The role of LINE-1 retrotransposition in the accumulating DNA damage of neural stem cells

By

Constance C. Huang

B.S., Rutgers University, 2017

Thesis

Submitted in partial fulfillment of the degree requirements for the

Degree of Master of Science in the Department of Molecular Pharmacology, Physiology & Biotechnology at Brown University

PROVIDENCE, RHODE ISLAND

May 2019

This thesis by Constance C. Huang is accepted in its present form by the Department of Molecular Pharmacology, Physiology, and Biotechnology as satisfying the thesis requirement for the Degree of Master of Science

Date________________ ____________________________________

Jill A. Kreiling, Advisor

Recommended to the Graduate Council

Date________________ ____________________________________

Robert Reenan, Reader

Date________________ ____________________________________

Judy Liu, Reader

Approved by the Graduate Council

Date________________ ____________________________________

Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Jill A. Kreiling, for her guidance and expertise throughout my project, and to Dr. Robert Reenan and Dr. Judy Liu for serving on my committee. I would also like to thank members of the Kreiling lab, Amy Elias and Emmanuel Verde for their help and support with conducting experiments, the members of the Sedivy lab for their guidance, and the Boeke Lab for providing the plasmid constructs for my experiments.

This project was supported by the Stem Cells and Aging Center for Biomedical Research Excellence award (P20GM119943) at the National Institute of General Medical Sciences.

Table of Contents

Signature Page…………………………………………………………………………………..i

Acknowledgements……………………………………………………………………………..ii

Table of Contents………………………………………………………………………………iii

List of Figures…………………………………………………………………………………..v

Introduction……………………………………………………………………………………..1

I. Cellular Senescence……………………………………………………………………1

II. LINE-1 Retrotransposable Elements…………………………………………….........2

III. Age-related increase in retrotransposable elements leads to accumulating DNA Damage…………………………………………………………………………………4

IV. Retrotransposable Elements in Neural Stem Cells……………………………………..5

V. Project Aims……………………………………………………………………………6

Methods and Materials…………………………………………………………………………7

Stem Cell Isolation…………………………………………………………………...7

Cell Culture…………………………………………………………………………..7

Plasmid Isolation……………………………………………………………………..7

Nucleofector Transfection…………………………………………………………...8

RNA Isolation………………………………………………………………………..9

RT-qPCR……………………………………………………………………….....9

Western Blotting……………………………………………………………………10

NSC Differentiation………………………………………………………………...10

Immunofluorescence Microscopy…………………………………………………..11

Results…………………………………………………………………………………………12

I. Induction of senescence in NSCs…………………………………………………………...12

II. Induction of DDR through the p53 pathway through L1 expression……………………15

III. Differentiation potential in L1-induced NSCs…………………………………………...17

Discussion…………………………………………………………………………………..18

Future Studies………………………………………………………………………………20

References...………………………………………………………………………………...22

List of Figures

Figure 1: DNA damage induces cellular senescence mediated by p53…………………………...1

Figure 2: Diagram of L1 replication into host genome……………………………………………3

Figure 3: Induction of L1 expression and SASP markers at 30 ng/ml of doxycycline…………13

Figure 4: Induction of L1 and SASP markers at low transfection pulse………………………...14

Figure 5: Phosphorylated p53 levels over 6-day treatment at1 ng/ml doxycycline..…………….16

Figure 6: Initial differentiation of untransfected NSCs……………………………………………18

Introduction

I. Cellular Senescence

Replicative cellular senescence is the process by which cells lose the ability to proliferate. This can stem from a variety of causes including, but not limited to, replicative exhaustion, DNA damage from various extrinsic and intrinsic stresses, and oncogene activation. Senescence naturally occurs with time through the shortening of telomeres. Telomeres shorten with each round of cell division until they reach a length that is too short to allow for further replication. Subsequently, the cell enters into senescence.

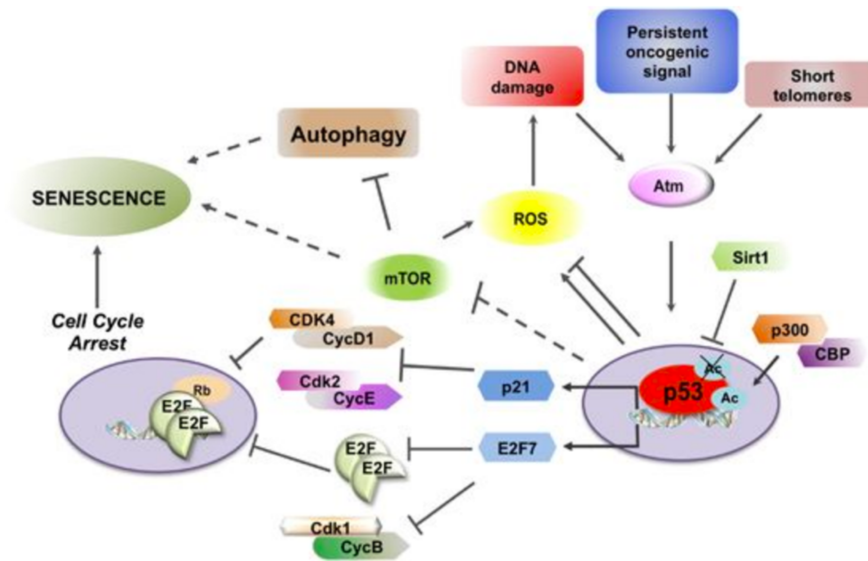


Figure 1. DNA damage induces cellular senescence mediated by p53 [1]

p53 is a transcription factor that plays a central role in cellular arrest in cancer. This function in turn translates to a significant role in aging. p53 is activated by ATM, an upstream kinase that is activated in response to DNA damage (Figure 1). Through ATM activation, p53 is phosphorylated and triggers a signal cascade that activates senescence. This pathway has been shown to be a key player in *in vivo* aging studies where p53 knockout mice showed signs of

accelerated aging and overexpression of p53 resulted in improved healthspan and subsequent extension of lifespan [2-3].

The detection of senescence in cells can be measure through increased levels of phosphorylated p53 as well as through increase in senescence associated secretory phenotypes (SASP) markers. p21, is a significant downstream target of p53 and is seen to be upregulated in senescent cells. Upregulation of p21, in turn results in the suppression of downstream proteins responsible for regulating cell cycle. Other SASP markers (not pictured), p19, p16, PAI1, play other critical factors in regulating cellular senescence and are, likewise, upregulated in senescent cells. As a result, these components are commonly used in the detection of senescence in cells.

Cellular senescence operates at a critical point between cancer and aging. Suppression of cell division is a key response to inhibiting tumor growth. However, senescence in aged tissues as a result of replicative exhaustion is an underlying cause of many age-related diseases. Therefore, studying age-related molecular changes that induce senescence offers the potential therapeutic opportunities to lessen the burden of aging.

II. L1 Retrotransposable Elements

The human genome contains a large portion of transposable elements (TEs), mobile genetic elements that are capable of moving within the genome. Initial sequencing data of the human genome estimates approximately 45% of the genome to be comprised of transposable elements [4]. A subset of TEs are retrotransposable elements (RTEs) that utilize a copy-and-paste mechanism to insert segments of DNA in other locations in the genome. The most abundant of RTEs are the long interspersed nuclear elements (LINEs), specifically LINE1 (L1) in mammals. L1 is considered autonomous in that it codes for proteins necessary for its own mobility. Proteins

coded by L1 are also aid in the mobility of non-autonomous RTEs, implicating L1 has a major role in shaping the mammalian genome due to its capabilities in replicating its own DNA, along with its role in the replication of other elements [5-6].

The L1 gene produces two proteins known as ORF1 and ORF2, translated by RNA polymerase II. Figure 2, taken from *Burns and Boeke, 2012* illustrates the sequence of action performed by L1 proteins [7]. ORF1 is an RNA binding protein that bears no homology with any proteins with known function. It was found to have binding affinity towards single stranded RNA and believed to perform chaperoning activity to facilitate the relocation of L1 RNA into the nucleus [8]. ORF2 is comprised of two domains, an endonuclease and reverse transcriptase, responsible for insertion of L1 RNA into the host genome. The endonuclease domain nicks the host DNA to create a double stranded break. The ends of the DNA are then extended by the reverse transcriptase domain which converts the L1 RNA into its DNA copy [9-10]. L1 is the most abundant retrotransposable element in the mammalian genome and, due to its autonomous nature and central role in other RTEs, is of particular interest for the study of aging.

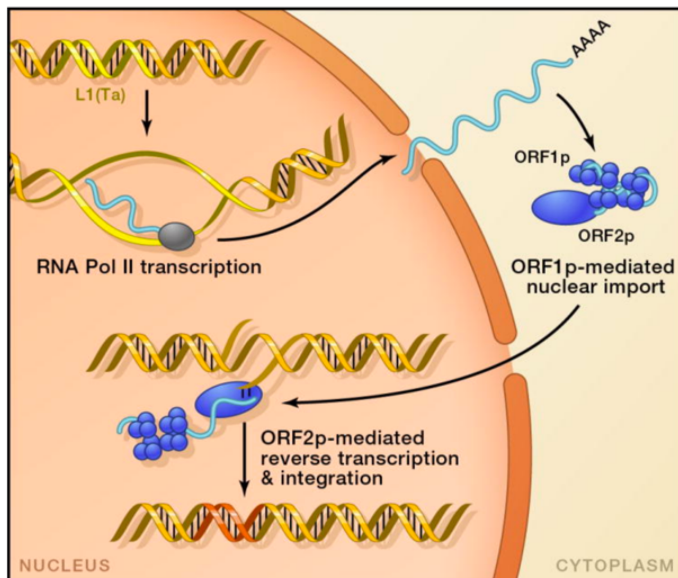


Figure 2. Diagram of L1 replication into host genome [8]

III. Age-related increase in retrotransposition leads to accumulating DNA damage

Retrotransposable elements are largely repressed throughout the human lifetime by means of heterochromatinization. In eukaryotic cells, DNA is wound around histone structures to allow for packaging into the nucleus. Post-translational modification of these histone structures determines the compactness of the DNA. Areas of DNA that are compact, called heterochromatin, are inaccessible and, consequently, transcriptionally inactive. Less condensed regions, call euchromatin, are more open and thus transcriptionally active.

In a previous study, heterochromatin distribution was shown to vary between early passage human diploid fibroblasts (HDFs) and senescent HDFs. The study shows a redistribution of heterochromatin where segments of the genome in early passage sells were later activated in senescent cells. Of particular interest, L1 RNA expression was found to have significantly increased in senescent cells [11]. Furthermore, L1 and other RTE expression is seen to increase in aging somatic tissues [12]. This evidence together demonstrates an age-related change in heterochromatin distribution can lead to the activation of RTEs that are dormant throughout the earlier stages of life.

The consequence of the activation of RTEs is a resulting accumulation of DNA damage. Previously described, the integration of DNA into the genome is initiated by a nick created by the endonuclease domain of ORF2. Evidence suggests that there are significantly more DSBs occurring than there are successful L1 insertions [13]. This DNA damage, much like that induced by ROS or other extrinsic stresses, triggers cellular senescence.

IV. Retrotransposable Elements in Neural Stem Cells

Many neurodegenerative diseases are tied to a decline in neurogenesis. Broadly speaking, these diseases are characterized by a loss of neurons and glial cells in the brain and spinal cord. Alzheimer's Disease is characterized by neuronal and synaptic loss across various areas of the brain. The main cause of Parkinson's Disease results from loss of dopaminergic neurons. Isechymal stroke results in tissue loss across the brain that leads to eventual cognitive and motor impairment for patients [14]. Suffice to say, there is an unmet need for the treatment of neurodegenerative disease that can greatly benefit from targeting the loss of neurogenesis as opposed to targeting disease specific pathologies.

Successful L1 retrotransposition in the brain is believed to be a key factor in neuronal mosaicism. A 2005 study of human L1 cassettes transfected into embryonic rat neuronal progenitor cells showed that successful L1 insertion may play a role in the diversification of the cell genome and ultimately determine cell fate. Furthermore, L1 levels were seen to be inversely correlated with Sox2 levels. Sox2 is a transcription factor known to maintain pluripotency in stem cells, thus is repressed during differentiation [15]. This demonstrates the special role of L1 elements in the differentiation of neuronal cells and could have possible ramifications for adult neurogenesis.

However, as previously mentioned, in human cell lines, these retrotransposable events are likely to cause DSB as opposed to successful insertion. DNA damage repair is also believed to decline over time as a result of chronic DDR necessary throughout the lifespan of an organism [16]. Neural stem cells (NSCs) will senesce into astrocytes as a result of DNA damage [17]. Thus, with the increase rates of L1 retrotransposition in the brain and the age-related decline of DDR, it is possible that NSCs lose differentiation capabilities later in life and senesce into astrocytic lineage

as opposed to the intended neuronal differentiation. Consequently, understanding the impact of retrotransposition on the brain will give insight to age-related changes that will give further insight into the onset of neurodegenerative diseases.

V. Project Aims

The goal of this project is to determine the role of L1 expression in accumulating DNA damage in neural stem cells that can result in the loss of differentiation potential in neural stem cells. Mouse NSCs were transfected with human L1 under control of a Tet-inducible promoter. Following activation of L1 expression using doxycycline, NSCs were analyzed for SASP and DNA damage markers. Transfected NSCs were subsequently placed into differentiation media to determine whether cells retained capability to differentiate into neurons.

Materials and Methods

Stem Cell Isolation

NSCs were isolated from a 4 month-old female mouse. Whole brain was digested using the gentleMACs Dissociator (Miltenyi #130-093-235), program ABDK_1. Myelin was removed using a 25% percoll (GE Healthcare #17-0891-01) gradient in DMEM/F12 (ThermoFisher #11320082) +10% FBS. Red blood cell removal was done with a 65% percoll gradient. Cells were subsequently washed with DMEM/F12 + 10% FBS and resuspended in NSC culture media consisting of Neurobasal-A (Gibco #10888022) + 2% B-27 (Gibco 12587010) +20 ng/ml of each growth factor, EGF (PeproTech #100-47) and bFGF (PeproTech # 100-18b).

Cell Culture

NSCs were seeded at 50,000 cells/ml in culture media + growth factors. Media was changed every 2 days. Cells were pelleted at 200 x g for 5 min. Half of the media was removed and replenished with fresh culture media and growth factors. Cells were passaged on a weekly basis using accutase (Fisher #50-112-9055) to dissociate.

Plasmid Isolation

L1 plasmids were cloned and characterized by the Boeke Lab [18]. pLD401 (wildtype human L1) and pLD567 (EN- H203A) were received in transfected *E. coli*. Cells were placed in 5 ml of LB (Luria Broth) + 100 ug/ml ampicillin (amp) and shaken overnight at 37C. Cells were then streaked onto LB + ampicillin plates and incubated overnight at 37C. Single colonies were selected and inoculated into 5 ml of LB broth + ampicillin. 5 ml of cell suspension was then split evenly into twelve 10 ml aliquots of LB + ampicillin and shaken overnight at 37C. Each culture

was separately prepared via QIAprep Spin Miniprep (Qiagen #27106), pooled together after elution, and measured on the Nanodrop.

2 ng of pMA2640 (Addgene #25434) containing rtTA was transfected into TOP10 competent cells (Thermo Fisher #C404003) by placing in a 42C waterbath for 30 seconds. After a 1-hour recovery period at 37C in S.O.C media provided by the kit, cells were plated on LB + amp and incubated overnight at 37C. Single colonies were selected and inoculated into 5 ml LB + amp and shaken overnight. The 5 ml suspension was subsequently added to 200 ml of LB + amp and shaken overnight. Plasmids were isolated using the HiSpeed Plasmid Maxi Kit (Qiagen #12663) and measured on the Nanodrop.

Nucleofector Transfection

This method was adapted from the Lonza Nucleofector I protocol for mouse embryonic stem cells [19]. Cells were dissociated and counted prior to transfection. 5,000,000 NSCs were resuspended in 100 µl of Nucleofector Solution + Supplement provided in the Amaxa Mouse NSC Nucleofector Kit (Lonza #VPG-1004). Cells were co-transfected at a 1:3 ratio of L1 plasmid to rtTA. Cell suspension was placed into a cuvette provided by the kit and pulsed with the nucleofector using program A-33. Suspension was immediately removed and placed into a 10 cm plate with culture media and growth factors and given a recovery period of 24 hours. 30 ng/ml of doxycycline was added to each culture for 48 hours. One 10 cm plate was harvested for each time point. For time points beyond 2 days, cells were pelleted and resuspended in NSC media and growth factors without doxycycline.

RNA Isolation

At each time point, cells were pelleted and resuspended in 1 ml Trizol reagent (Fisher Scientific #15-596-026). 200 µl of chloroform was added and centrifuged at 12,000 x g to create phase separation. Aqueous phase was removed and placed into 500 µl of isopropanol and RNA was precipitated overnight at -20C. RNA purification was performed following Qiagen RNeasy protocol. The concentration was determined by measuring 1 µl of RNA using the Qubit RNA HS Assay (ThermoFisher #Q32852).

RT-qPCR

cDNA was generated in a 50 µl RT reaction according to the Taq-Man Gold RT-PCR protocol [20]. qPCR was performed in 20 µl reactions containing 10 µl of SYBR Green master mix (Applied Biosystems #A25778), a final concentration of 300 nM of forward and reverse primers, 1 µl of cDNA, and 6.6 µl of water. Each sample was tested in triplicate on the plate and GAPDH was used to normalize. PCR cycles were as followed: 15 seconds at 95C for one cycle and 1 min at 60C for 40 cycles.

Primer sequences were as follows:

Target	Forward	Reverse
L1 UTR	GCACTCTGTCGATACCCCAC	CCCCCACGAACCATAAACCA
p21	CCTGGTGATGTCCGACCTG	CCATGAGCGCATCGCAATC
p19	TCCATTGAAGAAGGGAGTGG	ACCGTTTAGATGGCTGTTGC
MMP3	ATGGCCTTGCAAAAGATGTG	ATGGCCTTGCAAAAGATGTG
PAI1	CAACCGATTCGACCAGCTAT	CAACCGATTCGACCAGCTAT
GAPDH	AGGTTGTCTCCTGCGACTTC	TGTCATACCAGGAAATGAGCTTG

Western Blotting

Cells were resuspended in 50 µl of 1X Laemmli sample buffer with a final concentration of 100 mM of DTT, sodium orthovanadate, and protease inhibitors (3 µl per ml of loading buffer). Samples were boiled for 15 minutes and quantified using the Qubit Protein Assay kit (ThermoFisher #Q33211). Samples were aliquoted into 20 µg protein samples and kept at -80C until analysis.

Prior to analysis, 6 µl of DTT and 1X Laemmli sample buffer was added to a final volume of 30 µl. Samples were boiled for 5 min prior to loading onto gel. Samples were run at 60V through the upper gel and at 100V through a 10% acrylamide lower gel. Samples were transferred to an immobilon-FL membrane overnight at 30V at 4C.

The membrane was blocked with 4% BSA in 1X TBS. Probing was done with p53 (Cell Signalling #2524T), p535p (Cell Signalling #9284T), L1 ORF1 (Millipore #MABC1152), and GAPDH (Sigma #G8795), all diluted 1:1000 in antibody solution (1% BSA in 1X TBS + 0.1% Tween-20). Probing was done overnight at 4C. Secondary antibodies, goat anti-mouse (Li-Cor 926-32210) and goat anti-rabbit (Li-Cor 926-32211) diluted 1:1000 in antibody solution, were probed for 1 hour.

NSC Differentiation

Glass coverslips were coated in poly-D-lysine at a concentration of 50 µg/ml in 6-well plates. After a 24 hours recovery period post nucleofection, NSCs were seeded onto coverslips at 50,000 cells/ml in NSC culture media + growth factors. Cells were incubated for at least 4 hours to adhere to coverslips. After cell adhesion, NSC culture media was exchanged for

differentiation media (Neurobasal A + 2% B-27 + 5% FBS + 10 ng/ml bFGF). Media was changed every 2 days.

Immunofluoresence Microscopy

Cells grown on coverslips were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with PBS+0.1% Triton-X (PBST-0.1). Slides were blocked with 4% BSA + 2% donkey serum in PBST-0.1. NeuN (Millipore #MAB377X) and S100B (PA5-78161) were diluted 1:1000 in blocking buffer. Cells were incubated with primary antibody solution for 2 hours at room temperature. Donkey anti-rabbit Alexa Flour 546 (Invitrogen #A10040) was diluted 1:1000 in blocking buffer and incubated with the cells for 1 hour at room temperature. Cells were stained with 2 μg/ml DAPI for 10 min. Coverslips were mounted with Prolong Gold.

Results

I. *Induction of senescence in NSCs*

A Lonza Nucleofector I was used in the transfection of NSCs. Per Lonza protocol, 5,000,000 cells were used per transfection. NSCs were transfected with plasmids containing human L1 elements under a tetracycline inducible promoter. pLD401 contained a wildtype L1 ORFEUS and pLD567 contained a mutation (H203A) to deactivate the endonuclease domain of ORF2. Cells transfected with this plasmid were expected to show less indication of senescence. All NSCs were co-transfected with a pMA2640 plasmid containing a rtTA transactivator under a CMV promoter. Addition of doxycycline allows for the rtTA to bind to promoter and facilitate the expression of the L1 element. NSCs were transfected at a 1:3 ratio of L1 plasmid to rtTA. Optimal transfection ratio was determined by the level of cell survival post transfection.

After transfection, cells were placed into NSC culture media with growth factors for 24 hours to recover. The next day, cells were selected with 1 ug/ml of puromycin and blasticidin for 24 hours. Cells were subsequently washed with 1X PBS and placed into culture media and treated with doxycycline for 48 hours. Cells were sampled at T0, T1, and T2 post addition of doxycycline.

Various concentrations of doxycycline were tested to find an optimal concentration that would induce L1 expression. Taylor *et al* had previously demonstrated that the expression levels of L1 can be titrated by varying the concentration of doxycycline in HEK293T cells [18]. In experiments where cells were treated with 1 µg/ml of doxycycline, no RNA could be collected, suggesting that the levels of L1 expression was too high and inducing apoptosis rather than senescence in cells. Cells were also dosed with 30 ng/ml of doxycycline, which had been

demonstrated to be the lowest dosage that would yield significant L1 expression in a cell suspension.

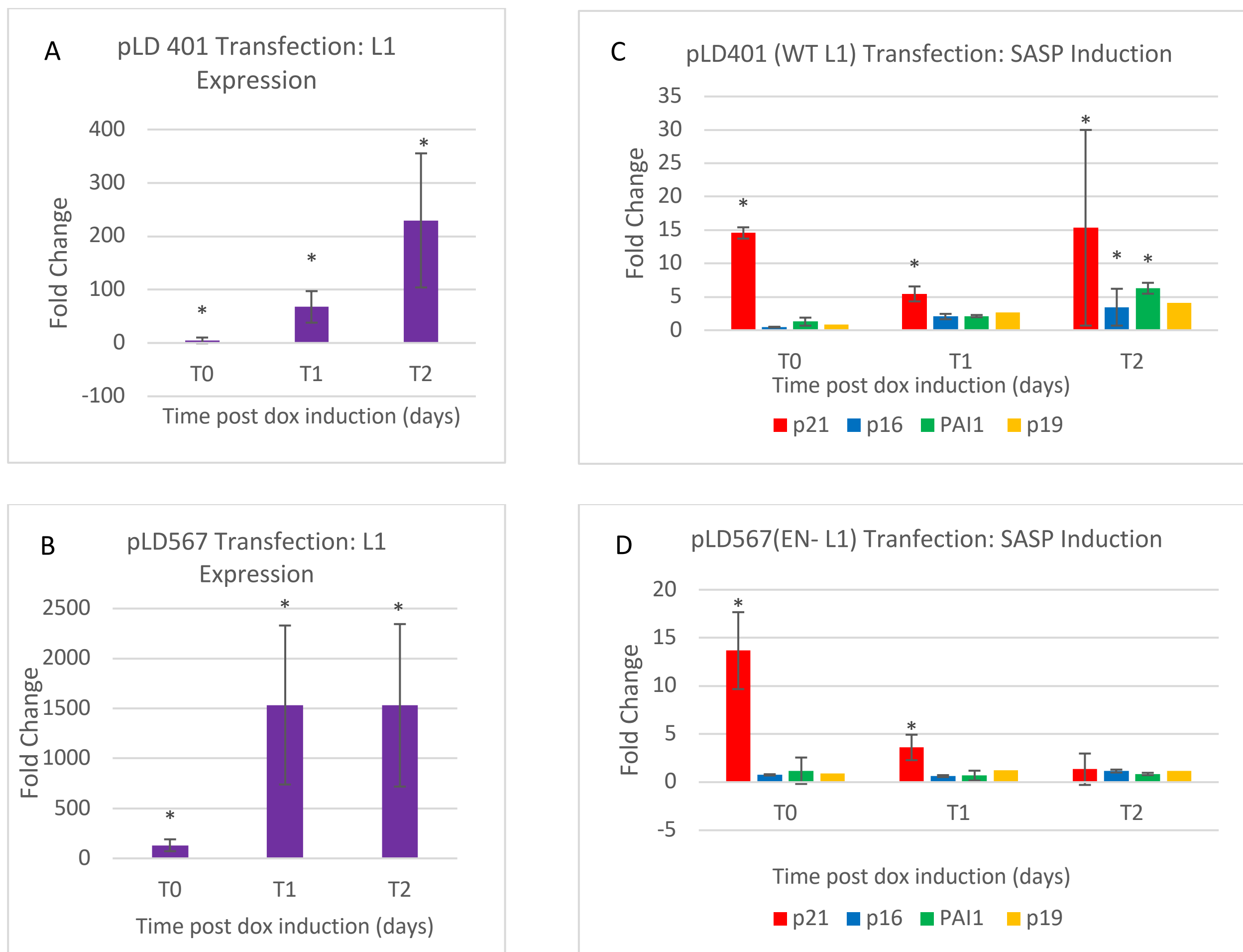


Figure 3. *Induction of L1 Expression and SASP Markers at 30 ng/ml of doxycycline.* Each sample was tested in triplicate across a PCR plate. With the exception of p19, each experiment was repeated twice. *p<0.05

L1 expression was induced significantly in both the pLD401 and pLD567 (Fig 3A-B). pLD401 samples showed a more gradual increase whereas pLD567 showed significant 1500-fold increase after 24 hours. In cells transfected with the wildtype L1, there was a gradual increase in SASP makers p16, PAI1 and p19 (Fig 3C). Markers showed statistically significant increase after 48 hours of doxycycline induction. Conversely, the same markers never showed significant

increase in cells transfected with the endonuclease deactivated L1 (Fig 3D) even with the substantially larger increase in L1 expression.

Of particular interest, p21 spiked at the T0 time point before doxycycline was added to the cultures. This was consistent in all samples transfected regardless of which L1 mutant was used. p21 is a DNA damage marker known to act directly downstream of p53 in DDR. p21 levels in cultures transfected with WT L1 spiked significantly and decreased after 24 hours. However, at the 48-hour mark, the p21 levels increased to higher levels than the initial spike at T0 (Fig 3C). In culture transfected with EN- L1, p21 spiked before doxycycline was added but decreased significantly after 48 hours (Fig 3D).

The early induction of p21 indicated possible DNA damage was occurring as a result of the transfection. A gentler electric pulse, referred to as A-20 in the nucleofector, was chosen to reduce chances of DNA damage from nucleofection.

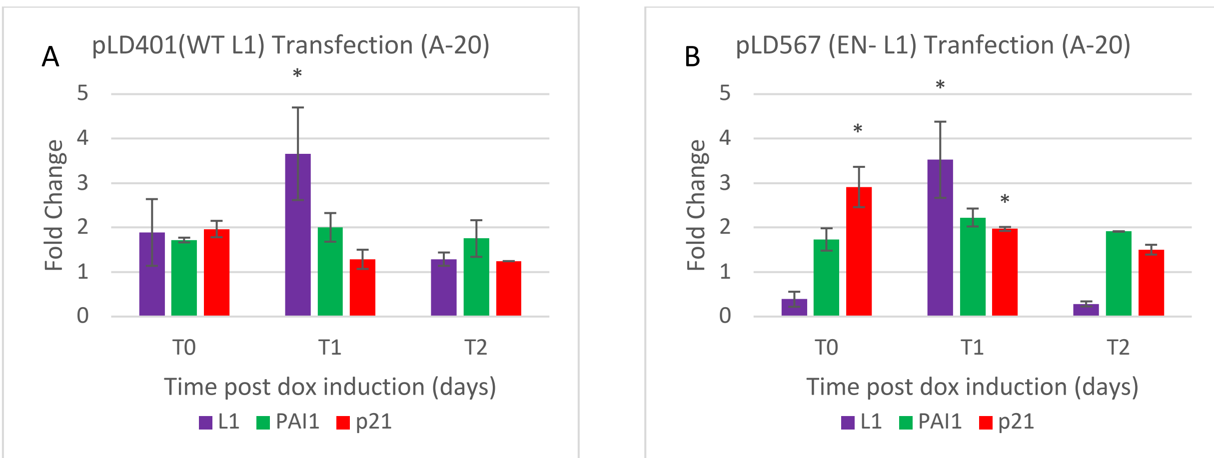


Figure 4. *Induction of L1 and SASP markers at low transfection pulse.* N=2, *p<0.05

At a lower pulse strength, L1 expression was significantly reduced from the 200-1500-fold increase previously observed in both the WT and EN- cultures (Fig 4). In both samples, L1 expression peaked at around 3.5-fold increase after 24 hours before declining at 48 hours. Only PAI1 and p21 were tested initially and there was no significant increase seen in the WT cultures.

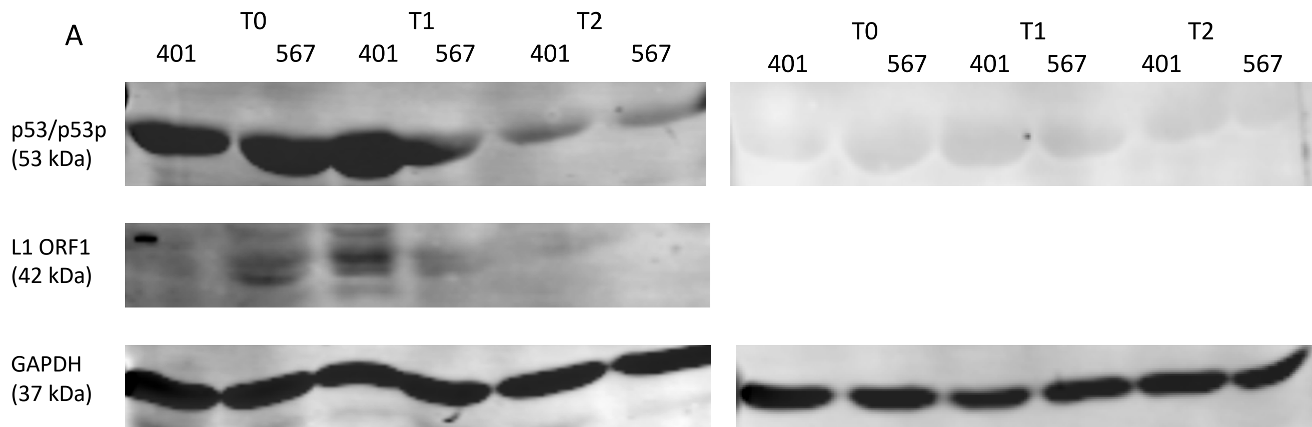
The EN- showed a significant increase in p21 once again starting at the T0 time point but it eventually lowers after 48 hours. As a result of the low levels of L1 expression and lack of initial SASP induction, subsequent SASP tests were not performed.

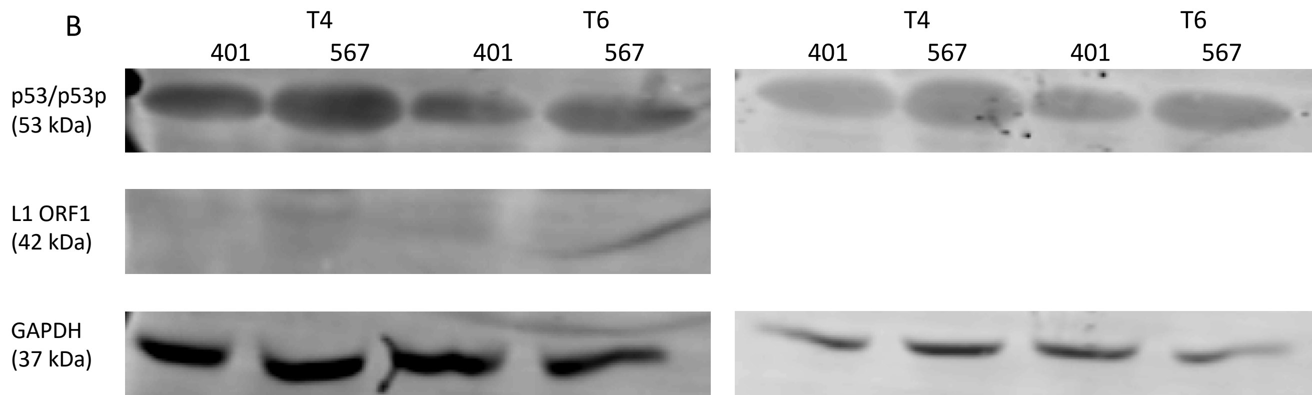
Initial data suggests that 30 ng/ml dosage is optimal for inducing L1 expression, but further work needs to be conducted to optimize transfection efficiencies.

II. *Induction of DDR through the p53 pathway through L1 expression*

Western blots were performed to detect the activation of phosphorylated p53, signs of activation of senescence as well as an indicator of DNA damage. 20 ug of each protein was run on the gel and separately probed for phosphorylated p53 (p53p) and total p53. The ratio of p53p to total p53 was calculated to indicate accumulation of DNA damage over time. Blots were also probed for L1 ORF1 as an additional indicator of L1 expression over time. This experiment was intended to extend over the course of 6 days. In order to facilitate an induction of senescence over a longer period of time than previously tested, the concentration of doxycycline was changed to 1 ng/ml and maintained for 48 hours in culture and were subsequently washed and resuspending in media without doxycycline for the later time points. All signals were normalized to GAPDH.

Phosphorylation of p53 appeared to steadily increase over the first 48 hours post addition of doxycycline in the WT L1 samples (Fig 5A). Later time points show evidence of decline in phosphorylation correlating with the removal of doxycycline (Fig 5B-C). Conversely, p53p levels are considerably lower in the EN- transfected cells, but show signs of increase at later time points. This can possibly be attributed to low GAPDH signal that would artificially increase the p53p signal when normalizing. Repeats are needed to verify the trend.





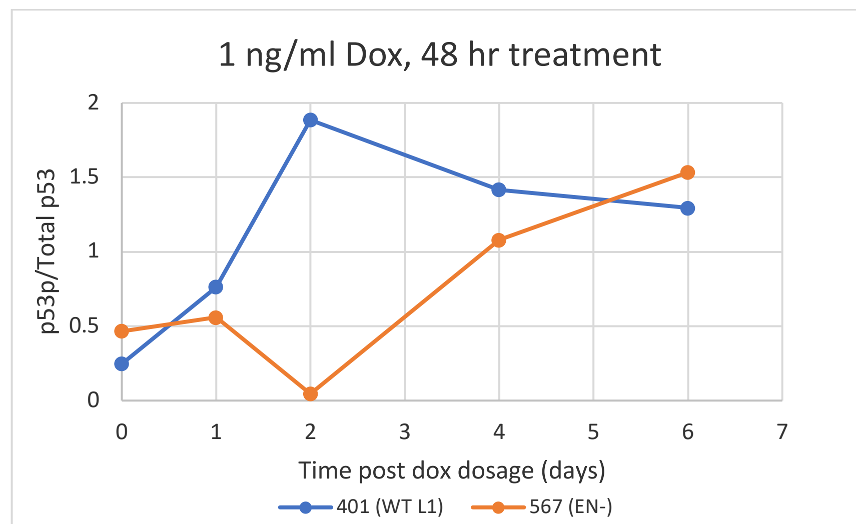


Figure 5. *Phosphorylated p53 levels over 6-day treatment at 1 ng/ml of doxycycline.* Total p53 (left) and phosphorylated p53 (right). L1 ORF1 was probed to verify expression. Values were normalized to GAPDH.

ORF1 levels are seen to be expressed at low levels T0-T2, peaking at T1. The signal subsequently decreases and is non-existent in later time points, corresponding to the removal of doxycycline.

III. *Differentiation potential in L1-induced NSCs*

Initial efforts to create differentiation media involved testing untransfected controls. NSCs were grown on poly-D-lysine coated glass coverslips 24 hours post transfection and placed in NSC culture media for at least 4 hours to allow for adhesion to coverslips. With the addition of doxycycline, cells media was then exchanged for a differentiation media to facilitate differentiation into neurons. Coverslips were fixed at each time point with 4% paraformaldehyde probed with a neuronal marker (NeuN) and astrocytic marker (S100B) to elucidate differentiation potential of NSCs expressing L1. At T0, prior to the addition of differentiation media, there is no evidence of neurons in culture (Fig. 6). However, after 6 days of in the differentiation media, evidence of neuronal differentiation began to emerge. Neuronal marker, NeuN (green), positive cells began to appear in areas of low cell density. In the same sample, areas of high cell density show a lack of neuron differentiation. S100B staining protocol is still under optimization but will eventually be used to stain for astrocytes. After the staining protocol has been optimized, NSCs transfected with L1 elements will be tested in the same manner to determine whether the expression of L1 will reduce the rates of neuronal differentiation in favor of the astrocytic lineage.

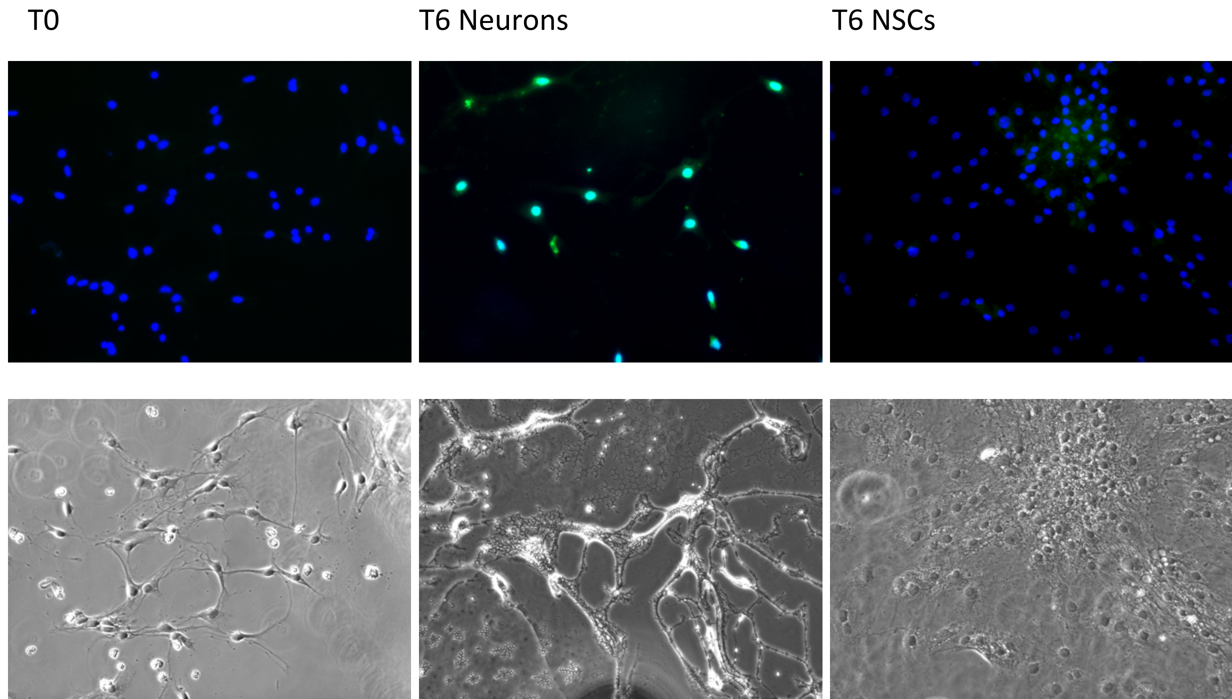


Figure 6. *Initial differentiation on untransfected NSCs.* Cells passage 4 and stained with NeuN (green) and DAPI (blue).

Discussion

There is accumulating evidence to suggest that unsuccessful retrotransposition events in aging tissues may result in increased rates of senescence due to accumulating DNA damage. These retrotransposable elements are found to be highly expressed in the brain and may even play a significant role in determining cell fate in the brain in early neurogenesis. However, this can also result in the likelihood that NSCs are susceptible to accumulating DNA damage with age which could be an underlying cause in many neurodegenerative diseases.

To assess a potential cause and effect relationship between retrotransposition and accumulating DNA damage, NSCs were transfected with either WT L1 elements or EN- L1 elements and assessed for signs of DNA damage and subsequent senescence. L1 elements were expressed at high levels immediately. A slow induction of SASP markers was apparent after 48

hours L1 activation. There was evidence that the protocol was inducing DNA damage in the cells based off of the spike in p21 seen before the addition of doxycycline. However, EN- transfected cells appear to show signs of recovery with subsequent reduction in p21 levels. WT transfected cells showed signs of recovery after 24 hours, however p21 levels spiked once again after 48 hours. This points to a possibility that DNA damage is induced with the increasing expression of L1. This is corroborated with evidence that other SASP primers began to show significant increase by 48 hours in WT cells, but never increase in EN- cells. L1 expression in EN- is also considerably higher than what is observed in WT transfected cells. This could point to the possibility that the EN- transfected cells are healthier and producing more protein. Compounded with the lack of SASP induction, it appears deactivating the endonuclease domain, and preventing DSBs from occurring, is improving the overall health of the NSCs compared to those transfected with WT L1 elements.

Unfortunately, efforts to prevent initial DNA damage resulting from nucleofection resulted in low transfection rates. L1 levels were low in both transfected cell cultures, never reaching the same levels seen in previous experiments. Future experimentation will be required to find an optimal electrical strength for transfection.

Activation of phosphorylated p53 increases significantly in WT L1 transfected cells when compared to those transfected with EN-. However, this effect was only evident in the first 48 hours while still in the presence of doxycycline. The initial intention was to maintain the doxycycline in culture for the full 6 days of experimentation, however, upon seeing the levels of L1 expression in the qPCR data, the decision was made to remove the doxycycline to prevent apoptosis of cells from high L1 levels. Considering the low levels of L1 in the later time points, future experimentation could benefit from maintain doxycycline for the entirety of the

experiment to further assess the DDR response. Phosphorylation of p53 also increased in the later time points of the EN- transfected cells, likely an artifact due to the low GAPDH signal. Further repeats of this experiment are needed to determine whether this is a persisting trend.

Differentiation potential of transfected NSCs were not fully examined. Through the process of conducting differentiation experiments, it was found that the antibiotic selection step may be compromising the health of the cells. NSCs treated with antibiotic selection were incapable of adhering to coverslips whereas non-treated control cells readily adhered. Subsequent efforts of adhere transfected cells that were not treated with antibiotics were successful, indicating that the nucleofection was not the cause. Previous dosages for the antibiotics were chosen based on experiments that had been conducted on HEK293T cells, which are much hardier than primary NSCs. As a result, future experimentation will be necessary to determine the best dosage of antibiotics for NSCs that will give adequate selection without causing detrimental health to the cell cultures.

It was also found that NSC differentiation was higher in areas of low cell density. This is corroborated with evidence seen that cell signaling pathways, specifically the SDF-1/CXCR4 pathway, is crucial to maintaining stemness in NSCs (1). Future experiments will require extra care dissociation to ensure dense cell areas are not formed.

Future Studies

Further experimentation is needed to determine reproducibility. The main limiting factor, and the reason time points mainly span between T0-T2, was because sample collection yielded incredibly low amounts of protein and RNA after 48 hours of induction. The volume of cells needed per nucleofection has also been a limiting factor. Thus the need to prolong the health of the transfected cultures is necessary. The western blotting experiment demonstrated that an

adequate amount of sample can be collected with at a low dosage of doxycycline. The differentiation experiments have also demonstrated the need to determine a proper antibiotic dosage is needed. Further optimization of protocols will allow for more replicates of experiments to ensure reproducibility.

Future experimentation could also incorporate 3TC, a drug commonly used in the treatment of HIV that has also been demonstrated to suppress retrotransposition. Coupling 3TC would provide an interesting negative control to further implicate the role of retrotransposition on the accumulating DNA damage being observed.

This study may also benefit from comparisons with NSCs cultured from old mice. Young mice had been chosen in this experiment because the relative difficulty in culturing NSCs from older animals prevents the cells from reaching larger passages. However, comparison of naturally occurring L1 expression in the brain and further titrating artificial L1 expression in young mouse cells will allow for a more biologically relevant model of retrotansposition in the brain.

References:

1. Rufini, A., Tucci, P., Melino, G. *Senescence and aging: the critical roles of p53*. Oncogene, 2013. **32**: 5129-5143
2. Armata HL, Garlick DS, Sluss HK. *The ataxia telangiectasia-mutated target site Ser18 is required for p53-mediated tumor suppression*. *Cancer Res* 2007; **67**: 11696–11703.
3. Matheu A, Maraver A, Klatt P, Flores I, Garcia-Cao I, Borras C *et al*. *Delayed ageing through damage protection by the Arf/p53 pathway*. *Nature* 2007; **448**: 375–379.
4. Lander, ES et al. Initial sequencing and analysis of the human genome. *Nature, 2001.* **409**: 860-921.
5. Ostertag, EM and Kazazian, HH. Biology of Mammalian L1 Retrotransposons. *Annual Review of Genetics, 2001*. **35**: 501-538.
6. Kazazian, HH. L1 Retrotransposons shape the mammalian genome. *Science,* 2000. **289(5482)**: 1152-1153.
7. Burns, KH and Boeke, JD. Human Transposon Tectonics. *Cell, 2012*. **149(4)**: 740-752.
8. Martin, SL. The ORF1 protein encoded by LINE-1: Structure and function during L1 retrotransposition. *J Biomed Biotechnol*, *2006*. 2006: 45621. Published online 2006. doi: 10.1155/JBB/2006/45621
9. Feng Q, Moran JV, Kazazian HH, Jr, Boeke JD. Human L1 retrotransposon encodes a conserved endonuclease required for retrotransposition. Cell. 1996; 87:905–916.
10. Mathias SL, Scott AF, Kazazian HH, Jr, Boeke JD, Gabriel A. Reverse transcriptase encoded by a human transposable element. Science. 1991; 254:1808–1810.
11. De Cecco, M et al. *Genomes of replicatively senescent cells undergo global epigenetic changes leading to gene silencing and activation of transposable elements*. Aging Cell, 2013. **12**: 247-256.
12. De Cecco, M et al. *Transposable elements become active and mobile in genomes of aging mammalian somatic tissues*. Aging, 2013. **5(12):** 867-883
13. Gasior SL, Wakeman TP, Xu B, Deininger PL. The human LINE- 1 retrotransposon creates DNA double-strand breaks. J Mol Biol 2006; 357:1383-139
14. *Neurodegenerative Diseases*. National Institute of Environmental Health Sciences. Last reviewed January 2019. Retrieved from: https://www.niehs.nih.gov/research/supported/health/neurodegenerative/index.cfm
15. Muotri, A.R., Chu, V.T., Marchetto, M.C.N., Deng, W., Moran, J.V., Gage, F.H. *Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition*. Nature, 2005. **435**: 903-910
16. Li, H., J.R. Mitchell, and P. Hasty, *DNA double-strand breaks: a potential causative factor for mammalian aging?* Mech Ageing Dev, 2008. **129**(7-8): 416-24.
17. Schneider, L. *et al. DNA damage in mammalian neural stem cells lead to astrocytic differentiation mediated by BMP2 signaling through JAK-STAT*. Stem Cell Reports, 2013. **1**: 123-138.
18. Taylor M.S., *et al. Affinity proteomics reveals human host factors implicated in discrete stages of LINE-1 retrotransposition*. Cell, 2013. **155(5)**: 1034-1048.
19. *Amaxa Mouse NSC Nucleofector Kit.* Lonza Colonge AG, 2009. Retrieved from https://bioscience.lonza.com/lonza_bs/CH/en/document/download/21385
20. *TaqMan Gold RT-PCR Kit*. Applied Biosystems, 2010. Retrieved from https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_040930.pdf

21. Ho, S.Y. *et al*. *SDF-1/CXCR4 signaling maintains stemness signature in mouse neural stem/progenitor cells*. Stem Cells Int, 2017. Published online doi: [10.1155/2017/2493752](10.1155/2017/2493752)