CELL SENESCENCE AND CENTROMERIC TRANSCRIPTION

AN ABSTRACT
SUBMITTED ON JULY 19TH, 2022
TO THE DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE GRADUATE SCHOOL
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Abstract

Cell senescence is an irreversible growth arrest, and senescent cells display extensive changes in chromatin structure and chromosome organization. Studies have shown that when cells enter senescence, global transcription profile is dramatically changed. However, how cell senescence alters centromeric transcription has never been studied. In my studies, I used DNA damage drug doxorubicin to induce cell senescence and then examined centromeric transcription using real-time PCR. I also used a non-DNA damage approach, MDM2 inhibitor Nutlin-3, to induce cell senescence and then examined centromeric transcription. I found a similar pattern for centromere transcription in senescent cells induced by these two approaches. Therefore, I hypothesize that senescence cells might show a unique pattern of centromeric transcription, which could provide another useful marker to define cell senescence.
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CHAPTER 1: BACKGROUND

1.1 Centromere, kinetochore and chromosome segregation

Properly passing genetic information from mother to daughter cells during the cell division is essential for the viability of cells and organisms. In eukaryotic cells, genetic information stored in DNA is packaged into chromosomes, which undergo replication and segregation. To achieve faithful segregation, chromosomes require a specialized region known as the centromere, upon which the kinetochore is assembled to dictate mitotic spindle microtubule attachments. Thus, the centromere and kinetochore are essential for accurate chromosome segregation. Abnormal centromere and kinetochore functions usually lead to chromosome missegregation and aneuploidy, which contribute to developmental defects or genetic diseases [1].

A key function of the centromere is to dictate kinetochore assembly. The kinetochore is a huge protein complex that provides the binding site to the mitotic spindle microtubules and acts as a hub to coordinate chromosome segregation [2]. Kinetochore assembly is initiated by CENP-A. CENP-A facilitates the assembly of the constitutive centromere-associated network (CCAN), which includes 16 proteins. Among CCAN, CENP-C is another key component in network assembly and is essential for recruiting all other CCAN subcomplexes to generate an extensive meshwork [3, 4]. CCAN then recruits or bridges other proteins to form outer kinetochore [2]. The KNL1–MIS12–NDC80(KMN) network is the major component in outer kinetochore and directly interacts with spindle
microtubules [5, 6]. Defects in mitotic spindle assembly activate the SAC (spindle assembly checkpoint) to arrest the cell cycle progression, thus allowing cells to repair the defects [7, 8]. Once all chromosomes are properly attached to the spindle, the SAC is silenced to allow chromosome segregation.

Timely removal of centromeric cohesion is crucial for proper mitotic progression. Premature loss of centromeric cohesion at early mitosis causes premature sister-chromatid separation and mitotic arrest, while delayed removal of centromeric cohesion results lagging chromosomes, leading to aneuploidy. Human sister chromatids adopt the iconic X shape at metaphase because cohesin on chromosome arms is released by mitotic kinases and the Wapl–Pds5 complex in prophase, but cohesin at centromeres is protected by Sgo1–PP2A [9, 10]. Sgo1 is initially recruited to kinetochore-proximal regions by histone H2A phospho-Thr120 catalyzed by mitotic kinase Bub1 [11-13] and then is driven to inner centromeres by RNA polymerase II-dependent transcription, where it directly binds cohesin and protects cohesion until the metaphase–anaphase transition [13].

1.2 Centromeric transcription

The centromere is a specialized region on a chromosome that dictates kinetochore assembly during mitosis and meiosis, which is essential for proper chromosome segregation. In most eukaryotes, centromeres contain tandemly repetitive non-coding DNA sequences. These DNA repeats were historically
viewed as heterochromatic, thereby transcriptionally silent; but recent studies showed that they are under active transcription mainly performed by RNA polymerase (RNAP) II [14]. It has been accepted that centromeric transcription plays an important role in proper centromere function [15].

Centromeric transcripts have been demonstrated to bind some kinetochore proteins, which can stabilize these kinetochore proteins on centromeric chromatin. For example, centromere RNAs can stabilize the binding of CENP-C on centromeric chromatin, a fundamental kinetochore protein that serves as an assembly platform for other kinetochore proteins [16]. Besides, CENP-C could also be associated with a-satellite RNA in the nucleolus, which could also facilitate its localization to the kinetochore [17, 18].

At metaphase, sister chromatids in human cells show the iconic X shape because they are primarily linked by the ring-shaped cohesin complex which is protected by Sgo1. Liu et al demonstrated mitotic kinase Bub1 phosphorylates histone H2A at Thr120, which recruits Sgo1 to mitotic kinetochores. In addition, H2A-pT120 is also required for the localization of RNAP II and active transcription at mitotic kinetochores. Active RNAP II transcription drives kinetochore-bound Sgo1 into inner centromeres [13]. Besides, ongoing transcription was reported to promote the deposition of CENP-A, the centromere-specific variant of histone H3 that defines centromeres, to centromeric chromatin,
and its transcripts could also contribute to the stabilization of CENP-A on centromeric chromatin in various types of eukaryotes [19-21].

1.3 The chromatin landscape of senescence cell

Cell senescence is a state of permanent cell cycle arrest caused by different stressors and plays important roles in normal physiology and prevention of carcinogenesis. It in aged organisms contributes to tumorigenesis and functional decline of tissues. Senescent cells undergo profound chromatin structural alterations that affect genome accessibility and transcriptional program, which include the emergence of SAHF (senescence-associated heterochromatin foci) [22], the distension of centromeres [23], and activation of retrotransposon [24].

One important alteration, which is most commonly observed in OIS (oncogene -induced senescence), is the emergence of SAHFs. SAHFs are dense, compact DNA, visible by DNA staining, which has been shown to be distinct from constitutive heterochromatin such as centromeres and telomeres regions and from other facultative heterochromatin such as inactivated X chromosomes (Xi) [22, 25]. SAHF is enriched with a number of molecular markers containing the high mobility group A (HMGA1 and HMGA2) proteins which associate with p16INK4a to induce SAHF formation [26], the histone variant mH2A which is known to contribute to gene silencing [27, 28], and heterochromatin markers H3K9Me3 [27], H4K20me3 [29]. SAHF plays a role in
contributing to the senescence-associated cell cycle exit by suppressing proliferation-promoting genes, including E2F target genes, which are mainly involved in promoting cell proliferation and the progression through S-phase of the cell cycle.

Centromeres also display dramatic structural alterations in senescence. These heterochromatic regions contain repetitive satellite DNA sequences in human that are normally repressed in heterochromatin, but in RS (replicative senescence) cells the pericentric Human Satellite II (HSATII) which locates in the pericentromeric region distends and displays increased chromatin accessibility [24]. In addition, it has been reported that in both RS and OIS cells, SADS (senescence-associated distension of satellites) was extensively observed and confirmed using 3D DNA FISH on HSATII and centromeric alpha satellite region [30, 31]. Interesting, these satellite DNA sequences are also hypomethylated, consistent with depression and distension [32]. Similar to SADS, retrotransposons, a highly unique group of transposable elements that are normally constitutively repressed, have also been shown to become more accessible, which facilitates RNA expression and the mobilization of retrotransposons in senescent cells and aged cells using mouse models [24, 33, 34].
1.4 Molecular pathways through which cells enter senescence

Cell senescence is characterized by different senescence-related phenotypes like cell size and morphological changes, chromatin remodeling, metabolic changes, and SASP (senescence-associated secretory phenotypes). Cellular aging and different stimuli including activated oncogenes, reactive oxygen species, DNA damage, or nucleotide depletion [35-37], will determine the ultimate fates of cells, which also depends on the cell type and the extent of the damage [38]. Mild DNA damage usually causes cell cycle arrest, while irreparable DNA damage could result in senescence program or apoptosis [39].

p53 plays an essential role in the regulation of cell cycle, apoptosis, and senescence. It is activated in a DNA damage response (DDR)-dependent way [40]. DNA double- or single-stranded breaks recruit serine/threonine protein kinase (ATM) or Serine/threonine-protein kinase (ATR) to activate the p53/p21cip1 axis, in which p53 is released from MDM2 inhibition and induces gene expression that triggers cell senescence.

Induction of cell senescence can be triggered in a DDR-independent way. Studies demonstrated that oncogene ras could cause an increase in both nucleoplasmic and nucleolar p19ARF, which in turn activates p53 by inhibition of MDM2-p53 interaction [41] to sequester Mdm2 in the nucleolus, allowing p53 to function without MDM2 antagonization in the nucleoplasm [42, 43]. These studies revealed important roles of p53 and of p53-induced senescence for tumor suppression after oncogene mutation.
1.5 Major knowledge gaps in centromeric transcription and cell senescence

As stated above, the structure of chromatin, including centromere, undergoes a significant change when cells enter senescence. Then I raised several questions. Firstly, does altered chromatin structure affect centromeric transcription? If so, how does it do so? Secondly, does change in centromeric transcription affect cell senescence and its cellular function.

In this thesis, I attempted to address if and how is centromeric transcription altered when cell enter senescence. I took advantage of two distinct approaches, DNA damage and MDM2 inhibitor Nutlin-3, to induce cell senescence and then examined centromeric transcription using real-time PCR.
CHAPTER 2: MATERIALS AND METHODS

2.1 Mammalian cell culture

HCT116 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum and 10mM L-glutamine.

The following drugs were used in this study. Doxorubicin (Santa Cruz Biotech), Nutlin3a (Selleckchem). These drugs were dissolved in DMSO and working concentrations were specified in each experiment.

2.2 Purification of RNAs

Cells were collected and dissolved in TRIzol solution (Invitrogen). Total RNAs were extracted, dissolved in nuclease-free water, and treated with TURBO DNase (Invitrogen) in the presence of RNase inhibitor (NEB) at 37°C for 1 hr. Total RNAs were then extracted with Phenol/Chloroform/Isoamyl alcohol (Invitrogen), precipitated with ice-cold ethanol solution containing glycogen (Roche) and sodium acetate (Invitrogen), and finally dissolved in nuclease-free water (Invitrogen)
2.3 Reverse transcription and real-time PCR analysis

Purified RNAs were mixed with iScript Reverse Transcription Supermix (Bio-Rad) and reverse transcription was performed according to the manufacturer's protocols. After being mixed with the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), the synthesized cDNA was subject to real-time PCR analysis using QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems).

The primers for human cells were used in this study: GAPDH-F, TGATGACATCAAGAAGGTGGTGAAG; GAPDH-R, TCCTTGAGGCCATGTGGGCCAT; Rpl30-F, CAAGGCAAAGCGAAATTGGT; Rpl30-R, GCCCGTTCAGTCTCTTCTCGATT; SAT-1-F: AAGGTCATGGCAGAAAAGAA; SAT-1-R, CAACGAAGGCACACAGATGTC; SAT-4-F: CATTCTCAGAAAACCTCTTTGTGATGTG; SAT-4-R, CTTCTGTCTAGTTTTATGTGATATA; SAT13/21-F, TAGACAGAAGCATTCTCAGAAA; SAT13/21-R, GCCGGCTTCAGTCTCTCCTACAC; Beta-actin-F, AGCGAGCATCCCCCAAGTT; Beta-actin-R, GGGCACGAAGGCTCATCATT; D19Z5-F, GCCTCAATGGGGTCAGAATG; D19Z5-R, TGGATCCCATCTCACTAAGATTCA; D18Z1-F, TGGGAAACGGGATTGTC; D18Z1-R, CTGCTCTCTCCAAATGTGAT; D21Z1-F, TGATGTGTTTACCCAGCCC; D21Z1-R, GCTATCCAAATATCCACC;
2.4 Beta-Gal assay

Cells were washed in PBS, fixed for 5 min (room temperature) in 2% formaldehyde, 0.2% glutaraldehyde in PBS, washed, and incubated at 37°C (no CO2) with fresh senescence-associated (SA-Beta-Gal) staining solution: 20 mg 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal) of dimethylformamide per ml; 0.2 M citric acid/sodium phosphate buffer, pH 6.0; 100 mM potassium ferrocyanide; 100 mM potassium ferricyanide; 5 M NaCl; 1 M MgCl2. Staining was evident in 2-4 hour and maximal in 12-16 hour.

2.5 Statistical analysis

Real-time PCR results were plotted and analyzed with the GraphPad Prism software using one-way ANOVA.
CHAPTER 3: CELL SENESCE NCE ALTERS CENTROMERIC TRANSCRIPTION

3.1 Introduction

To determine how cell senescence affects centromeric transcription, I took advantage of DNA damage agent doxorubicin, a well-accepted approach to induce cell senescence and then examined centromeric transcription using real-time PCR. To generalize my conclusion, I also applied a non-DNA damage approach, MDM2 inhibitor Nutlin-3, to induce cell senescence.

3.2 Results

Doxorubicin induces cell senescence

SA-β-gal assay is a most common method to measure cell senescence. SA-β-galactosidase, an endogenous lysosomal enzyme, is overexpressed and accumulated in senescent cells, its residual activity can be measured at pH 6.0. β-galactosidase will convert X-gal into a blue substance that can be visualized under light microscope [44].
To induce cell senescence, I plated HCT116 (p53 wild type) cells at day 0. At day 1, I treated HCT116 cells with 0.75 uM doxorubicin for 24 hr. At day 2, I washed off doxorubicin and further incubated cells with fresh medium. At day 7, I stained cells with SA-β-gal and then imaged them in color bright field (Figure 2.1B). As shown in Figure 1.1, the majority of doxorubicin-treated cells (90%) displayed enlarged round-shaped cell bodies, a typical phenotype of senescent cells, and exhibited a color of darker blueness; whereas the control nontreated (NT) proliferating cells barely showed these phenotypes (Figure 1.1A). Thus, I successfully induced cell senescence using doxorubicin-induced DNA damage.

**Doxorubicin induced-cell senescence dramatically alters centromeric transcription**

Human centromeres compromise a series of tandem repeats of 171 bp DNA sequences called α-satellite, which further forms higher-order repeat (HOR). They are under active transcription catalyzed by RNA polymerase (RNAP) II. In order to determine how cell senescence affects centromeric transcription, I examined centromeric transcription at different timepoints in the process of cell entering senescence in the above doxorubicin experiment. Total RNAs were extracted from HCT116 cells and real-time PCR was performed to evaluate centromeric transcription using primers for gene RPL30, and six pairs of centromere primers, α-Sat1, α-Sat4, and α-13/21, D19Z5, D18Z1, and D21Z1. These primers were validated for accurate quantification by the lab. As shown in Figure 2.1A, the expression of gene RPL30 at different timepoints was slightly
altered, whereas the expressions of different centromeres underwent dramatic change. At day 2 (1 day after doxorubicin treatment), the RNA levels of all the tested centromeric regions were significantly increased to varying extents. At days 4 and 6 (2 days and 4 days after release from doxorubicin), the RNA levels of α-Sat1, and α-13/21, D19Z5, D18Z1, and D21Z1 remained relatively unchanged except for α-Sat4, whose RNA amount was dramatically reduced. Interestingly, at day 7 when cells were senescent, all the tested centromere RNAs were significantly decreased compared with the ones at days 4 and 6. Remarkably, the amounts of α-Sat1 and α-Sat4 RNAs were further decreased dramatically to the levels that were even much lower than the ones in untreated (NT) proliferating cells (Figure 2.1A and Figure 2.1E), These interesting results suggest that centromeric transcription may fall into a specific pattern in senescent cells, which is very distinct from proliferating cells.

**Nutlin3 induces cell senescence**

In order to confirm the above pattern of centromeric transcription in senescent cells, I induced cell senescence using another type of approach and then examined centromeric transcription. The tumor suppressor p53, a stress-responsive transcription factor, plays an essential role in cellular senescence. MDM2 binds p53 and negatively regulates its stability and transcriptional activity. Nutlin3, a small-molecule inhibitor of MDM2, has been shown to be able to induce cell senescence by binding MDM2 in the p53-binding pocket so that it could activate p53 and some senescent phenotypes through bypassing the DNA
damage or other stress [45, 46]. HCT116 cells (P53 WT) were plated one day before addition of nutlin3a, treated with 10uM nutlin3a, and refreshed every other day. On day 7, the cells were stained with SA-β-gal and imaged in color bright field (Figure 2.1D). Compared with nontreated (NT) proliferating cells, 90% of HCT116 cells were positive, confirming that nutlin3a treatment is an efficient way to induce cell senescence (Figure 1.1B).

**Centromeric transcription is changed in Nutlin3-induced cell senescence**

I then examined centromeric transcription in HCT116 cells treated with nutlin3a. Cells were collected at days 2, 4, 6, and 7, and total RNAs were extracted for real-time PCR analysis using gene primers RPL30, and six pairs of centromere primers, α-Sat1, α-Sat4, and α-13/21, D19Z5, D18Z1, D21Z1. As shown in Figure 2.1C, the expression of gene RPL30 at different timepoints was marginally altered. Interestingly, the expressions of all the tested centromeric regions were not induced at all the timepoints, which is different from doxorubicin-treated cells where centromeric transcription was dramatically induced. Remarkably, a trend of decrease in centromeric transcription was also observed when cells enter senescence, which is similar to doxorubicin-induced cell senescence. Especially, α-Sat1 RNAs in both conditions were dramatically deceased. Notably, some centromeres behaved differently in these two conditions. Compared to untreated cells, D18Z1 RNA was much higher in doxorubicin-induced senescent cells, whereas it remained almost unchanged in Nutlin3a-induced cell senescence (Figure 2.1C and Figure 2.1E).
3.3 Discussion

When cells enter senescence, the global transcription profile is dramatically changed. However, how centromeric transcription is affected by senescence has never been studied. My thesis study here attempted to attack this question. To my knowledge, this is the first attempt. I found that the expression of some centromeres was greatly suppressed in senescent cells. This is strongly supported by using two totally distinct senescence-inducing approaches, DNA damage and non-DNA damage. Based on it, I hypothesize that there may be an “unique” pattern of centromeric transcription for senescent cells. This “uniqueness” could serve as another useful marker to define cell senescence, which could constitute informative molecular parameters for the studies of aging health and disease. Currently, I am performing RNA-Seq analysis to comprehensively study the relationship between centromeric transcription and cell senescence, thus testing this hypothesis. For future analyses, I will focus on determining whether these RNAs are also suppressed in physiologic and pathologic settings in vivo.

The next important question is what role decreased centromeric transcription plays in senescent cells. A detailed understanding of senescent cells will lead to the discovery of new targets used to specifically eliminate senescent cells and establish new strategies to reverse aging. As senescent cells usually do not enter cell cycle, it is unlikely that decreased centromeric transcription would affect chromosome segregation as it does in proliferating
cells. I speculate that decreased centromeric transcription might benefit cells for entering or maintaining the state of senescence. Anti-sense oligo-mediated knockdown and centromere RNA overexpression can be useful tools to test this speculation.

Figure 1.1. Doxorubicin or nutlin3a successfully induces senescence in HCT116 cell.

A. HCT116 cells were treated with 0.75uM doxorubicin for 24h at day 1 and further cultured with fresh medium without doxorubicin until day 7. Cells were then stained to assess their SA-βGal activity to evaluate the state of senescence.  
B. HCT116 cells were treated with 10uM nutlin3a and were refreshed every other day. At day 7, SA-βGal assay was performed to assess the state of senescence.
Figure 2.1. Cell senescence affects centromeric transcription

A. HCT116 cells were treated with 0.75uM doxorubicin for 24h and then washed at day 2. Cells were further incubated with fresh medium until day 7. At the indicated timepoints, cells were collected for RNA extraction followed by real-time-PCR analysis using the indicated primers. The details are recorded in the
materials and methods. The average and standard error calculated from at least three independent experiments are shown here. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. α-Sat, α-satellite.  

B. A workflow about how HCT116 Cell was treated with doxorubicin and collected on day 7. C. HCT116 cells were treated with 10uM nutlin3a and were refreshed every other day. At different timepoints described in E, RNAs were prepared and analyzed by real-time PCR using the indicated primers. The details are recorded in the materials and methods. The average and standard error calculated from at least three independent experiments are shown here. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. α-Sat, α-satellite.  

D. A workflow about how HCT116 Cell was treated with nutlin3a and collected on day 7. E. Experimental conditions that were used in A and C. The times listed here is the different day I collect the cell.
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