CHAPTER I

INTRODUCTION

1.1. Background and Literature Review

Aging and Senescence

Aging is an inevitable process that results in progressive loss of physiological function and impaired tissue regeneration of an organism. It also serves as a major risk factor for chronical disease development such as diabetes, Alzheimer's, and cardiovascular diseases (Fulop et al., 2010). Cellular senescence, one of the hallmarks of aging, is a form of irreversible cell-cycle arrest that can be both beneficial and detrimental. During aging, senescent cells accumulate in many tissues, including the skin and at pre-neoplastic lesions causing pleotropic effects (Dimri et al., 1995; Michalaglou et al., 2005; Herbig, 2006; Jeyapalan et al., 2007). Due to the accumulation of senescent cells during aging, it has been assumed that senescence contributes to aging. This theory, however, is contradictory with the primary purpose of senescence which is to prevent damaged cells from proliferating and trigger their clearance by immune system, hence protecting tissues from damaged and potentially cancer development. It is possible that during aging, the clearance of senescent cells or the regeneration of progenitor cells become inefficient thus results in the accumulation of senescent cells that contributes to aging process (López-Otín et al., 2013). Interestingly, the clearance of senescent cells has been found to restore tissue function and delay the aging process (Baker et al., 2011, 2016; Chang et al., 2015; Baar et al., 2017). These results demonstrate the importance of studying the role of senescence in cancer and organismal aging.

Causes of Cellular Senescence

The phenomenon of cellular senescence was originaly described by Hayflick and Moorhead in 1961 through their studies on cultured human fibroblasts. They observed that serially passaged human fibroblasts, after a finite number of division, enter an irreversible growth

arrest and lose their proliferative capacity (Hayflick & Moorhead, 1961). Only after several years later, the mechanism underlying the so-called replicative senescence (RS) or Hayflick limit was discovered.

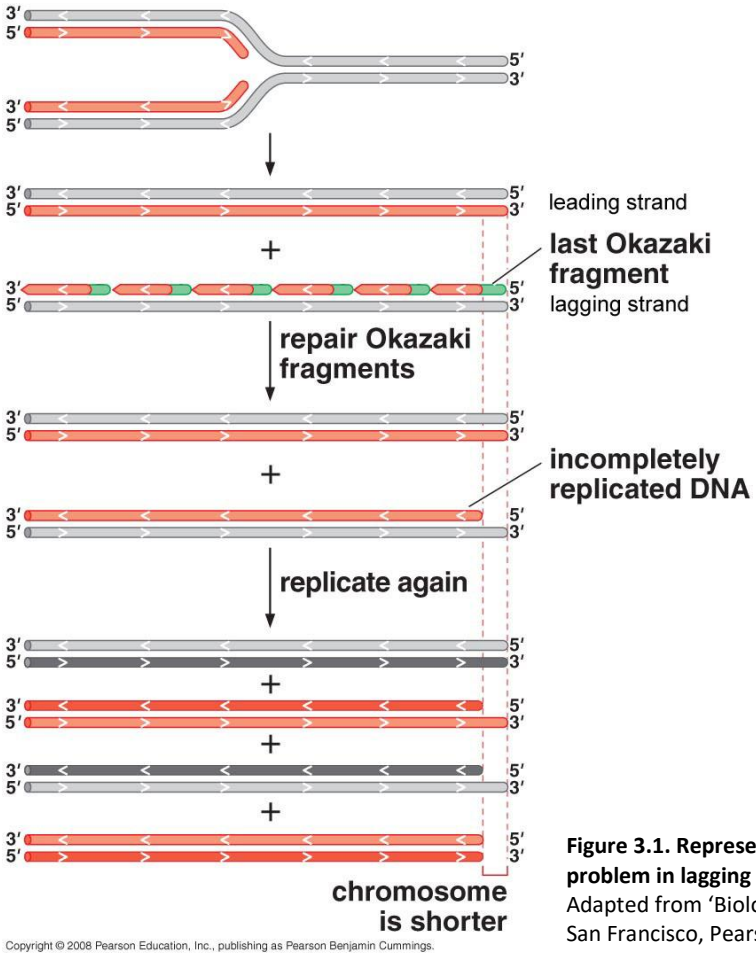

Figure 3.1. Representative figure of end replication problem in lagging stand during DNA replication. Adapted from 'Biology' by N. A. Campbell et al., 2008, San Francisco, Pearson, Benjamin Cummings

Telomere consists of repetitive TTAGGG sequence at chromosome ends that protects chromosome ends from chromosome fusions, translocations, and nondisjunction to ensure genomic stability (Blackburn, 1991). During DNA replication, DNA polymerase only works in one direction to copy DNA strands which produces leading and lagging strands. The lagging strand requires an RNA primer to initiate DNA synthesis but the primer will subsequently be removed thereby creating a gap that cannot be filled by conventional DNA polymerases (Olovnikov, 1973). Therefore, telomeres shorten in each replication cycle (Levy et al., 1992). Critically shortened telomeres elicit DNA damage response (Fumagalli et al., 2012) and p53 activation which enforce the cell to enter senescence (Vogelstein et al., 2000). This phenomenon, however, can be

prevented by telomerase, a reverse transcriptase enzyme which has the ability to elongate telomeres. In mammalian cells, telomerase elongates telomeres by adding TTAGGG repeats at the end of the chromosome by using its integral RNA template called telomerase RNA component (Harley, 1995). It is to be expected that immortal cells overexpress telomerase to maintain their telomere length and escape senescence (Shay et al., 1997).

Indeed, expression of telomerase can save some cells from RS, but it is now becoming clear that senescence can also be triggered by other aging-associated stimuli independently of this telomeric exhaustion process. For instance, DNA-damaged induced senescence (DIS) occurs in response to the accumulation of unrepaired DNA damage due to DNA damaging agents. Excessive ultraviolet (UV) exposure on dermal fibroblasts and keratinocytes *in vitro* and mouse skin *in vivo* results in increase of DNA damage and an accumulation of senescent cells within the skin epidermis (Wang et al., 2017). In addition to high UV exposure, DNA-damaging agents such as cytotoxic chemotherapies and oxidative stress are also potent inducers of senescence (Schmitt et al., 2002; Von Zglinicki, 2006).

Another trigger of senescence is the activation of oncogenes. Senescence caused particularly by oncogenic stimuli is referred to as oncogene-induced senescence (OIS). Oncogenic stimulus such as expression of mutant HRAS or BRAF in primary cells has been shown to cause premature senescence through the accumulation of p53 and $p16^{INK4a}$ (Serrano et al., 1997; Michaloglou et al., 2005). Induction of $BRAF^{V600E}$ expression in human melanocytes results in cell-cycle arrest accompanied by senescence markers such as increase of senescence-associated β-galactosidase (SA-β-gal) activity and $p16^{INK4a}$ expression. Human naevi (moles), benign lesions of melanocytes frequently caused by oncogenic mutation in $BRAF^{V600E}$, are also positive for SA-β-gal staining and immunohistochemistry (IHC) staining of $p16^{INK4a}$. Furthermore, it is negative for IHC staining of Ki-67, a proliferation marker, suggesting that oncogenic mutation can lead to senescence-like growth arrest (Michaloglou et al., 2005). Activating oncogenic signaling also

affects telomere structure and function, resulting in persistent DNA damage and an irreversible growth arrest (Suram et al., 2012).

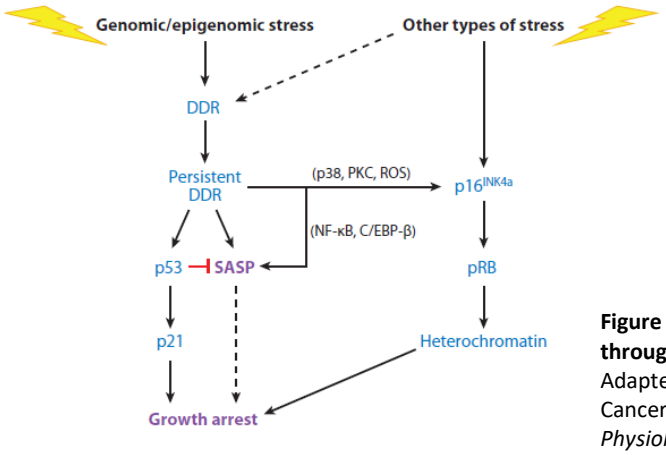

Figure 1.4. Regulations of cellular senescence through p53 and p16^{INK4a}/pRb pathways. Adapted from 'Aging, Cellular Senescence, and Cancer' by J. Campisi, 2013, *Annual Review Of Physiology*, 75(1), p. 688

Even though there are different types of senescence stimuli, senescence response appears to be regulated by two major pathways: p53 and p16^{INK4a}/pRb pathway. p53 has been long known as a crucial mediator for DNA damage response, including senescence. As mentioned previously, a critically shortened telomeres results in DNA damage and induce p53 activation. Expression of p53 provokes the expression of p21, an inhibitor of cyclin-dependent kinase (CDK) which are important for cell-cycle regulation. Inactivation of p53 has been shown to rescue human cells from RS (Gire & Wynford-Thomas, 1998; Beausejour et al., 2003). However, some cells fail to do so due to the activation of the cell cycle inhibitor p16^{INK4a} (Campisi, 2005). p16^{INK4a}, a tumor suprressor protein, regulates senescence through activation of pRb pathway and chromatin reoorganization. During p16^{INK4a}-induced senescence, the activated pRb will form a complex with E2F transcription factor which represses many of proproliferative E2F target genes through heterochromatinization (Narita et al., 2003; Campisi, 2005). Interestingly, once the heterochromatin foci is etablished, particularly due to p16^{INK4a}/pRb pathway activation, cellular senescence cannot be reversed even with inactivation of p53 or silencing of p16^{INK4a}. This shows the importance of p16^{INK4a}/pRb pathway to ensure senescence growth arrest is irreversible (Beausejour et al., 2003).

Senescence Biomarkers

In order to study cellular senescence, biomarkers to distinctly identify senescent cells are indispensable. Elevated SA-β-gal activity remains the gold standard to identify senescent cells both *in vitro* and *in vivo*. However, this marker only works well in fresh tissues which still retain its enzymatic SA-β-gal activity and the use of SA-β-gal alone is not enough to confirm that a cell is senescent as it can be detected in non-senescent cell types within hair follicles, sebaceous glands, and eccrine glands (Dimri et al., 1995). It is also difficult to quantify the expression as the SA-β-gal staining appears as smeared staining (Wang et al., personal communication/unpublished).

Cell cycle regulators such as $p16^{INK4a}$ and p53 are activated during cellular senescence. Expression of $p16^{INK4a}$, a selective inhibitor of CDK, is increased in senescent fibroblasts and aging tissue (Alcorta et al., 1996; Krishnamurthy, 2004; Michaloglou et al., 2005). Indeed $p16^{INK4a}$ experession is not commonly found in quiescent or terminally differentiated cells, however, due to its nature as a tumor suppressor, it is often mutated in various human cancers including melanoma (Hussussian et al., 1994; Serrano et al., 1996). Therefore, increased expression of $p16^{INK4a}$ is not entirely specific to senescence state hence it is difficult to distinguish $p16^{INK4a}$ as a marker for tumor progression or cellular senescence.

The nuclear lamina is a proteinaceous meshwork underneath the nuclear envelope which provides structural integrity for the nucleus. Its major protein components are type V intermediate filaments referred as nuclear lamins (Krohne and Benavente, 1986) and are essential for the regulation of DNA replication, transcription, and chromatin organization (Gerace and Blobel, 1980; Goldman et al., 2002; Guelen et al., 2008). Based on their isoelectric points, there are two main types of lamins: lamin A (lamin A, C) and lamin B (lamin B1, B2) (Krohne and Benavente, 1986). The A-type lamins are spliced isoforms of the *LMNA* gene and expressed only in somatic lineages. On the other hand, the B-type lamins arise from two different gene: *LMNB1* and *LMNB2,* and ubiquitously expressed in all cell types.

It has been shown previously that cells undergoing RS, DIS, and OIS lose lamin B1 (LMNB1) expression while lamin A/C (LMNA/C) expression remains unchanged (Shimi et al., 2011; Freund et al., 2012; Dereesen et al., 2013; Wang et al., 2017). The decline of LMNB1 expression in senescent cells is regulated at both transcription and translational level (Shimi et al., 2011; Freund et al., 2012; Dreesen et al., 2013). Through qRT-PCR analysis, mRNA level of LMNB1 decreased by ~20 folds in senescent fibroblasts due to mRNA stability (Freund et al., 2012; Dreesen et al., 2013). Micro RNA (miRNA) is a small non-coding RNA which regulates gene expression by inhibiting mRNA translation (Bushati & Cohen, 2007). Expression of miR-23a reduced LMNB1 protein level, but not LMNB1 mRNA level, by ~30% suggesting that miR-23a activity is responsible for LMNB1 protein reduction during cellular senescence (Dreesen et al., 2013). Another possible mechanism behind LMNB1 degradation is through autophagy mechanism (Dou et al., 2015). Autophagy is a catabolic mechanism to degrade cellular components that involves delivery of cytoplasmic constituents to lysosome (Levine & Kroemer, 2008). LC3, the autophagy protein, is present in the nucleus and directly interacts with LMNB1 (Dou et al., 2015). During OIS by activated RAS, LC3-LMNB1 interaction mediates LMNB1 degradation through nucleus-to-cytoplasm transport which delivers degraded LMNB1 to lysosome indicating that autophagy plays a role in LMNB1 degradation during senescence (Dou et al., 2015). Consistent with *in vitro* findings, LMNB1 level also declined in chronologically aged- and photoaged-human skin (Freund et al., 2012; Dreesen et al., 2013; Wang et al., 2017). Since LMNB1 is expressed in all cell types, co-staining with cell-type specific markers provides easy identification and quantification of different senescent cell types (Ivanov et al., 2013; Wang et al., 2017). Considering the increasing evidence of loss of LMNB1 downregulation in senescent cells, it can be employed as a biomarker to detect and quantify senescence *in vitro* and *in vivo*.

Besides its flattened and enlarged morphology and inability to proliferate, there is increasing evidences showing that senescent cells are not only passive bystanders but they can modulate cell function through changes in gene expression and chromatin reorganization (Campisi, 2013). These

changes include secretion of inflammatory cytokines, chemokines, growth factors, and matrix metalloproteinases (MMPs), also referred as senescence-associated secretory phenotypes (SASP) (Campisi, 2013; He & Sharpless, 2017). Various SASP components can be used to identify senescent cells *in vitro* and *in vivo* (Acosta et al., 2008; Kuilman et al., 2008; Pitiyage et al., 2011). High mobility group box 1 (HMGB1), a non-histone protein (Lotze & Tracey, 2005) binds to chromatin stabilizes nucleosome formation and regulates gene expressions by facilitating the access of transcription factor to the promotor regions (Grosschedl et al., 1994). During cell stress, HMGB1 can be secreted and acts as a cytokine to mediate the inflammatory response (Bianchi & Manfredi, 2007; Yamada and Maruyama, 2007). There are two mechanisms on how HMGB1 can be released into extracellular space: active and passive release. Active release of HMGB1 occurs when the protein is hyperacetylated in the nucleus (Bonaldi et al., 2003). Acetylated HMGB1 will then migrate to cytoplasmic secretory vesicles and relocate into the extracellular space. Passive release of HMGB1 happens in cells which undergo necrosis (Scaffidi et al., 2002). This is to be expected since HMGB1 is loosely bound to chromatin and when cells lose their membrane integrity, HMGB1 could diffuse out into the extracellular space. Besides necrotic cells, HMGB1 is also released by apoptotic cells depending on the cell type. Scaffidi et al. observed that only necrotic HeLa cells released HMGB1 while apoptotic cells retained nuclear HMGB1 (Scaffidi et al., 2002). However, Bell and colleagues showed contradictory results, in which apoptotic Jurkat cells released HMGB1 into the extracellular space (Bell et al., 2006). These studies indicate that HMGB1 release may vary with different apoptotic stimuli and cell types.

HMGB1 has been demonstrated to relocalize into the extracellular space by cells that undergo RS, DIS and OIS (Davalos et al., 2013). Based on immunoblotting result of whole-cell lysate and conditioned media of non-senescent and senescent cells, high HMGB1 level can be detected in the conditioned media of senescent cells (Davalos et al., 2013). Immunofluorescence staining also showed a decline in nuclear HMGB1 level in senescent cells (Davalos et al, 2013). In a time course experiment, senescent cells first underwent nuclear HMGB1 level depletion followed by increased

SA-β-gal activity, indicating that the loss of nuclear HMGB1 is an earlier event (Biran et al., 2017). Taken together, loss of nuclear HMGB1 and its relocalization to the extracellular space may be a potential marker to detect senescence state. However, the study of this senescence marker, particularly in skin cell types, is very limited and remains to be explored. Therefore, the objective of the following study is to characterize both LMNB1 and HMGB1 as senescence biomarkers particularly in skin cell types. The utilization of both markers will provide stronger evidence for the identification of senescent cells.

Skin Aging and Hair Depigmentation

Skin is one of the largest organ in human body and together with hair, nails, sweat glands, and sebaceous glands makes up the integumentary system. It functions as a protective barrier of the human body from outside environment and consists of multiple layers of cells. There are three main layers of the skin: hypodermis, dermis, and epidermis. The hypodermis is made of fatty connective tissue which connects the skin to the underlying tissues of bones and muscles. While dermis is a vascularized connective tissue where nerves, lymph vessels, hair follicles, and sweat glands reside. The epidermis is the most superficial layer of the skin, primarily made of keratinocytes, a cell that stores and produces keratin which gives skin its hardness and water-resistant properties. The basal layer of the epidermis or *stratum basale* is a single layer of basal cells, a precursor of keratinocytes of the epidermis. Basal cells are constantly dividing, producing new keratinocytes and pushing the existing cells towards the surface of the skin. Other than basal cells, pigment-producing melanocytes are also found at the basal layer and they give hair and skin its colour.

The most apperent pheonotypic changes of aging can be seen on the skin and its appendages (e.g. hair depigmentation). As the skin ages, its structure changes: fine lines and wrinkles start to become visible due to reduction of elastin and collagen fibers, the recovery rate of the skin slows down as immune function is reduced, and basal cells decrease its regeneration

causing the accumulation of senescent cells and thinning of the skin. The accumulation of senescent keratinocytes and fibroblasts had been demonstrated (Dimri et al., 1995; Herbig, 2006; Jeyapalan et al., 2007). However, how cellular senescence affects skin aging and hair depigmentation remains to be investigated.

Hair depigmentation or commonly known as hair greying often occurs as the result of aging. 74% of people between 45 and 65 years of age were affected by greying hair and regardless of ethnicity and geographical origin, the incidence of greying hair increased with age (Panhard, Lozano & Loussouarn, 2012). The hair follicle is a mini organ of the skin composed of many different cell types including keratinocytes and melanocytes. In most mammals, the hair has a regular growth cycle which consists of three phases: anagen (growth phase), catagen (regression phase), and telogen (rest phase) (Alonso, 2006). In anagen phase, melanocyte stem cells in the hair bulge migrate and differentiate into mature melanocytes that produce melanosomes (Slominski & Paus, 1993). Melanosomes, containing the pigment, then transfer melanin (pigment) to keratinocytes to form the pigmented hair shaft. During hair graying, one aspect of these process is disrupted, hence resulting in unpigmented hair shaft (Commo, Gaillard & Bernard, 2004; Nishimura, Granter & Fisher, 2005)

Stem cell exhaustion has been proposed as the explanation behind hair greying (Nishimura, Granter & Fisher, 2005). By using the melanocyte-targeted LacZ transgenic mice, Nishimura and colleagues show that incomplete maintenance of melanocyte stem cells in the bulge caused hair greying. This incomplete stem cell maintenance results in ectopic melanocyte pigmentation and differentiation within the stem cell niche of the hair follicle. These observations might support the previous study (Commo, Gaillard & Bernard, 2004) where there is a depletion of melanin content and melanocyte population in the hair bulb of greying hair. Even though the unpigmented hair shaft still contain some melanocytes in the outer root sheath (ORS), the population decreased compared to pigmented hair follicles (Commo, Gaillard & Bernard, 2004). It is possible that due to the incomplete stem cell maintenance, melanocyte stem cells do not migrate to the hair bulb and

instead differentiate in the stem cell bulge causing ectopic pigmentation and decrease of melanocyte population along the ORS and hair bulb. Even though the above studies have reported the possible mechanisms behind hair greying (Commo, Gaillard & Bernard, 2004; Nishimura, Granter & Fisher, 2005), the role of cellular senescence in hair depigmentation has not been reported before. For this reason, the following study intends to utilize the characterized senescence biomarkers (LMNB1 and HMGB1) to determine whether cellular senescence affects hair greying.

1.2. Research Objectives

Investigating the role of cellular senescence during aging is of scientific and clinical importance. However, the study of cellular senescence has been impeded due to the lack of specific and universal senescence biomarker that can be used both *in vitro* and *in vivo*. Due to this fact, this project aims to characterize LMNB1 and HMGB1 as senescence biomarkers in skin cell types through *in vitro* and *in vivo* studies. . *In vitro* studies will be performed on normal primary human dermal fibroblasts with four different senescence-inducing stimuli, including replicative senescence, p53-induced senescence, p16-induced senescence, and oncogenic BRAF-induced senescence. LMNB1 and HMGB1 levels will be quantified by western blotting and single cell immunofluorescence microscopy. In addition, *in vivo* studies will also be conducted, in which both LMNB1 and HMGB1 expression will be examined in human hair scalp biopsies to identify the presence of senescent cells in greying hair follicles. This will complement the *in vitro* cell culture-based findings.

1.3. Scope of work

To achieve the objectives of this study, several laboratory techniques will be employed including:

- Mammalian cell culture of human primary dermal fibroblasts
- Human genes over expression using DOX-inducible lentiviral system

- Protein quantification using western blot
- Senescence associated β-galactosidase staining
- Immunofluorescence and laser confocal scanning microscopy
- Fontana-Masson Staining
- Single cell image quantification using FIJI-ImageJ