antibiotic selection, stable cell lines were established for each background. Abnormal nuclear morphologies characteristic of progerin expression were verified using confocal microscopy (Figure 3.7)

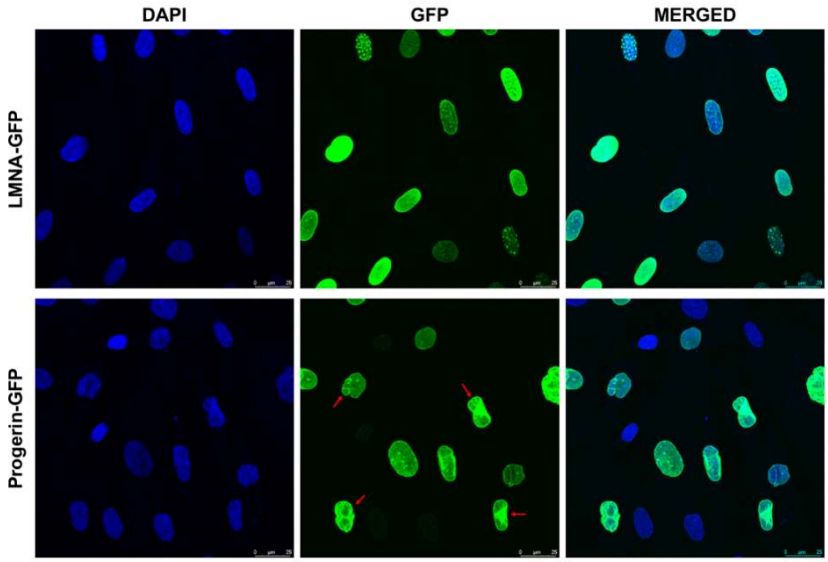

Figure 3.7: Abnormal nuclear morphology in progerin-expressing fibroblasts.

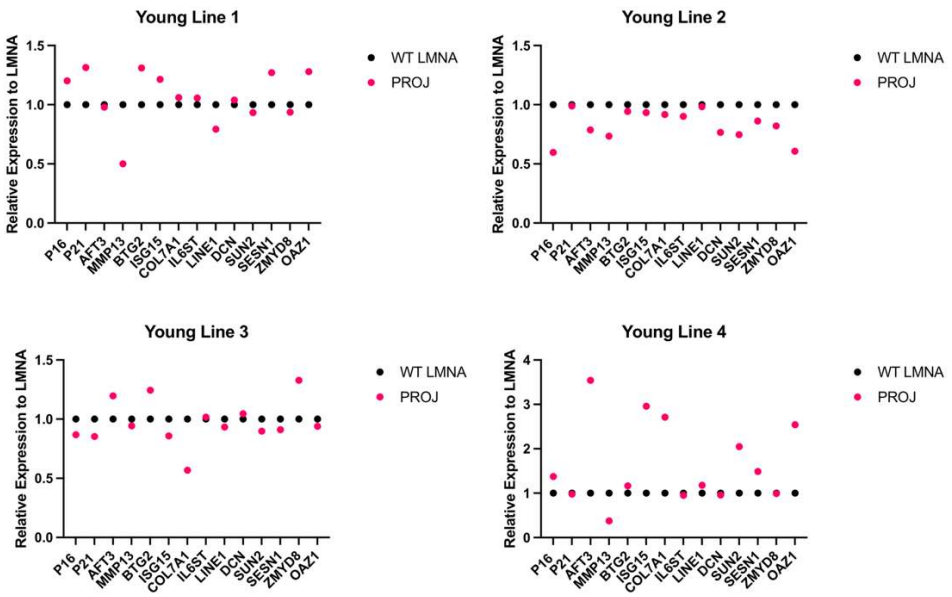

Figure 3.8: Gene expression profiles of WT LMNA vs. progerin-expressing young fibroblasts.

To characterize the effects of progerin expression in these lines, we compared the expression of age-associated genes in each progerin-expressing line relative to its matched wild-type LMNA control (Figure 3.8Figure 3.8). Only one donor background showed a robust activation of aging markers in the progerin group, while the others showed minimal or inconsistent changes. A similar pattern was observed with SA-β-gal staining, which was detected only in the same donor line, when comparing progerin-expressing cells to its own LMNA-GFP control (Figure 3.9).

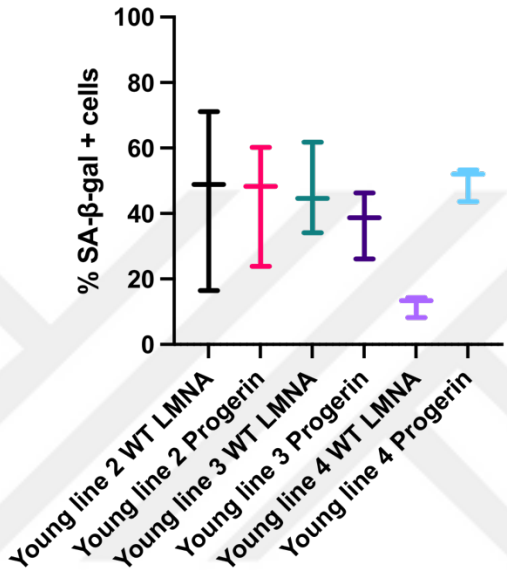

Figure 3.9: Percentages of senescent cells in WT LMNA and progerin-expressing young fibroblast lines.

Because this line consistently displayed strong induction of aging signatures across multiple assays, it was selected for further experiments. This line also had the highest relative progerin expression of all the tested cell lines (Figure 3.10).

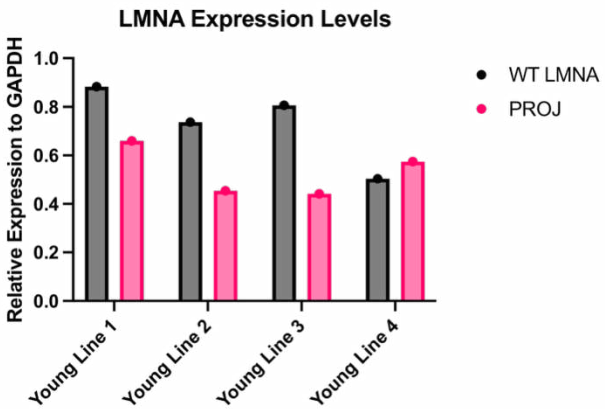

Figure 3.10: LMNA expression levels in WT vs. progerin-expressing young fibroblasts.

In-depth characterization of this selected line showed that progerin expression led to elevated levels of aging-associated genes (Figure 3.11). Higher mitochondrial ROS levels were also detected in the progerin-expressing line compared to the LMNA control (Figure 3.12). γH2AX levels were quantified to measure DNA damage however no significant differences were found (Figure 3.13). Similarly, histone marks H3K9me3 were unchanged while a significant decrease in H3K27me3 was observed suggesting a partial erosion of heterochromatin (Figure 3.14).

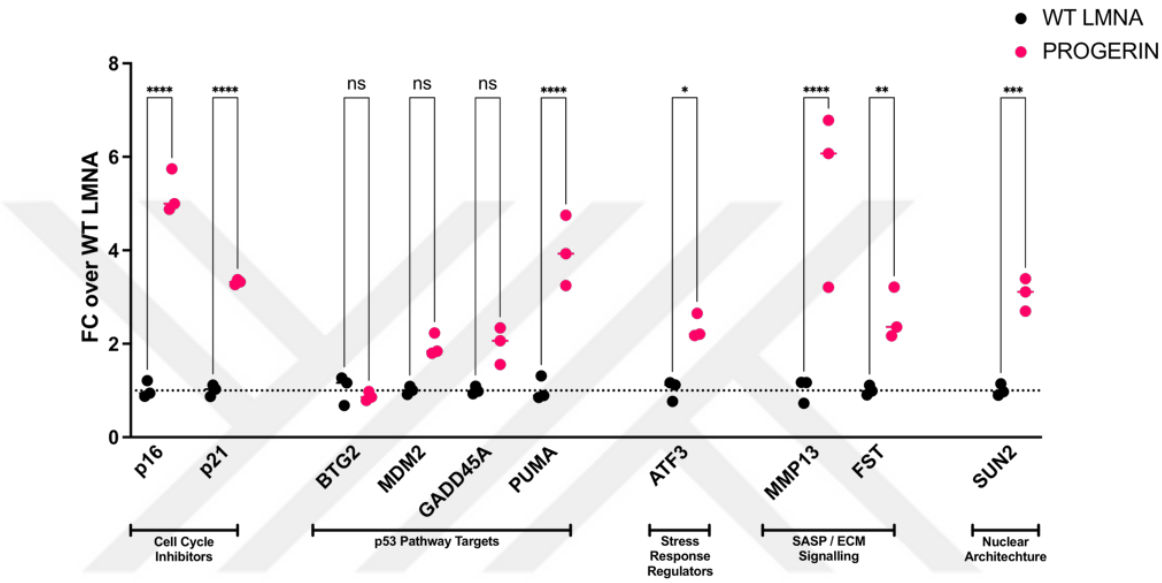

Figure 3.11: Expression of stress-related genes in WT LMNA and progerin-expressing fibroblasts.

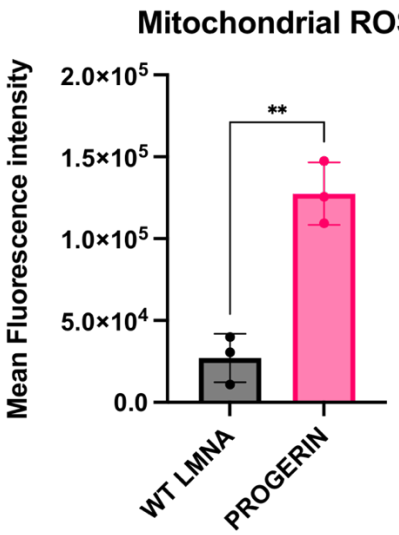

Figure 3.12: Mitochondrial ROS levels in WT LMNA and progerin-expressing fibroblasts.

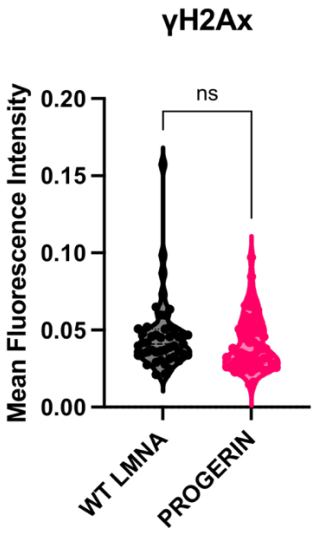

Figure 3.13: γH2AX levels in WT LMNA and progerin-expressing fibroblasts.

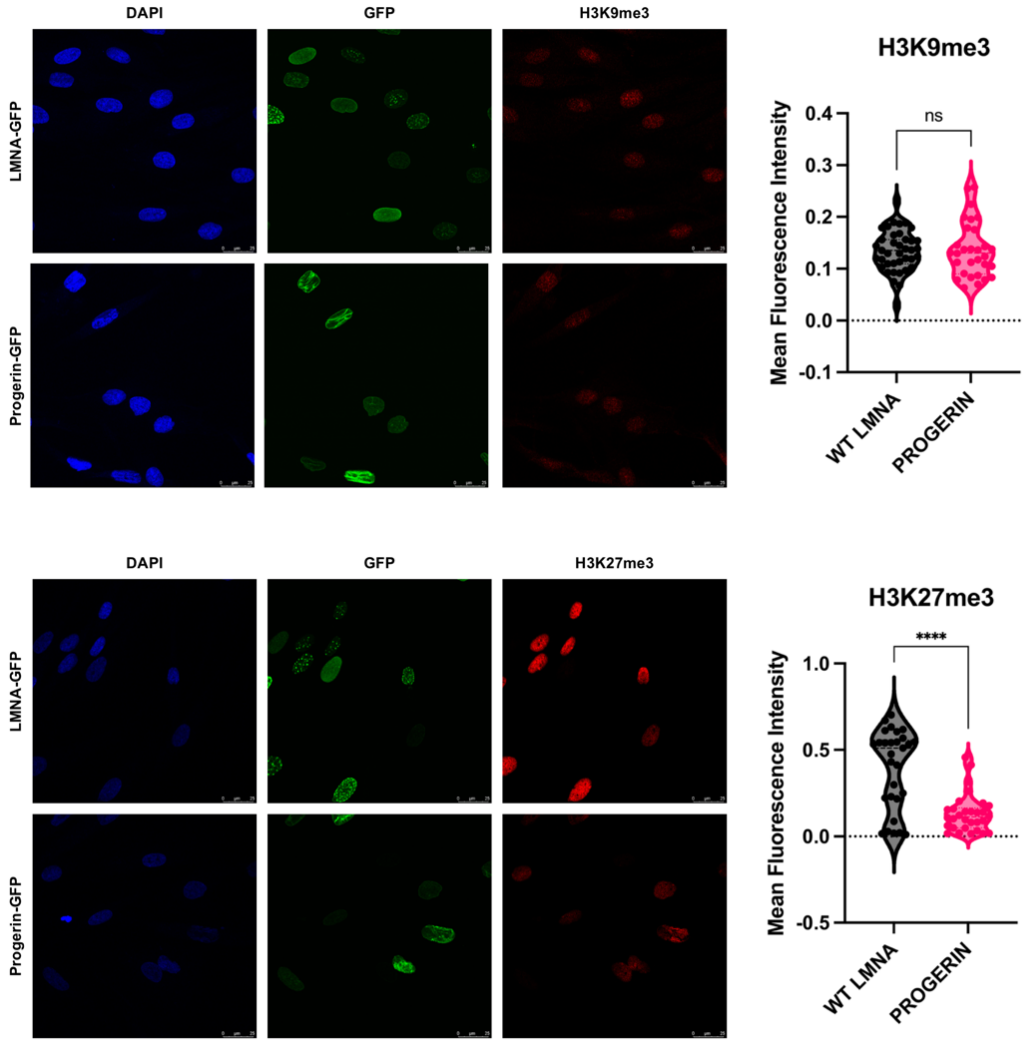

Figure 3.14: Quantification and representative images of H3K9me3 and H3K27me3 in WT LMNA and progerin-expressing fibroblasts.

3.4 Comparison of Initiation Dynamics Between Genetic and Chemical Reprogramming

We examined early cellular responses by tracking loss of fibroblast identity and acquisition of pluripotency markers. The fibroblast marker CD13 and the pluripotency marker TRA-1-60 were quantified over time (Figure 3.15). At day 2, no meaningful changes were detected in either condition. By day 4, TRA-1-60 positive cells emerged in the OSKM group, whereas no TRA-1-60 positive cells were observed under chemical reprogramming at this stage.

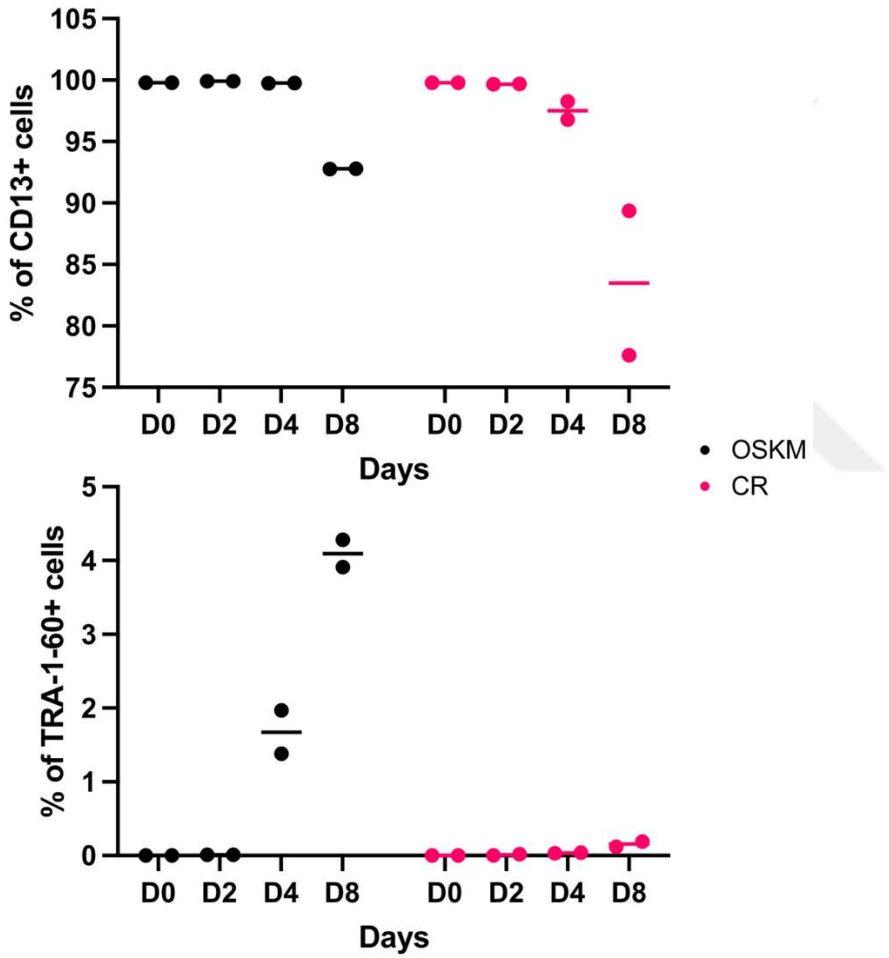

Figure 3.15: Dynamics of CD13+ and TRA-1-60+ populations during early reprogramming.

Despite lacking TRA-1-60 acquisition, chemically reprogrammed cells showed a greater loss of CD13 expression by day 4 compared to OSKM treated cells. This difference between reprogramming strategies was more pronounced by day 8, suggesting that chemical reprogramming exerts more homogeneous reprogramming pressure on cells. In this case, the population appears to progress in a “tighter” fashion. This was

evident in the histogram of CD13-stained cells on day 4: while almost all cells remained CD13+, chemically reprogrammed cells were uniformly CD13^low, whereas the OSKM group was heterogeneous, containing both CD13^low and CD13^high populations (Figure 3.16).

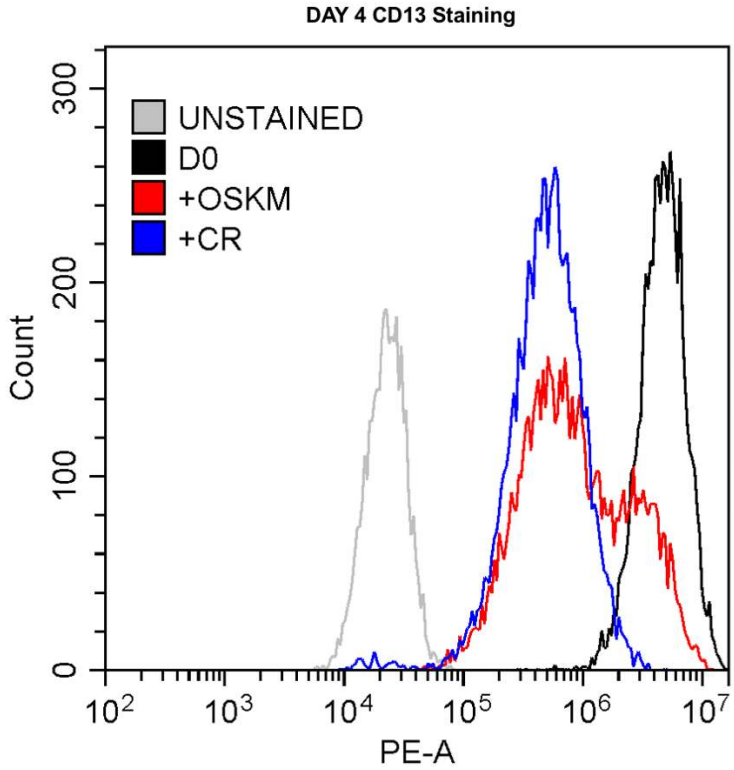

Figure 3.16: CD13 expression profiles of reprogrammed populations at day 4 (OSKM vs. chemical reprogramming).

3.5 Comparison of Stress Responses Between Genetic and Chemical Reprogramming

We analyzed stress induction in reprogrammed cells by examining both transcriptional and phenotypic markers. Gene expression was profiled at day 0, day 2, day 4, and day 8, focusing on canonical stress-related genes p16, p21, and ATF3. Beginning at day 4, OSKM-treated cells showed a significantly higher expression of these stress markers compared to chemically reprogrammed cells. This difference became more pronounced by day 8, suggesting a sustained stress response specific to genetic reprogramming.

To complement gene expression data with phenotypic readouts, we performed senescence-associated β-galactosidase (SA-β-gal) flow cytometry (Figure 3.17). By day

4, the OSKM group exhibited a marked increase in senescent cells relative to day 0. In contrast, chemical reprogramming led to a reduction in senescence, an outcome usually observed only after a chase period following partial reprogramming. On day 8 the OSKM-treated cells showed a further rise in senescence and the chemical reprogramming group displayed a lesser increase from its day 4 low point, though levels remained below day 0.

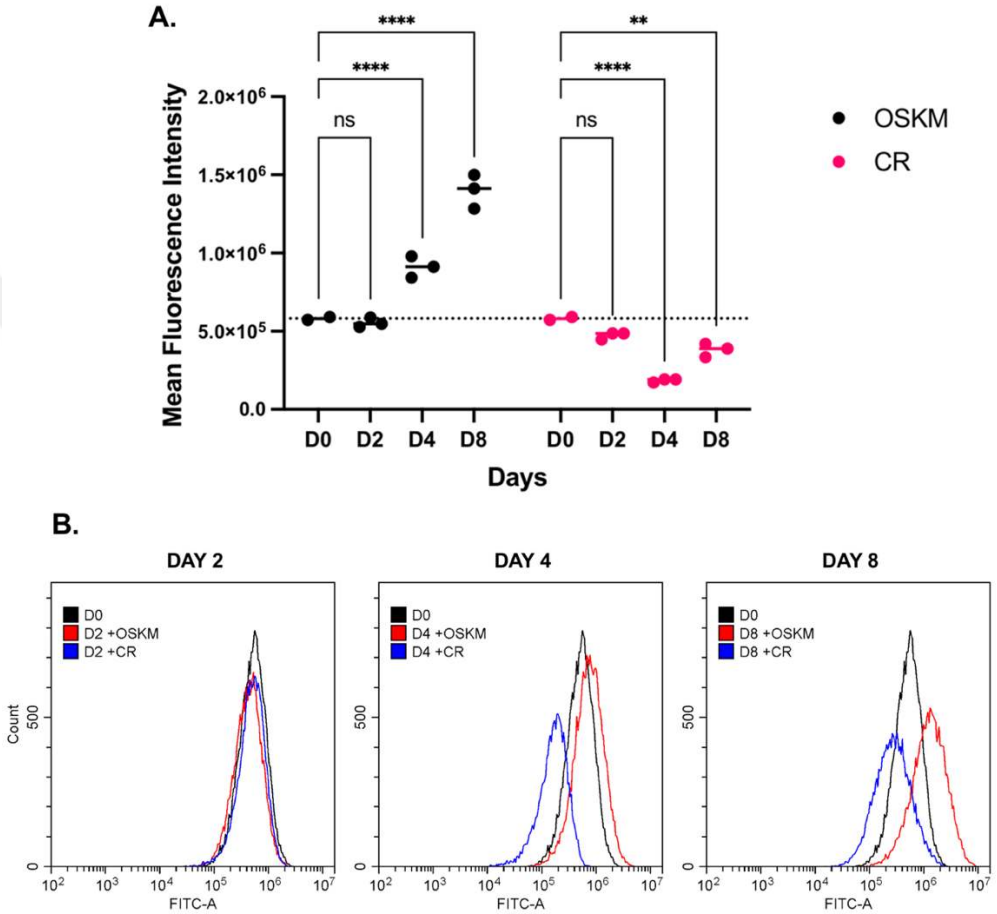

Figure 3.17: Senescence-associated β-galactosidase activity during early reprogramming. (A) Mean fluorescence intensity of β-gal staining quantified over time in OSKM and CR conditions. (B) Representative flow cytometry histograms of β-gal activity at day 2, 4, and 8 compared to baseline (D0).

To investigate whether the delivery method itself induced this stress response, we compared OSKM, chemical reprogramming, and a virus-control chemical reprogramming group, in which cells undergoing chemical reprogramming also received the same viral load (rtTA lentivirus) used for OSKM delivery. At day 4, this virus-control group showed increased senescence relative to chemical reprogramming alone, but the effect was considerably lower than that observed in OSKM (Figure 3.18). These results

suggest that transcription factor activity, rather than viral infection alone, accounts for much of the stress induced during OSKM reprogramming. Alternatively, some of the chemicals in the chemical reprogramming cocktail may negate the viral cellular stress induced by transduction.

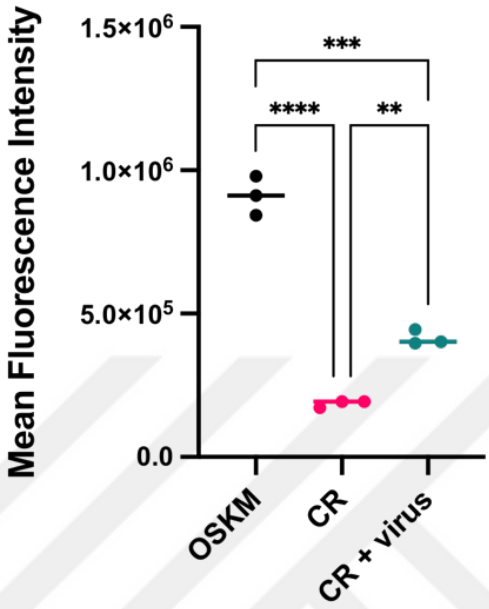

Figure 3.18: Effect of viral delivery on senescence during early genetic and chemical reprogramming.

3.6 Partial Reprogramming of Aged Fibroblasts

To test whether partial reprogramming could promote rejuvenation, aged fibroblasts were reprogrammed for two, four, or eight days, each followed by a four-day chase (Figure 3.19). This approach allowed us to evaluate whether transient exposure to reprogramming stimuli was sufficient to induce measurable changes once the treatment was withdrawn.

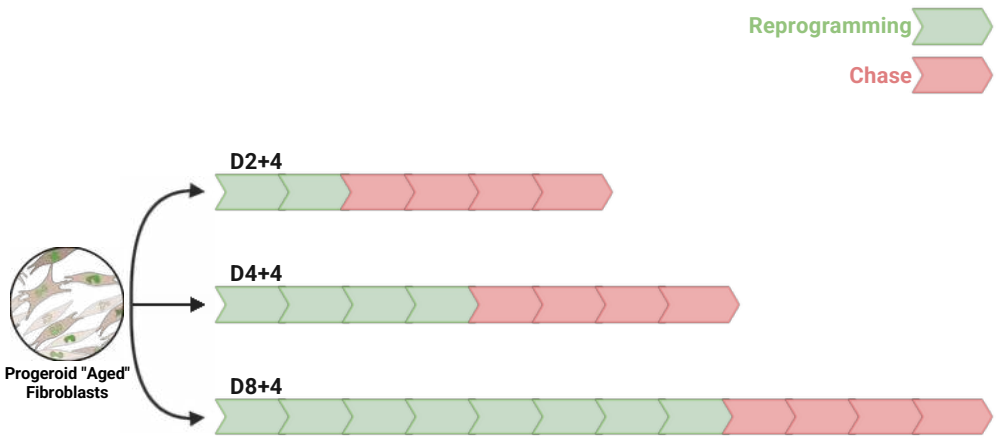

Figure 3.19: Partial reprogramming approach.

Analysis of age-associated gene expression revealed heterogeneous and inconsistent responses (Figure 3.20). Some markers showed transient increases followed by normalization, while others fluctuated at different stages depending on the reprogramming method. Early chemical reprogramming appeared to induce a stress response across several genes. Although the expression increase of these stress response genes was higher in chemical reprogramming, in cases where a rise was also observed in genetic reprogramming, chemical reprogramming displayed better recovery with extended reprogramming. Among the longer durations, stress decreased after two days but rose again with overly prolonged exposure; 8 days of reprogramming elicited a stress response, suggesting that 4 days is optimal for rejuvenation.

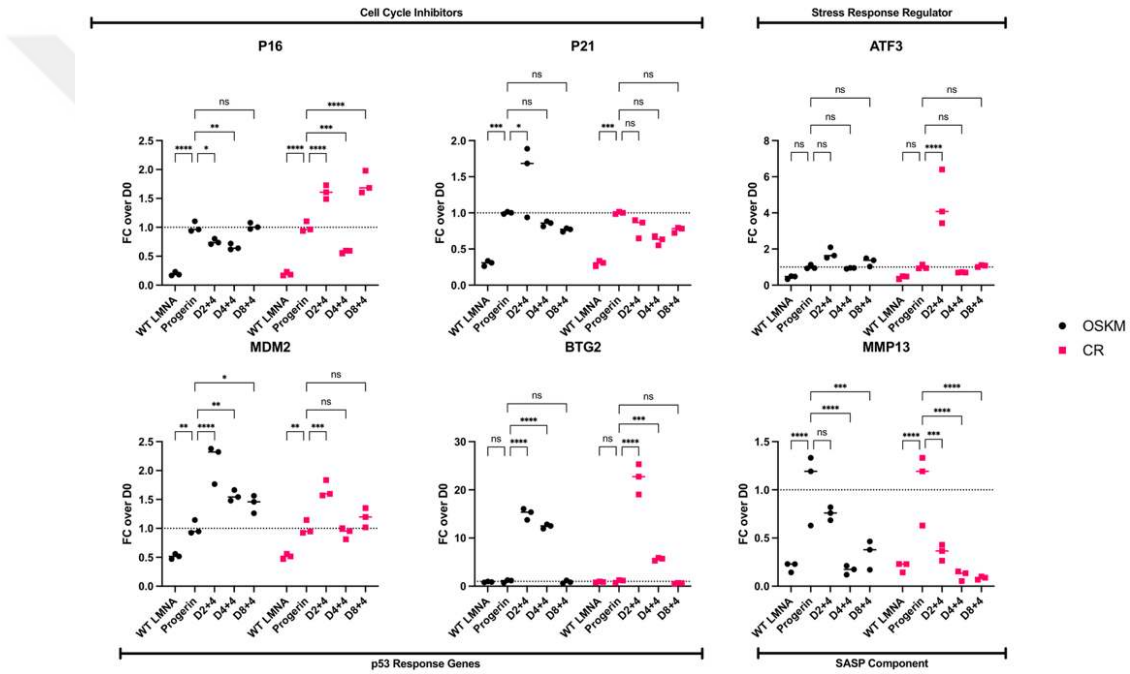

Figure 3.20: Stress/aging gene qPCR following chase-period partial reprogramming.

By contrast, senescence-associated β-galactosidase quantification yielded a clearer signal (Figure 3.21). Chemical reprogramming consistently reduced senescence across all conditions, with the strongest effect observed at the four-day reprogramming interval. This trend closely mirrored the data obtained without a chase, suggesting that chemical reprogramming directly alleviates senescence without requiring a recovery phase. OSKM reprogramming behaved differently. While the chase reduced senescence at the early time points, the effect was not durable, and by eight days senescence levels began to rise again.

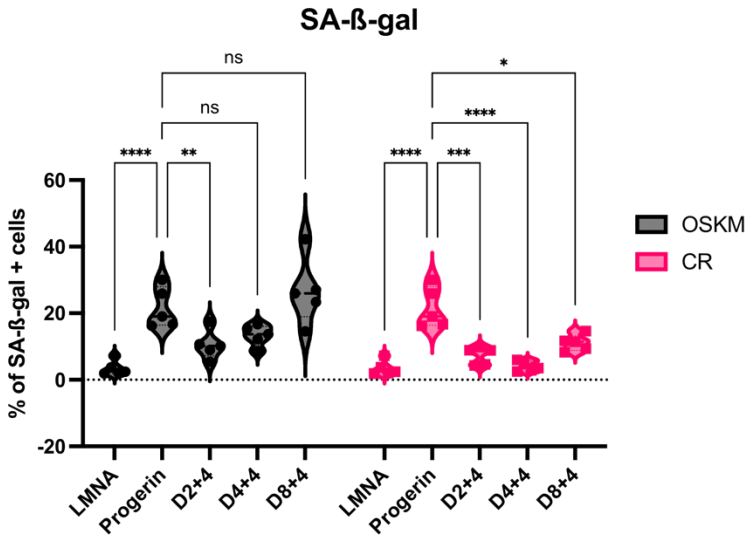

Figure 3.21: Senescence-associated β-galactosidase positive cell quantification following chase-period partial reprogramming.

Because of the clear difference in senescence levels between reprogramming modalities we examined the change in several p53 related genes (Figure 3.22). Expression of four target genes, p21, MDM2, GADD45A, and PUMA, showed a heterogeneous pattern between the two reprogramming modalities. In the OSKM group, all four genes increased by day 2, indicating a stronger activation of the pathway. In the chemical reprogramming group, only MDM2 and GADD45A increased, and both changes were smaller in magnitude than in OSKM.

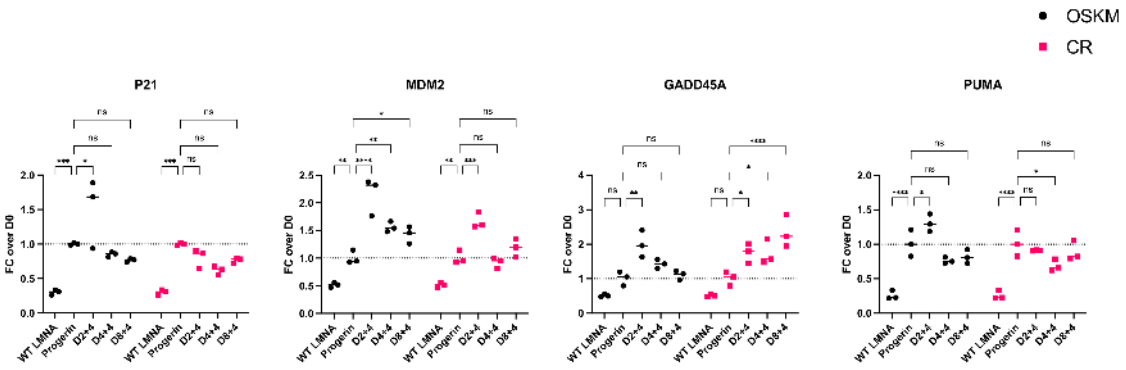

Figure 3.22: p53 response pathway gene qPCR following chase-period partial reprogramming

Mitochondrial ROS staining further distinguished the two strategies. Chemical reprogramming initially elevated mitochondrial ROS before returning to baseline, whereas OSKM reprogramming progressively reduced ROS levels, reaching significantly lower values than baseline by the later time points (Figure 3.23).

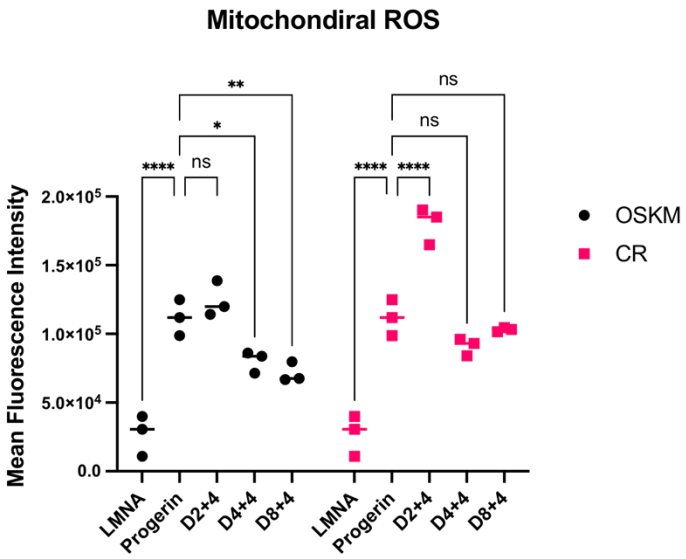

Figure 3.23: Mitochondrial reactive oxygen species levels after chase-period partial reprogramming.

These findings suggest that while chemical reprogramming is more effective at reducing senescence, it is less efficient at controlling mitochondrial oxidative stress. This raised the possibility that the duration and continuity of reprogramming exposure may influence the balance between rejuvenation and stress. To address this, we compared continuous eight-day reprogramming with a cyclic regimen in which cells underwent two independent four-day reprogramming periods separated by a chase phase (Figure 3.24). This design equalized the total reprogramming time but interrupted the exposure, allowing us to test whether phased reprogramming could sustain rejuvenation benefits without the adverse effects observed during prolonged continuous treatment.

Figure 3.24: Cyclic partial reprogramming setup.

Gene expression analysis of age-associated markers did not yield consistent or conclusive evidence for rejuvenation or for clear differences between protocols. The cyclic protocol did appear to increase stress-related markers, with this effect more pronounced in the chemical reprogramming condition (Figure 3.25).

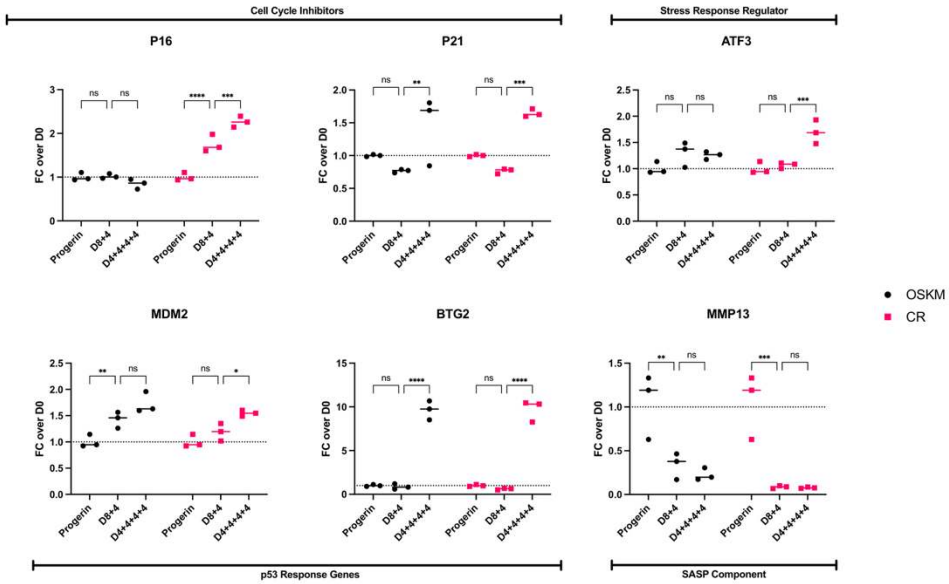

Figure 3.25: Stress/aging gene qPCR under constitutive versus cyclic reprogramming protocols.

By contrast, senescence-associated β-galactosidase staining revealed clear differences (Figure 3.26). In OSKM-treated cells, senescence levels did not significantly differ between day 0 and eight days of constitutive reprogramming, and the cyclic protocol produced no additional benefit, even leading to a mild increase in senescent cells. In chemical reprogramming, however, the cyclic protocol significantly enhanced the reduction of senescence beyond that observed with constitutive treatment. However, this may also be due to the longer overall experimental duration in the cyclic protocol, where rejuvenated cells had more time to outcompete senescent ones, rather than a greater intrinsic rejuvenation effect.

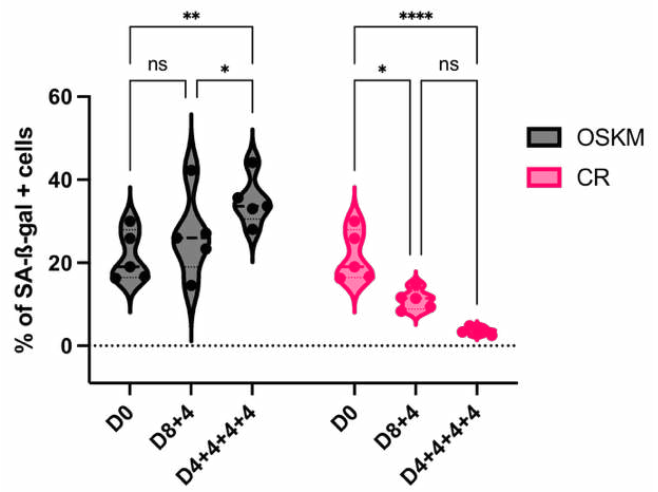

Figure 3.26: Senescence-associated β-galactosidase positive cell quantification under constitutive versus cyclic reprogramming protocols.

When we examined the p53 pathway under cyclic reprogramming, the results again showed a heterogeneous pattern (Figure 3.27). Both genetic and chemical reprogramming led to increased expression of p21 and MDM2. For GADD45A, expression rose under genetic reprogramming but decreased under chemical reprogramming. For PUMA, levels increased with genetic reprogramming but showed no significant change with chemical treatment. Overall, the cyclic protocol produced mixed outcomes across the four genes, with differences between modalities depending on the specific target.

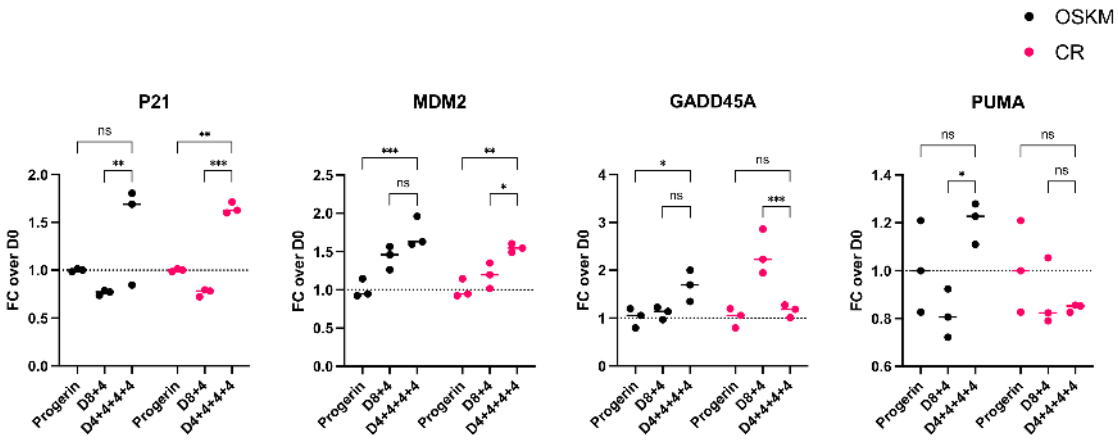

Figure 3.27: p53 response pathway gene qPCR under constitutive versus cyclic reprogramming protocols.

The cyclic application of partial reprogramming led to significant changes in mitochondrial ROS staining (Figure 3.28). While constitutive chemical reprogramming did not significantly reduce ROS compared to baseline, cyclic chemical reprogramming produced a marked decrease in mitochondrial ROS, even below the levels achieved with eight days of continuous OSKM reprogramming. OSKM-treated cells also showed reduced ROS further under the cyclic protocol, although the effect was less pronounced than in chemically treated cells. These results suggest that cyclic partial reprogramming particularly enhances the chemical regimen, effectively reducing both senescence and mitochondrial oxidative stress while providing limited additional benefit to OSKM-mediated reprogramming.

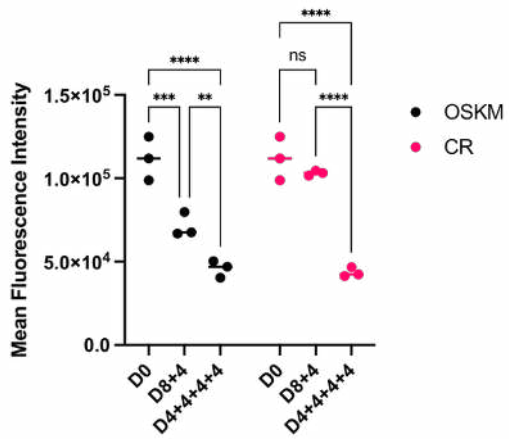

Figure 3.28: Mitochondrial ROS levels under constitutive versus cyclic reprogramming protocols.

Expression of fibroblast identity and tumorigenicity-associated genes was assessed at the conclusion of partial reprogramming experiments. COL1A1, a marker of fibroblast identity, showed no reduction under genetic reprogramming protocols. In fact, modest increases were observed at day 2+4 and in the cyclic protocol with two consecutive 4-day interventions. For chemical reprogramming, none of the standard continuous regimens produced a significant change in COL1A1 expression. Interestingly, the cyclic protocol yielded a clear reduction, suggesting that repeated shorter exposures exert a stronger effect on fibroblast identity than a single extended treatment (Figure 3.29).

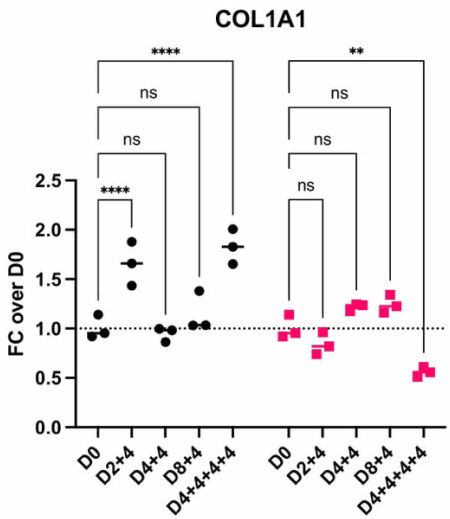

Figure 3.29: Expression levels of fibroblast gene COL1A1 across partial reprogramming timepoints.

Expression of hTERT, a key regulator of cellular immortalization, was also evaluated (Figure 3.30). No detectable transcripts were observed in any condition, including the longest (8-day) protocols. These findings indicate that partial

reprogramming did not activate telomerase and therefore did not trigger one of the most common routes toward uncontrolled proliferation.

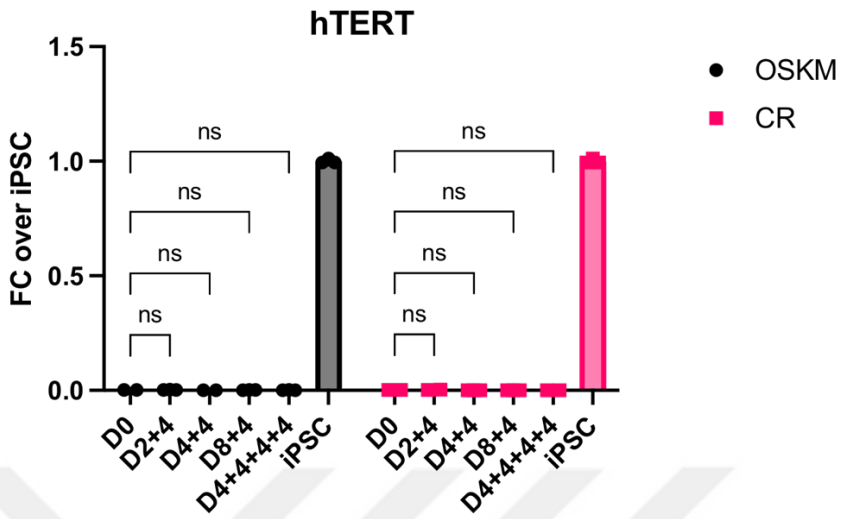

Figure 3.30: Expression levels of hTERT across partial reprogramming timepoints.

To test whether the senescence-reducing effects of partial reprogramming observed in progerin-expressing fibroblasts could also be detected in naturally aged cells, we performed a parallel experiment using four independent fibroblast lines isolated from elderly donors (Figure 3.31). Based on the earlier results, the protocol was limited to four days of reprogramming followed by a four-day chase, the condition that produced the strongest senescence reduction in the progerin background. SA-β-gal flow cytometry revealed a decrease in senescent cells under both OSKM and chemical reprogramming relative to baseline. However, unlike the progerin model, there was no significant difference between the two approaches in wild-type aged fibroblasts. The magnitude of senescence reduction in the chemical reprogramming group was consistent with previous results, whereas the reduction observed in OSKM-treated aged fibroblasts diverged from the progerin model. This discrepancy may reflect differences in baseline stress responses between naturally aged and progerin-expressing cells.

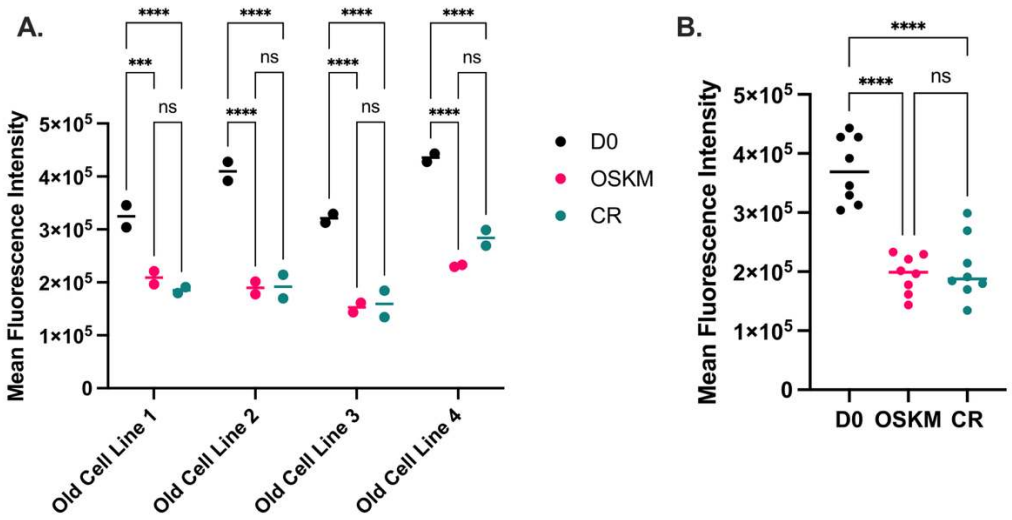

Figure 3.31: Senescence-associated β-galactosidase activity in fibroblasts from aged donors after partial reprogramming. (A) SA-β-gal flow cytometry results for four independent aged fibroblast lines after four days of OSKM or chemical reprogramming followed by a four-day chase. (B) Pooled quantification of senescence levels across the four lines.

3.7 Identification of Key Chemicals in Reprogramming Cocktail

To evaluate the contribution of individual compounds in the reprogramming cocktail, we first tested their effects on cell viability. Each of the 14 chemicals was applied separately to fibroblasts and compared to a DMSO control (Figure 3.32). Among all tested compounds, only CHIR99021, a GSK-3β inhibitor and Wnt pathway activator, produced a measurable increase in cell viability. Several other compounds, including DZNep, AKT kinase inhibitor, and TTNPB, significantly reduced viability, though none to the same extent as the full cocktail containing all components. This suggests that the marked reduction in viability observed under complete cocktail treatment is not attributable to any single agent but instead reflects cumulative or synergistic stress.

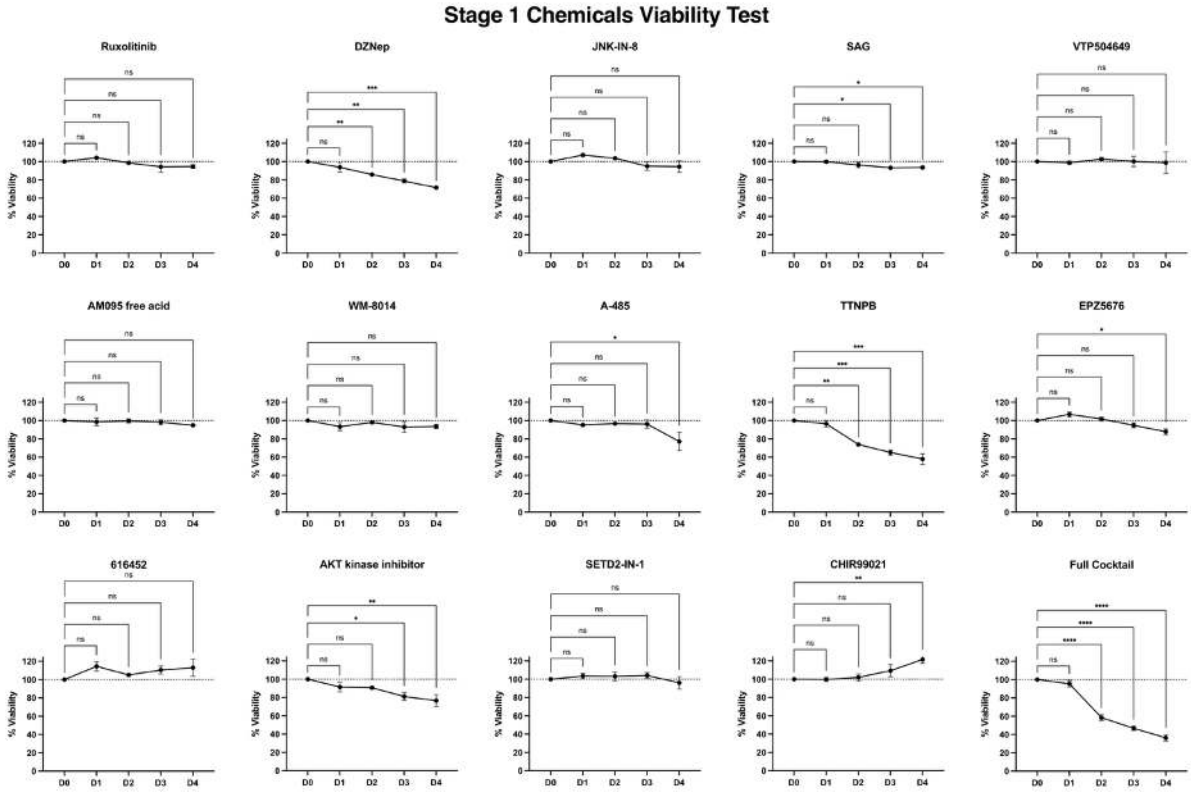

Figure 3.32: Individual effects of Stage 1 chemicals on fibroblast viability.

We next tested how removing individual compounds affected reprogramming readouts. Senescence-associated β-galactosidase and CD13 mean fluorescence intensities were quantified (Figure 3.33). The complete cocktail provided the reference profile. Several single-omission conditions deviated from this profile, while a subset closely matched it. Omission of TTNPB yielded the lowest CD13, whereas omission of RepSox increased CD13 and senescence signals relative to the cocktail.

Although individual bar plots of SA-β-gal and CD13 intensities provide marker-specific comparisons, significance was limited. To extract more information from the data, we plotted normalized CD13 against SA-β-gal. This coordinate system allowed us to visualize how single compound omissions alter both markers simultaneously. The full cocktail occupied a position characterized by relatively low CD13 and intermediate SA-β-gal. Several omissions, such as SETD2-IN-1, overlapped closely with this reference point, indicating minimal deviation. In contrast, omission of TTNPB produced the lowest CD13 signal while shifting strongly upward in SA-β-gal, placing it in the lower-right quadrant of the plot. Suggesting that it is essential for chemical reprogramming-based rejuvenation as without it SA-ß-gal levels remain high and CD13 levels are significantly less, both of which is undesirable for rejuvenation. Omission of VTP504649 showed the

opposite pattern, increasing both CD13 and SA-β-gal relative to the cocktail and occupying the upper-right quadrant. Suggesting that it is detrimental to rejuvenation and should be omitted. Other compounds, such as A-485 and AKT kinase inhibitor, shifted primarily along the SA-β-gal axis with less effect on CD13.

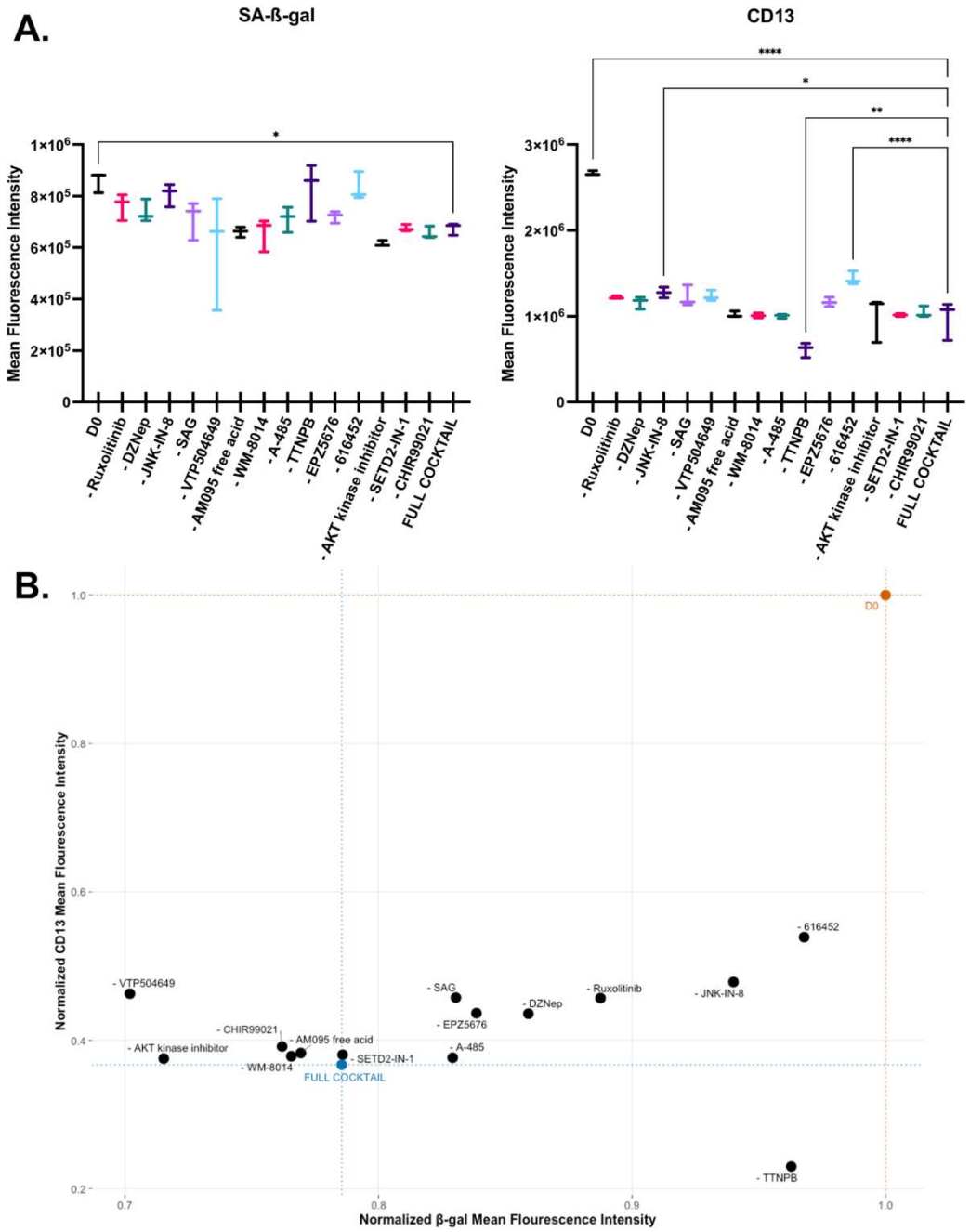

Figure 3.33: Effects of stage 1 chemicals on senescence and fibroblast identity. Senescence-associated β-galactosidase (SA-β-gal) and CD13 mean fluorescence intensities under single-compound omission conditions (no statistical significance was detected between unmarked columns) (A) and two-dimensional mapping of normalized CD13 vs. SA-β-gal intensities (B).

CHAPTER 4: DISCUSSION

In this study, we directly compared genetic and chemical approaches to partial reprogramming in the context of cellular rejuvenation. Our results demonstrate that both methods can induce markers of a more youthful state in aged human fibroblasts, but they do so with distinct dynamics and outcomes. The genetic approach produced rapid and pronounced changes in a subset of cells, whereas the chemical cocktail led to more gradual, population-wide changes. For example, we observed that OSKM induction quickly led to a population expressing pluripotency markers in a small fraction of cells while the majority of cells remained unchanged. In contrast, the chemical reprogramming protocol did not generate any fully pluripotent colonies within the same timeframe. Instead, it drove a coordinated shift in the whole cell population's phenotype without any single cell leaping ahead to an iPSC-like state. Consistently, fibroblasts undergoing chemical treatment showed a uniform partial loss of their somatic marker CD13 by day 4, whereas OSKM-treated cultures were highly heterogeneous at this early stage. A sub-population had downregulated CD13, and within that a smaller population had begun acquiring pluripotency related markers, while many others remained essentially in their original state. These findings confirm that the chemical regimen induces a more synchronous, homogeneous partial reprogramming, whereas the classical genetic method tends to produce a mixed population of unreprogrammed and fully reprogrammed cells.

Despite these differences, both approaches yielded signs of cellular rejuvenation. Partially reprogrammed aged fibroblasts exhibited improvements in several age-associated phenotypes. Together, these results underscore that transient reprogramming, driven either by OSKM or by small molecules can indeed rejuvenate somatic cells to a measurable extent. Furthermore, through systematic variation of the chemical cocktail, we identified specific components that are most crucial for this rejuvenation effect. In particular, systematic removal experiments revealed that removing certain epigenetic modulators from the cocktail diminished the reduction of senescence and led to greater losses of CD13, pointing towards key chemical drivers of the reprogramming process. The strongest drivers of rejuvenation in our subtraction experiments was the RAR pathway agonist TTNPB. Removal of TTNPB led to an impairment of senescence reduction and reduced CD13, indicating a central role for RAR signaling in senescence control and identity maintenance. However, TTNPB also produced a substantial viability loss by day 4 (≈58% of baseline). TTNPB is anti-proliferative and known to induce

apoptosis at high concentrations, its apparent effects on senescence may in part reflect selective elimination of damaged or senescent cells, rather than genuine rejuvenation (Sah et al., 2002; Colucci et al., 2024). Conversely, the removal of VTP504649 (menin–MLL interaction inhibitor) lowered β-gal and increased CD13 compared with the full cocktail. Its removal decreases senescence while increasing CD13, completely in line with rejuvenation goals, suggesting it should be removed from a rejuvenation cocktail. This is consistent with studies showing that the menin–MLL complex maintains transcriptional programs tied to lineage stability, and its inhibition can disrupt somatic gene expression (Janssens et al., 2024). The AKT kinase inhibitor and A-485 (p300/CBP inhibitor) illustrate opposite senescence effects with little CD13 change. Removing the AKT inhibitor decreased β-gal levels whereas removing A-485 increased β-gal levels, both with essentially unchanged CD13 levels. This supports the exclusion of the AKT kinase inhibitor and the inclusion of A-485 in an ideal rejuvenation cocktail. The role of p300/CBP in aging and reprogramming has been highlighted in recent work, where dysregulated acetyltransferase activity contributes to senescence-associated chromatin states (Sen et al., 2019; Di Fede et al., 2025). For other chemicals the inclusion and exclusion decision was more nuanced. TGF-β inhibition showed a clear trade-off. Removing 616452 increased β-gal and increased CD13, indicating that TGF-β blockade is required to suppress senescence but can erode fibroblast identity in this setting. It's effect on cell viability remained largely stable across 4 days. Suggesting that the reduction in senescence upon addition is not explained by cytotoxicity, but by specific pathway modulation. This is consistent with extensive evidence that TGF-β signaling promotes senescence, fibrosis, and impaired stem cell function in aging tissues (Tominaga & Suzuki, 2019). The centrality of TGF-β inhibition to reprogramming is also well established, including its ability to replace SOX2 and facilitate progression through intermediate states (Ichida et al., 2009; Katsuno & Derynck, 2021). The present data suggest that dose, timing, or cyclic application will be necessary to capture senescence benefits without excessive identity loss. Similarly, JNK-IN-8 led to an increase in senescence markers with a comparatively more modest increase in CD13, placing it in the same functional category as RepSox. Other notable effects included the removal of Ruxolitinib, which modestly raised β-gal levels while increasing CD13 relative to the full cocktail. DZNep (often used as an EZH2/H3K27me3-lowering agent) and EPZ5676 (DOT1L inhibitor) also contributed to senescence control. Their removal raised β-gal while increasing CD13. Taken together, these results indicate that the chemicals identified

here fall into distinct functional groups. Some, such as TTNPB and A-485, directly support rejuvenation by reducing senescence or stabilizing identity. Others, including RepSox and JNK-IN-8, exert trade-offs that require careful control of dose and timing. Still others, such as VTP504649 and AKT kinase inhibitor, appear detrimental in this context. Distinguishing between these roles clarifies which agents are required for reprogramming and which fine-tune rejuvenation outcomes. Future studies should focus on how these categories can be combined into cocktails that maximize rejuvenation while minimizing loss of identity and viability.

While both genetic and chemical approaches can elicit partial reprogramming, their cellular dynamics and mechanisms appear to differ significantly, and many unknowns remain about how each method operates over time. One difference we observed was in the induction of senescence and stress-related pathways during reprogramming. The OSKM-based approach triggered an acute stress response in fibroblasts: within days of factor induction, cultures showed elevated SA-β-gal activity as measured by increased mean fluorescence intensity in flow cytometry. By day 4 of OSKM expression this stress signal was clearly elevated, whereas chemically treated cells showed a decrease in senescence markers over the same period. This suggests that genetic reprogramming imposes a higher immediate stress burden on cells, likely due to the potent oncogenic stimuli from factors like c-MYC. Indeed, cell fate mapping studies have noted that OSKM reprogramming tends to provoke apoptosis or oncogene-induced senescence in a large fraction of cells, which is one reason why only a small minority (often <1-5%) of somatic cells successfully become iPSCs in these conditions (Zviran & Hanna, 2014). Our findings are consistent with those reports: OSKM overexpression can drive cells into a DNA-damage response or permanent arrest even as it pushes others toward pluripotency. In contrast, the chemical cocktail acted through multi-target modulation and avoided triggering such acute responses in any single pathway. Both OSKM and chemical reprogramming led to increases in p53 targets, but the extent of the response was greater under OSKM. In chemical reprogramming the p53 signal was milder and transient, suggesting a different form of regulation. Recent comparative analyses have similarly found that a 7-factor chemical cocktail upregulates p53 even as it rejuvenates mouse cells (W. Mitchell et al., 2024). This raises important questions about whether p53 activity is essential for reprogramming or rejuvenation, or whether its activation primarily reflects stress that could be mitigated. The contrasting senescence

changes with similar p53 responses between reprogramming modalities suggest it is possible that p53 serves as a safeguard to limit uncontrolled proliferation during chemical reprogramming, while in OSKM conditions it is more directly tied to oncogene-induced stress. Future studies will need to determine whether selective modulation of p53 can enhance rejuvenation outcomes by retaining its protective roles while minimizing excess stress on cells.

Another key difference in dynamics is related to cell proliferation. Efficient OSKM-mediated reprogramming typically requires several rounds of cell division. Cell cycle acceleration is thought to help remodel the epigenome and overcome barriers to pluripotency (Hu et al., 2019). However, chemical partial reprogramming can rejuvenate cells despite reducing their proliferation rate. It raises the possibility that the chemical cocktail engages more direct chromatin or metabolic remodeling processes that do not rely on passive dilution of epigenetic marks through cell division. By contrast, OSKM's reliance on rapid cell cycling may be one reason it operates in a more "hit-or-miss" fashion. Only the cells that manage to both proliferate and tolerate oncogenic stress can reset their epigenome, while others fall behind.

The exact sequence of molecular events triggered by the small molecules versus the Yamanaka factors remains incompletely understood. Both methods ultimately converge on activating some pluripotency-associated genes and silencing some age-associated genes, but the timing and degree of these changes vary. Failing to account for these differences may introduce confounding factors leading to false outcomes. One difficulty involves the synchronizing / equalizing of the "reprogramming pressure" between genetic and chemical methods. There is no simple way to quantify what, for example, a higher viral titer or stronger OSKM expression corresponds to in terms of small-molecule dosage or combination. In our study, we controlled obvious parameters (treating cells for the same duration, using optimized concentrations from literature for the cocktail, etc.), but we acknowledge that the two modalities might not have been perfectly matched in strength. It is possible that our OSKM protocol pushed cells closer to a pluripotent threshold than the chemical protocol did (or vice versa), simply due to intrinsic differences in how these interventions act. This underscores a broader issue in the field, reprogramming stimuli lack a common metric. Genetic factor induction is typically binary at the single-cell level, whereas chemical exposure is graded and affects all cells to some extent. Thus, even if the average outcomes are compared at a fixed time,

one method could be driving a subset of cells much further along the reprogramming trajectory. This is a limitation of our head-to-head comparison. Future work should seek better ways to calibrate reprogramming intensity across platforms. Perhaps by using intermediate biomarkers of reprogramming progression as a guide to adjust dosing or timing. Developing such quantitative measures would help ensure that when we compare genetic and chemical methods, we are doing so at equivalent stages of advancement. Until then, conclusions about one approach being "stronger" or "faster" than the other must be drawn with caution. In our data, we interpret differences in outcomes as largely method-inherent, but we cannot exclude that some disparities are due to one method simply overshooting or lagging behind the other under the conditions tested.

One of the most salient differences between the two reprogramming strategies lies in the heterogeneity of cell fate outcomes, and this has important implications for potential therapeutic use of partial reprogramming. As noted above, genetic OSKM reprogramming tends to be a stochastic, highly variable process at the single-cell level (Shakiba et al., 2019). Even in a controlled in vitro setting, only a small fraction of cells cross the threshold into an iPSC-like state, some fraction stalls in intermediate states, and many cells fail to reprogram (or undergo stress-induced cell cycle arrest). This scenario can be likened to a "catapult" effect: OSKM provides a powerful push that propels a few "lucky" cells over the wall into pluripotency, while the rest of the cells remain behind. In our experiments, by the end of an 8-day induction we could clearly identify a minority population in OSKM-treated cultures that had fully silenced somatic markers and turned on pluripotent markers, whereas the majority of cells still retained their original identity. This kind of heterogeneity is problematic for *in vivo* or regenerative therapy applications of reprogramming as if one or a few cells in a tissue were to overshoot into a pluripotent state, those cells could form teratomas or disrupt tissue architecture (Abad et al., 2013). Indeed, the safety of partial reprogramming *in vivo* hinges on preventing any cells from fully dedifferentiating. Past studies using transient OSKM expression in mice have shown that it is possible to reverse aging markers without apparent tumor formation, but this required tight temporal control (e.g. controlling duration through inducer drugs given in short pulses, or omission of the c-MYC factor) to stop the process before any cell went past the point-of-no-return (Lu et al., 2020; Ocampo et al., 2016). Longer continuous induction periods would lead to the risks sharply rising. Our findings reinforce this concern: OSKM expression must be terminated at an earlier at an early stage to achieve

“partial” reprogramming. If we had continued the genetic induction further, the heterogeneity of the population would have only grew. There is essentially no built-in brake with OSKM, a cell that is on a trajectory toward pluripotency will continue unless we actively intervene to halt factor expression. This necessitates extremely careful tuning in any therapeutic scenario. Chemical reprogramming, by contrast, behaved in our study like a more collective and controlled rejuvenation force. Continuing the earlier analogy, the chemical cocktail acts more as a “battering ram” that pushes the entire group of cells gradually forward, rather than a catapult that hurls a few cells far ahead. In practical terms, we did not observe any singular cells in the chemically treated populations that acquired overt pluripotent characteristics on their own. Even when extending the chemical treatment a bit longer, we saw the population as a whole tending toward a partially dedifferentiated state (for example, broad loss of the fibroblast surface marker CD13), but no individual cell broke out into a fully reprogrammed iPSC state. From a therapeutic perspective, the uniform partial reprogramming induced by small molecules is highly desirable. Ideally, one would want all cells in a tissue to rewind some aspects of their age, but no cell to lose its specialized identity entirely. Our results suggest that the chemical approach inherently leans toward this outcome. By requiring multiple coordinated molecular changes and having a lower overall potency per cell, the cocktail effectively raises the “dedifferentiation threshold” where cells only progress to an intermediate youthful state and do not easily jump to full pluripotency. This is supported by literature showing that purely chemical reprogramming passes through distinct intermediate cell states that are plastic but still lineage-committed, and only with additional steps and time do they eventually become iPSCs (Guan et al., 2022; Liuyang et al., 2023; Y. Wang et al., 2025). Thus, there are natural checkpoints in chemical reprogramming that may prevent overshooting. For instance, chemical-treated fibroblasts after one week expressed higher levels of certain early-development transcription factors (like KLF4 and ESRRB) but paradoxically had lower expression of late pluripotency genes like NANOG or endogenous OCT4 compared to controls, indicating a state that is neither fully somatic nor fully pluripotent (W. Mitchell et al., 2024a). This kind of state could be ideal for rejuvenation: cells might regain youthful gene expression and functionality while still “remembering” their original tissue identity. These observations highlight a potential advantage of chemical partial reprogramming: it may rejuvenate tissues more coherently, leading to incremental functional gains across the board, rather than creating a mosaic of highly rejuvenated and unchanged cells. For therapeutic use in aging or degenerative

diseases, this coherent action could translate to better tissue-wide outcomes. For instance, improving organ function without the risk of a few cells turning cancerous. Indeed, the principle has already been demonstrated in organismal models: a recent study in C. elegans showed that a two-drug partial reprogramming treatment extended lifespan by over 40% while reducing markers of aging like DNA damage and oxidative stress across the worm's tissues (Schoenfeldt et al., 2025). While, short-term OSK expression in mouse tissues also improved regenerative capacity without generating tumors, the safety profile of a chemical approach appears inherently more favorable, since it lacks any integrating viruses or transgenes and did not drive any cells into uncontrolled proliferation (Lu et al., 2020). However, an important caution is that the longer-term effects and potential hidden risks of chemical reprogramming are still unknown.

In previous chemical partial reprogramming studies a modest activation of p53 was observed (W. Mitchell et al., 2024a). One concern is that the mild activation of p53 could over extended treatment and lead to an accumulation of senescent cells or a growth disadvantage in certain cell types. While this can also be considered an advantage as p53 guards against neoplastic transformation it may promote a senescent phenotype if sustained. In our 1-2 week experiments, this was not a major issue, senescence did not overtly increase in the chemical group but for any future *in vivo* application, one must monitor whether repeated or chronic exposure to these chemicals could exhaust stem cell pools or induce premature senescence in certain compartments. Balancing rejuvenation and safety will thus require not only choosing the right method (genetic vs chemical) but also fine-tuning the treatment schedule to maximize benefits and minimize any heterogenous or adverse outcomes.

While our study provides a side-by-side look at genetic and chemical partial reprogramming, it has several limitations that must be acknowledged. Our work was done in vitro on fibroblast models (including both physiologically aged cells and a premature aging model). Cell culture conditions, especially for reprogramming, are reductive systems, they lack the complexity of a tissue environment that could modulate reprogramming outcomes. Thus, the absolute efficacy and safety profiles we observed may not translate directly to an *in vivo* setting. For instance, in an organism, there are immune responses, extracellular matrix constraints, and cell-cell interactions that could either impede reprogramming or conversely, amplify unwanted outcomes like fibrosis or tumorigenesis if a few cells stray. Another limitation is that we primarily focused on short-

term outcomes. We did not follow the partially reprogrammed cells long after the treatment cessation. It remains unclear whether the rejuvenation effects we induced are long-lasting or transient. A study suggest that cells can maintain the resetting of aging marks until periods up to 4 weeks after OSKM discontinuation (Gill et al., 2022). In our case, due to time constraints, we assessed markers soon after the intervention; a more thorough evaluation would involve letting the cells grow for additional population doublings post-treatment to see if, for example, epigenetic age measures remain low or if the cells begin to re-acquire age-related changes. The observed phenotype with OSKM mediated partial reprogramming may be different with chemical partial reprogramming. We also note that our analysis of rejuvenation was largely correlative, we demonstrated changes in molecular hallmarks of aging (like stress resistance, heterochromatin levels, and gene expression profiles), but we did not directly test functional rejuvenation at the organism or tissue level. In a therapeutic context, improved markers are only valuable if they confer functional benefits (e.g., improved wound healing, muscle strength, or cognitive function in an animal model of aging). Testing such functional outcomes was outside the scope of our cell-based study, but it is a crucial next step. Finally, there may be unforeseen risks that our in vitro system could not reveal. For instance, even if partial reprogramming is not mutagenic per se, it might select for pre-existing mutant cells that have a proliferative edge, potentially leading to clonal expansion of less healthy cells over time. This kind of subtle risk would only become evident in long-term studies or *in vivo* scenarios. Thus, while our comparison sheds light on immediate differences between genetic and chemical reprogramming, it is by no means a complete assessment of their safety or utility in a clinical sense.

Our findings open several avenues for further research. A clear priority is to better understand the mechanistic basis of the rejuvenation we achieved with each method. It will be informative to dissect how genetic factor overexpression versus small-molecule treatment reconfigures the epigenome and transcriptome over time. For example, applying single-cell multi-omics profiling throughout the reprogramming time course could map the trajectories and intermediate cell states unique to each method. Such studies might identify specific “rejuvenation nodes”, key regulatory genes or chromatin marks that are reset by both methods, as well as nodes that are uniquely engaged by the chemical cocktail or by OSKM. From a translational perspective, the obvious next step is to move beyond cell culture and test partial chemical reprogramming *in vivo*. The allure

of a drug-based rejuvenation therapy is significant, small molecules can in principle be delivered to specific tissues or systemically, avoiding the need for gene therapy vectors. Early proof-of-concept in simpler organisms is encouraging, but mammals present a far higher bar (Schoenfeldt et al., 2025). One challenge will be delivery: ensuring that the cocktail reaches target cells in an old animal at sufficient concentrations and with appropriate timing. The pharmacokinetics and potential off-target effects of each component will need careful evaluation. It may turn out that refining the cocktail for *in vivo* use (perhaps by using more potent or stable analogues, or by encapsulating the drugs for targeted release) will be necessary. Another challenge is monitoring the extent of reprogramming *in vivo*. Developing non-invasive biomarkers (such as blood-based epigenetic clocks or imaging tracers for rejuvenation) could greatly aid these studies. It is conceivable that partial reprogramming in situ will have different constraints; for example, certain tissues might be more permissive than others. Past experiments with OSK gene delivery in mice have shown mixed outcomes: some tissues can undergo cycles of reprogramming with functional improvements (e.g. muscle stem cell activation in aged muscle (C. Wang et al., 2021) or vision restoration in retinal neurons (Lu et al., 2020)), whereas others are prone to damage if pushed too far (Chen et al., 2021).Thus, a tissue-specific approach might be needed, tailoring how aggressively to induce partial reprogramming depending on the cell type. Chemical cocktails might offer more flexibility here; perhaps different formulations could be used for neural cells vs. connective tissue cells to achieve the right degree of rejuvenation.

It is also worth exploring repeated or cyclical partial reprogramming. In our study we applied a one-time intervention and measured outcomes shortly after. But aging is a continuous process; to truly counteract aging, one might need to periodically re-treat cells. Cyclic reprogramming using OSKM (with rest periods between inductions) has been shown to be beneficial in mouse models of progeria and natural aging (Macip et al., 2024; Ocampo et al., 2016). A similar cyclic paradigm could be attempted with chemical treatments, assuming the short-term effects are reversible and safe. For instance, perhaps a monthly dose of a reprogramming drug cocktail could keep aging markers at bay in a given tissue without overt side effects. Testing such regimens will require long-term animal studies, which are understandably complex, but they are crucial to determine whether partial reprogramming yields sustained rejuvenation or if cells eventually return to an old state (or become resistant to reprogramming stimuli). In parallel, more work is

needed to assess what aspects of aging are not addressed by partial reprogramming. Studies suggest that epigenetic and transcriptomic age can be rolled back, metabolic function can improve, and proteostasis and oxidative stress resistance can be enhanced (Cipriano et al., 2024). However, some hallmarks of aging might remain unresolved, for example, telomere attrition was not corrected by partial reprogramming in prior studies, and we likewise did not see any significant telomerase activation or telomere elongation in our short-term treatments (Paine et al., 2024). This means that even rejuvenated cells could still carry a form of "molecular clock" in their telomeres that limits their ultimate replicative lifespan. Combining partial reprogramming with telomere restoration strategies might be necessary for a more comprehensive rejuvenation outcome. Additionally, careful analysis of genomic stability in partially reprogrammed cells is needed. Although the process does not seem to inherently cause new mutations, it may select for certain cells as discussed, and any skewing of cell populations could have long-term consequences like fibrosis or cancer predisposition.

In conclusion, our comparative study highlights that genetic and chemical partial reprogramming each have unique strengths and weaknesses. Genetic OSKM induction is a powerful tool, capable of dramatic cell-state changes, but it comes with the liabilities of heterogeneity and a thin margin between rejuvenation and full dedifferentiation. Chemical reprogramming offers a gentler, more concerted rejuvenation of cells with inherently lower risk of producing fully pluripotent cells, which could make it better suited for therapeutic applications aimed at aging reversal. However, the chemical approach has yet to be firmly established, and its long-term effects are not fully charted. The unknowns about the dynamics of reprogramming, such as how each method navigates the epigenetic landscape and how we can precisely steer that navigation are exciting directions for research. By addressing these unknowns, we move closer to the ambitious goal of safely rejuvenating cells, tissues, or even whole organisms. The next steps will involve building on this knowledge to optimize partial reprogramming protocols and to ensure that any rejuvenation achieved can be translated into real-world health benefits. With careful refinement, partial reprogramming could emerge as a cornerstone of future regenerative medicine and anti-aging therapies. Repeated cycles of reprogramming may one day offer a way to preserve tissue function and radically extend human lifespan.

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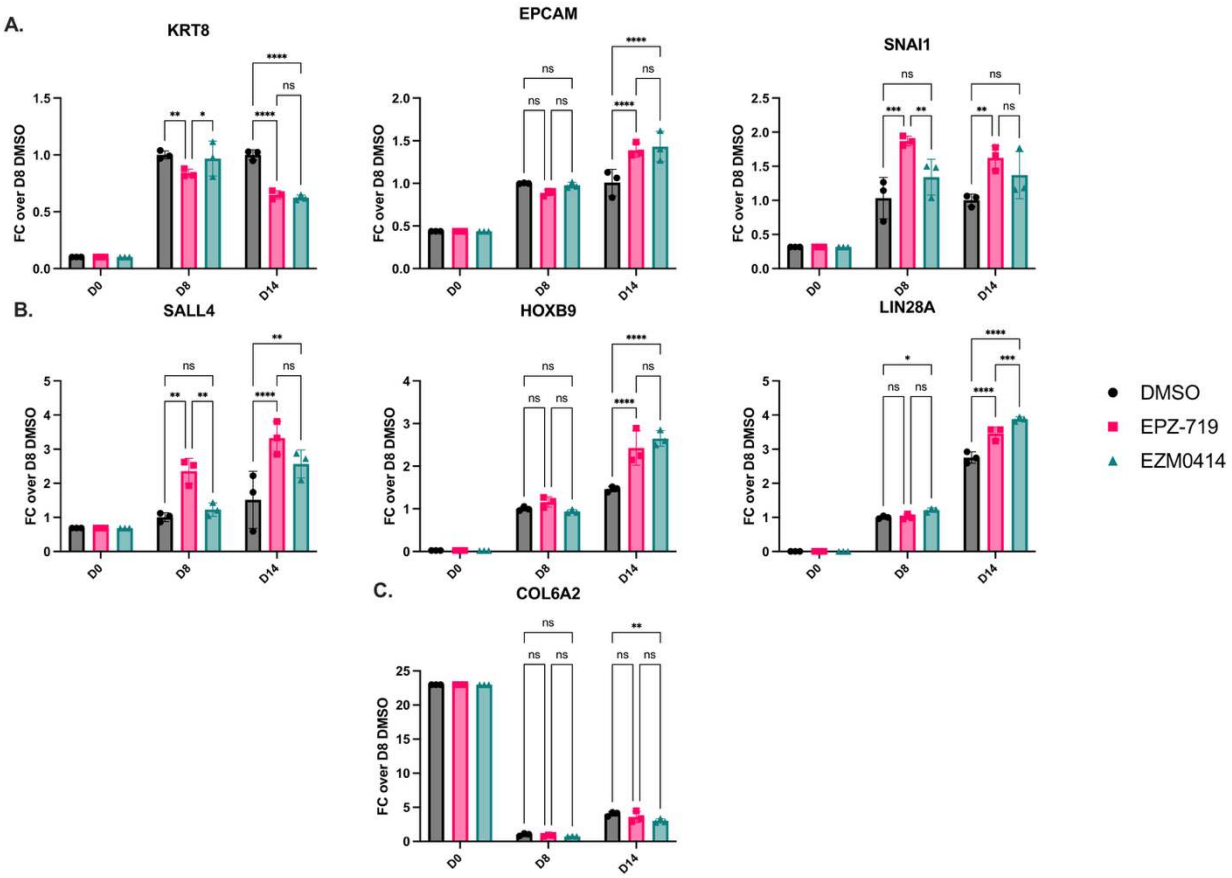
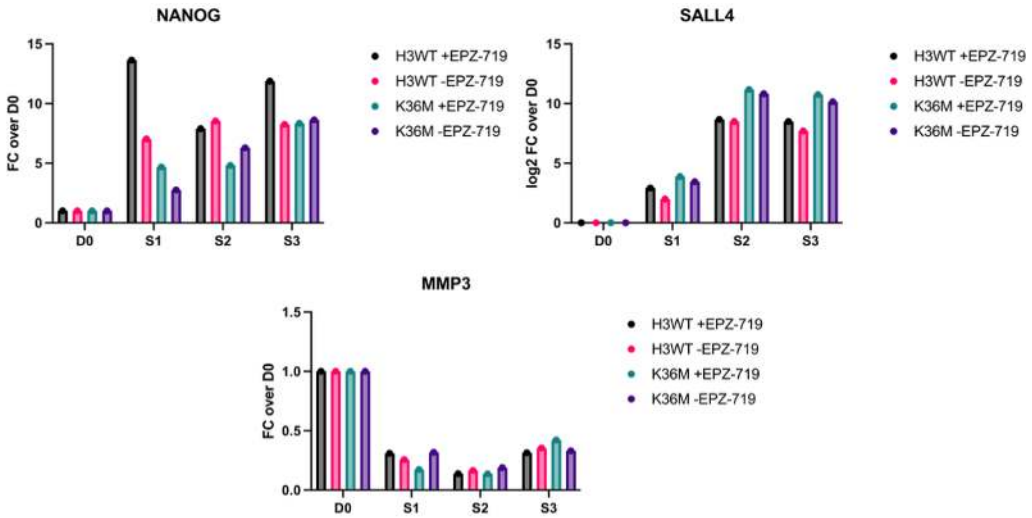
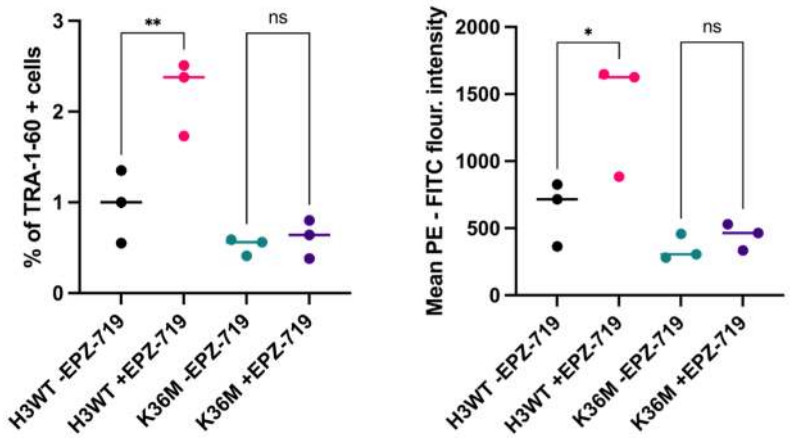
APPENDIX



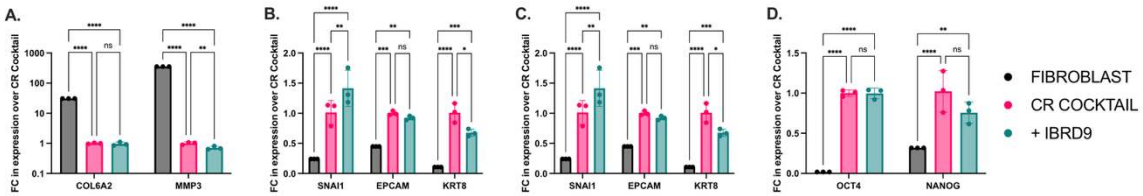
Appendix Figure 1: Effects of SETD2 inhibitors on epithelial, pluripotency, and fibroblast gene expression during Stage 1 chemical reprogramming. dH1f cells were treated with SETD2 inhibitors EPZ719 (0.09 µM) or EZM0414 (2 µM, protocol standard) alongside a DMSO control. Panel A shows the expression of epithelial-related genes, Panel B shows the expression of pluripotency-related genes, and Panel C shows the expression of the fibroblast marker gene COL6A2, highlighting the effects of SETD2 inhibition during the Stage-1 phase.



Appendix Figure 2: Effects of the SETD2 inhibitor EPZ719 on gene expression in histone mutant backgrounds during chemical reprogramming. dH1f cells expressing either H3 wild-type (control) or the unmethylatable H3K36M mutant were treated with EPZ719 throughout chemical reprogramming. Expression of NANOG, SALL4 and MMP3 was measured across all stages to assess the impact of SETD2 inhibition in the context of altered H3K36 methylation.



Appendix Figure 3: Effects of the SETD2 inhibitor EPZ719 on TRA-1-60 expression in histone mutant backgrounds at the end of chemical reprogramming. dH1f cells expressing either H3 wild-type or the unmethylatable H3K36M mutant were treated with EPZ719 during chemical reprogramming. Flow cytometry analysis was performed at the end of Stage 3 using TRA-1-60 antibody staining. The left panel shows the percentage of TRA-1-60–positive cells, while the right panel shows the mean normalized fluorescence intensity of TRA-1-60.



Appendix Figure 4: Effects of BRD9 inhibition on gene expression at the end of Stage 1 chemical reprogramming. dH1f cells were treated with the chemical reprogramming cocktail supplemented with a BRD9 inhibitor and analyzed at the end of Stage 1. Panel A shows fibroblast-related genes, Panel B shows epithelial-related genes, Panel C shows early pluripotency-related genes, and Panel D shows late pluripotency-related genes.