Abstract

ING4 was identified as a tumor suppressor in 2003 and shown to diminish colony-forming efficiency, induce p53-dependent apoptosis, and arrest cell cycle at G2/M phase. To investigate the role of ING4 in human cutaneous melanoma, we examined ING4 expression using tissue microarray and found that ING4 expression was significantly decreased in malignant melanoma compared with dysplastic nevi and reduced ING4 expression was correlated with melanoma thickness, ulceration and poor 5-year survival of melanoma patients. Multivariate analysis revealed that ING4 expression is an independent prognostic marker in melanoma patients. In melanoma cells, we found that overexpression of ING4 suppressed melanoma cell migration through RhoA-ROCK pathway and cell invasion by inhibiting MMP-2 and MMP-9 activity. We also demonstrated that ING4 inhibits endothelial cell growth and tube formation *in vitro* through suppressing NF-κB/IL-6 pathway. *In vivo* model revealed that ING4 inhibited blood vessel formation and recruitment of endothelial cells in matrigel plugs. Strikingly, we found that ING4 is induced by BRMS1. Further experiments showed that BRMS1 expression was significantly decreased in metastatic melanoma compared with primary melanoma or dysplastic nevi, and reduced BRMS1 staining was correlated with AJCC stages and worse 5-year survival of melanoma patients. Moreover, we demonstrated that BRMS1 overexpression inhibited endothelial cell growth and tube formation *in vitro* through suppressing NF-κB/IL-6 pathway and this BRMS1-mediated IL-6 expression is dependent on NF-κB. *In vivo* studies indicated that BRMS1 inhibited supportive blood vessel formation in matrigel plugs. Furthermore, we demonstrated that ING4 knockdown abrogated the suppressive effect of BRMS1 on HUVECs growth, while ING4 overexpression inhibited BRMS1 knockdown-induced angiogenesis, indicating BRMS1 as upstream regulator of ING4 in regulating tumor angiogenesis. Finally, we found that the integrate score of six-biomarker system, including ING4, BRMS1 together with other four
biomarkers, showed higher variations between melanoma with and without metastasis, and predicted melanoma patients outcome more accurately than individual biomarker. In summary, reduced ING4 expression in melanoma results in deficient suppression of melanoma cell migration and invasion. With BRMS1 as the upstream regulator, ING4 inhibits NF-κB/IL-6 to modulate melanoma angiogenesis. ING4 expression can be a prognostic marker and novel target for human melanoma treatment.
Preface

1. A version of chapter 3 has been published. [Li J], Martinka M and Li G. (2008). Role of ING4 in human melanoma cell migration, invasion and patient survival. Carcinogenesis 29(7): 1373-1379. Dr. G. Li supplied all the facilities and required materials and contributed to the experimental design. Dr. M. Martinka assisted us with scoring the immunohistochemical staining of tissue microarray slides. I designed and performed all the other presented experiments (including immunohistochemistry staining, tissue microarray analysis, and in vitro experiments), and I also prepared the manuscript.

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Medicine*. In press. With permission to reprint. This work is located in chapter 6.

Ethics certificate:
The use of human skin tissues in this study was approved by the Clinical Research Ethics
Board of University of British Columbia and the certificate number is H09-01321. The use
of animal in this study was approved by the Animal Care Committee of University of
British Columbia and the certificate number is A07-0471.
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<tbody>
<tr>
<td>6-4 PPs</td>
<td>(6-4) pyrimidine photoproducts</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ALM</td>
<td>Acral lentiginous melanoma</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternative reading frame</td>
</tr>
<tr>
<td>α-MSH</td>
<td>α-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-raf murine sarcoma viral oncogene homolog B1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BRMS1</td>
<td>Breast cancer metastasis suppressor 1</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinases</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CPD</td>
<td>Cyclobutane pyrimidine dimers</td>
</tr>
<tr>
<td>DTIC</td>
<td>Dacarbazine</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic motility shift assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor (FGF)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>H3K4Me2/3</td>
<td>Histone H3 lysine 4 di/tri methylated</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HBO1</td>
<td>Histone acetyltransferase binding to ORC 1</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Human head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPH</td>
<td>Hypoxia associated prolyl hydroxylase</td>
</tr>
<tr>
<td>HUVVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ING</td>
<td>Inhibitor of growth</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>LID</td>
<td>Lamin interaction domain</td>
</tr>
<tr>
<td>Liprin</td>
<td>LAR protein-tyrosine phosphatase- interacting protein</td>
</tr>
<tr>
<td>LMM</td>
<td>Lentigo maligna melanoma</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>LZL</td>
<td>Leucine-zipper like</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC1R</td>
<td>Melanocortin-1 receptor</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia transcription factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MOZ</td>
<td>Monocytic leukemia zinc finger protein</td>
</tr>
<tr>
<td>MORF</td>
<td>MOZ-related factor</td>
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</tbody>
</table>
NCI    National Cancer Institute
NCR    Novel chromatin regulatory
NER    Nucleotide excision repair
NF-kB Nuclear factor kappa B
NLS    Nuclear localization signal
NM    Nodular melanoma
NRAS Neuroblastoma RAS viral oncogene homolog
NTS    Nucleolar targeting signals
NuA4    Nucleosomal acetyltransferase of histone H4
PAG    Polyacrylamide gel
PBR    Polybasic region
PBS    Phosphate buffered saline
PCNA Proliferating cell nuclear antigen
PCR    Polymerase chain reaction
PDGF Platelet-derived growth factor
PHD    Plant homeodomain
PI    Propium iodine
PI3K Phosphatidylinositol 3-kinase
PIM Protein-interacting motif
PIP    PCNA-interacting protein domain (PIP)
PKB    Protein kinase B
PtdIns Phosphatidylinositol
PTEN    Phosphatase and tensin homolog
PVDF Polyvinylidene difluoride
RACE    Rapid amplification of cDNA ends
RGP    Radial growth phase
ROCK RHO-associated serine/threonine kinase
RR    Relative risk
RT-PCR Reverse transcriptase-polymerase chain reaction
SCLC Small-cell lung carcinoma
SDS Sodium dodecyl sulphate
siRNA Small interference RNA
SRB Sulforhodamine B
SSCP    Single strand conformation polymorphism
SSM    Superficial spreading melanoma
TCA    Trichloroacetic acid
TGF-β Transforming Growth Factor-beta
TKIs    Tyrosine kinase inhibitors
TMA    Tissue microarray
TMZ Temozolomide
TNF    Tumor necrosis factor
UV    Ultraviolet
VEGF Vascular endothelial growth factor
VGP Vertical growth phase
WASP Wiscott–Aldrich syndrome protein
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Dedication

This thesis is dedicated to my very caring and supportive parents and my wife. They have been supporting me with their love, understanding and encouragement throughout all these years.
1 General Introduction

1.1 Cutaneous melanoma

1.1.1 Melanocyte biology and transformation

Melanocytes are believed to be of neural crest origin based on in vivo studies of ablation and transplants of the neural crest, which produced changes in patterns of cutaneous pigmentation (Rawles, 1948). The majority of melanocytes reside in the skin, in particular within the epidermis and hair follicles, but some melanocytes are identified in visceral organs, the orbital cavity, leptomeninges, and inner ear (Nordlund, 1989). Epidermal melanocytes are present in all regions of the body, but their population density varies depending on different regions, with the ratio of melanocytes to keratinocytes in the basal layer ranging from 1:4 to 1:10 (Fitzpatrick and Szabo, 1959).

The unique property of melanocytes is the production of melanin pigments. Their major function in humans is to protect against potential toxic or carcinogenic effects of sunlight, which is attributed to broad spectral absorbance (Morison, 1985; Proctor and McGinness, 1986). In addition, melanin is also involved in trapping reactive oxygen species, sequestering metal ions, and binding certain drugs and organic chemicals (Riley, 1992, 1997). There are two major types of melanin: eumelanin, which is brown-black and insoluble, and pheomelanin, which is reddish-yellow and alkali soluble (Prota, 1980), and the type of melanin produced depends on the genotype of melanocortin-1 receptor (MC1R) gene (Rees, 2003). The MC1R gene encodes a transmembrane G-protein coupled receptor, which is activated by α-melanocyte stimulating hormone (α-MSH) or adrenocorticotropic hormone (ACTH) (Barsh et al., 2000; D’Orazio et al., 2006) and inhibited by agouti (Suzuki et al., 1997). Effects of α-MSH/ACTH on melanogenesis are mediated via microphthalmia transcription factor (MITF), the transcriptional factor controlling the
expression of several pigment enzymes including tyrosinase, the rate-limiting enzyme in melanogenesis (Levy et al., 2006).

Proliferation and behaviour of melanocytes is tightly controlled and regulated by their neighbouring keratinocytes under normal physiological conditions (Haass et al., 2004). It is believed that a complex system of paracrine growth factors and cell-cell adhesion molecules is responsible for this delicate regulation (Haass et al., 2004). Deregulation of this homeostasis may disturb the balance between melanocytes and keratinocytes, and trigger the continuous proliferation of melanocytes and finally results in development of malignant melanoma (Bissell and Radisky, 2001). In particular, there is a switch from the E-cadherin-mediated keratinocyte-melanocyte partnership to the N-cadherin-mediated intercellular communication between melanoma cells or between melanoma cells and fibroblasts (Haass et al., 2004). Alteration in the gap junctions formed between the melanocytes and keratinocytes and changes in the connexin expression, in particular the loss of connexin 43, also result in disrupted gap junctional activity, which contributes to melanoma progression (Hsu et al., 2000a; Hsu et al., 2000b). These changes release melanocytes from growth suppression, allow them to proliferate and self-aggregate to form nevi (Li et al., 2001).

A benign nevus is a lesion with increased number of nested normal melanocytes along the basal layer. In contrast, a dysplastic nevus exhibits architectural or cytologic atypia to different extent, but it is not considered malignant (Hussein, 2005). Dysplastic nevi can progress to the radial growth phase (RGP) melanoma, where the cells are able to proliferate intraepidermally, and instead of showing random atypia, these cells show cytomorphologic cancer throughout the lesion. RGP cells can then progress to the vertical growth phase (VGP), a more dangerous stage in which the cells invade the underlying dermis and form expansible nodules. The last step in melanoma progression is
development of metastatic properties, where the cells successfully spread to other areas of the skin or other organs (Miller and Mihm, 2006). Metastasis is the major reason for the death of melanoma patients.

1.1.2 Epidemiology of melanoma

Cutaneous melanoma is the most lethal form of skin cancers. Although it accounts for only 6% of all dermatological malignancies, it claims 80% of patients’ death from skin cancer (National Cancer Institute of Canada: Canadian Cancer Statistics 2007, Toronto, Canada, 2007). Epidemiological studies showed that the incidence of melanoma has been rising rapidly by as much as 3-8% yearly since 1960s and has doubled in the last decade (Dauda and Shehu, 2005; Thompson et al., 2005). In Canada, melanoma is the seventh most common cancer in men and the eighth most common cancer in women (Canadian Cancer Society: Canadian Cancer Statistics 2010), while in the United States, it represents the fifth most common cancer in men and the seventh most common cancer in women (Jemal et al., 2010). In 2010, an estimated 5,300 new cases of melanoma are expected to be diagnosed (300 cases, or 6% more than 2009), with 920 patients expected to die from the disease in Canada (Canadian Cancer Society: Canadian Cancer Statistics 2010). In British Columbia, melanoma is the sixth most common cancer in both male and female population. 460 and 330 new cases of melanoma are expected to be diagnosed in men and women, with 80 male and 50 female patients expected to die from this disease in British Columbia this year, respectively (Canadian Cancer Society: Canadian Cancer Statistics 2010).

The incidence of melanoma is different among different races (Crombie, 1979). Light-skin populations have an approximately 10-fold greater risk of developing cutaneous melanoma than Black, Asian, or Hispanic populations (Ries et al., 2000). Among Caucasians, people with red hair, pale skin and a tendency to freckle are three times more
likely to develop melanoma than others (Bliss et al., 1995; Thompson et al., 2005). This is presumably attributed to the less melanin produced and higher sensitivity of Caucasian’s skin to sun exposure. Besides race, geographic location is another factor that affects the incidence of melanoma (Thompson et al., 2005). The highest incidence rate of melanoma was found at the Australian Capital Territory with 28.9 per 100,000, while the lowest rate, 0.1, was reported among Kuwaitis in Kuwait (Parkin and Muir, 1992). The correlation between gender and melanoma incidence rate is still controversial. Some studies reported that men have approximately 1.5-fold higher chance to develop melanoma than females (Boyle et al., 1995; Tsai et al., 2005), but on the other hand, an earlier study showed that the incidence of melanoma is higher in Caucasian females than Caucasian males. The possible reason they offered to explain this higher incidence in Caucasian females is that many melanomas occurred on the female leg, which might reflect differences in habits of dress, tanning behavior rather than a greater susceptibility among females (Crombie, 1979).

1.1.3 Etiology of melanoma

Accumulated evidence showed that exposure to ultraviolet (UV) radiation in sunlight is the primary environmental factor in melanoma development (Gilchrest et al., 1999; Oliveria et al., 2006). It is also believed that the effect of UV radiation depends on both the UV wavelength and the pattern of exposure (Ehrhart et al., 2003; Oliveria et al., 2006; Cooper and Bowden, 2007). UV is separated into three spectrums: UVA, the longest wavelength (320-400 nm) with the lowest energy; UVC, the shortest wavelengths (below 280 nm) and the highest energy; and UVB, the middle range wavelength of 280-320 nm with intermediate energy. Since the ozone layer of the stratosphere of the earth absorbs UVC and part of UVB and thus prevents them from reaching the earth’s surface, UVA is the most abundant UV source in sunlight. However, UVB is believed to be dominantly responsible for inducing DNA lesions and its carcinogenic impact has drawn a lot of
attention in the past decades (Ehrhart et al., 2003; Cooper and Bowden, 2007). Besides the UV wavelength, the pattern of exposure to sunlight is also important in melanoma development. It is reported that history of sunburn in early age is correlated with melanoma risk, and intense exposure to sunlight intermittently is more melanoma-prone than continuous and mild exposure (Oliveria et al., 2006; Rees, 2008). UV irradiation promotes the generation of reactive oxygen species (ROS) that oxidizes melanin, which is normally anti-oxidant, leading to the generation of a redox-active tautomer, DNA damage, and suppression of apoptosis (Meyskens et al., 2004). Reduced apoptosis of DNA-damaged cells results in the accumulation of mutations, genetic instability, and eventually leading to the malignant transformation of melanocytes. Genetic alteration is another important risk factor in melanoma development, which will be discussed later in the thesis.

1.1.4 Staging and subtypes of melanoma

The current standard classification of melanoma staging was adopted in 2009 by the American Joint Committee on Cancer (AJCC). This standard is also called TNM standard where TNM stand for size of Tumor, lymph Nodes, and Metastasis, respectively. Ulceration status is also included in this standard for staging of primary melanoma. This is the 7th version of AJCC staging system which is revised from the 6th version in 2002, based on the multivariate analysis of 30,946 melanoma patients with I, II, and III stages plus another 7,972 patients from AJCC IV stage. The major difference between this new version and the old one is that mitotic rate replaces the invasion levels (Clark level) in defining the T1b stage (Balch et al., 2009). Basically, the final clinical or pathological classification of melanoma is defined based on the classifications of T, N and M together. Primary melanoma patients with tumor thickness <1.0 mm or between 1.01-2.0 mm without ulceration are considered stage I, primary melanoma patients with tumor thicker than 2.0 mm or between 1.01-2.0 mm with ulceration are in stage II. Those patients with
lymph nodes metastasis but without distant metastasis are in stage III, and finally those patients with distant metastasis are classified as stage IV. The AJCC staging is very important as it is closely correlated with patient survival. The 5-year survival rate is 85-99% for patients with stage I melanoma, it drops to 40-85% for stage II, 25-60% for stage III, and to the worst 9-15% for stage IV (Balch et al., 2001a). The main limit of this new version of melanoma staging standard is, as usual, that no molecular biomarkers have been implemented.

The subtypes of melanoma are distinguished by the clinical and pathological growth patterns as: superficial spreading, lentigo maligna, nodular, and acral lentiginous. Superficial spreading melanoma (SSM) represents approximately 70% of all melanomas and is the most common type of cutaneous melanoma occurring in light-skinned people (Su, 1997). It affects adults of all ages, with the peak incidence in the fourth and fifth decades of life. SSM can develop from a pre-existing melanocytic nevus, and most commonly affects intermittently sun-exposed areas, such as the upper backs of men and women and lower legs of women (Lens, 2008a). Nodular melanoma (NM) is the second most common type of melanoma and represents 10-15% of all melanomas (Lens, 2008a). The main characteristic of NM is rapid growth and the peak incidence of NM is in the middle age. It is seen most commonly on the trunk, head and neck (Barnhill and Mihm, 1993). Lentigo maligna melanoma (LMM) accounts for 5-10% of all melanoma (MacKie, 2000) and is mostly seen in elderly people, with a peak incidence in the their 70’s. This type of melanoma is typically found at sun-exposed sites, especially the cheeks, nose, and the neck. The last subtype of melanoma is acral lentiginous melanoma (ALM), which represents approximately 2-8% of all melanomas in Caucasians (Barnhill and Mihm, 1993). ALM is the most common form of melanoma in Asians and people with dark skin. Usually ALM is developed on the palms, soles and under the nail plate (Lens, 2008a).
1.1.5 Genetic alteration of melanoma

As mentioned in 1.1.3, genetic alteration is an important risk factor for melanomas (Miller and Mihm, 2006). 25-40% of melanoma-prone family members show mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A) (Miller and Mihm, 2006). Two protein products, p16\textsuperscript{INK4A} and p14\textsuperscript{ARF}, are encoded by CDKN2A. The p16\textsuperscript{INK4A} protein functions as regulator of G\textsubscript{1}/S-phase transition by inhibiting the activity of cyclin-dependent kinases (CDK) Cdk4 and Cdk6 (Ruas and Peters, 1998), while the p14\textsuperscript{ARF} regulates the activity of the p53 tumor suppressor protein by binding to Mdm2 and inhibiting Mdm2-mediated degradation of p53 (Prives and Hall, 1999). Another study showed that about 50% of melanomas have deletion of CDKN2A gene and 9% contain inactivated point mutations (Bennett, 2008). In 20% to 75% of melanomas, hyper-methylation of p16\textsuperscript{INK4A} promoter has been reported (Marini et al., 2006). These genetic or epigenetic changes of p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} may lead to functional loss and ultimately result in uncontrolled cell growth. This notion was also confirmed by a patients’ survival study, showing a significant correlations between losses of p16\textsuperscript{INK4A} or p14\textsuperscript{ARF} expression and melanoma progression as well as poor prognosis (Piepkorn, 2000; Dobrowolski et al., 2002; Sanki et al., 2007).

Unlimited growth of melanoma cells may be also stimulated by the abnormal activation of the mitogen-activated protein kinase (MAPK) signalling pathway, due to activating mutations of BRAF (v-raf murine sarcoma viral oncogene homolog B1) or NRAS (neuroblastoma RAS viral oncogene homolog) (Lin et al., 1998; Brunet et al., 1999; Welsh et al., 2001). The BRAF gene is the most frequent (60%-80%) mutation observed in human melanoma (Brose et al., 2002). Eighty percent of these mutations are found at exon 15, at a single amino acid residue. It is usually a substitution for valine by glutamic acid, V600E. This mutation causes increased kinase activation and signalling through the MAP kinase pathway and causes activation of the Brn-3 transcription factor (Goodall et al.,
Some other oncogenic mutations observed in *BRAF* gene are in the kinase domain of BRAF, such as the V600 lysine (V600K), aspartic acid (V600D), and arginine (V600R) mutations, and all of these mutations have been characterized as activating mutations, similar to V600E (Davies et al., 2002). Activation of this pathway can also be the result of somatic mutations of *NRAS*, which are found in about 15% of melanomas. These mutations, which occur exclusively of each other, cause constitutive activation of the serine–threonine kinases in the ERK–MAPK pathway (Albino et al., 1989; Davies et al., 2002; Omholt et al., 2003). Some *in vitro* experiments demonstrated that depletion of BRAF and NRAS from melanoma cells suppresses their growth (Hingorani et al., 2003; Eskandarpour et al., 2005; Hoeflich et al., 2006).

Another gene which is frequently affected by homozygous deletion in melanoma as well as other cancers is phosphatase and tensin homolog (PTEN) locus on chromosome 10 (Guldberg et al., 1997; Li et al., 1997; Steck et al., 1997). Under physiological condition, PTEN encodes a phosphatase that inhibits signalling pathways, such as phosphatidylinositol 3-kinase (PI3K)-PTEN-Akt pathway. In around 30% of melanomas, PTEN is found to be deleted and this loss of PTEN results in activation of protein kinase B (PKB, also called Akt), and promoted cell proliferation and survival (Wu et al., 2003).

The microphthalmia associated transcription factor (MITF) regulates the development and differentiation of melanocytes and maintains melanocyte progenitor cells in adults (Hodgkinson et al., 1993). In melanoma, it is reported that the copy number of the region on chromosome 3 that includes the *MITF* gene is increased and this is accompanied with the overexpression of MITF in melanoma (Garraway et al., 2005). Further experiments demonstrated that co-overexpression of both MITF and BRAF transformed primary cultures of human melanocytes, suggesting MITF as an oncogene. Consistently,
MITF amplification is correlated with poor prognosis and is associated with resistance to chemotherapy (Garraway et al., 2005).

1.1.6 Treatment of melanoma

Current treatment approaches for melanoma include surgery, chemotherapy, radiation therapy, biological therapy (immunotherapy), as well as relatively new cell and targeted therapy. The choice of different therapeutic strategy is based on stage, thickness, and location of the tumour, as well as the individual’s general health and personal situation. Surgery is still the primary treatment for localized melanoma, but adjuvant treatment, including high-dose of interferon (IFN) may be needed for those high-risk melanoma patients (Kirkwood et al., 1996). Radiation therapy is often used after surgical resection for patients with locally or regionally advanced melanoma or for patients with unresectable distant metastases. It may reduce the rate of local recurrence but does not prolong survival (Bastiaannet et al., 2005; Berk, 2008). Various chemotherapy agents are used, including dacarbazine (also termed DTIC), or immunotherapy (with interleukin-2 [IL-2] or IFN) as well as local perfusion are used by different centers. Some of these treatments can occasionally show dramatic success, but the overall success in metastatic melanoma is quite limited (Bajetta et al., 2002). For example, the response rates with single-agent DTIC did not exceed 12% (Gogas et al., 2007). Temozolomide (TMZ), an oral congener of DTIC, has similar efficacy as DTIC in the treatment of variety of solid tumors, especially in brain malignancies (Middleton et al., 2000; Gogas et al., 2007).

For cell and targeted therapy, two kinds of experimental treatments developed at the National Cancer Institute (NCI) have been applied to metastatic melanoma with moderate success. The first treatment used immune cells isolated from a patient’s own melanoma tumor. These cells are amplified in large numbers in a laboratory and returned to the patient after a treatment that temporarily reduces normal T cells in the patient’s body.
Together with lymphodepletion, this therapy resulted in complete responses in highly pretreated patients (Sotomayor et al., 2002). In the second treatment, adoptive transfer of genetically altered autologous lymphocytes into patients enabled these lymphocytes to recognize and bind to certain molecules found on the surface of melanoma cells and to kill them (Dudley et al., 2008). Vaccines developed utilizing the melanoma-associated antigens or humanized monoclonal antibodies targeting these markers have been developed and tested. However, clinical responses to melanoma vaccines are still poor and currently there is no melanoma vaccine with a proven efficacy (Terando et al., 2007; Lens, 2008b).

Since \textit{BRAF} is mutated in about 50\% of melanomas, it becomes an ideal target in melanoma treatment (Brose et al., 2002; Yazdi et al., 2003; Flaherty and McArthur, 2010). Early clinical trials suggest that BRAF inhibitors including Plexxicon can lead to substantial tumor regression in a majority of patients if their tumor contains the \textit{BRAF} mutation (Flaherty and McArthur, 2010). The exciting news about melanoma treatment via inhibiting BRAF pathway comes from a very recent publication in the New England Journal of medicine (Flaherty et al., 2010), reporting that treatment of metastatic melanoma with PLX4032, a selective BRAF inhibitor, in patients with tumors carrying the V600E BRAF mutation resulted in complete or partial tumor regression in the majority of patients. In this study, among 32 patients 24 showed partial response and two showed complete response. A phase III trial is undergoing right now to address whether this treatment can actually improve the survival of patients with metastatic melanoma. Another interesting finding previously reported is that the production of VEGF (Vascular endothelial growth factor) and IL-8, which were discovered to be decreased by ING4 and play important roles in the regulation of cancer angiogenesis (our unpublished data; Garkavtsev et al., 2004), is under the control of mutant BRAF (Sharma et al., 2005). In this
thesis, we found that ING4 inhibits melanoma angiogenesis. Therefore, combining BRAF inhibitor with possible agent targeting VEGF-mediated melanoma angiogenesis, such as restoration of ING4 expression, may achieve synergistic effect in treating metastatic melanoma.

1.2 Tumor cell migration and invasion

Tumor cells need the ability to migrate and invade to change position within the tissues. Cell migration is a central process in the development and maintenance of multicellular organisms. It is required not only by tumor cells, but also by normal cells, under physiological conditions, such as embryonic morphogenesis, wound healing and immune-cell trafficking (Friedl and Brocker, 2000). The ability of cancer cells to invade is a unique property which is critical in the process of malignant transformation (Hanahan and Weinberg, 2000). Invasion requires the penetration of tissue barriers, such as basement membrane and interstitial stroma, by cells (Friedl and Wolf, 2003b). In this thesis, I found that tumor suppressor ING4 inhibited melanoma cell migration and invasion.

1.2.1 Multiple steps of cell migration

Cell migration is a highly integrated, multi-step process that plays an important role in the progression of various diseases including cancer. A 5-step cell migration model was established previously (Lauffenburger and Horwitz, 1996), which starts with the protrusion of the leading edge. In this step, growing actin filaments connect to adaptor proteins and push the cell membrane in an outward direction (Rohatgi et al., 1999). Next step is the cell-matrix interaction and formation of focal contacts. Integrins interact with extracellular matrix (ECM) ligands and cluster in the cell membrane (Miyamoto et al., 1995; Zamir and Geiger, 2001). Then clustered integrins recruit adaptor and signalling proteins via their intracellular domains, and inducing phosphorylation and dephosphorylation signals into the cell (Hynes, 2002). Step 3 is the recruitment of surface
proteases to ECM contacts and focalized proteolysis. Surface proteases, matrix metalloproteinase (MMP), are concentrated at substrate binding sites (Mueller et al., 1999) and digest ECM components (Sameni et al., 2001; Friedl and Wolf, 2003a). This is followed by step 4, in which cells are contracted by actomyosin, which is composed of active myosin II and actin filaments (Verkhovsky et al., 1995; Kauffman et al., 2003) (Cramer, 1999). The last step is detachment of the trailing edge and this occurs through various mechanisms (Pfaff et al., 1999; Wear et al., 2000; Zeng et al., 2003).

1.2.2 Regulation of cell migration

Regulation of cell migration through protein–protein interactions and signaling events is focused on focalized adhesion dynamics and actomyosin polymerization and contraction. Cell protrusions binds to ECM via adhesion molecules, most notably transmembrane receptors of the integrin family (Hynes, 2002). Depending on the cell type and ECM substrate, focal contact assembly and migration can be regulated by different integrins: $\alpha_5\beta_1$ integrin binds fibronectin (Cukierman et al., 2001); $\alpha_6\beta_1$ or $\alpha_6\beta_4$ integrin bind laminin (Rabinovitz and Mercurio, 1997); $\alpha_i\beta_3$ integrin binds fibronectin or vitronectin (Leavesley et al., 1992); and $\alpha_2\beta_1$ integrin binds fibrillar collagen (Maaser et al., 1999). Besides integrins, proteases are also key regulator in cell migration. MMP-1 (a collagenase) binds to $\alpha_2\beta_1$ integrin (Dumin et al., 2001) and MMP-2 (a gelatinase) binds to $\alpha_i\beta_3$ (Brooks et al., 1998). Degradation of ECM facilitates cell expansion and migration as it provides more space (Friedl and Wolf, 2003a). Stress-fibre assembly and contraction are predominantly induced by the small G-protein RHO and its important downstream effector, the Rho-associated serine/threonine kinase (ROCK) (Kaibuchi et al., 1999; Katoh et al., 2001).

Cell migration speed is controlled by the turnover rates of adhesion and de-adhesion events (Lauffenburger and Horwitz, 1996), exhibiting an inverse relationship
between focal contact strength and migration rates. Stabilization of focal contacts increases attachment, reduces detachment and impairs migration rates, whereas weakening of adhesion strength, to a certain degree, propels migration (Lauffenburger and Horwitz, 1996; Palecek et al., 1997; Potter et al., 1998). Interestingly, previous studies also showed that ECM-degrading enzymes, such as MMPs and cathepsins, are frequently upregulated in tumor cells, and facilitate migration \textit{in vitro} (Deryugina et al., 1998; Rosenthal et al., 1999; Hofmann et al., 2000; Koblinski et al., 2000; Sameni et al., 2001), as well as dissemination and metastasis \textit{in vivo} (Rudolph-Owen et al., 1998; Maekawa et al., 2002). Similarly, the overexpression or activation of the RAC, RHO, ROCK signalling pathways have been correlated with \textit{in vitro} tumor-cell migration, as well as \textit{in vivo} invasion and progression (Itoh et al., 1999; Clark et al., 2000; Sahai et al., 2001). Collectively, the migration of tumor cells is activated by dominantly pro-migratory events, with absence of counteracting stop signals (Giannelli et al., 1997; Alper et al., 2001). This imbalance of signals allows cancer cells to become continuously migratory and invasive, leading to tumor expansion across tissue boundaries, followed by metastasis.

1.2.3 Two cell migration patterns and cell invasion

Tumor cells can infiltrate their surrounding tissues in two major patterns: individual cell migration and collective cell migration. In the former pattern, tumor cells spread as individual cells, while in the latter pattern, they migrate in solid cell strands, sheets, files or clusters (Friedl and Wolf, 2003b). Generally, tumor with lower differentiation stage is prone to expand via individual cells. Usually, individual migratory tumor cells originate from the interstitial stroma or bone marrow. Alternatively, cells that originated from a multicellular compartment, such as epithelium, lose their cell contacts, detach and migrate as individual cells through the adjacent connective tissue (Thiery, 2002). In contrast, in collective cell migration pattern, cell–cell adhesion that occurs in cell
groups is still present and results in the formation of a specific cortical actin filament assembly along cell junctions (Hegerfeldt et al., 2002). In the large contractile cell body, a subset of highly mobile cells at the front of the body, also called “path-generating cells”, generates migratory traction via pseudopod activity (Friedl et al., 1995; Hegerfeldt et al., 2002). Cells following path-generating cells are passively dragged behind (Friedl et al., 1995). The advantage of this kind of cell migration pattern is that the large cell mass can produce high concentrations of autocrine pro-migratory factors and matrix proteases, and protect inner cells from immunological assault by lymphocytes and natural-killer cells.

Individual cell migration, based on cell type, integrin engagement, cytoskeletal structure and protease production, can occur in different morphological variants: mesenchymal, amoeboid types, and cell chains. Mesenchymal movement is predominantly found in cells from connective tissue tumours, such as fibrosarcomas (Wolf et al., 2003), gliomas (Paulus et al., 1996), and in epithelial cancers following progressive dedifferentiation (Polette et al., 1998; Tester et al., 2000). This single cell migration pattern represents an efficient mechanism for tumor cell dissemination and metastasis (Putz et al., 1999). Amoeboid dissemination allows cancer cells to invade by undergoing early detachment and metastatic spread from a small primary tumor, and is most commonly observed in lymphomas and small-cell lung carcinomas (Rintoul and Sethi, 2002). Chain migration occurs in neural crest cells (Jacques et al., 1998), myoblasts (El Fahime et al., 2000) and melanomas (Friedl et al., 1997). The arrangement of invading tumor cells in chains represents a particularly effective penetration mechanism that confers high metastatic capacity and poor prognosis (Seftor et al., 2002). For collective migration, two variants have been described in tumours. In the first one, protruding sheets and strands maintain contact with the primary site, therefore result in local invasion. These characteristics are histologically detectable in invasive epithelial cancer such as oral squamous cell carcinoma and mammary carcinoma.
(Bell and Waizbard, 1986), colon carcinoma and others. In the second variant, a “nest” of cells detach from their origin and frequently extend along interstitial tissue gaps and paths of least resistance, as well as along perineural structures, as seen in epithelial cancer, melanoma and rhabdomyosarcoma (Bell and Waizbard, 1986) (Nabeshima et al., 1999). Using in vivo models, such tumour collectives can be detected at any stage of metastasis (Byers et al., 1995; Hashizume et al., 1996; Madhavan et al., 2001).

1.3 Tumor angiogenesis

Angiogenesis is the process of generating new blood vessels from pre-existing vessels (Lutsenko et al., 2003). Physiologically, it is a normal and vital process in growth and development, as well as in wound healing. However, angiogenesis also occurs under some pathological conditions such as cancer, macular degeneration, psoriasis and rheumatoid arthritis (Klagsbrun and Moses, 1999). Especially in cancer, it is believed that angiogenesis is a fundamental step required in the transition of tumors from a dormant state to a malignant one (Lutsenko et al., 2003). In this thesis, I found that ING4 and BRMS1 inhibit melanoma angiogenesis.

1.3.1 Formation of blood vessels through multiple steps

Angiogenesis is one of the hallmarks of cancer and a crucially acquired ability for cancer cells (Hanahan and Weinberg, 2000). It is required by solid tumors to grow beyond a certain size (1-2 mm) (Folkman et al., 1989). Tumor cells are dependent on angiogenesis because their growth and expansion require oxygen and nutrients, which are made available through the angiogenic vasculature. Accumulated evidence has shown that an alteration in the blood supply can significantly affect the tumor growth and its metastasis (Lutsenko et al., 2003).

Angiogenesis is a multiple-step process, which includes endothelial cell proliferation, migration and the formation of blood vessels (Kalluri, 2003; Mamou et al.,
First of all, VEGF and fibroblast growth factor (FGF), which are two important factors involved in angiogenesis (Cross and Claesson-Welsh, 2001), bind to their receptors on endothelial cells and activate signal transduction pathways. Then MMPs are activated and degrade the ECM, allowing the endothelial cells (ECs) to detach from the pre-existing capillary wall and proliferate. The Integrin $\alpha_v\beta_3$ on ECs membrane then facilitate their adhesion to the ECM as well as their migration. This step is followed by the binding of angiopoietin-1 Tie-2 receptors, which stimulate the recruitment of pericyte as well as vessel sprouting and stabilization. The final step is accomplished by platelet-derived growth factor (PDGF) -BB (PDGF isoform), which is released by the ECs and acts as a chemoattractant for pericyte precursors. Pericyte precursors join together with the ECs and differentiate into pericytes (Klagsbrun et al, 1999).

1.3.2 Characteristics of tumor angiogenesis

The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells to reside within 100 $\mu$m of a capillary blood vessel (Hanahan and Weinberg, 2000). However, experimental investigations demonstrated that the cells within aberrant proliferative lesions initially lack angiogenic ability, and so their expansion is limited. Therefore, incipient neoplasias must develop angiogenic ability to grow to a larger size (Bouck et al., 1996; Hanahan and Folkman, 1996; Folkman, 1997). Morphologically, blood vessels generated by tumor angiogenesis are different from normal blood vessels (McDonald and Baluk, 2002). Tumor blood vessels are irregular, heterogeneous and almost all of them are leaky. The capillaries are usually dilated, and the endothelial cells are often overlapped with each other and organized in a chaotic pattern. In addition they are characterized by lacking the normal smooth muscle layer due to the absence or detachment of pericytes. Interconnections and focal intercellular openings between the endothelial cells are very weak, which may
explain the reason that tumor blood vessels are extremely leaky compared to normal vasculature, which is another unique characteristic of tumor vessels (McDonald and Baluk, 2002). This leakiness contributes both to the extravasation of plasma proteins and erythrocytes, and to the transfer of tumor cells into the blood circulation and the formation of metastases (Maniotis et al., 1999). Moreover, the endothelial cells in tumor vessels do not form a normal monolayer, and thus the blood vessels show increased endothelial permeability for not only small molecules but also for large ones, which makes drug penetration very heterogeneous (Jain, 2001). Furthermore, the tumor vessels have irregular matrix protein composition, assembly and structures in the basement membrane, plus arterioles and venules are not clearly distinguished among tumor vessels. Blood flow is chaotic leading to a poorly oxygenated tumor tissue (Cao, 2004). All these characteristics of the tumor blood vessels are very important for anti-angiogenic cancer therapeutics, anticancer drug delivery and targeting approaches. Another interesting form through which tumor may acquire microcirculation is called “vasculogenic mimicry”, which was first described in uveal melanoma (Folberg et al., 1992; Folberg et al., 1993). The major difference between vasculogenic mimicry and conventional tumor angiogenesis lies in the composition of the vessels. Angiogenesis is new blood vessel formation from pre-existing ones and thus requires the participation of both endothelial cells and pericytes, while vasculogenic mimicry is basically composed of human tumor cells, with the absence of either endothelial cells or stromal cells. Uveal melanoma becomes an ideal model for studying vasculogenic mimicry because usually no induction of a stromal host response at the interface between the tumor and the surrounding host stroma was observed (Folberg et al., 2000). A very recent study from Tianjin Medical University Eye Center showed that curcumin, a major member of ginger family (Zingiberaceae), can inhibit tumor growth and vasculogenic mimicry through downregulating the EphA2/PI3K/MMP pathway in a
murine choroidal melanoma model. Further studies are needed to investigate the role of vasculogenic mimicry in human cutaneous melanoma and the possible value of targeting vasculogenic mimicry in melanoma therapy.

### 1.3.3 Regulation of tumor angiogenesis

Angiogenesis is usually regulated carefully by the balance between pro- and anti-angiogenic factors. Among these counterbalancing positive and negative signals, one group of them are soluble signals and their receptor, the latter displayed in the cell surface of endothelial cells. Another group of them are integrins and cell adhesion molecules that control cell-matrix and cell-cell communications (Hanahan and Weinberg, 2000). A long list of factors have been reported to initiate or sustain angiogenesis, and VEGF and FGF are two among the most important ones (Veikkola and Alitalo, 1999; Cross and Claesson-Welsh, 2001). Both VEGF and FGF bind to their corresponding transmembrane tyrosine kinase receptors on the surface of endothelial cells and activate the downstream signalling pathway.

VEGF can be induced by many factors through different pathways. One typical way is through hypoxia-inducible factor (HIF)-1α. Under hypoxic condition the degradation of HIF-1α was abolished and accumulated HIF-1α, together with HIF-1β, bind to the hypoxia response element (HRE) on the VEGF promoter and enhance the expression of VEGF (Semenza, 2003). It is also reported that blocking STAT3 signalling has been shown to inhibit SRC- and IL-6-induced VEGF upregulation (Niu et al., 2002). It is known that IL-6 is a downstream target of nuclear factor-kappa B (NF-κB) transcription factor (Coles et al., 2010). Therefore NF-κB/IL-6 may also play important roles in angiogenesis regulation. So far, it is not clear what molecules or signalling pathway is responsible for the secretion of FGF, but it is known that FGF promotes the proliferation and differentiation of ECs, smooth muscle cells as well as fibroblast (Kalluri, 2003).
Some other receptor tyrosine kinase ligands also function as activators of angiogenesis, such as PDGF and epidermal growth factor (EGF). They are involved in the endothelial cell proliferation and migration (Bergers and Benjamin, 2003). Integrin signalling also contributes to this regulatory balance. Quiescent vessels express one class of integrins, whereas sprouting capillaries express another. Interference with signalling from the latter class of integrins can inhibit angiogenesis (Varner and Cheresh, 1996; Giancotti and Ruoslahti, 1999), implying the important roles of cell adhesion molecules in angiogenesis regulation (Hynes and Wagner, 1996). Extracellular proteases are physically and functionally associated with angiogenic integrins, and both regulate the invasive capability of angiogenic ECs (Stetler-Stevenson, 1999). The first described and best studied angiogenic inhibitor is thrombospondin-1, which modulates endothelial cell proliferation and motility (Bull et al., 1994). In another well-designed experiment, thrombospondin-1 was found to be positively regulated by the p53 tumor suppressor protein in some cell types. Consequently, loss of p53 function, which occurs in most human tumors, causes downregulation of thrombospondin-1, liberating ECs from its inhibitory effects (Dameron et al., 1994b, a). Other inhibitors for angiogenesis are angioatatin (O'Reilly et al., 1994), and endostatin (O'Reilly et al., 1997).

1.3.4 Metastatic cascade

Metastasis is defined as the progressive growth of secondary tumor foci at sites discontinuous with the primary lesion (Kauffman et al., 2003). It is believed to be a late event but critical acquired capability for cancer (Hanahan and Weinberg, 2000). Melanoma can quickly metastasize to the regional lymph nodes or distant sites and the whole process consist of sequential and interrelated steps. After the initial transforming event, primary melanoma cells need to invade local tissue and intravasate into the bloodstream or lymphatic channel system. Then the melanoma cells in the circulation must
survive the transit process until they eventually arrest in the microvasculature of the secondary site, such lung, liver and brain. From there they may extravasate into the target tissue, followed by the final step of the cascade, growth into the metastatic lesion. This final step, also called as metastatic colonization, can be further subdivided into tumor cell survival and proliferation, both of which need the development of a blood supply through angiogenesis. The inability of a cancer cell to complete any single step in the metastatic cascade results in its failure to form clinically significant metastasis.

1.3.5 Angiogenesis and metastasis

Those early studies about tumor angiogenesis focused on the effects of the neovascularation on tumor expansion and the potential application of angiogenic inhibitors to tumor regression (Folkman, 1971, 1974). However, the impact of angiogenesis on tumor metastasis was relatively less well studied. Accumulated evidence indicate that highly vascular tumors have the potential to produce more metastasis than less angiogenic tumors, indicating that angiogenesis is a critical component of tumor metastasis (Zetter, 1998). In addition to its effects on tumor growth, angiogenesis can facilitate tumor metastasis by providing an efficient route for tumor cells to leave their primary site and enter the circulation. This is due to the characteristics of tumor vasculature: increased density, elevated permeability and less intercellular juctional complexes (Dvorak et al., 1995). Experimental investigation showed that as many as one million tumor cells can enter the circulation per gram of tumor per day (Chang et al., 2000). Although only few of these cells shedding into the blood vessels form metastases eventually, the number of metastases formed is generally proportional to the number of tumor cells shed. Angiogenesis may also facilitate cancer metastasis through supplying oxygen and nutrients to both the primary and secondary tumor sites. A primary melanoma needs oxygen and nutrient to grow and invade deep enough to reach the blood vessels and lymphatic system.
The tumor macro metastasis formation at the secondary site also requires angiogenesis to provide oxygen and nutrients (O'Reilly et al., 1994). The correlation between tumor angiogenesis and tumor metastasis is demonstrated by numerous experiments in animals. Taylor et al. showed in their early experiment that angiogenic inhibitor protamine sulphate inhibited angiogenesis as well as tumor metastasis (Taylor and Folkman, 1982). Similar results have since been obtained with nearly every angiogenic inhibitor identified, regardless of its mechanism of action.

The list of angiogenic inhibitors that also inhibit tumor metastasis includes the angiostatic steroids (Crum et al., 1985), thalidomide (D'Amato et al., 1994), the fumagillin analog TNP-470 (Ingber et al., 1990; Konno et al., 1995; Mori et al., 1995), thrombospondin (Weinstat-Saslow et al., 1994), angiostatin (O'Reilly et al., 1994), endostatin (O'Reilly et al., 1997), platelet factor 4 (Kolber et al., 1995), and the synthetic protease inhibitor BB94 (Watson et al., 1995). Decreased vascularity of the primary tumor is virtually always associated with decreased formation of metastatic colonies from all these studies. In addition to metastasis, the extent of angiogenesis was also shown to be correlated with patients’ survival in many different cancers. The first study in this line was from Weidner and colleagues who revealed that vascular density is correlated with metastasis of human breast cancer, and functions as an independent prognostic marker (Weidner et al., 1991). This report was then followed by a series of studies that confirmed this correlation in human breast cancer first (Ellis and Fidler, 1995; Gasparini and Harris, 1995; Hollingsworth et al., 1995), and then extended the finding to other type of cancers, including carcinoma of the prostate (Weidner et al., 1993; Brawer, 1996), lung (Yamazaki et al., 1994; Angeletti et al., 1996), stomach (Maeda et al., 1995), cervix (Wiggins et al., 1995), ovary (Hollingsworth et al., 1995), and head and neck squamous cell carcinoma.
(Gasparini et al., 1993), suggesting that angiogenesis is actually indicative of both increased metastasis and decreased survival.

1.3.6 Anti-angiogenesis tumor therapy

Anti-angiogenic tumor therapies are considered one of the most promising approaches in cancer treatment. Although previous clinical trial failed to obtain an overall survival benefit with anti-VEGF monoclonal antibodies which is used as monotherapy agent, the addition of a VEGF-specific antibody, bevacizumab to chemotherapy led to improved overall survival of colorectal cancer and lung cancer patients as well as progression-free survival in breast cancer (Jain et al., 2006). An unsuccessful data from a clinical trial using a VEGF antibody may only demonstrate that this specific agent does not benefit the patients. The dependence of tumor growth and metastasis on blood vessel formation still makes angiogenesis a rational target for cancer therapy. In a recent study, bevacizumab, a humanized monoclonal antibody which targets VEGF-A, was shown as an effective anti-angiogenic agent in treatment of ovarian granulosa cell tumor (Tao et al., 2009). A good example to illustrate the combined effect of anti-angiogenic and other therapies is the combination of bevacizumab and erlotinib in malignant pleural mesothelioma (Jackman et al., 2008). Intracellular tyrosine kinase inhibitors (TKIs) of angiogenesis, such as pazopanib, also demonstrated promises, especially with the advantage of their oral administration (Monk et al., 2010). However, some problems still remain to be resolved before anti-angiogenic therapy may accomplish major successes, including the poor drug-delivery (Jain, 2005), and the induced hypoxia by anti-angiogenic treatment in tumor (Ceradini et al., 2004).

1.4 Inhibitor of growth family of tumor suppressors

The Inhibitor of Growth (ING) genes were discovered during the past decades and identified as type II tumor suppressor genes (Russell et al., 2006). Previous studies
demonstrated that ING family members participate in various cellular stress responses and thus play important roles in the pathogenesis of various types of cancers, including melanoma (Campos et al., 2004a).

1.4.1 Gene location and splicing variants of INGs

ING family proteins are a group of tumor suppressors that are evolutionarily conserved and widely present in the eukaryotic proteomes (He et al., 2005). A summary of ING family members, their gene locations, associated variants, and reported functions are listed in Table 1.1. The founding member, ING1, was first discovered in 1996 using subtractive hybridization (Garkavtsev et al., 1996), and subsequently the other four ING family members were identified by searching the homologous sequence with ING1 (Shimada et al., 1998; Nagashima et al., 2003; Shiseki et al., 2003). All ING family members were mapped at independent chromosomes but they were all located in the subtelomeric region of their corresponding chromosomes except ING3 (Ythier et al., 2008). The exact gene location for all five ING family members are: ING1 (13q34), ING2 (4q35), ING3 (7q31.3), ING4 (12p13.3), and ING5 (2q37.3).

Splicing variants have been reported for several members and summarized as below (Li et al., 2009a). Five variants have been found for ING1 gene so far, including ING1v1 (p33ING1b), ING1v3 (p27ING1d), ING1v4 (p47ING1a), and ING1v2 and ING1v5 which encode the same protein p24ING1c (Walzak et al., 2008). The widely studied ING1b and ING1a showed different effects in many biological processes. ING1b associates with proteins with HAT activities and induces histone H3 and H4 acetylation, whereas ING1a associates with HDAC1 and inhibits histone acetylation (Vieyra et al., 2002). Upregulation of ING1b, not ING1a, induces apoptosis and sensitizes young fibroblasts to UV irradiation and hydrogen peroxide mediated apoptosis (Vieyra et al., 2002). There are two variants for ING3 gene in NCBI Genebank database, ING3v1 and
ING3v3; ING3v2 was removed from the NCBI Genbank database as a nonsense-mediated mRNA decay candidate. There are no further studies regarding the different functions between ING3v1 and ING3v3 so far (Walzak et al., 2008). ING4 gene encodes 4 variants, ING4v1, ING4v2, ING4v3, and ING4v4, and these variants exhibit distinct subcellular localization feature and play different roles in cellular functions. ING4v1 is the original ING4 and is mainly localized in the nucleus. ING4v1 negatively regulates cell proliferation and loss of contact inhibition (Nagashima et al., 2003; Kim et al., 2004). ING4v2 is mainly localized in cytoplasm and maintains the suppressive effect on cell migration, exerts weaker inhibition on cell proliferation, but loses its effect on cell spreading (Unoki et al., 2006). ING4v3 is a 9 bp skip form of the original ING4v1, and is expressed at a quite low level and only accounts for less than 10% of the expression of all ING4 variants. ING4v4, in contrast to ING4v1, plays a dominant negative role in the induction of p21 and in the suppression of cell motility by ING4v1 (Unoki et al., 2006). In a recent study, 5 alternative splicing variants of ING5 mRNA were discovered. Besides the full-length ING5, the other four variants are 182bp, 280bp, 392bp, and 517bp shorter than the full-length ING5 (Cengiz et al., 2010). So far there is no report about the different function of these ING5 variants.

1.4.2 Structure of ING family proteins

All ING proteins share highly homologous C-terminal structure, while each has a unique N-terminal structure. The domains of different ING family members and their corresponding binding partners are summarized in Figure 1.1. C-terminal of ING proteins is characterized by one or two nuclear localization signals (NLS) plus a plant homeodomain (PHD) (Schindler et al., 1993; He et al., 2005). The NLS motif is a strong basic region and essential for the transportation and accumulation of proteins in nucleus (Dingwall and Laskey, 1991; Garcia-Bustos et al., 1991; Makkerh et al., 1996). Three
potential nucleolar targeting signals (NTS) are found within the NLS motif of ING1 proteins, which has been shown to target ING1 to the nucleolus after UV damage (Scott et al., 2001a). Previous study indicated that deletion of three NTS-containing NLS motif can prevent ING1b from translocation into nucleolus, but was not able to abolish the localization of ING1b in the nucleus (Russell et al., 2008). However, deletion of both the NLS motif and a C-terminus NLS-like motif (residues 265-279) completely disrupted the nuclear import of ING1b (Russell et al., 2008), thereby pointing out the significance of basic acid-enriched C-terminus NLS motif in regulating the subcellular localization of ING proteins. The NLS motif of ING4 protein was also identified to be the region binding to p53, and thus necessary for p53-associated functions of ING4 (Chaturvedi et al., 2005).

The PHD finger, identified in 1993 as a Cys$_4$-His-Cys$_3$ motif in the homeodomain protein HAT3 in Arabidopsis thaliana (Schindler et al., 1993), is a Zn$^{2+}$-binding domain (Bienz, 2006) and is widely believed to be involved in protein-protein interactions and thus is commonly found in nuclear chromatin-binding proteins (Aasland et al., 1995; Pascual et al., 2000; Bordoli et al., 2001; Capili et al., 2001). In addition, a small conserved protein-interacting motif (PIM) is found along with PHD region of ING1 and ING2, which consists of acidic, basic and aromatic residues, and can bind to phosphatidylinositol monophosphates (Gozani et al., 2003).

Several unique motifs are reported at the N-terminus of different ING proteins. A specific PCNA (proliferating cell nuclear antigen)-interacting protein domain (PIP) was found at the amino-terminus of ING1, and through this PIP domain ING1 competes with p21 for binding to PCNA and regulates the cellular response to UV irradiation (Scott et al., 2001b). A leucine-zipper like motif (LZL) was described at the N-terminal of ING2 and is required for ING2-p53 interaction. LZL motif was shown to be crucial for ING2-enhanced DNA repair and apoptosis in response to UV irradiation (Wang et al., 2006b). Through
these domains, ING family proteins play significant roles in multiple critical cellular processes, which will be described in the next section.

1.4.3 Biological functions of ING proteins

ING proteins have been demonstrated to regulate a variety of cellular functions, including cell cycle progression, apoptosis, DNA repair, cell migration and invasion, and angiogenesis (Campos et al., 2004a; Russell et al., 2006).

1.4.3.1 ING proteins induce apoptosis

Apoptosis was first used as a term to describe the morphologically distinct form of cell death from necrosis in 1972 (Papathanasiou et al., 1991). Also known as type I programmed cell death, apoptosis serves either as an antagonist to mitosis for the controlled elimination of excessive cells that are no longer needed by the organism (e.g. during atrophy or tissue development), or as a critical defence mechanism against disease (e.g. to eliminate pre-neoplastic cells or damaged cells beyond repair after cytotoxic injury) (Bursch et al., 2000).

The initial experiments on the role of ING1 in apoptosis demonstrated that ING1 was expressed dominantly in regressing tails of Xenopus tadpoles but absent from growing hind limbs and the expression of ING1 was also induced in serum-starved P19 teratocarcinoma cells (Helbing et al., 1997; Wagner et al., 2001). Our group found that ING1b mediates UV-induced apoptosis in melanoma cells (Cheung and Li, 2002). While in MMRU, a melanoma cell line with functional p53, ING1b overexpression dramatically enhanced apoptosis induced by UVB irradiation, it did not increase the apoptosis in another melanoma cell line, MeWo, which carries a mutant p53. The effect of ING1b in apoptosis may be attributable to the interrelation between ING1b and p53, as ING1b can interact with p53 and subsequently stabilize p53 by sequestering p53 from MDM2, and enhance the transcriptional activity of p53 (Leung et al., 2002). An alternative explanation
for ING1b induced apoptosis is through the binding with PCNA. ING1b translocates to the nucleolus and binds to PCNA through its PIP domain (Scott et al., 2001b). This binding may interfere with or completely inhibit the binding of PCNA, a necessary factor for DNA replication, with p21. As the binding of PCNA with p21 causes G1 cell cycle arrest and thus the switch from DNA replication to DNA repair, the binding of ING1b with PCNA may inhibit DNA repair and cell cycle arrest, allowing the replication of damaged DNA, finally leading to apoptosis (Scott et al., 2001b).

Our group previously showed that ING2 enhanced UVB-induced apoptosis in melanoma MMRU cells in a p53-dependent manner (Chin et al., 2005). In this study, our group demonstrated that overexpression of ING2 significantly downregulated the expression of Bcl-2, resulting in an increased Bax/Bcl-2 ratio. Moreover, our laboratory found that ING2 promoted Bax translocation to mitochondria, altered the mitochondrial membrane potential, and induced cytochrome c release and thus the activation of caspase-9 and -3. In addition, our group showed that under non-stress conditions ING2 upregulates Fas expression and activates caspase-8, leading to the activation of extrinsic apoptotic pathway (Chin et al., 2005).

ING3 was reported to stimulate apoptosis of RKO cells under a non-stress condition by upregulating Bax and p21 expression in a p53-dependent manner (Nagashima et al., 2003; Doyon et al., 2004). Our group demonstrated that overexpression of ING3 significantly promoted UV-induced apoptosis in a p53-independent manner. Furthermore, ING3 did not change the expression of p53 target genes involved in intrinsic apoptotic pathways, such as Bax, but increased the cleavage of Bid and caspases-8, -9, and -3, instead. Moreover, ING3-enhanced apoptosis after UV irradiation was blocked by inhibition of caspase-8 or Fas activation. Knockdown of ING3 decreased UV-induced apoptosis remarkably (Wang and Li, 2006).
ING4 and ING5 were reported to enhance apoptosis under non-stress conditions in a p53-dependent manner (Shiseki et al., 2003). Another study by Wilhelm et al demonstrated that ING4 overexpression enhanced apoptosis triggered by serum starvation in HepG2 cells (Wilhelm et al., 2004). The same study also showed that exogenous ectopic expression of ING4 increased cell death when the cells were exposed to DNA damaging agents, such as etoposide and doxorubicin, and thus implying that ING4 may enhance the chemosensitivity to certain anticancer agents in HepG2 cells (Wilhelm et al., 2004).

1.4.3.2 ING proteins are involved in cell cycle progression

Cell cycle checkpoints are crucial control mechanisms that are required for the fidelity of cell division in eukaryotic cells. Many studies have shown that ING proteins are involved in the regulation of cell cycle progression. The first mechanism proposed to be responsible for the effect of ING1 to halt the cell cycle is cooperation between ING1 proteins and p53 to enhance the transcription of p53 target gene p21 (Garkavtsev et al., 1998).

p21 is a cyclin-dependent kinase inhibitor (CDKI) that plays an important role in G1-S cell cycle progression. As a mediator of the G1 cellular checkpoint, p21 binds and inactivates cyclin-Cdk2 or cyclin-Cdk4 complexes, resulting in hypophosphorylation of retinoblastoma protein (RB), sequestration of E2F, inactivation of E2F responsive genes, including cyclin A and cyclin E, and finally arresting the cell cycle at the G1-S transition (Nigg, 1995; Spellman et al., 1998; Abukhdeir and Park, 2008). p21 was previously reported as a downstream target of p53, but various lines of evidence showed that most ING proteins except ING1a have a positive effect on p21 expression or activity. Using luciferase assay, Kataoka et al showed that ING1b, ING1c, ING2 and ING3 all stimulated the p21 promoter, while ING1a showed the opposite effect (Kataoka et al., 2003). Overexpression of ING4 and ING5 can enhance the expression of p21, resulting in
decreased S phase population and increased G1 and G2/M phases 48 hours after transfection. Moreover, this induction of p21 and regulation of cell cycle by exogenous expression of ING4 or ING5 was observed in the RKO cell line with wild-type p53, but not in RKO-E6 cells with mutant p53, indicating these effects are p53-dependent (Shiseki et al., 2003).

Besides p21, other cell cycle regulators are also reported to be targets of ING proteins. Accumulated evidence indicates that cyclin B1, a key modulator in G2 phase, is also regulated by ING1b and ING1c (Garkavtsev and Riabowol, 1997; Nakamura et al., 2002; Takahashi et al., 2002; Porter and Donoghue, 2003). Simultaneous upregulation of p53 and ING1b significantly inhibited the transcription of cyclin B1 (Takahashi et al., 2002). ING2 interacts with SnoN, the Smad-interacting transcriptional modulator, and promotes the assembly of the protein complex containing SnoN, ING2 and Smad2, thereafter induces Transforming Growth Factor-beta (TGF-β) dependent transcription and cell cycle arrest in epithelial cells (Sarker et al., 2008). ING4 was reported to inhibit human lung adenocarcinoma cell growth both in vitro and in vivo by inducing p27, suppressing cyclin D1, Skp2, and Cox-2, and inactivating the Wnt-1/beta-catenin pathway (Li et al., 2008b).

1.4.3.3 ING proteins enhance DNA repair in response to UV irradiation

UV irradiation plays important roles in melanoma development by inducing two major types of DNA lesions, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidine photoproducts (6-4PPs), which are repaired by the nucleotide excision repair (NER) pathway. Previously we have shown that ING1b and ING2 are involved in NER (Bush et al., 2001; Campos et al., 2004a; Murr et al., 2006; Kuo et al., 2007). The physiological level of both ING1b and ING2 are required for NER in a p53-dependent manner (Wang et al., 2006a; Kuo et al., 2007), suggesting that ING proteins may
cooperate with p53 in NER. We also demonstrated that although ING1b and ING2 do not colocalize at UV lesions, these two proteins enhance rapid histone H4 acetylation, chromatin relaxation, and the recruitment of XPA to the DNA photolesions after UV irradiation (Wang et al., 2006a; Kuo et al., 2007), indicating that ING proteins are involved in global histone acetylation and chromatin remodelling. Furthermore, we found that the LZL domain of ING2 is crucial for proper functions of ING2 in NER, and deletion of the LZL domain abrogates the association between ING2 and p53 (Wang et al., 2006a).

### 1.4.4 Role of ING family proteins in melanoma progression

Previous studies showed that the expression of ING proteins are frequently deregulated in various human cancers and the altered expression levels are associated with cancer progression. ING1 expression has been reported to be downregulated or even lost in many human cancers, including lymphoid malignancies (Ohmori et al., 1999), bladder cancer (Sanchez-Carbayo et al., 2003), gastric cancer (Graham et al., 1999), breast cancer (Tokunaga et al., 2000), and non-small cell lung carcinoma (Kameyama et al., 2003). However, ING1 expression is not necessarily reduced in malignancies. Increased ING1 expression was reported in basal cell carcinoma (Chen et al., 2003), gliomas (Kataoka et al., 2003), as well as melanoma (Campos et al., 2002). ING1 expression is upregulated in 14 different melanoma cell lines at both mRNA and protein levels when compared with the normal human epithelial melanocytes. In the same study, single strand conformation polymorphism (SSCP) and direct DNA sequencing were also applied and confirmed the existence of nucleotide alterations in two melanoma cell lines, including one missense mutation detected in SK-mel-24 melanoma cell line (Campos et al., 2002).

Although the mechanism of ING1 overexpression in melanoma and how this upregulated expression is associated with melanoma progression remains elusive, studies showed that the expression and mutational status of the *ING1* gene in melanoma follows
that of p53. Neither ING1 nor p53 gene has frequent mutations in human melanoma (Sparrow et al., 1995b; Nouman et al., 2003). Both ING1 and p53 are overexpressed in most human melanoma biopsies (Bartek et al., 1991; Stretch et al., 1991). Nevertheless, the degree of p53 overexpression in melanoma is shown to be closely correlated with poor prognosis (Sparrow et al., 1995a; Essner et al., 1998). These concomitant alterations between ING1 and p53 suggest that the abnormal expression of ING1 may contribute to melanoma development through the physical interaction and interdependent functions between these two tumor suppressors (Garkavtsev et al., 1998; Leung et al., 2002). Another finding that can explain the association between ING1 overexpression and melanoma progression is that down-regulation of ING1 tumor suppressor sensitizes glioblastoma cells to cisplatin-induced cell death (Tallen et al., 2003). Therefore, it is possible that high ING1 expression constrains cancer chemotherapy through enhancing DNA repair (Campos et al., 2004b; Kuo et al., 2007).

Reduced ING2 expression was reported in hepatocellular carcinoma when compared with the matched non-tumor liver tissues, and this reduction of ING2 expression is correlated with tumor size, histological classification as well as serum AFP levels (Zhang et al., 2008). Kaplan-Meier curves and Cox analysis revealed that decreased ING2 expression can be used as an independent prognostic marker in hepatocellular carcinoma (Zhang et al., 2008). ING2 mRNA expression was reported to be downregulated in lung cancer cell lines (Okano et al., 2006). Our group demonstrated that nuclear ING2 expression was significantly reduced in primary and metastatic melanoma when compared with dysplastic nevi, but this reduction is not correlated with melanoma tumor thickness, ulceration or patient survival. This study suggested that reduced ING2 expression may contribute mostly to the initiation instead of progression of human melanoma (Beder et al., 2006).
Loss or decreased ING3 mRNA was reported in head and neck carcinoma and this reduction of ING3 mRNA is correlated with poorer patient survival (Gunduz et al., 2005; Gunduz et al., 2008). In melanoma, significantly reduced nuclear ING3 expression was detected in malignant melanomas compared with dysplastic nevi. Reduced nuclear ING3 expression was also correlated with increased ING3 level in cytoplasm, suggesting a nuclear-to-cytoplasm shift mechanism during melanoma progression. Furthermore, the reduction of nuclear ING3 expression was significantly correlated with a poorer prognosis in patients with primary melanoma (Kuo et al., 2007).

In a recent study, expression of ING5 was examined in head and neck squamous cell carcinoma (HNSCC) in which nuclear expression of ING5 in HNSCC was found to be significantly lower than that of non-cancerous epithelium, and was positively correlated with a well-differentiated status (Li et al., 2010b). In contrast, cytoplasmic expression of ING5 was significantly increased in HNSCC, and was inversely correlated with a well-differentiated status and nuclear ING5 expression. These data suggest that a decrease in nuclear ING5 localization and cytoplasmic translocation are involved in tumorigenesis and tumor differentiation in HNSCC (Li et al., 2010b). In another research study, mutation and mRNA expression status of ING5 were examined by RT-PCR and sequencing in oral squamous cell carcinoma (Cengiz et al., 2010). Three missense mutations within the LZL finger and novel chromatin regulatory (NCR) domains in ING5 protein were detected. In addition, it was revealed that ING5 mRNA expression decreased in 61% of the primary tumors as compared to the matched normal samples (Cengiz et al., 2010). Alteration in ING4 expression will be discussed in the next section.

1.4.5 Novel tumour suppressor ING4

ING4 is a relative novel member of ING family of tumor suppressors and is the major topic in this thesis. The human \( ING4 \) gene is located at 12p13.31 and consists of 8
exons, which encodes a 249-amino acid protein (Shiseki et al., 2003). Previous studies showed ING4 expression was decreased in various human cancers (Garkavtsev et al., 2004; Gunduz et al., 2005; Li et al., 2008a), and overexpression of ING4 diminished colony-forming efficiency, decreased cell population in S phase, and induced p53-dependent apoptosis (Shiseki et al., 2003). ING4 can physically interact with p300, a member of histone acetyltransferase (HAT) complexes, and consequently enhance p53 acetylation at Lys382 (Shiseki et al., 2003). ING4 was also shown to physically interact with the p65 subunit of NF-κB and regulate brain tumor angiogenesis through transcriptional repression of NF-κB-responsive genes, including IL-8, IL-6, COX2, and colony-stimulating factor 3 (Garkavtsev et al., 2004). In addition, ING4 acts as an adaptor protein to mediate the activity of hypoxia-inducible factor (Ozer et al., 2005).

1.5 BRMS1 and cancer metastasis

*BRMS1* (BReast cancer Metastasis Suppressor 1) was first identified by differential display comparing metastasis-suppressed chromosome 11 hybrids with metastatic, parental MDA-MB-435 human breast carcinoma cells in 2000 (Seraj et al., 2000b). The *BRMS1* gene is located on chromosome 11q13.1-13.2 and genomic *BRMS1* consists of 10 exons and 9 introns, spanning about 7 kb. The encoded BRMS1 protein is 246 amino acid long and carries two NLS that target this protein to the nucleus (Samant et al., 2000; Seraj et al., 2000b).

It is reported that BRMS1 mRNA expression level was high in melanocytes, but barely detectable in metastatic melanoma cell lines. Moreover, re-introduction of BRMS1 into highly metastatic melanoma cell line C8161.9 significantly suppressed the metastatic potential in both experimental and spontaneous metastasis assays without affecting tumor growth after orthotopic injection (Shevde *et al*., 2002). The effect of BRMS1 to inhibit metastasis was also demonstrated in other cancer models. BRMS1 expression is 5-fold
higher in the metastasis-suppressed hybrid neo11/435 cells when compared with the highly metastatic parental breast cancer cell line MDA-MB-435. Furthermore, BRMS1 transfected MDA-MB-435 cells showed significantly decreased incidence and number of metastases to the lung and regional lymph nodes when cells were injected orthotopically. However, the tumor growth rate of BRMS1-transfected MDA-MB-435 cells is similar to their parental controls, except a delay in growth for one week (Seraj et al., 2000b). BRMS1 was also shown to be expressed at lower level in a highly metastatic human bladder carcinoma cell line T24T compared with the less metastatic parental cell line T24 (Seraj et al., 2000a).

Different mechanisms may contribute to the reduction of BRMS1 expression in metastatic cancers. One possibility is that deletion at chromosome 11q, which includes the BRMS1 gene, occurs at very high frequency in various human cancers (Welch and Goldberg, 1997; Meehan and Welch, 2003; Zainabadi et al., 2005). Another study recently reported that the methylation of BRMS1 promoter might account for the loss of BRMS1 in breast cancer cells. The same study also demonstrated a direct correlation between methylation status of BRMS1 promoter in the DNA isolated from those tissues with loss of BRMS1 by immunohistochemistry, concomitantly (Metge et al., 2008). These studies imply that the loss of the BRMS1 expression may be a common event in tumor metastasis.

Another feature of BRMS1 protein is the large glutamic acid-rich region at the N-terminus, which is a putative acidic transcriptional transactivation domain and may stimulate transcription in various eukaryotic organisms (Struhl, 1995; Samant et al., 2000). Several metastasis-related genes were reported to be regulated by BRMS1, including epidermal growth factor receptor (Vaidya et al., 2008), osteopontin (Samant et al., 2007; Hedley et al., 2008), CXC chemokine receptor 4 (Yang et al., 2008), as well as microRNA-146 (Hurst et al., 2009). BRMS1 was also reported to interact with mSin3
chromatin remodelling complex and recruit HDACs to suppress downstream gene expression (Meehan et al., 2004). Cicek et al showed that BRMS1 physically interacts with RelA/p65 subunit of NF-κB and inhibits phosphorylation of IκBα, and thus negatively regulates NF-κB pathway (Cicek et al., 2005; Cicek et al., 2009). Through these mechanisms, BRMS1 has been shown to inhibit metastasis in xenograft models of breast cancer, melanoma and ovarian carcinoma (Seraj et al., 2000b; Shevde et al., 2002; Zhang et al., 2006). However, the underlying mechanism(s) through which BRMS1 functions to suppress metastasis still remains to be elucidated.

1.6 Objective

In this thesis, we sought to evaluate the prognostic significance of ING4 in human cutaneous melanoma and to examine the tumor suppressive roles of ING4 in melanoma cell migration, invasion and angiogenesis, as well as the mechanisms involved in these functions. We found that the expression of ING4 was significantly decreased in human melanoma compared with dysplastic nevi, and reduced ING4 expression was correlated with worse melanoma patient survival. We also demonstrated that ING4 inhibited melanoma cell migration and cell invasion. Finally we revealed that with BRMS1 as the upstream regulator, ING4 suppressed NF-κB/IL-6 to inhibit melanoma angiogenesis. These findings contribute to the better understanding of the pathways regulated by the tumour suppressor ING4, and therefore help to develop novel strategies for the prevention and treatment for human melanoma as well as other types of cancer.
<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Variants</th>
<th>length</th>
<th>Functions</th>
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<tbody>
<tr>
<td>ING1</td>
<td>13q33-34</td>
<td>ING1 v1 (p33ING1b)</td>
<td>279 a.a</td>
<td>Induce apoptosis, cell cycle arrest. Inhibit cell proliferation and angiogenesis.</td>
</tr>
<tr>
<td></td>
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<td>ING1 v2 and v5 (p24ING1c)</td>
<td>210 a.a</td>
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<td>ING1 v3 (p27ING1d)</td>
<td>235 a.a</td>
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<td></td>
<td></td>
<td>ING1 v4 (p47ING1a)</td>
<td>422 a.a</td>
<td>Show opposite functions to other ING1 variants</td>
</tr>
<tr>
<td>ING2</td>
<td>4q35.1</td>
<td>N/A</td>
<td>280 a.a</td>
<td>Induce apoptosis, cell cycle arrest, and DNA repair</td>
</tr>
<tr>
<td>ING3</td>
<td>7q31.3</td>
<td>ING3 v1</td>
<td>418 a.a</td>
<td>Induce apoptosis.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ING3 v3</td>
<td>98 a.a</td>
<td>Inhibit cell growth, loss of contact inhibition, cell migration and invasion. Induce apoptosis and cell cycle arrest.</td>
</tr>
<tr>
<td>ING4</td>
<td>12p13.3</td>
<td>ING1 v2</td>
<td>248 a.a</td>
<td>Maintain all functions of v1, except the inhibition of cell migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ING1 v3</td>
<td>246 a.a</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ING1 v4</td>
<td>245 a.a</td>
<td>Dominantly negative function of v1.</td>
</tr>
<tr>
<td>ING5</td>
<td>2q37.3</td>
<td>ING5 v1</td>
<td>Unknown</td>
<td>Induce apoptosis, cell cycle arrest.</td>
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<td></td>
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<td>ING5 v2</td>
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<td></td>
<td>ING5 v4</td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td>ING5 v5</td>
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Figure 1.1 Structural domains of ING family members and their binding partners. The domains of different ING family members and their corresponding binding partners are summarized.
2  Materials and Methods

2.1  Construction of tissue microarray

Formalin-fixed, paraffin-embedded tissues from 66 dysplastic nevi, 118 primary melanomas, and 53 metastatic melanomas were used for the construction of tissue microarray (TMA). All specimens were obtained from the 1990 to 1998 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 performed in accordance with the Declaration of Helsinki guidelines.

The most representative tumor areas were carefully selected and marked on the haematoxylin and eosin-stained slides. The TMA slides were assembled using a tissue-array instrument (Beecher Instruments, Silver Spring, MD) equipped with thin-walled stainless steel punches and stylets. Duplicate 0.6-mm diameter tissue cores were collected from each donor block and transferred into the recipient block at the designed positions. The purpose to use duplicate tissue cores is to avoid the limitations of the representative areas of the tumor. Multiple 4-µm sections were cut with a Leica microtome (Leica Microsystems Inc, Bannockburn, IL), and transferred to adhesive-coated slides using routine histology procedures. Haematoxylin and eosin were used to stain one section from each TMA. Other sections were kept at room temperature for immunohistochemical staining.

2.2  Immunohistochemistry

TMA slides were dewaxed at 55°C for 30 min and washed with xylene for 5 min, three times. Tissues were rehydrated by a series of 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 with 3% hydrogen peroxide for 20 min in room temperature. After blocking with
universal blocking serum (Dako Diagnostics, Missisauga, ON, Canada) for 30 min, slides were incubated with a polyclonal rabbit anti-ING4 antibody (1:50 dilution, Protein Tech Group, Chicago, IL), or a monoclonal mouse anti-BRMS1 antibody (1:200 dilution, kind gift of Dr. Danny Welch, University of Alabama at Birmingham, Birmingham, AL) (Hicks et al., 2006) at 4°C overnight. The sections were then incubated with biotin-labeled secondary antibody and streptavidin-peroxidase (Dako Diagnostics) for 30 min each, followed by developing with 3, 3′-diaminobenzidine substrate and counterstained with hematoxylin. Slides were finally dehydrated and sealed with coverslips. Negative controls were performed by omitting the primary antibody during the primary antibody incubation.

2.3 Evaluation of TMA immunostaining

The evaluation of ING4 and BRMS1 staining was made blinded by one dermatopathologist together with two other independent observers simultaneously, and a consensus score was reached for each core. For both ING4 and BRMS1, the staining intensity was scored into four grades: 0, negative; 1, weak; 2, moderate; 3, strong. Percentages of positive staining 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 different cores, the higher score was taken as the final score. The levels of ING4 and BRMS1 staining were evaluated by immunoreactive score (Remmele and Stegner, 1987), which is calculated by multiplying the scores of staining intensity and the percentage of positive cells. Based on IRS, ING4 staining pattern was defined as: negative (IRS: 0), weak (IRS: 1–4), moderate (IRS: 6–8), and strong (IRS: 9–12). BRMS1 staining pattern was defined as: weak (IRS: 0–6), and strong (IRS: 8–12).

2.4 Statistical analysis of TMA immunostaining

The SPSS version 11.5 software (SPSS, Chicago, IL) was used for the statistical analysis and all tests of statistical significance were two-sided. We used $\chi^2$ test to compare
ING4 and BRMS1 staining in different melanocytic lesions, as well as the correlation between ING4 or BRMS1 staining and the clinicopathological parameters of the primary melanoma patients. The Kaplan-Meier survival curve and log-rank test were used to evaluate the correlations between ING4 or BRMS1 expression and patient survival, or to evaluate the correlations between single or combined biomarker expression and patient survival in chapter 6. Cox regression analysis is a proportional hazards model which was used in this study to relate the patient survival to one or more covariates, including the immunostaining score of our interested genes and other clinicopathological parameters that may be associated with patient survival. The Cox multivariate analysis was performed by SPSS version 11.5 software.

The Graphpad PRISM version 5.0 and INSTAT 3 software (Graphpad Software, La Jolla, CA, USA) were used to compare the expression alteration of each individual biomarker or combined multiple-biomarker system between different stages or subgroups. The correlation coefficient (R) was calculated by correlation statistical analysis. \( P < 0.05 \) was considered statistically significant.

### 2.5 Cell lines and cell culture

The MMRU and MMAN melanoma cell lines were kind gifts from Dr. H.R. Byers (Boston University School of Medicine, Boston, MA, U.S.A.). The MeWo, SK-mel-24, SK-mel-93, and SK-mel-110 cell lines were kindly provided by Dr A.P. Albino (Memorial Sloan-Kettering Cancer Center, New York, U.S.A.). SK-mel-5 cell line was obtained from the Tissue Bank at the National Institutes of Health, U.S.A. HUVECs were purchased from BD bioscience (Mississauga, ON, Canada). All the melanoma cell lines 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 \( \mu \)g/ml streptomycin and 25 \( \mu \)g/ml amphotericin B. HUVECs were cultured
in Kaighn’s Modified Ham’s F-12K medium (Mediatech, Manassas, VA) supplemented with endothelial cell growth supplement (BD Biosciences) and 10% fetal bovine serum. All cells were maintained in 5% CO₂ atmosphere at 37°C.

2.6 Plasmids and transfection

pcDNA3-ING4 plasmid was kind gift from Dr. Remy Pedeux (National cancer institute, MD). BRMS1 plasmid was kindly provided by Dr. D.R. Welch (University of Alabama at Birmingham, Birmingham, Alabama). pFLAG-CMV-2 vector and p3×FLAG vector were purchased from sigma (Sigma-Aldrich, Oakville, Ontario, Canada). HA-tagged vector was kindly provided by Dr. M. Pagano at the New York University (New York, NY) (Busino et al., 2007). The cDNA of ING4 was obtained from pcDNA3-ING4 with polymerase chain reaction (PCR) and sub-cloned into pFLAG-CMV-2 vector between NotI and BglII restriction sites to generate the Flag-ING4, or sub-cloned into HA-tagged vector between KpnI and EcoRI restriction sites to generate the HA-ING4. The cDNA of BRMS1 was obtained from pIXSN-BRMS1 with PCR and sub-cloned into p3×FLAG vector between EcoRI and HindIII restriction sites to generate the Flag-BRMS1. For plasmid transfection, melanoma cells were grown to ~50% confluency and then transiently transfected with Flag-ING4, or HA-ING4, or Flag-BRMS1 plasmid with Effectene reagent (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s instructions. Twelve hours after transfection, the medium containing transfection reagents was removed. The cells were rinsed with PBS and then incubated in fresh medium.

2.7 Adenoviral vector and infection

A recombinant adenoviral vector expressing human ING4 gene was kindly provided by Dr. J. Yang (Soochow University, Suzhou, China). For adenoviral infection, melanoma cells were cultured until ~70% confluency and infected with control adenoviral
vector (adv) or adenoviral ING4 (adING4) at multiplicity of infection (MOI) of 100 (Xie et al., 2008).

2.8 Micro-RNA construction and transfection

The pcDNA EmGFP-miR vector (Invitrogen) was used to develop micro-RNA structures for ING4 (miING4) and BRMS1 (miBRMS1) knockdown according to the manufacture’s instruction, and two sequences were used for both ING4 and BRMS1. Sequences for these structures are listed in Table 2.1.

2.9 SiRNA and transfection

The siRNA targeting p50 and p65 subunits of NF-κB are purchased from Qiagen (ID numbers SI02654932 and SI02663094, respectively). Transfection of siRNA was performed when melanoma cells reach at 50-60% confluency, with SiLentFect reagent (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer’s instruction.

2.10 Antibodies

Normal mouse and rabbit serum IgG were purchased from Santa Cruz biotechnology. The primary antibodies included polyclonal rabbit anti-ING4 (Proteintech Group Inc, Chicago, IL. U.S.A.), polyclonal rabbit anti-ING4 (Invitrogen), monoclonal mouse anti-BRMS1 (kind gift from Dr. D.R. Welch, University of Alabama at Birmingham, Birmingham, Alabama) (Phadke et al., 2008), monoclonal mouse anti-Flag (Applied Biological Materials Inc, Richmond, BC, Canada), polyclonal rabbit anti-HA (ImmuneChem, Burnaby, BC, Canada), monoclonal mouse anti-RhoA, polyclonal rabbit anti-p50 or -RelA (p65) (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-Actin (Sigma).

2.11 Western blot and ELISA

Cells were washed with cold PBS three times, harvested by scraping on ice and pelleted by centrifugation at 2,500 g for 2 min. For whole lysate, cells pellets were lysed in
80 μl of modified RIPA buffer (50mM Tris-HCl, (PH 8.0), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1mM EDTA) containing freshly added protease inhibitors (100μg/ml phenylmethylsulfonyl fluoride, 1μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstatin A). The samples were then sonicated, incubated on ice for 30 min, and centrifuged at 12,000 g for 30 min at 4°C, before the supernatants were collected. For nuclear protein extraction, the cell lysate was incubated with 5× volume of 1× lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2 and 10 mM KCl). The cell pellet was resuspended gently and incubated in lysis buffer on ice for 15 min, allowing cells to swell. Then to the swollen cells in lysis buffer, IGEPAL CA-630 solution was added to a final concentration of 0.6%, and vortexed vigorously for 10 seconds. The sample was centrifuged immediately for 30 seconds at 10,000-11,000 g and the supernatant was transferred to a fresh tube. This fraction was the cytoplasmic fraction. 1 μl of 0.1 M DTT solution and 1 μl of the protease inhibitor cocktail (contains 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF), Pepstatin A, Bestatin, Leupeptin, Aprotinin, and trans-Epoxysuccinyl- L-leucyl-amido(4-guanidino)-butane (E -64)) was added to 98 μl of the extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.42 M NaCl, 0.2 mM EDTA, and 25% (v/v) Glycerol). The crude nuclei pellet was suspended in ~70 μl extraction buffer containing the DTT and protease inhibitor cocktail. The tube was agitated on a vortex mixer at medium to high speed for 15-30 min. This was followed by centrifuge for 5 min at 20,000-21,000 g. Finally we transferred the supernatant to a clean, chilled tube and stored at −70 °C until use. A Bradford assay was then performed to determine the protein concentration. 50 μg proteins were separated on 12% SDS-polyacrylamide gels (PAG) and blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The PVDF was then blocked with 5% Bovine serum albumin (BSA) in PBST (PBS containing 0.04% Tween-20) for 30 min at room temperature before incubating with primary
antibodies prepared in 5% BSA in PBST for 1 hour at room temperature. Blots were then washed in PBST three times, 5 min each and then incubated with secondary antibodies labelled with the near-infrared fluorescent dyes IRDye800 or IRDye 680 (LI-COR Biosciences, Lincoln, NE, U.S.A.) for 1 h at room temperature, followed by scanning on the Odyssey Infrared Imaging System to visualize proteins (LI-COR Biosciences). The protein expression levels were quantified with the ImageJ software (National Institutes of Health, U.S.A.). The fold-induction or reduction was normalized with actin loading control. For ELISA, the secreted IL-6 protein in the conditioned medium was measured with a human IL-6 ELISA kit (eBioscience) according to the manufacturer’s instructions.

2.12 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA of melanoma cells was extracted by TRIzol® reagent (Invitrogen). The RNA concentrations were measured with a spectrometer at 260 nm. 2.0 µg of total RNA was reversely transcribed into cDNA with the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's instruction. Real-time PCR reactions were performed in triplicate with SYBR Green PCR Master Mix using a 7900HT qPCR system thermal cycler (Applied Biosystems. Foster City, CA). All the primer sequences are listed in Table 2.2.

2.13 Wound healing assay

MMRU and SK-mel-5 melanoma cells were transfected with Flag-ING4 or empty vector, and cultured until confluent. Then the cells were washed with PBS and a standard 200 µl pipette tip was applied to drawn across and produce a 0.5-mm wide wound at the center of each well. The wounded monolayer was washed twice to remove non-adherent cells, and then incubated in fresh medium for another 24 hours. Photographs were taken at the same position of the wounds at 0 and 24-hour time points. The starting wound edges were defined in each photo by white lines based on the scratch at 0-hour time point and the
numbers of migrating cells across these white lines were counted at 5 random fields to quantify the rate of cell migration.

2.14  **RhoA pull-down assay**

MMRU melanoma cells were transfected with control vector or Flag-ING4 at 50% confluency for 24 hours. Then, cells were serum starved for 24 hours followed by serum stimulation with medium containing 10% FBS for 30 minutes. Cells were then lysed in 300 µl RIPA lysis buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 10 mM MgCl₂, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 µg/ml of aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were cleared by centrifugation at 12,000 g for 15 min at 4°C and 0.5 mg protein in 500 µl lysis buffer was incubated with 20 µg glutathione S-transferase fusion protein immobilized to glutathione-Sepharose 4B beads at 4°C for 1 hour. Bound proteins were then separated on 10% SDS-PAG and immunoblotted with monoclonal mouse anti-RhoA antibody (1:250; Santa Cruz Biotechnology). Total RhoA expression was used as control by running the total cell lysates under the same condition.

2.15  **Immunofluorescence staining with cells on coverslips**

MMRU cells were transfected with control vector or Flag-ING4 plasmid and subcultured onto coverslips in 6-well plate. After 6 hours, the cells were serum starved overnight and stimulated with complete medium containing 10% FBS for a designated period. Fixation solution (2% paraformaldehyde and 0.5% Triton X-100 in PBS) was then applied to the cells for 10 minutes at 4°C. After blocking with 1% BSA for 1 hour, the cells were incubated with polyclonal rabbit anti-Flag (1:50, Sigma) for 1 hour and Cy2-conjugated goat anti-rabbit antibody (1:500, Jackson ImmunoResearch, West Grove, PA) for 45 minutes. The cells were then stained with phalloidin-rhodamine (1 unit/cover slip; Invitrogen) for 30 minutes. Finally, the coverslips were incubated with 1:3,000 diluted
stock Hoechst 33258 (20 mM) for 10 minutes and the staining signals were visualized under a fluorescent microscope. Ten photos from each slide with each photo containing an average of three to five cells were taken with a cooled mono 12-bit Retiga-Ex camera equipped with Northern Eclipse imaging software. The intensity of F-actin was quantified using ImageJ software, and the mean of relative cellular fluorescent intensity was measured.

2.16 Cell invasion assay

Boyden chamber assay was used for the cell invasion analysis. 18 μl of 5 mg/ml Matrigel (BD Biosciences) in serum-free medium was applied to the upper compartment of 24-well Transwell culture chambers (with 8.0 μm pore size polycarbonate membrane) and was incubated in 37°C for 2 hour. MMRU cells (5×10^4) suspended in 250 μl of serum-free medium were loaded on the upper compartment, and the lower compartment was filled with 750 μl of complete medium. After 24 hours incubation, cells were fixed with 10% trichloroacetic acid (TCA) at 4°C for 1 hour. Non-invaded cells were removed from the upper surface of the filter carefully with a cotton swab. Invaded cells on the lower side of the filter were stained with 0.5% crystal violet for 2 hours. After wash with distilled water, the retained dye on the filters was extracted by 30% acetic acid followed by reading the absorbance at 590 nm.

2.17 Zymography

Zymography assay was carried out to examine the activity of MMP-2 and MMP-9. MMRU cells were transfected with empty vector or Flag-ING4 plasmid. Serum-free medium was applied to the cells overnight and the proteins in the conditioned medium were concentrated with YM-3 centricon membranes (Millipore) at 7,000 g for 4 hours at 4°C. Proteins (5 μg) were loaded on a 10% PAG containing 0.1% gelatin (Sigma). After electrophoresis, the gel was incubated in Triton X-100 exchange buffer (20 mM Tris-HCl
[pH 8.0], 150 mM NaCl, 5 mM CaCl₂, and 2.5% Triton X-100) for 30 min followed by 10 min wash with the incubation buffer (same as exchange buffer except without Triton X-100) three times. The gel was then incubated in incubation buffer overnight at 37°C, stained with 0.5% Coomassie blue R250 (Sigma) for 1 hour and destained with 30% methanol and 10% acetic acid for 1 hour. Gelatinolytic activity was shown as clear areas in the gel. Recombinant MMP-2 and MMP-9 (R&D Systems, Minneapolis, MN) were used as positive controls.

2.18 HUVECs growth and tube formation assay

Melanoma cells were cultured in 60mm plates with fresh complete medium for 24 hours, and 2 ml of conditioned medium was collected. For HUVEC growth assay, the endothelial cells were seeded in 24-well plate at 2 × 10⁴ cells/well and cultured in fresh F-12K medium for 24 hours, and then in 0.5 ml conditioned medium for another 24 hours before sulforhodamine B (SRB) assay was performed to test the rate of cell growth. Cells were first fixed with 10% TCA at 4°C for 1 hour, then were washed with tap water, air-dried and stained with 500 μl of 0.4% SRB (dissolved in 1% acetic acid) for 30 min at room temperature. The cells were then washed with 1% acetic acid, and air-dried. For quantification, the cells were incubated with 500 μl of 10 mM Tris (pH 10.5) on a shaker for 20 min to dissolve the bound dye, and followed by colorimetric determination at 550 nm for 100 μl aliquots. For IL-6 rescue experiments, we added 0.8 ng/ml recombinant IL-6 (eBioscience, San Diego, CA) in the conditioned medium from ING4-overexpressing melanoma cells and then performed the HUVECs growth assay. For IL-6 blocking assay, we added 320 ng/ml IL-6 antibody (eBioscience) in the conditioned medium from melanoma cells with ING4 knockdown treatment and then collected the conditioned medium for HUVECs growth assay. For tube formation assay, 96-well plate was coated with Matrigel and kept in 37°C for 2 hours. Then, 2×10⁴ HUVECs were suspended in 100
μl conditioned medium and applied to the pre-coated 96-well plate. After incubation at 37°C for another 24 hours, photos were taken under microscope and the tubular structures formed in the matrigel were counted in 5 random fields.

2.19 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from MMRU cells overexpressing ING4, BRMS1, or ING4 plus BRMS1 and the control sample as described above. Then we incubated the nuclear extract with oligonucleotide probe bearing NF-κB binding sequence on IL-6 promoter (Sequences are listed in Table 2.3). The probe was labeled with a γ-32P phosphate at its 5’ end and another oligonucleotide with the same sequence but without labeling was used as a competitive sequence at 100 or 500-fold concentration. Binding reaction and detection procedure were performed as described previously (Maffey et al., 2007). Basically, nuclear extract was incubated with probe for 15 min at room temperature in a buffer consisting of 0.13 mg/ml BSA, 0.013 μg/ml of polydI-dC. Then competitive probe was introduced in the binding reaction and incubated for another 15 min at room temperature. After incubation the mixtures were directly loaded onto a 5% native-PAG (acrylamide/bis acrylamide 29:1) in 0.5×TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA). Radioautography was then performed to examine the DNA binding activity.

2.20 In vivo angiogenesis assay and immunofluorescent staining on frozen sections

1×10^6 MMRU human melanoma cells were supported by 300 μl Matrigel and implanted subcutaneously into the flanks of 6-week-old male nude mice. Ten days later, the mice were sacrificed and the implanted matrigel plugs were excised, photographed and immediately embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek USA, Torrance, CA). After frozen at -80°C for 1 hour, 5 μm sections were cut from the embedded tissues using a CM1850 cryostat (Leica Microsystems, Richmond Hill, ON, Canada) and applied to glass slides. Immunofluorescent staining for CD31 expression was then performed using
rat anti-mouse CD31 antibody and FITC labeled goat anti-rat secondary antibody (Santa Cruz Biotechnology). Sections were counterstained with propidium iodide (Sigma-Aldrich). Photos were taken with a cooled mono 12-bit Retiga-Ex camera equipped with Northern Eclipse imaging software. The number of CD31 positive cells was counted in 5 random fields for both vector control and ING4- or BRMS1-overexpressing groups.

2.21 Immunoprecipitation

500 μg of nuclear extract was first pre-cleared by 20 μl Protein-G agarose beads (GE Healthcare Bioscience, Uppsala, Sweden) for 2 hours at 4°C. Then the samples were spin down and supernatant was collected incubated with either monoclonal mouse anti-Flag antibody or polyclonal rabbit anti-HA antibody overnight at 4°C, and then were pelleted down and washed by IP washing buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA) and boiled in 2 × Tris–glycine SDS sample buffer for Western blot analysis.

2.22 Statistical analysis for in vitro studies

The data were presented as the mean ± SD. Statistical analyses were performed using student’s $t$-test and $p$ value < 0.05 was considered significant.
Table 2.1 Sequences for miING4 and miBRMS1

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<th>Micro-RNA</th>
<th>Sequence</th>
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<td>miING4-S1</td>
<td>Top: 5’-TGCTGTGTCAGGTCCTCTGTTTGTGTTTTGGCCACTGACTGACACCAAAGAAGGACCTGAG-3’&lt;br&gt;Bot: 5’-CCTGTCAGGTCCTCTTCTTTGGTGTCAGTCAGTGGCCAAAACACCAAAGAAGGACCTGAC3’</td>
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<td>miING4-S2</td>
<td>Top: 5’-TGCTGTGTCAGGTCCTCTGTTTGTGTTTTGGCCAACCATCTCATGTCAGTCAGTGGCCAAAACACCAAAGAAGGACCTGAC-3’&lt;br&gt;Bot: 5’-CCTGTCAGGTCCTCTTCTTTGGTGTCAGTCAGTGGCCAAAACACCAAAGAAGGACCTGAC3’</td>
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<td>miBRMS1-S2</td>
<td>Top: 5’-TGCTGTGTCAGGTCCTCTGTTTGTGTTTTGGCCAACCATCTCATGTCAGTCAGTGGCCAAAACACCAAAGAAGGACCTGAC-3’&lt;br&gt;Bot: 5’-CCTGTCAGGTCCTCTTCTTTGGTGTCAGTCAGTGGCCAAAACACCAAAGAAGGACCTGAC3’</td>
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**Table 2.2** Sequences for all real-time PCR primers

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<th>For: 5’-CACAGACCTGGCCCGTTTT-3’</th>
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<td>IL-6 primer</td>
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<td>Rev: 5’-TGGCATTTGTGGTTGGGTCA-3’</td>
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<td>p65 primer</td>
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Table 2.3 Sequences of EMSA probe

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<th>EMSA probe</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Anti-sense: 5’-GACTCATGGGAAAATCCACATTTG-3’</td>
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3 Role of ING4 in Human Melanoma Cell Migration, Invasion, and Patient Survival

3.1 Rational and hypothesis

Previous studies indicated that ING family tumor suppressors might play an important role in melanoma development (Nouman et al., 2002). Mutations of the ING1 gene abrogate NER in melanoma cells, and ING1 expression has been reported to be downregulated or even lost in many human cancers (Campos et al., 2002; Campos et al., 2004b; Li et al., 2009a). Our group showed that ING2, a DNA damage-inducible gene, is downregulated in melanoma when compared with dysplastic nevi (Lu et al., 2006). Wang et al demonstrated that ING3 can induce melanoma cell apoptosis and nuclear ING3 expression is decreased in malignant melanoma compared with dysplastic nevi. We also demonstrated that reduced nuclear ING3 expression is reversely correlated with cytoplasmic ING3 expression as well as melanoma patient survival (Wang et al., 2007). To investigate the role of ING4 in melanoma pathogenesis, we used TMA and immunohistochemistry to assess the expression of ING4 in different stage of melanocytic lesions. We also analyzed the correlation between ING4 expression and melanoma pathogenesis, progression, clinicopathological parameters and patients survival. Finally we investigated the impact of ING4 on melanoma cell migration and invasion.

3.2 Results

3.2.1 Clinicopathological features of primary melanoma biopsies

The clinicopathological features of the 101 primary melanoma biopsies are summarized in Table 3.1. There were 62 men and 39 women, with age ranging from 21 to 93 years (median, 57 years). For the tumor thickness, 35 cases were ≤1.0 mm, 31 cases were 1.01-2.0 mm, 17 cases were 2.01-4.0 mm, and 18 cases were >4.0 mm. Tumor ulceration was present in 19 cases at diagnosis. For the histological subtype, there were 45
cases of superficial-spreading melanoma, 18 lentigo maligna melanomas, 16 nodular melanomas and other 22 cases unspecified. Nineteen melanomas were located in sun-exposed sites such as head and neck, and the other 82 were located in sun-protected sites.

### 3.2.2 ING4 expression was decreased in malignant melanoma

ING4 is a nuclear protein as it contains the nuclear localization sequence (Shiseki et al., 2003; Unoki et al., 2006). We found that ING4 staining was mainly localized in the nucleus in melanocytic lesions (Figure 3.1). Various levels of ING4 staining were observed in dysplastic nevi and malignant melanoma biopsies (Figure 3.1). Strong and moderate ING4 staining decreased from 98% in dysplastic nevi to 67% in primary melanomas and 53% in metastatic melanomas. Significant differences in ING4 staining were observed between dysplastic nevi and primary melanoma ($P = 0.000027, \chi^2$ test), and between dysplastic nevi and metastatic melanoma ($P = 0.000003, \chi^2$ test), but not between primary melanoma and metastatic melanoma ($P = 0.409, \chi^2$ test) (Figure 3.2A). Unlike ING1b and ING3 which showed nuclear-to-cytoplasm shift in melanoma cells (Nouman et al., 2002; Campos et al., 2004b; Wang et al., 2007), we did not observe nuclear-to-cytoplasm shift for ING2 from our previous data (Lu et al., 2006) or ING4 in melanoma cells in this study (data not shown).

### 3.2.3 ING4 expression was correlated with melanoma tumor thickness and ulceration

We conducted the correlation analysis between ING4 expression and clinicopathological parameters, including age, gender, tumor thickness, ulceration, histological subtype and tumor site in 101 primary melanoma biopsies. Reduced ING4 expression is significantly correlated with tumor thickness and ulceration ($P = 0.034$ and 0.002, respectively, $\chi^2$ test) (Figure 3.2B and C), but not correlated with patient’s age, gender, histological subtype or tumor site ($P > 0.1$ for all, $\chi^2$ test) (Table 3.1).
3.2.4 Correlation between ING4 expression and patient survival

To study whether ING4 expression is correlated with melanoma patient survival, we constructed Kaplan-Meier survival curves in patients with primary melanoma and metastatic melanoma, respectively. Our overall and disease-specific survival analysis data from primary melanoma group are shown in Figure 3.3A and B, patients with moderate to strong ING4 staining showed significantly better overall and disease-specific 5-year survival rate (85% and 91%) than patients with negative to weak ING4 staining did (52% and 67%) \((P = 0.0002\) and \(0.001\), respectively, log-rank test) (Figure 3.3A and B). In the metastatic melanoma group, both overall and disease-specific 5-year survival rate were similar in patients with moderate to strong ING4 staining (15% and 30%) and those with negative to weak ING4 staining (13% for both) \((P > 0.6\) for both, log-rank test) (Figure 3.4A and B). Next, we performed Cox regression multivariate analysis to study the effect of ING4 expression in patient survival, together with patient’s age, gender, tumor thickness, ulceration, histological subtype and tumor site in 101 primary melanomas. Our results indicated that ING4 expression is an independent prognostic factor for both overall \((RR = 0.40, 95\% CI = 0.17\) to 0.91, \(P = 0.031\)) and disease-specific 5-year survival \((RR = 0.337, 95\% CI = 0.12\) to 0.98, \(P = 0.045\)) (Table 3.2).

3.2.5 ING4 inhibited melanoma cell migration

As cell motility is an important factor for tumor progression and ING4 expression is significantly reduced in malignant melanoma, we investigated the role of ING4 in melanoma cell migration. First, we transfected melanoma MMRU cells with Flag-ING4 and found that ING4 was overexpressed in this cell line compared with vector control (Figure 3.5A). In the wound-healing assay, the control cells transfected with vector alone healed the wound completely 24 h after scratch, while the ING4 overexpressing cells were unable to heal the wound (Figure 3.5B). Quantitative analysis of the number of the cells
that migrated into the wound area indicated that ING4 inhibited cell migration by 63% in MMRU cell line ($P = 0.0006$, student’s $t$-test) (Figure 3.5C). This inhibition was not due to ING4 inhibition on cell proliferation, as the proliferation of ING4-transfected cells is only approximately 10% slower than the vector control (Figure 3.5D), although overexpression of ING4 started to show significant inhibition on cell proliferation 36h after transfection in this study ($P = 0.224$ and 0.015 for 24 and 36 h after transfection, respectively, student’s $t$-test). To confirm that ING4 inhibitory effect on cell migration is not cell line specific, we performed the cell migration assay in another wild type p53 melanoma cell line SK-mel-5. Our data showed that overexpression of ING4 inhibited SK-mel-5 cell migration by 66% compare with the vector control ($P = 0.001$, student’s $t$-test) (Figure 3.6).

3.2.6 ING4 inhibited RhoA activity and reduced ROCK-mediated stress fiber formation

RhoA-Rock pathway induces stress fiber reorganization and thus plays an important role in cell migration (Maekawa et al., 1999). To address whether the negative effect of ING4 on melanoma cell migration was associated with this pathway, we first used the RhoA pull-down assay to study the correlation between ING4 overexpression and RhoA activity. We found that active RhoA expression was greatly decreased in ING4 overexpressing cells compared with the vector-transfected cells (Figure 3.7A). Moreover, immunofluorescent staining revealed that serum stimulation induced much more stress fiber formation in vector-transfected MMRU cells than ING4 overexpressing cells. In addition, we found that ROCK inhibitor Y27632 abolished the effect of ING4 in stress fiber formation (Figure 3.7B). The relative fluorescence intensity of F-actin in cells transfected with ING4 was reduced by 50% compared to cells transfected with vector, while there is no significant difference between cells transfected with ING4 and vector after treatment with ROCK inhibitor Y27632 (Figure 3.7C).
3.2.7 ING4 regulated cell invasion and MMPs activity

To study the role of ING4 in melanoma cell invasion, we performed the Boyden chamber assay and zymography. We found that cell invasion was reduced by 43% in ING4 overexpressing MMRU cells compared with the vector control ($P = 0.006$, student’s $t$-test) (Figure 3.8A). Since MMPs play a crucial role in cell invasion (Schnaeker et al., 2004), we then carried out the zymography assay to compare the activity of MMP-2 and MMP-9 in ING4 overexpressing and vector control cells. As shown in Figure 3.8B, MMP-2 and MMP-9 gelatinolytic activity were decreased by 25% and 61% in ING4 overexpressing cells compared with the vector control, respectively ($P = 0.013$ and 0.007, respectively, student’s $t$-test) (Figure 3.8B and C).
Table 3.1 ING4 staining and clinicopathological characteristics of 101 primary melanomas

<table>
<thead>
<tr>
<th>ING4 staining</th>
<th>Variables</th>
<th>Negative</th>
<th>Weak</th>
<th>Moderate</th>
<th>Strong</th>
<th>Total</th>
<th>P value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≤57</td>
<td>0 (0%)</td>
<td>13 (26%)</td>
<td>24 (48%)</td>
<td>13 (26%)</td>
<td>50</td>
<td>0.071</td>
<td></td>
</tr>
<tr>
<td>Age &gt;57</td>
<td>6 (12%)</td>
<td>15 (29%)</td>
<td>18 (35%)</td>
<td>12 (24%)</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender Male</td>
<td>3 (5%)</td>
<td>15 (24%)</td>
<td>27 (44%)</td>
<td>17 (27%)</td>
<td>62</td>
<td>0.646</td>
<td></td>
</tr>
<tr>
<td>Gender Female</td>
<td>3 (8%)</td>
<td>13 (33%)</td>
<td>15 (38%)</td>
<td>8 (21%)</td>
<td>39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor thickness (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.034</td>
</tr>
<tr>
<td>≤1.0</td>
<td>0 (0%)</td>
<td>8 (23%)</td>
<td>19 (54%)</td>
<td>8 (23%)</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.01-2.0</td>
<td>1 (3%)</td>
<td>10 (32%)</td>
<td>9 (29%)</td>
<td>11 (36%)</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.01-4.0</td>
<td>1 (6%)</td>
<td>5 (30%)</td>
<td>6 (35%)</td>
<td>5 (29%)</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;4.0</td>
<td>4 (22%)</td>
<td>5 (28%)</td>
<td>8 (44%)</td>
<td>1 (6%)</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulceration Present</td>
<td>4 (21%)</td>
<td>8 (42%)</td>
<td>6 (32%)</td>
<td>1 (5%)</td>
<td>19</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Ulceration Absent</td>
<td>2 (2%)</td>
<td>20 (24%)</td>
<td>36 (44%)</td>
<td>24 (30%)</td>
<td>82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor subtype Nodular</td>
<td>1 (6%)</td>
<td>4 (25%)</td>
<td>7 (44%)</td>
<td>4 (25%)</td>
<td>16</td>
<td>0.796</td>
<td></td>
</tr>
<tr>
<td>SSM&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2 (5%)</td>
<td>11 (24%)</td>
<td>19 (42%)</td>
<td>13 (29%)</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMM&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1 (6%)</td>
<td>4 (22%)</td>
<td>7 (39%)</td>
<td>6 (33%)</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unspecified</td>
<td>2 (9%)</td>
<td>9 (41%)</td>
<td>9 (41%)</td>
<td>2 (9%)</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site&lt;sup&gt;2&lt;/sup&gt; Sun-protected</td>
<td>6 (7%)</td>
<td>20 (24%)</td>
<td>34 (42%)</td>
<td>22 (27%)</td>
<td>82</td>
<td>0.273</td>
<td></td>
</tr>
<tr>
<td>Site&lt;sup&gt;2&lt;/sup&gt; Sun-exposed</td>
<td>0 (0%)</td>
<td>8 (42%)</td>
<td>8 (42%)</td>
<td>3 (16%)</td>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>χ² test.

<sup>2</sup>Sun-protected sites: trunk, arm, leg and feet; Sun-exposed sites: head and neck.

<sup>3</sup>SSM: Superficial Spreading melanoma.

<sup>4</sup>LMM: Lentigo maligna melanoma
Table 3.2 Cox regression analysis of ING4 expression and other clinicopathological characteristics on 5-year survival of primary melanoma patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall survival</th>
<th></th>
<th>Disease-specific survival</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative risk</td>
<td>95% CI</td>
<td>P value</td>
<td>Relative risk</td>
</tr>
<tr>
<td>ING4</td>
<td>0.400</td>
<td>0.17 to 0.91</td>
<td>0.031</td>
<td>0.337</td>
</tr>
<tr>
<td>Thickness</td>
<td>4.525</td>
<td>1.89 to 11.11</td>
<td>0.001</td>
<td>6.098</td>
</tr>
<tr>
<td>Ulceration</td>
<td>2.632</td>
<td>1.15 to 5.88</td>
<td>0.022</td>
<td>4.329</td>
</tr>
<tr>
<td>Location</td>
<td>0.528</td>
<td>0.21 to 1.30</td>
<td>0.164</td>
<td>0.396</td>
</tr>
<tr>
<td>Subtype</td>
<td>0.609</td>
<td>0.20 to 1.85</td>
<td>0.384</td>
<td>0.933</td>
</tr>
<tr>
<td>Gender</td>
<td>1.901</td>
<td>0.84 to 4.30</td>
<td>0.123</td>
<td>1.217</td>
</tr>
<tr>
<td>Age</td>
<td>1.520</td>
<td>0.64 to 3.57</td>
<td>0.340</td>
<td>1.012</td>
</tr>
</tbody>
</table>

1 Coding of variables: ING4 was coded as 1 (negative and weak), and 2 (moderate and strong). 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). Age was coded as 1 (≤57 years), and 2 (>57 years). Gender was coded as 1 (female), and 2 (male).

2 CI: confidence interval.
**Figure 3.1** ING4 expression is significantly reduced in malignant melanomas. (A) Representative images of ING4 staining in dysplastic nevi with strong ING4 expression. (B) Primary melanoma with moderate ING4 expression. (C) Metastatic melanoma with weak ING4 expression. Bar, 10µm.
Figure 3.2  ING4 expression in different stages of melanocytic lesions.  (A) ING4 expression is significantly reduced in primary melanomas and metastatic melanomas compared with dysplastic nevi ($P = 0.000027$ and $0.000003$, respectively, $\chi^2$ test).  (B) ING4 expression is negatively associated with tumor thickness ($P = 0.034$, $\chi^2$ test).  (C) ING4 expression is significantly reduced in primary melanomas with ulceration ($P < 0.002$, $\chi^2$ test).
Figure 3.3 ING4 expression is correlated with 5-year survival of patients with primary melanoma. Primary melanoma patients with moderate to strong ING4 expression have a significantly better overall (A) and disease-specific (B) 5-year survival than those with negative to weak ING4 expression.
**Figure 3.4** ING4 expression is not correlated with 5-year survival of patients with metastatic melanoma in overall (A) and disease-specific (B) survival analysis.
Figure 3.5  Overexpression of ING4 inhibits melanoma cell migration. (A) MMRU melanoma cells were transfected with ING4 or control vector. 24 hours after transfection, cells were analyzed for the expression of ING4 by Western blot analysis. (B) Wound healing assay was done 24 hours after transfection. The photographs were taken 24 hours after the wounds were made. Magnification, ×100. (C) Quantitation of (B). Columns, mean of triplicates; bars, SD. Similar results were observed in three independent experiments. (D) Effect of ING4 on cell proliferation. MMRU cells were transfected with vector or Flag-ING4 and the sulforhodamine B cell survival assay was performed at various time points after transfection. Columns, mean of triplicates; Bars, SD. *P<0.05. ***P<0.001, Student’s t-test.
Figure 3.6 Overexpression of ING4 inhibits cell migration in melanoma SK-mel-5 cell line. (A) SK-mel-5 (SK-5) melanoma cells were transfected with Flag-ING4 or control vector. 24 hours after transfection, cells were analyzed for the expression of ING4 by Western blot analysis. (B) Wound-healing assay was performed 24 hours after transfection. The photographs were taken 24 hours after the wounds were made. Magnification, ×100. (C) Quantitation of (B). Columns, mean of triplicates; Bars, SD. Similar results were observed in three independent experiments. **P<0.01, Student’s t-test.
**Figure 3.7** ING4 reduces RhoA activity and stress fiber formation via ROCK. (A) ING4 reduces RhoA activity determined by RhoA pull-down assay (left panel). Total RhoA was used as loading control (right panel). (B) ING4 reduces stress fiber formation mediated by ROCK. Cells were transfected with vector or ING4, respectively, followed by serum starvation overnight and serum stimulation for 30 min. For ROCK inhibitor treatment, cells were pretreated with serum-free medium containing 10 µM Y27632 for 2 hours after transfection with vector or ING4, serum starved overnight and then incubated with complete medium containing 10% FBS and 10 µM Y27632 for 30 min. Magnification, ×400. (C) Quantitation of (B). *P<0.05, Student’s t-test.
Figure 3.8 ING4 overexpression inhibits melanoma cell invasion and the activity of MMP-2 and MMP-9. (A) MMRU cells were transfected with vector or ING4, respectively. Then cells were suspended in serum-free medium and seeded on matrigel, incubated at 37°C for 24 hours, stained by crystal violet and quantified. Columns, means from triplicate wells; Bars, SD. (B) ING4 inhibits MMP-2 and MMP-9 activity in MMRU cells by zymography. (C) Quantitation of (B). Columns, means from three independent experiments; Bars, SD. *P<0.05, **P<0.01, Student t test.
3.3 Discussion

ING family members play important roles in cellular stress response to ultraviolet irradiation and melanoma pathogenesis. We have previously shown that ING1b and ING2 enhance the repair of UV-damaged DNA and promote apoptosis by activating the p53-dependent mitochondrial pathway (Cheung et al., 2001; Cheung and Li, 2002; Chin et al., 2005; Wang et al., 2006b). ING3 can also promote UV-induced apoptosis by activating the p53-independent Fas/caspase-8 pathway (Wang and Li, 2006). In addition, we showed that nuclear expression of ING2 and ING3 is significantly reduced in human melanomas (Lu et al., 2006; Wang et al., 2007). In this study, we investigated the role of ING4 in human melanoma pathogenesis in vivo and in vitro. Our data demonstrated for the first time that ING4 expression is significantly decreased concomitant with melanoma progression and reduced ING4 expression is closely correlated with a poorer 5-year survival of primary melanoma patients. We also revealed that ING4 inhibits melanoma cell migration, cell invasion and the activity of MMP-2 and MMP-9.

Reduced ING4 expression in malignant melanoma described in this study is consistent with our previous findings that ING2 and ING3 expression are significantly reduced in human malignant melanomas (Lu et al., 2006; Wang et al., 2007), suggesting that ING tumor suppressors play important roles in melanoma tumorigenesis. Our data are also in agreement with the findings by Garkavtsev et al who showed that ING4 expression level is dramatically reduced in gliomas both at mRNA and protein levels (Garkavtsev et al., 2004). The reduction of ING4 expression in melanoma can possibly be explained by the findings that deletion or mutation at chromosome 12p12-13, which includes ING4 gene, occurs frequently in a number of human tumors (Gunduz et al., 2005). Hassler et al studied 18 glioblastoma multiforme patients and found that loss of the ING4 gene, together with VEGF and bFGF, were associated with neoangiogenesis (Hassler et al., 2006).
Gunduz et al reported that loss of heterozygosity at 12p12–13 region occurred in 66% head and neck squamous cell carcinoma patients, and that 76% tumor tissues had decreased ING4 mRNA expression compared with matched normal samples (Gunduz et al., 2005). In another study, Kim et al found deletion of the ING4 locus in 10–20% of human breast cancer cell lines and primary breast tumors by comparative genomic hybridization analysis (Kim et al., 2004). These studies imply that deletion of ING4 gene may be a common event in tumorigenesis. However, whether ING4 is mutated or deleted in human melanoma remains to be determined.

Our data showed significant differences for ING4 staining pattern between dysplastic nevi and primary melanoma, but not between primary melanoma and metastatic melanoma, suggesting that ING4 inactivation might be a critical requirement for the transformation from nevus to malignant tumor. Recent studies indicated that cellular senescence is a critical mechanisms for opposing neoplastic transformation by activation of oncogenic pathways (Chen et al., 2005b; Michaloglou et al., 2005; Braig and Schmitt, 2006; Miller and Mihm, 2006). NF-κB is shown to be involved in senescence of normal human epidermal keratinocytes by upregulating the expression of the manganese superoxide dismutase (MnSOD) (Bernard et al., 2004). Since ING4 was reported to downregulate NF-κB down stream genes (Garkavtsev et al., 2004), it is reasonable to assume that ING4 regulates cellular senescence by suppressing NF-κB activity, thus inhibiting malignant cell transformation.

In this study, we found that overexpression of ING4 inhibits melanoma cell migration in both melanoma cell lines, MMRU and SK-mel-5 (Figure 3.5B, Figure 3.6B). Cell migration is the central step in tumor metastasis and regulated by various factors (Ridley et al., 2003; Gassmann et al., 2004; Suyama et al., 2004). NF-κB, an important transcription factor, was shown to be involved in this regulation through activation of
RhoA GTPase (Gao et al., 2006). It was previously reported that ING4 binds to RelA subunit of NF-κB and acts as a negative regulator of NF-κB activity (Garkavtsev et al., 2004). Our data showed that ING4 inhibited RhoA activity (Figure 3.7A), which is believed to be able to promote reorganization of stress fiber during cell migration (Wheeler and Ridley, 2004). Therefore, it is possible that ING4 inhibits melanoma cell migration by downregulating RhoA activity through NF-κB pathway. Furthermore, we found that ROCK inhibitor abrogated the inhibition of ING4 on stress fiber formation, suggesting that ING4 mediates melanoma cell migration via the RhoA-ROCK signaling pathway (Figure 3.7B and C).

Our data showed that ING4 overexpression suppresses melanoma invasion which is consistent with the report by Shen et al who showed that ING4 interacts with liprin α1 to inhibit cell migration and cell invasion in RKO, HEK-293 and U 87MG cell lines (Shen et al., 2007). We also found that overexpression of ING4 inhibited the gelatinolytic activities of both MMP-2 and MMP-9. Previous study showed that the number of MMP-2-positive cells increased with increasing atypia in different stage of melanocytic lesions by immunohistochemical staining (Vaisanen et al., 1996), suggesting that MMP-2 expression is involved in melanomagenensis. MMP-9 was also found to be expressed in the vertical growth phase of primary melanomas, indicating that MMP-9 plays a key role in melanoma cell invasion (van den Oord et al., 1997) and thus in melanoma progression (Hofmann et al., 2000; Wong et al., 2007). The inhibitory effect of ING4 on MMP-2 and MMP-9 activities at least partially contribute to the suppression of melanoma cell invasion, and this is consistent with our tissue microarray data showing the negative correlation between ING4 expression and melanoma tumor thickness. The inhibitory effect of ING4 in cell invasion can also explain our observation that reduced ING4 expression significantly correlates with a poorer 5-year survival of patients with primary melanoma.
Other tumor suppressive functions of ING4 may also contribute to the inverse correlation between ING4 expression and patient survival. ING4 induces cell cycle arrest and enhances chemosensitivity (Zhang et al., 2004), and suppresses tumor angiogenesis (Garkavtsev et al., 2004), the loss of cell contact inhibition (Kim et al., 2004), and the activation of hypoxia inducible factor (Ozer and Bruick, 2005; Ozer et al., 2005). It is not surprising that inactivation of ING4 leads to the abrogation of these tumor suppressive functions and results in tumor progression and poorer survival outcome. As treatment history was not available, we were not able to determine whether chemoresistance of melanoma after ING4 inactivation was partially responsible for the poor patient survival. We found that ING4 expression is not correlated with the survival of patients with metastatic melanoma. This is because most patients with metastatic melanoma die within a few months (Kath et al., 1993). Currently there is no prognostic factors which can substantially predict survival rate by a few months in patients with metastatic melanoma (Balch et al., 2001b).

In summary, we demonstrated that ING4 plays an important role in human melanoma pathogenesis. Reduced ING4 expression may facilitate tumor progression by enhancing cell migration and cell invasion. Our results imply that ING4 may serve as a promising prognostic marker as well as a potential therapeutic target for malignant melanoma.
4 Prognostic Significance of BRMS1 Expression in Human Melanoma and Its Role in Tumor Angiogenesis

4.1 Rational and hypothesis

In the previous chapter we showed that ING4 expression is decreased in malignant melanoma when compared with the dysplastic nevi, and overexpression inhibits melanoma cell migration and invasion through suppressing RhoA-ROCK pathway and MMPs activity, respectively. It is very likely that this effect is accomplished through regulating NF-κB function, as ING4 was shown to inhibit NF-κB activity (Garkavtsev et al., 2004), and we previously demonstrated that NF-κB promotes melanoma cell migration also through RhoA-ROCK pathway (Gao et al., 2006). Therefore when we tried to find the upstream regulator or downstream target of ING4 in mediating melanoma development, BRMS1 became a good candidate, because previous studies revealed that BRMS1 also negatively regulated cell motility in breast cancer and non-small cell lung carcinoma (Yang et al., 2008; Hurst et al., 2009), and it is believed that this suppressive effect on cell migration is also through mediating NF-κB activity (Cicek et al., 2005; Cicek et al., 2009).

BRMS1 was first identified as a metastasis suppressor in breast cancer in 2000 (Seraj et al., 2000b). Genomic BRMS1 gene is located on chromosome 11q13.1-13.2 and comprised of 10 exons and 9 introns, spanning about 7 kb (Seraj et al., 2000b). The encoded BRMS1 protein is 246 amino acid long and carries two nuclear localization signals (NLS) that target this protein to the nucleus (Samant et al., 2000; Seraj et al., 2000b). BRMS1 mRNA expression is high in melanocytes, but very low in metastatic melanoma cell lines (Shevde et al., 2002). Further in vivo studies demonstrated that overexpression of BRMS1 significantly decreased the metastatic potential of breast cancer and melanoma cells (Seraj et al., 2000b; Shevde et al., 2002). To further investigate the role of BRMS1 in melanoma progression and prognosis, we used TMA and
immunohistochemistry to evaluate BRMS1 expression in different stages of human melanocytic lesions. We also examine the role of BRMS1 in melanoma angiogenesis regulation, as angiogenesis is a crucial pre-requisite for tumor metastasis. Finally we tested the effect of BRMS1 on NF-κB activity and IL-6, a downstream target of NF-κB as well as an important cytokine involved in angiogenesis.

4.2 Results

4.2.1 Decreased BRMS1 expression correlated with melanoma metastasis

The clinicopathological features of 137 melanoma biopsies examined in this study were summarized in Table 4.1. Various levels of BRMS1 staining were observed in different melanocytic lesions, but the staining was generally located in the nucleus, which was attributed to the two NLS that BRMS1 gene carries (Seraj et al., 2000b) (Figure 4.1). Strong BRMS1 staining decreased from 78% in dysplastic nevi to 61% in primary melanomas and further decreased to 40% in metastatic melanomas (Figure 4.2A). Significant differences for BRMS1 staining pattern were observed between metastatic melanoma and primary melanoma, or between metastatic melanoma and dysplastic nevi ($P = 0.021$ and 0.001, respectively, $\chi^2$ test). However, there was no significant difference in BRMS1 staining between primary melanoma and dysplastic nevi.

4.2.2 Decreased BRMS1 expression correlated with AJCC stage

In all 137 melanoma patients, we found that strong BRMS1 expression significantly decreased from 69% in AJCC stage I to 49% in stage II, and further deceased to 43% at stage III and IV ($P = 0.011$, $\chi^2$ test; Figure 4.2B). However, we did not find any correlation between BRMS1 staining and age or gender in patients with primary melanoma or metastatic melanoma. In addition, we also assessed the correlations between BRMS1 expression and other clinicopathological parameters in primary melanoma patients,
including tumor thickness, ulceration, histological subtype, and anatomic site. We found no significant correlation between BRMS1 staining and these parameters.

4.2.3 Decreased BRMS1 expression correlated with a worse patient survival

To study whether reduced BRMS1 expression is correlated with worse melanoma patient survival, we constructed Kaplan-Meier survival curves. Our data revealed that lower BRMS1 staining was significantly correlated with a poorer disease-specific 5-year survival of all melanoma patients. The survival rate dropped from 73% in patients with strong BRMS1 expression to 50% in those with weak BRMS1 expression (Figure 4.3A). Next, we examined whether BRMS1 expression was an independent marker for melanoma prognosis. We performed Multivariate Cox regression analysis to study the effect of BRMS1 expression in patient survival, together with AJCC stage as well as patient’s age and gender in all 137 melanomas. Our results indicated that BRMS1 expression was an independent prognostic factor for disease-specific 5-year survival (Table 4.2). Since there is no reliable prognostic marker for advanced melanoma, we further investigated the correlation between BRMS1 staining and disease-specific 5-year survival in metastatic melanoma and late stage primary melanoma with high metastatic potential (tumor thicker than 4 mm with ulceration). We found that strong BRMS1 expression was significantly associated with better 5-year survival in this group of patients. The survival rate decreased from 40% in patients with strong BRMS1 expression to 12.9% in those with weak BRMS1 expression (Figure 4.3B). However, BRMS1 was not significantly correlated with 5-year patient survival of melanomas with low metastatic potential (≤4 mm thick and >4 mm without ulceration) (Figure 4.3C).
4.2.4 Expression of BRMS1 in melanoma cells inhibited growth and tube formation of HUVECs

To test the effect of BRMS1 expression in melanoma cells on HUVECs growth and tube formation, we overexpressed BRMS1 in MMRU and SK-mel-110 cells or knocked down BRMS1 in MMRU and MMAN cells. The expression of BRMS1 was then tested by both RT-PCR (Figure 4.4) and western blot (Figure 4.5A). The expressions of p65 or p50 subunit of NF-κB were not affected by the manipulation of BRMS1 expression (Figure 4.5A). Then the conditioned medium was collected from melanoma cells and applied to either HUVEC growth assay or tube formation assay. The growth of HUVECs in conditioned medium from BRMS1-overexpressing MMRU and SK-mel-110 cells was inhibited by 48% and 51%, respectively, when compared with the corresponding vector control (Figure 4.5B). BRMS1 knockdown in MMRU and MMAN promoted the HUVECs growth by 1.85- and 1.56-fold, respectively, compared with the control (Figure 4.5B). The average number of complete tubular structures formed by HUVECs was significantly decreased in conditioned medium from BRMS1-overexpressing MMRU and SK-mel-110 compared with vector controls (Figure 4.5C).

4.2.5 BRMS1 inhibited IL-6 expression through suppressing NF-κB activity

We previously reported that NF-κB p50 subunit enhanced melanoma angiogenesis through upregulating IL-6 at both transcriptional and protein level (Karst et al., 2009). Therefore, we next investigated whether BRMS1 also exerts its inhibitory effect on melanoma angiogenesis through the NF-κB/IL-6 pathway. Our data revealed that BRMS1 overexpression in MMRU and SK-mel-110 cells decreased IL-6 mRNA expression by 45% and 41%, respectively, compared with vector control (Figure 4.6A). In contrast, BRMS1 knockdown in MMRU and MMAN cells resulted in elevated IL-6 mRNA level by 2.3-fold for both (Figure 4.6A). Furthermore, we found that ectopic expression of
BRMS1 in MMRU and SK-mel-110 cells decreased the secreted IL-6 protein in the medium from 1.6 and 1.2 ng/ml/10^5 cells to 0.7 and 0.4 ng/ml/10^5 cells, respectively (Figure 4.6B). On the other hand, the IL-6 protein was increased by 2.8- and 3.5-fold in conditioned medium from BRMS1 knockdown MMRU and MMAN cells, respectively (Figure 4.6B). To explore whether BRMS1 regulates IL-6 expression through the NF-κB pathway, we performed EMSA and demonstrated that BRMS1 overexpression dramatically decreased the DNA binding activity of NF-κB p65 subunit to the IL-6 promoter, whereas the unlabeled competitive sequence markedly inhibited this binding in a dosage dependent manner (Figure 4.6C). To confirm the role of IL-6 in BRMS1-regulated melanoma angiogenesis, we performed IL-6 rescue and IL-6 blocking assays. The addition of 0.8 ng/ml recombinant IL-6 to BRMS1-overexpressing MMRU and SK-mel-110 cells rescued the cell growth of HUVECs to the similar level of the corresponding vector control cells, while the application of sufficient IL-6 antibody abrogated the elevated HUVECs growth by BRMS1-knockdown in MMRU and MMAN cells (Figure 4.6D). We also performed the HUVEC tube formation assay after addition of IL-6 to the conditioned medium from BRMS1-overexpressing and vector control MMRU cells. We found that the inhibited tubular structure formation can be rescued by addition of IL-6 (Figure 4.6E and F). A parallel tube formation assay after addition of IL-6 was conducted in another cell line, SK-mel-110, and similar result was obtained (Figure 4.7).

4.2.6 BRMS1-mediated IL-6 expression is dependent on p65 and p50 subunits of NF-κB

To investigate whether the regulation of IL-6 expression by BRMS1 is dependent on NF-κB, we overexpressed or knocked down BRMS1 together with or without silencing of NF-κB p65 or p50 subunit in MMRU cells. The mRNA expression of p65 and p50 was successfully decreased by 75% and 60% by specific siRNA sequences (Figure 4.8A and
B). However, overexpression or knockdown of BRMS1 did not change p50 or p65 expression. Next, we examined the impact of BRMS1, p65 or p50 on IL-6 mRNA. Our data revealed that knockdown of either p50 or p65 decreased IL-6 expression drastically (Figure 4.8C). The concomitant overexpression of BRMS1 together with silencing of p50 or p65 did not further reduce the IL-6 mRNA expression than knockdown of p50 or p65 alone (Figure 4.8C). Our data also demonstrated that p50 or p65 knockdown abrogated the elevated IL-6 mRNA expression by BRMS1 knockdown (Figure 4.8C), suggesting that the suppressive effect of BRMS1 on IL-6 expression is dependent on NF-κB. Similar results for the dependency of BRMS1-regulated IL-6 expression on NF-κB were also obtained in another cell line, SK-mel-110 (Figure 4.9).

### 4.2.7 Expression of BRMS1 in melanoma cells inhibited angiogenesis in vivo

We performed the in vivo matrigel plug assay to investigate whether BRMS1 expression could inhibit the new blood vessel formation in a mouse model. Visual examination revealed obviously less vascularization in matrigel plugs containing BRMS1-overexpressing MMRU cells than the control plugs (Figure 4.10A). CD31 staining demonstrated that the control plugs contained much denser neovessels with a 7-fold higher number of CD31 positive cells compared with BRMS1-overexpressing plugs (Figure 4.10B and C). We then investigated the level of tumor-derived IL-6 in those matrigel plugs. qRT-PCR data demonstrated that the IL-6 mRNA level was significantly decreased by 61% in matrigel plugs containing BRMS1-overexpressing MMRU cells compared with the control plugs (Figure 4.10D).
Table 4.1 Clinicopathologic characteristics of 137 melanomas

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<th>Variables</th>
<th>No. of patients</th>
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</tr>
<tr>
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<tr>
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1Sun-protected sites: trunk, arm, leg and foot; Sun-exposed sites: head and neck.
Table 4.2  Multivariate Cox regression analysis of BRMS1 and AJCC stages on 5-year patient survival in 137 melanomas

<table>
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<th>Variable</th>
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<th>P</th>
</tr>
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<td>American Joint Committee on Cancer stage</td>
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<tr>
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<td>0.41</td>
</tr>
<tr>
<td>Gender</td>
<td>1.30</td>
<td>0.71 to 2.40</td>
<td>0.40</td>
</tr>
</tbody>
</table>

¹Coding of variables: BRMS1 expression was coded as 1, weak; and 2, strong. AJCC stage was coded as 1, stage I-III; and 2, stage IV. Age was coded as 1, ≤58 years; and 2, >58 years. Gender was coded as 1, male; and 2, female.

²CI: confidence interval.
Figure 4.1  Representative images of BRMS1 staining in human melanocytic lesions. (A,D) Dysplastic nevus with strong BRMS1 staining. (B,E) Metastatic melanoma with weak BRMS1 staining. (C,F) Negative control without the BRMS1 primary antibody. Magnification ×100 for A, B, C and ×400 for D, E, F.
Figure 4.2  BRMS1 expression was correlated with melanoma progression. (A) Significant differences for BRMS1 staining pattern were observed between metastatic melanoma (MM) and primary melanoma (PM) ($P = 0.021$, $\chi^2$ test), or between metastatic melanoma and dysplastic nevi (DN) ($P = 0.001$, $\chi^2$ test). (B) BRMS1 expression was significantly decreased with the progression of AJCC stages in 137 melanomas. ($P = 0.011$, $\chi^2$ test).
Figure 4.3  Correlation between BRMS1 expression and 5-year disease-specific survival of melanoma patients. (A) Survival of all (primary plus metastatic) melanoma patients. (B) Survival of patients with metastatic melanoma and late stage primary melanoma. (C) Survival of primary melanomas with low metastatic potential. Cum., cumulative.
**Figure 4.4** Overexpression of BRMS1 in MMRU melanoma cells. MMRU cells were transiently transfected with BRMS1 plasmid and BRMS1 expression levels were examined by semi-quantitative RT-PCR.
Figure 4.5 Expression of BRMS1 in melanoma cells negatively regulated HUVECs growth and tube formation. (A) BRMS1 overexpression in MMRU and SK-mel-110 cells and knockdown in MMRU and MMAN cells were confirmed by western blot. (B) BRMS1 overexpression in melanoma cells inhibited HUVECs growth, whereas BRMS1 knockdown promoted HUVECs growth. Data were presented as means ± s.d. from three independent experiments. (C) BRMS1 overexpression in melanoma cells inhibited HUVECs tube formation. Numbers of tubes formed per field were counted in five random fields for BRMS1-overexpressing and control groups (n=3/group). *P<0.05, **P<0.01; Student’s t-test.
Figure 4.6  BRMS1 expression in melanoma cells inhibited IL-6 expression and suppressed NF-κB activity. (A, B) BRMS1 suppresses IL-6 expression. MMRU and SK-mel-110 cells were transiently transfected with vector or Flag-BRMS1 plasmid for 48 hours. In a parallel experiment, MMRU and MMAN cells were transfected with vector control or miBRMS1 plasmid for 72 hours. IL-6 mRNA in the cells and secreted protein level in conditioned medium were determined with qRT-PCR (A) and ELISA (B). Data were presented as means ± s.d. from three independent experiments. (C) BRMS1 overexpression suppressed the binding activity of NF-κB p65 subunit to IL-6 promoter. Competitive sequence inhibited the binding reaction in a dosage dependent manner. NE, Nuclear extract; Com, Competitive sequence. (D) Addition of IL-6 recombinant protein (0.8 ng/ml) to the conditioned medium rescued BRMS1-suppressed endothelial cell growth, while application of IL-6 antibody (320 ng/ml) to neutralize the IL-6 in conditioned medium abrogated BRMS1 knockdown-enhanced endothelial cell growth (n=3/group). (E) Addition of IL-6 to conditioned medium rescued the tube formation inhibited by BRMS1 overexpression. (F) The numbers of tubes formed per field were counted for BRMS1-overexpressing and control groups in five random fields. *P<0.05, **P<0.01, ***P<0.001, Student’s t-test.
**Figure 4.7** Addition of IL-6 to conditioned medium rescued the tube formation inhibited by BRMS1 overexpression in SK-mel-110 cells. (A) Representative pictures for the tube formation under microscope. (B) The numbers of tubes formed per field were counted in 5 random fields. **P<0.01, Student’s t-test.
Figure 4.8 BRMS1-mediated IL-6 expression in MMRU cells was dependent on p65 and p50 subunits of NF-κB. MMRU cells were transiently transfected with p50 or p65 siRNA for 24 hours, then transfected with vector control, Flag-BRMS1 or miBRMS1 plasmid for 48 hours. Total RNA was extracted and subjected to qRT-PCR. (A) p50 mRNA level. (B) p65 mRNA level. (C) IL-6 mRNA level. Data were presented as means ± s.d. from three independent experiments. *P<0.05, **P<0.01; Student’s t-test.
Figure 4.9 BRMS1-mediated IL-6 expression in SK-mel-110 cells was dependent on p65 and p50 subunits of NF-κB. SK-mel-110 cells were transiently transfected with p50 or p65 siRNA for 24 hours, then transfected with vector control, Flag-BRMS1 for 48 hours. Total RNA was extracted and subjected to qRT-PCR. (A) p50 mRNA level. (B) p65 mRNA level. (C) IL-6 mRNA level. Data were presented as means ± s.d. from three independent experiments. *P<0.05, **P<0.01; Student’s t-test.
Figure 4.10 BRMS1 overexpression in melanoma cells inhibited blood vessels formation in vivo. (A) Photographs of matrigel plugs excised from mice after 10 days of growth in vivo. (B) The extent of host angiogenesis was examined by immunofluorescent staining for expression of CD31 and BRMS1 in matrigel plugs containing BRMS1-overexpressing or control MMRU cells. Propidium iodide and Hoechst nuclear staining indicated the overall cell density in each matrigel plug. (C) The number for CD31 positive cells was counted from 5 random fields for both vector control and BRMS1-overexpressing groups. (D) The expression of BRMS1 and IL-6 mRNA was examined in matrigel plugs (n=3/group). **P<0.01; Student’s t-test.
Figure 4.11  BRMS1 expression was decreased in melanoma cell lines. BRMS1 mRNA levels in nine melanoma cell lines were compared with normal human epithelial melanocytes (MC) by real-time RT-PCR (n=3 for each cell line, student's t-test). * $P<0.05$, ** $P<0.01$. 
4.3 Discussion

BRMS1 was first identified by Seraj et al in 2000 when studying the non-random amplifications and deletions in chromosome 11 using differential display (Seraj et al., 2000b). In this study, we investigated the role of BRMS1 in melanoma progression and the relevant mechanisms. We started with TMA technology and immunohistochemistry staining to test the BRMS1 expression in 178 melanocytic lesions at different stages. The results demonstrated that reduced BRMS1 expression was significantly correlated with melanoma metastasis and AJCC stage. We also found that weak BRMS1 staining was closely related to worse melanoma patient survival. We next investigated the role of BRMS1 in melanoma angiogenesis. We found that BRMS1 inhibited endothelial cell growth and tube formation by decreasing IL-6 expression through suppressing NF-kB activity in vitro, as well as inhibited neovessels formation in mice in vivo. To our knowledge, this is the first study to examine the expression pattern of BRMS1 and its association with patient survival in a cohort of melanoma patients. It is also the first report on the role of BRMS1 in tumor angiogenesis.

We found a significant difference in BRMS1 expression between primary melanoma and metastatic melanoma, but not between dysplastic nevi and primary melanoma (Figure 4.2A), suggesting that loss of BRMS1 expression was mainly involved in melanoma metastasis instead of tumor growth. This is consistent with the findings that BRMS1 mRNA expression level was high in melanocytes, but barely detectable in metastatic melanoma cell lines (Shevde et al., 2002). Our RT-PCR results also demonstrated reduced BRMS1 in melanoma cell lines compared to normal melanocytes (Figure 4.11). It has been shown that re-introduction of BRMS1 into highly metastatic melanoma cell line C8161.9 significantly suppressed the metastatic potential in both experimental and spontaneous metastasis assays without affecting tumor growth after
orthotopic injection (Shevde et al., 2002). The effect of BRMS1 to inhibit metastasis was also confirmed in other cancer models. BRMS1 was expressed 5-fold higher in the metastasis-suppressed hybrid neo11/435 cells when compared with the highly metastatic breast cancer cell line MDA-MB-435. Furthermore, BRMS1 transfected MDA-MB-435 cells showed significantly decreased incidence and number of metastases to the lung and regional lymph nodes when cells were injected orthotopically. However, the tumor growth rate of BRMS1-transfected MDA-MB-435 cells is similar to their parental controls, except a delay in growth for one week (Seraj et al., 2000b). BRMS1 was also shown to be expressed at lower level in a highly metastatic human bladder carcinoma cell line T24T compared with the less metastatic parental cell line T24 (Seraj et al., 2000a). The reduced expression of BRMS1 in metastatic melanoma can possibly be explained by the theory that deletion at chromosome 11q, which includes the \textit{BRMS1} gene, occurs at very high frequency in various human cancers (Welch and Goldberg, 1997; Meehan and Welch, 2003; Zainabadi et al., 2005). Another study recently reported that the methylation of BRMS1 promoter might account for the loss of BRMS1 in breast cancer cells. The same study also demonstrated a direct correlation between methylation status of BRMS1 promoter in the DNA isolated from those tissues with loss of BRMS1 by immunohistochemistry, concomitantly (Metge et al., 2008). These studies imply that the loss of the BRMS1 expression may be a common event in tumor metastasis.

We demonstrated that BRMS1 inhibited melanoma angiogenesis and this may at least partially explain the correlation between reduced BRMS1 expression and melanoma metastasis. A series of studies indicated angiogenesis was closely related to tumor metastasis in various tumors, including breast, lung, prostate, head and neck, as well as melanoma (Weidner et al., 1991; Weidner, 1995; Czubayko et al., 1996); therefore, the deficient suppression on angiogenesis by loss of BRMS1 will be critical for melanoma to
metastasize. In this study, we found that BRMS1 inhibited HUVECs growth and tube formation in vitro (Figure 4.5B and C). We previously reported that p50 subunit of NF-κB increased IL-6 expression and melanoma angiogenesis (Karst et al., 2009). It has also been shown that BRMS1 suppressed NF-κB activity and thus regulated NF-κB downstream gene expression (Cicek et al., 2005). Therefore, we next investigated whether BRMS1 inhibits endothelial cell growth through NF-κB/IL-6 pathway. Further experiments showed that BRMS1 did not affect the expression level of p65 or p50 subunits of NF-κB (Figure 4.5A), but inhibited NF-κB DNA binding activity (Figure 4.6C). BRMS1 overexpression decreased, whereas BRMS1 knockdown increased both IL-6 mRNA expression and protein secreted in the conditioned medium (Figure 4.8A and B). The role of IL-6 in BRMS1-regulated melanoma angiogenesis was further confirmed by IL-6 rescue and blocking assays (Figure 4.6D-F, Figure 4.7). These results indicated that the compensation of IL-6 loss caused by BRMS1 overexpression can neutralize the inhibitory effect of BRMS1, while blocking IL-6 activity by incubating with the IL-6 antibody can mimic the BRMS1 overexpression impact. Therefore, the reduction of IL-6 protein secreted by melanoma cells accounted for the inhibitory effect of BRMS1 on melanoma angiogenesis. To explore whether BRMS1 regulates IL-6 expression through NF-κB pathway, we concomitantly manipulated the expression of BRMS1 and p65 or p50 subunits of NF-κB, and then tested the expression of IL-6. We found that both overexpression of BRMS1 and knockdown of p65 or p50 alone decreased IL-6 mRNA expression, but combination of BRMS1 overexpression and p65 or p50 knockdown did not bring extra inhibitory effect. Silencing of BRMS1 caused higher IL-6 expression, which was abrogated by the knockdown of p65 or p50. These results indicated that BRMS1 inhibits IL-6 expression through suppressing NF-κB activity (Figure 4.8 and 4.9). Moreover, we showed that BRMS1 inhibited new blood vessels formation in mice in vivo (Figure 4.10 A-C), and
qRT-PCR result further revealed that the BRMS1 overexpression was still present and IL-6 expression was decreased in matrigel plugs overexpressing BRMS1, indicating the consistency between our in vitro and in vivo data (Figure 4.10D). Our results highlighted a novel mechanism through which BRMS1 regulates angiogenesis, which is critically important, as neovascularization is one of the essential steps required by tumor metastasis and progression (Folkman, 2002).

We found that reduced BRMS1 staining was correlated with AJCC stages (Figure 4.2B), but not with other clinicopathological parameters. AJCC melanoma stage is evaluated by taking melanoma tumor thickness, ulceration, regional lymph node metastasis and distant metastasis into account and thus it is an integrated measure of the progression status and outcome of human melanoma patients (Balch et al., 2001a). It is not surprising to see that BRMS1 expression is inversely correlated with AJCC stage because reduced BRMS1 expression showed close correlation with melanoma metastasis in this study. Furthermore, in primary melanoma patient group, BRMS1 expression showed significant difference between AJCC stages I and II (Figure 4.2B), but did not change significantly between thin melanoma (≤2.0 mm) and thick melanoma (>2.0 mm), or between melanoma with and without ulceration. These data suggested that BRMS1 staining was associated with the combination of tumor thickness and ulceration, which may be a better marker for melanoma metastatic potential than tumor thickness or ulceration alone.

Reduced BRMS1 expression was significantly correlated with melanoma metastasis in our study and with metastasis of breast and ovarian carcinomas from previous reports (Seraj et al., 2000b; Shevde et al., 2002; Zhang et al., 2006). Therefore, reduced BRMS1 expression is expected to correlate with poorer survival of melanoma patients due to the fact that metastasis is the major cause for the death of melanoma patients. Moreover, we found that strong BRMS1 expression is significantly correlated
with better prognosis in those patients with thick (>4 mm) ulcerated primary melanoma and metastatic melanoma. Since it was reported that both thick melanoma (≥4 mm) and presence of ulceration were associated with a high incidence of regional and distant metastasis (Mansfield et al., 1994; Ostmeier et al., 1999), our data suggested that BRMS1 could be one of the few prognostic markers for melanoma patients with existing metastases or with high metastatic potential. Furthermore, our multivariate Cox regression analysis indicated that the correlation between reduced BRMS1 expression and worse patient survival was independent of age, gender and AJCC stage of the patients (Table 4.2), further underscoring the important role of BRMS1 in melanoma prognosis. Combined with the previous reports on the crucial metastasis suppressive functions of BRMS1, our findings implied that BRMS1 may serve as a promising prognostic marker for melanoma and restoration of BRMS1 may provide a novel strategy for the treatment of metastatic melanoma.
5 ING4 is Regulated by BRMS1, and is a Suppressor of Melanoma Angiogenesis

5.1 Rational and hypothesis

In chapter 3, we showed that ING4 expression is decreased in melanoma when compared with dysplastic nevi and overexpression of ING4 inhibits melanoma cell migration and cell invasion. In chapter 4 we revealed that BRMS1 expression is decreased in metastatic melanoma and BRMS1 negatively regulates melanoma angiogenesis through NF-κB/IL-6 pathway. As both molecules are believed to be related to suppression of NF-κB activity, we hypothesize that ING4 may regulate melanoma angiogenesis and ING4 and BRMS1 may show correlated expression and/or have related function. To test this hypothesis, we examine the impact of ING4 on HUVECs growth and tube formation ability. We also investigated the effect of ING4 on NF-κB activity as well as IL-6 expression. Finally we also analyzed the correlation between ING4 and BRMS1 expression, and the pattern of their function in mediating melanoma angiogenesis.

5.2 Results

5.2.1 Expression of ING4 in melanoma cells suppressed growth and tube formation of HUVECs

We tested two miING4 sequences to knock down endogenous ING4 and both showed significant inhibition on ING4 expression by qRT-PCR and western blot (Figure 5.1). The sequence 2 was used for further experiments as it showed relatively better knockdown effect than the sequence 1. To test the role of ING4 on melanoma angiogenesis, we first overexpressed ING4 in MMRU and SK-mel-110 cells or knocked down ING4 in MMRU and MMAN cells. The expression of ING4 was then confirmed by western blot (Figure 5.2A). Then conditioned medium was collected from melanoma cells and applied to either HUVEC growth assay or tube formation assay. Compared with the
corresponding control, overexpression of ING4 in MMRU and SK-mel-110 cells resulted in decreased HUVECs growth by 71% and 46%, respectively. In contrast, ING4 knockdown in MMRU and MMAN cells led to 2- and 1.5-fold increase of HUVECs growth, respectively (Figure 5.2B). The average number of complete tubular structures formed by HUVECs was also decreased by 73% in conditioned medium from ING4-overexpressing SK-mel-110 cells, whereas increased by 1.6-fold in conditioned medium from ING4 knockdown MMRU cells, when compared with respective controls (Figure 5.2C and D).

5.2.2 ING4 mediated HUVECs growth by suppressing IL-6 expression

Previously our group reported that NF-κB p50 subunit enhanced melanoma angiogenesis through upregulating IL-6 at both transcriptional and protein level (Karst et al., 2009). Therefore, we next tested whether ING4 inhibits melanoma angiogenesis by suppressing NF-κB/IL-6 pathway. Our real-time RT-PCR data revealed that ING4 overexpression in MMRU and SK-mel-110 cells downregulated IL-6 mRNA expression by 49.5% and 47.2% compared with the vector control (Figure 5.3A, left panel). ING4 knockdown in MMRU and MMAN cells elevated IL-6 mRNA by 1.9- and 2.4- fold, respectively (Figure 5.3A, right panel). We then performed ELISA assay and found that ING4 overexpression in MMRU and SK-mel-110 cells decreased IL-6 protein in conditioned medium from 1.7 and 1.6 ng/mL/10^5 cells to 0.9 and 0.7 ng/mL/10^5 cells, respectively (Figure 5.3B, left panel). In contrast, IL-6 protein was increased by 3.4- and 2.9-fold in conditioned medium collected from ING4 knockdown MMRU and MMAN cells, respectively (Figure 5.3B, right panel). The role of IL-6 in ING4-regulated melanoma angiogenesis was then confirmed by IL-6 rescue and IL-6 blocking assays. The addition of 0.8 ng/ml recombinant IL-6 to ING4-overexpressing MMRU and SK-mel-110 cells rescued the HUVECs growth to the similar level of the corresponding vector control.
cells (Figure 5.3C), while the application of sufficient IL-6 antibody abrogated the elevated HUVECs growth by ING4-knockdown in MMRU and MMAN cells (Figure 5.3D).

### 5.2.3 ING4 expression in melanoma cells inhibited angiogenesis *in vivo*

We performed the *in vivo* matrigel plug assay to investigate whether ING4 expression in melanoma cells could inhibit the neovessel formation in a mouse model. Visual examination revealed obviously less vascularization in matrigel plugs containing ING4-overexpressing MMRU cells than the control plugs (Figure 5.4A). CD31 staining demonstrated that the control plugs had much denser neovessels with 3-fold CD31 positive cells compared with ING4-overexpressing plugs (Figure 5.4B and C). We then investigated the level of tumor-derived IL-6 in those matrigel plugs. qRT-PCR data demonstrated that the IL-6 mRNA level was significantly decreased by 51% in matrigel plugs containing ING4-overexpressing MMRU cells compared with the control plugs (Figure 5.4D).

### 5.2.4 ING4 expression is induced by BRMS1

Since we previously demonstrated that BRMS1 inhibited melanoma angiogenesis through decreasing NF-κB activity and IL-6 level (in Chapter 4), we next investigated whether these two molecules are related in expression and/or function. We first studied the expression of ING4 and BRMS1 in 173 melanocytic lesions using tissue microarray. Among all these biopsies, 63 showed weak ING4 expression while the other 110 had strong ING4 staining. The percentage of strong BRMS1 staining increased from 47% in weak ING4 group to 68% in strong ING4 group ($P = 0.011$, $\chi^2$ test, Figure 5.5A). We next performed RT-PCR to examine the expression of ING4 and BRMS1 in normal melanocytes and different melanoma cell lines. We found that expression of both two markers was decreased in all melanoma cells lines examined when compared with normal
melanocytes (Figure 5.6). Expression of ING4 and BRMS1 was also compared in 7 melanoma cell lines by western blot. Both ING4 and BRMS1 showed high expression in MMAN and MeWo cells, deficient expression in SK-mel-110 cell, and moderate expression in the other cell lines, exhibiting the similar expression trend in different melanoma cell lines (Figure 5.5B). We next examined whether the manipulation of BRMS1 expression regulated ING4 level, or vice versa. Our western blot result showed that when we overexpressed BRMS1 in MMRU and SK-mel-110 cells, we saw increased ING4 expression by 1.9- and 2.2-fold, respectively (Figure 5.5C, left panel). Moreover, BRMS1 knockdown in MMRU and MMAN cells resulted in decreased ING4 expression by 60% and 70% in MMRU and MMAN, respectively (Figure 5.5C, right panel). In contrast, neither overexpression of ING4 in MMRU and SK-mel-110 cells, nor knockdown of ING4 in MMRU and MMAN cells altered the endogenous expression of BRMS1 in these cell lines (Figure 5.5D). This induction of ING4 by BRMS1 was also confirmed by qRT-PCR. Knockdown of ING4 in MMRU and MMAN did not change BRMS1 mRNA expression (Figure 5.7A), whereas knockdown of BRMS1 in MMRU and MMAN cells resulted in 70.5% and 63.3% decrease of ING4 mRNA expression, respectively (Figure 5.7B). Immunoprecipitation analysis of BRMS1 and ING4 indicated that these two molecules are not in the same complex (Figure 5.8).

5.2.5 ING4 is the downstream target of BRMS1 in melanoma angiogenesis inhibition

I next examined whether ING4 and BRMS1 are functionally related. I overexpressed either ING4 or BRMS1 alone, or co-expressed both ING4 and BRMS1, and then tested their impact on HUVECs growth. I found that co-expression of ING4 and BRMS1 in MMRU cells did not further suppress HUVECs growth, but to the similar extent as overexpression of either ING4 or BRMS1 (Figure 5.9A). I also showed that
concomitant overexpression of ING4 and BRMS1 inhibited the tubular structure formation to the similar level as overexpression of either ING4 or BRMS1 (Figure 5.9B and C). Further experiments showed that ING4 knockdown abolished BRMS1-induced suppression of HUVECs growth, suggesting that ING4 is the downstream target of BRMS1 in regulating angiogenesis (Figure 5.9D). In a parallel experiment, both ING4 overexpression alone and ING4 overexpression plus BRMS1 knockdown inhibited HUVECs growth to the similar level, suggesting that BRMS1 knockdown did not interfere the effect of ectopic expression of ING4 on HUVECs growth (Figure 5.9E). We repeated HUVECs growth assay and tube formation assay in another melanoma cell SK-mel-110, and confirmed the finding that combination of ING4 and BRMS1 did not produce additional impact on angiogenesis (Figure 5.10). As both ING4 and BRMS1 inhibited melanoma angiogenesis through decreasing IL-6 expression, we next examined whether co-expression of ING4 and BRMS1 exert synergistic suppression on IL-6. qRT-PCR demonstrated that overexpression of ING4 plus BRMS1 in MMRU and SK-mel-110 cells decreased IL-6 mRNA expression to the similar level as individual overexpression of either one (Figure 5.11A). This result was supported by ELISA assay data, showing that overexpression of both ING4 and BRMS1 only decreased IL-6 protein in the conditioned medium to the similar level as individual overexpression (Figure 5.11B). Finally we performed EMSA to study the effect of ING4 and BRMS1 on the DNA binding activity of NF-κB to IL-6 promoter. Our data revealed that overexpression of ING4 or BRMS1 alone inhibited the binding of NF-κB to IL-6 promoter, while co-overexpression of both genes showed the inhibitory effect to the similar extent (Figure 5.11C).
Figure 5.1 ING4 knockdown in MMRU melanoma cells. MMRU cells were transiently transfected with miING4 sequence 1 or sequence 2 and then ING4 mRNA and protein expression were examined by qRT-PCR (A) and western blot (B). **$P<0.01$, ***$P<0.001$, Student’s $t$-test.
Figure 5.2 ING4 expression in melanoma cells inhibited growth and tube formation of HUVECs. Melanoma cells were infected by adv or adING4 to overexpress ING4, or transiently transfected with control vector or miING4 to silence ING4 expression. Then conditioned medium were collected for HUVECs growth or tube formation assay. (A) ING4 overexpression in MMRU and SK-mel-110 cells (upper panel), and ING4 knockdown in MMRU and MMAN cells (lower panel) were confirmed by western blot. (B) ING4 overexpression inhibited, whereas ING4 knockdown promoted the growth of HUVECs. Data were presented as means ± s.d. from three independent experiments. (C) ING4 overexpression in melanoma cells inhibited, whereas ING4 knockdown promoted HUVECs tube formation. (D) Numbers of tubes formed per field were counted in five random fields for ING4-overexpressing, knockdown and corresponding controls. *P<0.05, **P<0.01, ***P<0.001, Student’s t-test.
**Figure 5.3** ING4 expression in melanoma cells suppressed IL-6 level. (A and B) ING4 suppresses IL-6 expression. Melanoma cells were infected with adv or adING4, or transiently transfected with vector control or miING4. IL-6 mRNA in the cells and secreted protein level in conditioned medium were determined by qRT-PCR (A) and ELISA (B). (C) Addition of IL-6 recombinant protein (0.8 ng/ml) to the conditioned medium rescued ING4-suppressed HUVECs growth. (D) Application of IL-6 antibody (320 ng/ml) to neutralize the IL-6 in conditioned medium abrogated ING4 knockdown-promoted HUVECs growth. Data were presented as means ± s.d. from three independent experiments. *P<0.05, **P<0.01, ***P<0.001, Student’s t-test.
Figure 5.4 ING4 overexpression in melanoma cells inhibited blood vessels formation in vivo. (A) Photographs of matrigel plugs excised from mice after 10 days of growth in vivo. (B) The extent of host angiogenesis was examined by immunofluorescent staining for CD31 expression in matrigel plugs containing ING4-overexpressing or control MMRU cells. Propidium iodide staining indicated the overall cell density in each matrigel plug. (C) The number of CD31 positive cells was counted in 5 random fields for both vector control and ING4-overexpressing groups. (D) The expression of ING4 and IL-6 mRNA in matrigel plugs was determined by qRT-PCR. Data were presented as means ± s.d. from three independent experiments. *P<0.05, **P<0.01, Student’s t-test.
Figure 5.5 ING4 expression was induced by BRMS1. (A) Correlation of the expression between ING4 and BRMS1 in 173 melanocytic lesions. In strong ING4 staining group (n=110), the percentage of strong BRMS1 expression was also higher than that in weak ING4 staining group (n=63). (B) Expression of ING4 and BRMS1 were examined in 7 melanoma cell lines by western blot. (C) BRMS1 is the upstream regulator of ING4. Melanoma cells were transiently transfected with vector control or Flag-BRMS1 plasmid to overexpress BRMS1, or transfected with vector control or miBRMS1 to knockdown BRMS1. The expression of BRMS1 and ING4 was determined by western blot. (D) ING4 did not affect BRMS1 expression. Melanoma cells were transiently transfected with vector control or HA-ING4 plasmid to overexpress ING4, or transfected with vector control or miING4 to knockdown ING4. The expression of BRMS1 and ING4 was determined by western blot. Actin was used as a loading control.
**Figure 5.6** BRMS1 and ING4 expression was decreased in melanoma cell lines. BRMS1 and ING4 mRNA levels in nine melanoma cell lines were compared with normal human epithelial melanocytes by real-time RT-PCR (n=3 for each cell line, student's *t*-test).

* $P<0.05$, ** $P<0.01$. 
Figure 5.7 ING4 mRNA was decreased by BRMS1 knockdown. (A) MMRU and MMAN cells were transiently transfected with control vector or miING4, and then qRT-PCR was performed to examine the mRNA expression of ING4 and BRMS1. (B) MMRU and MMAN cells were transiently transfected with control vector or miBRMS1, and then qRT-PCR was performed to examine the mRNA expression of ING4 and BRMS1. **P<0.01, ***P<0.001, Student’s t-test. miC, miControl; mi4, miING4; miB, miBRMS1.
**Figure 5.8** ING4 and BRMS1 can not be immunoprecipitated in the same complex. MMRU cells were transfected with vector, Flag-BRMS1, HA-ING4, or Flag-BRMS1/HA-ING4. Nuclear extract from MMRU cells overexpressing BRMS1 or ING4 was immunoprecipitated with anti-Flag or anti-HA antibody, respectively. Nuclear protein from ING4 and BRMS1 co-expression MMRU cells was immunoprecipitated with anti-Flag and anti-HA antibodies separately. Western blot was then performed with BRMS1 antibody or ING4 antibody for the immunoprecipitated samples (A) and input control (B).
Figure 5.9  ING4 and BRMS1 overexpression in melanoma cells suppressed HUVECs growth and tube formation in a sequential pattern. (A and B) MMRU cells were transfected with Flag-BRMS1 plasmid, or infected with adING4, or treated with the combination of the two, then conditioned medium was collected and applied to HUVECs growth (A) and tube formation assay (B). (C) The number of tubes formed per field was counted in five random fields for ING4-overexpressing, BRMS1-overexpressing, or co-overexpressing and control group. (D) MMRU cells were transfected with Flag-BRMS1, together with or without miING4, and then conditioned medium was collected for HUVECs growth assay. (E) MMRU cells were infected by adING4, together with or without miBRMS1, and then conditioned medium was collected for HUVECs growth assay. *P<0.05, **P<0.01, Student’s t-test.
Figure 5.10 Co-overexpression of ING4 and BRMS1 in SK-mel-110 melanoma cells did not further suppress HUVECs growth and tube formation than overexpression of ING4 or BRMS1 alone. SK-mel-110 cells were transfected with Flag-BRMS1 plasmid, or infected with adING4, or co-expressed for both BRMS1 and ING4, then conditioned medium was collected and applied to HUVECs growth (A) and tube formation assay (B). (C) numbers of tubes formed per field were counted in five random fields for ING4-overexpressing, BRMS1-overexpressing, or co-overexpressing and control group. **P<0.01, ***P<0.001, Student’s t-test.
**Figure 5.11** ING4 and BRMS1 overexpression decreased IL-6 level in the same pathway. (A and B) Melanoma cells were infected with adv or adING4, together with or without Flag-BRMS1 transfection. IL-6 mRNA in the cells and secreted IL-6 protein level in conditioned medium were determined by qRT-PCR (A) and ELISA (B). Data were presented as means ± s.d. from three independent experiments. (C) ING4 and BRMS1 single overexpression or co-overexpression suppressed the binding activity of NF-κB p65 subunit to IL-6 promoter. Competitive sequence inhibited the binding reaction in a dosage dependent manner. NE, Nuclear extract; Com, Competitive sequence. **P<0.01, Student’s t-test.
5.3 Discussion

ING4 was first identified in 2003 by searching for homologous sequences with ING1, the founding member of ING family proteins, and followed by PCR and rapid amplification of cDNA ends (RACE) of placenta cDNA library (Shiseki et al., 2003). We have previously shown that ING4 expression was decreased in human cutaneous melanoma compared with dysplastic nevi, indicating its tumor suppressor role in melanoma pathogenesis (Li et al., 2008a). We also reported that ING4 expression inhibited melanoma cell migration and invasion (Li et al., 2009a). In this study, we investigated the role of ING4 in melanoma angiogenesis. Our data for the first time showed that ING4 inhibited melanoma angiogenesis in vitro and in vivo, through inhibiting NF-κB activity and IL-6 expression. Moreover, we demonstrated that ING4 is a downstream target of BRMS1 in the regulation of melanoma angiogenesis. To our knowledge, this is the first study showing that ING4 expression was controlled by BRMS1.

Angiogenesis is a multi-step process, which includes endothelial cell proliferation, migration and the formation of blood vessels (Kalluri, 2003; Mamou et al., 2006). In this study, we found that both the growth and tube formation of HUVECs were inhibited in conditioned medium collected from ING4-overexpressing melanoma cells. This effect was confirmed by ING4 knockdown experiments as silencing ING4 in melanoma cells resulted in enhanced endothelial cell growth and tube formation ability (Figure 5.2B-D). Furthermore, we demonstrated that ING4 overexpression in melanoma cells inhibited the supportive vasculature in vivo (Figure 5.4). Inhibition of melanoma angiogenesis by ING4 expression described in this study is consistent with, and partially explains our previous finding that ING4 expression was decreased in human melanoma when compared with dysplastic nevi, and this reduced ING4 staining was correlated with melanoma thickness and patient survival (Li et al., 2008a). Tumor growth can be separated into two stages,
avascular and vascular stage (Folkman, 1974). The invasive tumor growth can only be achieved when angiogenesis is “switched on” and new capillaries penetrate into the tumor (Folkman, 2002). Besides invasiveness, angiogenesis is also widely believed to be associated with tumor metastasis. A series of studies indicated that angiogenesis was closely related to metastasis in various tumors, including breast, lung, prostate, head and neck, as well as melanoma (Weidner et al., 1991; Weidner, 1995; Czubayko et al., 1996). Combined with the fact that metastasis is the major cause of melanoma patient death, it is not surprising to see that reduced ING4 is associated with tumor invasion, angiogenesis, metastasis, and eventually poorer outcome of melanoma patients.

We previously reported that p50 subunit of NF-κB promoted melanoma angiogenesis by upregulating IL-6 level (Karst et al., 2009). It is also known that ING4 inhibits NF-κB activity and thus its downstream gene expression (Garkavtsev et al., 2004; Nozell et al., 2008; Coles et al., 2010). Therefore, we hypothesized that ING4 may suppress melanoma angiogenesis through inhibiting NF-κB target gene IL-6 expression. Our data showed that ING4 overexpression in melanoma cells decreased, whereas ING4 knockdown increased IL-6 mRNA expression and protein in conditioned medium (Figure 5.3A and B). These data were consistent with the finding that IL-6 can be secreted by melanoma cells (Molnar et al., 2000), and the expression of IL-6, as a NF-κB target gene, can be inhibited by ING4 (Nozell et al., 2008; Coles et al., 2010). Furthermore, the role of IL-6 in ING4-mediated melanoma angiogenesis was confirmed by IL-6 rescue and blocking experiments. The inhibited HUVECs growth by ING4 overexpression can be rescued by adding recombinant IL-6 to conditioned medium, and the application of IL-6 antibody in conditioned medium abrogated ING4 knockdown induced HUVECs growth (Figure 5.3C and D). Collectively, these data indicated that ING4 regulated HUVECs growth through decreasing IL-6 level.
We next investigated whether ING4 collaborated with BRMS1 in this angiogenesis modulation, as both ING4 and BRMS1 inhibited melanoma angiogenesis through NF-κB/IL-6 pathway, and both were reported to inhibit NF-κB activity (Samant et al., 2007; Nozell et al., 2008; Cicek et al., 2009). The immunoprecipitation data showed that ING4 and BRMS1 can not be precipitated together, ruling out the possibility that they functioned in the same complex (Figure 5.8). Therefore, the possible correlation between ING4 and BRMS1 expression was tested. We previously examined the expression alteration of both ING4 and BRMS1 using the same tissue microarray, which enable us to compare the expression of these two biomarkers in the same group of patients. Our data revealed that higher ING4 expressing-group patients also demonstrated higher BRMS1 expression (Figure 5.5A). Endogenous expression of ING4 and BRMS1 also showed similar trend in 7 melanoma cell lines (Figure 5.5B). Further experiments revealed that BRMS1 overexpression induced ING4 expression, whereas BRMS1 knockdown resulted in decreased ING4 expression. In contrast, manipulation of ING4 expression did not affect BRMS1 expression, indicating that ING4 expression was under the control of BRMS1 (Figure 5.5C and D, Figure 5.7). So far, ING4 has been shown to inhibit the activity of several important transcription factors, including NF-κB (Garkavtsev et al., 2004; Nozell et al., 2008; Coles et al., 2010), HIF-1 (Ozer and Bruick, 2005; Ozer et al., 2005; Colla et al., 2007), and p53 (Shiseki et al., 2003; Zhang et al., 2005). To our knowledge, this is the first study showing that ING4 was regulated by BRMS1 at both mRNA and protein level, which may help to better understand the complete pathway of ING4 to regulate various biological functions. More experiments are needed to further elucidate the details of BRMS1 regulation on ING4 expression.

We then investigated whether ING4 and BRMS1 are functionally related. Our data demonstrated that co-expression of ING4 and BRMS1 did not show further inhibition on
HUVECs growth and blood tube formation than individual expression of ING4 or BRMS1 alone (Figure 5.9A-C). We also found that the inhibited HUVECs growth by BRMS1 expression can be abrogated by knockdown of endogenous ING4, whereas BRMS1 knockdown did not affect the inhibitory impact of ING4 expression on HUVECs growth (Figure 5.9D and E). These data were consistent with the finding that BRMS1 induced ING4 expression and suggested that they exerted their effect through the same pathway, with BRMS1 being the upstream regulator of ING4 to mediate melanoma angiogenesis. ING4 is under the control of BRMS1 and inhibits the activity of NF-κB either directly by interacting with the RelA subunit of NF-κB or its down stream gene promoter (Garkavtsev et al., 2004; Nozell et al., 2008), or indirectly by inducing IκB expression (Coles et al., 2010). Data from qRT-PCR and ELISA also supported that co-expression of ING4 and BRMS1 only decreased IL-6 mRNA or secreted protein to the similar level as single expression of individual genes (Figure 5.11A and B). Moreover, EMSA revealed that overexpression of ING4 or BRMS1 alone inhibited the binding activity of NF-κB to IL-6 promoter to the similar level as the co-expression sample (Figure 5.11C), suggesting that they inhibited NF-κB activity in the same pathway. All these data suggested that BRMS1 and ING4 inhibited melanoma angiogenesis through suppressing NF-κB activity and thus its target gene, IL-6 expression, in a sequential pattern.

In summary, we demonstrated that ING4 inhibited human melanoma angiogenesis. We identified BRMS1 as the upstream regulator and NF-κB/IL-6 as the downstream targets of ING4 in mediating melanoma angiogenesis. Our data indicated that the tumor suppressive role of ING4 in melanoma is partially attributed to its inhibitory effect on melanoma angiogenesis and imply that ING4 restoration may be a novel approach for anti-angiogenesis therapy for human melanoma.
6 Patient Outcome Prediction by Multiple Biomarkers in Human Melanoma

6.1 Rational and hypothesis

Malignant melanoma is associated with very high mortality rate, particularly for advanced diseases. Patients with metastatic melanoma have a very poor prognosis, with a median survival of only 6 to 10 months (Jemal et al., 2002). Despite this, advances in the prognostic assessment of melanoma are still essential to improve predictions for the survival of melanoma patients. One such way to improve the prognostic assessment of cancer patients is the use of molecular biomarkers. Unfortunately, so far no biomarker has been widely accepted by clinical practice or implemented in the classical TNM staging standard, possibly due to the limited value of individual biomarker. Previously we examined the expression of 10 biomarkers (Bim, BRG1, BRMS1, CTHRC1, ING4, NQO1, NF-κB-p50, PUMA, SNF5, and SOX4) in melanomas and most of them were found to be important for the prognostic assessment of melanoma patients to different extents (Cheng et al.; Lin et al.; Karst et al., 2005; Gao et al., 2006; Tang et al., 2006; Dai et al., 2008; Li et al., 2008a; Lin et al., 2009; Jafarnejad SM, 2010; Li J, 2010). In order to reveal whether the combination of multiple biomarkers can deliver better value for prognosis, we analyzed the expression of these 10 biomarkers in 73 primary melanomas, and 45 metastatic melanomas. We then focused on comparing the expression of each individual biomarker or the index score of the multi-biomarker system between AJCC I-II stages (without metastasis) and AJCC III-IV stages (with metastasis to either lymph node or distant organ) of melanomas. Finally we compared the capability of any individual biomarker or combined biomarker system to predict patient outcome.
6.2 Re-evaluating expression of each biomarker and calculating the index score for multiple biomarkers

The expression of ten biomarkers was examined using tissue microarray and immunohistochemistry previously. The detailed methodology for the staining of these biomarkers were reported (Cheng et al.; Lin et al.; Karst et al., 2005; Gao et al., 2006; Tang et al., 2006; Dai et al., 2008; Li et al., 2008a; Lin et al., 2009; Jafarnejad SM, 2010; Li J, 2010). The information on the antibodies used in these studies is listed in Table 6.1. We collected the raw readings for each individual biomarker and re-grouped the staining intensity and percentage of positive staining cells uniformly in this study. Staining intensity was defined as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), and the percentage of positive staining was scored into 3 categories: 1 (0-33%), 2 (34-67%), and 3 (68-100%) (Dai et al., 2005). The level of each biomarker staining was finally evaluated by immunoreactive score (IRS) (Remmele and Stegner, 1987), which is calculated by multiplying the score of staining intensity and that of the percentage of positive cells. Then the IRS was applied to the statistical analysis of expression variation among different stages of melanocytic lesions or various subgroups directly.

To assess the value of multiple biomarkers in melanoma prognosis, we calculated the index score for multiple biomarkers based on their individual IRS and how they changed from primary stages to advanced stages. The expressions of 6 biomarkers, including Bim, BRMS1, ING4, NQO1, PUMA, and SOX4, were all higher in primary stages (AJCC I and II) of melanomas than the advanced stages (AJCC III and IV), so the final index score is the sum of IRS of all 6 biomarkers. For the survival analysis using the 6-biomarker system, the final index score was grouped into 2 categories: low score group (0-16), and high score group (17-48).
6.3 Results

6.3.1 Clinicopathological features of melanoma biopsies

The clinicopathological features of all 118 melanoma biopsies analyzed in this study were summarized in Table 6.2. For 73 primary melanoma cases, there were 46 men and 27 women, with age ranging from 21 to 93 years (median, 59 years). In 47 cases, the tumors were ≤2.0 mm thick, and the other 26 tumors were >2.0 mm. Tumor ulceration was present in 15 cases at diagnosis. For the histological subtype, there were 30 cases of superficial-spreading melanoma, 16 lentigo maligna melanomas, 10 nodular melanomas, and other 17 cases unspecified. Sixteen melanomas were found in sun-exposed sites, including head and neck, while the other 57 were located in sun-protected area including arm, foot, leg, and trunk. Forty-five melanoma metastases were included in this analysis, including 31 men and 14 women, with age ranging from 27-89 (median age 60 years). We also applied AJCC criteria to this study in all melanoma patients. Among the 118 cases, 41 tumors at stage I, 29 at stage II, 26 at stage III, and 22 at stage IV (Table 6.2).

6.3.2 Six-biomarker system was correlated with melanoma metastasis

Various levels of staining were observed for each biomarker in different melanocytic lesions (Figure 6.1). We compared the expression profile of all 10 biomarkers in AJCC I-II and AJCC III-IV melanoma stages. Our data revealed that the expressions of six biomarkers, including Bim, BRMS1, ING4, NQO1, PUMA, and SOX4, were significantly higher in early stages (AJCC I-II) than advanced stages (AJCC III-IV), suggesting that these 6 biomarkers are correlated with melanoma metastasis (Table 6.3). We obtained the final index score for 6-biomarker system by summing up the IRS of all 6 biomarkers together, and then performed the correlation analysis using either each individual biomarker or the combined 6-biomarker system. The correlation coefficients for Bim, BRMS1, ING4, NQO1, PUMA, and SOX4 were 0.333, 0.146, 0.188, 0.146, 0.05,
and 0.208, respectively, whereas it reached 0.5 for the combined 6-biomarker system. We next investigated whether this 6-biomarker index score was correlated with other clinicopathological parameters. Our data showed that higher index score (17-48) was correlated with thin tumors (≤4.0mm), sun-protected sites and superficial spreading melanoma (P =0.008, 0.030 and 0.003, respectively, Mann-Whitney test; Figure 6.2A-C), but not correlated with patient age, gender, and ulceration.

### 6.3.3 Six-biomarker system provided better prognostic accuracy than any individual biomarker

Univariate Cox regression analysis showed that the 5 biomarkers, including Bim, BRMS1, ING4, NQO1, and PUMA, were all significantly correlated with both overall and disease-specific 5-year survival of the 118 melanoma patients, while SOX4 was correlated with disease-specific 5-year survival only. The relative risk (RR) and P value for each individual biomarker in overall survival analysis ranged from the lowest RR (0.461) (P = 0.003) for ING4, to the highest RR (0.626) (P = 0.076) for SOX4. In disease-specific survival analysis, the RR and P value for individual biomarker ranged from the lowest RR (0.408) (P = 0.002) for BRMS1, to the highest RR (0.527) (P = 0.029) for SOX 4 (Table 6.4). However, when we combined these 6 biomarkers and assessed the predictive ability for patient outcome with univariate Cox analysis, we found that the RR was decreased to 0.273 and 0.222 for overall and disease-specific survival (P = 0.00001 and 0.000002), respectively (Table 6.4). We next constructed the Kaplan-Meier curve in all 118 melanoma patients, and our data revealed that high index score of combined 6-biomarker system was significantly correlated with better 5-year patient survival in both overall and disease-specific survival analyses (P = 0.0000 for both, log-rank test). The overall and disease-specific 5 year survival rates dropped from 57% and 68% in those patients with high index score to 11% and 17% in low index score group, respectively (Figure 6.3). Next, we
examined whether this combined index score was an independent factor for melanoma prognosis. We performed multivariate Cox regression analysis to study the effect of this index score in patient survival, together with patient’s age and gender in all 118 melanomas. Our results indicated that it was an independent prognostic factor for both overall and disease-specific 5-year survival (RR = 0.237 and 0.208, 95% CI = 0.125 to 0.453 and 0.105-0.414, \( P = 0.00001 \) and 0.000007, respectively) (Table 6.5).
Table 6.1 Antibodies for 10 biomarkers studied

<table>
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<th>Full name</th>
<th>Supplier</th>
<th>Clone</th>
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¹The BRMS1 antibody was provided by Dr. Danny R. Welch, University of Alabama at Birmingham.
Table 6.2 Clinicopathologic characteristics of 118 melanomas

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<th>No. of patients</th>
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<td><strong>AJCC Stage</strong></td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>41</td>
<td>34.7</td>
</tr>
<tr>
<td>II</td>
<td>29</td>
<td>24.6</td>
</tr>
<tr>
<td>III</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>IV</td>
<td>22</td>
<td>18.7</td>
</tr>
</tbody>
</table>

<sup>1</sup>Sun-protected sites: trunk, arm, leg and foot; Sun-exposed sites: head and neck.
Table 6.3 Comparison of 10 biomarkers expression between melanomas with and without metastasis

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>IRS mean value</th>
<th></th>
<th></th>
<th>P value1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AJCC I-II (n=70)</td>
<td>AJCC III-IV (n=48)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bim</td>
<td>5.30</td>
<td>2.83</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRMS1</td>
<td>5.99</td>
<td>4.45</td>
<td>0.0030</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRG1</td>
<td>6.09</td>
<td>6.08</td>
<td>0.9253</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTHRC1</td>
<td>6.00</td>
<td>6.75</td>
<td>0.1342</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ING4</td>
<td>5.86</td>
<td>4.75</td>
<td>0.0239</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NQO1</td>
<td>4.96</td>
<td>2.75</td>
<td>0.0008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-κB-p50</td>
<td>4.84</td>
<td>5.71</td>
<td>0.1424</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PUMA</td>
<td>4.89</td>
<td>4.19</td>
<td>0.0379</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNF5</td>
<td>5.84</td>
<td>5.87</td>
<td>0.9430</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX4</td>
<td>5.77</td>
<td>4.35</td>
<td>0.0115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-biomarker system</td>
<td><strong>26.992</strong></td>
<td><strong>18.982</strong></td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 P value was calculated by Mann-Whitney test.

2 Mean value from 6 significantly altered biomarkers including Bim, BRMS1, ING4, NQO1, PUMA, and SOX4.
Table 6.4  Univariate Cox regression analysis of individual and multiple-biomarkers on 5-year patient survival in 118 melanomas

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Overall survival</th>
<th>Disease-specific survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>Bim</td>
<td>0.465</td>
<td>0.258-0.838</td>
</tr>
<tr>
<td>BRMS1</td>
<td>0.575</td>
<td>0.343-0.965</td>
</tr>
<tr>
<td>ING4</td>
<td>0.461</td>
<td>0.275-0.772</td>
</tr>
<tr>
<td>NQO1</td>
<td>0.473</td>
<td>0.279-0.803</td>
</tr>
<tr>
<td>PUMA</td>
<td>0.514</td>
<td>0.304-0.868</td>
</tr>
<tr>
<td>SOX4</td>
<td>0.626</td>
<td>0.373-1.049</td>
</tr>
<tr>
<td>6-biomarker</td>
<td>0.273</td>
<td>0.152-0.490</td>
</tr>
</tbody>
</table>
Table 6.5 Multivariate Cox regression analysis of 6-biomarker index score on 5-year patient survival of 118 melanomas

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall survival</th>
<th></th>
<th>Disease-specific survival</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR (95% CI)</td>
<td>P value</td>
<td>RR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Age</td>
<td>0.574 (0.337-0.979)</td>
<td>0.042</td>
<td>0.838 (0.457-1.535)</td>
<td>0.567</td>
</tr>
<tr>
<td>Gender</td>
<td>1.111 (0.635-1.943)</td>
<td>0.713</td>
<td>0.976 (0.523-1.822)</td>
<td>0.940</td>
</tr>
<tr>
<td>6-biomarker²</td>
<td>0.237 (0.125-0.453)</td>
<td>1×10⁻⁵</td>
<td>0.208 (0.105-0.414)</td>
<td>7×10⁻⁶</td>
</tr>
</tbody>
</table>

1 Coding of variables: Age was coded as 1, ≤59 years; and 2, >59 years. Gender was coded as 1, female; and 2, male. Score was coded as 1, low score (0-16); and 2, high score (17-48).

2 6-biomarker: Score represented 6-biomarker combined index score.
Figure 6.1 Representative images of immunostaining for 10 biomarkers. Positive and negative staining images are paired.
Figure 6.2  Correlations between 6-biomarker index score and clinicopathological parameters of primary melanoma patients. (A) The 6-biomarker index score is significantly lower in melanomas >4.0 mm thick than tumors ≤4.0 mm thick ($P = 0.008$, Mann-Whitney test). (B) Combined 6-biomarker score is significantly lower in primary melanomas located at sun-exposed sites (head and neck) than sun-protected positions ($P = 0.030$, Mann-Whitney test). (C) 6-biomarker system showed higher score in superficial spread (SS) primary melanomas than other histological subtypes ($P = 0.003$, Mann-Whitney test).
Figure 6.3 Correlation between 6-biomarker index score and 5-year survival of melanoma patients. High combined 6-biomarker index score is correlated with better overall 5-year patient survival (A) and disease-specific 5-year survival (B) (n=118; P < 0.001 for both, log-rank test).
6.4 Discussion

Previous studies have shown that the risk factors for malignant melanoma include 4 categories: genetic factors, previous melanoma, environmental factors, and immunosuppression, among which, the first 2 factors are believed to be the strongest factors (Miller and Mihm, 2006). We compared the expression of 10 biomarkers using TMA and immunohistochemistry staining between melanomas with and without metastasis. We found the expression of 6 biomarkers was significantly changed between AJCC I-II and AJCC III-IV stages of melanoma, although the effect was limited for individual biomarkers. Then we investigated the effect of each biomarker and combined multi-biomarker system in melanoma metastasis and prognosis. We found that the 6-biomarker combination showed closer correlation with melanoma metastasis and provided a better prognostic accuracy than any individual biomarker. To our knowledge, this is the first study to explore the impact of this 6-biomarker system on melanoma prognosis.

In this study, we found that the expressions of Bim, BRMS1, ING4, NQO1, PUMA, and SOX4 were all significantly decreased in those melanoma patients with metastasis (AJCC III-IV) compared with those without metastasis (AJCC I-II) (Table 6.3). Bim, as a member of BH-3 only proteins, is able to interact with all the pro-survival Bcl-2 members, and thus serves as a key factor in apoptosis regulation (Chen et al., 2005a). In melanoma, MAPK pathway was reported to be constitutively activated and Bim can be phosphorylated by ERK 1 and 2 kinases, and subsequently undergo degradation via the proteasome pathway (Smalley and Eisen, 2000; Luciano et al., 2003; Filipenko et al., 2005). These data suggested that loss of Bim was critical for the pro-survival effect of MAPK and thus promoted the progression of melanoma. BRMS1 was first reported as a suppressor of metastasis in breast cancer (Seraj et al., 2000b), and then in melanoma (Shevde et al., 2002) and ovarian cancer (Zhang et al., 2006). Several metastasis-related
genes were reported to be regulated by BRMS1, including epidermal growth factor receptor (EGFR) (Vaidya et al., 2008), osteopontin (OPN) (Samant et al., 2007; Hedley et al., 2008), CXC chemokine receptor 4 (CXCR4) (Yang et al., 2008), as well as microRNA-146 (Hurst et al., 2009). We previously reported that ING4 inhibited melanoma cell migration and invasion (Li et al., 2008a). ING4 was also known to promote apoptosis of A549 lung cancer cell (Li et al., 2009b) and RKO colorectal cancer cells (Shiseki et al., 2003), as well as suppress gliomas tumor growth and angiogenesis (Garkavtsev et al., 2004), all of which were prerequisites for tumor metastasis. NQO1 was demonstrated to enhance apoptosis induced by beta-lapachone treatment in prostate cancer cells (Planchon et al., 2001), while NQO1 null mice showed less p53 induction and decreased apoptosis, and thus resulted in increased sensitivity to chemical-induced skin carcinogenesis (Iskander et al., 2005). PUMA serves as a key factor in apoptosis regulation (Yu and Zhang, 2008). The loss of PUMA was linked to deficient apoptosis and uncontrolled tumor cell growth, which may finally contribute to the metastasis. We recently found that SOX4 inhibited melanoma cell migration through RhoA-ROCK pathway (unpublished data). SOX4 was also required to activate p53 by stabilizing p53 and enhancing p53 acetylation under DNA damage stress, suggesting its role in DNA damage response (Pan et al., 2009). The function of these six biomarkers in inducing apoptosis and inhibiting cell migration and invasion explains the correlation between the 6-biomarker index score and tumor thickness.

We also found that low 6-biomarker index score was correlated with sun-exposed tumor site of primary melanomas. This could be explained by the functions of these biomarkers in apoptosis and DNA repair. Decreased expression of Bim, ING4, NQO1 and PUMA could result in reduced apoptosis (Planchon et al., 2001; Shiseki et al., 2003; Chen et al., 2005a; Yu and Zhang, 2008; Li et al., 2009b), thus leading to inability to remove
severe DNA damage caused by UV. Furthermore, we have previously shown that NQO1 can inhibit the degradation of p33ING1b which plays an important role in repair of UV-damaged DNA (Cheung et al., 2001; Kuo et al., 2007; Garate et al., 2008; Li et al., 2009a). Therefore, reduced NQO1 may lead to faster degradation of p33ING1b, slower repair of UV-damaged DNA, genomic instability and skin cancer eventually. The reason for the correlation between higher 6-biomarker index score with superficial spreading melanoma is not yet known.

Statistical analysis demonstrated that these 6 biomarkers were all related to melanoma metastasis, although with limited correlation coefficient individually. The combination of 6 biomarkers together provided more accurate prediction of metastasis with the correlation coefficient R improved to 0.5, which may be attributed to the fact that these 6 biomarkers function to inhibit metastasis through different aspects, including inducing apoptosis, suppressing cell migration, invasion and angiogenesis, as well as promoting DNA repair. Thus the combination of these 6 biomarkers demonstrated a significant improvement to the predictive accuracy for metastasis. This 6-biomarker index score also showed enhanced correlation with better melanoma patient survival compared with any single biomarker using univariate Cox regression analysis (Table 6.4). Furthermore, multivariate Cox regression analysis revealed that this 6-biomarker index score is an independent prognostic factor for both overall and disease-specific 5-year survival of melanoma patients (Table 6.5).

Collectively, our results demonstrated that the 6-biomarker system is of great value to achieve stronger predictive capability for melanoma metastasis and patient survival. Tests of these biomarkers in clinical practice could provide important information for both prognosis and development of novel therapeutic targets for human melanoma.
7 General Conclusions

7.1 Summary of result and implications

Melanoma is the most lethal form of skin cancer. It attracts huge amount of attention because of its rapid increase in incidence (Dauda and Shehu, 2005; Thompson et al., 2005) and the ability of metastatic melanoma to resist all conventional chemo-, radio-, or immunotherapy (Grossman and Altieri, 2001; Ballo and Ang, 2003; Hersey, 2003; Soengas and Lowe, 2003; Tsao and Sober, 2005). Therefore, advances in the prognostic assessment and development of novel target for melanoma therapy are essential to conquer this notorious cancer eventually. ING family of tumor suppressors are down-regulated in various types of cancer, including melanoma, and participated in regulating extensive cellular functions, such as cell growth, apoptosis, DNA repair, and invasion (Campos et al., 2004a; Gong et al., 2005; Li et al., 2009a). In this thesis, I demonstrate that ING4 expression is significantly decreased in malignant melanoma, and this reduced ING4 expression is correlated with melanoma tumor thickness, ulceration and worse patient survival (Li et al., 2008a). These data support ING4 as a tumor suppressor in melanoma, and suggest that examination of ING4 expression in melanoma biopsies is valuable for melanoma prognosis.

ING4 was also shown to inhibit melanoma cell migration, cell invasion (Li et al., 2008a) and negatively regulate melanoma angiogenesis (Li and Li, 2010b) in this thesis. This observation at least partially explains the correlation between ING4 deregulation and melanoma progression, tumor thickness, and worse patient survival. Both invasive growth and sustained angiogenesis are believed to be the hallmarks of cancer (Hanahan and Weinberg, 2000). Malignant tumor cells need these acquired capabilities to grow at the primary site as well as to invade the adjacent tissue and metastasize to distant organs. Melanoma is a less common form of skin cancer, but the incidence of melanoma was
increasing rapidly during the past decades. Metastasis is believed to be the major reason for melanoma patient death and cell invasion and angiogenesis are prerequisites for melanoma metastasis. Therefore, extensive studies were performed to explore the potential targets which can regulate melanoma cell invasion, angiogenesis and in turn metastasis. In chapter 3, I showed that ING4 overexpression inhibits melanoma cell migration through decreasing RhoA activity and inhibiting stress fiber formation. Data presented here also indicates that ING4 suppresses MMP-2 and MMP-9 activity to retain melanoma cell from invasion. These functions of ING4 may be due to the inhibition of NF-κB pathway. ING4 was reported as a negative regulator of NF-κB activity (Garkavtsev et al., 2004; Coles et al., 2010), and our group previously showed that NF-κB promotes cell migration through increasing RhoA activity and stress fiber formation (Gao et al., 2006). In this thesis I further revealed that ING4 inhibits melanoma angiogenesis also through suppressing NF-κB activity and IL-6 expression (Li and Li, 2010b), which is consistent with the recent finding that ING4 negatively regulates NF-κB and downregulates its target gene IL-6 (Coles et al., 2010). All these functions of ING4 found in this study support the tumor suppressor role of ING4 in melanoma and contribute to the better understanding of the mechanisms through which ING4 exerts its tumor suppressive functions.

Data presented here also showed that ING4 is inducible by BRMS1 (Li and Li, 2010b), a metastasis suppressor first identified in breast cancer (Seraj et al., 2000b) and then extended to other cancers including melanoma (Shevde et al., 2002). I found the expression of ING4 and BRMS1 exhibit the similar trend in different melanoma cell lines (Li and Li, 2010b). TMA data indicated that BRMS1 expression is decreased in metastatic melanoma and significantly correlated with better patient survival. Further in vivo and in vitro experiments showed that BRMS1 also inhibits melanoma angiogenesis through NF-κB/IL-6 pathway (Li et al., 2010a). Our data finally showed that BRMS1 is an upstream
regulator of ING4 and IL-6 is a downstream target in ING4 mediated melanoma angiogenesis. These data provided novel information to the previous knowledge of BRMS1 functions. First of all, this is the first study performed to investigate the expression of BRMS1 in cohort of melanoma patients and the data presented BRMS1 as an independent prognostic marker for melanoma patients. Furthermore, BRMS1 also showed prognostic value for those late stage of melanoma, including metastatic melanoma and primary melanoma with highly metastatic potential (Li et al., 2010a). This is important because so far very few biomarkers have been demonstrated as prognostic marker in those late stage melanoma patients. Second, we for the first time showed that BRMS1 inhibits melanoma angiogenesis in vitro and in vivo (Li et al., 2010a). BRMS1 was identified as metastasis suppressor in various cancer, but the mechanisms through which BRMS1 suppress metastasis was not clear. Our data here offered a rational explanation that BRMS1 may inhibit melanoma metastasis through suppressing angiogenesis, as angiogenesis is a critical component of metastasis process. Moreover, this finding may be extended to other types of cancer because angiogenesis is widely required by many solid cancers to grow and metastasize. Of course, more experiments are needed to further elucidate the pathway involved in BRMS1-inhibited melanoma angiogenesis and the similar effect of BRMS1 in other cancer models. Third, we here for the first time revealed that BRMS1 induces ING4 expression (Li and Li, 2010b). This finding may lead us to better understanding of the whole pathway involved in ING4 and BRMS1 functions.

Finally in this study, we evaluated the multiple biomarker system in melanoma prognosis and obtained a 6-biomarker combination offering the better predictive capability for melanoma patient outcome (Li et al., 2010c). Recently, a group of scientists from University of Toronto and Queen’s University developed a microchip which can measure biomolecules using biopsies from prostate cancer in 30 min, providing a promising way to
diagnose cancer fast and accurately (Fang et al., 2009). One of the prerequisites for this technique is that we have to determine the most valuable biomarkers. This study is such an important step showing that a 6-biomarker system provides better prognostic value than any individual one. The test of this multi-biomarker system may help the pathologists and clinicians to predict the outcome of melanoma patients accurately, especially for those difficult cases in which the morphological variations are not typical at early stage of progression. Of course further tests are needed to confirm the significance of this system in clinical practice.

### 7.2 Limitations of this study and future directions

Our data showed that ING4 expression is significantly decreased in malignant melanoma, but the mechanism underlying aberrant ING4 expression in melanoma remains to be elucidated. The possible explanations include deletion or mutation at chromosome 12p12-13, which includes the *ING4* gene (Gunduz et al., 2005), or mRNA variant splicing (Unoki et al., 2006). Further experiments including genomic DNA and cDNA sequencing in paired normal and tumor biopsies, or methylation specific PCR may help answer this question. Another mechanism responsible for loss of ING4 in melanoma may be the reduced BRMS1 expression in melanoma revealed in this study. Our data showed that BRMS1 knockdown decreases ING4 expression at both mRNA and protein level. This finding may reflect the real situation in melanoma development, as our TMA data demonstrated that in melanoma patients with strong ING4 expression, the percentage of strong BRMS1 expression is also significantly higher than the patients with weak ING4 staining (Li and Li, 2010b). The finding that BRMS1 modulates ING4 expression helps describe a pathway underlying ING4 tumor suppressive functions, although more experiments are required to determine the detail of this regulation, such as ChIP and promoter activity assays.
Our data reported that BRMS1 expression is significantly decreased in metastatic melanoma, which also deserves more experimental studies to discover the underlying mechanisms. It was reported that reduced BRMS1 expression may be attributed the frequent loss of chromosome 11q, that includes the \textit{BRMS1} gene. (Welch and Goldberg, 1997; Meehan and Welch, 2003; Zainabadi et al., 2005). Another possible explanation is the hypermethylation of CpG island on BRMS1 promoter, and this notion was supported by the finding that methylation status of BRMS1 promoter is correlated with loss of BRMS1 protein in the same tissue (Metge et al., 2008). Further experiments are required to test the sequence of genomic DNA and cDNA of \textit{BRMS1} gene with melanoma biopies and cell lines, and a methylation specific PCR will help to clarify the epigenetic modification of \textit{BRMS1} in melanoma.

The inhibitory effects of ING4 on melanoma cell migration, invasion and angiogenesis were achieved through inhibiting NF-\kappa B pathway in this study, but the details of how ING4 suppresses NF-\kappa B activity still remains unclear. So far there are three different theories: 1) ING4 physically binds to p65 subunit of NF-\kappa B and inhibits its function (Garkavtsev et al., 2004); 2) ING4 and NF-\kappa B bind to the promoter of NF-\kappa B downstream gene simultaneously and suppress the transcription (Nozell et al., 2008); and 3) ING4 promotes the expression I\kappa B and thus inhibits the activation of NF-\kappa B (Coles et al., 2010). Although these theories are connected at some points, they are still quite different. Further experiments are needed to clarify the effect of ING4 on I\kappa B expression and phosphorylation, or to explore the binding site of ING4 to NF-\kappa B, or its downstream target promoter. ChIP assay, luciferase assay as well as qRT-PCR and western blot will be performed to answer these questions.

Collectively, this study demonstrated that ING4 functions as a tumor suppressor in human melanoma, through regulating melanoma cell migration, invasion as well as
angiogenesis. Our data implied that ING4 may serve as an ideal prognostic marker and restoration of ING4 in melanoma patients may rescue its tumor suppressive effects and thus contribute to develop novel strategy for melanoma treatment.
Bibliography


oncoprotein is a common feature of a wide spectrum of human malignancies. *Oncogene* 6, 1699-1703.


