REPUBLIC OF TURKEY
ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

JAK RELATED SIGNALING DURING CELLULAR SENESCENCE IN LONG-LIVED BLIND MOLE-RATS

NURCAN INCI KAVUM

M.Sc. THESIS

DEPARTMENT OF MOLECULAR AND TRANSLATIONAL BIOMEDICINE

SUPERVISOR
Asst. Prof. Dr. Perinur Bozaykut Eker

ISTANBUL-2022

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14.09.2022

"Nurcan Inci Kavum"

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LIST OF ABBREVATIONS

BMR	Blind Mole-Rat
CCD	Concerted Cell Death
CDK	Cyclin-Dependent Kinase Inhibitor
CDKN1a	Cyclin-Dependent Kinase Inhibitor 1A
COVID-19	Corona Virus Disease of 2019
CS	Cellular Senescence
CXCL	Chemokine (C-X-C Motif) Ligand
cDNA	Complementary DNA
DAPI	4′,6-Diamidino-2-Phenylindole
DNA	Deoxyribonucleic Acid
DDR	Dna Damage Response
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic Acid
et al.	and Others
FBS	Fetal Bovine Serum
GEO	Gene Expression Omnibus
GO	Gene Ontology
GRO	Growth-Regulated
GSEA	Gene Set Enrichment Analysis
HIF	Hypoxia-Inducible Factor
ICAM1	Intercellular Adhesion Molecule 1
IGFBP	Insulin-Like Growth Factor-Binding Protein
IFNβ	Interferon β
IL17	Interleukin 17
Il1α	Interleukin 1 Alpha
Il1β	Interleukin 1 Beta
IL6	Interleukin 6
IKK	Ikappa b Kinase
ILK	Integrin-Linked Protein Kinase
IRF9	Interferon Regulatory Factor 9

JAK	Janus Kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-ESI-MS/MS	Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry
MCP	Monocyte Chemotactic Protein
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metallo-Proteinases
mmu	Mus Musculus
mRNA	Messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
NES	Normalized Enrichment Score
NMR	Naked Mole-Rat
NF-kB	Nuclear Factor Kappa B
OIS	Oncogene Induced Senescence
P	Passage
PAI	Plasminogen Activator Inhibitor
p38 MAPK	P38 Mitogen-Activated Protein Kinases
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
pRB	Phosphorylated Retinoblastoma Protein
PTM	Post Translational Modification
p-value	Probability Value
qRT-PCR	Quantitative Real-Time PCR
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SASP	Senescence-Associated Secretory Phenotype
SA-β-Gal	Senescence-Associated B-Galactosidase
SD	Standard Deviation
SERPINs	Serine Protease Inhibitors
SIPS	Stress Induced Premature Senescence

SRSF1	Serine-/Arginine-Rich Splicing Factor 1
STAT	Signal Transducer and Activator of Transcription
TIMP	Tissue Inhibitor of Metalloproteinases
TLR	Toll-Like Receptor
TRIM32	E3 Ubiquitin-Protein Ligase
TUT4	Terminal Uridylyltransferase 4
TYK-2	Tyrosine Kinase 2
Uniprot	Universal Protein Resource
UV-Vis	Ultraviolet-Visible
X-Gal	5-Bromo-4-Chloro-3-Indolyl-Beta-D-Galacto-Pyranoside

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SUMMARY

Cellular senescence (CS) has emerged as a fundamental mechanism in developing aging-related diseases (1), and very recently, it has been shown that the Janus Kinase and Signal Transducer and Activator of Transcription (JAK-STAT) pathway plays an essential role during CS. Blind mole-rats (BMRs) are long-lived subterranean rodents showing cancer resistance (2). However, the mechanisms underlying their superior aging and anti-cancer phenotype remain elusive. Here, to understand the alterations in the JAK related signaling during CS in BMRs, replicative senescence was induced in primary lung fibroblasts. Senescent cells were validated with various senescence biomarkers, which are senescence-associated beta-galactosidase (SA-β-gal), morphological changes, and cell growth curve. Expression analysis of *Jak* and *Stat* genes of BMRs is comparatively analyzed to mice in early and late passage fibroblasts by reverse transcriptase qPCR method. Further proteomics analysis was performed in the BMR fibroblasts with LC-MS/MS. The results showed that the mRNA expressions of *Jak1* and *Jak2* genes were suppressed in BMR fibroblasts compared to mice in early passages. However, Jak expressions surprisingly increased in senescent BMR fibroblasts. Finally, the proteomics data analysis of senescent BMR fibroblasts demonstrated that cytokine-mediated signaling is activated during senescence in BMR fibroblasts. Therefore, our results suggest the alterations of JAK related signaling that could be associated with the cytokine-mediated signaling pathways through CS in long-lived BMRs may have a critical role in the adaptations of cancer and aging mechanisms.

Keywords: Aging, Blind mole-rat, Cellular senescence, Inflammation, Janus kinase

ÖZET

Uzun Ömürlü Kör Farelerde JAK Ile İlgili Sinyallerin Hücresel Senesansla Değişimi

Hücresel senesans (HS), yaşlanmaya bağlı olarak hücresel düzeyde öne çıkan temel mekanizmalardan biridir (1) ve son yapılan çalışmalar, Janus Kinaz ve Sinyal Dönüştürücü ve Transkripsiyon Aktivatörü (JAK-STAT) yolağı, HS sırasında önemli bir rol oynadığını gösterdi. Uzun ömürlü kör fareler (Blind mole-rat; BMR), yaşlanma ve kanser direnci gösteren yeraltı kemirgenleridir (2). Bununla birlikte, üstün yaşlanma ve kanser karşıtı fenotiplerinin altında yatan mekanizma belirsizliğini korumaktadır. Bu çalışmada, BMR'lerde HS sırasında JAK ile ilgili sinyalleşmedeki değişikliği anlamak için birincil akciğer fibroblastlarında replikatif senesans gerçekleştirilmiştir. Senesant hücrelerin doğrulanması amacıyla, senesans ile ilişkili beta-galaktosidaz (SA-β-gal), morfolojik değişiklikler ve hücre büyüme eğrisi gibi çeşitli senesans biyobelirteçleri kullanılmıştır. Ardından, BMR'lerin ve farelerin *Jak* ve *Stat* genlerinin erken ve geç pasaj fibroblastlarında karşılaştırılmalı ekspresyon analizi ters transkriptaz qPCR yöntemi ile analiz edilmiştir. Son olarak, JAK ile ilişkili daha ileri sinyal mekanizmaları, LC-MS/MS ile proteomik seviyesinde analiz edilmiştir. Elde edilen sonuçlar, *Jak1* ve *Jak2* genlerinin mRNA ifadelerinin erken pasajlarda farelere kıyasla BMR fibroblastlarında azaldığını ancak BMR fibroblastlarında HS sırasında bu genlerin artan ekspresyonunu gösterdi. Son olarak, senesant BMR fibroblastlardan elde edilen proteomik verilerinin analizi BMR fibroblastlarında sitokin aracılı sinyallemenin aktive olduğunu gösterdi. Sonuçlarımız, uzun ömürlü BMR'lerde JAK ve bağlantılı olarak sitokinle ilişkili sinyal yollarının senesans ile değişiminin, kanser ve yaşlanma mekanizmalarına karşı kritik bir rolü olabileceğini işaret etmektedir.

Anahtar Sözcükler: Hücresel senesans, İnflamasyon, Janus kinaz, Kör fare, Yaşlanma

1. AIM OF THE STUDY

Aging, characterized by a steady decline in cellular and physiological function, results in age-related diseases including neurodegenerative, cardiovascular diseases, and cancer (1). Developing technologies and new treatment methods enables expanded life for humans, yet pathologies related to aging restrict a healthy lifespan (3). Recently, cellular senescence (CS) was defined as one of the crucial hallmarks of aging and therefore has attracted attention in scientific research (4). Cell cycle arrest triggered by CS prevents the tumor growth of a cell. However, with the persistent release of numerous cytokines and chemokines through the senescence-associated secretory phenotype (SASP), CS is proposed to promote the development of several age-related pathologies during aging (5).

Despite living roughly six times longer than laboratory mice, blind mole-rats (BMRs) have been recognized as an advantageous rodent model for both aging and cancer research due to their lack of age-related pathologies and spontaneous malignancy (6). It is remarkable that BMRs, who live in severe environmental conditions and under constant stress, showed no direct aging phenotype during their 20 years of captivity, and no spontaneous tumor formation was reported. Besides, *in vitro* induced cancer resistance was reported recently(7) and, according to Gorbunova et al., the mechanism of cancer resistance in BMRs is regulated by an increase in necrotic cell death response with Interferon- β (IFN-β) release to prevent over proliferation of p53 and Rb pathways (8).

All IFN types are capable of activating The Janus kinase/signal transducer and activator of transcription (JAK-STAT) signaling pathway and two types of IFN, Type I (IFN-α, IFN-β, IFN-ω, and IFN-τ) and Type II IFNs bind to specific plasma membrane receptors, thereby initiating a cascade of events known as the JAK-STAT signaling pathway which affects circulating inflammatory markers, including SASP

factors. Therefore, the role of the JAK-STAT pathway has recently gained attention in aging and cellular senescence studies, in addition to cancer studies.

Previous studies suggest that IFN-β, one of the activators of JAK-STAT signaling, might have a role in the cancer resistance mechanisms of BMRs (8). Regarding the superior aging features and IFN-β-related cancer resistance mechanism, JAK-STAT signaling has the potential to be involved in the adaptive tools of BMRs. Based on this information, the proposed study aims to investigate variations in the protein expressions of the JAK-related signaling pathway in young and replicative senescent fibroblast cells of BMRs. Furthermore, considering aging is a complicated phenomenon that occurs through time, another goal of the proposed study is to acquire proteomics data for the first time to investigate numerous proteins and pathways linked with cellular signaling rather than targeting a single gene or signaling pathway. Proteomics research on BMR cells in the literature is limited, and there is no proteomics study on the specific species (*Nannospalax xanthodon*) to be employed in the study.

2. INTRODUCTION

2.1. Aging

The elderly population in the world is rapidly increasing accompanied by morbidity and mortality. Aging is a naturally occurring phenomenon that is strongly tied to the decline in genomic, cellular, and whole organism level homeostasis, which leads in various pathologies, including cancer and neurodegenerative diseases (9). Therefore, it is essential to understand the mechanisms behind aging. All living organisms experience biological aging and undergo certain biochemical changes, which eventually result in a loss of physiological integrity, decreased function, and death. These biochemical changes are known as the hallmarks of aging. Hallmarks of aging were firstly classified by Lopez-Otin and colleagues (10). They identified these hallmarks with three criteria; first, they should be clearly observed through aging. Second, one should be able to observe the increased aging during experimental stimulation. Third amelioration of the hallmark, experimentally, should slow down aging and improve longevity. As a result, nine hallmarks were defined and categorized into three groups according to their functional interconnections: 1. Primary hallmarks (genomic instability, epigenetic alterations, telomere attritions) are undoubtedly have negative impact on aging with causing damage including loss of telomere and chromosomal aneuploidies. 2. Antagonistic hallmarks (Mitochondrial dysfunction, cellular senescence (CS), and deregulated nutrient-sensing) have an adverse effect that depends on their intensity. 3. Integrative hallmarks (stem cell exhaustion and altered intercellular communication) occur when tissue homeostatic mechanisms are unable to repair the cumulative damage produced through primary and antagonistic aging hallmarks (10).

Analysis of the relationship between potential hallmarks and their proportional implications for aging is a significant challenge for identifying therapeutic targets to improve human health during aging with minimal adverse effects. With recent technological advancements, various experimental approaches have been developed

for detecting aging through different cellular and molecular activities. In this aspect, researchers' CS mechanisms in cellular or biological aging have gained attention as one of the significant aspects of aging (11).

2.2. Cellular Senescence

The word senescence is derived from the Latin word "*senex*", meaning old age (12) and was firstly described by Hayflick and Moorhead in 1961 as cell-cycle arrest of nonmalignant cells *in vitro* which is called as replicative senescence. During the experiments, Hayflick and Moorhead realized that cells stop dividing after around 50 serial passages, and this limitation is called the Hayflick limit (13). CS, a phenomenon in response to different types of stressors, is defined as a mostly irreversible and stable cell cycle arrest which is a dynamic and multi-step process due to their diversities of cell types and different context. The role of CS in in organisms can be both beneficial and detrimental. CS has a key role in developmental processes by conserving the tissue repair and homeostasis in addition to wound healing. Besides, cells prevent proliferation of tumors by limiting the proliferation of damaged cells. On the other hand, it has also deleterious side with hindering regeneration and tissue repair which contributes to organismal aging. The accumulation of senescent cells increases with chronological age and, therefore, they were observed in multiple age-related diseases such as diabetes and atherosclerosis (14). The high induction of senescent cells with age is supporting the hypothesis that CS itself can drive aging and is one of its key hallmarks.

CS can be categorized into two major types according to different stimuli: Replicative senescence and stress-induced premature senescence (SIPS). The primary trigger behind the replicative senescence was stated as a malfunction in telomere repair. Yet, there might be a heterogeneous mechanism such as rapid telomere shortening, altered gene expression, and induced oxidative stress for the induction of

replicative senescence (15). CS caused by different forms of stress is defined as SIPS and studies demonstrated that cells might undergo CS in response to several forms of DNA-damaging stressors, such as ionizing radiation, oxidative stress, oncogenic activation, and chemotherapeutic drug treatment (16).

Replicative senescence takes place as a result of telomere erosion, and reduced telomerase expression (17). Moreover, telomeres are nucleoprotein complexes that are composed of consecutive hexameric repeat units that are placed at the ends of linear chromosomes (18). Telomeric DNA consists of double-stranded DNA sequences followed by single-stranded guanine nucleotide repeats (G-overhangs). With the insertion of single-stranded G-overhangs into the cytosine-rich region in the double-chain part of the telomere, a structure called T-loop is formed and this structure ensures the protection of the telomere chromosome end (19). During replication, the 5' end of the new double-stranded DNA molecule is attached to the primary RNA resulting a gap. As a result, during S stage of the cell cycle, chromosomes lose 8-12 base pairs resulting in telomere shortening, and when the telomere length is shortened to a size that cannot form a T-loop, the cell cycle is terminated. Shortening in telomeres during aging in human fibroblasts were demonstrated in 90's, but the role of DNA loss in CS was unclear (18). Telomerase enzyme, a ribonucleoprotein, ensures that new telomere repeats are added to shortened telomeres, thus maintaining the protective properties of telomeres (20,21). Further studies revealed that lack of telomerase activity reduced replicative capacity of somatic cells due to loss of telomere capping function activating the DNA damage checkpoints and resulting in replicative senescence (22).

SIPS might occur in different types of cells such as normal or immortalized with induction of different form of cells. Due to occurrence of SIPS in earlier population doublings and stress induction is referred as "premature". SIPS is divided under subcategories according to the cause of stressor types including, oxidative stress, direct DNA damage, or exposure to chemotherapeutics (23).

Oxidative stress induced senescence occurs when cells are exposed to an oxidative agent or maintained under hypoxic circumstances, and mitochondrial activity is impaired (24). Besides, it has also been demonstrated that oxidative stress activates the tumor suppressor proteins p53 and retinoblastoma protein (pRb) in cells, resulting in SIPS (16).

Oncogene induced senescence (OIS) is another type of SIPS and occurs as a result of the activation of an oncogene or the weakening of a tumor suppressor mechanism (25). OIS induced by Rat sarcoma virus (RAS) was firstly observed in human fibroblasts as a result of RAS activation. With the increase of oncogenic RAS expression, CS occurs through the accumulation of p53 and p16 in the cells. In addition, inactivation of other tumor suppressors such as PTEN and NF1 are also shown as inducers of CS (26,27).

Although the CS inducing stress type differs, cell responses cannot be regarded independent from one another. Oncogene activation might generate oxidative stress, and reactive oxygen species (ROS) produced by oxidative stress can cause DNA damage. Moreover, unlike replicative senescence, this form of CS progresses rapidly, between a few hours to a few days (28).

2.3. Hallmarks of Cellular Senescence

Senescent cells exhibit many common morphological and biochemical features and these properties are used to detect senescent cells both *in vivo* and *in vitro*. However, due to the complexity of CS, it is difficult to find specific markers that can accurately identify and measure senescent cells (29). For this reason, it is suggested to combine two or more hallmarks to detect the CS. Seven subcategories can be defined for hallmarks of CS as shown in Figure 2.1.

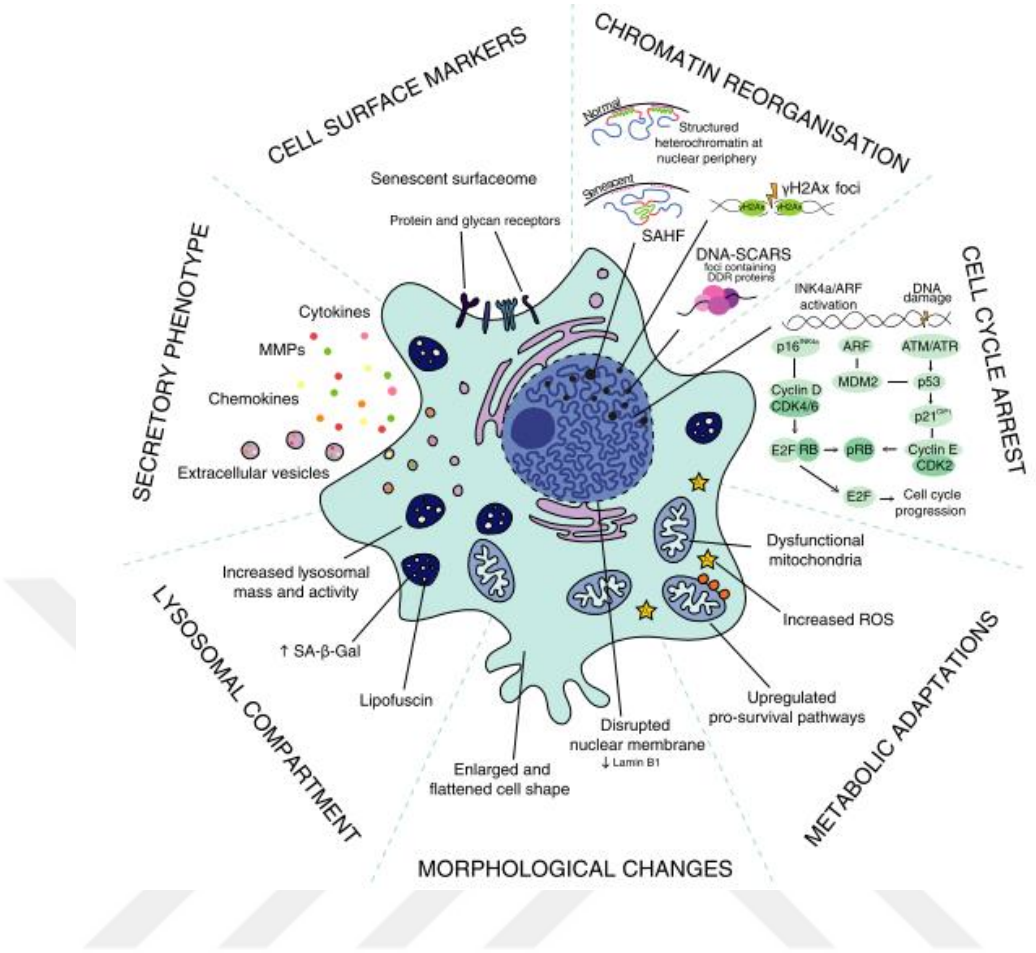

Figure 2.1: Summary of hallmarks of senescence is represented.

Various cellular mechanisms and alterations are used for detecting CS. The hallmarks for CS detection are categorized according to these mechanisms (Adapted from Gonzales 2021 et al.) (30).

2.3.1. Morphological Alterations

The senescent cells alter morphologically by becoming enlarged and flattened and having rather a large nucleus which represents their key physiological feature (13). Increase in granularity is another alteration in senescent cells, but might not be observed in all types of CS. The morphological changes can be detected using normal

or fluorescent microscopy for *in vitro* experiments (31,32). Multinucleation might occur during CS and can be detected using DAPI staining (30).

Lamin B1 protein, which is one of the major proteins in nuclear lamina, is a marker CS to detect nuclear alteration (33). During CS, Lamin B1 secretion is decreased due the loss of structural integrity in the nucleus. It should be noted that the changes in morphology and granularity of senescent cells are generally qualitative indicators. Therefore, it is suggested to use them to track the induction of the CS response *in vitro* as well as to properly evaluate the senescent phenotype by combining various identified markers as shown in Figure 2.1 (30).

2.3.2. Lysosomal Compartment

Senescence associated beta-galactosidase (SA-β-gal) activity is one of the first biomarkers discovered for CS detection and is essential in for the detection of senescent cells for various age-related diseases (34). SA-β-gal is a hydrolase enzyme and involved in the catalysis of hydrolysis of β-galactosides into monosaccharides. 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) is the widely known substrate of the SA-β-gal catalysis and dimerization of this substrates yields blue colored precipitation. Most of the SA-β-gal assays are colorimetric and use this substrate as chromogenic substrate. Since most β-galactosidases in cells are usually active at pH 4.0, the SA-β-gal assay is designed to detect senescent cells at a suboptimal pH which is 6.0 to distinguish normal and senescent cells (37). However, high β-galactosidase activity at pH 6.0 has also been found in cells under a variety of circumstances unrelated to CS and the use of other markers to determine CS is necessary for better detection (23).

2.3.3. Metabolic Adaptations

Increase in mitochondria amount due to lowered mitophagy activity occurs in senescent (35) and this accumulation results with generation of ROS which eventually causes to mitochondrial dysfunction (36). Besides, CS can be induced by mitochondrial-derived ROS that are released from senescent cells (37). However, more comprehensive analysis of mitochondrial function in various senescent cell types is required to use mitochondrial dysfunctions as a CS biomarker.

2.3.4. Cell Cycle Arrest

Cell division is the cascade of events that occur due to the cell cycle progress and is necessary for the continuous development of multicellular organisms (16). Another distinctive hallmark for CS, arrested-cell cycle is demonstrated through proteins p16, p21, and p53, besides to the decline of the phosphorylated pRb (30).

Cyclin-Dependent Kinase Inhibitors (CDKs) are part of cell cycle progression and they take role in phosphorylating and regulating various types of proteins during the cell cycle. CDKs that are encoded in CDKN2A (p16), CDKN2B (p15), and CDKN1A (p21) are the main regulators of the cell cycle arrest (38,39). Measuring the p16, p21, and p15 expressions is another most common hallmarks for CS.

Additionally, cell proliferation and/or DNA replication experiments might be performed to further investigate the cell cycle arrest. The growth curve of cells *in vitro*, can be analyzed by various methods such as live cell imaging and counting at multiple time-points across time can be utilized (40).

While improperly repaired DNA lesions might result in mutations, if the damage is not repaired DNA damage response (DDR) is activated by cells which can trigger CS or apoptosis by arresting cell cycle. Moreover, the dysregulation of DDR and repair mechanisms can result in a variety of severe pathologies, including accelerated aging and developmental defects. Continuous activation of DDR might phosphorylate different residues of *p53* gene(41). That's why increase in p53 is one of the most used markers of CS (42). However, it should be noted that DDR activation is a result of various stimuli which does not always lead to CS and DDR is not the only stimuli for CS.

2.3.5. Chromatin Reorganization

Another feature of senescent cells is condensation in heterochromatic regions known as senescence-associated heterochromatic foci (SAHF). These heterochromatic regions; Lysine 9 is rich in methylated histone H3, heterochromatin protein-1γ, and nonhistone chromatin proteins. SAHF positive cells can be identified by fluorometric staining of these proteins (43).

2.3.6. Cell Surface Markers

Although there is no specific surface marker is not defined for senescent cells, recent articles are focusing on surfaceome, all cell surface proteins, of senescent cells including ICAM-1, DEP1, and B2M are used to detect CS with histological staining method (30).

2.3.7. Secretory Phenotype

CS has the ability to influence cell environment and interact with neighboring cells through secreting a combination of secreted substances that can modify the activity of surrounding non-senescent cells and these factors are known as SASP (5). Senescent cells are able to communicate with neighboring cells and change the extracellular matrix through these secretory activities. Thanks to these properties, senescent cells show strong autocrine and paracrine effects. There are numerous soluble signaling factors that includes growth modulator, angiogenic factors, extracellular matrix components, and matrix metalloproteinases (MMPs) which are the components of SASPs (30,44).

Coppe et al., classified SASP factors in three categories which are soluble signaling factors (interleukins, chemokines, and growth factors), secreted proteases, and secreted insoluble proteins and extracellular matrix components (45). Senescent cells also release a variety of regulatory/inhibitory factors, such as tissue MMPs, plasminogen activator inhibitors (PAI), and insulin-like growth factor-binding proteins. Therefore, while investigating the SASP effectiveness; not only the number of factors included in SASP content, but also the combined effect of SASP containing regulator/inhibitor should be evaluated. A list of SASP factors with changes in their secretion is summarized in Table 2.1.

Table 2.1: SASP factors alterations during CS are summarized.

SASP factors[a]	Secretory profile of senescent cells[a]
IL-6	↑
IL-7	↑
IL-1a, -1b	↑
IL-13	↑
IL-15	↑
IL-8	↑
GRO-a,-b,-g[c]	↑
MCP-2	↑
MCP-4	↑
MIP-1a	↑
MIP-3a	↑
Eotaxin	×
MMP-1, -3, -10, -12, -13, -14	↑
IFN-γ	×
IGFBP-2, -3, -4, -6, -7	↑
TIMP-1	↓ or ×
TIMP-2	↑
PAI-1, -2; tPA; uPA	↑
ICAM-1, -3	↑

a: Increase in SASP factor at senescence are shown with upward arrow. Crosses represents no change and decrease is represented by downward arrows. Adapted from Coppe et al., 2010 (45).

SASP formation and release has so far been directly associated with two transcription factors: nuclear factor-κB (NF-κB) and CEBPβ (CCAAT/enhancer binding protein beta, C/EBPβ). Both of these transcription factors are known to be involved in the regulation of cellular stress and inflammatory signaling, which promotes the expression of many cytokines (46). In recent studies, it has been shown that these transcription factors induced are also activated through P38MAPK and mTOR signaling pathways (47).

NF-κB transcription factor that is mainly triggered by DDR mediates the large amount of SASP (48). Besides, NF-κB is stimulated by inflammatory cytokines in senescent cells and this stimulation can effectively stop apoptosis and preserve the senescent phenotype of the cells (49). NF-κB activation is connected to various pattern recognition receptor pathways, including TLRs and inflammasomes, along with signals from several upstream kinase cascades through canonical and non-canonical pathways (50,51). Although numerous kinases can directly affect the transcriptional capability of NF-kB proteins, IKK/ and NIK are the most essential upstream kinases (52,53). Moreover, JAK-STAT pathway is another triggering pathway for the upregulation of immunosuppressive cytokines including IL-10, IL-13, CXCL1 (GRO1), and CXCL2 (GRO2) (54).

SASP can be regulated by pharmacological or genetic inhibitions, thereby avoiding the harmful effects of SASP. The mTOR inhibitor rapamycin, one of the inhibitors used to manipulate SASP in the literature, inhibited the inflammatory effect of SASP by inhibiting IL1α transmission, which is responsible for the expression of pro-inflammatory cytokines such as IL6 (55).

2.4. Inflammation and Aging

Globally, infectious illnesses cause a large number of fatalities, and older persons are more susceptible to serious infections (56). Inflammation, a hallmark of aging, is known to be the defensive immune response for preserving metabolic homeostasis by removing harmful stimuli such as damaged cells and pathogens (57). Chronic low-grade inflammation throughout aging, which is commonly referred as "inflammageing," a hallmark of aging and may pose a health risk to elderly adults (58). Despite being a significant element in regulation of pro- and anti-inflammatory factors, the immune system is not the only source of these factors. Moreover, recent research in fibroblasts and epithelial cells indicated that CS is associated with a rise in the

production of 40-80 factors involved in intercellular signaling which are known as SASP. Regulated oncogene (GRO)α, monocyte chemotactic protein (MCP)-2, MMP-3, IL-6, IL-8, and IL-1 proteins trigger the inflammation and are also widely expressed by SASP (59).

The changes in immune system represents a decline in trigger of effective antibodies and cellular responses. This phenomenon is called as “immunosenescence” and affects both innate and adaptive immunity (60,61).

Many age-related disorders, including Alzheimer's and Parkinson's diseases, cancer, and atherosclerosis, have also been shown to have an immunological and inflammatory responses, as confirmed by different studies (62–64). Therefore, understanding associated genes and pathways in diverse species that contribute pleiotropically to phenotypes associated with ageing, longevity, and immunity might aid in the identification of shared molecular underpinnings mechanisms underlying the immunity-ageing cross-talk (65). JAK-STAT pathway is one of the critical pathways for immune system regulation specifically regulating cytokine receptors. Dysregulation of this pathway might result in multiple immune related diseases (66).

2.5. Januse-Kinase Signaling Pathway

Januse Kinases (JAKs) are member of non-receptor tyrosine kinase and has four different genes in this family, JAK1, JAK2, JAK3, and Tyrosine kinase 2 (TYK2). JAKs are generally localized in cytosol when the cytokine receptors are absent. Other than that, they are found in plasma membrane. JAK1 regulates vast variety of cytokines which employ common γ chain (γc) such as IL-4, IL-7, and IL-21. Besides, JAK1 and JAK2 has an important role in gp130, a common receptor subunit, and IFNs. TYK2 is another member of the family and firstly defined JAK protein for IFN

signaling. However, with recent research it is shown that although it is crucial for IL-12, it doesn't have any role in IFN-α/β signaling. STAT proteins are cytoplasmic transcription factors that are stimulated by extracellular signaling proteins. They are also involved in cytokine signaling and a part of family which have seven different proteins. When activated, variety of target genes transcripts are resulting in excessive cell proliferation and angiogenesis. Moreover, STAT3 increase is observed in oral cancer through increased transcription of JAK, IL-6 and EGFR pathways (67).

JAK-STAT pathway subsequently dimerizes and moves into the nucleus through the nuclear membrane to control the activation of associated genes (68). This pathway is involved in stem-cell maintenance and in other developmental process. Besides, it was found recently as a cytokine-stimulated signal transduction pathway (66). The JAK-STAT pathway has other roles in multiple biological processes such as apoptosis, cell proliferation, and immune regulation (69). One of the important activators of the JAK-STAT pathway is IFN proteins. Three classes of IFNs are defined that are Type I, Type II and Type III (70). JAK-STAT pathway can be triggered through all types of IFNs. The relation of IFNs and JAK-STAT pathway is demonstrated in Figure 2.2.

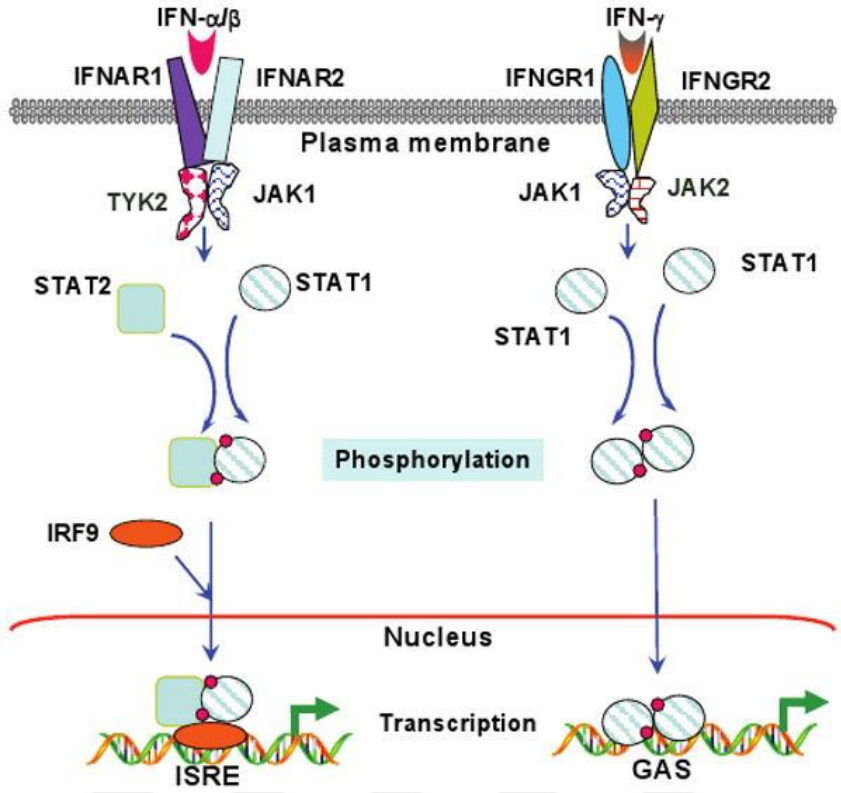

Figure 2.2: JAK-STAT signaling pathway is induced by IFNs.

The regulation JAK-STAT signaling pathway is employed by IFNs. JAK1 and JAK2 proteins are induced by Type I (IFN- α and IFN- β) and type III (IFN-γ) IFNs bind to different receptors, and involved in activation of transcription factors through JAK-STAT pathway (Adapted from Li et al) (71).

JAK-STAT pathway is one of the key mechanisms that is involved in cytokine production and induced in senescent cells by regulating SASP secretion. Targeting this pathway is suggested to be potential mechanism for CS (72). Besides, The JAK-STAT pathway is potential therapeutics for cancer treatment due to the evidence that it is activated in a significant portion of solid tumors and that this activation contributes to the malignant characteristics of cancer cells (73).

The precise molecular pathways of JAK stimulation stay largely unclear. More precise molecular knowledge on JAKs and the relation of JAK-cytokine-receptor is required to improve our understanding of the mechanism of JAK activation.

2.6. Proteomics Studies

The role of proteins as macromolecules in cellular systems is highly vital with their various functions in structure and function of cells. “Proteome” is defined as the all expressed proteins of the genome. Unlike genome, the expression of proteins greatly alternates through different cells(74). Besides, Pathological diseases including cancer and neurodegenerative diseases are the result of misregulations during the expression of protein and understanding the protein structure and conformations may lead to better treatment for pathologies. Despite a large amount of information available on the human genome, the prediction of post-translational modification (PTM) remains elusive. PTMs are covalent modifications that alter the protein properties during their synthesis. “Proteomics” is the study of proteome and used for identifying structure of proteins, amount of the proteins that are produced by specific cells or tissues and interactions of proteins (75).

Proteomic approaches are also important to identify the protein in senescent cells and to identify potential biomarkers of CS and aging. SASP profiles are thoroughly investigated and uncovered by contemporary proteomic techniques, which also revealed that SASP heterogeneity relies on type of the cells, temporal changes and CS inducing stimuli (76). As discussed above, a complex cocktail of proteins and molecules are involved in SASP and it is one of the hallmarks of CS. Besides, targeting SASP for therapeutic approaches is important to be able to determine these secretory factors (77). Proteomic studies are necessary to quantitatively analyze SASP factors, since transcriptome studies are not able to assessing the secreted proteins. For example, a recent article developed a proteomic database called as “SASP Atlas” for identifying

SASP factors across tissues and define the possible biomarkers for the detection of CS (78). Multiple studies on proteomics analysis of senescent cells were published (76,79,80), however there is no publicly available proteomics data on BMR senescence.

2.7 Model rodents for Cellular Senescence Studies

Various experimental and computational approaches can be used in CS studies. As it is important to choose the right biomarker for CS, the model organism for the studies also play a crucial role to study the CS. Ethical and practical issues of human models' direct researchers to find alternative models. These model organisms vary from the yeast cells which are the simplest eukaryotic cells to dogs and primates. Low level organisms (*Caenorabtidis elegans, Drosophila melanogaster* etc.) are widely used since their simple genome and are easy to manipulate (81). For example, Drosophila share %60 of genome with human. Furthermore, the importance and necessity of the mammalian models is accepted by scientists to understand the aging mechanisms more precisely.

The laboratory mouse *(Mus musculus)* and rat *(Rattus norvegicus)* are the most known and preferred models for CS studies due to their highly known background, ability to manipulate their genetic background and environments. Domestic mouse share 85% of protein-coding regions with human. However, to understand the aging and CS and uncover novel mechanisms, superior aging models have gained interest that have long-life span. These species are phylogenetically close to *Murinae* and studies with these models might help understanding the evolutionary longevity mechanisms. Although nearly 50 years passed since their discovery, there are many unknowns in their molecular mechanisms related to longevity (6).

Among long-lived models, two mole-rats; naked mole-rats (NMRs) and blind mole-rats (BMRs) display superior longevity and cancer-resistance mechanisms. The NMRs mostly live in Africa and they are eusocial, while BMRs generally live in the Middle East and have solitary life style (82).

2.7.1 Blind Mole-Rats

BMRs are extraordinary underground rodents belong to the subfamily of Spalacinae and can live up to 21 years old (Figure 2.3) (83). Besides, in 40 years of monitoring, there has never been a single case of spontaneous cancer in BMRs, which makes them extraordinarily immune to external carcinogens. Considering that tumors are a major cause of death in other aging rodent species (including mice, rats, and hamsters), the lifespan of BMRs has aroused attention in the tumor suppression mechanisms(84).

Figure 2.3: Blind mole-rats are subterranean rodents.

A recent finding claimed that after 7-20 population doublings, BMR fibroblasts begin to release IFNβ which initiates a mechanism called as concerted cell death (CCD), that is defined by a high proportion of necrotic cells. The cell-death

characteristic was reversed by repressing the tumor suppressors Rb and p53.Through dramatically promoting the necrotic cell-death response with the induction of IFNβ in response to hyperproliferation, BMR might develop a novel anticancer strategy(85). Besides, Type I interferons including IFNβ are one of the main stimulators of the JAK signaling pathway(66). However, the exact mechanism of the inflammation pathways of BMRs remain elusive.

Overall, JAK-STAT pathway is involved in multiple critical regulation in cells especially in immune system. Any dysregulation in the proteins related to JAK-STAT pathway might cause several diseases. Moreover, there are various treatments that are under development targeting this pathway(86,87). To understand the role of JAK-STAT pathway in BMRs, it is aimed to elucidate the change in mRNA and protein expressions of the JAK-STAT pathway in young and replicative senescent fibroblast cells of extraordinary long living BMRs, which has not yet been fully elucidated.

3. MATERIALS AND METHODS

3.1. Cell culture

Primary BMR and mouse lung fibroblast cells were isolated in our previous studies. Briefly; lungs were collected from both mouse and BMR and to get rid of remaining blood tissues were washed with Phosphate Buffer Saline (PBS). and were cut into small pieces using collagenase-A solution. Then, tissues were transferred into 15 ml tubes and incubated for 4 hours at 37°C, 5% CO_2 for digestion. After 4 hours of incubation, collagenase-A was replaced with culturing medium. Pipetting was done repeatedly to generate a firm cell suspension. Finally, the suspensions were cultured into plates and incubated in the standard incubator. Different cell mediums were used for BMR and mouse fibroblasts. Dulbecco's Modified Eagle's Medium DMEM/F12(ATCC) medium containing 15% Fetal Bovine Serum (FBS, Thermo Fisher, Waltham, MA, USA) was applied to BMRs, while Dulbecco's Modified Eagle's Medium DMEM (ATCC) medium containing 10% FBS (Thermo Fisher, Waltham, MA, USA) was used for mouse. After formation and proliferation of fibroblast cells, they were trypsinized and frozen for further use.

Frozen BMR cells were thawed quickly, and slowly pipetted into 15ml falcon tube that has 9 ml of related medium, and 1% penicillin/streptomycin (Thermo Fisher, Waltham, MA, USA). Then, the tubes were centrifuged at 1500 rpm for 5 min. Obtained cell precipitation was dissolved in the fresh medium and incubated in T25 flasks at 37°C, 5% CO_2 conditions. When the density of flasks was over 90%, cells were collected with the help of trypsin enzyme (0.25% EDTA) and centrifuged at 1500 rpm for 5 min. Cell pellets were dissolved with the cell medium and cells were counted by Thoma's improved chamber and seeded into new flasks.

3.2. Confirmation of Replicative Senescence

Induction of replicative senescence was achieved by serial passaging of BMR fibroblasts. When isolated primary fibroblast flask (passage(P)-0) reached confluency, cells were serially passaged. P2-3 cells were described as the early passage or young fibroblast cells and cells from P7-9 were described as the late passage or senescent fibroblasts. To confirm CS, different biomarkers related to morphological and signaling pathway are used.

3.2.1. β-Galactosidase Staining

To detect SA-β-gal enzyme in BMR cells, SA-β-Gal assay was performed. CellEvent™ Senescence Green Detection Kit (C10850, Waltham, MA, USA) was performed according to the Manufacturer's protocol. Both early and late passage cells were seeded into 96 well plates as 10^4 in triplicates. For adherence of cells, the plate was incubated 24 hours in the incubator. After 24 hours, fixation solution was applied for each well as 100 ul, which was prepared as 2% Formaldehyde solution in PBS for 10 minutes as suggested in the kit. The working solution of the β-gal staining which is obtained from the kit was prepared as a 1:1000 ratio. Then, each well was washed with PBS and the working solution was added as 100 ul to each well, and the plate was incubated at 37℃ without CO_2 supply which is important due to the sensitivity of pH of the solutions for 2 hours. After the incubation, wells were washed three times with PBS solution and images from early and late passages of fibroblasts in triplicates were the images captured using Axio Inverted Fluorescence Microscopy (Zeiss, New York, NY, USA) and Zeiss Software.

3.2.2 Cellular Growth Curve

To observe the slowed proliferation and cell-cycle arrest, early and late passage BMR fibroblasts were seeded as 4×10^4 cells per well in a 24-well plate in triplicates. After 24-, 48-, 72-, 96-, and 120-hours cells were collected and cell numbers for each day was counted using Thoma's improved chamber. Then, a cellular growth curve graph was created in Microsoft Office Excel program with error bars (n=3).

3.3. mRNA expressions of Fibroblast cells

3.3.1. RNA isolation

The Quick-RNA Miniprep RNA kit (R1054, Zymo Research) was used to isolate RNA from cells. DNA is degraded on the membrane by rDNase treatment that was provided by the kit. The purified RNA is then eluted in low ionic strength conditions with RNAse and DNAse free water. Total RNA from samples was stored at -80℃ for further experiments.

RNA samples' purification and quantification were done with NanoDrop 2000 UV-Vis spectrophotometers at 260 nm absorbance and the calculations of required RNA amounts were cDNA synthesis was done using the absorbance results.

3.3.2. cDNA synthesis

To obtain cDNA from RNA samples, Sensifast cDNA synthesis kit (BIO-65053, Bioline, Tennessee, TN, USA) was used. In each reaction tube, 0.1µg of RNA, 4 µl of

TransAmp buffer, and 1 µl of reverse transcriptase enzyme were added. Then, mixture was completed up to 20 µl with nuclease free-H_2O. The reaction tubes were incubated at 25 °C for 10 min for primer annealing, then 42 °C for 15 min for reverse transcription. Finally, to inactivate enzyme, tubes were incubated at 85 °C for 5 min and put into ice immediately.

3.3.3. Reverse Transcriptase Quantitative Polymerase Chain Reaction

3.3.3.1.Primer synthesis and Optimization

Using the online Primer3 program (https://primer3.ut.ee/), primer sets were generated with optimum 60℃ degree annealing temperature. mRNA FASTA sequence for BMR was obtained from the NCBI database (accessed on 06.12.21). Table 3.1 indicates the primer list used in the research.

Table 3.1: Designed primers for each gene are demonstrated.

	Gene	Left Primer	Right Primer
Blind mole-rat	*Jak1*	AATCTTCTTCTGGCCCGTGA	AGCAGCCACACTCAGATTCT
	Jak2	GGCAATGACAAGCAAGGACA	TGAAGGAGGGACGCTGATTT
	Il6	GCTTGAACAATGACGAGGCC	GACATTGGCCTGCACGTTTT
	Actb	CCACCATGTACCCAGGCATT	CGGACTCATCGTACTCCTGC
Mouse	*Jak1*	TCTGTCACAACCTCTTCGCC	CATCAAGGAGTGGGGTTGCT
	Jak2	AGACGAGTCAACCAGGCATG	TAACACCGCCATCCCAAGAC
	Actb	CCACCATGTACCCAGGCATT	CGGACTCATCGTACTCCTGC

3.3.3.2. RT-qPCR

Each qRT-PCR was performed with the CFX96 Touch Real-Time PCR Detection System (BIO-RAD, California, CA, USA) using nuclease-free PCR tubes. For every reaction tube contained 10 µl SYBR green (BIO-98005, Bioline), 3.2 µl dH2O, 0.8 µl of the forward and reverse primer (400 nM), and 2 µl of the cDNA template to complete the reaction to 20 ul. The following RT-qPCR protocol was applied; 95℃ for 2 min, 40 cycles of 95℃ for 5 sec, optimized annealing temperature for 10 sec, 72℃ for 5 sec. A melting curve was created with 60 to 95℃ and 1℃ increase in every 5 sec.

3.3.3.3. Gel extraction for quantitative analysis

Agarose gel electrophoresis was performed to check that if the products were at the desired size. Then, the bands were observed in the gel at the desired size for each primer using ChemiDoc system. The bands were cut from the gel and gel extraction was performed with the Hibrigen Gel Extraction kit to create a standard curve. The concentration of the obtained double-stranded DNA was measured by Nanodrop spectrophotometer and standards from 10^7 to 10^3 were used for further analysis. The samples were run with standard curve samples and copy number of mRNA expressions was calculated. Then, mRNA expressions were normalized to β-actin mRNA expression. Control groups' mRNA expression was accepted as 100% to calculate changes in other samples' mRNA expressions.

3.4. Extraction of proteins and the analysis of Nano LC ESI MS MS

Protein samples from both early and late passage fibroblasts were collected for the protein extraction (n=4). Cell pellets were sent to SZAomics for LC-ESI-MS/MS analysis. Briefly, cell pellets were sonicated and centrifuged at max rpm for 10 min. Supernatants were collected and using the Bradford method concentrations of proteins were determined. To extract peptides, FASP Protein Digestion Kit (Expedeon-44250) with addition of protein digesting trypsin enzyme (Pierce-90057) were used. Raw data analysis for detection of proteins of LC-ESI-MS/MS results was performed by SZAomics.

The raw data was analyzed by SZAomics which was identified from Uniprot *Mus musculus* databases. For further analysis, the protein quantities acquired from BMRs were normalized. Then, EdgeR package was used for differential expression analysis. To calculate logarithmic fold changes for each protein, generalized linear model was applied. The enrichment analyzes were performed using the obtained proteins fold changes and Gene set enrichment analysis (GSEA) tool was used for further analysis to obtain KEGG, and gene ontology (GO) gene sets. The ClusterProfiler package was used to generate bar, and dot graphs showing. Network graphs were produced using the Cytoscape EnrichmentMap and Autoannotation tools containing sets and annotations of clustering findings were produced.

3.5. Statistical analysis

GraphPad Prism 9.3.1 was used for statistical calculations and generating graphs (GraphPad Software, CA, USA). The non-parametric Mann-Whitney unpaired t-test was used to compare and assess the means of two groups for qPCR experiments. When the *p*-value was less than 0.05, the difference was accepted as statistically significant.

4. RESULTS

4.1. Primary lung fibroblasts observation in BMR and mice

After isolation of both BMR and mice lung fibroblast cells, images were taken and presented in Figure 4.1 from passage 1 cells at day 3. After isolation, cells were seeded into t25 flasks and were proliferated in the flasks until they reach confluency.

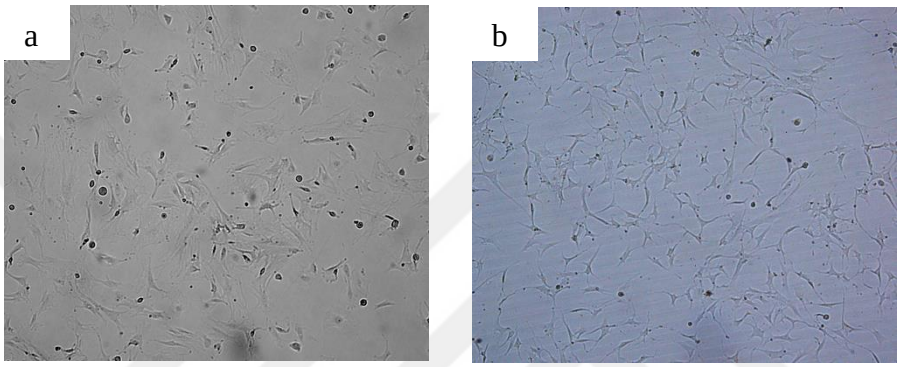

Figure 4.1. Migration of BMR and mouse fibroblasts from lung tissue.
Each image was captured on day 6 of culturing, using a lens of 10x. a) BMR lung fibroblasts b) Mouse lung fibroblasts.

After confirming fibroblast structure in cell culture, cells were serially passaged in flasks to obtain replicative senescence *in vitro.* To compare nonsenescent and senescent cells in cell culture early passages of BMR fibroblasts (P2-3) and late passages of BMRs (P7-8) were used for further experiments.

4.2. Confirmation of Replicative Senescence in BMR Fibroblasts

Replicative senescence was confirmed in the late passage fibroblast by CS biomarkers. First, microscopic images were captured from both early and late passages of fibroblasts after culturing plates for 3-4 days in the incubator. As seen in Figure 4.2a, cell structures were visualized for both early and late passage fibroblasts. The figure demonstrates the enlarged and flattened shapes in late passage cells. Further, to confirm the cell-cycle arrest with replicative senescence, a cell cycle growth figure was obtained by culturing cells from both early and late passage of BMRs in 24 well plate as triplicate. Cells were counted every 24 hours for 5 days and results were recorded (Fig 4.2b). Slowed proliferation in late passage fibroblasts compared to early passage fibroblasts were observed with cellular growth curve. To confirm the CS; SA-β-gal activity was detected both in early and late passage of BMR fibroblasts. The results showed positive staining is higher in late passage fibroblasts compared to early passages as shown in Figure 4.2c. Finally, mRNA expression of *Il6*, one of the major SASP component, analyzed with qPCR and the results showed that its expression was increased. However, no significant change was observed in *Il6* expression as shown in Figure 4.2d.

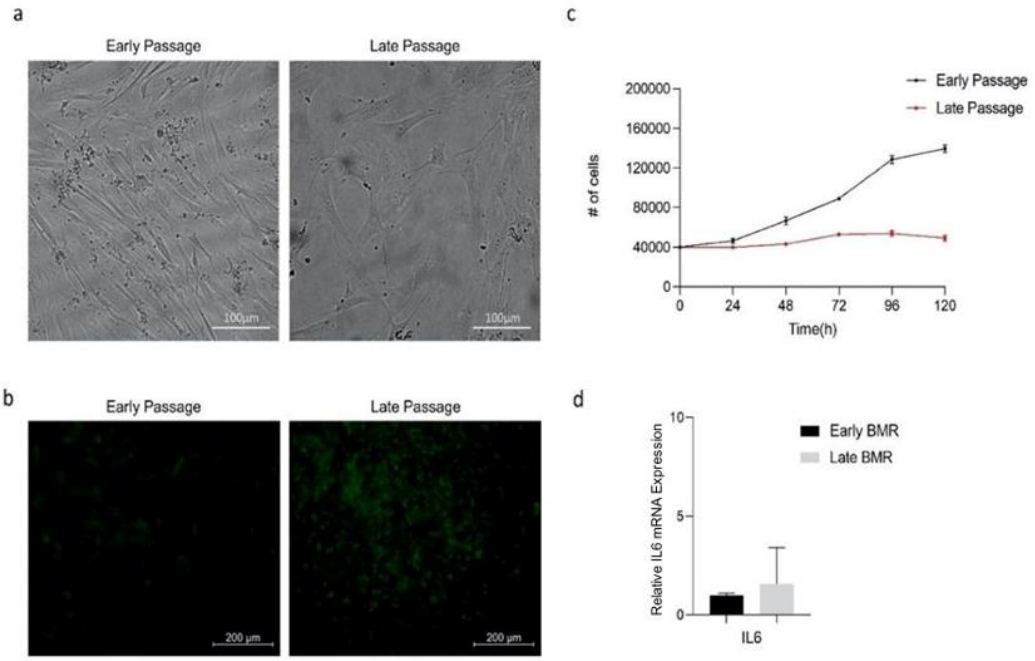

Figure 4.2: Observation of cellular senescence in late passages of BMR fibroblasts undergoing replicative senescence.

a) Morphological differences are identified among early and late passage fibroblasts. Each image was captured on day 3 of culturing, using a lens of 20X and a bar of 100 um. BMR fibroblast cells were planted at a density of 5 x 10^5 cells per T25 flask. b) SA-β-gal-stained fluorescence images of cells in early and late passage cells are compared. Early passage cells showed low light of SA-β-gal staining (Lens, 10X; Bar, 200 m), however late passage cells showed higher green staining than early passage cells. c) The cell growth curves of early and late passage cells were examined. The graph of the number of cells counted every 24, 48, 72, 96, and 120 hours represents the cellular growth curve. d) In early and late passage fibroblasts, the level of IL6 gene expression was examined. The levels of expression were determined by qRT-PCR, and representative data for IL6 was shown with fold change. The bars show the mean and standard deviation (SD) from three repeated experiments. The fold change was determined using the mean expression values from three replicates.

4.3. mRNA expressions of *Jak1* and *Jak2* in senescent BMR fibroblasts

After confirming the replicative senescence in BMR fibroblasts, comparative analysis of JAK-STAT pathway related gene expressions which are *Jak1 and Jak2* mRNA expressions were analyzed with RT-qPCR for both mice and BMR fibroblast samples (Figure 4.3). Comparison of *Jak1* and *Jak2* gene expressions between BMR and mouse fibroblasts showed that the mRNA expressions for both of the genes were lower in early passages of BMR when compared to mouse. However, in the late passage fibroblasts no significant change was observed between mouse and BMR (Figure 4.3a). We further compared the gene expressions for each species to observe the change of the *Jak1* and *Jak2* expressions through CS. Interestingly, the results showed that the expression levels were significantly increased during the replicative senescence in late passage BMRs compared to early passage BMRs (Figure 4.3b).

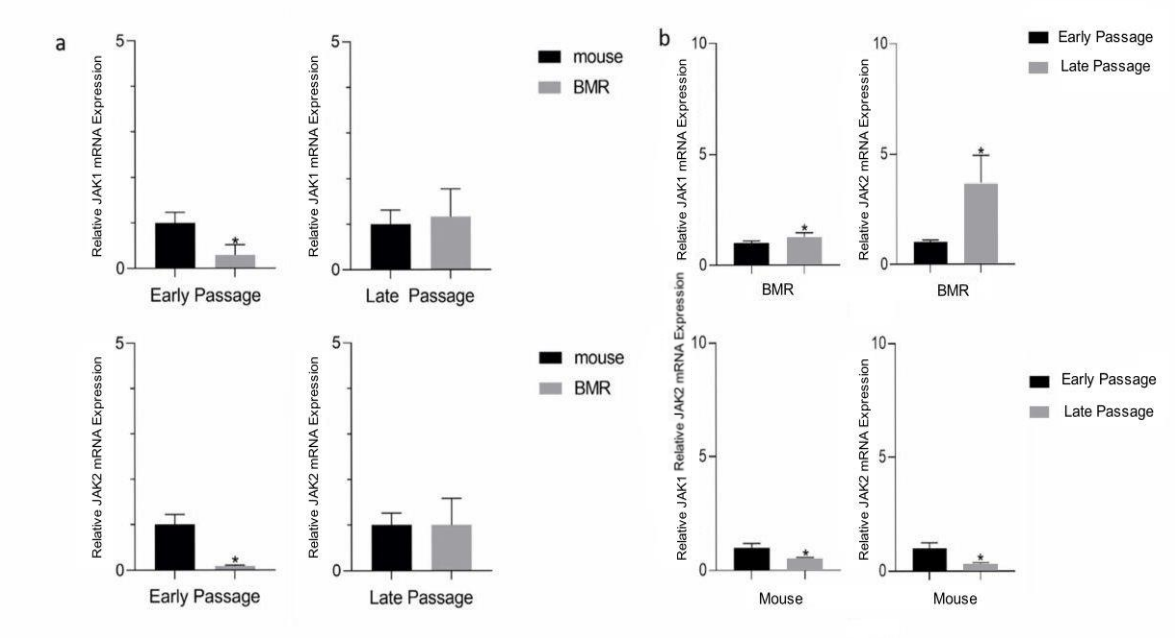

Figure 4.3: mRNA Expressions of *Jak1* and *Jak2* are compared with qPCR analysis in BMR and mice during replicative senescence.

a) Quantification of *Jak1* and *Jak2* gene expressions in BMR and mouse fibroblasts. Representative data of *Jak1* and *Jak2* in early mouse and BMR fibroblasts is shown with fold change. JAK expressions are downregulated in early BMR cells compared to mouse cells. *Jak1* and *Jak2* expressions did not show any significant changes in late passages. The expression levels were quantified with qRT-PCR. b) *Jak1* and *Jak2* gene expressions were quantified in early and late passage fibroblasts of BMR and mouse, individually. The expression levels were compared with qRT-PCR and representative data for *Jak1* and *Jak2* is shown with fold change. For calculation of *p* value Mann-Whitney test (n=3, * $p < 0.05$) was used. (BMR: Blind Mole-Rat). Calculations for the fold change and standard deviation (SD) are done with the mean expression values of three replicates. Three independent experiments repeated and bars represent mean ± SD.

4.4. Proteomics data analysis in BMR during replicative senescence

To reveal the further mechanism related to CS signaling pathways in BMR fibroblasts, it is important to extend the analysis in protein levels. We investigated the protein expressions of early and late passage of BMR fibroblasts using LC-MS/MS analysis. 818 total proteins were identified after MS analysis. After filtering and normalizing the protein data, the analysis was performed using RStudio. Number of proteins were 766 after filtrations.

4.4.1. Gene Ontology Analysis

The results of overrepresented analysis of Gene Ontology (GO) enrichment pathway identified the biological process, cellular components, and molecular functions of high and low expressed proteins in senescent BMR fibroblast. GO enrichment analysis showed main enriched pathways in senescent BMR fibroblasts are represented in Figure 4.4. Moreover, cytokine-mediated signaling pathway is positively enriched in late passage of BMR cells. The proteins identified in this pathway were found to be integrin-linked protein kinase (ILK), E3 ubiquitin-protein ligase (TRIM32), serine/arginine-rich splicing factor 1 (SRSF1), and terminal uridylyltransferase 4 (TUT4).

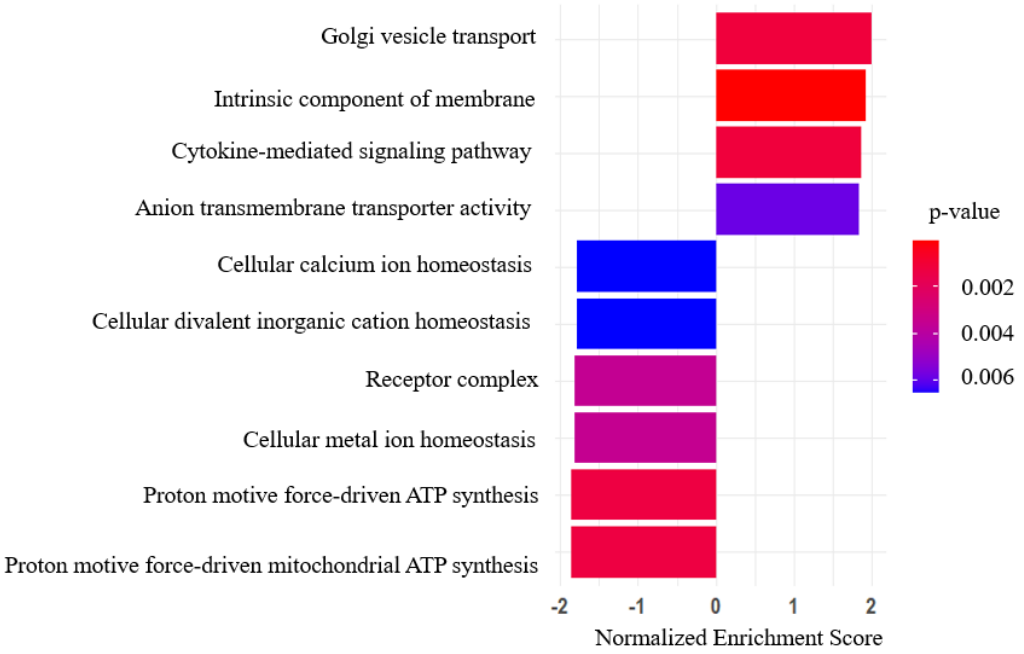

Figure 4.4: GO pathway enrichment analysis of BMR fibroblasts proteomics data.

The pathway enrichment analysis in GO is shown. Visualization process overrepresented in related pathways in late passage fibroblasts compared to early passage with normalized enrichment score.

GO enrichment analysis was performed for specific pathways and detail analysis with the proteins to further understand and further analyze significantly regulated pathways (Figure 4.5).

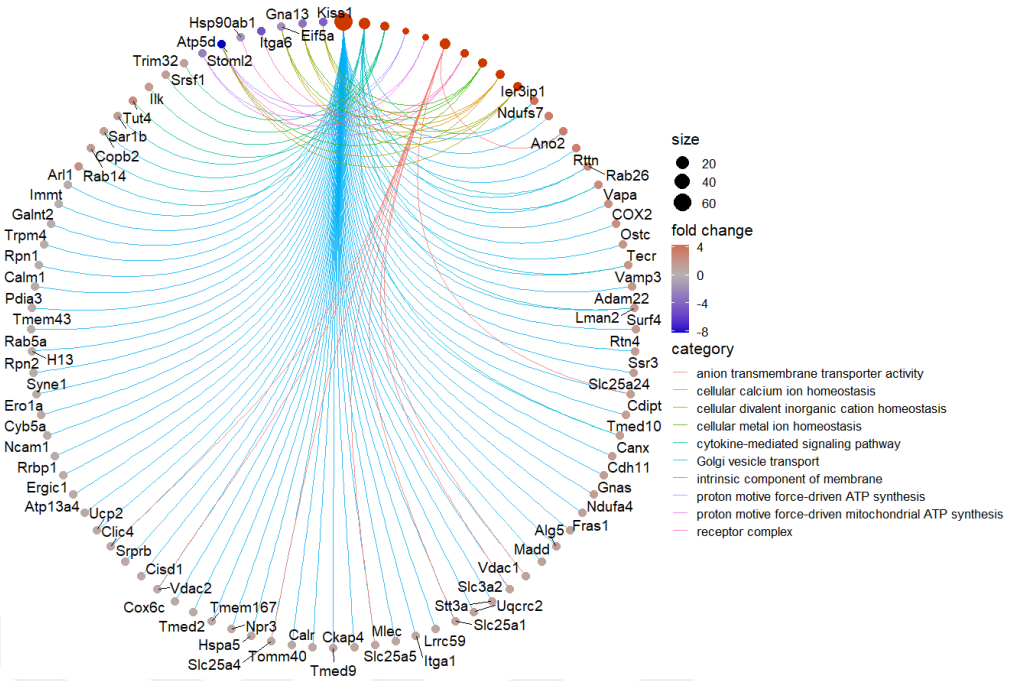

Figure 4.5: Significant pathways enriched in GO pathway analysis of BMR fibroblasts with detailed proteins were represented as cnet plot.

Gene association are shown with colored lines. Circle size is used to show the gene ratio. Threshold for significancy of pathways were taken as $p < 0.05$ ($n = 4$).

4.4.2. KEGG pathway enrichment Analysis

Results of overrepresented analysis of KEGG enrichment pathway that were identified in high and low expressed proteins in senescent BMR fibroblast are shown in Table 4.1 with the enrichment scores, normalized enrichment scores and p-values. P-value cut-off was selected as $p<0.1$.

Table 4.1: KEGG pathway enrichment analysis results of proteomics data

ID	Pathway	enrichmentScore	NES	pvalue
mmu05146	Amoebiasis	0.544615464	1,571676	0,031216
mmu00053	Ascorbate and aldarate metabolism	0,800009556	1,469935	0,050967
mmu04976	Bile secretion	0,774626336	1,692504	0,008589
mmu04514	Cell adhesion molecules	-0,806148996	-1,46882	0,057143
mmu04110	Cell cycle	-0,69154047	-1,61486	0,024879
mmu05230	Central carbon metabolism in cancer	-0,631028653	-1,51123	0,054692
mmu05171	Coronavirus disease - COVID-19	-0,579325428	-2,07964	0,000332
mmu04927	Cortisol synthesis and secretion	0,848493694	1,430326	0,052081
mmu04934	Cushing syndrome	0,672521486	1,469412	0,072393
mmu00270	Cysteine and methionine metabolism	-0,755042166	-1,53424	0,044383
mmu05418	Fluid shear stress and atherosclerosis	-0,665450115	-1,69462	0,01156
mmu00051	Fructose and mannose metabolism	-0,912256267	-1,53639	0,018279
mmu00010	Glycolysis / Gluconeogenesis	-0,545820951	-1,44529	0,075992
mmu04912	GnRH signaling pathway	0,682833245	1,432655	0,089387
mmu04640	Hematopoietic cell lineage	-0,839463212	-1,4138	0,07431
mmu05161	Hepatitis B	-0,64068361	-1,45336	0,083512
mmu04066	HIF-1 signaling pathway	-0,600137563	-1,52829	0,04567
mmu04390	Hippo signaling pathway	-0,601516262	-1,44055	0,087315
mmu04911	Insulin secretion	0,73362097	1,445573	0,071183
mmu01232	Nucleotide metabolism	-0,704728244	-1,43201	0,091738
mmu04740	Olfactory transduction	0,833201893	1,404548	0,06475
mmu00030	Pentose phosphate pathway	-0,737550023	-1,42961	0,089579
mmu04974	Protein digestion and absorption	0,843687504	1,770144	0,00222
mmu00230	Purine metabolism	-0,704728244	-1,43201	0,091738
mmu00620	Pyruvate metabolism	-0,659249374	-1,49547	0,064045
mmu04810	Regulation of actin cytoskeleton	-0,456123445	-1,48899	0,03376
mmu04926	Relaxin signaling pathway	0,673247592	1,68761	0,015022
mmu00830	Retinol metabolism	0,883620834	1,48954	0,028554
mmu03010	Ribosome	-0,550716678	-1,98309	0,000498
mmu03008	Ribosome biogenesis in eukaryotes	-0,651553041	-1,42993	0,096717
mmu00140	Steroid hormone biosynthesis	0,825285547	1,516377	0,029613
mmu04918	Thyroid hormone synthesis	0,673668235	1,531919	0,049539
mmu03250	Viral life cycle - HIV-1	-0,784819477	-1,42996	0,077778

NES: Normalized enrichment score p-value <0.1

In addition, dot plot for KEGG pathway enrichment analysis was done to compare the activated and suppressed pathways in late passage of BMR fibroblasts compared to early passage cells (Figure 4.6). The results showed that cell cycle and HIF-1 signaling pathways were suppressed in late passages of BMR fibroblasts.

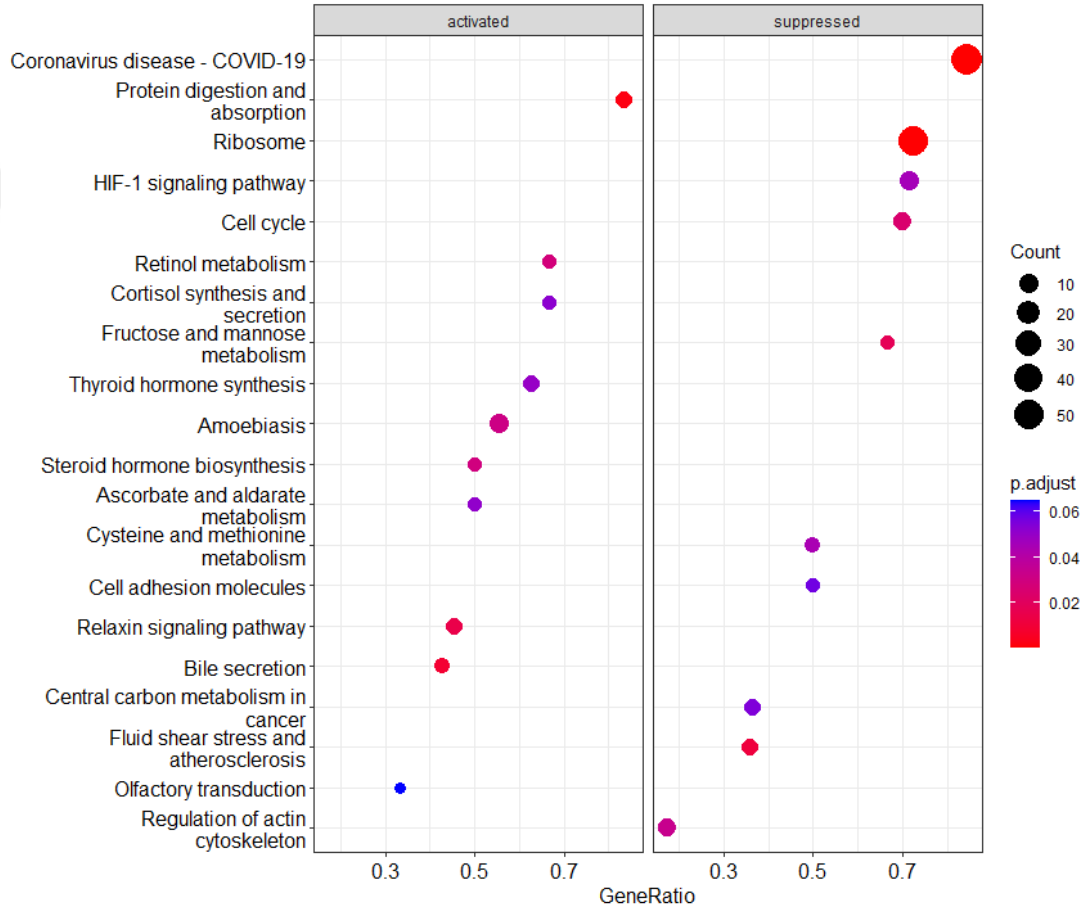

Figure 4.6: KEGG pathway enrichment analysis of BMR fibroblasts proteomics data. KEGG pathway enrichment analysis is represented with dot plot to show the activated and suppressed pathways in senescent BMR fibroblasts. GSEA was performed with Benjamini-Hochberg multiple test corrections. The dot size is based on gene count enriched in the pathway, and the color of the dot shows the pathway enrichment significance. Threshold for significany of *p value* was taken as 0,1 (n=4).

5. DISCUSSION AND CONCLUSION

Progressive deterioration of an organism's physiological survival mechanisms is defined as aging. CS is, one of the hallmarks of aging, the process f irreversible cell cycle arrest with contributing to age- related diseases including cancer and neurodegenerative diseases (1). Previous studies demonstrated that each long-lived rodent adapted their own novel mechanisms (88). BMR is an underground long-lived rodent that mostly lives in Middle East region, and they are regarded as a unique model for aging and cancer research due to their long lifespan and resistance to both age-related phenotypic alterations and tumor growth (89). Recent research revealed the possible mechanism for the anticancer resistance mechanism of BMRs. It is demonstrated that IFNβ secretion is responsible for triggering concerted cell death mechanism which is observed after 7-20 population doublings *in vitro*. IFNs have crucial role in inflammatory pathways and is highly crucial for induction of CS (90). Besides, they are also involved in the activation of the JAK-STAT signaling pathway.

In this study, an adaptation of JAK-related pathway in BMRs with replicative senescence was shown by investigating mRNA expressions of *Jak1* and *Jak2* genes. Additionally, proteomics data that were obtained from the early and late passage of BMR cells were correlated with the experimental results. Primary lung fibroblasts of BMR and mice were obtained from our previous studies and were stored in liquid nitrogen. These cells were thawed and cultured to perform replicative senescence in BMRs and mice. Replicative senescence is widely used CS type for *in vitro* studies with performing serial passaging. Moreover, CS studies with BMR fibroblasts are very few and thus, the exact passages for generating replicative senescence are required to be confirmed with various CS biomarkers. For this purpose; cellular growth curve, morphological observation, SA-β-gal staining, and mRNA expression of Il6 gene were analyzed. To confirm the status of CS, we observed the expanded and flattened shape, increased SA-β-Gal staining, and slowed proliferation in late passage fibroblasts compared to early passage fibroblasts. Il6 is one of the SASP factors and its expression

level is mostly used for the detection of CS. However, our results did not show any significant increase in the expression of this gene which is also consistent with a recent study(83). In this previous study, it is shown that IL-6 expression is not constantly increasing through CS of BMRs. On the other hand, a specific pathway known as IL-6/JAK/STAT3 has been previously found in multiple tumors as the induction pathway for their formation. Loss of IL6 expression might affect the tumor formation through this mechanism. Therefore, IL6 expression in BMRs might need further investigation to understand this pathway in detail.

After validation of CS in BMR samples, the *Jak1* and *Jak2* gene expressions were compared with qPCR both in mouse and BMR. RT-qPCR was used to obtain the copy number of the genes. *Actb* was used as housekeeping gene for normalizing expression levels for each sample. The results of the *Jak1* and *Jak2* mRNA expression comparison between mouse and BMR revealed that *Jak1* and *Jak2* levels in early passage of BMR fibroblasts were significantly low. However, during CS nonsignificant difference was observed between these two species. Moreover, when the expression of *Jak1* and *Jak2* gene expressions were compared in BMR through CS, significantly increased expressions was observed. Our results correlates with the increased expression of IFN-β, an inducer of JAK-STAT pathway, which was also increasing through CS in a recent research (84).

To confirm the elevation in CS related signaling at the protein level, proteomics analysis was performed. Our research, is the first in the literature that performs proteomics analysis for comparison of early and late passage of BMR fibroblasts. To analyze the proteomics data, GO and KEGG pathway analysis were used. GO is the highly preferred method for enrichment analysis (91) that can be used for up and down regulated gene sets through GO terms that are over or under-represented. Consistent to the alterations in JAK signaling, GO enriched pathway analysis of senescent BMR fibroblasts showed an increase in the cytokine-mediated signaling. The proteins observed in the proteomic data related to this signaling pathways are TUT4, SRSF1,

TRIM32 and ILK. Recently, TUT proteins are found to be a modulator for inflammation of mammalian cells and TUT4 is claimed to be involved in IL-6 degradation (92). Therefore, the increase of TUT4 protein expression might be related to the lack of expression in IL-6. NF-kB signaling pathway is one of the regulator mechanisms for SASPs and in our proteomics data NF-kB signaling pathway related proteins ILK and TRIM32 are elevated in senescent BMR fibroblasts. Although there are not so many studies for these proteins related to CS, one research claimed that TRIM32 overexpression is directly related with the induction of virus-triggered IFN-β. TRIM32 is also involved in IL-17 mediated signaling with other elevated protein SRSF1(93). As it is known, cytokine mediated signaling increase with CS and our results suggest that the increased protein expression of cytokine-mediated signaling through CS might be related with the JAK-STAT pathway increase in BMRs. Yet, to understand the mechanism of BMR's anti-aging mechanism it is important to identify and further investigate the proteins related with IFN-β.

Finally, KEGG pathway analysis of proteomics data revealed that HIF-1 signaling pathway is suppressed in senescent BMR fibroblasts. The proteins identified in the data related to HIF-1 signaling are: Rps6 mTOR, Pgk2, Eno1, Pgk1, Ldha, Aldoa, Gapdh, Eloc, and Ldhb. BMRs are resistant to hypoxic conditions, and a previous study showed that in normoxia conditions HIF-1a level in BMRs is 2-fold higher than rat (94). Furthermore, HIF-1 is defined as one of the longevity factor and stabilization of this protein might increase lifespan around 50%. However, most studies are performed in *Caenorhabditis elegans* and the role of hypoxia in mammalians showed that stabilizing HIF-1a might result in cancer development(95). Therefore, the role of hypoxia might be both harmful and beneficial and needs further investigation. The results of suppressed HIF-1 mechanism in protein level in senescent BMRs might help us to understand the role of hypoxia related aging mechanisms.

Protein analysis by MS is a potent tool in proteomics to explore the protein profiles produced across various physiological and pathological situations. However, due to

the lack of protein database of blind mole-rats, proteins fragments of the LC-MS/MS results were identified using *Mus musculus* protein database. For this reason, the number of proteins identified after data analysis were low and more experiments should be performed to understand and confirm the mechanism of cytokine-mediated signaling mechanisms of BMRs during CS, in future.

Mediterranean BMRs are known with their unique mechanism and one of the longest-lived rodents. Although they live around 15 years, they demonstrated CS, a hallmark of aging, in our *in vitro* experiments. A recent report claimed that BMR fibroblasts have adapted mechanism for CS with yielding increased expression of p21 and p53, and lowered expression for Some SASP factors including IL6, IL8, and ICAM1. Our study revealed that, *Jak1* and *Jak2* expressions increase with CS in long-lived BMRs, although it is significantly low in early passage fibroblasts compared to mouse. Besides, considering our recent findings, which reveals that BMR shows an elevated mRNA expression of SASP factors during CS unlike mouse, these extraordinary rodents might adapt its cytokine-mediated signaling pathway through JAK related signaling (96). However, further *in vitro* analysis required to enlighten the mechanisms of BMRs' unusual cancer and aging adaptations that are related to JAK signaling pathways. The separation of the inflammatory phenotypes of CS for future studies might offer a novel strategy for the creation of new inflammation inhibitors that may be utilized to prevent cytokine storms and prevent inflammaging.

6. REFERENCES

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7. CURRICULUM VITAE

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