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Insulin-like growth factor binding protein-3 (IGFBP-3) plays an essential role in cellular senescence: molecular and clinical implications.

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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ASK1	Apoptosis stimulating kinase 1
β2M	Beta-2-microglobulin
BrdU	Bromodeoxyuridine
cdk	Cyclin-dependent kinase
CR	Caloric restriction
DCF	2'7'-dichlorofluorescein diacetate
DMF	Dimethylformamide
DTT	Dithiothreitol
EV	Empty vector
FACS	Fluorescence-activated cell sorting
GH	Growth hormone
GSH	Glutathione
HBSST	Hanks buffered salt solution- Triton-X-100
HDF	Human dermal fibroblast
HFL-1	Human embryonic lung fibroblast cells

IEE	IGFBP-3 enhancer element
IGF	Insulin-like growth factor
IGFBP-3	Insulin-like growth factor binding protein-3
IGFBP-3R	Insulin-like growth factor binding protein-3 Receptor
MAPK	Mitogen-activated protein kinase
MOI	Multiplicity of Infection
PBS	Phosphate Buffer Saline
PD	Population doubling
pRB	Retinoblastoma protein
ROS	Reactive oxygen species
SA-β-gal	Senescence associated beta galactosidase
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNS
SIPS	Stress-induced premature senescence
SOD	Superoxide Dismutase
TGFβ	Transforming growth factor beta
TMB	(3,3',5,5'-tetramethylbenzidine)

Trx	Thioredoxin
WS	Werner Syndrome

Abstract

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Normal somatic cells have a limited proliferative capacity *in vivo* and *in vitro*, termed senescence and later, thought to contribute to molecular and cellular organismal aging. There are several studies that demonstrate the importance of the GH/IGF axis in longevity, aging and cellular senescence. One primary component of the IGF signaling involves IGFBP-3. It is well documented that IGFBP-3 levels are significantly increased in senescent human

diploid fibroblasts however IGFBP-3 function is not known in this system. Interestingly, Werner syndrome fibroblasts, commonly used as a model of cellular aging, have upregulated IGFBP-3 levels in young and late passage cells compared to age matched normal fibroblasts. It is known that suppression of p38 MAPK activity in WS fibroblasts can reverse the senescence and promotes cell proliferation. As increased IGFBP-3 expression is associated with cellular senescence, and suppression of p38 MAPK can reverse senescence in WS fibroblasts, it is hypothesized that "IGFBP-3 can induce senescence, by activating the p38 MAPK signaling pathway." Our studies demonstrate IGFBP-3 and novel IGFBP-3R can induce senescence in young fibroblasts, while suppression of IGFBP-3 in pre-senescent fibroblasts, can delay the onset of replicative senescence. We identified ROS accumulation in IGFBP-3/IGFBP-R-induced senescent cells which we speculated may be signaling p38 MAPK activation. Inhibition of ROS accumulation suppressed p38 signaling and prevented IGFBP-3/IGFBP-3R-induced senescence. To evaluate the sequence of activation we inhibited p38 activity prior to senescence induction. Interestingly, p38 inhibition prevented IGFBP-3/IGFBP-3R-induced senescence, suggesting IGFBP-3 signals ROS induction which activates p38 signaling. We next examined the significance of IGFBP-3R in IGFBP-3-induced senescence. Suppression of endogenous IGFBP-3R inhibits IGFBP-3-induced senescence. We aimed to identify a possible regulatory mechanism for IGFBP-3 upregulation. Using sequence analysis software we identified 3 possible highly conserved miRNA sequences aligned to IGFBP-3. miR-19a appeared to have the most

significant downregulated expression in late passage fibroblasts compared to early passage. Furthermore, overexpression miR-19a in late passage cells, significantly decreased IGFBP-3 expression, suggesting miR-19a may silence IGFBP-3 expression in senescence. Making a direct mechanistic connection between senescence and aging is significant and unraveling how IGFBP-3/IGFBP-3R can induce senescence could prove beneficial in understanding the aging process.

Chapter 1

Introduction and Literature Review

1.1 Cellular senescence and aging

It was originally believed that cells *in vitro* could proliferate indefinitely and maintaining cells in culture was a matter of identifying the proper conditions (Ben-Porath & Weinberg, 2005). It was the work of Hayflick and Moorhead in the early 1960's who initially demonstrated that despite, culturing conditions, cells cease to proliferate and enter a senescent state (Hayflick & Moorhead, 1961). The term 'senescence' is defined as a series of cellular changes often associated with aging and inducible by various signaling pathways leading to irreversible cell growth arrest (Campisi, 2008). It is difficult to identify an exact definition of cellular senescence. The most widely accepted definition of senescence is an irreversible growth arrest triggered by, continuous telomere shortening with each cell replication, oxidative damaging agents, stress stimuli or cell cycle deregulations following overexpression of proto-oncogenes (Toussaint, *et al* , 2002). The complexity of senescence is revealed by the fact that different cell types can undergo senescence by multiple, divergent pathways.

There are currently two classifications of senescence, replicative senescence and stress-induced premature senescence (SIPS). Although different mechanisms are responsible for inducing either replicative senescence or SIPS, the phenotypic and genotypic changes associated with both are nearly indistinguishable from one another.

Even though senescent cells lose their proliferative capacity, they remain metabolically active and viable for extended periods of time in culture. Senescent cells are characterized as being postmitotic and incapable of proliferating in response to mitogenic stimuli.

One of themore well understood inducers for replicative senescence in normal cells, is significantly shortened telomeres, as most human somatic cells do not produce the telomere-synthesizing enzyme telomerase and consequently suffers telomere shortening with each round of DNA synthesis (Harley, *et al.,* 1990 and Ferenac, *et al.,* 2005). SIPS was originally identified when normal young cells exposed to various types of subcytotoxic stress, such as UV radiation, reactive oxygen species, nutrient imbalances or suboptimal culture conditions could promote irreversible growth arrest, regardless of their remaining replicative lifespan (Ishikawa, 2006). The SIPS-like phenotype and telomere-dependent replicatively senescent cells share basic similarities, such as irreversible growth arrest and resistance to apoptosis, which are known to signal different pathways; yet, the ultimate result remains the same. As summarized in Figure 1, senescence is a complex cellular phenomenon that can be induced by various signals to activate diverse signaling pathways, all of which ultimately lead to irreversible cell growth arrest.

The phenotypic changes associated with replicative senescent cells are relatively indistinguishable from prematurely induced senescent cells. Cells exhibit decreased cell saturation density, increased cell surface and volume, and a distinct flat morphology. The morphological changes are attributed to an increasing size of the nucleus and

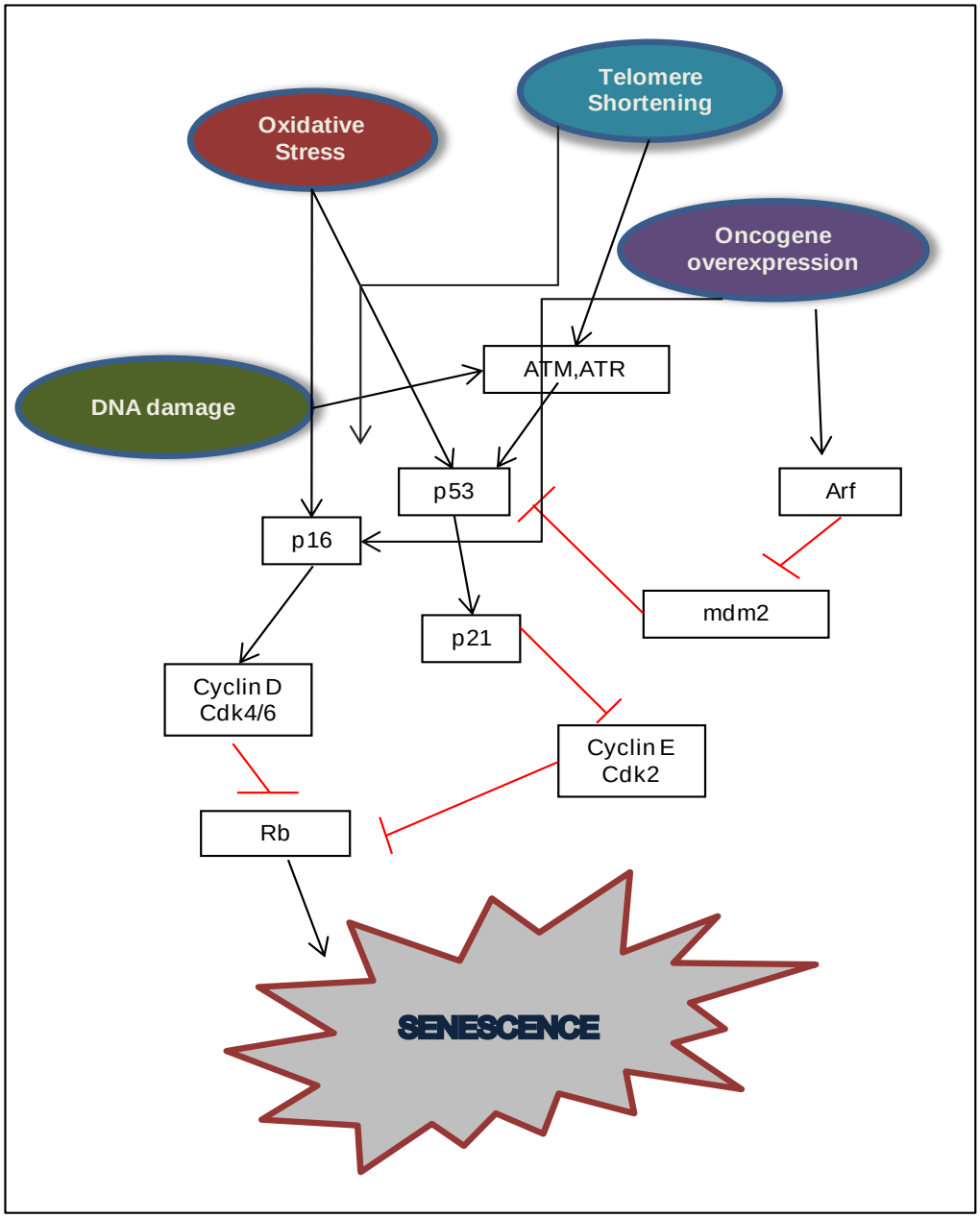


Figure 1: Senescence can be induced by various stimuli and signaling various pathways all leading to senescence.

nucleoli, with an increase in the number and/or size of lysosomes, vacuoles and mitochondria (Busuttil, *et al.,* 2004). The fundamental morphological changes of senescent cells are well known to be associated with cytoskeletal regulatory proteins, including integrins, focal adhesion complexes, comprised of integrins beta-1, paxillin, focal adhesion kinase (FAK), small Rho GTPases, caveolin-1, actin stress fibers and Rb family proteins (Cho and Park, 2004). The identification of 'senescence-associated genes,' that induce and/or maintain senescence has also been crucial in unmasking pathways involved in tumorigenesis (Campisi *et al.,* 2003).

A major limitation of studying cellular senescence is the lack of specific biomarkers that can distinguish senescent cells from quiescent or terminally differentiated cells. Currently, the presence of senescence associated beta-galactosidase (SA-β-gal) activity is the most commonly used biomarker of senescence, resulting from residual lysosomal β-galactosidase activity at pH 6.0 (Dimri, *et al*., 1995). Additional markers have been identified and fall into three broad categories: factors of signal transduction pathways involved in induction and/or maintenance of senescence, DNA-damage markers coincident with telomeres, and markers of focal heterochromatin (Dierick, *et al.*, 2002). A prominent characteristic of senescent cells is a diminished response to serum or growth factors. However, the underlying mechanisms for the hyporesponsiveness to mitogenic stimuli is not well understood (Dierick, *et al.*, 2002).

The limited mitotic lifespan of cells has been interpreted as a manifestation of cellular aging. Senescent cells continually display a number of molecular changes observed during the process of aging in vivo (Campisi, *et al*., 1997; Campisi, *et al*., 1996). For example, skin biopsy samples from elderly individuals compared to young

people shows increased SA-β-gal cells. Ever since Hayflick and Moorhead first reported the limited replicative lifespan of dermal fibroblasts in culture, senescence has been used as a model for aging studies. Some theories suggest that normal accumulation of the by-products of normal metabolism can be detrimental to cell function.

One of the oldest theories proposes that aging results from a senescence phenotype generated by accumulation of point mutations, deficiencies of enzymes and/or mutations in protein synthesis leading to increase in faulty proteins (Orgel, 1963). DNA repair plays an important role in preventing senescence, and perhaps cells with inefficient DNA repair mechanisms may undergo senescence. Several premature aging disorders result from mutations or deletions in genes that are responsible for producing DNA repair enzymes. The cause of senescence and its correlation to organismal aging are addressed at many levels; at the cellular level in particular there is substantial evidence for many different theories without a distinct consensus.

1.2 Molecular mechanisms of senescence

1.2.1 p53, p21 and p16 signaling in cellular senescence:

Despite the various stimuli that can induce senescence, the current belief is that both replicative and stress-induced senescence are dependent on two major signaling pathways, which includes the p53 and retinoblastoma (RB) tumor suppressor proteins , as well as the $p16^{Ink4a}$ (from hereon, p16) (Zdanov, 2007).

Under normal conditions retinoblastoma protein (Rb) binds to E2F transcription factors to prevent the induction of genes required for the completion of the cell cycle,

whereas the p53 transcription factor induces a number of genes that block proliferation (Beausejour, *et al.,* 2003). Rb must be hyperphosphorylated for cells to proceed to S phase of the cell cycle, and senescent cells display no phosphorylation of Rb regardless of growth factor stimulation (Beausejour, *et al.,* 2003). Under normal physiological conditions, p53 remains low, due to Mdm2-induced degradation. p53 can be phosphorylated and activated by various kinases such as casein kinase, DNA-PK or ATM. p53 activation can lead to protein accumulation, posttranslational modifications and subcellular translocation to induce cell cycle arrest or apoptosis by a variety of pathways including IGF-1-AKT, Rb-E2F, and p38 MAPK. DNA damage, chromosomal defects, telomere shortening, cellular hypoxia and oncogene overexpression are known to activate p53 and its downstream targets, specifically $p21^{waf-1}$. Both *in vitro* and *in vivo* experiments have repeatedly demonstrated that the anti-oncogenic function of p53 is mainly due to the ability to induce apoptosis or cell cycle arrest. The significance of the p53-p21 pathway in replicative senescence was first suggested when tumor-virus-encoded oncoproteins that interfere with p53 function extend the replicative life span of human cells, essentially by allowing them to ignore signals from short dysfunctional telomeres (Itahana, *et al.*, 2004). Experimentally, inhibition of p53 function by expression of human papillomavirus type 16 EV6 or disruption of a key downstream effector p21 by homologous recombination can extend replicative life span. Although p21 down regulation is *sufficient* to extend lifespan, is not *necessary,* as expression of a dominant-negative mutant p53 can increase life span without decreasing p21 expression (Beausejour, *et al.,* 2003). It was often believed that functional wild-type p53 was required for human fibroblasts to undergo replicative senescence. However,

more recent data has shown cells can undergo premature senescence independent of p53 signaling. Even though p53 is one of the primary signals activated when telomere length significantly shortens.

The second pathway believed to be crucial in cell cycle arrest is the activation of p16 signaling. CDK1/p16 is also responsible for regulating RB activity, yet the mechanism for cell cycle arrest is different from that of p53. Ectopic expression of telomerase does not protect cells from replicative senescence, suggesting p16 function is independent of telomere status (Kiyono, *et al*., 1998, and Rheinwald *et al.,*, 2002). The transcriptional regulation of p16 is complex and not well characterized. p16 expression is known to increase with age in normal tissues of humans, while p16 is be downregulated in mice subjected to caloric restriction, resulting in increased lifespan. Although many investigators report presenescent fibroblasts display upregulated p21 mRNA and protein levels following p53 activation, several opposing reports demonstrate once cells enter a senescent state, levels of p21 return to normal. Furthermore, p16 mRNA and protein levels are known to remain elevated following senescence induction, suggesting an irreversible second barrier of cell proliferation inhibition induced by p16 upregulation (Takahashi, *et al.,* 2006 and Vaziri, *et al.,* 1999). The regulation of cell cycle progression and/or inhibition by p53, p21 and p16 signaling pathways, has categorized these genes as 'senescence-associated' genes (SA-genes).

1.2.2 Telomeres and cellular senescence:

Telomeres are repeated TTAGGG DNA sequences that form essential protective structures at chromosomes ends; however, they cannot be fully replicated by

conventional DNA polymerase. Telomeres are maintained by a specialized RNA-templated polymerase, telomerase reverse transcriptase (Allsopp, *et al.,* 1992). The repeated DNA sequence forms a protective T-loop structure stabilized by telomere-binding proteins (Griffith *et al*, 1999), thereby inhibiting chromosome ends from being recognized as DNA breaks and preventing their degradation or end-to-end fusions (de Lange, 2002). Most mammalian somatic tissues lack telomerase and therefore can shorten telomeres by 50-200 bp with each replication. As telomeres shorten they can be mistaken for damaged DNA and identified for repair or produce telomeric end fusions, both of which result in senescence induction.

Although telomere shortening in replicative senescence is well documented, it is now widely accepted that replicative senescence is not exclusively triggered by telomere shortening nor does telomerase overexpression prevent senescence (Ferenac, *et al.,* 2005). Telomerase can be induced at very low levels during S phase, yet this activation is insufficient to prevent telomere shortening (Allsopp, RC, *et al.,* 1995). Interestingly, telomerase is expressed in a larger number of cancer cells, which are known to acquire an ability to ignore or bypass senescence-induced signals to become fully malignant (Kim, *et al.,* 1994). While the causal role of telomere erosion in senescence has been firmly established, and the ability of telomerase to prevent senescence in normal human fibroblasts indicates senescence is a very complicated cell program mediated by multiple pathways and mechanisms (Bodnar *et al,* 1998). The senescence response observed independent of telomere length appears to depend on the cell type, signaling effector and the level of its activation or overexpression (Harely, *et al.,* 1990).

1.2.3 Oxidative Stress and Free-radical theory of aging:

A substantial amount of experimental evidence has shown intracellular reactive oxygen species (ROS) contribute to senescence and aging. The free-radical theory of aging was first proposed over 50 years ago and has since been studied extensively in an effort to identify the benefits or detrimental effects of ROS to aging. The free radical theory proposes that normal aging is a consequence of random deleterious damage to tissues by oxidative stress produced as a by-product of normal metabolism. Under normal conditions, approximately 2-3% of oxygen atoms are taken up by the mitochondria and not sufficiently reduced, thereby producing ROS, which include, superoxide ion, hydroxyl radicals and hydrogen peroxide. ROS serve various cellular functions such as second messenger, anti-bacterial agent, aging accelerant and growth stimulants (Colavitti, *et al.,* 2005). To prevent ROS accumulation, enzymes that degraded ROS are upregulated, such as catalase and superoxide dismutase. Increased oxidative stress, chronic or acute, can induce premature senescence *in vitro*; however, the kinetics of the growth arrest is often dependent on the anti-oxidative capacity of the cells. In depth studies in Drosophila and *C.elegans* indicate that overexpression of ROS degrading enzymes or mutations in certain genes that signal pathways to modulate ROS stress response can significantly prolong lifespan. The exposure of cells to a variety of stresses, such as UV-irradiation, hyperoxia, H_2O_2, *tert*-butylhydroperoxide or ethanol triggers young normal human diploid fibroblasts to enter into a premature senescent state via different signaling pathways; however all stimuli involved significant

accumulation of ROS (Feippiat, *et al.,* 2002). The stimuli and mechanisms involved in ROS production, and the downstream effects are complex and diverse (Figure 2).

Cells are commonly maintained in culture at 20% atmospheric oxygen levels, which is well above oxygen to which cells are exposed in the human body at only 3%. Interestingly, normal cell cultures maintained in low oxygen environments have an increased proliferation capacity (Muller, 2009). The biochemical characteristics of aged tissues are the consequences of an increase in their pro-oxidant state, suggesting that this state may affect the activity and function of key proteins that signal transduction pathways that regulate stress responses (Muller, 2009).

Caloric restriction (CR) has been well studied to expand the life span of rodents and suppress the development of diverse age-associated disease such as cancer, diabetes and renal disease (Sun, *et al.,* 2001). One possible explanation for how CR extends life span is through alterations in oxidative damage/oxidative stress (Sun, *et al.,* 2001 and Richardson, *et al.,* 2004). CR mice are known to be more resistant to oxidative stress, as the activity of one or more major antioxidant enzymes have been reported to be significantly increased in tissue of CR rodents. Although there is no overall trend in the expression of antioxidant enzymes with CR, it has been repeatedly demonstrated the CR can reduce the production of ROS in mitochondria isolated from various tissue of rodents (Bokov, *et al, 2004)*.

Several studies have proposed ROS production may lead to increased activation of p38 MAPK and consequently cellular senescence. More specifically, a recent study evaluated the ROS-mediated p38 MAPK pathway activation in aged livers and the long-

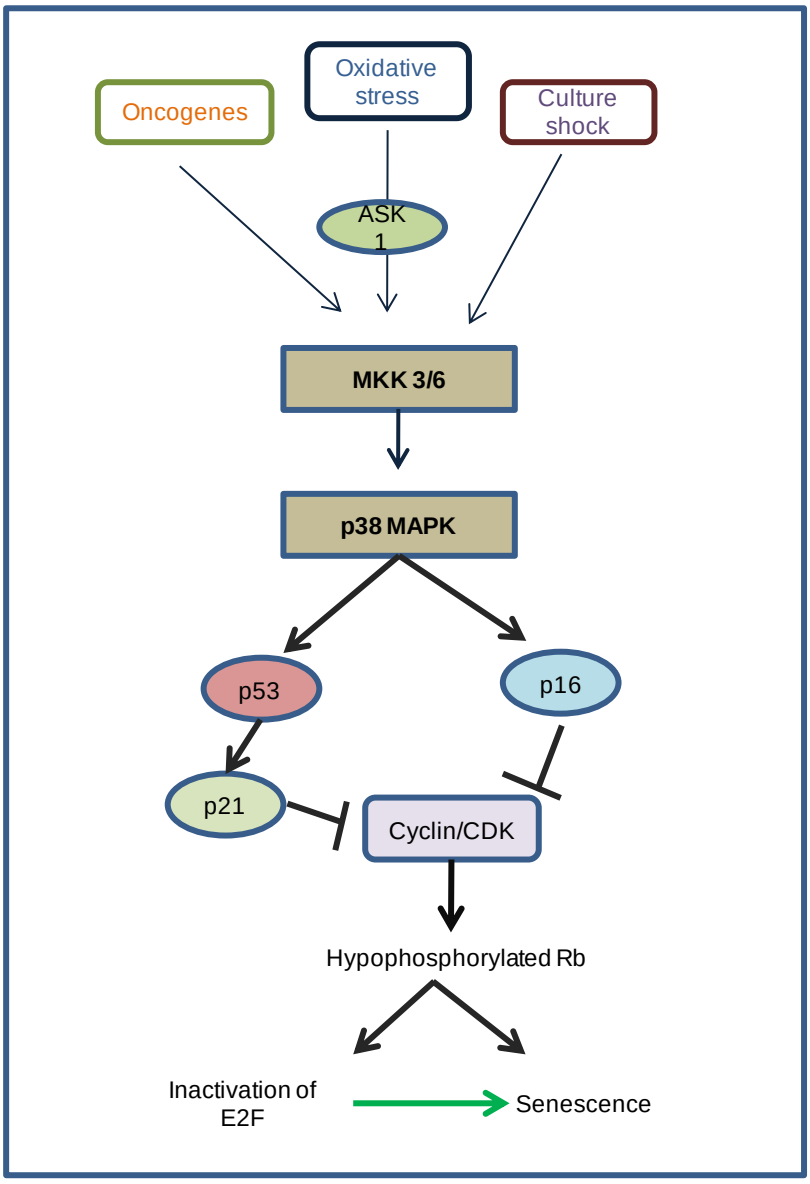


Figure 2 Schematic diagram of p38 MAPK signaling. Multiple stimuli can activate p38 signaling which in turn can elicit various downstream signaling pathways.

lived Snell dwarf mouse model (Hsiech and Papaconstantinou, 2006) The longevity of Snell dwarf mice has been attributed to their resistance to oxidative stress (Hsiech and Papaconstantinou, 2006). The activation of p38 MAPK pathway by ROS production, is based on the ability of reduced Thioredoxin (Trx) to bind to and inhibit apoptosis stimulating kinase 1 (ASK1). ROS production activates the dissociation of the Trx-ASK1 complex, thereby increasing p38 MAPK activity (Hsiech and Papaconstantinou, 2006). The balance between free versus ASK1 regulates the level of p38 MAPK pathway activation (Hsiech and Papaconstantinou, 2006). In addition, the levels of Trx-ASK1 complex in young and aged dwarfs are higher than their age-matched controls. These results suggest ROS production may alter the ratio of ASK1 and Trx-ASK1, and subsequently increase the age-associated basal level of p38 MPAK activity (Hsiech and Papaconstantinou, 2006, Hsiech and Papaconstantinou, 2002 and Hsiech, *et al.,* 2004).

1.3 Werner Syndrome, model of *in vivo* aging

In vitro studies of senescence have provided a wealth of information relevant to the *in vivo* processes of aging. Senescent cells have long been postulated to contribute to normal human aging as a whole organism, as accumulation of senescent cells increases with age and senescent cells are known to contribute to age-associated pathologies at the cellular level. Several premature aging disorders have been identified in humans, such as Cockayne syndrome, Rothmund Thompson and Hutchinson Guilford progerias. The early onset of aging disorders in these patients is useful for studying the aging process. Although these disorders have been characterized to be associated with single gene mutation, the biological consequences are vast. One of

the most well characterized premature aging disorders is Werner syndrome (WS). Patients with WS, described as a segmental Progeroid syndrome, show the premature onset of many clinical features of aging and therefore provide a significant model of normal organismal aging (Davis, *et al.,* 2007). WS is a homozygous recessive disease manifested by a mutation in the *WRN* gene. *WRN* belongs to the RecQ family of DNA helicases that encodes the WRN protein, which shares a strong homology to DNA/RNA helicases. Helicases regulate numerous DNA metabolic functions such as transcription, replication and DNA repair. Dermal fibroblasts derived from individuals with inherited premature aging syndromes, such as Werner syndrome, have reduced replicative life span *in vitro* compared to unaffected persons at the same age, thereby presenting a potential *in vivo* model for studying possible *in vitro* effects.

1.4 p38 MAPK signaling

The mitogen-activated protein kinase (MAPK) pathway is comprised of proteins known to transduce a variety of external stimuli to elicit various cellular responses, such differentiation, inflammation, apoptosis or cell growth (Figure 2). In the mammalian system three major MAPK pathways have been characterized: 1-MAPK/ERK 2-SAPK/JNK and 3-p38 MAPK. Interestingly, the result of recent studies suggested a significant role for p38 MAPK signaling in the accelerated aging phenotype of WS fibroblasts in culture. It has been demonstrated that upregulation of p38 MAPK activation leads to the stabilization of p21 and the senescence phenotype *in vitro*. WS fibroblasts treated with SB203580, a cytokine-suppressive anti-inflammatory drug which targets p38 activity reverses the aged morphology of young WS fibroblasts similar to

that observed in normal fibroblasts of the same donor age. In addition to increasing the life span and growth rate to within normal range, a reduction in p21 expression is observed after SB203580 treatment, suggesting p21 activation by p38 is an essential regulator (Davis, *et al.,* 2007)

mRNA and protein expression levels of various genes known to be upregulated in replicative and premature senescence have been examined WS fibroblasts in an effort to identify crucial genes that are be responsible for the senescence phenotype. Interestingly, IGFBP-3 mRNA levels are significantly upregulated in young and old WS fibroblasts compared to age matched normal fibroblasts (Murano, *et al.,* 1991). Other studies have demonstrated that stable expression of an activated form of mitogen activated protein kinase-kinase-kinase 6 (MKK6), a direct activator of the stress-induced p38 MAPK pathway, is sufficient for inducing features of senescence in normal young fibroblast (Zarubin, *et al.,* 2005).

1.5 The GH/IGF axis in aging:

Signaling through the growth hormone/Insulin-like growth factor (GH/IGF) axis has long been known to play a major role in determining the rate of aging in variety of species. The GH/IGF signaling pathway is one of the most well conserved and most consistent processes of aging in organisms ranging from yeast to mammals (Longo and Finch, 2003). Hormonal changes, controlled by the endocrine system, have long been studied as possible regulators in inducing cellular aging. The levels of GH and its downstream target IGF-I are known to be significantly downregulated with increasing aging; yet restoring hormonal levels does not delay the onset or reverse the phenotype,

but can actually accelerate the process. The mechanism or role of GH/IGF signaling is still widely debated. Some studies support an antioxidant protective role, while others suggest the mitogen effect causes decreased cellular proliferation. In mammals, decreased or dysregulated GH/IGF-1 signaling encourages longevity. Longevity genes are classified into two categories, genes relevant to nutrient-sensing systems and genes associated with mitochondrial function or redox regulation.

From a clinical perspective one of the most influential aspects of aging and longevity is caloric restriction. It is widely accepted that caloric restriction retards the aging process in laboratory mice and rats by delaying the occurrence or complete prevention of a broad spectrum of age-associated pathophysiological changes (Goldstein, *et al.,* 1993). CR is known to suppress the GH/IGF-1 axis, further implicating the significance of this signaling pathway (Keyon, 2005). The characterization of redox regulation and mitochondrial function in CR is still unclear.

The molecular pathways involved in the regulation of chronological lifespan have been identified in various organisms. Longo and Finch (2003) have diagramed a conserved regulation of longevity in yeast, worms, and flies, demonstrating how the conserved glucose or insulin/IGF-I-like pathways down-regulate antioxidant enzymes and heat shock proteins, reduce the accumulation of glycogen or fat, and increase growth and mortality. Mutations that reduce the activity of these conserved pathways have been shown to extend longevity by simulating caloric restriction or more severe forms of starvation. The induction of stress-resistance genes is required for longevity extension in yeast and worms. In mice, IGF-I activates signal transduction pathways analogous to the longevity regulatory pathways in lower eukaryotes and increases

mortality (Itahana, *et al.,* 2002). However, the intracellular mediators of lifespan extension in GH- or IGF-I-deficient mice are not entirely understood. In humans, GH or IGF-I deficiencies caused by mutations or deletions can lead to dwarfism, obesity, and other adverse effects. Mammals with stress resistance capabilities have reduced IGF-I signaling, while increased levels of IGF-1 have been associated with increased risk of cancer (Hursting, *et al.,* 2003). The effects of GH or IGF-1 deficiencies remain unclear with regard organismal aging and longevity.

The IGFs were originally described as mediators of the effects of growth hormone on somatic growth. The IGF system plays a critical role in integrating growth, development and lifespan determined by metabolic conditions, subsequently serving as endocrine, autocrine and paracrine stimulators of mitogenesis, survival and cellular transformation (Rudman, *et al.,* 1990 and Stewart, *et al.,* 1996). The IGF system is comprised of IGF-I, IGF-II, a family of transmembrane receptors including the IGF-I and IGF-II receptors, and insulin-like growth factor binding proteins (IGFBPs) (Walker, *et al.,* 2003) (Figure 3). The IGFBP superfamily is comprised of high and low affinity IGF binding proteins. High-affinity IGFBPs are essential for regulating the bioactivity of IGFs, either in circulation or the immediate extracellular environment by prolonging their half-lives and regulating the availability of free IGFs to interact with IGF receptors. Six high affinity binding proteins have been identified, cloned and sequenced, IGFBP-1 through 6. IGFBP-3 is the most abundant IGFBP in serum and is responsible for carrying 75% of IGF-I and IGF-II in the heterotrimeric ternary complex with an acid-labile subunit. The ternary complex is acted upon by proteases, whereby IGF-1 is released and available for interaction with IGF-IR. IGF-I activation of the IGF-IR can elicit diverse biologic

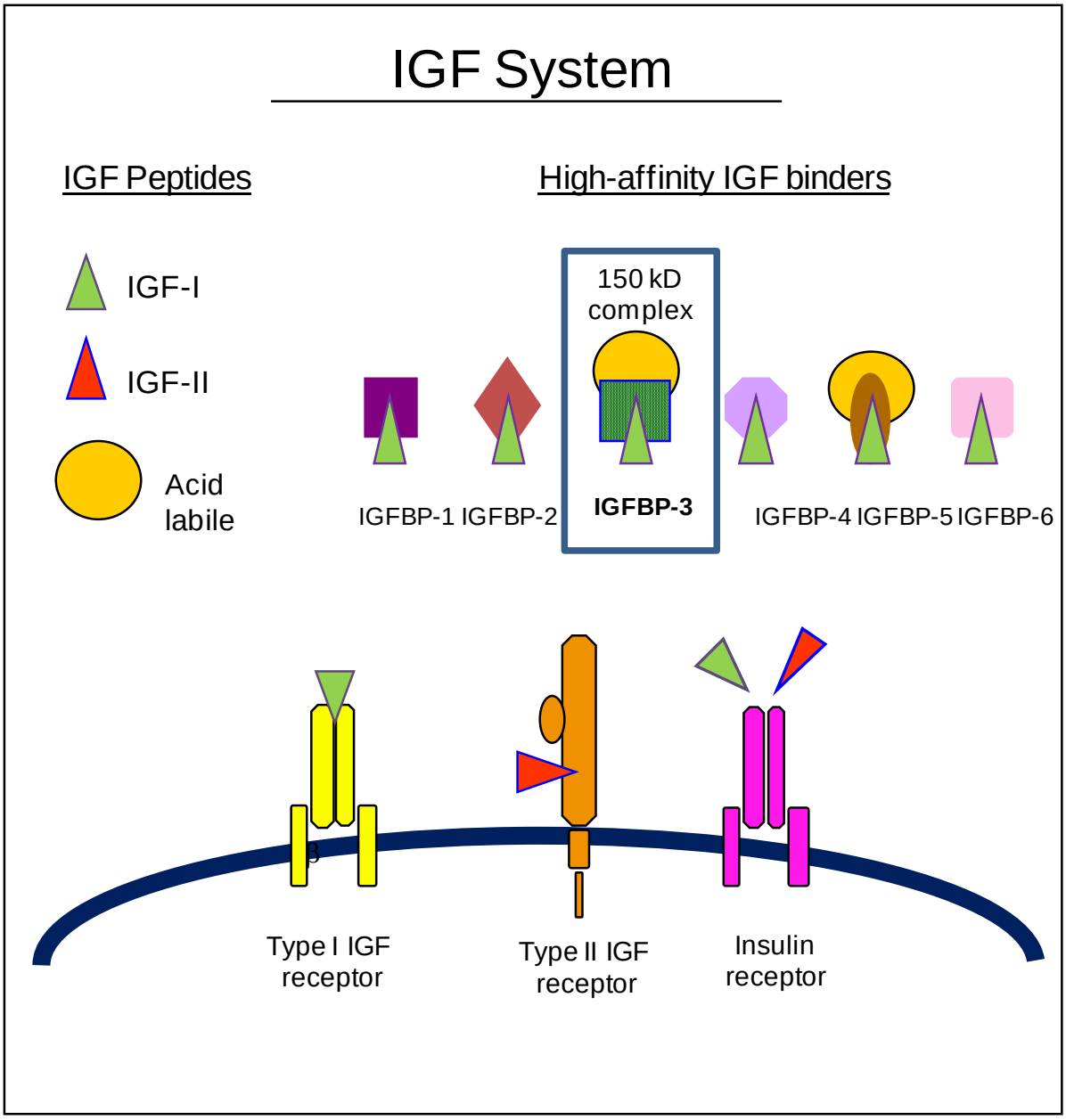


Figure 3: Schematic diagram of IGF system. There are several constituents of the IGF system, including IGF-I , IGF-II, their respective receptors and insulin receptor. In addition, there are 6 high affinity binding proteins. IGFBP-3 binds IGF-1 in complex with acid labile subunit.

effects, such as cell proliferation and differentiation, increased metabolic activation, and cell survival via antiapoptotic pathways, depending on cell type (Walker, *et al.,* 2003)

1.6.5 IGFBP-3 signaling in senescence

Although IGFBP-3 is the principal carrier of IGFs in circulation, it is also an important component to the GH/IGF axis, playing a role in autocrine and paracrine growth control and apoptosis. The interaction of IGFs and IGF-IR is well characterized. However, it is widely accepted that IGFBP-3 has distinct biological effects independent of the IGF-IGF-R axis. Numerous reports indicate IGFBP-3 can exert its biologic actions through a specific cell surface receptor or by interaction with intracellular proteins (Firth and Baxter, 2002 and Jogie-Brahim, *et al.,* 2005) (Figure 4). The IGF-independent actions of IGFBP-3 have revealed a large array of signaling proteins with links to cell cycle control and apoptosis (Oh, *et al.,* 1993). First, IGFBP-3 is a well-documented inhibitor of cell growth and/or promoter of apoptosis in human cancer cells, primarily through the attenuation of the IGF-/IGF-R interaction. However, it is now know that these bioactivities can occur by IGF-independent pathways (Blat, *et al.,* 1989). The IGFBP-3 promoter contains a p53 binding site that upregulates its transcription (Jogie-Brahim, *et al*., 2005).

Evidence suggests an interaction between IGFBP-3 and TGF-β signaling pathways. It has been demonstrated that IGFBP-3's growth inhibitory signal may require an active TGF-β signaling pathway and implicates Smad 2 and 3 in IGFBP-3 signal transduction. Furthermore, IGFBP-3 has been proposed as a functional ligand for the serine/threonine kinase type V TGF-β receptor, which is characterized to inhibit

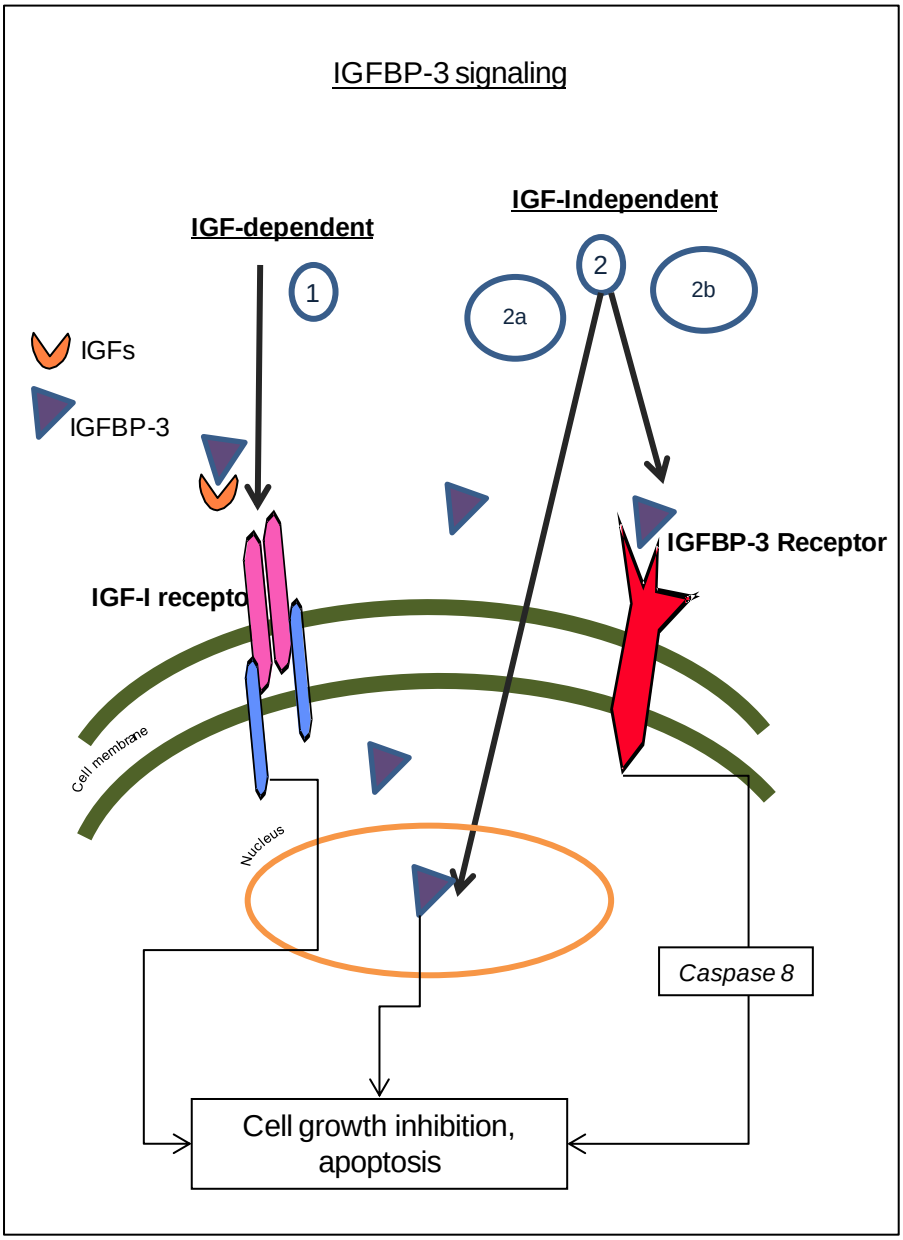


Figure 4: Schematic diagram of IGBP-3 signaling. The principal function of IGFBP-3 is to protect IGF from degradation, until IGF can bind to its targeted receptor. IGFBP-3 also has IGF-independent effects as illustrated in (2) pathway. IGFBP-3 is known to enter the cell via endocytosis and more recently the identification of novel cell death receptor, IGFBP-3R mediates IGFBP-3 signaling to induce caspase-8-dependent apoptosis.

cell growth (Barger, *et al.,* 2003). These studies confirm and support IGFBP-3's involvement in cell signaling pathways independent of IGF and the IGF-I receptor interactions.

Debacq-Chainiaux *et al*, (2008) identified IGFBP-3 upregulation when screening for senescence-associated genes following repeated exposure to sublethal concentrations of *tert*-butylhydroperoxide (*t*-BHP) and ethanol. Further analysis revealed TGF-β1 mRNA levels were upregulated after senescence induction, and surprisingly, inhibition of TGF-β1 suppressed IGFBP-3 upregulation after *t*-BHP or ethanol-induced premature senescence (Debacq-Chainiaux, *et al.,* 2008). Finally, suppression of IGFBP-3 by siRNA inhibited premature senescence induced by *t*-BHP or ethanol. This group has previously published work showing many senescence-related genes are overexpressed in fibroblasts after exposure to a variety stress agents, including fibronectin, an essential extracellular matrix component in cell adhesion, cytoskeletal organization and mediation of external mitogenic signals (Debacq-Chainiaux, *et al.,* 2008).

Caveolin-1 protein is known to be a major regulator of IGFBP-3 endocytosis following protein processing and secretion. Interestingly, caveolin-1 is known to play a critical role in senescence-associated morphological changes by regulating focal adhesion kinase activity and actin stress fiber formation (Cho and Park, 2004). The upregulation of caveloin-1 may be having a positive effect on the efficiency of IGFBP-3 to be internalized following secretion and signaling senescence induction.

Further evidence for a potential role of IGFBP-3 in *in vivo* aging has been demonstrated as gradually increasing IGFBP-3 levels were observed in rat liver

homogenates in correlation with increasing age (Keyon, *et al.,* 2005). Another supportive finding for the potential significance of IGFBP-3 in senescence was the unmasking of a novel transcriptional regulatory element, IGFBP-3 enhancer element (IEE). The IEE was identified in the 5' untranslated region of the IGFBP-3 gene and can differentially activate IGFBP-3 expression in senescent when compared to young fibroblasts. Site-directed mutagenesis within IEE abolished binding activity of an as yet to be indentified binding partner, selectively decreased IGFBP-3 promoter activity in presenescent cells, ultimately leading to inhibition of senescence. This data suggests the IEE as being a positive transcription regulatory element that contributes to the up-regulation of IGFBP-3 during cellular senescence (Lu, *et al.,* 2005).

Finally, Kim *et al*, (2007) suggest a potential role of IGFBP-3 in the senescence of human umbilical vein endothelial cells (HUVEC), as IGFBP-3 suppression inhibited senescence. Moreover, an inverse correlation with Foxo3a activity was indentified when overexpression of IGFBP-3 accelerated senescence, and downregulation of IGFBP-3 by siRNA rescued the growth arrested induced by p53 overepxression (Kim, *et al.,* 2007). Foxo3a belongs to the family of Forkhead transcription factors that appear to transcriptionally up-regulate antioxidant defenses, including superoxide dismutase and catalase (Kim, *et al.,* 2007). Foxo3a protein levels were increased in aged cells following IGFBP-3 knockdown. Downregulation of Foxo3a is known to accelerate senescence in human dermal fibroblasts, suggesting a possible relationship between IGFBP-3 and Foxo3a. Furthermore, in cell culture, constitutive expression of Akt activity leads to leads to inhibition of Foxo3a transcriptional activity, resulting in a rise in intracellular ROS. This rise in ROS is known to induce a p53-dependent senescent

arrest. Although the exact interaction between Akt phosphorylation and IGFBP-3 remains unclear, is has been reported that IGFBP-3 can induce activity of cellular phosphate known to activate Akt signaling.

1.7 IGFBP-3/IGFBP-3R axis

To extend our understanding of the IGF-independent action of IGFBP-3, we initiated an investigation to identify an IGFBP-3 receptor (IGFBP-3R) using the yeast two-hybrid system and identified a cDNA not identified in the database representing a novel gene/protein designated Clone 4-33 (Ingermann, *et al*., 2010). Genomic database analysis identified that the gene covered a segment of 9-kb and spanned 4 exons on chromosome band16q13. The 4-33 cDNA encodes a predicted protein of 240 amino acids (Ingermann, *et al*., 2010). The protein sequence contains a cAMP-dependent phosphorylation site, a single leucine-zipper motif, and a putative transmembrane domain near the C-terminus (Ingermann, *et al*., 2010). The existence of 4-33 in the plasma membrane and its binding to IGFBP-3 but not to other IGFBPs suggested that 4-33 is a putative IGFBP-3R (Ingermann, *et al*., 2010). The IGF-independent senescence inducing mechanism(s) of action of IGFBP-3 in fibroblasts has not been entirely elucidated; however, the identification of this novel IGFBP-3R suggests there may be an unidentified pathway signaling senescence. Our laboratory has recently published the identification of this novel binding partner for IGFBP-3 (Ingermann, *et al*., 2010). IGFBP-3R characterization and signaling mechanism has been described in a variety of cancer cells lines, showing apoptotic signaling by

activation of caspase signaling cascade (Ingermann, *et al*., 2010). The function of this IGFBP-3R in normal cells remains to be investigated.

1.8 Significance of research

A growing body of evidence has demonstrated that IGFBP-3 is an important growth-suppressing factor in various cell systems through an IGF-dependent mechanism. Recent studies have also revealed that IGFBPs may have specific biological effects in various cell systems, including fibroblasts, many of which are not mediated through interaction with IGFs (IGF-independent actions). Studies from our laboratory and others have demonstrated that IGFBP-3 is upregulated in senescent fibroblasts and can induce premature senescence in early passage fibroblasts. Upon replicative senescence *in vitro*, a significant upregulation of IGFBP-3 protein in the cultured medium is observed. Furthermore, several investigators have identified IGFBP-3 to be upregulated at both the protein and mRNAs during replicative and premature senescence. IGFBP-3 mRNA expression levels are consistently elevated in both SIPS fibroblasts and from cells obtained from WS patients, regardless of patient age. These data are suggestive of an adaptive or causal role for IGFBP-3 protein in the senescent growth arrest of human diploid fibroblasts. IGFBP-3-transfected fibroblasts display morphological changes; upregulated senescence-associated genes, and increased SA-β-gal staining. The mechanism(s) for upregulation of endogenous IGFBP-3 in senescent human diploid fibroblasts and IGFBP-3 induced-senescence in early passage fibroblasts have yet to be entirely elucidated. Since elevated IGFBP-3 mRNA and protein levels, along with increased p38 MAPK activity and ROS production, are all

upregulated in replicative and/or prematurely induced senescence, it is of interest to characterize the significance of the IGFBP-3/IGFBP-3R system in senescence and the aging process. Several studies have demonstrated IGFBP-3 upregulation following senescence induction; however, the specific biochemical/molecular mechanisms involved in IGFBP-3's action have yet to be identified. We therefore hypothesize that this novel IGFBP-3/IGFBP-3R axis regulates senescence by activation of p38 MAPK signaling pathway following ROS accumulation (Figure 5).

The presence of senescence-associated markers at sites of age-related pathologies has proven helpful in linking *in* vitro senescence with *in vivo* aging. Currently, the identification and quantification of senescent cells *in vivo* is possible, although the physiological consequences of senescent cells *in vivo* is only beginning to be understood. Compelling data demonstrates a link between increasing rates of cellular senescence and accelerated aging. The extent to which cellular senescence contributes to the natural life span of any particular species remains to be demonstrated. Establishing a link between cellular senescence and aging would inevitably provide a wealth of information towards identifying possible therapeutics for age-associated pathologies. Identification of the pathways activated or suppressed in the induction of senescence will provide valuable insight into numerous molecular mechanisms believed to be protective or detrimental to organism survival. Therefore, unraveling the cellular senescence signaling pathway(s) is extremely vital and will undoubtedly advance this field of study.

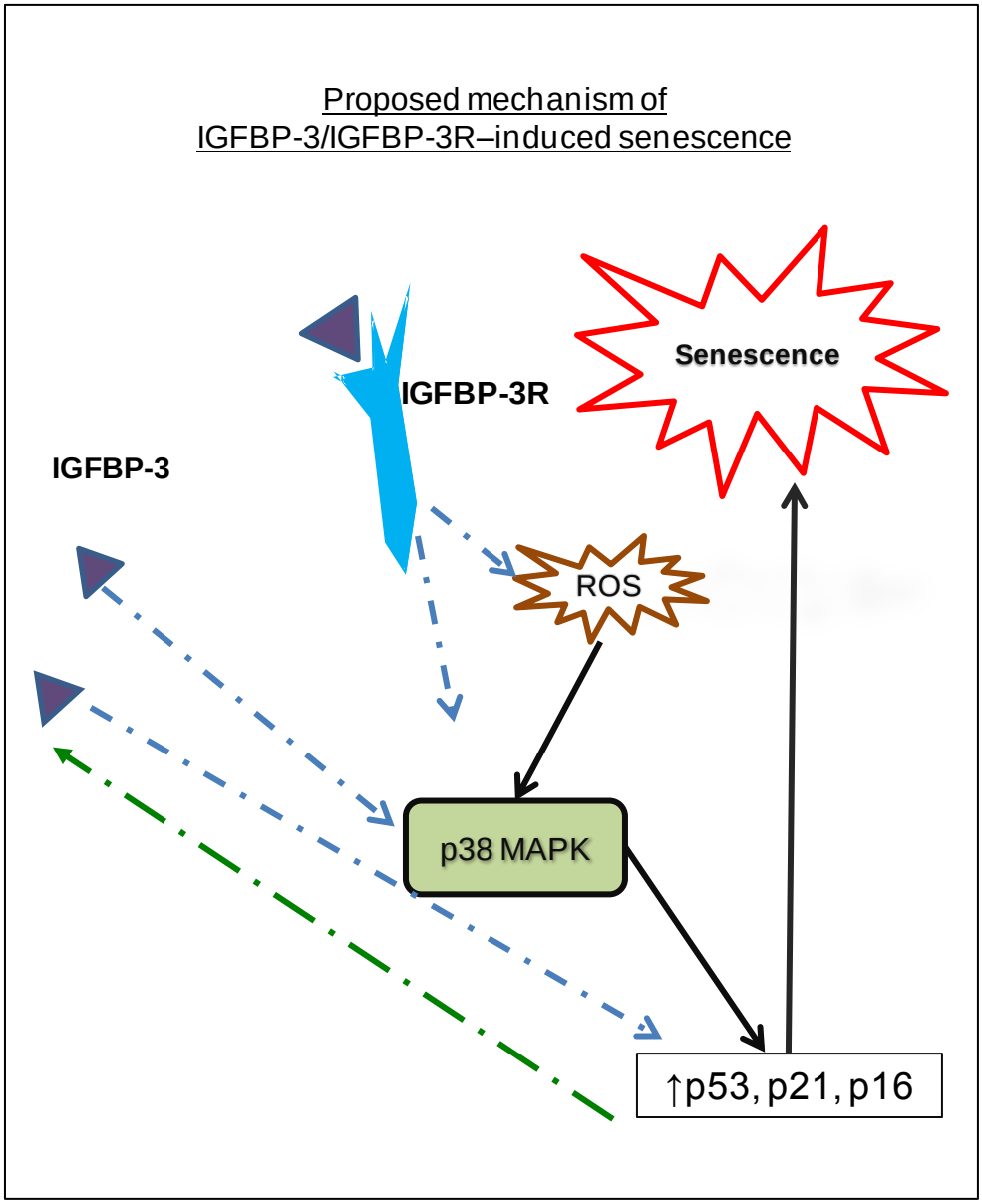


Figure 5: Proposed mechanism for IGFBP-3 induced senescence. Dotted lines are hypothesized signaling pathways and solid lines represent known function.

Chapter 2

Materials and Methods

Cells Culture and lysates

Human lung fibroblasts (HFL-1) (Manassas, Va, ATCC) were maintained in F-12 nutrient media (Carsbald, Ca, Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). AG003141 and AG00781 Werner Syndrome fibroblasts (WS) (Coriell Institute, Camden, NJ) were maintained in MEM (Sigma, St. Loius, Mo) and supplemented with 2mM L-glutamine and 10% FBS. BJ and BJ/hTERT fibroblasts were cultured in DMEM (Manassas, Va, Cellgro) medium supplemented with 10% fetal bovine serum 10% DMEM. Cells were split in a 1:2 or 1:3 ratio depending on experiment using 0.05% trypsin-EDTA (Gibco). All cell cultures were incubated at 37^0C in 5% CO_2 and 100% humidity. Cells were lysed in HBSST (Hanks buffered with 0.035% sodium bicarbonate, 0.5% Triton X-100, 1 mM Mg SO_4 and 1mM $CaCl_2$, ph 7.4) supplemented with Halt ^{TM} protease inhibitor (Thermo Scientific). Cell lysates were cleared by centrifugation at

12,000 rpm for 10 minutes. Totalprotein isolated in the supernatant was quantified using BCA-Protein Assay Kit (Pierce) and spectrophotometer analysis.

Passage doubling

Passage doubling was calculated using the equation: *n = 3.32 (LOG UCY-log I) + x*. n = final PD at the end of a given subculture, UCY = cell yield at a specific point, I = cell number used as an inoculum at the start of subculture, x = doubling level of the incoulum used to initiate the subculture being quantitiated.

Chemicals and Reagents

The following materials were purchased from Sigma Chemical (St. Loius, Mo): trypan blue solution, dimethlyformamide (DMF) and reduced L-glutathione. Glutaraldeyde and formaldeyde were purchase from Fisher Scientific. p38 inhibitor SB203580 (4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4-yl]pyridine) was purchased from Tocris Bioscience (Ellisville, Mo).

Real-time RT-PCR.

Total RNA was extracted from cultures using a TRIzol (Invitrogen, Carlsbad, Ca) extraction technique as recommended by the manufacturer. Total RNA was reverse transcribed using ThermoScriptTM RT-PCR System (Invitrogen). Quantitative PCR (qPCR) reactions assay contained 1x SYBR green PCR mastermix (Applied Biosystems, Carlsbad, Ca), 200 nM primers concentration. A hot start at 95^0C for 5 minutes, followed by 40 cycles at 95^0C for 15 seconds and 65^0C for 1 minutes using a 7900 HT Thermal cycler (Applied Biosystems). Each sample was tested in duplicate or triplicate, as indicated in the text. Table 1 lists qPCR primers used.

miRNA

Total RNA was transcribed using miRNA specific primers, 34a, 19a or 381. Pre-miR precursor was purchased from Ambion (Carlsbad, Ca). Cells were seeded at 65% confluency and trasnfected using siPORT^{TM} Neo FX^{TM} agent (Applied Biosystems) in 10% serum containing media with pre-miRNA or FAM-labeled control precursors for a final 25nM concentration. Cell protein and mRNA levels were analyzed by qPCR or western blot, 60 hours following transfection.

Table 1: qPCR Primers

β2M	For 5'-GTG CTC GCG CTA CTC TCT CT-3' Rev 5'-TCA ATG TCG GAT GGA TGA AA-3'
IGFBP-3	For 5'-CAG AGC ACA GAT ACC CAG AAC TTC-3‘ Rev 5'-CAC ATT GAG GAA CTT CAG GTG ATT-3'
p53	For 5'-CGA GTA CGA CCA GTG CTT CA-3' Rev 5'-CTG GAC TTG CGA AAT CCT TC-3'
p21	For 5'-GGA AGA CCA TGT GGA CCT GT-3’ Rev 5'-GGC GTT TGG AGT GGT AGA AA-3’
p16	For 5'-TGG ACC TGG CTG AGG AGC T-3‘ Rev 5'- GAC CTT CCG CGG CAT CTA T-3'

Western Blot Analysis and Quantitation

Cells were washed in PBS and lysed in HBSST buffer. Lysates were mixed with 4x Sample preparation buffer (SPB, 125mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue) for 10 minutes at 95^0C with or without DTT. Cell lysates were loaded into 5% stacking gel and 10, 12 or 14% SDS-PAGE gel run at 180V for 1 hour. Proteins were then transferred to PVDF membrane at 350 mAmps for 70 minutes. Blots were blocked at room temperature for 1 hour in 1x TBS/0.05% Tween 20 with 5% non-fact dried milk. Primary antibodies were added in blocking buffer for 2% BSA. p53 (#9282), phospho p38 (#9212) and total p38 (#9212) antibodies were purchased from Cell Signaling (Denver, Ma). Primary antibodies p21 (SC-756), p16 (sc-9968) and SOD-1 (sc-11407) were purchased from Santa Cruz (Santa Cruz, Ca). An α- tubulin antibody was purchased Sigma-Aldrich. Blots were washed 3 times in 1xTBS/ .05% Tween20 for 5 minutes and incubated and rocked for 1 hour at room temperature with secondary antibodies conjugated to horseradish peroxidase, α-rabbit (#7074) or α-mouse (#7076) (Cell signaling). An α-goat (sc-2056) antibody was purchased from Santa Cruz. Protein was detected by chemiluminescence reaction Western Lightning (Perkin Elmer, Covina, Ca) according to the manufacture's instructors. Quantitation was performed by densitometry analysis.

siIGFBP-3R generation

Synthesis of siIGFBP-3R-Double-stranded RNAs targeting IGFBP-3R were purchased from Dharmacon, Inc. (Thermo Scientific). Four sets of IGFBP-3R siRNAs were used consisting of a 21-nucleotide sense and antisense strand (Table 2). An siCONTROL non-targeting siRNA #1 (Dharmacon) was used as a nonspecific control. A mixture of 4 sets of IGFBP-3R siRNAs (total 20nM) were transfected for 6 days into M12 prostate cancer cells. Two target sequences corresponding to siRNAs #1 and #3 were further selected to construct shRNA plasmids using pSUPER RNAi SystemTM (OligoEngine) (Table 3). The complementary sequences was annealed together, and inserted into the BamHI and HindIII site of pSRN, generating pSRN-IGFBP-3R shRNA #1, or pSRN-IGFBP-3R shRNA #3, respectively.

Generation of adenoviral IGFBP-3 and IGFBP-3R

NotI-XbaI restriction fragments from pcDNA3/wild-type IGFBP-3 cDNA and EcoR1-Xho1 restriction fragments from CS2-IGFBP-3RF were used to ligate into a NotI-XbaI digested pShuttle-CMV and EcoRV digested pShuttle-CMV, respectively. We used SalI-NotI restriction fragments from pcDNA3.1/LacZ (Invitrogen) were used to ligate into

SalI-NotI-digested pShuttle-CMV. Recombination into the pAdEasy viral backbone was accomplished in E. coli strain BJ5183 according to the manufacturer's instructions. The recombination was verified and the adenoviral recombinant DNA was transferred to DH5. Recombinant pAdEasy plasmids containing CMV-cDNA inserts were purified QIAGEN columns (QIAGEN), and transfected QBI-293A cells with 5 µg of PacI-digested DNA using the calcium phosphate method (Promega, Sunnyvale, Ca). Cells were seeded at 2 x 10^6 cells per 150-mm culture dish 24 hours prior to transfection. Lysis of transfected cells, indicating adenoviral growth, occurred within 4 days. Following amplification, lysates containing clonal recombinant adenovirus were prepared from 150-mm culture dishes and purified by CsCl gradient centrifugation. Recovered virus was aliquoted and stored at -20°C in 5mM Tris (pH 8.0) buffer containing 50mM NaCl, 0.05% bovine serum albumin (BSA), and 25% glycerol. A viral titrate assay was performed using a serial dilution infection of QBI-293A cells and counting plaques under an overlay of 0.3% agarose, 10% FBS, and 1x DMEM. The AdEasy system (Quantum Biotechnologies, Canada) was used to generate Ad:IGFBP-3 and Ad:IGFBP-3R.

Table 2: IGFBP-3R siRNA nucleotide strands

#1	Sense 5'-GUG AGG AAU GUG UUA GUG UUU-3' Antisense 5'-ACA CUA ACA CAU UCC UCA CUU-3
#2	sense 5'- GAC AAC UGG UCC UUA UCA CUU-3' antisense 5'-GUG AUA AGG ACC AGU UGU CUU-3'
#3	sense 5'- GGA ACA AGA CCC GGA CAU UUU-3' antisense 5'-AAU GUC CGG GUC UUG UUC CUU-3'
#4	sense 5'-GGA ACC UGC CUA UAU UUU AUU-3', antisense 5'-UAA AAU AUA GGC AGG UUC CUU-3'

Table 3: Target sequences of IGFBP-3R siRNAs #1 and #3 for generation of pSRN constructs

#1 (+523 to +544)	sense 5'-A TCC CCG TGA GGA ATG TGT TAG TGT TTC AAG AGA A CA CTA ACA CAT TCC TCA CTT TTT A-3', antisense 5'-AGC TTA AAA AGT GAG GAA TGT GTT AGT GTT CTC TTG AAA CAC TAA CAC ATT CCT CAC GGG-3'
#3 (+300 to +318)	sense 5'-GAT CCC CGG AAC AAG ACC CGG ACA TTT TCA AGA GAA ATG TCC GGG TCT TGT TCC TTT TTA-3' antisense 5'-AGC TTA AAA AGG AAC AAG ACC CGG ACA TTT CTC TTG AAA ATG TCC GGG TCT TGT TCC GGG-3'

IGFBP-3, IGFBP-3^{GGG}, and IGFBP-3R- induced senescence

Cells were infected with adenovirus at multiplicity of infection (MOI) as indicated in the text in 1% media. After 24 hours infection, the cells were washed once and allowed to recover in 10% media for 48 hours. A second adenovirus infection was performed in 1% media for 24 hours followed by one time wash. The cells were incubated in 10% FBS media for 3 days. Cells were in culture for 7 days total, at which time they were harvested for protein or RNA isolation.

Generation of pSRN- IGFBP-3 and IGFBP-3R shRNA supernatants

pSRN-IGFBP-3 shRNA viral vector was a generous gift from (Penn State). siIGFBP-3R-double stranded RNAs targeting IGFBP-3R were purchased from Dharmacon, Inc. IGFBP-3R. To produce retroviral supernatants, a 293T packaging cell line was seeded at 35% confluency in 10mm plates and co-transfection with pCL ampho, pME VSVg and pSRN plasmid, an empty vector, IGFBP-3 or IGFBP-3R shRNA using Fugene 6 reagent (Roche, Inianapolis, In). Tissue culture medium was collected 48 hours post transfection, and fresh media was added. After an additional 48 hours, cell medium was pooled, filtered through a 0.45 mm filter and aliquoted. Viral

supernatants were stored at -80^{o}C or supplemented with 4 μg/ml polybrene and were used immediately for infection of cells combined with target cell media in a 1:1 or 1:2 ratio.

Senescence associated β-galactosidase staining (SA-β-gal).

Upon completion of experimental treatment, cells were washed in PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde for 10 minutes at room temperature. Plates were stained as described previously (Dimir et al., 1995). Briefly, cells were incubated overnight ith 1 mg/ml β-galactosidase substrate (X-gal, Gold BioTechnology, St. Louis, MO) prepared fresh in 40 mM citric acid/NaP buffer, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl at 37^{0}C. Microscope images were captured at 20x, phase 2 for morphology analysis. SA-β-gal stained cells were captured at 10x, Phase 1 contrast. A total of 100 cells from 3 random fields were counted and scored for positive staining.

Cell Viability Assay (MTT Assay) and population doubling rate

Cell viability was measured by 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (ATCC). Cells were seeded in triplicate in 96-well plates, grown to 50% confluency, and treated as indicated. MTT was added to the cell culture and incubated for 2 hours at 37^0C. Detergent reagent was then added and incubated in the dark for 2 hours at room temperature. MTT is reduced by metabolically active cells to produce intracellular purple formazan that can be quantified by spectrophotometer; the absorbance was measured at 570nm. To measure cell PD, cells were seeded at 40% confluency and cultured in 10% FBS. Cells were harvested by trypsinization at time intervals indicated, stained with trypan blue, and the viable cells enumerated by use of a hemocytometer. Triplicate wells of cells were counted separately and the results averaged.

Cell Death ELISA Assay

Apoptotic Cell death was measured using the Cell Death Detection ELISA (Roche Applied Sciences) according to the manufacturer's instructions. Equivalent cell

populations were analyzed and absorbance measured at 405nm (reference wavelength, 49nm).

BrdU cell proliferation assay

A bromodeoxyuridine (BrdU) cell incorporation assay was performed according to the manufacturer's instructions (Chemicon, Temecula, Ca). Cells were plated $1x10^4$ per well into 96 well cultures plates. Prior to the end of the incubation time period indicated for each experiment, cells were incubated with BrdU for 2 hours. Cells were then fixed and denatured, washed in incubated with anti-BrdU for 1 hour at room temperature. Following three washes, the cells were incubated with goat anti-mouse IgG peroxidase conjugate for 30 minutes at room temperature, followed by additional washings. TMB peroxidase substrate was then added to cells for 30 minutes at room temperature in the dark. Positive cells were visible by blue color, where intensity was measured using a spectrophotometer at wavelength 450/540 nm. The amount of BrdU incorporation in proliferating cells is proportional to color intensity.

DCF Staining

Dichlorofluorescein diacetate (DCF) is used to detect ROS following senescence induction. After cells were treated and incubated as indicated, cells were incubated for 30 minutes with medium containing 5mM DCF. Media was then removed and fresh media was added. The cells were then immediately visualized for fluorescence. For Fluorescence-activated cell sorting (FACS) analysis cells were washed twice with PBS, trypsinzed and collected in 1 ml of PBS. Flow cytometric analysis was performed using the FC500 combined with CXP software (Beckman Coulter Fullerton, CA).

Immunocytochemistry

HFL-1 cells were seeded on glass cover slips in 6-well plates and infected with adenovirusto induce senescence. Cover slips were acid cleaned and stored in 70% ethanol prior to use. Following senescence induction, cells were rinsed twice with PBS, then fixed in 3.7% paraformaldeyde for 10 minutes at RT. After fixation, the cells were washed twice with PBS at room temperature and permeabilzed in 0.5% NP-40 in PBS for 10 minutes at RT. Following two additional washings, the cells were blocked for 30 minutes in PBS. The slides were then incubated with yH2AX antibody overnight at 4^0C,

followed by incubation with fluorochrome conjugated secondary antibody for 1 hour at RT in the dark. After washing, DAPI was added to PBS to all the nucluei to be visualized. After 2 additional washes for 3 minutes, a drop of VECTASHIELD® mounting media (Vector Laboratories, Burlingame, Ca) was added to microscope slide, with coverslips being added. The immuostained cells were visualized by a Nikon Eclipse 8600 20x magnification, with representative images being captured by Nikon Digital Camera DXM1200G, using Nikon ACT-1 image editing program.

Subcellular fraction preparations

Cell lysates were harvested by washing monolayer cultures in ice-cold PBS. For subcellular fraction preparations, plasma membrane, cytosolic and nuclear fractions were isolated by homogenizing cells using membrane protein extraction kit (Bio Vision, Moutain View, Ca) and centrifuging successively at 700xg and 100,000X g for 10 and 60 minutes, respectively. All pellets were solubilized in 50mM HEPES, pH 7.4, 150mM NaCl, 2mM MgSO4, 1% Triton X-100. Equal amounts of protein from each fraction were analyzed by SDS-PAGE, followed by Western blotting for fractionation specific markers.

Statistical analysis

Analyses of protein or mRNA expression analyzed by western blot or qPCR, respectively were evaluated using a student's t-test with an alpha level set at 0.05.

Chapter 3

Characterization of the role of IGFBP-3 in cellular senescence, replicative and prematurely induced senescence

3.1 Introduction and rationale

The limited proliferative capacity observed in many eukaryotic cell types is often interpreted as a manifestation of cellular aging. Irreversible growth arrest at the G1/S phase of the cell cycle is due to the overexpression of CDK inhibitors including p21 and p16, leading to hypophosphorylation of the Rb protein. Human diploid fibroblasts that enter into replicative senescence are characterized by an enlarged cell shape, SA-β-gal activity, short telomeres and changes in senescence-associated gene expression. Prematurely induced senescence in human diploid fibroblast can display similar characteristics, although the mechanism and signaling to enter the senescent state may be different.

GH/IGF axis has long been regarded as one the most conserved pathways in aging and longevity. IGF signaling is known to both promote and inhibit cell growth. IGFBP-3 is the most abundant IGFBP in serum and is responsible for carrying 75%

IGF-I and IGF-II in a heterotrimeric ternary complex with an acid-labile subunit. The ternary complex is acted upon by proteases, whereby IGF-1 is released and available or interaction with IGF-IR. A growing body of evidence has demonstrated that IGFBP-3 is an important growth-suppressing factor in various cell systems through an IGF-dependent mechanism. Although IGFBP-3 is the principal carrier of IGFs in circulation, it is also an important component to the GH/IGF axis, playing a role in autocrine and paracrine growth control and apoptosis. The IGF-independent actions of IGFBP-3 have revealed a large array of signaling proteins with links to cell cycle control and apoptosis. The IGFBP-3 promoter contains a p53 binding site that upregulates its transcription (BJogie-Brahim *et al*., 2005). Debacq-Chainiaux *et al*, identified IGFBP-3 upregulation when screening for senescence-associated genes following repeated exposure to sublethal concentrations of *tert*-butylhydroperoxide (*t*-BHP) and ethanol [50]. Furthermore, several studies have identified IGFBP-3 to be upregulated at both the protein and mRNA levels in both replicative and premature senescence (Debacq-Chainiaux *et al*, 2008).

Cells that undergo replicative or prematurely induced senescence are currently indistinguishable from one another, suggesting a common downstream signaling influenced by multiple extracellular and intracellular signal transduction pathways. Identifying pathways by which senescence is replicatively and prematurely, induced and maintained, may be critical to understanding the mechanistic causes and consequences of organismal aging.

3.2 Model of replicative senescence in HFL-1 cells

Normal human diploid fibroblasts are in incapable of replicating indefinitely (Hayflick and Moorehead, 1961). To investigate the phenotypic and genotypic changes associated with cellular senescence, we initially generated an *in vitro* model of replicative senescence using human embryonic lung fibroblasts, HFL-1. HFL-1 cells were originally plated beginning at PD 2 and grown under normal conditions with media replaced every two days. Cells were split in 1:4 ratio with each passaging when they reached confluency. At PD24 cells began displaying a senescent morphology but continued to proliferate, however by PD50 cells cease to proliferate, but remained viable for at least 16 days in culture. Upon generating various cultures of multiple population doublings, cells from PD4, 24 and 50 were seeded at 40% confluency in triplicate. On day 3, 5, 7 and 9 the cells were trypsinized and the cell number counted. PD 4 cells became confluent after 3 days and had to be split in a 1:2 ratio on days 3 and 5. A cumulative growth curve demonstrating replicative capacity of cells ranging from early to late passage is shown in Figure 6A. This data indicates a progressive decrease in the proliferation potential with increased population doublings. To further confirm our replicative senescence model we evaluated the morphological changes and percentage of SA-β-gal positive cells (Figure 6B). Compared to early passage cells (PD 4), PD 24 had approximately 30% positive staining, while PD 50 had 75% positive SA-β-gal stained cells.

Activation of p53 is known to upregulate p21 signaling and both CDK inhibitors, p21 and p16, prohibit cell cycle progression from G1 to S phase. Semi-quantitative

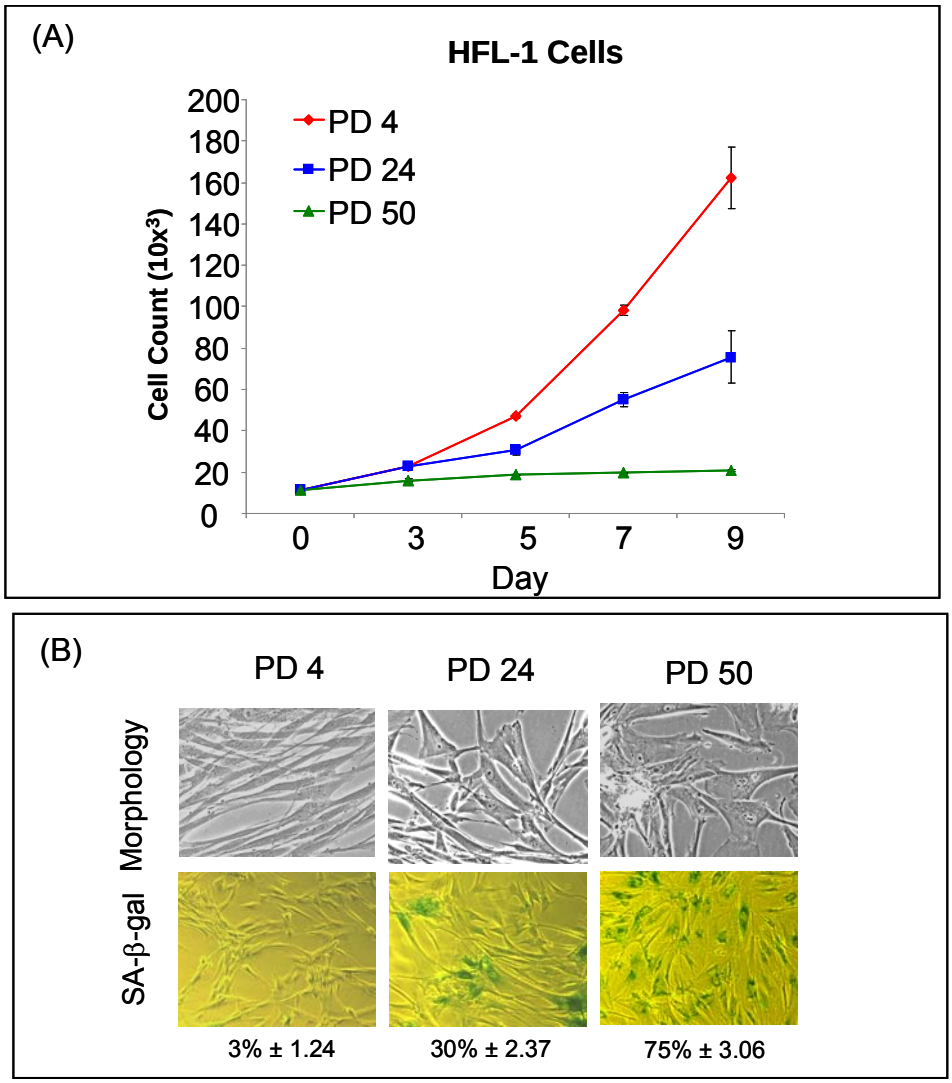


Figure 6: Replicative senescence in HFL-1 cells. (A) Cumulative growth curve of cells cultured for 9 days with fresh medium every 2 days. Cells were seeded 11 x10^3 per well of a 12 well plate in triplicate, n=2. (B) HFL-1cells at PD 4, 24 and 50 were cultured under normal conditions. When cells reached 95% confluenty morphological changes were analyzed by phase contrast microscopy, 20 x (top panel) and stained for evaluation of SA-β-gal activity (bottom panel). Percentage represents 3 counts of 100 cells, n=3. The continual passing of HFL-1 decreases replicative capacity and increase SA-β-gal positive cells and morphology changes associated with senescence. PD 24 cells referred to as pre senescent cells and PD 50 cells are replicatively senescent.

real-time RT-PCR was performed with RNA extracted from each PD 4, 24 and 50, with β2M being used as a loading reference. The expression of p16, p21 and p53 in PD24 increased 2, 3.2 and 7.5 fold, respectively compared to PD 4 cells. Senescent HFL-1 cells at PD 50 had an even greater mRNA fold expression of p16, p21 and p53 at 7.2, 5.8 and 14 fold, respectively (Figure 7A). Western blot analysis of senescence associated genes from each PD indicated a significant upregulation of protein production correlated to decreases in replicative capacity. As mentioned earlier, the role of p38 MAPK in senescence is emerging as playing a critical role in senescence, therefore we examined both the phosphorylated levels, indicative of increased p38 signaling, and total p38 protein levels (Figure 7B). Indeed, both total and phosphorylated p38 MAPK signaling is upregulated in replicatively senescent HFL-1 cells.

IGFBP-3 mRNA levels and proteins have been shown to be increased in both replicative and prematurely-induced senescent cells (Pascal, *et al.,* 2005). As our laboratory has recently published the identification of a novel IGFBP-3R, this is the first data identifying upregulation of IGFBP-3R in senescent fibroblasts. Semi-quantitative RT-PCR revealed a significant increase in both IGFBP-3 and IGFBP-3R with increased PD (Figure 8A). As IGFBP-3 is known to be secreted, we incubated cells in serum free media for 1, 3 or 5 days before collecting media and harvesting cell lysate. With increased cell incubation time, there is an increased accumulation of IGFBP-3 production. However, an upregulation of IGFBP-3 protein can initially be detected in

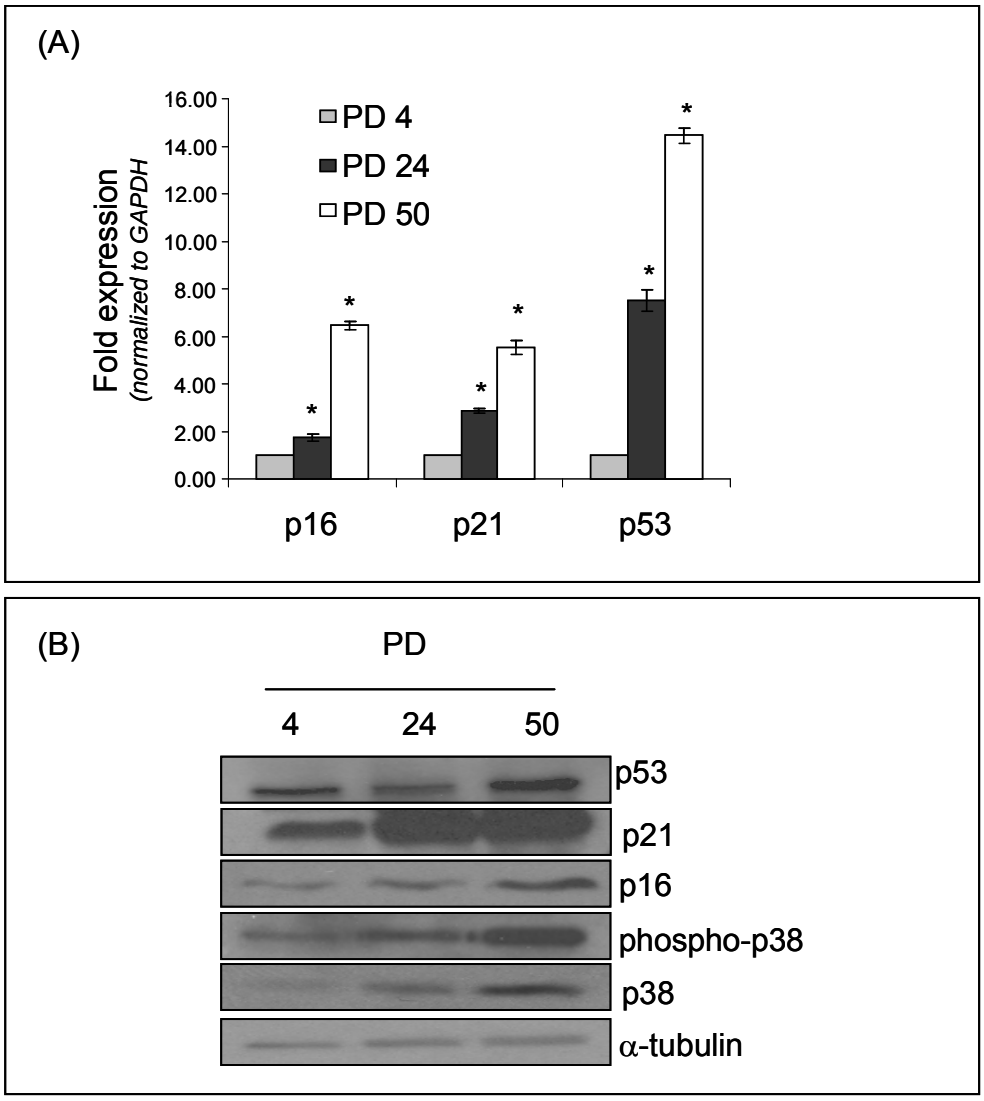


Figure 7: SA-associated gene expression in HFL-1 cells in replicative senescence. Cells were seeded at 70% confluency and 3 days later RNA and protein levels evaluated in triplicate , n=3. (A) qPCR evaluation of p21, p16 and 53 mRNA levels, GAPDH was used as a loading control. (B) Western blot analysis of expression levels of SA-genes and activation of p38 by phosphorylation of p38, α-tubulin was used as a loading control. With increasing population doubling there is a significant increase in SA-associated gene expression on both mRNA protein levels.(*, $p \leq 0.05$)

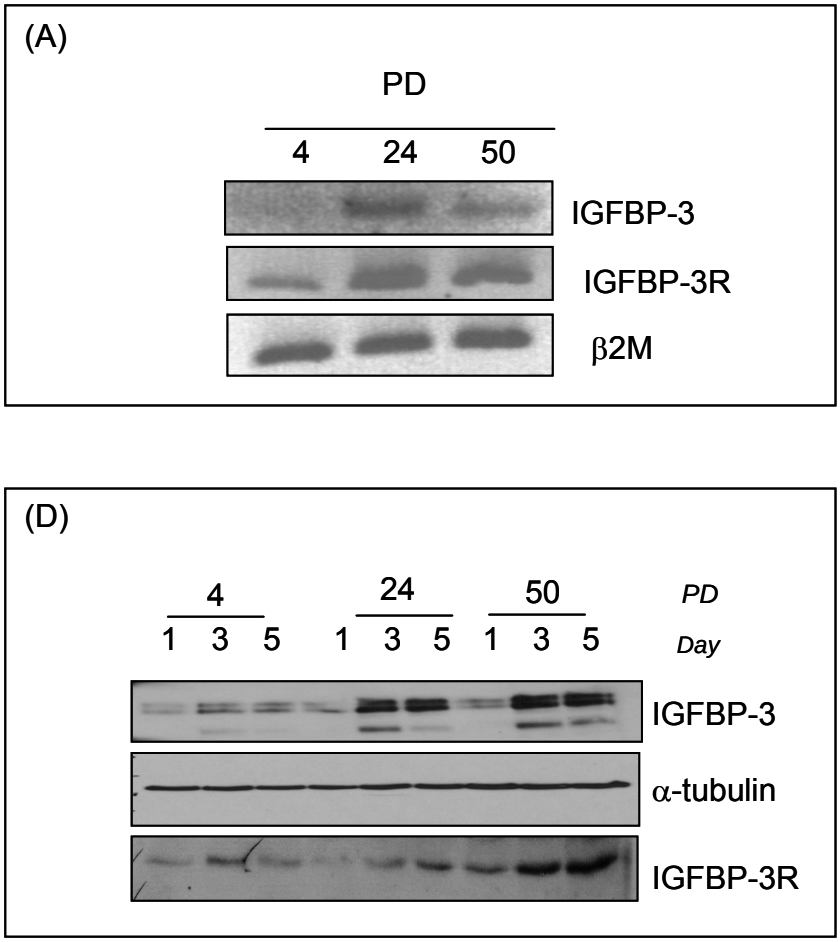


Figure 8: IGFBP-3 and IGFBP-3R mRNA and protein expression levels in HFL-1 cells at PD 4, 24 and 50. Cells were seeded in triplicate at 75% confluency and RNA was isolated 60 hours later, n=3. (A) RT-PCR evaluation of IGFBP-3 and IGFBP-3R, and β2M was used as a loading control. (B) Cell lysates were harvested for protein analysis after cells were incubated for 1, 3 or 5 days in serum free media, α-tubulin was used as a loading control. The mRNA and protein levels of IGFBP-3 and IGFBP-3R are upregulated with increasing PD.

presenescent (PD24) fibroblasts (Figure 8B). In addition, IGFBP-3R production was also observed with increased PD. IGFBP-3/R levels are increased way before cells undergo senescence, suggesting IGFBP-3/R may be responsible for inducing senescence and not entirely for maintenance. By evaluating proliferative potential, morphological changes, SA-β-gal activity and senescence-associated gene expression, we established an *in vitro* model of replicative senescence and from hereon will, designate PD4 cells as young, PD24 as presenescent cells and PD 50 as senescent cells.

3.2.2 Suppression of endogenous IGFBP-3

Since IGFBP-3 is upregulated in replicatively senescent cells, we aimed to evaluate the effects of suppressing endogenous IGFBP-3 in presenescent, PD24, HFL-1 cells. To test the efficiency of suppressing endogenous IGFBP-3, we generated viral supernatants of either pSRN empty vector or pSRN-IGFBP-3^{shRNA} specifically targeting IGFBP-3 mRNA. HFL-1 cells were incubated with viral supernatants of pSRN-EV or pSRN-IGFBP-3^{shRNA} for 24 hours, allowed to recover in 10% serum containing media for 24 hours and incubated for 2 or 4 days in serum-free media before harvesting. Endogenous IGFBP-3 protein secretion was significantly suppressed in cells infected with IGFBP-3^{shRNA} compared to control and empty vector infected cells, as indicated by western blot (Figure 9A). The effect of suppressing endogenous IGFBP-3 in presecent cells was evaluated over a 14 day time point. Cells were seeded at 50% confluency and infected for 24 hours. Cells were then grown in 10% media for additional 4 days before

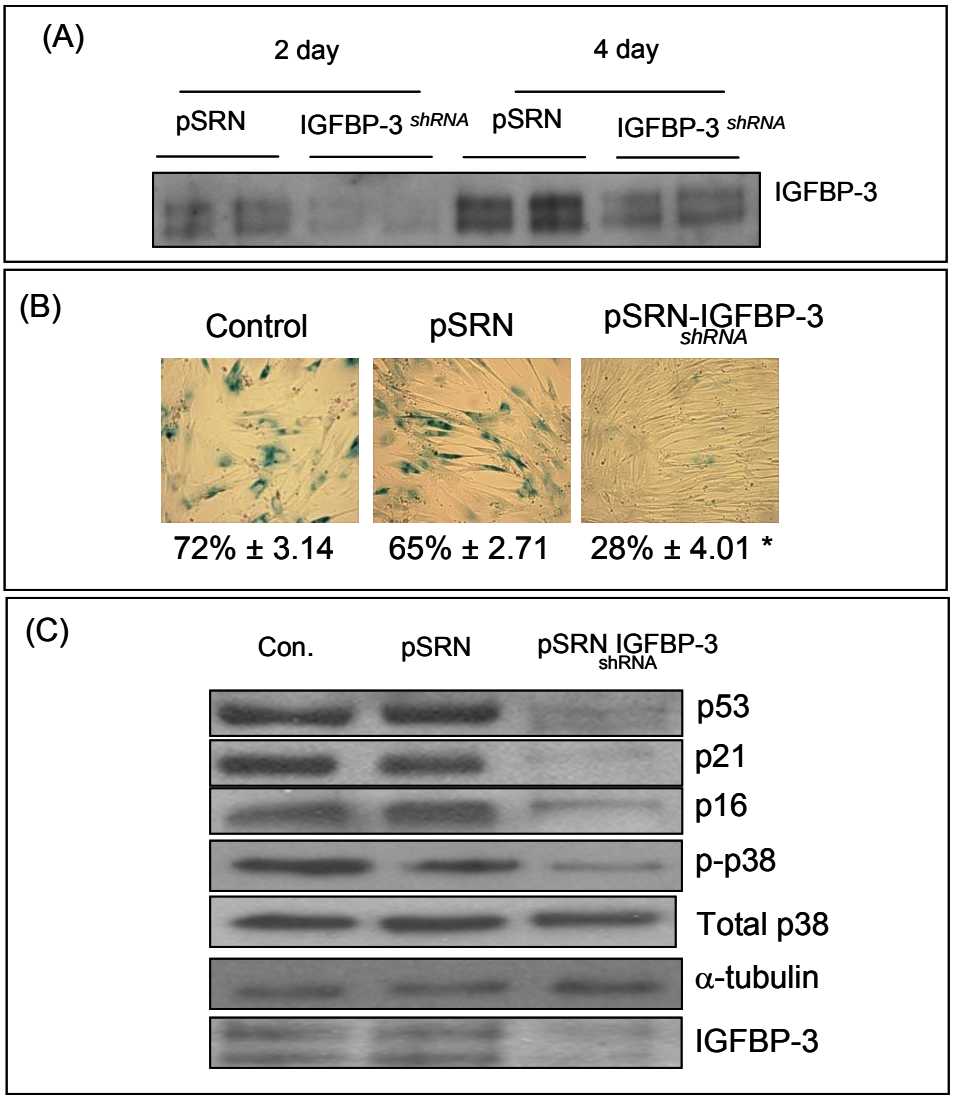


Figure 9: **Suppression of endogenous IGFBP-3 inhibits senescence**. (A) Presenescent HFL-1 (PD 24) cells were exposed to pSRN IGFBP-3 siRNA or empty vector retrovirus supernatant for 24 hours before fresh media was added for an additional 24 hours. (A) Secretion of IGFBP-3 in culture media measured by western blot, following 2 days or 4 days in serum free media. (B) SA-β-gal staining following suppression of endogenous IGFBP-3, (200x). (C) Protein expression of senescence associated genes analyzed by immunoblot following IGFBP-3 suppression. Endogenous suppression of IGFBP-3 decreases the number of SA-b-gal positive cells and inhibits the upregulation of senescence associated genes and p38 MAPK signaling. (*, $p \leq 0.05$)

being split in 1:2 ratio. Since cells were not stably transfected, a second viral infection was performed and cells were recovered in 10% serum containing media for 4 more days before being passaged in 1:2 ratio. A third and final supernatant infection was performed and cells were incubated for an additional three days before cell lysates were collected or SA-β-gal activity was measured (Figure 9B). Comparing control, empty vector and IGFBP-3 shRNA treated cells at day 14, significant morphological differences and decreased SA-β-gal staining. Control and empty vector had 82% and 68% SA-β gal staining, respectively, and IGFBP-3 shRNA treated cells had significantly less stained cells at 28% (Figure 9B). The increased abundance of senescence associated genes p53, p21 and p16 is significantly reduced in cells infected with IGFBP-3^{shRNA} (Figure 9C). The inhibition of senescence by suppressing endogenous IGFBP-3 in presenscent cells establishes an essential role of IGFBP-3 in senescence.

3.2.3 IGFBP-3 signaling in Werner Syndrome derived fibroblasts.

Werner syndrome (WS), a premature aging disorder, is commonly used as a model of normal human aging. Recently, it has been reported that WS fibroblasts exhibit phenotypes similar to senescent fibroblasts, including increased IGFBP-3 expression and phosphorylated p38, suggesting IGFBP-3 may play a key role in senescence (Davis, *et al.,* 2005). WS fibroblasts provide a valuable model for allowing *in vitro* senescence mechanisms to be examined *in vivo.* IGFBP-3 mRNA is known to be upregulated in WS dermal fibroblasts. However, its upregulation compared to age matched fibroblasts has not been fully investigated (Murano, *et al.,* 1991). Initially, we

compared the morphology of normal human dermal fibroblasts to WS fibroblasts from 2 different donors, AG03141 and AG00781, at similar PDs. It is clearly evident that the WS cells exhibit an enlarged, flattened irregular shape compared to the HDF with a slender spindle-like shape (Figure 10A). As p38 MAPK signaling has recently emerged as possibly regulating the senescence phenotype of WS cells, we evaluated the phosphorylation status of p38 MAPK in addition to IGFBP-3 secreted protein levels (Figure 10B). IGFBP-3 reverses the senescence phenotype similar to early passage fibroblasts and surprisingly, there is significant suppression phosphorylated of p38. Total p38 protein does not appear to be downregulated, suggesting senescence signaling is regulating p38 activity and not protein production in WS cells.

While it is currently known that SB203580, a p38-specific inhibitor, reverses the accelerated senescence phenotype observed in WS fibroblasts (Davis *et al.,* 2005), we initiated studies to investigate the effect of IGFBP-3 siRNA on WS cells. Surprisingly, our data shows decreased levels SA-β-gal staining following suppression of endogenous IGFBP-3 (Figure 11A). Furthermore, p38 MAPK phosphorylation and senescence associated genes were downregulated following IGFBP-3 shRNA treatment (Figure 11B). Interestingly, as we have shown IGFBP-3-induced senescence can upregulate p38 MAPK activation and endogenous suppression of IGFBP-3 inhibits p38 activation, we aimed to identify the mechanisms between these two interacting pathways. Taken together, suppression of endogenous IGFBP-3 inhibits p38 MAPK activity, whereas inhibition of p38 MAPK activation inhibits IGFBP-3 production. Our

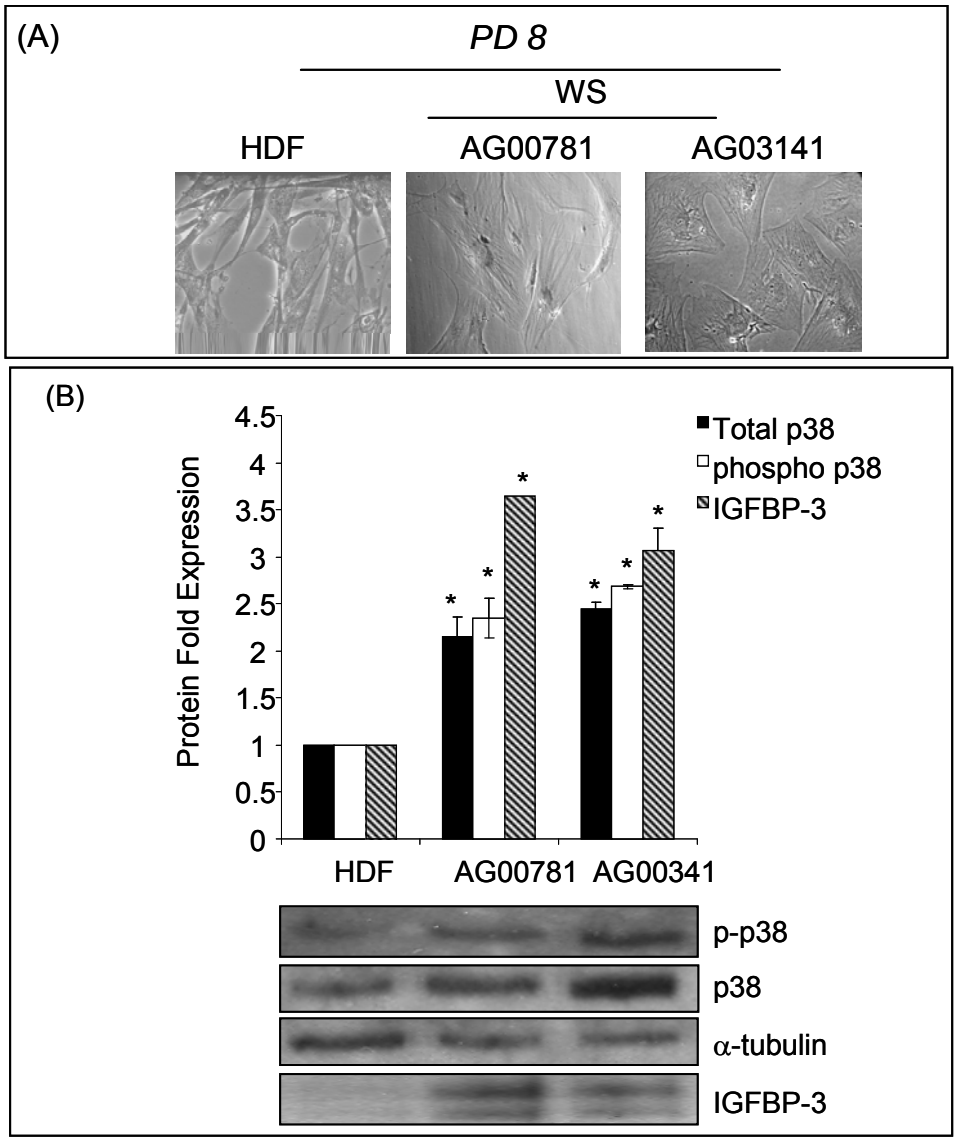


Figure 10: **Expression of IGFBP-3 levels in Werner Syndrome cells and normal Human dermal fibroblast (HDF) at same population doubling (PD8).** HDF and WS cells derived from two different donors, AG03141 and AG00718 were seeded at 80% confluency. (A) analyzed for morphological changes by phase contrast microscopy, 20 x (top panel) and (B) Cell lysates and culture media were harvested and measured by western blot following incubation for 3 days in serum free media. Densitometric analyses of phosphorylated p38, total p38 and IGFBP-3, α-tubulin was used as a loading control to indicate protein changes, n=2. (*,$p \leq 0.05$). WS fibroblasts appear to resemble rpelicative senescence cells compared to normal HDF at similar PD. WS cells also have upregualated IGFBP-3 and p38 MAPK protein expression levels compared to HDFs.

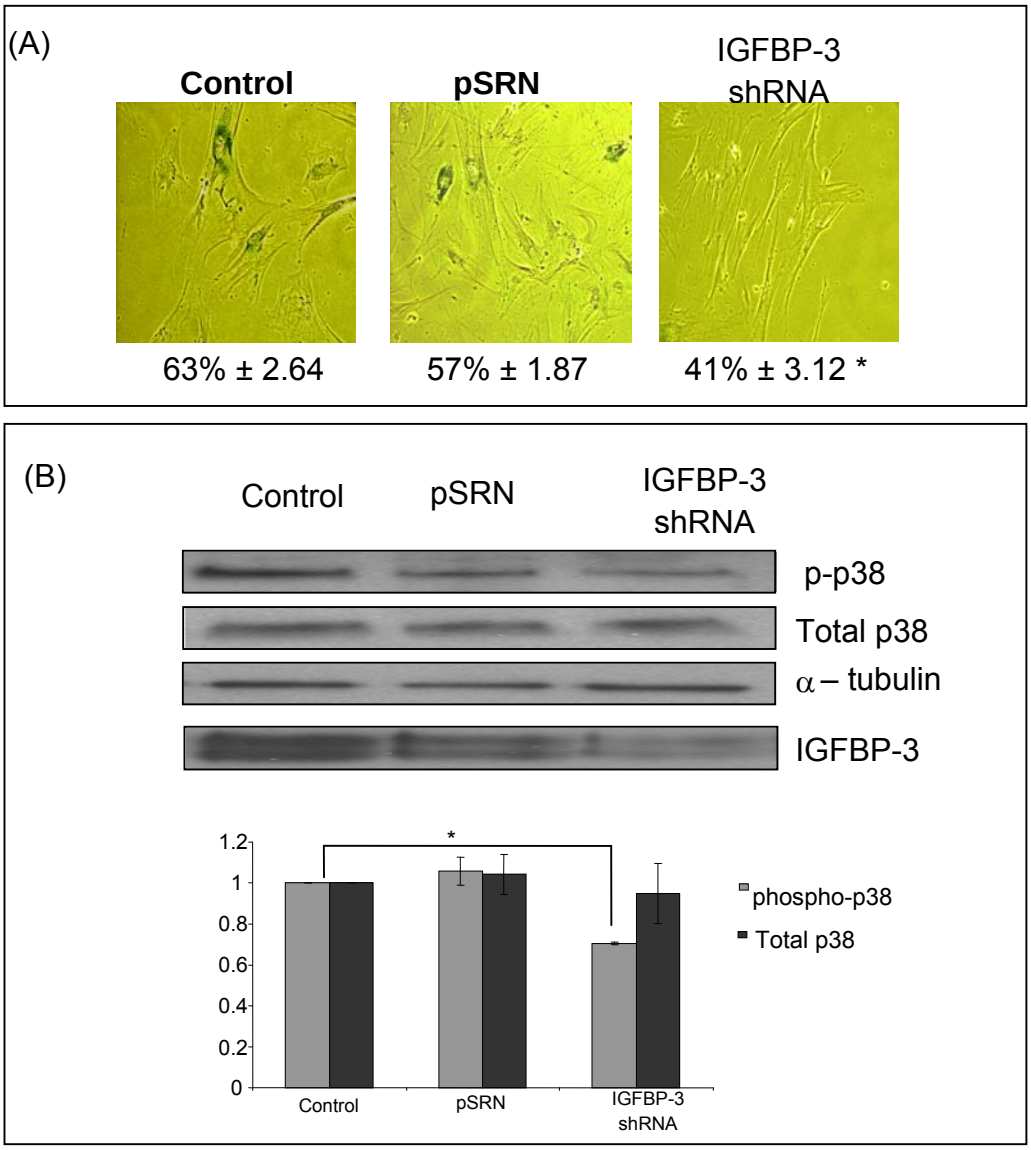


Figure 11: **Suppression of endogenous IGFBP-3 in WS fibroblast reverses senescence phenotype**. AG00781 WS cells were infected with IGFBP-3 shRNA or empty vector retrovirus supernatant for 12 hours allowed to recover for 48 hours in fresh 10% media. Protocol was repeated 3 times for a total of 8 days in culture. (A) SA-β-gal staining cells was performed 60 hours after final infection, 100 cells per count in 3 counts, n=2 , magnification at 20x). (B) Serum free media replaced serum-containing media following final infection and cell media and lysates were collected 48 hours. Secretion of IGFBP-3 and p38 MAPK expression were analyzed by western blot, α-tubulin was used as loading control. IGFBP-3 shRNA decrease the number of SA-β-gal positive cells and decreases p38 MAPK phosphoyrlation. Densitometric analysis compares control versus treated cells for statistical significance (*, $p \leq 0.05$).

findings strongly suggest IGFBP-3/IGFBP-3R axis may be a major signaling pathway in cellular aging.

3.2.4 IGFBP-3 overexpression induces premature senescence.

IGFBP-3 upregulation in senescence appears to be critical, as its endogenous suppression can inhibit and reverse senescence. To further evaluate an essential role of IGFBP-3 in senescence, we investigated the effects of overexpressing IGFBP-3 and its novel receptor in early passage HFL-1 cells. Moreover, we aimed to investigate the effects of IGFBP-3 independent of IGF. Therefore, we utilized mutated IGFBP-3, IGFBP-3^{GGG}, which has no binding affinity for IGF. We have published reports regarding the proapoptotic effect of IGFBP-3 and IGFBP-3^{GGG} in various cancer cell lines. However, the present study is novel as it investigates the effects of IGFBP-3 upregulation in normal non-immortalized cells. Early passage, PD4, fibroblasts were infected with adenovirus expressing IGFBP-3, IGFBP-3^{GGG}, IGFBP-3R or empty vector. After two infections and a total of 7 days in culture, cells were analyzed for viability by MTT assay (Figure 12A) and apoptosis by Cell Death ELISA (Figure 12B). MTT analysis indicated IGFBP-3, IGFBP-3^{GGG} nor IGFBP-3R remained viable, in contrast to what has previously been observed in cancer cells. Furthermore, adenovirus treatment did not induce apoptosis, compared to positive control etoposide treatment. At MOI 250, cells death occurred indicated cell toxicity of virus rather than IGFBP-3 or IGFBP-3R induced apoptosis. Adenovirus treatment at MOI 100 did not significantly increase Cell Death ELISA absorbance measurements. This suggests IGFBP-3 can activate

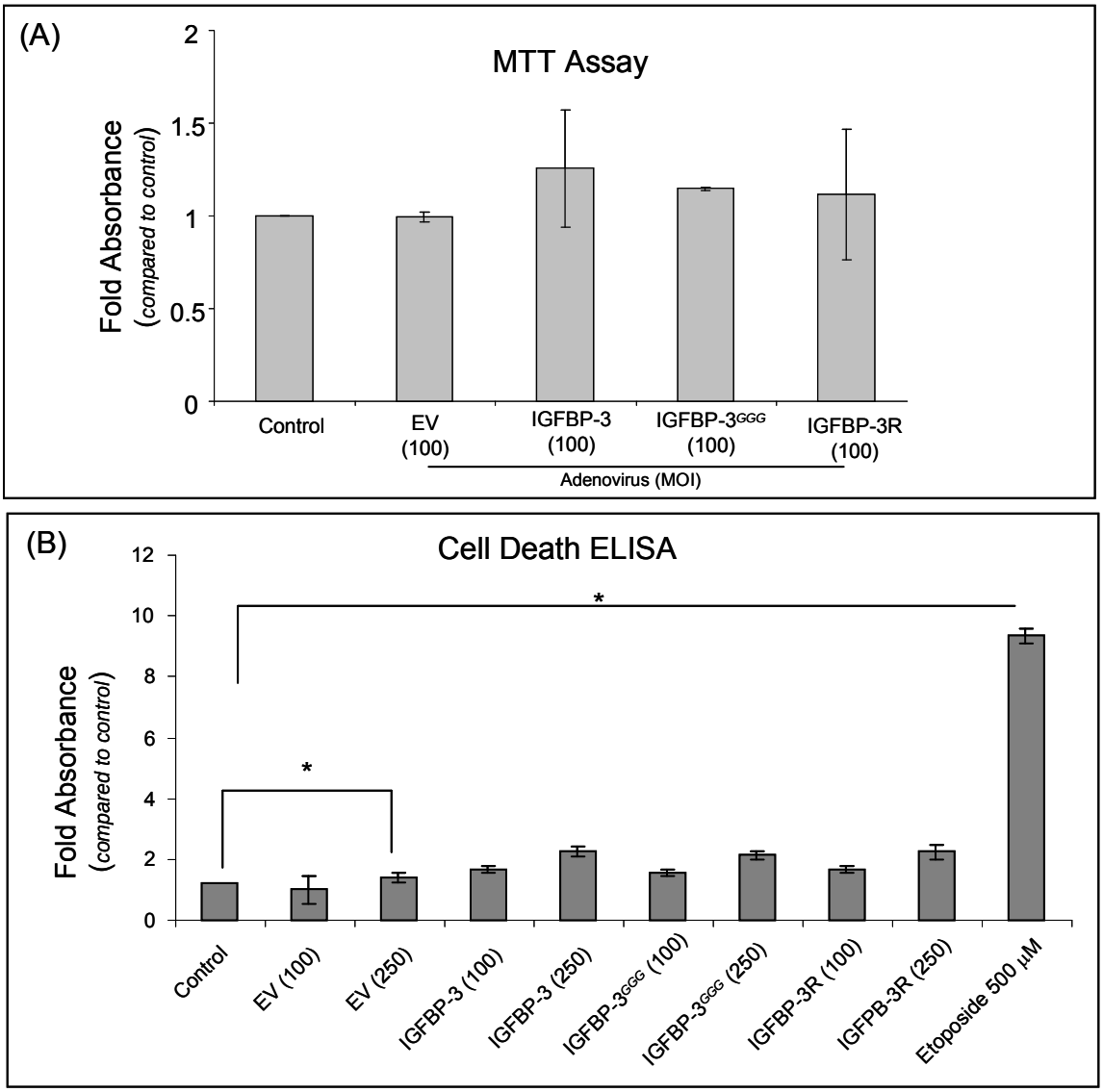


Figure 12: **Overexpression of IGFBP-3 in HFL-1 cells does not induce apoptosis.** Early passage cells were adenovirus infected with EV, IGFBP-3, IGFBP-3^{GGG} or IGFBP-3R at MOI 100 on day 0 and day 3 of a 7 day incubation. (A) Cell viability was measured by MTT assay in triplicate, n=3. (B) To assess apoptosis induction after final treatment, equal cell numbers were analyzed by Cell Death ELISA in triplicate, n=3. As a positive control cells were treated 24 hours with 500μM etoposide. As EV (250) had significantly increased absorbance compared to control, adenovirus IGFPB-3, IGFBP-3^{GGG} and IGFBP-3R treated samples were analyzed for statistical significance compared to EV treated samples same similar MOI. (*, p ≤0.05) Overepxression of IGFBP-3 and IGFBP-3R does not induce apoptosis.

divergent signaling pathways in normal versus cancer cells. If IGFBP-3 did not promote cell death and cells remained viable for 7 days, we proceeded to determine the effects on proliferation capacity and any possible hallmark characteristics of senescence. HFL-1 PD4 cells were seeded at 40% confluency in triplicate and infected twice with adenovirus. On days 0, 3, 5, and 7 cell numbers were calculated and a cumulative growth curve was generated (Figure 13A). Non-treated and empty vector treated cells both exhibited normal proliferation capability, as cell numbers doubled every two days. However, IGFBP-3, IGFBP-3 ^{GGG}, IGFBP-3R adenovirus infection caused a significant inhibition of cell proliferation beginning on day 3. Although there appears to be a substantial inhibition in replication, not all cells undergo growth arrest as the growth curve does not plateau. Another experimental method used to confirm inhibition of cell replication is BrdU incorporation. As cells replicate, BrdU becomes incorporated into DNA synthesis and therefore increased BrdU staining is indicative of DNA replication. IGFBP-3 and IGFBP-3R infected cells showed significantly decreased BrdU incorporation compared to empty vector virus and control cells, confirming a cell growth arrest (Figure 13B). IGFBP-3 does not induce apoptosis in normal cells contradictory to cancer cell signaling. However, IGFBP-3 does induce cell growth inhibition in normal cells as demonstrated by lack of BrdU incorporation and decrease cell replication.

Adenovirus treated cells were fixed and stained for SA-β-gal analysis and morphological changes were observed (Figure 14, bottom panel, top panel, respectively). As control and EV treated PD4 cells displayed slender spindle-like morphology, IGFBP-3, IGFBP-3 ^{GGG}, IGFBP-R displayed a large flat, irregular shape

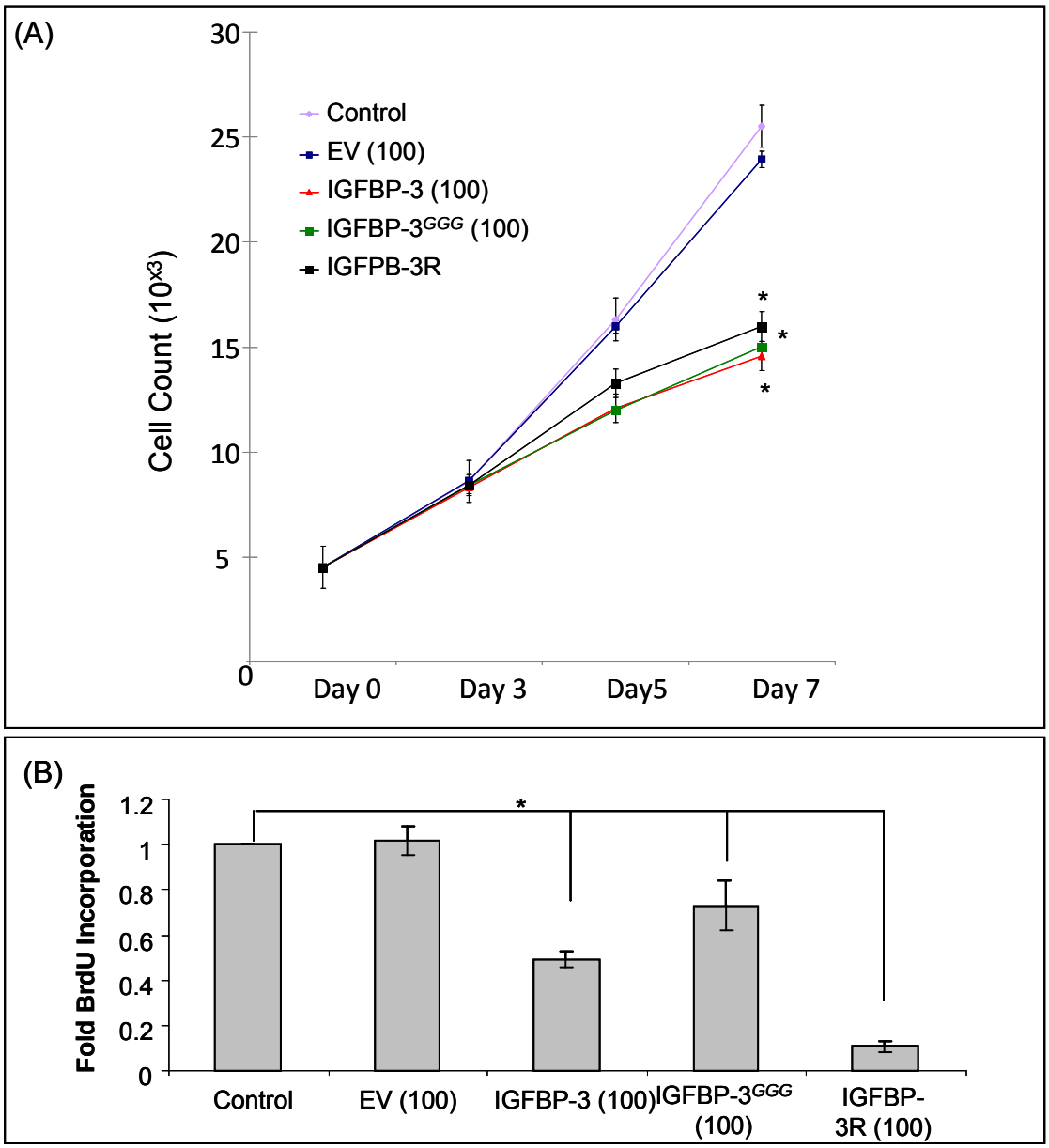


Figure 13: **Overexpression of IGFBP-3 in HFL-1 cells prohibits cell proliferation and inhibits DNA synthesis.** HFL-1 (PD5) cells were seeded in 2.2 x10^5 per 12 well plate in triplicate and infected twice with adenovirus empty vector, IGFBP-33, IGFBP-3^{GGG} or IGFBP-3R at MOI 100 on day 0 and day 3 of a 7 day incubation. (A) On days 3,5 and 7 cells were trypsinzed, stained with trypan blue and counted. Cumulative growth curve was generated, each time point represents average of triplicate samples, n=3. (B) On day 5 cells were trypsinzed and seeded in triplicate in 96 well plate for determination of DNA synthesis by BrdU incorporation. (*,p ≤0.05) IGFBP-3 and IGFBP-3R overexpression inhibits replicative capacity and inhibits BrdU incorporation.

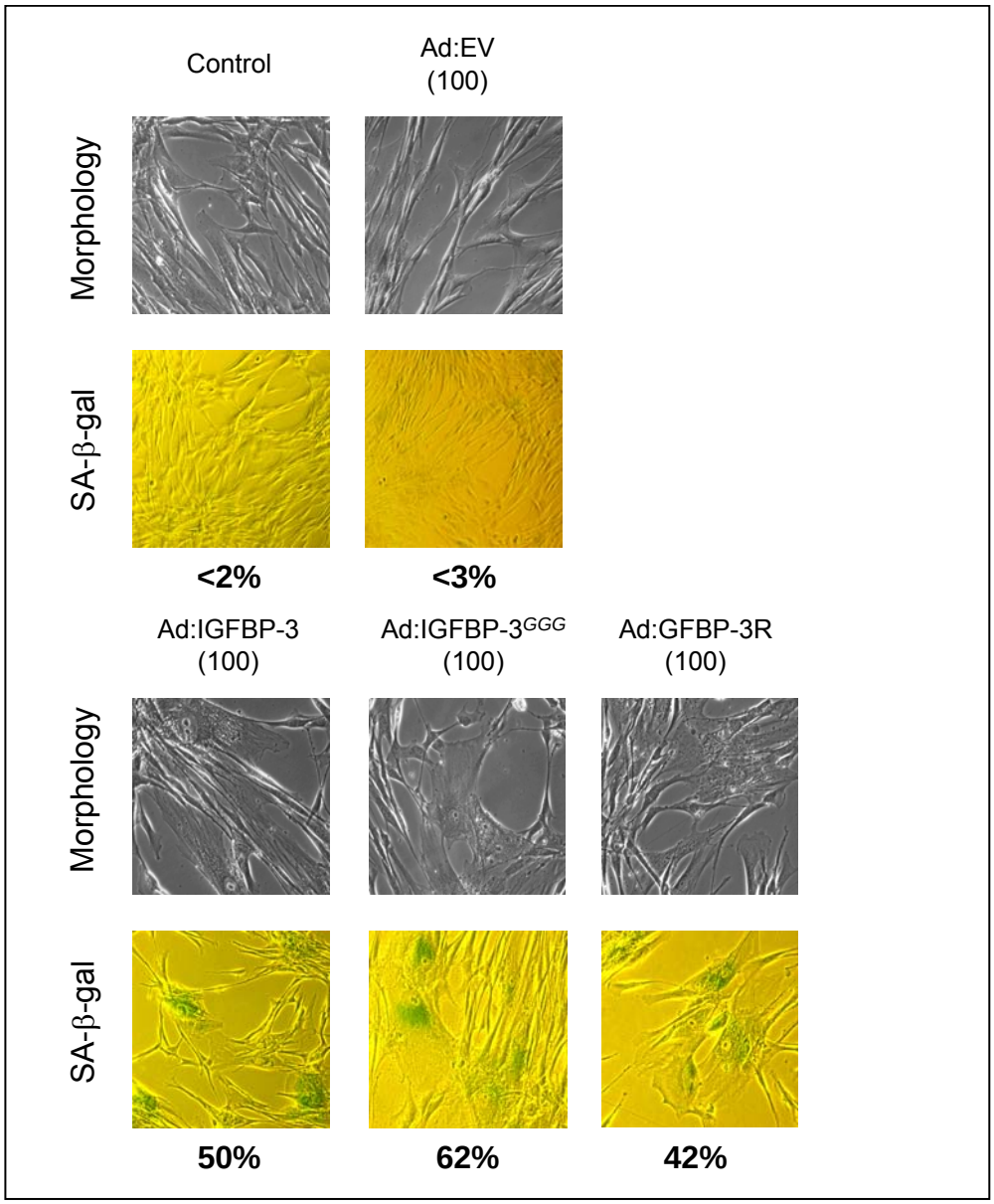


Figure 14: **Overexpression of IGFBP-3 induces senescence.** Early passage cells infected 2x with adenovirus empty vector, IGFBP-3 or IGFBP-3^{GGG} at MOI 100 on day 0 and day 3 of a 7 day incubation. (A), Morphology observations were photographed at 20x phase contrast magnification. SA-β-gal staining was performed, and percentages represent average of 100 cells from 3 fields, n=2. Overexpression of IGFBP-3 and IGFBP-3R increases SA-β-gal positive cells indicative of senescence induction.

and significantly increased SA-β-gal activity, 50% and 62% and 42% respectively compared to 2% control or 3% empty vector adenovirus (Figure 14). Here we demonstrated IGFBP-3, IGFBP-3 ^{GGG} or IGFBP-R can cause early passage cells to exhibit a similar phenotype observed in replicative senescence and significantly increased SA-β-gal positive cells (Table 1).

Overexpressing IGFBP-3, IGFBP-3 ^{GGG}, IGFBP-R resulted in the significant upregulation of senescent-associated genes p53, p21, p16 and phosphorylated p38 (Figure 15 and Table 1). Many stimuli that can trigger replicative or premature senescence signaling are known to upregulate SA gene expression. However, we have clearly demonstrated IGFBP-3 may possibly be an upstream target of these cell cycle regulator genes. These data support our hypothesis that IGFBP-3 upregulation does not induce apoptosis or alter cell viability, as previously seen in cancer cells, but more importantly induces growth arrest defined as cellular senescence.

3.2.5 IGFBP-3-induced senescence independent of telomerase.

Telomere shortening is universally accepted as a major regulator of replicative senescence, yet no information currently indicates a role of IGFBP-3 in telomere shortening or telomere end fusion. Numerous studies have demonstrated, senescence induction is not exclusively dependent on telomere length as despite the presence of telomerase, cells can still enter a senescence state (Allsopp, *et al.,* 1992 and 1995 and Itahana *et al.,* 2004).

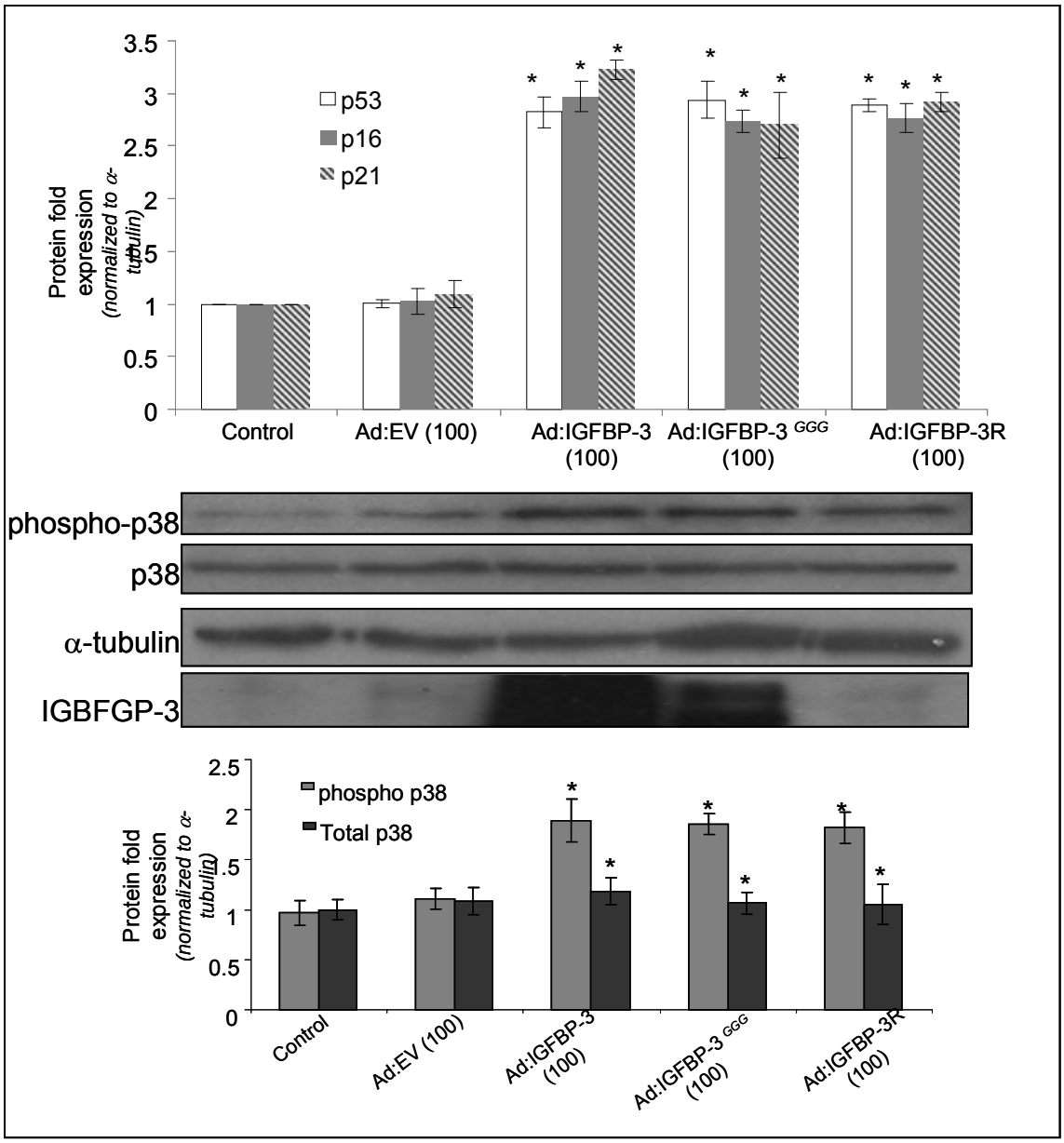


Figure 15: Regulation of expression of SA-genes following IGFBP-3-induced senescence. Early passage cells infected twice with adenovirus empty vector, IGFBP-3 or IGFBP-3^{GGG} at MOI 100 on day 0 and day 3 of a 7 day incubation. (A) Cells lysates were collected and analyzed by western blot, α-tubulin was used as a loading control. Senescence associated genes were measured by densiometric analysis. Statistical analysis compared control cells versus adenovirus infected cells (*, $p \leq 0.05$). Overexpression of IGFBP-3 and IGFBP-3R increases SA gene expression indicative of senescence induction.

Table 1: Overexpression of IGFBP-3 and IGFBP-3R significantly upregulates SA-□-gal staining and SA gene expression.

	% SA-□-stain positive cells	p53	*p value*	p16	*p value*	p21	*p value*
Control	2.1 ± 0.2 %	1		1		1	
Ad: EV (100)	2.82 ± 0.17 %	1.01 ± 0.037	*0.442*	1.02 ± 0.120	*0.807*	1.09 ± 0.126	*0.338*
Ad: IGFBP-3 (100)	50.03 ± 3.01 %	2.82 ± 0.144	*0.002*	2.968 ± 0.147	*0.002*	3.221 ± 0.090	*0.001*
Ad:IGFBP-3^{GGG} (100)	62.4 ± 1.54 %	2.94 ± 0.176	*0.002*	2.732 ± .102	*0.001*	2.70 ± 0.314	*0.012*
Ad:IGFBP-3R (100)	41.43 ± 0.85 %	2.89 ± .061	*0.003*	2.768 ± 0.135	*0.001*	2.920 ± 0.090	*0.011*

To investigate if telomere end fusions were generated following IGFBP-3-induced senescence in HFL-1 cells, we initially attempted to prepare metaphase chromosomes from IGFBP-3- induced senescent cells. Unfortunately the dilemma with this assay is IGFBP-3 treated cells cease proliferating and do not enter mitosis, preventing one from harvesting metaphase chromosomes. As a result we could not proceed with this assay.

We then aimed to examine if IGFBP-3 induced senescence was dependent on telomerase activity. Using BJ and BJ/hTERT, which are immortalized and have stably transfected telomerase, respectively, we analyzed the results of overexpressing IGFBP-3. BJ fibroblasts undergo replicative senescence at approximately PD65 therefore; we used PD27 in our experimentation. BJ (PD27) cells were infected with adenovirus 2x and cultured for a total of 7 days before analyzing for senescence induction. The cells were fixed and stained for SA-β-gal analysis and morphological observations (Figure 16, bottom panel, top panel, respectively). The control and EV treated BJ cells displayed normal fibroblast morphology, while IGFBP-3, IGFBP-3 ^{GGG}, IGFBP-R displayed a large flat, irregular shape and significantly increased SA-β-gal activity, 62% 56% and 72% respectively compared to 26% control or 28% empty vector adenovirus (Figure 16). Here we demonstrated IGFBP-3, IGFBP-3 ^{GGG} or IGFBP-R can causes BJ (PD24) cells to exhibit a similar phenotype observed in replicatively senescent cells. Next we examined if telomerase activity would inhibit IGFBP-3-induced senescence. Surprisingly, BJ/hTERT (PD53) entered into a senescence phenotype with significant morphology changes and increased SA-β-gal activity following adenovirus infection (Figure 17). Unexpectedly, it appeared BJ/hTERT had more SA-β-gal stained cells than

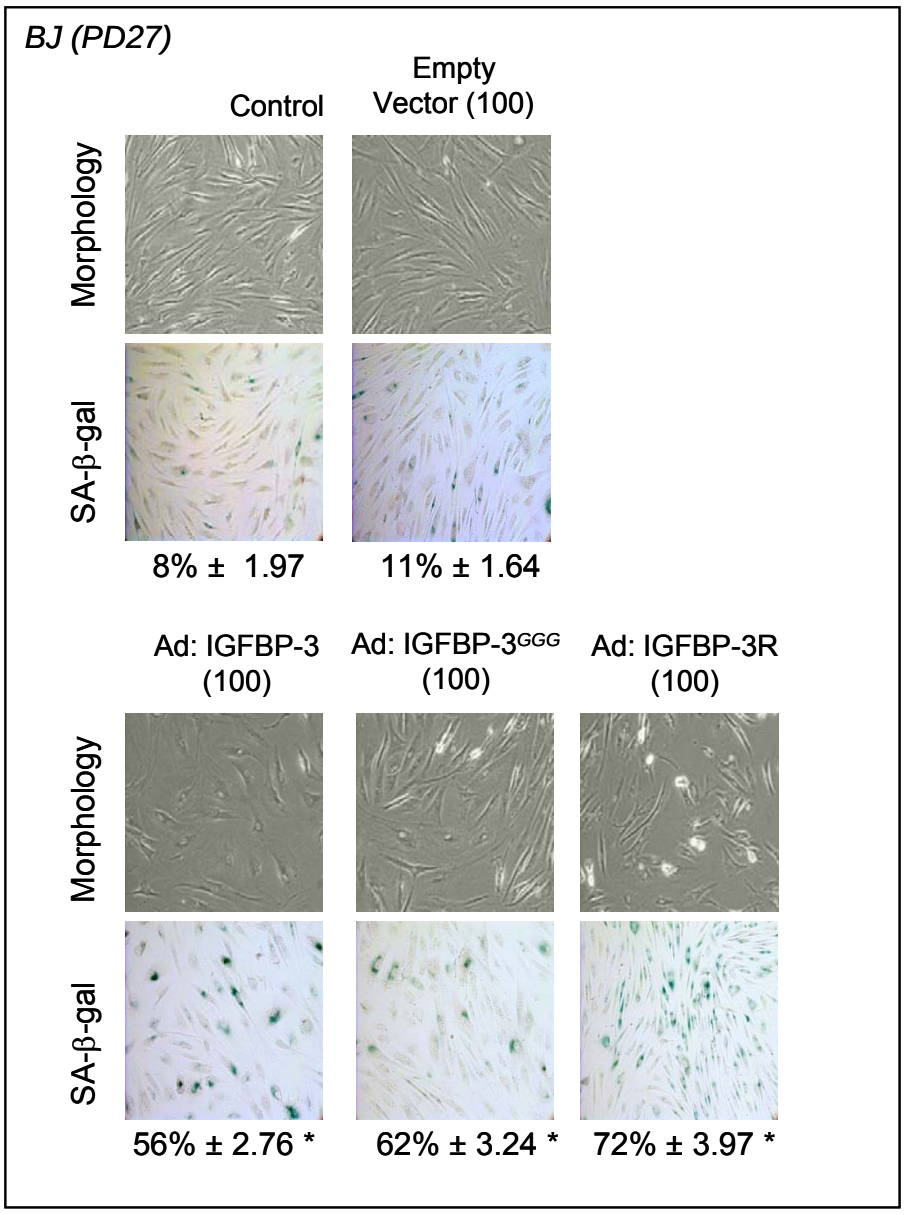


Figure 16: Overexpression of IGFBP-3 in human foreskin fibroblasts (BJ) at PD 27. BJ cells were seeded at 30% confluency and infected with adenovirus in 1% serum containing media for 24 hours. Cells were incubated for 48 hours in 10% media followed by a second adenovirus infection for 24 hours. Serum free media was added and cells were incubated for an additional 60 hours. Cell were fixed for analysis of morphological observations (top panel) or stained for SA-β-gal positive cells (bottom panel). Cells were photographed at 20x magnification phase contrast microscopy. Percentages represent average of 3 100 cells counts, n=2. (*,p ≤0.05) Overexpression of IGFBP-3/R can induce senescence as detected by increased SA-β-gal positive cells.

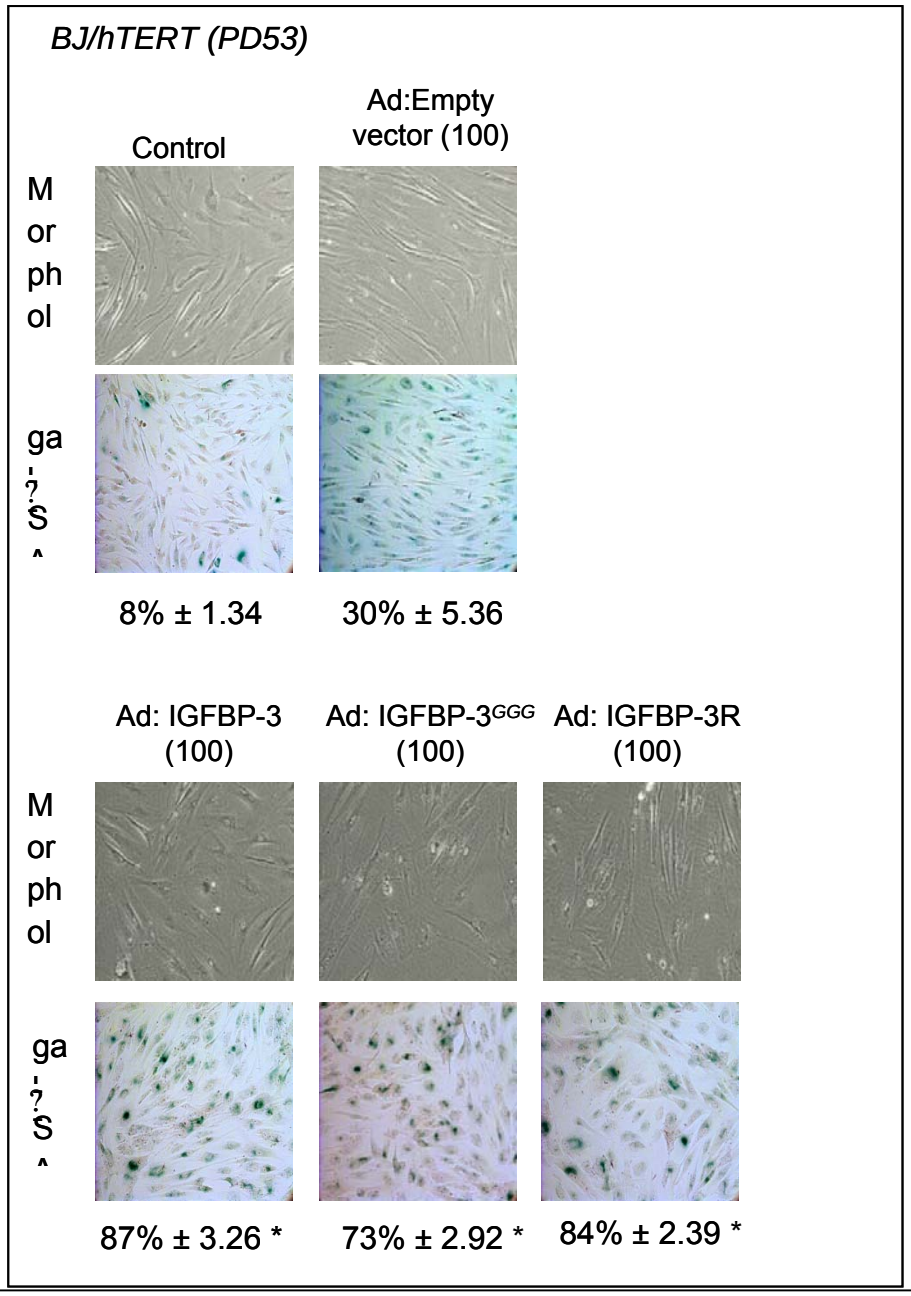


Figure 17: Overexpression of IGFBP-3 hTERT immortalized human foreskin fibroblasts (BJ/hTERT) at PD 53. Cells were seeded at 30% confluency and infected with adenovirus in 1% serum containing media for 24 hours. Cells were incubated for 48 hours in 10% media followed by a second adenovirus infection for 24 hours. Serum free media was added and cells were incubated for an additional 60 hours. Cell were fixed for analysis of morphological observations (top panel) or stained for SA-β-gal positive cells (bottom panel). Cells were photographed at 20x magnification phase contrast microscopy. Percentages represent average of 3 100 cells counts, n=2. (*,p ≤0.05) Overexpression of IGFBP-3/R can induce senescence despite expression of telomerase, as detected by increases SA-β-gal positive cells.

BJ cells at PD27. This finding suggests differential regulation of IGFBP-3 signaling based on cell type. From a gene expression perspective, overexpressing IGFBP-3, IGFBP-3 ^{GGG}, IGFBP-R resulted in the significant upregulation of senescent associated genes p53, p21, and p16. Surprisingly, we could not detect phosphorylated p38 in both BJ and BJ/hTERT fibroblasts although there does not appear to be a change in total p38 protein (Figure 18A, B, respectively). This may suggest IGFBP-3 induced senescence in BJ fibroblasts is not entirely dependent on p38 MAPK or the time point to evaluate p38 activity needs to be investigated. Another speculation is p38 levels are difficult to detect in these particular cells.

In conclusion, we have identified upregulation of IGFBP-3 and IGFBP-3R in replicatively senescent HFL-1 cells. We further demonstrated IGFPB-3, IGFBP-3^{GGG} and IGFBP-3R can induce premature senescence, as indicated by senescent morphology changes, increased SA-β-gal positive cells, and increased expression of senescence-associated genes. Moreoever, we demonstrate IGFBP-3/IGFPB-3R can signal p38 MAPK pathway activation. IGFBP-3/IGFBP-3R can also induce senescence independent of telomerase activity. The suppression of endogenous IGFBP-3 inhibits senescence induction in HFL-1 cells and reverses the senescence phenotype in WS fibroblasts. For the first time, we have demonstrated IGFBP-3 regulation on p38 MAPK signaling, as IGFBP-3 upregulation increases p38 activity and suppression of IGFBP-3 inhibits its upregulation. How IGFBP-3 is upregulated in senescent cells and how IGFBP-3 interacts with p38 signaling pathway remains to be elucidated.

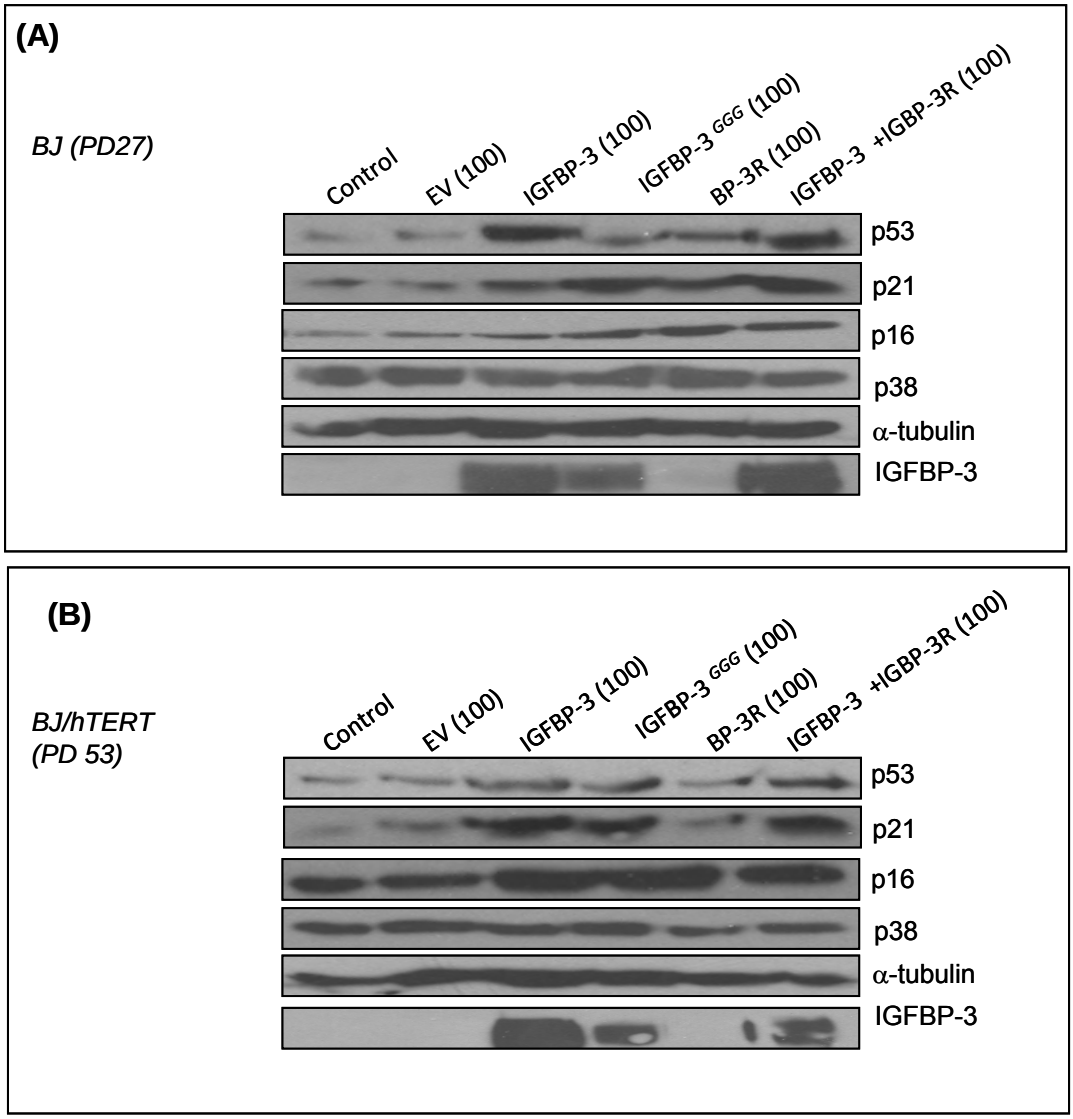


Figure 18: **Expression of senescence associated genes in IGFBP-3-induced senescence BJ (PD27) cells and BJ/hTERT (PD53) immortalized.** BJ and BJ/hTERT cells were seeded at 30% confluency and adenovirus infected in 1% serum containing media for 24 hours. Cells were incubated for 48 hours in 10% media followed by a second adenovrius infection for 24 hours. Serum free media was added and cells were incubated for an additional 60 hours. Cell lysates were harvested and analyzed for SA gene expression by western blot, n=2. (A) BJ cells (B) BJ/hTERT cells Both BJ and hTERT expression BJ cells have upregulated expression of SA-genes following senescence induction by overexpression of IGFBP-3 and IGFBP-3R.

Chapter 4

Potential mechanisms for IGFBP-3-induced senescence and IGFBP-3 upregulation

4.1 Introduction and Rationale

Senescence is a complex cell process, therefore it is unlikely to result from activation of single pathway, but rather from collaborative changes in multiple pathways, that can be triggered by diverse stimuli. Although the characteristics of senescence, such as growth arrest, morphological changes, and changes in gene expression are indistinguishable, the mechanisms that induce these characteristics have not been entirely elucidated.

In this study, we demonstrated IGFBB-3 and IGFBP-3R expression is upregulated in replicative senescence and furthermore, overexpression of IGFBP-3 and/or IGFBP-3R can induce premature senescence in normal diploid fibroblasts. Although several other studies suggest involvement of IGFBP-3 in senescence, the mechanism(s) by which IGFBP-3 expression is regulated senescence or what pathway(s) IGFBP-3 can signal to induce senescence have yet to be identified. Interestingly, when we induced senescence by overexpressing IGFBP-3 or IGFBP-3R, in addition to upregulated senescence associated genes, we identified p38 MAPK activation in our cell system p38 has been described as causative and a conserved

pathway in senescence signaling, as it's activation is signaled by diverse stimuli, including telomere shortening, oncogene overexpression and oxidative-stress. Although the upstream stimuli induced activation of different pathways, p38 appears to be a conserved downstream target. However, p38 signaling can induce multiple downstream effectors depending on its upstream inducer in order to elicit a range cell process such as cell growth inhibition, apoptosis or senescence (Iwasa, *et al.,* 2003). The conserved characteristic of p38 signaling is further confirmed p38 activation appears to be a critical regulator of senescence in both mouse and human fibroblasts, despite the fact that both these cells have significantly different contributions of p53 and Rb signaling (Iwasa, *et al.,* 2003). Characterization p38 signaling, as being conserved, induced by multiple senescence stimuli and its ability to signaling various downstream targets, suggest a possible relationship with IGFBP-3/IGFBP-3R signaling as IGFBP-3/IGFBP-3R to have similar biological functions. IGFBP-3 is known to be upregulated by multiple senescence stimuli; however, the downstream effects have yet to be unmasked.

As suggested in the free radical theory of aging, several studies have confirmed the significance of oxidative stress and/or the decreased antioxidant defense in regulating biological aging (Harman, 2006). ROS, such as hydrogen peroxide and hydroxyl radicals have various effects vital to biological processes, including cell growth, apoptosis and senescence (Finkkel, 2003). The ability of cells to maintain a balance is crucial for cell viability, as am imbalance between ROS and antioxidants can lead to detrimental effects, such as aging. However, cells aim to maintain a balance between oxidative stress and anti-oxidant defenses (Finkkel, 2003). Several studies have shown that oxidative-stress-induced senescence can increase IGFBP-3 production, but

it is not entirely known whether IGFBP-3 is an upstream activator of ROS or a downstream effecter of ROS signaling. (Debacq-Chainiaux, *et al.,* 2008).

IGFBP-3 has long been studied as a primary regulator of IGF signaling; however IGFBP-3's IGF-independent effects are known signal growth cell inhibition and apoptosis in various cell lines. Our laboratory recently published the identification of novel IGFBP-3R that regulates IGFBP-3-induced caspase-8 dependent apoptosis in cancer cells (Ingermann, *et al.,* 2010). The data from this publication indicates IGFBP-3 signaling is chiefly mediated through IGFBP-3R, as IGFBP-3R suppression can inhibit the apoptotic effects of IGFBP-3.

By exploiting the significance of ROS signaling and p38 signaling, we aimed to determine how the IGFBP-3/IGFBP-3R axis may be interacting between these two senescence regulating processes. In our initial studies, we demonstrate a significant role of IGFBP-3/IGFBP-3R axis in senescence both replicative and prematurely induced. In order to better understand this pathway, we will need to examine how IGFBP-3 is being upregulated and/or suppressed in senescent cells and young normal cells, respectively. Taking into account the significance of ROS in organismal aging and senescence signaling and the identification of IGFBP-3/IGFBP-3R regulation of p38, we propose a possible relationship between ROS, p38 and IGFBP-3/IGFBP-3R signaling.

4.2 Results

4.2.1 Upregulation of ROS production in IGFBP-3 induced senescence

In Chapter 3, we clearly demonstrate IGFBP-3/IGFBP-3R can induce senescence, and more over cause p38 MAPK activation. Next we aimed to investigate if IGFBP-3-induced senescence could cause an increase in intracellular ROS production. To measure ROS production we quantified levels of 2'7'-dichlorofluorescein diacetate (DCF-diacetate). DCF-A can readily cross the cell membrane and become intracellular hydrolyzed by esterases to non-fluorescent DCF, which is then rapidly oxidized by ROS to produce the fluorescent DCF product that can be quantified by flow cytometry. Fluorescence emitted by the oxidation products of DCF allows for the estimation of ROS levels *in vitro*. IGFBP-3 was induced as previously described, and following senescence induction cells were treated with DCF for 30 minutes and analyzed by FACS. Control and empty vector treated cells showed 15% and 16% fluorescence, respectively (Figure 19). IGFBP-3, IGFBP-3^{GGG} and IGFBP-3R adenovirus treated cells showed significantly higher percentages of fluorescence at 68%, 51% and 33%, respectively. Treatment of cells with H_20_2 for 2 hours prior to DCF treatment was used as a positive control, showing 73% fluorescence (Figure 19). We have shown for the first time that IGFBP-3/IGFBP-3R-induced senescence can significantly increase ROS production.

Under normal cellular regulation, ROS accumulation can be combated by antioxidant enzymes used to detoxify free radical species, such as catalase and MnSOD. Glutathione (GSH) is an additional antioxidant that prevents ROS damage by

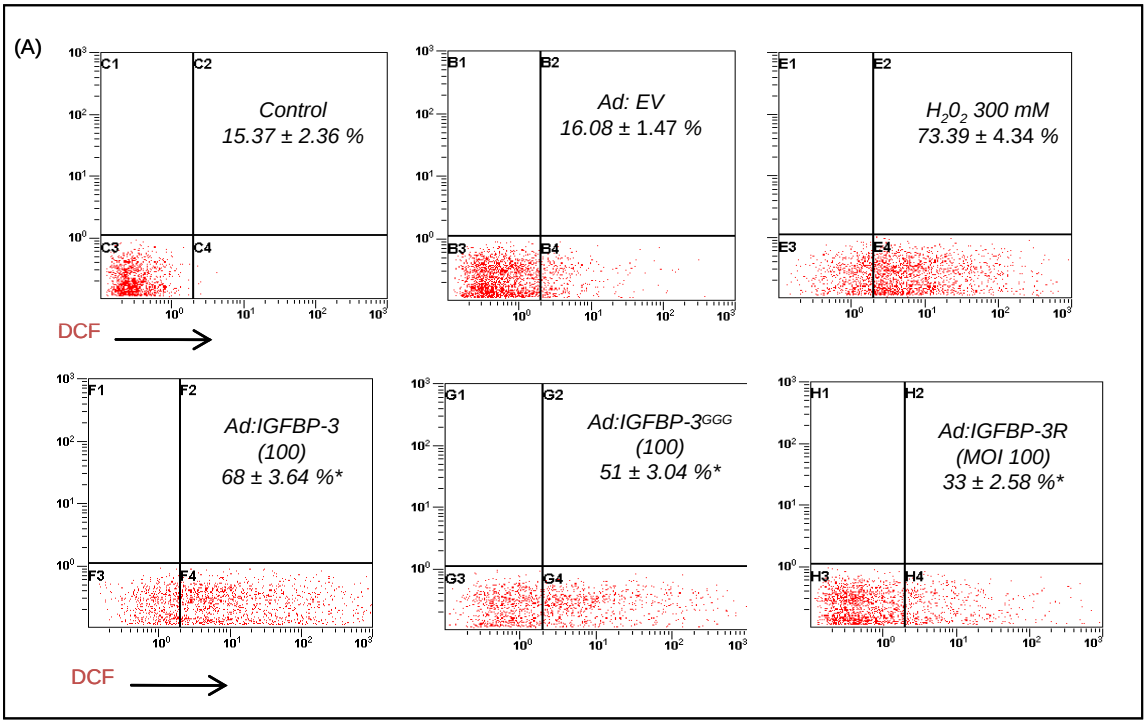
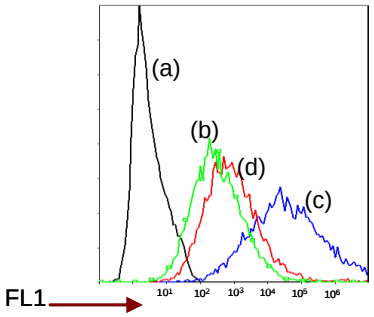


Figure 19: IGFBP-3-induced senescence in HFL-1 cells increases intracellular ROS production. HFL-1 (PD7) cells were adenovirus infected twice with EV, IGFBP-3, IGFBP-3^{GGG} or IGFBP-3R at MOI 100 for a total of 7 days in culture. (A) Intracellular ROS levels were measured by FACS analysis after staining with the fluorescent probe DCF. A 2 hour treatment with 300 μM H_20_2 was used as positive control. Percentage represents average fluorescence of duplicate samples, n=3. Statistical significance was measured between control and treated cells. (*, p≤0.05) IGFBP-3/IGFBP-3R-induced senescence causes an increased accumulation of ROS production detected by FACS.

scavenging free radicals. GSH is commonly used in vitro to study the antioxidant tide that can donate an electron to unstable molecules, such as ROS.

If IGFBP-3 and IGFBP-3R increased ROS production following senescence induction, we examined if GSH treatment could prevent both ROS accumulation and IGFBP-3 induced senescence. Two hours prior to adenovirus treatment and on day 6 of 7 day treatment, cells were incubated with 10mM GSH. FACS analysis indicated that GSH could inhibit ROS production. As seen in Figure 20, cells infected with adenovirus IGFBP-3 or IGFBP-3^{GGG} in the presence of GSH had a shift towards the left at similar levels to control and empty vector infected. The shift represents a decreased accumulation of ROS compared to cells that did not received GSH, but underwent senescence following adenovirus treatment. To evaluate senescence inhibition cells were stained for activity. In the presence of GSH, IGFBP-3 or IGFBP-3^{GGG} infected cells showed decreased number of SA-β-gal positive cells, from 80% to 48% and 75% to 46%, respectively (Figure 21, bottom panel). Moreover, senescence morphological changes were not exhibited with ROS inhibition by GSH treatment (Figure 21, top panel). This data indicates GSH inhibits ROS accumulation and prevents IGFBP-3-induced senescence.

As we have demonstrated IGFBP-3-induced senescence caused upregulation of senescence-associated genes, we investigated if this upregulated expression would be inhibited by GSH treatment. GSH treatment significantly inhibited IGFBP-3-induced upregulation of p53, p21 and p16 compared to cells without GSH treatment (Figure 22A-C). We speculated ROS upregulation was inducing p38 activation; therefore we

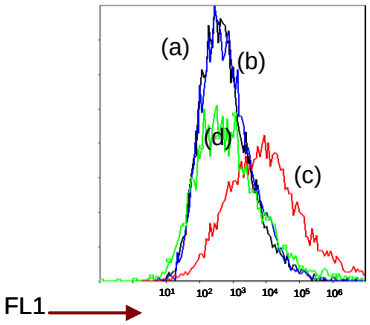


(a) Unstained cells

(b) Control

(c) H202 300 μM

(d) H202 300μM + GSH 10 mM

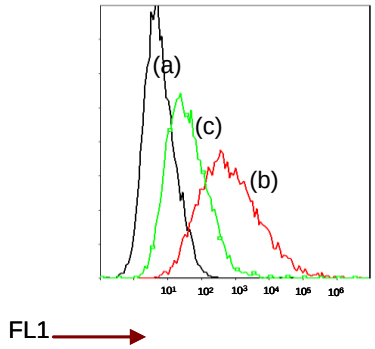


(a) Control

(b) Ad: EV (100)

(c) Ad: IGFBP-3 (100)

(d) Ad: IGFBP-3 (100) + GSH (10 mM)



(a) Ad: EV (100)

(b) Ad: IGFBP-3^{GGG} (100)

(c) Ad: IGFBP-3^{GGG} (100) + GSH (10 mM)

Figure 20: ROS accumulation in IGFBP-3-induced senescence is inhibited by treatment with 10 mM GSH, analyzed by FACS. HFL-1 (PD7) cells were adenovirus infected twice with EV, IGFBP-3 , IGFBP-3^{GGG} or at MOI 100 for a total of 7 days in culture.2 hours prior to each adenovirus infection and on day 6, cells were incubated with 10 mM GSH. (A) Intracellular ROS levels were measured by FACS analysis after staining with the fluorescent probe DCF. A 2 hour treatment with 300 μM H_20_2 was used as positive control. Graph represents n=3, of duplicate samples. ROS accumulation following IGFBP-3-induced senescence is inhibited by GSH treatment.

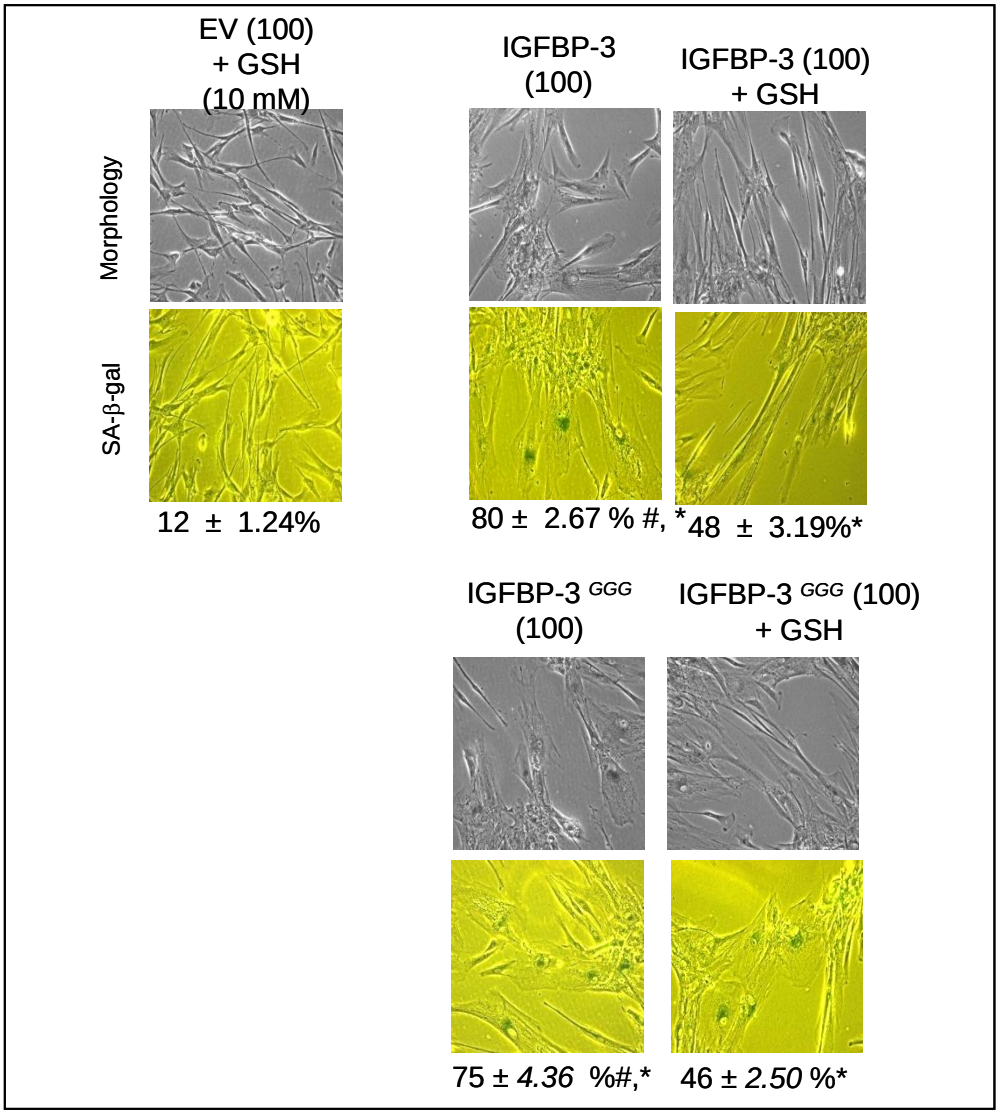


Figure 21: Inhibition of ROS accumulation, by treatment with 10 mM GSH, in IGFBP-3-induced senescence. HFL-1 (PD7) cells were adenovirus infected twice with EV, IGFBP-3, IGFBP-3^{GGG} all at MOI 100 for a total of 7 days in culture. 2 hours prior to each adenovirus infection and on day 6, cells were incubated with 10 mM GSH. Cell were fixed for analysis of morphological observations (top panel) or stained for SA-β-gal positive cells (bottom panel). Cells were photographed at 20x magnification phase contrast microscopy. Percentages represent average of 3 100 cells counts, n=2. ($p \leq 0.05$) #, represent statistical significance compared to empty vector infected cells. *, represents significant suppression compared to adenovirus infection without GSH treatment. GSH ihibits IGFBP-3-induced senescence, as demonstrated by decreased SA-β-gal positive cells.

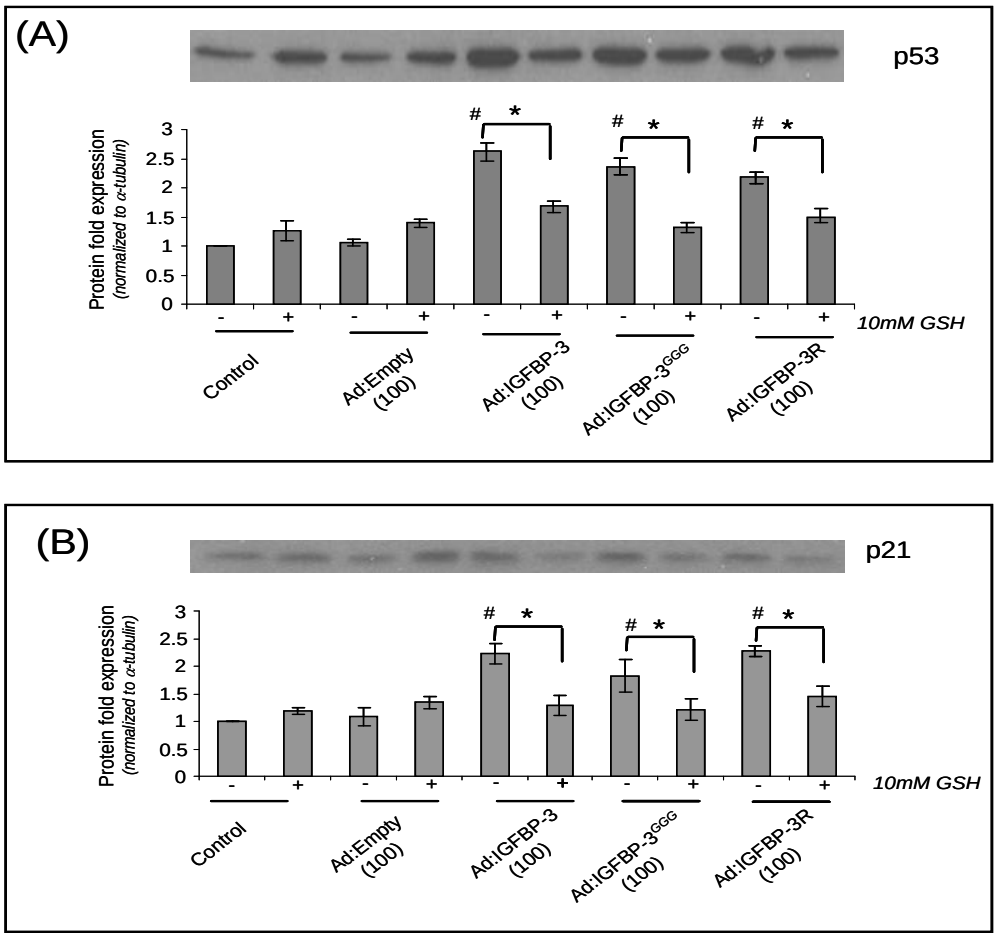


Figure 22: Senescence associated protein expression with 10 mM GSH, in IGFBP-3-induced senescence. HFL-1 (PD7) cells were adenovirus infected twice with EV, IGFBP-3 , IGFBP-3*GGG* or IGFBP-3R at MOI 100 for a total of 7 days in culture.2 hours prior to each adenovirus infection and on day 6, cells were incubated with 10 mM GSH. Cell lysates were harvested and analyzed for SA gene expression (A) p53,and (B) p21by western blot. Densitometric analyses of triplicate samples were measure and graphed. α-tubulin was used as loading control. ($p \leq 0.05$) #, represent statistical significance compared to control cells. *, represents significant suppression compared to adenovirus infection without GSH treatment. SA-gene are upregulated in IGFBP-3/R-induced senescence, however GSH treat can prevent this upreulation and inhibit senescence.s

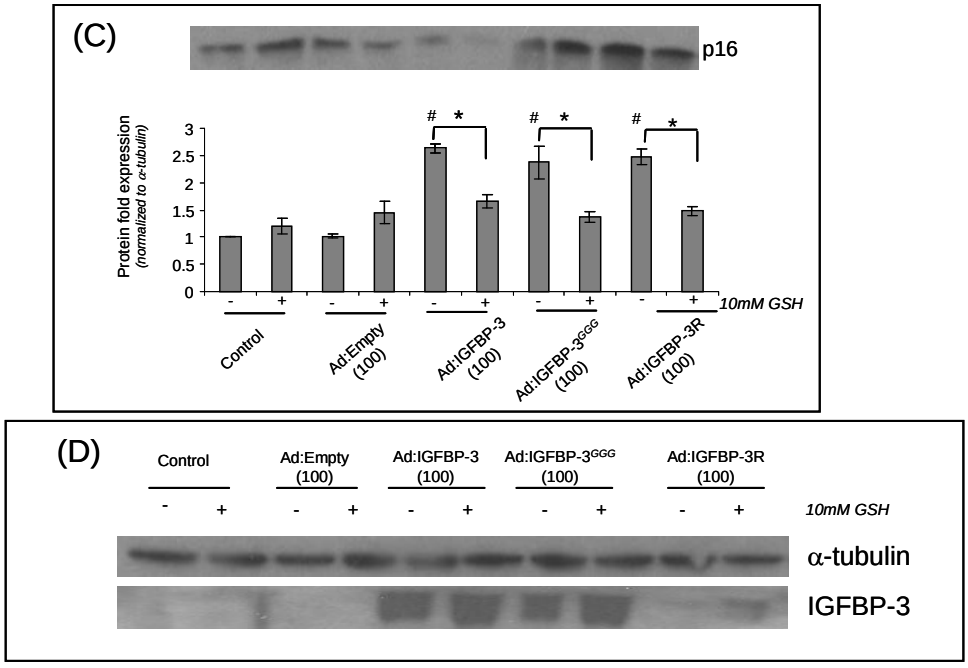


Figure 22: Senescence associated protein expression with 10 mM GSH, in IGFBP-3-induced senescence. HFL-1 (PD7) cells were adenovirus infected twice with EV, IGFBP-3, IGFBP-3^{GGG} or IGFBP-3R at MOI 100 for a total of 7 days in culture.2 hours prior to each adenovirus infection and on day 6, cells were incubated with 10 mM GSH. Cell lysates were harvested and analyzed for SA gene expression (C) p16 and (D) IGFBP-3 by western blot. Densitometric analyses of triplicate samples were measure and graphed. α-tubulin was used as loading control. ($p \leq 0.05$) #, represents statistical significance compared to control cells. *, represents significant suppression compared to adenovirus infection without GSH treatment. SA-gene expression is upregulated in IGFBP-3/R-induced senescence, however GSH treat can prevent this upreulation and inhibit senescence.

examined both total and phosphorylated p38 in the presence of GSH (Figure 23). Densitometry measurement revealed a significant increase in phosphorylated p38, as the levels of total p38 remained unchanged (Figure 23). These data suggest IGFBP-3/IGFBP-3R signaling pathway can induce ROS accumulation and downstream p38 signaling. This signaling sequence, IGFBP-3→ROS→p38→senescence is confirmed as inhibition of ROS accumulation using GSH treatment inhibits p38 signaling and subsequently, senescence.

To evaluate the significance of p38 signaling in IGFBP-3-induced senescence, we examined if p38 inhibition could inhibit IGFBP-3–induced senescence. Similar to treatment with GSH, the p38 inhibitor SB203580 was used two hours prior to adenovirus infection and a third treatment on day 6. Cell lysates were analyzed by western blot. We observed a significant decrease in the SA genes, (Figure 24). In Figure 25, we show a suppression of phosphorylated p38, but not total p38 protein, following IGFBP-3 induced in the presence of SB203580. Taken together, we demonstrated IGFBP-3/IGFBP-3R-induced senescence was inhibited by p38 inhibition, suggesting p38 signaling is a crucial downstream target of in IGFBP-3 signaling pathway.

4.2.2 miRNA involved in IGFBP-3 regulation

The regulation of gene expression in response to senescence stimuli are numerous and complex, from transcription and translational levels to protein stability.

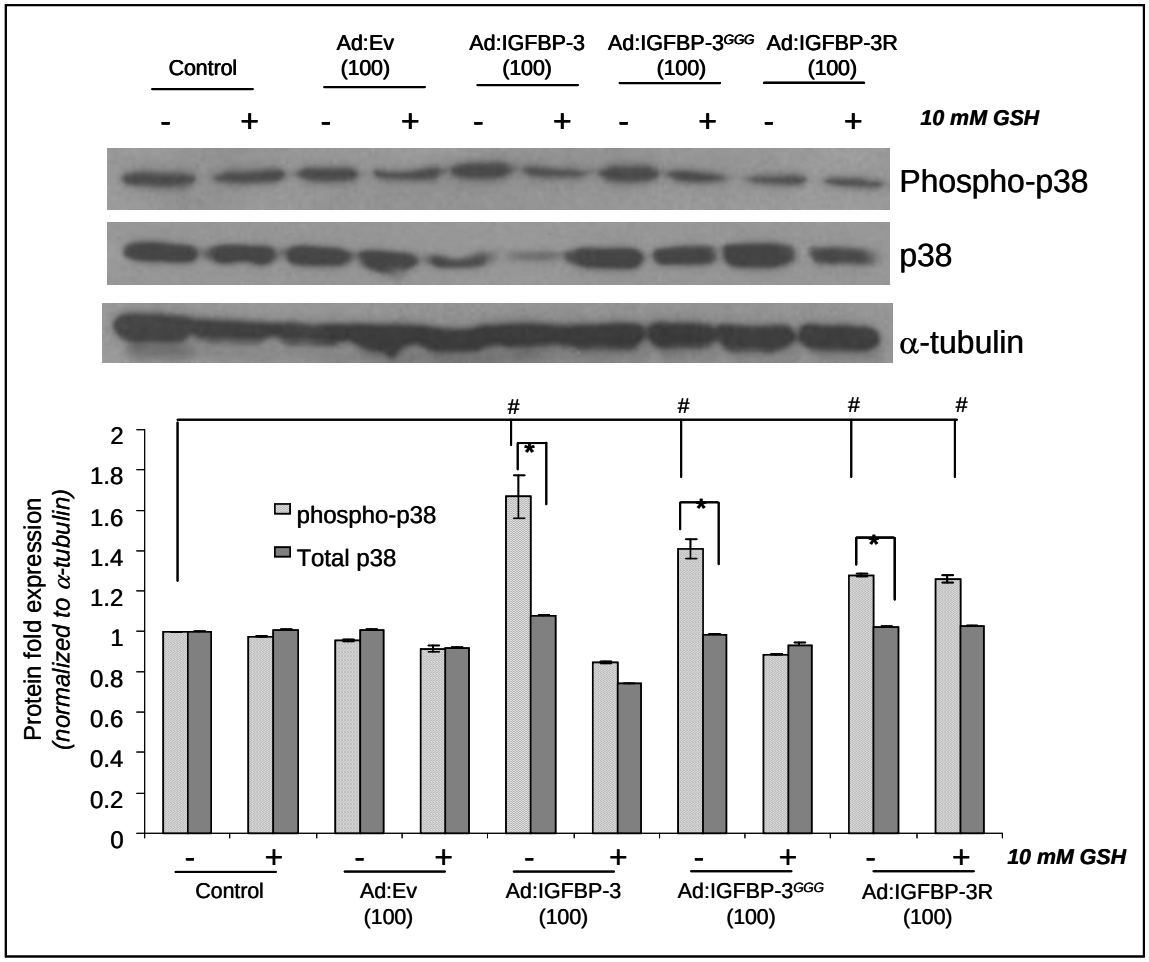


Figure 23: p38 MAPK signaling with 10 mM GSH during IGFBP-3-induced senescence. HFL-1 (PD7) cells were adenovirus infected twice with EV, IGFBP-3, IGFBP-3^{GGG} or IGFBP-3R at MOI 100 for a total of 7 days in culture.2 hours prior to each adenovirus infection and on day 6, cells were incubated with 10 mM GSH. Cell lysates were harvested and analyzed for SA gene expression p38 MAPK signaling by western blot. Densitometric analysis of triplicate samples were measured and graphed. α-tubulin was used as loading control. ($p \leq 0.05$) #, represents significance compared to control cells, and *, represents statistical analysis between adenovirus treated in absence or presence of GSH. #, represent statistical significance compared to control cells. *, represents significant suppression compared to adenovirus infection without GSH treatment. p38 MAPK signaling is upregulated in IGFBP-3/R-induced senescence, however GSH treat can prevent this upreulation and inhibit senescence

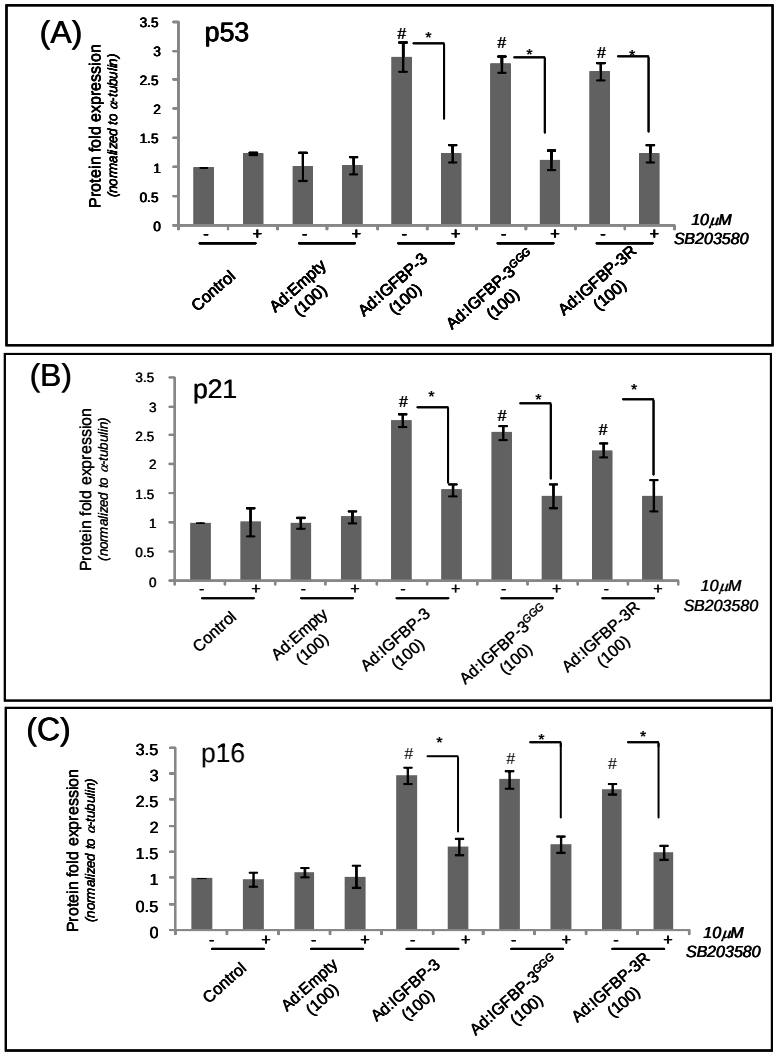


Figure 24: Senescence associated protein expression with 10 μM pSB203580, in IGFBP-3-induced senescence. HFL-1 (PD7) cells were adenovirus infected twice with EV, IGFBP-3, IGFBP-3^{GGG} or IGFBP-3R at MOI 100 for a total of 7 days in culture. 2 hours prior to each adenovirus infection and on day 6, cells were incubated with 10 mM GSH. Cell lysates were harvested and analyzed for SA gene expression (A) p53, (B) p21, and (C) p16. Densitometric analyses of triplicate samples were measure and graphed. α-tubulin was used as loading control. ($p \leq 0.05$) #, represent statistical significance compared to control cells. *, represents significant suppression compared to adenovirus infection without SB203580 treatment. SA-gene expression is upregulated in IGFBP-3/R-induced senescence; however inhibition of p38, by SB203580, can prevent this upreulation and inhibit senescence.

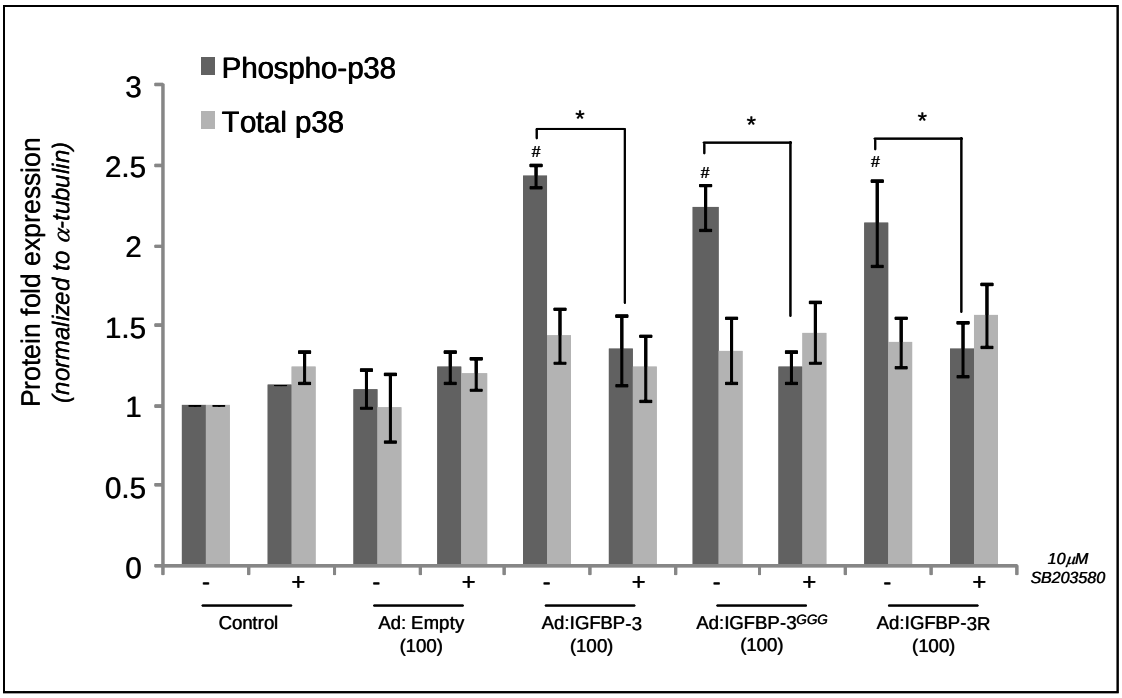


Figure 25: p38 MAPK signaling after expression with 10 mM pSB203580, in IGFBP-3-induced senescence. HFL-1 (PD7) cells were adenovirus infected twice with EV, IGFBP-3 , IGFBP-3^{GGG} or IGFBP-3R at MOI 100 for a total of 7 days in culture.2 hours prior to each adenovirus infection and on day 6, cells were incubated with 10 mM GSH. Cell lysates were harvested and analyzed for SA gene expression p38 MAPK signaling by western blot. Densitometric analyses of triplicate samples were measure and graphed. α-tubulin was used as loading control. ($p \leq 0.05$) #, represent statistical significance compared to control cells. *, represents significant suppression compared to adenovirus infection without GSH treatment. p38 MAPK signaling is upregulated in IGFBP-3/R-induced senescence, however inhibition of p38, by SB203580, can prevent this upreulation and inhibit senescence.

Although it has been almost 20 years since microRNAs (miRNA) have been discovered, it has only been in the last 10 years that miRNAs were recognized to have distinct post-transcriptional regulatory functions. miRNas are small, noncoding RNAs that regulate gene expression by targeting mRNAs for translational repression (Bartel, 2009). The recent surge in biological functional studies of miRNAs presents an additional mechanism by which genes with diverse function on multiple pathways may be regulated. miRNAs regulate gene expression at the post-trasncriptional level affecting translational and stability of miRNAs (Bartel, 2009). RNA piolymerase II transcribes miRNA and the primary transcript is cleaved by Drosha ribunuclease III enzyme to produce an approximately 70-nt stem loop (Bartel, 2004). Subsequently, the stem loop is cleaved by cytoplasmic Dicer to generate a noncoding mature miRNA product, approximately 20-24 nt long (Bartel, 2009). miRNAs are incorporated into RNAs to induce silencing complex (RISC) which target the 3' UTR of target mRNAs through base pairing with the miRNA (Bartel, 2004). Although there are few exceptions, most miRNAs inhibit translational or destabilization of the target mRNA.

Lafferty-Whyte *et al*., (2009) recently identified senescence-associated miRNA targets that suggested a common process that regulates different senescence induction. After analysis of miRNAs upregulated following various senescence inducing pathways, miR-499 and miR34a were the two common regulators of all senescence stimuli (Lefferty-Whyte, *et al.,* 2009).

Using TargetScanS and PicTar software, sequence alignment predictions identified four highly and three poorly conserved miRNAs targets of the 3'-UTR region of

IGFBP-3 (Figure 26). p53 is known to be a primary regulator of cellular senescence, yet the mRNA expression pattern differs between replicative senescence and premature senescence (Shelton, *et al.,* 1999 and Komarova, *et al.,* 1998). miR-34a is commonly referred to as tumor suppressor and known to be regulated by p53 (Kumamoto, *et al.,* 2008). Studies have shown that miR-34s (*mir-34a, mir-34b*, and *mir-34c*) are downstream effectors of p53-dependent senescent and apoptotic pathways (He *et al.,* 2007 and Chang, *et al.,* 2007). To date little data has been published regarding the biological function of miR-381, although there are over 300 predicated target genes. Currently, there are over 600 genes with predicated miR-19a target sequences. miR-19a is a member of miR-17–92 cluster of seven miRs (miR-17–5p, miR-17–3p, miR-18, miR-19a, miR-20, miR-19b-1, and miR-92-1) (Quin, *et al*., 2009).

After sequence analysis, we selected 3 of the highly conserved predicated miRNA targets, miR-19a, miR-381 and miR-34 and examined the expression levels of each miRNA in HFL-1 cells at PD6 and PD48 (Figure 27). Although previous reports have shown increased miR34a expression in replicative and prematurely-induced senescent cells, we found no significant upregulation in our cell system. In addition, miR-381 did not appear to be differentially expressed between cell PD. We did however note an 80% significant decrease in miR-19a expression correlated to increased thatIGFBP-3 mRNA expression in late passage HFL-1 cells (Figure 27).

As miR19a was significantly downregulated in correlation to increased IGFBP-3 levels, we aimed to evaluate the effects of upregulated miR-19a in late passage fibroblast. HFL-1 (PD45) cells were transfected with pre-miR19a or pre-miR-FAM

(A)

Position 43-49 of IGFBP3 3' UTR	Predicted consequential pairing of IGFBP-3(top) and miRNA (bottom)	Seed Match
Position 43-49 of IGFBP3 3' UTR	5' ...UCAAAUAUGCCUUAUUUUGCACA... ||| ||||||| * hsa-miR-19b 3' AGUCAAAACGUACCUAAACGUGU	8mer
Position 43-49 of IGFBP3 3' UTR	5' ...UCAAAUAUGCCUUAUUUUGCACA... ||| ||||||| * hsa-miR-19a 3' AGUCAAAACGUAUCUAAACGUGU	8mer
Position 55-61 of IGFBP3 3' UTR	5' ...UAUUUUGCACAAAAGACUGCCAA... |||||| * hsa-miR-34a 3' UGUUGGUCGAUUCUGUGACGGU	7mer-1A
Position 131-137 of IGFBP3 3' UTR	5' ...ACUGAUUUUUUUUAAACCAAAGU... ||||||| * hsa-miR-9 3' AGUAUGUCGAUCUAUUGGUUUCU	7mer-m8
Position 55-61 of IGFBP3 3' UTR	5' ...UAUUUUGCACAAAAGACUGCCAA... |||| |||||| hsa-miR-449a 3' UGGUCGAUUGUUAUGUGACGGU	7mer-1A
Position 55-61 of IGFBP3 3' UTR	5' ...UAUUUUGCACAAAAGACUGCCAA... |||| |||||| hsa-miR-449b 3' CGGUCGAUUGUUAUGUGACGGA	7mer-1A
Position 55-61 of IGFBP3 3' UTR	5' ...UAUUUUGCACAAAAGACUGCCAA... |||||| hsa-miR-34c-5p 3' CGUUAGUCGAUUGAUGUGACGGA	7mer-1A

(B)

	PicTar score	PicTar score per species	microRNA	Probabilities	Nuclei mapped to alignment	Nuclei mapped to sequence	Free Energies kcal/mol	Structure of predicted duplex
*	2.36	2.39	hsa-miR-34a	0.91	70	55	-19.9	__GCC_____GC___AAGAC__UGCCA :__UGG_____CG___UUCUG__ACGGU_
*	2.27	2.13	hsa-miR-19a	0.88	59	44	-18.6	_____UGC____UUUGCACA :_____ACG____AAACGUGU_
*	2.08	1.61	hsa-miR-381	0.8	1290	1122	-24.6	_GCAGAGA_G___UGUUCUUGU___:_UGUCUCU_C___ACGGGAACA___
*	2.27	2.13	hsa-miR-19b	0.88	59	44	-18.6	_____UGC____UUUGCACA :_____ACG____AAACGUGU_

Figure 26: The predicted sequence alignment of miRNAs targeted to the 3'-UTR region of IGFBP-3. Predicated targest generated from (A) TargetScanS and (B) PicTar software (*, highly conserved).

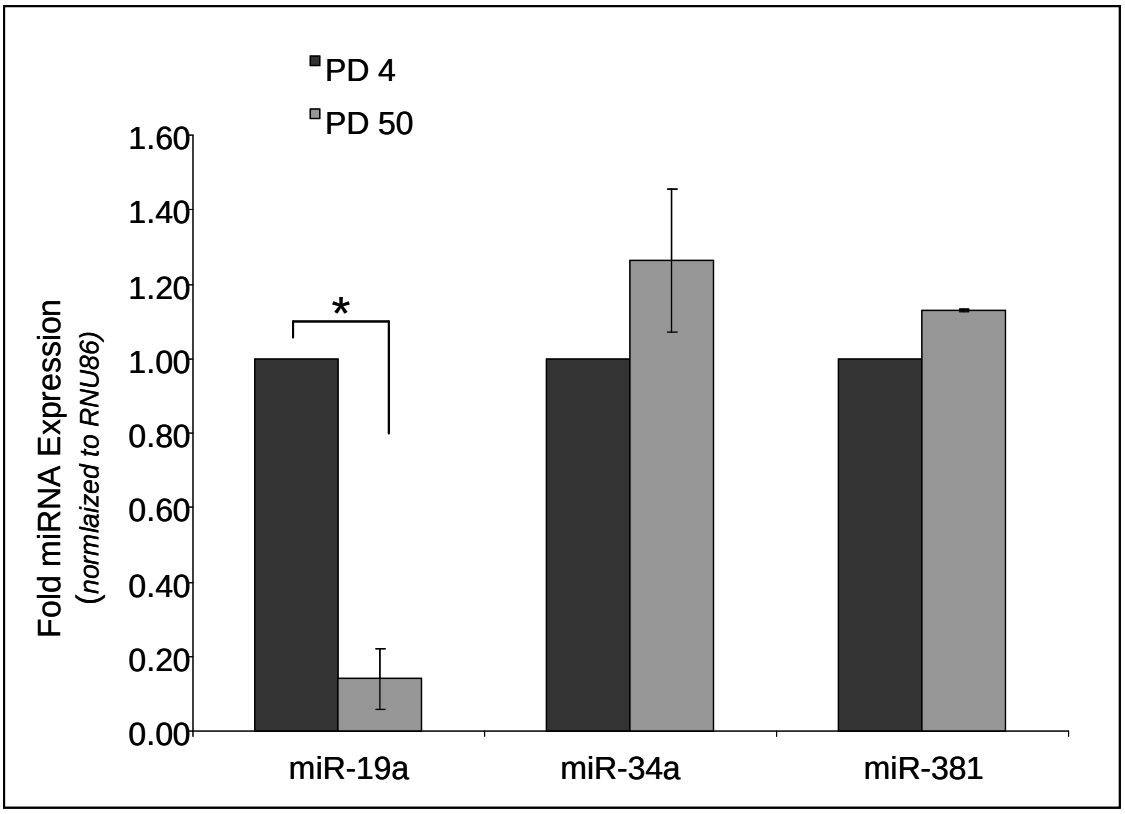


Figure 27: miRNA expression levels in normal versus replicatively senescent cells. HFL-1 cells at PD 6 and PD 48 were seeded at 60% confluency. Two days later RNA was isolated and miRNA levels were evaluated in triplicate, n=3. miRNA RNU6B was used as loading control. (*, p≤0.05) miR-19a is significantly decreased in senescent HFL-1 cells.

labeled negative control for 12 hours before media was replaced with serum-free media for an additional 48 hours. mRNA expression measured by qRT-PCR indicated almost 25-fold increase in miR19a expression (Figure 28A). Interestingly, IGFBP-3 mRNA expression was significantly decreased by nearly 30% compared to controls (Figure 28B). The secretion of IGFBP-3 protein was analyzed by western blot following culture media collection (Figure 28C). Taken together, the data supports miR-19a can suppress IGFBP-3 expression and appears to be downregulated in replicatively senescent cells where IGFBP-3 expression is significantly upregulated. The next possible experiment to analyze the regulation of mir-19a on IGFBP-3-induced senescence would be to evaluate if constitutively expressed mir-19a could significantly suppress IGFBP-3 inhibiting replicative senescence. Furthermore, can mir-19a expression inhibit IGFBP-3-induced senescence in early passage fibroblasts?

4.2.3 IGFBP-3R signaling in IGFBP-3-induced senescence

IGFBP-3 has long been studied to have IGF-independent biological functions, although the mechanism involved in independent effects are largely unknown. Furthermore, IGFBP-3 IGF-independent effects appear to be dependent on cellular localization and cell type, further complicating our understanding. Our lab has recently reported the identification a new cell death receptor, IGFBP-3R, which is a single-span membrane protein that binds IGFBP-3 specifically, and no other IGFBP species in breast cancer (MDA231) and prostate cancer cells (M12) (Ingermann, *et al.,*2010). The study concluded IGFBP-3/IGFBP-3R axis is impaired in breast and prostate cancer,

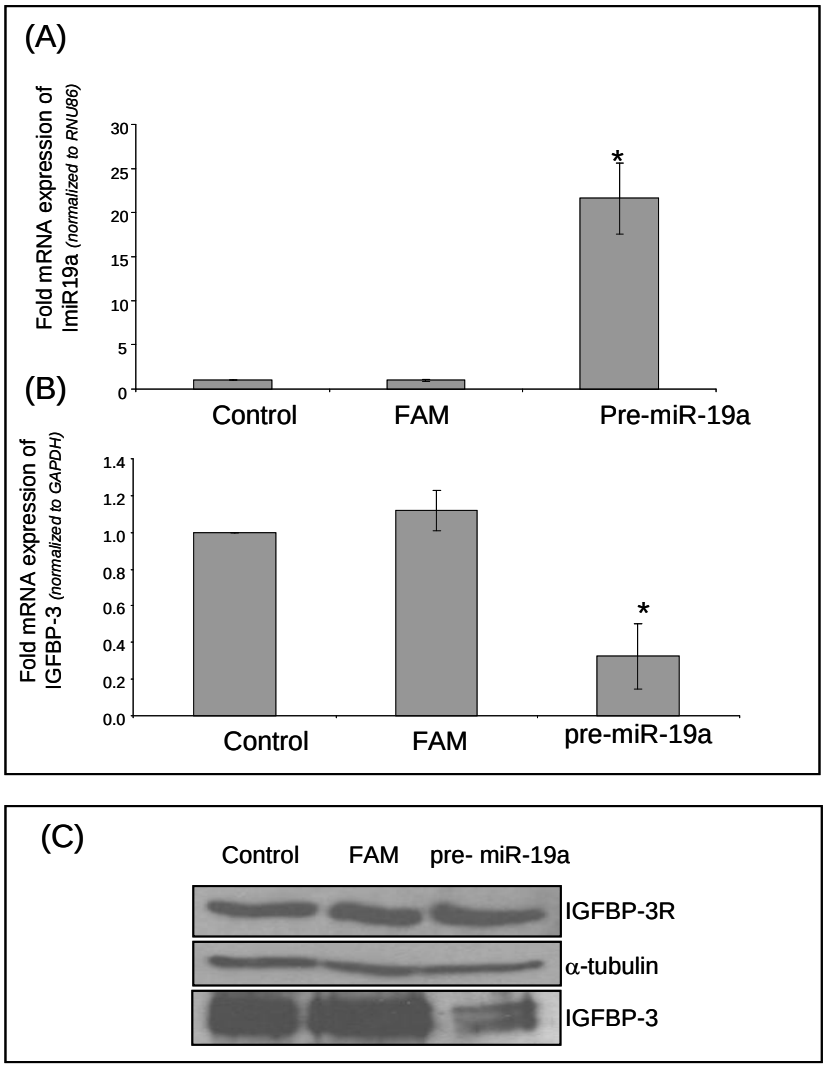


Figure 28: Transfection of pre-miR-19a in HFL-1 (PD 45). HFL-1 cells were seeded at 70% confluency. Cells were transfected with either FAM-labeled control miRNA or pre miR19a for 12 hours in serum containing. Media was replaced with serum free media and cells were incubated for an additional 48 hours before RNA isolation or cell harvesting. (A) qRT-PCR analysis of miR-19a, using RNU86 as an internal control and IGFBP-3 expression, using GAPDH as an internal control levels,n=2. (C) Proteins were analyzed by western blot, α-tubulin was used as a loading control. IGFBP-3 secretion was measured from culture media. Statistical significance was noted between the control and treated samples. (*, p≤0.05) miR-19a suppression IGFBP-3 expression.

supporting previous data for the anti-proliferative effect of IGFBP-3 in cancer cells (Ingermann, *et al.,* 2010). Furthermore, IGFBP-3R mediates IGFBP-3 induced capase-8-dependent apoptosis. Suppression of IGFBP-3R prior to overexpressing IGFBP-3 inhibits the proapoptotic effect of IGFBP-3 in MDA231 cells (Ingermann, *et al.,* 2010). This data established a novel IGFBP-3 binding partner that plays an essential role in IGFBP-3 signaling. The role of IGFBP-3R in normal cells has yet to bee investigated, until now.

The ability of the IGFBP-3/IGFBP-3R axis to induce or maintain senescence is likely relevance to aging. The principal function of IGFBP-3 is to transport IGFs in order to protect them from rapid degradation until they can bind to IGF receptors and modulate downstream IGF/IGFR signaling. However, reports have shown no significant correlation between aged human species and the ratio of IGF and IGFBP-3 (Fontana, L*et al.,* 2008), contradicting what has previously been reported in rodent studies (Shimokawa, *et al.,* 2008), which studies suggest IGFBP-3 upregulation in aged tissues and cells may be IGF-independent.

The data generated thus far suggest that IGFBP-3/IGFBP-3R axis is an essential pathway regulating senescence replicative and prematurely induced senescence, possibly through ROS production and activation p38 MAPK signaling. With significant expression of IGFBP-3R at PD24 in HFL-1 cells, we employed two different constructs of shRNAs against the novel IGFBP-3R. Knock-down of IGFBP-3R was analyzed by real time-PCR and western blot (Figure 29A and 29B, respectively). An almost 60% and 80% suppression was achieved using IGFBP-3 shRNA 1-3 and 5-3 constructs,

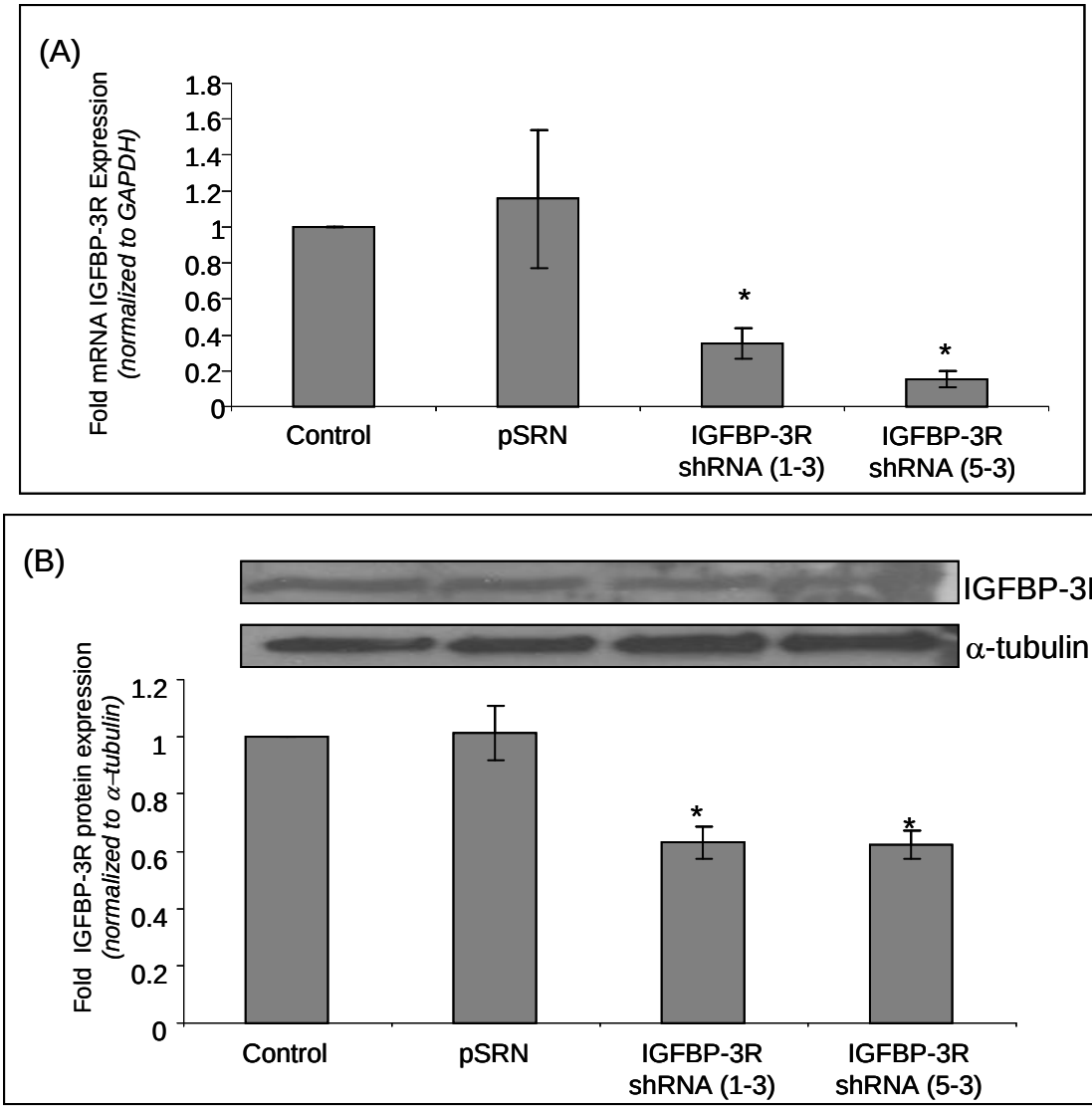


Figure 29: Suppression of IGFBP-3R using viral supernatant. Presecenct cells HFL-1 (PD 24) cells seeded at 50% confluency and exposed to pSRN IGFBP-3R siRNA 1-3 or 5-3 or empty vector retrovirus supernatant for 24 hours before fresh serum containing media was added for an additional 24 hours. Infection was repeated with a second before RNA was isolated and cell lysates were harvested. (A) qRT-PCR analys is IGFBP-3R mRNA expression, GAPDH was used a loading control. (B) Western blot analysis of IGFBP-3R expression, α-tubulin was used a loading control and densitometric measurements were quanitified.IGFBP-3R shRNA (1-3) and (5-3) plasmids suppress endogenous IGFBP-3R expression.

respectively. IGFBP-3R protein levels did not have as large a decrease when analyzed by western blot but still had significant decrease of almost 40% for each shRNA compared to control (Figure 30B).

HFL-1 (PD24) cells were subjected to IGFBP-3R shRNA or empty vector virus supernatant for 24 hours before fresh serum containing media was added for an additional 24 hours. A second infection was performed for an additional 24 hours. Cells were infected with IGFBP-3 adenovirus for 2 days followed by a third shRNA infection for 24 hours. Cells were treated with second IGFBP-3 adenovirus infection and incubated for an additional 48 hours. The number of SA-β-gal positive cells were counted in 3 fields of 100 cells per field, n=2 (Figure 30A). IGFBP-3 overexpressed cells had increased SA-b-gal positive cells, however in the effects of IGFBP-3 were inhibited in the presence of IGFBP-3R shRNA. Furthermore, we evaluated the downstream IGFBP-3-upregulated senescence associated genes p21 (Figure 30B). IGFBP-3R suppression does not completely knockdown IGFBP-3R signaling, as indicated by only a moderate decrease in protein levels. The effects of IGFBP-3 overexpression were not completely inhibited by IGFBP-3R shRNA, however, this may be explained a significant ligand to receptor ratio. As IGFBP-3R overexpression alone can induce premature senescence in early passage HFL-1 cells, this may be possible as early passage cells have low levels of endogenous IGFBP-3. To further evaluate the essential function of IGFBP-3R in IGFBP-3-induced senescence, a complete knockdown of IGFBP-3R expression will need to be achieved, to investigate if IGFBP-3 can still signal senescence.

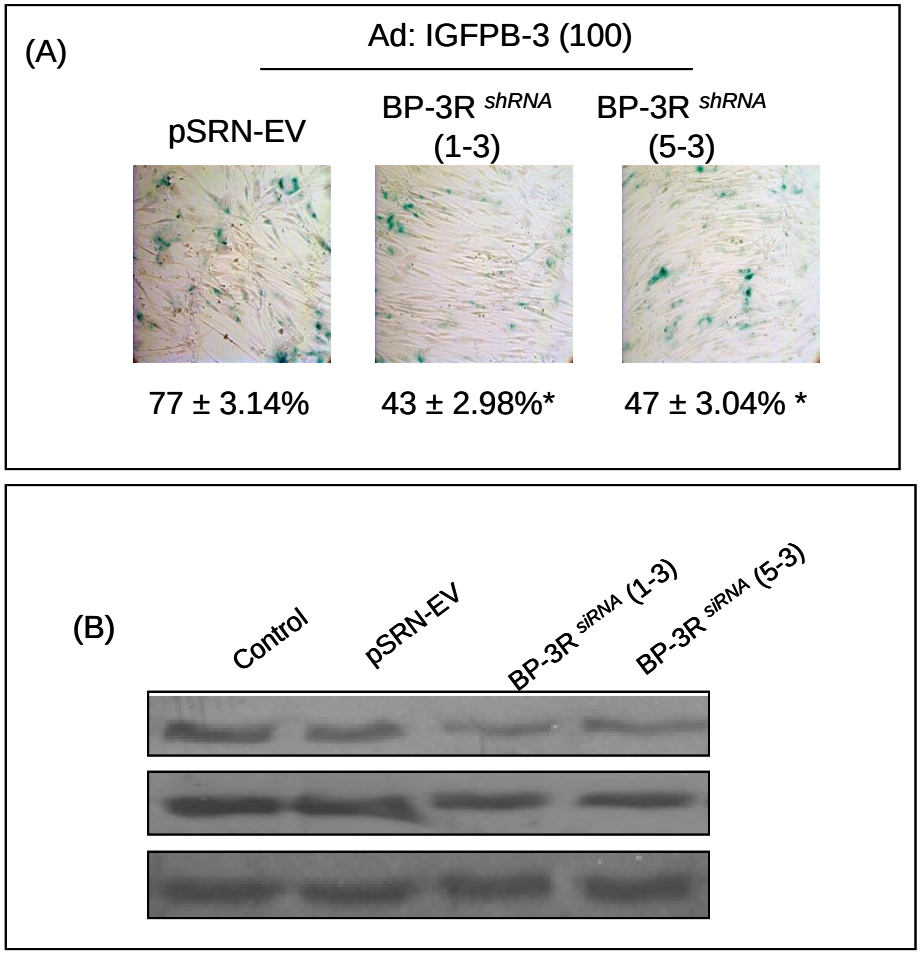


Figure 30: Suppression of IGFBP-3R using shRNA virus supernatant. Presecenct cells HFL-1 (PD 24) cells seeded at 50% confluency and exposed to pSRN IGFBP-3R siRNA 1-3 or 5-3 or empty vector retrovirus supernatant for 24 hours before fresh serum containing media was added for an additional 24 hours. A second infection was performed for an additional 24 hours. Cells were IGFBP-3 adenoviurs infected for 2 days followed by a third shRNa infection for 24 hours. Cells were treated with second IGFBP-3 adenovirus infection. 2 days later cells were either fixed an stained for SA-β–gal (A) activity or lysates were harvested for protein analysis, α-tubulin used as loading control (B). IGFBP-3R shRNA can significnatly inhibited IGFPB-3-induced increases in SA-β-gal positive cells and SA gene, p21. (*, p≤0.05)

4.3 Discussion

This study identifies IGFBP-3/IGFBP-3R-induced senescence can cause an upregulation of ROS production and activation of downstream SA genes. Suppression of ROS accumulation by GSH inhibits IGFBP-3/R-induced senescence and downstream p38 MAPK signaling. Furthermore, IGFBP-3-induced senescence can also be inhibited by suppression p38 MAPK activation. For this first time, we have investigated the role of newly identified IGFBP-3R in regulating senescence. IGFBP-3R overexpression can induce senescence similar; however suppression of endogenous IGFBP-3R inhibits IGFBP-3-induced senescence. This data suggests IGFBP-3R axis is an essential mediator in the IGFBP-3 senescence signaling cascade. As IGFBP-3 is known to be upregulated we investigated a possible regulator targeted responsible for this upregulation. The expression of miR-19a is significantly downregulated in senescent in comparison early passage HFL-1 cells and inversely to IGFBP-3 expression. Interestingly, overexpressing miR-19a in senescent cells suppresses the expression of IGFBP-3 at both mRNA and protein levels, suggesting a role in regulating IGFBP-3 expression. We provide sufficient evidence to propose that IGFBP-3/IGFBP-3R axis is upregulated in senescence and this upregulation can induces senescence via ROS accumulation and p38 MAPK activation. Figure 31 presents a model proposing IGFBP-3 can signaling IGFBP-3R induce ROS accumulation which in turn activates p38 MAPK signaling and downstream senescence associated gene expression, ultimately leading to senescence.

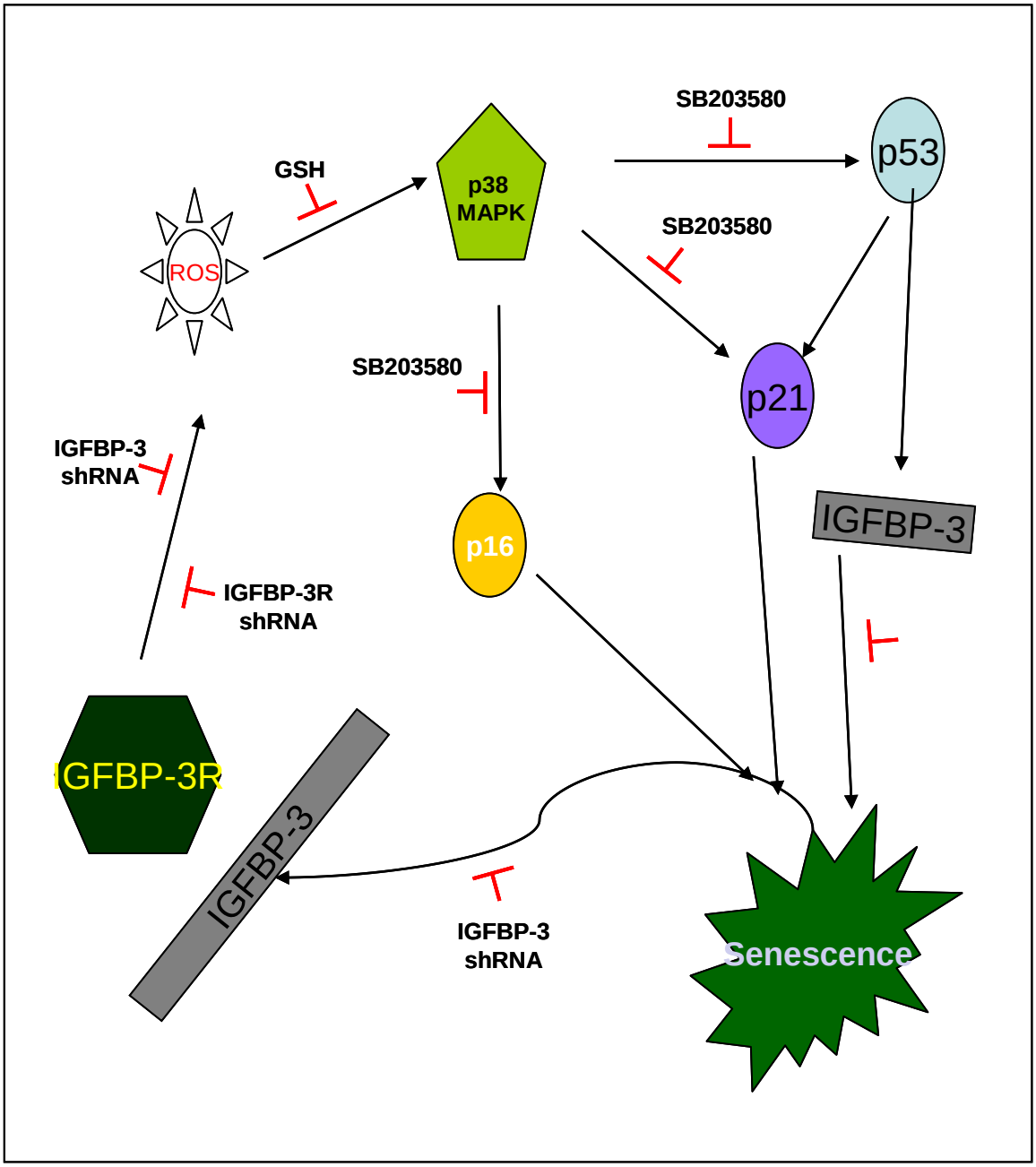


Figure 31: IGFBP-3/IGFBP-3R axis signaling in cellular senescence. IGFBP-3 and IGFBP-3R expression is upregulated in replicative and prematurely induced senescence. IGFBP-3 upregulation induces ROS accumulation which activates p38 signaling and other downstream senescence associated genes. Suppression of endogenous IGFBP-3R, ROS accumulation and p38 phosphorylation inhibits IGFBP-3-induced senescence.

Chapter 5

Discussion

5.1 Preface

Aging presents a worldwide social and financial challenge, and by the year 2050, over two billion people will be at least 65 year of age (Lutz, *et al.,* 1997). Human aging is very often related to variety of diseases including but not limited to cardiovascular disorders, cancer, diabetes, and muscular degeneration. During this century the effects of aging are estimated to be a major global health care challenge (Mills, 1998). Understanding the mechanisms of aging are necessary to provide insights into the pathogenesis of a range of age-associated diseases and more importantly, potential interventions. Aging has long been studied in a wide range of species leading to the identification of longevity genes, tumor suppressive genes, and multiple pathways of cellular aging, also termed senescence (Patridge, *et al.,* 2002; and Martin, 2005). Numerous molecular pathways have been proposed to contribute to aging, however the complex nature of genetics, molecular and cellular signaling, have prevented a detailed understanding of the aging process. One of the most widely studied mechanisms of aging was proposed nearly 50 years ago with the identification of cellular senescence.

5.2 IGFBP-3/IGFBP-3R signaling in senescence

Aging is an evolutionary byproduct of millions of years of selection for reproductive success. The aging of organisms occurs at virtually every level of complexity, from cells to tissue to organ systems. Two consistent interventions used to increase lifespan in both invertebrates and vertebrate animal systems are caloric restriction and suppression of GH/IGF/insulin axis. Animal models have established IGF signaling pathway as a pivotal regulator of aging in rodents and invertebrates (Berryman, *et al*.,2008). GH/IGF signaling cascade, or homologous pathways in other species, are evolutionarily conserved and undoubtedly affect the basic phenotypic characteristics of aging and longevity in a wide range of organisms. In humans specifically, decreased GH/IGF activity is linked to increased morbidity in adulthood and increased risk of age-associated pathologies including cardiovascular disease, diabetes and neurordegenerative disease (Perrini, 2010). However, the understanding of IGF and aging becomes more complex as there appears to be a tightly controlled regulation and maintenance of IGF to prevent disease, as IGF activity suppression and overexpression can drastically alter the onset of age-associated diseases and overall longevity. IGFBP-3 is the major binding protein involved in IGF signaling; however, IGFBP-3 homology is not observed across species identified to have GH/IGF conserved signaling. Therefore, despite being a key regulatory protein in IGF function, IGFBP-3's role in aging may be different than what has previously been described in regards to IGF signaling cascade and aging. Although IGFBP-3 primarily functions to regulate IGF signaling, IGFBP-3 has distinct IGF-independent biological functions. Cell growth inhibition, via apoptosis, has long been viewed as the primary physiological response to

overexpression of IGFBP-3 in various cancer cell lines, independent of IGF. A role of IGFBP-3 independent of IGF has yet to be examined in normal cells.

Our laboratory has recently published the identification of a novel IGFBP-3R, which characterizes and describes how IGFBP-3 effects can be mediated independent of IGF (Ingermann, *et al.,* 2010). IGFBP-3R is described as a single-span membrane cell death receptor that binds specifically to IGFBP3 and no other IGFBPs (Ingermann, *et al.,* 2010). By examining the IGFBP-3/IGFBP-3R axis in a variety of breast and prostate cancer cell lines, the published data demonstrated an anti-tumor effect mediated by caspase-8-dependent apoptosis. Furthermore, IGFBP-3 effects were dependent on IGFBP-3R, as suppression of IGFBP-3 prevented IGFBP-3-induced apoptosis (Ingermann, *et al.,* 2010). The biological function of IGFBP-3R has been primarily characterized in various cancer cells lines. The published data regarding IGFBP-3R brings to the forefront a potential mechanism by which IGFBP-3 can mediate senescence and in aging, independent of IGF signaling.

The link between human aging and cellular senescence has long been studied and confirmed to be highly correlated. Evidence of increased accumulation of senescence cells in aged patients and from patient exposed to stress stimuli such as oxidative-stress and UV exposure suggest a highly plausible hypothesis that states cellular senescence contributes to biological organismal aging. By identifying how senescence is induced and the effects of accumulating senescence cells *in vivo* will provide tremendous insight into biological aging, age-associated diseases and possibly, therapeutic interventions.

Interestingly several groups have been reported IGFBP-3 mRNA and protein expression to be upregulated in both replicative and premature-induced senescence, *in vivo* and *in vitro* (Debacq-Chainiaux, *et al.,* 2008; Goldstein *et al.,* 1993; Kim *et al.,* 2007). However, there are no proposed mechanisms for how IGFBP-3 is being upregulated or what may be the consequences of this upregulation. In this current study, we aimed to characterize and identify the role if IGFBP-3 and its newly identified IGFBP-3R in cellular senescence in order to understand their roles in biological aging.

We initially developed a model of replicative senescence induced by serial passage of HFL-1 cells. Our model clearly demonstrates upregulation of senescence associated genes, at both the mRNA and protein levels during replicative senescence. Furthermore, p38 MAPK signaling appears to be activated as indicated by increased phosphorylation of p38. We show both IGFBP-3 and its novel receptor, IGFBP-3R, have significantly increased mRNA and protein expression levels which were correlated to decreased cell replicative capacity.

As we identified an upregulation of IGFBP-3 and IGFBP-3R in replicative senescence, we aimed to determine possible mechanisms for upregulation and signaling induced by IGFBP-3 upregulation. In order to examine the role of IGFBP-3, we evaluated the effects of overexpressing IGFBP-3 in young fibroblasts and used a mutant IGFBP-3^{GGG} to establish IGF-independent effects. Overexpression of IGFBP-3, IGFBP-3^{GGG} and IGFBP-3R in early passage fibroblasts (PD4) induces senescence morphological changes, increased SA-β-gal staining and increasing mRNA and protein levels of senescence associated genes, p16, p53 and p21. Furthermore, using a

mutant form of IGFBP-3 (IGFBP-3^{GGG}), which has lost binding affinity to IGFs but retains IGF-R binding capability, confirms IGF-1-independent effects. Overexpression of IGFBP-3 or IGFBP-3R increased ROS accumulation and p38 MAPK activity. This data demonstrated that overexpression of IGFBP-3, its mutant or IGFBP-3R triggers premature senescence of early passage normal fibroblasts, implicating a crucial role of IGFBP-3 in the induction of senescence.

The significance of IGFBP-3 expression in replicative senescence was further evaluated, as endogenous suppression of IGFBP-3 prohibited presenescent cells from entering a senescent state. Next, fibroblasts derived patients with Werner syndrome which have a limited *in vitro* replicative lifespan, clearly show upregulated IGFBP-3 expression. We demonstrated that suppression of endogenous IGFBP-3 protein using shRNA techniques reversed the senescence phenotype and increased the proliferation potential compared to non-treated cells. Endogenous suppression of IGFBP-3 in HFL-1 and WS cells illustrates the impact of this signaling pathway and generates confidence that IGFBP-3 may be explored as a possible therapeutic target.

Shortened telomeres and/or decreased telomerase activity were originally described as the primary signaling pathway for replicative senescence induction. The capability of cells to undergo premature senescence highlighted a novel hypothesis that senescence was not solely triggered by replicative exhaustion and subsequent telomere shortening. In our cell system, we demonstrated IGFBP-3/IGFBP-3R could induce premature senescence in the presence of telomerase. This data demonstrates IGFBP-3/R-induced senescence cannot be inhibited by telomerase activity, suggesting the

signaling pathway of IGFBP-3/R axis is different than that triggered by significantly decreased telomere length.

Our published data clearly demonstrates IGFBP-3R plays an integral role in IGFBP-3 induced apoptosis in cancer cells; however, we demonstrate for the first time that IGFBP-3R is also vital to IGFBP-3 signaling in normal cells. To determine if IGFBP-3 required IGFBP-3R signaling, we tested the effects of suppressing endogenous IGFBP-3R expression. Suppression IGFBP-3R followed by overexpression of IGFBP-3 decreased the number of cells that underwent senescence; however senescence was not completely abrogated. The inability of IGFBP-3R suppression to completely inhibit IGFBP-3-induced senescence suggests IGFBP-3 can signal other pathways or more specifically internalized. IGFBP-3 is known to be internalized via distinct endocytosis mediated caveolin-1 (Lee, *et al.,* 2004). IGFBP-3 possesses a caveolin-scaffolding domain consensus sequence at the C-terminal end (Lee, *et al.,* 2004). It has been widely demonstrated that caveolin-1 acts as an antiprolifierative protein, as its overexpression in mouse embryonic fibroblasts induces G1 phase cell cycle arrest and activates p53/p21 signaling (Galbiati, *et al.,* 2001). Given the role of caveloin-1 in regulating cell cycle arrest and that IGFBP-3 internalization requiring caveloin-1, tthese results suggest IGFBP-3 may be able to elicit a senescence response independent of IGFBP-3R signaling and confirms the extensiveness by which IGFBP-3 can induce cell growth inhibition. Although it appears that IGFBP-3/IGFBP-3R axis signaling is complex, inhibition of senescence can be achieved by suppression of endogenous IGFBP-3 or IGFBP-3R expression. All together, our results indicated that IGFBP-3/IGFBP-3R axis is a key regulator in cellular senescence.

To better understand how IGFBP-3 functions in senescence signaling, we aimed to examine what upregulates its expression in senescent cells and/or what inhibits its expression in young replicating cells. The recent emergence of functional studies regarding miRNAs brought forward a novel opportunity to investigate possible miRNA regulation of IGFBP-3. miRNA regulation of gene activity may provide crucial information towards unraveling a specific role for genes involved in senescence signaling pathways. Of the 4 highly conserved predicted miRNA targets for IGFBP-3, miR-19a appeared to be most differentially expressed compared to IGFBP-3 expression. We have demonstrated that miR-19a is downregulated in senescence cells, which is inversely correlated with IGFBP-3 expression. Upregulating miR-19a expression by pre-miR-19a transfection caused decreased IGFBP-3 mRNA and protein expression. One group has reported that miR-19a can attenuate the progression of endothelial cells from G1 to S phase, and subsequently induce senescence (Qin, *et al.,* 2009). Furthermore, they demonstrate miR-19a appears to suppress cyclin D1 expression, which is a key regulator of cell cycle progression. Our findings, taken together with published data, suggest miR-19a may be vital in regulating IGFBP-3 suppression, thereby inhibiting its growth inhibitory effects in young early passage cells. Ultimately, this supports the complexity of senescence and more importantly how senescence phenotype and gene expression may not be indistinguishable from various senescence stimuli, but the role and signaling pathways of regulator genes is diverse.

5.3 ROS upregulation in IGFBP-3/IGFBP-3R-induced senescence

ROS are oxygen free radicals that are highly reactive toward various cellular contents including DNA, protein and lipids. ROS are generated in various biological systems and known to be important determinants in regulating cell signaling pathways involved in proliferation, apoptosis and senescence (Gamaley, 1999; Powis, 1995). Evaluation of ROS levels, oxidation of macromolecules, and a shift in cellular redox status suggest ROS production increases with age (Kregel, *et al.,* 2007). Although previous studies show oxidative stress-induced senescence increases IGFBP-3 secretion, to date there has been no evidence to suggest IGFBP-3 could signal increased ROS production. The cellular processes that produce ROS are vast and complex and the downstream pathways activated by ROS are even more complicated and numerous. Under normal conditions ROS production is essential for normal cellular functions. The damaging effects of ROS occur with increased production and/or increased accumulation due to decreased antioxidant effects. One of the primary pathways signaled by ROS production is p53. p53 can be signaled by DNA damage induced by ROS or other oxidative stress stimuli initiated by increased ROS production. Interestingly, it has been reported that p53-induced senescence can be inhibited by silencing IGFBP-3, and furthermore there is an inverse correlation between IGFBP-3 and Foxo3a in this system (Kim, *et al.,* 2004). Foxo3a is known to be involved in the upregulation of catalase and MnSOD, two enzymes involved in the elimination of toxic ROS (Czymai, *et al.*, 2010). Foxo3a is a member of FOXO family of forkhead transcription factors regulated by PI3K/Akt signaling pathway. (Kim, *et al*., 2004) PI3K/Akt signaling is regulated by insulin and IGF signaling pathways and known to

contribute to life span in mammals. Foxo3a protein is known to be lower in aged HDFs cells compared to younger cells (Kim, *et al*., 2004). This regulation of Foxo3a by IGF signaling brings forth the significance of maintaining a tight balance of IGF regulation. As downregulation of Foxo3a protein is known to accelerate senescence, the inverse relationship between IGFBP-3 and Foxo3a may further support our hypothesis between ROS upregulation and IGFBP-3 induced senescence (Kim, *et al.,* 2005).

5.4 p38 MAPK signaling in IGFBP-3/IGFBP-3R-induced senescence

As we have demonstrated IGFBP-3 and IGFBP-3R induced senescence can signal p38 activation by ROS accumulation, the next step will be to investigate how ROS signals p38 phosphorylation. As discussed in the introduction, the Trx-ASK1 complex is known to regulate senescence by activation of p38 MAPK; therefore, it might be essential to characterize the Trx-ASK1 complex in our cell system. ROS has been shown to be involved in Akt signaling, which can then phosphorylate apoptosis signal-regulatory proteins (ASK1) (Kim, 2001; Mochizuki, 2006). Recently, ASK1 has been indentified to be complexed with thioredoxin1 (Trx1), and upon ROS oxidation, ASK1 can phosphorylate p38 MAPK, thereby inducing downstream senescence transcription factors.

5.5 Werner Syndrome and IGFBP-3

There are two canonical progeroid disorders, Werner's Syndrome and Hutchinson-Gilford progeriod syndrome (Cox and Faragher, 2008). These disorders recapitulate rapid phenotypic aging, which occurswell before normal aging. Advancing our understanding of the pathogenesis of these disorders must be examined in parallel, as this will aid in understanding the normal aging process (Cox and Faragher, 2008). Mutations of specific genes that characterize and define each progeriod syndrome have been identified and well studied to participate in a verity of pathways and processes (Cox and Faragher, 2008). It is important to note that although the mutated gene products contribute to in vital biological processes, their participation may not be the pivotal player,by which the accelerated aging pathologies are derived. These disorders demonstrate the integral relationship between genotype and phenotype and furthermore, the extensiveness and complexity between aging and senescence.

To date there has been no investigation into the role of IGFBP-3 protein in people having Werner syndrome, despite reports of endogenous upregulation. We demonstrate that the senescence phenotype observed from WS derived fibroblasts could be reversed by suppression of endogenous IGFBP-3 similar to that observed when WS cells were exposed to long-term treatment with SB203580 (Davis, *et al.,* 2005). Although previous studies demonstrate that blocking p38 inhibits only p21, expression, we demonstrate a decrease in p53 and p16 in addition to p21, suggesting that IGFBP-3 may convered on p21 expression directly related to p38 signaling but also influce pathways mediated directly by p53 and p16. From a clinical perspective, the significance of IGFBP-3 to regulate *in vivo* aging process has not been determined.

The identification of p38 MAPK as a potential therapeutic target in WS patients suggests IGFBP-3/IGFBP-3R inhibition in this system may also have beneficial effects.

Although IGFBP-3 appears to be important for inducing apoptosis in cancer cells, the advantages or disadvantages of upregulated IGFBP-3 expression in normal cells and tissues needs to be explored. We hypothesize that the balance between upregulated IGFBP-3/IGFBP-3R axis signaling is tightly controlled. By all accounts, cells enter into a senescent state to prevent the replication of damaged cells, and as result senescence is classified as a tumor suppressive mechanism. In this study we have demonstrated for the first time the effects of overexpressing IGFBP-3/IGFBP-3R signaling in normal diploid fibroblast, and the effects of suppressing endogenous signaling. It would be advantageous to explore the tight balance between upregulation of IGFBP-3/IGFBP-3R signaling that can induce senescence in normal cells but induce proapoptotic signaling in cancer cells. The complexity of this pathway suggests that the response elicited by this signaling axis may be dependent on the molecular and genetic composition of cells.

5.6 Perspective

In summary, the results presented here demonstrate a novel role for the upregulation of IGFBP-3/IGFBP-R signaling in both replicative and premature-induced senescence. IGFBP-3/IGFBP-R can induce premature senescence by increasing intracellular ROS production, activating downstream p38 MAPK, and subsequently inducing senescence-associated genes, all of which leads to cell growth arrest in

normal cells. Inhibition of ROS inhibits IGFBP-3/IGFBP-3R-induced senescence. p38 MAPK signaling is activated downstream of ROS production and may be signaling downstream senescence-associated genes for upregulation. Suppression of endogenous IGFBP-3 expression can inhibit replicative senescence induction and furthermore, reverse the senescence phenotype typical of Werner Syndrome fibroblasts. The novel IGFBP-3R also plays a vital role in the signaling of IGFBP-3 as IGFBP-3R shRNA can inhibit IGFBP-3- induced senescence.

The last 20 years has produced an exponential rise in the understanding of cellular and molecular aging. The mechanisms by which progeriod syndromes are caused may have little to no relevance to normal aging; however, this suggest that the most likely cause of aging phenotype is cellular senescence. The aging of organisms occurs at virtually every level of complexity, from cells to tissue to organ systems. These studies address a critical, emerging field of IGFBP-3's role in the signaling of cellular senescence. The ability of IGFBP-3 to induced senescence is of much relevance to unraveling organismal aging, and we have clearly demonstrated the IGFBP-3/IGFBP-3R axis plays an essential role in cellular senescence (Figure 31).

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Vita

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