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Partial Reprogramming: A Shortcut to Rejuvenation

A Thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in

Biology

by

David Du Lam

Committee in Charge:

Professor Juan Carlos Izpisua Belmonte, Co-Chair
Professor Deborah Yelon, Co-Chair
Professor Alysson Muotri

2019

The Thesis of David Du Lam is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Co-Chair

University of California San Diego

2019

Dedication

I dedicate this thesis to my loving parents Van Lam & Julie Du, my brother Danton Lam, and my incredibly supportive girlfriend Kelly Kha. Thank you for your endless encouragement and unconditional support, I would not be who I am today without you.

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Abstract of the Thesis

Partial Reprogramming: A Shortcut to Rejuvenation

by

David Du Lam

Master of Science in Biology

University of California San Diego, 2019

Professor Juan Carlos Izpisua Belmonte, Co-Chair
Professor Deborah Yelon, Co-Chair

There is an exponential increase in the incidence of disease with age such as cancer, cardiovascular disease, and neurological disorders [1, 2]. Investigating novel approaches to address aging should be given serious consideration since age is a major common risk factor for chronic diseases. Aging and development of progeny to adults has typically been considered a unidirectional process. However, that notion is no longer clear since terminally differentiated cells can now be reprogrammed to an embryonic-like

cell—termed induced pluripotent stem cells (iPSC) [3, 4]. This suggests that at the cellular level, development is not limited to one direction and can be reversible[3, 4]. Lapasset and colleagues conducted experiments showing improvements in markers of aging when fibroblasts from centenarians were reprogrammed to iPSCs and subsequently re-differentiated into "rejuvenated" fibroblasts [5]. This experiment and others suggested that reprogramming rejuvenated the cells [5–8]. However, it remains unclear when the "rejuvenation" occurred—during the reprogramming process or when the cell was pluripotent. Here, we show that partial reprogramming rejuvenated cells through several reduced hallmarks of aging. Furthermore, partial reprogramming via short term cyclical induction of Yamanaka factors also extended the lifespan of our animal model. Reprogramming may be useful for understanding the molecular basis of aging and this study is a proof of concept that reprogramming has the potential for therapeutic application.

Introduction

Aging is the most common risk factor for chronic disease [1, 2]. If the biological factors of aging could be targeted and ameliorated, it may be possible to reduce the monetary costs and biological consequences of aging. Therapies targeting aging may reverse aging, extend lifespan, or improve quality of life—all potential desirable outcomes.

Due to the vastness of literature available in aging and reprogramming, it is not possible to present a truly comprehensive review and it is probably beyond the scope of this thesis. However, the following brief literature review will be a refined overview of aging, reprogramming, and the intersection between these two topics.

Aging can be defined as a decline in resistance to stress, damage, mutation and maintaining homeostasis [2]. This decline has been associated with hallmarks of aging that include: telomere attrition, cellular senescence, stem cell exhaustion, mitochondrial dysfunction, and epigenetic alteration [9–13]. Telomeres are the nucleic acid ends of chromosomes which protect the genomic DNA from shortening after each round of DNA replication [9, 10]. As we age, these telomeres continue to shorten and are eventually eroded [11]. At which point, genomic DNA becomes vulnerable to attrition after the loss of telomeres [12]. Cells will undergo senescence or apoptosis to prevent erosion of genomic DNA or loss of critical genes [10, 12, 14]. A driving factor of aging is the senescence or apoptosis of cells, particularly adult stem cells [15].

The loss of adult stem cells is another player that must be considered as a driver of aging [13]. More adult stem cells reside in younger tissues and subsequently these tissues retain a greater capacity for regeneration than older tissues [16–19]. The decline in regenerative capacity during aging can be partially attributed to an increase in senescence

of adult stem cell populations [13]. Normally, adult stem cells replicate and replenish damaged and mutated cells, so a depletion in this active stem cell population reduces an organism's capacity to maintain homeostasis and resist stress and damage.

Reactive oxygen species (ROS) cause stress and damage [20]. ROS are a byproduct of oxidative phosphorylation. These harmful free radicals are typically contained within mitochondria where they are less likely to damage DNA, protein, and other critical cellular structures. With age, there is a decline in mitochondrial function and mitochondrial membrane integrity, which leads to an increase in ROS leakage [21]. The ROS can damage the structure of DNA and proteins, and disrupt their function because the structure of these molecules is tightly linked to their function. Phenotypes of aging are more apparent with age, in part, because of a synergistic effect between ROS damage and a reduced ability to replace damaged cells.

Alongside an accumulation of damaged cells, there is also an accumulation of pathological proteins observed with age [22]. Alzheimer's disease (AD) and Parkinson's disease (PD) are diseases directly linked to a loss of proteostasis [23]. It is well known that Alzheimer's disease (AD) is associated with an abundance of abnormal proteins: intracellular neurofibrillary tau tangles and extra cellular amyloid-beta plaques (a-Beta) [24]. Interestingly, a-Beta has been observed to accumulate with normal aging, even in the absence of an AD diagnosis [23, 24]. This suggests that there may be a link between an accumulation of abnormal protein and aging. This trend is further illustrated with the misfolded nuclear envelope protein, progerin. The protein was first discovered in patients with Hutchinson Gilford Progeria Syndrome (HGPS), a rare disease that causes pre-mature aging [25]. Progerin has also been observed to accumulate in healthy individuals

as they age [2, 25]. Progerin's tight association with aging has led to its use as a tool to model aging in vitro [26]. Together, these examples highlight the decline of proteostasis as a hallmark of aging.

Aging is an incredibly complex process that is affected by multiple different processes like those mentioned above: telomere attrition, cellular senescence, stem cell exhaustion, mitochondrial dysfunction, and loss of proteostasis [2]. Despite aging's complexity, it is difficult to imagine that all its processes are all occurring independently. Epigenetic alterations seem to serve as a hub that connects all these different processes [27]. Epigenetics are heritable genetic expression patterns that do not change the DNA sequence. Specifically, epigenetic alterations are chemical (methylation/acetylation) changes to the DNA or histone proteins that affect gene expression. Every cell in the human body contains the same genome, yet there are many different highly specialized cell types and biological systems. Epigenetic modifications allow for the differential expression of the genome resulting in cellular specialization.

Epigenetics is linked to all the hallmarks of aging through alterations in expression patterns [27]. Telomere attrition is a direct consequence of DNA replication, and the suppression of telomerase expression (the enzyme responsible for telomere extension) [15]. Moreover, in order to change cellular states (eg. mitotic to senescent), epigenetic modifications must direct a cell to upregulate genes such as senescence associated genes (eg. P16, P21, P53) [28, 29]. Furthermore, it has been observed in aged-mice that there are changes in HIF-1alpha expression and H3K4me2/3 deposition [30, 31]. These are markers closely linked to mitochondrial function. Lastly, key transcription factors like FOXO/DAF-16 that help maintain the proteome can become dysregulated

with age leading to a disruption in proteostasis [31]. Epigenetic alteration is surely one of the most critical hallmarks of aging, since it acts as the connecting hub between the other hallmarks of aging and may even be grossly considered as the molecular basis of aging [27].

Having completed a brief review of the hallmarks of aging above, I will now proceed to present a brief review of reprogramming. All mammalian cells originate from a single embryonic cell. During the initial stages of embryonic development, the cells are identical with respect to their genomic DNA and expression pattern. Expression patterns change as development proceeds and cells begin to form specialized populations [32]. These changes can be mediated by epigenetic changes. The path of development from pluripotent embryonic cells, to multi-potent progenitors, and terminally differentiated cells is determined by a series of "cellular-decisions". Conrad Waddington, in 1957 illustrated this idea as the epigenetic landscape, whereby a ball (metaphor for a cell) rolling down a hill with various peaks and valleys (different cell lineages) can take specific permitted paths (cell fates) [33]. This metaphor of a ball rolling downhill for development implies cell fate is irreversible once determined. Half a century later, Yamanaka showed that terminally differentiated cells could be induced or "reprogrammed" to a pluripotent state similar to embryonic stem cells—termed induced pluripotent stem cells (iPSC). The terminally differentiated cells were reprogrammed by forced induction of the Yamanaka factors, four genes: Oct4, Sox2, Klf4, and c-Myc [3, 4]. This highlighted that the developmental process and cell fate could be reversed.

The reprogramming of specialized cells to iPSC was not a direct conversion between cell types, rather the reprogramming process was more similar to a reversal of

embryological development [34, 35]. For example, terminally differentiated fibroblasts first lost markers specific to fibroblasts, became more similar to progenitors, and then lost their lineage specificity as they continued to finally become iPSC [34, 35]. Lapasset et al. similarly reprogrammed fibroblasts, but the cells in their study were particularly unique because they were obtained from centenarians (individuals around 100 years old) [5]. Interestingly, the group re-differentiated the iPSC derived from centenarian fibroblasts back into fibroblasts again. They observed restored telomere length with improved oxidative stress and mitochondrial metabolism [5]. Furthermore, the reprogramming process appeared to have reset the physiologic age set point of the fibroblasts, as re-differentiated fibroblasts from centenarians underwent a comparable number of cellular divisions as fibroblasts derived from young donors before senescing [5]. These experiments suggested that the fibroblasts were rejuvenated through reprogramming.

Human development and reproduction provide clues that the physiologic age set point can be reset. Sperm and egg can be contributed by older individuals, and still be capable of producing an embryo that is as objectively young as an embryo conceived by two younger individuals. The physiologic set point of age seems to reset during fertilization or gametogenesis, and may also be reset during cellular reprogramming. Human reproduction and the study with centenarian fibroblasts suggest that the redirection of aging phenotypes is possible. Reprogramming may offer insights into manipulating aging. Re-differentiated fibroblasts from iPSC appear rejuvenated, but it is unknown when the rejuvenation occurs, during the process of reprogramming or during pluripotency. Here, we report that hallmarks of aging are ameliorated in vitro during partial reprogramming, and life span was increased in a premature aging model after

cyclic short term induction of Yamanaka factors. We defined partial reprogramming as reprogramming without acquiring pluripotency.

Results

In Vitro Studies

In order to effectively initiate reprogramming in cells, we isolated primary tail tip fibroblasts (TTFs) from mice with a doxycycline inducible transgene of the Yamanaka factors (Oct4, Sox2, Klf4, c-Myc or OSKM) [36]. We called these mice 4F mice. The mice used in our experiments were heterozygous for the transgene. Heterozygotes were necessary because previous studies showed that induction of TTFs from 4F homozygous mice generated pluripotent cells [37, 38]. We wanted to avoid generating iPSC because we were studying how aging phenotypes are affected by the reprogramming process prior to reaching pluripotency. We first needed to confirm that OSKM were indeed being expressed upon exposure to doxycycline, and we used quantitative PCR to confirm this (Figure 1). It was also important that the expression of OSKM could be modulated by discontinuation of doxycycline (Figure 1). Short term induction of OSKM for four days did not result in the loss of cellular identity, as evidenced by Thy1 positivity, a marker for fibroblasts (Figure 2). Importantly, continuous induction of OSKM in heterozygous 4F TTF for 12 days did not result in iPSC. This was evident in their lack of expression of NANOG, a marker of pluripotency (Figure 3). Collectively, these experiments reinforced that the phenotypic changes observed would be a consequence of the reprogramming process and not specifically pluripotency.

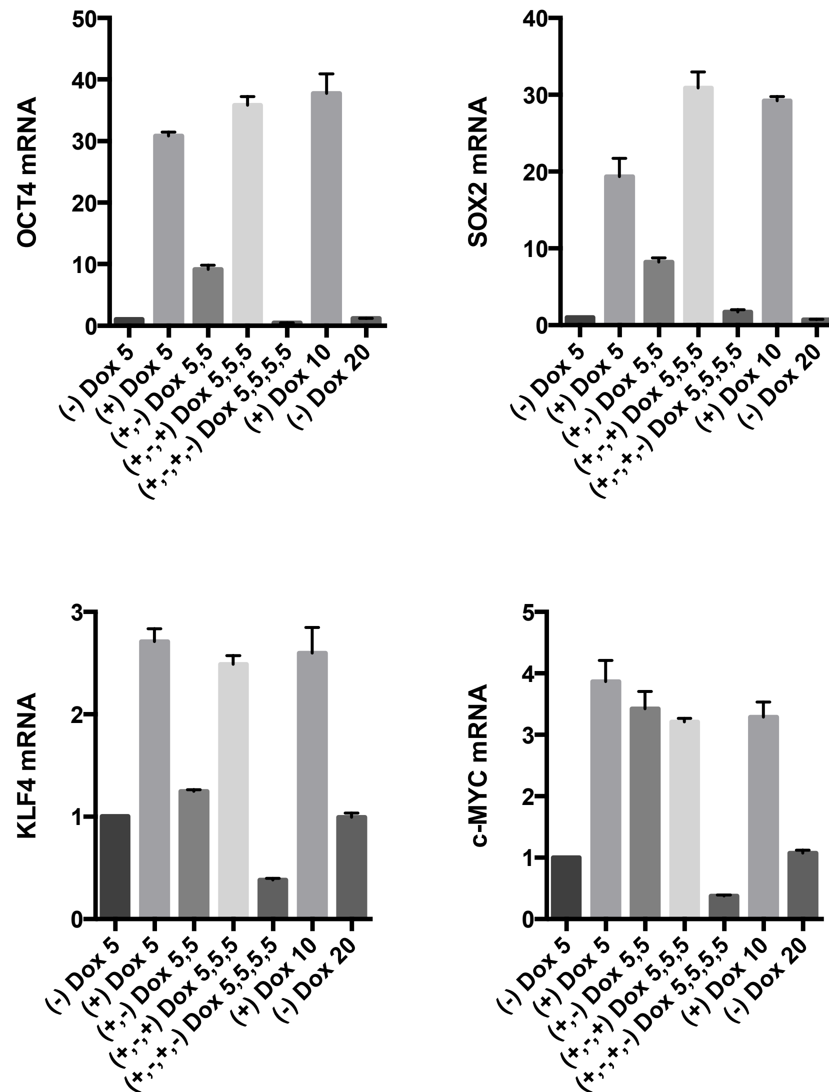

Figure 1: qPCR analysis of OSKM expression during doxycycline induction and after induction withdrawal. Expression of OSKM can be modulated in vitro by cyclic short term induction of OSKM in TTFs. Quantitative PCR was used to measure changes in OSKM expression in a repeated 5 day alternating schedule of doxycycline induction vs. withdrawal. Removal of doxycycline results in a significant drop in OSKM expression. GAPDH was used as a loading control. OSKM are present on a doxycycline inducible polycistronic transgene in our transgenic 4F mice.

OSKM – OCT4, SOX2, KLF4, and c-MYC

TTF – Tail tip fibroblast

4F Mice – Mice with OSKM dox inducible transgene

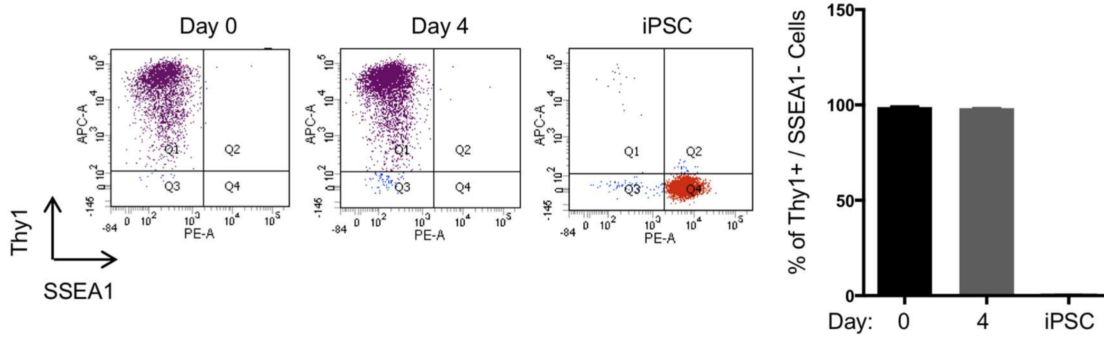

Figure 2: Flow cytometry analysis of cell identity after induction of OSKM. Short term induction of OSKM for 4 days does not result in a loss of fibroblast cell identity. Flow cytometry was used to assess loss of fibroblast lineage marker (Thy1) and acquisition of a marker of intermediate cellular reprogramming (SSEA4). iPSC were used as a positive control.

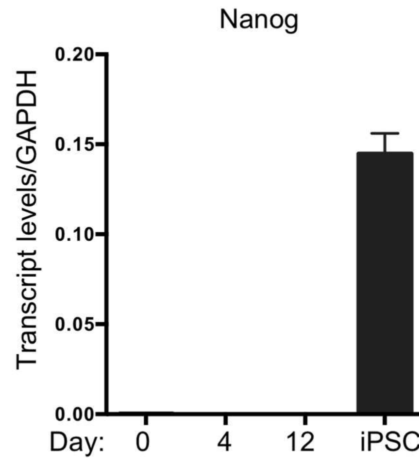

Figure 3: qPCR analysis for pluripotency after extended period of induction. Continuous in vitro induction of OSKM does not result in pluripotency, even after 12 days. Quantitative PCR was used to measure levels of NANOG a marker of pluripotent stem cells. iPSC were used as a positive control.

In order to study the effect of partial reprogramming on aging, we decided to use cells from a pre-mature aging mouse model. The pre-mature aging model we used is a mouse with a G609G mutation of LaminA that produces a truncated form of a nuclear envelope protein, also known as progerin [26, 39]. A similar mutation in the LaminA gene in humans is responsible for Hutchinson Gilford Progeria Syndrome, a disease of pre-mature aging [39]. We called these LAKI mice. Crossing 4F mice with LAKI mice, allowed us to induce reprogramming in mice with accelerated aging (4F LAKI mice).

TTFs were isolated from 4F LAKI mice to study the effects of reprogramming in cells that age prematurely.

Accumulation of DNA damage is a known driver of aging [40]. Short term induction of OSKM in TTFs isolated from 4F LAKI mice showed decreased DNA damage as evidenced through decreased expression of P16, P21, and P53 (Figure 4). Moreover, cellular senescence is known to contribute to aging, and partially reprogrammed 4F LAKI TTFs showed decreased senescence associated B-galactosidase activity [41] (Figure 5). We also saw improved nuclear envelope architecture in cells that had short term induction of OSKM (figure not shown). Continued cycles of short term induction of OSKM also increased expression of telomerase (Figure 6).

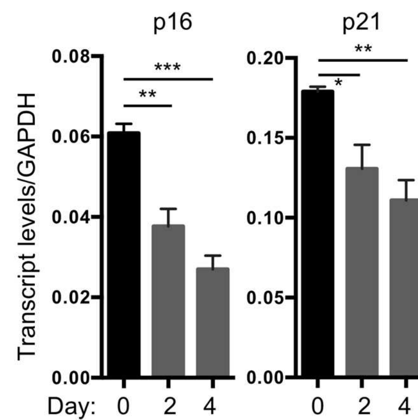

Figure 4: qPCR analysis of age related stress response genes with OSKM induction. In vitro induction of OSKM showed a graded decrease in expression of age related stress response genes, specifically P16 and P21. Quantitative PCR was used to measure levels of P16 and P21 after 0, 2 and 4 days of induction.
*p<0.05, **p<0.005, and ***p<0.0005

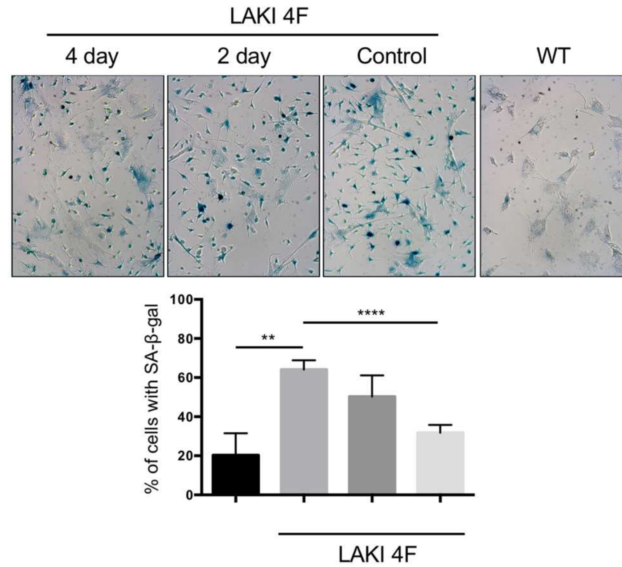

Figure 5: Analysis of senescence associated beta-galactosidase activity with OSKM induction. In vitro induction of OSKM in TTFs from LAKI 4F mice showed a decrease in senescence associated Beta-galactosidase (B-gal) staining. Cellular B-gal staining was quantified.
p=0.005,*p<0.0005
LAKI mice – Hutchinson Gilford Progeria Syndrome transgenic mice that are a model of premature aging.

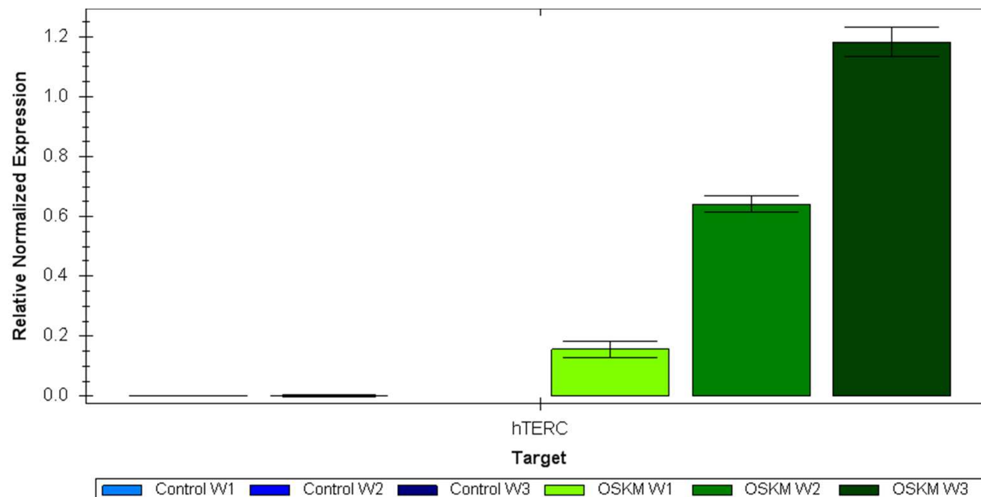

Figure 6: qPCR analysis of telomerase expression during continuous induction of OSKM. Three week continuous in vitro induction of OSKM in TTFs from 4F mice shows a graded increase in expression of telomerase. Quantitative PCR was used to measure changes in telomerase expression in TTFs from wild type mice and 4F mice after 1, 2, and 3 weeks of continuous doxycycline induction.

We looked at markers of DNA damage after halting doxycycline induction to assess if the amelioration of aging phenotypes was maintained. DNA damage, H3K9me3, and nuclear envelope abnormalities reaccumulated when cells were cultured for a few days after the induction of OSKM was stopped (data not shown). However it is important to note, there was a reversal in the markers of DNA damage observed after another short term induction cycle of OSKM. This suggests that short term induction of OSKM can prevent or reset the accumulation of age associated phenotypes in vitro.

In Vivo Studies

After observing an improvement in aging phenotypes in vitro after short term induction of OSKM, we were curious about how short term induction of OSKM and partial reprogramming would affect an organism as a whole. We decided to use the LAKI 4F mice as an in vivo model for premature aging where induction of OSKM could be induced systemically [36, 39].

Teratomas have been shown to develop during reprogramming in transgenic mice that were homozygous for a doxycycline inducible polycistronic transgene of OSKM (4F) and homozygous for rtTA (reverse tetracycline-controlled transactivator) [37, 38]. Our in vitro studies with primary cells from mice heterozygous for both 4F and rtTA did not form pluripotent cells even after continuous induction of OSKM for many days (Figure 3). For this reason we decided to use heterozygous 4F and rtTA mice to study the effect of partial reprogramming as we hypothesized that a similar genotypic schema would be least likely to develop teratomas in vivo.

We initially began in vivo induction of OSKM with addition of doxycycline in mouse drinking water. We observed a high rate of mortality, weight loss, and decreased

activity after four days of induction (Figure 7). The induction of OSKM for 4 days was likely too long, and resulted in dedifferentiation of highly specialized cells causing loss of function in vital systems. For this reason, we implemented cyclic short term induction of OSKM and it resolved the initial issues. The mice were cyclically given doxycycline for two days, and normal water for five days (Figure 8). With such a short timeframe of induction, it was important to confirm that OSKM was indeed still measurably expressed. After two days of induction, quantitative PCR confirmed OSKM expression in mouse whole blood, skin, and liver—suggesting systemic induction of OSKM (Figure 9).

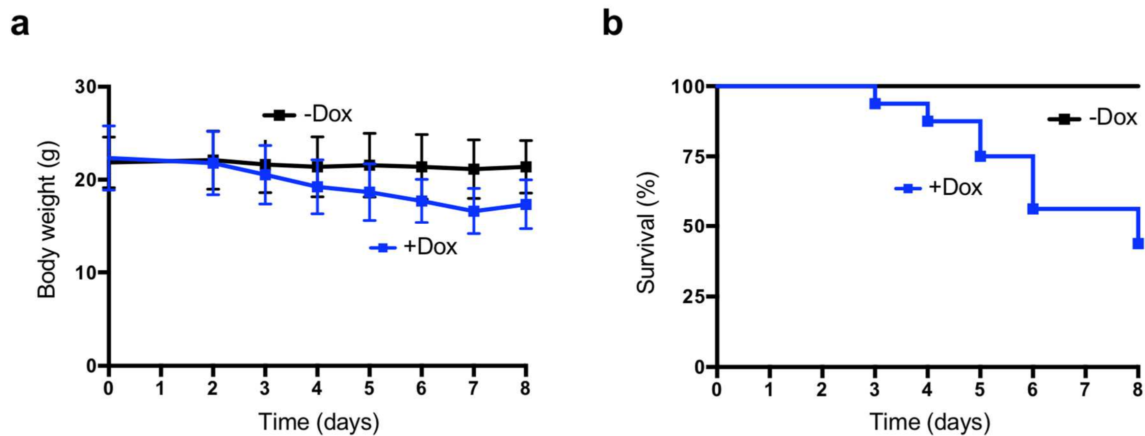

Figure 7: Body weight and survival of WT 4F mice with continuous induction of OSKM. Heterozygous 4F mice were provided drinking water with doxycycline (1mg/ml) for continuous in vivo induction, however the mice began to lose weight (a) and die (b) by day 4. Wild type mice without the doxycycline inducible transgene for 4F were not affected by the doxycycline in their drinking water as they maintained their weight and did not die. (WT: n=4, 4F: n=16)

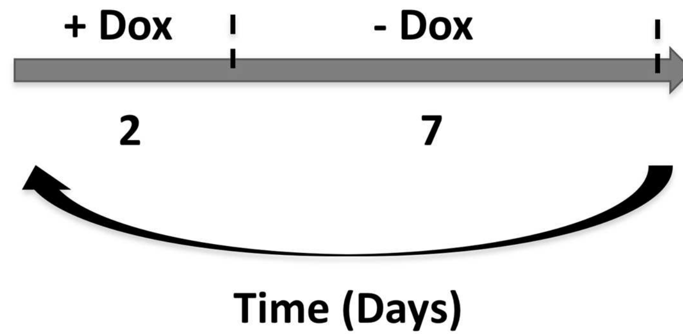

Figure 8: Schematic for short term cyclic induction of OSKM, where mice are given doxycycline water (1mg/ml) for 2 days, and then it is replaced with normal water for 5 days. This dosing cycle is then repeated with the mice, weekly.

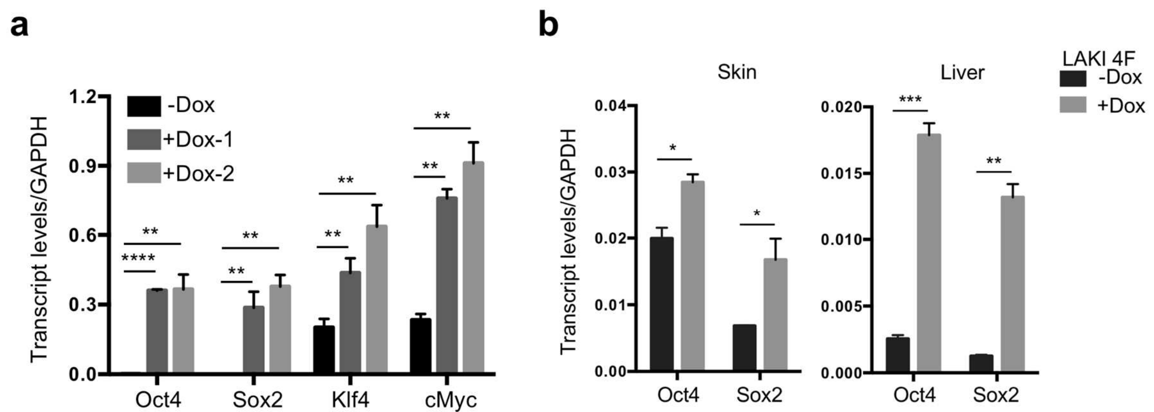

Figure 9: OSKM expression during short term induction. OSKM is expressed in mouse blood (a), skin (b), and liver (b) even with 1 and 2 day durations of doxycycline induction. Quantitative PCR was used to measure changes in OSKM expression in mouse whole blood from mice that did not receive doxycycline, and those that received induction for 1 and 2 days.

(a) **p<0.001, ****p<0.0001

(b) *p<0.05, **p<0.005, ****p<0.0005

Short term cyclic induction of OSKM was carried out for 35 cycles of doxycycline in 4F mice. The 4F mice receiving cyclic short term induction of OSKM were better able to maintain their weight, however there was no improvement in mortality compared to controls (Figure 10). Short term cyclic induction of OSKM was also carried out in our pre-mature aging model, LAKI 4F mice. Unfortunately, the partial reprogramming was unable to rescue the trend of weight loss observed in LAKI mice

(Figure 11). However, these mice showed a striking improvement in lifespan (Figure 11). Furthermore, LAKI 4F mice that received cyclic doxycycline treatment, showed significantly improved external features such as improved fur, kyphosis, and general activity (Figure 12).

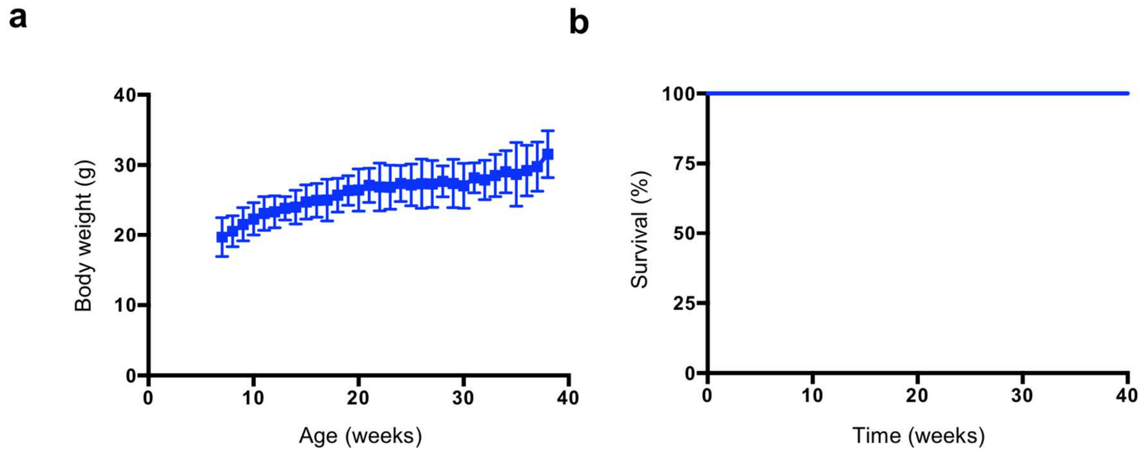

Figure 10: Body weight (a) and survival (b) of WT 4F mice with short term induction of OSKM. Utilizing short term cyclic induction of OSKM in heterozygous 4F mice, normal weight was maintained. There was no improvement in mortality in 4F mice compared to control after 35 cycles of induction. (WT: n=5, 4F: n=3)

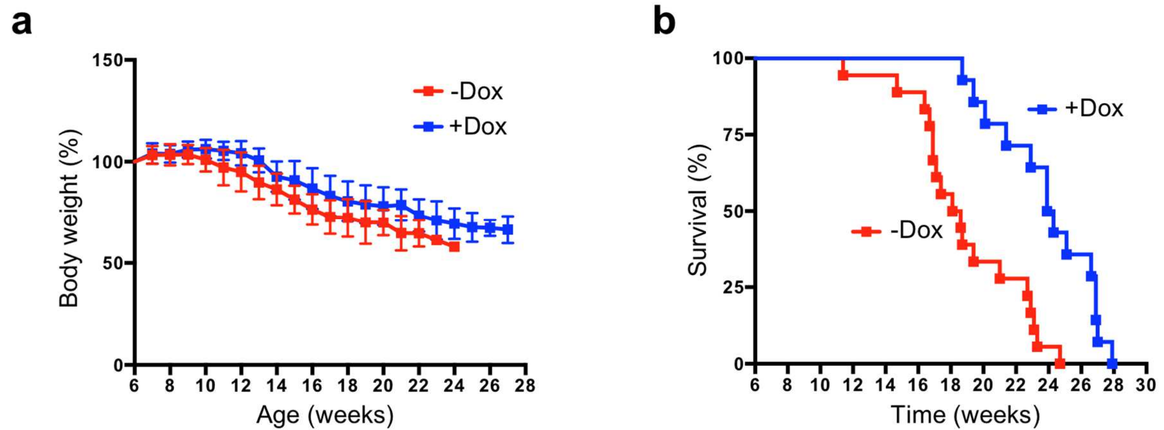

Figure 11: Body weight (a) and survival (b) of LAKI 4F mice with short term induction of OSKM. Short term cyclic induction of OSKM in LAKI (-/-) 4F (-/+) mice continued to experience a trend in weight loss similar to controls. Short term cyclic induction however showed a dramatic improvement in extension of lifespan in LAKI 4F mice compared to controls. (LAKI 4F -Dox: n=18, +Dox: n=15)

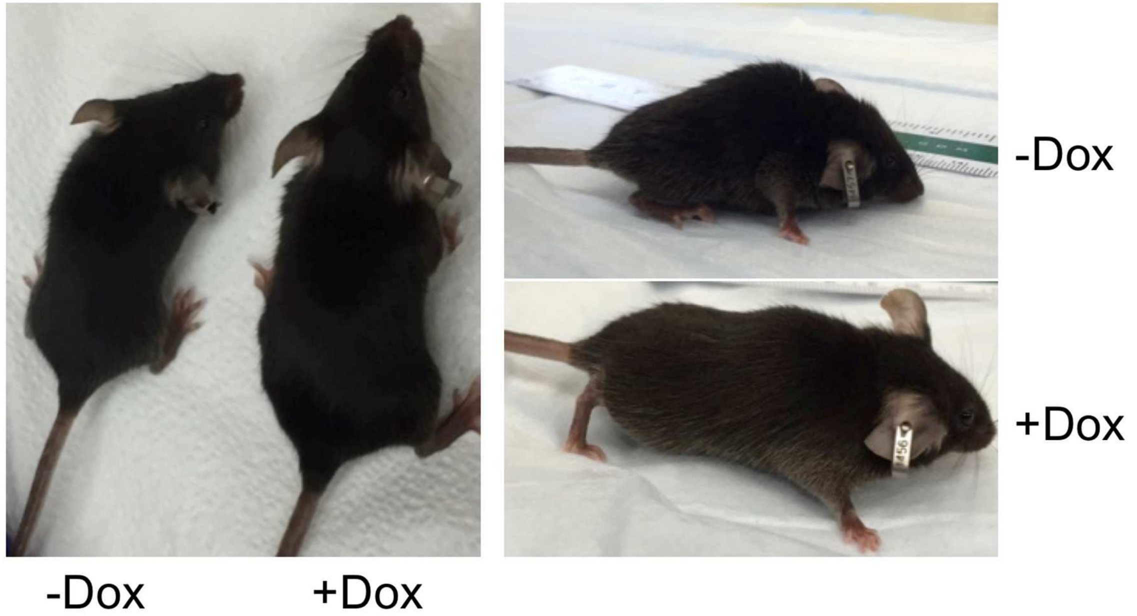

Figure 12: Representative images of 3 month old LAKI 4F mice that have received short term induction of OSKM. Note increased curvature of spine in mice not induced with doxycycline. Note improved overall appearance of mouse that received induction with doxycycline.

Discussion

Age is one of the largest risk factors for the development of disease [1, 2]. A thorough understanding of this complex physiologic process may help elucidate how diseases manifest and suggest new therapeutic strategies. Aging and development occur in parallel and previously could only be observed unidirectionally. The introduction of iPSC has changed this paradigm and we are now able to revert terminally differentiated cells into pluripotent stem cells [3, 4]. The iPSC is not generated through a direct conversion of cell type, but rather the reprogramming seems to occur through a step wise process that resembles a reversal of normal development [34, 35]. Previous studies report in vitro reprogramming rejuvenates various hallmarks of aging [5–8]. However, this process of rejuvenation has been poorly understood, and the effect of reprogramming on aging has never been investigated at the organismal level.

Our findings indicate that the hallmarks of aging are ameliorated during the reprogramming process and a pluripotent cell state is not necessary for rejuvenation. Specifically, it suggests that the rejuvenation we observed occurred during the initial stages of reprogramming. Partial reprogramming via short term induction of the Yamanaka factors was sufficient to reduce several hallmarks of aging in vitro. Observing reduced aging hallmarks in vitro logically progressed to a question of reducing aging hallmarks in vivo through reprogramming. Previous studies investigating in vivo reprogramming observed increased cancer development, teratoma formation, and mortality[37, 38]. The relatively rapid reprogramming process via induction likely easily overshoots a cellular state that could be therapeutic. Utilizing a cyclical short term induction protocol for the Yamanaka factors, we were able to avoid many of the

previously described problems with in vivo reprogramming. Importantly, we did not observe any teratoma formation with this induction schema. Furthermore, a significant extension of lifespan was observed after cyclical short term induction of the Yamanaka factors in a mouse model of premature aging. Our findings suggest that partial reprogramming improves hallmarks of aging and may confer an overall organismal benefit leading to lifespan extension and potentially an improved baseline level of health.

Our work is a proof of concept that partial reprogramming improves aging both at the cellular level and organismal level. It remains unclear what is responsible for the lifespan extension observed. Our preliminary studies suggest that epigenetic remodeling has a large role in aging and rejuvenation. Understanding the epigenetic mechanism can help elucidate how to directly manipulate the epigenome to promote rejuvenation and ultimately avoid the use of Yamanaka factors. This research was limited to a mouse model of premature aging, therefore future studies are necessary to determine how partial reprogramming may affect normal physiologic aging.

Materials and Methods

Mouse Strains

The mouse model for Hutchinson Gilford Progeria Syndrome (HGPS), a disease characterized by accelerated aging in humans, was generated by Carlos López-Otín at the University of Oviedo, Spain and donated by Brian Kennedy at the Buck Institute. These mice carry the LMNA mutation G609G (LAKI) which models HGPS in mice.
Mice carrying the OSKM polycistronic transgene and reverse tetracycline-controlled trans-activator (rTta) were obtained from The Jackson Laboratory (WT 4F). Wild type (WT) C57BL/6 mice were obtained from The Jackson Laboratory. LAKI 4F mice had one gene for OSKM (4F/+) and one gene for rTta (rTta/+) and were homozygous for LMNA mutation (LAKI) were generated by crossing 4F mice and LAKI mice. All mice were C57B/6 background. Experiments with WT and WT 4F mice ranged in age from 2-12 months. Experiments with LAKI and LAKI 4F mice ranged in age from 7-8 weeks. Mice were housed in a 12 hour light/dark cycle between 0:600 and 18:00 in a temperature controlled setting with free access to water (doxycycline 1mg/ml) and food.

In vitro primary tail tip fibroblast culture and induction of OSKM

Tail tip fibroblasts (TTFs) were isolated from WT 4F and LAKI 4F mice and cultured at 37˚C in DMEM (Invitrogen), 10% fetal bovine serum, Gluta-MAX, and non-essential amino acids. TTFs from WT 4F mice were treated with doxycycline (2ug/ml) for induction of OSKM at passage 8, and LAKI 4F TTFs were induced at passage 6. TTFs were induced for varying amounts of time.

In vivo induction of OSKM

Induction of OSKM in mice was done via administration of doxycycline (Sigma) (1mg/ml) in drinking water that is freely accessible to the mice. The mice were given

short cyclical induction cycles where doxycycline was given for 2 days followed by 5 days of doxycycline withdrawal. This cyclical induction was repeated for the duration of experiments. For our lifespan experiments, the mice began cyclical OSKM induction at age 8 weeks until death.

RNA analysis via qPCR

RNA was isolated from in vitro cultured TTFs or from mouse whole blood (collected via submandibular/facial veins), liver, and skin using TRIzol (Invitrogen). Subsequently, cDNA synthesis was done using iScript Reverse Transcription Superix for RT-PCR (Bio-Rad). Gene expression was quantified via qPCR and was done with SsoAdvanced SYBR Green Supermix (Bio-Rad). Samples were normalized with GAPDH.

Senescence associated beta-galactosidase assay

The senescence associated beta-galactosidase assay (SA- βgal) was conducted as previously described [42]. The cells were fixed in 4% paraformaldehyde for 5 minutes, and then washed twice with PBS. The cells were incubated at 37˚C overnight in a solution containing 40 mM citric acid/Na phosphate buffer, 5 mM K4[Fe(CN)6] 3H2O, 5 mM K3[Fe(CN)6], 150 mM sodium chloride, 2 mM magnesium chloride and 1 mg/ml X-gal. The cells were then washed twice with PBS, and dehydrated with MeOH. Cells were imaged via brightfield microscopy.

Flow Cytometry

In vitro cultured TTFs were analyzed via flow cytometry. Cells were first harvested using TrypLE (Invitrogen), washed with PBS and then incubated by corresponding antibodies in conjunction with FACS blocking buffer (1x PBS/10% FCS)

for one hour on ice, shielded from light. Cells were then washed 3x with FACS blocking buffer and then resuspended to be analyzed using an LSRII instrument. Results were calculated by subtracting isotype control.

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