ROLE OF SWI/SNF CHROMATIN REMODELING COMPLEX IN MELANOMA DEVELOPMENT

by

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Abstract

Human cutaneous malignant melanoma is an aggressive form of skin cancer, for its ability to metastasize rapidly and its resistance to conventional radiotherapy and chemotherapy. The mammalian SWI/SNF complex mediates ATP-dependent chromatin remodeling processes that are critical for transcriptional regulation, control of cellular processes, and involvement in DNA repair. Therefore, aberrant expression of SWI/SNF chromatin remodeling complex is involved in cancer development. To investigate the role of SWI/SNF complex in the development of melanoma, we used tissue microarray technology and immunohistochemistry to evaluate the expression of SNF5, the common core subunit, and BRG1, the ATPase subunit, in melanocytic lesions at different stages, and we analyzed the correlation between SNF5 and BRG1 expression and clinicopathologic variables and patient survival. In addition, we also investigated the role of SNF5 in nucleotide excision repair (NER), a type of DNA repair mechanism that removes ultraviolet-induced DNA lesions. We found that reduced SNF5 expression is significantly associated with melanoma progression and a worse patient survival, and that SNF5 is an independent prognostic factor for human melanoma. Furthermore, we showed that downregulation of SNF5 protein level in melanoma cell lines enhanced chemoresistance of melanoma cells. This suggests that SNF5 plays an important role in melanomagenesis and may serve as a promising prognostic marker for melanoma. BRG1 expression, on the other hand, was found to be increased in primary and metastatic melanoma compared to dysplastic nevi. Knockdown of BRG1 in human melanoma cell lines reduced cell proliferation due to G1 phase arrest. This suggests that BRG1 might play a role in the initiation stage of melanomagenesis. As for the role of SWI/SNF complex in NER, our current observation demonstrated that in human keratinocytes, SNF5 is required for efficient removal of CPD and is required for UV-induced histone acetylation. In human melanoma

cells, SNF5 does not seem to play a major role in NER, for it is not required for removal of CPD and UV-induced global chromatin relaxation. Taken together, these data implicate that SWI/SNF complex plays an essential role in melanoma development and may serve as a promising therapeutic target for melanoma.

Preface

Chapter 3 and chapter 4 were co-authored by Ronald Wong and Dr. M. Martinka. Ronald contributed significantly to the experimental designs of *in vitro* studies. Dr. M. Martinka assisted us with scoring the immunohistochemical staining of tissue microarray slides. I designed and performed the majority of the experiments in chapter 3 and 4 (including immunohistochemical staining, tissue microarray analysis, and *in vitro* studies), and I also prepared the whole manuscripts for chapter 3 and 4.

List of publications:

- Hanyang Lin, Ronald P.C. Wong, Magdalena Martinka and Gang Li (2009). Loss of SNF5 Expression Correlates with Poor Patient Survival in Melanoma. *Clinical Cancer Research*. 15(20): 6404-11. With permission to reprint, this work is located in chapter 3.
- Hanyang Lin, Ronald P.C. Wong, Magdalena Martinka and Gang Li (2010). BRG1 Expression Is Increased in Human Cutaneous Melanoma. *British Journal of Dermatology.* In press. With permission to reprint, this work is located in chapter 4.

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Abbreviations

Abbreviation	Definition
6-4PP	Pyrimidine (6-4) pyrimidone photoproducts
AAF-G	Acetylaminofluorene-guanosine
AcH4	Acetylation of histone H4
AJCC	American Joint Committee on Cancer
Apaf	Apoptotic protease activating factor
ARF	Alternate open reading frame
ATP	Adenosine triphosphate
BAF	BRG1- or BRM- associated factors
Bcl-2	B-cell lymphoma 2
BER	Base excision repair
BRCA1	Breast cancer 1, early onset
BRG1	Brahma-related gene-1
BRM	Brahma
CDC	Cell division cycle
CDK	Cyclin-dependent kinases
CDKN 1A	Cyclin-dependent kinase inhibitor 1A
CPD	Cyclobutane pyrimidine dimmers
DSB	Double-strand break
DTIC	Dacarbazine
ES	Epithelioid sarcomas
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
hHR23B	Human Rad23 homolog
HIC1	Hypermethylated in cancer 1 protein
IFN	Interferon-α2B
IHC	Immunohistochemistry
ING	Inhibitor of growth
MEF	Murine embryonic fibroblasts
MITF	Microphthalmia-associated transcription factor
MNase	Micrococcal nuclease
MRT	Malignant rhabdoid tumors
NER	Nucleotide excision repair
N-RAS	Neuroblastoma RAS viral oncogene homolog
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
RGP	Radial growth phase
RhoA	Ras homolog gene family, member A
siRNA	Small interfering RNA
SRB	Sulforhodamine B
SWI/SNF	SWItch/Sucrose NonFermentable
TMA	Tissue microarray
TMZ	Temozolomide
UV	Ultraviolet

VGP	Vertical growth phase
Wnt	Wingless-type MMTV integration site family
XPC	Xeroderma pigmentosum, complementation C

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Most importantly, I would like to thank my supervisor Dr. Gang Li. He has shown me the way of doing quality research. His teaching, critical remarks, and constructive comments have made me develop critical scientific thinking. I would especially like to thank his diligence. He always replies my e-mail promptly and always gives me feedback on my manuscripts and scholarship applications in a surprisingly efficient manner.

Dedication

This work is dedicated to my very supportive and caring parents, my loving sisters, and the twos I have only loved.

1. General Introduction

1.1 Cutaneous malignant melanoma

1.1.1 Melanoma incidence and epidemiology

Cutaneous malignant melanoma is the most aggressive and lethal form of skin cancer. Although melanoma accounts for only 4% of all dermatological cancers, it is responsible for 80% of deaths from skin cancer (Miller and Mihm 2006). Melanoma is curable through early diagnosis and surgical excision (Balch *et al.,* 2001), but up to 20% of patients will develop metastatic tumor due to its invasive and metastatic properties (Houghton and Polsky 2002). Consequently, metastatic melanoma patients have a poor prognosis, with median survival of only 6 to 10 months and <5% of the patients surviving over 5 years (Balch *et al.,* 2001; Manola *et al.,* 2000; Sampsel and Barbera-Guillem 2004).

The incidence of melanoma is rising steadily and rapidly in the last four decades in the countries with a large light-skin population. In the United States, melanoma is the fifth most common cancer in men and the sixth most common cancer in women (Jemal *et al.,* 2009). In Canada, melanoma is the eighth most common cancer in both men and women (Canadian Cancer Society: Canadian Cancer Statistics 2009). Australia has the highest incidence rates worldwide. In Queensland, Australia, the cumulative incidence in the population older than 50 years is 1 in 19 for men and 1 in 25 for women (Goldstein and Tucker 1993). In 2009, an estimated 5,000 new cases of melanoma are expected to be diagnosed, with 940 patients expected to die from the disease in Canada (Canadian Cancer Society: Canadian Cancer Statistics 2009). The incidence of melanoma increases

at a faster rate than any other neoplasm, with the exception of lung cancer in women (Brochez and Naeyaert 2000; Howe *et al.,* 2001). In the United States, the incidence increased by 270% from 1973 to 2002 (Ries *et al.,* 2000). In Canada, the incidence increased in males and females by 1.6% and 1.0% per year respectively between 1996 and 2005 (Canadian Cancer Society: Canadian Cancer Statistics 2009).

The incidence of melanoma is strongly affected by race and geographic location (Thompson *et al.,* 2005). Light-skin populations have an approximately 10-fold greater risk of developing cutaneous melanoma than black, Asian, or Hispanic populations (Ries *et al.,* 2000). This presumably relates to the higher sensitivity of white skin to sun-exposure. In fact, the primary environmental risk factor for melanoma is exposure to sunlight (Jemal *et al.,* 2001). Therefore, males are approximately 1.5 times more likely to develop melanoma than females, and the most common areas are the back for men, and the arms and legs for women (Boyle *et al.,* 1995; Tsai *et al.,* 2005). In addition, a history of childhood severe episodic sunburn has been shown to be an important risk factor for melanoma (Whiteman *et al.,* 2001).

1.1.2 Melanocyte transformation and melanoma progression

Melanocytes are specialized pigmented cells that produce melanins predominantly in the skin and the eyes. Cutaneous melanocytes originate from the embryonic neural crest. They migrate through the dermal layer of the skin to their niche at the dermal/epidermal junction during fetal development (Takeda *et al.,* 2007). At the basal layer of dermal/epidermal junction, melanocytes are found interspersed among every 5 keratinocytes (Jimbow 1995). The basal keratinocytes give rise to multiple layers of

overlying epidermal keratinocytes, which form the major component of the epidermis. Terminally differentiated keratinocytes form a dead layer of cells that protects skin from foreign matter (Eckert and Rorke 1989). Melanocytes play a key role in protecting keratinocytes from cellular damage by supplying them with melanin (Lin and Fisher 2007). Melanins are photo-protective pigments that absorb ultraviolet (UV) radiation from the sun to reduce free-radical generation and DNA damage of the Skin (Agar and Young 2005). Each melanocyte delivers melanins to approximately 40 keratinocytes via dendritic processes extending from melanocyte cell body (Yamaguchi *et al.,* 2007). Consequently, people lacking functional melanocytes in pigmentary disorders such as vitiligo and albinism are hypersensitive to UV radiation (Boissy and Nordlund 1997).

Proliferation of melanocytes is strictly controlled and regulated by keratinocytes. However, mutations in critical growth regulatory genes, production of autocrine growth factors, and loss of adhesion receptor all contribute to disrupted intracellular signaling in melanocytes, allowing them to escape from regulation by keratinocytes (Haass *et al.,* 2004). As a result, melanocytes can proliferate and spread, leading to development of nevi, or commonly known as moles (Kuchelmeister *et al.,* 2000). Nevi have well-defined borders, they do not interfere with surrounding cutaneous structures, and they appear as nest-like melanocytic cell clusters confined in the dermal/epidermal junction. Some nevi are classified as “atypical” or “dysplastic”, referring to the presence of abnormal clinical or histological features. They exhibit a degree of architectural and cytologic atypia, but are not considered to be malignant. Dysplastic nevi are considered potential melanoma precursors, but this hypothesis remains controversial, as many dysplastic nevi never develop into melanoma (Hussein 2005). Deregulated nevi can progress to the radial

growth phase (RGP) melanoma, where the cells spread through the epidermis. RGP cells can then progress to the vertical growth phase (VGP), a more dangerous stage in which the cells invade the underlying dermis and have metastatic potential. Downward vertical growth facilitates direct contact with vascular and lymphatic vessels providing a route for melanoma metastasis (Gray-Schopfer *et al.,* 2007). It is important to note that not all melanomas pass through these individual phases. RGP and VGP can both develop directly from melanocyte or nevi, and both can progress directly to metastatic melanoma (Miller and Mihm 2006).

1.1.3 Melanoma staging and subtypes

Melanoma staging has been established by the American Joint Committee on Cancer (AJCC), which incorporates important prognostic indicators, including Breslow thickness, Clark's level, ulceration, number of lymph nodes, and anatomic site of metastases (Balch *et al.,* 2009). The AJCC system classifies melanoma into four stages: stage I, tumor thickness ≤2.0 mm without ulceration; stage II, tumor thickness >2.0 mm; stage III, regional metastasis to lymph nodes; and stage IV, distant metastasis (Markovic *et al.,* 2007). Breslow thickness measures tumor depth in millimeters from top of the granular layer of the epidermis to the deepest point of tumor involvement (Breslow 1970). Clark level refers to deepest portion of the skin invaded by tumor: level I, the outermost epidermis; level II, the papillary dermis; level III, between the papillary and reticular dermis; level IV, the reticular dermis; and level V, the subcutaneous fat (Clark *et al.,* 1969). Ulceration is defined as the absence of an intact epidermis overlying a major

portion of the primary melanoma based on microscopic examination of the histologic sections (Balch *et al.,* 2009).

Subtypes of melanoma are distinguished by clinical and pathologic growth patterns. The four major subtypes of melanoma are superficial spreading melanoma, lentigo maligna, nodular melanoma, and acral lentiginous melanoma. Superficial spreading melanoma is the most common subtype and represents approximately 70% of melanomas. Lentigo maligna is the least common subtype and represents for 4-15% of melanomas. Nodular melanoma represents 15%, and acral lentiginous melanoma represents 10% of melanomas (Runkle and Zaloznik 1994).

1.1.4 Current melanoma treatments

The standard therapy for localized melanoma is surgical resection. If surgery is not an option, radiation therapy is usually used for locally advanced melanoma (Berk 2008). Most primary melanomas are curable by surgical resection. However, the relatively high rate of recurrence in subgroups of patients suggests the need for adjuvant therapy (Treisman and Garlie 2010). Unfortunately, in the past three decades, there has been little progress on the chemotherapeutic development for advanced melanoma. Dacarbazine (DTIC) is one of the only Food and Drug Administration (FDA)-approved cytotoxic agents that produce partial response in only 13-20% of patients, with a median duration of 5-6 months (Chapman *et al.,* 1999). Many attempts have been made to combine DTIC with other chemo- or immuno- therapeutic agents, such as platinum analogues, microtubule inhibitors, and temozolomide (TMZ). However, no consistent survival advantage has emerged from such studies (Eggermont and Kirkwood 2004). In

fact, 2-agent regimens show little superiority to single-agent DTIC (Atallah and Flaherty 2005; Chapman *et al.,* 1999; Rosenberg *et al.,* 1999).

There has been some success in developing adjuvant immuno-based therapy. A study from the German Dermatologic Cooperative Group treated 444 patients with stage III disease with low-dose subcutaneous interferon-α2B (IFN), and found a significant improvement in overall survival in patients treated with IFN compared with observation (59% vs 42%, P=0.0045) (Garbe *et al.,* 2008). This and other data with IFN have led to FDA approval of the agent for the adjuvant therapy for patients with intermediate- or high-risk melanoma, and has now been accepted as a standard agent for patients with melanoma larger than 4 mm or with lymph node involvement (Treisman and Garlie 2010). Despite this, the recommendation is still not universal, and many other investigators have not accepted IFN and have concluded that there is currently still no standard adjuvant therapy following resection of melanoma (Kefford 2003; Lens and Dawes 2002). In conclusion, the development of novel therapeutic strategies for this disease is urgently needed.

1.2 Melanomagenesis

1.2.1 UV radiation

Many epidemiological studies suggest that exposure to UV radiation from sunlight is the primary environmental factor in melanoma development (Gilchrest *et al.,* 1999; Oliveria *et al.,* 2006). UV radiation can be further subdivided into UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm) wavebands. UVC, though highest energy, contributes to the development of skin cancers the least, since it is prevented from reaching the surface of the earth by ozone which blocks wavelength below 300 nm. UVA is the most predominant UV source in sunlight to which humans are exposed, but its role in skin cancer, including melanoma, is not nearly as well documented as UVB and is currently controversial. UVA is poorly absorbed by DNA, but still produces oxidative lesions by photosensitization mechanisms (Cadet *et al.,* 1997). UVB, on the other hand, is considered to represent the most carcinogenic waveband historically associated with skin cancer risk. Nucleic acids and proteins both absorb light within the UVB range, peaking at 260 and 280 nm, respectively. UVB causes two types of DNA lesions: the 6-4 photoproducts (6-4PPs), generated between adjacent pyrimidine residues, and cyclobutane pyrimidine dimmers (CPDs), formed specifically between adjacent thymine or cytosine residues. The oxidative lesions induced by UVA are repaired by base excision repair (BER), and 6-4PPs and CPDs induced by UVB are repaired by nucleotide excision repair (NER) (Jhappan *et al.,* 2003).

UV radiation causes genetic changes in the skin, impairs cutaneous immune function, increases the local production of growth factors, and induces the formation of DNA-damaging reactive oxygen species that affect keratinocytes and melanocytes

(Gilchrest *et al.,* 1999; Thompson *et al.,* 2005). Melanocytes contain several prosurvival and antiapoptotic proteins. Once damaged, melanocytes may inhibit UV-induced apoptosis and allow the heavily damaged melanocytes to survive (Klein-Parker *et al.,* 1994; Morales-Ducret *et al.,* 1995; Plettenberg *et al.,* 1995). Failure to repair the UV-induced DNA lesions leads to the accumulation of genetic mutations and eventually contributes to the malignant transformation of melanocytes, which in turn contributes to the development of melanoma.

1.2.2 Deregulation of apoptosis and chemoresistance

Melanoma is notoriously chemoresistant. Several *in vitro* and *in vivo* studies indicated that the chemoresistant characteristic of melanoma cells is either intrinsic or acquired during application of chemo-drugs (Kern *et al.,* 1997; Osieka 1984; Schadendorf *et al.,* 1994). Although the molecular mechanism for drug resistance in melanoma is still poorly understood, it appears that the low therapeutic efficacy in melanoma is likely due to its inability to induce apoptosis (Soengas and Lowe 2003). Apoptosis is the mechanism of programmed cell death by which the body rids itself of damaged, genetically defective, or superfluous cells (Willis and Adams 2005). Apoptosis plays an important role in anticancer protection by preventing the accumulation of damaged cells with tumorigenic potentials. Melanoma cell lines have been shown to be highly resistant to drug-induced apoptosis (Li *et al.,* 1998). Melanoma tumors have also been shown to exhibit lower rates of spontaneous apoptosis than other solid tumor types (Gilchrest *et al.,* 1999). In addition, it is widely believed that melanocytic cells acquire a

resistance to apoptosis during the progression from normal melanocyte to melanoma cell (Alanko *et al.,* 1999; Hussein *et al.,* 2003).

In general, cells respond to chemotherapy by activating the p53 tumor suppressor and Bcl-2 proapoptotic factors. These will block Bcl-2 and other prosurvival factors, release cytochrome *c*, and finally lead to apoptosis (Soengas and Lowe 2003). *p53* was the first tumor suppressor gene linked to apoptosis. p53 mutations were found to be associated with advanced tumor stage, chemoresistance, and poor patient survival in a broad spectrum of human malignancies (Lowe and Lin 2000; Schmitt and Lowe 1999; Wallace-Brodeur and Lowe 1999). However, melanomas display a low frequency of p53 mutations despite their extreme chemoresistance (Albino *et al.,* 1994). One explanation for this paradox is that p53 does not influence treatment response in this tumor type. Alternatively, p53 function could be disabled by lesions that disrupt other components of the pathway. Disruption of the upstream p53 regulator p14ARF can functionally replace p53 loss during melanomagenesis (Chin *et al.,* 1997; You *et al.,* 2002). Disruption of apoptosis downstream of p53 may also alleviate pressure to mutate p53 and simultaneously decrease drug sensitivity (Schmitt *et al.,* 2002a; Schmitt *et al.,* 2002b). For example, Apaf-1, a proapoptotic effector downstream of p53-induced apoptosis pathway, is frequently found downregulated in melanomas (Soengas *et al.,* 2001). Therefore, the fate of a cell does not solely depend on p53 status but depends on a net balance of other p53 associated proapoptotic effectors and antiapoptotic effectors, which will determine whether or not apoptosis is ultimately engaged.

1.2.3 Deregulation of cell cycle progression

The molecular mechanism of transition from primary melanoma to metastatic melanoma is not entirely clear. Recent gene profiling studies shed light onto the complexity of pathogenesis of melanoma progression, and suggest that an interaction between cell cycle signaling, adhesion pathways, and epithelial-mesenchymal transition program appears to be critical in the development of this metastatic disease. DNA microarray technology has shown certain promise in identifying molecular patterns and gene abnormalities, which may be responsible for progression of melanomas. All microarray analyses invariably point at two groups of genes involved in tumorigenicity: cell cycle genes and adhesion genes (Danilov *et al.,* 2008). In fact, prior to gene profiling studies, several signaling pathways involved in cell cycle progression had been implicated in melanocyte transformation and melanoma progression. These include B-RAF/N-RAS and PTEN/Akt, CDKN2A ($p16^{INK4}$), p53/Apaf-1, cyclin D1/Cdk4, and Wnt5a (Dissanayake *et al.,* 2007; Haluska and Ibrahim 2006; Kalinsky and Haluska 2007; Monzon *et al.,* 1998; Weeraratna *et al.,* 2002). Microarray analyses further showed that metastatic melanomas have Cdc6, Cdk1, mitosin, Cdc28 protein kinase 2, CDC2/Cdk1, cyclin B1, and CDKN1a upregulated (Alonso *et al.,* 2007; Jaeger *et al.,* 2007; O'Brien *et al.,* 2007; Zhu *et al.,* 1995). Taken together, the primary abnormalities of melanoma may be found in deregulated cell cycle, whereas impairment of cell adhesion becomes responsible for these abnormalities by association. To be able to succeed in controlling this metastatic disease, all signaling pathways associated in cell cycle progression may need to be targeted at the same time.

1.3 SWI/SNF chromatin remodeling complex

1.3.1 Biological functions of SWI/SNF complex

SWI/SNF chromatin-remodeling complex is a 2-MDa multisubunit complex first identified in yeast and highly conserved among eukaryotes (Peterson 1996). The ATP-dependent chromatin remodeling factors utilize the energy from ATP hydrolysis to alter the contact between histone and DNA. Several models have been proposed to account for the ability of SWI/SNF to modify chromatin structure (Hassan *et al.,* 2001; Peterson and Workman 2000). Models include ATP-dependent movement of histone octamers in *cis* along the DNA, transfer of histone octamers from one nucleosomal array to another, or replacement of nucleosomal histones (Saha *et al.,* 2006; Smith and Peterson 2005). The net result is an altered structure that is hypersensitive to nuclease digestion and increased affinity for transcription factors (Schnitzler *et al.,* 1998). The SWI/SNF complex, thus, was predominantly believed to play a role in regulating gene expression because massive *in vitro* and *in vivo* data demonstrated that both transcription activators and repressors physically interact with the complex to recruit the complex to target genes in order to remodel the chromatin structure (Becker and Horz 2002; Holstege *et al.,* 1998; Natarajan *et al.,* 1999; Neely *et al.,* 1999; Yudkovsky *et al.,* 1999). Other than transcriptional regulation, the complex is critical for control of cellular processes, including differentiation and proliferation, and involvement in DNA repair by altering the accessibility of UV-damaged DNA-binding proteins to DNA lesions (Dinant *et al.,* 2008; Klochendler-Yeivin *et al.,* 2002; Martens and Winston 2002; Roberts and Orkin 2004; Sif *et al.,* 2001). SWI/SNF complex does not regulate an exclusive signalling pathway. It serves as a fundamental component of various essential and often unrelated pathways.

The complex incorporates either the Brahma-related gene-1 (BRG1), also known as SMARCA4, or Brahma (BRM) as the ATPase subunit. BRG1 is approximately 74% identical to BRM (Khavari *et al.,* 1993). In addition to BRG1 or BRM, the complex contains 8-10 subunits, referred to as BRG1- or BRM- associated factors, BAFs. BAF47, also known as SNF5/INI1/SMARCB1, is the core subunit and is present in all complexes. The stoichiometry of the SWI/SNF complex has not been resolved, but it is likely that no single complex contains all the subunits (Fig. 1.1). For example, an individual SWI/SNF complex contains either BRM or BRG1, but not both. The BRM/BAF complexes are structurally distinct from BRG1/BAF complexes (Wang *et al.,* 1996a; Wang *et al.,* 1996b). The BRG1/BAF complexes are further divided into those that contain BAF250 or BAF180 protein (Wang 2003). Whether these complexes are functionally distinct is still under debate and active investigation.

1.3.2 SWI/SNF complex and cancer

The SWI/SNF complex plays a major role in a variety of cellular processes and DNA repair. It is not surprising that loss of SWI/SNF subunits has been reported in a number of malignant cell lines and tumors, and a large number of experimental observations suggest that this complex functions as a tumor suppressor. Much genetic evidence has defined *SNF5* as a tumor suppressor gene in humans and mice. In mice, homozygous deletion of SNF5 is embryonic lethal, and heterozygous mice are predisposed to develop tumors due to loss of heterozygosity (Guidi *et al.,* 2001; Klochendler-Yeivin *et al.,* 2000; Roberts *et al.,* 2000). In humans, homozygous inactivating mutations or deletions of the SNF5 gene are associated with malignant

rhabdoid tumors (MRTs) (Biegel *et al.,* 2002; Sevenet *et al.,* 1999; Versteege *et al.,* 1998). Indeed, SNF5 has been convincingly defined as a *bona fide* tumor suppressor (Biegel *et al.,* 2002; Guidi *et al.,* 2001; Klochendler-Yeivin *et al.,* 2000; Roberts *et al.,* 2000; Sevenet *et al.,* 1999; Versteege *et al.,* 1998). Similarly, much genetic evidence has indicated that *BRG1* functions as a potential tumor suppressor gene. Knockout mice studies revealed that $BRG1^{-/-}$ is embryonically lethal, and heterozygous $BRG1^{+/-}$ is viable and cancer prone (Bultman *et al.,* 2000). Also, reduced BRG1 expression was found in breast, ovarian, lung, and bladder cancer cell lines, and was associated with poor prognosis for non-small cell lung cancer patient survival (Decristofaro *et al.,* 2001; Fukuoka *et al.,* 2004; Reisman *et al.,* 2002; Reisman *et al.,* 2003; Wong *et al.,* 2000).

Other then acting as a tumor suppressor, SNF5 is also involved in other ceullar processes. Studies have shown that SNF5 exhibits an anti-proliferative activity because SNF5 re-expression in MRT cell lines induces G1 cell cycle arrest associated with an increase in p16INK4a and repression of E2F and Cyclin D1 (Oruetxebarria *et al.,* 2004; Tsikitis *et al.,* 2005; Versteege *et al.,* 2002). SNF5 is also required for various differentiation pathways, including hepatocyte differentiation *in vivo* (Gresh *et al.,* 2005), and neural, or adipocyte differentiation *in vitro* (Albanese *et al.,* 2006; Caramel *et al.,* 2008a). This suggests that SNF5 is able to regulate the balance between cell proliferation and differentiation. Furthermore, SNF5 is shown to play a role in cell migration. MRT's invasive property is dramatically reduced upon SNF5 expression in a RhoA-dependent manner (Caramel *et al.,* 2008b). In addition, SNF5 is involved in apoptosis. Inactivation of SNF5 would cause murine embryonic fibroblasts (MEFs) and HeLa cells to undergo G1 cell cycle arrest, followed by a p53-dependent apoptotic response (Kato *et al.,* 2007).

UV-induced apoptosis is also enhanced in cells lacking SNF5 (Klochendler-Yeivin *et al.,* 2006). Finally, SNF5 regulates mitotic checkpoint control to mantain chromosomal stability (Vries *et al.,* 2005).

BRG1 plays critical roles in the control of cell proliferation. Most studies demonstrated that BRG1 acts as a tumor suppressor because a large number of tumor cells have either silenced or mutated *BRG1* genes (Decristofaro *et al.,* 2001; Glaros *et al.,* 2007; Wong *et al.,* 2000), and re-expression of BRG1 inhibits growth of such cells in culture (Khavari *et al.,* 1993; Muchardt *et al.,* 1998). This is because retinoblastoma (Rb) family members requires BRG1 activity to regulate cell cycle (Dunaief *et al.,* 1994; Strober *et al.,* 1996). Rb protein is one of the major cell cycle regulators that controls the G1/S transition as well as progression through S phase (Sicinski *et al.,* 1995). BRG1 contains the Rb-binding motif LxCxE and interacts with Rb family members p107 and p130 (Dahiya *et al.,* 2000; Dunaief *et al.,* 1994; Strober *et al.,* 1996). In vitro studies showed that constitutively active Rb protein does not induce G1 arrest in cells lacking BRG1, and re-expression of BRG1 reconstitutes Rb growth inhibition in such cells (Reisman *et al.,* 2002; Strobeck *et al.,* 2000; Strobeck *et al.,* 2001; Strobeck *et al.,* 2002; Zhang *et al.,* 2000). Other than interacting with Rb protein, BRG1 is also required by a number of tumor suppressors to be functional. BRG1 is found to be recruited by HIC1 tumor suppressor to control cell growth (Zhang *et al.,* 2009). LKB1, a tumor suppressor found deleted in lung cancers, requires BRG1 for growth arrest (Marignani *et al.,* 2001). BRCA1, a tumor suppressor associated with breast and ovarian cancers, was found to co-purify with BRG1 (Bochar *et al.,* 2000).

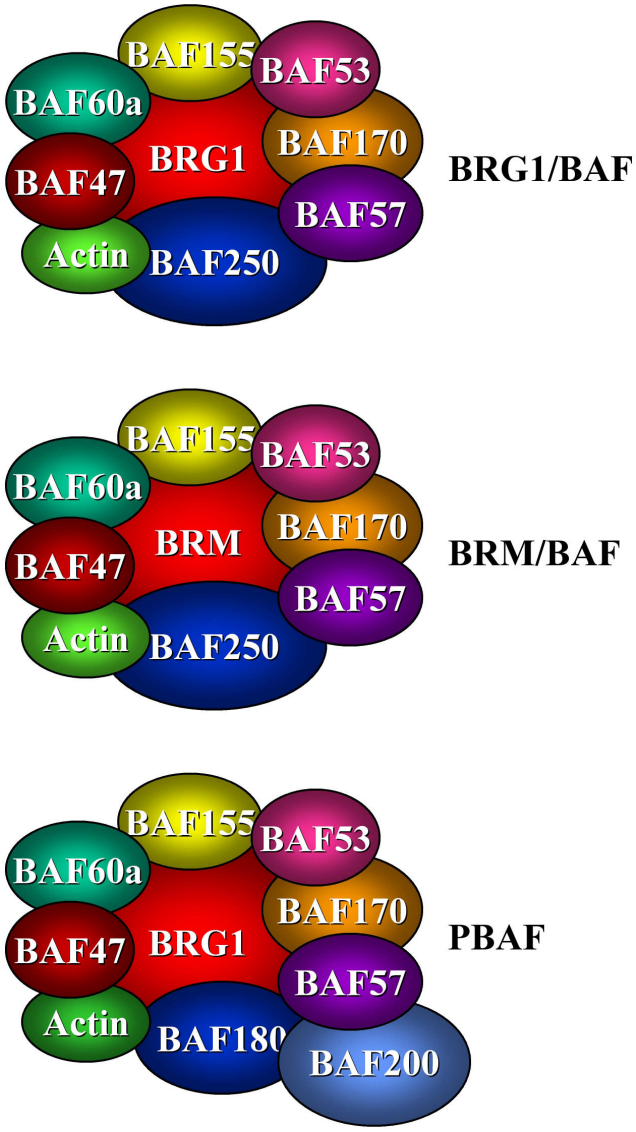
1.3.3 SWI/SNF complex and DNA repair

UV radiation from sunlight is the major environmental factor for the development of melanoma, and it causes two types of DNA lesions: cyclobutane pyrimidine dimmers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP). These two types of DNA lesions are removed by nucleotide excision repair (NER) (Gillet and Scharer 2006). There are two subpathways of NER. Global genome NER (GG-NER) removes damage from the entire genome. Transcription-coupled NER (TC-NER) removes damage in the transcribed strand of active genes (de Laat *et al.,* 1999; Hanawalt 2002). The mechanism of both subpathways is very similar. They consist four sequential steps: damage detection, excision of the damaged segment, repair synthesis, and ligation to restore the intact DNA (de Laat *et al.,* 1999; Sancar 1996; Sugasawa 2006). Because the DNA-damaged recognition proteins, XPC-hHR23B complex, and DNA repair factors are relatively big, (up to thirty polypeptides) every step of NER is believed to require the relaxation of compact structure of chromatin (Smerdon 1991). It has been implicated that the relaxation process, also known as chromatin remodeling, is allowed either through post-translational modifications of histones, or through SWI/SNF chromatin remodelling complex, which disrupts the interaction between DNA and histones (Chodaparambil *et al.,* 2006; Eberharter *et al.,* 2005).

It has long been known that the relaxed DNA has better NER efficiency than more condensed DNA. NER occurs more frequently in naked DNA than in chromatin, and is more efficient in the linker region of chromatin than in the nucleosome (Hara *et al.,* 2000; Smerdon and Thoma 1990; Wang *et al.,* 1991; Wellinger and Thoma 1997). Several studies have indicated that the SWI/SNF complex plays an essential role in NER.

The complex enhances CPD repair in nucleosome as measured by lesion specific phage T4 endonuclease (Lee *et al.,* 2004). In yeast, it facilitates the removal of 6-4PPs and enhances accessibility to repair factors *in vivo* (Hara and Sancar 2002; Yu *et al.,* 2005). More recently, Gong *et al.* demonstrated that the core subunits of SWI/SNF complex, SNF5 and SNF6, interact with NER lesion detection complex Rad4-Rad23 (yeast homologues of human XPC-hHR23B complex) *in vivo* (Gong *et al.,* 2006). The same group also showed that in mammalian cells, the depletion of SNF5 and BRG1 results in defects in CPD repair in HeLa and primary fibroblast cells (Gong *et al.,* 2008). These data suggest pivotal roles of SWI/SNF complex in NER. However, it is still unclear if SWI/SNF complex directly affects NER in response to UV damage in mammalian cells. The molecular mechanisms of the roles of SWI/SNF complex in NER in mammalian cells warrant further investigations.

Fig. 1.1. The different components in the mammalian SWI/SNF complex. The complex incorporates either BRG1 or BRM as the ATPase subunit. In addition to BRG1 or BRM, the complex contains 8-10 subunits, referred to as BRG1- or BRM- associated factors, BAFs. BAF47 is the common core subunit present in all complexes. The BRG1/BAF complexes are further divided into those that contain BAF250 or BAF180 protein. *Modified from Reisman et al. (2009).*



1.4 Objectives

The objectives of this study are to evaluate the prognostic significance of the SWI/SNF complex and its role in melanoma tumorigenesis and progression. We used tissue microarray technology and immunohistochemistry to evaluate the expression of SNF5 and BRG1 in different stages of human melanocytic lesions and correlate the expression pattern with the clinicopathological parameters. Since understanding the molecular mechanisms of how UV-damaged DNA is repaired can contribute to the design of effective strategies for skin cancer prevention, we also examined the role of SNF5 in nucleotide excision repair in human keratinocytes.

2. Materials and Methods

2.1 Construction of tissue microarray (TMA)

Formalin-fixed, paraffin-embedded tissues from 66 dysplastic nevi, 118 primary melanomas, and 53 metastatic melanomas were used for this study. All specimens were obtained from 1990 to1998 archives of the Department of Pathology. Vancouver General Hospital. The use of human skin tissues was approved by the Clinical Research Ethics Board of the University of British Columbia and was done in accordance with the Declaration of Helsinki guidelines. The most representative tumor area was carefully selected and marked on the hematoxylin-eosin-stained slide. The TMAs were assembled using a tissue-array instrument (Beecher Instruments, Silver Spring, MD). Duplicate 0.6-mm-thick tissue cores were taken from each biopsy specimen. Two composite high-density TMA blocks containing 126 and 111 cases from a total of 237 patients were designed. Using a Leica microtome (Leica Microsystems Inc, Bannockburn, IL), multiple 4-μm sections were cut and transferred to adhesive coated slides using regular histology procedures. Hematoxylin and eosin were used to stain one section from each TMA. Other sections were kept at room temperature for immunohistochemical staining.

2.2 Immunohistochemistry of TMA

TMA slides were dewaxed at 55°C for 20 min followed by three 5-min washes with xylene. The rehydration of tissues was performed by 5-min washes in 100%, 95%, and 80% ethanol and distilled water. Antigen retrieval was performed by heating the

samples at 95°C for 30 min in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 30 min. After 30 min blocking with the universal blocking serum (Dako Diagnostics, Missisauga, ON, Canada), the sections were incubated with monoclonal mouse anti-SNF5 antibody and polyclonal rabbit anti-BRG1 antibody (1:200 dilution and 1:100 dilution, respectively) at 4°C overnight. The sections were then incubated for 30 min each with a biotin-labeled secondary antibody and then streptavidin-peroxidase (Dako Diagnostics). The samples were developed using 3,3'-diaminobenzidine substrate (Vector Laboratories, Burlington, ON, Canada) and counterstained with hematoxylin. Dehydration was then performed following a standard procedure and the slides were sealed with coverslips. Negative controls were performed by omitting the primary antibodies during the primary antibody incubation.

2.3 Evaluation of immunostaining

The evaluation of immunohistochemical staining was examined by one dermatopathologist and two other observers simultaneously, and a consensus was reached for each core. Microarray positivity for SNF5 was defined as any detectable nuclear or cytoplasmic staining (Massoumi *et al.,* 2009). The positive reaction of BRG1 was scored into four grades according to the intensity of the staining: 0, 1+, 2+, and 3+. The percentages of BRG1–positive cells were scored into five categories: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). In the cases with a discrepancy between duplicated cores, the higher score from the two tissue cores was taken as the final score. The multiplication of the intensity and percentage scores is used as the final staining

score. The final immunoreactive score (IRS) for the staining was defined as negative (0), weak (1-4), moderate (5-8) or strong (9-12).

2.4 Statistical analysis of TMA immunostaining

Statistical analysis was performed with SPSS 11.5 software (SPSS, Chicago, IL, USA). The χ^2 test was used to compare the quantitative differences of staining in different stages of melanoma progression. The association between SNF5 and BRG1 staining and the clinicopathologic parameters of the primary melanoma patients, including age, gender, tumor thickness, ulceration, histological subtype, location and Clark's level, was also evaluated by χ^2 test. The Kaplan-Meier method and log-rank test were used to evaluate the correlation between SNF5 and BRG1 expression and patient survival. Cox regression model was used for multivariate analysis. A *P* value of <0.05 was considered significant.

2.5 Cell lines and cell culture

All melanoma cell lines (MMRU, MEWO, and Sk-mel-3), HaCaT cell line, and HEK293 cell line were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada) in the presence of 100 units/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin B. Normal human epidermal melanocytes were cultured in melanocyte basal medium supplemented with 0.4% bovine pituitary extract, 50 ng/ml amphotericin B, 1.0 ng/ml basic fibroblast growth factor, 50µg/ml gentamicin, 5.0 µg/ml hydrocortisone, 5.0 µg/ml

bovine insulin, and 10 ng/ml phorbol 12-myristate 13-acetate (PromoCell, Heidelberg, Germany). All cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

2.6 siRNA and transfection

Cells were grown to 50% confluency before small interfering RNA (siRNA) transfection. Non-specific control siRNA or SNF5 and BRG1 siRNA (Qiagen, Missisauga, ON, Canada) was transfected by siLentFect Lipid Reagent (Bio-Rad, Missisauga, ON, Canada) according to the manufacturer's instructions. Twenty hours after transfection, the medium containing transfection reagents was removed. The cells were rinsed twice with PBS and incubated in fresh medium. Forty-eight hours after transfection, cells were lysed for Western blot analysis, and subjected to *in vitro* studies.

2.7 Antibodies

The primary antibodies included rabbit anti-BRG1 rabbit anti-cyclin B1, rabbit anti-cyclin D1, mouse anti-p21, mouse anti-p27 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-SN5 (Abcam, Cambridge, MA, USA), mouse anti-*β*-actin (Sigma, St. Louis, MO, USA), rabbit anti-histone H4, rabbit anti-acetylated histone H4 (Upstate, Charlottesville, VA), mouse anti-cyclobutane pyrimidine dimer (CPD), and mouse anti–pyrimidine(6-4)pyrimidinone (6-4PP) (MBL, Naka-ku Nagoya, Japan).

2.8 Western blot analysis

Cells were harvested and washed thrice with PBS. Whole cell proteins were extracted by triple detergent buffer (20 mM Tris-Cl [pH 8.0], 150 mM NaCl. 0.1%

sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing protease and phosphatase inhibitors (Roche Diagnostics, Laval, QC, Canada) (Wang and Li 2006). The samples were then sonicated, incubated on ice for 10 min, and centrifuged at 12,000× *g*. The supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad, Mississauga, Ontario, Canada). Proteins were separated on SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Mississauga, Ontario, Canada). The PVDF was blocked with 5% skim milk for 30 min at room temperature before incubating with primary antibodies prepared in 5% bovine serum albumin overnight at 4°C. Blots were washed three times in PBS containing 0.04% Tween-20 (PBST) for 5 min each and then incubated with secondary antibodies labelled with the near-infrared fluorescent dyes IRDye 800 or IRDye 680 for one hour at room temperature. The signals were detected with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) (Chin *et al.,* 2005).

2.9 SRB cell survival and cell proliferation assays

Cells were transfected with SNF5 or BRG1 siRNA for 48 hours, and then treated with or without various doses of doxorubicin, etoposide, and camptothecin (Sigma, St. Louis, MO, USA), or UV irradiation. Twenty-four hours after drug treatments or UV irradiation, cell survival was determined by sulforhodamine B (SRB) staining (Li *et al.,* 1998). Briefly, cells were washed with PBS and fixed with 10% trichloroacetic acid at 4°C for one hour. Residual acid was washed with tap water. The cells were then air-dried and stained with 0.4% SRB (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada)

dissolved in 1% acetic acid for 30 min at room temperature, followed by destaining with 1% acetic acid. For quantification, the bound dye was dissolved in 10 mM Tris (pH 10.5) and measured by reading absorbance at 550 nm.

2.10 Drug-induced apoptosis analysis with flow cytometry

Cells were transfected with SNF5 siRNA for 48 hours and then treated with 0.25 μg/ml doxorubicin, 5 μM etoposide, and 25 nM camptothecin. Twenty-four hours after drug treatments, cells were collected by trypsinization and pelleted by centrifugation at 2,000× *g* for 5 min. After washing twice with cold PBS, cells were fixed with 70% ethanol at 4°C for one hour, and cells were stained with 40 μg/ml propidium iodide (PI) in hypotonic fluorochrome buffer (0.1% Triton X-100, 0.1% sodium citrate, and 25 μg/ml RNase A) for 30 min. Samples were then analyzed using a FACSCanto flow cytommeter (BD Biosciences, Mississauga, Ontario, Canada). About 10,000 cells of each sample were analyzed each time. To examine the apoptotic cells after drug treatments, both floating and adherent cells were collected for the flow cytometry analysis. Cells in Sub-G1 phase were considered apoptotic.

2.11 Cell cycle analysis with flow cytometry

Twenty-four hours after transfection with BRG1 siRNA, MMRU and Sk-mel-3 cells were serum-starved for 24 hours to synchronize cells at G_1 phase. The cells were then released from G_1 by rinsing them thrice in 10% FBS-containing DMEM and incubating them in this media containing 50 ng/ml nocodazole for different time points.

The efficiency of synchronization and the cell progression after release was assessed by flow cytometry as described in section 2.10.

2.12 UV irradiation

UV irradiation was performed by removing culture media and exposing cells to controlled doses of UVB (290–320 nm) light using a bank of four unfiltered FS40 sunlamps (Westinghouse, Bloomfield, NJ, USA) or UVC (254 nm) light using an UltraLum crosslinker (Claremont, CA, USA). The intensity of the UV light was measured by an IL 700 radiometer fitted with a WN 320 filter and an A127 quartz diffuser (International Light, Inc., Newburyport, MA, USA).

2.13 Host-cell-reactivation assay

The pRL-CMV plasmid, encoding *firefly luciferase* gene*,* was irradiated with UVC at 1,600 J/m^2 using UltraLum crosslinker. The irradiated or non-irradiated *firefly* plasmids and pRL-CMV plasmid encoding *renilla luciferase* gene, serving as a transfection control, co-transfected to cells at a ratio of 20 to 1. Forty hours after transfection, cells were lyzed by passive lysis buffer, and reporter enzyme level was analyzed with a luciferase assay kit (Promega, Madison, WI, USA). All reactions were performed in triplicates. The percentage luciferase activity was calculated as the fraction of irradiated plasmid over non-irradiated control plasmid and normalized with the *renilla* plasmid. Plasmids were transfected into cells at 50-70% confluency using the Effectene transfection kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's procedures.

2.14 Slot-western analysis of cyclobutane pyrimidine dimers (CPD) and 6–4 photoproducts (6-4PP)

The slot-western analysis was performed as previously described (Li *et al.,* 1996). Briefly, 1 µg of genomic DNA extracted from HaCaT or HEK293 cells was suspended in 50 µl of 6× standard saline citrate and filtered onto a nitrocellulose membrane through a slot-blot apparatus (Schleicher and Schuell, Keene, NH, USA). The membrane was incubated *in vacuo* at 80 °C for 30 min, blocked with 5% milk at 4°C for 30 min and sequentially hybridized with mouse anti-CPD or mouse anti-6-4PP overnight at 4°C. Infrared IRDye-labeled secondary antibody was applied to the blot for one hour at room temperature. The signals were detected with Odyssey Infrared Imaging System as described in section 2.8.

2.15 Micrococcal nuclease digestion assay

Microccocal nuclease digestion assay was performed as previously described (Smith *et al.,* 1998). Briefly, cells were resuspended in lysis buffer (10 mM Tris–HCl [pH 8.0], 10 mM MgCl2, 1 mM DTT) with 0.1% NP-40. Nuclei were pelleted by 1,200× *g* for 10 min and washed twice with the lysis buffer. For digestion, nuclei were resuspended in the micrococcal nuclease digestion buffer (10 mM Tris–HCl [pH 8.0], 50 mM NaCl, 300 mM sucrose, 3 mM MgCl2). Samples were then digested with 1 U of micrococcal nuclease for 5 min at 37 °C (Sigma). The reaction was stopped with 1% SDS and 20 mM EDTA. Samples were centrifuged at 12,000×g for 15 min. DNA was then extracted twice with phenol/chloroform, dissolved in distilled water and electrophoresed in 1.5% agarose

gel and visualized under UV light after ethidium bromide staining. pCI-ING1b transfected cells served as a positive control (Kuo *et al.,* 2007).

2.16 Histone extraction

Cells were resuspended in lysis buffer (10mMTris–HCl [pH 7.5], 1 mM MgCl2, 1 mM DTT) with 0.5% NP-40, protease and phosphatase inhibitors, and TSA. Nuclei were pelleted by 1,200× *g* for 5 min and washed twice with the lysis buffer at 4°C. The histones were extracted by resuspending the insoluble fraction in extraction solution (0.5 M HCl or 0.25 M H_2SO_4, 10% glycerol, 0.1 M 2-mercaptoethylamine-HCl). The samples were then sonicated, incubated on ice for 1 hour, and centrifuged at 12,000× *g*. The supernatants were collected, neutralized with 2N NaOH, and subjected to Western-blot analysis as described in chapter 2.8.

2.17 Statistical analysis for *in vitro* studies

The data were presented as the mean ± SD. Statistical analyses were performed using student's *t* test was used. A *P* value of <0.05 was considered significant.

3. Loss of SNF5 Expression Correlates with Poor Patient Survival in Melanoma[1]

3.1 Rationale and hypothesis

SNF5 is the core subunit of mammalian SWI/SNF complex. Much genetic evidence has defined *SNF5* as a tumor suppressor gene in humans and mice. In mice, homozygous deletion of SNF5 is embryonic lethal, and heterozygous mice are predisposed to develop tumors due to loss of heterozygosity for SNF5 (Guidi *et al.,* 2001; Klochendler-Yeivin *et al.,* 2000; Roberts *et al.,* 2000). In humans, homozygous inactivating mutations or deletions of the *SNF5* gene are associated with malignant rhabdoid tumors (MRTs) (Biegel *et al.,* 2002; Sevenet *et al.,* 1999; Versteege *et al.,* 1998). Furthermore, studies showed that SNF5 exhibits an anti-proliferative activity in MRTs because SNF5 overexpression leads to a G1 cell cycle arrest associated with an increase in p16INK4a, E2F, and Cyclin D (Oruetxebarria *et al.,* 2004; Tsikitis *et al.,* 2005; Versteege *et al.,* 2002). The invasive property of MRT is also dramatically reduced upon SNF5 expression in a RhoA-dependent manner (Caramel *et al.,* 2008b).

A number of studies using immunohistochemistry focused on the ATPase subunit of SWI/SNF complex, BRG1 and BRM. Reduced BRG1 and BRM expression was found in breast, lung, and prostate cancer cell lines, and associated with poor prognosis for the patients with non-small cell lung cancer (Decristofaro *et al.,* 2001; Fukuoka *et al.,* 2004; Reisman *et al.,* 2002; Reisman *et al.,* 2003; Sun *et al.,* 2007; Wong *et al.,* 2000). However, few studies have focused on SNF5, the core subunit of SWI/SNF complex, in cancers. Although similar phenotypes of *SNF5* and *BRG1* knockout mice might suggest

[1] A version of this chapter has been published. Lin H, Wong RP, Martinka M, Li G (2009). Loss of SNF5 expression correlates with poor patient survival in melanoma. *Clin Cancer Res* **15:** 6404-6411.

that both genes function in partially redundant pathways, it has been shown that loss of *SNF5* does not affect the expression of BRG1 target genes or the assembly of SWI/SNF complex (Doan *et al.,* 2004). This indicates that SNF5 and BRG1 might not have the exact same functions. In order to investigate the role of SNF5 in the development of melanoma, we used tissue microarray technology and immunohistochemistry to evaluate SNF5 expression in different stages of human melanocytic lesions.

3.2 Results

3.2.1 Clinicopathological features of TMAs

Fifty-one cases of dysplastic nevi, 88 cases of primary melanomas, and 48 cases of metastatic melanomas are evaluated for SNF5 staining. As shown in Table 3.1, for the 88 cases of primary melanomas, there were 54 males and 34 females, with age ranging from 21 to 93 years (median = 57). For primary melanoma staging, Breslow thickness and Clark's level were used as criteria for evaluating SNF5 expression: 26 tumors were ≤1.0 mm, 30 tumors were 1.01-2.0 mm, 14 tumors were 2.01-4.0 mm, and 18 were > 4.0 mm; 20 tumors were at Clark's level II, 25 tumors were at level III, 31 tumors were at level IV, and 12 tumors were at level V. Ulceration was observed in 18 cases. For the histological subtype, 13 tumors were nodular melanomas, 39 tumors were superficial spreading melanomas, 15 tumors were lentigo maligna melanomas, and 21 tumors were non-specified. 17 melanomas were located in sun-exposed sites (head and neck), and 71 were located in sun-protected sites (other locations).

3.2.2 Reduced SNF5 expression correlates with melanoma progression

To investigate if SNF5 expression is changed in pigmented melanocytic lesions, immunohistochemistry staining of dysplastic nevi, primary melanoma, and metastatic melanoma were done using TMA technique (Fig. 3.1). Positive SNF5 staining was recorded in 98%, 90%, and 81% of the biopsies in dysplastic nevi, primary melanoma, and metastatic melanoma respectively (Fig. 3.1E). Melanocytes in all five cases of normal skin tissues and five benign nevi showed strong positive SNF5 staining (Fig. 3.1A and B). Although the majority of the biopsies from different stages of melanocytic lesions

have positive staining, negative SNF5 staining was significantly increased in metastatic melanoma when compared to dysplastic nevi ($P = 0.005$, χ^2 test). However, there is no significant difference in SNF5 staining between dysplastic nevi and primary melanoma ($P = 0.069$, χ^2 test), or between primary melanoma and metastatic melanoma ($P = 0.161$, χ^2 test). We have also compared SNF5 expression in ten melanoma cell lines with melanocyte and found that seven melanoma cell lines have reduced SNF5 expression (Fig. 3.2).

3.2.3 Correlation of SNF5 expression with clinicopathological parameters

Because Clark's level of invasion is known to be an important prognostic marker for patients with primary melanoma, we first studied if SNF5 expression correlates with Clark's level. We found a significant difference in SNF5 expression between Clark's level V and Clark's level II (Fig. 3.3A; $P = 0.019$, χ^2 test). All the tumors in Clark's level II had positive SNF5 staining, while only 75% of tumors in Clark's level V had positive SNF5 expression.

As UV radiation is the main environmental factor for melanoma formation, we analyzed the SNF5 staining in sun-exposed or sun-protected sites. Reduced positive SNF5 staining significantly correlated with the location of primary melanomas (Fig. 3.3B; $P = 0.044$, χ^2 test). While 93% of tumors from sun-protected sites (trunk, arm, leg and feet) had positive SNF5 staining, SNF5 staining was reduced to 76% in tumors from sun-exposed sites (head and neck). We did not find significant correlations between

SNF5 expression with other clinicopathologic variables, including Breslow tumor thickness, subtype, ulceration, or patient's age or gender (Table 3.1).

3.2.4 Reduced SNF5 expression correlates with poor patient survival

To evaluate whether reduced SNF5 staining in human primary and metastatic melanomas correlate with a worse prognosis, Kaplan-Meier survival curves were constructed using overall or disease-specific 5-year survival to evaluate the biopsies with positive SNF5 staining to those with negative SNF5 staining. Our data revealed that positive SNF5 staining correlated with both overall and disease-specific 5-year survival in primary melanomas (Fig. 3.4A and B; $P = 0.016$ and $P = 0.049$, respectively, log-rank test). The correlation is also significant when primary and metastatic melanoma cases are combined for the analysis; both overall and disease-specific 5-year survival rates are significantly better for patients with positive SNF5 expression in their tumor tissues compared to those with negative SNF staining (Fig. 3.4C and D; $P = 0.029$ and $P = 0.040$, respectively, log-rank test).

In addition, we examined whether positive SNF5 expression is an independent prognostic marker for melanoma. We performed a multivariate analysis including SNF5 expression, age, gender, tumor thickness, ulceration, location, and subtype for 88 primary melanomas. We found that similar to tumor thickness and presence of ulceration, which have been widely accepted as independent prognostic factors for melanoma patient survival, SNF5 expression is also an independent prognostic factor for both overall (relative risk, 5.145; 95% confidence interval, 1.48-17.89; $P = 0.010$; Table 3.2) and disease-specific 5-year survival (relative risk, 4.637; 95% confidence interval, 1.15-

18.63; $P = 0.031$; Table 3.2). Our results clearly indicate that negative SNF5 expression in either primary or metastatic melanoma is associated with poor prognosis, suggesting that SNF5 reduction may serve as a molecular prognostic marker for this aggressive disease.

3.2.5 Resistance to chemotherapeutic drugs increases in SNF5 knockdown melanoma cell lines

A major obstacle in treating melanoma is its resistance to drug-induced apoptosis (Soengas and Lowe 2003). Because negative SNF5 expression strongly correlates with poor patient survival, we investigated the involvement of SNF5 in chemoresistance of melanoma cells. We first transiently transfected MMRU and MEWO melanoma cells with SNF5 siRNA or control siRNA. Forty-eight hours after transfection, cells were harvested for Western blot analysis (Fig. 3.5A) or subjected to cell survival and apoptosis assays (Fig. 3.5B and C). Western blot indicated that at least 75% knockdown of SNF5 protein expression in both MMRU and MEWO cells transfected with SNF5 siRNA compared to those transfected with control siRNA. In SRB cell survival assay, cell survival of both MMRU and MEWO SNF5 knockdown cells treated with doxorubicin, etoposide, and camptothecin was significant higher than those of control cells (Fig. 3.5B). However, we did not observe a significant difference in cell proliferation upon SNF5 knockdown in both MMRU and MEWO cells when compared to the control cells without drug treatment (Fig. 3.6). To investigate if reduced chemosensitivity of SNF5 knockdown cells is due to reduced apoptosis, we performed flow cytometry analysis in parallel with SRB cell survival assay. Our data showed that both drug-treated MMRU and MEWO

SNF5 knockdown cells have significantly lower sub G1 populations compared to control cells (Fig. 3.5C). Thus, these data indicate that downregulation of SNF5 expression reduces drug-induced apoptosis in melanoma cells. This might suggest that melanoma resistance to chemotherapy is at least partially due to the reduction of SNF5 protein level.

Table 3.1. SNF5 staining and clinicopathological characteristics of 88 primary melanomas.

	SNF5 staining			
Variables	**Negative**	**Positive**	**Total**	***P* value***
Age				
≤57	3 (7%)	41 (93%)	44	0.291
>57	6 (14%)	38 (86%)	44	
Gender				
Male	7 (13%)	47 (87%)	54	0.286
Female	2 (6%)	32 (94%)	34	
Tumor thickness (mm)				
≤1.0	2 (8%)	24 (92%)	26	0.288‖
1.01-2.0	2 (7%)	28 (93%)	30	
2.01-4.0	2 (14%)	12 (86%)	14	
>4.0	3 (17%)	15 (83%)	18	
Ulceration				
Present	1 (6%)	17 (94%)	18	0.463
Absent	8 (11%)	62 (89%)	70	
Tumor subtype				
Nodular	0 (0%)	13 (100%)	13	0.187**
Superficial spreading	3 (8%)	36 (92%)	39	0.484**
Lentigo maligna	3 (20%)	12 (80%)	15	0.170**
Unspecified	3 (14%)	18 (86%)	21	0.482**
Site‡				
Sun-protected	5 (7%)	66 (93%)	71	0.044
Sun-exposed	4 (24%)	13 (76%)	17	
Clark's level				
II	0 (0%)	20 (100%)	20	0.019§
III	3 (12%)	22 (88%)	25	
IV	3 (10%)	28 (90%)	31	
V	3 (25%)	9 (75%)	12	

* χ^2 test.

‖ Comparison of thickness ≤2 mm and >2 mm.

** Comparison of the specified group with all the other groups.

‡ Sun-protected sites: trunk, arm, leg and feet; Sun-exposed sites: head and neck.

§ Comparison of Clark's level II and V.

Fig. 3.1. Correlation between SNF5 expression and melanoma progression. *A-D,* Representative images of SNF5 immunohistochemical staining. *A*, positive staining in normal skin (arrows indicate melanocytes); *B*, positive staining in benign nevus; *C*, positive SNF5 staining in dysplastic nevus; and *D*, negative SNF5 staining in primary melanoma. Magnification, x400. *E*, reduced SNF5 expression correlates with melanoma progression. A significant difference of SNF5 staining was observed between dysplastic nevi (*DN*) and metastatic melanoma (*MM*) ($P = 0.005$, χ^2 test).

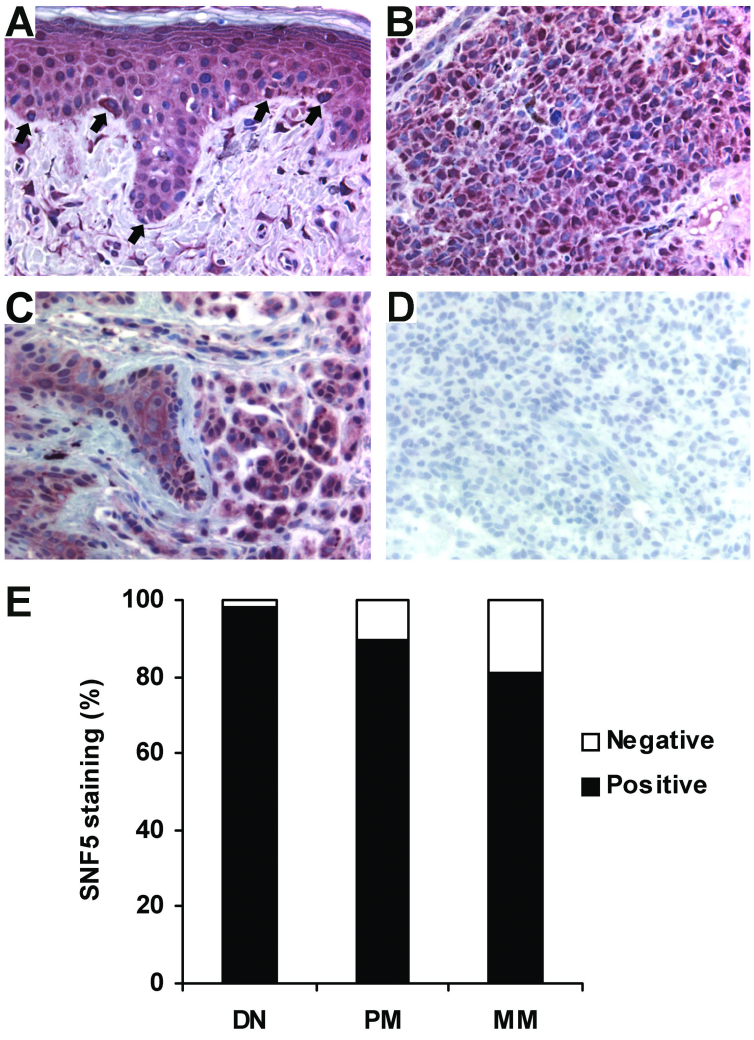


Fig. 3.2. Reduced SNF5 expression in melanoma cell lines. Melanocyte and ten melanoma cell lines were cultured in DMEM at 37°C, and subjected to whole cell protein extraction and Western blot. Compared to melanocyte, seven out of ten melanoma cell lines showed reduced SNF5 expression.

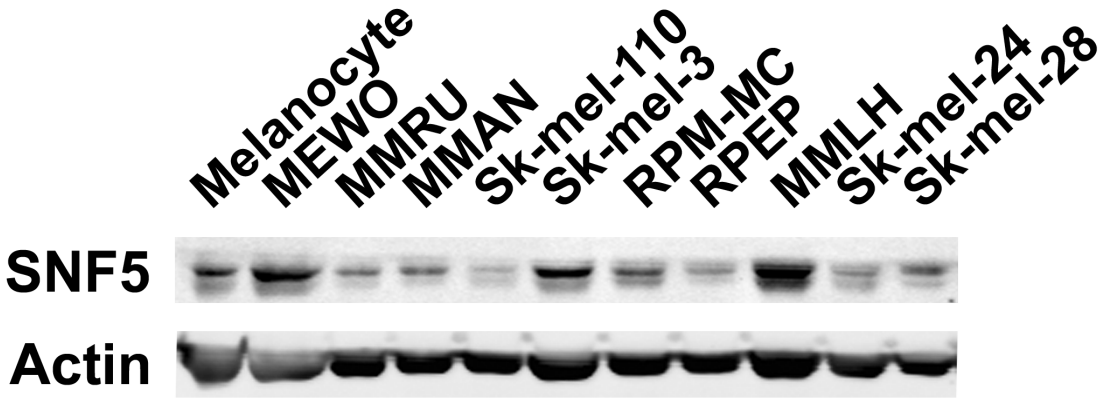


Fig. 3.3. Correlation between SNF5 expression and other clinicopathologic parameters in primary melanoma. *A*, reduced SNF5 expression is correlated with Clark's level (P = 0.019, χ^2 test, comparison between level II and V). *B*, reduced SNF5 expression is correlated with sun-exposed tumor sites (P = 0.044, χ^2 test).

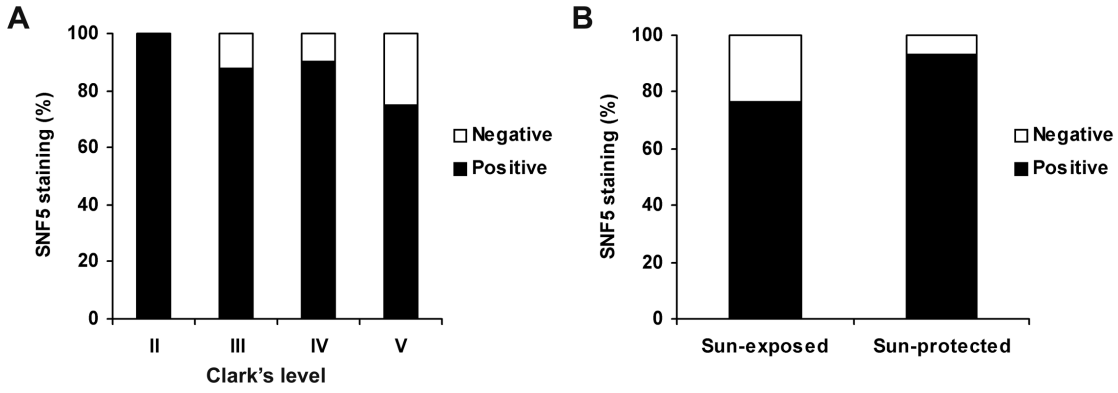


Fig. 3.4. Kaplan-Meier survival analyses of melanoma patients. Patients with negative SNF5 expression have a significantly worse 5-year survival than those with positive SNF5 expression. *A*, overall survival for primary melanoma patients ($P = 0.016$, log-rank test). *B*, disease-specific survival for primary melanoma patients ($P = 0.041$, log-rank test). *C*, overall survival for all melanoma patients (including primary and metastatic cases) ($P = 0.029$, log-rank test). *D*, disease-specific survival for all melanoma patients ($P = 0.040$, log-rank test). *Cum.*, cumulative.

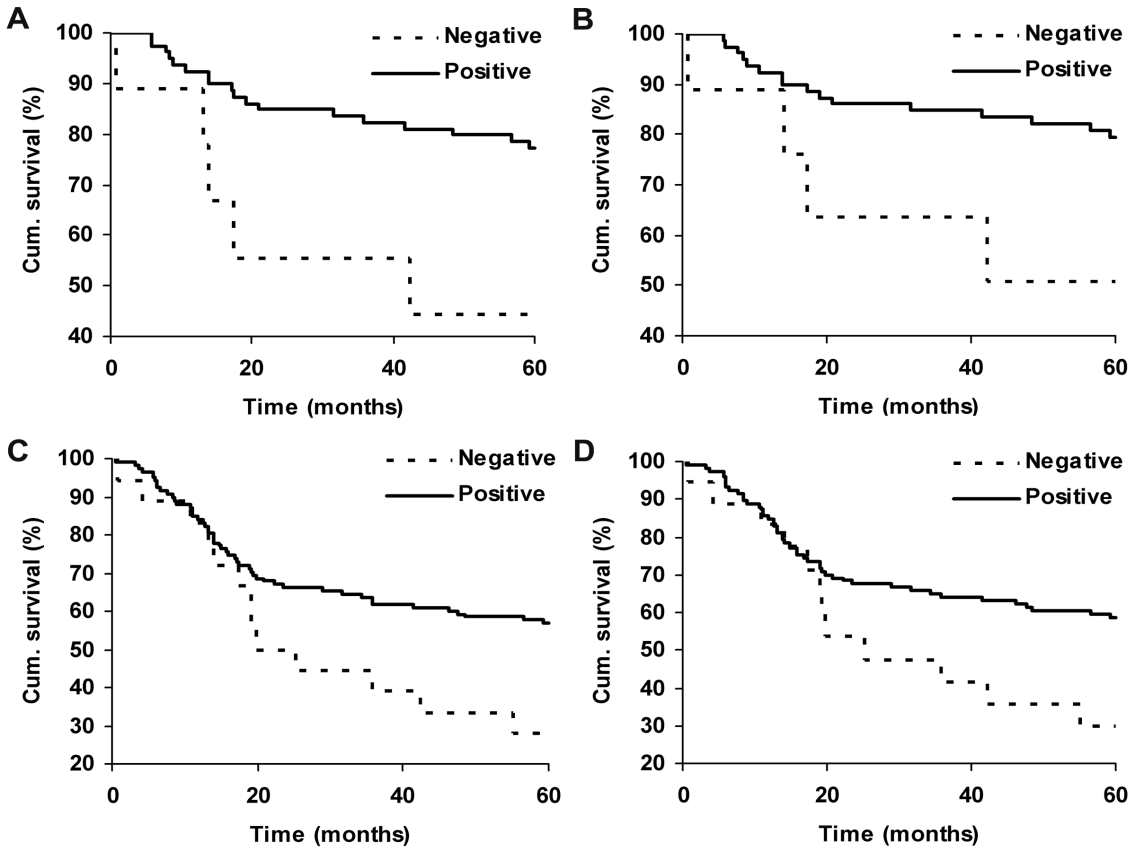


Table 3.2. Cox regression analyasis of SNF5 expression and other clinicopathological characteristics on 5-year survival of 88 primary melanoma patients.

Variable*	Overall survival			Disease-specific survival		
	Relative risk	**95% CI†**	***P* value**	**Relative risk**	**95% CI**	***P* value**
SNF5	5.145	1.48 to 17.89	0.010	4.637	1.15 to 18.63	0.031
Age	0.775	0.28 to 2.12	0.620	0.870	0.29 to 2.57	0.802
Gender	3.215	1.29 to 8.00	0.012	2.804	1.06 to 7.44	0.038
Thickness	0.296	0.09 to 0.94	0.039	0.233	0.06 to 0.87	0.030
Ulceration	0.293	0.10 to 0.85	0.024	0.243	0.08 to 0.76	0.015
Location	0.705	0.26 to 1.93	0.497	0.601	0.21 to 1.71	0.340
Subtype	0.476	0.13 to 1.81	0.276	0.531	0.14 to 2.07	0.362

*Coding of variables: SNF5 was coded as 1 (negative), and 2 (positive). Age was coded as 1 (≤57 years), and 2 (>57 years). Gender was coded as 1 (female), and 2 (male). Thickness was coded as 1 (≤2 mm), and 2 (>2 mm). Ulceration was coded as 1 (absent), and 2 (present). Location was coded as 1 (head and neck), and 2 (others). Subtype was coded as 1 (nodular) and 2 (others).

† CI: confidence interval.

Fig. 3.5. Chemotherapeutic drug-induced apoptosis is repressed in SNF5 knockdown melanoma cells. *A*, SNF5 protein knockdown level detected by Western blot (arrow indicates SNF5 band from previous blot). *B*, drug-induced cell survival by SRB staining. 48 hours after transfection with SNF5 siRNA or control siRNA, MMRU and MEWO cells were treated with doxorubicin, etoposide, or camtothecin. 24 hours after drug treatments, cells were fixed with 10% trichloroacetic acid for 1 hour and quantitated by SRB staining. *C*, drug-induced apoptosis assayed by FACS. 48 hours after transfection with SNF5 siRNA or control siRNA, MMRU and MEWO cells were treated with 0.25 μg/ml doxorubicin (*DOX*), 5 μM etoposide (*EP*), 25 nM camptothecin (*CPT*) for 24 hours or without drug treatment as control (*Ctrl*). Cells were then stained with 40 μg/ml PI for 30 min, and the percentage of apoptotic (sub-G1) cells was measured by flow cytometry. All experiments were carried out in triplicate. Data are shown as mean ± standard error. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

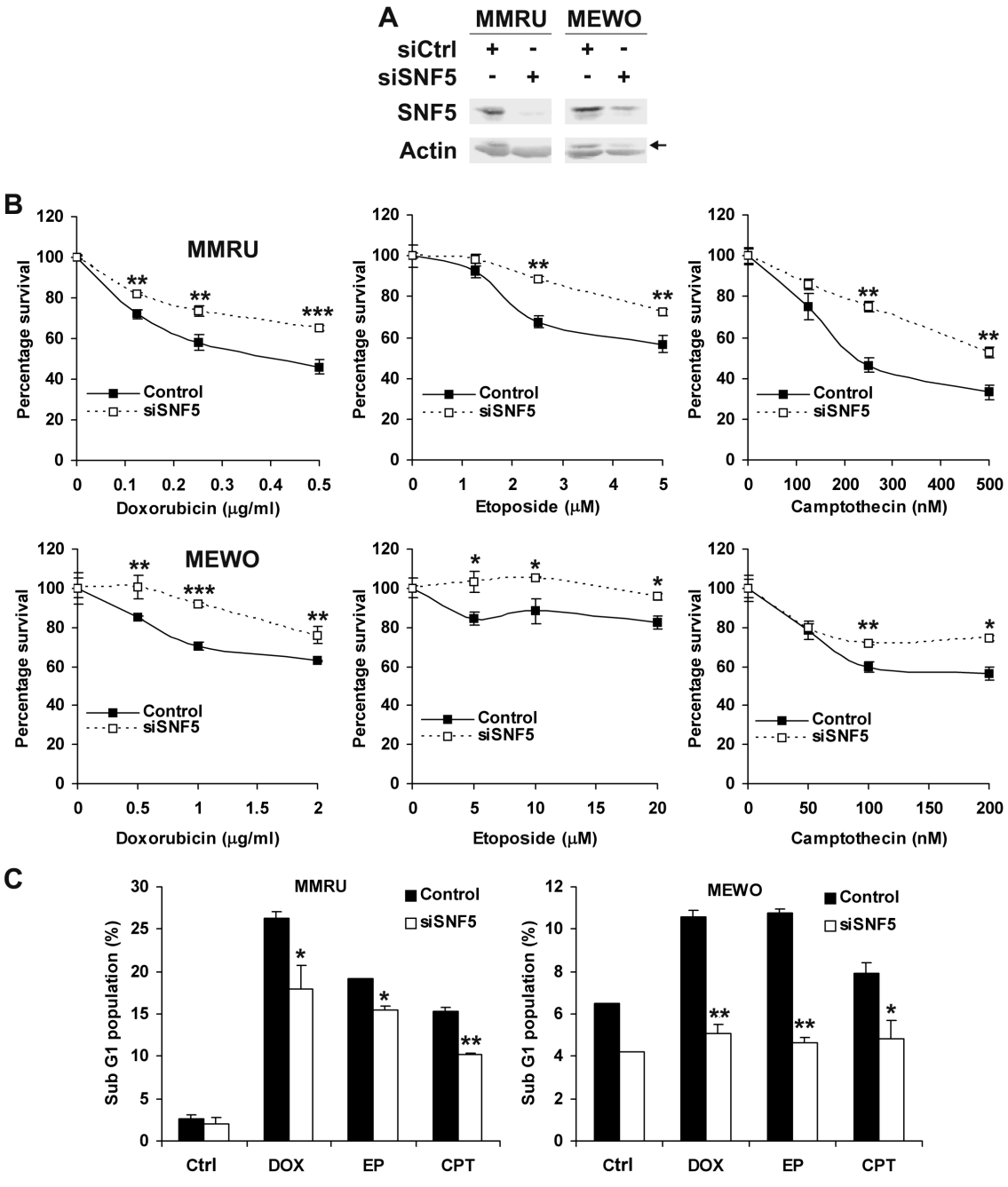
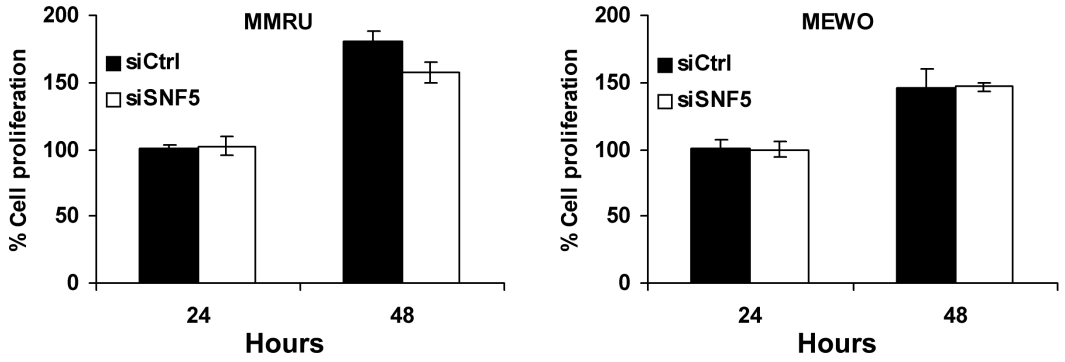

A
MMRU
MEWO
siCtrl
siSNF5
SNF5
Actin
B
MMRU
MEWO
Percentage survival
Control
siSNF5
Doxorubicin (μg/ml)
Etoposide (μM)
Camptothecin (nM)
C
Sub G1 population (%)
Ctrl
DOX
EP
CPT

Fig. 3.6. Cell proliferation is not altered in SNF5 knockdown melanoma cells. 48 hours after transfection with SNF5 siRNA or control siRNA, MMRU and MEWO cells were incubated for 24 and 48 hours. Cells were then fixed with 10% trichloroacetic acid for 1 hour and quantitated by SRB staining. No difference in cell proliferation was observed between control and SNF5 knockdown for both MMRU and MEWO cells.



3.3 Discussion

Several lines of evidence indicate that aberrant expression of the SWI/SNF chromatin remodelling complex proteins is involved in tumorigenesis. Deletion or mutation of the *BRG1* gene was found in lung, breast, prostate, and melanoma cancer cell lines (Becker *et al.,* 2009; Decristofaro *et al.,* 2001; Reisman *et al.,* 2002; Reisman *et al.,* 2003; Wong *et al.,* 2000). The *SNF5* gene was also found inactivated in MRTs and down-regulated in epithelioid sarcomas (ES) (Caramel *et al.,* 2008b; Hornick *et al.,* 2009). Our result reveals a significant correlation between reduced SNF5 expression and melanoma progression (Fig. 3.1E). However, our finding is in contrast with the report by Hornick *et al*. showing SNF5 expression is intact in metastatic melanoma (Hornick *et al.,* 2009). We argue that the discrepancy may be due to lack of proper control and the smaller sample size in their study. They tested only 20 cases of metastatic melanomas without comparison to nevi or primary melanoma biopsies. Therefore, it is inaccurate for these authors to claim that SNF5 expression is indeed intact in metastatic melanoma. Different antibodies used in the immunohistochemical studies could also be the cause of the discrepancy.

Although the difference in SNF5 expression between dysplastic nevi and primary melanoma only reaches a borderline significance (Fig. 3.1E), which might due to insufficient sample size (P = 0.069, χ^2 test), a trend of decreased SNF5 staining in primary melanoma was observed (2% negative SNF5 staining in dysplastic nevi vs 10% in primary melanoma). This suggests a possible role of SNF5 in the initiation of melanoma. It is also worth to note that reduced SNF5 expression correlates with tumor location at sun-exposed sites (Fig. 3.3B), indicating a crucial role of UV radiation in

regulating SNF5 level. Reduction of SNF5 level could be due to UV-induced mutation at the *SNF5* locus at 22q11.2, which is found homozygously deleted or mutated in MRTs (Roberts and Orkin 2004). Besides the role of SNF5 in melanoma initiation, SNF5 has been shown to play a role during nucleotide excision repair (NER) pathway, which repairs UV-induced DNA photolesions (Dinant *et al.,* 2008). In yeast, Snf5 is found to interact with Rad4, a DNA-damaged recognition protein, to enhance NER after UV irradiation (Gong *et al.,* 2006). Therefore, reduced SNF5 expression may enhance accumulation of mutations and genomic instability which drive the subsequent progression of the disease.

In agreement with the correlation between reduced SNF5 expression and melanoma progression, reduced SNF5 expression also correlates with overall and disease-specific 5 year patient survival (Fig. 3.4), and is likely an independent factor predicting patient outcome (Table 3.2). Although the difference in SNF5 expression between primary melanoma and metastatic melanoma did not reach significance (P = 0.161, χ^2 test), a linear trend of decreased SNF5 staining in metastatic melanoma was still observed (negative SNF5 staining in 10% primary melanomas compared with 19% in metastatic melanomas). Furthermore, SNF5 expression was reduced significantly in Clark's level V compared to level II. These stage-specific expression patterns suggest that reduced SNF5 activity might be required for the progression from primary tumors to metastatic tumors. We did not find statistical difference in the correlation between reduced SNF5 expression and Breslow tumor thickness (P = 0.288, χ^2 test). Invasiveness of cancer cells is defined by their ability to migrate and invade through extracellular matrix and neighboring cells (Alexandrova 2008). In fact, the role of SWI/SNF in

melanoma cell migration and invasion is not clear. One study demonstrated that over-expression of SNF5 reduces cell migration properties in a RhoA-dependent manner in MRT cells (Caramel *et al.,* 2008b), while Sun *et al.* showed that over-expression of BRG1 and BRM enhances prostate cancer cell invasion (Sun *et al.,* 2007). Therefore, more studies are required on the role of SNF5 in melanoma cell invasion, for instance, whether SNF5 regulates the expression and activity of matrix metalloproteinases.

Acquired resistance to apoptosis is a hallmark of cancer (Hanahan and Weinberg 2000), which allows cancer cells to escape drug-induced apoptosis and enables the establishment of metastasis. Metastatic melanoma is particularly resistant to conventional radiotherapy and chemotherapy (Buzaid 2004; Terando *et al.,* 2003). A study by Oh *et al*. indicating that doxorubicin-induced apoptosis is repressed in NIH3T3 cells transduced with dominant-negative form of BAF60a (Oh *et al.,* 2008). BAF60a, a mediating subunit of SWI/SNF complex proteins, interacts with p53 and uncoupling of p53 with the SWI/SNF complex resulted in repression of apoptosis and cell cycle arrest. Since melanomas rarely harbour p53 mutations (Chin *et al.,* 1998; Serrone and Hersey 1999), SNF5 might play a role in regulating apoptosis in melanoma cells. Indeed, our *in vitro* data revealed that knocking down SNF5 in melanoma cells with siRNA triggered stronger resistance to chemotherapeutic drugs (Fig. 3.5). Combined with patient survival data, this finding led us to speculate that reduced SNF5 expression may contribute to chemoresistance and thus decreased survival in melanoma patients. Nevertheless, the molecular mechanisms of SNF5 and its relations to BAF60a in apoptosis of melanoma cells after chemodrug treatment warrant further investigation.

In conclusion, the data from this study demonstrate that SNF5 expression is reduced in human cutaneous melanoma and significantly correlated with patient survival, suggesting that SNF5 plays an important role in melanomagenesis and it may serve as a promising prognostic marker and the therapeutic for malignant melanoma.

4. BRG1 Expression is Increased in Human Cutaneous Melanoma[2]

4.1 Rationale and hypothesis

The SWI/SNF complex incorporates either the Brahma-related gene-1 (BRG1) or Brahma (BRM) as the ATPase subunit. The role of BRG1 in cancer development is not well understood. In fact, there is a major discrepancy between the early studies and the recent ones. Early studies support the notion that BRG1 acts as a tumour suppressor because a large number of tumour cells have either silenced or mutated *BRG1* genes (Decristofaro *et al.,* 2001; Glaros *et al.,* 2007; Wong *et al.,* 2000), and re-expression of BRG1 inhibits growth of such cells by interacting with retinoblastoma (Rb) family members, which are responsible for G1 checkpoint and cell growth (Dunaief *et al.,* 1994; Khavari *et al.,* 1993; Muchardt *et al.,* 1998; Sicinski *et al.,* 1995; Strober *et al.,* 1996). Constitutively active Rb protein does not induce G1 arrest in cells that lack BRG1, and re-expression of BRG1 reconstitutes Rb growth inhibition (Reisman *et al.,* 2002; Strobeck *et al.,* 2000; Strobeck *et al.,* 2001; Strobeck *et al.,* 2002; Zhang *et al.,* 2000). Furthermore, knockout mice studies revealed that $BRG1^{-/-}$ is embryonically lethal, and heterozygous $BRG1^{+/-}$ is viable and cancer prone (Bultman *et al.,* 2000). Reduced BRG1 expression was also found in breast, lung, and bladder cancer cell lines (Decristofaro *et al.,* 2001; Fukuoka *et al.,* 2004; Reisman *et al.,* 2002; Reisman *et al.,* 2003; Wong *et al.,* 2000).

However, recent studies suggest otherwise. Wang *et al.* showed that SNF5-deficient human tumour cell lines require residual BRG1-containing SWI/SNF complex for cell growth (Wang *et al.,* 2009). Naidu *et al.* showed that BRG1 cooperates with CBP,

[2] A version of this chapter has been accepted for publication. Lin H, Wong RP, Martinka M, Li G. BRG1 expression is increased in human cutaneous melanoma. *Br J Dermatol* in press.

a histone acetyltransferase protein, to constrain p53 activity and permit cancer cell proliferation (Naidu *et al.,* 2009). In addition, BRG1 is found to interact with the microphthalmia-associated transcription factor (MITF), an oncoprotein, to promote melanoma survival and proliferation (de la Serna *et al.,* 2006; Keenen *et al.* 2010). Finally, increased BRG1 expression was found in gastric cancers and prostate cancers (Sentani *et al.,* 2001; Sun *et al.,* 2007).

We previously demonstrated that reduced SNF5 expression is significantly associated with melanoma progression and a worse patient survival (Lin *et al.,* 2009). However, it was unclear whether the catalytic subunit of the complex, BRG1, plays a role in melanoma development. Many lines of evidence suggest SWI/SNF subunits do not function equally. Loss of SNF5 does not affect the expression of BRG1 target genes or the assembly of SWI/SNF complex (Doan *et al.,* 2004). Also, loss of SNF5 is associated with a small subset of tumour types, whereas loss of BRG1 is observed in a wide variety of solid tumours (Decristofaro *et al.,* 2001; Grand *et al.,* 1999; Manda *et al.,* 2000; Muchardt and Yaniv 2001; Yuge *et al.,* 2000). To investigate the role of BRG1 in melanomagenesis, we employed tissue microarray technology and immunohistochemistry to evaluate BRG1 expression in different stages of human melanocytic lesions.

4.2 Results

4.2.1 Clinicopathological features of TMAs

Forty-eight cases of dysplastic nevi, 90 cases of primary melanomas, and 47 cases of metastatic melanomas are evaluated for BRG1 staining. As shown in Table 4.1, for the 90 cases of primary melanomas, there were 55 males and 35 females, with age ranging from 21 to 93 years (median 57). For primary melanoma invasion, Breslow thickness and Clark's level were used as criteria for evaluating BRG1 expression: 29 tumours were ≤1.0 mm, 30 were 1.01-2.0 mm, 13 were 2.01-4.0 mm, and 18 were >4.0 mm; 22 tumours were at Clark's level II, 25 level III, 31 level IV, and 12 level V. Ulceration was observed in 18 cases. For the histological subtype, 13 tumours were nodular melanomas, 39 superficial spreading melanomas, 17 lentigo maligna melanomas, and 21 non-specified. 18 melanomas were located in sun-exposed sites (head and neck), and 72 in sun-protected sites (other locations).

4.2.2 BRG1 expression is increased in melanoma

To investigate if BRG1 expression is changed in pigmented melanocytic lesions, immunohistochemistry staining was performed in TMA slides containing dysplastic nevi, primary melanoma, and metastatic melanoma biopsies (Fig. 4.1). Moderate-to-strong BRG1 nuclear staining was recorded in 37.5%, 73.3%, and 78.7% of the biopsies in dysplastic nevi, primary melanoma, and metastatic melanoma, respectively (Fig. 4.2). A significant difference in BRG1 staining was observed between dysplastic nevi and primary melanoma ($P < 0.0001$, χ^2 test), and between dysplastic nevi and metastatic melanoma ($P < 0.0001$, χ^2 test). However, there is no significant difference in BRG1

staining between primary melanoma and metastatic melanoma (P = 0.488, χ^2 test). We have also compared BRG1 expression in nine melanoma cell lines with melanocyte and found that seven melanoma cell lines have increased BRG1 expression (Fig. 4.3).

4.2.3 Correlation of BRG1 expression with clinicopathological parameters and melanoma patient survival

Clark's level of invasion, tumour thickness, and ulceration are known to be important prognostic markers for patients with primary melanoma. We studied if BRG1 expression correlates with these markers. We did not find significant correlations between BRG1 expression and Clark's level, tumour thickness, or ulceration (Table 4.1). Interestingly, we found a significant correlation between BRG1 expression and patient age. The moderate-to-strong BRG1 staining was significantly increased in age group >57 yr compared with age group ≤57 yr (P = 0.042, χ^2 test, Fig. 4.4). We did not find significant correlations between BRG1 expression with other clinicopathologic variables, including tumour subtypes and patient gender (Table 4.1). We then examined if BRG1 expression was associated with the survival of primary melanoma patients and all melanoma patients (including primary and metastatic cases) by Kaplan-Meier survival analysis. We divided BRG1 staining into two groups, negative-to-weak and moderate-to-strong. Although no significant correlation of BRG1 expression with 5-year overall and disease-specific patient survival in primary melanoma (Fig. 4.5A and B; P = 0.900 and 0.502, respectively, log-rank test), or in all melanoma cases (Fig. 4.5C and D; P = 0.490 and 0.364, respectively, log-rank test) was found, there is a trend that negative-to-weak BRG1

staining group has a better patient survival. The statistical insignificance might be due to insufficient sample size.

4.2.4 Silencing of BRG1 in melanoma cell lines reduces cell proliferation

Deregulated cell proliferation is a hallmark of cancer (Hanahan and Weinberg 2000). Since BRG1 expression is strongly increased in malignant melanoma compared to dysplastic nevi, we investigated the involvement of BRG1 in melanoma cell proliferation. We first transiently transfected MMRU and Sk-mel-3 melanoma cells with BRG1 siRNA or control siRNA. Twenty-four hours after transfection, cells were harvested for Western blot analysis (Fig. 4.6C) or subjected to cell proliferation assays and cell cycle analysis (Fig. 4.6A and B). Western blot indicated that at least 80% knockdown of BRG1 protein expression in both MMRU and Sk-mel-3 cells transfected with BRG1 siRNA compared with those transfected with control siRNA. In SRB cell proliferation assay, knockdown of BRG1 in both MMRU and Sk-mel-3 cells significantly reduced cell proliferation (Fig. 4.6A). To investigate if the reduced cell proliferation of BRG1 knockdown is due to cell cycle arrest at G1 phase, we performed flow cytometry analysis. Our data showed that knocking down BRG1 in both MMRU and Sk-mel-3 cell lines increased cell population in G1 phase, indicating G1 cell cycle arrest, which lead to decreased S and G2/M phases compared with control cells (Fig. 4.6B). Because cell cycle G1 phase progression is controlled by cyclin D1, and cyclin-dependent kinases (CDK) inhibitors, p21 and p27, and G2/M phase progression is regulated by cyclin B1 (Hochegger *et al.,* 2008; Lee and Yang 2003; Li *et al.,* 2006; Reed 2003), we examined the expression of cyclin D1, cyclin B1, p21, and p27 expression in BRG1 knockdown cells. Our results indicated that

knockdown of BRG1 downregulated cyclin D1 and cyclin B1 expressions, but did not affect p21 and p27 expression (Fig. 4.6C). These data suggest that downregulation of BRG1 expression reduces cell proliferation in melanoma cells, at least partially due to cell cycle arrest at G1 phase and downregulation of cyclin D1 which subsequently lead to decreased G2/M phases and downregulation of cyclin B1.

4.2.5 BRG1 does not affect melanoma resistance to chemotherapeutic drugs

In our previous studies, we showed that SNF5 plays a role in drug-induced apoptosis. Therefore, we investigated if BRG1 is also involved in chemo-resistance of melanoma cells. We first transiently transfected MMRU and MMAN melanoma cells with BRG1 siRNA or control siRNA. Twenty-four hours after transfection, cells were treated with chemotherapeutic drugs. Forty-eight hours after transfection, cells were harvested and subjected to SRB cell survival assays. However, we did not observe a significant difference in the survival assay (Fig. 4.7). This suggests that BRG1 might not play a role in melanoma resistance to chemotherapy and might not have the same function as SNF5.

Table 4.1. BRG1 staining and clinicopathological characteristics of 90 primary melanomas.

Variables	BRG1 staining			Total	*P* value[a]
	Weak	Moderate	Strong		
Age					
≤57	16 (36%)	20 (46%)	8 (18%)	44	<0.05
>57	8 (17%)	22 (48%)	16 (35%)	46	
Gender					
Male	11 (20%)	30 (55%)	14 (25%)	55	>0.05
Female	13 (37%)	12 (34%)	10 (29%)	35	
Tumour thickness (mm)					
≤1.0	9 (31%)	15 (52%)	5 (17%)	29	>0.05
1.01-2.0	9 (30%)	13 (43%)	8 (27%)	30	
2.01-4.0	2 (15%)	6 (46%)	5 (39%)	13	
>4.0	4 (22%)	8 (44%)	6 (33%)	18	
Ulceration					
Present	4 (22%)	10 (56%)	4 (22%)	18	>0.05
Absent	20 (28%)	32 (44%)	20 (28%)	72	
Tumour subtype					
Nodular	6 (46%)	4 (31%)	3 (23%)	13	>0.05
Superficial spreading	12 (31%)	19 (48%)	8 (21%)	39	
Lentigo maligna	3 (18%)	8 (47%)	6 (35%)	17	
Unspecified	3 (14%)	11 (52%)	7 (33%)	21	
Site[b]					
Sun-protected	20 (28%)	32 (44%)	20 (28%)	72	>0.05
Sun-exposed	4 (22%)	10 (56%)	4 (22%)	18	
Clark's level					
II	8 (36%)	10 (46%)	4 (18%)	22	>0.05
III	7 (28%)	11 (44%)	8 (21%)	25	
IV	7 (23%)	14 (45%	10 (32%)	31	
V	2 (17%)	7 (58%)	3 (25%)	12	

[a] χ^2 test for (1+) *versus* moderate-strong (2+, 3+) BRG1 expression.
[b] Sun-protected sites: trunk, arm, leg and feet; Sun-exposed sites: head and neck.

Fig. 4.1. Representative images of BRG1 immunohistochemical staining in human melanocytic lesions. *A* and *D*, weak BRG1 staining in dysplastic nevi. *B* and *E*, moderate BRG1 staining in primary melanoma. *C* and *F*, strong BRG1 staining in metastatic melanoma. Magnification ×100 for *A*-*C*; ×400 for *D*-*F*.

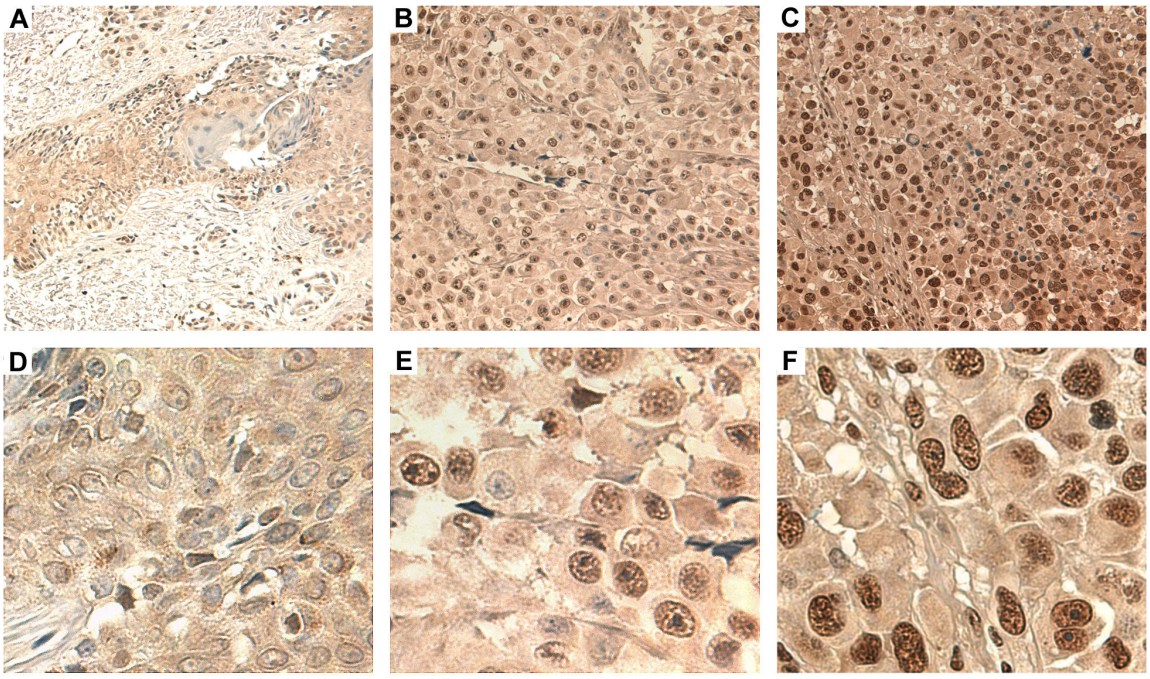


Fig. 4.2. BRG1 expression in different stages of melanocytic lesions. A significant difference of BRG1 staining was observed between dysplastic nevi (DN) and primary melanoma (PM), and between dysplastic nevi and metastatic melanoma (MM) ($P < 0.0001$ for both, χ^2 test).

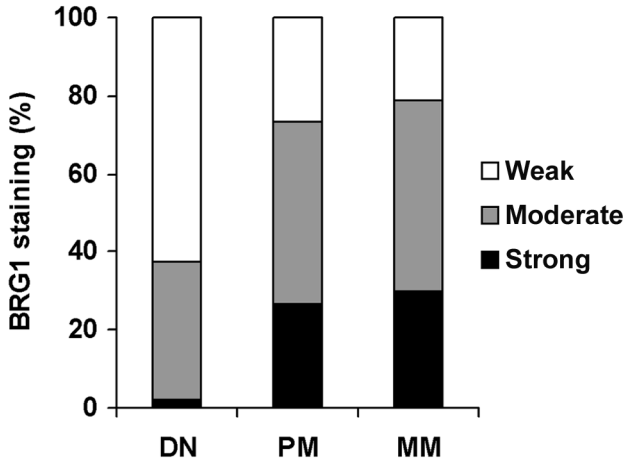


Fig. 4.3. Increased BRG1 expression in melanoma cell lines. Melanocyte and nine melanoma cell lines were cultured in DMEM at 37°C, and subjected to whole cell protein extraction and Western blot. Compared to melanocyte, seven out of nine melanoma cell lines showed increased BRG1 expression.

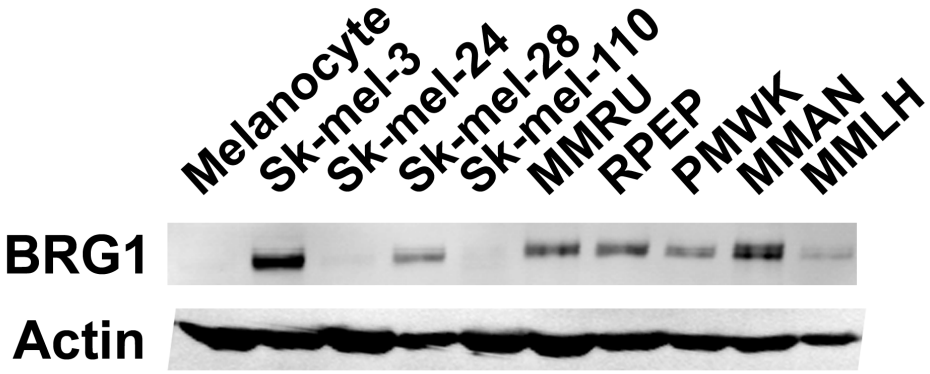


Fig. 4.4. Increased BRG1 expression correlates with patient age in primary melanoma ($P = 0.042$, χ^2 test).

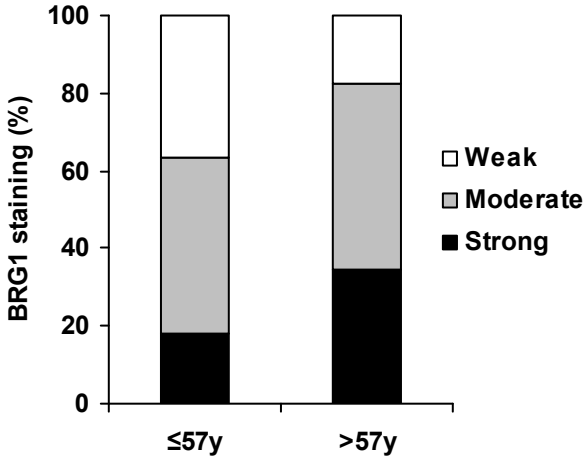


Fig. 4.5. Kaplan-Meier survival analyses of melanoma patients. There is no significant correlation of BRG1 expression with *A*, overall survival for primary melanoma patients ($P = 0.900$), *B*, disease-specific survival for primary melanoma patients ($P = 0.502$), *C*, overall survival for all melanoma patients (including primary and metastatic cases) ($P = 0.490$), and *D*, disease-specific survival for all melanoma patients ($P = 0.364$,). *Cum.*, cumulative.

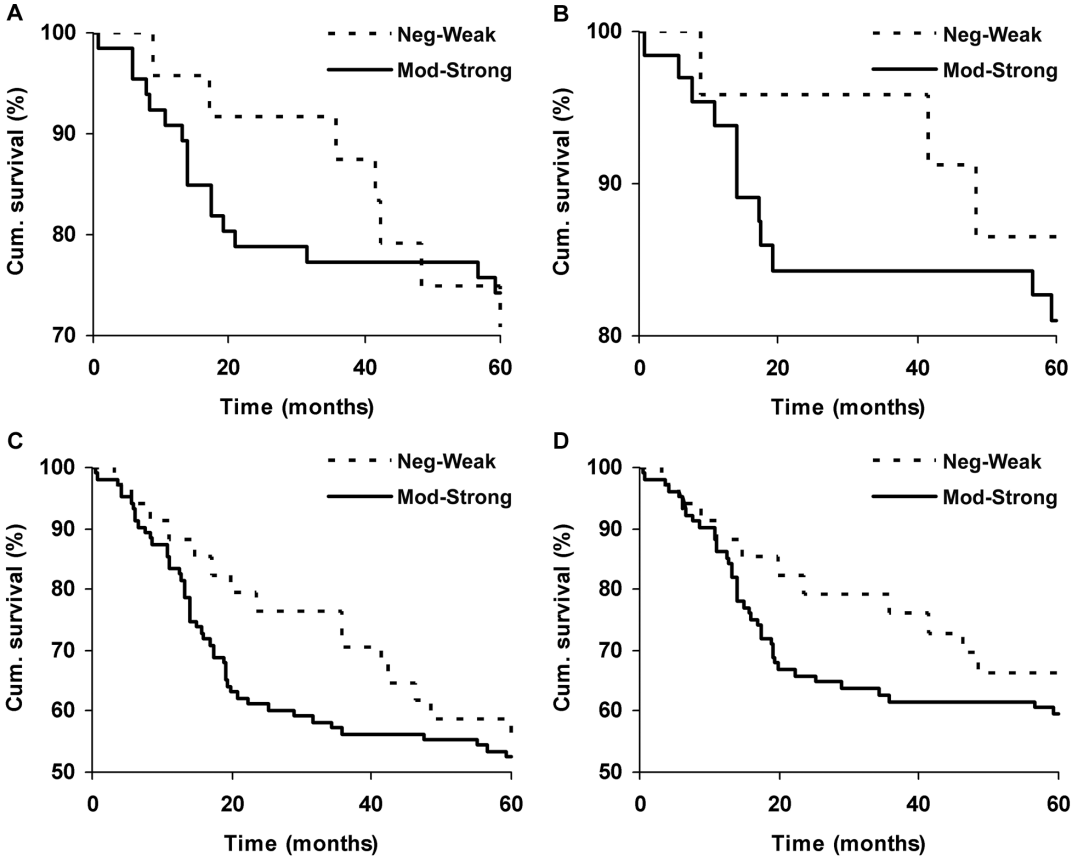


Fig. 3.

Fig. 4.6. Knockdown of BRG1 melanoma cells reduces cell proliferation. *A*, cell proliferation assay by SRB staining. 24 hours after transfection with BRG1 siRNA or control siRNA, MMRU and Sk-mel-3 cells were incubated for 24, 48, and 72 hours. Cells were fixed with 10% trichloroacetic acid at 4°C for one hour and the cell proliferation was quantitated by SRB staining. *B*, cell cycle analysis by fluorescence-activated cell sorting (FACS). 24 hours after transfection with BRG1 siRNA, MMRU and Sk-mel-3 cells were serum-starved for 24 hours to synchronize cells at G_1 phase. The cells were then released from G_1 by rinsing them three times in 10% FBS-containing DMEM and incubating them in this media containing 50 ng/ml nocodazole for different time points. Cells were then stained with 40 μg/ml PI for 30 min, and the percentage of G1, S, and G2/M population cells was measured by flow cytometry. All experiments were carried out in triplicate. Data are shown as mean ± standard error. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. *C*, Western blot analysis for the protein levels of BRG1, cyclin D1, cyclin B1, p21, and p27 in BRG1 knockdown and siRNA control for both MMRU and Sk-mel-3 cell lines.

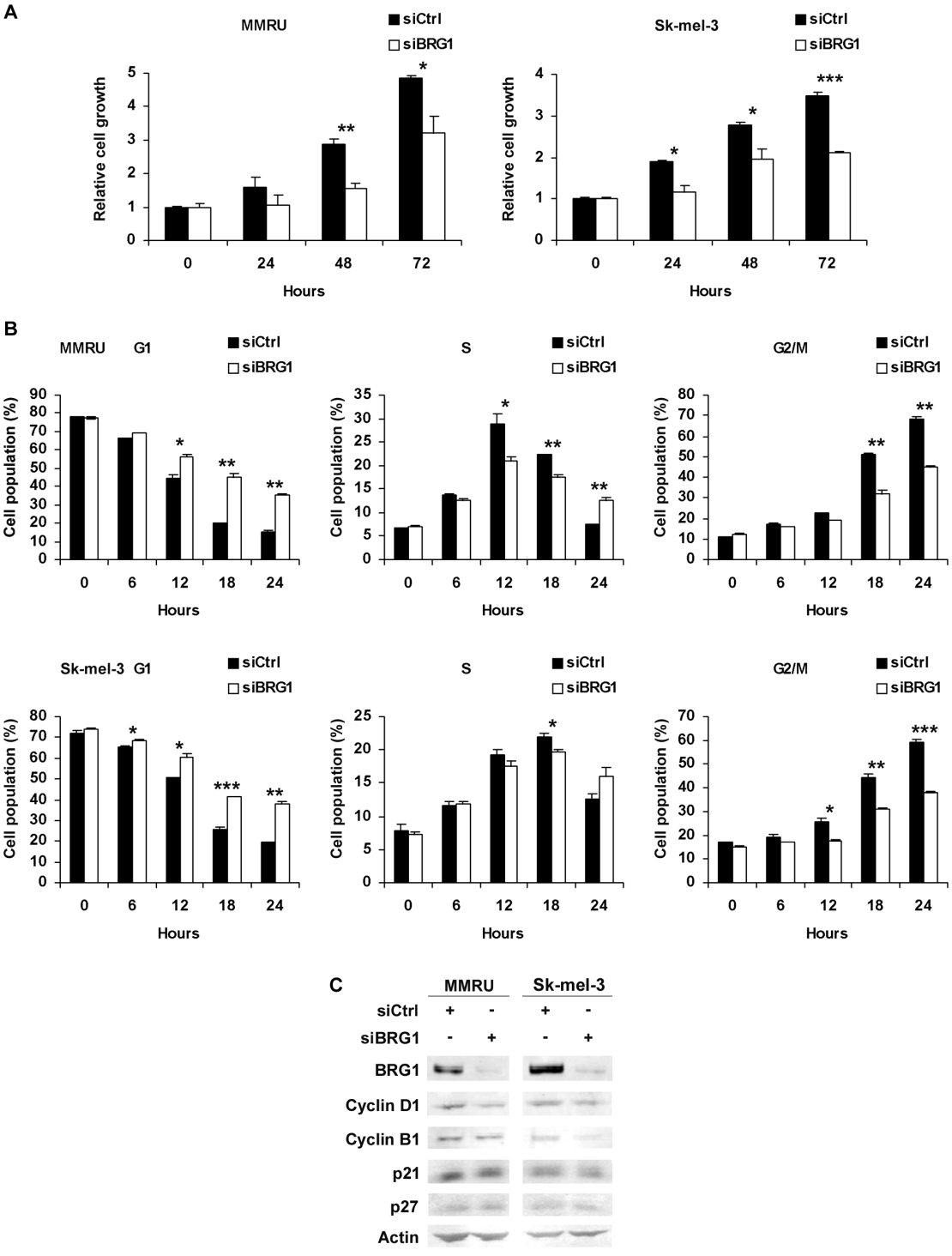

A
MMRU
siCtrl
siBRG1
Relative cell growth
Hours
Sk-mel-3
B
MMRU G1
S
G2/M
Sk-mel-3 G1
Cell population (%)
C
MMRU
Sk-mel-3
siCtrl
siBRG1
BRG1
Cyclin D1
Cyclin B1
p21
p27
Actin

Fig. 4.7. Drug-induced cell survival by SRB staining. Chemotherapeutic drug-induced apoptosis is not affected in BRG1 knockdown melanoma cells. 48 hours after transfection with SNF5 siRNA or control siRNA, MMRU and MMAN cells were treated with doxorubicin, etoposide, or camtothecin. 24 hours after drug treatments, cells were fixed with 10% trichloroacetic acid for 1 hour and quantitated by SRB staining.

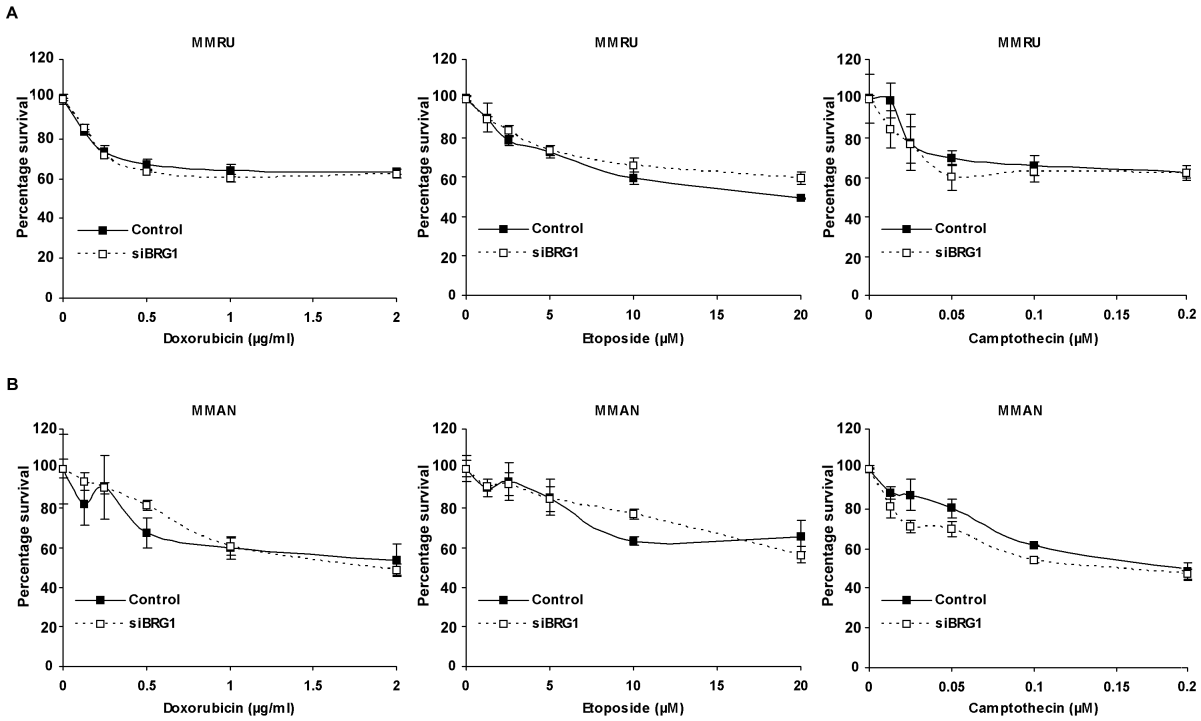
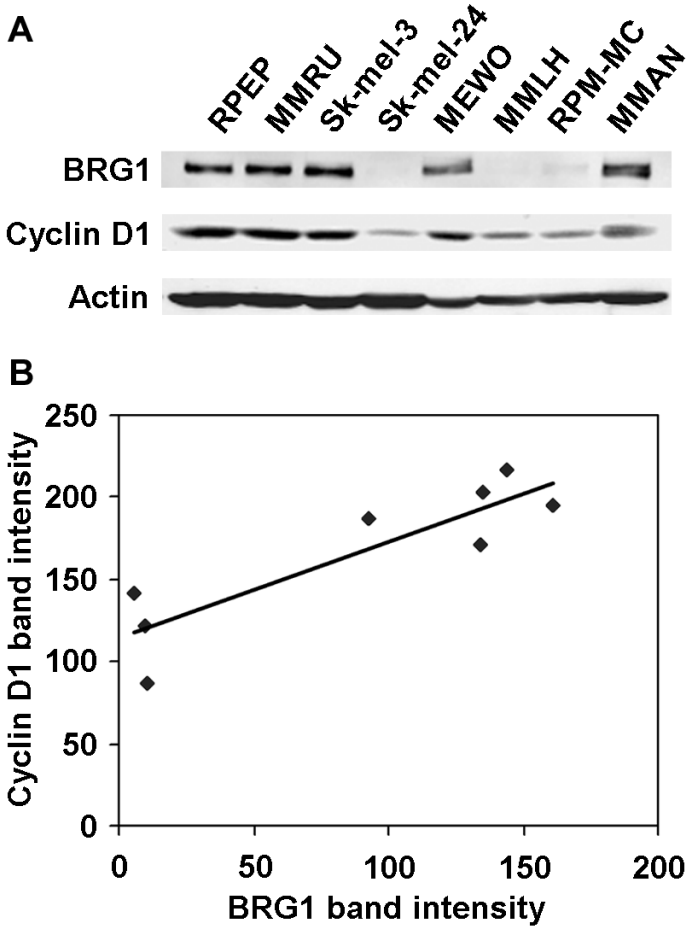


Fig. 4.8. Correlation of BRG1 expression and cyclin D1 expression in eight melanoma cell lines. *A*, eight melanoma cell lines were cultured in DMEM at 37°C, and subjected to whole cell protein extraction and Western blot. *B*, BRG1 and cyclin D1 band intensities were quantified by ImageJ, and the correlation was analyzed by SPSS 11.5 software (Spearman's rank correlation coefficient = 0.8095, $P = 0.0218$).



4.3 Discussion

A substantial body of evidence indicated that deregulated expression of several subunits of the SWI/SNF complex are involved in tumourigenesis. The *SNF5* gene was found inactivated in MRTs and downregulated in epithelioid sarcomas (Caramel *et al.,* 2008a; Hornick *et al.,* 2009). BRG1 and BRM are concomitantly lost in 15-20% of primary non-small cell lung carcinomas (Fukuoka *et al.,* 2004; Reisman *et al.,* 2003). BRG1 expression in gastric and prostate cancers, however, is upregulated and correlated with tumour progression (Sentani *et al.,* 2001; Sun *et al.,* 2007). To better understand the role of BRG1 activity in melanoma development, we used TMA technology and immunohistochemistry to investigate BRG1 activity in 185 cases of pigmented skin lesions at different stages. Our results show that BRG1 expression is significantly increased in both primary and metastatic melanoma compared to dysplastic nevi (Fig. 4.2). Our finding is in contrast with the report by Becker *et al.* claiming that BRG1 expression in primary and metastatic melanoma is frequently lost (Becker *et al.,* 2009). We argue that the discrepancy maybe be due to the smaller sample size in their study. They included 18 cases of primary melanoma and 20 cases of metastatic melanomas, which might represent only a subset of melanoma with downregulation of BRG1. Our study has a much larger sample size and shows an upregulation of BRG1 in both primary and metastatic melanoma with comparison to dysplastic nevi serving as control.

In our progression model (Fig. 4.2), we found that BRG1 expression increased significantly in primary melanoma compared to dysplastic nevi but did not further increase in metastatic melanoma. This suggests that BRG1 may play an important role in the initiation stage of melanoma development but might not be required for the

progression from primary tumours to metastatic tumours. Consistent with the progression model, we did not find any significant correlation of BRG1 expression with clinicopathological parameters (Table 4.1), such as Clark's level, tumour thickness, and patient survival, suggesting that BRG1 might not be required for cell migration and tumour invasion to drive the subsequent progression of the disease. Interesting, we found that BRG1 expression is significantly increased in patient age group >57 yr compared with age group ≤57 yr (Fig. 4.4). This might be due to changes of gene expressions with aging in tumourigenesis, as seen in the inactivation of oestrogen receptor gene in aging colorectal mucosa (Issa *et al.,* 1994). The increased BRG1 expression in aging might also affect the overall patient survival analysis since aging is likely to increase patients' risk of death from non-melanoma causes.

Uncontrolled cell proliferation is a hallmark of cancer. Studies showed that cancer cells ignore antigrowth signals to progress through G1 phase (Hanahan and Weinberg 2000). Many studies defined BRG1 as a tumour suppressor. Not only is BRG1 found inactivated in many human cancers and cell lines (Klochendler-Yeivin *et al.,* 2002; Roberts and Orkin 2004), but BRG1 is also found to be interacting with tumour suppressors, such as Rb protein, BRCA, LKB1, and HIC1, to induce growth arrest by downregulation of E2F-targeted genes and upregulation p21 (Bochar *et al.,* 2000; Dunaief *et al.,* 1994; Marignani *et al.,* 2001; Zhang *et al.,* 2009). However, recent studies demonstrated that some cancer cell lines actually require BRG1 for them to proliferate (Bourgo *et al.,* 2009; Naidu *et al.,* 2009; Wang *et al.,* 2009), and BRG1 interacts with oncoprotein MITF to promote melanoma proliferation (de la Serna *et al.,* 2006; Keenen *et al.* 2010). In short, paradoxically, loss of BRG1 leads to cancer formation both *in vivo*

and *in vitro* (Bultman *et al.,* 2000; Glaros *et al.,* 2008), but deletion of BRG1 also results in diminished tumour cell growths (Bourgo *et al.,* 2009; Naidu *et al.,* 2009; Wang *et al.,* 2009). Therefore, it is rather difficult to conclude whether BRG1 is indeed a *bona fide* tumour suppressor.

Our *in vitro* studies reveal that BRG1 knockdown in melanoma cells with siRNA reduces melanoma cell proliferative ability. The reduced proliferative ability is due to G1 cell cycle arrest through downregulation of cyclin D1 activity but not through upregulation of p21 and p27 pathway (Fig. 4.6). In fact, we also found that BRG1 expression is correlated with cyclin D1 expression in eight melanoma cell lines (Fig. 4.8). Combined with our TMA data, this finding led us to speculate that increased BRG1 expression in primary melanoma may contribute to melanoma cell proliferative ability in the early stage of this disease. Our finding is consistent with the study by Wang *et al*. showing that cancer formation in the absence of SNF5 is dependent on the activity of the residual BRG1 (Wang *et al.,* 2009). We previously showed that SNF5 expression is reduced in melanoma (Lin *et al.,* 2009), and this study demonstrated that increased BRG1 expression is required for early stage of melanomagenesis. However, our finding contradicts with the reports showing reduced BRG1 expression in other types of cancers and re-expression of BRG1 induce G1 cell cycle arrest through upregulation of p21 (Decristofaro *et al.,* 2001; Fukuoka *et al.,* 2004; Reisman *et al.,* 2002; Reisman *et al.,* 2003; Sif *et al.,* 2001; Wong *et al.,* 2000). This may be due to tissue/cell-type specificity. In fact, a study by Zhang *et al.* demonstrated that BRG1 can act as either a co-activator or a co-repressor of transcription at the same promoter depending on its binding partners (Zhang *et al.,* 2007). Since BRG1 is able to interact with either tumour suppressors or

oncoproteins (Bochar *et al.,* 2000; de la Serna *et al.,* 2006; Dunaief *et al.,* 1994; Marignani *et al.,* 2001; Zhang *et al.,* 2009), we postulate that BRG1 can either promote or inhibit cell cycle progression depending on its binding partners and cell types. The exact molecular mechanism of how BRG1 regulate melanoma cell proliferation warrants further investigation.

In conclusion, the data from this study show that BRG1 expression is increased in human cutaneous melanoma, suggesting that BRG1 may play an important role in early stage of melanomagenesis.

5. Role of SNF5 in Nucleotide Excision Repair in Human Keratinocytes and Melanoma Cells

5.1 Rationale and hypothesis

Ultraviolet (UV) radiation from sunlight is the major environmental factor for the development of melanoma. In order to design effective strategies for melanoma prevention, the molecular mechanism of UV-damaged DNA repair needs to be understood. UV-damaged DNA is repaired by nucleotide excision repair (NER) pathway, which involves up to thirty polypeptides (Aboussekhra *et al.,* 1995; de Laat *et al.,* 1999). For this number of repair factors to gain access to the lesion sites, chromatin's compact structure needs to be relaxed (Smerdon 1991). This relaxation process, called chromatin remodeling, can be allowed either through post-translational modifications of histones, or through the SWI/SNF ATP-dependent chromatin remodeling complex (Chodaparambil *et al.,* 2006; Eberharter *et al.,* 2005), which utilizes the energy from ATP hydrolysis to alter the contact between histone and DNA by nucleosome sliding, chromatin structure alteration, or eviction of nucleosomes (Kassabov *et al.,* 2003).

Recent studies have implicated the role of the SWI/SNF chromatin remodeling complex in NER. The complex enhances repair of a mononucleosome reconstituted with an acetylaminofluorene-guanosine (AAF-G) lesions in the core (Hara and Sancar 2002). It enhances CPD repair in nucleosome as measured by lesion specific phage T4 endonuclease (Lee *et al.,* 2004). Moreover, it enhances accessibility to repair factors *in vivo* (Yu *et al.,* 2005). More recently, it was also demonstrated that the core subunits of SWI/SNF complex, SNF5 and SNF6, interact with NER lesion detection complex Rad4-

Rad23 (yeast homologues of human XPC-hHR23B complex) *in vivo* (Gong *et al.,* 2006). Also, our recent published data showed that SNF5 expression was reduced in sun-exposed skin tumors, indicating a crucial role of UV radiation in regulating SNF5 level (Lin *et al.,* 2009). These data suggest pivotal roles of SWI/SNF chromatin remodeling complex in NER. However, the molecular basis of cross-talk between SWI/SNF and NER in mammalian cells is not fully understood. In this study, we hypothesize that SWI/SNF complex plays essential roles of chromatin remodeling in NER to maintain genomic stability after UVR in human skin cells. To investigate the role SWI/SNF complex in NER, we performed a series of DNA repair assays in both human keratinocytes and melanoma cells.

5.2 Results

5.2.1 Role of SNF5 in UV sensitivity

Because persistence of unrepaired DNA lesions can signal the initiation of apoptosis (Harris and Levine 2005), and the SWI/SNF complex may play a role in the UV damage response by repairing CPD to suppress UV-induced apoptosis, we first focused on monitoring UV-induced apoptosis in melanoma cells and HaCaT cells depleted of SNF5. Unfortunately, we did not detect significant difference between cells transfected with control siRNA and cells transfected with SNF5 siRNA in both MMRU and HaCaT upon UVB or UVC treatments (Fig. 5.1 and Fig. 5.2.). This suggests that SWI/SNF complex might not play a role in the UV damage response by suppressing UV induced apoptosis. In Fig. 5.1, SNF knockdown MMRU cells actually showed slightly more resistance to UV-induced apoptosis compared to control cells. This might be due to the role of SNF5 in tumor cell apoptosis discussed in chapter 3.2.5.

5.2.2 SNF5 is required for the repair of UV-damaged DNA lesions in human keratinocytes but not in melanoma cells

To investigate if SWI/SNF complex directly affects the removal of UV-induced DNA damage in human melanoma and normal cells, we knocked down SNF5 expression in both MMRU and HaCaT cells and performed host-cell-reactivation assay and slot-western analysis. In MMRU cells, we did not observe significant difference between control cells and SNF5 knockdown cells in host-cell-reactivation assay (Fig. 5.3), nor in slot-western analysis (Fig. 5.4). Interestingly, in HaCaT cells, we observed a retardation of CPD removal in SNF5 knockdown cells compared to control cells (Fig. 5.5). The

difference in the rate of repair between control cells and SNF5 knockdown cells is maximal at 24 h as CPD remaining was 45% and 80%, respectively. These results show that SWI/SNF complex is required for the repair of UV-damaged DNA lesions in normal human cells but not in melanoma cells.

5.2.3 SNF5 is required for UV-induced global H4 acetylation in human keratinocytes

Previous studies showed that UV irradiation could induce global histone hyperacetylation (Gong *et al.,* 2005). This in turn is able to relax chromatin structure to facilitate NER (Murr *et al.,* 2006; Yu and Waters 2005). To study if SWI/SNF complex repair CPD in HaCaT cells through relaxation of chromatin, we knocked down SNF5 expression in HaCaT cells, irradiated them with UVC, extracted the histones, and used antibodies that recognize acetylation on lysine 5, 8, 12, and 16 of histone H4 (AcH4) to examine the histone modification post-UV irradiation. Compared to control cells, SNF5 knockdown HaCaT cells did not show induction of AcH4 upon UV irradiation. AcH4 level actually reduced at 30 min post-UV compared with no UV in the knockdown cells. This result indicated that SWI/SNF complex plays a role in UV-induced histone hyperacetylation and suggested that SWI/SNF complex might repair CPDs through chromatin relaxation, which is in part due to histone acetylation.

5.2.4 Role of SNF5 in UV-induced global chromatin relaxation in melanoma cells

Because SWI/SNF does not affect CPD and 6-4PP removal rates in melanoma cells, we investigated if SWI/SNF is required in UV-induced chromatin remodeling in melanoma cells at all. To affirm the role of SWI/SNF complex in UV-induced chromatin relaxation, MMRU cells were transfected with SNF5 siRNA, UVC irradiated at 20 J/m^2, and allowed to repair for 30 min prior to MNase digestion. Cells transfected with ING1b expression plasmid served as a positive control (Kuo *et al.,* 2007). As expected, overexpression of ING1b resulted in less condensed chromatin structure as evidenced by more nuclease digestion. UV irradiated cells showed slightly more relaxation compared to non-UV treated cells. However, we did not observe difference between SNF5 knockdown cells and control cells in both UV and non-UV irradiated MMRU cells. This result indicates that SWI/SNF complex might not be required for UV-induced chromatin relaxation nor essential for NER in human melanoma cells.

Fig. 5.1. Sensitivity of MMRU and MMRU SNF5 knockdown cells to UV irradiation. Cells were transfected with SNF5 siRNA for 48 hours, and then exposed to various doses of UV irradiation. Twenty-four hours after UV irradiation, cells were fixed with 10% trichloroacetic acid, stained with SRB, and measured by reading absorbance at 550 nm.

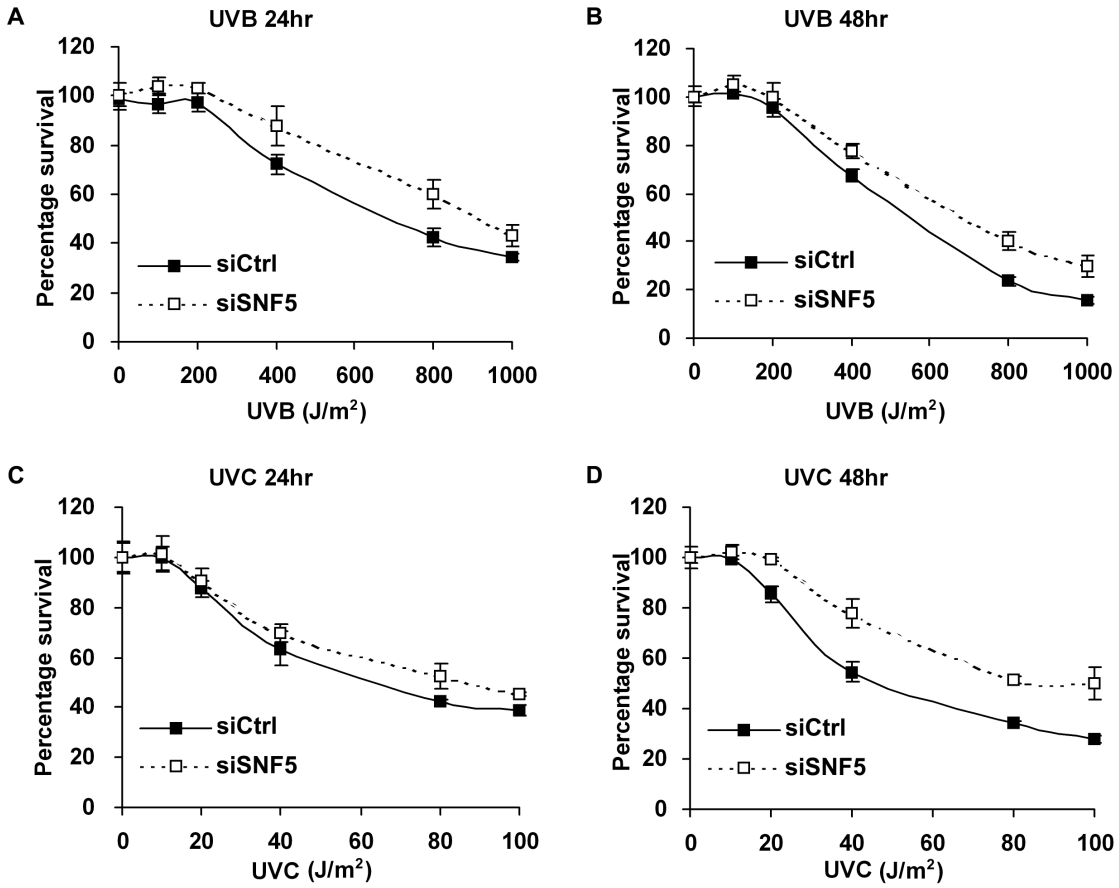


Fig. 5.2. Sensitivity of HaCaT and HaCaT SNF5 knockdown cells to UV irradiation. Cells were transfected with SNF5 siRNA for 48 hours, and then exposed to various doses of UV irradiation. Twenty-four hours after UV irradiation, cells were fixed with 10% trichloroacetic acid, stained with SRB, and measured by reading absorbance at 550 nm.

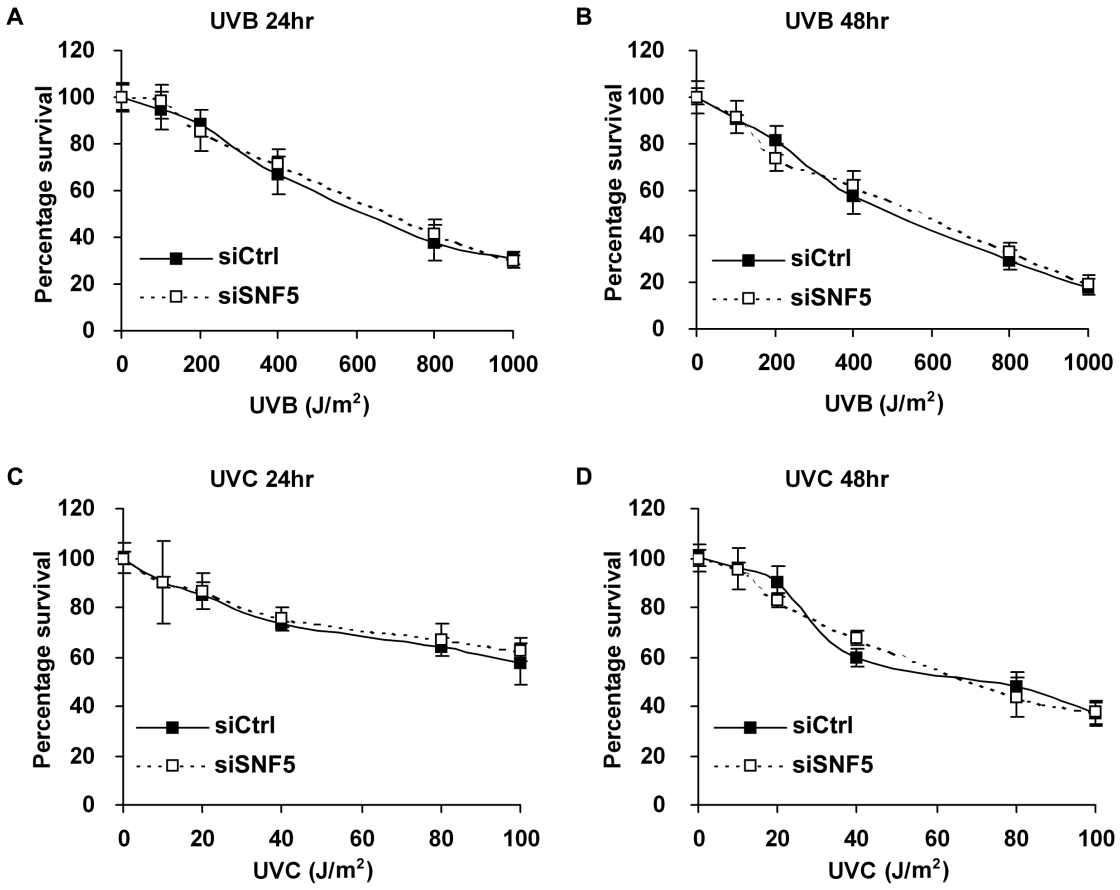


Fig. 5.3. SNF5 is not required for the repair of UV-damaged DNA lesions in MMRU cells. Cells were tranfected with either control siRNA or SNF5 siRNA together with non-damaged or 1,600 J/m^2 UVC-irradiated pRL-CMV firefly luciferase plasmid. Thirty or forty hours after transfection, the activity of the damaged reporter was assayed and normalized to the values obtained from the undamaged reporter plasmids to evaluate the repair efficiency.

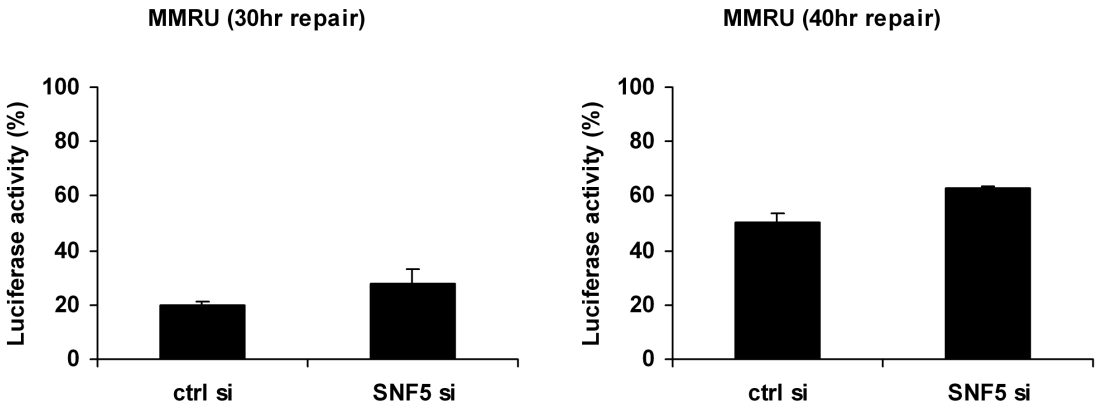


Fig. 5.4. SNF5 is not required for efficient removal of UV-induced photolesions in genomic DNA of MMRU cells. Cells were tranfected with either control siRNA or SNF5 siRNA for twenty-four hours and then exposed to 10 J/m^2 UVC irradiation. Cells were then harvested for slot-western analysis of UV-induced photo lesions with antibodies against CPD and 6-4PP.

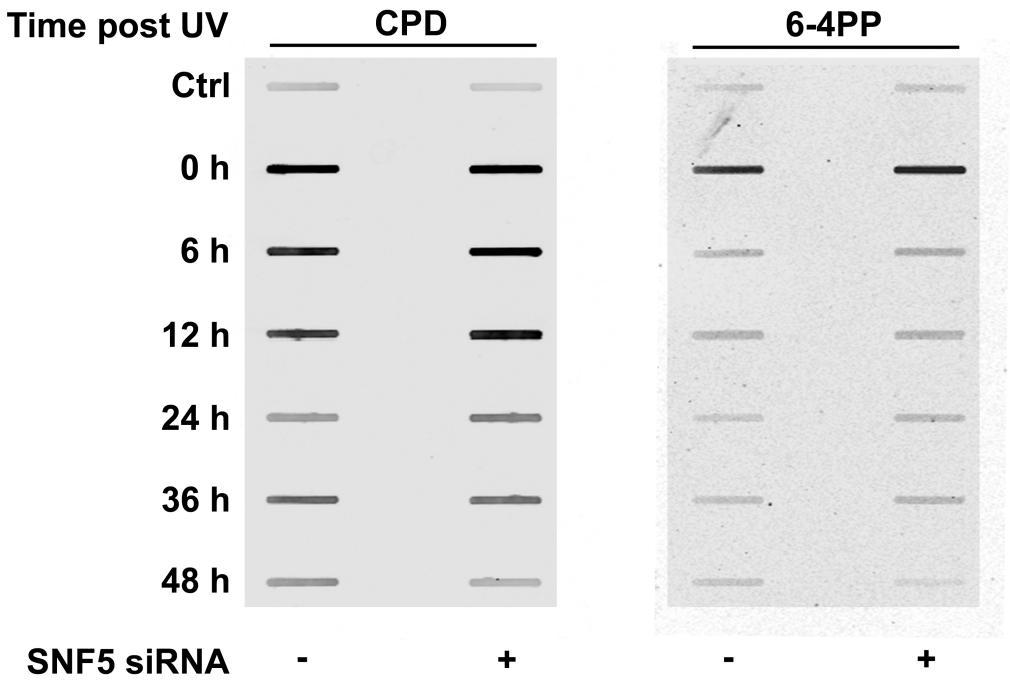


Fig. 5.5. SNF5 is required for efficient removal of CPD in genomic DNA of HaCaT cells. Cells were tranfected with either control siRNA or SNF5 siRNA for twenty-four hours and then exposed to 10 J/m^2 UVC irradiation. Cells were then harvested for slot-western analysis of UV-induced photo lesions with antibodies against CPD. Data presented in the graph on the right represent the relative CPD remaining in each sample. Percentage CPD was calculated from the intensity relative to initial irradiated sample. The data points represent an average of three independent measurements with error bars representing standard deviation.

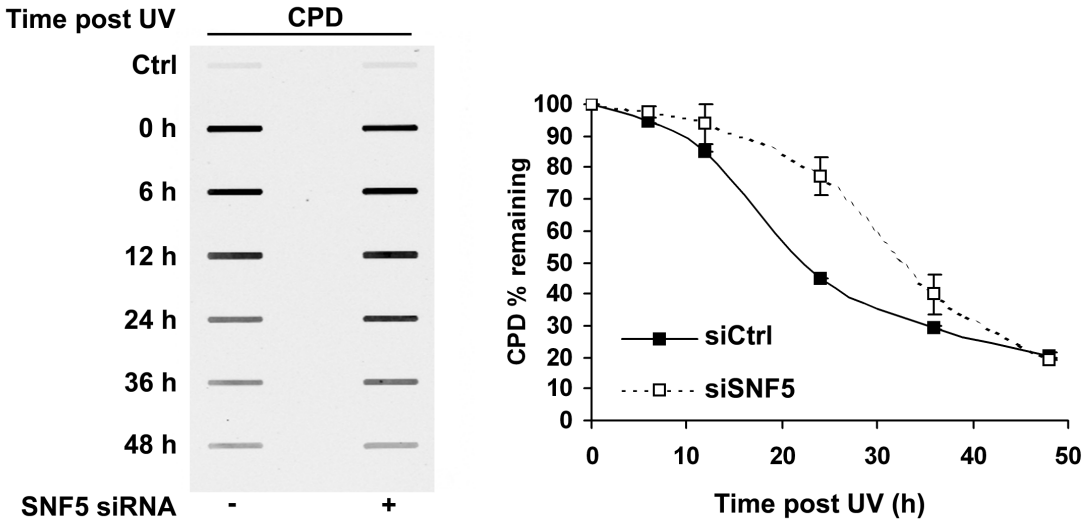


Fig. 5.6. SNF5 knockdown prevented histone H4 hyperacetylation after UV irradiation in HaCaT cells. Cells were tranfected with either control siRNA or SNF5 siRNA for twenty-four hours and then exposed to 20 J/m^2 UVC irradiation. Cells were then harvested for histone extraction and subjected to Western-blot analysis.

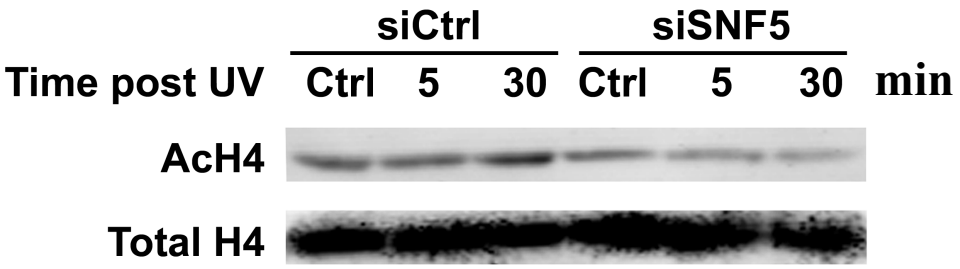
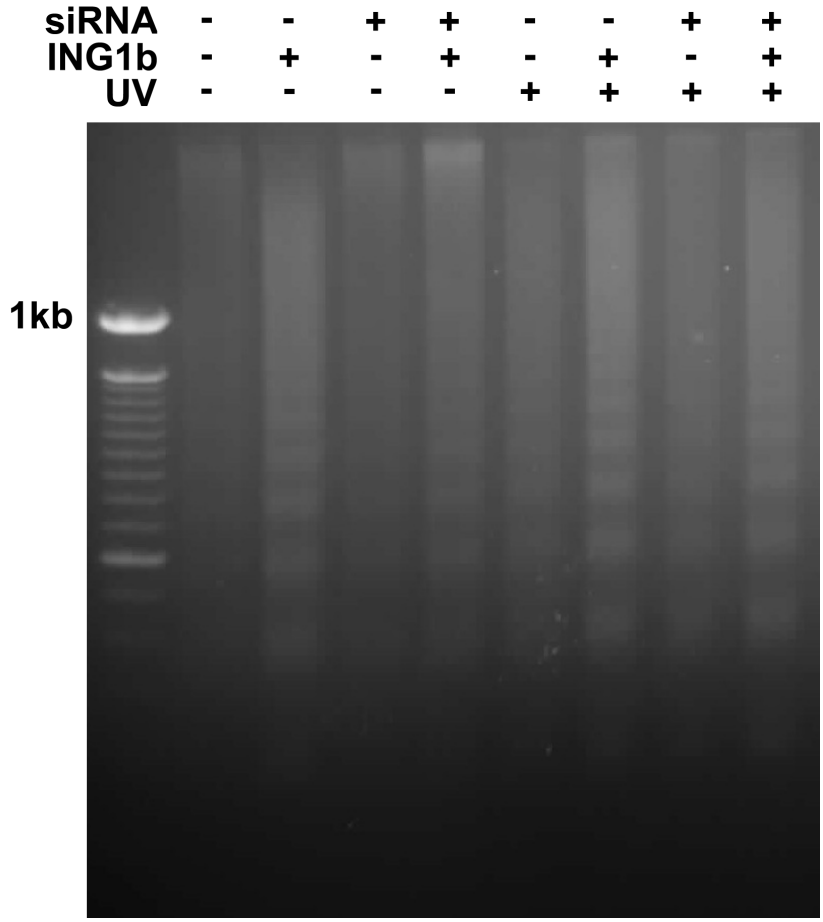


Fig. 5.7. SNF5 knockdown did not affect chromatin accessibility to micrococcal nuclease (MNase) digestion in MMRU cells after UV irradiation. MMRU cells were tranfected with either control siRNA or SNF5 siRNA for twenty-four hours and then exposed to 20 J/m^2 UVC irradiation. DNA was collected 30 min following UVC irradiation and subjected to 1 U MNase digestion. Cells transfected with ING1b expression plasmid were included as a positive control. DNA ladder of 100 bp was used as a marker for the agarose gel.



5.3 Discussion

SWI/SNF chromatin remodelling complex is known to be an important player in DNA repair pathways, such as NER, BER, and DSB repair (Dinant *et al.,* 2008). Although SWI/SNF complex has been implicated in regulating the chromatin relaxation process in NER by a number of *in vitro* excision assays (Hara and Sancar 2002; Hara and Sancar 2003), its exact role in NER has not been investigated in mammalian cells. In this study, we provide some evidence that SNF5 is essential for efficient removal of CPD and is required for UV-induced histone acetylation in human keratinocytes but not in MMRU cells.

When the DNA lesions are left unrepaired, cells will signal the initiation of apoptosis (Harris and Levine 2005). Because SWI/SNF is involved in UV-damaged DNA lesions, SWI/SNF should play a role in the suppression of UV-induced apoptosis. In fact, Gong *et al.* showed that human carcinoma cells overexpressed with BRG1 are more resistant to UV-induced apoptosis (Gong *et al.,* 2008). Park *et al.* indicated that BRG1 knockdown cells are more sensitive to UV-induced apoptosis (Park *et al.,* 2009). Also, Klochendler-Yeivin *et al.* showed that primary fibroblasts lacking SNF5 have increased DNA damage sensitivity and apoptosis (Klochendler-Yeivin *et al.,* 2006). However, our finding contradicts with these reports. This may be due to tissue/cell-type specificity. In fact, a study by Oh *et al.* demonstrated that BAF60a knockdown in SaOS-2 cells alleviates the p53-mediated apoptosis and cell cycle arrest, and BAF60a knockdown in NIH3T3 cells repress doxorubicin-induced apoptosis (Oh *et al.,* 2008). Moreover, a report by Napolitano *et al.* indicated that BRG1 overexpressed mesenchymal stem cells show induction of cell cycle arrest and apoptosis (Napolitano *et al.,* 2007). This leads us

to speculate that SWI/SNF complex might have dual roles: it is responsible for both DNA-lesions repair and p53-mediated apoptosis. Therefore, we did not observe significant difference in UV sensitivity between control and SN5 knockdown HaCaT cells. SNF5 knockdown MMRU cells showed more resistant to UV-induced apoptosis, which might be because SNF5 plays more roles in apoptosis than it does in DNA repair in melanoma cells (Fig. 5.1 and 5.2).

In this report, we found that SNF5 is required for the removal of UV-induced DNA lesion CPD in HaCaT cells (Fig. 5.5). This is expected since *in vitro* studies have suggested that SWI/SNF plays an important role in chromatin remodeling in NER (Hara and Sancar 2002; Hara and Sancar 2003). Also, studies in both yeast and mammalian cells indicated that SWI/SNF is indispensable for NER (Gong *et al.,* 2006; Gong *et al.,* 2008). Moreover, a recent study by Zhao *et al.* showed that BRG1 directly affects efficient removal of CPD in human fibroblast (Zhao *et al.,* 2009). Our study in HaCaT cells agrees with the current literature that SWI/SNF complex is essential for NER. However, in MMRU cells, SNF5 does not seem to have an important role in DNA repair (Fig. 5.3 and 5.4). This could be due to the fact that SNF5 protein level in MMRU cells is relatively low compared to that of melanocyte control (Fig. 3.2). Knocking down SNF5 protein level in MMRU might not affect DNA repair significantly. The host-cell-reactivation assay and the slot-blot western analysis might not be sensitive enough to detect the minute difference. Furthermore, as mentioned earlier, SNF5 may play more roles in regulating apoptosis than it does in NER in MMRU cells.

Histone acetylation has been found to be stimulated by UV and enhances the accessibility of chromatin and recruitment of repair factors (Ramanathan and Smerdon

1986; Ramanathan and Smerdon 1989; Smerdon *et al.,* 1982). In other words, immediately after UV irradiation, the surge of histone acetylation could initiate chromatin decondensation to make lesion sites more accessible to damage recognition factors. Depletion of endogenous SNF5 in HaCaT cells resulted in a significant decrease of basal AcH4 level and dramatically abrogated the AcH4 induction of post-UV irradiation (Fig. 5.6). It has been demonstrated that BRG1 is not involved in the recognition step of NER but is required to protect recognition protein, XPC, from UV-induced degradation in order to complete the subsequent steps of NER successfully (Zhao *et al.,* 2009). This leads us to speculate that SNF5, also, might be required for continuous chromatin relaxation by protecting AcH4 from deacetylation upon UV irradiation. In fact, a recent study showed that SWI/SNF complex is able to physically interact with acetylated histones through BRG1's bromodomain (Lee *et al.* 2010). Taken together, we propose a model for the mechanism of NER. The initial chromatin relaxation upon UV irradiation is due to histone acetylation. This then leads to the recruitment of XPC, which assists the recruitment of the SWI/SNF complex. The SWI/SNF complex, in turn, maintains histone acetylation level, protects XPC from degradation, and further remodels the chromatin structure to allow more repair factors to bind to the lesion sites in order to complete NER. It is also important to note that the chromatin relaxation ability of the complex might only work in normal cell lines but not in melanoma cell lines (Fig. 5.7). The exact molecular mechanism of how SWI/SNF is not functioning properly in melanoma cells warrants further investigation.

In summary, our current observation demonstrates that in human keratinocytes, SNF5 is required for efficient removal of CPD. SNF5 is also required for UV-induced

histone acetylation. In human melanoma cells, SNF5 does not seem to play a major role in NER, for it is not required for removal of CPD and UV-induced global chromatin relaxation.

6. General Conclusions

6.1 Summary and future directions

SWI/SNF chromatin remodeling complex has been shown to be deregulated in a broad variety of cancer types and may play an important role in cancer development and progression. The *SNF5* gene was found inactivated in MRTs and downregulated in epithelioid sarcomas and cutaneous melanoma (Caramel *et al.,* 2008a; Hornick *et al.,* 2009; Lin *et al.,* 2009). BRG1 and BRM are concomitantly lost in 15-20% of primary non-small cell lung cancers (Fukuoka *et al.,* 2004; Reisman *et al.,* 2003). Analysis of more than 100 cell lines revealed that both BRG1 and BRM are lost or mutated in about 10% of established cancer cell lines, including breast, ovarian, lung, bladder, pancreas, melanoma, and colon cancers (Decristofaro *et al.,* 2001; Wong *et al.,* 2000). Besides downregulation of the SWI/SNF subunits in cancers, some studies showed that BRG1 expression is upregulated and correlated with tumor progression in gastric and prostate cancers (Sentani *et al.,* 2001; Sun *et al.,* 2007). In this study, we used tissue microarrays and immunohistochemistry to evaluate the expression patterns of SNF5 and BRG1 in melanocytic lesions at different stages. Our data indicated that SNF5 expression is reduced in melanoma and significantly correlates with patient survival, indicating an important role of SNF5 in melanoma progression. BRG1, on the other hand, is significantly increased in human melanoma but the increase of BRG1 is not associated with patient survival, suggesting that BRG1 may only be involved in melanoma initiation. Although SNF5 and BRG1 belong to the same complex, their protein

expression levels vary dramatically. This suggests that each subunit of the SWI/SNF complex might play different roles in tumorigenesis.

In fact, many lines of evidence suggest SWI/SNF subunits do not function equivalently. Loss of SNF5 does not affect the function and the assembly of SWI/SNF complex (Doan *et al.,* 2004). Also, loss of SNF5 is rare and is associated with a small subset of tumor types, whereas loss of BRG1 is more common and is observed in a wide variety of solid tumors (Decristofaro *et al.,* 2001; Grand *et al.,* 1999; Manda *et al.,* 2000; Muchardt and Yaniv 2001; Yuge *et al.,* 2000). Furthermore, even though BRG1 is approximately 74% identical to BRM (Khavari *et al.,* 1993), the phenotypes of BRG1 knockout and BRM knockout mice are strikingly different (Bultman *et al.,* 2000; Reyes *et al.,* 1998). In addition, our *in vitro* studies clearly indicated that SNF5 and BRG1 show different activities in melanoma development. SNF5 knockdown significantly suppresses drug-induced apoptosis in melanoma cells while BRG1 knockdown does not affect melanoma chemoresistance at all (Fig. 3.5 and 4.7). Knockdown of SNF5 in melanoma cells does not affect cell proliferation whereas knockdown of BRG1 in melanoma cells reduces cell proliferation (Fig. 3.6 and 4.6).

Combined with our TMA data and *in vitro* studies, we demonstrated that SNF5 plays an essential role in melanoma progression, and BRG1 plays an essential role in melanoma initiation. This implicates that SNF5 and BRG1 may serve as promising prognostic markers and therapeutic targets for malignant melanoma. The highly metastatic potential and resistance to conventional radio- and chemo-therapy are the major reasons for which melanoma patients succumb to the disease. Therefore, strategies to restore reduced SNF5 expression or to reduce upregulated BRG1 expression may be a

potential approach for melanoma therapy. Moreover, analyzing SNF5 or BRG1 expression in melanoma patients may predict the response to chemotherapy. However, the exact roles of SNF5 and BRG1 in melanomagenesis warrant further investigations.

Our current study raises many questions that need to be further investigated. Since each subunit of SWI/SNF complex might not have the same function in melanoma development, it would be of interest to perform tissue microarray and immunohistochemistry to evaluate all the remaining subunit expression in different stages of human melanocytic lesions, and to look for possible correlations between each subunit. It is then possible to characterize which subunits are tumor suppressor like and which are potential oncoproteins. Also, since our *in vitro* data showed that melanoma cells depleted with SNF5 expression renders cells resistant to chemotherapeutic drugs, it would be of interest to look at the correlation between SNF5 expression and the expression of other apoptosis factors, such as the Bcl-2 family. In addition, since our *in vitro* data indicated that knockdown of BRG1 in human melanoma cell lines reduced cell proliferation due to G1 phase arrest, we could also perform immunohistochemical staining on cyclin proteins and examine any possible correlations. As for the functional studies, we could investigate if SNF5 regulates drug-induced apoptosis in a p53-dependent manner since BAF60a has been shown to interact with p53 and this interaction is required for doxorubicin-induced apoptosis (Oh *et al.,* 2008). Another biological function that SNF5 might be involved in melanoma is cell migration since Caramel *et al.* showed that the invasive property of MRTs is significantly reduced upon SNF5 re-expression in a RhoA-dependent manner (Caramel *et al.,* 2008b). The molecular mechanisms of how BRG1 regulates melanoma cell proliferation could also be further studied. Since BRG1 is able to interact with either

tumor suppressors to inhibit cell growth, or with oncoproteins to promote cell proliferation (Bochar *et al.,* 2000; de la Serna *et al.,* 2006; Dunaief *et al.,* 1994; Marignani *et al.,* 2001; Zhang *et al.,* 2009), we could study if BRG1 interacts with MITF or other oncoproteins to promote cell cycle progression in melanoma. BRG1 might also regulate cyclin D1 expression by interacting with cyclin D1 upstream regulators, such as Beta-Catenin and $p42/p44^{MAPK}$ (Lavoie *et al.,* 1996; Shtutman *et al.,* 1999).

SWI/SNF chromatin remodeling complex has been implicated in regulating chromatin structure during NER. Our study showed that SNF5 is required for efficient removal of CPD and UV-induced histone acetylation in human keratinocytes. Since UV radiation is the major environmental factor of melanoma development, our data shed light on the molecular mechanisms of how UV-damaged DNA is repaired which may eventually enable us to design effective strategies for skin cancer prevention. However, several questions remained unanswered. As mentioned earlier, each subunit of the SWI/SNF complex might not have the same function. It is, therefore, worth to investigate the role of other key subunits, such as BRM and BRG1, in NER. It might also be more appropriate to knockdown BRG1 protein level rather than to knockdown SNF5 protein level when elucidating the role of SWI/SNF complex in melanoma cell lines since melanoma cells have elevated BRG1 expression compared to that of melanocyte (Fig. 4.3). Another possible pathway of NER that is worth to study is the interaction between ING family proteins and the SWI/SNF complex. Our group has previously showed that ING proteins are involved in NER: ING1b and ING2 enhance rapid histone H4 acetylation, chromatin relaxation, and the recruitment of XPA to the photolesions after UV irradiation (Kuo *et al.,* 2007; Wang *et al.,* 2006). Also, other groups have showed

that SNF5 is present in both purified ING1b and ING2 complex (Doyon *et al.,* 2006; Kuzmichev *et al.,* 2002). Therefore, further studies are needed to see if ING proteins interact with and recruit SWI/SNF complex to lesion sites to facilitate NER in both normal and melanoma cell lines.

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