CHARACTERISATION OF NOVEL MARKERS AND EFFECTORS OF SENESCENCE AND THEIR ROLE IN CANCER AND AGEING

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ABSTRACT

CHARACTERISATION OF NOVEL MARKERS AND EFFECTORS OF SENESCENCE AND THEIR ROLE IN CANCER AND AGEING

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Cellular senescence is a reversible cell cycle arrest that has been shown to play a role in aging and cancer. Senescent cells accumulation can occur prematurely, as seen in response of stress and oncogenic insults, or normally, as observed in organismal aging. Thus, identification and studying senescent cells in vivo and in vitro have an important diagnostic and therapeutic potential. In addition, the molecular mechanisms involved in establishing and maintaining senescence are not fully understood. Consequently, the current approach that is used for senescent identification has limitations. For this reason, we characterized a list of potential markers of senescence from a proteomic screening of plasma membrane of senescent cells. From the list, we have validated 10 of them, namely, DEP1, NTAL, EBP50, STX4, VAMP3, ARMCX3, B2MG, LANCL1, VPS26A and PLD3 that are differently expressed in different model of cell senescence. These markers can be combined to detect senescent cells in vitro or in tissue sections. We also proposed a FACS based method using two markers (DEP1 and B2MG) with known extracellular epitopes. This could facilitate the senescence detection and studying.

From the proteomic screening, we also found that BTK is elevated in senescent cells. BTK was induced in response of p53 activation due to different stimuli. Moreover, phosphorylation of ATM, p53 at ser15 and γH2AX was increased after BTK overexpression, which suggested the involvement of BTK in DNA damage pathways. BTK was able to phosphorylate p53 directly at N-terminus. BTK inhibition with chemicals or RNAi depletion was sufficient to bypass senescence. In addition, BTK inhibitors were also able to extend life span in flies. p53 KO flies did not show any difference in life span after using BTK inhibitors, which showed the importance of BTK in p53-mediated senescence. BTK inhibition also decreased the senescent cells accumulation in flies. All these data collectively suggest the possibility of using BTK inhibitors to ameliorate aging-associated disorders.
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<tbody>
<tr>
<td>ADC</td>
<td>Antibody–drug conjugate</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternate reading frame protein</td>
</tr>
<tr>
<td>ARMCX3</td>
<td>Armadillo repeat-containing X-linked protein 3</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3-related</td>
</tr>
<tr>
<td>B2MG</td>
<td>Beta-2 microglobulin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum</td>
</tr>
<tr>
<td>BTK</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>BTN2A1</td>
<td>Butyrophilin, superfamily 2, Member A1</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CXCR2</td>
<td>Chemokine (C-X-C motif) receptor 2</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCR2</td>
<td>Decoy receptor 2</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Double-distilled water</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DEP1</td>
<td>Density-enhanced phosphatase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DP</td>
<td>Dimerization partner</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>E2F</td>
<td>E2 factor</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetra acetic acid</td>
</tr>
<tr>
<td>EV</td>
<td>Empty vector</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box O</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H3K9Me</td>
<td>Histone H3 methylated Lys9</td>
</tr>
<tr>
<td>HDF</td>
<td>Human dermal fibroblast</td>
</tr>
<tr>
<td>HNRNPU</td>
<td>Heterogenous nuclear ribonucloprotein U</td>
</tr>
<tr>
<td>ICAMI1</td>
<td>Intercellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor-1</td>
</tr>
<tr>
<td>IGFβ</td>
<td>Insulin-like Growth Factor- β</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LANCL1</td>
<td>lantibiotics synthase component c like 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCL</td>
<td>Mantle cell lymphoma</td>
</tr>
<tr>
<td>MEF</td>
<td>Murine embryonic fibroblast</td>
</tr>
<tr>
<td>MIPs</td>
<td>Molecularly imprinted polymers</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>MUNC18-1</td>
<td>Mammalian uncoordinated -18-1</td>
</tr>
<tr>
<td>NIS</td>
<td>Nuclear import signalling</td>
</tr>
<tr>
<td>NK</td>
<td>Nature killer</td>
</tr>
<tr>
<td>NOTCH3</td>
<td>Neurogenic locus notch homolog protein-3</td>
</tr>
<tr>
<td>NTAL</td>
<td>Non-T cell activation linker</td>
</tr>
<tr>
<td>OIS</td>
<td>Oncogene-induced senescence</td>
</tr>
<tr>
<td>PA-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<td>PI</td>
<td>Propidium Iodide</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol bisphosphate</td>
</tr>
<tr>
<td>PLD3</td>
<td>Phospholipase D 3</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia protein</td>
</tr>
<tr>
<td>PPP1A</td>
<td>Protein phosphatase PP1-alpha catalytic subunit</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>PRC</td>
<td>Polycomb repressor complex</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SAHF</td>
<td>Senescence-associated heterochromatin foci</td>
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<tr>
<td>SASP</td>
<td>Senescence associated secretory phenotype</td>
</tr>
<tr>
<td>SA-β-Gal</td>
<td>Senescence-associated β galactosidase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>shRNA</td>
<td>Small hairpin ribonucleic acid</td>
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<td>Simian vacuolating virus 40</td>
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<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNFSF-11</td>
<td>Tumour necrosis factor (ligand) superfamily, member 11</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>VAMP3</td>
<td>Vesicle associated membrane protein 3</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
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<tr>
<td>VPS26A</td>
<td>Vacuolar protein sorting associated protein 26 A</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle</td>
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<tr>
<td>WM</td>
<td>Waldenstrom macroglobulinemia</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>XLA</td>
<td>X linked agammaglobulinemia</td>
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</table>
CHAPTER 1. INTRODUCTION

1.1 Cellular senescence

Normal primary cells extracted from mammalian tissue undergo a definitive number of divisions before they stop proliferation. In 1961, Hayflick and Moorhead noticed that cells in vitro were not immortal but they divided for a certain number of passages before they completely stopped division. The non-proliferative cells stayed alive for many weeks, but were unable to grow in spite of the presence good culture conditions. Later studies have illustrated the role of cell senescence as a tumour suppressive barrier against early malignant transformation (Larsson, 2011). This role has increased the interest in senescence research. In addition, aging is another consequence of senescence; accumulation of senescent cells with age can exaggerate aging symptoms. Targeting senescent cells has shown to ameliorate aging symptoms in premature aging mice (Baker et al., 2008; Baker et al., 2011). Indeed, Hayflick and his colleague firstly proposed the role of senescent cells in aging and this theory has recently been confirmed (Campisi, 2013).

Recent observations have shown an emerging role of senescent cells as a wider mechanism rather than tumour suppression or aging processes only. Senescence can participate in many physiological mechanisms, for instance tissue repair, wound healing and embryogenesis (Xue et al., 2007; Krizhanovsky et al., 2008; Munoz et al., 2013; Storer et al., 2013).
Figure 1.1 Senescence stimulates many signals involve in permanent cell cycle arrest. Activation senescence through DNA damage, telomere shorten and oncogenic upregulation leads to increase in p16 expression, which results in inhibition CDKI that subsequently activates pRB to induce senescence. In addition, upregulation of p14ARF inhibits p53 mdm2 binding, which leads to p53 and p21 activation that leads ultimately to senescence induction (Campisi and d’Adda di Fagagna, 2007).

The main mechanisms of preventing cancer formation or progression are cellular senescence and/or apoptosis (Lowe et al., 2004). Mutations in different genes may lead to the initiation of these two mechanisms. Apoptosis, otherwise known as a programmed cell-death, is the program where cell enters into obligatory suicide (Lowe et al., 2004). On the other hand, cellular senescence is defined as a stable cell cycle arrest in G1 phase in which cells remain metabolically active without dividing abilities, these normally occur through upregulation different genes, such as p53, p21, p16 and pR, figure 1.1 (Collado and Serrano, 2010).

Senescent cells have distinctive features, which are used usually as markers for identification and determination of cellular senescence. Senescent cells normally appear as multinucleated, large, flat and extended and this depends primarily on the cell type, genetic background and the inducers of cell senescence. Some cells exhibit spindle, vacuolization features. Another feature
of cell senescence is a DNA replication capacity, which is very low. It can be easily measured by incorporation of 5-bromodeoxyuridine and measurement of Ki-67 level. However, these approaches cannot differentiate permanent or temporary cell cycle arrest; so it is improper to be using them alone to detect senescent cells (Kuilman et al., 2010). Therefore, normally a combination of more than two markers is used to identify cell senescence in vitro and in vivo.

1.2 Senescence-associated secretory phenotype (SASP)

Cellular senescence is usually associated with changing in the expression of several genes, which leads to the secretion of growth factors, chemokines and cytokines (Kuilman et al., 2010). This phenotype is known as senescence-associated secretory phenotype (SASP), which was first noticed in fibroblasts with replicative senescence (Kuilman et al., 2010). Different cell types have different secretomes, which are specific for each cell. The SASP is compound of biologically active compounds and it has influence on the surrounding microenvironment of senescent cells. These factors can trigger inflammatory responses and may also facilitate new tumour growth (Coppe et al., 2010).

Secretion of inflammatory cytokines can have an effect on cell behaviour and responses. For example, secretion of high level of GROα from senescent cells triggers proliferation of mammary cells (Coppe et al., 2010). Also, SASP can promote migration of leukocytes and tumour cells, which in turn may induce tumour metastasis (Mantovani, 2004).

Examples of senescent cells that are highly active and can secrete active molecules are liver stellate cells (Schnabl et al., 2003) and endothelial cells (Shelton et al., 1999). In addition, epithelial cells of mammary glands, prostate cells and others have shown increasing secretion of active compounds during senescence (Schwarze et al., 2005; Coppe et al., 2008).

Although it is believed that SASP from senescent cells can stimulate tumour growth and also alter surrounding microenvironment, many studies have revealed the role of these proteins in cellular senescence and tumour growth arrest. According to Wajapeyee et al, lack of IGFBP7 in nevi lesions can promote melanoma progression (Wajapeyee et al, 2008). In addition, Acosta et al showed that senescent cells induced by oncogenes, express CXCR2, which
maintains cellular senescence via a self-stimulating mechanism with its ligand (Acosta et al., 2008). These inflammatory cytokines also can create an immune response against senescent cells and then eradicate them (Xue et al., 2007).

Mechanisms by which senescent cells attract the immune system are not fully understood and thought to be through the SASP. Xue et al conducted the first study to show the relationship between the immune response and senescent cells. They showed that certain inflammatory cytokines from senescent cells have the ability to call the innate immune response, which facilitates senescent cells phagocytosis (Xue et al., 2007).

Recently, Kang et al investigated the interaction between precancerous senescent hepatocytes and the adaptive immune response. Secreted cytokines and chemokines from those senescent cells attract CD4⁺ for clearance. Senescent hepatocytes that expressed Nras\(^{G12v}\) were cleared by specific Th\(_1\), hence “senescence surveillance” was relayed on effective adaptive immune clearance (Kang et al., 2011). In parallel with these observations, senescent cells that expressed Nras\(^{G12v}\) in mice lacking CD4⁺ or have severe combined immunodeficiency (SCID), were not cleared by adaptive immune action. This led to initiation and progression of carcinoma (Kang et al., 2011).

### 1.3 Markers of senescence

Identification of cell senescence is not easy; none of available markers is able to detect senescence exclusively. So far, no single marker or character that has been identified is particularly specific for senescence phenomena. Thus, rather than single markers, senescent cells normally are identified by a collective of a group of markers that would help to identify cellular senescence.

For instance, activation of tumour suppressor genes, p16, p21, p53, p14 and p27 are not used singly but used as surrogate markers of senescence. SA-β-Gal activity at pH 6.0 is normally used as a first identification method in combination with other surrogate markers (Collado and Serrano, 2010).

#### 1.3.1 Senescence associated β- galactosidase

Senescence associated β-galactosidase (SA-β-Gal) is frequently used as marker because its presence is very high in senescent cells (Dimri et al., 1995).
In normal cells, β-galactosidase displays activity at pH 4.0 within lysosomes; however, its action can also be determined at pH 6.0 in senescent cells (figure 1.2).

![Image](image.png)

**Figure 1.2 Senescent cells show a SA-β-gal staining at pH 6, compared to the growing cells on the left** (Narita *et al.*, 2003).

This may be due to enlargement in the structure of lysosome in these cells (Lee *et al.*, 2006). Despite this activity in cellular senescence, SA-β-Gal has not been shown to have any role in the senescence phenotype. SA-β-Gal also was noticed at pH 6.0 in certain proliferating cells, for instance highly confluent growing cells (Yang and Hu, 2005). Moreover, SA-β-Gal activity was also seen in non-proliferative cells such as quiescent cells at low serum concentration (Yegorov *et al.*, 1998). In addition, serum starvation and H$_2$O$_2$ treatment of culture cells led to an increase in the activity of β-Gal at pH 6.0 (Yang and Hu, 2005). These studies clearly showed the inability of SA-β-Gal to be used to detect senescent cells without other surrogate markers of cell senescence. These disadvantages and others have shown the need to find out more reliable markers to identify senescence.

### 1.3.2 Senescence associated heterochromatin foci (SAHF) and other Chromatin related markers

During cell senescence, the chromatin structure undergoes several alterations that appear as a dot like pattern as seen in figure 1.3. They make senescent cells less susceptible for digestion by nucleases (Narita *et al.*, 2003). In contrast, DNA staining of growing cells normally appears as uniform colour outlines. Therefore, SAHF is a very specific feature for senescence, and cannot be observed in reversible cell cycle arrest such as quiescence (Narita *et al.*, 2003).
The modifications in chromatin methylated on lysine 9 of histone H3 (H3K9Me), histone H2A variant macroH2A and heterochromatin protein1 (HP1) promote foci formation at many different genes that are essential for cell growing (Narita et al., 2003; Narita et al., 2006). Zhang et al demonstrated the role of histone repressor A (HIRA) and anti-silencing function 1A (ASF1a), which enforces heterochromatin formation, in senescent cells (Zhang et al., 2005). Murno et al, (2004) found that treating fibroblasts with histone deacetylase inhibitors which stimulate chromatin relaxation induces cell senescence through up-regulation of p16, which lead to senescence associated heterochromatin foci formation (Murno et al, 2004). These illustrate the role of the heterochromatin foci in senescence regulation.

On the other hand, SAHF is not a universal marker of senescence; its features cannot be observed in all cell types that undergo senescence. In pre-malignant lesions, senescent cells do not always form SAHF (Kosar et al., 2011). However, it is mostly seen during replicative senescence, and some cells that undergo oncogenic-induced senescence (OIS) (Narita et al., 2003). In addition, some fibroblast cells that undergo OIS do not form SAHF. It is normally observed in senescence cells that are induced by doxorubicin, bacterial intoxication and telomere erosion (Kosar et al., 2011).

Recently, Swanson and colleagues revealed senescence associated distension of satellites (SADS) as a new marker, which is a distinguishing character for all senescence types including progeria cells. In addition, this unravelling takes place in early stages of senescence formation, and different from SAHF, it
affects both p16 and p21 mechanisms of senescence, which makes SADS feature of chromatin more specific (Swanson, et al., 2013).

1.3.3 Change in genes expression during senescence

Cell senescence is normally associated with alteration in gene expression, including some CDKs and their inhibitors (Stein et al., 1990). Cyclin-dependent kinase inhibitors that are upregulated during senescence are p21 and p16. These inhibitors are part of tumour suppressor networks that are usually controlled by transcription factors such as p53 and Rb. These proteins are able to initiate and maintain cell cycle arrest, which is the main feature of senescent cells (Stein et al., 1990; Atadja et al., 1995). p53 induces p21, which leads to senescence; however, the effectors that stimulate p16 levels are not fully understood (Gil and Peters, 2006).

During senescence, many genes that are involved in cell cycle progression are suppressed, for example, PCNA (proliferating-dependent cell nuclear antigen), E2F, cyclin A&B and others (Coppe et al., 2010). However, this is not only the situation; many alterations in gene transcription seem to be linked to cell cycle arrest. Many studies have shown alterations in expression of genes that code for secreted proteins, which probably could modify the microenvironment of these tissues. Some senescent cells secrete proteins that can change the structure of extracellular matrix and even enhance inflammatory status (Coppe et al., 2010).

Senescent markers can be also measured in blood cells, which make its molecular identification and monitoring easier. A study conducted by Liu et al showed that expression of p16 increased with age in CD3 T cells and was not related to gender; however, its expression was remarkably linked to tobacco consumption and lack of physical activity (Liu et al., 2009). These molecular markers of aging also can be used to monitor cell senescence in patients who undergo chemotherapy. Sanoff et al showed that chemotherapy of patients who had breast cancers had a strong up-regulation in p16 and p14 and other cytokines that are normally associated with senescent cells such as VEGFA and MCP1. Patients who underwent chemotherapeutic treatment showed increase in senescence markers similar to normal volunteers of the same age.
More aggressive doses were associated with higher increase in these markers (Sanoff et al., 2014).

1.4 The physiological role of senescence

Cell senescence is a program of permanent cell cycle arrest. It is normally induced to prevent malignant transformation of the tissue, so it can be a tumour suppressive mechanism (Chen et al., 2005; Collado et al., 2005). In addition, senescence has also shown a role in wound healing and fibrosis (Xue et al., 2007; Krizhanovsky et al., 2008). Recent works have also shown a new physiological function of senescence, through its role in embryonic development (Munoz et al., 2013; Storer et al., 2013).

1.4.1 Senescence as a tumour suppressor mechanism

Several experiments have been conducted to illustrate the role of cellular senescence in tumour progression. Senescent cells are more often observed in the pre-malignant stages of the tumour, and they completely disappear in the malignancy stage (Chen et al, 2005). Senescent cells have been found in pre-malignant tumours such as prostatic intraepithelial neoplasia, lung adenomas and melanocyte nevi (Michaloglou et al., 2005). On the other hand, there was a lack of senescent cells in prostate adenocarcinoma, lung adenocarcinoma and melanoma, which are the corresponding malignancy stages (Chen et al, 2005; Collado et al., 2005; Michaloglou et al., 2005). In addition, the anti-malignant property of senescent cells in vivo was first noticed in a mouse model lacking PTEN expression in prostate and high NRAS expression in the presence of p53 (Chen et al, 2005). However, in the presence of inactive PTEN and p53 together led to invasive prostate cancer in early age, where no senescence-like phenotype was seen, compared to inactivation of PTEN only (Chen et al, 2005).

This clearly shows the role of senescence as a barrier against malignant transformation. Remarkably, loss of senescent cell mediators such as p53, led to malignancy progression and also resulted in losing markers of cellular senescence, such as heterochromatin foci formation (Narita et al., 2003).

In order for a premalignant tumour to become malignant, it needs to escape from senescence. This can be achieved by losing downstream tumour suppressors such as pRb and p53 (Larsson, 2011). The main role of pRb is to
inhibit the E2F transcription process, which is crucial for DNA replication and cell cycle progression (Chicas et al., 2010). PRb can be inactivated by oncogenes that are encoded by viruses, for instance, SV40 virus and adenovirus E1A that inactivate pRb and release E2F, which results in senescence bypass (Land et al., 1983; Burkhart and Sage, 2008). Moreover, cyclin-dependent kinase (CDK) can repress pRb; cyclin E/CDK2 and Cyclin D/CDK4 can also phosphorylate and inactivate pRb, resulting in releasing E2F and cell proliferation, and subsequently senescence suppression (Burkhart and Sage, 2008). The functions of CDK4/6 can be effectively down regulated by CDK inhibitors (CDKIs) p16 and p15, which leads to senescence suppression and tumour formation (Beausejour et al., 2003).

Cellular senescence can be also bypassed by losing the p14ARF/p53/p21 pathway. In normal cells, p53 levels are very low due to mouse double-minute-2 homolog (Mdm2) activity. It regulates p53 stability through induction of p53 ubiquitination, which subsequently leads to its degradation (Prives and Hall, 1999).

However, upon oncogenic activation, Arf is induced, which in turn inhibits Mdm2 and therefore stabilises p53 activity, and it becomes more effective in regulation of different genes expression included in senescence that can work as tumour suppression (Levine and Oren, 2009). Part of p53-mediated senescence is accomplished through p21 activation. Human cells lacking p53/p21 do not induce replicative senescence (Levine and Oren, 2009; Vousden and Lane, 2007). Another way of inactivation of p53 upon oncogenic activation is through methylation of p14 gene locus (Dirac and Bernards, 2003; Gil and Peters, 2006).

p21 can also be activated in a p53 independent manner through, for example, Stat1, IFN and TGFβ (Agrawal et al., 2002; Arany et al., 1996; Datto et al., 1995) These signals are able to activate p21 and signal for cancer suppression through senescence induction or cell cycle arrest. Therefore, p21 inactivation is an additional way to bypass cellular senescence. In fact, myc oncogene is able to inactivate p21 expression (Wu et al., 2003; Larsson, 2011).
1.4.2 Replicative senescence

Normal human diploid cells can grow in vitro for a period of time before they become senescent and arrest irreversibly. This is called replicative senescence. Telomere length shortens during cell division, until a DNA damage response leads to upregulations of CDKIs, which then induce senescence (Cristofalo et al., 2004). Telomeres are located at end of each chromosome, which are composed of repeat of kilo bases of TTAGGG double strand (Hemann and Greider, 1999). In cells that have infinite cell division, when telomere get shorter telomerase enzyme adds TTAGGG repeat to telomeres sites which keep cells dividing (Cech, 2004). In replicative senescence, downregulation of telomerase activity is thought to have a role in senescence induction (Cech, 2004). Consistent proliferative propagation of cells leads to shortening in telomeres, which also causes proliferative arrest (Kuilman et al., 2010). In cancer cells, a telomerase enzyme prevents telomeres to become short and subsequently avoids replicative cellular senescence (Kim et al., 1994).

Once telomeres become very short and their organisation is interrupted, this will induce a DDR, which leads to stimulation of several DDR kinases (D'Adda et al., 2003). These kinases trigger cell cycle inhibitors such as p53 and p16 that promote cell cycle arrest and permit cells to repair damages. Further, replicative senescence is strongly associated with RB tumour suppressor and cyclin dependent kinase inhibitor p16 (Shay et al., 1991; Fujita et al., 2009).

In vivo, replicative senescence has been also observed in old individuals. SA-β-Gal levels are increased in tissue of aged people (Dimri et al., 1995). Furthermore, reduction in telomere length also has been noticed in some human tissue during aging (Takubo et al., 2000; Jiang et al., 2008; Song et al., 2010).

These findings and others support the idea that replicative senescence also occurs in vivo. Replicative senescence has been shown to be associated to pathological changes that are linked with aging. Senescence associated β-gal staining and telomere erosion have been observed in atherosclerosis (Minamino et al., 2002; Foreman and Tang, 2003), diabetes (Minamino et al., 2009; Gardner et al., 2005), ocular diseases (Zhu et al., 2009; Zhu et al., 2009),
osteoporosis and bone health (Martin et al., 2004) and others (Naesens, 2001; Braun et al., 2012).

1.4.3 Premature Senescence

Premature senescence occurs when a cell with normal telomere length becomes senescent due to different stimuli. There are many studies showing the occurrence of premature senescence in vitro and in vivo (Serrano et al., 1997; Collado et al., 2005; Michaloglou et al., 2005; Coppe et al., 2008; Schmitt et al., 2002).

1.4.3.1 Oncogene-induced senescence

Oncogene-induced senescence is premature senescence and is initiated when certain types of oncogenes are upregulated in the presence of normal tumour suppressors genes. These tumour suppressors are usually upstream of these oncogenes (Collado and Serrano, 2010). Examples of these oncogenes are Ras, PI3K, HIFα and E2F, which are normally supressed by neurofibromin 1 (NF1), PTEN, VHL and RB respectively (Collado et al., 2007). Abnormal activation of these oncogenes may lead to activation of corresponding tumour suppressors that may lead to senescence. One of the main features of replicative and oncogenic induced senescence is the critical participation of both p53-p21 and RB-16 pathways. In a mouse model where p53 downregulated or upstream effector p19ARF reduced enough to prevent senescence induction by RAS oncogene (Serrano et al., 1997).

In human fibroblasts such as IMR90, p16 appears to have a vital role for oncogenic-induced senescence more than p53 as those cells rely primarily on p16 for senescence induction (Serrano et al., 1997). However, in rodent cells such as REF52, p53 showed an important role in senescence induction after RAS activation (Serrano et al., 1997). This clearly shows that type of the cell is important to determine which tumour suppressor could be essential for oncogenic-induced senescence.

In fact, OIS usually does not have the same mechanistic pathways and this depends on cellular and genetic types (Kuilman et al., 2010). In addition, conditional expression of KrasV12 in mice leads to initiation of multiple lung
adenomas, in association with senescence markers. However, senescence markers were not present during lung adenocarcinoma in K-ras$^{V12}$ expressing mice (Collado et al., 2005). Another example is melanocyte nevi, which are commonly associated with active BRAF and NRAS mutations, where SA-β-Gal stating and p16 markers are high; however, telomere length is commonly same as normal skin cells, which exclude the role of replicative senescence (Michaloglou et al., 2005). This underlies the role of premature senescence or OIS in vivo in cancer prevention.

1.4.3.2 Other forms of premature senescence

Other stresses can induce senescence in cells with normal telomere length. For example, chemotherapy, radiation, and H$_2$O$_2$ treatment can also induce senescence in vitro and in vivo (Te Poele et al., 2002; Chen and Ames, 1994). However, the role of senescence induction in vivo after chemotherapy has not been studied extensively. Interestingly, although senescence induction after cancer therapy has been shown by many studies (Roberson et al., 2005; Te Poele et al., 2002; Coppe et al., 2008; Schmitt et al., 2002), the role of apoptosis in vivo after cancer chemotherapy of solid tumour has not been reported until now. This suggests the potential function of senescence in cancer chemotherapy, which should be investigated deeply. Accumulation of cell senescence has been noticed after cancer treatment with chemotherapy and with radiation (Gewirtz et al., 2008; Roberson et al., 2005; Te Polele et al., 2002). For senescence induction after cancer therapy, it normally takes days for full induction, opposite of apoptosis, which normally occurs within 24 hours (Campisi et al., 2007; Chang et al., 1999).

The decision between apoptosis or senescence that a cell takes after therapy depends on the level of the stress, among many other factors. High stress normally induces apoptosis, and low doses of the stress induce senescence, with some apoptotic effects on some cells (Chang et al., 1999). In cancer cell lines such as PC3, 250 nM of doxorubicin induces apoptosis, but 25nM induces senescence (Schwarze et al., 2005; Ewald et al., 2009). Senescence also can occur after cancer therapy when the apoptotic signalling is blocked in cells by, for example, overexpression of anti-apoptotic factors such as bcl-2 or by
inhibition apoptotic proteins such as caspase (Crescenzi et al., 2003; Rebbaa et al., 2003).

Senescence-inducing agents can induce DNA damage to generate single or double strand breaks, which suggests the link between genomic integrity and senescence (Schwarze et al., 2005; Ewald et al., 2009). Like replicative senescence and OIS, therapy-induced senescence is mainly initiated through DNA damage. For example, Aurora kinase inhibitor can induce senescence in many cancer cell lines through targeting chromatid segregation during mitosis (Huck et al., 2010).

Sirtinol (sirt1 inhibitor) is another example of an agent that induces senescence. It inhibits histone DNA deacetylase, which changes chromatin structure. Treatment of cancer cells with 0.1µM of Sirtinol induces senescence within 7 days (Ota et al., 2006).

Cytotoxic agents are also able to induce premature senescence in normal cells. Human fibroblasts, for example, lose ability to proliferate after a number of passages. However, treatment of these cells in early passages with 200µM of H₂O₂ leads to a senescence-like cell cycle arrest, induction of SA-β-Gal staining and upregulation in p53, p16 and p21 genes (Chen and Ames, 1994).

1.4.4 Other roles of senescence

Studies have shown that senescence has a role in developmental processes (Munoz et al., 2013; Storer et al., 2013; Jun et al., 2010). Involvement of senescence in embryonic development, wound healing and fibrosis are examples of these roles.

1.4.4.1 Involvement in Embryonic development

Early findings by Hayflick and his group revealed that normal diploid cells can proliferate to a limited number of cycles before reaching a condition of replicative senescence where cells are unable to divide anymore (Hayflick and Moorhead, 1961). For long time, many scientists had believed that the role of senescence was limited to adult cells until two articles published recently surprisingly revealed a role of senescence in embryo development. These articles propose that cellular senescence takes place during murine development. Munoz et al concentrate on the inner ear and retraction of
mesonephric tubules (Munoz et al., 2013), while Storer et al focused on the apical ectodermal ridge (AER) during limb development. The mechanisms of developmental senescence are not completely the same one that control senescence in adults (Storer et al, 2013).

In order to explore the exact mechanism of cell senescence in embryo, p21, p16 and p53 knockout mice were studied. SA-β-Gal staining of p53 and p16 knockout mice appeared during developmental senescence, conversely, p21 knockout mice showed low SA-β-Gal staining cells, and the embryo was developmentally not normal comparing to the controls. Therefore, up-regulation of p16 and p53 pathways has not shown any role in senescence during embryo development. Both studies believe that developmental senescence is mainly triggered by p21 through a TGFβ/SMAD and PI3K/FOXO dependant mechanism, and independently of the p53 pathway (Munoz et al., 2013; Storer et al, 2013). Both studies showed that Inhibition of senescence resulted in developmental malformation even in the presence of apoptotic mechanism.

1.4.4.2 Liver fibrosis and wound healing

During senescence, cells lose ability to proliferate and differentiate, but they actively secrete a vast amount of cytokines, which help cancer suppression (Xue et al., 2007). Current research has revealed the involvement of these secretions in wound healing. Senescent fibroblasts aggregate as normal mechanism of wound healing and tissue repair (Jun et al., 2010; Demaria et al., 2014). Krizhanovsky and his colleague showed that senescent myofibroblasts in mice liver are able to control fibrosis formation (Krizhanovsky et al., 2008; ).

After liver injury, accumulation of myofibroblasts leads to excessive extracellular matrix (ECM) secretion, which leads to liver fibrosis and finally cirrhosis (Krizhanovsky et al., 2008). However, senescence induction in these myofibroblasts can reduce liver fibrosis (Krizhanovsky et al., 2008). In parallel, mice without p53 and or p16 suffered from extreme fibrosis and delay in wound healing (Demaria et al., 2014)

These observations can be explained by the ability of senescent cells to secret many ECM degradation components to degrade ECM and prevent fibrosis and enhance wound healing. In addition, senescent myofibroblasts are unable to
proliferate further, which again limits fibrosis. After cell senescence induction, they are removed by NK cells (Krizhanovsky et al., 2008).

1.4.4.3 Adult cells

In addition to involvement of senescence in embryo development and wound healing, it is also involved in adult organisms during normal physiological development. As part of platelets production, megakaryocytes undergo senescence program for maturation process (Besancenot et al., 2010). In addition, senescence also was noticed during syncytiotrophoblasts mechanism for normal maturation (Chuprin et al., 2013). In these mechanisms, senescent cells were identified by growth arrest and positivity of SA-β-Gal (Besancenot et al., 2010).

1.5 Mechanisms of senescence

Cellular senescence is usually associated with one of two main pathways: p53 and/or pRb pathways. After oncogenic stress, telomere shorten, oxidative stress or DNA damage, different signals can exert their effects to induce senescence (Lowe et al., 2004). For example, p14ARF, which is induced by OIS, leads to p53 accumulation and in turn p21 induction, which ultimately promotes senescence (Lowe et al., 2004). Another proposed pathway is through p16, which activates pRb that arrests cell proliferation and stimulates cell senescence (Lowe et al., 2004). There are also other genes that have displayed senescent effects, for instance, PPP1A, Smurf 2, and others (Castro et al., 2008; Kondoh et al., 2005; Wang et al., 2002; Zhang and Cohen, 2004).

PPP1CA is one of the effector of senescence after RAS activation in the presence of p53. In the absence of PPP1CA, oncogenic upregulation of RAS was unable to induce senescence even in the presence p53/p21 pathway. RAS activation associated with increasing in PPP1CA expression, which suggested its role as a tumour suppressor. In many of renal and colorectal carcinoma at least one copy of the allele was lost, which suggests the importance role of this gene as tumour suppressor probably through oncogenic-induced senescence (Castro et al., 2008).

SMURF2 is an E3 ubiquitin ligase that has a role in controlling replicative senescence. Its activation in early passage fibroblasts was enough to induce
senescence. During replicative senescence, the expression of SMURF2 is high in parallel with telomere attrition and senescence-associated phenotypes such as SA-B-Gal and p16 upregulation. In addition, it recruits Rb and activates p53 pathway for senescence induction. In the absence of p21, it can induce senescence, which suggests that senescence induction by SMURF2 is independent of p21 (Zhang and Cohen, 2004).

1.5.1 p16-Rb

Cell cycle checkpoints are necessary for cells in order to complete normal cell cycle. p16-Rb pathway controls mainly G1 to S transition, through down-regulation of CDK4/6 activity and consequently inhibiting Rb phosphorylation. This leads to association of Rb with E2F and hinders activation of E2F target genes, which are important for G1/S transition (Lazzerini et al., 2005).

On chromosome 9p21, p16 gene is located along with p14ARF gene; two different reading frames are involved for p16 and p14 gene transcription. Methylation of p16 promoter through polycomb repressor complexes 1 and 2 (PRC) is involved in inhibition of p16 expression (Krishnamurthy et al., 2004).

Rb is a vital tumour suppressor protein that regulates transition phase of G1/S phase, and its down-regulation is associated with many cancers (Li et al., 2009). p16 expression is also controlled by Rb phosphorylation, which leads to induction in p16 expression and subsequently decreases in CDK4/6 activity and Rb dephosphorylation leads to reduction in p16 level (Lazzerini et al., 2005). Interestingly, p16 expression is also upregulated in Rb null cells, proposing other unknown pathways that regulate p16 gene (Li et al., 2009).

Rb can interact and regulate many effectors of chromatin structure (Chinnam and Goodrich, 2011). Chicas and his colleagues showed the ability of Rb and other Rb’s family members such as p130 and p107 can downregulate the E2F target genes activity that inhibits quiescence. In addition, they showed that upregulation of cyclin E1 after losing Rb can stop senescence induction (Chicas et al., 2010).

Rb’s family (p130 and p107) can control chromatin modifications during senescence through its interactions with DP and MuvB transcription factors (Litovchick et al., 2001).
Any signals that initiate DDR can also upregulate the p16-Rb pathway, which leads to senescence induction in association with p53-p21 activation. However, some senescence activation works mainly through p53-p21 pathway and abrogation of p16 delays senescence in fibroblasts but does not prevent it. Conversely, some epithelial cells are more susceptible to becoming senescent through p16 signalling than fibroblasts (Shay and Wright, 2005).

In addition, this can be different depending cell type or even species. For example, replicative senescence is mainly conducted through p53 pathway in murine, however, in human cells both the p16-Rb and p53 pathways are involved (Smogorzewska et al., 2002). The p16-Rb is also important for formation of SAHF, as a result of silencing proliferative genes (Narita et al., 2003).

Many other results have suggested the involvement of Rb in regulation of genes during senescence. For instance, promyelocytic leukemia (PML), is a marker of senescence, and during senescence the number of PML nuclear bodies are elevated (Bischof et al., 2005). Rb showed ability to recruit PML nuclear bodies during senescence (Bischof et al., 2005).

1.5.2 Role of p53 in senescence

p53 is one of the master regulator of cell senescence and its role in senescence has been extensively studied. In this section, p53 structure, function and role in regulating senescence and its effectors will be discussed.

1.5.2.1 p53 structure and function

p53 is a crucial tumour suppressor protein that is involved in many cellular functions. It can act as regulator of gene transcription. It contains 393 amino acids and is composed from five main domains (Cho et al., 1994). In the N-terminal region, the transactivation domain comprises the first 42 amino acids. This region has a role in regulation of p53 inducible genes. It also plays a role in p53 stability. MDM2 regulates p53 stability through binding to the transactivation domain of p53. In the central part of p53 is a DNA binding domain where p53 responsive elements of the target genes normally bind. Many human cancers are associated with mutation in the central domain of p53. There is a proline-rich domain between the transactivation domain and the DNA binding domain.
This region has a role in the apoptotic activity of p53 (Prives and Hall, 1999). After the DNA binding domain, a tetramerization domain is located as shown in figure 1.4. This domain is important for binding with other proteins that make dimers and tetramers. At tetrameric form, p53 can bind to DNA at high level. After the tetramerization domain, a regulatory domain starts, which is the c-terminus end of p53. Post-translational modifications are normally taking place in this domain, which regulates p53 activation (Prives and Hall, 1999).

![Figure 1.4 Schematic diagram of p53. It composed of main five regions (From C-terminal to N-terminal). Regulatory region is post translational modifications region which regulates p53 activity. Tetramerization region facilities interaction of p53 with other proteins. DNA binding domain is important for DNA binding function. Proline rich domain is next region which is important in apoptosis inducing function. The last region is a tetramerization domain which is essential in the activation of p53 target genes (Prives and Hall, 1999).](image)

The activity of p53 is tightly regulated at different levels. The stability of p53 is an important mechanism by which its function is regulated. Dissociation of p53 from important downregulators proteins such as MDM2 and MDM4 is an essential step for p53 pathway induction. MDM2 is an E3 ubiquitin ligase that induces p53 ubiquitination, which results in its degradation. Different stresses or DNA damage lead to phosphorylation of the transactivation region of p53 by many kinases such as ATM and ATR that are induced during DNA damage responses. This leads to increasing in the half-life of p53 and accumulation of p53 in stressed cells (Stommel and Wahl, 2004). Phosphorylation of p53 at N-terminus at ser15 generally stabilises p53 and inhibits its interaction with Mdm2 (Shieh et al., 2000).

p53 cellular localisation is also an important factor of p53 regulation and function. p53 is localised in the nucleus after cellular DNA damage, and loss of p53 activity is associated with its localization in cytoplasm (Bosari et al., 1995;
Moll et al., 1995). Increase of nuclear export is one form of p53 constraint, so it cannot exerts its effects. Nuclear import and export of p53 is tightly controlled. After DNA damage, p53 normally is imported into the nucleus through nuclear import signalling (NIS), where tetramerization takes place, as seen in figure 1.4 (El-Deiry et al., 1992). Activation of DNA damage responsive elements leads to p53 tetramerization and prevents its exportation to the cytoplasm by nuclear export system (NES). Many proteins that affect p53 exportation have been identified. Mdm2 is an example of a protein that promotes NES of p53 (Stommel et al., 1999).

p14\textsuperscript{ARF} can directly bind to and inactivate Mdm2, so it leads to p53 stabilisation and activation (Kamijo et al., 1998). It isolates Mdm2 in the nucleoli, which results in decreasing in the amount of Mdm2 binding to and ubiquitination of p53 (Weber et al., 1999). Furthermore, p14\textsuperscript{ARF} is also able to prevent Mdm2-p53 complex exportation to the cytoplasm, thereby preventing p53 degradation by proteasome (Xirodimas et al., 2001). These show the importance role of p14\textsuperscript{ARF} in p53 regulation through direct action on Mdm2 activity.

1.5.2.2 p53 –p21

DNA damage response (DDR) is triggered by damage that takes place in genomic or telemetric DNA, due to extracellular or intracellular factors. Extracellular stress, such as ionization radiation and chemotherapeutic drugs, internal factors, such as endogenous ROS, and oncogenic upregulation can initiate DDR. DNA repair can take place and the cell may re-enter cell cycle again. If the damage is unrepairable, apoptosis or senescence normally is initiated (Te Poele et al., 2002; Chen and Ames, 1994; Sherr and McCormick, 2002). Any causes of double strand breaks initiate DDR, which in turn activates ATM and ATR that leads to stimulation of p53-p21 pathway activity (Sherr and McCormick, 2002). The p53-p21 pathway is controlled at different levels by many regulators, for example (MDM2).

In relation to carcinogenesis, up-regulation of oncogenes is another main factor of cell senescence activation through p53 (Chen et al., 2005; Collado et al., 2005). These oncogenes trigger DNA replication, which leads to DDR and cell senescence induction; this mechanism is abolished in cells with ATM.
inactivation (Di et al., 2006). Interestingly, p21 also can induce senescence in a p53 independent manner through TGFβ (Wu et al., 2003).

1.5.3 The role of reactive oxygen species (ROS) in senescence

ROS has shown a role in senescence activation. Many studies have linked the role of ROS in telomere attrition (Von Zglinicki, 2002), which can induce DDR and senescence (Lu and Finkel, 2008; Rai et al., 2009). Accumulation of ROS does not only induce cellular senescence, but also leads to aging (Muller et al., 2007). Other studies suggest that ROS can be accumulated in response to activation of main effector of senescence, as activation of main downstream effectors of the DDR lead to ROS accumulation in both stress and replicative senescence. Activation of p53 (Macip et al., 2003), p21 (Macip et al., 2002), p16 (Takahashi et al., 2006), RAS (Lee et al., 1999) and BRAFV600E (Kaplon et al., 2013) lead to ROS accumulation. Other studies have linked the ROS elevation to initial mitochondrial dysfunction, which leads to dysfunction in the metabolic pathways that result in ROS accumulation (Allen et al., 1999; Zwerschke et al., 2003).

1.5.4 Senescence modulators

Recently, identification and characterisation of novel regulators of cellular senescence has become a primary target for many scientists. These regulators directly or indirectly control two tumour suppressor proteins, p53 and pRB, which consequently lead to cell cycle arrest and senescence induction. Examples of these regulators are PML, PTEN and others that can either stabilise p53 and pRB and therefore induce cellular senescence or degrade them and contribute to cell cycle progression (Mallette et al., 2004; Bischof et al., 2005).

Promyelocytic leukemia, otherwise known as PML, is an important regulator, which has an essential role in tumour suppression through modulation activity of p53 and pRB. This gives it a major role in proliferation suppression, apoptosis and senescence. This can be achieved through binding targets to nuclear bodies (NBs) (Salomoni and Pandolfi, 2002). RasV12 is the first reported oncogene that was able to induce senescence through a PML dependent mechanism (Pearson et al., 2000). PML is enhanced by Ras that in turn causes
recruitment of pRB and pRB in NBs. This ultimately leads to modification in the structure of these tumour suppressors. Phosphorylation and acetylation of p53 lead to cell cycle arrest, and cellular senescence in a PML-dependent manner. Histone deacetylase is an enzyme that is upregulated by PML and can regulate pRB gene transcription, which promotes cell cycle progression and senescence (Ferbeyre et al., 2000; Pearson et al., 2000). PML has also been shown to have down-regulation effects on p53: cells that lack PML exhibited impairment in senescence inducement by the p53-dependent pathway (De Stanchina et al., 2004).

Casein kinase 2 (CK2), it is a ubiquitous kinase which has a role in cancer and cell growth (Ahmed et al., 2002). Many researches have demonstrated the role of CK2 in tumour progression when it is stimulated, which leads to p53 down regulation. Moreover, CK2 also can phosphorylate and induce PML degradation; this lead to impairment in senescence induction (Scaglioni et al., 2006).

Bcl-2 is another example of modulator of senescence. Senescent cells have a distinctive feature, which is the ability to survive and resist the apoptotic mechanism. This phenomenon can be explained by a rising in gene expression of bcl-2 (Wang, 1995). As bcl-2 is anti-apoptotic protein and prevents apoptosis, therefore, senescence is mainly upregulated in this situation (Crescenzi et al., 2003) The transcription factor CREB has shown to increase bcl-2 transcription; in non-senescent cells, this transcription factor is seen at very low concentration (Ryu et al., 2007).

Plasminogen activator inhibitor-1 (PAI-1) is another example of senescence modulator. Silencing the PAI-1 gene bypasses senescence in primary human and murine fibroblasts (Kortlever et al., 2006). Suppression of its expression can permanently induce PI3K-PKB pathway, which is involved in cell senescence suppression (Kortlever et al., 2006). Overexpression of PAI-1 in p53 deficient cells is also able to induce senescence in these cells. This shows that the expression of this gene is critical for senescence induction and an important regulator of p53 pathway (Kortlever et al., 2006).
1.6 The negative effects of senescence

Senescence is a mechanism to prevent malignant transformation of cells after oncogenic upregulation or DNA damage signals (Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). However, accumulation of senescent cells in vivo has shown to have negative effects. This accumulation leads to impairment of organ function due to the fact that senescent cells cannot perform their normal roles, which can influence organ function (Baker et al., 2011; Van Deursen, 2014). In addition, SASPs are secreted from senescent cells, which affect the surrounding microenvironment. They can enhance senescence formation, calling immune cells for clearance and even can induce malignant transformation (Shelton et al., 1999; Kang et al., 2011; Coppe et al., 2008).

Is cell senescence a bad or good thing? Many genes controlling senescence, such as p53, have an important role in preventing cancer transformation in the organism during early life span. However, they clearly contribute to the accumulation of senescent cells later in life, which leads to organismal aging. Cell senescence and its genes could have antagonistic pleiotropic properties, by which they have a good role early in life and a bad role afterwards (Williams, 1957; Campisi, 2013). Therefore, knowing the links between these two different features of senescence could help in understanding senescence in depth and providing better way to bypass the dark side of senescence.

1.7 Senescence and aging

Lack of specific markers of senescent cells has delayed the characterisation of these cells in vivo. Conventional markers of senescence such as p16, p21, p53, p14 and SA-β-Gal are widely used in combination with at least more than one marker to evaluate the percentage of senescent cells in aged tissue. It is clear that senescent cells accumulate in different tissues with time and becomes clearer in advanced age (Campisi, 2013). In addition, in age-associated diseases, such as, atherosclerosis and Alzheimer senescent cells normally accumulate, which contribute to the symptoms of these diseases (Bhat et al., 2012; Wang and Bennett, 2012).

In fact, the connection between senescent cell accumulation and age-related illness is not fully understood, as the main downstream modulators of
senescence such as p53, p21 and p16 are tumour suppressors. Mutations in these genes can result in death from cancer even at early ages (Sherr et al., 2000). In addition, p53 has a role in cell apoptosis as well, which again makes its role in cell senescence and aging not clear because of its opposite dual function (Rodier and campisi, 2011).

Nevertheless, two studies on p16 in vivo clearly linked senescence and aging. The first one showed that BubR1p16-/- KO mice have less senescent cells in different tissues comparing to BubR1p16+/+ KO mice, which leads to delay of age-associated changes such as sarcopenia and can extend life span (Baker et al., 2008).

The second one was performed by the same group and showed that targeting senescent cells in a p16 dependant manner improves ageing (Baker et al., 2011). They conducted a novel method of clearing senescent cells from BubR1 KO mice, which are expressing p16. This procedure relies primarily on transgenic mouse model in which senescent adipocyte, skeletal muscle and eye tissues can be cleared by apoptosis once administrated AP20187, as shown in figure 1.5. This drug can work upon caspase 8 expression through a short Fabp4 promoter, as shown in figure 1.5. This promoter was replaced with the p16 promoter; hence an increase in p16 expression will activate the apoptotic pathway, which leads to cell death. This effectively eliminated the senescent cells that expressed p16, which led to improve the functions of many tissues that showed impairment during p16 induction (Baker et al., 2011).

Figure 1.5 Diagram illustrates construction of INK-ATTAC and the stimulation process of apoptosis (Baker et al., 2011).
Performing senescent cells clearance in humans is challenging. This is because each cleared cell needs a replacement one; otherwise the integrity of human tissue might be affected. However, senescent cells replacement with progenitors to re-build the number of lost cells could be sufficient for sustainable cell senescence clearance (Van Deursen, 2014). Another approach to delay senescent cell accumulation in tissue is using anti-senescence drugs such as Rapamycin, which is effective in maintaining the proliferative capacity of oral epithelial cells after radiation, leading to tissue protection from mucositis (Iglesias et al., 2012). This strategy could delay senescent cell accumulation in human tissue, but it might contribute to malignant transformation, as senescence is a tumour suppressor mechanism and any imbalance in its role could be dangerous.

1.7.1 Senescence as a hallmark of aging
Aging is a complex deterioration on the function of the human organs with age. The Hallmarks of aging, coined in 2013 by Lopez et al, try gathering the factors that contribute into aging phenomena (Lopez-Otin et al., 2013). These hallmarks should appear during normal ageing, and increase of these processes in the lab should accelerate ageing. Moreover, delaying their manifestation should extend healthy life span (Lopez-Otin et al., 2013).
Among hallmarks of aging as seen in figure 1.6, senescence was selected due to many reasons. Firstly, cell senescence accumulates normally in human tissue during aging (Campisi, 2013). In addition, accumulation of senescent cells \textit{in vivo} contributes to aging associated disorders (Baker \textit{et al}., 2008; Baker \textit{et al}., 2011). Lastly, clearance of senescent cells from tissues ameliorates symptoms of aging, and leads to healthy aging (Baker \textit{et al}., 2011). So, these clearly demonstrate the importance of senescence as a hallmark of aging.

1.7.2 Contribution to age-associated illnesses

There are evidences that have been shown the role of senescence in different age-associated diseases. The followings are examples of aging related diseases, in which senescence has shown a direct contribution.

1.7.2.1 Cardiovascular related disorders

In atherosclerosis, there are some evidences of a link to senescence of endothelial cells. The cellular components of vessels in younger are different form older people. Vascular smooth muscle cells (VSMC) and endothelial cells
(EC) are the main cells that acquire senescent features (Wang and Bennett, 2012). Examples of these features are changes in proliferative ability, induction in senescence markers and telomeres attritions. These characteristics can be noticed in lesions of atherosclerosis (Wang and Bennett, 2012).

Delaying cell senescence would be helpful for preventing or at least delaying atherosclerosis formation. Examples of drugs that delay atherosclerosis through senescence are chloroquine, a classical drug for Malaria treatment, which can delay atherosclerosis formation through a p53 dependant mechanism (Razani et al., 2010). Same result was seen with pioglitazone, an anti-diabetic drug, a PPAR agonist, which showed increase in telomeres integrity and length. This led to a decrease in senescence associated markers expression and delay atherosclerosis formation (Wang and Bennett, 2012).

Cell senescence is also induced in myocardial ischemia and hypoxia. A study conducted by Zhu and colleges showed increasing in deposition of myofibroblasts after myocardial infarction. Expression of a main regulator of senescence such as p53 was noticed. Knockdown of p53 decreases cytokine upregulation such as MCP1, IL1, IL6 etc., which are normally induced during senescence (Zhu et al., 2013). Therefore, senescence fibroblasts could be a source of fibrosis and collagen accumulation after myocardial infarction. Thus, senescence cells targeting through modulation p53 activity could be a useful therapeutic method for fibrosis after myocardial infarction.

1.7.2.2 Ocular diseases

Accumulation of senescent cells has been associated with ocular disorders. A study reported that incidence of cataract in BubR1 insufficient mice, associated with senescent cell accumulation was high comparing to wild type (Baker et al., 2008). Consistent with this observation, senescent cells also accumulate in individuals with glaucoma comparing to controls. SA-β-Gal staining of cornea and sclera of glaucomatous individuals was significantly higher compared to the normal (Liton et al., 2005). In addition, cell senescence deposition also increases in retinal pigmentation, which could explain some etiological factors of this disease (Zhu et al., 2009). These studies collectively confirm the role of
senescence in ocular associated diseases, and suggest a strategy for senescent cell modulation in order to treat or prevent ocular diseases.

1.7.2.3 Diabetes and metabolic diseases

Aging and organismal senescence has a contribution in increased incidence of metabolic diseases. The relationship between telomere shortens and insulin resistance has been reported before (Gardner et al., 2005).

In addition, senescence has been shown to be involved in type2 diabetes. This is through increase in insulin resistance in adipose tissue through p53 dependent mechanism. Caloric consumption leads to accumulation of ROS, which contributes to insulin resistance and leads to senescence-like changes and increase in SA-β-gal activation through p53 stimulation. Downregulation of p53 activity in adipose tissue leads to decrease in pro-inflammatory cytokines secretion, which leads to increase insulin sensitivity (Minamino et al., 2009). In addition, SA-B-gal activity and the level of p53 and p21 mRNA is higher in visceral fat from diabetic patients compared to non-diabetic individuals. These observations clearly underline the role of cell senescence on insulin resistance and type2 diabetes (Minamino et al., 2009; Bernet et al., 2014).

Another study also showed that with aging muscle stem cells become senescent, and ability to delay senescence increases the potential of regeneration (Sousa et al., 2014).

1.7.2.4 Others

There are other age-related diseases that associate with accumulation of cell senescence. Senescent chondrocyte, for example, deposits in articular cartilage of people with osteoarthritis. In addition, chondrocyte exposed to DNA damaging agents became senescent rapidly. As mechanical stress to cartilage induces oxidation, it could be one way of inducing chondrocyte senescence in cartilage (Martin et al., 2004).

Senescence has also shown to contribute to the pathology of other diseases. In kidney transplantation, presence of cell senescence in grafted kidneys associates with poor prognosis of transplantation. Presence of high level of
senescence markers such as p16 also associates with unsuccessful renal transplantation (Naesens, 2001). In addition, renal transplantation of transgenic mice (Cdkn2α) with low senescent cells in kidney correlates with higher survival rate compared to mice received kidneys from normal mice (Braun et al., 2012).

The above evidences clearly demonstrate the role of senescence in the pathology of many aging related diseases. Ability to modulate senescence could potentially contribute for treatment of these disorders.

1.8 Senescence as a therapeutic target

It has been shown that senescent cells accumulation leads to impairment in the functions of many tissues. Therefore, the aims of scientists are to delay age-associated illnesses by delaying or targeting cell senescence.

Senescent cells targeting could be a good method to ameliorate senescence-associated disease such as cataract, diabetes and atherosclerosis. The concept of this idea is promising. However, this method is challenging, as it is not simple to translate this transgenic approach that described by Baker and his colleague to human. Therefore, is it possible to target senescent cells in human? One possible way would be to use antibody-drug conjugate (ADC) approach, by which conjugated antibody against specific plasma membrane marker is directed. This leads to binding of ADC to this marker and internalisation of ADC and toxin release, which results in death of the target cells (Law et al., 2004; Smith et al., 2006). For senescent cells, there is a lack of the specific plasma membrane marker. Therefore, characterisation of novel markers of plasma membrane of senescent cells are the first demand in order to use this method for senescent cells clearance.

1.8.1 Approaches for life span extension

Aging is generally defined as deterioration in tissue function throughout a period of time. Recently, it has become one of the most important healthcare concerns. Therefore, it has been a continuous aim to extend lifespan with good health and to ameliorate aging symptoms. In addition, aging is the main cause for all major health-threatening illnesses, and the number of individuals suffering from aging-associated illnesses is predicted to approximately double in the future.
Moreover, pathologies associated with aging have psychological and economic impacts, which urge for interventions that could decrease its consequences.

As mentioned previously, a recent review from Lopez Otin et al, has proposed the hallmarks of aging that contribute to aging process, (figure 1.6). All of these hallmarks are present during normal aging, and when they are experimentally eliminated, this should ameliorate aging symptoms (Lopez-Otin et al., 2013). Telomere attrition is one of these hallmarks; telomere shortening underlies the stop of cell proliferation in vitro and leads to cell senescence (Hayflick and Moorhead, 1961). Presence of exogenous telomerase is enough to keep cells dividing and delay cell aging without cancerous changing (Bodnar et al., 1998).

Impairment in proteostasis has been associated with some aging linked disorders (Powers et al., 2009). Regulators of protein homeostasis normally act through repairing or excising misfolded proteins. This will ensure no more accumulation of damaged proteins is taking place, and also will keep synthesis of new intact proteins (Koga et al., 2011). Some age-associated diseases such as Alzheimer’s, muscle atrophy and Parkinson’s diseases are the result of impairment in folding mechanisms of some proteins (Powers et al., 2009). Impairment in the folding of Hsp72, for example, leads to Muscle atrophy, and its activation maintains muscle function and delays muscle dystrophy (Gehring et al., 2012).

Increased consumption of calories also has been shown to accelerate aging symptoms. Therefore, controlling of caloric intake without extending to level of malnutrition has been associated with longevity and healthy life (Colman et al., 2009).

Delaying aging through caloric restriction is mediated through many nutrient signalling mechanisms, such as GH, insulin receptor, IGF-1 and mTOR pathways. Decreases in these factors have been shown to increase in life span in vivo (Mattison et al., 2012). Interestingly, the level of IGF-1 and GH are very high age and in progeria. Therefore, a reduction in insulin and IGF-1 signalling is widely observed in normal and premature aging (Garinis et al., 2008). A pharmacological inhibition of mTOR by rapamycin can delay aging in mice models (Harrison et al., 2009). However, this drug cannot be used to achieve
healthy aging, as it is also immunosuppressant drug (Law, 2005). Thus, looking for safer drug that increases life span with fewer side effects is demanded.

**1.8.2 ADC as a therapeutic approach**

Antibody drug conjugate (ADC) is a new class of the drugs that is used to target cancer cells. This approach needs very specific marker that is expressed on surface of cancer cell. So, an antibody conjugated with a cytotoxic drug can be directed to a specific marker, which leads to ADC binding and internalisation and toxin releasing that results in cell death. This approach could be used to clear senescent cells, which probably lead to decrease in senescent cell accumulation in different tissue. The potency of ADC can be increased and modulated by different factors depending on which tissue or cancer type is treated (Kovtun and Goldmacher, 2007).

Many cytotoxic drugs that target different cancer cells have been linked to antibodies. Many conjugated cytotoxic drugs that have been used to target cancer cells are tubulin specific toxins. Non-dividing cells are very resistant to these toxins, which make them reliable for human application. An example of these drugs is auristatin, which targets the β-subunit on tubulin and inhibits microtubules polymerisation (Jordan and Wilson, 2004). For senescent cells, these classes of the drugs are not effective, as senescent cells are non-dividing cells and are highly resistance for many classical cytotoxic agents comparing to dividing cells (Hampel et al., 2005).

Binding of conjugated antibody to a target protein on the plasma membrane is normally followed by internalisation of the complex into cytoplasm. The rate of this process is crucial, as some complexes have very low internalisation rate, which could affect the amount of delivered toxins. It has been shown that auristatin conjugates with anti-CD20 and anti-melanotransferrin antibodies have higher internalisation rate than their antibodies without conjugates (Law et al., 2004; Smith et al., 2006).

Releasing of cytotoxic drug after internalisation is also important for optimum cytotoxic effects and cell targeting. Thus, the conjugated drug only has an effect after release and dissociation from antibodies into the cytoplasm.
Therefore, several linkers that are moderately stable in the extracellular environment but fragile inside the cells have been developed. Hydrazone is an example of a linker that is stable at pH 7.4 but dissociates in lower pH (Wu and Senter, 2005). A disulphide bond linker is also used for conjugation of drug to the antibody. It can be dissociated with thiols and reducing compounds, for instance reduced glutathione, which is present at very high concentration inside the cell comparing to the blood (Lambert, 2005; Mansoor and Svardal et al., 1992).

1.8.3 Molecularly Imprinted polymer Nanoparticles
A Molecularly Imprinted Polymer (MIP) is a polymer particle that has been generated using a molecular imprinting approach. Nanoparticles can be generated when small templates such as peptides are present during the process of polymerisation. Monomers holding many functional groups are assembled around the target through covalent and non-covalent bindings, which lead to fixation into positions by polymerisation with high degree of crosslinking. Removal of target from polymers leads to the formation of grooves that are complementary to the target, as shown in figure 1.7(Haupt and Mosbach, 1998).

![Figure 1.7 Schematic of the process of MIP preparation. Monomers are mixed with template peptide (Melittin). After polymerisation of the mixture, cavities are formed which are complementary to the peptide in amino acid sequence and functional group (Hoshino et al., 2010).](image)

Hoshino and his colleagues reported the use of MIPs in blood stream of mice for the clearance of a toxin. MIPs were generated by mixing monomers and melittin, after polymerisation, the melittin was removed and MIPs were purified (figure 1.7). Melittin is a peptide that is one of the bee venom components. It
has the ability to induce renal failure and cardiac arrest at high dose (Hoshino et al., 2010).

To test the ability of MIPs in melittin clearance from blood stream, melittin was injected intravenously into mice alone or with MIPs against mellittin that are generated after mixing with mellittin. Complete mice death was noticed in mice that received mellittin alone. Nevertheless, administering MIPs with mellittin decreased the mortality rate. In addition, the distribution of fluorescent- labelled melittin was dramatically decreased after 15 min of MIPs injection comparing to the control (Hoshino et al., 2010). These observations clearly suggest the possibility of using MIPs in vivo and the ability of the mice systemic organ to clear the by product.

Therefore, MIPs can be used as alternative of ADC for targeting and killing cells. This can be an advantage over antibodies due to easy process of production and its cost. However, antibody has higher affinity for binding to antigen compared to MIPs (Haupt and Mosbach, 1998).

1.9 Bruton’s Tyrosine Kinase (BTK)

1.9.1 Background

BTK is a non-receptor tyrosine kinase that is mutated in the inherited immunodeficiency disease X-linked, agammaglobulinemia (XLA) (Vetrie et al., 1993). In XLA patients, there are complete deficiency of immunoglobulins and B cells in the blood. However, there is no clear defect in other immune cells (Conley et al., 1994). BTK is also expressed in other myeloid and lymphoid cells but not in T cells (Conley et al., 1994).

The role of BTK in the B cell receptor (BCR) pathway has been studied extensively. This is because of the involvement of its action in XLA phenotypes and also in B cells development and function. Nonetheless, BTK is also involved in other pathways of many receptors, such as G-protein chemokine receptors and Toll-like receptors (TLR). In addition, except of T lymphocytes, BTK is expressed in all hematopoietic cells (De Weers et al., 1993; Smith et al., 1994).
1.9.2 BTK structure and activation

BTK is one of the Tec family kinases members that have been highly conserved (Bradshaw, 2010). As shown in figure 1.8, the structure of BTK is homologous to the SRC family kinases. It has the SRC homology domains, SH2 and SH3 and a kinase domain for catalytic activity. BTK contains an amino-terminal pleckstrin homology domain (PH domain) and a domain with a high number of prolines that has a zinc finger motif, which is crucial for the activity and stability of BTK (Hyvonen and Saraste, 1997; Bradshaw, 2010).

BTK stimulation and activation is started after an antigen binds to BCR on the cell membrane, this leads to BTK phosphorylation at tyrosine 551 by SRC family kinases such as Lyn and Fyn or by Syk. This results in BTK stimulation and autophosphorylation at tyrosine 223 in the SH3 domain (Park et al., 1996; Rawlings et al., 1996).

BTK is mainly in the cytoplasm; however, it also can be detected in the nucleus of B cells (Gustafsson et al., 2012). It has been suggested that BTK could regulate transcription in B cells through regulating different nuclear proteins such as ARIDA and MLLT (Hirano et al., 2004; Rajaiya et al., 2006). Despite these proposed roles, the exact function of BTK in the nucleus is not fully understood.

Figure 1.8 Schematic diagram of different region of BTK. (Hendriks et al., 2014).
1.9.3 BTK pathway in B cells

After antigen binding to BCR in B cells, Src protein kinases such as Lyn and Fyn phosphorylate the BCR complex, which results in activation Igα and Igβ of immunoreceptor tyrosine-based activation motifs (ITAM). This leads to formation of binding sites for SYK (figure 1.9) (Rolli et al., 2002). After BCR activation, PI3K is recruited that results also in PIP3 formation (Okada et al., 2000), which attracts BTK to the plasma membrane and permits LYN and SYK to stimulate BTK phosphorylation at Y55140. In addition, BTK can also be activated by PTEN, which dephosphorylates PIP3 and then down-regulates BTK (Hendriks et al., 2014). BTK activation leads to phosphorylation of PCLγ2 (Kim et al., 2004).

PCLγ2 generates inositol triphosphate (IP3) and diacylglycerol (DAG), which stimulate different downstream cascades. DAG is involved in activation of the RAS pathway, which leads to phosphorylation of MAPK (Hashimoto et al., 1998).

BTK and PCLγ2 are also recruited by SLP65 along with other proteins (Weber et al., 2008). BTK mainly phosphorylates PCLγ2 at position Y753 and Y759, which is important for the catalytic activity of PCLγ2 (Kim et al., 2008). In addition, BTK can also be involved in phosphatidylinositol-4-phosphate-5-kinase (PIP5K) recruitment without its enzymatic activity. This leads to the production phosphatidylinositol-4-5-bisphosphate (PIP2) that works as a substrate for PCLγ2 and PI3K (Saito et al., 2003).
1.9.4 Involvement of BTK in other pathways

BTK has shown involvement in B cells adhesion and migration; it is an important molecule for chemokine receptors such as CXCR5 (De Gorter et al., 2007). In addition, stromal cells express CXCL12 (SDF1) in bone marrow, which stimulates BTK through direct association (De Gorter et al., 2007).

The effect of BTK in the signalling of chemokines was studied in mice lacking BTK in B cells. In this model, B cells migration into lymph node was significantly reduced (De Gorter et al., 2007). Moreover, Inhibition of BTK activation using BTK inhibitors in patients with CLL leads to increase of the exporting of malignant B cells into circulation, which emphasises the role of BTK in B cells migration into lymph nodes (Byrd et al., 2013; Wang et al., 2013).

1.9.5 BTK in B cells malignancies and inflammatory diseases

Many studies have shown the role of BTK upregulation in different B cell malignancies, such as chronic lymphocytic leukaemia (CLL), Mantle cell
lymphoma (MCL), and multiple myeloma (MM) (Herman et al., 2011; Chang et al., 2013; Kuehl and Bergasagel, 2012).

CLL is normally associated with the presence of high amount of CD5 B cells in the blood. B cells in CLL normally have low level of IgM and also have anergic response (inability to response to antigen binding) to BCR stimulation. This can be explained by sustained signalling of BCR (Muzio et al., 2008). Development of cancerous CLL cells takes place in lymph nodes, where different factors are provided, such as chemokines, T cells stimulation and antigens (Herishanu et al., 2011).

Continuous BTK induction has been implicated in initiating and maintaining CLL. BTK is highly expressed in B cells of CLL patients, and is extremely phosphorylated in CLL patients (Herman et al., 2011). In vivo BTK<sup>−</sup> mice with CLL showed less cancer development and less cancer formation; however, overexpression of BTK raised cancer formation chances and decreased survival rates (Kil et al., 2013). In addition, ibrutinib, which is irreversible BTK inhibitor, is able to reduce the survival and the growing of B cells in vitro, and also stops activation of AKT and ERK (De Rooij et al., 2012; Ponder et al., 2012).

In MCL, BTK is highly expressed, and it is also associated with phosphorylation of the protein. BTK inhibitors have shown positive effects on MCL cells, same as with CLL (Chang et al., 2013). BTK inhibition in MCL cells also reduces the cells migration and adhesion, which showed the importance of BTK in MCL pathogenesis (Chang et al., 2013).

Autoimmune disease such as rheumatic arthritis usually manifests with aggressive proliferation of synovial membrane and accumulation of immune cells such as macrophages and B cells (Di Paolo et al., 2011). B cells are able to produce autoantibodies; this leads to inflammatory complexes formation, complement stimulation and immune cells accumulation (Di Paolo et al., 2011). Therefore, B cells down regulation through BTK inhibitors are one the option in to manage rheumatic arthritis (Honigberg et al., 2010). This shows the role of BTK activation in some autoimmune diseases such as rheumatic arthritis.

1.9.6 BTK inhibitors

Due to the involvement of BTK activation in different B cells malignancies and in some autoimmune disease, small molecules to target and inhibit its activation
were developed. Ibrutinib is the first BTK inhibitor has received approval (in 2014 to treat mantle lymphoma and chronic lymphocytic leukaemia) (Wang et al., 2013).

1.9.6.1  Ibrutinib

Ibrutinib (otherwise known as PCI-32765) is orally taken. It irreversibly binds covalently to C481 of BTK (Honigberg et al., 2010). It inhibits the stimulation of BTK in tyrosine 223. It is produced by Pharmacyclics under Imbruvica® name (Honigberg et al., 2010; Byrd et al., 2013; Wang et al., 2013). Through caspase3, ibrutinib can induce cell apoptosis in CLL cells (Herman et al., 2001). In vitro, ibrutinib can supress the autophosphorylation of BTK and its downstream targets PLCγ2, Akt and ERK. It can exert the effect at very low concentration, about 10nM, which is enough to obtain a complete suppression of BTK and its downstream pathway in B cells (Aalipour and Advani, 2014). Ibrutinib has shown promising result in clinical trials. In 2012, FDA announced the results of ibrutinib as major breakthrough in the treatment of CLL and MCL. As consequence of this, the drug received the approval later (Aalipour and Advani, 2014).

1.9.6.2  CGI-1746

CGI-1746 is another BTK inhibitor, developed by CGI Pharmaceuticals to target BTK. It reversibly binds to the SH3 domain, which blocks BTK phosphorylation at Y551. It is in preclinical phase and has not received the approval yet (Hendriks et al., 2014). It has ability to downregulate the transactivation of BTK at Y227, which leads to fix the protein at inactive form. (Di Paolo et al., 2011). In vitro, CGI-1746 inhibits B cells proliferation through blocking BCR signalling, it also supresses cytokines production in macrophages through suppression FCyRIII signalling. In a mouse model of rheumatic arthritis, CG1-1746 strongly supressed cytokines and antibodies productions; this lead to a decrease in the erosion area of the joints (Di Paolo et al., 2011).

1.10  Aims

Cellular senescence is a crucial process that has an important role in limiting cancer cells progression and tumour propagation (Lowe et al., 2004). This
project is focused on the characterisation of novel membrane markers of cell senescence. In addition, we propose a method of senescent cells clearance using targeted approaches against our novel membrane markers. The last aim of this project is to understand the significance of one of these novel markers, BTK, in cell senescence and aging.

1.11 Specific objectives

1. To characterise novel membrane markers of senescent cells

2. To clear senescent cells using targeted approaches.

3. To understand the role of BTK in cell senescence and aging.
CHAPTER 2. MATERIALS AND METHODS

2.1 Cell lines culture

Different cancer cell lines with inducible systems and also normal cell lines were used to characterise novel cell senescence markers and modulators. Normal fibroblasts also were used in this study to check the expression of senescence markers and effectors. These varieties of cell senescence models helped us to categorise these novel markers depending on different senescence pathways and origins. Cell lines were maintained in culture using Dulbecco’s Modified Eagle Medium (MDEM) supplemented with 10% foetal Bovine serum (FBS) (GIBCO) and 50 units/ml of Penicillin- Streptomycin (GIBCO).

EJp21 (Fang et al., 1999), EJp16 (Macip et al., 2002) and EJp53 (Sugrue et al., 1997) cancer cells, table1, have tetracycline off system by which the inserted plasmid can express p21, p16 and p53 respectively when tetracycline is removed. This leads to senescence induction in these cells (See the list of the cell lines used in the project, appendix, table 6). These cell lines, which have been used previously in senescence studies, provide clear ideas of which marker is upregulated in response of p21, p16 or p53.

To downregulate BTK phosphorylation, two different small molecules inhibtirs were used. Ibrutinib (PCI-32765, Selleck) and CGI-1746 (Axon, MedChem) were used in this study. These helped us to see the effects of BTK inhibition on senescence and p53.

The culture media for these cancer cell lines contained 1µg/ml of tetracycline. It was also supplemented with hygromycin (100 µg/ml) and geneticin (750 µg/ml) for EJp21 and EJp53 and hygromycin (100 µg/ml) and puromycin (2 µg/ml) for EJp16 as selective antibiotics for these cell lines. For starting gene expression, tetracycline was removed from the culture media by washing cells 3 times with 1x Phosphate buffer saline (PBS).

HT1080-p21-9 cells are another cancer cells that were used in this project, which have Isopropyl β-D-1-thiogalactopyranoside (IPTG) on system. These cells have a p21 plasmid; by removing IPTG from the media the p21 plasmid
was suppressed. To induce p21 expression 25 µM of IPTG was added for 3 days to induce senescence (Porter et al., 2012).

Human fibroblasts were also used in this project. Foetal lung fibroblast IMR90, normal human fibroblasts 501T and RD, which is a fibroblasts from restrictive dermopathy patient were cultured in Dulbecco’s Modified Eagle Medium (MDEM) supplemented with 10% foetal Bovine serum (FBS) (GIBCO) and 50 units/ml of Penicillin- Streptomycin (GIBCO). To induce senescence, the cells were passaged until permanent cell cycle arrest was seen and SA-B-Gal was observed.

2.1.1 Cell passaging
When cells reached 80 % confluence in 10 cm plate, old medium was removed from the plate and it was washed twice with 4 ml of sterile1xPBS (GIBCO). 1 ml of 0.25% Trypsin-EDTA (GIBCO) was then added to the plate and was placed in the incubator for 2-5 min. Cells were then collected with 5 ml of media a 15-falcon tube and centrifuged for 5 min at 200 g. After that, calculated amount of media was then added to cells pellet and was split into many plates, depending on each experiment.

2.1.2 Cell line long-term freezing
To freeze cells, confluent 10 cm plates were used. Cells were collected as described in section (2.2.1). Total amount of freezing media is 1 ml for each cryovial (Freezing media: 90% DMEM media, supplemented with 10 FBS and penicillin streptomycin, 10% DMSO). The vial was put into freezing container (Nalgene), which contained isopropanol to decrease the temperature under control. It was then placed at -80°C, and after 24 hours the vial was inserted into liquid nitrogen for long-term storage.

2.1.3 Cell counting
Cells were counted before splitting. Two methods for counting were used in this project an automated machine counter (BIO-RAD, Automated Cell Counter) and a Bright Line Counting Chamber with an improved Neubauer ruling pattern. 10 µl of cells in suspension were mixed with the same amount of Trypan blue solution, 0.4% (Thermo Fisher Scientific) and 10 µl of the mixture was put into Bright Line Counting Chamber and covered with coverslip. The number of the
cells was then counted in each circle under microscope at 10x objective. For the automated machine, 10 μl of the mixture was added to a specific slide (BIO-RAD, Counting Sides, Dual Chamber for Cell Counter) and was inserted into the machine, which reads the number of the cells automatically. For growing experiments, cells were split and were counted every 3 or 4 days. Fresh media was added after cell counting.

### 2.2 Protein extraction, separation and purification

#### 2.2.1 Whole cell lysate

Medium was removed and plates were washed twice with 1x PBS and trypsinized, collected and kept on ice. 100 μl of RIPA buffer (50 mM Tris HCl, 1% NP40, 0.5% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, pH 7.4) and 1 μl of Protease Inhibitor Cocktail Set III (Calbiochem) and 1 μl of phosphatase Inhibitor (Sigma Aldrich) were added and cell pellet suspended and incubated for 20 min on ice. Cells were ruptured with syringe 5 times and centrifuged for 15 min at 12,000 rpm and the supernatant was transferred into labelled tubes where 35μl 4X loading buffer (8% SDS, 20% 2-mercaptoethanol, 40% glycerol, 0.008% bromophenol blue, 250 M Tris HCl, pH 6.8) was added into 100 μl of the sample. Samples were then stored at -80°C.

For protein extraction from *Drosophila melanogaster*, 10 flies were used. Cold RIPA buffer was used and flies were homogenised using Dounce homogenizer 20 times and left for 30 min on ice before spinning for 20 min at maximum speed.

#### 2.2.2 Extracellular membrane protein separation and purification

Cells were collected by scraping in cold 1x PBS and then centrifuged 500 x g for 5 min at 4°C, and then washed once in 1 ml of ice cold PBS. Approximately, 5-10x10⁸ cells were used for plasma membrane extraction according to abcam65400.

Cells were then resuspended in 1 ml of the homogenize buffer Mix (1/500, protease inhibitor cocktail/ homogenize buffer) in ice-cold Dounce homogenizer. Cells were then homogenized on ice for 30-50 times. To check the efficiency of
homogenization, 2-3 µl of the homogenized suspension was pipetted onto cover slip and observed under microscope.

The homogenate was transferred to a 1.5 ml microcentrifuge tube and then centrifuged in 700 x g for 10 min at 4°C. The supernatant was collected to a new vial and centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was the cytosol fraction, and the pellet was total cellular membrane protein.

The total membrane proteins (pellet) were resuspended in 200 µl of the Upper Phase Solution. 200 µl of the Lower Phase Solution was added and mixed well and then incubated on ice for 5 min (Tube A). A fresh phase tube without samples was prepared. 200 µl of the Upper Phase Solution was added and 200 µl of the Lower Phase Solution was also added (Tube B). Both A and B tubes were centrifuged in a microcentrifuge at 1000 x g for 5 min. The upper phase from tube A was carefully transferred to a new tube (C) and kept on ice.

To maximize the yield, the tube A lower phase was extracted again by adding 100 µl of the upper phase solution from tubeB. And then was mixed well centrifuged at 1000 x g for 5 min. The upper phase was carefully collected from tube A, and combined with the upper phase phase of tube C. the combined upper phase C was extracted, by adding 100µl of the lower phase solution from tube B and was mixed well and centrifuged at 1000 x g for 5 min. the upper phase of C was carefully collected to the new tube D and was diluted with cold water, and centrifuged at 20,000 for 10 min at 4°C. The supernatant was removed, the pellet was the plasma membrane protein which was resuspended in 1x PBS.

2.2.3 Cell fractionation using sucrose gradient
Cells were washed twice at 300xg for 5 min with ice –cold PBS-MC (1x PBS, MgCl₂, 1 mM CaCl₂). Then, cells were resuspended in 1 ml ice-cold Hypotonic buffer (RSB: 10mM HEPES-KOH, 10mM KCl, 1.5 mM, MgCl₂, pH 7.5) containing complete Protease Inhibitor Cocktail (EDTA), 1mM activated Na₃VO₄, 10 mM NaF, 10 µM MG132, 5 mM N-ethylmaleimide and cells were left to swell for 10 Min.
Cells were ruptured using an ice-cold dounce homogenizer. Approximately 40 strokes (monitored by cell disruption using trypan blue/haematocytometer) were done. The homogenate was then centrifuged at 500 x g for 10 min at 4°C.

Approximately 12ml Sucrose Density Gradients for SW40 Ti Rotor were prepared using automated gradient marker. 10-50% Sucrose gradient was used, buffer 1: 10mM HEPES-KOH, 1mM MgCl$_2$, 10% (W/V) Sucrose, pH 7.4; buffer 2: 10mM HEPES-KOH, 1mM MgCl$_2$, 50% (W/V) Sucrose, pH 7.4.

Sucrose Gradients were kept on ice for 10 min before loading the homogenate carefully to top of gradient to minimize gradient disruption. Then, was balanced and loaded tubes into SW40 Ti buckets and centrifuged at 100,000x g for 18 hours at 4°C. After that, the sample was then fractionated into 24x0.5 ml fractions. 50-100 µl was Transferred to 96-well plate and 30 µl of 4x Laemmli Sample Buffer was added, before loading the sample into 10 % polyacrylamide gel and see section 2.5.1.

2.2.4 SDS polyacrylamide gel electrophoresis
The Bio-Rad gel glasses were used to prepare Polyacrylamide gels. After assembling the gel unities, a 10% resolving gel was prepared: 4.8 ml of ddH$_2$O, 2.5 ml of 40% Acrylamide mix, 2.5 ml of 1.5M Tris (pH 8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% Ammonium persulfate and 4 µl of TEMED was added into the gel unit where a space of 1.5 cm was left for the stacking gel. After adding the resolving gel, 2ml of dH2O was added. When the resolving gel was set, the water was removed and roughly 3 ml of stacking gel: 2.23 ml of ddH$_2$O, 380 µl of 40% Acrylamide mix, 380 µl of 1.0M Tris (pH 6.8), 30 µl of SDS, 30 µl of 10% ammonium persulfate, 3 µl of TEMED, was added on the top and 1.5mm/15 wells comb (Bio-Rad) was inserted directly and removed later after the gel setting.

The sample was prepared as described in 2.3.1 and 2.3.2 and 100µl of protein samples was mixed with 35 µl loading buffer. The sample was then boiled at 95°C, and then was allowed to cool down at room temperature before was spun down and loaded into the SDS-Polyacrylamide gel.
2.3 Colony Formation Assay

Colony formation assay is a cell survival assay that is based in the ability of single cells to form a colony. To evaluate the capacity of cells to form colonies, the cells were split into 60 mm plates (200 cells) and treated with different BTK inhibitors or DMSO in triplicate. Cells were placed in incubator and left to grow for 14-15 days. Media was changed every 5 days with fresh BTK inhibitors.

2.3.1 Giemsa Staining

Plates were washed with 3 ml of 1× PBS and fixed with 2 ml of 10% neutral buffered formalin (SIGMA-ALDRICH™). Plates were incubated at room temperature for 30 min. Formalin was removed by aspiration and plates were washed twice with 1× PBS. Plates were allowed to completely air dry with the lids removed. 5 ml of Staining Reagent was added to the plates and they were incubated at room temperature for 5 hours. The stain was poured off and the plates were rinsed with ddH₂O. Plates were allowed to air dry and the number of colonies from each plate was counted and recorded.

Staining reagent: 6.4 ml of PO₄ buffer (67mM), 89.6 ml of dH₂O, 4 ml Giemsa stain (Fluka). Prepare fresh for each stain

1M PO₄ buffer: 1M Sodium Phosphate monobasic, 1M Sodium Phosphate dibasic mixed in a 2 (dibasic) to 1 (monobasic) ratio; pH to 7.0 with NaOH.

2.4 Flow cytometry

Flow cytometry is a technique that can measure the optical and fluorescence of single cells. It can also measure the fluorescence of DNA (through PI staining), apoptosis (through annexin V staining), extracellular markers such as CD in WBCs and others (Brown and Wittwe, 2000). It can also differentiate sort cells population through their size and surface markers (Brown and Wittwe, 2000).

2.4.1 Propidium iodide

Propidium iodide (PI) is a fluorescent molecule that intercalates into nucleic acids. It binds to DNA and fluoresces red when excited with 488nm wavelength light. To assess the percentage cell death, a PI staining followed by a FACS analysis was normally performed in this project. Cells were split into 6-well plates and left to grow for 24 hours before treatment. All samples were made in
duplicate. Media from each sample was aspirated and collected in labelled 15 ml falcon tubes. To wash the cells, 2 ml of 1x PBS was added to the plates and kept in the same tube of the media. 1 ml of trypsin was added to the plates and cells were collected in the same tubes of the media and 1x PBS. The tubes were then centrifuged at 200g for 5 min and the pellet was then washed twice with 1x PBS. For fixation, 1 ml of 70% ethanol was mixed with the pellet and place at -20 °C for at least 30 min.

Cells were taken out of the freezer and were centrifuged at 200 g for 5 min. The cells were then washed once with 1 ml of 1x PBS. The pellet was resuspended in 300 μl of PI buffer: 50 μg/ml of Propidium Iodide, 10 μg/ml RNase A, 1 xPBS, and transferred to polystyrene round-bottom tubes. The tubes were incubated for 30 min at 37 °C in the dark. 10,000 events were recorded for each sample using the Beckton Dickinson FACSCanto II and FACSDiva 6.0 software (Beckton Dickinson) for acquisition and analysis.

2.4.2 Annexin V
Adherent and floating cells were collected and washed with 1x binding buffer without fixation. Cells were then resuspended at 1x10^6 cells /ml in 1x binding buffer. Approximately, 0.5µl of annexin V- FITC was added for each 1 ml of 1xbinding buffer (1:2000). The mixture was then incubated for 15 min at room temperature in dark. The samples were then ready for reading with FACS.

Annexin binding buffer 5x: 50mM HEPES, 750mM NaCl, 5mM MgCl₂, 9mM CaCl₂, pH 7.4.

2.4.3 Senescent cell detection using extracellular markers using conjugated antibody or MIPs particles.
This experiment normally started with 90% confluent 6 well plates. The cells were washed with cold 1x PBS, and then collected by gently scraping them in 0.5 ml cold 1x PBS. The cells were then kept on ice. Then, cells were centrifuged at 200 x g for 5 min at 4ºC, supernatant was then discarded.

The cells were then resuspended in 200µl of Blocking Buffer and incubated 15 min on ice. After that, the cells were transferred into 96 rounded bottom multiwell plates. The cells were spun down 500 x g for 5 min at 4ºC, supernatant was discard and resuspended with Antibody Mix or MIPs particles (1:4 and 1:9
diluted with blocking buffer) and Incubated at 4°C in the dark for 30-45 min, then the cells were wash 2 times with Blocking Buffer (150µl per well). The pellet was resuspended in 300-500µl of Blocking Buffer and transferred to FACS tube then, read fluorescence at cytometer.

- Blocking Buffer: 0.5%BSA+1xPBS

- The concentration of the MIPS particles is 0.3mg/ml

- Antibody Mix: combination of 2 to 4 fluorescently tagged primary antibodies in the blocking buffer (final staining volume: 25-40 µl). Please look for the list of the antibody used for flow cytometry in this project in table.5, appendix).

2.4.4 Antibody internalisation assay (CypHer5E Mono NHS Ester)

CypHer5E is a red excited fluorescent, pH sensitive cyanine dye. It is non-fluorescent at basic pH and it is fluorescent at acidic pH. Monoclonal antibody from ascetic fluid should be dialysed in 1x PBS at room temperature for 4 hours. Antibody was removed from dialysis cassette and the antibody concentration was determined.

Determine antibody concentration, use the formula:

\[
Molar \ Concentration\ of\ Ab = \frac{Absorbance\ of\ antibody\ at\ 280nm}{Molar\ extension\ coefficient\ of\ Ab}
\]

A= Absorbance of antibody at 280 nm
B= Molar Extension coefficient of antibody=210000 M⁻¹CM⁻¹.

2.4.4.1 Determination of CypHer5E Mono NHS Ester concentration

The whole vial of CypHer5E Mono NHS Ester dye was dissolved in sterile freshly opened DMSO, 500µl for 5mg pack and mixed and sonicated for a few second to ensure the solution was homogenous. 5µl of reconstituted dye was added to 4ml of PBS/0.5 M Sodium carbonate buffer pH 8.3 (9:1) in a glass vial and mixd well and at 500 nm the absorbance was determined and 0.125% DMSO was used as a blank. Then, the concentration of dye was calculated using the formula:
\[ Molar \text{ concentration of } \text{CypHer5E} = \frac{\text{Absorbance of dye at 500nm x D.F (800)}}{\text{Extension coefficient of dye}} \]

Dilution factor = 4ml %5 µl = 800
Extension coefficient of the dye at 500nm = 40 000 M\(^{-1}\)CM\(^{-1}\)

2.4.4.2 Conjugation of CypHer5E Mono NHS Ester to an antibody

The antibody was diluted to 1 mg/ml in 1XPBS and 0.5 M Sodium carbonate buffer pH 8.3 (9:1); (v/v), and the amount of dye to be added to the 1mg/ml antibody solution was calculated using the following equation:

Molar concentration of the antibody \times \text{Volume of the antibody solution} \times \text{Molar excess} = \text{Moles of CypHer5E required}

20 molar excess was recommended by the product.

Therefore, the volume of dye to be added to the antibody solution can be calculated using the following equation:

\[ \text{Volume of CypHer5E Mono NHS Ester} = \frac{\text{Moles of CypHer5E required}}{\text{Molar concentration of CypHer5E}} \]

Add the required amount of CypHer5E Mono NHS Ester to 1mg/ml antibody solution and mix. The solution should be left in the dark for approximately 1 hour, then it was dialyzed for at least 4 hours at 4\(^{\circ}\)C to separate labelled antibody from unconjugated dye. The conjugated antibody which was inside the dialysis tube was collected into a separate Eppendorf tube.

2.4.4.3 Estimating the final dye/protein (D/P) ratio

Dye antibody ratio (D/P) optimally performing value is between 7 to 12.

The estimated D/P ratio was calculated as follows:
\[
\frac{D}{P} = \frac{(A)\text{Dye} \times \varepsilon \text{Protein}}{(A_{280} - 0.16(A)\text{Dye})\varepsilon \text{ dye}}
\]

\(A_{\text{Dye}}\) is the absorbance of the labelled antibody conjugate @500nm

\(A_{280}\) is the absorbance of the labelled antibody conjugate @280

\(\varepsilon_{\text{Dye}}\) is molar coefficient of the dye (40 000 M\(^{-1}\)CM\(^{-1}\) @ 500nm)

\(\varepsilon_{\text{Protein}}\) is molar coefficient of the antibody (210 000 M\(^{-1}\)CM\(^{-1}\) @ 280nm)

### 2.4.5 Study the internalisation of B2MG and DEP1 using senescent EJp16 cells

EJp16 senescent cells were collected and blocked as described in section 2.5.3., normally 500,000 cells were used. 100 µl of the labelled antibody (DEP1 and B2MG) was mixed 500,000 senescent EJp16 cells (as a pellet). The mixture (labelled-Abs and the cells) was kept on ice in dark. In time dependant manner, 10 µl of the mixture (labelled-Abs and the cells) was mixed then added to 200 µl of the blocking buffer and was read by FACS on APC channel. After 15 min, another 10 µl was mixed with 200 µl of the blocking buffer and was read by FACS on APC.

### 2.5 Protein Analysis

#### 2.5.1 Immunoblotting

A piece of Immobilon-P membrane (Millipore) was cut to the dimensions of the gel and wet in the transfer buffer : (1L of 1x transfer buffer, 2.9 g Tris Base, 5.8 g glycine and 0.37g SDS). The transfer tank was then assembled and was covered with cold transfer buffer. One piece of the sponge was placed on the cassette holder and one filter paper sheet was placed on the top. Then the gel containing the protein placed one top of the filter paper after immersing in the transfer buffer. The membrane was then placed on the top of the gel, and another filter paper was then placed on the top of the gel. Then, the cassette holder was closed and placed into transfer tank so that the side of the cassette with gel was facing the cathode (\(-\)). After covering the tank with transfer buffer the system was switched on at 120 V for 80 min.
The membrane was then removed from the cassette holder and the protein side was then labelled. The membrane was then blocked with 5%BSA+ 1xPBS-0.05% tween for 30 min before washing 3 times with 1xPBS-0.05% tween each time 10 min. The membrane was then incubated with primary antibody (Table 1, appendix). The primary antibody was diluted in 5ml of the blocking buffer and incubated overnight at 4°C on a shaker. In the next day, the membrane was washed 3 times with 1xPBS-0.05% tween each time 10 min, before incubating with the corresponding secondary antibody (Table 4, appendix) for 1 hour at room temperature on a shaker. After that, the membrane was washed 3 times with 1xPBS-0.05% tween each time 10 min.

The visualization of the proteins was performed by Pierce ECL plus western blotting substrate (Thermo Scientific). From the substrate kit, equal amount of the solution 1 and solution 2 were mixed and the membrane was then incubated for 5 min, covered with cling film and was placed in a film cassette with the protein side up. In the dark room, a sheet of autoradiography film was placed on top of the membrane and was exposed for 30 seconds and 1 min, after that the film was inserted into the film processor for film developing.

2.2.1 Immunofluorescence
Cells were split into 6 multiwall plates containing sterile coverslips and allowed to grow. The medium was then aspirated and the cells were washed 3 times with cold 1xPBS, and fixed with 10% formalin for 10 min. Cells were then washed 3 times 1xPBS and permeabilized with 1 ml of 0.1% Triton X-100 for 10 min. After that, cells were washed 3 times with 1x PBS and blocked with 1% BSA for 30 min. Then the coverslips containing cells were incubated with primary antibody (Table 2, appendix) overnight in the fridge. The next day, the coverslips were washed 3 times with 1xPBS and incubated with corresponding secondary antibody (Table 2, appendix) for 1 hour in the dark. After the incubation, the coverslips were then washed 3 times 1xPBS and stained with DAPI for 10 min. The slides were labelled and the coverslips were mounted and sealed. The slides were then analysed using the Nokia TE300 semi-automatic microscope.
2.6 Mass spectrometry

Senescent and growing EJ p21 and EJp16 plasma membrane samples were separated by 10% SDS-PAGE. After staining with the Instant blue, Gel lanes were cut sequentially into slices of approximately 1.5mm and transferred to a 96 well low binding PCR plate. Each slice was washed/swollen with ammonium bicarbonate (80ul, 50mM) for 30 minutes, after this time the buffer was aspirated off using a Gilson. Each slice was destained with acetonitrile (80ul) for 30 minutes, the solvent was removed.

After aspiration of the final acetonitrile trypsin (Promega, sequencing grade modified trypsin V5111, 20ug/1.8ml 25mM ammonium bicarbonate, 15ul) was added to each dehydrated gel piece. The plate was sealed and heated at 30oC overnight. The sealing film was removed and extraction buffer added to each well (80ul, 97% TFA (0.2%) 3% acetonitrile). The samples were extracted at room temperature for 1 hour. The extracted samples were transferred to low-binding Eppendorf tubes and concentrated to dryness in a speedvac. The samples were re-dissolved in injection solvent (40ul, 5% TFA) and analyzed by mass spectrometry (Shevchenko et al., 2006). Nanoscale LC was used to separate the complex peptide mixtures using a Waters nanoACQUITY UPLC. Chromatography was performed using a 50 minute reversed-phase gradient (formic acid (0.1%)/acetonitrile) and a 75 µm x 25 cm C18 column (Waters, BE130) operated at 300 nL/min. Mass spectrometry analysis was performed using a SYNAPT G2S (Waters Manchester UK) operated in a data-independent (MSE) manner. The selected analysis mode enabled precursor and fragment ions from the tryptic digest to be analyzed simultaneously. The data acquired was processed and searched using ProteinLynx Global Server (Waters) and visualized and reanalyzed using Scaffold (Proteome Software, Oregon, USA). This work was performed kindly by Rebekah Jukes-Jones, MRC Toxicology unit, University of Leicester.

2.7 In vivo longevity study using Drosophila Melanogaster

The longevity experiments using *Drosophila Melanogaster* were performed in ten tubes which contain the food (maize 504g, glucose 555g, brewer’s yeast 350g, agar 62.5g, nipagin 20%, 99% propanoic acid 21ml, in 7L of H2O) with
DMSO as controls and ten tubes, which contain the food with the ibrutinib (10µM). Each tube contained ten flies, so the total was 100 flies for each condition. The food was prepared in the new tube with fresh ibrutinib (10µM) and DMSO and the flies were transferred. Flies number was counted every day.

For food changing, the food was heated until become liquid, then 1200 µl of the food was transferred to each tube. The ibrutinib, CGI and DMSO were added and mixed in the tubes with food then were kept at room temperature for 4 hours until the steam disappeared. The flies were then transferred to the new tube, and left in the flies room at 25°C.

2.8 Detection of senescence-associated β-galactosidase activity

Cells were washed twice with cold 1xPBS, and fixed with 3% formaldehyde for 5 min at room temperature and washed twice with cold 1xPBS 5 min each time, and then staining solution was added (X-gal in dimethylfomamide I mg/ml, citric acid/ Na phosphate buffer (pH = 6.0) 40mM, potassium ferrocynide 5mM, potassium ferricyanide 5mM, NaCl 150mM, KCl 2mM). Cells were then incubated at 37°C without CO₂. After 24 hours, blue colour of cells was used as marker of senescent cells.

For flies staining, flies were fixed in 4 % formaldehyde for 15 min at room temperature, and then washed one with PBS then stained with working solution for 48 hours at 37°C. The flies’ age was 14 and 40 days old.

2.9 Transfection and culture of bacterial cells

BTK plasmid was used to either overexpress or silence BTK. To have enough plasmids for cell transfection, plasmids were grown using competent DH5α cells. The bacteria cells were put on ice to thaw and mixed with 0.5 µg of plasmid. They were incubated on ice for 30 min and heat shocked at 42°C for 30s. After the heat shock, they were immediately placed on ice and 900 µl of LB media was added to each tube. Then, cells were left to grow for 1 hour at 37°C in the shaker. The bacterial cells were spun down and resuspended in 50 µl and plated in agar plates containing ampicillin. Plates were placed in the 37°C oven overnight. The next day, a colony was picked and placed in 5 ml of TB supplemented with the desired antibiotic and left to grow overnight in the shaker at 37°C.
The E.Z.N.A. ® Plasmid Midiprep Kit (OMEGA Bio-Tek, Inc.) was used for the isolation of plasmids from the 5 ml bacterial cultures. Bacteria were pelleted by centrifugation at 4000 rpm for 10 min; the following steps were performed according to the manufacturer protocol. DNA was eluted in 1 ml of sterile ddH$_2$O and quantify using a spectrophotometer. Plasmids were stored at -20°C until further use.

2.9.1 Plasmid Transfection
Transfection of plasmids was made using Lipofectamine™ 2000 (Invitrogen). Cells were split one day before transfection and were 70-80% confluent on the day of transfection. For each 60 mm plate, 0.5 ml of serum-free media were mixed with 20 μl of lipofectamine™ 2000 in one tube and incubated at room temperature. In another tube, 8 μg of DNA plasmid (Mission pLKO.1 Empty vector control plasmid DNA, Sigma Aldrich SHC001) or a BTK plasmid (OriGene RG211582) were diluted in 0.5 ml of serum-free medium. After the 5 min incubation, the diluted DNA was combined with the diluted Lipofectamine™ 2000. The solution was mixed gently and incubated for 20 min at room temperature to allow the formation of the complexes. In the meantime, the medium in the cells was aspirated and changed for serum-free media. After the incubation time 1 ml of the complexes was added to each plate and the cells were placed in the incubator at 37°C. Medium was changed after 5 hours and cells were left for 18-24 hours before using them for further experiments. For transfection using other size of plates, volumes were scale down accordingly.

2.9.2 ShRNA Transfection
The use of shRNA allows the down regulation of expression of specific mRNAs; shRNA against BTK that was used in this project. BTK shRNA plasmid (h) from sana cruz (sc-29841-SH) was used to knock BTK gene down, which allowed to see the effect of BTK gene in cell senescence. Each plasmid contained a puromycin resistance gene.

shRNA was transfected into EJp53 cells using Lipofectamine™ 2000. Cells were split the day before transfection and were 70%-80% confluent at the moment of transfection. For each 60mm plate, 10 μl of Lipofectamine™ 2000 were mixed with 0.5 ml of media and incubated for 5 min. On a separate tube,
1µg of the shRNA was mixed with 0.5 ml of media. After the incubation time, the diluted shRNA was mixed with the diluted Lipofectamine™ 2000 and incubated at room temperature for 20 min. During this time, the medium in the plates was aspirated and changed for serum-free media. 1 ml of the shRNA/Lipofectamine complexes was added to each plate and the plates were placed in the incubator at 37°C. The medium in the plates was changed the next morning and the cells were kept with selective antibiotic (Puromycin). The cells were selected for 2 weeks, and BTK depletion was confirmed by western blot.

2.10 Immunohistochemistry
Dissected tissue sections fixed with 10% formalin for 24 hours at room temperature, sections then were rinsed with water for 30 min and dehydrated through increasing concentration of ethanol, 45 min each. Tissues then were cleared 2 changes of xylene, 1 hour each, so tissue sections can be miscible with paraffin later. After that, tissue sections were immersed in 3 changes of paraffin, 1 hour each, and were embedded paraffin block (can be stored at room temperature for years). Paraffin embedded tissue was cut on microtome and float in 40°C water bath. Sections then were transferred into slides (superfrost plus slide). Then, slides were deparaffinised in 2 changes of xylene, 5 min each, and 2 changes in 100% ethanol 10 minutes each before blocking endogenous peroxidase activity by incubating slides in 3% H₂O₂ solution in 100% methanol for 10 min.

For antigen retrieval, slides were kept in 10mM citrate buffer, pH 6.0 and incubated at 100°C. Slides were left to cool down at room temperature before wash them two times with 1xPBS for 5 min. Then, tissue sections were marked with aPap pen and then they were blocked with 5%BSA fetal bovine serum in 1xPBS, and incubated at 4°C in a humid chamber for 1-2 hours. Then, primary antibody was applied, and was incubated in humid chamber again at 4°C overnight. Slides were then washed 3 times with 1xPBS for 5 min. Streptavidin-peroxidase (Steptavidin, Horseradish Peroxidase, Vector ®-SA-5004) was applied for at least 30 min. Biotinylated secondary antibody (Appendix) was added for 1 hour, and slides were then washed 3 times with 1xPBS for 5 min, and then slides were washed 3 times with 1xPBS for 5 min before adding Peroxidase substrate (DAB Peroxidase (HRP), Vector ®-SK4100). Slides were
rinsed with H₂O before immersing in Hematoxylin for 15 seconds and were rinsed with H₂O.
Tissues were dehydrated through four gradual changes of ethanol (70%, 90%, 100% and 100%) and also were cleared through two changes of xylene before applying mounting on slides.

2.11 Separation of white blood cells from whole blood
35 ml of whole blood were added carefully and slowly to 15 ml of histopaque solution (SIGMA-10771-500ml), (avoiding disrupting and mixing blood into histopaque layer). Sample was then centrifuged at 4000 RPM for 30 min without acceleration or deceleration. White layer above the red blood cells and below histopaque solution is White blood cells. This layer was then collected into new tube and 30 ml of RPMI media was added and then centrifuged at 2000 RPM for 10 min. Supernatant was then discarded and pellet was stored at -80 °C or re-suspended for further analysis.

2.12 Immunoprecipitation

2.12.1 Immobilising p53 antibody
100 µl of protein A beads was spun down and the supernatant was discarded. The beads were washed 3 times with 500 µl of 1xPBS-0.05% tween and were resuspended in 100 µl of the same buffer. Approximately, 5µg of p53 antibody (DO-1, sc-126) was added to the beads and was incubated for 1 hour at 4ºC in the rotator. Magnetic rack was used to remove the supernatant.

2.12.2 Immunoprecipitation
EJp53 cells with inducible gene for p53 were cultured without tetracycline for 4 days. Cells were washed with cold 1xPBS and scraped on ice. 1ml of IP buffer (150mM NaCl, 0.1% Triton, 20mM HEPES, 1mM EDTA, Protease inhibitor and NaF 1;100, 1mM DTT) was added, mixed and incubated for 30 min. The mixture was then centrifuged for 15 min at maximum speed. Supernatant was collected and protein concentration was determined. 250µg of cell extract was added to the p53-bead mixture and incubated overnight at 4ºC. Beads were pulled down using magnetic rack and washed 3 times with cold IP buffer.
2.13 *In Vitro* Kinase assay

Full length of Recombinant BTK (Invitrogen, PV3363) and different regions of p53 were used in this study. Different p53 regions (Mammalian p53, recombinant full length p53, 1-80, 100-300, 300-393) were provided kindly from Dr Nickolai Barlev Department of Biochemistry, University of Leicester.

To examine the ability of BTK to phosphorylate p53, 0.5 µg of BTK and 0.5 µg different regions of p53 were added in tube contained 25 µl of the kinase buffer (12.5 mM (pH7.5), 10mM MgCl₂, 1 mM EGTA, 0.5mM Na₃VO₄, 5mM β-Glycerophosphate, 0.01% Triton X-100, 2 mM DTT, 200 µM ATP). Approximately, 0.37 MBq of [³²P] γ-ATP was added to each tube for 30 min at 30 °C. Loading buffer was then added to each sample, after that the samples were boiled for 5 min and loaded into 10% polyacrylamide gel. The gel was then dried using (Model v583, Gel drier (Biorad) for 1 hour. Phosphor screen (Care of GH Health care) was then putted into the dried gel for 1 hour. Phospho-image of dried gel was then developed using (Typhoon TRIO+ Variable mode manger) typhoon.
CHAPTER 3. CHARACTERISATION NOVEL MARKERS OF CELL SENESCENCE

3.1 Extracting the plasma membrane fraction of EJp16 and EJp21 cells

In order to identify novel plasma membrane proteins of senescent cells that could be used as markers, EJp16 and EJp21 cancer cell lines were used. These cells have a tetracycline off expression system for p16 (Macip et al., 2002) and p21 (Fang et al., 2002) expression respectively. Figure 2.3 (A) shows induction of p21 and p16 expression in EJp21 and EJp16 respectively after removing tetracycline.

![Figure 2.3](image)

**Figure 3.1 Plasma membrane separations of EJp21 and EJp16 cells.** A) Expression of p16 and p21 upregulated in senescent EJp16 and EJp21, after 6 days of tet removal. B) Plasma membrane of EJp21 and EJp16 were fractionated using abcam 65400 protocol (Plasma membrane extraction kit). Calnexin was used as a control for the plasma membrane, and MAPK was used as a control for the cytosol. The experiment was performed two times. (+) with Tetracyclne, (-) without tetracycline, (C) Control, (S) Senescence.
Plasma membrane and cytosolic fractions of senescent and control EJp21 and EJp16 were loaded in 10% polyacrylamide gel, as seen in figure 3.1(B), and processed kindly by Mrs Rebekah Jukes-Jones (Proteomics group, MRC Toxicology unit, Leicester). Calnexin, a marker of the membrane fraction, showed a clear separation of plasma membrane from cytosolic fraction, which is indicated by the presence of MAPK (Figure 3.1B).

A list of proteins upregulated in plasma membrane of senescent EJp21 and EJp16 cells was obtained. DEP1, EBP50 and VAMP were selected from a common list of the proteins expressed in senescent EJp21 and EJp16 but not in growing EJp16. STX4, NTAL and ARMCX3 were selected from common list of the proteins expressed in senescent EJp21 and EJp16 but not in the growing EJp21. PLD3 and VP26A were selected from the list of proteins expressed in senescent EJp21 but not in growing EJp21. B2MG and LANCL1 were selected from list of proteins expressed in senescent EJp16 but not in the growing EJp16. ICAM was found in list of proteins expressed in senescent EJp21 and EJp16 but not in growing EJp21, and it has been described previously as a marker of senescence (Gorgoulis et al., 2005). Please see the discussion section for more details on these proteins. None of these proteins had been linked to senescence and all of them were predicted to be located on the plasma membrane using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

3.2 Validation of potential markers of senescence using EJp16 and EJp21

Validation of the selected markers of cell senescence was performed. Western blot was the first confirmation approach. In order to induce senescence in these cells, tet was removed for at least 5 days. As seen in figure 3.2, in EJp16 cells, DEP1, NTAL, EBP50, STX4 VAMP3 and ARMCX3 levels were very low in growing cells. However, in senescent cells, the amounts of these proteins were highly upregulated, except for VAMP3, where no change was noticed. In EJp21 cells, the level NTAL and DEP1 were clearly increased, however, EBP50, STX4 and ARMCX3 showed low level of expression after entering senescence.
VAMP3 displayed increase in senescent EJp21 cells, but not in EJp16 cells. The levels of B2MG, VPS26A and LANCL1 and PLD3 were not increased significantly in any model of senescence. Lastly, as expected, DCR2 a known marker of senescence (Collado et al., 2005), showed higher expression in senescent EJp21 and EJp16 but not in growing cells; however, it was clearer in EJp16 cells.

Figure 3.2 Validation of potential markers of senescence in EJp21 and EJp16 using western blot. For senescence upregulation, tet was removed for 7 days, media and fresh tet were added after 3 days. After 7 days, cells were washed with cold 1xPBS, and the cells were collected using scraper. For plasma membrane proteins purification, the kit from abcam 65400 (Plasma membrane extraction kit) was used. DEP-1, NTAL, EBP50, VAMP3 and ARMCX3 were highly upregulated in EJp21 and EJp16 after senescence induction. No change was noticed in expression of LANCL1, B2MG, VPS26A and PLD3 in both models. DCR2 was used as a marker of senescence. Calnexin and Na/K ATPase were used as loading controls for plasma membrane proteins. The experiment was performed twice independently. (C) Control, (S) Senescence.
In order to quantify each band accurately, Image J software was used. Protein expression in EJp16 showed very high quantification after senescence comparing to the same protein in EJp21 after senescence induction (figure 3.3). DCR2 showed at least 10 fold increase in senescent EJp16 compared to growing cells. DEP1 and ARMCX3 showed roughly 8 fold increase in EJp16 after cells entering senescence. NTAL and EBP50 showed 6 and 5 folds in increase in senescent EJp16 cells respectively. STX4 and B2MG showed only from 3 to 2 fold increase. In EJp21 cells, EBP50 showed more than 10 fold increase after EJp21 entering senescence. STX4 and ARMCX3 showed 4 and 3 folds increase respectively. The remaining markers showed either very low increase or no differences in expression between senescence and growing EJp21 cells. Although the percentage of SA-βGal positive cells is higher in EJp21 than EJp16, potential markers of senescence showed higher expression levels in EJp16 comparing to EJp21 after senescence induction, as seen in figure 3.5.

Figure 3.3 Quantification of EJp21 and EJp16 bands using Image J. EJp16 showed higher expression after senescence induction comparing to EJp21, except for EBP50 and STX3 that showed higher expression in EJp21 after senescence. VPS26A, PLD3, B2MG and LANCL1 showed very low expression in both models. Error bar represents standard deviation.
Figure 3.4 Senescence-associated-β-galactosidase staining of EJp21 and EJp16 cells. (A) SA-β-Gal for EJp21 and EJp16 was performed after 7 days of tet removal. The cells were fixed with 3% formaldehyde before washing with 1×PBS twice and adding the staining solution for 24 hours at 37°C. (B) Quantification of the percentage of the positive SA-β-Gal of EJp16 and EJp21 cells from two experiments. Error bars represent standard deviations. The experiment was performed twice.

3.3 Western blot analysis of markers of senescence using EJp53
After validation of the expression of these markers in EJp16 and EJp21 cells, we tested their expression of the putative markers in EJp53 cells. EJp53 has tet off system that normally become senescence after 5 to 7 days of tet removal (Sugrue et al., 1997). The expression of NTAL, EBP50, B2MG and VAMP3 were highly upregulated in senescent EJp53 comparing to growing cells, as seen in figure 3.5. No change in the expression of DEP1 and ARMCX3 were noticed. As expected, higher expression of known marker of senescence DCR2 was seen after cells entering to senescence. These results confirm the
suitability of the characterised markers in senescence identification. However, the experiment was performed once, so confirmation of these results should be performed.

**EJp53**

<table>
<thead>
<tr>
<th>Marker</th>
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<td>NTAL</td>
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<td>B2MG</td>
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<td>Calnexin</td>
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<td>DCR2</td>
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<td>Na/K ATPase</td>
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<td>EBP50</td>
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- **EJp53**

**Figure 3.5 The expression of senescence markers in EJp53.** For senescence induction, EJp53 cells were incubated without tet for 4 days, after that cells were washed with cold 1xPBS, and the cells were collected using scraper. For plasma membrane proteins purification, the kit from abcam 65400 (Plasma membrane extraction kit) was used. NTAL, B2MG and EBP50 were induced in senescent EJp53 compared to the growing cells, However, for VAMP3, the quality of the blot was very low, so the upregulation cannot be confirmed. DCR2 was used as a marker of senescence, and Na/K ATPase and Calnexin were used as loading controls. The experiment was performed once. (-) Control, (+) Senescence.

### 3.4 Study the expression of putative of markers of senescence using HT1080p21-9

The new putative markers of cell senescence were validated in EJp21, EJp16 and EJp53. The next aim was to confirm the expression of these markers in
senescent cells from different cell background. HT1080p21-9 cells lines were used (Porter et al., 2012). These osteosarcoma cells have IPTG on system where expression of p21 is induced after IPTG addition. To induce senescence, IPTG was added for at least 4 days until permanent cell arrest was seen, and SA-B-gal was positive.

The expression of NTAL and STX4 was highly increased in senescent HT1080p21-9 cells, as seen in figure 3.6, compared to non-senescent cells. DEP1, EBP50 and NOTCH3 showed moderate expression in senescence comparing to non-senescence cells. Very low changes or no changes in ARMCX3, B2MG and VAMP3 were noticed.
Figure 3.6 Expression of putative markers of senescence in HT1080p21-9. For senescence induction; HT1080p21-9 cells were treated with IPTG for 3 to 4 days until permanent cell arrest was observed. (A) The cells were washed with cold 1xPBS, and the cells were collected using scraper, and plasma membrane proteins were purified using the kit from abcam 65400. STX4, NTAL and EBP50 were highly upregulated during senescence in HT1080p21-9. Mild upregulation was seen in ARMCX3 and no change was seen for VAMP3. (B) For whole cell lysate, the cells were collected with trypsin and resuspended in RIPA buffer before spending down at 12,000g speed. The pellet was the whole cell lysate. DEP1 and B2MG were more expressed in senescence than in the control. NOTCH3 was used as a control for senescence. (C) Control, (S) Senescence.
3.5 Western blot analysis of potential markers of senescence using normal and progeria fibroblasts

In order to confirm the suitability of these markers in identification more physiological model of senescence, normal human fibroblasts were used. 501T human fibroblasts and human dermal fibroblast from progeria patients with restrictive dermopathy (RD) were used. RD cells were obtained kindly from Dr Sue Shackleton, Department of Biochemistry, Leicester University. The cells were cultured and passaging until permanent cell cycle arrest was seen. NTAL and DEP1 were increased in 501T after cells entering to senescence comparing to none senescent cells, as shown in figure 3.8. In RD cells, DEP1 was upregulated in senescent cells, while NTAL showed very small induction in senescent RD comparing to the growing cells. Others markers could not be detected or tested due to lacking of the sample. However, the experiment was performed once, so confirmation of these results should be performed.

![Western Blot Image]

**Figure 3.7 Expression of senescence markers in 501T and progeria cells.** Cells were allowed to grow in culture until permanent cell cycle arrest and SA-β-Gal were observed. After that, The cells were washed with cold 1xPBS, and the cells were collected using scraper, and plasma membrane proteins were purified using the kit from abcam 65400. NTAL and DEP1 were upregulated moderately in 501T, however, in progeria cells, only DEP1 was upregulated, and mild changes were seen in NTAL. (NHDF) Normal human dermal fibroblasts, (PC) Progeria cells (restrictive dermopathy cells), (-) Control, (+) Senescence.
3.6 The expression of senescence markers in normal fibroblasts infected with RAS

After studying the expression of senescence markers in different models of senescence, the level of these markers were tested in an oncogene-induced senescence model. Human dermal fibroblasts (HDF) cells were kept in standard culture conditions and infected with adenovirus that containing the RAS gene (Gift of S. Aaronson, Mount Sinai School of Medicine, New York, USA). The cells were kept in culture until permanent cell cycle arrest was noticed. To confirm senescence induction, known markers of senescence were measured. NOTCH3 was highly upregulated in HDF infected with RAS comparing to non-infected cells (figure 3.9). In addition, ERK, which is a known downstream target of RAS, was highly phosphorylated.

Two novel markers of senescence were examined, as seen in figure 3.9. STX4 and LANCL1 were increased highly in senescent HDF cells after RAS infection. However, others markers of senescence could not be. This could mean that these two markers are involved in oncogenic senescence.

![Image of protein expression](image)

**Figure 3.8** The expression of the potential markers of senescence in HDF infected with RAS. The cells were infected with a retroviral construct containing Ras for 3 days. After that, the cells were washed with cold 1xPBS, and the cells were collected with trypsin, and were resuspended in RIPA buffer before spending down at 12,000g, the pellet was the whole cell lysate. LANCL1 and STX4 were highly induced after RAS expression in HDF. NOTCH3 was used as a marker of senescence, and p-ERK indicated the upregulation of the RAS-MEK-ERK pathway.
3.7 Cellular localisation of potential markers of senescence

In order to confirm the localization of these markers in the plasma membrane, fractionation by sucrose gradient of whole-cell lysates of senescent EJp16 was performed. DEP1, NTAL, EBP50, B2MG, STX4 and ARMCX3 localize in the same fraction as cell membrane markers Na/K ATPase and Calnexin (figure 3.10). MAPK, HDAC1 and SOD1 are the markers of cytosol, nucleus and mitochondria, respectively, which were used to make sure no contamination was occurred during fractionation. So, this clearly confirmed that the markers are on plasma membrane of senescent cells, and also showed the validity of plasma membrane separation method and mass spectrometry result.

![Image of western blot](image)

**Figure 3.9 Localization of potential markers of senescence in plasma membrane of EJp16.** EJp16 Cells were cultured in the absence of tet for 7 days before the lysate was extracted. The lysate of EJp16 was then applied into the sucrose gradient before centrifugation at 100,000g for 18 hours at 4°C. After that, the sample was fractionated into 24 fractions (100µl for each sample) and mixed with 35 µl of 4x loading buffer before loading into 10% polyacrylamide gel for western blot. Senescence markers localised in the same lanes of Na/K ATPase and Calnexin (the markers of plasma membrane). MAPK, HDAC1 and SOD are the markers of cytosol, nucleus and mitochondria respectively. The experiment was performed three times independently.
3.8 Immunofluorescence study the expression level of potential markers

After validating the upregulation of senescence markers in different models of cell senescence using Western blots, we further validated these markers using immunofluorescence in EJp16 and EJp21. DEP1, NTAL, EBP50 and STX4 showed induction in senescent EJp16 comparing to growing cells (figure 3.10). DCR2, VAMP3 and ARMCX3 also showed increase in the expression, but at lower level, In EJp21, all markers were highly upregulated, except STX4, which only showed a low upregulation, and EBP50 showed the opposite result. In IMR90 fibroblasts, STX4 and VAMP3 increased after senescence induction comparing to non-senescent cells. DCR2 was used as a marker of cell senescence.

These results correlated with results obtained by Western blot. Upregulation of the markers was seen in EJp21 and EJp16, consistent with what was noticed in western blot. However, the expression of EBP50 in EJp21 decreased in immunofluorescence comparing to result obtained by Western blot, for reasons that are not understood.
Figure 3.10 The expression of the potential markers of senescence using immunofluorescence. For senescence induction, EJp21 and EJp16 were incubated without tet for 7 days. For IMR90, the cells were cultured until permanent cell cycle arrest and SA-β-Gal were observed. The expression of EBP50, NTAL, DEP1 and STX4 was highly induced in senescent EJp16, and all markers were expressed in senescent EJp21. STX4 and VAMP3 were induced in senescent IMR90, compared to growing cells. The localization of the proteins was not as expected, as some of them such as VAMP3 localized in nucleus as well. DCR2 was used as a control for senescence.

3.9 The expression of senescence makers in blood cells from different age groups

It has been shown that senescence markers can be detected from blood samples. The expression of p16 can be measured and monitored in CD3 T cells in healthy volunteers (Liu et al., 2009). Therefore, we aimed to test the markers of senescence in WBCs of different group ages after separation from whole blood samples. EBP50 was highly induced in three samples, two were from more than 55 years old, and one was between 35 and 55 years (figure 3.12).
The significant upregulation showed the suitability of using EBP50 to monitor the biological aging in WBCs. However, none of the remaining markers were detected in these samples.

Figure 3.11 The expression of EBP50 in WBCs was induced during aging. (A) Blood samples were added to the histopaque solution before centrifugation at 4000 RPM for 30 min. The white layer was then collected into a clean tube and washed with RPMI media and the pellet was then used for the testing the expression of the senescence markers using Western Blot. The levels of EBP50 increased in two samples from >55 years donors and one sample from 35-55 years age group (p<0.01). (B) The quantifications are from three experiments from the same sample. Error bars represent standard deviations.

3.10 The level of selected senescence markers are upregulated in mice lung adenoma, from a conditional V600E BRAF knock-in mouse model and human nevi

After testing the expression of senescence markers in vitro and in blood samples using different cell biology techniques, mice lung adenoma from a conditional V600E BRAF knock-in mouse model were used (Acosta et al., 2013). Senescent cells are present at very high quantity as reported previously (Dankort et al., 2007), BRAF overexpression leads to senescent cells
accumulation and adenoma formation. We aimed to examine the relevance of using the potential markers of senescence in mice tissue. Indeed, the level of DEP1, STX4 and B2MG were highly upregulated in these tissue, as shown in figure 3.13, p19 was used as a known marker of senescent cells in murine tissue.

![Image](https://example.com/image.png)

**Figure 3.12 The expression of senescence markers in mice lung adenoma.** The tissues of lung adenoma were deparaffinised in xylene before dehydrating the tissue in ethanol. The endogenous peroxidase activity was then blocked by 3% H$_2$O$_2$ solution. For the antigen retrieval, the slides were kept in citrate buffer and incubated at 100 ° C, the slides were then blocked with BSA and incubated with antibodies and after that were stained for H&E. STX4 was highly expressed in this tissue, while DEP1 and B2GM showed a moderate expression. NTAL did not show any expression. p19 was used as a marker of senescent cells in mice tissue.

3.11 The expression of markers of senescence cell in human nevi

It was also important to examine the ability of these markers for expression in senescent cells of human tissue. For this, we used human nevi, which normally contain high amount of senescent cells (kindly provided from Dr Gerald Saldanha, Consultant of Histopathology at Royal Infirmary, Leicester).

DEP1 and STX4 were induced in human nevi samples in two different samples, as seen in figure 3.14. B2MG showed only a mild increase, where NTAL showed a complete negative result. p16 was used as a marker for senescent cells. These demonstrate the validity of using some of the markers described for
tracking senescent cells in human nevi, and potentially could be used to detect senescent cells in human body generally.

Figure 3.12 The expression of senescent markers in human nevi. The human nevi samples were treated as described in Figure 3.1.2. STX4 and DEP1 expressed highly in human nevi, where B2MG expressed at low level. NTAL did not express in these tissue. p16 was used as a marker of senescent cells in human nevi. The work was performed in two different samples of nevi from two different patients as indicated (A) and (B).

3.12 Relationship between the level senescent markers and survival in patients with breast cancer

After investigating the expression of the markers of senescence in vivo and in vitro in different models of senescence, we studied the correlation between the rate of expression of the markers and survival rate of patients with cancer. This is because cells senescence is a tumour suppressor mechanism. Therefore,
accumulation of senescent cells should correlate with high survival rate in patients with cancers. So, we expected high expression of these markers would associate with good prognosis in patients with cancer.

The results were obtained from public database using PPISURV, bioinformatics software (www.bioprofiling.de). Each single graph represents one data set.

Of all the cases explored, survival rate of patients with breast cancer showed very strong relationship with expression of the markers of senescence (figure 3.16). The expression of EBP50 showed the strongest association with the survival rate of breast cancer. These observations clearly demonstrated the role of these markers as tumour suppressors in the context of cell senescence. This data was obtained by Dr Nickolai Barlev, Department of Biochemistry, Leicester University of Leicester.
Figure 3.14 Correlation between senescent markers expression and survival rate of patients with breast cancer. Kaplan-Meier curves of breast cancer patients. Red represents high expression, while green represents low expression. These data were obtained from public databases through bioinformatics analysis using PPISURV (www.bioprofiling.de). LNACL1, VAMP3, STX4, EBP50 and NTAL have all two graphs from different GEO data sets.
3.13 Discussion

Primary cells extracted from mammalian tissue undergo a definitive number of divisions before they stop proliferation (Hayflick and Moorhead, 1961). Although senescence has been studied for years, the molecular mechanism that initiates and regulates senescence is not fully known, and the current markers of senescence identification are not sufficient for its studying and monitoring in vitro and in vivo. For this reason, we have screened the plasma membrane of senescent cells in order to define new markers of senescence. This screening is important because proteins on the surface of senescent cells have not been characterised and studied previously. In addition, during senescence, cells undergo intensive shape remodelling that leads of appearing of senescent cells as flat and spindle in shape. Therefore, screening of plasma membrane of senescent cells could help in discovering proteins that contribute to these morphological changes. This will also help in detection of senescent cells in the lab using extracellular epitopes of the markers of external epitopes. In addition, we propose that this screen could be used to define ways to clear senescent cells using antibody or MIPs conjugated with cytotoxic drugs.

None of the proteins identified had been shown to have any role in senescence. For instance, DEP-1 is a phosphatase enzyme that has been shown to play role in many biological activities at the cellular level. It plays a role in tumour suppressor mechanisms (Sacco et al., 2009). Activation of DEP1 associated with a decrease in progression of pancreas colon and breast malignancies. DEP1 binds to p27 to induce cell cycle arrest (Trapasso et al., 2000; Trapasso et al., 2004). This mechanism could explain of role of DEP1 as an effector of cell senescence, especially since it induces cell cycle arrest at G1 (Trapasso et al., 2000).

Recently, Chun and his colleague have reported a huge reduction in DEP1 expression in tissue of patients with cervical cancer (Chun et al., 2015). They tested the silencing and overexpression of DEP1 in cervical carcinoma cell lines and they noticed a decrease in cell death after DEP1 depletion, and increasing in the cell death and cell migration after DEP1 expression in these cells. These observations supported our view of the potential role of DEP1 in senescence, which will have to be further analysed.
STX4, PLD3 VPS26A and VAMP3 are involved in the cellular trafficking of the cell, which could facilitate the process of SASP secretion. STXs are membrane proteins that belong to t-SNARE family and help intracellular vesicles trafficking in different cell types. (Kean et al., 2009; Chen and Scheller, 2001). STX4 plays an important function in IgE secretion from plasma cells (Rahman et al., 2013). This could suggest the potential role of STX4 in secretion phenomena of senescent cells. It also downregulates apoptotic pathway through inhibition of A-SMase activity (Perrotta et al., 2010). A study showed the role of STX4 in keratinocyte cornification: blocking STX4 downregulated the process of cornification (Kadono et al., 2012). This suggests that STX4 could be involved in the process of wound healing through senescence.

VAMP3 is also known as Cellubrevin is involved in adhesion and migration of granule in cells, and it also facilities insulin trafficking into cells (Polgar et al., 2002; Olson et al., 1997). It plays a role in secretion and trafficking of matrix metalloproteinases (MMPs) (Kean et al., 2009). Senescent cells secrete IL1, IL6 and MMPs that facilitate tissue repair and fibrosis elimination after wound healing (Xue et al., 2007; Krizhanovsky et al., 2008). VAMP3 could facilitate the secretion of these factors. Another study showed the role of VAMP3 in endocytosis of chemotactic factors (Riggs et al., 2012). This could be the role of this protein in secreting chemotactic mediators in senescence cells by which cell senescence can interact with the immune system.

VPS26A is a part of a multimeric complex, which participates in protein transport to the Golgi apparatus (Bugarcic et al., 2011). Again it could have role in transportation in senescent cells, and SASP secretion. In addition, mutations in VPS35, from the same family of VPS26, are associated with late onset of Parkinson’s disease (Zimprich et al., 2011). It might due the involvement of family in senescence directly or indirectly, which results in senescence misregulation.

PLD3 is a phospholipase enzyme, which is involved in adhesion and transport of vesicles, it also participate in myotube formation (Osisami et al., 2012). Recent work has shown that the coding variant in PLD3 may have an effect on
Alzheimer’s pathogenesis (Cruchaga et al., 2014). Work should be performed to see if this pathogenesis is related to senescence cells accumulation, as there was an evidence of senescent cells deposition in the brain increases the risk for in Alzheimer’s disease (Bhat et al., 2012).

The remaining potential senescence markers, LANCL1, NTAL and EBP50 are involved in many cellular signalling pathways that may be associated to cell senescence (Brdicka et al., 2002; Mayer et al., 2001).

NTAL is induced in myeloid cells and after FcεRI assembling, which leads to its phosphorylation (Brdicka et al., 2002). In mast cells, NTAL shows a role in the production of chemotactic components (Halova et al., 2013). This could be its role also in senescent cells, where NTAL may be involved in calling the immune system for cell senescence targeting. Many data has shown the ability of many SRC and Tec family kinases to phosphorylate NTAL at different regions in B-cells and mast cells (Wang et al., 2005; Iwakia et al., 2008). This could open a new window of relationship between these enzymes and NTAL in senescence induction. Investigation of this association in the context of senescence and the p53 pathway will help to reveal a possible involvement of p53 and its target protein in this mechanism. Since there have been studies demonstrating the ability of some Tec family kinases, such as SYK and BTK, to regulate p53 and senescence, NTAL could play a role in this pathway of senescence (Bailet et al., 2009; Althubiti et al., unpublished data).

Another putative marker of senescence is EBP50, which has shown its involvement in ion exchange, cancer promotion and ability to assemble apical microvilli (Yun et al., 1997; Georgescu et al., 2008; Shenolikar et al., 2001). Many studies have shown the role of this protein in cancer and apoptosis. For instance, a study showed the role of EBP50 in decreasing cancer cell invasion and metastasis in vivo (Li et al., 2014). This supports our view of a tumour suppressive mechanism of this protein. Another study showed the overexpression of EBP50 in different human cell lines decreased cell proliferation, promoted apoptosis and increased cell cycle arrest at G1 (Ji et al., 2014). Wang and his colleagues showed that higher EBP50 expression correlated with less tumour progression in patients with oesophageal squamous
carcinoma (ESC). In addition, they showed that survival rate of patients with ESC correlated with high expression of EBP50 (Wang et al., 2014). These evidences strongly suggest the role of EBP50 as a tumour suppressor and its ability to induce cell cycle arrest at G1 and decrease cancer progression could be through senescence induction.

LANCL1 is another marker of senescence that has been characterised in this study. It was originally explored in red blood cells, and its role thought to be in regulation of G protein-coupled receptor activity (Mayer et al., 1997; Mayer et al., 2001). Recent work has shown that LANCL1 is an antioxidant gene, and its genetic deletion lead to ROS production in the brain, which resulted in mitochondrial dysfunction and DNA damage (Huang et al., 2014). Therefore, LANCL1 activation could mostly be induced during premature senescence as a result of ROS accumulation, which is typical of this phenotype.

B2MG is a membrane plasma protein, which is one of the HLA-I complexes. With this complex, B2MG interacts with immune system (Winchester et al., 2003; Groves and Greenberg, 1982). Therefore, it could play role in regulation of immune cells for recognising and clearing senescent cells. In addition, it is also induced in many autoimmune and inflammatory disorders. Wang and his colleagues have revealed unexpected links between the components of MHC-I and p53 expression (Wang et al., 2013). They have shown that p53 has an effect on the expression of these components on the surface of the cancer cell, where absence of p53 dramatically reduced the expression of MHC-I. As B2MG is a part of the MHC-I, p53 could have a direct effect on it, and thereby may be on cell senescence as well. B2MG deficient mice, which are available commercially, can be used to see if this gene has any effects on senescence and aging (Koller et al., 1990).

Recently, Smith et al., showed that B2MG has a direct role in impairing the function of the brain in age-dependent manner (Smith et al., 2015). In addition, they found the levels of B2MG in blood of mice and human were high during aging. Injection of B2MG intravenously affected the neurogenesis and accelerated neurodegeneration in young mice. In the absence of B2MG expression in the mice, there was an improvement in the neurogenesis and
abrogation in age-associated cognitive degeneration (Smith et al., 2015). This study did not check senescent cell accumulation after injection of B2MG systemically. The direct effect could be because of the senescence induction in the brain, which could lead to impairment in brain functions. In addition, investigating the effects of B2MG administration or abrogation on other aging-related diseases also will be essential.

ARMCX3 (ALEX3) is among the family of armadillo proteins that have been associated to carcinogenesis and developmental mechanism (Kurochkin et al., 2001; Serrat et al., 2013). Overexpression of ARMCX3 leads to mitochondrial modifications (Serrat et al., 2013). It could play a role in this modification in senescence as well, since it has been shown that senescent cells associate with distinctive features in mitochondria, in which mitochondria appears as a condensed compact comparing to mitochondria in normal cells (Sugrue et al., 1997).

In this project, the majority of the work of the validations was performed in inducible cell lines. Future proposed work could include a bigger panel of physiological senescence models such as replicative and oncogenic induced senescence. Moreover, testing the expression of the markers in mice tissue from different ages would give a wider idea about the feasibility of using the markers in vivo for monitoring of senescent cells accumulation and aging studying.

These markers were also validated in some clinical samples. Human nevi and lung adenoma of mice with conditional V600E BRAF knock in samples are usually composed of high number of senescent cells. The expression of the markers in senescent cells in these samples shows the relevance of using them as markers for cell senescence detection from human and mice tissues. Future studies could include fresh samples, which SA-β-Gal staining can be obtained from frozen sections. So, quantification of positive SA-β-gal cells can be counted, and the percentage of the senescent cells express the markers also can be counted, which would give better estimate of relevance of using these markers for senescence detection in vivo.
We also assessed the expression of potential markers of senescence in blood samples obtained from healthy donors. Notably, EBP50 highly expressed in blood cells of elderly people, which could be used in the future as a marker of biological aging in human, which can be determined easily from blood sample. Future work should test the markers in blood samples from larger number of population. As many factors can accelerate the biological aging even in people at the same age, the age of some person may not compatible with his/her biological aging (Liu et al., 2009; Sanoff et al., 2014). This will have a potential impact on testing the biological aging for individuals using blood samples. FACS can detect extracellular epitopes of the senescence markers and the result can be obtained rapidly. This could be an application in the future; such rapid technique has the potential to be used in monitoring senescence and aging in blood cells before and after chemotherapy, for example, or in aging associated diseases.

None of the other markers tested were detected in the blood samples. This could be because of using whole white blood cells for detection of the markers. Previous studies have shown that p16 can be detected mainly in CD3 T cells. Therefore, investigation the expression of the putative markers in these cells and compare them with the expression of p16 could show some relevant results.

We used the external epitope of some characterized markers as a target for senescence detection using a FACS-based approach. This approach allows senescence identification within two hours and can give an estimate of the amount of senescent cells in the sample, comparing to the classical marker of senescence, SA-β-Gal, which takes 24 hours. However, the FACS based approach could be expensive due to the cost of the antibodies. On the other hand, SA-β-Gal associates with false positive in some conditions: high confluence of cells, medium starvation, and cells treatment with H2O2 can give false positive staining (Yegorov et al., 1998; Yang and Hu, 2005). All these are enough for looking for a new detection method, and our FACS based approach is a good candidate.
We have shown that these markers correlated with better survival rate in patients with breast cancer. Proposed work in the future could be testing the prognostic potential of these markers in different types of cancer in clinic. For example, testing the expression of the markers using blood samples from different cancer patients before and after treatment. This will help to evaluate the possibility of using them as prognostic markers in clinic. Work could be extended to include testing the prognosis of them using biopsies from solid tumours.

However, testing the prognostic potential of these markers in clinic could have some difficulties. Ability to obtain consent for samples patients is not simple. Moreover, number of patients to be included in this study to give a good image about the prognostic potential of these markers may be not available. In addition, the presence of a huge number of prognostic markers that have been showed prognostic potential for different cancer types in clinic and not been used or approved now is high. In total, these could be challenges in testing and using these markers for cancer prognosis in clinic.

In the future, therapeutic strategies could be applied to inhibit these proteins if they are shown to contribute to senescence. For example, in some age-associated diseases such as cataract and diabetes where there is high amount of senescent cells (Liton et al., 2005; Zhu et al., 2009; Minamino et al., 2009; Bernet et al., 2014). This would help ameliorate of symptoms of these disorders. However, inhibition of senescence should be tested carefully, as such approach could initiate neoplastic transformation.

In summary, our data propose the ability of these markers to be used for senescence detection in parallel with previously described markers (p53, p14, p16 and p21).
CHAPTER 4. ESTABLISHING A METHOD FOR CLEARING SENESCENT CELLS

It has been shown that clearing senescent cells from the tissue leads to improvement in function of many organs. Baker et al., described a method in which senescent cells expressing p16 were cleared by the apoptotic pathway (Baker et al., 2011). This study showed the importance of senescence cell clearance in body fitness and improvement of organ function (Baker et al., 2011).

Clearance of senescent cells in humans could delay and ameliorate aging symptom and leads to better life. However, the method of the previous study cannot be translated into clinic, so looking for therapeutic approaches that can be used for human in the future is essential.

Antibody/ drug conjugate (ADC) is a new class of the drugs that is being used to target cancer cells. This approach needs a very specific marker that is expressed in the outer surface of cancer cell, which is used as epitope for ADC. So, a conjugated antibody with a cytotoxic drug can be directed to a specific marker, which leads to ADC binding and internalisation and toxin releasing that result in cell death. We hypothesised that this approach could be used to clear senescent cells, which will probably lead to decrease in senescent cell accumulation in different tissues. Our newly identified markers could potentially be used for this end.

4.1 Assessment of the expression external epitopes of the potential markers of senescence using FACS

In order for ADC to kill senescent cells, it should firstly bind to a specific marker on the plasma membrane of the cell. A FACS based approach was used to study the binding of different commercially available antibodies against our markers, labelled with fluorescent tags. This allowed us to measure the percentage and the quantity of bound antibody to the proteins on the plasma membrane. Antibodies against DEP-1, B2MG and NOTCH3 were used. Only these proteins were chosen because of their long predicted external domain they have. Antibodies can bind without need for permeabilisation, as this step preserves the integrity of the cells and the plasma membrane.
In the initial method, the cells were collected by trypsinisation with or without permeabilisation. However, no change in fluorescence intensity was seen. Sodium Azide was used to help for antigen retrieval after using trypsin (Jiao et al., 2009). After that, cells were scraped gently with cold PBS and trypsin was not used. A significant change was noticed in the intensity of the fluorescence. This showed that using trypsin could affect plasma membrane proteins expression.

Three cell lines were used in this study, HDF, HT1080p21-9 and EJp16. Generally, there was increase in the fluorescent intensity in these cell lines, as seen in figure 4.1, HT1080p21-9 and EJp16 showed higher increase and shift the curves into the right in senescence comparing to controls. The expression of DEP1, B2MG and NOTCH3 was very low in senescent HDF comparing to the controls. However, their levels were higher in senescent EJp16 and HT1080p21-9 cells where p16 and p21 were induced respectively. There were increased at least three folds in mean fluorescent intensity of senescent HT1080p21-9 and EJp16 comparing to the controls. This shows binding of the antibodies to the selected markers of senescent cells.
**Figure 4.1 Assessment of Ab binding to potential markers of senescence.** The cells were gently collected by scraping before washing with cold PBS and then were blocked with BSA. The cells were then incubated with the primary antibodies tagged with fluorescence. The expression of the markers was then detected by FACS. Three different cell lines were used, EJp16, HT1080p21-9 and HDF. The expression of B2MG and DEP1 induced in EJp16 and HT1080p21-9 with at least three folds increase in mean fluorescence intensity (MFI), but only 1.7 folds in MFI in HDF. NOTCH3 was used as a marker of senescence. The number in each curve represents MFI for each one. Error bar represents standard deviation. The experiment was performed three times independently.
4.2 The cytotoxic effect of different toxins on viability of senescent cells

In order for ADCs to be effective and kill target cells, conjugated toxins should be released from the complex to exert its effects. There are many cytotoxic drugs that have been used, depending on sensitivity of the cancer type and or cell type for death by these drugs (Jordan and Wilson, 2004). For this reason, the sensitivity of EJp21 and EJp16 to 3 different cytotoxic drugs was investigated.

The sensitivity of these cells in senescent and growing status for cell death by mitomycin C, doxorubicin and actinomycin D was measured using PI staining. In EJp16, after 48 hours of adding drugs, there was high cell death in growing EJp16 after mitomycin C and actinomycin D addition at 3.5 µg/ml, which was around 30% cell death, as seen in figure 4.2. However, in senescent EJp16 cells, the percentage of cell death was lower, around 20%. Doxorubicin showed the opposite effect at 3.5 µg/ml: it induced cell death around 10% in growing cells and around 15% in senescent EJp16 cells.

In EJp21, the effect of the drugs was noticed with lower concentration. After two days of addition of the drugs, growing cells showed around 25% cell death at approximately 1 µg/ml of the drug’s concentration. As showed in figure 4.2, doxorubicin had the lowest effect: around 10% cell death. In senescent cells, doxorubicin, however, had high cell death effect, around 50%. Therefore, the initial senescent cells targeting experiments could be done using DEP1 or B2MG antibodies conjugated with doxorubicin.
Figure 4.2 Measurement of cytotoxic effects of different toxins in EJp21 and EJp16 cells. EJp21 and EJp16 were treated with different concentrations of doxorubicin, mitomycin C and actinomycin D in the presence or absence of tet for 48 hours or with tet removed for 7 days. Cell death was then measured by PI staining. T. EJp21 cells were sensitive for cell death by doxorubicin, mitomycin C and actinomycin D at lower concentration comparing to EJp16 cells. Doxorubicin showed high cell death in growing EJp16 and EJp21 comparing to the controls. The experiment was performed once.
4.3 Internalisation rate of antibodies of the markers of senescence

Internalisation rate of ADC after binding to protein on the plasma membrane is a crucial step for targeting a specific cell type. Antibody conjugated with CypHer5E, which is non-fluorescent at basic pH and is fluorescent at acidic pH was used. The internalisation rate of two antibodies DEP1 and B2MG were assessed using EJp16 senescent cells.

The mean fluorescence intensity was measured using FACS after short time intervals. CypHer5 dye is not fluorescent at basic pH, however, after internalisation and due to the acidity of the cytosol, it can give fluorescence, which can be measured at 550 wavelengths.

For DEP1, there was an increase in MFI after 15 min up to three folds, and the maximum increase was seen after 90 min. For B2MG, less increase in MFI was seen, after 15 min only 1.5 folds increase was achieved. The maximum fluorescent signal was noticed after 120 min, figure 4.3.

This result showed the suitability of conjugation these antibodies with toxin for targeting senescent cells.
Figure 4.3 Internalisation rate of B2MG and DEP1 antibodies into EJp16 cells.
Cells were collected gently by scraping before washing with cold PBS and blocking with blocking buffer. The cells were kept on ice in the dark and were resuspended into 100µl of the conjugated antibody, in time dependent manner, 10µl of the labelled antibody was added into 200µl of the blocking buffer and the reading was measured in time dependent manner. Fold changes in Mean Fluorescence Intensity (MFI) are plotted. (t) After the conjugation of the CypHer5E to the antibodies, the maximum internalisation rate of DEP1 was seen after 90 minutes after adding conjugated antibody to cell suspension. For B2MG, the highest internalisation rate achieved after 120 minutes. The experiment was performed three times independently. Error bars represent standard deviation.
4.4 Assessment of expression of B2MG in senescent cells using MIPs

Antibody-drug conjugate is an efficient method for attacking and clearing target cells (Kovtun and Goldmacher, 2007). However, the process of production and conjugation is complicated, expensive and time consuming. In the future, high amount of drug conjugate may be needed to use in vitro and in vivo, so the cost of ADC is extremely high. Therefore, finding a cheaper method to produce mimics of ADC was demanded.

Another method that can also be exploited is molecularly imprinted polymers conjugated with cytotoxic drugs. MIPs are nanoparticles that are produced when small templates such as peptides are present during the mechanism of polymerisation. During polymerisation, monomers holding many functional groups are assembled around the template through covalent and non covalent bindings, which lead to fixation into points by polymerisation with high degree of crosslinking. After template removing from polymers leads to form grooves that are complementary to the target template (Haupt and Mosbach, 1998).

Molecularly imprinted polymers (MIPs) against B2MG were produced by Prof Sergey Piletsky, Chemistry Department, Leicester University. B2MG was chosen because it has very short amino acid sequence, so it is easier for production. To study the ability of MIPs to bind to B2MG, EJp16 cells were collected by scraping as previously described, and cells were resuspended in different dilutions of MIPs (0.3mg/ml) 1:9 and 1:4 in the 0.5%BSA+1xPBS. Therefore, if B2MG is expressed at the surface of senescent EJp16, MIP tagged with FITC will bind to the complementary region of B2MG and this can be measured by FACS. At 1:4 dilutions, there was shift to the right in senescent cells comparing to the control (figure 4.4). At lower dilution, there was a less shift to right. This clearly showed the ability of MIPs to bind to B2MG protein on the surface of senescent cells. As shown in figure 4.4, the average fold increase in the fluorescent intensity in senescence EJp16 was higher than non-senescence at 1:4 dilutions of MIPs. The average was around 3 folds increase, and only around 2 folds with lower dilutions 1:9.
Figure 4.4 Measuring the expression of B2MG in EJp16 using MIPs. EJp16 cells were cultured with or without tet for 7 days, and then were gently collected by scraping before washing with cold PBS and blocking. Cells were then incubated with the different dilutions of the MIPs (1:4 and 1:9 diluted with blocking buffer). At high dilution of MIPs (1:4), there was high expression of B2MG in senescent EJp16 comparing to control. However, at lower dilution (1:9) very low difference was seen between senescence and non-senescence EJp16 cells. The average of increase in the fluorescent intensity was 3 folds increase at 1:4 dilution, and 2 folds at 1:9. The experiment was performed two times independently. Error bars represent standard deviation.

DEP1 and B2MG showed high expression, which suggests the possibility of using them to target senescent cells using ADC or MIPs-conjugated with cytotoxic drugs. In addition, the internalization rate of B2MG and DEP1 in EJp16 was very high, but this did not show the percentage of the protein that internalizes. Therefore, measuring the percentage of protein internalisation is
also important. Future work also should involve other cell lines. Further, studying other markers with external epitopes will be also important to expand the candidates of potential markers that can be used for senescence identification and targeting.

4.5 Discussion
The ability of antibody binding to the markers of senescence opens a new window for targeting senescent cells using antibodies or MIPs conjugated with cytotoxic drug. Senescent cell accumulation in tissue contributes to aging associated symptoms and tumour promotion (Baker et al., 2008; Van Deursen, 2014). In addition, senescent cell clearing from mice tissues ameliorate the symptoms of aging (Baker et al., 2011). Therefore, this approach could help in targeting senescent cells and subsequently improve functions of human organs in aging individuals.

Many studies have shown that senescent cell accumulation contributes to cancer progression (Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). A recent work by Zacarias et al., showed that cell senescence was upregulated due to ERBB2 overexpression in patients with advanced breast cancer and contributed to cancer cells progression through secretion of cytokines that were necessary for senescent cells survival (Zacarias et al., 2015). Blocking these cytokines downregulated cancer cells growth. This is an example of how senescent cells accumulation due to oncogenic induction could have a harmful effect. Therefore, senescent cell targeting using an ADC approach could be used during cancer treatment to help diminish cancer progression due to senescence effects. Previous work by the Campisi group and others demonstrated that SASP can induce malignant transformation. Secretion of SASP by senescent cells has shown to induce malignant transformation in nude mice after injection of senescent fibroblasts. SASP compound such as MMP3, VEGF and other cytokine factors can induce cancer progression through tumour migration and angiogenesis (Coppe et al., 2006; Krtolica et al., 2001; Liu and Hornsby, 2007)

Senescent cell targeting using ADC will face many challenges. First of all, the drug may be metabolised in the blood stream before reaching the optimum
action. So, pharmacokinetics of any proposed drug should be evaluated at different levels from administration until the clearance. To minimise this drawback, targeting senescent cells through ADC can be applied locally, for example, on the site of primary tumour, and also in the site of glaucoma or fibrosis where senescent cells are highly present.

In addition, senescent cells targeting through markers of senescence could associate with side effects, especially if the markers are expressed highly in some normal tissue. Therefore, the selection of the marker to be used as a target for ADC is important. The marker should not only be expressed in senescent cell, but also it should have a low basal level in all tissues. Otherwise, ADCs could have side effects by targeting normal cells that have high level of the markers alongside with senescent cells. A good example of senescent marker that was characterised in this study is DEP1. According to public available data from BioGps it showed roughly the same low basal expression level across many human tissues (figure 6.1). Another example of a marker that could be used for senescent cell targeting is B2MG. It is located on the extracellular surface of cells, and does not have any internal part. Therefore, using this candidate for senescent cells targeting should also be tested. However, according public available data from BioGps, B2MG has variable expressions among different human tissues, which could be associated which side effects if B2MG is targeted (figure 6.1).

We have shown that DEP1 and B2MG are upregulated in senescence and could have tumour suppressive mechanism, as explained in Chapter. 3. They are also associated with positive prognosis in patients with breast cancer. We hypothesized that they good candidates to target senescence through ADC or MIPs

There are many reported data of treatment of cancers with ADCs (Panowski et al., 2014). However, no research works have used MIPs as a therapeutic approach. Hence, work should be focused on examining the possibility of using MIPs for targeting senescent cells in vitro. The affinity of MIPs binding to an antigen is weak comparing to an antibody (Hoshino et al., 2008), so it may not be an ideal system to be used clinically. However, it was used in this study due
to its low cost and availability, as a way to explore a marker to be used for senescent targeting.

**Figure 4.5 Basal levels of DEP1 and B2MG mRNA in different human tissues.** The graph shows approximately equal levels of DEP1 expression across many tissues. This suggests the suitability of using DEP1 as a target for senescent cells clearing. B2MG, however, shows high expression in different tissues (especially blood cells and intestine), which suggests senescent cells targeting through B2MG could be difficult.

This graph was obtained from public available data, BioGPS (http://biogps.org/#goto=welcome)
CHAPTER 5. BTK PHOSPHORYLATES P53 AND REGULATES SENESCENCE AND AGING

BTK is a tyrosine kinase, which is involved in BCR signalling pathway in B cells. Various BTK inhibitors have been developed to treat chronic lymphocytic leukaemia (CLL). Ibrutinib is an example of the drug that has received licence recently to treat CLL. Others BTK inhibitors are under test in different clinical trials stages (Wang et al., 2013). From the initial proteomic screening (please see the appendix), we found that BTK is upregulated in senescence. However, it is not a good candidate for senescent cells targeting, as it is an internal plasma membrane protein without any extracellular part (Hendriks et al., 2014). However, since there is a small molecule that can inhibit its activity, further experiments were proposed.

4.6 BTK is upregulated in response to p53 and may act as a tumour suppressor

To validate the mass spec result, the induction of BTK was confirmed in EJp53. BTK protein levels were highly upregulated in EJp53 induced to express p53, as shown in figure 5.1A. Since BTK was upregulated after p53 expression, we hypothesised that BTK could also be expressed after p53 expression due to DNA damage in wild type p53 cell lines. Indeed, colon cancer cell line HCT116, which has wild type p53, showed BTK upregulation after induction of DNA damage by different inducers such hydrogen peroxide and radiation, meaning that BTK may be contributed in p53-mediated responses to stress (figure 5.1.B).
Figure 5.1 BTK levels increase in response to p53 upregulation. A) Tet was removed from the media in EJp53 for 6 days to induce p53 expression. BTK upregulation was associated with increasing in p53 levels. Actin was used as a loading control. B) HCT116 cells were treated with 200µM of tert-butyl hydroperoxide (tBH) for 2 hours or irradiated with 10 gray (IR) and then incubated for 24 hours. There was a clear upregulation in BTK expression in both conditions, and an associated increase in p53 levels. Actin was also used as a loading control. The experiment was performed two times independently.
In B-cell malignancies, BTK has an oncogenic role that has been extensively studied. However, the previous data suggests that it could have a non-discovered function of its tumour suppression activity through p53 pathway. To reveal this contradiction, publicly available data PPISURV was used to analyse the survival rates of cancer patients. In a chronic lymphocytic leukaemia GEO dataset, poor prognosis was associated with high expression of BTK. However, good prognosis of patients with breast cancer correlated with high expression of BTK, figure 5.2. These observations clearly showed that BTK has dual effects as a tumour suppressor in some solid tumours and oncogenic effects in B-cell malignancies. This data was obtained by Dr Nickolai Barlev, Department of Biochemistry, Leicester University of Leicester.

Figure 5.2 Kaplan-Meier survival curves of patients with chronic lymphocytic leukaemia, breast and lung cancer. The data is presented into (red) high expression and (green) low expression of BTK. This data was obtained from public databases through a bioinformatics analysis of PPISURV. Each graph represents a different GEO dataset.
4.7 BTK expression induced senescence in EJp53 cells

In order to investigate the functional role of BTK expression in EJp53, a colony formation assay was performed. Colony formation assay is widely used to assess the ability of single cell to form colonies, hence its ability to proliferate.

Cells were transfected with BTK and seeded into 6 cm plates and either incubated with or without tetracycline to regulate p53 expression. The plates were left for two weeks, changing medium and tetracycline every three days.

BTK expression in EJp53 was able to decrease the colony number in the absence of p53, as shown in figure 5.3, comparing to control cells that did not have BTK. Presence of p53 is enough to prevent cell proliferation and colony formation in presence or absence BTK expression. This result showed the ability of BTK expression in preventing cell proliferation.
Figure 5.3 Colony formation assay of EJp53 cell transfected with BTK. EJp53 cells were transfected with BTK or with an empty vector (EV). The following day, roughly 100 cells were split into 6 cm plates (in triplicates), and were allowed to grow in the presence or absence of tet for 2 weeks before fixing and staining with Giemsa staining. A) BTK transfection reduced colony formation in the absence of p53. B) The percentage of colonies in the experiments. In the absence of p53, colony number was very low, which showed the role of BTK in preventing cell proliferation even in the absence of p53 (p<0.01). This experiment was performed two times independently, each one in duplicate. Error bars represent standard deviations.

Colony formation assay (CFA) clearly demonstrated the ability of BTK expression to decrease the proliferation in EJp53 even in the absence of p53. This suggested the possibility of a role BTK as a tumour suppressor in these cells. To confirm this, EJp53 cells were transfected with BTK and incubated for
8 days in the presence and absence of p53. EJp53 without p53 and BTK grow and reached ~10 million cells in average, however, as shown in figure 5.4, cells with BTK reached only half a million in average after 8 days. This clearly supported the idea that BTK could be a novel tumour suppressor that can suppress cell proliferation independently of p53.

Figure 5.4 EJp53 count after transfection with BTK. EJp53 cells were transfected with BTK or with empty vector (EV). The following day, an equal amount of cells were plated in triplicates in the presence or absence of tet and were counted at 4 and 8 days. For that, 10 µl of the cell suspension was mixed with equal amount of the trypan blue. The cells were counted using an automated Bio-Rad system. In the absence of p53, the cells grow very fast, but after BTK expression the cells number is reduced significantly (p<0.01). The experiment was performed once in triplicate. Error bars represent standard deviations.

4.8 BTK expression induces cancer cell death independently of p53

It is clear that BTK is upregulated in after p53 is induced. The next aim was to explore the role of BTK in the p53 pathway. The expression of p53 was not
upregulated after BTK transfection in the presence of tetracycline, as seen in figure 5.5. However, BTK expression increased p53 expression after induction by tetracycline.

Figure 5.5 The effects of BTK overexpression on p53. EJp53 cells were transfected with BTK or EV and incubated for 2 days in the presence or absence of tet. T Western blot was performed to see the effect of BTK overexpression on p53 levels. Actin was used as a loading control. BTK overexpression had a robust effect in increasing p53 levels; however, no effect was seen in the absence of p53. The experiment was performed more two times independently.

The mechanism by which BTK decreases EJp53 growth is unknown. To address this point, a PI staining was carried out to determine the percentage of cell death after BTK expression. EJp53 cells in the presence and absence of p53 were transfected with BTK. PI staining was performed after 24 hours of transfection. As shown in figure 5.6, induction of cell death in EJp53 after BTK expression was about 35% of the cells. In the presence of p53, there was only 20% cell death. The reason of a higher cell death in the absence of p53 could be that senescent cells are known to be resistant to cell death.
Figure 5.6 BTK expression induced cell death in EJp53. EJp53 cells were transfected with BTK or EV and were allowed to grow for 2 days in the presence (Control) or absence (+p53) of tet. The cells were then collected and washed with PBS and fixed with 70% ethanol for 30 min. The cells were then resuspended in PI staining solution for 30 min before measuring the cell death by FACS. BTK expression increase cell death in EJp53 cells in absence p53 from 4% to 35%, whereas in the presence, of p53 BTK was able to induce cell death from 2% to 21%. The experiment was performed once in duplicate.

PI staining suggested that BTK prevented cell proliferation mainly through cell death. To explore if BTK expression induces apoptosis, an annexin V staining was performed. In the absence of p53, there was an increase in apoptosis in EJp53 cells transfected with BTK to about 27%, comparing to cells transfected with a control plasmid which was only 6%. In the presence of p53, apoptotic cells were also seen in the transfected cell with BTK, but it was only 23% comparing to non-transfected cell, which was only 11%, figure 5.7. These observations clearly suggested the role of BTK in apoptosis in the absence of p53.
Figure 5.7 BTK expression induced apoptosis in EJp53. EJp53 cells were transfected with BTK or EV and were allowed to grow for 2 days in the presence or absence of p53. The cells were then collected and washed with annexin V buffer. The cells were then resuspended in annexin V staining solution for 15 min before transferring to FACS tubes for analysis. In the absence of p53, BTK expression was able to increase apoptosis from 6% to 27%, but in presence of p53, BTK increased apoptosis from 11% to 23% only. The experiment was performed in duplicate.

4.9 BTK expression induces Apoptosis in HCT116 cells

BTK expression showed anti-proliferative effects in EJp53 cells. BTK expression clearly stopped colony formation in these cells. In addition, it induced cell death and apoptosis after overexpression. To confirm these findings, a PI staining was performed in HCT116 (colon cancer cells) after 24 hours of BTK transfection. There was a clear cell death effect of BTK (figure 5.8). At least 30% cell death was seen in HCT116 after BTK transfection comparing to 10% in the control. This result showed a consistent effect of BTK in different cell lines.
BTK expression induced cell death in HCT116. HCT116 cells were transfected with BTK or EV (Control) and incubated for 2 days. (A) T Western Blot showed the expression of BTK after transfection comparing to the control (C). F (B) The cells were resuspended in PI staining solution for 30 min before measuring the cell death by FACS. After BTK expression, there was an increase in cell death from 10% to 31%. The experiment was performed in duplicate.

BTK expression induced cell death in HCT116, but the exact mechanism of it is not confirmed. Annexin V staining for HCT116 after 24 hours of BTK transfection was performed. The result demonstrated the ability of BTK to induce apoptosis in HCT116 (figure 3.9). 20% apoptotic HCT116 after BTK expression was seen, and only 5% of apoptosis was in the control. Our results suggest that BTK caused apoptosis of EJp53 and HCT116 independently of p53. To further confirm this, molecular effectors of apoptosis were measured after BTK expression in HCT116. Caspase 3 and PARP cleavage induction after BTK expression showed the role BTK in apoptosis, as shown in figure 3.10. This is consistent with the previous observations in EJp53, which shows the ability of the BTK to induce apoptosis.
Figure 5.9 BTK induced apoptosis in HCT116. HCT116 cells were transfected with BTK or EV and were allowed to grow for 2 days. The cells were then collected and washed with annexin V buffer. The cells were then resuspended in annexin V staining solution for 15 min before transferring to FACS tubes for analysis. BTK expression increased apoptotic cells from 5% to 21%. The experiment was performed in duplicate.

Figure 5.10 Measuring caspase3 and cleaved PARP in HCT116 transfected with BTK. The cells were transfected with BTK or EV (C) and were allowed to grow for 2 days. Actin was used as a loading control. BTK expression induced caspase3, and PARP cleavage as indicated by arrows.
4.10 BTK induces DNA damage responses through binding and phosphorylation p53

4.10.1 BTK expression induced DNA damaging pathway

BTK was able to induce p53 level due to DNA damage; however, the mechanism of p53 upregulation was not known. The effect of BTK inhibitors on EJp53 was not immediate, as after three days no difference was seen between cells with or without BTK inhibitors figure 5.11. Therefore, BTK inhibitors (ibrutinib 0.5µM or CGI-1746 2µM) were added to EJp53 for 3, 7 and 13 days. After 3 days, there was no effect observed from BTK inhibitors on p53 expression, as seen in figure 5.11. However, after 7 days the effect of the inhibitors was clear, but there was a slight p53 level after treatment with ibrutinib for 7 days. A complete absence of p53 was achieved after adding the BTK inhibitors for 13 days. This clearly showed a time dependent action of these inhibitors on p53 expression. From this observation, we hypothesized that BTK could play a role in p53 posttranslational modifications, mainly phosphorylation, which leads to the stabilisation of p53 by preventing proteasomal degradation (Stommel and Wahl, 2004)

![Figure 5.11 Treatment of EJp53 with BTK inhibitors in time-dependent manner.](image)

EJp53 cells were incubated with 0.5µM of Ibrutinib or 2µM of CGI-1746 for 3, 7 and 13 days in the presence or absence of p53. Western blot was performed to see the effects of BTK inhibitors on p53 at different time points. A reduction in p53 expression was seen after 7 days of treating EJp53 with ibrutinib or CGI-1746. There was no effect when they treated for 3 days. Actin was used as a loading control. The experiment was performed once.
To test the effects of BTK expression on the targets that phosphorylate p53 after DNA damage, BTK was transfected into HCT116. Upregulation of ATM phosphorylation and H2AX was seen, which suggested the role of BTK in this pathway (figure 5.12). In addition, phosphorylation at Ser15 of p53 was also seen. These observations proposed that BTK may induce DNA damaging pathway and in the same time increasing the total and phosphorylated p53 levels.

Figure 5.12 BTK expression induced the DNA damaging pathway. HCT116 cells were transfected with BTK or EV and were allowed to grow for 2 days. Western Blot shows an increase in the phosphorylation of ATM, H2AX and p53 after BTK expression. No effect was seen on the total levels of p53 for reasons that are not clear. Actin was used as a loading control. The experiment was performed twice independently.
4.10.2 BTK phosphorylates p53
BTK has a kinase activity and can phosphorylate and activate several targets. However, it has not been shown any previous link to p53 phosphorylation. We proposed that BTK induces p53 and the DNA damage pathway directly through binding and phosphorylating p53.

For this purpose, an in vitro kinase assay was performed to see if BTK was able to phosphorylate p53. Recombinant BTK and mammalian p53 were used in vitro in the presence of γP\textsuperscript{32}. If BTK could phosphorylate p53, it would directly bind to it. (ATP) γP\textsuperscript{32} was used for this reaction and could be detected using phosphor-image.

In the presence of p53 alone, there was no phosphorylation, as seen in figure 5.13. In the second lane, BTK was able to phosphorylate itself and it was used as positive control. In the third lane, it was not clear may be because of presence contamination in the sample or failing in pulling p53 down, or because of hyperphosphorylation of p53 which appeared as a smear.

![BTK phosphorylates p53](image)

**Figure 5.13e BTK phosphorylaties p53 from mammalian cells.** p53 protein obtained from mammalian cells (EJp53 cells) after pulling down with magnetic beads was incubated for 10 min at 30 C with recombinant BTK in the presence of γP\textsuperscript{32}. Loading buffer was then added to each sample, and loaded into 10% polyacrylamide gel. The gel was then dried for 1 hour. Phosphor screen was then placed into the dried gel for 1 hour. Phospho-image of dried gel was then developed using a Typhoon scanner.
To address this problem, recombinant full length p53 and different parts of p53 (1-80, 100-300 and 300-393 amino acids) were incubated with recombinant BTK in the presence of γP$^{32}$. As seen in figure 5.14, no phosphorylation was seen in the presence of p53 alone, which was used as a negative control. BTK was able to phosphorylate itself and this was used as positive control. Interestingly, BTK phosphorylated full-length p53, which appears to be higher than 53 KD because of the fact that it was tagged with GST. Specifically, BTK was able to phosphorylate the N-terminus of p53 (1-80), but no phosphorylation was observed in 100-300 and 300-393 parts of p53. In the future, experiment should be performed to see which amino acids of p53 are phosphorylated by BTK.
Figure 5.14 BTK phosphorylated full-length recombinant p53 and its N-terminus.

(A) Recombinant full-length p53 (FL) and different parts of p53 (1-80, 100-300, 300-393) were incubated for 10 min at 30°C with recombinant BTK in the presence of γP\textsuperscript{32}. Loading buffer was then added to each sample, and loaded into 10% polyacrylamide gel. The gel was then dried for 1 hour. Phosphor screen was then placed into the dried gel for 1 hour. Phospho-image of dried gel was then developed using A Typhoon scanner. The experiment was performed twice independently. (A) no phosphorylation of p53 was seen in lane (1), which was used as a negative control. In lane (2), BTK was able to phosphorylate itself in the absence of p53, which was used as a positive control. In lane (3), BTK was able to phosphorylate itself and full-length p53. In lane (4), BTK was also able to phosphorylate itself and p53 (1-80) fraction. In lanes (5 and 6), BTK was able to phosphorylate itself, but there were no phosphorylation seen on the 100-300 and 300-393 fragments of p53. (B) Picture of the gel stained with instant blue. FL-p53, 1-80 and 300-393 were labelled, however, recombinant BTK, and 100-300 fragment of p53 could not identified.

4.11 BTK contributes to cell senescence

There are many commercial available drugs that can inhibit BTK activity (Wang et al., 2013). One of this inhibitor is ibrutinib, which has recently received approval to be prescribed for human to treat many types of leukaemia (Hendriks et al., 2014). Ibrutinib inhibits the enzymatic activity of BTK at cysteine-481 irreversibly and prevents the full activation of BTK through the suppression of tyrosine-223 autophosphorylation (Hendriks et al., 2014).

Since BTK expression was activated in cell senescence, ibrutinib was used to see whether BTK is important for p53-induced senescence. As shown in figure 3.15, Ibrutinib was not only able to inhibit BTK activation, but also was able to suppress BTK expression. In addition, ibrutinib was also able to completely abolish p53 expression. This result showed an undiscovered role of ibrutinib in suppressing p53 expression.
Figure 5.15 The effect of ibrutinib on p53 expression in EJp53. EJp53 cells were treated with 0.5 μM ibrutinib for 6 days in the presence or absence of tetracycline. Western blot shows that, in the presence of p53, BTK was induced in EJp53 cells, but in the presence of ibrutinib, BTK levels were reduced, and p53 levels were very low comparing to the control. This experiment was performed three times independently.

To exclude any possible interaction of ibrutinib with the tet-regulatable system, EJp53 cells were infected with an adenovirus expressing p53. Ibrutinib also was able to decrease p53 expression after adenovirus infection. This clearly confirmed the results, as shown in figure 5.16. Moreover, phosphorylation of ERK, which is a downstream target of BTK pathway, also decreased after ibrutinib addition confirming the effects of the inhibitor on BTK activity.

Figure 5.16 The effect of ibrutinib on adenovirus expressing p53. EJp53 cells were treated with 0.5µm of ibrutinib in the presence and absence of tet for 6 days. Other EJp53 cells were infected with an adenovirus construct containing p53, in the presence or absence 0.5µM ibrutinib for 3 days. Ibrutinib was able to decrease p53 levels after tet removal. After p53 overexpression using adenovirus construct containing p53, ibrutinib was also able to reduce p53 levels. Downregulation of ERK phosphorylation confirmed that the pathway below BTK was inhibited. Actin was used as a loading control.
In order to confirm these observations in other cells, primary malignant B cells were incubated with CD154 and IL4 to induce B cell proliferation and p53 expression were used. Ibrutinib was able to completely decrease p53 expression in this model, as shown in figure 5.17. This experiment was performed by Dr Jesvin Samuel, Department of Biochemistry, University of Leicester. This result strongly confirms the observation in other cell lines, and the ability of ibrutinib in affecting physiological p53 level. Hence, BTK may have a role in p53 stabilisation of p53 protein levels, which could explain its effect on p53.

**Figure 5.17 Ibrutinib was able to inhibit p53 expression in primary B- cells.** p53 expression was stimulated using IL4 and CD154 (S: p53 stimulation); and after adding 0.3 um of ibrutinib (S+I: p53 stimulation plus ibrutinib), p53 levels completely decreased. (P: peripheral blood). Actin was used as a loading control.

Ibrutinib reduced p53 levels in EJp53 cells and in primary B malignant cells. In a cell count experiment, EJp53 cells expressing p53 were unable to proliferate and became senescent, and EJp53 cells without p53 were able to grow, as expected (figure 5.18). With ibrutinib, EJp53 cells grew highly comparing to EJp53 without ibrutinib. In EJp53 expressing p53, ibrutinib addition was capable of keeping cells growing but at slow rate, compared to the cells without ibrutinib. This result demonstrated that ibrutinib can bypass senescence in EJp53. To confirm this, CGI-146 a more specific BTK inhibitor was used (Di Paolo et al., 2011). CGI-1676 was able to bypass senescence in EJp53 (figure 5.19). This confirmed the ability of BTK inhibitors to maintain cells growing even in the presence of p53.
EJp53 counting after ibrutinib treatment. EJp53 cells were split into equal numbers and treated with 0.5 µM of ibrutinib in the presence or absence of tet and then counted every two days by an automated Bio-Rad counter. The media was replaced with fresh drugs every 3 days. There was a significant (p< 0.01) increase in cell growth in presence of ibrutinib and p53, compared to p53 only. Error bars represent standard deviations. The experiment was done in triplicate.

Figure 5.18 EJp53 counting after CGI-1746 treatment. Same as 5.18 using 2µM of CGI-1746. There was a significant (p< 0.01) increase in cell growth in presence of CGI and p53. Error bars represent standard deviations. The experiment was done in triplicate.

Chemical inhibitors of BTK stopped senescence and increased the rate of cells growing, but this could be due to off target effects. To explore this, a shRNA plasmid against BTK was introduced into EJp53 to permanently silence the BTK
gene. shRNA against Luciferase was also introduced into EJp53 cells as a control. EJp53 Luci and EJp53 shBTK were counted in the presence and absence p53. As shown in figure 5.20, BTK depletion increased growing of EJp53 in the presence of p53. This is consistent with the results that BTK inhibition results in bypass of senescence.

![Cell count graph](image)

**Figure 5.20 EJp53 counting after BTK depletion.** EJp53 cells were transfected with shBTK or shLuci. The following day, the cells were selected with Puromycin for 2 week. Fresh media and puromycin were added every 3 to 4 days. After selection, the cells were split into equal number and were counted for 2 weeks in the presence or absence of tet. For cell counting, equal amount of cell suspension was mixed with same amount of trypan blue and counted by Bio-Rad automated counter. There was a significant increase (p< 0.01) in cell growth after BTK knockdown in the presence of p53. Error bars represent standard deviation. The experiments were performed in triplicate.

Senescence cells have distinctive morphological features: they appear long, flat in shape and spindle-like (Li et al., 2012). These features were completely disappeared after BTK inhibition. In presence of p53 and BTK inhibitors, the morphological features completely disappeared (figure 5.21 (A)). The same result was observed after transfecting the cells with shRNA against BTK (figure 5.21, B). The efficiency of the transfection was checked by Western blot, as shown in figure 5.12, C.
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A

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**p53**

**Actin**
B

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Figure 5.21 The morphological features of EJp53 cells after BTK inhibition or depletion. A) Western blot showing BTK and p53 expression in EJp53 cells stably expressing shBTK (+) or shLuci (-), cultured in the presence or absence of tet for 6 days. BTK depletion was enough to downregulate p53 expression. B) EJp53 cells were split and treated with 0.5 µM of Ibrutinib 6 days in the presence or absence of tet. EJp53 shBTK cells were also split and incubated for 6 days in the presence or absence of tet. EJp53 cells with p53 showed low count, large spindle shape, in the presence of Ibrutinib or after BTK depletion, the morphology of the cells did not change.

Cells treated with Ibrutinib and CGI-1746 were next plated for a colony formation assay. In addition, EJp53 shBTK was used in this study. As shown in figure 5.22, in the presence of p53, the number of colonies decreased to 10%. However, EJp53 in the absence of BTK were able to form colonies from 60 to 80 % even in the presence of p53, which supported the previous results. The number of the colonies was also high after BTK silencing with shRNA in the presence of p53.

The previous results from figure 5.18,19,20 and 21 are clearly demonstrated that BTK inhibition or depletion are enough to bypass senescence in EJp53 on p53.
Figure 5.22 Colony formation of EJp53 treated with Ibrutinib, CGI-1746 or shBTK. In A) 200 EJp53 cells were plated in 6 cm plates and treated with 0.5 μM of Ibrutinib or 2μM of CGI-1746 for 2 weeks in the presence or absence of tet. Alternatively EJp53 stably expressing shBTK were used. A) The number of the colonies in the presence of p53 was low, but in the presence of Ibrutinib, CGI-1746 or shBTK, the number of the colonies was high. B) The percentage of the colonies from 3 experiments. The average of the colonies after treatment with ibrutinib or CGI-1746 was significantly higher than the controls (p< 0.01). The number of colonies after BTK knockdown was significantly (p< 0.01) higher than the controls (shLuci). Error bars represent standard deviations.

4.12 The expression of BTK in blood samples of healthy donors from different ages

We have shown in the previous chapter that senescence markers can be measured in blood cells, section 3.9. For instance, p16 a known marker of
senescence increases normally in blood samples of healthy donors (Liu et al., 2009).

BTK is activated and expressed highly in blood cells (Wang et al., 2013); however, no previous studies have shown the expression of BTK in different ages of healthy volunteers.

Since BTK is highly expressed in blood cells (Wang et al., 2013), it was worth to see if its expression is increased during aging. For this purpose, blood samples from different age groups were used to measure BTK levels using Western blot.

In order to see the expression levels of BTK in blood cells of different group ages, consent was obtained for blood samples collection by Dr Sandrine Jayne, The Ernest and Helen Scott Haematological Research Institute, University of Leicester. Samples from <25, 25-35, 35-55 and >55 years old healthy volunteers were received. White blood cells were then separated from whole blood cells using histopaque solution. The lysate from white blood cells was used to measure BTK expression using western blot.

There was an increase in the BTK expression in the WBCs from the elderly healthy volunteers, as seen in figure 5.23, comparing to younger donors. However, this increase is not significant. EBP50 was used as a marker of senescence.
Figure 5.23 BTK levels r in WBCs of elderly people compared to younger donors.

Blood samples from healthy volunteers were added to the histopaque solution before centrifugation at 4000 RPM for 30 min. The white layer was then collected into a clean tube and washed with RPMI media and the pellet was then used for the testing the expression of the senescence markers using Western Blot. BTK expression increased up two folds in samples from 35-35 and >55 years old donors compared to samples from <25 and 25-35 years. However the changes were not significant (p> 0.01). Actin was used as a loading control, and EBP50 was used as a molecular marker of senescence.
4.13 BTK inhibitors ameliorate aging and extend lifespan \textit{in vivo}

The previous results (sections 5.3, 5.4, 5.10, 5.11, 5.12, and 5.14) strongly suggested the role of BTK in phosphorylation, regulation and stabilisation of p53 \textit{in vitro}. It also demonstrated the role of BTK inhibitors in p53 expression and cell senescence bypassing.

The next aim was to translate these observations into an organismal model and test the effectiveness of BTK inhibitors \textit{in vivo}. \textit{Drosophila melanogaster} was chosen for this experiment, due to many reasons. Firstly, its lifespan is very short comparing to mice. It is a widely accepted model for studying aging and many target genes of aging have been identified firstly in this model (Helfand and Rogina, 2003). In addition, BTK has been studied in fruit fly and its effects in development of \textit{Drosophila} have been tested (Hamada et al., 2014). These collectively show the suitability of this model in our experiments.

Longevity experiments in \textit{Drosophila melanogaster} were conducted in collaborations with Dr Eran Tauber, Department of Genetics, University of Leicester.

\textit{Drosophila melanogaster} was treated with 5 µM of ibrutinib and 5µM of CGI-1746. The drugs were mixed with 1.5 ml of the flies’ food each tube. Each concentration included 10 tubes and each tube contained 10 flies, so each concentration contained 100 flies. 200 flies were treated with DMSO and used as controls in two sets.

Flies fed the drugs throughout their lifespan lived longer than controls, as shown in figure 2.4. Ibrutinib at 5 µM and CGI-1476 showed strong effect on life span extension (p< 0.01).
Figure 5.24 BTK inhibitors extended life span of Drosophila melanogaster.
Survival curves, as percentage of live flies with time. 100 flies were used for each drug treatment. 10 flies were incubated in each tube, the total number of the tubes were 10 for each treatment. The food of the flies was changed and mixed with fresh drugs every 2 days and the number of the flies was recorded during food changing. A) 5 µM of Ibrutinib was able to extend flies life span significantly (p< 0.01) to at least 20% comparing to the controls. B) 10 µM of CGI-1746 extended life span significantly (p< 0.01) comparing to the controls. The experiment was performed twice independently.
Since BTK inhibitors bypassed senescence through modulation of p53 in vitro, flies with knock out p53 gene were used to see the effect of ibrutinib after p53 deletion. To see the effect of p53 on lifespan extension of p53KO flies treated with BTK inhibitors, 100 flies were treated ibrutinib at 5 µM (the concentration gave the optimum life span extension), and 100 flies were treated with DMSO. As shown in figure 5.25, there was no extension on lifespan in p53KO flies with ibrutinib, meaning that BTK inhibitors extended lifespan through p53 action and p53 knocking out was sufficient to abolish ibrutinib effect on flies. This result was a consistent with the previous results, which showed p53 role in senescence bypassing in vitro.

![Figure 5.25 Ibrutinib was unable to extend life span in p53 KO flies.](image)

**Figure 5.25 Ibrutinib was unable to extend life span in p53 KO flies.** Survival curves, as percentage of live flies with time. 100 flies were used for each drug treatment. 10 flies were incubated in each tube, the total number of the tubes were 10 for each treatment. The food of the flies was changed and mixed with fresh drugs every 2 days and the number of the flies was recorded during food changing. 5µM of ibrutinib was added to p53 KO flies food, no change in life span was noticed comparing to the control (p>0.01).

We next assessed the amount of senescent cells in flies after BTK treatment. Flies were fed 5 µM ibrutinib or DMSO for 40 days before they were fixed in 10% formalin for 10 minutes. After that they were immersed in x-gal staining for 48 hours at 37ºC. There was accumulation of β-Gal staining in the heads of flies.
after 40 days; however, less β-Gal were seen after the treatment with ibrutinib, which potentially correlates with senescence accumulation (figure 5.26). No β-Gal staining was noticed after two weeks of life, which suggests the absence of cell senescence in flies head at this age. This result showed that BTK inhibition was capable of reducing β-Gal staining and potentially senescent cell accumulation in vivo, as expected, and also demonstrated the possibility of flies head to be used for monitoring senescent cell accumulation.

**Figure 5.26 BTK inhibitors decreased senescent cells accumulation in vivo.** Flies treated with 5µM of ibrutinib or DMSO for 40 days were fixed in 4% formaldehyde for 15 min before washing and incubating with β-Gal staining solution for 48 hours at 37°C. Flies treated with ibrutinib showed less SA-β-Gal in the head after 40 days comparing to the control, which could suggest less senescent cells. No β-Gal staining was seen in the flies head after two weeks of the life, which also suggest the decrease of senescent cells after ibrutinib treatment. The experiment was performed in 7 flies two times independently.

To confirm this result, the levels of p53 also measured using flies whole tissue. For this, 10 flies at 40 days treated with 5µM ibrutinib and DMSO were homogenised and resuspended in the RIPA buffer for 20 minutes before centrifugation at maximum speed. Supernatants were used for proteins detection. p53 level was higher in DMSO treated flies comparing to 5 µM ibrutinib. In addition, BTK inhibitor was able to reduce armadillo
phosphorylation, which is a downstream target of BTK in flies, figure 5.27 (Hamada et al., 2014). This result showed that BTK inhibition also blocks p53 expression \textit{in vivo}, as observed in human cells.

![Western Blot Image](image)

**Figure 5.27 p53 level decreased in flies treated with ibrutinib.** 10 40-days old flies treated with 5µM of ibrutinib or DMSO for 40 days were mixed in RIPA buffer and were homogenised and used for Western Blot. A reduction in p53 level was seen in flies treated with ibrutinib. Inhibition of BTK also reduced phosphorylation of armadillo (P-arm), a downstream member of the BTK pathway. Ponceau staining was used as a control for the loading. The experiment was performed once.

Weight loss and muscle wasting are normally associated with aging (Doherty, 2003). In addition, clinicians prescribing ibrutinib for CLL patients observed weight gain in these patients (Dyer, personal communication, July, 2014). To test the ability of BTK inhibitors to ameliorate symptoms associated with aging and weight increasing, flies either treated with DMSO or 10 µM of ibrutinib were selected for weight measuring randomly in mid-age. Flies treated with ibrutinib had higher weight of average 0.97g, comparing to weight DMSO treated flies, which had average 0.84g. Mann-Whitney test was performed, which showed a significant difference between these conditions, figure 5.28. These can exclude
any caloric restriction that could lead to accidental longevity in BTK inhibitors treated flies. This experiment was kindly performed by Dr Kamaldeep Chana.

![Bar graph showing weight difference between control and ibrutinib treated flies.](image)

**Figure 5.28 Flies treated with ibrutinib gained weight.** 6 flies from DMSO treated and 5 flies from ibrutinib treated groups were weighted. Error bars show standard deviation.

### 5.9 Discussion

From the proteomic screening we performed (Althubiti *et al.*, 2014), we have found that BTK is upregulated in senescence. We discovered a new role of BTK in senescence and we also characterised a link between BTK and p53 signalling, which illustrated a vital role of BTK in senescence and apoptosis. We also showed that BTK inhibitors were able to delay senescence and aging in a p53-dependent manner.

BTK is a tyrosine kinase that is involved in B-cell signalling. It also plays a role in B-cell maturation. Inhibition of BTK phosphorylation by small molecules has shown to be successful in CLL management (Honigberg *et al.*, 2010; Wang *et al.*, Byrd *et al.*, 2013). Here we show that BTK could have a role in tumour suppression in other situations. BTK is upregulated after p53 activation, and its overexpression is enough to increase cell death. BTK overexpression
associates with phosphorylation of ATM, p53 at serine 15 and γH2AX, which shows its important role in DNA damaging signalling pathway.

This could elucidate the way BTK induces p53. This suggested a feedback loop between BTK and p53 activation, which requires more explanation. We showed that BTK was able to phosphorylate p53 directly at N-terminal region, which has amino acids that are binding site for mdm2, which could explain the mechanism by which BTK regulates p53. This is because p53 phosphorylation stabilises p53 and prevents its degradation, which enhances its activity. The opposite effect was seen on p53 after blocking BTK phosphorylation using BTK inhibitor. Further work should be conducted to examine which amino acids of p53 are phosphorylated by BTK, and if more than one residue is involved. Work could be done also to examine the binding site of p53 by which BTK is bound, this would be important confirm the direct interaction between p53 and BTK. In addition, it is important to check if mdm2 involves in this loop.

BTK could regulate other effector of cellular senescence other than p53. p21 and p16 are examples of effectors by which senescence can be induced (Lowe et al., 2004). Testing senescence in human fibroblasts or MEFs with knockout p53 is an important work. This will help to see if BTK can modulate senescence effectors in the absence of p53.

5.9.1 BTK serves as a tumour suppressor

We have shown that overexpression of BTK in epithelial cells in culture increases apoptosis. This could explain the dual role of BTK as a tumour suppressor in senescence and apoptosis. Induction of apoptosis or permanent cell cycle arrest depends on many factors. The amount of tumour suppressor expression has an effect on the fate of cell. Senescence tends to be induced after minimal expression of p53 and p21, but apoptosis normally induces after sharp induction of p53 (Chang et al., 1999; Schwarze et al., 2005; Ewald et al., 2009). This could be the case of BTK, where minimal BTK upregulation induces senescence, and higher induction leads to apoptosis. Further experiments to evaluate role of different expression levels of BTK on cell fate will be important.

BTK has shown oncogenic effects on B-cells malignancies (Herman et al., 2011; Chang et al., 2013; Kuehl and Bergasagel, 2012). However, its
expression correlated with better survival rate in solid tumour. Therefore, inhibition of BTK with chemical inhibitors in mouse model of solid tumour would be essential to see the role of BTK activity on solid cancer progression. This will help in understanding of BTK function in solid tumours.

5.9.2 BTK inhibition bypasses senescence in vitro

We also showed that cell senescence induced by p53 could be bypassed in culture using either chemical inhibitor of BTK or shRNA against BTK. These methods allowed portion of cells to continue proliferation and escaping from cell cycle arrest by p53. BTK has not been shown to have any role in cell cycle arrest before. Without BTK, p53 stabilisation would be affected, which leads to a reduction in p53 levels. This could compromise cell cycle arrest induction, and successfully bypass senescence. Including other models of cell senescence would be important. For example oncogenic induced senescence, overexpression RAS or RAF in human fibroblasts or MEFs in the presence and absence of BTK inhibitors will clarify possibility of BTK involvement in this oncogenic pathway, and if its inhibition affects oncogenic-induced senescence induction in the presence and absence of p53.

5.9.3 BTK inhibitors extend life span in vivo

BTK inhibition also showed effectiveness in Drosophila melanogaster, which has a BTK gene homolog. There was a life span extension after using ibrutinib or CGI-1746. In addition, aging symptoms, such as inability to climb or weight loss, were improved after BTK inhibition. This clearly shows that BTK inhibitors not only extend life span but also contribute to maintenance of a healthy life. It was difficult to measure the amount of the drug received by flies. In addition, BTK inhibitors could have effects on many kinases, which could have an effect on the lifespan of the flies. So, studying lifespan extension after BTK deletion from flies will confirm whether this gene has an effect or the effect of BTK inhibitors is off target.

A functional p53 was required for life span extension by BTK inhibitors. This was consistent with the hypothesis that BTK acts as an enhancer of the p53-induced senescence. In addition, the SA-β-Gal that used previously to assess senescent cells in flies (Nakamura et al., 2014) was also reduced in brains of
flies treated with BTK inhibitor, which supports the idea that the role of BTK inhibitors on aging is triggered by delaying senescence induction. Consistent with this observation, it has been shown that p53 inhibition in flies neurons extend life span (Bauer and Helfand, 2006). However, percentages of positive SA-β-gal staining cells in the flies head should be counted to give a better indication of the percentage of positive cells for each fly.

5.9.3.1 BTK inhibition ameliorate aging symptoms

Increasing in the weight of flies treated with ibrutinib could be linked to improved fitness, but the number of the measured flies is not enough. Thus, increasing the number of the flies and using different concentrations of the drug will be important. In addition, measurement of the flies weight in an age dependent manner will help to reveal at which age the drug improves the fitness.

In the future, effectiveness of BTK inhibitor as anti-aging should be tested in mammals. For example testing BTK inhibitors on accelerated aging mice will be easier to conduct. Taking into account that ibrutinib is already being prescribed for patients with B cells malignancies; it could be used for life span extension and ameliorating the symptoms associated with aging. It could be tested firstly in population who are at high risk, such as people with progeria and others premature aging syndromes or people older than 85 years (Clegg et al., 2013). This could prevent further accumulation of senescent cells and may result in healthier aging and probably extend life span of these groups.

Testing of role of BTK inhibitors in specific senescence associated diseases will be important, for example in increased insulin sensitivity in mice with type2 diabetes (Minamino et al., 2009). Using BTK inhibitors in very specific situation in very short time could avoid its potential oncogenic side effects.

Using BTK inhibitors as anti-aging drugs could associate with side effects. They inhibit p53 activity, and downregulation of the guardian of the genome activity increases the susceptibility for cancers formation (Haferkamp et al., 2008). Therefore, any future use of BTK inhibitors should be focused and for a specific group of people, for example, local treatment of atherosclerotic plaque where senescent cells are highly located and contribute to its pathogenesis (Wang and
Bennett, 2012). In the case of glaucoma, using BTK inhibitor as drops could minimise its side effects instead of using it systematically.

Many drugs with anti-aging effects are now under examination. But, none of them has been licensed yet for human use. For example, rapamycin is used as chemotherapeutic agent for cancer therapy can also prolong life span of mammals (Harrison et al., 2009). However, it suppresses immune system that stops its using for treatment aging-associated diseases (Law, 2005). Conversely, very low side effects have been seen in patients with ibrutinib treatment (Advani et al., 2013).

To sum up, BTK seems to be upregulated in response of p53 activity due to many DNA damaging insults. Phosphorylation of p53 by BTK seems to occur at N-terminus (1-80) of p53. This part is very important for p53 stabilisation, as mdm2 ubiquitinates p53 at this region (Serine 15). A potential phosphorylation of p53 at this region by BTK could delay p53-mdm2 interaction, which would lead to p53 stabilisation. p53 phosphorylation by BTK can stabilise it, which in turn results in increasing functional activity of p53. Inhibition of BTK through chemical inhibitors de-stabilise p53 that leads to its degradation, which subsequently results in senescence and apoptosis in vitro and life span extension in vivo (figure 6.2).
Figure 5.29 A summary of the role of BTK in p53 regulation. BTK phosphorylates BTK directly at N-terminus, which results in p53 stabilisation and subsequently senescence and/or apoptosis. In addition, BTK inhibitor inhibits p53 phosphorylation by BTK (as seen in the box), which leads in p53 degradation and later to delaying senescence and life span extension.
6. Concluding points

1) We have found that DEP1, NTAL, EBP50, STX4, VAMP3, ARMCX3, B2MG, LANCL1, VPS26A and PLD3 are induced in senescence in vitro, dependent on the cell type and the inducer.

2) These markers are associated with better survival rate in patients with breast cancer.

3) B2MG and DEP1 are known to have extracellular epitopes, which can be used for senescent cells detection and targeting.

4) BTK is induced in response to p53 upregulation, and it could serve as a tumour suppressor.

5) BTK expression is induced in apoptosis, and correlates with better survival rate in patients with solid tumours.

6) BTK phosphorylates p53 at N-terminus, which could explain the mechanism of its tumour suppressive activity.

7) BTK inhibitors bypassed senescence and extended life span in vivo. They also showed some effects on aging symptoms.


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p21CIP1 expression via Miz-1 dependant interaction with the p21 core promoter, *Oncogene*, 22; 351-360.


Appendix
<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Dilution</th>
<th>Molecular Weight (KDa)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Actin</td>
<td>Mouse</td>
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<td>42</td>
<td>Abcam</td>
</tr>
<tr>
<td>Calnexin</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>95</td>
<td>Cell Signalling #2433</td>
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<td>Na/K ATPase</td>
<td>Mouse</td>
<td>1:4000</td>
<td>100</td>
<td>Ab7671</td>
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<tr>
<td>STX4</td>
<td>Mouse</td>
<td>1:1000</td>
<td>33</td>
<td>Ab77037</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mouse</td>
<td>1:1000</td>
<td>120</td>
<td>Cell Signalling #9102S</td>
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<tr>
<td>p21</td>
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<td>53</td>
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<tr>
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<td>220</td>
<td>Santa Cruz: sc-376749</td>
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<tr>
<td>DCR2</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>40</td>
<td>Abcam[EPR3588(2)]</td>
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<td>NTAL</td>
<td>Mouse</td>
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<td>33</td>
<td>Ab3992</td>
</tr>
<tr>
<td>EBP50</td>
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<td>1:500</td>
<td>50</td>
<td>Ab3452</td>
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<td>ARM CX3</td>
<td>Rabbit</td>
<td>1:500</td>
<td>36</td>
<td>Ab98938</td>
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<td>Novus Biologicals (NBP1-59921)</td>
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<td>Rabbit</td>
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<td>13</td>
<td>Ab68833</td>
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<tr>
<td>LANCL1</td>
<td>Rabbit</td>
<td>1:500</td>
<td>40</td>
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<td>Cell signalling (9102)</td>
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<td>H1-s (DSHB)</td>
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<td>15</td>
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Table 2. Antibodies used for Immunofluorescence

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<th>Supplier</th>
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<td>Santa Cruz: sc-376749</td>
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<tr>
<td>DCR2</td>
<td>Rabbit</td>
<td>1:100</td>
<td>40</td>
<td>Abcam[EPR3588(2)]</td>
</tr>
<tr>
<td>NTAL</td>
<td>Mouse</td>
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<td>33</td>
<td>Ab3992</td>
</tr>
<tr>
<td>EBP50</td>
<td>Rabbit</td>
<td>1:200</td>
<td>50</td>
<td>Ab3452</td>
</tr>
<tr>
<td>ARMCX3</td>
<td>Rabbit</td>
<td>1:100</td>
<td>36</td>
<td>Ab98938</td>
</tr>
<tr>
<td>VAMP3</td>
<td>Rabbit</td>
<td>1:100</td>
<td>13</td>
<td>Ab68833</td>
</tr>
<tr>
<td>B2M</td>
<td>Mouse</td>
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<td>Ab759</td>
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Table 3. Antibodies used for Immunohistochemistry

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<th>Name</th>
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<th>Supplier</th>
<th>Reacts with</th>
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<td>p16&lt;sup&gt;Rk4&lt;/sup&gt;a</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Santa Cruz SC-1207</td>
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<tr>
<td>p16&lt;sup&gt;Rk4&lt;/sup&gt;a</td>
<td>Mouse</td>
<td>1:100</td>
<td>Abcam 54210</td>
<td>H</td>
</tr>
<tr>
<td>p19&lt;sup&gt;ARF&lt;/sup&gt;</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Abcam[EPR3588(2)]</td>
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<td>NTAL</td>
<td>Mouse</td>
<td>1:100</td>
<td>Ab3992</td>
<td>H/M</td>
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<td>PTPRJ(DEP1)</td>
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<td>B2M</td>
<td>Mouse</td>
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Table 4  A list of secondary antibodies used in the project

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<th>Used for</th>
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<tr>
<td>Mouse IgG</td>
<td>1:10,000</td>
<td>Horseradish peroxidase</td>
<td>Thermo Scientific #314130</td>
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<tr>
<td>Rabbit IgG</td>
<td>1:10,000</td>
<td>Horseradish peroxidase</td>
<td>Thermo Scientific #31460</td>
<td>WB</td>
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<tr>
<td>Mouse IgG</td>
<td>1:10,000</td>
<td>IR Dye-800cw</td>
<td>Li-COR #926-32210</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>1:10,000</td>
<td>IR Dye-680 cw</td>
<td>Li-COR #926-68071</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>1:500</td>
<td>Alexa Fluor 550</td>
<td>Thermo Scientific #31430</td>
<td>IF</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>1:500</td>
<td>Alexa Fluor 488</td>
<td>Thermo Scientific #31460</td>
<td>IF</td>
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<tr>
<td>Mouse IgG</td>
<td>1:200</td>
<td>Biotinylated</td>
<td>DAKO # E0413</td>
<td>IHC</td>
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<tr>
<td>Rabbit IgG</td>
<td>1:200</td>
<td>Biotinylated</td>
<td>DAKO # E0353</td>
<td>IHC</td>
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Table 5  A list of antibodies used for flow cytometry

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<tr>
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<td>1:5</td>
<td>R&amp;D Systems, FAB1934P</td>
<td>PE</td>
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<td>B2MG</td>
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<td>NOVUS BIOLOGICALS, NB100-77981</td>
<td>FITC</td>
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<td>DCR2</td>
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<td>R&amp;D Systems, FAB633G</td>
<td>FITC</td>
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<td>NOTCH3</td>
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<td>eBIO SCIENCES, 17-5787</td>
<td>APC</td>
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# Table 6. A list of cell lines used in the project

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<td>IMR90</td>
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<td>501T</td>
<td>Human fibroblast</td>
<td>Normal cells</td>
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<tr>
<td>HDF</td>
<td>human dermal fibroblast</td>
<td>Normal cells</td>
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<td>HCT116</td>
<td>Colon cancer cells</td>
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<tr>
<td>RD</td>
<td>Restrictive dermopathy cells</td>
<td>Mutation in ZMPSTE24</td>
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<tr>
<td>EJp16</td>
<td>Bladder carcinoma cells</td>
<td>Tet-off p16 inducible system</td>
</tr>
<tr>
<td>EJp21</td>
<td>Bladder carcinoma cells</td>
<td>Tet-off p21 inducible system</td>
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<tr>
<td>EJp53</td>
<td>Bladder carcinoma cells</td>
<td>Tet-off p53 inducible system</td>
</tr>
<tr>
<td>HT1080-p21-9</td>
<td>Fibrosarcoma</td>
<td>IPTG on p21 inducible system</td>
</tr>
<tr>
<td>PC3-p53</td>
<td>Prostate cancer cells</td>
<td>Tet-off p53 inducible system</td>
</tr>
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</table>

Proteins expressed in plasma membrane of EJp16 senescent cells but not in EJp16 growing cells
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<thead>
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<th>Gene Symbol</th>
<th>Description</th>
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<td>K1C14_HUMAN</td>
<td>Keratin, type I cytoskeletal 14 OS</td>
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<td>MYO1B_HUMAN</td>
<td>Myosin-la OS=Homo sapiens GN</td>
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<td>K1C16_HUMAN</td>
<td>Keratin, type I cytoskeletal 16 OS</td>
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<tr>
<td>DDX17_HUMAN</td>
<td>Probable ATP-dependent RNA helicase DDX17 OS</td>
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<td>LYN_HUMAN</td>
<td>Tyrosine-protein kinase Lyn OS=Homo sapiens GN</td>
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<td>STXB1_HUMAN</td>
<td>Syntaxin-binding protein 1 OS=Homo sapiens GN</td>
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<td>MYO1A_HUMAN</td>
<td>Myosin-la OS=Homo sapiens GN=MYO1A PE=1 SV=1</td>
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<td>AL4A1_HUMAN</td>
<td>Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial</td>
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<tr>
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<td>Isoleucyl-tRNA synthetase, mitochondrial OS=Homo sapiens GN</td>
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<td>ODPA_HUMAN</td>
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<td>CATA_HUMAN</td>
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<td>ADRO_HUMAN</td>
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NUDC_HUMAN  Nuclear migration protein nudC OS=Homo sapiens GN=NUDC PE=1 SV=1
ATD3B_HUMAN  ATPase family AAA domain-containing protein 3B OS=Homo sapiens
NLTP_HUMAN  Non-specific lipid-transfer protein OS=Homo sapiens GN=SCP2 PE=1 SV=2
ARC1B_HUMAN  Actin-related protein 2/3 complex subunit 1B OS=Homo sapiens GN
HSDL1_HUMAN  Inactive hydroxysteroid dehydrogenase-like protein 1 OS=Homo sapiens
TPM4_HUMAN  Tropomyosin alpha-4 chain OS=Homo sapiens GN=TPM4 PE=1 SV=3
TMM43_HUMAN  Transmembrane protein 43 OS=Homo sapiens GN=TMEM43 PE=1 SV=1
DEFM_HUMAN  Peptide deformylase, mitochondrial OS=Homo sapiens GN=PDF PE=1 SV=1
AAKG1_HUMAN  5’-AMP-activated protein kinase subunit gamma-1 OS=Homo sapiens GN
VP26A_HUMAN  Vacuolar protein sorting-associated protein 26A OS=Homo sapiens
NMT1_HUMAN  Glycylpeptide N-tetradecanoyltransferase 1 OS=Homo sapiens GN
LMAN2_HUMAN  Vesicular integral-membrane protein VIP36 OS=Homo sapiens GN
P4HA2_HUMAN  Prolyl 4-hydroxylase subunit alpha-2 OS=Homo sapiens GN=P4HA2
T106B_HUMAN  Transmembrane protein 106B OS=Homo sapiens GN=TMEM106B
GBB2_HUMAN  Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 OS
VATD_HUMAN  V-type proton ATPase subunit D OS=Homo sapiens GN=ATP6V1D PE
NQO1_HUMAN  NAD(P)H dehydrogenase [quinone] 1 OS=Homo sapiens GN=NQO1 PE
RS10_HUMAN  40S ribosomal protein S10 OS=Homo sapiens GN=RPS10 PE=1 SV=1
S10AG_HUMAN  Protein S100-A16 OS=Homo sapiens GN=S100A16 PE=1 SV=1
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GNAQ_HUMAN  Guanine nucleotide-binding protein G(o) subunit alpha OS
SPEE_HUMAN  Spermidine synthase OS=Homo sapiens GN=SRM PE=1 SV=1
MPRD_HUMAN  Cation-dependent mannose-6-phosphate receptor OS=Homo sapiens
G45IP_HUMAN  Growth arrest and DNA damage-inducible proteins-interacting protein
RT05_HUMAN  28S ribosomal protein S5, mitochondrial OS=Homo sapiens GN=MRPS5
PLD3_HUMAN  Phospholipase D3 OS=Homo sapiens GN=PLD3 PE=1 SV=1
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Proteins expressed in plasma membrane of EJp21 senescent cells but not in EJp21 growing cells

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<td>SPCS3_HUMAN</td>
<td>Signal peptidase complex subunit 3</td>
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<td>Gene Symbol</td>
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<td>MDHC_HUMAN</td>
<td>Malate dehydrogenase, cytoplasmic</td>
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<td>RT05_HUMAN</td>
<td>28S ribosomal protein S5, mitochondrial</td>
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<td>PSB5_HUMAN</td>
<td>Proteasome subunit beta type-5</td>
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<td>PLD3_HUMAN</td>
<td>Phospholipase D3</td>
<td>Homo sapiens</td>
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<td>EXD2_HUMAN</td>
<td>Exonuclease 3'-5' domain-containing protein 2</td>
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<td>Acylglycerol kinase, mitochondrial</td>
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<td>Beta-hexosaminidase subunit alpha</td>
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<td>Putative tubulin beta-4q chain</td>
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<td>Twinfilin-1</td>
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<td>Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial</td>
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<td>Acid ceramidase</td>
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<td>Ras-related protein Rab-6B</td>
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<td>Caveolin-2</td>
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<td>DnaJ homolog subfamily B member 1</td>
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<td>Carnitine O-acetyltransferase</td>
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<tr>
<td>DNJA3_HUMAN</td>
<td>DnaJ homolog subfamily A member 3, mitochondrial</td>
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<td>PLD2_HUMAN</td>
<td>Phospholipase D2</td>
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<td>B2MG_HUMAN</td>
<td>Beta-2-microglobulin</td>
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<td>MIRO2_HUMAN</td>
<td>Mitochondrial Rho GTPase 2</td>
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HBB_HUMAN  Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2
IPO4_HUMAN  Importin-4 OS=Homo sapiens GN=IPO4 PE=1 SV=2
RM39_HUMAN  39S ribosomal protein L39, mitochondrial OS=Homo sapiens GN=MRPL39 PE=1 SV=3
BCS1_HUMAN  Mitochondrial chaperone BCS1 OS=Homo sapiens GN=BCS1L PE=1 SV=1
AASS_HUMAN  Alpha-aminoadipic semialdehyde synthase, mitochondrial OS=Homo sapiens
ERGI3_HUMAN Endoplasmic reticulum-Golgi intermediate compartment protein 3 OS=Homo sapiens
MAOX_HUMAN  NADP-dependent malic enzyme OS=Homo sapiens GN=ME1 PE=1 SV=1
RT15_HUMAN  28S ribosomal protein S15, mitochondrial OS=Homo sapiens GN=MRPS15 PE=1 SV=1
SERC_HUMAN  Phosphoserine aminotransferase OS=Homo sapiens GN=PSAT1 PE=1 SV=2
TTL12_HUMAN Tubulin--tyrosine ligase-like protein 12 OS=Homo sapiens GN=TTLL12 PE=1 SV=2
GLOD4_HUMAN Glyoxalase domain-containing protein 4 OS=Homo sapiens GN=GLOD4 PE=1 SV=1
NDUBA_HUMAN NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10 OS=Homo sapiens
SIAS_HUMAN  Sialic acid synthase OS=Homo sapiens GN=NANS PE=1 SV=2
6PGL_HUMAN  6-phosphogluconolactonase OS=Homo sapiens GN=PGLS PE=1 SV=2
MTX2_HUMAN  Metaxin-2 OS=Homo sapiens GN=MTX2 PE=1 SV=1
NUCG_HUMAN  Endonuclease G, mitochondrial OS=Homo sapiens GN=ENDOG PE=1 SV=4

Proteins expressed in plasma membrane of EJp21 and EJp16 senescent cells, but not EJp21 control cells

ICAM1_HUMAN Intercellular adhesion molecule 1 OS=Homo sapiens GN=ICAM1 PE=1 SV=2
ITB3_HUMAN  Integrin beta-3 OS=Homo sapiens GN=ITGB3 PE=1 SV=2
ERBB4_HUMAN Receptor tyrosine-protein kinase erbB-4 OS=Homo sapiens GN=ERBB4 PE=1 SV=1
STX4_HUMAN  Syntaxin-4 OS=Homo sapiens GN=STX4 PE=1 SV=2
NEUR1_HUMAN Sialidase-1 OS=Homo sapiens GN=NEU1 PE=1 SV=1
NTAL_HUMAN  Linker for activation of T-cells family member 2 OS=Homo sapiens GN=LAT2 PE
RAB23_HUMAN Ras-related protein Rab-23 OS=Homo sapiens GN=RAB23 PE=1 SV=1
BT2A1_HUMAN Butyrophilin subfamily 2 member A1 OS=Homo sapiens GN=BTN2A1 PE=1 SV=3
BTK_HUMAN  Tyrosine-protein kinase BTK OS=Homo sapiens GN=BTK PE=1 SV=3
DNJC5_HUMAN DnaJ homolog subfamily C member 5 OS=Homo sapiens GN=DNAJC5 PE=1 SV=1
ARMX3_HUMAN Armadillo repeat-containing X-linked protein 3 OS=Homo sapiens GN=ARMCX3
Proteins expressed in plasma membrane of EJp16 and EJp21 senescent cells, but not EJp16 control cells

MYO1B_HUMAN  Myosin-1b OS=Homo sapiens GN=MYO1B PE=2 SV=3
LYN_HUMAN    Tyrosine-protein kinase Lyn OS=Homo sapiens GN=LYN PE=1 SV=3
STXB1_HUMAN  Syntaxin-binding protein 1 OS=Homo sapiens GN=STXBP1 PE=1 SV=1
MYO1A_HUMAN  Myosin-1a OS=Homo sapiens GN=MYO1A PE=1 SV=1
NHRF1_HUMAN  Na(+)/H(+) exchange regulatory cofactor NHE-RF1 OS=Homo sapiens GN
DESP_HUMAN   Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3
HNRPU_HUMAN  Heterogeneous nuclear ribonucleoprotein U OS=Homo sapiens GN
RAP1B_HUMAN  Ras-related protein Rap-1b OS=Homo sapiens GN=RAP1B PE=1 SV=1
XRP2_HUMAN   Protein XRP2 OS=Homo sapiens GN=RP2 PE=1 SV=4
SNX9_HUMAN   Sorting nexin-9 OS=Homo sapiens GN=SNX9 PE=1 SV=1
VAMP3_HUMAN  Vesicle-associated membrane protein 3 OS=Homo sapiens GN=VAMP3
PTPRJ_HUMAN  Receptor-type tyrosine-protein phosphatase eta OS=Homo sapiens GN
S27A4_HUMAN  Long-chain fatty acid transport protein 4 OS=Homo sapiens GN=SLC27A4
PAR6B_HUMAN  Partitioning defective 6 homolog beta OS=Homo sapiens GN=PARD6B PE=1 SV=1
T106B_HUMAN  Transmembrane protein 106B OS=Homo sapiens GN=TMEM106B PE=1 SV=2
GNAO_HUMAN   Guanine nucleotide-binding protein G(o) subunit alpha OS=Homo sapiens GN
ARMX3_HUMAN  Armadillo repeat-containing X-linked protein 3 OS=Homo sapiens GN=ARMCX3