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INVESTIGATING MOLECULAR PATHWAYS OF ROS-INDUCED PRO-INFLAMMATORY SENESCENCE IN PREADIPOCYTES

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By

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Abstract

Obesity has been associated with chronic low-grade inflammation, which is considered to be a major cause for insulin resistance and type 2 diabetes (T2D). The therapeutic interventions for T2D are lacking due to the unclear understanding of the molecular mechanisms of obesity driven T2D. The ultimate focus of current project is to investigate and reveal the molecular mechanisms of this association to discover novel therapeutic targets for obese individuals living with diabetes. The adipose tissue in the obese state is characterized by the accumulation of reactive oxygen species (ROS), which will lead to DNA damage. The cell undergoes senescence, when the DNA is severely damaged and unable to be repaired. An increased secretion of pro-inflammatory cytokines characterizes senescent cells and it's known as senescence associated secretory phenotype (SASP) as reported by *in vitro* and *in vivo* studies. The knowledge about SASP components and the determination of pro-inflammatory phenotype is lacking especially in obesity and T2D. The main goal of this study is to investigate the role of the novel transcription factors, including STAT1, DDIT3 and C/EBPδ, in pro-inflammatory senescence. A new role for the transcription factor STAT1 has been revealed in senescence. It seems to promote cell cycle via p21 pathway and regulates inflammation by affecting IL6 expression when encountered by genotoxic agent such as H_2O_2. These results will enhance our understanding about the molecular details that will help in the development of effective therapeutic interventions.

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List of Abbreviations

C/EBPδ	CCAAT/Enhancer BindingPprotein (C/EBP), Delta
DDIT3	DNA Damage Inducible Transcript 3
DSB	Double Strand Breaks
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
H_2O_2	Hydrogen Peroxide
HFD	High Fat Diet
IL-6	Interleukin-6
ROS	Reactive Oxygen Species
RNA	Ribonucleic Acid
RT-qPCR	Real Time- Quantitative Polymerase Chain Reaction
SASP	Senescence Associated Secretory Phenotype
STAT1	Signal Transducer and Activator of Transcription 1
SA-β-gal	Senescence Associated Beta- Galactosidase
shRNAs	Short Hairpin RNA
T2D	Type 2 Diabetes
TNF-α	Tumor Necrosis Factor -Alpha
WCMC-Q	Weill Cornell Medical College- Qatar
WHO	World Health Organization

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Chapter 1. Introduction

1. Introduction

Obesity has been associated with chronic low-grade inflammation, which is considered to be a major cause for insulin resistance and T2D (Xu et al., 2003; Hotamisligil, 2006 & 2010; Konner & Bruning, 2011). The therapeutic interventions for T2D are lacking due to the unclear understanding of the molecular mechanisms of obesity driven T2D. The ultimate focus of this project is to investigate and reveal the molecular mechanisms of this association to discover novel therapeutic targets for obese individuals living with diabetes. The adipose tissue in the obese state is characterized by the accumulation of ROS, which will lead to DNA damage and activation of DNA damage responses (DDR) (Ahima, 2009). The cell undergoes senescence, when the DNA is severely damaged and unable to be repaired. Senescence is defined as an irreversible cell cycle arrest and results from an activated stress response (Minamino et al., 2009).

Several studies have proposed that, senescence is associated with age related degenerative disorders (Campisi, 2013) and tumor progression (Minamino et al., 2009). It has been reported in *in vitro* and *in vivo* studies, that cellular senescence will result in secretory phenotypic changes by senescent cells characterized by a significant increased secretion of pro-inflammatory cytokines (Baker et al., 2011). Senescence associated secretory phenotype (SASP) is characterized by an increased secreted levels of more than 40 factors involved in intercellular signaling, such as cytokines, chemokines, growth factors, secreted proteases and secreted insoluble components (Baker et al., 2011). The knowledge about SASP components and the determination of pro-inflammatory phenotype is lacking especially in obesity and T2D. Current study by Mazloum's group Weill Cornell Medical College-Qatar (WCMC-Q), have designed a reliable and robust

protocol for hydrogen peroxide (H_2O_2) induced cellular senescence in mice 3T3-L1 pre-adipocytes. To investigate critical pathways that promote pro-inflammatory senescence, RNA sequencing using Illumina next-generation sequencing in combination with pathway analysis on RNA isolated from control and treated cells was performed.

1.1 Hypothesis

Novel transcription factors STAT1, DDIT3 and C/EBPδ, play a role in ROS-induced pro-inflammatory senescence in preadipocytes.

1.2 Significance of the problem

The incidences of Obesity and diabetes in Qatar ranks among the highest in the world and the morbidity of these metabolic disorders creates a major public health problems. The evidence that chronic inflammation is a major contributor in the progression of metabolic diseases has been shown, however the molecular details of this relationship are largely unknown. Understanding the molecular mechanism of these diseases could help in developing effective therapeutic intervention.

1.3 Research Aims

- Validation of upregulated potential SASP molecules in H_2O_2-induced cellular senescence in pre-adipocytes by qPCR and dot blot.

- Validation of up-regulated nuclear transcription factors genes in H_2O_2-induced cellular senescence in pre-adipocytes.

- Functional characterization of identified nuclear transcription factors in promoting SASP in H_2O_2-induced cellular senescence in pre-adipocytes.

Chapter 2. Review of the literature

2. Review of the literature

2.1 Obesity and inflammation

The rise in obesity is caused by increased calorie intake and decreased calorie expenditure; it is a major risk factor for T2D and closely associated with life-threatening cardiovascular disorders and obesity related cancers (Minamino et al., 2009). The morbidity of these metabolic disorders constitutes a major health problem not only in high-income countries, but also contributing to an alarming rise in the developing world (Hotamisligil, 2006 & 2010; Konner & Bruning, 2011). T2D is characterized by insulin resistance/hyperglycemia and is associated with a group of metabolic and cardiovascular disorders including dyslipidemia, visceral obesity, glucose intolerance, hypertension and endothelial dysfunction (DeFronzo, 2010; Hotamisligil, 2010; Konner & Bruning, 2011; Gregor & Hotamisligil, 2011).

In recent years, extensive research has evolved for the observed association between obesity and T2D. This enhanced our understanding of the link between these diseases and highlighted the mechanisms for the development of T2D and associated complications. An example of this connection is the prevalence of chronic low-grade inflammation among obese individuals, which is considered as a possible cause of insulin resistance and T2D (Hotamisligil, 2010; Gregor & Hotamisligil, 2011; Konner & Bruning, 2011). Published genetic studies with inflammatory mediators on insulin action support the above hypothesis (Kang et al., 2011).

The inflammatory responses are generated from metabolic signals that damage metabolic homeostasis (Weisberg et al., 2003). Components of the innate immune system such as the pro-inflammatory M1 macrophages infiltrate the inflamed adipose tissue in

obese states, and a range of pro-inflammatory cytokine levels of tumor necrosis factor - alpha (TNF-α) and interleukin-6 (IL-6) are increased in the adipose and muscle tissues of obese human and cause insulin resistance and diabetes (Xu et al., 2003; Weisberg et al., 2003; Ahima, 2009; Gregor & Hotamisligil, 2011).

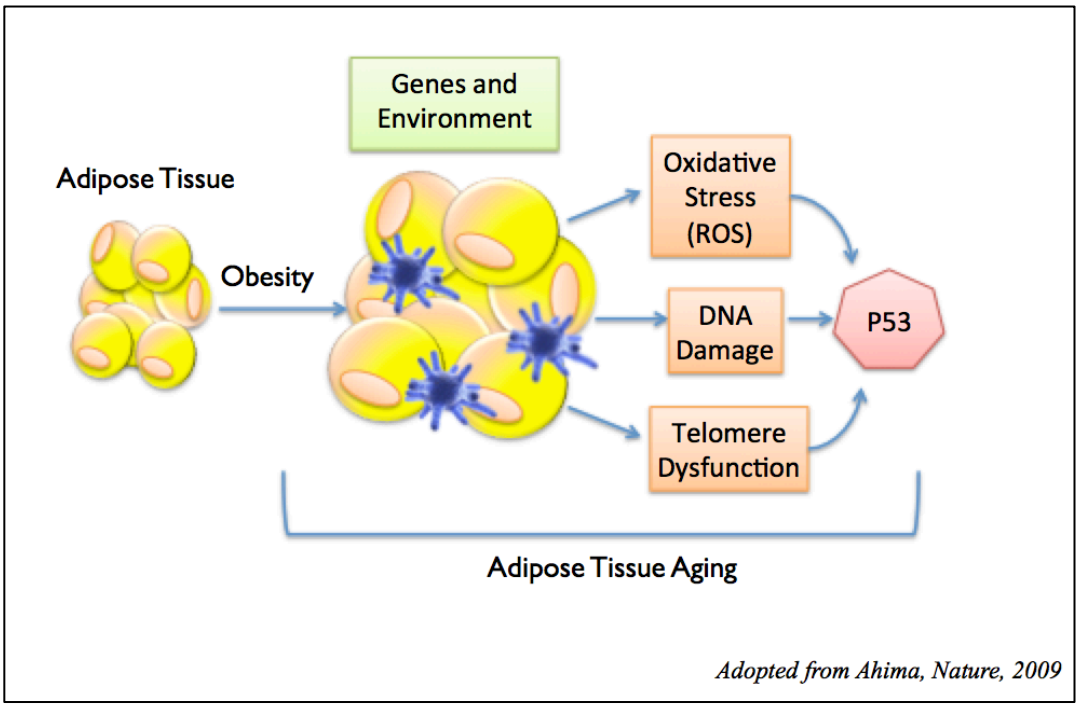


Figure 2.1 The connection between obesity-mediated aging of adipose tissue and diabetes.

2.2 High Fat Diet and Inflammation

The first evolution in the discovery of adipose tissue inflammation showed increased cytokine levels (e.g. TNF-α) in obese mice versus lean controls (Campisi, 2013). This was followed by enormous studies to investigate the molecular events leading to adipose tissue inflammation by focusing on effects of high fat diet (HFD in rodents (Xu et al., 2003; Suganami et al., 2005; Nishimura et al., 2009). The intensive investigation about the upstream expression of inflammatory cytokines identified certain kinases [c-jun N-terminal Kinase, inhibitor of K kinase] as the main intracellular players

in the induction of inflammation in metabolic diseases (Mortazavi et al., 2008; Trapnell et al., 2010). Increased activation of these kinases was observed in obese adipose tissue and their role in mediating inflammation in obesity was pointed following their deletion in animal studies (Mortazavi et al., 2008; Trapnell et al., 2012).

In metabolic tissue, the inflammatory state in obesity has an additional feature characterized by markedly increased infiltration of immune cells, such as macrophages (Olefsky & Glass, 2010). Macrophages have been increased in adipose tissue of HFD compared with lean mice and contributed to increased cytokine release (Olefsky & Glass, 2010).

The state of activation of macrophages is affected by metabolic factors and directed toward being pro-inflammatory or anti-inflammatory activations, referred to as M1 and M2 respectively. M1 macrophages promote insulin resistance and inflammation whereas M2 macrophages regulate tissue repair and improve insulin sensitivity (Fujisaka et al., 2009). HFD promotes the differentiation of the pro-inflammatory adipose tissue macrophage type M1 macrophages and thus alters the ratio of M1/M2 (Fujisaka et al., 2009). The increase in inflammation promoting factors in HFD animal models is not only restricted to macrophages but has also been linked to other immune cells. It has been reported that HFD leads to an increase in mast cells and natural killer T cells and to an imbalance in T-lymphocyte subsets in adipose tissue (Feuerer et al., 2009; Nishimura et al., 2009).

Interestingly, lymphocyte activation and proliferation in co-culture assays was shown to be induced in adipose tissue from obese mice (Nishimura et al., 2009), indicating that production of chemokines by adipocytes could precede changes in T-

lymphocyte subsets. Following disturbance in T-lymphocyte subsets and mast cells buildup during development of obesity, adipose tissue becomes infiltrated by classically activated pro-inflammatory M1 macrophages (Liu et al., 2009; Nishimura et al., 2009). This is possibly in response to chemokines productions from T lymphocytes and mast cells (Weisberg et al., 2003; Xu et al., 2003; Liu et al., 2009; Winer et al., 2009; Tchkonia et al., 2010).

2.3 Pre-adipocyte senescence and obesity-associated chronic inflammation

Obesity is considered to be the major trigger of insulin resistance and is likely to be caused by the combination of increased calorie intake and genetic predisposing factors (Ahima, 2009). T2D progresses due to insufficient insulin production from the beta cells. In addition, inflammation has emerged in recent years as a major contributor for the development of insulin resistance and obesity.

During aging, organs undergo deterioration in structure and function and are associated with genetic instability and oxidative stress (Ahima, 2009). These changes will create a microenvironment within the adipose tissue that is susceptible to the accumulation of ROS and subsequent inflammation (Ahima, 2009). This evidence was achieved in a study by (Minamino et al., 2009), who analyzed the adipose tissue from the genetically obese agouti mice and found elevated levels of ROS and DNA damage as compared to control mice. Recent evidence suggests that obesity promotes adipose tissue aging due to accumulation of senescent adipocytes in response to elevated ROS levels in the obese state (Ahima, 2009). This was accompanied by the activation of tumor suppressor p53 *in vivo* that was proposed to trigger an inflammatory response leading to insulin resistance and glucose intolerance (Ahima, 2009; Minamino et al., 2009).

Furthermore, it was proposed that excessive calorie intake led to the accumulation of oxidative stress in the adipose tissue of mice with T2D-like disease. This consecutively resulted in the senescence-like features, such as positive staining with senescence-associated beta-galactosidase (SA-β-gal), increased expression of the tumor suppressor p53, and the cell cycle inhibitor p21, along with elevated levels of pro-inflammatory cytokines (Minamino et al., 2009). Conversely, marked enhancement in the senescence changes and decreased cytokine expression was achieved upon the inhibition of p53 activity in adipose tissue that resulted in improved insulin sensitivity (Minamino et al., 2009).

It is known that different fat depots make distinct contributions to the pro-inflammatory and clinical consequences of obesity (Tchkonia et al., 2010). Unlike subcutaneous fat, the visceral fat depot is more strongly associated with obesity linked inflammation and cytokine production (Tchkonia et al., 2010). These visceral depots can be pathologically deregulated in the obese state through the production of different inflammatory cytokines and chemokines such as, TNF-α and IL-6, and may contribute to insulin resistance (Starr el at., 2009). The stromal vascular fraction of visceral fat is thought to be the major contributor to chronic inflammation in the obese state. This fraction of adipose tissue is comprised of pre-adipocytes, endothelial cells, immune cells, and other cell types (Wu et al., 2007; Gustafson et al., 2009; Fain et al., 2010).

Pre-adipocytes comprise 15–50% of cells in fat and reside mainly in fat depot (Tchkonia et al., 2010). Though their main role is to differentiate into fat cells, their gene expression profile is different from fat cells and is more related to macrophages (Charriere et al., 2003). Pre-adipocytes express toll-like receptors and have the potential

of full innate immune response (Lin et al., 2000; Chung et al., 2006; Vitseva et al., 2008). Upon activation, the macrophage participates in the inflammatory cycle by releasing even more chemokines and cytokines (Tchkonia et al., 2010). Obesity or serial passage of pre-adipocytes was proposed to lead to an increased cell division and accumulation of senescent pre-adipocytes. This might subsequently initiate the infiltration of immune cells that commonly occurring in obesity (Tchkonia et al., 2010).

2.4 Cellular senescence and inflammation

Normal cells can divide for only finite number of times before they reach a state of replicative cellular senescence. Senescence is described as an irreversible state of cell cycle arrest, where cells undergo a global cellular "reprogramming" resulting in morphological and phenotypic changes (Campisi & Fagagna, 2007). It was first discovered *in vitro* as a limit to cell division controlled by replicative senescence, as reported by (Hayflick, 1965), in diploid human fibroblasts. He was the first to report that the cells stop proliferating after a limited number of cell division, yet remaining alive and metabolically active. These cells reaching the senescence state down-regulate certain genes involved in cell cycle and various components of the extracellular matrix. On the other hand, it has a role in up-regulating cell cycle inhibitor genes, degrading enzymes and several cytokines (Banito & Lowe, 2013).

It has been proposed that cellular senescence results from an activated stress response, which suppresses early age tumor formation and promotes tissue repair (Campisi, 2013). This stress response program is also described to be associated with tumor progression (Campisi, 2013) and advanced age related degenerative diseases (Baker et al., 2011). It has been reported that cellular senescence is manifested both *in*

vitro and *in vivo* in a wide variety of cells and tissues (Jeyapalan & Sedivy, 2008). The most significant phenotypic change is the acquisition of a secretory phenotype. This phenotype involves a remarkable increase in the secretion of pro-inflammatory cytokines by senescent cells (Davalos et al., 2010). This secretory phenotype has been described being induced by genotoxic agents and oncogene.

SASP is characterized by an increase in the secreted levels of more than 40 factors involved in intercellular signaling, such as inflammatory cytokines (e.g., IL-1, IL-6), chemokines (IL-8), growth factors, secreted proteases, and secreted insoluble components (Davalos et al., 2010). These secreted pro-inflammatory cytokines by senescent cells support the development of cancer and cellular senescence (Cichowski & Hahn, 2008). Among all of these secreted factors, IL-6 has been reported as the most prominent cytokine of the SASP (Sansoni et al., 2008).

Moreover, different groups of cells from the immune system such as (macrophages, natural killer cells and T-cells) of the adaptive and innate immune system can be recruited as a result of the pro-inflammatory secretion of cytokines. These cells are believed to play a role in curbing the initial inflammation by clearing senescent cells as well as oncogene-transformed cells (Xue et al., 2007; Krizhanovsky et al., 2008; Kang et al., 2011; Campisi, 2013). Recent emerging evidence from studies on premature aging animal models has shown that the specific targeting of senescent cells delays premature aging related pathologies (Baker et al., 2011). This was considered as the first direct evidence that supports the notion that senescent cells can drive age-related pathologies (Baker et al., 2011; Campisi, 2013).

Besides the SASP, senescent cells are characterized by the appearance of several cellular and molecular markers. The cellular alterations include altered morphology (enlarged flat cells, multi-vacuoled and multi-nucleated) and increased positive staining for SA-β-gal (Sikora et al, 2011). The molecular alterations include the accumulation of DNA damage foci, activation of DDR proteins, and increased nuclear staining of PML nuclear bodies and senescence-associated heterochromatin foci (Sikora et al, 2011). Senescence can be induced by different means, including persisting DNA double strand breaks (DSBs), oncogene-induced mitogenic signals, epigenomic disturbances and tumor suppressor gene activation (Campisi, 2013).

Cellular senescence is driven and sustained by at least two major axes, the p53/p21^{CDKN1A} or the pRB/p16^{CDKN2A} signaling pathways (Campisi, 2013). Evidence suggests that the persistent DNA damage and DDR activation are characteristic features of cellular senescence, which is driven by p53/p21^{CDKN1A} pathway (Campisi, 2013; Fagagna, 2008). It is known that DDR is activated by dysfunctional telomeres as well as DSBs elsewhere in the genome, which typically induces cell cycle arrest and DNA repair (Jackson & Bartek, 2009). However, if the DNA damage is too severe and cannot be repaired, cells may acquire a persistent DDR activation and senescence states (Sikora et al., 2011).

2.5 Significance

The incidences of Obesity and diabetes in Qatar rank among the highest in the world and the morbidity of these metabolic disorders creates a major public health problems. Over the past three decades, there was an observed increase in obesity prevalence at an alarming rate and it was estimated by the World Health Organization

(WHO) that the number of obese individuals will increase dramatically (Konner & Bruning, 2011). The causes of obesity are associated with increased calorie intake combined with genetic predisposing factors. Obesity is a major risk factor for T2D and life-threatening cardiovascular disorders among other illnesses (Hotamisligil, 2006 & 2010; Konner & Bruning, 2011). The current epidemic of T2D is being driven by the obesity epidemic. The number of patients with diabetes world wide is expected to reach 300 million by 2025 and thus the worldwide health impact of T2D and its complications is enormous (DeFronzo, 2010).

There is compelling evidence that chronic inflammation is a major contributor in the progression of metabolic diseases, yet the molecular details of this relationship are largely unknown, hampering the development of effective therapeutic intervention. This proposed project is driven by the hypothesis that cellular senescence by oxidative stress is governed by the interaction between DDR proteins that in turn play a critical role in triggering inflammation and insulin resistance observed in obesity and T2D. In this regard, high-throughput transcriptomics and gene silencing technologies were applied by Mazloum's group at WCMC-Q to identify novel therapeutic targets for the rapidly growing population of individuals living with diabetes.

2.6 Preliminary data supporting the hypothesis

It is well established that DDR induced by dysfunctional telomeres and DSBs (Di Micco et al., 2008) are features of cellular senescence. The activation of DDR results from the eroded telomeres. As the telomeres reach a critical length, the eroded telomeres are recognized as the DNA double strand ends. This damage is sensed by the DDR machinery and attempts to repair the damage through the activation of a reversible form

of cell cycle arrest. If the damage is persistent, it often leads to apoptosis through the activation of p53 or replicative senescence, a highly reversible form of cell cycle arrest, through transactivation of p21. In the same context, is was established by Campisi's group, that DDR activation and p53/p21 signaling cascades are required to promote cellular senescence-associated growth arrest phenotype and to induce a subset of the pro-inflammatory SASP molecules (Campisi., 2013).

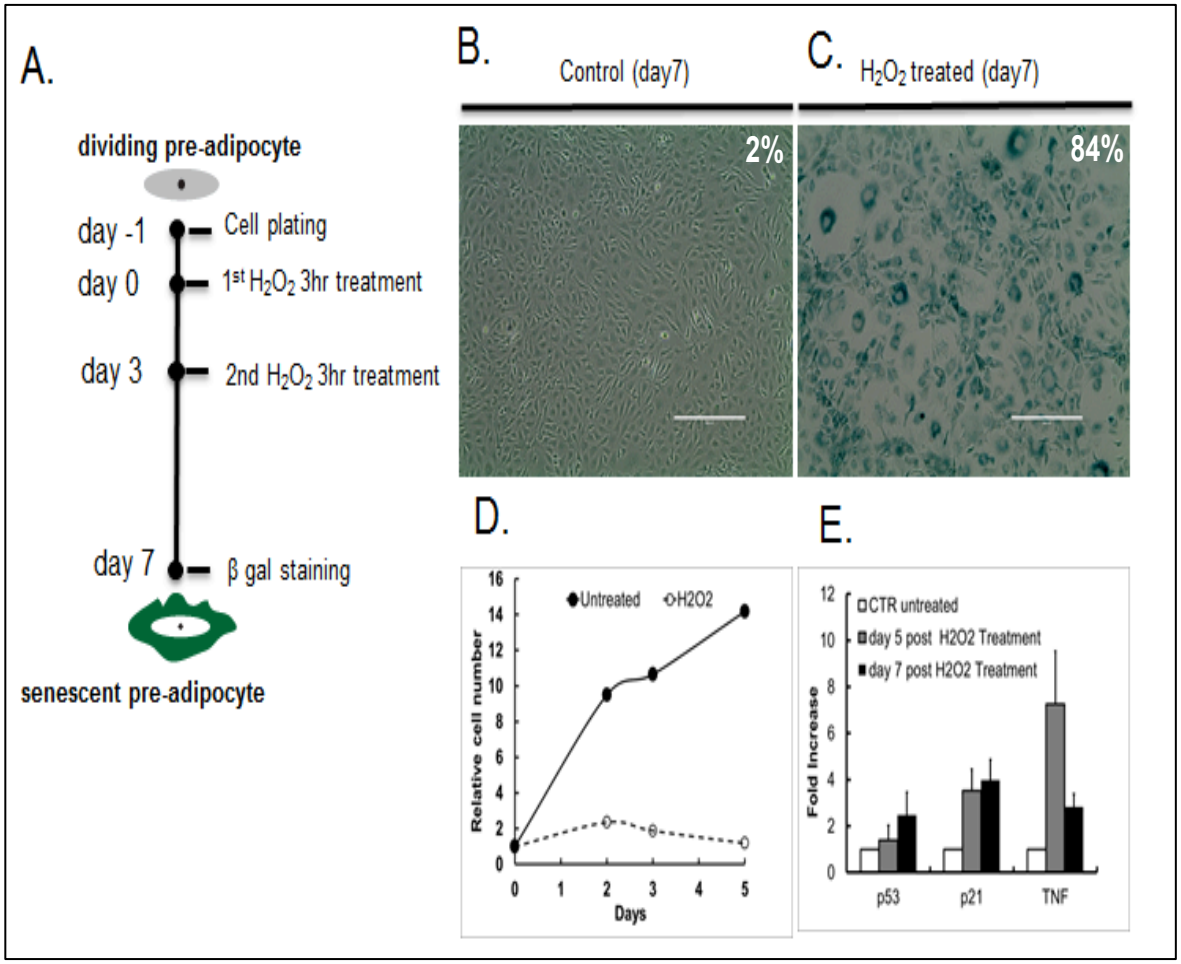


Figure 2.2 Establishment and characterization of H_2O_2 induced premature senescence treatment protocol in mice 3T3-L1 pre-adipocytes. A) Schematic representation of time course experimental conditions of H_2O_2 induced premature senescence program. **B)** β-gal staining of 3T3-L1 cells, control (untreated). 3T3-L1 cells were grown in parallel with treated cells and were grown in parallel with treated cells and were split when the cells reach 70% confluency and stained on day7. **C)** β-gal staining of 3T3-L1 cells. H_2O_2 treated (200uM as shown in panel A. SA-β-gal positive cells were quantified from four different fields and presented as percentage figure in the upper corner of every panel. **D)** Growth curve of 3T3-L1 cells treated with or without H_2O_2. The number of live cells was taken at different days post first treatment (days 2, 3, and 5) and presented as folds increase from the number of plated cells at pre-treatment day0.

The knowledge on SASP components and what drives the robust pro-inflammatory phenotype is lacking especially in obesity and T2D. To investigate pre-adipocytes senescence promoting factors, Mazloum's research lab first generated and characterized an *in vitro* experimental model for cellular senescence in mice pre-adipocytes using H_2O_2 as a source of ROS. Previous work from *Chen's* lab has provided evidence that sub lethal concentrations of H_2O_2 (100-250 μM) induce senescence and growth arrest in normal human fibroblasts (Chen et al., 1998). Commercially available mouse 3T3-L1 pre-adipocytes were acquired to establish the *in vitro* model system. These cells are fibroblast-like cells and have been well characterized in studying adipogenesis *in vitro* (Green & Meuth, 1974).

The investigation of the mechanisms of ROS-induced pro-inflammatory senescence in pre-adipocytes began by screening for optimal conditions that enrich for cellular senescence using SA-β-gal staining kit. Briefly, cells were treated as shown in (Figure 1A). At the conclusion of the experiment (day 7), cells were stained with X-gal solution. β-gal stained-positive cells have a strong enrichment in senescent cells upon repeated H_2O_2 treatment compared to untreated control (Figure 1B; 2% β-gal stained-positive cells and 84 % β-gal stained-positive cells).

Growth arrest is the most prominent hallmark of cellular senescence (Martin et al., 2012; Campisi, 2013; Tchkonia, 2013). As further evidence of clear senescence phenotype, growth curve analysis was performed for H_2O_2-treated (200μM) showing that H_2O_2 treatment (200 μM) resulted in growth inhibition relative to untreated control (Figure 1C).

To monitor gene expression levels of known DDR and inflammation factors in senescent 3T3-L1 cells, qPCR and Western blot analysis were performed. Results in (Figure 1E) illustrated that the gene expression levels of DDR genes (p53, p21) and the pro-inflammatory cytokines (TNF-α) are upregulated at days 5 and 7 as compared to untreated control. Western blot analysis of representative set of DDR and stress response genes (p53, p21, Chk2, NFκB) were also upregulated or activated by phosphorylation and/or acetylation upon H_2O_2 treatment (data not shown).

Taken altogether, these preliminary data from Mazloum's group showed the establishment of a robust *in vitro* model system to begin uncovering mechanisms underlying pro-inflammatory pre-adipocyte senescence.

RNA-sequencing (RNA-seq) in combination with pathway analysis on RNA isolated from control and H_2O_2 treated cells was performed, to unravel novel factors and investigate critical pathways that promote and sustain pro-inflammatory senescence as shown in (Figure 1A). RNA sequencing was accomplished on three biological replicates of control and treated 3T3-L1 RNA preparations. Sequencing of the RNA-seq cDNA library was performed at the Genomics Core Facility at WCMC-Q using Illumina next-generation sequencers. Mapping of reads was performed at the Bioinformatics Core Facility at WCMC-Q, using TopHat version 2.0.6 and Cufflinks, 2.0.2 (Mortazavi et al., 2008; Trapnell et al., 2010 & 2012). Cufflinks software was also employed to calculate significant differential gene expression. The significance threshold was set at a q-value of 0.05. Furthermore, the cutoff for differential expression ratio value was set of at least 2, to emphasize relatively high differential expression values.

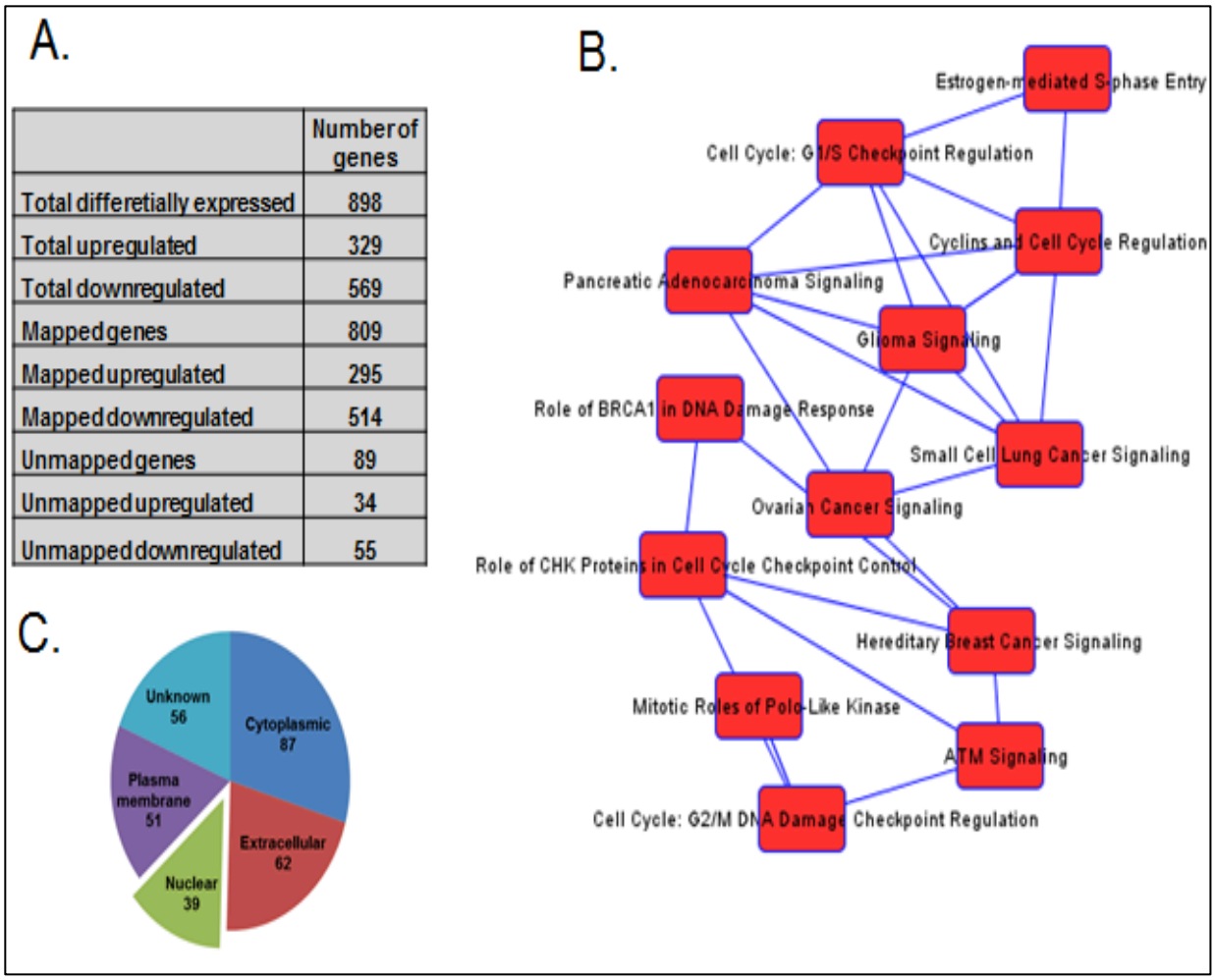


Figure 2.3 Transcriptomic analysis of senescent mice 3T3-L1 pre-adipocytes reveals differential regulation of DDR and cell cycle regulation pathway. A) Table shows number of differentially expressed genes in senescent pre-adipocytes treated as shown in 1A compared to untreated controls**. B)** Network of differentially regulated canonical pathways was extracted from Ingenuity Pathways Analysis of mapped differentially expressed genes in senescent pre-adipocytes. **C)** Pie chart shows number of annotated genes that were identified as upregulated during cellular senescence.

Significant differential expression was analyzed for H_2O_2 treated cells relative to the control. Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, http://www.ingenuity.com) was used to identify signaling pathways that are differentially regulated in the two different gene sets. Figure 2A shows the total number of differentially expressed genes including upregulated and downregulated genes that were either mapped or unmapped to known mouse genes.

To gain knowledge on regulatory pathways of differentially expressed genes during H_2O_2-induced senescence of 3T3-L1 pre-adipocytes, the genes were analyzed by IPA. Shown in Figure 2B is a network of predicted overlapping canonical pathways, which included estrogen mediated S-phase entry, cell cycle checkpoint regulation pathways, cancer signaling, mitotic roles of polo-like kinase, and DSB repair by homologous recombination among others. It was evident that this initial analysis revealed enrichment in canonical DDR pathways, which validated the in vitro cell-based system and prompted to further unravel key regulatory molecules in promoting and sustaining cellular senescence.

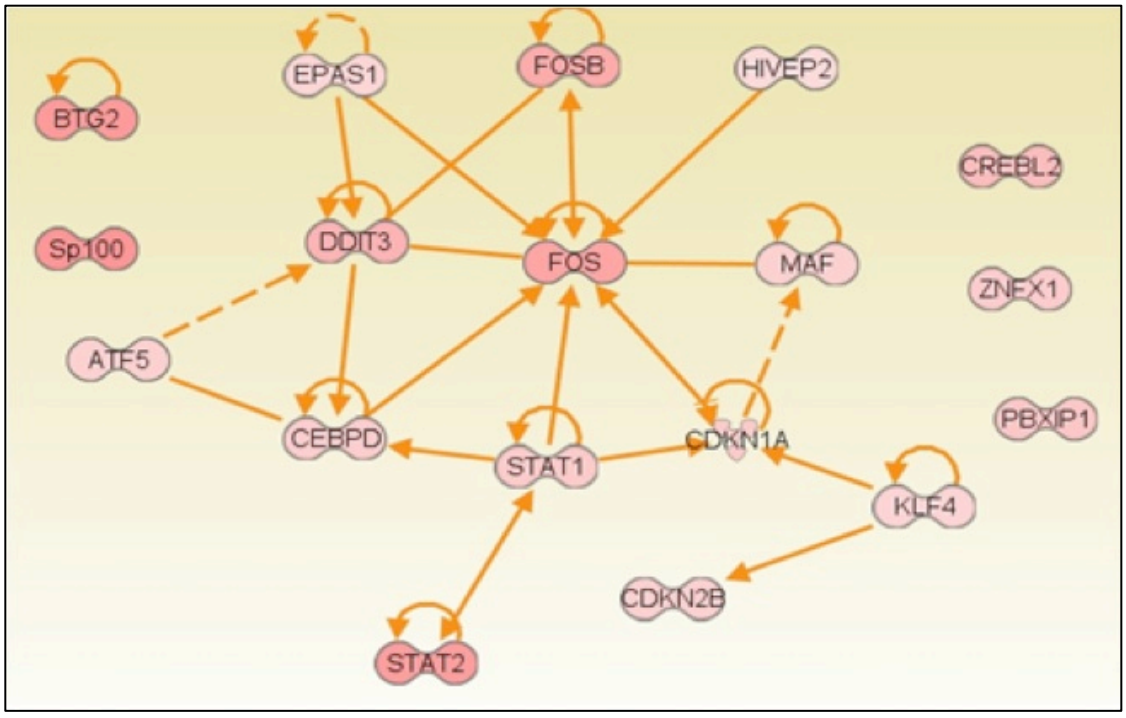


Figure 1.4 IPA analysis driven validation and functional characterization of novel transcription factors for senescence signaling molecules in ROS induced senescent pre-adipocytes. Network of up-regulated transcription factors in RNA-Seq dataset reveals novel critical players in cellular senescence.

The employment of RNA sequencing and bioinformatics analyses led to the discovery of hundreds of novel up-regulated genes, which were differentially expressed genes in H_2O_2-induced cellular senescence in pre-adipocytes. More importantly, this study allowed the identification of 62 upregulated and extracellular annotated genes, which were predicted to be potential SASP molecules in H_2O_2-induced senescent 3T3-L1 as shown in Figure 2C. The 10 most expressed genes were MMP13, APOD, AGT, CXCl10, IGF2, DCN, ISG15, C3, CP, DPT, and ISLR, whose fold change ranged from 8 up to 64; some of which are established components of SASP molecules. So far the validation for about 14 potential SASP components was done by real-time qPCR. Further validation is necessary to reevaluate their secretory profiles by ELISA based assays.

In addition 39 upregulated nuclear genes were discovered, 17 of which are annotated as transcription factors. Shown in Figure 3 is a network of the discovered transcription factors that were differentially expressed in H_2O_2-induced pre-adipocyte senescence. Their expression values ranged from 2 fold induction up to 8 folds when compared to untreated control. Except for CDKN1A (p21) and CDKN2B genes (Campisi, 2013), little is known about the roles they might play in cellular senescence. Further experiments are needed to validate some of these factors and to explore their roles in pro-inflammatory senescence especially in obesity driven T2D.

Based on the premise that up-regulated nuclear factor could promote pro-inflammatory senescence SASP, in this project we will:

1. Validate the gene expression and secretion profile of SASP molecules and a subset of the discovered nuclear transcription factors.

2. Validated transcription factors will be chosen as candidates using stable lentiviral knockdown system for functional characterization.

The validation and characterization of these genes will bear great significance in identifying novel therapeutic targets for the rapidly growing population of obese individuals living with diabetes.

2.7 Transcription factors upregulated in ROS-induced pro-inflammatory senescence

2.7.1 Signal transducer and activator of Transcription 1

Signal transducer and activator of Transcription 1 (STAT1) is a transcription factor and a member of STAT family. It is involved in cellular responses induced by cytokines and growth factors, because of its function as signal messengers and transcription factors (Schindler et al., 2007). Tyrosine phosphorylation is the most common mode of action of STATs family by members of JAK kinase in response to cytokine signaling. Upon phosphorylation, the phosphorylated STATs dimerize and migrate to the nucleus and bind to the promoter of responsive genes (Levy & Darnell, 2002). They play an important role in mediating a wide range of biological responses (apoptosis, cell survival, proliferation and differentiation) (Schindler et al., 2007).

There are seven members of STATs, among them only STAT1 and STAT3 have been well studied so far. They have opposite effects in the regulation of cell survival and apoptosis. Activation of STAT3 protects the cell from apoptosis while STAT1 activation promotes apoptosis through the regulation of the transcription of genes involved in controlling the cell cycle (Dimberg et al., 2003; Xiao et al., 2006). However, the

knowledge about the role of STAT1 in pro-inflammatory senescence and its influence on pathophysiology of senescent cells in preadipocytes have not been investigated.

2.7.2 DNA damage inducible transcript 3

DNA damage inducible transcript 3 (DDIT3), also known as CHOP, is a member of the CCAAT/enhancer binding protein (C/EBPs). CEBPs contain a transcriptional activation domain and a basic Leucine Zipper Domain region, for DNA binding and dimerization (Averous et al., 2004).

Previously, it was reported that DDIT3 plays an oncogenic role in tumorigenesis in several cancers (Crozat, 1993; Rabbitts, 1993). Also, it was well established that DDIT3 is a key mediator of the Endoplasmic Reticulum stress induced apoptosis in murine fibroblast cells (Zinszner et al., 1998). Its expression is increased at the transcriptional level and translational levels as well with highlighting its important role in cell growth and differentiation (Ma & Hendershot, 2003). Knowledge is still lacking about the role of DDIT3 in cellular senescence and if it has a role in promoting inflammation associated with senescence or not.

2.7.3 CCAAT/Enhancer binding protein

CCAAT/Enhancer binding protein (C/EBPδ) gene is a member of the C/EBP transcription factor family. It encodes for the C/EBPδ protein (Tsukada, 2011). The importance of C/EBPδ is associated with its function both as a tumor suppressor (Porter et al., 2003; Zucchi et al., 2004), a tumor promoter (Gery, 2005; Ikezoe et al., 2005) and strongly liked with conditions that correlate with cancer progression.

It is well established that under stress condition as in hypoxia, C/EBPδ forms heterodimers in the presence of the key candidates CHOP and ATF, which are stress response factors (Rouschop et al., 2010). Although the expression of C/EBPδ is considered to be low to undetectable, this expression can be induced by extracellular signals including cytokines, which confirms its role in stress responses (Takiguchi, 1998). Moreover, the expression of C/EBPδ is induced in response to stress in various cell types and tissues including tumors and the proliferation effect can be cell type dependent as well (Balamurugan et al., 2010; Min et al., 2011).

In addition to C/EBPδ's effect in regulating proliferation, it has been associated in the differentiation of preadipocytes (Hishida et al., 2009). The relationship between C/EBPδ and inflammation have been studied, an induction in the expression of C/EBPδ have been reported by inflammatory cytokine including IL-6, thus directly activating the gene expression of IL-6 (Poli, 1998). These entire studies highlight the importance of C/EBPδ, however, the role of C/EBPδ in senescence and inflammation have not been yet studied and needs more investigation.

2.8 Research Design

2.8.1 Validation of up-regulated potential SASP molecules in H_2O_2-induced cellular senescence in pre-adipocytes by qPCR and dot blot

Predicted SASP molecules were validated on the mRNA level by RT-qPCR. The next step is to perform dot blot and/or antibody microarray validation of secretion profile of predicted SASP molecules from preliminary data. Protein expression and secretion profile levels of predicted SASP molecules in 3T3-L1 pre-adipocytes treated with or without H_2O_2 were monitored in conditioned media using dot blot and/or antibody

microarrays following the published experimental conditions (Coppe et al., 2010). In brief, at the end of the experiment, medium is replaced with serum free medium and after 24 hours the medium from every condition will be analyzed for secreted protein profile. Proteins will be subjected to dot blot and/or antibody microarray.

2.8.2 Validation of up-regulated nuclear transcription factors genes in H_2O_2-induced cellular senescence in pre-adipocytes

The transcriptomics analysis has allowed the identification of 17 nuclear transcription factors that were differentially upregulated in H_2O_2-induced cellular senescence in pre-adipocytes (Figure 3). The current project studied was based on the validation of 3 transcription factors by real time PCR and Western Blot analyses.

To monitor the gene expression and protein levels during early stages of senescence programming, the transcription factors (DDIT3, C/EBPδ and STAT1) were subjected to detailed time course analysis to monitor their expression level at the protein and mRNA levels. 3T3-L1 pre-adipocyte cells were treated with H_2O_2 and harvested at different time intervals along with an untreated control. Total RNA and protein lysates were obtained for real-time PCR and Western blot analyses using validated primers and commercially available antibodies respectively.

2.8.3 Functional characterization of identified nuclear transcription factors in promoting SASP in H_2O_2-induced cellular senescence in pre-adipocytes

Stable lentiviral shRNA silencing technology was employed for the three chosen transcription factors to characterize them further. Gene silencing was assessed by Western blot analysis.

Gene-silenced pre-adipocytes were induced to senesce by repeated H_2O_2 treatment as described in (Figure 1A). Growth curves, SA-β-gal staining assays were performed as shown in (Figure 1). Cell numbers were obtained at different time points post treatment (day 5 and day 7 post treatments) to establish a growth curve and assess the cell growth arrest phenotype in shRNA knockdown versus shRNA scramble transfected cells treated with or without H_2O_2. In a time course experiments, 3T3-L1 pre-adipocytes were fixed and stained for SA-β-gal to measure senescence phenotype in shRNA knockdown versus shRNA scramble transfected cells treated with or without H_2O_2 at the following intervals (day 5 and day 7 post treatments). DDR and inflammation markers such as p53, p21, TNF-α and IL-6 mRNA levels were monitored in the silenced cells. Western blot analysis for protein levels of total and activated DDR proteins were evaluated such as, total protein levels of p53 and p21 along with actin as loading control.

Chapter 3. Materials and methods

3.1 Materials and reagents

Table 1-7 contains a list of all the materials and their resources used in the research.

Table 3.1 List of chemicals

Description	Catalog Number	Company
Agrose for routine Use	A9539-250G	Sigma Aldrich
Ammonium Persulphate	A3678-100G	Sigma Aldrich
Calcium Chloride	C5670-100G	Sigma Aldrich
EDTA	E4884-500G	Sigma Aldrich
EGTA	E4378-100G	Sigma Aldrich
Bovine Serum Albumin	A9647-500G	Sigma Aldrich
Glycine	G8898-1KG	Sigma Aldrich
LB agar	L2897-1KG	Sigma Aldrich
LB broth	L3022-1KG	Sigma Aldrich
Nictinamide	N0636-100G	Sigma Aldrich
Phenylmethanesulfonyl fluoride (PMSF)	P7626-100G	Sigma Aldrich
Potassium Chloride	P5405-1KG	Sigma Aldrich
Sodium azide	S8032-100G	Sigma Aldrich
Sodium hydroxide	S8045-500G	Sigma Aldrich

Sodium dodecyl sulfate	L3771-500G	Sigma Aldrich
Sodium fluoride	01148-500G	Sigma Aldrich
Trizma base	T1503-1KG	Sigma Aldrich
Sodium Chloride	S5886-5KG	Sigma Aldrich
Paraformaldehyde	16005-1KG-R	Sigma Aldrich
Sigma fast protease inhibitor cocktail, EDTA free	S8830-20TAB	Sigma Aldrich
Sodium orthovanadate	S6508-10G	Sigma Aldrich
Benzamidine hydrochloride	434760-5G	Sigma Aldrich
Calpin inhibitor	A6185-5MG	Sigma Aldrich

Table 3.2 List of molecular reagents

Description	Catalog Number	Company
DMEM High Glucose	42430025	Gibco
Hydrogen Peroxide	H3410	Sigma Aldrich
ECL Western Blotting Substrate	32106	Thermo Scientific
Super Signal West Dura Extended Duration Substrate	34075	Thermo Scientific
Super Signal West Femto Maximum Sensitivity Substrate	34095	Thermo Scientific

Lipofectamine 2000 Reagent	11668019	Invitrogen
TRIzol Reagent	15596018	Ambion
Acrylamide/ Bis- Acrylamide 30% Solution	A3574	Sigma Aldrich
Bovine Serum	16170078	Gibco
Antibiotic Antimycotic Solution	A5955	Sigma Aldrich
Precision Plus Protein Dual Color Standard	1610374	Biorad
Gene Ruler 1kb Plus	SM1333	Fermentas
6X DNA Loading Dye	R0611	Fermentas
Puromycin 10mg/ml	A1113803	Gibco
RNAse Inhibitor 2000 Units 20U/ul	N8080119	Applied Bio-systems
TaqMan Gene Expression Master Mix	4369016	Applied Bio-systems
Dimethyl Sulphoxide DMSO	D2438-50ML	Sigma Aldrich
Ethidium bromide solution 10mg/ml	E1510-10ML	Sigma Aldrich
Glycerol 99%, puriss Glycerin 99%	15523-1L	Sigma Aldrich
Tween 20	P9416-100ML	Sigma Aldrich
N,N,N,N-tetramethylethylenediamine	T9281-100ML	Sigma Aldrich
Hydrochloric acid 37%	07102-1L	Sigma Aldrich

Ethanol Anhydrous	277649-2L	VWR
Methanol	24229-2.5L	Sigma Aldrich
2-propanol, anhydrous 99.5%	278475-2L	Sigma Aldrich
Hydrogen Peroxide	H3410-500m	Sigma Aldrich
2 betamercaptoethanol	M6250-500Ml	Sigma Aldrich
Phenol Chloroform	77617-500ml	Sigma Aldrich
Antibiotic Antimycotic Solution (100X)	A5955-100ML	Sigma Aldrich

Table 2.3 List of kits

Description	Catalog Number	Company
Senescence Beta Galactosidase Staining Kit	9860S	Cell Signaling
Gene Elute Plasmid Mini Prep Kit	PLN350	Sigma Aldrich
Endo-Free Plasmid Maxi Kit	12362	Qiagen
High Capacity cDNA Reverse Transcription Kit	4368814	Applied Bio-systems

Table 3.4 List of primers

Description	Catalog Number	Company

Mouse Cyclophilin A (PPIA) VIC	Mm02342430_g1	Life Technologies
Mouse STAT1 FAM	Mm00439531_m1	Life Technologies
Mouse CEBPD FAM	Mm00786711_s1	Life Technologies
Mouse DDIT3	Mm01135937_g1	Life Technologies
IGFBP3	Mm01187817_m1	Life Technologies
C3	Mm0043838_m1	Life Technologies
DCN	Mm00514535_m1	Life Technologies
AGT	Mm005996662_m1	Life Technologies
MMP3	Mm00440295_m1	Life Technologies
IGFBP4	Mm00494922_m1	Life Technologies
IL-6	Mm00446190_m1	Life Technologies
TNF-α	Mm00443258_m1	Life Technologies
MMP13	Mm00439491_m1	Life Technologies
GAS6	Mm00490378_m1	Life Technologies
CDKN1A	Mm04205640_g1	Life Technologies
VEFGB	Mm00442102_m1	Life Technologies
DPT	Mm00498111_m1	Life Technologies

IGF2	Mm00439564_m1	Life Technologies
CXCL10	Mm00445235_m1	Life Technologies
CXCL1	Mm04207460_m1	Life Technologies
TRP53	Mm01731287_m1	Life Technologies
CXCL5	Mm00436451_g1	Life Technologies

Table 3.5 List of antibodies

Description	Catalog Number	Company
Mouse monoclonal p53	Ab26	Abcam
Mouse monoclonal to TNF-α	Ab1793	Abcam
Monoclonal anti Beta actin	A2228	Sigma Aldrich
Anti-p21 Rabbit	Ab7960	Abcam
Mouse monoclonal [52B83] to TNF-α	Ab1793	Abcam
Anti-IGFBP4 Antibody	Ab77350	Abcam
Anti-IGFBP3 Antibody	Ab86461	Abcam
Anti-IP10 Antibody	Ab8098	Abcam
Anti- IGF2 Antibody	Ab9574	Abcam
Anti- C3 Antibody	Ab11887	Abcam

Anti-MMP3 Antibody	Ab53015	Abcam
Anti-MMP13 Antibody	Ab39012	Abcam
Anti-GAS6 Antibody	Ab86059	Abcam
Anti-ISG15 Antibody	Ab131119	Abcam
Anti-Decorin Antibody	Ab137508	Abcam
Anti-Dermatopontin Antibody	Ab118710	Abcam
Anti-Ceruloplasmin Antibody	Ab48614	Abcam
Anti-CEBP Delta Antibody	Ab65081	Abcam
Stat1 Antibody	9172	Cell Signaling
CHOP (L63F7) Mouse mAb	2895	Cell Signaling

Table 3.6 List of plasmids

Description	Catalog Number	Company
Mouse DDIT3	TRCN0000324349	Sigma Aldrich
Mouse STAT1	TRCN0000054923	Sigma Aldrich
Mouse CEBPD	TRCN0000321288	Sigma Aldrich

Table 3.7 List of buffer recipes

RIPA Lysis Buffer	50 mM Tris pH 7.5 150 mM NaCl 1 mM EDTA 1 mM EGTA 1% Triton 0.5 % NaDoc 0.1% SDS
5 x SDS Loading Sample Buffer	250 mM Tris HCl pH 6.8 10% SDS 30% Glycerol 5% Beta-Mercaptoethanol 0.04 % Bromophenol Blue
10% Ammonium Persulfate-10 ml	Dissolve 1 g ammonium persulfate (APS) in 8 ml H_2O. Adjust volume to 10 ml with H_2O. Solution is stable at 4°C for two weeks.
10 x SDS PAGE Running Buffer-1 liter	Dissolve 10 g SDS, 30.3 g Tris and 144.1 g glycin in 800 ml H_2O. Adjust volume to 1 liter with H_2O. Store at room temperature.
10 x PBS-1 liter	Dissolve 80 g NaCl, 2 g KCl, 26.8 g Na_2HPO_4, 2.4 g KH_2PO_4 in 800 ml H_2O. Adjust to pH 7.4 with HCl. Adjust volume to 1 liter with H_2O. Divide in aliquots and sterilize by autoclaving. Store at room temperature.
1x Transfer Buffer/ wet blots-1 liter	Dissolve 2.9 g Glycine, 5.8 g Tris and 0.37 g SDS in 200 ml methanol.

	Adjust volume to 1 liter with H_2O. Store at 4°C.
1 x TBS(Tris Buffered Saline)-1 liter	Dissolve 6.05 g Tris (50 mM) and 8.76 g NaCl (150 mM) in 800 ml H_2O. Adjust pH to 7.5 with 1 M HCl (~ 9.5 ml). Adjust volume to 1 liter with H_2O. TBS is stable at 4°C for three months.
1 x TBST(Tris Buffered Saline Tween) – 1 liter	Dissolve 1 ml Tween 20 in 1 liter TBS buffer. TBST is stable at 4°C for three months.
0.5 M EDTA, pH 8:	For 500 mL Re-suspend 93.05 g Na_2•EDTA•$2H_2O$ (disodium dyhydrate) in about 400 mL of ddH_2O Add about 9 g solid NaOH Once all the NaOH dissolves, slowly adjust the pH with 10 *N* NaOH Bring up the volume to 500 mL with ddH_2O
50x TAE- 1 liter	Dissolve 242.2 g Tris base in around 600 mL of ddH_2O Slowly add 57.1 mL glacial acetic acid Add100mL0.5MEDTA, pH8 Bring up the volume to 1 L with ddH_2O
1.5 M Tris, pH 8.8 Stock Buffer for Separating Gel- 1 liter	Dissolve 181.65 g Tris base in around 800 mL of ddH_2O Adjust the pH to 8.8 with concentrated HCl Bring up the volume to 1 L with ddH_2O

1.5 M Tris, pH 6.8 Stock Buffer for Stacking Gel- 1 liter	Dissolve 181.65 g Tris base in around 700 mL of ddH_2O Adjust the pH to 6.8 with concentrated HCl Bring up the volume to 1 L with ddH_2O
10x Tris-glycine Running Buffer 4 Liters	121.1 g Tris base 576 g glycine 200 mL 20% SDS Bring up the volume to 4 L with ddH_2O

3.2 Methods

3.2.1 Mammalian cell culture

Cryopreserved 3T3-L1 were purchased as passage 8 from (Zen-Bio, USA) and used for experiment between passage 10 and 15. Cells were maintained in high-glucose, dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum (GIBCO®, USA), penicillin and streptomycin antibiotics (Sigma Aldrich, USA). Human Embryonic Kidney 293T cells (HEK-293T) were gifted from Dr. Arash Rafii (WCMC-Q). Cells were maintained in high-glucose, dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (Sigma®, USA), penicillin and streptomycin antibiotics (Sigma Aldrich, USA). All cells were grown at 37°C in 5% CO2. Medium was changed every 2–3 days until cells achieved 70–80% confluency.

3.2.2 H_2O_2-induced cellular senescence treatment protocol

A robust protocol has been established in Mazloum's Lab for H_2O_2-induced cellular senescence in mice 3T3-L1 pre-adipocytes at Weill Cornell Medical College- Qatar (WCMC-Q). The cells were treated with 200uM H_2O_2, allowed for a time course treatment in parallel with control cells (Untreated) and harvested at the time interval post the first 3 hours treatment (30 minutes, 3 hours, day 1, day 3, day 5 and day 7).

3.2.3 Growth Curve

For growth curve analysis, 3T3-L1 (100×10^3 cells per well) were plated in 6 well plates or 1×10^6 cells in 10cm plate. The number of the cells were reordered at different time points post plating using TC10 Trypan blue dye/ automated cells

counter) from (Bio-Rad). Then a growth curve was generated to illustrate the rate of cell growth in each condition compared to controls.

3.2.4 RNA silencing and generation of lentiviral particles

Stable lentiviral particles expressing small hairpin interfering RNAs (shRNA) targeting mouse STAT1, DDIT3, C/EBPδ mRNA in 3T3-L1 pre-adipocytes were generated using cDNA lentiviral shRNA vectors (MISSION® shRNA Plasmid DNA, Sigma Aldrich).

The sequence for STAT1 was:

5'CCGGGCCGAGAACATACCAGAGAATCTCGAGATTCTCTGGTATGTTCTCGGCTTTTTG-3'. The sequence for DDIT3 was:

5'CCGGGAAACGAAGAGGAAGAATCAACTCGAGTTGATTCTTCCTCTTCGTTTCTTTTTG -3'. The sequence for C/EBPδ was:

5'CCGGTCGACTTCAGCGCCTACATTGCTCGAGCAATGTAGGCGCTGAAGTCGATTTTTG -3'. A scramble non-sense RNAi sequence was used, which was directed against a sequence with no corresponding part in the mouse genome (shScramble) to control the unspecific effects of shRNA (Ereneret al., 2012). In brief, 293T cells were co-transfected with shRNA lentiviral plasmid or shScramble lentiviral plasmid plus the lentiviral packaging and envelope plasmids, using lipofectamin2000 following manufacturer instruction. Medium containing generated viral particles was collected three days post transfection, followed by purification step (Millipore). Generated (shStat1, ShDDIT3, ShC/EBPδ) lentiviral particles were used to infect 3T3-L1 pre-adipocytes using 4μg/ml polybrene in order to generate stable (shStat1, ShDDIT3, ShC/EBPδ) expressing cells. Puromycin selection (2

μg/ml) was used to select the infected cells.

3.2.5 Dot Blot

Cells were treated with H_2O_2 at the time interval post the first 3 hours treatment. At the end of (Day 5) post H_2O_2 treatment, medium was replaced with serum free medium for 24 hours. The conditioned medium was collected, centrifuged to remove cell debris. Cell lysate were subjected to Bio-Dot SF Microfiltration Apparatus (Bio-rad) to monitor the protein expression levels of the predicted SASP molecules. 100μl of the condition medium was applied on the Nitrocellulose membrane after normalizing it to the number of the cells. Non-specific sites were blocked using 4% BSA/TBST for 1 hour at room temperature. The membrane was incubated with the primary antibody dissolved in 2% BSA/TBST for 1 hour at room temperature. Then washed 3 times with TBST for 5 minutes. After that the membrane was incubated with the secondary antibody dissolved in 2% BSA/TBST and then washed 3 times with TBST for 5 min. ECL reagent was used to develop the membrane, covered with transparent plastic cover and exposed in the Gel-Doc system.

3.2.6 Western blotting

The cell pellets were lysed in ice cold RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 0.5% NaDoc (Sigma), 1% Triton X-100) supplemented with 1X protease inhibitor cocktail (Sigma), 10mM NaF (Sigma), 1mM Sodium orthovanadate (Sigma), 1mM PMSF, 5mM benzamide (Sigma), 20μg/ml calpin inhibitor (Sigma), 5 mM NAM (Sigma) and 3mM TSA (InvivoGen). Then, homogenized using sonicator cell disrupter and centrifuged for

10 minutes at 4°C and 15,000 g. Protein concentrations were determined using Biorad reagents according to manufacture's protocol. A 15-20 μg of proteins were separated by SDS-PAGE followed by electroblotting onto PVDF membranes (Biorad) and Western blot analysis. Western blots were probed with the following antibodies: rabbit anti-STAT1, rabbit anti-C/EBPδ, mouse anti-DDIT3, mouse anti-p53 and rabbit anti-p21. Quantitation of Western bands was performed using ImageJ software, by selecting each band area and integrating the mean intensity and pixel value, then dividing the product by that of the standard band, which was either actin or GAPDH. Relative intensity was then normalized with the control treatment or the initial time-point as 1.

3.2.7 Quantitative RT-PCR

Total RNA was prepared with the RNeasy Mini Prep Kit (Qiagen). First strand cDNA was synthesized using cDNA synthesis kit (Roche) following manufacturer's instructions. The cDNA was subject to quantitative RT-PCR using Quat Studio 6Flex thermal cycler (Applied Biosystems). Exon-exon junction TaqMan primers were used to amplify the studied genes (Applied Biosystems) (STAT1: Mm00456425_m1, DDIT3: Mm00461298_m1, C/EBPδ: Mm00503579_m1). Target gene expression was normalized to the endogenous control. The average Ct value from each triplicate was used to calculate fold induction of the gene, with the control group normalized to 1.

3.2.8 SA-β-Gal staining

Cells were fixed on day 7 post-treatment and stained for β-gal (Cell Signaling, protocol following manufacturer's instructions). Positively stained cells were quantified from multiple fields of at least three independent experiments to evaluate the knockdown effect on senescence phenotype upon H_2O_2 treatment in 3T3-L1 pre-adipocytes, when compared to shmock control.

3.2.9 Statistical analysis

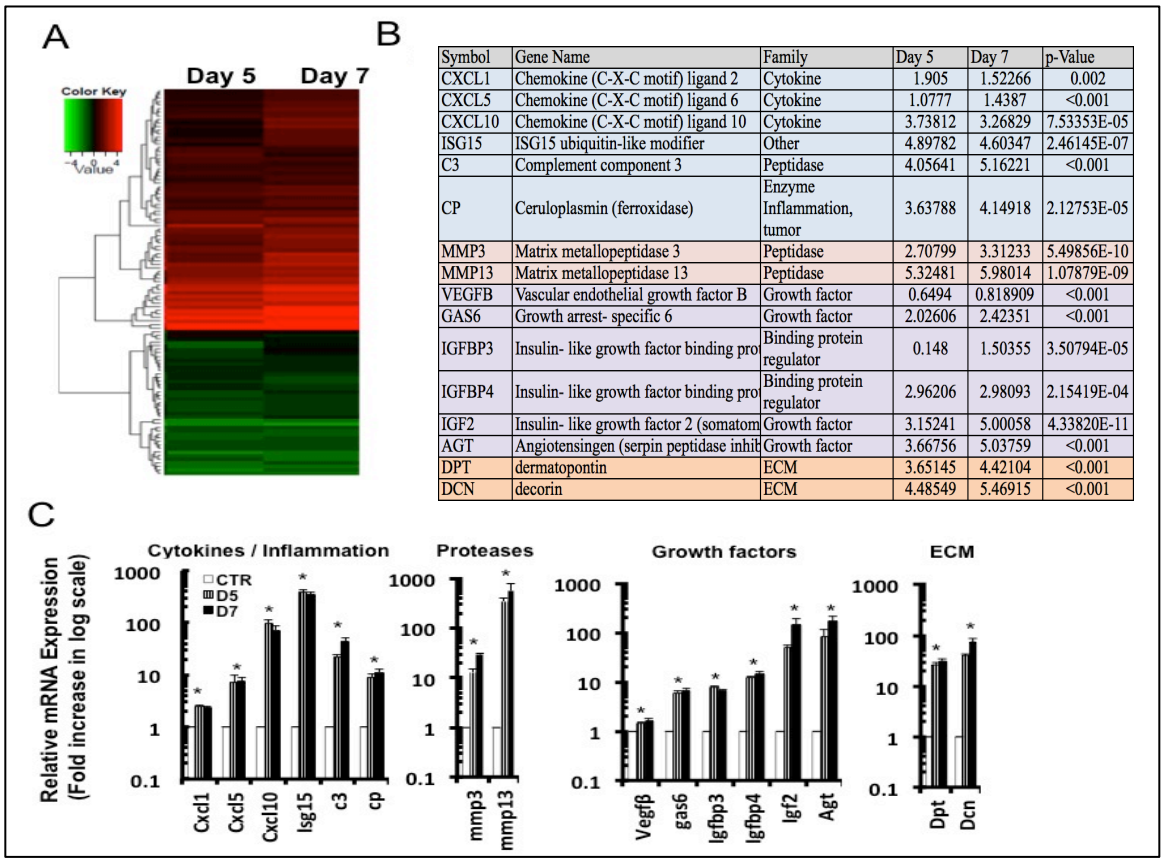
The results are expressed as mean ± SEM experiments were repeated at least 3 times. Statistical analyses were performed from a minimum of 3 separate experiments carried out independently. The data was analyzed using widely accepted statistical software. Paired Student's T-test was used. Significant difference was defined as P value<0.05.

Chapter 4. Results

4. Results

4.1 Validation of up-regulated potential SASP molecules in H_2O_2-induced cellular senescence in pre-adipocytes by RT-qPCR.

Figure 4A shows the hierarchal clustering of common genes among the different conditions at day 5 and day 7. The list of the predicted SASP in H_2O_2-induced senescent pre-adipocytes from the transcriptomic analysis are shown in Figure 4B. Figure 4C summarizes the qPCR validation for the predicted SASP in transcriptomic analysis. These group of genes were significantly upregulated at day 5 and day 7 post H_2O_2 treatment.



Symbol	Gene Name	Family	Day 5	Day 7	p-Value
CXCL1	Chemokine (C-X-C motif) ligand 2	Cytokine	1.905	1.52266	0.002
CXCL5	Chemokine (C-X-C motif) ligand 6	Cytokine	1.0777	1.4387	<0.001
CXCL10	Chemokine (C-X-C motif) ligand 10	Cytokine	3.73812	3.26829	7.53353E-05
ISG15	ISG15 ubiquitin-like modifier	Other	4.89782	4.60347	2.46145E-07
C3	Complement component 3	Peptidase	4.05641	5.16221	<0.001
CP	Ceruloplasmin (ferroxidase)	Enzyme Inflammation, tumor	3.63788	4.14918	2.12753E-05
MMP3	Matrix metallopeptidase 3	Peptidase	2.70799	3.31233	5.49856E-10
MMP13	Matrix metallopeptidase 13	Peptidase	5.32481	5.98014	1.07879E-09
VEGFB	Vascular endothelial growth factor B	Growth factor	0.6494	0.818909	<0.001
GAS6	Growth arrest- specific 6	Growth factor	2.02606	2.42351	<0.001
IGFBP3	Insulin- like growth factor binding pro	Binding protein regulator	0.148	1.50355	3.50794E-05
IGFBP4	Insulin- like growth factor binding pro	Binding protein regulator	2.96206	2.98093	2.15419E-04
IGF2	Insulin- like growth factor 2 (somatom	Growth factor	3.15241	5.00058	4.33820E-11
AGT	Angiotensingen (serpin peptidase inhib	Growth factor	3.66756	5.03759	<0.001
DPT	dermatopontin	ECM	3.65145	4.42104	<0.001
DCN	decorin	ECM	4.48549	5.46915	<0.001

Figure 4.1 A) Hierarchal clustering of common genes among the different conditions. **B)** Table that lists the number of the statistically significant differentially expressed SASP factors in H_2O_2-induced senescent pre-adipocytes. **C)** Validation of the predicted SASP factors in H_2O_2-induced senescent pre-adipocyes on the mRNA level. Statistical analysis were performed using student's t-test analysis, * $p<0.05$.

Validation of up-regulated potential SASP molecules in H_2O_2-induced cellular senescence in pre-adipocytes

Figure 5A is the Dot Blot results for the secreted SASP in conditioned medium in day 5 post H_2O_2 treatment. Figure 5B is the ImageJ quantification for the dot blot. These SASP were significantly upregulated at day 5 post H_2O_2 treatment.

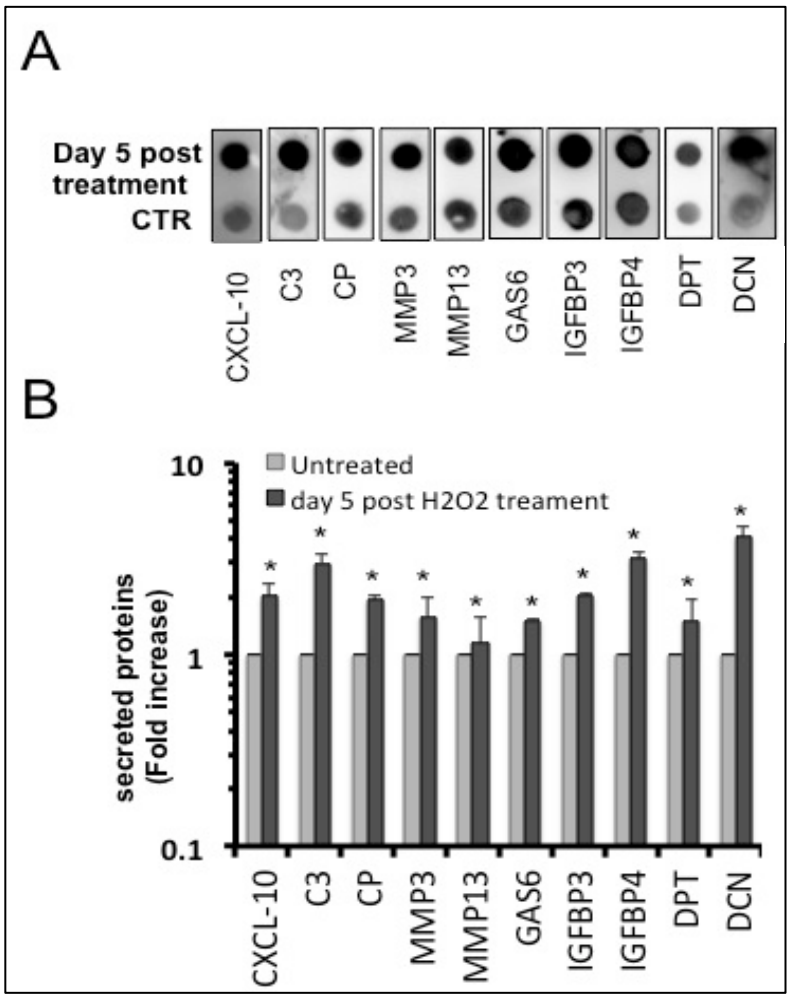


Figure 4.2 A) Validation of the predicted SASP factors in H_2O_2-induced senescent pre-adipocyes on the level of secreted proteins using Dot Blot. **B)** ImageJ quantification for the dot blot in A panel. Statistical analysis were performed using student's t-test analysis, * $p<0.05$.

Validation of 3 up-regulated nuclear factors in H_2O_2-treated 3T3-L1 pre-adipocytes

Figure 6 summerizes the RT-qPCR results for the expression of the 3 tested nuclear factors (STAT1, DDIT3 and C/EBPδ) post H_2O_2 treatment. Selected genes were significantly upregulated at day 5 and day 7 post H_2O_2 treatment. Thus, these results validate the transcriptomics data for the corresponding genes shown in Figure 3.

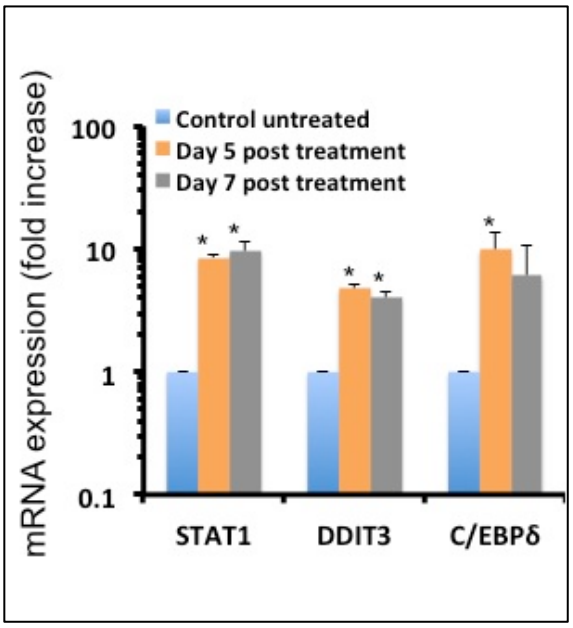


Figure 4.3 mRNA levels of STAT1, DDIT3 and C/EBPδ were analyzed by RT-qPCR at the indicated time points post H_2O_2treatments. CyclophilineA was used as endogenous control. Results are presented as means + SEM of three independent experiments. Statistical analysis were performed using student's t-test analysis, * $p<0.05$.

4.4 Time course assessment of mRNA levels of STAT1, DDIT3, and C/EBPδ

The mRNA levels in Figure 7A, 7B showed that STAT1 and DDIT3 were upregulated at (30 min, day 3, day 5 and day 7). However, C/EBPδ shown in Figure 7C was upregulated only at day 5 and day 7. The difference in the observed time of onset of gene levels might be strictly controlled to be functionally involved in certain stages of senescence and inflammation development. Figure 7D is a representative data for Western blot using antibodies against the proteins products of the 3 studied genes. STAT1 and DDIT3 were increasingly upregulated from 30 min to day7. Attempts to examine C/EBPδ protein were not successful due to quality of the antibody. Therefore it requires more investigation in the future using a new antibody.

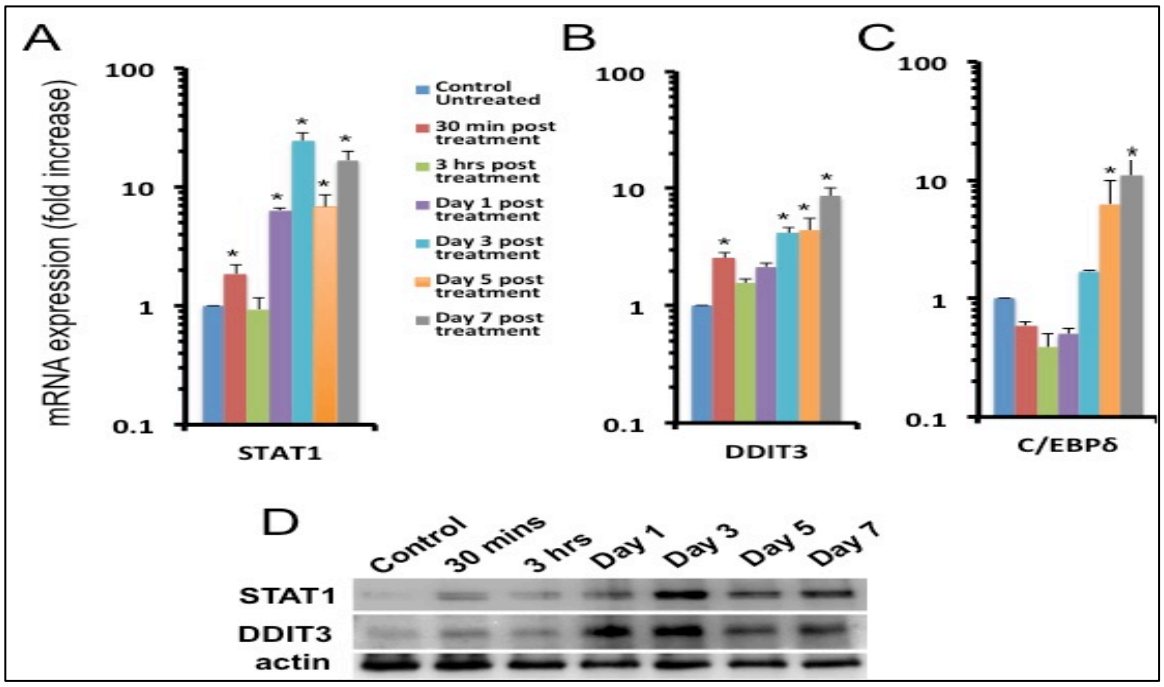


Figure 4.4 A,B,C) Time course assessment of mRNA levels of STAT1, DDIT3 and C/EBPδ by RT-qPCR at the indicated time points post H_2O_2 treatments. Results are presented as means + SEM of three independent experiments. Statistical analysis were performed using student's t-test analysis, * $p \leq 0.05$.**D)** Western blot analysis was performed on cell lysates of the indicated time points using antibodies against of STAT1and DDIT3 . Actin was used as a loading control.

4.5 Unlike DDIT3 knockdown, STAT1 knock down leads to increased cell number

Among the three genes chosen for the senescence study, we were only able to knockdown STAT1 and DDIT3 using shRNA lentiviral system as shown in Figure 8A. The knockdown was very strong as almost no proteins were detected by Western blotting in shSTAT1- or shDDIT3- infected cells compared to shScramble- or non-infected- cells.

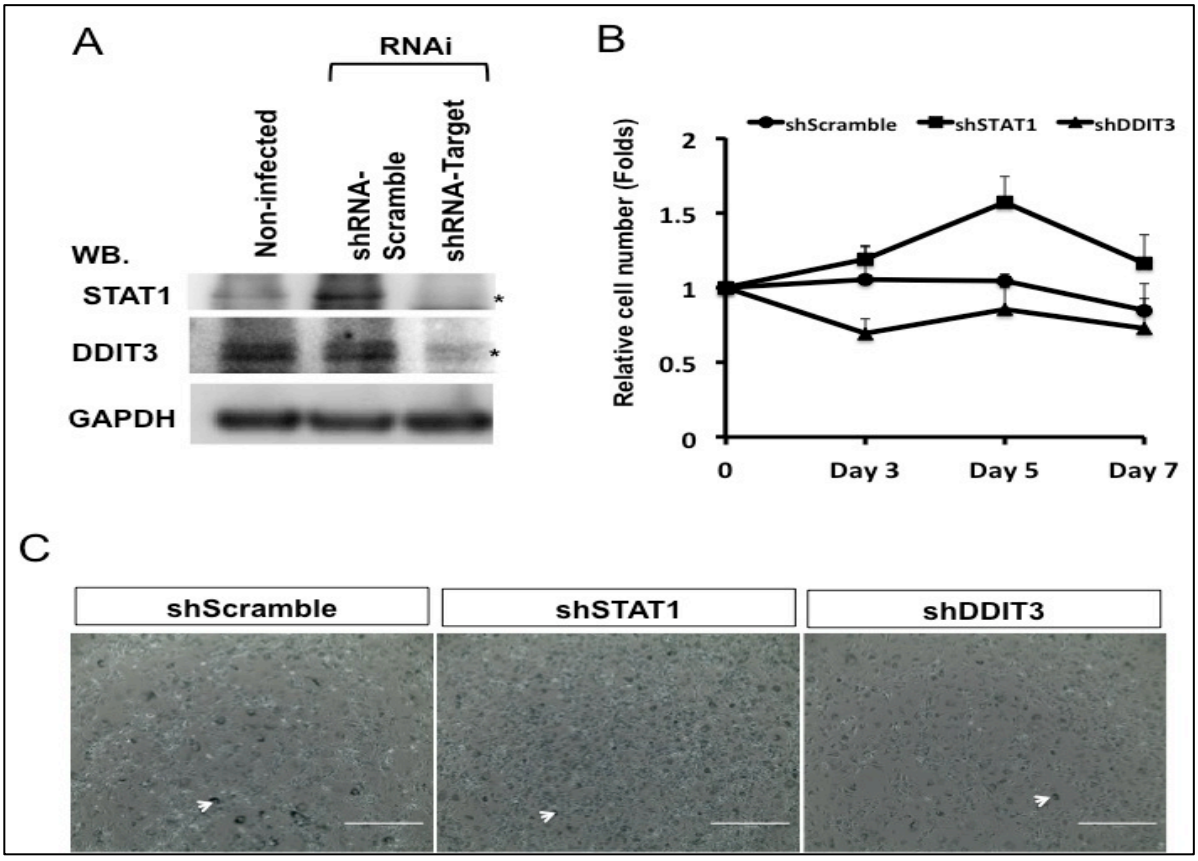


Figure 4.5 A) Western blot analysis on protein lysates of shSTAT1- and shDDIT3- silenced 3T3-L1 cells. shScramble and non-infected cells were used as controls. Expressed proteins were detected using antibodies against of STAT1 and DDIT3. **B)** Growth curve for untreated and H_2O_2 treated shSTAT1 and shDDIT3 at different time points. **C)** SA-β-gal staining for shSTAT1, shDDIT3 and shScramble at day 7 post H_2O_2 treatment. Scale bar, 1mm. Results are presented as means + SEM of three independent experiments.

shSTAT1- and shDDIT3- and shScramble- infected- cells were induced to senesce by using H_2O_2 treatment and growth curve for each cell line was plotted in Figure 8B. shSTAT1 silenced cells continued to divide and were less sensitive to the H_2O_2 treatment when compared to shScramble cells. Unlike shScramble, growth did not arrested in shSTAT1, at least up to day 5 post-treatment in Figure 8B. On the other hand, DDIT3 knockdown showed the opposite effect to the STAT1 knockdown in Figure 8B. shDDIT3 silenced cells exhibited slow growth curve compared to shScramble cells (30 min – day 1) as shown in Figure 8B.

By using the senescence biomarker β-gal, no obvious difference was found in β-gal staining between shSTAT1 and shScramble or between shDDIT3 and shScramble as shown in Figure 8C. Most of the cells were positive for the βgal staining in shScramble, shSTAT1 and shDDIT3. But interestingly, when comparing the size and number of cells between the different conditions, shSTAT1 silenced cells were higher in number and smaller in size compared to shScramble silenced cells as shown in Figure 8C. So far DDIT3 knockdown cells did not seem to be different in size but less in number compared to shScramble cells.

4.6 STAT1 knockdown leads to decreased p21 expression

To get insight in to the molecular mechanisms of STAT1 and DDIT3 effect on preadipocytes senescence, the gene expression of senescence markers (p21 and p53) was assessed on both mRNA and protein levels as shown in Figure 9.

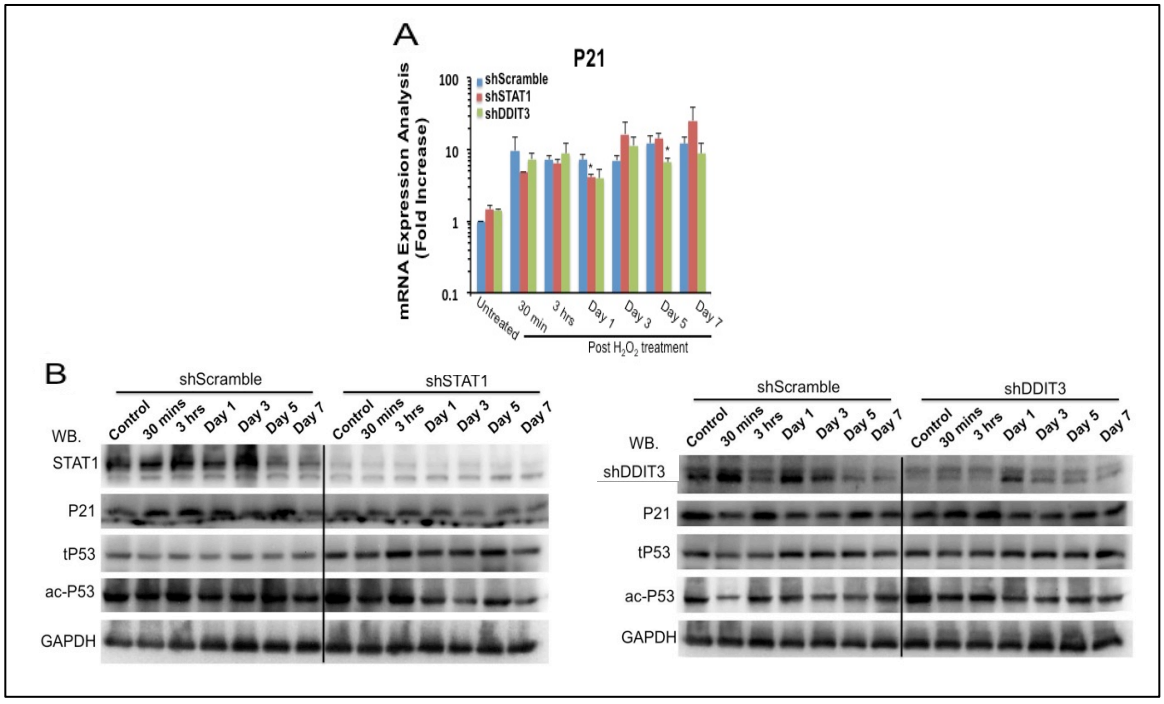


Figure 4.6 A) mRNA expression analysis of senescence markers for shSTAT1, shDDIT3. RT-qPCR analysis for p21 gene expression in untreated and H_2O_2 treated shSTAT1, shDDIT3 and shScramble in a time course experiment. **B)** p21 protein expression is downregulated in shSTAT1 silenced cells. Western blot analysis on protein lysates of shSTAT1-, shDDIT3- silenced 3T3-L1 cells. Cell were either untreated or treated with H_2O_2 in a time course experiment (30 min, 3 hrs, day 1, day 3, day 5 and day 7 post treatment). shScramble cells were used as control. Expressed proteins were detected using antibodies against STAT1, DDIT3 and p21. GAPDH was used as loading control. Results are presented as means + SEM of three independent experiments. Statistical analysis were performed using student's t-test analysis, * $p \leq 0.05$.

As shown in Figure 9A, p21 mRNA level increased with H_2O_2 treatment as compared to shScramble. Furthermore, both shSTAT1 and shDDIT3 have downregulated p21 mRNA level as shown in Figure 9A. P53 mRNA expression was not changed in both shSTAT1 & shDDIT3 infected cells compared to shScramble (results not shown).

The protein expression of senescence markers was also analyzed in a time course experiment of H_2O_2 treated cells as shown in Figure 9B. The p21 protein level was upregulated in shScramble cells following H_2O_2 treatment to induce senescence related cell cycle arrest. Possibly p21 was downregulated in H_2O_2 treated shSTAT1 silenced cells, as compared to control shScramble. These findings showed that both STAT1 could affect cellular senescence by regulating p21 expression.

Regarding total p53, when quantifying their expression between 3 independent experiments, no significant difference between the shSTAT1, shDDIT3 and shScramble- silenced cells was found. On the other hand, the quantification of acetylated p53 showed significant decrease in the shSTAT1 silenced cells.

4.7 STAT1 knockdown leads to increased IL-6 expression

To get insight in the molecular mechanisms of STAT1 and DDIT3 effect in preadipocytes inflammation, the gene expression of the inflammatory markers (IL-6 and TNF-α) was assessed on the mRNA levels as shown in Figure 10A and 10B.

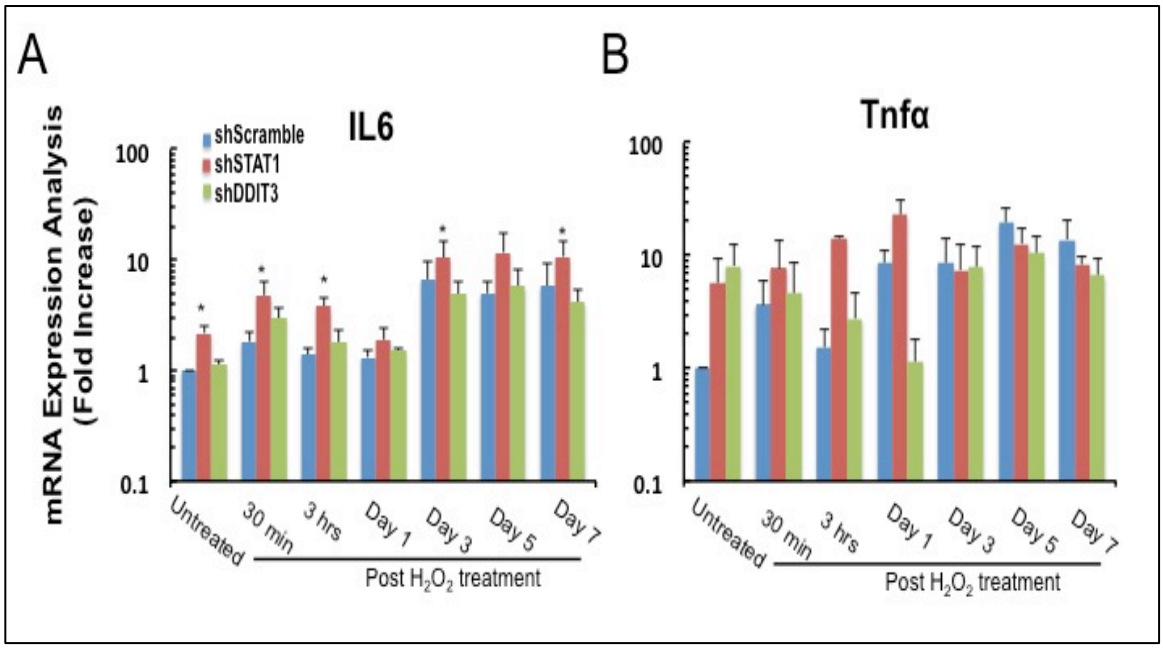


Figure 4.7 A,B) mRNA expression analysis of inflammatory markers for shSTAT1, shDDIT3. RT-qPCR analysis for IL6 and TNF-α gene expression in untreated and H_2O_2 treated shSTAT1, shDDIT3 and shScramble in a time course experiment. Results are presented as means + SEM of three independent experiments. Statistical analysis were performed using student's t-test analysis, * $p \leq 0.05$.

Pro-inflammatory gene IL-6 was upregulated in both H_2O_2 and untreated and treated shSTAT1 silenced cells as compared to shScramble cells in Figure 10A. Similarly, mRNA expression was also increased in untreated shSTAT1 silenced cells. There was a trend to increase TNF-α in shSTAT1 cells at lower time of exposure to H_2O_2 (30 min – day 1). This effect was lost at higher times of treatment as shown on Figure 10B. Figure 10A, also illustrate that IL-6 gene expression profile was not affected upon silencing DDIT3. This suggests that DDIT3 has no effect on IL-6 mRNA expression as shown in Figure 10A. However, increased TNF-α mRNA was observed upon silencing

DDIT3 in untreated cells as shown in Figure 10B. Treatment of shDDIT3 cells with H_2O_2 caused an initial decrease in TNF-α expression in short time treatment followed by an increase in TNF-α expression back to the shscramble levelat (day 3 – day 7).

Chapter 5. Discussion

Inflammation has emerged in recent years as a major contributor for the development of insulin resistance and obesity (Ahima, 2009). Thus obesity is suggested to promote adipose tissue aging due to accumulation of senescent adipocytes in response to elevated ROS levels in the obese state. Cellular senescence is involved in major pathophysiological processes including tumor progression and advanced age related degenerative diseases (Baker et al., 2011; Campisi, 2013). It is characterized by the acquisition of a secretory phenotype termed SASP that is considered the most remarkable feature of many senescent cells.

SASP is characterized by elevation in the secretion of factors including cytokines, chemokines, growth factors, secreted proteases, and secreted insoluble components (Davalos et al., 2010), that could contribute to the development of cancer and cellular senescence. It can also give an explanation about the role of senescent cells in aging and age related pathologies (Coppe et al., 2010). In addition, there is compelling evidence that chronic inflammation is a major contributor in the progression of metabolic diseases (Ahima, 2009). Therefore, our current study contributes several findings that allow better understanding of the molecular details of this relationship that will help in developing effective therapeutic interventions.

First, we evaluated the secretory profile that accompanied senescence and were upregulated in trascriptomics analysis in H_2O_2 induced cellular senescence in pre-adipocytes. A significant induction was observed in the levels of cytokines (CXCL10, C3, CP), proteases (MMP3, MMP13), growth factors (GAS6, IGFBP3, IGFBP4), and ECM molecules (DPT and DCN). Some of which are established components of SASP molecules. It is well established that senescent cells secrete inflammatory growth factors

that can affect surrounding cells including IL-6. This cytokine is a prominent cytokine of SASP and is associated with DNA damage and oncogenic stress (Kuilman et al., 2008; Coppe et al., 2010). Overexpression of chemokines, including members of CXCL family and insulin-like growth factor in senescent cells has been reported (Wang et al., 1996; Castro et al., 2003). These SASP components are suspected to exert variety of effects on senescent cells or may have beneficial effects. Thus, the presented evaluation for the secretory profile extends the recent findings on SASP especially in senescence H_2O_2 induced preadipocytes.

Following an established protocol for H_2O_2 induced cellular senescence in pre-adipocytes, the initial validation of the selected transcription factors STAT1, DDIT3 and C/EBPδ, showed upregulation on the mRNA level that validates the trascriptomics data for the corresponding nuclear factors. The time course assessment of the transcription factors showed variations in the upregulation at the mRNA and the protein levels. These different levels of genes regulation might be strictly controlled to be functionally involved in certain stages of senescence and inflammation development.

Several studies have reported about the members of STAT family that they have contradictory information about its expression during aging. STAT1 expression was shown to be down regulated in rat macrophages (Yoon et al., 2004), whereas STAT3 expression is up-regulated in senescent fibroblast (Wang et al., 2006) and down regulated in rat brain cells (Fulop et al., 2006). These contradictions in the expression of STAT members could be cell type dependent or could vary with aging rate among different species and organs.

DDIT was reported as a nuclear protein and also expressed as a cytoplasmic protein that can be induced in response to stress in human mouse fibroblasts and sarcoma cells (Jauhiainen et al., 2012). As for C/EBPδ, it was well established that in response to stress condtions its expression can be induced (Takiguchi, 1998). This induction was observed in various cell types and tissues including tumors (Balamurugan et al., 2010; Min et al., 2011).

In an attempt to functionally characterize these genes, stable knockdown of these genes using ShRNA lentiviral system was employed. shSTAT1 silenced cells were still dividing and were less sensitive to H_2O_2 treatment when compared to shScramble cells. Therefore, STAT1 might promote cell arrest when expressed in H_2O_2 treated cells. On the other hand, DDIT3 knockdown showed the opposite effect of the STAT1 knockdown. ShDDIT3 silenced cells exhibited a slow growth and seem to senesce earlier than the shScramble cells. Interestingly, this data shows that DDIT3 may play an anti-senescence role when 3T3-L1 cells are treated with H_2O_2. Further investigation to understand the mechanisms underlying the proliferation of senescent cells after the knockdown of STAT1 and DDIT3 is required.

Most of the cells were positive for the βgal staining in shScramble, shSTAT1 and shDDIT3 by using the senescence biomarker βgal. This biomarker is still considered as the first marker that allows the detection of senescent cells (Kurz et al., 2000). The comparison of the size and number of cells between the different conditions, showed that shSTAT1 silenced cells were higher in number and smaller in size compared to shScramble silenced cells. Senescent cells are known to be morphologically bigger in size (Coppe et al., 2010) hence, this data provide the evidence that probably most of cells

are not going into senescence, as they should become bigger in size. DDIT3 knockdown cells did not seem to be different in size but less in number compared to shScramble cells.

To understand the molecular events of STAT1 and DDIT3 to affect preadipocytes senescence and inflammation, the senescence markers (p21 and p53) as well as inflammatory markers (IL-6 and TNF-α) were measured. The activation of DDR upon the damage caused by ROS is responsible for engaging the p53/p21 pathway (Campisi, 2013). The levels of p21 seems to be significantly downregulated at day 1 post H_2O_2 treatment in shSTAT1 treated cells compared to shScrambled cells. Thus, p21 down-regulation might prevent cell cycle arrest and therefore counteract senescence. However, p21 is significantly reduced in day 5 post treatment of shDDIT3. This expression might be related to anti-apoptotic effect of p21 rather than its pro-cell cycle arrest effect since there is less cell number at days 5 and 7 in shDDIT3 cells compared to shScramble. It will be interesting to study apoptosis in this context. The expression levels of p53 on the mRNA and protein were not significant following H_2O_2 treatment, more experiments are needed in order to get statistical significant differences. Instead, the acetylated p53 levels were reduced in shSTAT1 silenced cells which correlates with the fact that acetylated p53 is involved in promoting cellular senescence (Furukawa et al. 2007).

Pro-inflammatory gene IL-6 was upregulated in shSTAT1 silenced cells compared to shScramble cells. Accordingly, STAT1 may not only promote cell cycle arrest but regulates inflammation too. On the other hand, shDDIT3 didn't show any difference in the expression of IL-6.

In conclusion, first we validated the secretory profile in H_2O_2 induced cellular

senescence in pre-adipocytes. Second, we validated three transcription factors during senescence and used gene-silencing approach to functionally characterize these transcription factors in regulating senescence in H_2O_2 treated pre-adipocytes. Our findings propose that STAT1 is upregulated during senescence to promote cell growth arrest through p21 regulation, while DDIT3 regulate cell survival. We also propose that STAT1 may control inflammation. These findings may have important implication with respect to better understanding the mechanisms underlying inflammation in STAT1during senescence. Thus it will help in the development of effective therapeutic interventions.

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Appendix

A. Institutional Biosafety Committee Approval (WCMC-Q)

Weill Cornell Medical College in Qatar

Qatar Foundation

Institutional Biosafety Committee (IBC)
Weill Cornell Medical College in Qatar (WCMC-Q)
Qatar Foundation - Education City
PO Box 24144, Doha, Qatar

Phone: +974-4492-8132
Fax: +974-4492-8144
Email: ehs@qatar-med.cornell.edu
Website: http://qatar-weill.cornell.edu/ehs/ibcPolicy.html

Date: October 17, 2012

PI:	Nayef Mazloum, PhD
WCMC-Q IBC Registration #:	NE12-003
Project Title:	Investing DNA damage response and PARP-1 activation in obesity associated pro-inflamatory senescence

Dear Dr. Najafi:

The institutional Biosafety Committee (IBC) of Weill Cornell Medical College in Qatar (WCMC-Q) met on October 11, 2012 and conducted a review of your IBC Laboratory Registration for the above named project. The IBC approved this project at **Biosafety Level 2** for the WCMC-Q location. The scope of IBC approval for this project is limited to use of the rDNA materials described in your IBC Laboratory Registration Document dated September 30, 2012 submitted for this project.

Please note that WCMC-Q IBC approval is contingent upon your adherence to the following WCMC-Q IBC Guidelines:

- Ensuring compliance with WCMC-Q Safety Plans and applicable regulations (e.g., NIH Guidelines, http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm).
- Determining if experiments require IBC approval.
- Ensuring experiments that require prior IBC approval are not conducted until IBC approval is obtained.
- Making initial determination of containment levels required for experiments.
- Notifying the IBC of any changes to rDNA and other hazardous material experiments previously approved by the IBC.
- Reporting any significant problems, violations of WCMC-Q Safety Plans and applicable regulations/guidelines, or any significant research-related accidents and illnesses to EHS (4492-8132) and the IBC.
- Ensuring personnel receive appropriate orientation and specific training for the safe performance of the work. This training must include:
  - Communication about the potential hazards of the work.
  - Work practices and instruction on engineering controls to minimize exposure.
  - Suitable personal protective equipment.
  - Familiarity with laboratory Standard Operating Procedures, and the content of the Registration filed with the IBC.
  - What to do in case of personal exposure or accidental release of contaminated materials.
- Determining appropriate medical surveillance required for this work. .

Please do not hesitate to contact the IBC if you have any questions or need assistance in complying with the terms of this approval. Best wishes for a productive and rewarding research project.

Sincerely,

B. Shykind

Benjamin Shykind, Ph. D.
Chairman, Institutional Biosafety Committee

B. Project Reference QUUG-CAS-DBES-13/14-14

Qatar University
Office of Academic Research

UNIVERSITY GRANT APPLICATION

ACADEMIC YEAR (2013/2014)

Marwan Abu Madi and Nayef A. Mazloum

Oct 3, 2013

ملخص الدراسة

الكشف عن المسارات الجزيئية الناجمة عن (مواد الأكسجين التفاعلية ROS) في التهابات الخلايا الدهنية الهرمة

تعد حالات السمنة والسكري في دولة قطر من بين أعلى المعدلات انتشاراً في العالم، وتسبب العديد من الاضطرابات الأيضية التي تخلق مشاكل صحية عديدة. وقد ارتبطت معها بدرجة منحفضة التهابات مزمنة ، والتي تعد سبباً رئيسياً لمقاومة الأنسولين والسكري (T2D). المحاولات العلاجية الجديدة لهذا النوع من السكري لا يمكن الوصول اليها نظراً لعدم فهم المسارات الجزيئية للسمنة المرتبطة بمرض السكري (T2D). لذلك يتحتم التركيز على دراسة هذه المسارات لاكتشاف أهداف علاجية جديدة للبدانة المتعايشة مع مرض السكري. حيث تتميز الأنسجة الدهنية في الذين يعانون من السمنة المفرطة من تراكم (مواد الأكسجين التفاعلية ROS)، والتي تؤدي إلى اضطراب الحمض النووي وهرم الخلايا. ولقد أثبتت العديد من التجارب المخبرية والدراسات المجراة أن الخلايا الهرمة سوف تؤدي الى تغييرات مظهرية متمثلة على شكل افرازات، وعلى الأخص زيادة افراز السيتوكينات الموالية لهذه الالتهابات المعروفة باسم (SASP). ولا توجد معرفة كاملة حول مكونات (SASP) والاتهابات المرتبطة معها على الأخص في حالات السمنة والسكري (T2D). ومن هنا ينبع الهدف من هذه الدراسة في استكشاف المسارات الجزيئية في الخلايا الدهنية الهرمة، حيث أن فهم هذه التفاصيل الجزيئية من شأنها أن تساعد في تطوير وسائل علاجية جديدة.

الكشف عن المسارات الجزيئية الناجمة عن (مواد الأكسجين التفاعلية ROS) في التهابات الخلايا الدهنية الهرمة

مقدمة من

عائشة يعقوب مدني

بكالوريوس علوم الحيوية الطبية، جامعة قطر، ٢٠٠٩

للحصول على درجة الماجستير

في

العلوم الحيوية الطبية

لجنة المناقشة والحكم: موافق

١. د.مروان أبو ماضي (محكم داخلي)

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