1-1-2012

Induction of Cellular Senescence As A Novel Therapeutic Strategy for Melanoma Treatment

Gao Zhang
University of Pennsylvania, gaozhang@seas.upenn.edu

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Induction of Cellular Senescence As A Novel Therapeutic Strategy for Melanoma Treatment

Abstract
Oncogene-induced senescence (OIS) is a well-documented phenomenon both in vitro and in vivo as a tumor-suppression mechanism. It functions as a barrier to tumor initiation and progression. However, tumor cells develop a sophisticated yet not well-understood mechanism to overcome and subsequently repress cellular senescence to facilitate its progression. Here we report our findings on oncogene-induced senescence and therapy-induced senescence in human melanoma cells. Mutated BRAF or NRAS oncogenes can induce senescence in some but not all melanoma cells, whereas some small molecules are more potent triggers of premature senescence in melanoma cells than others. We established an integrated FACS approach to quantify the heterogeneous response of melanoma cells to therapies. Importantly, we identified two distinct forms of therapy-induced senescence, which were dependent on the type of therapy. By implementing a systems biology approach, we identified core senescence components that were sufficient and necessary for the induction of therapy-induced senescence in melanoma cells. Furthermore, we showed that oncogene-induced senescence in normal cells resembled therapy-induced senescence in cancer cells based on gene expression profiles. Our work unraveled an intimate link between autophagy and senescence in melanoma cells undergoing therapy-induced senescence. We proved that combination therapy might not necessarily lead to synergy but antagonism, depending on which pathways were targeted. Thus we present the rationale for utilizing pro-senescence therapy as a novel therapeutic strategy for patients with advanced melanoma, and suggest that pro-senescence therapy should be combined with other targeted therapies or immunotherapies to maximize cell death.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Bioengineering

First Advisor
Meenhard Herlyn

Keywords
Melanoma, Senescence, Therapy-induced senescence

Subject Categories
Biomedical

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/599
INDUCTION OF CELLULAR SENESCENCE AS A NOVEL THERAPEUTIC STRATEGY FOR MELANOMA TREATMENT

GAO ZHANG

A DISSERTATION

in

Bioengineering

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2012

Supervisor of Dissertation

_________________________________
Meehard Herlyn, D.V.M., D.Sc., Professor, The Wistar Institute

Graduate Group Chairperson

_________________________________
Beth A. Winkelstein, Ph.D., Professor, Bioengineering

Dissertation Committee

Matthew Lazzara, Ph.D., Assistant Professor, Chemical and Biomolecular Engineering
Katherine L. Nathanson, MD, Associate Professor, Medicine
Casim A. Sarkar, Ph.D., Assistant Professor, Bioengineering
Lynn M. Schuchter, MD, Professor, Medicine
ACKNOWLEDGEMENTS

First of all, I would like to give my deepest gratitude to my mentor Dr. Meenhard Herlyn. I regard Dr. Herlyn as my scientific father as he guided me into this exciting field of melanoma research and has tirelessly directed me all the way until now. I am extremely impressed with his enthusiasms and curiosity for science, his dedication to translational research and his extensive knowledge. I am very grateful for the opportunity that I can work in this prestigious melanoma research lab and really appreciate that he gives me inspiring advices, warm encouragements, and scientific freedom during all the time of my research. I will benefit from his mentoring throughout my entire scientific career.

Secondly, I really appreciate all the members in my thesis committee: Dr. Matthew Lazzara, Dr. Katherine L. Nathanson, Dr. Casim A. Sarkar, and Dr. Lynn M. Schuchter. They have committed so much time and a lot of efforts to guide my research, provided me with the most critical comments and have always been a great source of constructive suggestions and encouragements.

I have enjoyed the wonderful time that I have spent with all the previous and current colleagues in Dr. Herlyn’s lab. They are not only my scientific partners, but also my incredible friends. I want to thank all other lab members for their assistance, constant discussions and encouragements. Special thanks also go to several undergraduates who have worked with me in the lab to provide experimental assistance. Without them, this work could not have been done.

I would also like to extend thanks to my colleagues in The Protein Expression and Library Facility, Microscopy Facility, FACS Facility and Genomics Facility at the Wistar
Institute. The have provided professional assistance, for which this work has benefited a lot. Our close collaborator, Dr. Zhi Wei, has also supported this work tremendously, for which I am very grateful.

Finally, I would like to give my special thanks to my parents. I thank them with my heart for their love, support and encouragement throughout my life. Without them, I cannot cross the finish line.
ABSTRACT

INDUCTION OF CELLULAR SENESCENCE AS A NOVEL THERAPEUTIC STRATEGY FOR MELANOMA TREATMENT

Gao Zhang

Supervisor: Dr. Meenhard Herlyn

Oncogene-induced senescence (OIS) is a well-documented phenomenon both in vitro and in vivo as a tumor-suppression mechanism. It functions as a barrier to tumor initiation and progression. However, tumor cells develop a sophisticated yet not well-understood mechanism to overcome and subsequently repress cellular senescence to facilitate its progression. Here we report our findings on oncogene-induced senescence and therapy-induced senescence in human melanoma cells. Mutated BRAF or NRAS oncogenes can induce senescence in some but not all melanoma cells, whereas some small molecules are more potent triggers of premature senescence in melanoma cells than others. We established an integrated FACS approach to quantify the heterogeneous response of melanoma cells to therapies. Importantly, we identified two distinct forms of therapy-induced senescence, which were dependent on the type of therapy. By implementing a systems biology approach, we identified core senescence components that were sufficient and necessary for the induction of therapy-induced senescence in melanoma cells. Furthermore, we showed that oncogene-induced senescence in normal cells resembled therapy-induced senescence in cancer cells based on gene expression profiles. Our work unraveled an intimate link between autophagy and senescence in melanoma cells undergoing therapy-induced senescence. We proved that combination therapy might not
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CHAPTER 1 INTRODUCTION

Melanoma is the deadliest skin cancer that accounts for 80% of mortalities related to skin cancer. About 160,000 new cases of melanoma are diagnosed and 48,000 melanoma-related deaths occur worldwide each year (Flaherty et al., 2012). In Chapter 2, we discuss the cellular origin of melanoma, melanoma types and progression. We then briefly review targeted therapies and immunotherapies currently available, which have dramatically improved patient survival. In Chapter 3, we review current knowledge about oncogene-induced senescence in normal cells and therapy-induced senescence in cancer cells, and discuss the underlying molecular mechanisms. In Chapter 4, we discuss our finding that rare melanoma cells undergo spontaneous senescence in vitro and in vivo. In Chapter 5, we report that some not all melanoma cells undergo oncogene-induced senescence in vitro. In addition to genetic approaches, we report that small molecules are potent in triggering premature senescence in melanoma cells by focusing on Doxorubicin, Nutlin-3a and PD 0332991. We discuss the application of two FACS approaches to quantifying the therapy-induced senescence in melanoma cells and show that some subpopulations of melanoma cells, such as CD20+ or Jarid1B+ cells may be intrinsically resistant to therapy-induced senescence. In Chapter 6, we identify Aurora kinase inhibitors as the most potent senescence triggers and discuss that Aurora kinases may be novel therapeutic targets in melanoma. We further refine the FACS approach and apply it to profiling the heterogeneous response of melanoma cells to therapies. Remarkably, we identify and demonstrate that there are two distinct forms of therapy-induced senescence in melanoma cells. While Aurora kinase inhibitors induce irreversible senescence, BRAF
inhibitors induce transient senescence. The transient senescence might be a survival mechanism whereby melanoma cells evade therapy-induced apoptosis. In Chapter 7, we report that autophagy and the endoplasmic reticulum stress response precede and are required for therapy-induced senescence, identified by a computational biology approach. Furthermore, we show that there are some common pathways that mediate both oncogene-induced senescence in normal cells and therapy-induced senescence in tumor cells. We then report that impairment of autophagy converts therapy-induced senescence into cell death, suggesting that autophagy is a survival pathway for melanoma cells undergoing therapy-induced senescence. Finally, we demonstrate that combination therapy may lead to antagonism. Lastly, in Chapter 8, we conclude by briefly summarizing our key findings and presenting our future directions.
CHAPTER 2 FUNDAMENTALS OF MELANOMA BIOLOGY

Cutaneous melanomas account for 80% of mortalities of skin lesions. The estimated new cases and deaths from melanoma in the United States are 76,250 and 9,180, respectively in 2012. The recently FDA approved targeted therapy, Vemurafenib and immunotherapy, Ipilimumab, show great promises to improve melanoma patient survival but challenges ahead should be addressed to circumvent drug resistance that inevitably occur. To develop better therapeutic strategies for treating patients with advanced melanoma, it is essential to study and understand the cellular origin of melanomas and how malignant melanomas initiate, develop and metastasize. Here we briefly discuss the origin of melanoma and review the fundamental biology of malignancy by focusing on melanoma types, progression, and current therapies.

2.1 The Cellular Origin of Melanomas

Mature melanocytes are specialized pigment cells and reside in the junction of skin epidermis and dermis. Basal melanocytes make close contact with epidermal keratinocytes and the ratio of melanocytes and keratinocytes is roughly 1:8. Upon ultraviolet (UV) irradiation, melanocytes transport melanin to surrounding keratinocytes, as a protection from UV-induced DNA damage. Skin epidermal melanocytes and keratinocytes communicate through cell-cell adhesion molecules, such as E-cadherin. Loss of E-cadherin allows melanocytes to escape from the tight control by keratinocytes, which is one of prerequisites of malignant transformation of melanocytes. Melanocytes
can be transformed into malignant melanoma cells through a variety of means, which are discussed below.

2.1.1 *In Vivo* Malignant Transformation of Melanocytes

Transgenic mice inappropriately expressing hepatocyte growth factor/scatter factor (HGF/SF) in the skin have been an experimental model to study the effect of UV radiation on melanoma genesis. HGF/SF transgenic mice develop primary cutaneous melanoma with a mean onset age of approximately 21 months (Noonan et al., 2000). HGF/SF transgenic mice with UV radiation (60% UVB and 40% UVA) failed to accelerate the development of melanoma but did develop other types of skin tumors. However, over-expression of basic fibroblast growth factor (bFGF) in combination with UVB irradiation developed melanoma in human skin grafted to mice (Berking et al., 2001). Over-expression of bFGF led to activated melanocytes, which is over-expressed in melanoma and can be detected in fibroblasts within the tumor microenvironment. Remarkably, when UVB was combined with several growth factors including bFGF, stem cell factor, and endothelin-3, melanoma was induced in human skin grafted to mice within 4 weeks (Berking et al., 2004). Clearly, UVB is an important environmental carcinogen as it promotes the incidence of melanoma. Data from the genome sequencing of malignant melanoma patients suggest a mutational signature of DNA damage due to UV exposure (Berger et al., 2012; Pleasance et al., 2010). However, the precise mechanism of UV exposure and its association to melanoma genesis remains elusive. One possibility is that activated melanocytes, upon irradiation, display an interferon-γ related gene signature, which facilitate inflammation, immune evasion and survival mediated by macrophages present in the tumor microenvironment (Zaidi et al., 2011).
2.1.2 *In Vitro* Experimental Malignant Transformation of Melanocytes

Several studies explored the over-expression of a mutated oncogene, down-regulation of tumor suppressor(s) and different combinations that could transform melanocytes into malignant skin lesions. One example demonstrated that introduction of mutationally activated \( \text{N-Ras}^{G12V} \), \( \text{hTERT} \), \( \text{CDK4}^{R24C} \), and \( \text{p53}^{R248W} \) in human melanocytes was sufficient to give rise to human melanocytic neoplasia (Chudnovsky et al., 2005). Similarly, introduction of activated \( \text{N-Ras}^{G12V} \), \( \text{hTERT} \), and \( \text{SV40ER} \) also transformed human melanocytes as assessed by anchorage-independent growth assay and *in vivo* tumor formation assay (Gupta et al., 2005). The results so far, indicate that to transform primary human melanocytes, it is essential that cells acquire an oncogenic mutation, become immortal, and lose certain functions of tumor suppressor pathways, including \( \text{p16-Rb} \) and \( \text{p53-p21} \), which are frequently altered in melanoma cells. Roughly 50% of melanoma patients harbor \( \text{BRAF}^{V600E} \) mutation, suggesting that \( \text{BRAF}^{V600E} \) is an oncogenic driver that can transform human melanocytes. Indeed, a subpopulation of melanocytes expressing \( \text{BRAF}^{V600E} \) and \( \text{shTP53} \) were able to grow anchorage-independently in soft agar assay and became weakly tumorigenic *in vivo* (Yu et al., 2009). This subpopulation of melanocytes was unique, as they did not undergo \( \text{BRAF}^{V600E} \)-induced senescence. Knock-down of \( \text{p53} \) further enhanced the proliferation of melanocytes that bypassed OIS.

2.1.3 Neural Crest Stem Cells as Cell Origin of Melanoma?

It is not clear whether differentiated mature melanocytes are the only source for malignant transformation. The precursor of mature melanocytes, dermal stem cells, is
located deep in the dermis, not epidermis (Li et al., 2012; Li et al., 2010). Dermal stem cells represent a reservoir of mature melanocytes and did not undergo OIS in response to $\text{BRAF}^{\text{V600E}}$ (personal communication with X. Xu, University of Pennsylvania). Melanocytes transduced with active Notch1 exhibited increased proliferation and anchorage-independent growth, indicating that Notch activation can give rise to a transformed phenotype (Pinnix et al., 2009). However, transduced melanocytes with activated Notch1 were not tumorigenic, implying that additional factors were required to fully transform melanocytes in cooperation with Notch expression. Melanocytes expressing Notch1 survived and grew in stem cell medium as spheres, which are termed as “induced neural crest stem-like cells” (Zabierowski et al., 2011). Dermal stem cells and neural crest stem-like cells share some common gene expression signatures with a subset of malignant melanoma cells as revealed by gene expression microarray data. It has been postulated that cancer cells can re-express developmental pathways that are normally inherent to stem cells, by providing survival and proliferation advantages. It will be interesting to investigate whether dermal stem cells or neural crest stem-like cells could represent alternative cell origin for malignant transformation into melanoma cells.

2.2 Melanoma Types and Progression

Malignant melanoma can fall into four categories: a superficial-spreading melanoma (SSM), lentigo-maligna melanoma (LMM), nodular melanoma (NM), and malignant melanoma with an unclassified radial-growth phase (Clark et al., 1975).
The frequency of malignant melanoma of the superficial-spreading type is 60% and the development usually starts with a neoplasm that takes years to progress. The initial growth phase is called radial-growth phase. In a focal area, a compact collection of epithelioid melanoma cells protrude deeply into underlying tissues to form a nest (Clark et al., 1975). During the second growth phase that is termed vertical-growth phase, cells first fill the papillary dermis, and impinge on and invade reticular dermis. During the vertical-growth phase, if there are two or more populations of cells that are different from those of radial-growth phase, it is closely associated with the development of metastasis (Clark et al., 1975). This special case is termed “intra-lesional transformation”. One clear instance of “intra-lesional transformation” shows two neighboring nests of cells, one deeply pigmented and the other pigment-free (Clark et al., 1975).

Malignant melanoma of the lentigo-maligna type (Clark et al., 1975) constitutes around 10% of all human primary cutaneous melanomas. The most prominent histologic feature of LLM at the radial-growth phase is the absence of invading of cells into papillary dermis. The radial-growth phase can be separated into two developmental stages, noninvasive and invasive into papillary dermis. In the noninvasive stage, abnormal melanocytes are found in the basilar portion of epidermis, either individually or in nests. Immediately adjacent to abnormal melanocytes, there are normal melanocytes in appearance. This combination of large abnormal melanocytes and smaller normal melanocytes reflects the cell pleomorphism (Clark et al., 1975). When the presence of invasion into papillary dermis is observed, the lesion is referred to LMM.

Nodular melanoma accounts for 12% of all melanomas (Clark et al., 1975). It is characterized as the lack of radial-growth phase and is comprised exclusively of a
vertical-growth phase. Melanoma cells of nodular melanoma can invade deeply into, survive and replicate.

It remains controversial whether skin neoplasm arise from the intra-epidermal component of acquired pigmented nevi. Now it is becoming clear that SSM, LMM, NM and malignant melanoma of the unclassified radial-growth phase may develop either from a pre-existing acquired nevus or “de novo” from apparently normal epidermal melanocytes (Clark et al., 1975). Regardless of its origin, the subsequent development of melanoma is not influenced by whether the tumor arose within a nevus or normal melanocytes.

The first step of melanoma development depicted in the Clark model starts with the outgrowth of structurally normal melanocytes leading to the formation of a benign nevus. The growth of a nevus is limited, presumably controlled by oncogene-induced senescence. Accompanied by other genetic lesions, the development of aberrant growth occurs either within a benign nevus or a new location, leading to dysplastic nevi (Miller and Mihm, 2006). The next step is defined as radial-growth phase, during which cells replicate intraepidermally. Following that, cells invade deeply into dermis, characterized as vertical-growth phase. The last step describes a subpopulation of cells being capable of colonizing a new site and aberrantly proliferating there (Miller and Mihm, 2006).
2.3 Current Therapies

2.3.1 Targeted Therapies for Melanoma

It is not clear if differentiated mature melanocytes are the only source for malignant melanoma. Identification of melanomas that harbor a \( \text{BRAF}^{\text{V600E}} \) mutation in 50% of patients since 2002 has changed the paradigm of melanoma research, providing an enormously important therapeutic target (Davies et al., 2002). \( \text{BRAF} \) mutation sustains a constitutively activated \( \text{RAF-MEK-ERK} \) pathway, which is important for cancer cell proliferation and survival. Knock-down of \( \text{BRAF} \) by genetic approaches in human melanoma cells led to decreased growth and increased cell death both \textit{in vitro} and \textit{in vivo}, suggesting that \( \text{BRAF}^{\text{V600E}} \) positive melanoma cells are addicted to MAPK pathway (Hingorani et al., 2003; Hoeflich et al., 2006). MEK inhibitor AZD6244 induced cytostatic effects in melanoma cells both \textit{in vitro} and \textit{in vivo}, manifested by cell cycle arrest in G1 and decreased phosphor-ERK level (Haass et al., 2008). A screen of a chemical compound library for small molecule compounds that selectively inhibit \( \text{BRAF}^{\text{V600E}} \). PLX4720 was identified to efficiently suppress the activity of mutant \( \text{BRAF} \) \textit{in vitro} and \textit{in vivo} by inducing cell cycle arrest and apoptosis (Bollag et al., 2010; Lee et al., 2010; Tsai et al., 2008). However, in melanoma cell lines that are wild-type for \( \text{BRAF} \), PLX4720 can in turn transactivate \( \text{RAF} \) dimers and \( \text{ERK} \) signaling to adversely promote growth, implicating the importance of stratification of patients for targeted therapy (Halaban et al., 2010; Hatzivassiliou et al., 2010; Poulidakos et al., 2010). Among \( \text{BRAF}^{\text{V600E}} \) positive melanoma cell lines, a subset of cell lines showed intrinsic resistance to PLX4720. PTEN is a tumor suppressor gene that negatively regulating the PI3K-AKT pathway. Loss of PTEN expression in \( \text{BRAF}^{\text{V600E}} \) melanoma cells was
correlated with the intrinsic resistance to PLX4720, which was mediated by the suppression of BIM expression (Paraiso et al., 2011). Similarly, over-expression of Akt3 in BRAFV600E melanoma cells conferred the resistance to PLX4720, mediated by inhibition of BH3-only proteins Bim-EL and Bmf (Shao and Aplin, 2010). In a subset of BRAFV600E melanoma cell lines with acquired CDK4 mutation, over-expression of cyclin D1 contributed to the resistance to BRAF inhibitor SB590885 (Smalley et al., 2008).

In clinical trials, PLX4032 shows great promise in BRAF mutant melanoma patients by improving patient progression-free and overall survival (Chapman et al., 2011; Flaherty et al., 2010; Sosman et al., 2012). Unfortunately, acquired resistance to mutant BRAF inhibitors inevitably occurs not only in preclinical studies but also in melanoma patients, leading to tumor relapses. Acquired resistance was also observed in experimental studies and can be recapitulated in vitro by chronically treating melanoma cells with BRAF inhibitors. A variety of approaches were employed by different groups to uncover molecular mechanisms underlying acquired resistance to BRAF inhibitors, which include up-regulation of receptor tyrosine kinases such as IGFR and PDGFR, acquisition of de novo N-RAS or K-RAS mutations, up-regulation of PI3K pathway, reactivation of MAPK pathway through COT, and B-RAF amplification or splicing variants (Johannessen et al., 2010; Nazarian et al., 2010; Poulikakos et al., 2011; Shi et al., 2012; Su et al., 2012; Villanueva et al., 2010). The most pressing issue is how cells succeed to circumvent the drug resistance. Experimental investigations suggest that blocking IGFR, PDGFR and PI3K pathway, respectively, could achieve the greater efficacy in combination with BRAF inhibitors. A recent study suggested the HSP90
inhibitor XL888 was potent in overcoming acquired resistance to BRAF inhibitors mediated by different mechanisms (Paraiso et al., 2012).

A recent study reported improved survival with MEK inhibition in patients with advanced melanoma harboring BRAF mutation (Flaherty et al., 2012). MEK is a downstream target of BRAF, functioning to amplify the signals by BRAF mutation. Previous clinical trials showed MEK is a promising target in melanoma patients harboring BRAF mutation. Median progression-free survival was 4.8 months in melanoma patients treated with MEK inhibitor, trametinib, whereas 1.5 months in melanoma patients treated with dacarbazine or paclitaxel (Flaherty et al., 2012). In pre-clinical studies, melanoma cells also acquired resistance to MEK inhibitor, suggesting that acquired resistance inevitably occurs to targeted therapies (personal communication with J. Villanueva, The Wistar Institute). It should be noted that melanoma cells that had acquired resistance to BRAF inhibitors also displayed resistance to MEK inhibitor or the combination of BRAF and MEK inhibitors (Gowrishankar et al., 2012).

Clinical trials involving the combination of BRAF inhibitors with MEK inhibitors, or MEK inhibitors with PI3K/mTOR inhibitors are currently ongoing. This warrants further investigations on whether acquired resistance could be eliminated or delayed if multiple pathways are targeted simultaneously at the very beginning to prevent the emergence of rare clones that become resistant. A clinical trial involving both of BRAF and MEK inhibitors is underway to explore this idea.
2.3.2 Immunotherapies for Melanoma

In addition to FDA approved targeted therapy, Vemurafenib for treating BRAF<sup>V600E</sup> melanoma patients, Ipilimumab was also approved by the FDA as tumor immunotherapy for treating advanced melanoma patients. Ipilimumab is a monoclonal antibody that targets cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is a key negative regulator of cytotoxic T-lymphocyte (CTL) immune activity. Ipilimumab has shown promising clinical benefits in patients with advanced or metastatic melanoma (Trinh and Hwu, 2012). Ipilimumab improved the overall survival in patients with previously treated metastatic melanoma, with the median overall survival being 10.0 months, compared to the median progression-free survival being 6.8 months for melanoma patients treated with Vemurafenib (Hodi et al., 2010).

Recently, there have been encouraging results on tumor immunotherapy targeting programmed death-1 (PD-1) in advanced metastatic melanoma patients. PD-1 is another inhibitory receptor expressed on activated T cells in addition to CTLA-4, which can suppress antitumor immunity as an immune checkpoint molecule (Ribas, 2012). By blocking one of these immune-intrinsic checkpoints, it is hypothesized that immune function can be restored (Brahmer et al., 2010). In a recent clinical trial in which patients were enrolled with advanced melanoma, non–small-cell lung cancer, castration-resistant prostate cancer, or renal-cell or colorectal cancer, objective responses, defined as complete or partial responses, were observed in 18% of non–small-cell lung cancer patients, 28% melanoma patients, and 27% renal-cell cancer patients (Topalian et al., 2012). Immunohistochemical analysis revealed that if patients lacked PD-1 ligand (PD-L1), there was no response to this treatment, suggesting the selectivity of this
immunotherapy is based on the expression of PD-L1 in tumors (Topalian et al., 2012). In an accompanying phase 1 clinical trial, anti-PD-L1 also showed some objective responses in patients with advanced melanoma, renal-cell cancer, non–small-cell lung cancer, and ovarian cancer (Brahmer et al., 2012). These two clinical trials also demonstrated that patients with colon and pancreatic cancers had no tumor responses to either anti-PD-1 or anti-PD-L1 treatment. It will be interesting to study which molecular pathways will determine the responses to either anti-PD-1 or anti-PD-L1.

In summary, in patients with advanced metastatic melanoma, resistance to targeted therapy inevitably occurs following the initial response. It is critical to simultaneously block multiple pathways so that cancer cells can no longer re-route to different signaling pathways to re-emerge, survive, and proliferate. Furthermore, there are currently no targeted therapies available for patients with advanced melanoma that are either harboring NRAS\textsuperscript{Q61R} mutation or are wild type for both of BRAF and NRAS. There is an urgent need to develop therapies for this subset of melanoma patients that accounts for 50% of all melanoma patients. Immune therapy Ipilimumab or anti-PD1 alone or in combination with other targeted therapies may show promise for treating this subset of melanoma patients.
CHAPTER 3 OVERVIEW OF SENESCENCE

Cellular senescence is a physiological pathway by which cells permanently exit the cell cycle progression. Cells undergoing senescence gradually lose their proliferation potential without giving rise to any progenies. Senescent cells, however, are metabolically active and secret many cytokines. To date, three major types of senescence have been reported and extensively studies, including telomere-dependent replicative senescence in normal cells, oncogene-induced senescence in normal and cancer cells, and therapy-induced senescence in cancer cells. How senescence is exactly invoked remains to be fully elucidated. The induction of senescence is quite complex, mainly depending on the source and intensity of stress stimuli. Cellular types may also play a role. The current view is that cellular senescence is a fail-safe program as an integrated stress response to stimuli by preventing or delaying tumorigenesis. Thus it presents a promising concept of utilizing pro-senescence as a novel therapeutic strategy for treating cancer patients. A new wave of data, however, suggests that there is a dark side of senescence, in which the secretory phenotype of senescent cells can alter and remodel the microenvironment. While this is still controversial and further experiments are warranted, we focus here on illustrating different forms of cellular senescence in normal and cancer cells and discuss the underlying molecular mechanisms that initiate, execute and maintain the senescence machinery.
3.1 Replicative Senescence in Normal Cells

It was first discovered in 1960s that serially cultured human diploid cells including fibroblasts and keratinocytes have a definite lifespan \textit{in vitro} (Hayflick, 1965; Hayflick and Moorhead, 1961; Rheinwald and Green, 1975). Roughly, after $50 \pm 10$ doublings, cells cease the proliferation in culture. It was hypothesized at that time that there was a cellular intrinsic threshold of mitosis. When this threshold was reached in response to a triggered signal due to the accumulation of damages or unknown internal targets, cells were forced to stop division. Furthermore, it was postulated that an expression of aging or senescence at the cellular level underlies this \textit{in vitro} phenomenon. Now it is well documented that serially cultured normal cells lose their proliferation potential over a period of time as a result of the erosion of telomere, triggering a DNA damage signal to halt the cell division (Bodnar et al., 1998; d'Adda di Fagagna et al., 2003; Harley and Goldstein, 1978). This physiological consequence has been later referred to as replicative senescence.

3.2 Oncogene-Induced Senescence in Normal Cells

Phenotypically similar to replicative senescence, forced expression of the oncogene H-RAS$^{V12}$ in fibroblasts can rapidly trigger premature senescence in cultured normal cells, which acquire many features indistinguishable from replicative senescence. This phenomenon has been coined as oncogene-induced senescence (OIS) (Serrano et al., 1997). Fibroblasts expressing H-RAS$^{V12}$ were arrested in the G1 stage of the cell cycle, accompanied by the up-regulation of two tumor suppressor genes, p53 and p16. In the early phase of the discovery of OIS in normal cells, there was once a debate on this
finding as to whether this was an *in vitro* artifact mainly due to the oncogene shock or it did faithfully recapitulate the role of oncogenic mutation *in vivo* (Perez-Mancera and Tuveson, 2006). Accumulated evidence had later suggested that OIS not only exists *in vitro* but also *in vivo*, constituting an intrinsic tumor suppressive pathway, similar to oncogene-induced apoptosis. Normal cells undergoing OIS generally become larger and flattened, form heterochromatin foci, accumulate in G1 or G2 cell cycle, express senescence-associated β-galactosidase activity (SA-β-gal), remain metabolically active and do not respond to mitogen. Oncogenic H-RAS$^{V12}$ triggered senescence in fibroblasts by up-regulating p53-p21 and p16$^{INK4a}$–Rb pathways (Lin et al., 1998; Narita et al., 2003). Inactivation of either p53 or p16$^{INK4a}$ efficiently prevented the induction of senescence triggered by oncogenic RAS, supporting that OIS is intimately linked to tumor suppressor genes (Beausejour et al., 2003).

### 3.2.1 Molecular Pathways Leading to OIS *In Vitro*

As illustrated in Figure 3.1, the major molecular mechanisms underlying OIS include, but are not limited to, the establishment of heterochromatin foci, DNA damage response (DDR) through DNA hyper-replication, and the reinforcement of an inflammatory program that leads to the senescence-associated secretory phenotype (SASP) (Acosta et al., 2008; Bartkova et al., 2005; Bartkova et al., 2006; Coppe et al., 2008; Di Micco et al., 2006; Gorgoulis et al., 2005; Kuilman et al., 2008; Lin et al., 1998; Rodier et al., 2009). These three mechanisms eventually converge on numerous downstream pathways mediating OIS, which not only play important roles for initiating, establishing and executing the senescence program, but also work as *bona fide* markers of senescence.
They include activation of p53-p21 and Rb/p16^{INK4a} pathways, up-regulation of PML nuclear bodies, formation of senescence-associated heterochromatin foci (SAHF), and recruitment of Rb to E2F targets (Chicas et al., 2010; de Stanchina et al., 2004; Ferbeyre et al., 2000; Narita et al., 2006). There are two phases following the forced expression of an oncogene both in vitro and in vivo. The initial phase is characterized by a proliferation burst, in which DNA damage response is provoked due to DNA hyper-replication, followed by the onset of premature senescence triggered by different mechanisms dependent on the source of oncogene stimuli and cell types (Sarkisian et al., 2007). Mutant RAS, including H-RAS^{V12}, K-RAS^{V12}, NRAS^{Q61R}, NRAS^{G12D}, and BRAF, such as BRAF^{V600E}, and MEK, like MEK1^{Q56P}, are among those extensively studied as oncogenic triggers that are very potent in activating premature senescence in normal cells. Mutant RAS and BRAF activate a sustained signaling cascade, leading to the up-regulation of the RAS-RAF-MEK-ERK pathway. The activity of MAPK pathway as a result of the forced expression of an oncogene dictate a threshold, under which cell proliferation is accelerated and promoted, above which premature senescence is promptly triggered (Sarkisian et al., 2007). Inhibition of the MAPK pathway by a MEK inhibitor prevents the oncogene-induced cell cycle arrest, suggesting that this pathway is essential for establishing and maintaining the senescence program (Lin et al., 1998).

Oncogenes are potent drivers by orchestrating tumor development. DNA damage response (DDR) is observed in a series of tumor types including lung cancer, bladder cancer, skin lesions, breast cancer and colon cancer at early stages. At the advanced stage, the genetic deficiencies in repairing the damage will result in genomic instability and tumor progression. At the very early stage, the presence of an oncogene will lead to
the DNA hyper-replication, which confers stress on cells, referred to as “oncogene stress”.

**Figure 3.1 Diagram of molecular mechanisms underlying oncogene-induced senescence.** DNA hyper-replication and activation of an inflammatory program are two major underlying pathways to trigger OIS as a result of hyper-activation of MAPK pathway in normal cells. DNA damage response and secretion of cytokines are induced leading to a senescence phenotype.

The “oncogene stress” is mediated by DNA hyper-replication, which further leads to the activation of the ATR/ATM-regulated DDR program, culminating the senescence phenotype (Bartkova et al., 2005; Bartkova et al., 2006; Di Micco et al., 2006; Gorgoulis et al., 2005). The induction of senescence is a tumor suppressive mechanism by delaying, preventing or suppressing tumorigenesis *in vivo* (Chen et al., 2005; Guo et al., 2009;...
Ventura et al., 2007; Xue et al., 2007). In addition to DNA hyper-replication as a result of the hyper-activation of MAPK pathway, the reinforcement of an inflammatory program represents the second major molecular mechanism that underlies OIS. It remains to be elucidated how exactly the inflammatory program is invoked in response to oncogenic stress. One possibility is that persistent DNA damage response leads to the activation of inflammatory genes. Indeed, depletion of key DDR genes such as ATM, NBS1, or CHK2 decreased cytokine secretion, such as IL-6 and IL-8 (Rodier et al., 2009). This suggests that DNA damage response and inflammatory program are intertwined to mediate the induction of senescence. Several key up-stream regulators were identified to regulate this inflammatory program, including NF-κB, C/EBPβ and STAT3, which formed a sub-network to coordinately drive initiation and maintenance of the senescence associated inflammatory program (Acosta et al., 2008; Freund et al., 2011; Kuilman et al., 2008; Rodier et al., 2009).

3.2.2 Physiological Processes Leading to OIS In Vitro

Depending on the source and level of oncogenic stimuli and the cellular context, either apoptosis or premature senescence is triggered to eliminate pre-cancerous clones from the malignant transformation. For instance, H-RAS\textsuperscript{V12} mainly triggered autophagic cell death to limit clonogenic survival in human ovarian epithelial cells, while few cells entered the senescence state representing a subpopulation of surviving cells (Elgendy et al., 2011). In contrast, H-RAS\textsuperscript{V12} dominantly triggered premature senescence in human fibroblasts. The mechanisms underlying differential responses to H-RAS\textsuperscript{V12} have not been well addressed. It is hypothesized that the effect is largely due to the source and intensity of
oncogenic stimuli. Interestingly, in the scenario of H-RAS^{V12}–induced premature senescence in fibroblasts, autophagy preceded and was required to mediate the transition from mitosis to senescence (Narita et al., 2011; Young et al., 2009). Depletion of key autophagy genes in fibroblasts induced bypassing of H-RAS^{V12}–induced senescence. Thus, it further underscores how oncogenic stress can channel into either cell death or senescence via the activation of autophagy, although the key mechanisms determining the response, either cell death or senescence are not known. H-RAS^{V12} is a more potent trigger for the induction of premature senescence in human melanocytes, compared to oncogenic BRAF or NRAS (Denoyelle et al., 2006). Unlike DNA hyper-replication or the inflammatory program identified in normal fibroblasts, unfolded protein response (UPR) was required for the induction of senescence in response to over-expression of H-RAS^{V12} (Denoyelle et al., 2006). Depletion of UPR genes blunted oncogene-induced senescence in melanocytes. Together, this suggests that the same oncogenic mutation can lead to different downstream molecular mechanisms that underlie OIS. The consequences might also be dependent on the cellular phenotype.

3.2.3 In Situ Evidence of OIS

A V600E mutation in oncogene BRAF has been identified in approximately 50% of malignant melanoma patients and 40% of human benign nevi (Davies et al., 2002; Pollock et al., 2003). Nevi are growth arrested for decades and rare clones can progress into melanoma cells by the acquisition of additional genetic aberrations (Maldonado et al., 2004; Patton et al., 2005). BRAF^{V600E} induced senescence in normal human melanocytes in vitro, and mouse and zebrafish melanocytes in vivo (Ceol et al., 2011;
Dankort et al., 2009; Denoyelle et al., 2006; Dhomen et al., 2009; Michaloglou et al., 2005; Wajapeyee et al., 2008). Nevi stained positively for a variety of senescence markers, including SA-β-gal, PML and H3K9Me3, and negatively for cell proliferation marker Ki-67 (Gray-Schopfer et al., 2006; Michaloglou et al., 2005; Tran et al., 2012). Expression of BRAFV600E also induced benign lung tumors that rarely progressed into lung adenocarcinoma, manifested with the hallmarks of senescence (Dankort et al., 2007). Thus, bypassing BRAFV600E-induced senescence contributed to the transition from benign to advanced tumors. Supporting this view, KRASV12 induced lung adenomas in mice and mammary tumorigenesis, and NRASG12D induced benign lymphoma in mice (Braig et al., 2005; Collado et al., 2005; Sarkisian et al., 2007).

### 3.3 Reactivation of Cellular Senescence in Cancer Cells

To facilitate malignant transformation of normal cells, it is critical to overcome the barrier of OIS. Additionally, in established cancer cells, intact senescence pathways are suppressed. Thus, blockage of senescence suppression can result in the reactivation of senescence in cancer cells. In this session, we summarize and discuss current approaches to restoring senescence in cancer cells. We primarily focus on pharmacological methods.

Exposure of HCT116 colon cancer cells to Doxorubicin at a moderate dose induced senescence-like growth arrest. Knock-out of p53 or p21 significantly decreased therapy-induced senescence, implicating that p53 and p21 are important regulators of therapy-induced senescence in cancer cells (Chang et al., 1999b). Exposure of a panel of cancer cell lines derived from different organs to Doxorubicin at moderate doses led to the
induction of senescence-like phenotypes (Chang et al., 1999a). A FACS approach was used to indirectly identify cancer cells undergoing therapy-induced senescence, which forms a basis for our own thesis work. Shortly before the treatment, cancer cells were labeled with the cell membrane dye PKH2, which is used to monitor cell division. The integration of PKH2 fluorescence with side scatter (SSC), which is an indicator of cellular granularity, identified a subset of cancer cells that did not divide as quickly as control cells while acquiring the enlarged cell morphology phenotype. Subsequently, senescent cancer cells were identified as a subpopulation of cells of PKH2$^{hi}$/SSC$^{hi}$. The advantage of this approach is two-fold. First, unlike conventional SA-β-gal staining method, in which cells were fixed, the combination of PKH2 and SSC permits the separation of senescent cancer cells from non-senescent cancer cells. Upon cell sorting to derive senescent and non-senescent cancer cells, the proliferation of sorted populations was monitored to investigate their proliferation capacity. Secondly, while some cancer cells senesced and others underwent cell death, some cells did not respond to the treatment. FACS sorting can allow the subsequent study of non-senescent cancer cells in comparison with senescent cancer cells at gene expression level to pinpoint which molecular pathways could determine differential responses (Chang et al., 2002). The ability of cells undergoing chemotherapy-induced senescence led to a better prognosis.

Therapy-induced senescence (TIS) in cancer cells not only occurred in vitro, but was observed in vivo. Tissues from lung cancer patients treated with chemotherapy stained positively for SA-β-gal, whereas tissues from lung cancer patients receiving only surgical excision did not (Roberson et al., 2005). Murine lymphomas responded to cyclophosphamide (CTX) treatment by engaging the p53 and p16-dependent senescence
program (Schmitt et al., 2002). Subsequently, the NF-κB pathway was identified to mediate the response to CTX-induced senescence (Chien et al., 2011). Disruption of the NF-κB pathway by depletion of the p65 subunit resulted in chemoresistance and impaired survival. Similarly, using the same B-cell lymphoma mouse model, treatment with another chemotherapy reagent, Doxorubicin also led to induction of senescence that was NF-κB dependent. B-cell lymphoma patients with high NF-κB activity had a better survival rate compared to patients with low NF-κB activity, implicating NF-κB-dependent senescence mechanisms attributed to patients’ survival (Jing et al., 2011).

Some rare clones inevitably escaped from therapy-induced senescence in cancer cells, although the molecular mechanisms remain elusive. Escaping cells re-entered the cell cycle, expressed senescence markers and shared similar gene expression profiles with parental cells (Roberson et al., 2005). These cells over-expressed Cdc2/Cdk1, which cooperated with Survivin to facilitate escape (Roberson et al., 2005; Wang et al., 2011). Another study also confirmed this result by identifying an intrinsically resistant clone from MCF-7 breast cancer cells to Doxorubin-induced senescence (Elmore et al., 2005). This clone expressed substantially more Cdc2 than parental cells, further implicating a role of Cdc2 in overriding therapy-induced senescence in cancer cells.

It should be cautioned, however, that the frequency of reversing therapy-induced senescence is rare. Current methods limited the separation of cancer cells that evade therapy-induced senescence from cancer cells that are intrinsically resistant to therapy-induced senescence. Based on our knowledge from oncogene-induced senescence in normal cells, it is reasonable to postulate that rare cancer cells can emerge from therapy-
induced senescence through intrinsic or acquired resistance mechanisms, which will ultimately lead to tumor recurrence.

The results based on therapy-induced senescence prompted a screen from a chemical compound library for identifying small molecules that are potent in inducing senescence in cancer cells as a therapeutic strategy for treating cancer patients. To date, two small molecule inhibitors have been identified to show the ability of restoring senescence in cancer cells, in addition to chemotherapies. Skp2 is an E3 ligase that is over-expressed in cancers. Inactivation of Skp2 by a small molecule MLN4924 evoked senescence by involving p27 and p21 in a prostate xenograft mouse model (Lin et al., 2010). Interestingly, a PTEN inhibitor, VO-OHpic, induced senescence in prostate cancer using mouse models by modulating the p53 pathway, which was further enhanced by p53 activator Nutlin-3a (Alimonti et al., 2010). Therapy-induced senescence by MLN4924 or VO-OHpic is distinct from chemotherapy-induced senescence or oncogene-induced senescence, as DNA damage response or hyper proliferation is not activated, suggesting that these two small molecules can be utilized as a potential strategy for treating patients with advanced cancer.

3.4 Reactivation of Cellular Senescence in Melanoma Cell Lines

Since senescence has been implicated as a tumor suppressive mechanism, it is hypothesized that reactivation of cellular senescence can be exploited as a therapeutic strategy for melanoma patient. Three approaches will be briefly discussed here that are used to reactivate senescence in melanoma.
3.4.1 Reactivation of Senescence in Human Melanoma Cell Lines Through Genetic Approaches

Investigations on oncogene-induced senescence in normal cells suggest that the intensity of MAPK pathway determines the differential response, proliferation, cell death or senescence. In malignant melanoma cells, the MAPK pathway is constitutively activated, controlling cell proliferation and survival. Elevating this pathway to a higher degree through over-expression of wild-type c-RAF or BRAF\textsuperscript{V600E} in mutant BRAF melanoma cells triggers cell cycle arrest via induction of senescence (Houben et al., 2009; Maddodi et al., 2010). Inhibition of MAPK pathway with the MEK inhibitor U0126 can suppress the induced senescence. This indicated that hyper-activation of MAPK pathway can determine and trigger the induction of senescence in melanoma cells. Similarly, this pathway can also be augmented by over-expression of N-RAS\textsuperscript{Q61R}, which is frequently detected in around 20% melanoma cell lines and patients. Forced expression of N-RAS\textsuperscript{Q61R} in melanoma cell lines harboring BRAF\textsuperscript{V600E} mutation induces G\textsubscript{0}-G\textsubscript{1} cell cycle arrest, promoting senescence (Petti et al., 2006). Co-existence of BRAF\textsuperscript{V600E} and RAS\textsuperscript{Q61R} is rare in melanoma cell lines, indicating that it is lethal to have both mutations in melanoma cells. It is clear that induction of senescence as a result of co-existence of BRAF\textsuperscript{V600E} and NRAS\textsuperscript{Q61R} can lead to synthetic lethality, mediated by the induction of senescence, which restrain the further propagation of all clones. A recent study has also shown transduction of melanoma cells with PP2A-B56\textalpha, which is a negative c-Myc regulator, induces senescence, underscoring the role of c-Myc in suppressing OIS to facilitate melanoma development (Mannava et al., 2012). Over-expression of histone deacetylase (HDAC1), in melanoma cells drives the formation of
heterochromatin foci, concurrent with induction of senescence (Bandyopadhyay et al., 2007).

Conversely, reactivation of cellular senescence in melanoma can also be achieved via down-regulation of several genes. For instance, depletion of c-Myc induced senescence in melanoma cells, regardless of its mutation status. The induced senescence can be rescued by pharmacological inhibition of MAPK or PI3K-AKT pathway, indicating that c-Myc may function to block OIS by suppressing the activity of MAPK or PI3K-AKT pathway (Zhuang et al., 2008). Indeed, over-expression of c-Myc prevented BRAF\(^{V600E}\)-induced senescence in normal melanocytes (Zhuang et al., 2008). This suggests that c-Myc can counter the effect of BRAF\(^{V600E}\) in melanocytes and functions to suppress BRAF\(^{V600E}\)-induced senescence in melanoma cells. Similarly, depletion of Twist 1 or 2 in melanoma cells can reactivate senescence. Cooperation between H-RAS\(^{V12}\) and Twist 1 or 2 can transform mouse embryonic fibroblasts, by abrogating p53- and Rb-dependent OIS program (Ansieau et al., 2008).

Expression of dominate-negative TBX2, or knock-down of MITF, DEK, EZH2 or MDM2 in melanoma cells readily promotes the establishment of heterochromatin foci, up-regulates p53-p21 pathway, and engages the DNA damage response program leading to reactivation of senescence (Fan et al., 2011; Giuliano et al., 2010; Khodadoust et al., 2009; Vance et al., 2005; Verhaegen et al., 2012).

In summary, induction of senescence can be achieved by genetic approaches. The above-mentioned factors facilitate melanoma cell proliferation by actively suppressing the intrinsic senescence program, mainly mediated by mutant BRAF or NRAS induced senescence.
3.4.2 Reactivation of Senescence in Human Melanoma Cell Lines Through Pharmacological Approaches

In addition to genetic approaches, several chemotherapeutics and small molecules are very potent in triggering senescence in human melanoma cells, referred to as therapy-induced senescence (TIS). Temozolomide (TMZ) is a DNA alkylating agent, which was used as a first-line chemotherapy for melanoma treatment. Treatment of human melanoma cells with TMZ can induce a moderate induction of senescence, depending on p53 status. Cells are arrested in G2/M phase, concurrent with activation of p53-p21 pathway (Mhaidat et al., 2007). Similarly, hydroxyurea, fotemustine, or H2O2 can also induce senescence in human melanoma cell lines, presumably through the engagement of DNA damage response genes or reactive oxygen species (ROS) (Giuliano et al., 2010).

The diterpene ester PEP005 is a novel anticancer agent extracted from the sap of Euphorbia peplus. It is an activator of protein kinase C and leads to induction of senescence in human melanoma cell lines through PKC-dependent hyper-activation of the MAPK pathway (Cozzi et al., 2006). Cells are arrested in G1 and G2/M phases, along with up-regulation of p21 and dephosphorylation of Rb.

Interestingly, reactivation of p53 by Nutlin-3a can effectively induce senescence in human melanoma cells, agreeing with the role of the p53/p21 pathway in mediating senescence (Korotchkina et al., 2010; Verhaegen et al., 2012). PD 0332991 is a potent CDK4/6 inhibitor and also shows promise in triggering senescence in human melanoma cells (Anders et al., 2011).
In summary, small molecules can reactive senescence in human melanoma cells, which is continuously suppressed by a subset of genes. The underlying molecular mechanisms mediating therapy-induces senescence in human melanoma cells include the activation of DNA damage, increased MAPK pathway activity and restoration of p53/p21 and Rb pathways.

### 3.5 Markers for the Detection of Senescence

SA-β-gal is a “gold standard” marker for the detection for senescent cells *in vitro* and *in situ* (Dimri et al., 1995). Senescent cells have a higher lysosome β-galactosidase enzymatic activity than normal cells. β-galactosidase can catalyze X-gal to produce an insoluble blue product, which can be visualized using bright field microscopy. In addition to SA-β-gal, numerous markers have been reported to date to faithfully identify senescent cells, including PML, H3K9Me3, HP1γ, macroH2A, Dec1, HMG2A, and Ki-67Neg. Recently it has been debated whether SA-β-gal is a good marker for senescence. The authors failed to use current senescence marker to faithfully distinguish nevi from normal melanocytes and metastatic melanoma cells, questioning whether SA-β-gal and others are good senescence markers (Tran et al., 2012). Forced expression of an oncogene in normal cells engages the formation of heterochromatin foci to mediate the induction of senescence. Knock-down of a DNA damage gene such as ATM can bypass oncogene-induced senescence. However, in these bypassing cells, populations still retain staining of markers for the heterochromatin foci (Di Micco et al., 2011). Thus, for normal cells undergoing oncogene-induced senescence, they should be positive for senescence markers including SA-β-gal and heterochromatin foci. For normal cells and cancer cells
undergoing therapy-induced senescence when DNA damage is activated, they should be positive for senescence markers including SA-β-gal, p53, 53BP1, and γH2AX but not markers for heterochromatin foci.

A recent study characterized a panel of OIS markers. Only SA-β-gal activity can distinguish human and murine pancreatic intraepithelial neoplasia, which are benign tumors, from pancreatic ductal adenocarcinoma, which are more aggressive tumors (Caldwell et al., 2012). Our data also support the view of SA-β-gal as a senescence marker but it should be noted that SA-β-gal should be multiplexed with other senescence markers to stain cells for the detection of senescence. Perhaps, a more stringent functional assay should follow to assess cell proliferation and colony formation ability.
CHAPTER 4 SPONTANEOUS SENESCENCE OF MELANOMA IN VITRO AND IN VIVO

4.1 Introduction

By definition, cancer cells are immortal and have an indefinite proliferative capacity in vitro and in vivo. The aberrant cell growth is due to the dysfunction of cell cycle checkpoints. In cell culture of human melanoma cell lines established from metastatic melanoma patients, we observed rare cells that resembled normal cells undergoing replicative senescence or OIS, displaying the enlarged cell morphology. When metastatic melanoma cells were seeded at a clonal density, after a few days of cell culture, most of cells formed colonies, which is indicative of their proliferative potential. On the other hand, a few cells existed as single cells and never divided once. We hypothesized that those cells that failed to form colonies and displayed the enlarged cell morphology were in a senescence stage.

4.2 Results

4.2.1 Spontaneous Senescence in Melanoma In Vitro

To test that hypothesis, we performed SA-β-gal staining in combination with Ki-67 immunofluorescence staining in 8 metastatic melanoma cell lines. SA-β-gal staining showed that there were SA-β-gal positive cells (SA-β-gal+) in every metastatic melanoma cell line, albeit the frequency of SA-β-gal+ cells was low (Figure 4.1). SA-β-gal+ cells
were negative for Ki-67 staining, confirming that these rare melanoma cells in each cell line were senescent. By using C8161 cells as a representative, co-staining cells with SA-β-gal, Ki-67 and PML

![Image of senescence analysis](image)

**Figure 4.1** Melanoma cells undergo spontaneous senescence in vitro. Melanoma cells of 8 metastatic melanoma cell lines were seeded in 24-well plates and allowed to grow for two days. The senescence was assessed by SA-β-gal staining. Total cells were indicated by Hoechst 33342 staining.
Figure 4.2 Spontaneous senescence was observed in C8161 metastatic melanoma cells. C8161 melanoma cells were seeded in 24-well plates and allowed to grow for two days. Senescent cells were assessed by SA-β-gal, Ki-67, and PML staining. Total cells were indicated by Hoechst 33342 staining.

These results demonstrated that rare SA-β-gal\(^+\) cells were also stained positively for PML and negatively for Ki-67 (Figure 4.2).

4.2.2 Spontaneous Senescence in Melanoma In Vivo

Having confirmed the existence of rare senescent cells in human metastatic melanoma cell lines, we decided to explore further samples derived from human melanoma xenografts and human metastatic melanoma patients. Human metastatic melanoma cells \((10^6)\) 1205Lu, A375, UACC-62, 451Lu and C8161 were inoculated into immunodeficient mice, respectively. When tumors were established, they were harvested for SA-β-gal staining. The staining showed that there were SA-β-gal\(^+\) cells in melanoma cells grown in
mice, although the frequency was low (Figure 4.3). Also, tissues derived from a human metastatic melanoma patient were subject to SA-β-gal staining, and we observed similar rare SA-β-gal$^+$ cells, confirming the data from in vitro and human xenografts (Figure 4.4).

**Figure 4.3 Spontaneous senescence was observed in melanoma xenografts.**

Melanoma (1205Lu) cells ($10^6$) were inoculated in mice to form tumors. Frozen sections of 1205Lu xenografts were stained with SA-β-gal and Hoechst 33342. Senescent cells, as indicated with positive SA-β-gal staining, are identified above.
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Figure 4. 4 Spontaneous senescence was observed in a human metastatic melanoma patient. Frozen sections of a human metastatic melanoma patient were stained with SA-β-gal and Hoechst 33342.

4.3 Discussion

In summary, we identified melanoma cells undergoing spontaneous senescence both in vitro and in vivo. It is not known by which mechanisms melanoma cells undergo spontaneous senescence. Melanoma cells are highly tumorigenic, suggesting their high proliferative capacity. These senescent cells are rare but they do exist. The results have laid a foundation for our subsequent study of chemotherapies and targeted therapies to accelerate the senescence process. A recent study compared tissues from normal melanocytes, nevi, and metastatic melanoma patients by performing SA-β-gal staining. In
contrast to our findings, the percentage of SA-β-gal positive cells was high in 3 out of 8 human metastatic melanoma patients (Tran et al., 2012). Some aspects have also been discussed in Chapter 3. In summary, future experiments are warranted to investigate this issue by increasing the number of tissues to be examined.

4.4 Materials and Methods

Cell Culture Methods

Human metastatic melanoma cell lines have previously been described (Satyamoorthy et al., 1997). A375 cells were purchased from American Type Culture Collection. UACC-62 and UACC-903 cells were kind gifts from Dr. Marianne B. Powell (Stanford University). Melanoma cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) Media (GIBCO) supplemented with 10% fetal bovine serum (Atlanta Biologicals), or MCDB 153 medium (Sigma) containing 20% Leibovitz's L-15 medium (Cellgro), 2% fetal bovine serum, 5 µg/mL insulin (Sigma) and 1.5M CaCl$_2$ (Sigma). All cells were cultured at 37° C in a humidified incubator supplied with 5% CO$_2$.

SA-β-gal and Immunofluorescence Staining

Cells were fixed with 1X DPBS containing 2% formaldehyde (Sigma) and 0.2% glutaraldehyde (Sigma). SA-β-gal staining was performed in 24-well plates as previously described (Dimri et al., 1995). Subsequently, SA-β-gal staining buffer was removed and cells were permeabilized and blocked with 1X DPBS supplemented with 0.3% Triton X-100 and 3% BSA for 1 hour. Cells were incubated with primary antibodies PML (Santa
Cruz) and Ki-67 (Vector Lab) overnight at 4° C. After rinse, cells were incubated with secondary antibodies at room temperature for 1 hour. Cells were counterstained with 2 ug/ml Hoechst 33342 (Invitrogen) in 1X DPBS for 10 minutes and images were acquired with Nikon TE2000 Inverted Microscope.

**Melanoma Xenotransplantation, Patients’ Samples and Histology**

Melanoma cells (10⁶) were harvested from cell culture and resuspended with culture medium and Matrigel at 1:1 ratio. Cells were subcutaneously injected. Mice were sacrificed when the tumor volume reached 1cm³. Solid tumors were collected and immediately embedded in OCT medium in a disposable base mold on crushed dry ice. OCT embedded samples were allowed to freeze completely and the base mold was wrapped with tin foil. Frozen blocks were stored at -80 °C and cut 4-8 µm thick cryostat sections for staining. All animal experiments were performed in accordance with Wistar IACUC protocol 112330 in NOD.Dg-Prkdc scidIL2rg tm 1 Wjl/SzJ mice. Fresh melanoma tissues from metastatic lesions were procured and embedded in OCT and then frozen in liquid nitrogen. OCT embedded blocks were cut into 5 µm sections for additional staining. The protocol was approved by the Institutional Review Board of the University of Pennsylvania.
CHAPTER 5 ACCELERATING PREMATURE SENESCENCE IN MELANOMA CELLS BY GENETIC AND PHARMACOLOGICAL APPROACHES

5.1 Introduction

Having shown that there is spontaneous senescence in melanoma cells that exists not only in vitro but also in vivo, we explored further to investigate whether induction of senescence can be accelerated in melanoma cells through genetic and pharmacological approaches. The current literature shows that reactivation of senescence in human melanoma cells has been successfully achieved through genetic manipulations such as the over-expression of mutant B-RAF or N-RAS, wild-type C-RAF or PP2A-56α, or the knock-down of MITF, c-Myc, TBX2, TWIST, MDM2 or DEK (Ansieau et al., 2008; Giuliano et al., 2010; Houben et al., 2009; Khodadoust et al., 2009; Maddodi et al., 2010; Mannava et al., 2012; Peres et al., 2010; Petti et al., 2006; Vance et al., 2005; Verhaegen et al., 2012; Zhuang et al., 2008). This indicates that intact senescence pathways can be reactivated as a response to the hyper-activation of the MAPK pathway or restored in tumor cells that are originally suppressed to facilitate tumor development.

Furthermore melanoma cells can readily undergo therapy-induced senescence, especially in vitro, in response to chemotherapies or small molecules, including temozolomide, hydroxyurea, fotemustine, H2O2, PEP005, PD 0332991, and Nutlin-3a (Anders et al., 2011; Cozzi et al., 2006; Giuliano et al., 2010; Korotchkina et al., 2010; Mhaidat et al., 2007; Ohanna et al., 2011). As a proof-of-principle, we investigated the
ability of triggering senescence in human melanoma cells by over-expressing BRAF\textsuperscript{V600E} or NRAS\textsuperscript{Q61R}, or using the chemotherapy reagent, Doxorubicin. Subsequently, we developed a FACS approach that can be used to quantify the induction of senescence.

5.2 Results

5.2.1 Oncogene-Induced Senescence (OIS) in Human Melanoma Cells

Current studies show that wild type c-Raf, mutant BRAF\textsuperscript{V600E} or NRAS\textsuperscript{Q61R} can induce senescence in human melanoma cell lines. The underlying molecular mechanisms include hyper-activation of the MAPK pathway, engagement of autophagy, and synthetic lethality as a result of co-existence of BRAF\textsuperscript{V600E} and NRAS\textsuperscript{Q61R}. In our study, we focused on BRAF\textsuperscript{V600E} and NRAS\textsuperscript{Q61R}.

Ten melanoma cell lines were chosen for this experiment, representing melanoma cell lines of different genetic background (Table 5.1). Normal skin fibroblast and melanocyte cell lines were also included as controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell lines</th>
<th>BRAF</th>
<th>Nras</th>
<th>PTEN</th>
<th>C-KIT</th>
<th>CDK4</th>
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<tr>
<td>1b</td>
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<td>V600E</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
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<td>D549G</td>
<td>G12D</td>
<td>WT</td>
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<td>WT</td>
</tr>
<tr>
<td>4</td>
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<td>V600E</td>
<td>WT</td>
<td>Mu/Hom Del</td>
<td>WT</td>
<td>K22Q</td>
</tr>
<tr>
<td>4</td>
<td>1205Lu</td>
<td>V600E</td>
<td>WT</td>
<td>Mu/Hom Del</td>
<td>WT</td>
<td>K22Q</td>
</tr>
<tr>
<td>4</td>
<td>SK-MEL-28</td>
<td>V600E</td>
<td>WT</td>
<td>Mu</td>
<td>WT</td>
<td>R24C</td>
</tr>
<tr>
<td>5</td>
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<td>WT</td>
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<td></td>
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</tr>
<tr>
<td>5</td>
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<td>WT</td>
<td>WT</td>
<td></td>
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<tr>
<td>5</td>
<td>WM8</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
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</tr>
<tr>
<td>5</td>
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</tr>
<tr>
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<td>V600E</td>
<td>WT</td>
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</tr>
</tbody>
</table>
Table 5.1 A summary of 10 human melanoma cell lines used in this study. Mutation status for BRAF, NRAS, PTEN, C-KIT and CDK4 was also included.

Melanoma cell lines were transduced with lentiviral vectors that constitutively express the empty vector control and BRAF$^{V600E}$, respectively. Following 3-day puromycin selection to establish stably infected cell cultures, cells were then subject to a cell proliferation assay and SA-β-gal staining. Interestingly, we observed there were two groups of melanoma cell lines in response to over-expression of BRAF$^{V600E}$, resistant to BRAF$^{V600E}$-induced senescence and sensitive to BRAF$^{V600E}$-induced senescence (Figure 5.1 and 5.2).

**Figure 5.1** A sub-group of melanoma cell lines showed resistance to BRAF$^{V600E}$-induced senescence. Stably infected melanoma cells were seeded in 96-well plates and cell proliferation was monitored at different time points.
Figure 5.2 A sub-group of melanoma cell lines were sensitive to BRAF<sup>V600E</sup>-induced senescence. Stably infected melanoma cells were seeded in 96-well plates and cell proliferation was monitored at different time points.

Cells from the resistant group continued to grow in the presence of BRAF<sup>V600E</sup> as control cells, whereas cells from the sensitive group slowed down proliferation in response to BRAF<sup>V600E</sup> expression, suggesting that over-expression of BRAF<sup>V600E</sup> induced cell cycle arrest. To further corroborate the data from cell proliferation assays, we used the same infected cells to carry out time-course BrdU-incorporation assay. BrdU is only incorporated into DNA of proliferating cells, not quiescent cells or senescent cells. The sensitive group showed decreased BrdU labeling, whereas there was little or no change in the resistant group (Figure 5.3). Finally, we performed SA-β-gal staining and confirmed that in the sensitive group, melanoma cells underwent BRAF<sup>V600E</sup>-induced senescence (data not shown).
However, the underlying molecular mechanism that determines differential responses among different melanoma cell lines to the over-expression of BRAF<sup>V600E</sup> was not understood. At least this pattern was not correlated with BRAF or NRAS mutation status. This further implicates the heterogeneity among melanoma cell lines.

**Figure 5.3** A sub-group of melanoma cell lines were sensitive to BRAF<sup>V600E</sup>-induced senescence-like cell cycle arrest. Stably infected melanoma cells were seeded in 96-well plates and BrdU incorporation was monitored at different time points. Those sensitive cell lines were indicated by ★.

To replicate the finding that showed mutant NRAS<sup>Q61R</sup> induced senescence-like cell cycle arrest in BRAF<sup>V600E</sup> positive melanoma cell lines, we chosen metastatic 1205Lu cell line (Petti et al., 2006). Our previous data showed that 1205Lu was among the sensitive group showing response to BRAF<sup>V600E</sup>-induced senescence. For this study, we used an ER inducible vector in which expression of NRAS was regulated in a 4-
Hydroxytestosterone (4-OHT) -dependent manner. We first titrated the dose of 4-OHT in the medium and found that the minimal dose of 4-OHT that induced the expression of NRAS was 100nM. Following three days of expression of NRASQ61R, phosphor-AKT was increased, in agreement that NRAS is an up-stream regulator of the PI3K/AKT pathway (Figure 5.4). After 9 days of expression of NRASQ61R, we observed that over-expression of NRASQ61R triggered senescence in 1205Lu cells compared to EtOH-treated control cells (Figure 5.5).

**Figure 5.4 Expression of mutant N-RASQ61R was under the regulation of 4-OHT.** 1205Lu melanoma cells were infected with an ER inducible vector that over-expressed N-RASQ61R. Cells were cultured in the presence of 4-OHT at different titrations for 3 days.
NRAS\textsuperscript{Q61R} was over-expressed in 1205Lu melanoma cells for 9 days, followed by SA-\(\beta\)-gal staining. Together, this data suggested that oncogenic BRAF or NRAS can induce senescence in a group of melanoma cell lines, presumably through the hyper-activation of the MAPK pathway. Not all melanoma cell lines are responding to oncogene-induced senescence, suggesting that senescence pathways might be defective in those melanoma cell lines. In melanoma cell lines undergoing oncogene-induced senescence, we found that not all cells underwent oncogene-induced senescence, reflecting intra-tumoral heterogeneity of melanoma cells or suggesting that not all melanoma cells responded to OIS at the same kinetic.
5.2.2 Therapy-Induced Senescence (TIS) in Human Melanoma Cells

Our previous data showed that a subgroup of melanoma cell lines could undergo OIS mediated by BRAF$^{\text{V600E}}$ or NRAS$^{\text{Q61R}}$. However, there were two disadvantages of this approach. First, not all melanoma cells within the same line were triggered to undergo senescence. Second, the rate of the induction of senescence by genetic approaches was slow. Thus, we decided to focus on therapy-induced senescence by using Nutlin-3a and Doxorubicin for proof-of-principle. Nutlin-3a is a p53 activator by inhibiting MDM2 (Tovar et al., 2006; Vassilev et al., 2004). As the p53/p21 signaling pathway is up-regulated in normal cells undergoing OIS, we hypothesize that reactivation of p53 in p53 wild-type melanoma cells induces senescence. Doxorubicin has been widely used as a chemotherapy reagent to study therapy-induced senescence in cancer cell lines, such as MCF7 breast cancer cells and HCT116 colon cancer cells. Therapy-induced senescence by Doxorubicin was mainly mediated by the activation DNA damage, which further up-regulated the p53-p21 signaling pathway.

5.2.2.1 Nutlin-3a-induced senescence in human melanoma cells

About 90% of melanoma cell lines harbor wild-type p53, although its tumor suppressor function is inhibited. Thus, p53 remains a promising target for melanoma therapy. Nutlin-3a stabilizes p53 by inhibiting its negative regulator MDM2. Reactivation of p53 in other types of cancer either induces cell cycle arrest or apoptosis, and this might be dose dependent. We hypothesized that Nutlin-3a can induce senescence in melanoma cells by restoring p53 function in wild-type p53 melanoma cell lines. We first selected two p53 wild-type melanoma cell lines 1205Lu and WM35, and two p53 mutant
melanoma cell lines 451Lu and WM164. Nutlin-3a inhibited the proliferation of p53 wild-type melanoma cell lines but not p53 mutant cell lines, suggesting that Nutlin3a only worked for p53 wild-type cells (Figure 5.6).

Figure 5.6 Nutlin-3a suppressed cell proliferation of p53 wild type melanoma cell lines but not mutant melanoma cell lines. Four melanoma cell lines were treated with DXR at 250nM for 24 hours, U0126 at 5uM and Nutlin-3a at 2.5uM, 5uM and 10uM, respectively. Cell proliferation was monitored on Days 2, 5 and 8 by using MTS assay.

We then asked whether inhibition of cell proliferation in p53 wild type melanoma cells by Nutlin-3a could lead to the induction of senescence. WM35 cells were treated with Nutlin-3a at 5uM for 5 days, followed by SA-β-gal staining. The percentage of SA-β-gal positive cells in control cells was 10.76%, whereas the percentage of SA-β-gal
positive cells in Nutlin-3a treated cells was 46.85%, indicating that Nutlin-3a induced senescence in p53 wild type melanoma cells (Figure 5.7). 1205Lu cells were treated with DMSO, Nutlin-3a at 2.5uM and 7.5uM for 16 days. The percentage of SA-β-gal positive cells was 0, 81.2% and 98.5% in 1205Lu cells treated with DMSO, Nutlin-3a at 2.5uM, and 7.5uM.

Figure 5.7 Nutlin-3a induced senescence in p53 wild type WM35 melanoma cells. (A) SA-β-gal and Hoechst 33343 staining for DMSO treated control cells. Images were acquired from three separate fields. (B) SA-β-gal and Hoechst 33343 staining for Nutlin-3a treated cells. Images were acquired from three independent fields.

Figure 5.8 Nutlin-3a induced senescence in p53 wild type 1205Lu melanoma cells. 1205Lu melanoma cells were treated with DMSO, Nutlin-3a at 2.5uM and 7.5uM. SA-β-gal and Hoechst 33343 staining was done on Day 16.
Next, we investigated whether therapy-induced senescence in melanoma cells could be quantified by using a previously reported FACS approach (Chang et al., 1999a; Chang et al., 2002; Chang et al., 1999b). This FACS approach indirectly interrogated senescence by taking into account cell size and cell division. Cells were first labeled with a cell membrane dye, PKH26, before drug treatment. At certain time points following drug treatment, cells were harvested for FACS analysis by integrating PKH26 and Side Scatter. We then used this approach to study Nutlin-3a induced senescence using 1205Lu melanoma cells and profiled the induction of senescence at different time points. The results showed that as melanoma cells underwent therapy-induced senescence, Nutlin-3a treated cells became larger and underwent cell division more slowly than control DMSO treated cells. By Day 8, Nutlin-3a induced 93% senescent cells as indicated by PKH26<sup>hi</sup>/SSC<sup>hi</sup>, as compared to control DMSO treated cells (5.6% senescent cells) (Figure 5.9).

Figure 5.9 Nutlin-3a induced senescence in p53 wild type 1205Lu melanoma cells.
Cells were treated with DMSO or Nutlin-3a at 10uM. Cells were harvested on Day 0, 2, 5, and 8 for FACS analysis. Senescent cells were labeled as PKH26$^{hi}$/SSC$^{hi}$.

5.2.2.2 Inhibition of cyclin-dependent kinase 4/6 induced senescence in human melanoma cells

PD 0332991 is a potent inhibitor to inhibit the activity of cyclin-dependent kinase 4/6 (CDK4/6), which results in exclusive G1 cell cycle arrest (Fry et al., 2004). The cell cycle arrest was concomitant with decreased phosphorylation of Rb, indicating that the Rb pathway was activated. We investigated whether inhibition of CDK4/6 in melanoma cells induced senescence. Melanoma cells were treated with PD 0332991 at different doses and senescence was significantly induced, albeit all cells were killed when treated with PD 0332991 at 8uM (Figure 5.10). This suggested that the induction of senescence in melanoma cells by inhibition of CDK4/6 was dose dependent.

We further titrated the dose of PD 0332991 between 7uM and 8uM and confirmed that PD 0332991 at 8uM was very cytotoxic. However, cells survived the treatment of PD 0332991 between 7.4uM and 7.8uM but they did not undergo therapy-induced senescence (Figure 5.11). This suggested that PD 0332991 at high dose induced cell death, but triggered a non-senescence population of melanoma cells at sub-lethal dose. It is not fully understood how the same inhibitor could induce different phenotypes. This warrants further investigations on exploring signaling pathways that mediate these three distinct phenotypes, senescence, non-senescence and survival, and cell death.
5.2.2.3 Doxorubicin-induced senescence in human melanoma cells

Doxorubicin (DXR) is a chemotherapy reagent used for treating patients with advanced cancer. At high dose, cells rapidly undergo apoptosis and cell death as a result of DNA damage response. However, at low to medium dose, some cells die but the rest undergo therapy-induced senescence. We treated melanoma cells with DXR at 50nM and

Figure 5.10 CDK4/6 inhibition induced senescence in 1205Lu melanoma cells. 1205Lu cells were treated with PD 0332991 at different doses and SA-β-gal staining was done on Day 7. Note that PD 0332991 at 8uM killed all cells (data not shown here).

Figure 5.11 CDK4/6 inhibition at sub-lethal did not induce senescence in 1205Lu melanoma cells but cells survived. 1205Lu cells were treated with PD 0332991 at different doses between the range of 7uM and 8uM and SA-β-gal staining was done on Day 7. Note that PD 0332991 at 8uM killed all cells (data not shown here).
250nM for 24 hours, followed by extensive washing. We then monitored cell proliferation at different time points and found that melanoma cells slowed down cell proliferation at later time points (Figure 5.12). To confirm that melanoma cells undergo DXR-induced senescence, we performed SA-β-gal staining. Indeed, SA-β-gal staining on Day 9 showed that DXR was very effective in triggering senescence in human melanoma cells (Figure 5.13).

**Figure 5.12 DXR induced growth arrest in melanoma cell lines.** WM793 and 451Lu melanoma cells were treated with DXR at 0, 50nM and 250nM for 24 hours. Cell proliferation was assessed by cell proliferation assay at different time points.

**Figure 5.13 Doxorubicin-induced senescence in WM88 melanoma cells.** WM88 is a BRAFV600E/p53wt melanoma cell line. WM88 cells were treated with DXR at 250nM for 24 hours and stained with SA-β-gal on Day 9.
5.2.2.4 Establishment of a flow-cytometry based system to quantitatively detect therapy-induced senescence.

SA-β-gal is the most widely used marker for detection of cellular senescence. However, this cytochemical staining approach necessitates the fixation of cells, during which the substrate X-gal would be catalyzed by its enzyme, SA-β-gal, to produce an insoluble blue derivative. To quantify the induction of senescence, we first developed an approach that involves another chemical substrate of SA-β-gal, namely C₁₂FDG. C₁₂FDG is easily permeable to cells, and is subsequently hydrolyzed by its enzyme SA-β-gal to produce a green fluorescent product, amenable for FACS analysis.

Cell tracing agents such as CellVue Claret, which is a far red fluorescent probe, can be used to monitor cell proliferation over multiple cell divisions. The cell tracing agent is equally distributed when cells divide to give rise to two daughter cells. Thus, the mean fluorescence intensity (MFI) of CellVue Claret can be used to monitor cell doublings.

Our approach is based on the above two fluorescent stains, CellVue Claret and C₁₂FDG to quantitatively detect senescent cells. This approach allows the subdivision of all cells into four subpopulations based on fluorescent dyes, CellVue Claret and C₁₂FDG (Figure 5.14A). Cells within Gates 1 and 4 were senescent cells that differ due to the MFI of CellVue Claret. We hypothesized that cells within Gate 1 were rapidly undergoing cell-cycle arrest in response to drug treatment, followed by the induction of cellular senescence, whereas cells within Gate 4 undergo several divisions as indicated by dilution of CellVue Claret, and then underwent senescence, which mirrors the replicative senescence in normal cells. Cells within Gates 2 and 3 were non-senescent cells as they were C₁₂FDG low or negative. We report that cells within Gate 2 remained in a quiescent
state as they slowly proliferated and never underwent senescence. In contrast, cells within Gate 3 bypassed or escaped therapy-induced senescence and showed no response to the drug. Furthermore, this integrated FACS system was verified by performing SA-β-gal staining on all four subpopulations on day 9 after Doxorubicin treatment (Figure 5.14B).

**Figure 5.14 A flow-cytometry based system identified four subpopulations of tumor cells in response to drug treatment.** (A) A schematic representation of the flow-cytometry based senescence detection system. (B) Human melanoma cell line 1205Lu were labeled with CellVue Claret and treated by Doxorubicin at 250nM for 24 hours. Four subpopulations of treated cells based on CellVue Claret and C_{12}FDG were sorted on Day 9 post treatment. Sorted cells were allowed to adhere to plates overnight, followed by SA-β-gal staining. Note that percentage of SA-β-gal positive cells cannot be determined for cells within Gate 2 as the cell number was very low after sorting.

Sorted cells were seeded in 96-well plates for a time-course cell proliferation assay up to day 15. Gates 1 and 4 cells never grew, while Gates 2 and 3 cells grew (data not shown). This further strengthens our conclusion that our proposed FACS system is...
successful in detection and separation of senescent and non-senescent cells in response to drug treatment.

5.2.2.5 Existence of distinct subpopulations of cells in response to drug treatment that may become resistant to senescence

Previous studies from our laboratory have identified and characterized two distinct subpopulations of melanoma cells that can survive drug treatment at high dose. These two subpopulations of cells might not overlap and are enriched as CD20 positive and Jarid1B positive cells, respectively (Roesch et al., 2010, Somasundaram et al., personal communication). Thus, we hypothesize that CD20 positive and Jarid1B positive cells, respectively, are resistant to therapy-induced senescence.

5.2.2.5(A) A subset of CD20 positive cells that are non-senescent cells in response to drug treatment

Our previous study has suggested that a small subpopulation of cells (1%) can survive cisplatin at high dose treatment for three weeks. Among surviving clones, about 10% cells were CD20 positive cells. Thus, we hypothesized that a subset of CD20 positive cells becomes resistant to therapy-induced senescence. 1205Lu cells were treated with DXR at 100nM for three days. FACS analysis was then performed on Day 11 post treatment to interrogate cell proliferation indicated by CellVue Claret, induction of senescence indicated by C₁₂FDG, and CD20 expression by a CD20 antibody. About 9.4% of DXR-treated surviving cells were CD20 positive cells while they were C₁₂FDG negative cells. CD20 positive cells were further subdivided into two subsets. 8% of CD20
positive cells were CellVue\textsuperscript{High} (non-dividing) and 1.4% of them were CellVue\textsuperscript{Neg} (proliferating) cells (Figure 5.15). This may suggest that CD20 might be a marker for labeling non-senescent cells following drug treatment.

Figure 5.15 The existence of two subpopulations of cells that are CD20\textsuperscript{Pos}/C\textsubscript{12}FDG\textsuperscript{low}. Human melanoma 1205Lu cells were first labeled with CellVue Claret, treated with Doxorubicin at 100nM for 72 hours. The substrate of SA-\(\beta\)-gal, C\textsubscript{12}FDG was incubated with treated cells for one hour to allow hydrolysis. Subsequently, cells are harvested to incubate with the conjugated CD20-PE antibody for 30 minutes before FACS analysis.

5.2.2.5(B) A subset of Jarid1B positive cells that were non-senescent cells in response to drug treatment
Our previous study suggested that a small subpopulation of slow-proliferating Jarid1B positive cells can survive a variety of drug treatment at high doses (Roesch et al., 2010). Thus, we hypothesized that a subset of Jarid1B positive cells might become resistant to therapy-induced senescence. Human melanoma WM3734 cells were engineered to emit a green fluorescent light when they expressed *Jarid1B* encoding histone H3 lysine 4 demethylase (Figure 5.16A).

Engineered WM3734/Jarid1B<sup>eGFP</sup> cells were treated with Doxorubicin at 100nM for three days. FACS analysis was performed on Day 16 post treatment to interrogate cell proliferation indicated by CellVue Claret, senescence status by C<sub>12</sub>RG, and Jarid1B expression by GFP.

Indeed, a subset of Jarid1B positive cells were non-senescent cells (5.73%) while another subset of Jarid1B positive cells had undergone senescence (15.6%). Another two subsets of non-senescent WM3734 cells included cells labeled as CellVue<sub>Low</sub>/C<sub>12</sub>RG<sub>Low</sub>/Jarid1B<sub>Neg</sub> (5.47%) and CellVue<sub>Low</sub>/C<sub>12</sub>RG<sub>Low</sub>/Jarid1B<sub>Low</sub> (15.1%), respectively (Figure 5.16B). This results suggest that there were six distinct subpopulations of cells, based on C<sub>12</sub>RG and GFP staining, in response to DXR treatment.
Figure 5.16 The existence of three non-senescent subpopulations of WM3734 cells after Doxorubicin treatment. (A) A schematic representation of JARID1B reporter vector. Jarid1B promoter is annealed to GFP and this insert is subcloned into a pLU-MCS-PGK-Blasticidin vector with a selectable marker Blasticidin. WM3734 cells were infected with this vector to express eGFP when they expressed Jarid1B. (B) Distinct subpopulations of cells were indicated by C12RG and GFP (Jarid1B). Infected WM3734 cells were first labeled with CellVue Claret and treated with Doxorubicin at 100nM for 72 hours. The substrate of SA-β-gal, C12RG was incubated with treated cells for one hour to allow hydrolysis. Subsequently, cells were harvested to incubate with the PE-conjugated CD20 antibody for 30 minutes before FACS analysis on Day 16.
5.3 Discussion

We first studied oncogene-induced senescence in human melanoma cells by over-expressing either oncogenic BRAF\textsuperscript{V600E} or NRAS\textsuperscript{Q61R}. We identified there were two subgroups of melanoma cell lines in response to oncogene-induced senescence. The resistant group does not respond to OIS, whereas the sensitive group does respond by undergoing OIS. Also, within the sensitive group, not all cells in the same melanoma cell line undergo OIS. Thus, this indicates that melanoma cells show inter-tumor heterogeneity. Hyper-activation of MAPK pathway is one molecular mechanism underlying OIS in melanoma cells, although more signaling pathways remain to be identified. We then investigated whether senescence could be induced when cells were treated with different drugs. For this purpose, we treated cells with Nutlin-3a, PD 0332991 and Doxorubicin. The data suggested that all three drugs significantly induced senescence in human melanoma cells. The therapy-induced senescence was dosage dependent. Particularly for the CDK 4/6 inhibitor PD 0332991, senescence was induced in low and medium doses, whereas cell death was induced in higher doses. When cells were treated with PD 0332991 at sub-lethal doses, cells survived and did not undergo senescence. This result suggests that the same inhibitor at different doses can activate different signaling pathways.

With Nutlin-3a, we used the FACS approach that can indirectly quantify the induction of senescence by taking into account cell division and morphology of cells treated with drugs.
Doxorubicin (DXR) was a potent trigger of therapy-induced senescence in melanoma cells, similar to its effect in other types of cancer cells. Presumably, therapy-induced senescence by Doxorubicin was mediated by DNA Damage Response (DDR), which led to engagement of the p53-p21 signaling pathway.

We then developed a FACS approach to directly quantify the degree of senescence in cancer cells by taking advantage of two fluorescent probes, C\textsubscript{12}FDG or C\textsubscript{12}RG, and CellVue Claret. Based on the fluorescence of C\textsubscript{12}FDG and CellVue Claret, we identified four subpopulations of melanoma cells following DXR treatment, characterized as senescent subpopulation, replicative senescence subpopulation, quiescent subpopulation and proliferative subpopulation. SA-β-gal staining confirmed the results based on FACS sorting.

Our attention was then focused on two major types of subpopulations of melanoma cells emerging from therapy-induced senescence. The first type, senescent melanoma cells are labeled as C\textsubscript{12}FDG- or C\textsubscript{12}RG-positive while they can be further subdivided based on the fluorescence intensity of CellVue Claret. The second, non-senescent melanoma cells are indicated as C\textsubscript{12}FDG- or C\textsubscript{12}RG-negative, among which a further subset of cells we hypothesized becomes intrinsically resistant to therapy-induced senescence. This integrated FACS system allows us, in the future, to sort the two types of melanoma cells, which can be subsequently analyzed by genome-wide expression microarrays. We expect to identify a core transcriptional network of senescence that functions to initiate and maintain the senescence machinery utilized by a variety of pro-senescence therapies. We are intrigued by two questions, i) what is the common signaling pathway that pro-senescence therapies engage to trigger premature senescence in human
melanoma cells? ii) by which molecular mechanisms does a subset of melanoma cells become intrinsically resistant to therapy-induced senescence? We hypothesize that this phenotypic heterogeneity within the same melanoma cell line might reflect intra-tumoral heterogeneity that determines differential responses of subpopulations of cells to therapy-induced senescence. Thus our study has the potential to a greater understanding of transcriptional organization of a core cellular senescence network that is reactivated in tumor cells following therapy-induced senescence. Subsequently, we continue to explore the molecular basis of intrinsic resistant mechanism(s) to therapy-induced senescence displayed by a subpopulation of melanoma cells. Will this subpopulation also show cross-resistance to targeted therapy like BRAF or MEK inhibition?

5.4 Materials and Methods

Cell Culture Methods
Human metastatic melanoma cell lines have previously been described (Satyamoorthy et al., 1997). Melanoma cells were maintained in MCDB 153 medium (Sigma) containing 20% Leibovitz's L-15 medium (Cellgro), 2% fetal bovine serum, 5 μg/mL insulin (Sigma) and 1.5M CaCl₂ (Sigma). Human skin fibroblasts were maintained in DMEM (Cellgro) supplemented 10% fetal bovine serum. Human skin melanocytes were maintained in 254CF medium (Invitrogen). All cells were cultured at 37°C in a humidified incubator supplied with 5% CO₂.

SA-β-gal Staining
Cells were fixed with 1X DPBS containing 2% formaldehyde (Sigma) and 0.2% glutaraldehyde (Sigma). SA-β-gal staining was performed in 6-well plates as previously described (Dimri et al., 1995). Cells were counterstained with 2 ug/ml Hoechst 33342 (Invitrogen) in 1X DPBS for 10 minutes and images were acquired with Nikon TE2000 Inverted Microscope.

**Plasmids, Lentiviral Production and Transduction**

mER-NRAS<sup>Q61R</sup> is a gift from Dr. Maria Soengas (CNIO, Spain). pLU-Jarid1B(prom)-PGK-Blast, pLU-TREmin-MCS-PGK-Puro and pLU-TREmin-BRAF<sup>V600E</sup>-PGK-Puro lentiviral vectors were constructed by the Protein Expression and Libraries Facility at the Wistar Institute. BRAF mutation was verified by DNA sequencing. 293T cells were transfected with pCMV, pVsvg and plasmids using Lipofectamine 2000 (Invitrogen). Lentiviral particles were harvested for three days. Cells were transduced overnight with lentiviral particles in the presence of Polybrene at 8 ug/ml. Stably infected cells were selected using Puromycin at 2 ug/ml or Blasticidin at 6 ug/ml for 6 days.

**Chemicals**

PD 0332991 (CDK 4/6 inhibitor) was purchased from Selleck Chemicals (Houston, TX); U0126 was purchased from LC Laboratories (Woburn, MA); Nutlin-3a was purchased from Cayman Chemical (Ann Arbor, MI); Doxorubicin was purchased from Sigma (St. Louis, MO); 4-OHT was purchased from LKT Laboratories (St. Paul, MN).

**Cell Labeling and Antibody Staining**
Adherent melanoma cells were harvested with 0.05% Trypsin-EDTA and washed once with 1X DPBS. Cells were labeled with PKH26 or CellVue Claret (Sigma) according to the manufacturer’s protocol and allowed to adhere to tissue culture plates overnight. Following drug treatment, adherent cells were harvested, stained with ImaGene Green™ C12FDG or ImaGene Red™ C12RG (Invitrogen). Cells were stained with CD20-PE (BD Biosciences) on ice for 45 minutes. Cells were immediately subject to FACS analysis using BD LSRII and at least 5,000 cells were acquired. FACS data were analyzed using FlowJo software.

**FACS Data Analysis**

FACS data were imported and analyzed with FlowJo software (Tree Star, Inc.). Unlabeled cells were used to set up the gating to contain 100% negative cells for PKH26 or CellVue Claret. DMSO treated cells were used to set up the gating to contain 5% positive cells for C12FDG, C12RG, CD20, or Jarid1(prom)-eGFP.

**BrdU Incorporation Assay and Cell Growth/Viability**

Quantitative determination of DNA synthesis in cell cultures was determined using Cell Proliferation ELISA, BrdU (chemiluminescent) kit (Roche) according to the manufacturer’s protocol. Cell Viability was measured by MTT assays as previously described (Smalley et al., 2009).

**Western Blotting and Antibodies**

Cells were washed with ice-cold PBS containing 100uM Na3VO4 and scraped off culture dishes. After centrifugation, cell pellets were lysed in a buffer containing 10 mM Tris-HCl pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1mM Na3VO4 and protease
inhibitors (Roche complete protease inhibitor tablets). Lysates were cleared by micro-
centrifugation and protein concentration was determined with Protein Assay Dye Reagent
Concentrate (Bio-Rad). For western blots, 25 ug lysates were run on 15-well 10% or 12%
SDS-PAGE gels and transferred using a dye fast Trans-Blot® Turbo™ transfer system
(Bio-Rad) onto nitrocellulose membranes. Blots were blocked in SEA BLOCK Blocking
Buffer (Thermo Scientific) diluted with 1X TBS at 1:1 ratio at RT for 1 h, incubated
overnight with primary antibody at 4° C, stained with secondary antibodies conjugated to
IRDye® Infrared Dyes (LI-COR Biosciences), and visualized using an Odyssey flat bed
scanner (LI-COR Biosciences). All antibodies used were from Cell Signaling Technology
(Beverly, MA), except β-Actin, which was purchased from Sigma (St. Louis, MO),
CHAPTER 6 IDENTIFICATION OF TWO DISTINCT FORMS OF THERAPY-INDUCED SENESCEENCE IN HUMAN MELANOMA CELLS

6.1 Introduction

Our preliminary data showed that human melanoma cells were readily undergoing oncogene-induced senescence mediated by BRAF<sup>V600E</sup> or NRAS<sup>Q61R</sup>, or therapy-induced senescence by Doxorubicin, Nutlin-3a and PD 0332991. This suggested that melanoma cells retain the intact senescence pathway, which can be reactivated through genetic and pharmacological approaches. The drawback of the genetic approach was twofold. First, it could not be used as a therapeutic method for the clinical treatment as most of melanoma cells have already harbored BRAF or NRAS mutation. Second, the rate of induction of senescence was much slower, compared to the pharmacological approach. Among these three tested drugs, Doxorubicin is a chemotherapeutic reagent that acts by inducing DNA damage. It is very difficult to pinpoint which pathways are mediators of drug-induced senescence. Nutlin-3a is a p53 activator, which does not show efficacy for p53 mutant melanoma cell lines. PD 0332991, which targets CDK 4/6, is thus far the most potent drug that induces senescence in human melanoma cells with known targets. Hence, we decided to include more targeted therapies. By using the FACS approach, we have observed that tumor cells from the same cell line respond differently to the drug, demonstrating heterogeneity among melanoma cells. Some cells underwent senescence, while the others continued to proliferate or remained in a quiescent state. In addition to
cell fates including senescence, proliferation and quiescence, we decided to also investigate early apoptosis, cell death and autophagy.

6.2 Results

6.2.1 Inhibition of Aurora Kinase Induced Senescence in Human Melanoma Cells

It has been reported that a selective Aurora A kinase inhibitor, MLN8054, triggered senescence in different types of cancer cells both in vitro and in vivo (Huck et al., 2010). Aurora kinase inhibitors are of particular interest in that Aurora kinases A and B were up-regulated in melanoma’s advanced stages compared to normal skin, nevi and melanomas of early stages (Wang et al., 2010). We first determined expression of Aurora kinase A and B at the protein level in a panel of human melanoma cell lines and found them to express more Aurora kinase A and B than normal human skin cells, suggesting they could be therapeutic targets (Figure 6.1). We therefore investigated the use of Aurora kinase inhibitors using a panel of human melanoma cell lines.
Figure 6.1 Melanoma cells express more abundant Aurora A and B kinases than normal skin cells. Human melanoma cells and normal skin cells were harvested to assess the expression of Aurora A and B kinases by western blotting.

We then treated 1205Lu cells with Aurora A kinase inhibitor MLN8237 at 0.25uM and Aurora B kinase inhibitor AZD1152 at 0.25uM for 7 days, followed by SA-β-gal staining. Cells were also treated with DMSO for 7 days as a control. The staining showed that both Aurora kinase inhibitors were very potent in inducing senescence in melanoma cells (Figure 6.2A). The induction of senescence was also quantified by FACS analysis based on the staining of C12FDG. The percentage of C12FDG+/7-AAD− was 68.9 and 64.3, respectively, in cells treated with MLN8237 and AZD1152 at 0.25uM for 5 days (Figure 6.2B), while the percentage of C12FDG+/7-AAD− was only 4.08 in cells treated with DMSO
Figure 6.2 Aurora kinase inhibition induced senescence in melanoma cell. (A) 1205Lu cells were treated with MLN8237 at 0.25uM and SNS-314 at 0.25uM for 7 days. DMSO treated cells were used as a control. The induction of senescence was measured by SA-β-gal staining. (B) 1205Lu cells were treated with MLN8237 at 0.25uM and SNS-314 at 0.25uM for 5 days. DMSO was again used as a control treatment. The induction of senescence was measured by FACS analysis using 7-AAD and C12FDG.

We treated two metastatic melanoma cell lines, UACC-903 and A375, with AZD1152 at 0.25uM and 1uM. Cells were treated with DMSO as a control comparison. The induction of senescence was assessed by SA-β-gal staining on Day 5 and showed that the drug is a potent trigger for induction of senescence in both melanoma cell lines (Figure 6.3).
Figure 6.3 Aurora B kinase inhibition induced senescence in melanoma cells. UACC-903 and A375 melanoma cells were treated with AZD1152 at 0.25 uM and 1 uM for 5 days. Concurrently, cells were treated with DMSO as a control. The induction of senescence was measured by SA-β-gal staining. All cells were visualized by Hoechst 33342 staining.

To further validate the induction of senescence in A375 cells by Aurora B kinase inhibition, treated cells were stained for PML and p21 immunofluorescence. PML is a “classic” senescence marker, and p21 is marker of cell cycle arrest. The co-staining results showed that AZD1152 treated cells were positive for both of PML and p21 staining (Figure 6.4). Moreover, the percentage of Ki-67 positive cells was decreased in AZD1152 treated cells compared to DMSO treated cells (Figure 6.5). Taken together,
SA-β-gal staining and immunofluorescence staining using PML, p21 and Ki-67 confirmed that AZD1152 treated cells underwent therapy-induced senescence.

Figure 6.4 Treated melanoma cells stained positive for senescence markers. A375 melanoma cells were treated with DMSO, and AZD1152 at 0.25 uM and 1 uM for 5 days. Treated cells were stained for PML and p21 expression by immunofluorescence staining. All cells were visualized by Hoechst 33342 staining.
Figure 6.5 Treated melanoma cells stained negative for Ki-67. A375 melanoma cells were treated with AZD1152 at 0.25 uM and 1 uM for 5 days. Cells were also treated for 5 days with DMSO as a control condition. Treated cells were stained with cell proliferation marker Ki-67 by immunofluorescence. All cells were visualized by Hoechst 33342 staining.

Senescence is an irreversible form of cell cycle arrest. To demonstrate that Aurora B kinase inhibitor induced senescence in human melanoma cells as an irreversible process, three metastatic melanoma cells, 1205Lu, A375 and UACC-903, were treated with AZD1152 at 0, 0.25uM and 1uM, respectively, for five days. Treated cells were then harvested, re-seeded in 96-well plates for cell proliferation assay and 12-well plates for clonogenicity assay. For all three lines investigated, while DMSO pre-treated cells
continued to grow, AZD1152 pre-treated cells generally lost proliferation potential and did not form colonies. However, A375 cells treated with AZD1152 at 0.25uM showed growth after 8 days (Figure 6.6). Altogether, this suggested that senescence induced in melanoma cells with Aurora B kinase inhibitor was an irreversible process in most but not all cell lines.

**Figure 6.6 Aurora B kinase inhibitor-induced senescence is irreversible.** (A) 1205Lu, UACC-903, and A375 melanoma cells were treated with AZD1152 at 0.25 uM and 1 uM for 5 days. DMSO treatment was also used as a control. Treated cells were then harvested and re-seeded in 96-well plates. The cell proliferation MTT assay was done on Days 0, 2, 4, 6, and 8. (B) The same cells were re-seeded in 12-well plates. Cells were fixed and subject for crystal violet staining on Day 14.
6.2.2 Aurora Kinase Inhibitors Were More Potent in Triggering Senescence than other Inhibitors

In preliminary studies, we used the human metastatic melanoma cell lines 1205Lu and A375 to investigate a panel of small molecule signaling modulators, some of which have shown the potential for triggering senescence in other types of cancers. These signaling modulators include (1) Nutlin-3a (p53 activator), (2) PD 0332991 (CDK4/6 inhibitor), (3) MLN8237 (Aurora A kinase inhibitor), (4) AZD1152 (Aurora B kinase inhibitor), (5) SNS-314 (pan Aurora kinase inhibitor), (6) VX-680 (pan Aurora kinase inhibitor), (7) PLX4032 (BRAF kinase inhibitor), and (8) Doxorubicin (DNA damage reagent). Nutlin-3a and PD 0332991 are known senescence triggers in melanoma cells and were tested in our preliminary studies (Anders et al., 2011; Korotchkina et al., 2010; Verhaegen et al., 2012). In agreement with a previous report, we have also found that Doxorubicin at low to medium dose can induce senescence concurrent with apoptosis in melanoma cells (Chang et al., 2002). GDC0879 and PLX4720 are selective mutant BRAF kinase inhibitors, and we were intrigued if inhibition of the MAPK pathway may also induce senescence in BRAF\(^{V600E}\) positive melanoma cells. Selective Aurora A kinase inhibitor MLN8054 or MLN8237 induces senescence in other types of cancer, and we showed that Aurora A or B kinase inhibitors potently induced senescence in melanoma cells (Gorgun et al., 2010; Huck et al., 2010). We were also interested in the degree of induced senescence in melanoma cells when treated with pan Aurora kinase inhibitors.

After exposing 1205Lu melanoma cells to a panel of signaling inhibitors at titrated doses for 4 days, we monitored therapy-induced senescence based on the fluorescence staining of C\(_{12}\)FDG. Since, by definition, senescent cells are still viable, early apoptotic
and dead cells were excluded from the subsequent analysis based on the fluorescence of PSVue 550. We arbitrarily set up the gate for $C_{12}FDG$ fluorescence based on cells that are maintained in culture medium to give rise to 2.5% $C_{12}FDG$ positive cells among all live cells, reflecting the phenomenon of spontaneous senescence. Surprisingly, Aurora kinase inhibitors, especially an Aurora B kinase inhibitor (AZD1152) and three pan Aurora Kinase inhibitors (SNS-314, AMG 900 and AT9283) were generally more potent than the other drugs tested (Figure 6.7). Interestingly, BRAF inhibitors PLX4720 and GDC-0879 also induced moderate but significant senescence in 1205Lu melanoma cells (Figure 6.7).

**Figure 6.7** Melanoma cells undergo senescence in response to a panel of signaling inhibitors. Human melanoma 1205Lu cells were treated with a panel of signaling inhibitors at titrated doses for 4 days. Cells were then harvested for $C_{12}FDG$ and PSVue 550 staining.
6.2.3 Establishment of An Integrated FACS Approach that Profiles Phenotypic Cellular Responses of Cancer Cells to Therapies

SA-β-gal staining is a “gold standard” for the detection of senescent cells in vitro and in vivo (Dimri et al., 1995). However, limitations of this technique are hindrance to the quantitative and simultaneous interrogation of heterogeneous responses of cells to senescence stimuli. In our preliminary studies, we used two FACS approaches that can quantify the induction of senescence either directly or indirectly. The first FACS approach is based on PKH26 and SSC labeled senescent cells as PKH26\textsuperscript{hi}/SSC\textsuperscript{hi}, based on the fact that senescent cells stopped cell division and become larger. The second FACS approach is based on CellVue Claret and C\textsubscript{12}FDG labeled senescent cells as CellVue\textsuperscript{hi}/C\textsubscript{12}FDG\textsuperscript{hi} and CellVue\textsuperscript{hi}/C\textsubscript{12}FDG\textsuperscript{hi}, dependent on the fact that senescent cells expressed high activity of β-galactosidase enzyme.

Cell fates following drug treatment include live, cell death, apoptosis, senescence, quiescence, proliferation and autophagy. We first aimed to develop a method that will quantify all phenotypic responses of cancer cells to therapies, except autophagy due to the limitation of the assay. We envisioned that this method will analyze samples in a “semi-high-throughput” manner. To achieve this goal, we adapted the FACS approach by taking advantage of fluorescent probes such as Propidium Iodide (PI), which labels dead cells, PSVue, which detects apoptotic cells, CellTrace Violet, which is a cell membrane dye for monitoring cell proliferation, and C\textsubscript{12}FDG, which is a substrate of SA-β-gal enzyme. Parameters inherent to FACS analysis such as forward scatter (FSC) and side scatter (SSC) enable us to assess the size and granularity of cells. Senescent cells generally become larger (Figure 6.8A). Combination of FSC and SSC helps us exclude
cell debris. It was also useful to demonstrate that cell size and granularity are significantly increased upon stress stimuli. Combination of PI and PSVue staining could separate cells into three major subpopulations: live cells (PI$^{\text{Neg}}$/PSVue$^{\text{Neg}}$), early apoptotic cells (PI$^{\text{Neg}}$/PSVue$^{\text{Pos}}$), and dead cells, including late apoptotic cells (PI$^{\text{Pos}}$/PSVue$^{\text{Pos}}$) (Figure 6.8B). We then focused our attention only on live cells to discern different phenotypic responses of cancer cells to drug treatment. Multiplexing of CellTrace Violet with C$_{12}$FDG further segregated live cells into four subpopulations, including cells in an irreversible senescence state (CellTrace Violet$^{\text{hi}}$/C$_{12}$FDG$^{\text{hi}}$), cells in a quiescence state (CellTrace Violet$^{\text{hi}}$/C$_{12}$FDG$^{\text{lo}}$), cells in a proliferation state (CellTrace Violet$^{\text{lo}}$/C$_{12}$FDG$^{\text{lo}}$), and cells in a transient senescence state (CellTrace Violet$^{\text{lo}}$/C$_{12}$FDG$^{\text{hi}}$) (Figure 6.8C).

**Figure 6.8** An integrated FACS approach to quantify phenotypic cellular responses of cancer cells to therapy. (A) Cell debris is excluded from the analysis based on FSC and SSC. (B) Live cells, early apoptotic cells and dead cells are identified based on PI
and PSVue 643 fluorescence. (C) Irreversible senescence, transient senescence, quiescence and proliferation are identified based on C12FDG and CellTrace Violet fluorescence.

The current senescence detection methods including SA-β-gal staining are unable to distinguish irreversible senescence from transient senescence. It is believed that senescence is irreversible, during which cells are permanently arrested and can not re-enter the cell cycle. Intriguingly, the integrated FACS approach, in contrast, implies that some cells do not remain in a robust but instead a transient senescent state.

To further validate this integrated FACS approach, A375 melanoma cells were treated with the pan Aurora kinase inhibitor SNS-314 at 0.05uM for three days along with DMSO as a control.
Figure 6.9 Most melanoma cells treated with an Aurora kinase inhibitor underwent irreversible senescence identified by the integrated FACS approach. A375 cells were labeled with CellTrace Violet and treated with DMSO or SNS-314 at 50nM for three days. Cells were harvested, stained and analyzed by the integrated FACS approach.

Upon drug treatment with SNS-314, cells became larger in size, compared to DMSO treated cells based on FSC and SSC. After excluding cell debris, in SNS-314 treated cells, there were 13.8% dead cells and 11.9% early apoptotic cells, compared to 3.37% and 1.77% in DMSO treated cells. Then live cells were further separated into four subpopulations based on the fluorescence of CellTrace and C\textsubscript{12}FDG. 71.4% of SNS-314 treated cells were in irreversible senescence state, whereas only 2.68% of DMSO treated cells were in an irreversible senescence state (Figure 6.9). SNS-314 did induce different phenotypes including reversible senescence, proliferation and quiescence, which were dominated by irreversible senescence (Figure 6.9).

6.2.4 Two Distinct Therapy-Induced Senescence States Form in Melanoma Cells, Irreversible and Transient Senescence

1205Lu and A375 melanoma cells harbor the BRAF\textsuperscript{V600E} mutation. Figure 6.7 had shown that BRAF inhibitors PLX4720 and GDC-0879 induced senescence in 1205Lu cells, albeit the degree of the induction of senescence was less than the one induced by different Aurora kinase inhibitors. We then turned to another BRAF\textsuperscript{V600E} melanoma cell line, A375 to confirm the result by cross comparing the induction of senescence by
BRAF inhibitors and Aurora kinase inhibitors. The integrated FACS approach revealed that BRAF inhibitors induced predominantly transient senescence, whereas Aurora kinase inhibitors induced predominantly irreversible senescence (Figure 6.10).

To gain a better understanding of induced transient senescence by BRAF inhibitors, we performed a time-course FACS analysis by interrogating cells treated with Aurora kinase inhibitor or BRAF inhibitors for 3 days and 5 days, respectively. FACS analysis showed that cells treated with BRAF inhibitors gradually acquired the transient senescence phenotype by initially continuing to proliferate. Transient senescence peaked on Day 5, whereas cells treated with Aurora kinase inhibitor acquired the irreversible senescence phenotype on Day 3, which remained on Day 5 (Figure 6.11).

To confirm the results of the FACS approach, we turned to SA-β-gal/Ki-67 co-staining. SA-β-gal staining showed that cells treated with Aurora kinase inhibitors for 5 days retained the SA-β-gal staining when cells were allowed to recover in drug-free medium for 5 days, whereas cells treated with the BRAF inhibitor for 5 days reversed staining and regained proliferative potential, as evidenced by Ki-67 staining (Figure 6.12).
Figure 6.10 BRAF inhibitors induced transient senescence in melanoma cells, whereas Aurora kinase inhibitors induced irreversible senescence. A375 cells labeled with CellTrace Violet were treated with DMSO, AZD1152 at 0.5uM, SNS-314 at 0.05uM, GDC-0879 at 2.5uM and 7.5uM, and PLX4720 at 2.5uM and 7.5uM for 5 days. Cells were harvested, stained and analyzed by the integrated FACS approach.

A cell proliferation assay confirmed that cells treated with BRAF inhibitors regained the proliferative capacity when grown in drug-free medium (Figure 6.13).
Figure 6.11 BRAF inhibitors induced transient senescence in melanoma cells. A375 cells were labeled with CellTrace Violet and treated with DMSO, SNS-314 at 0.05uM, and PLX4720 at 2.5uM and 7.5uM for 5 days. Cells were harvested, stained and analyzed by the integrated FACS approach.
Figure 6.12 BRAF inhibitor-treated cells showed reversal of the senescence, whereas Aurora kinase inhibitor-treated cells did not. A375 cells were treated with DMSO, AZD1152, SNS-314 and PLX4720 for 5 days. Cells were allowed to recovery in drug-free medium for five days, followed by SA-β-gal/Ki-67 co-staining.
A375 cells were treated with DMSO, BRAF inhibitor GDC-0879 at 5uM and 10uM, and BRAF inhibitor PLX4720 at 5uM and 10uM for five days. Then cells were allowed to grow in drug-free medium and cell proliferation assay was carried out at each time point.

6.2.5 Senescent Cancer Cells Over-produce Reactive Oxygen Species (ROS) with Mitochondria as the Source of ROS

Having identified two distinct forms of therapy-induced senescence, we decided to primarily focus on Aurora kinase inhibition induced senescence because of its irreversible process. Dysfunction of mitochondria contributes to oncogene-induced senescence in normal cells due to ROS. We first asked whether oxidative stress was also detected in senescent melanoma cells by inhibition of Aurora kinases. For detection of ROS, we used CellROX Deep Red, which is an indicator of oxidative stress. Melanoma cells that underwent therapy-induced senescence either by AZD1152 or SNS-314 over-produced ROS, which was evidenced by the overlap between C12FDG positive cells and CellROX Deep Red positive cells (Figure 6.14).
Next, we searched for source of ROS in senescent cancer cells using MitoSox Red, which is a mitochondria superoxide sensor. We observed a strong overlap between C$_{12}$FDG positive cells and MitoSox Red positive cells, indicating that mitochondria were the source of ROS in senescent cancer cells (Figure 6.15). It should be noted that ROS was not detected in melanoma cells treated with BRAF inhibitor PLX4720, suggesting that ROS might be required for the induction of irreversible senescence (data not shown).

Figure 6.14 Melanoma cells over-produce ROS when undergoing therapy-induced senescence. 1205Lu cells were treated with DMSO, AZD1152 at 0.5uM and SNS-314 at 0.05uM for five days. Cells were stained with C$_{12}$FDG and CellROX Deep Red for FACS analysis.
Figure 6.15 Mitochondria are the source of ROS for cancer cells undergoing therapy-induced senescence. 1205Lu cells were treated with DMSO, AZD1152 at 0.5uM and SNS-314 at 0.05uM for five days. Cells were stained with C$_{12}$FDG and MitoSox Red for FACS analysis.

6.3 Discussion

Aurora kinases are emerging therapeutic targets in cancer (Gautschi et al., 2008). There are three isoforms of Aurora kinases, Aurora A kinase, Aurora B kinase and Aurora C kinase. Aurora kinases A and B are over-expressed or amplified in a variety of cancers. Our data and those of others have shown that Aurora kinases A and B are over-expressed in malignant melanoma compared to normal skin. By screening a panel of signaling pathway modulators, we found that selective Aurora kinase inhibitors or pan
Aurora kinase inhibitors were more potent in triggering senescence in melanoma cells than any other small molecules. Indeed, it has been shown that a selective Aurora A kinase inhibitor induces senescence in breast and colon cancer cells. BRAF inhibitors also induce senescence in melanoma cells and the degree of the induction is cell line and dose dependent. By using the integrated FACS approach to profile heterogenous phenotypic responses of cancer cells in response to drugs, we identified two distinct forms of therapy-induced senescence, irreversible senescence represented by inhibition of Aurora kinase vs. transient senescence represented by inhibition of mutated BRAF. Functional assays confirm that melanoma cells treated with BRAF inhibitors first undergo transient senescence and regain cell proliferation capacity upon the withdrawal of the drug, whereas melanoma cells treated with Aurora kinase inhibitors undergo irreversible senescence and that process is irreversible in most but not all cell lines. Furthermore, we demonstrate that ROS is over-produced in senescent cancer cells induced with Aurora kinase inhibitor but not BRAF inhibitors, implicating that ROS may also distinguish irreversible from transient senescence.

6.4 Materials and Methods

Cell Culture Methods

Human metastatic melanoma cell lines have previously been described (Satyamoorthy et al., 1997). A375 cells were purchased from American Type Culture Collection. UACC-62 and UACC-903 cells were kind gifts from Dr. Marianne B. Powell (Stanford University). Melanoma cells were maintained in Dulbecco's Modified Eagle Medium:
Nutrient Mixture F-12 (DMEM/F-12) Media (GIBCO) supplemented with 10% fetal bovine serum (Atlanta Biologicals), or MCDB 153 medium (Sigma) containing 20% Leibovitz's L-15 medium (Cellgro), 2% fetal bovine serum, 5 µg/mL insulin (Sigma) and 1.5M CaCl₂ (Sigma). All cells were cultured at 37° C in a humidified incubator supplied with 5% CO₂.

**SA-β-gal and Immunofluorescence Staining**

Cells were fixed with 1X DPBS containing 2% formaldehyde (Sigma) and 0.2% glutaraldehyde (Sigma). SA-β-gal staining was performed in 24-well plates as previously described (Dimri et al., 1995). Subsequently, SA-β-gal staining buffer was removed and cells were permeabilized and blocked with 1X DPBS supplemented with 0.3% Triton X-100 and 3% BSA for 1 hour. Cells were incubated with primary antibodies overnight at 4° C. After rinse, cells were incubated with secondary antibodies at room temperature for 1 hour. Cells were counterstained with 2 ug/ml Hoechst 33342 (Invitrogen) in 1X DPBS for 10 minutes and images were acquired with Nikon TE2000 Inverted Microscope.

**Chemicals**

MLN8237, AZD1152, SNS-314, GDC-0879, AT9283 and PD 0332991 were purchased from Selleck Chemicals (Houston, TX); PLX4032 and PLX4720 were provided by Plexxikon; AMG 900 was provided by Amgen; Nutlin-3a was purchased from Cayman Chemical (Ann Arbor, MI); Rapamycin was purchased from LC Laboratories (Woburn, MA);
Cell labeling

Adherent melanoma cells were harvested with 0.05% Trypsin-EDTA and washed once with 1X DPBS. Cells were labeled with CellTrace™ Violet (Invitrogen) according to the manufacturer’s protocol and allowed to adhere to tissue culture plates overnight. Following drug treatment, floating and adherent cells were pooled at certain time points, stained with Imagen Green™ C12FDG (Invitrogen) alone, or in combination with CellROX Deep Red (Invitrogen) or MitoSox Red (Invitrogen). Cells were then stained with PSVue® 550 (MTTI) or PSVue® 643 (MTTI) alone, or propidium iodide (Sigma) together with PSVue® 643 or PSVue® 550. Cells were immediately subject to FACS analysis using BD LSRII and at least 5,000 cells were acquired.

FACS Data Analysis

FACS data were imported and analyzed with FlowJo software (Tree Star, Inc.). Unlabeled cells were used to set up the gating to contain 100% negative cells for CellTrace Violet. DMSO treated cells were used to set up the gating to contain 5% positive cells for C12FDG, CellROX Deep Red, MitoSox Red or PSVue.

Cell Growth/Viability and Assessment of Cell Clonogenicity

Cell Viability was measured by MTT assays as previously described (Smalley et al., 2009). For the assessment of cell clonogenicity, drug treated cells were harvested and seeded into 12-well tissue culture plates at a density of 5X10² cells/well as triplicates in drug-free medium. Medium was refreshed every three or four days for 14 days. Colonies were then stained with methanol containing 0.05% crystal violet for overnight. After extensive wash with PBS, cells were air-dried and subject to image acquisition.
CHAPTER 7 AUTOPHAGY IS A SURVIVAL MECHANISM FOR MELANOMA CELLS UNDERGOING THERAPY-INDUCED SENESCENCE

7.1 Introduction

Our previous studies showed that in addition to cell death, apoptosis, quiescence and proliferation, surviving melanoma cells undergo either irreversible senescence or transient senescence in response to drug treatment. We focused on irreversible senescence and investigated the underlying molecular mechanisms that mediated this process.

7.2 Results

7.2.1 Identification of Autophagy and ER Stress Response in Therapy-Induced Senescence by Microarray Analysis

To understand the molecular mechanisms underlying therapy-induced irreversible senescence in melanoma cells in response to Aurora kinase inhibitors, we decided to carry out a time-course gene expression microarray analysis to assess the dynamics of gene expression in melanoma cells. We first carried out a preliminary time-course microarray experiment by profiling gene expression of 1205Lu melanoma cells treated with AZD1152 at 0, 6, 12, 36, 60, 72, 96, and 120 hours. The goal was to identify optimal time points for cell harvesting. The results suggest that there is no significant difference
in gene expression level between 36 hour and any later time points (data not shown). Experimental evidence showed the prominent SA-β-gal staining in treated melanoma cells on Day 5, and we reasoned that gene expression changes that were responsible for the induction and maintenance of the senescence program would occur earlier, prior to the induction of senescence. Indeed, by performing co-staining of treated melanoma cells with SA-β-gal together with Ki-67 and PML at different time points, we started to see a few senescence positive cells on Day 3, when cells were treated with AZD1152 or SNS-314 (Figure 7.1). The number of SA-β-gal positive cells peaked by Day 5 (Figure 7.1). We profiled gene expression changes at 0, 6, 12, 24, 36 and 48 hours for 1205Lu cells treated with AZD1152 at 0.5uM and SNS-314 at 0.05uM.

![Image of cell staining results](image_url)
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Figure 7.1 Aurora kinase inhibitor induced senescence occurred on Day 3 and peaked on Day 5. 1205Lu cells were treated with DMSO (Panel A) for 5 days as a control, along with with AZD1152 at 0.5uM (Panel B) and SNS-314 at 0.05uM (Panel C) for 5 days. Cells were fixed at each time point and subject to SA-β-gal/Ki-67/PML co-staining.

We compared gene expression levels before treatment and at different time points after treatment. There was a dramatic increase of the numbers of up- and down-regulated genes between early time points and 48 hours. This suggested that the effective drug response occurred after 36 hours and peaked at 48 hours (Figure 7.2).
Figure 7.2 The dramatic effects of AZD1152 and SNS-314 were seen at 48h. 1205Lu cells were treated with AZD1152 and SNS-314 and gene expression at each time point was compared to 0 hours.

We then used the Gene Set Enrichment Analysis (GSEA) to interrogate the most significantly up-regulated genes. We identified 16 significantly up-regulated pathways under Bonferroni corrected p value <0.01 in one of the two treatments (Figure 7.3). The top ones included lysosome and unfolded protein responses. The significances of these up-regulated pathways were consistent between the two drug treatments.
Figure 7.3 Identification of significant pathways in response to AZD1152 and SNS-314. Significant pathways were identified based on the most significantly up-regulated genes (Bonferroni corrected P value <0.01 in one of the two treatments).

Next, we compared the top 50 significantly up-regulated pathways of melanoma cells in response to AZD1152 and SNS-314, and we found that 37 pathways were shared, which was highly significant (Hypergeometric test P value = 4.2x10^{-42}) (Figure 7.4).

Lysosomes fuse with autophagosomes at the late stage of autophagy to form autolysosome, where degradation of captured substrates by autophagosomes occurs. Our microarray data reveal that the lysosome pathway is highly enriched among significantly up-regulated genes in response to Aurora kinase inhibition. We hypothesize that the autophagy pathway might also be activated because it is closely linked with and dependent on lysosomes. There is a fast growing body of evidence suggesting autophagy and senescence are functionally intertwined (Narita, 2010; White and Lowe, 2009; Young and Narita, 2010).
Autophagy mediates mitotic transition into senescence for normal fibroblasts expressing oncogenic $H-RAS^{V12}$ (Narita et al., 2011; Young et al., 2009). Loss of autophagy-related genes could contribute to the bypass of $H-RAS^{V12}$-induced senescence, implying autophagy is required for OIS (Young et al., 2009). When oncogenic $BRAF^{V600E}$
was re-introduced in BRAF$^{V600E}$ positive melanoma cells, cells underwent senescence-like cell-cycle arrest, accompanied by the engagement of autophagy (Maddodi et al., 2010). There is also evidence implicating a link between autophagy and senescence in cancer cells undergoing senescence in response to a variety of therapies (Lin et al., 2010; Luo et al., 2012; Mosieniak et al., 2012; Yang et al., 2012). Inhibition of autophagy leads to a decrease of senescence. Young et al. 2009 and Nariata et al. 2010 utilized the time-course gene expression microarray analysis to reveal that the autophagy, lysosome and senescence pathways are significantly up-regulated when fibroblasts expressing H-RAS$^{V12}$ transition into senescence. Day 4 is the most critical time point to study gene expression changes in fibroblasts expressing H-RAS$^{V12}$. The microarray data at Days 0 and 4 have been made publicly available. We determined that 48 hours was the most critical time point for 1205Lu cells treated with AZD1152 or SNS-314, having the most significant gene expression changes compared to controls. We compared these two data sets to study if there were any commonly regulated genes and pathways. Both experiments used Illumina HumanHT-12 chips. We first identified significantly up- and down-regulated genes for each data set to compare data at the late time point to one of the early time points. We then performed Gene Set Enrichment Analysis (GSEA) by interrogating significantly expressed genes and discovered the top 50 up- and down-regulated gene sets that were enriched. We then cross compared GSEA results from H-RAS$^{V12}$, AZD1152 and SNS-314 and found there were 10 up-regulated gene sets and 36 down-regulated gene sets in common. Among 10 enriched up-regulated gene sets, there were “unfolded protein response”, “activation of chaperon IRE1α” and “lysosome” sets (Figure 7.4). Most of 36 down-regulated gene sets were associated with cell cycle
checkpoints, S phase, and DNA replication (Figure 7.5 and data not shown). These results are intriguing because senescence induced in different cell types by different stress stimuli may share some common effector programs leading to the induction of senescence.

![Figure 7.5](image)

**Figure 7.5** Significantly down-regulated pathways overlapped in response to AZD1152 and SNS-314 treatment of melanoma cells and over-expression of H-RAS\(^{V12}\) in fibroblasts. The top 50 enriched pathways of AZD1152 and SNS-314 significantly overlap (42 in common, \(P\) value\(=2.6\times10^{-54}\); 36 out these 42 common enriched pathways were significantly overlapped in the top 50 enriched pathways of H-RAS\(^{V12}\) (\(P\) value\(=1.14\times10^{-46}\)).

### 7.2.2 Validation of Autophagy in Therapy-Induced Senescence

To confirm the results of time-course gene expression microarrays, we treated 1205Lu cells with AZD1152 and determined activation of autophagy using an LC3B antibody. The conversion of LC3B-I to LC3B-II occurred at 24 hours and persisted, concomitant
with inhibition of phosphorylation of Rb (Figure 7.6). These data suggest that autophagy was up-regulated prior to the induction of senescence. To demonstrate that autophagy is actively engaged, we treated 1205Lu and A375 cells with DMSO, PES-Cl, AZD1152 and SNS-314 for 48 hour in the presence of NH₄Cl, which blocks fusion between autophagosome and lysosome to inhibit autophagy flux. PES-Cl is a HSP70 inhibitor, and it in turn inhibits autophagy (data not known). When cells were treated with PES-Cl, p62 was increased, confirming that PES-Cl is an autophagy inhibitor. In the presence of NH₄Cl, p62 was also increased in cells treated either with AZD1152 or SNS-314, compared to cells treated with Aurora kinase inhibitor alone, suggesting the autophagy flux was inhibited (data not shown). Under the same condition, LC3B was increased, indicating that autophagy was active in response to the inhibition of Aurora kinase (data not shown).

Furthermore, 1205Lu cells stably expressing mCherry-LC3B lentiviral construct also showed punctate staining at 48 hours when treated with AZD1152 or SNS-314 compared to DMSO-treated control cells (Figure 7.7A). The punctate staining became more prominent at 72 hour (Figure 7.7B). Taken together, these findings show that inhibition of Aurora kinase when cells are undergoing therapy-induced senescence is engaging the autophagy pathway.
Figure 7.6 Autophagy was activated in melanoma cells in response to Aurora B kinase inhibition. 1205Lu cells were treated with AZD1152 at 1uM and cell lysates were collected at each time point.

Figure 7.7 Autophagy was activated in melanoma cells in response to Aurora kinase inhibition. 1205Lu cells expressing mCherry-LC3B were treated with DMSO, AZD1152 and SNS-314 for 48hr and 72hr. Cells treated with HCQ were used as a positive control.
7.2.3 Inhibition of Autophagy Abrogates Therapy-Induced Senescence and Enhances Cell Death But Paradoxically Promotes Quiescence or Cell Proliferation

As depletion of autophagy genes could bypass \(H\text{-}RAS^{V12}\)-induced senescence in fibroblasts, reported by Young et al. 2008, we investigated whether inhibition of autophagy could have any effect on therapy-induced senescence in melanoma cells. We first focused on two well-known autophagy inhibitors, bafilomycin A1 (BafA1) and hydroxychloroquine (HCQ). BafA1 or HCQ does not directly inhibit autophagy, but inhibits fusion between autophagosomes and lysosomes by altering the pH of the lysosomes to block their function. In 1205Lu cells, AZD1152-induced senescence was blunted by BafA1 or HCQ as shown by SA-\(\beta\)-gal staining (Figure 7.8).

![Image showing SA-\(\beta\)-gal and Hoechst staining in 1205Lu cells treated with different combinations of AZD1152, BafA1, and HCQ.]

**Figure 7.8** Senescence induced by Aurora kinase inhibition was blunted by lysosome inhibitors. 1205Lu cells were treated with DMSO and AZD1152 at 1\(\mu\)M for three days in combination with BafA1 or HCQ. Cells were fixed and stained with SA-\(\beta\)-gal and Hoechst 33342.
We then examined the role of Spautin-1 for therapy-induced senescence. Spautin-1 has recently been identified as a specific and potent autophagy inhibitor (Liu et al., 2011). It inhibits autophagy by promoting degradation of Beclin-1 and the Vps34 complex, which initiate the formation of autophagosome. We first verified the effect of Spautin-1 when 1205Lu cells were starved in EBSS medium. Starvation of 1205Lu cells activated autophagy as indicated by increased LC3B expression. When starved cells were treated with Spautin-1 at 1uM and higher doses, increased LC3B levels significantly diminished, and cell death increased as indicated by the expression of cleaved PARP (Figure 7.9). This suggests that Spautin-1 is a very effective autophagy inhibitor, and cancer cells rely on autophagy for survival when they are growth arrested under starvation.

![Figure 7.9 Spautin-1 inhibits starvation-induced autophagy.](image)

A375 cells were cultured in normal growth medium or EBSS for 24 hours in combination with Spautin-1 at different doses. Autophagy and apoptosis were assessed by immunoblotting.
We then treated A375 cells with DMSO, AZD1152 and SNS-314 for 3 days in combination with Spautin-1 at titrated doses. Spautin-1 significantly inhibited therapy-induced senescence by AZD1152 or SNS-314 and enhanced cell death. The percentage of irreversible senescence was decreased from 58% for cells treated with AZD1152 alone, to 5.5% for cells treated with AZD1152 and Spautin-1 at 5µM (Figure 7.10), whereas the percentage of cell death including apoptosis was increased from 10.6% to 44.6%. Of note, combination therapy of AZD1152 and Spautin-1 paradoxically promoted a subpopulation of proliferating cells, whereas combination therapy of SNS-314 and Spautin-1 promoted a subpopulation of quiescent cells. For example, the percentage of proliferating cells was increased from 5.8% for cells treated with AZD1152 alone to 20.4% for cells treated with AZD1152 and Spautin-1 at 5µM (Figure 7.10).
Figure 7.10 Spautin-1 converted therapy-induced senescence into cell death, but paradoxically promoted cell proliferation or quiescence. A375 cells were treated with
DMSO, AZD1152 and SNS-314 in combination with Spautin-1 at indicated doses for 3 days. Cell death/apoptosis, transient senescence, irreversible senescence, quiescence and proliferation were assessed by the integrated FACS approach.

**Figure 7.11** Rapamycin decreased the effectiveness therapy-induced senescence.

1205Lu cells were treated with DMSO and AZD1152 in combination with Rapamycin at indicated doses for 5 days. Senescence was quantified based on fluorescence of $C_{12}$FDG. “A” stands for AZD1152 and “R” for rapamycin.

Since autophagy mediated therapy-induced senescence, we hypothesized that the autophagy inducer rapamycin may accelerate therapy-induced senescence. Surprisingly, rapamycin, in turn, impaired the ability of AZD1152 to induce senescence in 1205Lu cells (Figure 7.11). We inferred they were in a quiescence stage. Another study has implicated that senescence, induced by the p53 activator Nutlin-3a, was converted into quiescence by rapamycin in melanoma cells (Korotchkina et al., 2010). These results suggest that the mTOR signaling pathway is essential for therapy-induced senescence.
In summary, autophagy is a survival pathway for cancer cells undergoing therapy-induced senescence. Inhibition of autophagy converts therapy-induced senescence into cell death, but paradoxically also promotes cell proliferation or quiescence. The underlying mechanisms are not clear. Autophagy induction also impairs therapy-induced senescence by inhibiting the mTOR pathway suggesting that two inhibitors may not always synergize but antagonize.

7.2.4 Inhibition of ER Stress Response Converts Therapy-Induced Senescence into Quiescence

GSEA analyses identified two ER stress response related gene sets, “unfolded protein response” (UPR) and “activation of chaperone by IRE1α” that were enriched in 1205Lu cells treated with Aurora kinase inhibitors and fibroblasts expressing H-RAS\(^{V12}\). Intriguingly, in human melanocytes expressing H-RAS\(^{V12}\), neither B-RAF\(^{V600E}\) nor N-RAS\(^{Q61R}\) senescence was mediated by the ER-associated unfolded protein response (Denoyelle et al., 2006). Inactivation of UPR in melanocytes expressing H-RAS\(^{V12}\) decreases the induction of senescence dramatically.

We thus tested whether pharmacological inhibition of UPR would block therapy-induced senescence in melanoma cells. As a result of the identification of “activation of chaperone by IRE1α”, we tested a specific IRE1α inhibitor, Irestatin 9389, for its effect on therapy-induced senescence (Feldman and Koong 2007). Irestatin alone did not induce senescence in melanoma cells, although cell death was induced when treating cells with Irestatin at higher dose. SA-β-gal staining showed that AZD1152 induced senescence in melanoma cells was abrogated when cells were treated with Irestatin (Figure 7.12). We
then quantified the degree of inhibition using FACS. As with SA-β-gal staining, Irestatin blocked therapy-induced senescence by AZD1152 or SNS-314, while inducing minimal cell death. For example, the percentage of senescence was decreased from 46.3% for cells treated with AZD1152 to 25.7% for cells treated with AZD1152 in combination with Irestatin at 40uM (Figure 7.13).

The results, along with the data about melanocytes from Denoyelle et al. 2006, suggest that ER stress response is a shared effector mechanism for the induction of senescence in response to oncogenic mutation or therapies.

**Figure 7.12 Senescence induced by Aurora kinase inhibition was blunted by IRE1α inhibition.** 1205Lu cells were treated with DMSO and AZD1152 at 0.5uM for 5 days in combination with IRE1α inhibitor at titrated does by inhibiting the ER stress response. Cells were fixed and stained with SA-β-gal (upper row) and Hoechst 33342 (lower row).
Figure 7.13 Senescence induced by Aurora kinase inhibition was down modulated by IRE1α inhibition. 1205Lu cells were treated with DMSO, AZD1152 at 0.5uM and SNS-314 at 0.05uM for 5 days in combination with the IRE1α inhibitor Irestatin at titrated doses, which inhibited the ER stress response. Cells were harvested and stained for FACS analysis. Senescence was assessed by C_{12}FDG. Cell death was assessed by Annexin V.

7.3 Discussion

We utilized a computational approach to identify autophagy and the ER stress response as induced in response to therapy-induced senescence in melanoma cells. By comparing with the microarray data of fibroblasts expressing H-RAS^{V12}, we observed that there was a significant overlap between melanoma cells treated with Aurora kinase
inhibitor and fibroblasts expressing H-RAS\textsuperscript{V12}. This suggests there are common senescence effector programs not only for OIS in normal cells, but also for therapy-induced senescence in cancer cells. Indeed, autophagy is also engaged in mediating oncogene-induced senescence-like cell cycle arrest in melanoma cells.

Experimental evidence confirmed that autophagy preceded and was required for therapy-induced senescence. When autophagy was inhibited, therapy-induced senescence was converted into cell death in some but not all cells. This suggests that autophagy is a survival mechanism for senescent cells under stress. During Aurora B or Aurora A and B inhibition, in combination with autophagy inhibition, a subpopulation of proliferating or quiescent cells was enriched. Co-targeting two pathways may result in antagonism not synergy. This has broad implications for the design of combination therapies.

BRAF inhibitor apparently also activates autophagy and/or the ER stress response. It warrants further investigations which checkpoints will determine the routes to either transient senescence or irreversible senescence.

7.4 Materials and Methods

Cell Culture Methods

Human metastatic melanoma cell lines have previously been described (Satyamoorthy et al., 1997). Melanoma cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) Media (GIBCO) supplemented with 10% fetal bovine serum (Atlanta Biologicals), or MCDB 153 medium (Sigma) containing 20% Leibovitz's L-15 medium (Cellgro), 2% fetal bovine serum, 5 µg/mL insulin (Sigma) and 1.5M CaCl\textsubscript{2} (Sigma). To starve cells, melanoma cells were maintained using EBSS medium (Thermo). All cells were cultured at 37° C in a humidified incubator supplied
with 5% CO₂.

**SA-β-gal and Immunofluorescence Staining**

Cells were fixed with 1X DPBS containing 2% formaldehyde (Sigma) and 0.2% glutaraldehyde (Sigma). SA-β-gal staining was performed in 24-well plates as previously described (Dimri et al., 1995). Subsequently, SA-β-gal staining buffer was removed and cells were permeabilized and blocked with 1X DPBS supplemented with 0.3% Triton X-100 and 3% BSA for 1 hour. Cells were incubated with primary antibodies, Ki-67 (Vector Labs) and PML (Santa Cruz) overnight at 4° C. After rinse, cells were incubated with secondary antibodies at room temperature for 1 hour. Cells were counterstained with 2 ug/ml Hoechst 33342 (Invitrogen) in 1X DPBS for 10 minutes and images were acquired with Nikon TE2000 Inverted Microscope.

**Chemicals**

AZD1152 and SNS-314 were purchased from Selleck Chemicals (Houston, TX); Rapamycin, and Bafilomycin A1 were purchased from LC Laboratories (Woburn, MA); Spautin-1 was kindly provided by Dr. Junying Yuan (Harvard University) and also purchased from Cellagen (San Diego, CA); Hydroxychloroquine (HCQ) was kindly provided by Dr. Ravi K. Amaravadi (University of Pennsylvania); Irestatin 9389 was purchased from Axon Medchem (The Netherlands); PES-Cl was synthesized by J. Winkler (University of Pennsylvania).

**Cell labeling**
Adherent melanoma cells were harvested with 0.05% Trypsin-EDTA and washed once with 1X DPBS. Cells were labeled with CellTrace™ Violet (Invitrogen) according to the manufacturer’s protocol and allowed to adhere to tissue culture plates overnight. Following drug treatment, floating and adherent cells were pooled at certain time points, stained with ImaGene Green™ C_{12}FDG (Invitrogen) alone, or in combination with CellROX Deep Red (Invitrogen) or MitoSox Red (Invitrogen). Cells were then stained with Annexin-V APC (Invitrogen), PSVue® 550 (MTTI) or PSVue® 643 (MTTI) alone, or propidium iodide (Sigma) together with PSVue® 643 or PSVue® 550. Cells were immediately subject to FACS analysis using BD LSRII and at least 5,000 cells were acquired.

**FACS Data Analysis**

FACS data were imported and analyzed with FlowJo software (Tree Star, Inc.). Unlabeled cells were used to set up the gating to contain 100% negative cells for CellTrace Violet. DMSO treated cells were used to set up the gating to contain 5% positive cells for C_{12}FDG.

**Time-Course Gene Expression Microarray and Data Analysis**

Adherent 1205Lu melanoma cells were washed twice with Hank's Buffered Salt Solution (HBSS) and harvested with 0.25% trypsin-EDTA at certain time points (0, 6, 12, 18, 24 and 48 hour) post drug treatment. Cells were washed again with HBSS and lysed directly in TRI Reagent® RNA Isolation Reagent (Sigma) and stored at -20°C. RNA isolation and cDNA synthesis was performed in Wistar Genomics Facility. Illumina HumanHT-12 v3 Expression BeadChip were hybridized, labeled and processed (San Diego, USA)
according to the manufacturer's protocol. Microarray data were obtained from 3 independent biological replicates per time point. Microarray raw data generated from Illumina Chip were normalized, background-corrected, and summarized using the R package “lumi” (Du et al., 2008). To reduce false positives, the unexpressed probes were removed, leaving 23,569 probes that were examined in all experiments described herein. Meanwhile, when multiple probe IDs interrogate the same gene, we took the maximum value as the gene expression level, which was used in the downstream analysis. Fold changes were calculated to compare the difference of mean gene expression levels between before treatment and at different time points after treatment. Meanwhile, student’s T-test was applied to obtain the parametric p values for measuring the significance of gene expression level differences. Genes with p value < 0.01 and fold change >1.2 (up or down regulated) were claimed to be significantly differentially up or down regulated, which were then subject to hypergeometric test based gene set enrichment analysis (GSEA). We obtained the 1453 canonical pathways from the Molecular Signatures Database (MSigDB) (http://www.broadinstitute.org/gsea/msigdb/index.jsp) for GSEA. We only considered pathways that contain at least 10 genes and at most 150 genes profiled and expressed in the experiment. Two-way hierarchical clustering was performed and plotted using the “heatplot” function in the R package “made4”, with the basis of Euclidean distance, centering log-transformed gene expression by mean and single linkage clustering. The genes up- or down-regulated at 1 or more time points were subjected to the two-way clustering analysis for generating the heat map.
Data Access

All time-course gene expression microarray data of 1205Lu melanoma cells were submitted to the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE38466. Time course gene expression microarray data of fibroblasts expressing \( H-RAS^{V12} \) on Day 0 and 4 used in this study have previously been submitted to GEO under the accession number GSE28464 (Narita et al., 2011).

Plasmids and Lentiviral Production

pLVO-Puro-Cherry-LC3 has been previously described (Tormo et al., 2009). 293T cells were co-transfected with plasmid, pCMV and pVsvg together with Lipofectamine 2000 (Invitrogen). 30% DMEM medium containing lentiviral particles were collected everyday for three days. 1205Lu cells were infected with lentivirus in the presence of Polybrene at 8 ug/ml. Stably infected cells were selected with Puromycin at 2 ug/ml.

Western Blotting and Antibodies

Cells were washed with ice-cold PBS containing 100uM Na\(_3\)VO\(_4\) and scraped off culture dishes. After centrifugation, cell pellets were lysed in a buffer containing 10 mM Tris-HCl pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1mM Na\(_3\)VO\(_4\) and protease inhibitors (Roche complete protease inhibitor tablets). Lysates were cleared by micro-centrifugation and protein concentration was determined with Protein Assay Dye Reagent Concentrate (Bio-Rad). For western blots, 25 ug lysates were run on 15-well 10% or 12% SDS-PAGE gels and transferred using a dye fast Trans-Blot\textsuperscript{R} Turbo\textsuperscript{TM} transfer system (Bio-Rad) onto nitrocellulose membranes. Blots were blocked in SEA BLOCK Blocking Buffer (Thermo Scientific) diluted with 1X TBS at 1:1 ratio at RT for 1 h, incubated
overnight with primary antibody at 4° C, stained with secondary antibodies conjugated to IRDye® Infrared Dyes (LI-COR Biosciences), and visualized using an Odyssey flat bed scanner (LI-COR Biosciences). All commercial antibodies were purchased from Cell Signaling except LC3B and beta-actin from Sigma.
CHAPTER 8 CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Conclusions

Our long-term goal is to advance therapy for patients with advanced melanoma, which is exceptionally refractory to conventional chemotherapeutic agents. Current targeted therapies and immunotherapies have shown promise and improvement in overall survival for a subset of patients. Acquired resistance to targeted therapies inevitably occurs, prompting us to search for better anti-cancer therapies.

Senescence is an intrinsic fail-safe defense mechanism against tumorigenesis and serves as a potential therapeutic strategy for treating cancer patients. Previous studies by our group and others have shown that over-expression of mutated oncogenic BRAF or NRAS leads to oncogene-induced senescence in normal melanocytes.

In this study, we demonstrated that, in a panel of human melanoma cell lines, premature senescence can be induced via i) over-expression of mutated BRAF$^{V600E}$; ii) administration of low to moderate dose of a chemotherapeutic agent, Doxorubicin (DXR); iii) a small molecule, Nutlin-3a, which is a p53 activator by negatively regulating MDM2; and iv) CDK4/6 inhibitor, PD 0332991. Using two FACS approaches, we quantified therapy-induced senescence in melanoma cells and observed that not all cells within the same cell line responded to the drug equally. We then refined the FACS approach to quantitatively profile the heterogeneous response of cancer cells to therapy. By screening small molecules that modulate a variety of signaling pathways, we identified the most potent inhibitors, Aurora Kinase A or B inhibitors, that induced senescence in melanoma cells most significantly. We then identified two distinct forms of
therapy-induced senescence through the integrated FACS approach, irreversible senescence and transient senescence. BRAF inhibitors induced transient senescence, whereas Aurora kinase inhibitor induced irreversible senescence. Using a computational biology approach, we discovered that autophagy and the unfolded protein response preceded and was required for therapy-induced senescence mediated by Aurora kinase inhibition. Gene expression profiles of therapy-induced senescence in cancer cells resembled those of oncogene-induced senescence in normal cells, indicating that common pathways are utilized to execute the senescence program by both stress stimuli. Furthermore, we found that senescence can be a survival mechanism for cancer cells undergoing therapy. Targeting autophagy could convert therapy-induced senescence into cell death, but it also enriched a subpopulation of either proliferating or quiescent cells. These results suggest that targeting two pathways might not always lead to synergy but antagonism. Our data have profound implications for utilizing pro-senescence as an anti-cancer therapy and the design of combination therapies.

8.2 Future Directions

8.2.1 What underlies the difference between two distinct forms of therapy-induced senescence?

It remains unclear which checkpoints determine the downstream route leading to either irreversible or transient senescence. We hypothesize that BRAF inhibitors induce transient senescence as a survival mechanism to evade apoptosis. Inhibition of BRAF induced autophagy. Because autophagy and irreversible senescence are also tightly linked in the context of Aurora kinase inhibition induced senescence, it is important to tease out
further how autophagy and senescence cooperate, leading to different responses. We propose to utilize RNA-seq and Reverse phase protein arrays (RPPA) to compare irreversible and transient senescence, expecting to identify new pathways for therapy.

8.2.2 How to design novel combination therapies for BRAF inhibitors that lead to cell death and not transient senescence?

BRAF inhibitors improve patient survival but tumors relapse within a year for most patients with metastatic melanoma. About 90% of melanoma patients retain the wild-type p53, but its tumor suppressor function is disabled due to loss at CDKN2A locus and interaction with HDM2 (Lee and Herlyn, 2012). Pharmacological restoration of p53 in melanoma cells induces apoptosis (Smalley et al., 2007). It has been hypothesized that p53 activators aid in combination therapies for melanoma patients. Combining Nutlin-3a with a MEK inhibitor, U0126 or AZD6244 effectively suppressed melanoma growth in vitro (Ji et al., 2012). Since BRAF kinase inhibitors are more potent in specifically targeting BRAF<sup>V600E</sup> positive melanoma cells, we propose to test the combination of BRAF kinase inhibitors with Nutlin-3a in melanoma cells to study whether such combination strategy can lead to greater synergy.

8.2.3 Is pro-senescence therapy a valid therapeutic method to be used in patients?

We further found that inhibition of senescence induction can lead to apoptosis, but often to survival or even proliferation, suggesting that current strategies are not sufficiently advanced to propose them for patients. Two conditions need to be met: a) the treatment should lead to irreversible senescence alone or in conjunction with the induction of apoptosis; b) novel combinations of therapies need to be found
which will inhibit cells from entering transient senescence, quiescence or non-response.

8.2.4 Is cell-cell variability determining the heterogeneous response of cancer cells to therapy?

Our data suggest that tumor cells within the same cell line respond to the therapy unevenly. What is the underlying mechanism for this phenomenon? Does genetic heterogeneity determine the phenotypic heterogeneity? We propose to integrate gene expression profiles, time lapse imaging for live cells, and FACS sorting techniques to tackle this question by interrogating single melanoma cells.
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