Role of Sox4 Transcription Factor in Human Cutaneous Melanoma

by

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Abstract

Cutanous melanoma is an aggressive malignancy with very few effective treatment strategies in the early stages and virtually no successful cure in the late stages. So far many aspects of biology of melanoma, especially mechanisms responsible for its metastasis have remained undiscovered. The SRY-related HMG box4 (Sox4) protein is aberrantly expressed in several types of tumors. In this study we investigated the role of Sox4 in human cutaneous melanoma. We hypothesized that expression of this protein is changed during melanoma progression with functional consequences on progression of melanoma. We revealed that Sox4 expression is reduced in metastatic melanomas and this loss of expression correlates with poorer patients survival. We found that Sox4 expression is required for suppression of melanoma cell migration and invasion. We determined that Sox4 uses at least two distinct pathways to suppresses melanoma cell migration and invasion. First, through binding to the regulatory regions and inhibiting the transcription of NF-κB p50. Secondly, it also regulates the miRNA biogenesis pathway at least partially through upregulation of the pre-miRNA processor, Dicer. Moreover, we showed that expression of Sox4 inversely correlates with that of NF-κB p50 in melanoma biopsies but positively correlates with Dicer expression which further supports our *in vitro* observations. We also revealed that expression of Dicer, similar to its upstream regulator Sox4, decreases in metastatic melanoma and this reduced expression inversely correlates with patient survival. In addition to Dicer, we also found that expression of the pre-miRNA processing enzyme Drosha is reduced in early stages of melanomagenesis. Dicer and Drosha demonstrate different expression patterns which imply differential regulatory mechanisms. Nevertheless, samples that lost expression of both Dicer and Drosha represented worse survival outcome in contrast to those

with positive expression of both markers. Finally, we revealed that the subcellular localization of Dicer and Drosha may be deregulated in melanocytic lesions and possibly has relevance to the biology of melanoma.

The data presented in this thesis elucidated a hitherto unknown mechanism responsible for suppression of metastasis which is malfunctioned in melanoma. A better understanding of this pathway may help toward treatment or prevention of metastatic melanoma.

Preface

Contributions

1. A version of chapter 1 has been accepted for publication in Cellular and Molecular Life Sciences. [Jafarnejad SM], Ardekani GS, M. Ghaffari and Li G. Pleiotropic function of SRY-related HMG box transcription factor 4 in regulation of tumorigenesis. Dr. Li supplied all the facilities and contributed to the overall outline of the manuscript and review of the content. Dr. G.S. Ardekani and M. Ghaffari participated in the literature review and writing of the section "Sox4" and "concluding remarks". I performed the literature review, design of the outline and prepared the manuscript
2. A version of chapter 3 has been published in American Journal of Pathology. [Jafarnejad SM], Wani AA, Martinka M, and Li G. Prognostic significance of Sox4 expression in human cutaneous melanoma and its role in cell migration and invasion. Dr. G. Li provided all the required facilities and materials and contributed to the design of experiments. Dr. A Wani performed the RT-PCR experiments. Dr. M. Martinka assisted with scoring the immunohistochemistry staining of TMA slides. I contributed to the design, performed all other presented experiments and prepared the manuscript.
3. A version of chapter 4 has been accepted for publication in Oncogene. [Jafarnejad SM], Ardekani GS, Ghaffari M, Martinka M and Li G. Sox4-mediated Dicer expression is critical for suppression of melanoma cell invasion. Dr. G. Li provided all the required facilities and materials and contributed to the design of experiments. Dr. G.S. Ardekani performed the western blots in Figures 4.2, M. Ghaffari performed the IPA analysis. Dr. M. Martinka assisted with scoring the immunohistochemistry staining of TMA slides. I contributed to the design, performed other presented experiments and prepared the manuscript.

4. A version of chapter 5 has been accepted for publication in Pigment Cell & Melanoma Research. [Jafarnejad SM], Sjoestroem C, Ardekani GS, Martinka M and Li G. Reduced expression of nuclear Dicer correlates with melanoma progression. Dr. G. Li provided all the required facilities and materials and contributed to the design of experiments. C. Sjoestroem performed The IHC staining in Figure 5.6. Dr. G.S. Ardekani participated in TMA data analysis. Dr. M. Martinka assisted with scoring the immunohistochemistry staining of TMA slides. I contributed to the design, performed other presented experiments and prepared the manuscript.
5. A version of chapter 6 has been accepted for publication in Modern Pathology. [Jafarnejad SM], Sjoestroem C, Martinka M and Li G. Expression of the RNase III enzyme Drosha is reduced during progression of human cutaneous melanoma. Dr. G. Li provided all the required facilities and materials and contributed to the design of experiments. C. Sjoestroem performed the IHC staining in Figure 6.1. Dr. M. Martinka assisted with scoring the immunohistochemistry staining of TMA slides. I contributed to the design, performed other presented experiments and prepared the manuscript.

List of publications

1. Jafarnejad SM, Ardekani GS, Ghaffari M and Li G. Pleiotropic function of SRY-related HMG box transcription factor 4 in regulation of tumorigenesis. Cellular and Molecular Life Sciences. Manuscript accepted for publication. This work is located in chapter 1.
2. Jafarnejad SM, Wani AA, Martinka M, Li G. (2010) Prognostic significance of Sox4 expression in human cutaneous melanoma and its role in cell migration and invasion. Am J Pathol. 177(6):2741-52. This work is located in chapter 3.

3. Jafarnejad SM, Ardekani GS, Ghaffari M, Martinka M, Li G. (2012) Sox4-mediated Dicer expression is critical for suppression of melanoma cell invasion. Oncogene. Manuscript accepted for publication. This work is located in chapter 4.
4. Jafarnejad SM, Sjoestroem C, Ardekani GS, Martinka M and Li G. Reduced expression of nuclear Dicer correlates with melanoma progression. Pigment Cell & Melanoma Research. Manuscript accepted for publication. This work is located in chapter 5.
5. Jafarnejad SM, Sjoestroem C, Martinka M and Li G. Expression of the RNase III enzyme Drosha is reduced during progression of human cutaneous melanoma. Modern Pathology Manuscript accepted for publication. 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 (certificate number is H09-01321).

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List of abbreviations

Abbreviation	Definition
ACC	Adenoid Cystic Carcinoma
Ago	Argonaut
AJCC	American Joint Committee on Cancer
AKT	Protein Kinase B-α
Ap-1	Activator protein-1
Apaf-1	Apoptotic protease activating factor-1
ARF	Alternative Reading Frame
Arp2/3	Actin-related protein 2/3
Bax	Bcl2- associateed X protein
Bcl2	B-cell lymphoma 2
BNIP3	BCL2/adenovirus E1B 19 kDa protein-Interacting Protein 3
BRAF	Ras Associated Factor B
BRCA1	BReast CAncer 1
BSA	Bovine Serum Albumin
C19MC	Chromosome 19 MicroRNA Cluster
cAMP	cyclic Adenosine Monophosphate
CD56	Cluster of Differentiation 56
Cdc42	Cell division control protein 42
CDK	Cyclin Dependent Kinase
CDKN1B	Cyclin-Dependent Kinase Inhibitor 1B
cDNA	complementary DNA
CED-3	C. elegans cell Death gene-3
ChIP	Chromatin ImmunoPrecipitation
c-Myc	cellular Myelocytomatosis viral oncogene
CREB	cAMP Responsive Element Binding protein
CtBP2	C-terminal-Binding Protein 2
DCL1	Dicer-Like protein 1
DGCR8	DiGeorge Syndrome Critical Region 8
DMEM	Dulbecco's Modified Eagle Medium
DN	Dysplastic Nevi
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dsRBD	double-strand RNA Binding Domain

DUSP4	Dual-Specificity Phosphatase 4
ECM	Extra-Cellular Matrix
EDTA	EthyleneDiamineTetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
eIF	eukaryotic Translation Initiation Factor
ERG	ETS-Related Gene
ERK	Extracellular Regulated Kinase
ETS1	Erythroblastosis virus E26 oncogene homolog 1
ETV1	ETS Translocation variant 1
F-actin	Filamentous- actin
FISH	Fluorescence In Situ Hybridization
FOS	FBJ Osteosarcoma Oncogene Homolog
GEO	Gene Expression Omnibus
GPCR	G Protein–Coupled Receptor
GTP	Guanosine Triphosphate
H&E	Hematoxylin and Eosin
H_2O_2	Hydrogen Peroxide
HDAC1	Histone Deacetylases 1
HER-2	Human Epidermal Growth Factor Receptor-2
HIF-1	Hypoxia Inducible Factor-1
HMG	High Mobility Group
hMSCs	human Mesenchymal Stem Cells
IFN	Interferon
IKK	IκB Kinase
ING4	Inhibitor of Growth 4
INK4a	Cylin-dependent kinase inhibitor 2A
IκB	Inhibitor of κB
kb	kilobases
KD	knockdown
kDa	kilo Dalton
LEF	Lymphocyte Enhancer Factor
LOH	Loss of Heterozygosity
MAPK	Mitogen Activated Protein Kinase
MC1R	Melanocortin 1 Receptor
MDM2	Mouse Double Minute 2
MEK	Mitogen-activated protein kinase Kinase
miR*	Complementary strand of guide miRNA
miRNAs	microRNAs

MITF	Microphthalmia Transcription Factor
Mitrons	Intron-Derived miRNAs
MM	Metastatic Melanoma
MMP	matrix metalloproteinase
mRNA	messenger Ribonucleic Acid
MSH	Melanocytes-Stimulating Hormone
MT-MMPs	Membrane-Tethered MMPs
mTOR	Mammalian Target of Rapamycin
NER	Nucleotide Excision Repair
NF-κB	Nuclear Factor-κB
NN	Normal Nevi
nt	nucleotide
ORF	Open Reading Frame
p21	protein 21
p53	protein 53
p56lck	Lymphocyre-specific protein tyrosine Kinase
p63	protein p63
PACT	Protein Activator of PKR
PBS	Phosphate Buffered Saline
PBST	PBS containing 0.05% Tween-20
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PI3K	Phosphoinositide 3-Kinase
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PM	Primary Melanoma
Pol II	Polymerase II
Pre-miRNAs	Precursor miRNAs
Pri-miRNAs	Primary miRNAs
PTEN	Phosphatase and Tensin Homolog
PTH/PTHrP	Parathyroid Hormone /Parathyroid Hormone-related Protein
PUMA	p53 Upregulated Modulator of Apoptosis
PVDF	Polyvinylidene Difluoride
qPCR	quantitative PCR
Rac	GTPase-activating protein
Ran-GTPase	Ras-Related Nuclear protein – GTP phosphatase
RB	Retinoblastoma
rDNA	ribosomal DNA
RelA	Reticuloendotheliosis viral oncogene homolog A

RGP	Radial Growth Phase
RHD	Rel Homology Domain
Rho	Ras homolog
RIPA	Radio-Immunoprecipitation Assay
RISC	RNA-Induced Silencing Complex
RLC	RISC-Loading Complex
RNAse	Ribonuclease
RNASEN	Ribonuclease III, Nuclear
ROCK	RHO-associated serine/threonine kinase
rRNA	ribosomal RNA
SDS-PAGE	Sodium Dodecyl Sulfate -Polyacrylamide Gels Electrophoresis
Sfpi1	Spleen focus forming virus proviral integration oncogene
siRNA	small interfering-RNA
Sox	SRY-related HMG box
Sp-1	Specificity protein 1
SRB	Sulforhodamine B
SRY	Sex-determining Region Y
TCF	T Cell-specific Factors
TGF-β	Transforming Growth Factor Beta
TMA	Tissue Microarray
TMPRSS2	Transmembrane Protease, Serine 2
TOPO2A	Topoisomerase IIA
TRBP	Tar RNA-Binding Protein
TrxR1	Thioredoxin Reductase 1
Ubc9	Ubiquitin-conjugating enzyme 9
UBF	Ubiquitous Binding Factor
uPA	urokinase-type Plasminogen Activator
UV	Ultraviolet
VASP	Vasodialator-Stimulated Phosphoprotein
VEGF	Vascular Endothelial Growth Factor
VGP	Vertical Growth Phase
WAF1	Wild type p53-Activated Fragment 1
XRCC1	X-ray Repair Complementing defective repair in Chinese hamster cells 1

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Dedication

I dedicate this work to the lovely memory of my sister and her everlasting smile.

Chapter 1. Introduction

1.1. Cutaneous melanoma

1.1.1. Biology of melanocytes and melanoma

Cutaneous melanoma is a type of skin malignancy arising from melanocytes, pigmented cells which are responsible for the skin colour (Gray-Schopfer et al, 2007). During embryonic development melanocytes originate from the neural crest progenitors (melanoblasts), which are transient multipotent cells arising by delamination from the developing dorsal neural tube (Ernfors, 2010). Melanoblasts migrate, proliferate and differentiate en route to their final destinations in the epidermis and hair bulbs of the skin, the uveal tract of the eye, the stria vascularis (the upper portion of the spiral ligament in ear), the vestibular organ and the endolymphatic sac of the ear and the leptomeninges of the brain (Lin and Fisher, 2007). In human, cutaneous melanocytes reside in the basal keratinocyte layer of the epidermis and in the hair follicles in which epidermal keratinocytes regulate their homeostasis (Tanimura et al, 2011).

Mature melanocytes synthesize and package melanin within lysosome-like organelles called melanosomes which are transferred or secreted via melanocytic dendrites to the surrounding keratinocytes of the epidermis and hair follicles. UV radiation stimulates keratinocytes to produce factors that induce production of melanin pigments from melanocytes which in turn are actively transferred to keratinocytes and distributed towards the surface of the epidermis or in hair shafts, therefore darken the skin color (Miyamura et al, 2007). Other than contribution to the skin pigmentation, the key role of melanin is to protect against UV damage especially to the DNA (Brenner and Hearing, 2008). Genetic or epigenetic changes in critical growth regulatory genes can allow the melanocytes to escape their tight regulation by

keratinocytes. Consequently, melanocytes can proliferate and spread in limited amount, leading to formation of a nevus (common mole). The control of growth in neval cells is disrupted but in general, nevi are benign and their growth potential is limited. As a consequence nevi rarely progress to cancer (Clark et al, 1984). These lesions histologically present an increased number of nested melanocytes along the basal layer (Miller and Mihm, 2006). A more advanced lesion is the dysplastic nevi developed from the pre-existing nevi or appearing in new locations. Dysplastic nevi are characterized by random proliferation of atypical nevomelanocytes, lentiginous or epithelioid-cell pattern, neovascularization, and enhanced inflammatory response (Clemente et al, 1991). Occasionally neval cells can progress to the radial growth-phase (RGP) melanoma, in which the cells acquire the ability to proliferate intra-epidermally. At this stage, cells tend to grow horizontally within epidermis and sometimes achieve local micro-invasion of the dermis (Piris and Mihm, 2009). Once the RGP melanoma cells acquire a more aggressive invasive phenotype, it is considered to have entered the vertical growth phase (VGP). VGP represents a more advanced stage in melanoma development, characterized by emergence of cells with metastatic potential and nodules or nests of cells invading the dermis (Piris and Mihm, 2009). Usually, RGPs have good prognosis and are curable by excision. However, once the lesion enters the VGP, it is considered to have metastatic potential with poor prognosis (Clark et al, 1984). Notably, not all cases of primary melanoma pass through each individual phase. Indeed, some cases progress directly to malignant melanoma without any visible earlier stages (Clark et al, 1984). The final stage of melanoma progression (i.e. metastatic melanoma) is characterized by spread of cell into other areas of the skin and/or other organs in which they will form secondary tumors (Miller and Mihm, 2006).

1.1.2. Staging and subtypes of melanoma

Currently a standard method adopted by American Joint Committee on Cancer (AJCC) is being used for staging of melanoma (Balch et al, 2001). This standard is also called TNM standard where TNM stand for extent of the tumor (T), whether cancer cells have spread to nearby lymph nodes (N), and whether distant metastasis (M) has occurred. In addition, tumor thickness and ulceration status is also included in this method for staging of primary melanoma (Balch et al, 2001). In brief, 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. Stage III consists of patients with lymph nodes metastasis but without distant metastasis, and patients with distant metastasis are classified as stage IV (Balch et al, 2001). The AJCC staging is very important for the prognostic purposes, and is inversely correlated with melanoma patient survival (Balch et al, 2009).

There are four main clinical subtypes of primary melanoma. 1) Superficial spreading melanoma is usually flat with an intra-epidermal component, particularly at the edges, and is associated with severe sunburn, especially at an early age. Superficial spreading melanoma is the most common form (about 70%) of melanomas (Gray-Schopfer et al, 2007). 2) Nodular melanoma which consists of raised nodules without a significant flat portion, accounts for 15% to 30% of all melanomas. 3) Acral lentiginous melanoma that is mainly found on the palms of the hands, the soles of the feet and in the nail bed accounts for 5% of melanomas. Interestingly, this type of melanoma is not associated with UV exposure and is more common in non-Caucasian populations (Gray-Schopfer et al, 2007). 4) Lentigo maligna comprises roughly 5% of

all melanomas and is generally flat in appearance and is associated with chronic sun exposure; therefore mainly occurs on sun-exposed regions in the elderly individuals.

1.1.3. Epidemiology of melanoma

In the last few decades the incidence of melanoma in western populations has been rising steadily. The incidence and mortality rates of melanoma have increased at annual rates of 2%–3% for the last 30 years (Perlis and Herlyn, 2004). Although melanoma accounts for only 4% of all types of skin cancer incidences, it is responsible for 80% of death from skin cancer. Accordingly, only 14% of patients with metastatic melanoma survive for five years (Miller and Mihm, 2006). According to Canadian Cancer Society, melanoma is the eights most common cancer in men and the seventh most common cancer in women in Canada (Canadian Cancer Statistics 2012). In 2012, an estimated 5800 (13 per 100000 capita) new cases of melanoma are expected to be diagnosed in Canada with 970 patients expected to die from the disease (Canadian Cancer Statistics 2012). The same study also predicted an estimated 910 cases of melanoma diagnosis in British Columbia in 2012.

Among the risk factors for melanoma are a family history, a previous melanoma, multiple benign or atypical nevi, immunosuppression, sun sensitivity and exposure to ultraviolet radiation (Miller and Mihm, 2006). Therefore, a combination of genetic predisposition and environmental factors contribute to the genesis of the melanoma. Asserting this notion are the findings that people with fair skin have higher risk of developing melanoma than their dark skin counterparts and among Caucasians, people with red hair, pale skin and a tendency to freckle are more likely to develop melanoma (Bliss et al, 1995; Ries et al, 2000).

1.1.4. Etiology of melanoma

Despite the higher prevalence of sporadic melanomas (Skolnick et al, 1994), studies delineating the genetic basis of familial melanomas provided much critical information regarding the molecular pathogenesis of this disease. Accordingly, mutations in several components of some prominent signaling pathways and regulatory mechanisms have been discovered in familial melanomas which predispose their member to this malignancy. So far several melanoma-predisposing genes with varying degree of penetrance have been identified based on the discovery of germline mutations in multiple-case families.

Cyclin-dependent kinase inhibitor (CDKN2A) is the best-characterized high-penetrance gene, whose loss or inactivating mutation occurs in 25% to 40% cases of familial melanoma (Borg et al, 2000; Kamb et al, 1994; Nobori et al, 1994). CDKN2A encodes two distinct melanoma predisposing genes, p16 (INK4a) and p14 (ARF) (Quelle et al, 1995). The INK4a protein binds to Cyclin Dependent Kinase 4/6 (CDK4/6) and inhibits its interaction with cyclin D, preventing the subsequent phosphorylation of the retinoblastoma protein (pRB), which is necessary for progression through the G1 cell cycle checkpoint, resulting in suppression of G1 phase of the cell cycle (Alcorta et al, 1996). ARF was initially found to function in a cyclin D-independent but p53 dependent manner (Quelle et al, 1995) (Kamijo et al, 1997) to induce a cell-cycle arrest and inhibit cell transformation. It was later demonstrated that ARF inhibits the ubiquitination of p53 by binding to the mouse double minute 2 (MDM2) and ARF-BP1/Mule ubiquitin ligases (Chen et al, 2005; Kamijo et al, 1998), thereby stabilizes this critical tumor suppressor protein and suppress cell cycle progression and tumorigenesis. Interestingly, genetic change in other component of these pathways, such as CDK4 and MDM2, were described in human melanomas without concurrent loss of INK4a and ARF, indicating that overexpression of

CDK4 and MDM2 may substitute for loss of INK4A and ARF function in a subset of melanomas (Muthusamy et al, 2006b).

CDK4 itself is another melanoma predisposing gene (Zuo et al, 1996), which encodes the cyclin-dependent kinase 4 protein and is a key regulator of the cell cycle progression. Activation of CDK4 allows the cell to bypass the G1/S cell-cycle checkpoint through phosphorylation and inhibition of the pocket protein, pRB that result in release and activation of the E2F transcription factor, leading to initiation of DNA replication and progression of cell cycle (Kato et al, 1993). Germ line CDK4 mutations have been reported in several cases of familial melanoma (Soufir et al, 1998). The majority of these mutations affect the critical Arg-24 amino acid that is essential for INK4a–CDK4 interaction. When this amino acid is mutated INK4a can no longer bind to and inactivate the CDK4 protein (Coleman et al, 1997) allowing uncontrolled cell proliferation.

Polymorphism in melanocortin 1 receptor (MC1R) is also reported to be associated with melanoma (Stratigos et al, 2006). *MC1R* is a melanoma susceptibility gene that encodes a seven transmembrane G protein–coupled receptor (GPCR) expressed on epidermal melanocytes. In normal melanocytes, binding of the melanocytes-stimulating hormone (α-MSH) to MC1R stimulates adenylyl-cyclase and leads to generation of cAMP, which in turn enhances transcription of microphthalmia transcription factor (MITF), tyrosinase, and other enzymes necessary for production of melanin through cAMP responsive element binding protein (CREB) (Schaffer and Bolognia, 2001).

It is believed that mutations of the Extracellular Regulated Kinase/Mitogen Activated Protein Kinase (ERK/MAPK) pathway are the most well-defined genetic events in melanoma. In this pathway, ligand-mediated activation of receptor tyrosine kinases triggers guanosine

triphosphate (GTP) loading of the Ras GTPase, which can then recruit Raf (Raf-1, B-Raf and A-Raf) kinases to the plasma membrane for activation. Raf kinases phosphorylate and activate the Modulator of Extracellular regulated Kinases (MEK1 and MEK2) that in turn phosphorylate ERK (ERK1 and ERK2) (Chang and Karin, 2001). Active ERKs would exert their functions through phosphorylation of downstream effectors such as fos, MITF, myc and Ap-1 (Yang et al, 2003). Mutations in *N-ras* are relatively prevalent events in earlier stages of melanocytic lesions, with 56% rates in congenital nevi (Papp et al, 1999). However, this rate declines in later stages of melanoma with 33% in primary melanomas, and 26% in metastatic melanomas (Demunter et al, 2001). Similarly, *BRAF* mutations have been detected in considerable subset of malignant melanomas (Davies et al, 2002), with a single base substitution, V600E, accounting for 80% of these mutations. Interestingly, like *N-ras*, mutations in *BRAF* were also found to be particularly prevalent in nevi (Pollock et al, 2003), indicating that activation of this pathway may be important for earlier stages of melanoma progression and additional changes are required for progression of melanoma toward later stages. This notion is further affirmed by the observations that although activation of the RAS signaling pathway is very common in melanoma, it does not occur in isolation and is usually associated with other alterations such as loss of PTEN expression (Dankort et al, 2009).

The PTEN protein is also involved in the Phosphoinositide 3-Kinase (PI3K) pathway that is frequently activated in melanoma. PI3K phosphorylates the 3-O-hydroxyl group of phosphoinositides producing the phosphatidylinositol-3,4,5-trisphosphate or PIP3, a critical second messenger which recruits multiple downstream mediators such as AKT for activation of growth, proliferation and survival signaling through activation of several prominent downstream cascades such as mTOR and MAPK pathways (Cantley, 2002). As a check and balance measure,

the amount of PIP3 is also negatively regulated through dephosphorylation by PTEN phosphatase (Cantley, 2002). PTEN was initially identified as a candidate tumor suppressor after its positional cloning from a region of small arm of chromosome 10, known to exhibit loss in a wide range of tumors (Steck et al, 1997). Loss of PTEN expression due to chromosomal deletion has also been observed in melanoma (Guldberg et al, 1997). In addition, mutations of *PTEN* have been detected in a variety of human cancer including melanoma (Guldberg et al, 1997; Teng et al, 1997). Consistent with a tumor suppressor role for PTEN, artificial overexpression of PTEN in melanoma cells lacking PTEN protein suppressed tumor development in mice (Stahl et al, 2003), evidently through suppression of AKT activity, with consequent upregulation of the apoptotic pathway of melanoma cells. Mutation, overexpression or alteration in activity of other components of the PI3K pathway have also been frequently observed in melanoma (Meier et al, 2005).

The Nuclear Factor κB (NF-κB) is another molecular pathway whose deregulation has been frequently observed in melanoma. In mammals, there are five NF-κB subunits RelA/p65, RelB, p105/p50, c-Rel, and p100/p52 and each subunit contains a Rel homology domain (RHD), in their N-termini mediating DNA-binding and dimerization (Perkins, 2000). Typically, in non-stimulated normal cells, NF-κB subunits are held in an inactive cytoplasmic form bound to a member of IκB protein family, IκB α, β or ε. In response to stimulation, such as exposure to the inflammatory cytokine TNFα, signalling pathways are initiated that result in the activation of IκB kinase (IKK) complex. IKK complex phosphorylates members of the IκB family (α, β or ε), resulting in their ubiquitination and proteasomal degradation (Ghosh et al, 1998). Upon degradation of IKB, NF-κB translocates to the nucleus where it can regulate expression of its target gene (Ghosh et al, 1998). NF-κB pathway is able to induce several oncogenic mechanisms,

and is believed to be constitutively activated in multiple types of cancer cells, including melanoma (Davis et al, 2001; Gilmore, 2003; Suh et al, 2002; Yang and Richmond, 2001). A variety of mechanisms have been found by which NF-κB pathway is uncoupled from its normal modes of regulation, thereby promoting cancer development (Karin et al, 2002). Previous studies have shown that the expression and DNA binding ability of NF-κB is elevated in melanoma cells relative to normal melanocytes (Dhawan and Richmond, 2002; McNulty et al, 2001) and the level of its inhibitor IκB is significantly lower in metastatic melanoma biopsies (McNulty et al, 2004). Moreover, recently we reported that NF-κB p50 expression increased with melanoma progression and upregulation of NF-κB p50 enhanced melanoma cell migration (Gao et al, 2006) and angiogenesis (Karst et al, 2009; Wani et al, 2011).

1.1.5. Melanoma invasion and metastasis

Acquisition of invasive potential is the main transition in progression of benign melanocytic lesions to malignant melanoma. This invasive behaviour is normally associated with enhanced cell motility and altered extracellular matrix composition and structure (Haass et al, 2005). Different proteolytic enzyme systems, such as the plasminogen activator system, cystein proteinases and matrix metalloproteinases, have been identified in cutaneous melanoma progression (Frohlich, 2010). There is compelling evidence that melanoma cells produce increased amounts of matrix metalloproteinases such as MMP2 and MMP9 (Hofmann et al, 2003; Rotte et al, 2012), which are required for tumor growth and invasion by digesting the extracellular matrix surrounding the tumor tissue.

Among the genes whose expression is altered during progression of melanoma (Haqq et al, 2005), some are required for the escape of tumor cells from the primary tissue. Melanomas can induce angiogenesis and lymphangiogenesis as a venue for escape through secretion of factors

such as Interleukin-6 and VEGF (Rinderknecht and Detmar, 2008; Wani et al, 2011). Some other changes are required for melanoma cells to adapt to the environments other than epidermis which host the secondary tumors. For instance, reduced E-cadherin and increased N-cadherin expression enables cells to dissociate from epidermal keratinocytes and make interactions with stromal fibroblasts that facilitate survival outside the epidermis (Smalley et al, 2005). Interestingly, a major deregulation of genes involved at various steps of cell motility as well as invasion is observed in melanoma (Bittner et al, 2000) which could reflect activation of signaling cascades and transcriptional programs or post-transcriptional regulatory mechanisms that promote pro-invasive changes and escape of the melanoma cells from the original epidermal site either through enhanced activity of positive regulators or inactivation of negative regulators of cell motility and invasion.

1.2. Tumorigenesis and cancer

Cancer development is a multistep process characterized by genetic or epigenetic alterations that result in sustained proliferative signalling, evading cell death, resisting growth suppressor signals, enabling replicative immortality, sustained angiogenesis, genomic instability, reprogramming of energy metabolism, evading immune destruction and activating invasion and metastasis mechanisms, which characterize malignant growth (Hanahan and Weinberg, 2011). In normal tissues the functions of single cells are tightly controlled by means of surveillance and repair mechanisms directed by their genetic programming and environmental constraints, maintaining the tissue homeostasis (Chow et al, 2011; Jackson and Bartek, 2009). Nevertheless, due to endogenous errors or exogenous stimuli, on some occasions these mechanisms fail and result in activation of tumorigenic pathways or otherwise inactivation of the restraining measures. The output of these changes is reflected by progressive genetic alterations that drive

the transformation of normal cells into highly malignant derivatives. Progression of cancer is associated with several genetic or epigenetic changes in multiple genes. Each change will confer one or more new features to the cancer cell, enabling it to defy the internal and external constraints and continue in gradual increases in size, number, disorganization and malignancy (Vogelstein and Kinzler, 1993).

At least three important classes of genes play key roles in tumor initiation and progression: proto-oncogenes, tumor suppressor genes and DNA damage response and genetic stability genes; any alteration in these genes may lead to malfunctioning of the control mechanisms.

1.2.1. Oncogenes

Genes whose activation or overexpression could contribute to initiation or progression of cancer are called oncogenes. Activation or overexpression of oncogenes generally renders a growth advantage to the harbouring cells compared with their neighbours. Depending on the specific oncogene of interest, they can cause formation of new tumor from previously normal cells or promote invasion and metastasis of a hitherto benign tumor. To prove the oncogenic status of a gene in any particular type of cell, it should be demonstrated that experimental activation of the candidate gene *in vitro* cell culture or *in vivo* animal model enhances tumorigenicity or metastatic potential and more importantly the candidate oncogene is either overexpressed or activated in human cancer tissues.

Overexpression of oncogenes can be achieved in different ways. Gene amplification will result in presence of more than two copies of a gene in the normal diploid genome, resulting in enhanced expression of the encoded mRNA and, in most cases, protein. Overexpression of HER-2 in breast cancer is a well-documented example of such a mode of oncogenic activation (Meng

et al, 2004). Enhanced transcription of the coding gene, either through activating mutation in its regulatory sequences or constitutive activation of other genes involved in modulation of its transcription can also result in overexpression of an oncogene. Overexpression of matrix metalloproteinase (MMP)-9 by Sp-1, Ets-1 and Nuclear Factor-κB (NF-κB) is the example of such a mechanism (Jorda et al, 2005). Stabilization of the transcript or protein due to mutation in the coding sequences (Wei et al, 2005), altered physical interaction with other regulatory factors (Lee and Dutta, 2007) and post-translational modifications (Sears et al, 2000) are also among important mechanisms involved in upregulation of some oncogenes. On some occasions, upon chromosomal translocation due to genomic rearrangement, the DNA sequences which encode an oncogene (or in some cases part of) will merge with another gene that is highly expressed in the cells, creating a hybrid gene structure which encodes massive amount of the oncogene owing to the promoter activity of the recipient gene. Such genomic rearrangements often happen in chronic myeloid leukemia in which the ABL tyrosine kinase proto-oncogenes fuse to the break point cluster (BCR) gene, mediating its overexpression (Michor et al, 2005).

Enhanced expression of an oncogene could naturally result in increased cumulative activity; however other mechanisms can also augment activity of an oncogene without altering its expression status. For example, gain-of-function mutation could be a point mutation that enhances the function of a proto-oncogene. Valine to a glutamate mutation at codon 600 in BRAF kinase is a well-studied example of gain-of-function point mutation which enhances the enzymatic activity of BRAF protein in multiple types of malignancies (Davies et al, 2002; Wojciechowska and Lewinski, 2006). Chromosomal rearrangements may also result in creation of new hybrid onco-protein with higher constitutive activity or altered substrate specificity which enhances the activity of the oncogene without increased transcription of the coding gene

(Lacronique et al, 1997; Soda et al, 2007). Notably, due to activating nature of oncogenes, their mutations are dominant. Therefore, mutation in only one allele of these genes is sufficient to increase the tumorigenicity of the cells.

1.2.2. Tumor suppressor genes

Genes whose downregulation or loss of function contributes to promotion of malignancy are called tumor suppressor genes. This set of genes includes but is not limited to negative regulators of cell growth and proliferation such as cyclin dependent kinase inhibitors (e.g. p21/WAF1) and RB tumor suppressor protein (Zauberman et al, 1997), promoters of cell death such as PUMA (Nakano and Vousden, 2001) or suppressors of tumor cell migration, invasion, and metastasis such as p63 and ING4 (Barbieri et al, 2006; Li et al, 2008).

It is believed that tumors must contain two dysfunctional alleles of the tumor suppressor gene according to the "two-hit" hypothesis, which suggest that both alleles must be lost in order to form the permissive environment for formation or progression of cancer (Knudson, 2001). Therefore, classical tumor suppressor genes can be identified by the loss of both alleles in cancer cells such as RB1 in retinoblastoma (Scrable et al, 1990). However, such definition will exclude those genes that require two functional alleles to sufficiently suppress cancer and thus only one inactivated allele confers a selective advantage for tumor growth. One well-defined example of such genes, which are called haploinsufficient, is PTEN tumor suppressor, inactivation of one allele of which promotes progression of prostate cancer (Kwabi-Addo et al, 2001).

Similar to activation of oncogenes, inactivation of the wild-type tumor suppressor genes can occur due to a variety of events. For instance, loss of the whole chromosome or deletion of part of any given chromosome can remove the whole or a section of the coding sequence of a

tumor suppressor gene. Deletion of *CDKN1B* in metastatic prostate carcinoma (Kibel et al, 1999) is one example of this scenario. Otherwise, a single mutation in the regulatory or coding region of a tumor suppressor gene, such as *BRCA1* in breast cancer (Hashizume et al, 2001), can abrogate its expression or function. Deletion or single mutation in the wild-type allele in an individual who already carries another mutated or dysfunctional allele can cause a phenomenon called "loss of heterozygosity" (LOH) that will result in diminution of the tumor suppressor gene activity altogether.

Sometimes, a mutation does not inactivate a tumor suppressor gene or shut down its expression but instead converts its function in a fashion that gives a growth advantage to the tumor cells. Conversion of p53 tumor suppressor to an oncogene by a single mutation in some types of cancer (Iggo et al, 1990; Kern et al, 1992) is one well-investigated case of this category.

In many cases, there may not be a genetic change in the regulatory or coding sequences of any given tumor suppressor gene, but rather some epigenetic mechanisms that interferes with its expression or function. For instance, methylation of the gene promoter which suppresses its transcription (Mirmohammadsadegh et al, 2006), destruction of the mRNA or suppression of its translation by non-coding RNAs (Lou et al, 2010), enhanced proteasomal degradation rate (Wang et al, 2007), or abnormalities in other proteins that may interact with the tumor suppressor protein of interest (Schmitt et al, 1999) can all result in diminution of its activity, hence enhanced tumorigenesis. Aberrant subcellular localization is another mechanism that cancer cells use to achieve their goal of inactivating a tumor suppressor. For instance, breast cancers that contain the wild-type p53 protein may inactivate its tumor-suppressing activity by excluding it from the nucleus and sequestering this protein in the cytoplasm, away from its site of action in the cell

nucleus. Indeed, this mechanism may explain how some breast cancer tissues inactivate p53 function without mutation (Moll et al, 1992).

It should also be noted that attributing an oncogene and tumor suppressor function to a particular gene is context dependent. In other words, some genes function as an oncogene in certain types of tissues or stages of tumorigenesis while acting as a tumor suppressor in another type of tissue or tumorigenesis stage. For instance, in normal tissue the suppressor activities of TGF-β dominate, whereas during tumorigenesis, changes in TGF-β expression and cellular responses favor its oncogenic activities (Wakefield and Roberts, 2002).

1.2.3. DNA damage response and genetic stability genes

In addition to the conventional tumor suppressor genes whose products can inhibit formation or progression of tumors, there are other genes whose products do not directly suppress cancer development but instead their absence or malfunction can result in enhanced mutation rate of proto-oncogenes or tumor suppressor genes, thus leading to enhanced susceptibility totumorigenesis or progression of cancer. One prominent group of such genes includes those involved in DNA damage response and preservation of genome stability (Negrini et al, 2010). Individuals with mutations affecting these genes, products of which are responsible for detecting or repairing DNA damage, are especially prone to cancer. For instance, xeroderma pigmentosum is a rare disease, characterized by extreme sensitivity of the skin to sunlight. In individuals affected by this disease, the repair of ultraviolet (UV)-induced DNA damage is impaired due to mutations in genes involved in the DNA-damage response mechanism known as nucleotide excision repair (NER). Compared to normal individuals, xeroderma pigmentosum patients are 1000-time more likely to develop skin cancer in sun-exposed areas (van Steeg and

Kraemer, 1999). It is noteworthy that in these patients, the dysfunctional DNA repair pathway does not directly contribute to development of cancer but instead due to this malfunction, the environmentally (in this case UV) induced DNA damages in proto-oncogenes and/or tumor suppressor genes are not properly repaired which can result in their respective activation and inactivation, hence development of cancer (van Steeg and Kraemer, 1999).

1.2.4. MicoRNAs and cancer

In the last two decades, a great deal of attention has also been drawn to the extent which some previously unknown or less-known factors such as non-coding RNAs play in cancer. Numerous studies have indicated that noncoding RNAs play key roles in initiation and progression of cancer. MicroRNAs (miRNAs) are small, non-coding RNAs with important regulatory roles in almost all cellular functions implicated in cancer initiation and progression such as cell differentiation (Tay et al, 2008), regulation of cell cycle (Linsley et al, 2007), apoptosis (Cimmino et al, 2005), DNA repair (Lal et al, 2009), and cell motility (Korpal et al, 2008).

With some exceptions, the majority of miRNAs are transcribed by RNA polymerase II (Pol II) as evidenced by their polyadenylated and capped structures (Cai et al, 2004). Transcription of miRNAs is under the control of an intricate network of signalling pathways and transcription factors. Several lines of evidence indicate regulation of transcription of various miRNAs by tumor suppressors and oncogenes. For instance, the p53 tumor suppressor directly enhances transcription of miR-34 (He et al, 2007), while the c-Myc proto-oncogene binds to and activates the transcription of the miR-17-92 cluster (Dews et al, 2006).

The products of transcription are called primary miRNAs (pri-miRNAs), which could encompass several kilobases (kb) and include at least one stem-loop structure (Lee et al, 2002).

These pri-miRNAs are then cleaved in the nucleus by the so-called microprocessor complex which contains an RNAse III enzyme called Drosha (RNASEN) (Lee et al, 2003) working in collaboration with several accessory factors such as DGCR8 (DiGeorge Syndrome Critical Region 8), RNA helicases, etc (Gregory et al, 2004). The products of this cleavage step, called precursor miRNA (pre-miRNA) that are RNAs with ~ 70-nt, will keep the ~22-nt stem and terminal loop structure in addition to a 2 nt overhang at their 3′ end (Lee et al, 2003). Pre-miRNAs are then exported to the cytoplasm by a complex containing Exportin-5 and Ran guanosine triphosphate-dependent (Ran-GTPase) (Lund et al, 2004).

Once in cytoplasm, the RNase III Dicer cleaves off the loop of the pre-miRNA and generates the mature miRNA as a roughly 22-nt miRNA duplex with two nucleotides protruding as overhangs at each 3′ end (Lee et al, 2002). The mature miRNA then function by means of the RNA-Induced Silencing Complex (RISC) a large protein complex that include Argonaut (Ago) proteins as their core subunit (Hammond et al, 2000). It is noteworthy that prior to cleavage by Dicer, the RISC-loading complex (RLC) is generated to provide a platform for RISC assembly and cytoplasmic processing. RLC is composed of Dicer, Tar RNA-binding protein (TRBP), protein activator of PKR (PACT) and pre-miRNA (Wang et al, 2009a). Following production of the mature miRNA, RLC dissociates and the miRNA duplex is separated into the guide strand that will direct RISC toward the target mRNAs and the passenger strand (commonly referred to as, miR*) (Gregory et al, 2005). The guide or passenger strands will be distinguished based on thermodynamic stability of the two terminal base pairs. The guide strand is the one with less stable base pair at the 5′ (Khvorova et al, 2003).

It is worth mentioning that although the aforementioned mechanism for biogenesis of miRNAs is very common, it is not universal. There are some miRNAs whose biogenesis path

bypasses one or more of these steps. For instance, Pol-III instead of Pol-II is known to be responsible for transcription of miRNAs encoded by C19MC miRNA cluster (Borchert et al, 2006). Also, processing of pri-miRNAs by Drosha is not obligatory. Mitrons, the intron-derived miRNAs (such as mir-877) are released from their host transcripts through the common RNA splicing mechanism, instead of the nuclear micro-processor (Berezikov et al, 2007; Ruby et al, 2007). In addition, biogenesis of at least some miRNAs is independent of Dicer. Pri-miRNAs such as pri-miR-451 are processed by Drosha to produce pre-miRNA, which are then loaded into RISC and cleaved by Ago (Cheloufi et al, 2010).

miRNAs silence their target mRNAs by different mechanisms: target cleavage resulting in induction of degradation of target mRNA, induction of mRNA deadenylation and decay, inhibition of translational initiation via competition with eIF6 or eIF4E and suppression of translation-elongation by blocking the progression of protein synthesis by ribosoms (Filipowicz et al, 2008). In recent years some other mechanisms for inhibition of gene expression such as triggering heterochromatin formation and DNA methylation at the site of the target gene in the nucleus (Yu et al, 2008) and even enhancement of translation of target miRNAs (Orom et al, 2008) have been suggested by which miRNAs can affect expression of their target genes.

Aberrant expression and/or function of miRNAs are considered as frequent events in many diseases including cancer (Lu et al, 2005). In fact expression profile of miRNAs can be used to classify poorly differentiated tumours with a better accuracy than the messenger RNAs expression profiles (Lu et al, 2005). The growing list of miRNAs and types of cancer that they are aberrantly expressed in suggests that a large number of miRNAs are bona-fide tumor-suppressors or oncogenes (oncomiRs). Indeed several miRNAs have been shown to suppress (Kumar et al, 2008; Trang et al, 2011) or promote tumorigenesis (Gabriely et al, 2011; Ma et al,

2010) at different stages, indicating the enormous potential of these small molecules in regulation of cancer-related cellular functions. In addition, deregulated expression of some factors required for biogenesis and function miRNAs such as Dicer, Drosha and Argonauts have also been observed in different tumors (Adams et al, 2009; Merritt et al, 2008). The global deregulation of miRNA expression in multiple types of tumors also points toward processing defects in cancer affecting the general expression level of mature miRNAs (Faggad et al, 2010; Lu et al, 2005; Ozen et al, 2008).

The functional output of any miRNA depends on the expression profile of its target mRNAs in the cell. Therefore, any change in the expression profile of the target mRNAs, either in different cell types or in the same type of cell under different physiological or pathological conditions might affect the final effect of miRNAs on the cell behaviour. Inevitably, based on the nature of mRNAs which are targeted by any given miRNA, that miRNA can play a pro or anti-tumorigenic role in cancer. Accordingly, some miRNAs play tumor suppressive role in one type while having oncogenic function in another type of cancer. For instance, miRNA-211 expression was shown to promote colorectal cancer (Cai et al, 2012) but suppress melanoma invasion (Levy et al, 2010a). These observations highlight the intricate network of miRNAs and their versatile role in different cancers. They also imply the critical role which the factors that regulate the expression and function of these RNA species play in cancer biology.

1.2.5. Cancer invasion and metastasis

The neoplastic growth usually initiates with mutations which cause clonal expansion of the mutant cells (Sieber et al, 2005). Subsequent mutations will result in additional clonal expansion and tumor progression. In the case of epidermal carcinomas, unhindered progression of tumor may result in metastasis. Metastasis is a generic term for the process by which cancer cells leave

the primary tumour, disseminate through the blood vessels or lymphatic system and form secondary tumours at anatomically distant sites (Brooks et al, 2010). It is a serious clinical problem, which is often impossible to eradicate successfully and despite all the recent progress in understanding its mechanisms, metastasis remains the cause of 90% of deaths from solid tumors (Gupta and Massague, 2006). It is believed that metastases originate from a subpopulation of highly invasive cells that reside in a biologically heterogeneous primary tumor (Langley and Fidler, 2011). During the process of metastasis these invasive tumor cells detach from the extracellular matrix that they normally reside in and invade the surrounding tissue. This phenomenon requires the localized proteolysis at the tumor cell-basement membrane interface which signifies the transition from a benign carcinoma *in situ* to a malignant invasive tumor (Langley and Fidler, 2011). Expression and function of extracellular proteases such as matrix metalloproteinase (MMPs) is crucial for this process. Accordingly, MMP-mediated extracellular matrix (ECM) degradation has long been identified to lead to cancer cell invasion and metastasis (Kessenbrock et al, 2010). Similarly, cell motility and chemotaxis can make important contributions to this process (Kedrin et al, 2007). At this stage, cancer cells migrate toward and penetrate into a nearby blood or lymphatic vessels so as to gain access to the systemic circulation.

In the majority of solid tumors, cancer cells induce expansion of the existing vascular system or formation of new vessels through several modes such as recruitment of bone-marrow derived and/or vascular-wall-resident endothelial progenitor cells that differentiate into endothelial cell, vessel splitting, vascular mimicry (tumour vessels lined by tumour cells), or vessel co-option, in which tumour cells hijack the existing vasculature (Carmeliet and Jain, 2011).

Tumors employ a multitude of genes and molecular pathways to induce the neo-angiogenesis process. For instance, under hypoxic conditions, which is a common phenomenon in tumors with poor vascularity (Hockel and Vaupel, 2001), the Hypoxia Inducible Factor-1 (HIF-1) transcriptional complex becomes stabilised that in turn activates expression of genes coding for angiogenesis-inducing factors such as Vascular Endothelial Growth Factor (VEGF) (Buchler et al, 2003) and angiopoietin-2 (Pichiule et al, 2004). For a metastasis to happen, cancer cells must be able to survive in circulation and reach the distal microvascular beds by passive mechanical or active mechanisms, after which they need to once again penetrate the capillaries and extravasate into the surrounding parenchyma (Langley and Fidler, 2011). Tumour cells as well as their surrounding non-cancerous stromal cells secrete pro-inflammatory factors such as tumour necrosis factor alpha, and interleukin-8. These factors can bind to their receptors on the endothelial cells that linen the blood vessels, and on the tumour cells themselves, and stimulate production of adhesion molecules, such as integrins that facilitate their binding to the endothelial cells when in circulation (Avraamides et al, 2008). Once inside the tissue parenchyma, tumor cells establish an extensive niche that involves the surrounding stromal cells and extracellular matrix to promote their growth in the secondary site (Langley and Fidler, 2011).

In addition to the intrinsic features of cancer cells, extrinsic factors in the tumour microenvironment can also promote the metastasis of cancer cells (Kopfstein and Christofori, 2006). Several lines of evidence demonstrated that stromal cells, especially fibroblasts and infiltrating macrophages, enhance cancer cell motility and invasion by producing growth factors and cytokines (Goswami et al, 2005; Olumi et al, 1999). Moreover, fibroblasts and macrophages can produce MMPs which promote cancer cell motility by degrading the ECM (Hagemann et al, 2006; Poulsom et al, 1992).

1.2.6. Cancer cell migration

Since the process of metastasis includes relocation of cells from one site to another, many steps of the metastasis cascade require cell motility, which is driven by cycles of actin polymerization, cell adhesion and acto-myosin contraction (Olson and Sahai, 2009). The process of cell migration is essential for tissue development and homeostasis, including migration of neurons and melanoblasts in early embryos (Ernfors, 2010; Metin et al, 2008). It is a highly integrated, multi-step operation that requires orchestrated function of a plethora of proteins inside and on the surface of the cells. Beside the normal physiological situations, cell migration is also essential during pathological events, such as wound healing (Tremel et al, 2009). Tumor cells are believed to recruit or hijack these normal physiological and pathological processes and use them for metastatic spread.

Among other models of cell migration, a 5-step cell migration model has been suggested for single cell migration which comprises cell polarization and protrusion of the leading edge followed by attachment of the leading edge to the substrate, proteolytic degradation of tissue components that physically surround the cell, actomyosin contraction and forward sliding of the cell's rear (Lauffenburger and Horwitz, 1996). Acquisition of an asymmetric morphology with defined leading and trailing edges is required for an efficient migration by the cells. This phenomenon is achieved through the process of chemotaxis which enables the cells to detect a gradient in the concentration of the chemo-attractant (migration-promoting agent) and move toward it (Jin et al, 2008). The initial response of a cell to a chemo-attractant is to polarize and extend protrusions in the direction of migration. These protrusions can be large, broad lamellipodia or spike-like filopodia, which are usually driven by actin polymerization, stabilized by adhering to the extracellular matrix or adjacent cells via transmembrane receptors linked to

the actin cytoskeleton (Ridley et al, 2003). The gradient of the chemo-attractant also induces a polarized intracellular signalling cascade which orients protrusion of the leading edge, integrin-mediated adhesion to the underlying substrate, and contraction and detachment at the far end of the cell to orchestrate cell motility (Ridley et al, 2003).

The membrane protrusions, lamellipodia or filopodia, which endow the cells with the capacity to push the plasma membrane in the direction of chemo-attractant are formed by polarization of actin filaments (Ridley et al, 2003). It should be noted that the organization of filaments depends on the type of protrusion (i.e. in lamellipodia, the filaments form a branching network, while in filopodia they organize into parallel bundles) (Welch and Mullins, 2002). Reorganization of the actin cytoskeleton is the primary mechanism of cell motility which endows the cells with the flexibility to move around the neighboring cells and the ECM.

The Rho family of small GTPases such as Rho, Rac, and Cdc42 are the main regulators of actin reorganization (Raftopoulou and Hall, 2004). These factors can transmit the extracellular chemotactic signals to downstream effectors by functioning as molecular switches. They are in the "on" state when bound to GTP. However, their intrinsic phosphatase activity hydrolyzes the GTP to GDP, turning the protein "off" (Heasman and Ridley, 2008). This process is accelerated by the interaction with GTPase Activating Proteins (GAPs), whereas interaction with Guanine-nucleotide Exchange Factors (GEFs) facilitates the exchange of GDP to GTP those turning the protein "on" (Heasman and Ridley, 2008). The downstream targets of Rho proteins include several kinases such as the Rho-associated coiled-coil kinase1/2 (ROCK) and the p21-activated kinase (PAK), as well as several actin cytoskeleton related factors such as WASp proteins, and other scaffolding molecules which have direct effects on actin cytoskeleton rearrangements,

hence are important to cell motility (Spiering and Hodgson, 2011). Therefore, inhibition of Rho family small GTPase signaling suppresses cell migration.

There are two general models of single cell migration, mesenchymal and amoeboid (Huttenlocher and Horwitz, 2011). Cells that undergo mesenchymal migration model adopt a fibroblast-like spindle shaped morphology and rely on integrin mediated adhesion and require the activity of the membrane-tethered MMPs (MT-MMPs) and other types of proteases such as uPA (Friedl and Wolf, 2003). This mode of cell migration is Rac GTPase-dependent (Sahai and Marshall, 2003), hence inhibition of Rac activity would result in decreased rate of cell migration (Kurisu et al, 2005). With respect to amoeboid migration, actin cytoskeleton is reorganized along the membrane, causing dynamic membrane blebbing along the surface. This mode of cell migration is Rho GTPase-dependent (Sahai and Marshall, 2003). Rho GTPase exerts its function mainly through the Rho-associated kinase, ROCK. Rock phosphorylates LIM-kinase, which in turn phosphorylates cofilin (Maekawa et al, 1999) and induces stabilization of filamentous actin (F-actin) (Arber et al, 1998). ROCK activity is sensitive to the Y-27632 reagent; therefore treatment of the cells with Y-27632 will abrogate Rho dependent F-actin formation and amoeboid cell migration (Maekawa et al, 1999).

In addition to the individual cell migration there is another pattern of cell migration, called collective cell migration, in which cells migrate in solid cell strands, sheets, or clusters (Rorth, 2009). Collective cell migration retains the same five-step principles of the single cell migration; with one exception that in this mode the cells remain attached to each other by cell-cell junctions (Ilina and Friedl, 2009). Collective cell migration occurs in many physiological and pathological processes, including formation of mammary ducts (Ewald et al, 2008), wound healing (Poujade

et al, 2007) and cancers in which cells are not completely de-differentiated, including rhabdomyosarcoma and breast cancer (Friedl et al, 1995; Friedl and Gilmour, 2009).

1.3. SRY-related HMG box (Sox) family

In addition to their critical roles in embryonic development, cell fate decision and differentiation, members of Sox family of transcription factors have been implicated in various cancers. The Sox gene family is found throughout the animal kingdom. In human, at least 20 members of this family have been identified so far (Bowles et al, 2000), showing a diverse and dynamic pattern of expression throughout embryogenesis and in a variety of adult tissue types (Stros et al, 2007). Sry (for sex-determining region Y), the founding member of this family was identified through searches for conserved sequences among translocated Y chromosomal DNA from XX male patients (Sinclair et al, 1990) and was later confirmed to be involved in male sex differentiation (Berta et al, 1990).

All Sox proteins are characterized by possession of a high mobility group (HMG) DNA-binding domain. The 79-amino-acid HMG domains bind to the consensus target sequence (A/T)ACAA(T/A) in the minor grooves of DNA (Stros et al, 2007) and modify the chromatin structure to generate a conformation that facilitates various DNA-dependent activities. This domain is shared with some other DNA binding proteins, including those that bind DNA without sequence specificity such as HMG-1 protein and ubiquitous binding factor (UBF) as well as several sequence-specific DNA-binding proteins, such as the T cell-specific factors TCF/LEF (Farr et al, 1993).

The DNA-binding domains of Sox genes are at least 60% similar or 50% identical to the HMG box domain of SRY (Prior and Walter, 1996). The nomenclature of this family is based on

the order of gene discovery (Schepers et al, 2002). It is interesting that 9 of the 20 human Sox genes are single exons, likely reflecting the mechanism of expansion of this ancient gene family via non-tandem duplication and retroposition (Schepers et al, 2002). This family is sub-grouped into 6 distinct classes (A–F), based on homology within the HMG domain and other structural motifs as well as functional properties. These classes include: A, Sry; B, Sox1, 2, 3, 14, 15 and 19; C, Sox4, 11, 12 and 20; D, Sox5, 6, and 13; E, Sox8, 9, and 10; F, Sox7, 17, and 18 (Prior and Walter, 1996).

Despite their common features, each Sox protein selectively interacts with and regulates a unique set of target genes. Moreover, some other possible types of targets binding sequences, at least for some members of the family such as Sox4, have also been suggested (van Beest et al, 2000). Each member of this family is expressed in several different types of cells, and any given cell type can co-express many Sox factors (Wegner, 1999). Adding an extra level of complexity, it is also believed that Sox proteins regulate different target genes in a cell type-dependent manner (Kamachi et al, 2000). It appears that binding of Sox proteins to their consensus sequences in the promoter or enhancer regions of the target genes is not sufficient to exert a regulatory function, but also requires other binding partners that cooperate with the Sox protein after binding to the nearby sequences (Kamachi et al, 1999). Therefore, due to the cell-specific distribution of the partner factors as well as positioning of their corresponding binding sequences in the regulatory regions of the target genes, the action of Sox proteins may show variations based on the gene, type of the cell or tissue of interest.

Since the original discovery of SRY, many other Sox family members were also implicated in regulation of critical functions in various developmental processes, such as sex differentiation,

neurogenesis, skeletogenesis, hematopoiesis, angiogenesis, cardiogenesis, melanogenesis and hair development (Chew and Gallo, 2009; Harris et al, 2010; Lefebvre et al, 2007).

1.3.1. Sox protein as tumor suppressors and oncogenes

Several lines of evidence indicate that Sox proteins are implicated in cancer. For instance, amplification of *Sox2* at chromosome 3q was detected in prostate cancer (Sattler et al, 2000). Gain of chromosomal region 20q13, which contains Sox18 coding gene, has also been detected in breast (Collins et al, 1998) and colon cancers (Korn et al, 1999). Overexpression of several Sox family members was also identified at mRNA or protein levels in different types of cancer. For instance, Sox2 is overexpressed in at least some types of gastric adenocarcinomas (Tsukamoto et al, 2005). Similar observations was later reported in immature teratomas of central nervous system, melanoma, breast and colon cancer (Laga et al, 2011; Neumann et al, 2011; Phi et al, 2007; Rodriguez-Pinilla et al, 2007). Although, in some reports Sox2 was shown to be downregulated during cancer progression (Tsukamoto et al, 2004). Deregulated expression of other members of the Sox family in malignancies has also been demonstrated (Cheng et al, 2001; Lee et al, 2002; Ueda et al, 2007).

Many members of Sox family show characteristics of a typical oncogene in one type of malignancy, while acting as a tumor suppressor in another type. For example, Sox7 mRNA was reported to be significantly upregulated in pancreatic, primary gastric and esophageal cancer cell lines, while it was downregulated in primary colorectal, primary breast and primary kidney tumors (Katoh, 2002). Similarly, gain of Sox9 copy number is detected in primary colorectal cancers, in which it promotes proliferation, inhibit senescence, and collaborate with other oncogenes in neoplastic transformation (Matheu et al, 2012). On the other hand, its expression is

increasingly downregulated as melanocytic lesions progress towards malignancy and its overexpression in melanoma cell lines induced cell cycle arrest (Passeron et al, 2009).

1.3.2. Role of Sox proteins in melanocyte development and melanoma

Involvement of Sox proteins in melanocyte development was first discovered in mice, in which a truncating mutation in *Sox10* was linked to the Hirschsprung disease, a disorder of the gut which is caused by the failure of the neural crest cells migration, especially in cases where it was associated with features of Waardenburg syndrome (Herbarth et al, 1998). The Waardenburg syndrome is characterized by deafness and pale skin, hair, and eye color caused by physical absence of melanocytes from the skin, hair, eyes, or the stria vascularis of the cochlea (Read and Newton, 1997). Indeed, *Sox10* mutant mice serve as models for Waardenburg syndrome and Hirschprung disease (Herbarth et al, 1998). This function of Sox10 was later attributed to its role in regulation of transcription of the master regulatory gene for melanogenesis, microphthalmia associated transcription factor (MITF) (Potterf et al, 2000) which is required for melanocyte development, function and survival by modulating various differentiation and cell-cycle progression genes (Levy et al, 2006). In addition to MITF, Sox10 has the ability to transcriptionally regulate a number of genes that are required for melanin synthesis such as tyrosinase (Hou et al, 2006). Expression of Sox10 is reported in human melanoma and silencing of its expression in human melanoma cells decreased proliferation and cell survival, and completely abolishes in vivo tumour formation (Shakhova et al, 2012).

Sox9 is also expressed in neonatal and adult human skin melanocytes and is upregulated by UVB exposure mediated by cAMP and protein kinase A (Passeron et al, 2007). Similar to Sox10, Sox9 regulates MITF and tyrosinase expression at transcription level, leading to an increase in the expression of these key melanogenic proteins and production of melanin (Passeron et al,

2007). Interestingly, upregulation of Sox9 inhibits the human and mouse melanomas growth and its expression was found to be increasingly downregulated as melanocytes progressed to the premalignant and then the malignant and metastatic states (Passeron et al, 2009). Consistently, overexpression of Sox9 in both human and mouse melanoma cell lines induced cell cycle arrest by increasing p21 transcription and restored their sensitivity to retinoic acid (Passeron et al, 2009). Sox5 is also expressed in early neural crest cells and neural crest-derived peripheral glia in chicken (Perez-Alcala et al, 2004). Later on it was demonstrated that Sox5 modulates Sox10 activity. However, unlike Sox9, Sox5 binds to the promoter regions of Sox10 and recruits CtBP2 and HDAC1 to the regulatory regions of Sox10 target genes in melanocytic cells and suppress its activity. Therefore, Sox5 inhibits the Sox10-dependent promoter activation in melanocyte lineage (Perez-Alcala et al, 2004).

Sox2 is preferentially expressed in cells that interfaced and infiltrated dermal stroma (Girouard et al, 2012). In addition, knockdown of Sox2 expression resulted in decreased invasion of melanoma cells, whereas its overexpression increased their invasiveness (Girouard et al, 2012). Sox18 has been found to be expressed in mouse hair follicles and participate in the control of pheomelanin pigmentation in hair shafts and coat pigmentation (Pennisi et al, 2000). Recent findings revealed that Sox18 contributes to lymphangiogenesis and metastasis of melanoma and abrogation of its function suppresses these processes (Duong et al, 2012).

1.4. Sox4

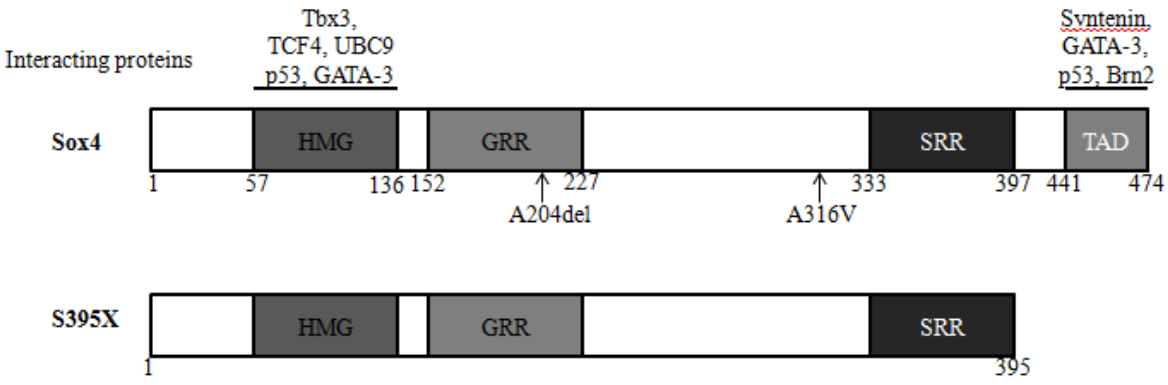
The human *Sox4* gene was first identified based on its homology with its mouse homologue (Farr et al, 1993). The location of *Sox4* was determined by metaphase fluorescence *in situ* hybridization (FISH) at chromosome 6p23 (Farr et al, 1993). Sox4 is a member of the SoxC

class and contains a single exon (Prior and Walter, 1996). All SoxC proteins have a high degree of similarity in the HMG domain. The HMG box is 84% identical and 95% similar among SoxC proteins across all vertebrate, whereas their TADs share 67% identity and 94% similarity (Dy et al, 2008).

Another distinguished feature of the SoxC class is a conserved proline, serine, and acidic residues-rich transactivation domain (TAD) at the C-terminal (Dy et al, 2008). This domain is crucial and sufficient for the activity of SoxC proteins and its deletion completely abrogates their transactivation capacity (Dy et al, 2008). Transactivating function of Sox4 TAD is independent of its HMG domain which is responsible for DNA binding as evident from its sustained activity upon grafting onto a GAL4 DNA-binding domain (Vandewetering et al, 1993). SoxC proteins seem to compete with each other to bind to their target sequences, since the activity of any full-length SoxC protein is inhibited by expressing equivalent amounts of Sox4, 11 or 12 proteins lacking the TAD (Dy et al, 2008). The exact mechanism by which TAD regulates transcriptional activation of target genes is not fully understood. One possible mechanism is that it is achieved through direct interaction with other transcription factors such as p53 (Pan et al, 2009) and β-catenin/TCF4 complex (Sinner et al, 2007b). In melanoma cells, Sox4 TAD also interacts with Syntenin resulting in prevention of proteasomal degradation of Sox4, therefore enhancing Sox4 stability (Beekman et al, 2012b).

In addition to HMG (aa 57–136) and TAD (aa 441–474) domains, Sox4 also contains a glycine rich region (aa 152–227) at the centre and a serine rich region (SRR, aa 333–397) adjacent to the TAD (Figure 1). A study in HEK293 cells revealed that the central domain containing a glycine rich region has pro-apoptotic activity which is independent of the transcriptional activity of Sox4 (Hur et al, 2004a) (Figure 1.1).

Figure 1.1. Structure of the human Sox4 protein, its binding partners, mutations and truncated isoform (S395X) detected in human lung cancer. GRR, glycine-rich region; SRR, serine-rich region; TAD, transactivation domain.



Similar to many other members of the family, the Sox4 protein has been identified as a necessary factor in embryonic development. Accordingly, *Sox4* knockout mice have been shown to die halfway through gestation due to severe heart defect (Schilham et al, 1996). Sox4 is highly expressed in thymocytes and induces their differentiation besides promoting pro-B lymphocyte expansion (Schilham et al, 1996; Schilham et al, 1997). Later findings revealed that Sox4 is also important for development of endocrine pancreas and insulin secretion (Goldsworthy et al, 2008; Wilson et al, 2005) as well as development of musculoskeletal system (Nissen-Meyer et al, 2007) and central nervous system (Potzner et al, 2007). Importantly, Sox4 is required for maintenance of the stemness through integration of the Oct4/Sox2 axis (Ikushima et al, 2011) which is required for the continuous self-renewal and potential to produce specialized progenies in stem cells, hence formation and upkeep of normal tissues in embryos and adult individuals.

1.4.1. Regulation of Sox4 expression

Regulation of Sox4 expression, especially in the pathological contexts, is a field that has remained largely unknown and requires further investigation. Initially, Clarke and colleagues (Graham et al, 1999b) reported that Sox4 is a progesterone-regulated gene in breast cancer cells and its expression is induced by progestins. Consistently, treatment of T-47D breast cancer cells with the synthetic progestin ORG-2058 increased Sox4 transcription within few hours of treatment. Notably, ORG-2058 had no detectable effect on the expression of other *Sox* genes, suggesting that in this system the observed phenomenon was specific to Sox4 (Graham et al, 1999b). In osteoblast-like cells, physiological concentrations of human parathyroid hormone stimulate Sox4 mRNA expression in a time-dependent manner, indicating involvement of the PTH/PTHrP receptor (Reppe et al, 2000). Prostaglandin A2 and delta12-PGJ2 also induce Sox4 mRNA expression in hepatocarcinoma cells (Ahn et al, 1999). Del Giacco and colleagues found that IFN-beta/all-trans retinoic acid treatment induced the expression of Sox4 in a thioredoxin reductase 1 (TrxR1) enzyme dependent manner in hepatocarcinoma cells (Gorreta et al, 2005). However, in none of these observations is it clear whether these hormones affect the activity of the Sox4 promoter or they exert their function at other levels (e.g. mRNA stability).

Nonetheless, other studies have revealed details on the regulation of the Sox4 promoter. In hematopoietic progenitor cells, Sox4 expression is regulated at transcription level by the HOXB4 transcription factor (Lee et al, 2010). The HOX gene family members are important regulators of hematopoiesis. In T-cells, expression of Sox4 is upregulated at both mRNA and protein levels by TGF-β. This regulation is mediated by downstream mediators of TGF-β, Smad2 and Smad3, which bind to the regulatory region of the *Sox4* gene (Kuwahara et al, 2012). Overexpression of Sox4 transcript upon binding of the Smad2/3 complex to its promoter after

TGF-β treatment has also been observed in glioma cell lines (Ikushima et al, 2009). On the other hand, inhibitor of differentiation 2 (ID2) and REST/NRSF repress expression of Sox4 in neural progenitor cells (Bergsland et al, 2006). Since REST/NRSF is a transcriptional repressor, it is pertinent to assume that it could suppress Sox4 promoter activity, although this assumption requires further experimental verification. The retinoic acid receptor-related orphan receptor α (RORα) is also suggested to upregulate Sox4 transcription (Wang et al, 2012b). RORα plays a critical role in regulation of the circadian clock and enhances Sox4 transcription by direct binding to the promoter sequences after activation by its antagonist (Wang et al, 2012b).

Sox4 is transcriptionally regulated by Sox7 in endometrial cancer cells (Saegusa et al, 2012). Accordingly, overexpression of Sox7 activates the Sox4 promoter, leading to the increased expression at both mRNA and protein levels, seemingly due to the presence of Sox7-responsive elements in the promoter region of Sox4 gene. Interestingly, Sox7 inhibits the activity of its own promoter (Saegusa et al, 2012), further confirming that the same Sox protein can simultaneously enhance and suppress the activity of different promoters in the same cell, consistent with the context-dependent manner of function for Sox protein. Sox4 expression may further be influenced by other members of the SoxC class. In fact, whereas ablation of *Sox4* in the sympathoadrenal lineage did not have any significant effect on expression of Sox11, knockdown of Sox11 markedly reduced Sox4 protein expression (Potzner et al, 2010), although it is not clear whether or not this effect of Sox11 is exerted through direct binding to the Sox4 promoter. In addition, Sox4 may regulate its own expression as is evident by binding of the Sox4 protein to its promoter sequence in prostate cancer cells (Lai et al, 2011).

At post-transcriptional level, Sox4 is regulated by several miRNAs including miR-129-2 (Huang et al, 2009) miR-335 (Tavazoie et al, 2008) and miR-93 (Liu et al, 2012). Furthermore,

Sox4 protein level is regulated by proteasome-mediated degradation pathway (Beekman et al, 2012a).

1.4.2. Sox4 in tumorigenesis

1.4.2.1. Oncogenic functions of Sox4

The first indirect evidence identifying a role for Sox4 in cancer came from studies in colon carcinoma, in which Sox4 was shown to enhance transcription of p56lck, a non-receptor protein tyrosine kinase that is aberrantly expressed in colon and small lung carcinoma cell lines (McCracken et al, 1997). Enhanced expression of Sox4 transcript in colorectal cancer samples was reported later which correlated with higher incidence of recurrence and a shorter recurrence-free survival (Andersen et al, 2009).

Amplification of chromosomal region 6p22 in at least some types of tumor (Grasemann et al, 2005; Hurst et al, 2004) was also another indirect evidence for a tumorigenic function of Sox4. Although, at least in some cases there was no significant correlation between expression of Sox4 and amplification of 6p22.3 (Bruch et al, 2000). Nevertheless, an increased expression of Sox4 was observed in lung primary tumours and lung cancer cell lines (Medina et al, 2009). The same study also identified a somatic mutation at the 395 residue of Sox4 that introduced a premature stop codon at the C-terminal domain, predicting a shorter Sox4 protein which abrogated its transactivation potential (Fifgure 1.1). While neither wild-type Sox4 nor its truncated isoform could induce oncogenic transformation of the NIH3T3 mouse fibroblasts, wild-type Sox4 could enhance tumorigenicity of the NIH3T3 cells expressing the activated HRAS. However, the truncated isoform significantly reduced the tumorigenecity of the HRAS expressing cells (Medina et al, 2009).

Sox4 is overexpressed in human hepatocellular carcinoma (Hur et al, 2010), classical medulloblastomas (Lee et al, 2002), non-small cell lung carcinoma (Friedman et al, 2004), adenoid cystic carcinoma (ACC) (Pramoonjago et al, 2006) and bladder tumors (Aaboe et al, 2006). Huang and colleagues revealed that Sox4 is overexpressed in endometrial tumors (Huang et al, 2009). They also showed that Sox4-knockdown suppresses endometrial cancer cells growth. They further confirmed that Sox4 is a target of miR-129-2, expression of which leads to decreased Sox4 expression and reduced proliferation of cancer cells (Huang et al, 2009). Downregulation of Sox4 by miR-129-2 is also described in gastric cancer in which Sox4 expression is shown to be aberrantly induced (Shen et al, 2010). Sox4 is also a target of miR-335, that has been shown to suppress metastasis and migration of breast cancer cells and restoration of which decreased the lung colonizing activity of these cells (Tavazoie et al, 2008).

In prostate cancer, an increased expression of Sox4 has been detected in transcript (Vanaja et al, 2006) and protein levels (Liu et al, 2006). Sox4-knockdown induces apoptosis in LNCaP prostate cancer cell line and its overexpression induces transformation of RWPE-1 cells. Depletion of Sox4 expression also results in stabilized p53 protein and loss of survivin expression in prostate cancer cells (Liu et al, 2006). In addition, a genome-wide promoter analysis of the Sox4 transcriptional network in prostate cancer cells found a plethora of genes including some prominent proto-oncogenes such as Tenascin-C, Frizzled-5, Patched-1, Delta-like1, and EGFR whose promoters are subject to Sox4 binding (Scharer et al, 2009).

In human glioblastoma, Sox4 expression is increased by TGF-β stimulation and expression of both factors is elevated (Lin et al, 2010). Sox4 is also highly expressed in glioma-initiating cells (GICs) and is required for TGF-β induced expression of Sox2 that is essential for retention of stemness of GICs (Ikushima et al, 2009). Sox4-knockdown cells show less sphere-forming

ability and self-renewal capacity. Inhibitors of TGF-β signaling deprive tumorigenicity of GICs by promoting their differentiation, an effect which is attenuated in GICs transduced with Sox2 or Sox4 (Ikushima et al, 2009).

Sox4 can also enhance β-catenin/TCF activity by increasing the stability of β-catenin. It increases the protein level of β-catenin and wnt signaling pathway activity. The enhanced β-catenin/TCF activity by Sox4 is caused by stabilization of the β-catenin protein, through induction of CK2, a kinase involved in regulation of β-catenin stability (Lee et al, 2011). Sinner and colleagues observed that Sox4 enhances beta-catenin/TCF activity and cell proliferation (Sinner et al, 2007a). Sox4 physically interacts with TCF/LEF family members via its HMG-box domain and stabilizes β-catenin and TCF/LEF proteins (Sinner et al, 2007a). In malignant melanoma, Sox4 is reported to activate the wnt/β-catenin signaling pathway (Cai et al, 2011).

By a sophisticated approach, using oncogenic-retrovirus-induced insertional mutagenesis, Copeland and colleagues (Du et al, 2005) showed that replication-defective retrovirus carrying Sox4 can induce myeloid leukemias after transplanting into mice bone marrow cells in cooperation with Mef2c, a transcription factor involved in regulation of homing and invasiveness of leukemic cells. The same group later revealed that Sox4 represses Sfpi1 transcription by binding to a critical Sfpi1 upstream DNA element (Aue et al, 2011). Additionally, by analyzing 285 acute myeloid leukemia patient samples, they found a significant negative correlation between Sox4 and Sfpi1 mRNA expression providing convincing evidence for the involvement of Sox4 in promotion of at least a subset of myeloid malignancies (Aue et al, 2011). In myeloid cells Sox4 binds to the promoter sequences of CREB and enhances its expression (Sandoval et al, 2012). Transduction of CREB transgenic mouse bone marrow cells with a Sox4 retrovirus increases the *in vitro* survival and self-renewal. In addition, leukemic blasts from the majority of

acute myeloid leukemia (AML) patients have higher CREB, phosphorylated CREB, and Sox4 protein expression, indicating that Sox4 and CREB cooperate and contribute to increased proliferation of hematopoietic progenitor cells and myelogenesis.

1.4.2.2. Sox4 as a tumor suppressor

By using mRNA differential display and Northern blot analysis, Ahn *et al* (Ahn et al, 1999) found that expression of Sox4 mRNA is enhanced during apoptosis induced by prostaglandin (PG)A2 and Delta12-PGJ2 in human hepatocellular carcinoma cells. The same group later observed that Sox4-induce apoptosis in these cells is through the caspase-mediated pathway (Ahn et al, 2002). They further revealed that antisense oligonucleotide against Sox4 blocks the apoptosis induced by PGA(2) and delta(12)-PGJ(2) in these cells (Ahn et al, 2002). Caspase-dependent manner of Sox4 for induction of apoptosis was also confirmed by other researchers (Kim et al, 2004). The apoptosis-inducing function of Sox4 was later shown to be dissociated from its transcriptional activity. Accordingly, the central domain (amino acid 166-342) of Sox4 is critical for induction of apoptotic cell death in HEK293 cells. Deletion of the DNA-binding domain or trans-activation domain in Sox4 did not significantly affect the pro-apoptotic activity of Sox4, whereas overexpression of a construct containing the central domain induced the apoptotic activity comparable to that of the full-length protein (Hur et al, 2004b).

Human ubiquitin-conjugating enzyme 9 (Ubc9) interacts with HMG-box of Sox4 and repress Sox4 transcriptional activity in HEK293T cells (Pan et al, 2006). Nevertheless, the SUMOylation-deficient mutant of UBC9 does not abolish its ability to inhibit Sox4 activity. Elevated Ubc9 expression has been detected in at least some types of malignancies such as primary and metastatic melanomas in which Ubc9 plays a crucial role in suppression of apoptosis (Moschos et al, 2007).

Reduced expression of Sox4 has been recently reported in primary gallbladder carcinoma (Wang et al, 2012a), in which its expression is associated with low histological grade, low pathologic T stage, and early clinical stage of this malignancy. In addition, the expression of Sox4 in tumors with positive nodal metastasis was lower than those without metastasis. Consistently, Sox4 expression is positively correlated with a better overall and disease-free survival (Wang et al, 2012a).

In colon cancer cell lines, Sox4 induces p53 stability and activity (Pan et al, 2009). Sox4 interacts with and stabilizes p53 protein by repressing Mdm2-mediated ubiquitination and degradation of p53. Furthermore, Sox4 interacts with p300/CBP and enhances p300/CBP/p53 complex formation and p53 acetylation which increases p53 stability. Expression of Sox4 protein but not mRNA is increased upon treatment of these cells with doxorubicin which is required for activation of p53 in response to doxorubicin-induced DNA damage (Pan et al, 2009). Consistently, Sox4 promotes cell cycle arrest and apoptosis, and can inhibit colon cancer cells' tumorigenesis in a p53-dependent manner (Pan et al, 2009). In line with these observations, ionizing radiation induces Sox4 expression paralleled with the induction of p53 protein in medulloblastoma cell line (Chetty et al, 2012). In addition, Sox4-knockdown dramatically blocks the radiation-induced increases in p53 (S-15) phosphorylation and XRCC1 protein expression along with an increase in levels of phospho-c-H2AX compared with cells subjected to radiation alone (Chetty et al, 2012), suggesting that Sox4 functions upstream of p53-mediated DNA repair.

As opposed to the aforementioned role of Sox4/β-catenin axis in promotion of tumorigenesis, a recent report revealed that Sox4 could indeed enhance β-catenin/TCF4 transcription, through upregulation of TCF4 at the transcription level, without any direct β-

catenin association, resulting in significant decreases in proliferation rate, along with increases in expression of p21, as well as TCF4 in endometrial carcinoma cells, while Sox4 knockdown increases cell growth in the same model (Saegusa et al, 2012).

1.5. Objective and hypotheses

Aberrant expression of Sox4 has been found in several types of human malignancies. It has also been associated with survival of cancer patients. In addition, Sox4 was reported to differentially modulate different features of tumorigenesis such as apoptosis, cell cycle arrest and attachment-independent cell growth in various types of tissues. However, so far no study has been conducted on Sox4 expression pattern in different stages of human melanoma, its possible correlation with tumor progression or patient survival, and its biological functions in melanoma cells. **We hypothesized that Sox4 is involved in regulation of melanoma tumorigenesis.** We investigated the expression pattern of Sox4 in melanocytic lesions, its functions in regulation of melanoma tumorigenesis and the downstream mechanisms by which Sox4 regulate melanoma tumorigenesis.

Previously, other groups reported the possible regulation of expression of miRNA biogenesis factors by Sox4. **We hypothesized that control of expression of Dicer, a critical miRNA biogenesis factor, by Sox4 is important for Sox4-medidated regulation of melanoma progression.** We also investigated the expression pattern of Dicer in melanoma, its correlation with patients' survival and role of Sox4 in control of biogenesis of melanoma.Subcellular localization of Dicer has recently been shown to change in certain types of cancer. **We hypothesized that Dicer is not uniquely expressed in cytoplasm of melanocytic cells and that changes in nuclear expression of Dicer may have some**

significance in melanomagenesis. We studied the expression pattern of nuclear Dicer and its correlation with clinicopathological features of melanoma.

In addition to Dicer, aberrant expression or function of several other miRNA biogenesis factors have been implicated in tumorigenesis. **We hypothesized that expression and subcellular localization of Drosha, a component of the nuclear microprocessor complex, is deregulated in melanoma.** We used TMA and immunohistochemistry to study the expression pattern of Drosha and its correlation with clinicopathological features of melanoma.

Chapter 2. Materials and methods

2.1. Cell lines and cell culture, antibodies, reagents and expression plasmids

The MMRU (a metastatic melanoma cell line), RPEP (derived from a recurrent melanoma) and SK-mel-3 (a primary melanoma cell line) cells were kind gifts from Dr. H.R. Byers (Boston University School of Medicine, Boston, MA, U.S.A.). These cells were selected among a number of cell lines with similar features representing different stage of melanoma progression. All melanoma cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Fisher Scientific, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Fisher Scientific) in the presence of 100 units/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin B (Invitrogen, Burlington, ON, Canada) in 5% CO_2 humidified atmosphere at 37^{o}C.

Anti-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA); mouse anti-Flag from (Applied Biomaterial, Richmond, BC, Canada); rabbit anti-Sox4 from Abcam (Cambridge, MA), Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Sigma-Aldrich ; rabbit anti-NF-κB p50 antibody from Santa Cruz Biotechnology ; Cy2-conjugated goat anti-rabbit secondary antibody from Jackson ImmunoResearch (West Grove, PA, USA); polyclonal rabbit anti-Dicer from Sigma or monoclonal Rabbit anti-Dicer antibody and the synthetic immunogenic peptide from CloneGene (Hartford, CT, USA); polyclonal rabbit anti-Drosha antibodies from Abcam or Santa Cruz Biotechnology . Phalloidin-rhodamine was purchased from Invitrogen and ROCK inhibitor Y27632 from Tocris Bioscience (Bristol, United Kingdom).

Sox4-specific siRNAs were purchased from Santa Cruz Biotechnology or Qiagen (Mississauga, ON, Canada); Dicer and NF-κB p50 siRNAs from Qiagen. Effectene transfection reagent was purchased from Qiagen and Silenfect siRNA transfection reagent from Bio-Rad (Mississauga, Ontario, Canada).

The Sox4 open reading frame was subcloned from pOTB7-Sox4 (ImaGegen, Berlin, Germany) into 3×Flag-CMV-7.1 using PCR primers harboring EcoRI and BamHI restriction sites. pCDNA3-Flag-Dicer was kindly provided by Dr. Ian J. MacRae (The Scripps Research Institute). The human Dicer promoter reporter construct (pGL3-DICER-Prom) was provided by Dr. D.E. Fisher (Harvard Medical School, MA) through Addgene (plasmid 25851).

2.2. Construction of tissue microarrays (TMAs)

All specimens were obtained from the 1990 to 1998 archives of the Department of Pathology, Vancouver General Hospital. The use of human skin tissues in this study was approved by the Clinical Research Ethics Board of The University of British Columbia. The most representative tumor area from each sample was carefully selected by a dermatopathologist and marked on the H&E-stained slide. The TMAs were assembled using a tissue-array instrument (Beecher Instruments, Silver Spring, MD). Duplicate 0.6-mm thick formalin-fixed paraffin-embedded tissue cores were taken from each biopsy specimen and spotted on five high-density TMA blocks. Duplicate tissue cores were used to reduce the limitations of the representative areas of the tumor. 4-μm sections were cut with a Leica microtome and transferred to adhesive coated slides. Tissues from 49 normal nevi, 100 dysplastic nevi, 402 primary melanomas, and 162 metastatic melanomas were used in construction of the tissue microarray construct. We also used a smaller construct (two-slide) with 66 dysplastic nevi, 117 primary melanomas, and 53

metastatic melanomas. The location of primary and metastatic melanoma cases in each array is displayed in Table 2.1-2.4.

Table 2. 1. Tissue locations of 402 primary melanoma cases in 5-slide TMA construct

Location	No. of cases
Arm	62
Back	68
Foot	17
Head	100
Leg	60
Neck	12
Trunk	73
Unspecified	3
Vulva	7

Table 2. 2. Tissue locations of 162 metastatic melanoma cases in 5-slide TMA construct

Location	No. of cases
Arm	5
Axillary	28
Bowel	1
Brain	13
Breast	1
Duodenum and Esophagus	1
Eye	1
Femoral Lymph Nodes	1
Foot	1
Frontal Sinus	1
Gastric	1
Groin	6
Hard Palate	1
Head	10
Hematoma	1
Iliac	1
Ilio-obturator	1
Inguinal	29
Leg	7
Liver	1
Medistinal	1
Neck	21
Parptidectomy	2
Peritoneal	1
Periumbilical	1
Pleural	2
Postauricular Node	1
Spleen	2
Subumilical	1
Temporal Hematoma	1
Trunk	11
Unspecified	6
Vulva	1

Table 2. 3. Tissue locations of 117 primary melanoma cases in 2-slide TMA construct

Location	No. of cases
Arm	21
Foot	3
Head	17
Leg	22
Neck	5
Trunk	49

Table 2. 4. Tissue locations of 53 metastatic melanoma cases in 2-slide TMA construct

Location	No. of cases
Arm	2
Axillary	12
Brain	3
Breast	1
Cheek	1
Femoral Lymph Nodes	1
Foot	1
Frontal Sinus	1
Groin	1
Inguinal	7
Inguinal Node	2
Leg	1
Lobe	1
Lung	2
Neck	11
Omentum	1
Right Frontal	1
Shin	1
Spleen	1
Upper Jugular vein Region	1

2.3. Immunohistochemistry

TMA slides or single tumor slides were dewaxed at 55°C for 30 minutes and three consequent washes with xylene. Tissues were rehydrated by a series of washes in 100%, 95%, and 80% ethanol, followed by two washes in distilled water. Antigen retrieval was done by heating the samples at 95°C for 30 minutes in 10 mM sodium citrate (pH 6.0). After inactivating the endogenous peroxidise by incubating in 3% H_2O_2 for 30 minutes and blocking with universal blocking serum for 30 minutes, slides were incubated with the relevant first antibodies at 4°C overnight. Slides were then incubated with biotin-labeled secondary antibody and streptavidin-peroxidase for 30 minutes each, followed by developing with diaminobenzidine substrate kit (Vector Laboratories, Burlington, Ontario, Canada) and counterstained with hematoxylin. Negative control stainings were performed by omitting the primary antibody during the primary antibody incubation. For blocking experiment, the relevant antibody was incubated with 10 times concentration of its corresponding synthetic immunogenic peptide at 4°C overnight before immunohistochemical staining.

2.4. Evaluation of TMA immunostaining

The evaluation of nuclear Sox4, cytoplasmic Dicer, nuclear Dicer, cytoplasmic Drosha and nuclear Drosha expression levels was made by two independent observers (including one dermatopathologist) simultaneously. In cases with a discrepancy between different cores, the higher score was taken as the final score. The corresponding staining for each core was scored into four grades according to the following staining intensities: 0, 1+, 2+, and 3+. Percentages of positive nuclei or cytoplasm were also scored into five categories: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The immunoreactive score, which is calculated by

multiplying the scores of staining intensity and the percentage of positive cells, was used as the final staining score.

2.5. Plasmids and siRNAs transfection

For plasmid transfection, cells were grown to ~50% confluency and transiently transfected with POTB7-Sox4, 3xF-Sox4 or Flag-Dicer plasmids with Effectene reagent according to the manufacturer's instructions. Twelve hours after transfection, the medium containing transfection reagent was removed. Cells were rinsed with PBS and then incubated in fresh medium. Cells were harvested at 24 hours after transfection and lysed for Western blot assay or further processed for indicated analysis. Transfection of siRNA was performed when melanoma cells reach ~50% confluency, with SiLentFect reagent according to the manufacturer's instruction. Twelve hours after transfection, the medium containing transfection reagent was removed. Cells were rinsed with PBS and then incubated in fresh medium. Cells were harvested at 72 hours after transfection and lysed for Western blot assay or further processed for indicated analysis.

2.5. Protein extraction and western blot

Cells were washed with PBS, harvested by scraping on ice and pelleted by centrifugation at 3000 g for 3 min. For whole lysate, cells pellets were lysed in modified RIPA buffer (50 mM Tris-HCl, (pH 8.0), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1mM EDTA) containing freshly added protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). Samples were then sonicated, incubated on ice for 30 min, and centrifuged at 12,000 g for 10 min at 4°C, before the supernatants were collected. For cytoplasmic/nuclear fractionation, cells were lysed in cell lysis buffer (10 mM Tris-HCl [pH

7.5], 1 mM $MgCl_2$, and 0.5% NP-40) for 5 min on ice. Nuclei were pelleted by centrifugation at 10,000 *g* for 10 seconds at 4^{o}C. Nuclei were resuspended in modified RIPA buffer, sonicated and incubated on ice for 30 min. Samples were centrifuged at 12,000 g for 10 min at 4°C, before the supernatants were collected.

Protein concentration was determined by Bradford assay (Bio-Rad) according to manufacturer's instructions. 50 μg proteins of each sample were separated on 8, 10 or 12% SDS-polyacrylamide gels (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membrane was then blocked with 1.5% Bovine serum albumin (BSA) in PBST (PBS containing 0.05% Tween-20) for 1 hour at room temperature before incubating with primary antibodies prepared in 1.5% BSA in PBST for 1 hour at room temperature. Blots were then washed in PBST three times, 5 min each and incubated with secondary infrared dye-conjugated antibodies IRDye 800 or IRDye 680, at room temperature for 1 h before washing by PBST three times, 5 min each and scanning on the Odyssey Infrared Imaging System to visualize proteins (LI-COR Biosciences) equipped with Odyssey 2.1 software. Expression of β-actin was used as loading control.

2.6. Reverse transcription and real-time quantitative polymerase chain reaction (qPCR)

Total RNA was prepared by Qiazol extraction (Qiagen) according to manufacturer's protocol. RNA concentrations were measured with a spectrometer at 260 nm. 2 μg of total RNA was reverse-transcribed into cDNA with the SuperScript First-Strand Synthesis System (Invitrogen) or Transcriptor cDNA Synthesis System (Roche, Indianapolis, IN, USA) according to the manufacturers' protocols. qPCR was performed with SYBR Green Master mix system (Applied Biosystem, Carlsbad, CA, USA) or SYBR Green Master mix system (Roche). Using a 7900HT

qPCR system thermal cycler (Applied Biosystems). Expression of human β-actin mRNA was used as an internal control. All primer sequences that were used in this study are listed in Table 2.5.

2.7. Wound healing assay

Forty-eight hours after transfection with siRNAs, cells were washed with PBS and a standard 200 µl pipette tip was used to draw across the well to produce a wound at the center of each well. The monolayers were then washed three times with PBS to remove floating cells and incubated in fresh complete medium for another 18 hours. Photographs were taken at the same position of the wounds at 0 and 18 hours time points. The starting wound edges were defined in each photo by a black lines based on the scratch at 0 hours time point and the numbers of migrating cells across these lines were counted to quantify the rate of cell migration.

2.8. Immunofluorescence

Cells were transfected with siCTR or siSox4 and subcultured onto coverslips in six-well plates in complete medium. Forty-eight hours after transfection, the medium was removed, cells were washed with PBS and a new serum-free medium applied for overnight. Then, cells were collected after stimulation with complete medium containing 10% fetal bovine serum for 30 minutes. In case of ROCK inhibitor (Y27632) treatment, the inhibitor was added to the serum free medium (10 µM) and applied overnight. Then cells were collected after stimulation with complete medium containing 10% fetal bovine serum and Y27632 for 30 minutes. Cells were fixed with 2 ml of fixative (1% paraformaldehyde and 0.5% Triton X-100 in PBS) for 20 minutes at room temperature. After washing with PBS, cells were incubated with BSA for 1 hour

followed by staining with phalloidin-rhodamine (1 unit) for 30 minutes, then incubated with rabbit anti-Sox4 primary antibody for 2 hours and Cy2-conjugated goat anti-rabbit secondary antibody (1:2000) for 1 hour. Finally, the coverslips were incubated with 1:3000 dilution of stock Hoechst 33258 (20 mM) for 5 minutes and the cells were visualized under a fluorescent microscope. Photos were taken with a cooled mono 12-bit Retiga-Ex camera equipped with Northern Eclipse imaging software. To quantify the intensity of F-actin staining, 10 images per slide were taken under a fluorescent microscope. Images were analyzed using ImageJ software (NIH, Bethesda, MD), and the mean of relative cellular fluorescent intensity was measured.

2.9. Cell invasion assay

Cell invasion analysis was performed using the Boyden chamber. Forty µl of 5 mg/ml matrigel (BD Biosciences, Mississauga, ON, Canada) in serum-free medium was added to the upper compartment of 24-well Transwell culture chambers (with 8.0 µm pore size polycarbonate membrane). 4 x 10^4 cells suspended in 250 µl of serum-free medium were seeded on the upper compartment, and 750 µl of complete medium was added to the lower compartment. After 24 hours incubation, cells were fixed with 10% trichloroacetic acid at 4°C for 1 hour. Non-invaded cells were removed from the upper surface of the filter with a cotton swab. Invaded cells on the lower side of the filter were stained with 0.5% crystal violet for 2 hours at room temperature and the retained dye on the filters was extracted by 30% acetic acid followed by reading the absorbance at 590 nm.

2.10. Sulforhodamine-B assay

To compare cell growth rates, cells were seeded in 24-well plates 24 hours after transfection with siRNAs. At each time point, cells were fixed with 10% trichloroacetic acid, stained with 0.4% sulforhodamine B in 1% acetic acid for 15 min at room temperature, and then de-stained with 1% acetic acid. Cell density was quantified by dissolving bound dye in 10 mM Tris (pH 10.5) followed by colorimetric determination at 550 nm. The initial time point (0 hour) was measured by fixing cells immediately after they had attached to the tissue culture plate, 6 hours after seeding. Subsequent time points were measured by fixing cells 24, 48, and 72 hours later. Relative rates of cell growth were calculated as a ratio of the cell density at each time point over the cell density at 0 hour.

2.11. Chromatin immunoprecipitation assay

MMRU cells were transfected with 3×Flag-Sox4 construct. Twenty four hours after transfection, formaldehyde-fixed cells were immunoprecipitated overnight with mouse anti-Flag or rabbit anti-Sox4 and the associated genomic DNA was analyzed by PCR and agarose gel electrophoresis. We designed four sets of primers, spanning a distance from 2.5 kb to 850 bp of the NF-κB p50 transcription start site and another four sets of primers, spanning a distance from -3 kb to +1000 bp of the Dicer transcription start site. A list of the sequences of primers and the site of their corresponding amplicons is shown in Table 2.6.

2.12. Luciferase reporter assay

siCTR or siSox4 treated MMRU cells were plated in 12-well plates and co-transfected with pGL3-DICER-Prom and Renilla pRL-CMV plasmids. Firefly luciferase activities in the lysates

were determined after 24 h using the Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA) and were normalized with Renilla pRL-CMV plasmid transfection.

2.13. miRNAs profiling assay

The miRNA profiling array was carried out using Applied Biological Materials (ABM)'s miRNA profiling service (ABM C201). Total RNAs from siCTR, siCTR/Flag-vector, siSox4, or siSox4/Flag-Dicer transfected MMRU cells were prepared with Qiazol extraction followed by Poly-A Tailing reactions and miRNA cDNA synthesis (ABM C204). Real-time qPCR reactions and instrumental analysis was performed using Roche LightCycler480. The expression profile of miRNAs in all four groups is deposited and publically available in GEO database (accession number: GSE36715). Functional analysis of the miRNA profiling data was performed using the Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA). Lists of miRNAs were generated by pair-wise comparison of our expression data sets (siSox4 vs siCTR and siSox4/F-Dicer vs CTR). miRNAs with 2-fold up- or down-regulation after Sox4-knockdown were subjected to the Ingenuity knowledge proprietary database to identify the biological functions that were most significant to the data sets.

2.14. Statistical analysis

The Kruskal-Wallis and Mann-Whitney test were applied to compare the staining between normal nevi, dysplastic nevi, primary melanoma and metastatic melanoma using the GraphPad prism version 4 or 5. Spearman test was used to analyse the correlation between two markers using the GraphPad prism version 4 or 5. Other statistical analyses were performed with the SPSS 11.5 software. The correlations between protein expression and clinicopathologic

variables, including age, gender, tumor thickness, location, and ulceration were analyzed by χ^2 test. Student t-test was used to evaluate the differences in RT-PCR results as well as cell migration, invasion and growth assays. Fisher's exact test was used to estimate the significance of the incidence of biological functions by calculating a *P* value determining the probability that the association between the miRNA dataset and these biological functions is significant. A *P* value of less than 0.05 was considered significant.

Table 2.5. Sequences for all primers used in qPCR assays.

Sox4	forward	5'-GGTCTCTAGTTCTTGCACGCTC-3'
	reverse	5'-CGGAATCGGCACTAAGGAG- 3'
Dicer	forward	5'-GTACGACTACCACAAGTACTT-3'
	reverse	5'-ATAGTACACCTGCCAGACTGT-3'
NF-êB p50	forward	5'-GGATTTCGTTTCCGTTATGT-3'
	reverse	5'-TGTCCTTGGGTCCAGCAGTT-3'
β-actin	forward	5'-CCCTGAGGCACTCTTC-3'
	reverse	5'-AGGTCTTTGCGGATGT-3'

Table 2.6. Sequences of the primers used for Chromatin Immunoprecipitation (ChIP) assay and the distance of the amplicons to the corresponding transcription start site.

p50-A	forward	5'-AGGAATGGAGGAGGGTTAATC-3'	-2181
	reverse	5'-GACACCAGTCATTGGATTTAGAG-3'	
P50-B	forward	5'-ACTGGAGGAGGAGGATGGAG-3'	-936
	reverse	5'-TCATTGTTTCACTACTGGGTGG-3'	
P50-C	forward	CTGTGAAGAGATGTGAATGTAACTG-3'	-392
	reverse	5'-GTGCCGCTGATAGAGTCATG-3'	
P50-D	forward	5'-TCATTTCTCTTCACGTCCCTC-3'	+701
	reverse	5'-ACCACCTTCCCTCTCTCCTC-3'	
Dicer-A	forward	5'-TAACACGGGCTGAAATATAGG-3'	-3019
	reverse	5'-CTGTATCCGTTCTAATGGTCTATC-3'	
Dicer-B	forward	5'-ACTAGGACAGGTGTGAGGGAC-3'	-2378
	reverse	5'-TCAGTAGAGACGGGGTTTCAC-3'	
Dicer-C	forward	5'-AAATTAGCTGGGTGTGGTGG-3'	-1269
	reverse	5'-GGTGCTGAAACTGCTTCCTG-3'	
Dicer-D	forward	5'-CAGAGAGTCTGCCAAACTTAGC-3'	+208
	reverse	5'-CTGGCGGTGAAAGGTTAATC-3'	

Chapter 3. Prognostic significance of Sox4 expression in human cutaneous melanoma and its role in cell migration and invasion

3.1. Background and rationale

Members of the Sox transcription factors family have been shown to play important roles in embryonic development, cell fate decision, and differentiation (Kiefer, 2007; Wegner, 1999). Aberrant expression or function of members of Sox family has also been reported in various cancers (Lu et al, 2008; Wang et al, 2009b). Similarly, aberrant expression of Sox4 has been found in several human malignancies. It has been shown that Sox4 is upregulated in cancers of lung (Bangur et al, 2002), salivary gland (Frierson et al, 2002), brain (de Bont et al, 2008), prostate (Liu et al, 2006), adenoid carcinoma (Pramoonjago et al, 2006), and endometrial cancer (Huang et al, 2009) at mRNA or protein levels. Nevertheless, reduced Sox4 expression is reported in primary gallbladder carcinoma (Wang et al, 2012a). Moreover, others have shown that expression of Sox4 induces apoptosis, promote cell cycle arrest and inhibit tumorigenesis in human colon cancer cell lines (Pan et al, 2009), suggesting that Sox4 has a potential tumor-suppressive function in certain tissues. However, so far no study has been conducted on Sox4 expression in different stages of human melanoma, its possible correlation with tumor progression or patient survival, and its biological functions in melanoma cells. In the present study we examined the expression pattern of Sox4 protein in different stages of melanocytic lesions and explored the mechanism by which it regulates melanoma progression. We also analyzed the possible correlation between Sox4 expression and patients' survival and clinicopathological parameters.

3.2. Results

3.2.1. Reduced sox4 expression is correlated with melanoma progression

Microscopic analysis of immunostained cores revealed a distinct nuclear staining of Sox4 in dysplastic nevi (Figure 3.1A) with faint cytoplasmic expression in few samples. Notably, significant differences in nuclear Sox4 staining were observed between dysplastic nevi and metastatic melanomas and between primary melanomas and melanoma metastases ($P < 0.05$ and $P < 0.01$, respectively, Kruskal-Wallis test; Figure 3.1B). Nevertheless, there was no significant difference in Sox4 staining between primary melanomas and dysplastic nevi ($P > 0.05$, Kruskal-Wallis test; Figure 3.1B). Similarly, when we grouped the samples into negative-weak (0 to 3) or moderate-strong (4 to 12), a significant reduction of nuclear Sox4 expression was observed between metastatic melanomas and dysplastic nevi ($P = 0.012$, χ^2 test) and also between metastatic melanomas and primary melanomas ($P = 0.005$, χ^2 test), but not between primary melanomas and dysplastic nevi ($P = 0.708$, χ^2 test) (Figure 3.1C). We did not observe any significant correlation between nuclear expression of Sox4 and patient sex, age, tumor thickness, location, subtype, or ulceration status (Table 3.1).

Figure 3.1. Expression of Sox4 protein in cutaneous melanoma. (**A**) Representative images of dysplastic nevi (DN) with strong nuclear staining, primary melanoma (PM) with moderate staining, and metastatic melanoma (MM) with negative staining. Scale bar = 100 μm. (**B**) Kruskal-Wallis test for differences in Sox4 staining among DN, PM, and MM. The median is depicted as a horizontal line inside each box. (**C**) χ^2 for differences in Sox4 staining in DN, PM, and MM. **P* < 0.05; ***P* < 0.01.

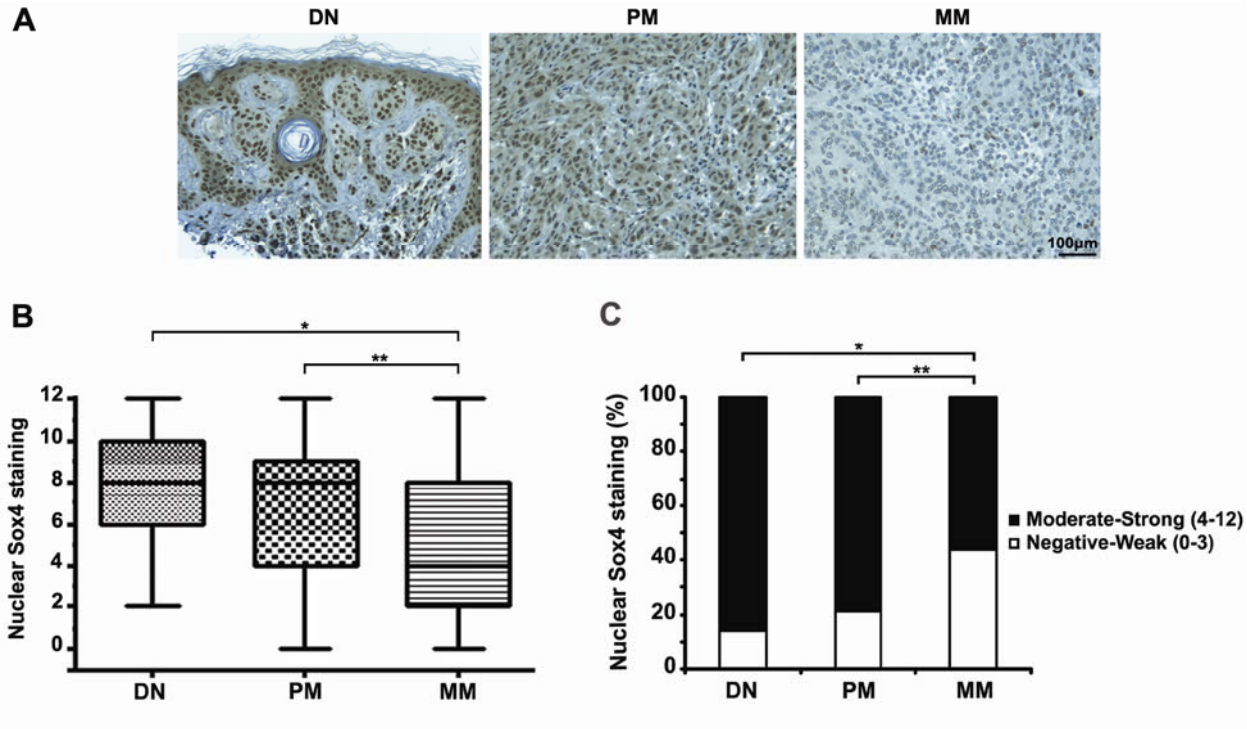


Table 3.1. Sox4 nuclear staining and clinicopathological characteristics of 89 cases of primary melanomas.

	Sox4 staining			
Variables	Negative-weak	Moderate-strong	Total	P value*
Age				
≤58	14 (30.4%)	32 (69.6%)	46	>0.05
>58	5 (11.6%)	38 (88.4%)	43	
Gender				
Male	10 (17.9%)	46 (82.1%)	56	>0.05
Female	9 (27.3%)	24 (72.7%)	33	
Tumor thickness (mm)				
≤1.5	12 (26.7%)	33 (73.3%)	45	>0.05
>1.5	7 (15.9%)	37 (84.1%)	44	
Ulceration				
Present	1 (5.5%)	17 (94.5%)	18	>0.05
Absent	18 (25.3%)	53 (74.7%)	71	
Tumor subtype				
Superficial spreading	12 (31.6%)	26 (68.4%)	38	>0.05
Lentigo maligna	1 (5.9%)	16 (94.1%)	17	
Other†	6 (17.6%)	28 (82.4)	34	
Site‡				
Sun-protected	17 (24.3%)	53 (75.7%)	70	>0.05
Sun-exposed	2 (10.5%)	17 (89.5%)	19	

* χ^2 test.

† Other: unspecified subtype.

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

3.2.2. Stronger nuclear Sox4 staining correlates with better 5-year survival of melanoma patients

To evaluate the correlation between nuclear Sox4 expression and 5-year survival of melanoma patients, we constructed Kaplan-Meier survival curves using overall or disease-specific 5-year patient survival data to analyze the biopsies stained negative-weak versus moderate-strong for Sox4. Our data revealed that overall survival in moderate-strong Sox4 staining group was 55.7%

compared with 45% in negative-weak group. However, the log rank analysis indicates that this difference is not significant (P = 0.119; Figure 3.2A). The disease-specific 5-year survival of the patients was significantly reduced from 65.2% in moderate-strong Sox4 group to 49.2% in negative-weak Sox4 group (P = 0.039; Figure 3.2B). We also performed multivariate Cox regression analysis including Sox4 nuclear staining, age, and gender to examine whether the nuclear Sox4 expression is an independent prognostic marker for melanoma. The results showed that nuclear Sox4 expression is an independent factor for predicting the disease-specific, but not the overall patient survival (P = 0.049 and 0.095, respectively; Table 3.2) when we combined all primary and metastatic melanoma cases for the analysis.

As shown in Figure 3.2A, the nuclear Sox4 staining is not significantly correlated with overall 5-year patient survival, although a trend toward a poorer overall patient survival with negative-weak Sox4 staining is evident. This may be due to the fact that a small number of deaths occurred in the low-risk (< 1.5 mm) melanoma patients within 5 years. When we combined the metastatic melanoma with high risk primary melanoma cases (thickness <1.5 mm) and excluded the low risk (>1.5 mm) cases of primary melanoma, we found that nuclear Sox4 expression has a significant positive correlation with both overall and disease-specific 5-year survival (P = 0.012 and 0.007, respectively; Figure 3.2C and D). Accordingly, overall survival reduces from 42.2% in moderate-strong group to 21.4% in negative-weak group and the disease-specific survival declines from 50.3% in moderate-strong group to 26.5% in negative-weak group (Figure 3.2C and D). Furthermore, the multivariate Cox-regression analysis revealed that nuclear Sox4 staining is an independent factor for predicting both overall and disease-specific 5-year patient survival (P = 0.026 and 0.025, respectively; Table 3.2) of metastatic melanoma and high-risk primary melanoma.

Figure 3.2. Kaplan-Meier analysis of correlation between Sox4 expression and 5-year survival. (**A**) Overall and (**B**) disease-specific 5-year survival of all primary and metastatic melanoma patients. (**C**) Overall and (**D**) disease specific 5-year survival of metastatic melanoma and high-risk primary melanoma patients (thickness > 1.5 mm).

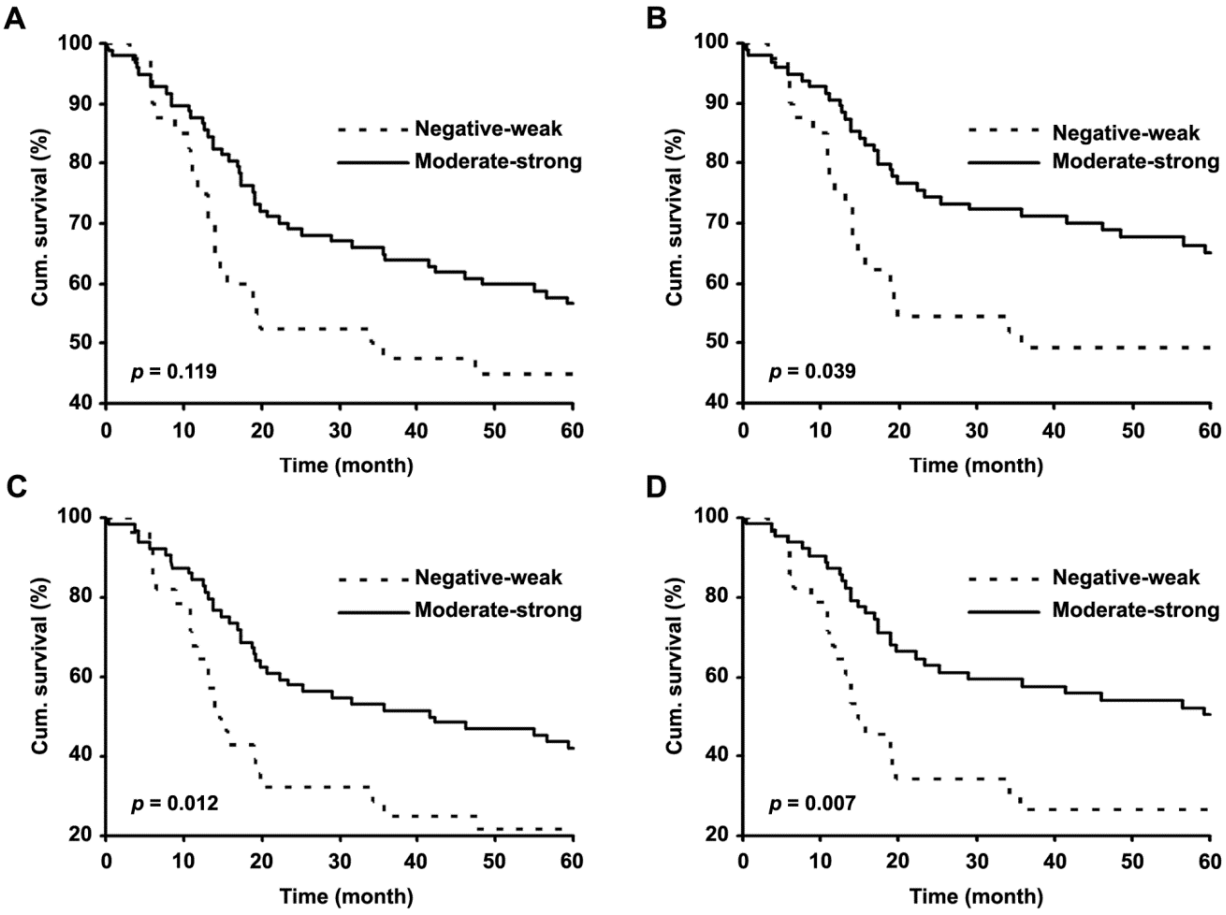


Table 3.2. Multivariate Cox regression analysis of Sox4 nuclear expression in 48 cases of metastatic melanomas and 89 cases of primary melanomas.

Variable*	All primary and metastatic melanomas			High-risk melanomas (thickness >1.5mm)		
	Relative risk	95% CI†	P value	Relative risk	95% CI†	P value
Overall survival						
Sox4	1.571	0.924 - 2.673	0.095	1.904	1.078 - 3.362	0.026
Age	0.676	0.407 - 1.122	0.122	0.974	0.574 - 1.650	0.922
Gender	1.238	0.736 - 2.084	0.419	1.075	0.606 - 1.906	0.802
Disease specific survival						
Sox4	1.780	1.001 - 3.163	0.049	1.994	1.087 - 3.658	0.025
Age	1.780	0.540 - 1.662	0.850	1.337	0.753 - 2.373	0.320
Gender	1.100	0.618 - 1.958	0.744	1.011	0.547 - 1.870	0.970

*Coding of variables: Sox4 nuclear staining was coded as 1 (negative-weak), and 2 (moderate-strong). Age was coded as 1 (≤58 years), and 2 (>58 years). Gender was coded as 1 (female), and 2 (male).

† CI: confidence interval.

3.2.3. Sox4 knockdown enhances melanoma cell migration

Increased ability of the cells to migrate is one of the critical requirements of cancer cell invasion, resulting in higher metastasis potential and shorter survival of melanoma patients (Condeelis et al, 2005; Friedl and Wolf, 2003). To investigate whether decreased expression of Sox4 can affect the migration of melanoma cells, we used Sox4-specific siRNA to knockdown (KD) its expression (Figure 3.3D) and examined the rate of cell migration by wound healing assay. Our results revealed that Sox4-KD MMRU cells have a marked increase in cell migration compared with their control counterparts (Figure 3.3A). Accordingly, Sox4-KD resulted in a 70% increase

of migrated cells into wound area compared with the control siRNA-transfected cells (Figure 3.3B). We also observed a 37% increase in cell migration of another melanoma cell line, RPEP, after Sox4-KD compared with the control siRNA (Figure 3.4). It is noteworthy that although diminished expression of Sox4 enhances melanoma cells migration, it does not significantly affect the growth rate of these cells based on the cell proliferation assay (Figure 3.5).

Figure 3. 3. Enhancement of melanoma cell migration and invasion upon Sox4-KD in a NF-κB p50 dependent manner. (**A**) Wound-healing assay was performed on monolayers of MMRU melanoma cells 48 hours after transfection. Original magnification, X100. (**B**) Quantitation of (A). (**C**) Boyden chamber assay. $*P < 0.05$; $**P < 0.01$, $***P < 0.001$. (**D**) Detection of expression of Sox4 and NF-κB p50 by western blot after Sox4-KD. β-actin was used as a loading control.

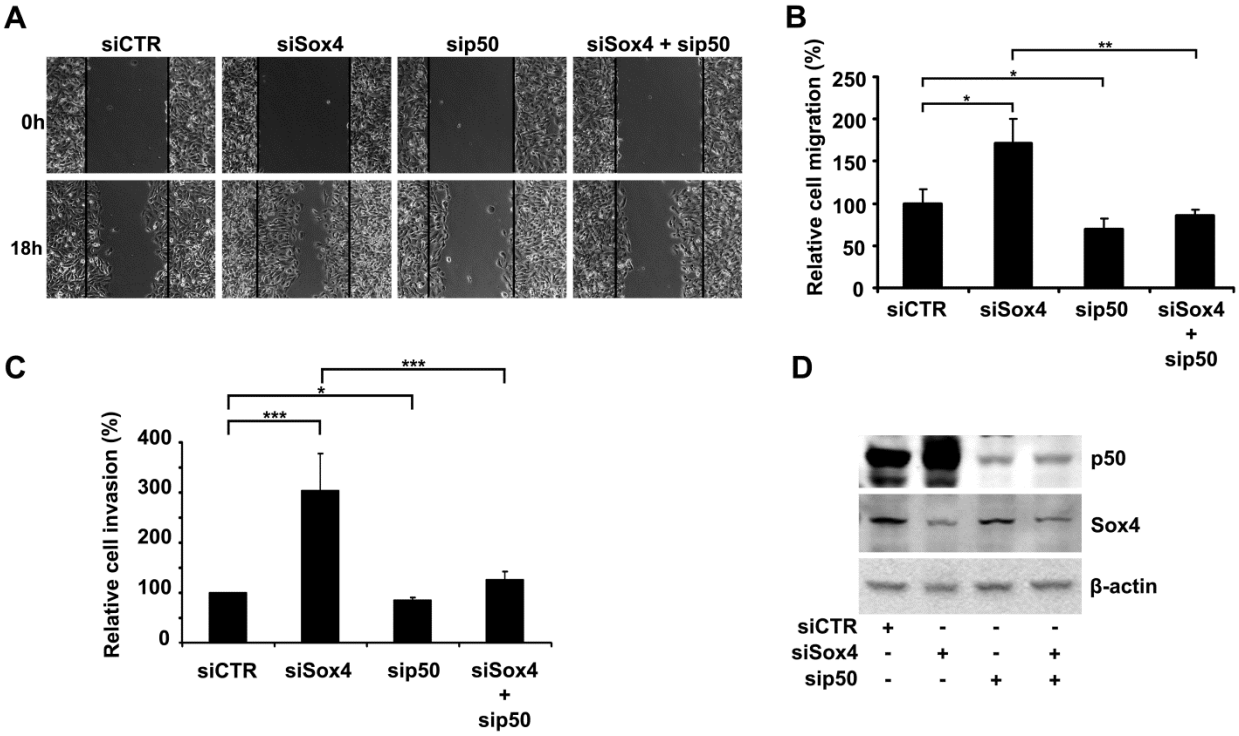


Figure 3.4. Increased migration of RPEP melanoma cells after Sox4-KD. (**A**) Wound healing assay was performed on monolayers of RPEP cells. Magnification: ×100. (**B**) Quantitation of (A), * $P < 0.05$. (C) Western blot analysis of Sox4 expression. β-actin was used as a loading control.

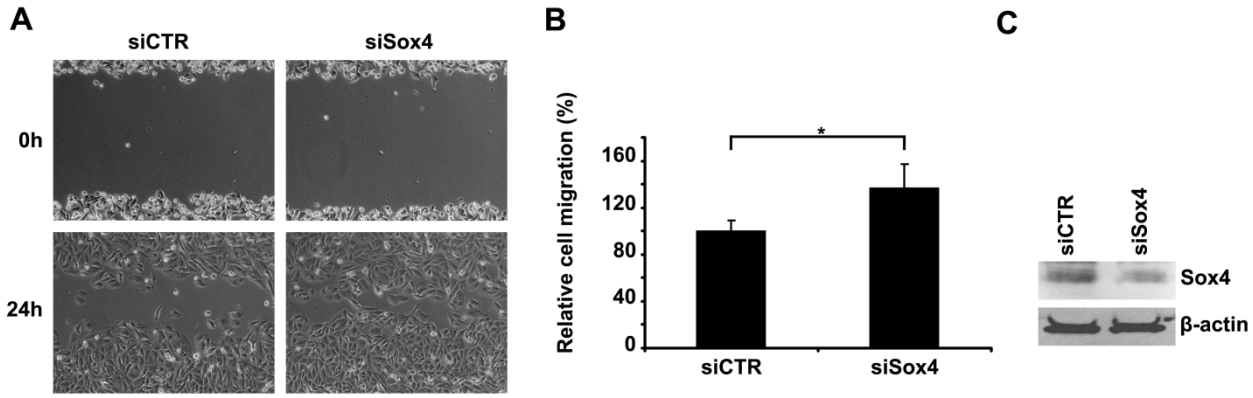
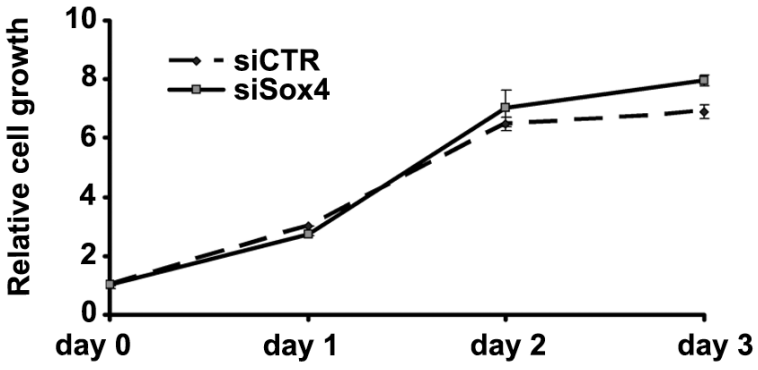


Figure 3.5. Sulforhodamine B (SRB) assay shows no significant difference in MMRU cell growth rate after Sox4-KD.



3.2.4. NF-κB p50 is required for Sox4 knockdown-induced migration of melanoma cells

Previous studies in our lab revealed that NF-κB p50 enhances melanoma cell migration (Gao et al, 2006). Therefore, we sought the possible role of NF-κB p50 in Sox4 KD-mediated melanoma cell migration. For this purpose we used specific siRNAs to concomitantly knockdown Sox4 and p50 and measured cell migration rate by wound healing assay (Figure 3.3A). Our results showed that while Sox4-KD induced a 70% increase in cell migration, p50 knockdown reduced cell migration by 30% comparing to the control group. Strikingly, when we co-knocked down Sox4 and p50 in MMRU cells, we observed a rate of cell migration comparable to the control siRNA treatment (Figure 3.3A and B). These results indicate that Sox4-KD mediated increase in cell migration requires the expression of NF-κB p50 in melanoma cells.

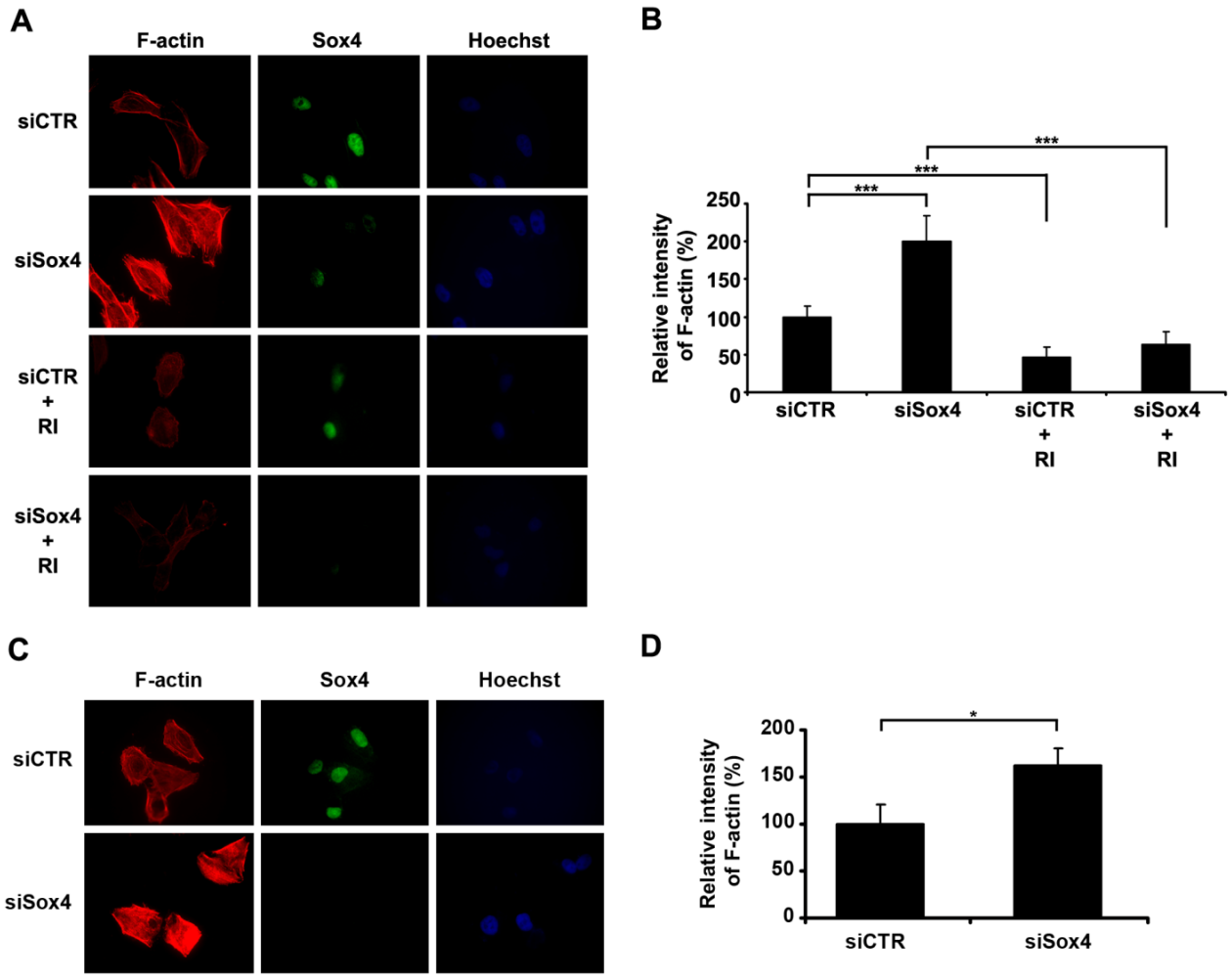
3.2.5. Sox4 regulates melanoma cell invasion in a NF-κB p50 dependent manner

Elevated potential of the cells to invade through basal membrane is another pivotal characteristic of metastatic melanoma (Friedl and Wolf, 2003). To study the role of Sox4 in melanoma cell invasion, we examine the ability of MMRU cells to invade through matrigel matrix using the Boyden chamber assay. We found that cell invasion was induced by three-fold in Sox4-KD cells, compared with the control (Figure 3.3C). On the other hand, p50-KD reduced cell invasion by 18% compared with the control group (Figure 3.3C). Similar to the migration assay, when concomitantly knocked down Sox4 and p50, we observed a rate of cell invasion comparable to the control siRNA treatment (Figure 3.3C), indicating the requirement of NF-κB p50 in Sox4-KD-mediated increased melanoma cell invasion. We also observed a remarkable increase in the expression of NF-κB p50 protein (Figure 3.3D) upon Sox4-KD, which further explains the observed increased cell migration and invasion after depletion of Sox4 protein and abrogation of these effects by co-knockdown of p50 and Sox4.

3.2.6. Sox4 knockdown requires ROCK activity to increase the F-actin filaments formation in melanoma cells

Our lab previously reported that NF-κB p50 induces cell migration through increased RhoA/ROCK activity and formation of F-actin filaments (Gao et al, 2006). To further delineate the mechanisms involved in Sox4-KD induced cell migration, we starved the Sox4-KD and control MMRU cells overnight with serum depletion and stained the cells with rhodamine-conjugated phalloidin (Figure 3.6A). On average, cells transfected with siSox4 had a 99% increase in F-actin filaments formation compared with control siRNA after 30 minutes of serum stimulation (Figure 3.6A and B). Similarly, knockdown of Sox4 elevates formation of F-actin filaments in RPEP melanoma cell line by over 62% (Figure 3.7A and B). Interestingly, when treated with ROCK inhibitor, Y27632, the formation of F-actin filaments was effectively abrogated in both control and Sox4-KD cells, indicating the requirement of ROCK activity in Sox4-KD induced F-actin filaments formation (Figure 3.6A and B).

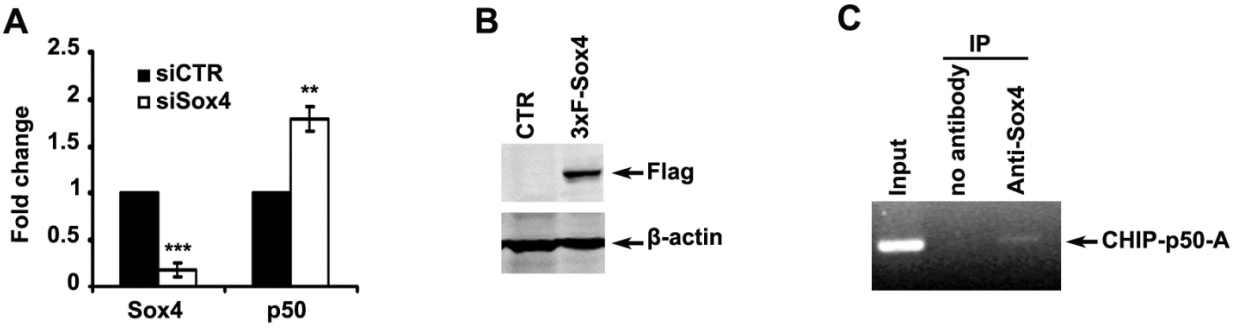
Figure 3.6. Sox4-KD induces F-actin formation. (**A**) Enhanced formation of actin filaments upon Sox4-KD compared with control MMRU cells. Treatment with ROCK inhibitor Y27632 (RI) reverts the Sox4-KD effect. (**B**) Quantitation of (**A**). (**C**) Enhanced formation of F-actin filaments upon Sox4-KD in RPEP cells. Magnification: ×630. (**D**) Quantitation of (**C**). **P* < 0.05, ****P* < 0.001.



3.2.7. Inhibition of NF-κB p50 transcription by Sox4 in melanoma cells

As shown in Figure 3.4D, Sox4-KD induces the expression of NF-κB p50 protein, implying an inhibitory role of Sox4 in regulation of NF-κB p50 expression. However, it is not clear whether this regulation happens at the transcription level or at the levels of translation and/or protein stability. To further investigate these possibilities, we performed a qPCR assay measuring the expression of NF-κB p50 mRNA after Sox4-KD in MMRU cells. Our results revealed a marked overexpression of NF-κB p50 mRNA after Sox4-KD (Figure 3.7A), implying that Sox4 can regulate the expression of NF-κB p50 at least in part at transcript level. To further study the mechanistic details of this effect, we conducted a chromatin immunoprecipitation assay using MMRU cells transiently transfected with a construct encoding 3×Flag-Sox4 (Figure 3.7B). Using the specific polyclonal anti-Sox4 antibody, we were able to pull down nucleoprotein complexes containing Sox4 protein. By using specific primers designed for various regions of NF-κB p50 promoter, we were able to detect precipitation of a genomic fragment corresponding to the upstream sequences of NF-κB p50 promoter (Figure 3.7C).

Figure 3.7. Inhibition of NF-κB p50 transcription by Sox4. (**A**) qPCR analysis of NF-κB p50 and Sox4 mRNAs expression after Sox4-KD. β-actin was used as loading control. $^{**}P < 0.01$, $^{***}P < 0.001$. (**B**) Overexpression of 3×Flag-Sox4 in MMRU cells. β-actin was used as a loading control. (**C**) chromatin immunoprecipitation assay to demonstrate binding of Sox4 protein binds the NF-κB p50 promoter sequence in MMRU cells.

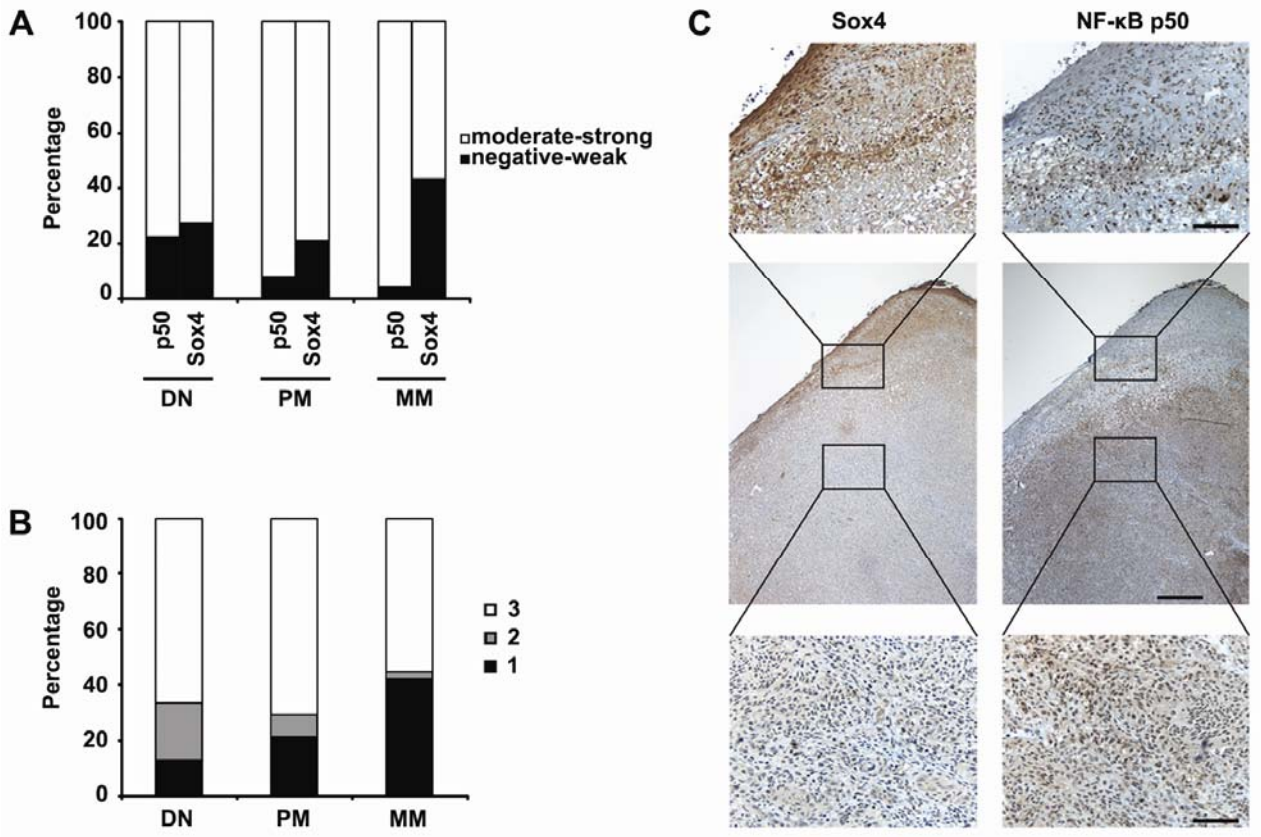


3.2.8. Inverse correlation between expression of Sox4 and NF-κB p50 in melanoma

Based on the observed upregulation of NF-κB p50 on Sox4-KD and its critical requirement for enhancement of melanoma cell migration and invasion on Sox4-KD, we sought to analyze the protein expression pattern of both Sox4 and NF-κB p50 in the same 169 melanocytic lesions. When all of the cases were analyzed, we found that whereas Sox4 staining is significantly decreased in metastatic melanomas compared with dysplastic nevi and primary melanomas ($P = 0.0260$, χ^2 test; Figure 3.8A), the expression of NF-κB p50 increases in primary melanoma and metastatic melanoma cases comparing to dysplastic nevi ($P = 0.0147$, χ^2 test; Figure 3.8A). Next, we divided each sample based on the concurrent expression level of Sox4 and NF-κB p50 into three groups: 1, low (negative-weak) Sox4/high (moderate-strong) p50; 2, high Sox4/low p50;

and 3, high Sox4/high p50. No sample with low Sox4 and low p50 staining was available. Interestingly, we found a remarkable increase in percentage of samples with low Sox4 and high NF-κB p50 in metastatic melanoma compared with primary melanoma and dysplastic nevi (P = 0.0001, χ^2 test; Figure 3.8B). The percentage of samples with high Sox4 and low NF-κB p50 significantly declines in metastatic melanoma comparing with the primary melanoma and dysplastic nevi (P = 0.0002, χ^2 test; Figure 3.8B). However, no statistically significant changes were observed in the percentage of samples with high Sox4 and high NF-κB p50 staining among different stages of melanocytic lesions. We further assessed a number of single tissue slides from high risk nodular melanoma and superficial spreading melanoma patients for expression of Sox4 and NF-κB p50 proteins. As shown in Figure 3.8C, in samples which were positively stained for Sox4, the strongest staining was contained to the epidermis region as opposed to the deeper tumor mass which is mostly negative for Sox4 staining. However, the expression of NF-κB p50 was mainly restricted to the tumor area with very weak or negative staining in the epidermis. These data further indicate an inverse expression pattern of Sox4 and NF-κB p50 in melanoma samples.

Figure 3.8. Inverse correlation between expression of Sox4 and NF-κB p50. (**A**) Significant reduced expression of Sox4 (P = 0.026, χ^2 test) and elevated expression of NF-κB p50 (P = 0.014, χ^2 test) in metastatic melanoma compared with the primary melanoma and dysplastic nevi. (**B**) Combined analysis of Sox4 and NF-κB p50 staining. Each sample was categorized based on the expression of Sox4 and NF-κB p50 as follows: 1, low (negative-weak) Sox4/high (moderate-strong) p50; 2, high Sox4/low p50; 3, high Sox4/high p50. (**C**) Representative images of serial sections of a high-risk nodular primary melanoma stained for NF-κB p50 and Sox4. Scale bar = 100 μm for left and right panels and 400 μm for the middle panels.



3.3. Discussion

Deregulated expression of multiple members of Sox family is associated with various human malignancies (Gangemi et al, 2009; Huang et al, 2008; Nonaka, 2009; Wang et al, 2009b). Sox4 has been shown to be aberrantly expressed at either transcript or protein levels in different cancers (Aaboe et al, 2006; Bangur et al, 2002; de Bont et al, 2008; Frierson et al, 2002; Huang et al, 2009; Liu et al, 2006; Pramoonjago et al, 2006). However, to our knowledge, no study on the expression and possible functions of Sox4 in human melanoma and its correlation with tumor progression or patient survival has been conducted so far. In the present study we analyzed Sox4 expression in 43 cases of dysplastic nevi, 89 cases of primary melanoma, and 48 cases of melanoma metastases by TMA and investigated its role in regulating the migration and invasion abilities of melanoma cells.

Microscopic analysis revealed that Sox4 is mainly a nuclear protein (Figure 3.1A) which is consistent with previous reports in addition to its function as a transcription factor (Boyd et al, 2006; Pan et al, 2006). Statistical analysis of the tissue microarray immunoreactivity scores revealed a significant reduction in Sox4 expression in metastatic melanomas compared with primary melanomas and dysplastic nevi. However, we did not detect any significant difference between dysplastic nevi and primary melanoma (Figure 3.1B and C). These results suggest that reduced or lost expression of Sox4 may be relevant to the process of melanoma metastasis.

Despite previous reports on elevated expression of Sox4 in cancers of lung (Bangur et al, 2002), salivary gland (Frierson et al, 2002), brain (de Bont et al, 2008), and prostate (Liu et al, 2006), we observed a reduced expression of Sox4 in metastatic melanoma compared with primary melanoma and dysplastic nevi. This discrepancy could be due to intrinsic differences in the biology of various types of tissues. Accordingly, Sox4 is reported to have anti-apoptotic

function in prostate cancer cells which may explain its elevated expression in tumor biopsies (Liu et al, 2006). It is noteworthy that similar contradictory expression pattern and functions have been also reported for other members of the Sox family. For instance, Sox9 is suggested to be an oncogene in prostate cancer (Wang et al, 2008), while it can suppress formation of melanoma (Passeron et al, 2009). Other studies have shown elevated apoptosis rate after overexpression of Sox4 in HEK293, bladder, and colon cancer cells (Aaboe et al, 2006; Hur et al, 2004b; Pan et al, 2009). Furthermore, Ahn and colleagues showed that up-regulation of Sox4 after treatment with Delta12-prostaglandin J2 induces apoptosis and antisense-mediated knockdown of Sox4 abrogated apoptosis in hepatocellular carcinoma (Ahn et al, 2002). The p53 up-regulated modulator of apoptosis (PUMA) has also been reported as a direct transcription target of Sox4 (Liu et al, 2006), indicating the role of Sox4 in the tumor suppression pathways. Nevertheless, there has been no study dealing with the possible role of Sox4 in cancer metastasis so far. In this report we revealed that Sox4 expression is reduced or lost in the course of progression of primary melanoma to metastatic melanoma (Figure 3.1).

Moreover, we demonstrated a positive correlation between nuclear Sox4 expression and 5-year disease-specific survival of melanoma patients as well as 5-year overall and disease-specific survival of high-risk melanoma cases (Figure 3.2). Sox4 knockdown significantly stimulated melanoma cell migration (Figure 3.3A and B, Figure 3.4) and invasion (Figure 3.3C), which are two of the most critical events in the process of cancer progression (Gupta and Massague, 2006). These observations suggest that Sox4 may function as an inhibitor of melanoma metastasis, which by far is the most important cause for the high rate of mortality of melanoma (Miller and Mihm, 2006), explaining the observed positive correlation between Sox4 expression and higher survival rate in patients. This observation is also in line with the previous report of positive

correlation between Sox4 expression and survival of patients with bladder cancer (Aaboe et al, 2006) and hepatocellular carcinoma (Hur et al, 2010).

NF-κB has been regarded as one of the main regulators of several cellular processes including cell proliferation, inflammation, cell survival, migration, and metastasis (Bollrath and Greten, 2009; DeLuca et al, 2007; Shen and Tergaonkar, 2009). Our lab previously reported that expression of NF-κB p50 is elevated during melanoma progression and that abrogation of its expression by RNA interference reduces cell migration in vitro (Gao et al, 2006). A positive role of NF-κB p50 in facilitating the invasion of melanoma has also been reported by others (Amiri and Richmond, 2005; van de Stolpe et al, 1994). Here we showed that knockdown of NF-κB p50 in melanoma cells reduces the cell migration and invasion (Figure 3.3). Strikingly, co-knockdown of NF-κB p50 abolished Sox4-KD-induced cell migration and invasion (Figure 3.3). Furthermore, we observed a marked upregulation of NF-κB p50 expression after Sox4-KD (Figure 3.3D), implying that Sox4 functions upstream of NF-κB p50 and can suppress the expression of p50 and probably the activity of the NF-κB pathway. Moreover, we observed a considerable increase in the formation of actin filaments after Sox4-KD in MMRU and RPEP cells (Figure 3.6A-D). Formation of actin filaments is commonly considered as a critical event for cell motility and metastasis (Yamazaki et al, 2005). Our lab previously reported induced RhoA activity and ROCK-mediated formation of actin filaments on overexpression of NF-κB p50 in melanoma cells (Gao et al, 2006). Here we revealed that Sox4-KD has a stimulatory effect on F-actin filaments formation and treatment with ROCK-inhibitor, Y27632, abrogates this effect, providing further evidence for the involvement of NF-κB p50-RhoA/ROCK-actin filaments formation pathway in the Sox4-KD-induced cell migration.

Despite the observed overexpression of NF-κB p50 protein after Sox4-KD, it was not understood at what step of the gene expression this regulation occurs. To address this question, we performed quantitative PCR assay after Sox4-KD and revealed a significant overexpression of NF-κB p50 mRNA (Figure 3.7A), indicating that Sox4 regulates the expression of p50 at least in part at the transcript level. Since the Sox4 protein is a DNA binding factor, we sought to further examine its ability to bind to NF-κB p50 promoter sequence by chromatin immunoprecipitation assay. We were able to pull down a genomic fragment upstream of NF-κB p50 promoter (Figure 3.7C). The promoter region of the human NF-κB p50 has been previously defined to be located at the first 1 kb nucleotides of the transcription start site as well as part of the first exon (Cogswell et al, 1993). We were not able to detect binding of Sox4 to this promoter area; nevertheless, our PCR results showed binding of Sox4 to a genomic sequence adjacent to this promoter region (approximately 2 kb from start site). In line with our observation, this fragment contains a distinctive Sox4 binding site AACAAAG, which can explain the observed binding of Sox4 to this specific sequence.

It is worth noting that although Sox4 has been initially identified as a transcription factor, it is possible that it can also inhibit transcription of its target genes depending on the binding partners present. This mechanism has been previously described for members of the Sox family (Kamachi et al, 2000; Wilson and Koopman, 2002). It can also explain the observed contradictory functions of Sox4 in different cancer types. However, the exact mechanistic details of Sox4-mediated inhibition of NF-κB p50 transcription and also its potential binding partner(s) to the promoter region is yet to be revealed. Collectively, these data suggest a significant influence of Sox4 on the NF-κB p50 pathway in melanoma cells.

Pan and colleagues showed a direct role of Sox4 in stabilization of p53 protein after UV irradiation or application of genotoxic agents and subsequent induction of apoptosis, cell cycle arrest, and suppression of tumorigenesis (Pan et al, 2009). p53 has long been considered as a main hub in the cellular stress response to UV radiation (Latonen and Laiho, 2005; Park et al, 2009), which is the most prominent environmental factor for melanoma development (Atillasoy et al, 1998; Gilchrest et al, 1999). It is also known that p53 can inhibit NF-κB-mediated tumorigenesis (Meylan et al, 2009) and absence or aberrant function of p53 may result in activation of NF-κB pathway, hence formation or promotion of cancer. Nevertheless, the frequency of p53 mutation in melanoma is significantly lower than other types of human malignancy (Chin et al, 1998; Hartmann et al, 1996). It has been suggested that melanoma cells compensate for lack of p53 mutation by mutation or reduced expression of other factors involved in p53-mediated tumor suppression, such as Apaf-1 (Chin et al, 1998; Chin et al, 2006; Monzon et al, 1998; Soengas et al, 2001). Therefore, reduced expression of Sox4, as an upstream stabilizer of p53, in metastatic melanoma, may indeed help the tumor cells to activate the NF-κB pathway and acquire further ability to invade other tissues. This idea may provide another explanation for our observed reverse pattern of Sox4 and NF-κB p50 expression (Figure 3.8).

Sinner and colleagues have previously observed that Sox4 protein may interact and stabilize β-catenin protein which is a main hub of wnt signaling pathway in colon carcinoma cells (Sinner et al, 2007a). A similar observation has been made in prostate cancer cells (Scharer et al, 2009). Wnt signaling is a prominent signaling pathway involved in various features of tumorigenesis including epidermal-mesenchymal transition and metastasis (Larue and Delmas, 2006), cell attachment, cell motility, and matrix metalloproteinase expression and activity (Crampton et al, 2009). Considering the invasion and migration suppressor function of Sox4 in

melanoma and its reported interaction with β-catenin protein, it would also be of interest to investigate the effect of Sox4 on the wnt signaling pathway and its possible influence on the cell detachment as a main feature of epithelial mesenchymal transition and metastasis.

In conclusion, our data indicate that Sox4 expression is significantly reduced in metastatic melanoma and reduced Sox4 is correlated with a poorer 5-year patient survival. Furthermore, we demonstrated the inhibitory effect of Sox4 on NF-B pathway and melanoma cell migration and invasion *in vitro*. We also reported a reverse pattern of expression of Sox4 and NF-κB p50 in different stages of melanocytic lesions, as well as mutually exclusive expression pattern of Sox4 and NF-κB p50 in high risk primary melanoma. These data suggest that Sox4 plays an important inhibitory role in melanoma progression and metastasis.

Chapter 4. Sox4-mediated Dicer expression is critical for suppression of melanoma cell invasion

4.1. Background and rationale

Except for a few types of malignancies, a general downregulation of miRNA expression in cancers has been observed, mainly due to chromosomal abnormality, epigenetic changes, or aberrant expression and function of the miRNA biogenesis factors such as Dicer (Visone and Croce, 2009). In addition, reduced expression of Dicer was also reported to promote tumorigenesis in animal models (Kumar et al, 2009; Lambertz et al, 2010; Sekine et al, 2009). Nevertheless, the expression status of most miRNAs and their biogenesis factors, as well as the mechanisms which regulate the expression of miRNAs in melanoma is mainly unknown.

In the previous chapter, we showed a significant correlation between reduced Sox4 expression and melanoma metastasis. Sox4 has also been reported to bind to the promoter sequences of certain miRNA biogenesis factors and components of the RISC such as Dicer, RNA Helicase A, and Ago 1 in prostate cancer cell lines (Scharer et al, 2009). Nevertheless, the relevance of the interaction between Sox4 and miRNA biogenesis factors in suppression of melanoma invasion is not understood. We hypothesized that Sox4 controls the expression of Dicer in melanoma cells and this event is important for Sox4-medidated regulation of melanoma progression.

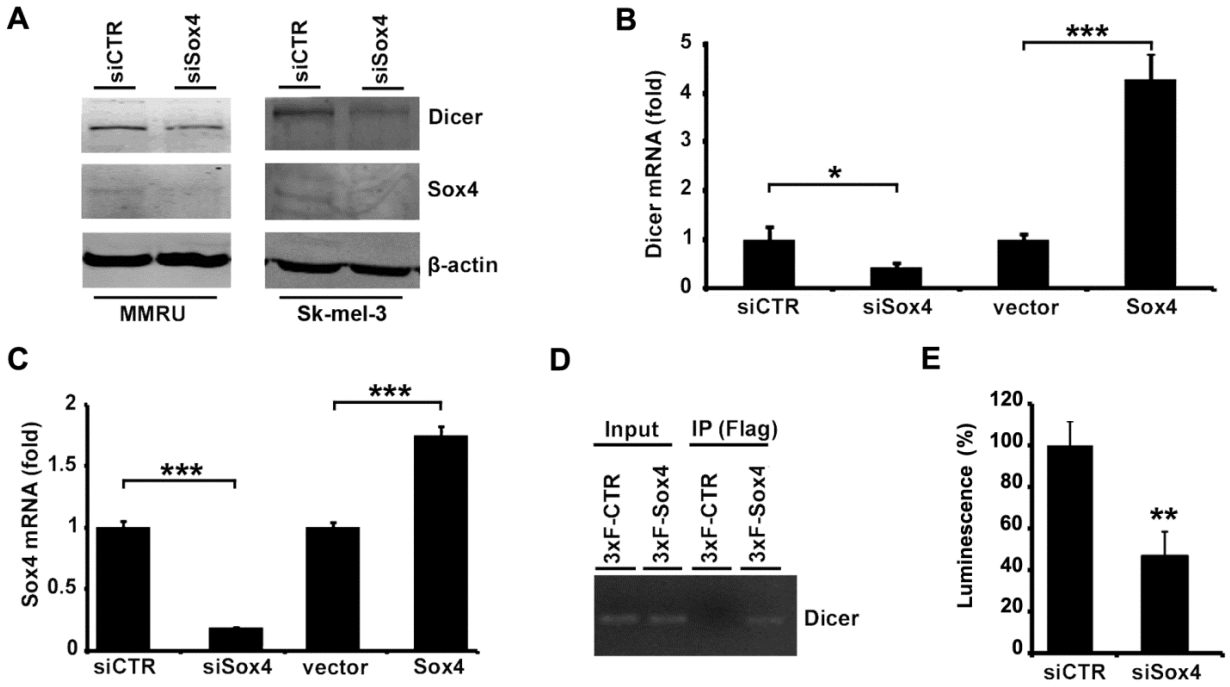
Here we investigated the regulation of Dicer expression by Sox4 in melanoma and its significance in suppression of melanoma invasion. In addition, we examined the expression pattern of Dicer and its role in melanocytic lesions.

4.2. Results

4.2.1. Regulation of Dicer expression by Sox4

To determine whether Sox4 is able to regulate expression of Dicer in melanoma cells, we knocked down Sox4 expression in two different melanoma cell lines and observed a considerable reduction in Dicer expression at protein level (Figure 4.1A). To further investigate the mechanism by which Sox4 regulates Dicer expression, we used real-time qPCR and found that Sox4-KD reduces Dicer mRNA expression by three folds, while a modest increase in Sox4 expression mediated by transfection of MMRU cells with POTB7-Sox4 increases Dicer mRNA expression by four folds (Figure 4.1B and C). In addition, through ChIP assay using anti-Flag antibody mediated pull-down of 3×Flag-Sox4, we confirmed that Sox4 is able to bind to sequences about 2200bp upstream of Dicer transcription start site (Figure 4.1D). Using a luciferase reporter construct under control of the Dicer promoter, we further showed that Sox4-KD resultes in over 50% reduction in Dicer promoter activity (Figure 4.1E). These data indicate that Sox4 positively regulates the expression of Dicer at the transcriptional level by binding to and activating its promoter region.

Figure 4.1. Regulation of Dicer expression by Sox4. (**A**) Western blot assay, showing the reduced expression of Dicer after Sox4-KD. (**B, C**) qPCR showing the reduced expression of Dicer mRNA after Sox4-KD or Sox4 overexpression. β-actin was used as a loading control. (**D**) Flag-Sox4 protein binds to the *Dicer* promoter sequence in MMRU cells, demonstrated by ChIP assay. (**E**) Dicer promoter luciferase reporter activity of control and Sox4-KD MMRU cells. All values are expressed as mean±s.d. **P* < 0.05, ****P* < 0.001, Student's *t*-test.



4.2.2. Suppression of melanoma cell invasion by Sox4 in a Dicer-dependent manner

To study the role of Dicer in melanoma invasion, we examined the ability of MMRU and Sk-mel-3 cells to invade through matrigel using the Boyden chamber assay. We found that Dicer-KD (Figure 4.2A) enhances matrigel invasion of MMRU cells by 2-fold and Sk-mel-3 cells by 1.7-fold compared with the respective controls (Figure 4.2B), indicating that Dicer suppresses invasion of melanoma cells. It might be argued that the enhanced invasion by Dicer-KD might be due to increased cell growth rate and not enhanced matrigel invasion ability. To address this concern we used cell growth assay and found that Dicer-KD MMRU and Sk-mel-3 cells grow slightly, but significantly, slower than their siCTR transfected counterparts (Fig. 4.3A and B), refuting the possibility that the Dicer-KD-induced invasion was due to higher cell growth.

We have showed that Sox4-KD enhanced MMRU cell invasion by 3-fold (Figure 3.3). To understand the significance of Sox4-regulate Dicer expression in suppression of melanoma invasion we overexpressed Flag-Dicer in Sox4-KD cells (Figure 4.2C) and observed that overexpression of Flag-Dicer reduces invasion in Sox4-KD cell by 30 percent (Figure 4.2D), indicating that Flag-Dicer is able to at least partially revert the Sox4-KD phenotype.

Figure 4.2. Dicer expression is required for suppression of cell invasion by Sox4. (**A**) Reduced expression of Dicer after Dicer-KD. (**B**) Enhanced invasion ability of melanoma cells after Dicer-KD. (**C**) Rescued expression of Dicer after transfection of Sox4-KD MMRU cells with Flag-Dicer. (**D**) Overexpression of Dicer reverts the Sox4-KD enhanced MMRU cell matrigel invasion. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$,

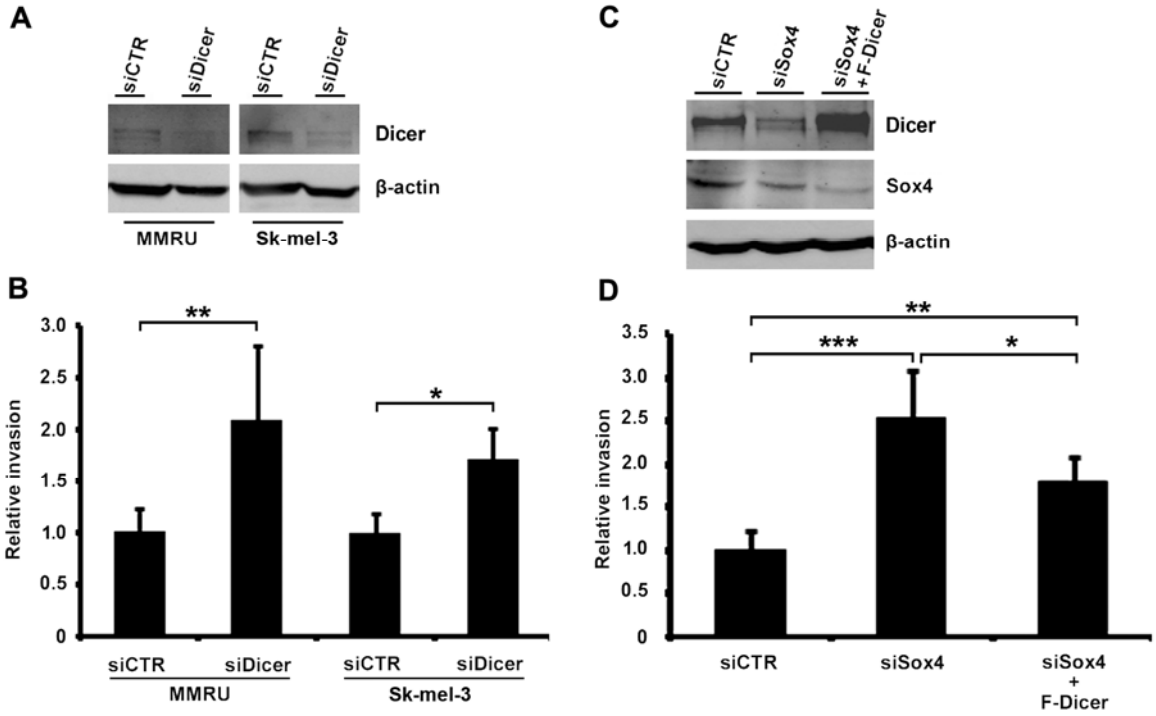
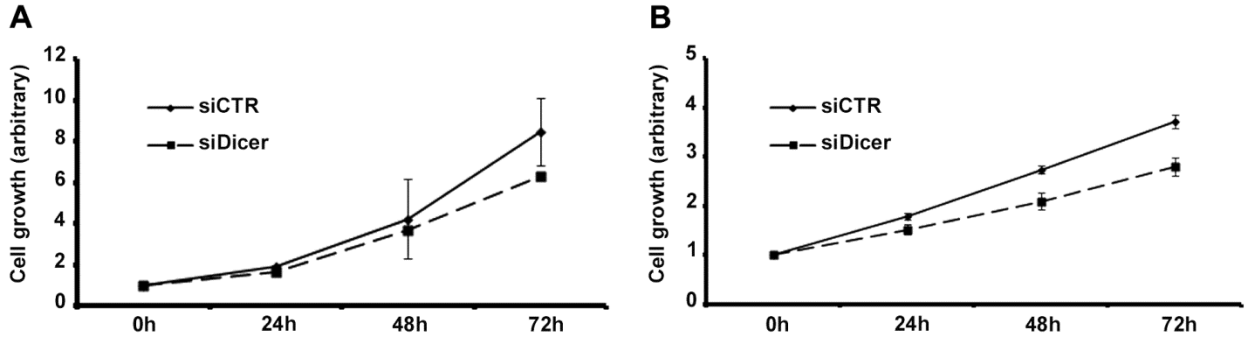


Figure 4.3. SRB assay shows a minor reduction in cell growth of (A) MMRU and (B) Sk-mel-3 cells upon Dicer knockdown.



4.2.3. Reduced cytoplasmic Dicer expression is correlated with melanoma progression

We used TMA technology to investigate the expression pattern of Dicer in melanoma biopsies and its possible correlation with melanoma progression using a polyclonal Dicer-specific antibody (Sigma) (Figure 4.4A). Notably, a significant difference in cytoplasmic Dicer staining was observed between different stages of melanoma. Kruskal-Wallis test revealed a clear reduction in the expression of Dicer in metastatic melanoma compared with other stages of melanocytic lesions ($P = 0.003$; Figure 4.4B), but not among normal nevi dysplastic nevi and primary melanomas. In addition, when we grouped the samples into negative, weak, or strong cytoplasmic Dicer staining, we found a significant difference between primary melanomas and metastatic melanomas ($P = 0.0001$, χ^2 test; Figure 4.4C), although the differences among normal nevi, dysplastic nevi, and primary melanomas were not significant ($P > 0.05$). Our data further demonstrated that Dicer expression is also inversely correlated with American Joint Committee on Cancer (AJCC) stages ($P = 0.003$, χ^2 test; Figure 4.4D).

In addition, we found that expression of cytoplasmic Dicer is positively correlated with lymphocytic response in primary melanoma cases ($P = 0.036$, χ^2 test; Table 4.1). Furthermore, primary melanoma samples from male individuals showed less Dicer expression compared to their female counterparts ($P = 0.009$, χ^2 test; Table 1). We did not find any significant correlation between cytoplasmic Dicer expression and patient age, tumor thickness, location, subtype, or ulceration status (Table 4.1).

To further validate these findings we used a monoclonal Dicer-specific antibody (Clonegene) to probe for the expression of Dicer in a smaller TMA construct, containing 31 cases of dysplastic nevi, 71 cases of primary and 46 cases of metastatic melanomas (Figure 4.5A). Our analysis confirmed that expression of cytoplasmic Dicer is significantly lost in

metastatic melanomas compared with dysplastic nevi and primary melanomas ($P = 0.001$, χ^2 test; Figure 4.5B). We did not observe any significant correlation between the expression of cytoplasmic Dicer and clinicopathologic parameters using this antibody (Table 4.2).

Table 4.1. Cytoplasmic Dicer staining and clinicopathologic characteristics of 262 cases of primary melanomas.

	Cytoplasmic Dicer staining				
	Negative	Weak	Strong	Total	*P* value*
Age					
≤62	15 (11.2%)	31 (23.1%)	88 (65.7%)	134	0.702
>62	15 (11.7%)	35 (27.3%)	78 (61.0%)	128	
Sex					
Male	23 (16.7%)	32 (23.2%)	83 (60.1%)	138	0.009
Female	6 (4.8%)	35 (28.2%)	83 (67.0%)	124	
Tumor thickness (mm)					
≤2 mm	12 (8.2%)	34 (23.1%)	101 (68.7%)	147	0.073
>2mm	18 (15.7%)	32 (27.8%)	65 (56.5%)	115	
Ulceration					
Present	8 (14.8%)	14 (25.9%)	32 (59.3%)	54	0.648
Absent	22 (10.6%)	52 (25.0%)	134 (64.4%)	208	
Lymphocytic response					
Present	12 (12.8%)	15 (15.9%)	67 (71.3%)	94	0.036
Absent	18 (10.7%)	51 (30.4%)	99 (58.9%)	168	
Tumor subtype					
Lentigo maligna	10 (21.3%)	9 (19.1%)	28 (59.6%)	47	0.256
Superficial spreading	8 (8.8%)	24 (26.4%)	59 (64.8%)	91	
Nodular	5 (10.9%)	9 (19.6%)	32 (69.5%)	46	
Other†	7 (9.0%)	24 (30.8%)	47 (60.2%)	78	
Site‡					
Sun-exposed	13 (13.8%)	22 (23.4%)	59 (62.8%)	94	0.632
Sun-protected	17 (10.1%)	44 (26.2%)	107 (63.7%)	168	

*÷^{2} test.

†Other: unspecified subtype.

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

Figure 4.4. Reduced expression of cytoplasmic Dicer correlates with melanoma progression. (**A**) Representative images of NN and DN with strong cytoplasmic staining, PM with moderate and MM with negative cytoplasmic Dicer staining. (**B**) Kruskal-Wallis test for differences in Dicer staining among NN, DN, PM, and MM. The median is depicted as a horizontal line inside each box (P = 0.003). (**C**) χ^2 test for differences in Dicer staining in NN, DN, PM, and MM. Significant difference was found between PM and MM (P = 0.0001). (**D**) Cytoplasmic Dicer expression is negatively associated with AJCC stage of melanoma cases (P = 0.003, χ^2 test).

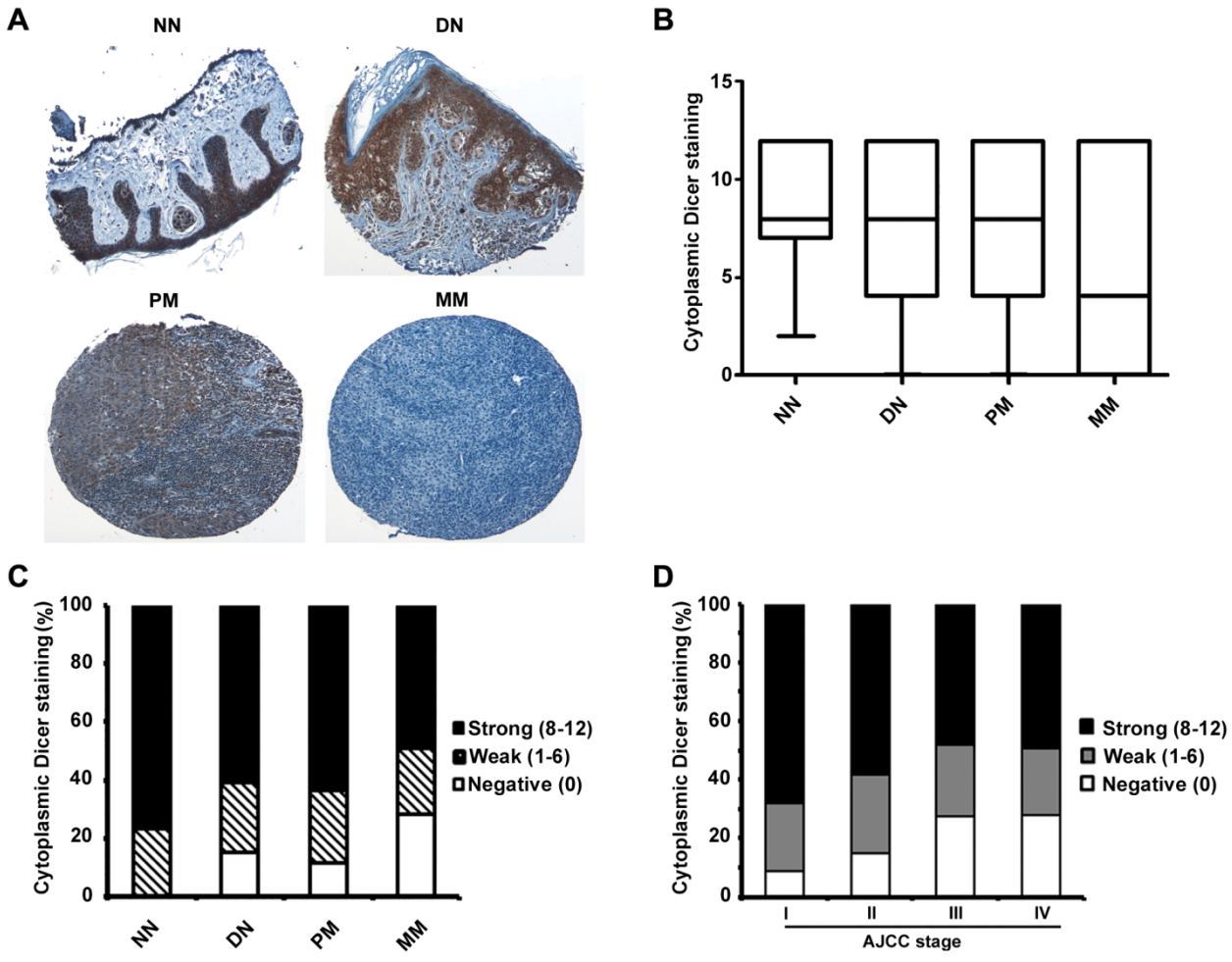


Figure 4. 5. Tissue microarray using a second Dicer-specific monoclonal antibody. (**A**) Representative images of DN and PM with strong cytoplasmic Dicer staining and MM with weak Dicer staining. (**B**) Statistical analysis shows a significant reduction in Dicer expression in metastatic melanoma compared with dysplastic nevi and primary melanoma ($P = 0.007$, χ^2 test).

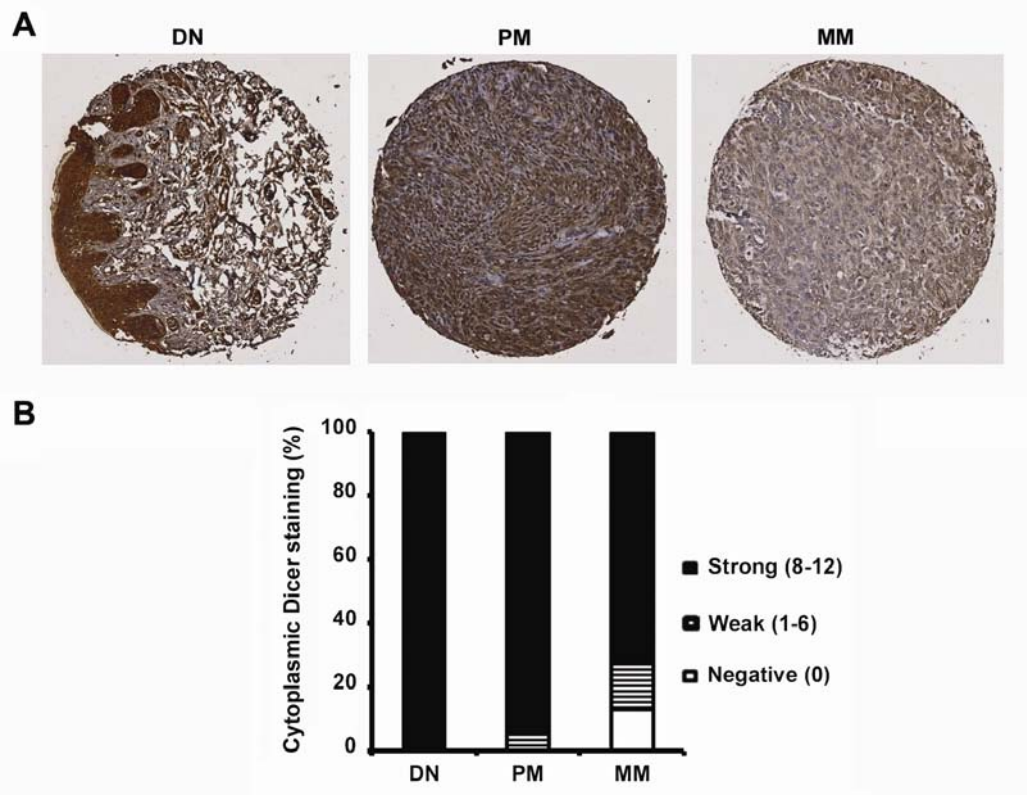


Table 4.2. Cytoplasmic Dicer staining and clinicopathologic characteristics of 71 primary melanomas.

	Cytoplasmic Dicer staining*			
	Weak	Strong	Total	P value#
Age				
≤59	3 (8.1%)	34 (91.9%)	37	0.346
>59	1 (2.9%)	33 (97.1%)	34	
Sex				
Male	4 (8.7%)	42 (91.3%)	46	0.129
Female	0 (0%)	25 (100%)	25	
Tumor thickness (mm)				
≤2	3 (7.3%)	38 (92.7%)	41	0.472
>2	1 (3.3%)	29 (96.7%)	30	
Ulceration				
Present	0 (0%)	16 (100%)	16	0.267
Absent	4 (7.3%)	51 (92.7%)	55	
Lymphocytic response				
Present	0 (0%)	4 (100%)	4	0.665
Absent	3 (4.5%)	64 (95.5%)	67	
Tumor subtype				
Lentigo maligna	0 (0%)	15 (100%)	15	0.235
Superficial spreading	3 (11.5%)	23 (88.5%)	26	
Other†	1 (3.3%)	29 (96.7%)	30	
Site‡				
Sun-exposed	1 (6.3%)	15 (93.7%)	16	0.903
Sun-protected	3 (5.5%)	52 (94.5%)	55	

*There was no core with negative cytoplasmic Dicer staining for primary melanoma cases.
χ^2 test.
† Other: unspecified subtype.
‡ Sun-protected sites: trunk, arm, leg and feet; Sun-exposed sites: head and neck.

4.2.4. Cytoplasmic Dicer staining positively correlates with better patient survival

We evaluated the correlation between cytoplasmic Dicer expression and 5-year survival of primary and metastatic melanoma patients by constructing Kaplan-Meier survival curves. Overall survival in strong Dicer staining group was 63.6% compared with 52.1% in weak and

46.2% in negative Dicer staining group (P = 0.007, log rank test; Figure 4.6A). In addition, the disease-specific 5-year survival of the patients was significantly reduced from 67.1% in strong Dicer staining group to 62.5% in weak and 47.7% in negative Dicer staining group (P = 0.008, log rank test; Figure 4.6B), indicating a significant positive correlation between Dicer expression and melanoma patients survival. We also performed multivariate Cox regression analysis including cytoplasmic Dicer staining, age and sex. The results revealed that Dicer staining is able to predict both overall and disease-specific patient survival independent of age or sex of the patients (P = 0.002 and 0.007, respectively; Table 4.3). Using data obtained with the second anti-Dicer antibody, we also observed a clear positive correlation between Dicer expression and 5-year patient survival. Although the correlation between Dicer expression and overall survival was not significant (P = 0.077, log rank test; Figure 4.7A), Dicer expression was significantly correlated with disease-specific survival (P = 0.037, log rank test; Figure 4.7B).

Figure 4.6. Dicer expression positively correlates with 5-year survival of all primary and metastatic melanoma patients. (**A**) Overall and (**B**) disease-specific 5-year survival of melanoma patients.

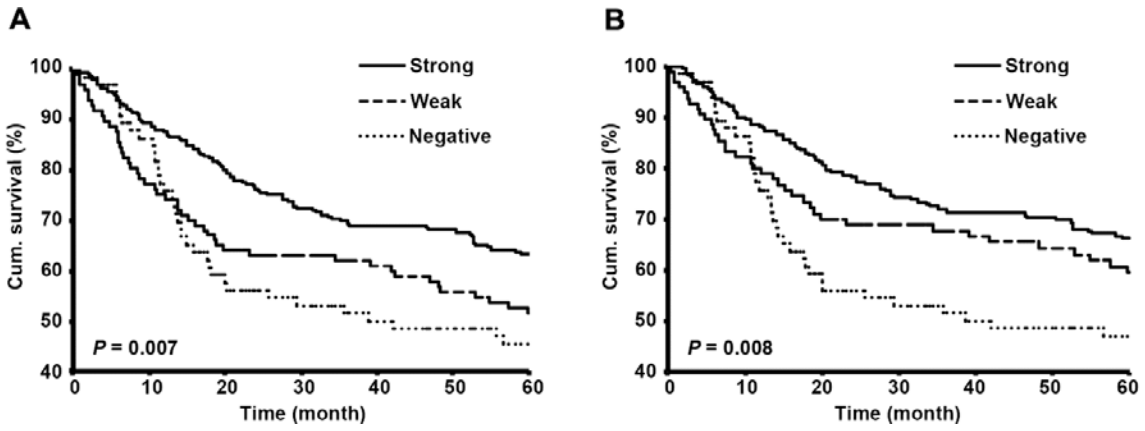
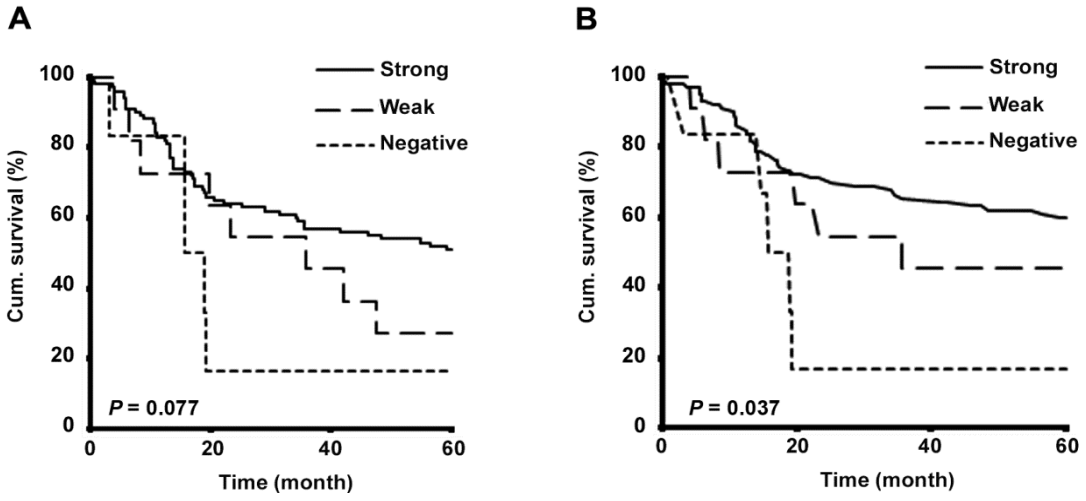


Table 4.3. Multivariate Cox regression analysis of cytoplasmic Dicer expression in 262 primary melanomas and 135 metastatic melanomas.

	Overall survival			Disease-specific survival		
Variables*	Hazard ratio	95% CI†	*P* value	Hazard ratio	95% CI†	*P* value
Dicer	0.618	0.455-0.839	0.002	0.639	0.462-0.885	0.007
Age	1.068	0.786-1.451	0.670	1.087	0.786-1.502	0.612
Sex	0.985	0.723-1.341	0.924	1.007	0.726-1.398	0.963

*Coding of variables: Cytoplasmic Dicer staining was coded as 1 (negative-weak) and 2 (strong). Age was coded as 1 (>60 years), and 2 (<60 years). Gender was coded as 1 (female) and 2 (male).
†CI, confidence interval.

Figure 4.7. Expression pattern of cytoplasmic Dicer using the second Dicer specific antibody positively correlates with patients' survival. (**A**) Although not statistically significant, a trend in positive correlation between cytoplasmic Dicer expression and overall 5-year survival of melanoma patients is evident. (**B**) Dicer expression positively correlates with disease-specific 5-year survival of melanoma patients.



4.2.5. Positive correlation between expression of Sox4 and Dicer in melanoma patients

We used our previous data for Sox4 TMA and obtained the cytoplasmic Dicer expression for the corresponding cases from the current TMA data set to compare the expression pattern of both Sox4 and Dicer in the same melanocytic lesions. We divided all cases based on the expression pattern of Sox4 into weak Sox4 (42 cases) or strong Sox4 group (102 cases) and found that expression of Dicer is considerably higher in cases with strong Sox4 staining than those with weak Sox4 staining ($P = 0.004$, χ^2 test; Figure 4.8A). Accordingly, the percentage of cases with strong Dicer staining increased from 9% in weak Sox4 group to 34% in strong Sox4 group while the percentage of cases with negative Dicer staining decreases from 45% in weak Sox4 group to 24% in strong Sox4 group.

4.2.6. Sox4 regulates the expression of a large subset of miRNAs

Considering the regulation of Dicer expression by Sox4 and to investigate whether Sox4 has a role in biogenesis of miRNAs in melanoma cells we used a qPCR based miRNA profiling method to detect the changes in expression of all validated and predicted mature human miRNAs after Sox4-KD in MMRU cells. Our results showed at least 2-fold downregulation of 34.9% of miRNAs in Sox4-KD cells compared with siCTR transfected control cells. Sox4-KD also increased the expression of 26.5% of miRNAs by at least 2-fold and had no significant effect on the other 38.6% miRNAs (Figure 4.8B). These results indicated a major change in the expression pattern of more than 60% of miRNAs after Sox4-KD in melanoma cells, implying the critical role of Sox4 in this process. Next, to determine the most important biological functions that are affected by Sox4-KD mediated change in miRNA expression we used Ingenuity Pathway Analysis (IPA) software and analyzed the miRNAs with at least 2-fold up- or down-regulation after Sox4-KD. Our results demonstrated that reproductive system diseases-related miRNAs

show the most significant change after Sox4-KD in melanoma cells (Figure 4.8C). Interestingly, cancer related miRNAs also showed a very significant change (Figure 4.8C). According to IPA's database, 61 cancer related miRNAs were affected by Sox4-KD, 53 of which were at least 2-fold downregulated (Table 4.4). In addition, between 21 metastasis-related miRNAs which were affected by Sox4-KD, 19 miRNAs showed at least 2-fold downregulation (Table 4.5). Moreover, 26 out of 28 melanoma-related miRNAs identified by this software were at least 2-fold downregulated after Sox4-KD (Table 4.6).

We also screened for expression profile of miRNAs in Sox4-KD MMRU cells after rescuing Dicer expression by exogenous Flag-Dicer. Consistent with a downstream role of Dicer in Sox4-regulated miRNA expression, we found a reversal in the expression of a majority of miRNAs which showed either reduced or increased expression after Sox4-KD with overexpression of Flag-Dicer (GEO database; accession# GSE36715). For instance, of 307 miRNAs with more than two-fold reduced expression after Sox4-KD, expression of 198 miRNAs were either comparable or more than control group after rescue of Dicer expression (Table 4.7). Interestingly, rescue of Dicer expression also revert the Sox4-KD induced changes in expression of majority miRNAs involved in cancer, metastasis or melanoma (Tables 4.4, 4.5 & 4.6).

Figure 4.8. Regulation of miRNAs expression by Sox4 in melanoma. (**A**) Positive correlation between Sox4 and Dicer expression in 144 melanocytic lesions at different stages. Dicer expression was compared between weak or strong Sox4 expressing cases ($P = 0.004$, χ^2 test). (**B**) Pair-wise comparison of miRNAs in siSox4 vs siCTR cells based on their expression fold-change. (**C**) Functional analysis of the miRNA profiling data using the Ingenuity Pathway Analysis. The horizontal line indicate the *p*-value = 0.05 threshold.

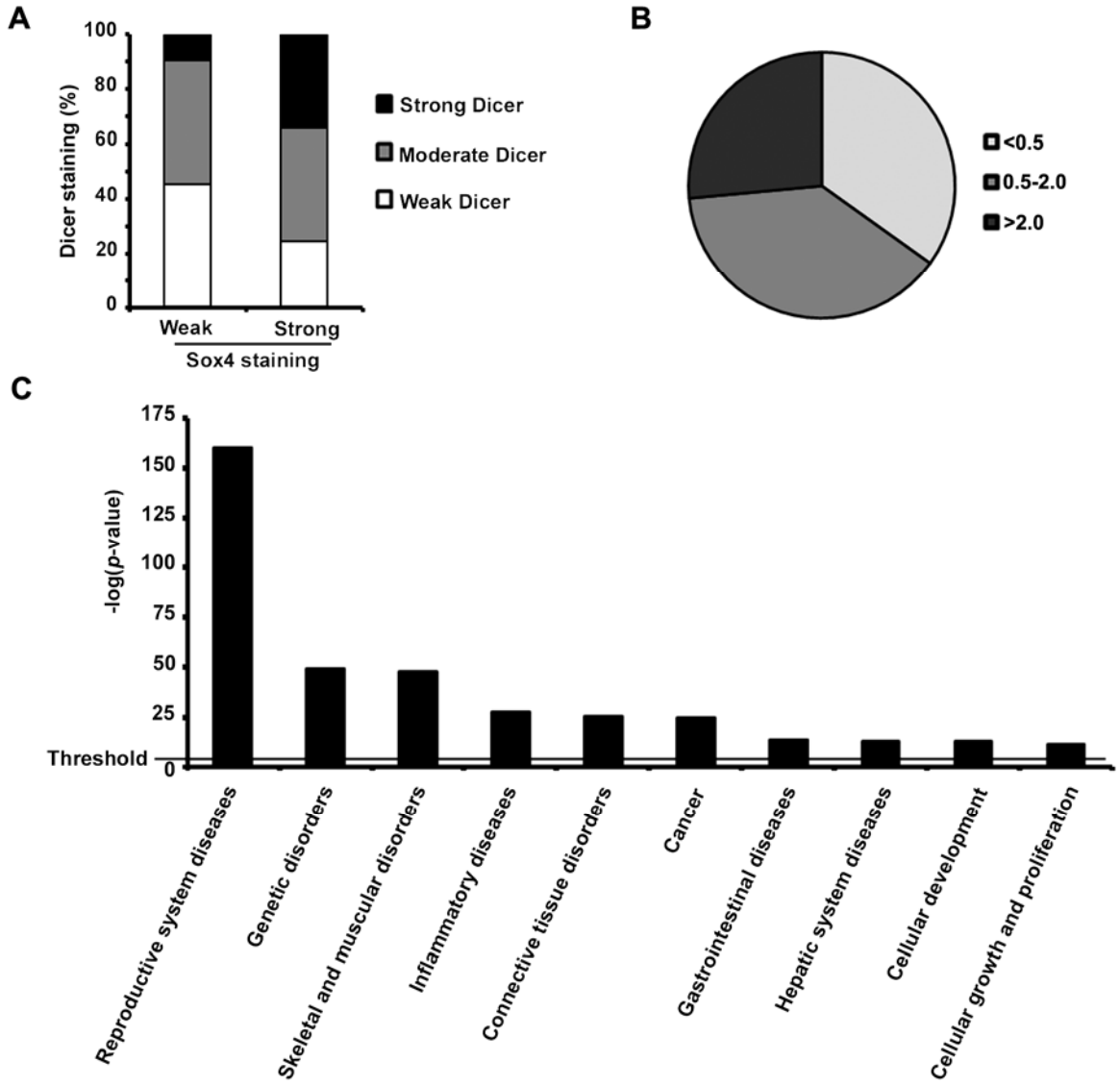


Table 4.4. Expression of cancer-related miRNAs in Sox4-KD MMRU cells and in Sox4-KD cell in which expression of Dicer is rescued.

miRNA	siSox4/siCTR*	siSox4+F-Dicer/siCTR**
let-7g	-4.925	-2.341
miR-1	-3.138	-21.321
miR-8	-6.646	-1.184
miR-9	-5.251	-0.604
miR-10a	-6.498	-1.457
miR- 15b	-4.199	-17.642
miR-17	-7.781	-0.600
miR-19b	-6.589	-0.461
miR-21	-3.972	-0.761
miR-22	-2.462	-0.686
miR-23	-2.809	-0.416
miR-24	-2.266	-0.721
miR-25a	-1401.251	-1.411
miR-26a	-897.644	-5.681
miR-27b	-3.945	-0.363
miR-29c	-51.984	-43.188
miR-30b	-4.257	-1.337
miR-31	-3.580	-0.891
miR-32	-5.657	-2.607
miR-34c-3p	-7.013	-1.333
miR-95	4.555	-1.918
miR-99a	-2.147	-0.095
miR-101	-4.199	-12.075
miR-103	-4.724	-1.524
miR-122	-3.972	-1.511
miR-125b	-7.781	-10.575
miR-130	-4.823	-1.101
miR-132	-2.014	-1.723
miR-133	-3.758	-1.197
miR-135	-5.736	-1.054
miR-136	-2.158	-1.097
miR-143	-2.204	-1.229
miR-146b-5p	-2.129	-64.988
miR-149	-2.657	-1.655
miR-150	-2.158	-2.131
miR-154	-8.014	-1.382
miR-155	-3.272	-1.283
miR-181a	-5.657	-1.047
miR-182	-2.639	-0.902
miR-183	4.823	-1.057
miR-188	2.863	-3.022

miRNA	siSox4/siCTR*	siSox4+F-Dicer/siCTR**
miR-191	-2.158	-0.344
miR-193a-5p	-2.000	-1.445
miR-194	-3.387	-7.924
miR-196a	-2.479	-0.652
miR-199a-5p	177.294	-1.801
miR-204	-4.056	-1.325
miR-205	-2.071	-3.097
miR-210	-3.482	-3.007
miR-218	-3.706	-1.217
miR-290	-4.141	-1.007
miR-324	-2.521	-10.045
miR-329	19.260	-7.990
miR-342-5p	-2.888	-1.961
miR-345	-3.387	-3.946
miR-363	-2.099	-0.320
miR-375	-10.004	-2.171
miR-449c	98.873	-1.957
miR-490-5p	3.965	-0.793
miR-499-5p	-10.144	-0.719
miR-515-3p	67.066	-1.712

*Expression ratio in Sox4-KD cells comparing to control cells.

** Expression ratio in Sox4-KD+F-Dicer cells comparing to control cells

Table 4.5. Expression of metastasis-related miRNAs in siSox4-treated MMRU cells.

miRNA	siSox4/siCTR*	siSox4+F-Dicer/siCTR**
let-7g	-4.925	-2.341
miR-1	-3.138	-21.321
miR-8	-6.646	-1.184
miR-23a	-2.809	-0.416
miR-27b	-3.945	-0.363
miR-32	-5.657	-2.607
miR-34c-3p	-7.013	-1.333
miR-122	-3.972	-1.511
miR-125b	-7.781	-10.575
miR-130	-4.823	-1.101
miR-133	-3.758	-1.197
miR-135	-5.736	-1.054
miR-146b-5p	-2.129	-64.988
miR-149	-2.657	-1.655
miR-150	-2.158	-2.131
miR-155	-3.272	-1.283
miR-183	4.823	-1.057
miR-196a	-2.479	-0.652
miR-199a-5p	177.294	-1.801
miR-205	-2.071	-3.097
miR-363	-2.099	-0.320

* Expression ratio in Sox4-KD cells comparing to control cells.
** Expression ratio in Sox4-KD+F-Dicer cells comparing to control cells

Table 4.6. Expression of melanoma-related miRNAs in siSox4-treated MMRU cells.

miRNA	siSox4/siCTR*	siSox4+F-Dicer/siCTR**
let-7g	-4.925	-2.341
miR-1	-3.138	-21.321
miR-8	-6.646	-1.184
miR-9	-5.251	-0.604
miR-21	-3.972	-0.761
miR-23a	-2.809	-0.416
miR-27b	-3.945	-0.363
miR-29c	-51.984	-43.188
miR-30	-4.257	-1.337
miR-32	-5.657	-2.607
miR-34 c-3p	-7.013	-1.333
miR-99a	-2.147	-0.095
miR-125b	-7.781	-10.575
miR-130	-4.823	-1.101
miR-132	-2.014	-1.723
miR-133a	-3.758	-1.197
miR-135a	-5.736	-1.054
miR-146b-5p	-2.129	-64.988
miR-149	-2.657	-1.655
miR-150	-2.158	-2.131
miR-155	-3.272	-1.283
miR-183	4.823	-1.057
miR-196a	-2.479	-0.652
miR-199a-5p	177.294	-1.801
miR-205	-2.071	-3.097
miR-218	-3.706	-1.217
miR-290	-4.141	-1.007
miR-363	-2.099	-0.320

*Expression ratio in Sox4-KD cells comparing to control cells.
** Expression ratio in Sox4-KD+F-Dicer cells comparing to control cells

Table 4.7. Expression profile of 307 miRNAs with reduced expression in Sox4-KD cells after Dicer is rescued by exogenous expression of Flag-Dicer.

siSox4/siCTR*	No. of miRNAs	siSox4+-F-Dicer/CTR**	No. of miRNAs
< 0.1	27	< 0.1	16
0.1-0.249	101	0.1-0.249	24
0.25-0.4.99	179	0.25-0.4.99	69
		0.5-1.99	175
		≥2	23

*Expression of miRNAs in siSox4 cells normalized by their expression levels in siCTR control group. The siCTR group cells were transfected with control siRNA.
** Expression of miRNAs in siSox4+F-Dicer cells normalized by their expression levels in CTR control group. The CTR group cells were transfected with control siRNA followed by transfection with empty Flag vector.

4.3. Discussion

Metastasis is the leading cause of melanoma-related death. Therefore, identifying the factors which are involved in regulation of melanoma invasion and metastasis is of outstanding priorities. We previously showed that expression of Sox4 is reduced in metastatic melanoma and revealed that Sox4 suppressed melanoma cell migration and invasion at least partially through suppression of NF-κB p50 expression. Different groups investigated the pattern of Sox4 binding to the promoters or regulation of expression of its target genes in human genome (Castillo et al, 2011; Liao et al, 2008; Scharer et al, 2009) which created a wealth of knowledge regarding Sox4's target network. However, these high-throughput screenings did not return consistent results. For instance, Sox4 was shown to bind to the promoter of Dicer in a prostate carcinoma cell line (Scharer et al, 2009), while the same observation was not made in small cell lung cancer

(Castillo et al, 2011), nor was Sox4 knockdown shown to change Dicer expression in hepatocellular carcinoma cell lines (Liao et al, 2008). Although the actual reason for this discrepancy is not known, differential sensitivity of the methods and variations in the biology of different tissues might play a role.

In this study, we investigated the regulation of Dicer expression by Sox4 in melanoma and its significance in Sox4-mediated suppression of melanoma invasion. We observed that Sox4 is able to regulate expression of Dicer at both mRNA and protein levels (Figure 4.1A and B). Using overexpression of 3×Flag-Sox4 construct and pull-down with anti-Flag antibody, we were able to confirm by ChIP assay that Sox4 binds to *Dicer* promoter sequences in melanoma cells (Figure 4.1D). Interestingly, the region which showed the highest degree of amplification after pull-down of 3×Flag-Sox4, contains two adjacent consensus Sox4 binding sites (AACAAAG and AACAATA) (Vandewetering et al, 1993; Wotton et al, 1995), which may explain the significance of Sox4 binding to this region. Reduction of the Dicer promoter activity upon Sox4-KD, as shown by luciferase reporter assay (Figure 4.1E), further confirmed the Sox4-regulated regulation of Dicer transcription. To understand the role of Dicer in regulation of melanoma invasion, we used matrigel invasion assay and for the first time revealed that knockdown of Dicer augments invasion ability of two different melanoma cells (Figure 4.2B). Consistently, Dicer expression had been previously proven critical in suppression of metastasis in multiple types of cancer, downregulation of which would enhance tumor metastasis (Martello et al, 2010; Su et al, 2010). We also observed that overexpression of Dicer in melanoma cells can revert the Sox4-KD phenotype (Figure 4.2D), indicating the downstream role of Dicer in Sox4-mediated suppression of melanoma invasion.

By using immunohistochemistry (Figure 4.4A), we observed a significant reduction in cytoplasmic Dicer expression in metastatic melanoma compared with primary melanoma, dysplastic nevi and normal nevi (Figure 4.4B and C), suggesting that reduced or lost expression of Dicer may be relevant in the process of melanoma metastasis. Interestingly, we also observed an inverse correlation between Dicer expression and AJCC staging of the melanoma cases (Figure 4.4D). Accordingly, the main and only significant difference in cytoplasmic staining was between AJCC stage II and III, which corresponds to transition from primary melanoma to lymph node metastasis. This observation is consistent with our Kruskal-Wallis and χ^2 tests (Figure 3B and C), in which Dicer expression is mainly reduced in metastatic melanomas compared with the earlier stages of melanocytic lesions. Altogether, these data suggest that Dicer expression is reduced during the course of melanoma progression, implying an inhibitory role for Dicer in this process.

Aberrant expression of Dicer has been reported in multiple types of tumors. For instance, reduced expression of Dicer was shown in ovarian, lung, hepatocellular and basal cell carcinomas (Faggad et al, 2010; Karube et al, 2005; Merritt et al, 2008; Sand et al, 2010; Wu et al, 2011). Overexpression of Dicer was reported in prostate cancer, colon and squamous cell carcinomas (Chiosea et al, 2006; Faber et al, 2011; Sand et al, 2010). This obvious discrepancy between different reports highlights the possibility of tissue specific function for Dicer in cancer development.

In a recent report, (Ma et al, 2011) Dicer protein was found to be upregulated in melanoma compared to other skin cancers such as carcinomas and sarcomas. When compared among all examined cutaneous malignancies, they found Dicer upregulation in a tumor-type specific manner, namely in melanoma compared with melanocytic nevi (Ma et al, 2011). We believe that

this discrepancy might be due to differences in sample size (521 cases in our study compared with 223 cases used by Ma *et al*) which could significantly affect the power of the study, inherent differences in the source populations from which the samples were collected or possible differences in the immunoreactivity of the antibodies. To further address this concern, we performed immunohistochemistry on a TMA construct containing 31 cases of dysplastic nevi, 71 cases of primary melanomas and 46 cases of metastatic melanomas using the same antibody mentioned in Ma et al report (Figure 4.5). We observed that cytoplasmic Dicer staining using this antibody closely resembles our original TMA analysis (Figure 4.4), as opposed to what Ma et al observed. This further verifies our original findings that Dicer expression is decreased during melanoma progression which is in line with our *in vitro* results that suggest Dicer as a suppressor of melanoma cell invasion. Furthermore, we found that Dicer expression positively correlates with overall and disease-specific survival of melanoma patients (Figure 4.6A and B), confirmed by a second anti-Dicer antibody (Figure 4.7). The survival analysis using data produced by both antibodies revealed a positive correlation between Dicer expression and disease specific 5-year survival (Figure 4.6B and Figure 4.7B). However, despite an observed significant correlation between cytoplasmic Dicer expression and overall patient survival (P = 0.007; Figure 4.6A) using the first antibody, we found no significant correlation between overall survival and Dicer expression using the second antibody (P = 0.077; Figure 4.7B). We believe this could be due to smaller sample size with the second antibody (n = 117) compared with the analysis with the first antibody (n = 397).

This is the first study on the correlation between Dicer expression and melanoma patient survival. As shown in Figure 4.2B, Dicer-KD significantly stimulated melanoma cell invasion which is one of the most critical events in the process of cancer progression toward metastasis

(Gupta and Massague, 2006). This suggests that Dicer acts as an inhibitor of melanoma metastasis, which is well-recognized as the most important cause of melanoma related death. Moreover, we found a positive correlation between expression of Dicer and lymphocytic response in primary melanomas (Table 4.1). Lymphocytic response in melanoma is known to be responsible for killing tumor cells and may induce spontaneous regression. In addition, lymphocytic response in melanoma vertical growth phase is a prognostic factor associated with better patient survival (Spatz et al, 2010). Suppression of melanoma invasion and enhancement of lymphocytic response may explain the observed positive correlation between Dicer expression and higher survival rate in patients.

Interestingly, we also observed less expression of cytoplasmic Dicer in male samples compared with female individual ($P = 0.009$, Table 4.1). Using the second anti-Dicer antibody, we found a similar trend, although not statistically significant possibly due to smaller sample size (Table 4.2). This difference in Dicer staining between different sexes may be due to unequal representation of each gender in different stages of melanoma. In fact, we had 70 female and 67 male samples with tumors at the AJCC stage I and 53 female and 72 male in AJCC stage II. Therefore, the percentage of female samples in the less advanced stage I melanoma was more (57%) than that of more advanced stage II (43%), whereas the percentage of male samples in stage I melanoma was less (48%) than that of stage II (52%). However, we cannot exclude the possible gender-specific differences in Dicer expression, especially during melanomagenesis.

By comparing the expression status of Sox4 and Dicer proteins in our TMA database, we found that there is a significant positive correlation between their expression patterns (Figure 4.8A), further confirming our observation that Dicer expression is regulated by Sox4 in melanoma cells. As a matter of fact, this phenomenon may not be exclusive to melanoma. For

instance, independent studies demonstrated overexpression of both Dicer and Sox4 in prostate cancer (Chiosea et al, 2006; Liu et al, 2006) and downregulation of both proteins in gallbladder carcinoma (Shu et al, 2012; Wang et al, 2012a). Considering the observed upregulation of Dicer by Sox4 in prostate cancer cell lines (Scharer et al, 2009), it would be interesting to study the correlation between their expressions in these types of malignancy.

Finally, we investigated the role of Sox4 in biogenesis of mature miRNAs in melanoma cells *in vitro*. Our data demonstrated that Sox4-KD alters the expression of the majority of miRNAs expressed by these cells (Figure 4.8B) implying involvement of Sox4 in the process of miRNA biogenesis. Consistent with our other observations, we found that rescued Dicer expression can revert the Sox4-KD induced changes in the expression of a majority of miRNAs. Our analysis showed a clear reversion in expression of those miRNAs which were either up- or down-regulated upon Sox4-KD toward normalcy after rescue of Dicer expression indicating the requirement for Dicer in regulation of miRNAs expression by Sox4 (Table 4.4, 4.5, 4.6, 4.7). It is noteworthy that, although expression of majority miRNAs shifted upon overexpression of Dicer in Sox4-KD cells, a considerable number of miRNAs did not show a significant change which indicates existence of mechanisms other than Dicer upregulation by which Sox4 can modify the expression of these miRNAs. Regulating the expression of other miRNA biogenesis factors such as Drosha and Ago1 by Sox4, direct effect of Sox4 on promoters which control the expression of some pri-miRNAs and indirect effect of Sox4 on transcription of pri-miRNAs through downstream transcription factors such as p53, the expression of which has been shown to be regulated by Sox4 (Pan et al, 2009), are a few possible scenarios which need verification in future studies. We also studied the cellular functions that are regulated by those miRNAs, expression of which were affected by Sox4, using Ingenuity Pathway Analysis software. Our

data revealed that reproductive system diseases represented the most relevant function in our analysis. This observation is not surprising, as Sox4 has been previously shown to be involved in development of reproductive system (Graham et al, 1999a; Hunt and Clarke, 1999). Cancer related miRNAs were also one of the most significantly changed groups in our study (Figure 4.8C). Further analysis revealed that Sox4-KD affects the expression of cancer, metastasis and melanoma-related miRNAs, the majority of which are more than 2-fold down-regulated (Table 4.4, 4.5, 4.6). To our knowledge, this was the first study to investigate the role of Sox4 in regulation of miRNAs biogenesis and demonstrated the significant effect of this gene in the expression profile of miRNAs in melanoma cells.

Although we showed that Sox4 and Dicer regulates invasion of melanoma cells *in vitro* and that their reduced expression levels are exhibited in advanced stages of melanoma, it is not known whether low expression levels of Sox4 and or Dicer would promote melanoma metastasis *in vivo*. We believe metastasis assays in mice, either using xenografts or tail vein injection of melanoma cells with Sox4 and Dicer knockdown would address this question in the future.

In conclusion, our data suggest a reduction in expression of Dicer in melanoma which could be due to downregulation of its upstream regulator Sox4, resulting in perturbation of the basic machinery controlling miRNA biogenesis in melanoma cells. We found that similar to Sox4, Dicer expression is positively correlated with melanoma patient survival, revealing the prognostic value of Dicer in melanoma. We further showed that alterations in expression of Sox4 and Dicer significantly affect the invasive potential of melanoma cells and may play a key role in molecular pathogenesis of melanoma.

Chapter 5. Reduced expression of nuclear Dicer correlates with melanoma progression

5.1. Background and rationals

Dicer is a highly conserved protein in eukaryotes (Murphy et al, 2008). Some organisms such as *D. melanogaster* encode multiple Dicer proteins, whereas human and mice encode only one Dicer (Lee et al, 2004). Since the initial discovery of Dicer, it was believed that Dicer protein is strictly localized in the cytoplasm (Lee et al, 2002). However, in the last few years new functions have been assigned to Dicer in both lower and higher eukaryotes which require its localization in the nucleus (Nakagawa et al, 2010). For instance, DCL1, the Arabidopsis homologue of Dicer, has been shown to be localized to nucleus, in which it functions as part of the miRNA production complex (Fujioka et al, 2007). Others reported that mammalian Dicer associates with ribosomal DNA (rDNA) in cells, probably contributing to maintaining the integrity of rDNA (Sinkkonen et al, 2010). In addition, Dicer was recently shown to interact with nuclear pore complex proteins and reside in the nucleus in human cells (Ando et al, 2011).

In previous chapter we showed a reduced expression of cytoplasmic Dicer in metastatic melanoma and requirement of Dicer expression for suppression of melanoma invasion. Nevertheless, so far very little is known about the expression pattern of nuclear Dicer and its role in malignancies. We hypothesized that Dicer is not uniquely expressed in cytoplasm of melanocytic cells and that changes in nuclear expression of Dicer may have some significance in melanomagenesis. To validate this hypothesis, we analyzed the expression pattern of nuclear Dicer in melanocytic lesions in our tissue microarray database.

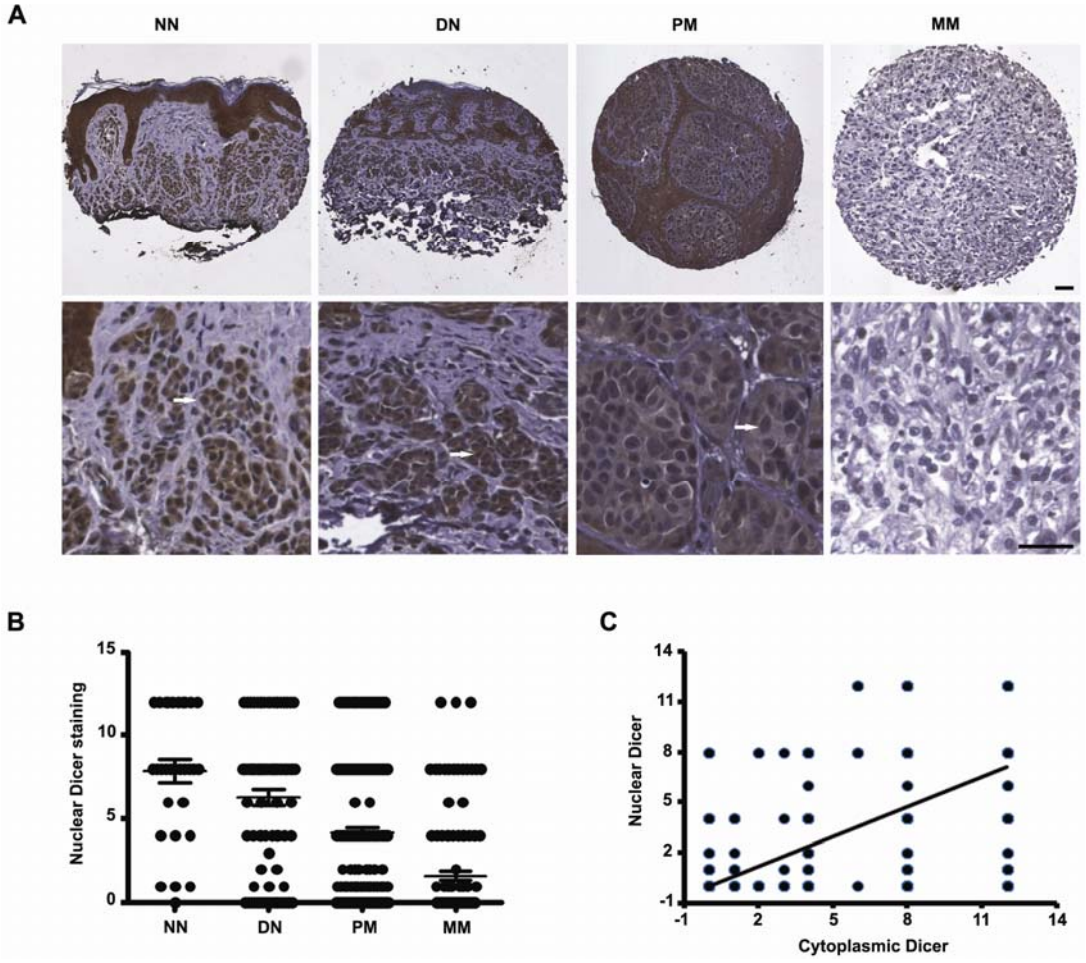
5.2. Results

5.2.1. Reduced nuclear Dicer expression is correlated with melanoma progression

We analyzed the expression pattern of nuclear Dicer in melanocytic lesion in our tissue microarray data consisted of 514 melanocytic lesions including 30 normal nevi, 87 dysplastic nevi, 262 primary melanomas, and 135 metastatic melanomas stained by a polyclonal anti-Dicer antibody (Sigma) (Figure 5.1A). We observed a significant reduction in nuclear expression of Dicer protein during melanoma progression (P = 0.0001, Kruskal-Wallis test; Figure 5.1B). Although we did not observe a significant difference between normal nevi and dysplastic nevi (P = 0.074, Mann-Whitney test), our data revealed significant reduction in nuclear Dicer expression from dysplastic nevi to primary melanomas (P = 0.001) and from primary melanomas to metastatic melanomas (P = 0.0001). Also, when we divided the samples into negative or positive nuclear Dicer groups, we observed a similar result that reduced nuclear Dicer expression significantly correlated with melanoma progression (P = 0.0001, χ^2 test; Figure 5.2A).

Moreover, we found nuclear Dicer expression to be inversely correlated with American Joint Committee on Cancer (AJCC) stages (P = 0.001, χ^2 test; Figure 5.2B). Importantly, the nuclear Dicer expression was also inversely correlated with tumor thickness and ulceration status (Table 5.1), two prominent prognostic markers of melanoma. We also found more nuclear Dicer expression in female patients and individuals under 61 years old (Table 5.1). It is notable that we had observed a similar pattern for correlation between expression of cytoplasmic Dicer and sex of the patients (Table 4.1). As mentioned earlier, this difference may be due to unequal representation of samples from each gender or age group in different stages of melanoma. Nevertheless, we are not aware of the functional significance or the mechanism responsible for this differential pattern of expression in patients with different sex or age.

Figure 5.1. Reduced expression of nuclear Dicer correlates with melanoma progression. (**A**) Representative images of normal nevi (NN) with strong nuclear Dicer staining, dysplastic nevi (DN) with moderate nuclear staining, primary melanoma (PM) with weak staining, and metastatic melanoma (MM) with negative Dicer staining. Scale bar = 50 μm. (**B**) Kruskal-Wallis test for differences in nuclear Dicer staining among NN, DN, PM, and MM (P = 0.0001). The median is depicted as a horizontal line in each group. (**C**) Spearman correlation test was used to estimate the correlation between expression of the nuclear and cytoplasmic forms of Dicer in melanocytic lesions (P = 0.0001, r = 0.53).



5.2.3. Correlation between cytoplasmic and nuclear Dicer expression in melanocytic lesions

We compared the expression of nuclear and cytoplasmic Dicer in the same melanocytic lesions and found a modest positive correlation in their expression patterns (spearman r value = 0.53, *P* = 0.0001; Figure 5.1C). It is noteworthy that although our data indicate that expression of cytoplasmic and nuclear Dicer is correlated, it is not a perfect correlation as judged by the Spearman r value of 0.53. This highlights the possibility of differential regulation of expression of nuclear and cytoplasmic Dicer or perhaps differential regulation of subcellular localization of Dicer in the cytoplasm and nucleus at least in a considerable subset of the melanocytic lesions.

Figure 5.2. Chi-square test for differences in nuclear Dicer staining at different stages of melanoma progression. **(A)** Significant difference was found between normal nevi and dysplastic nevi (*P* = 0.034), between dysplastic nevi and primary melanomas (*P* = 0.001), and between primary melanomas and metastatic melanomas (*P* = 0.0001). **(B)** Nuclear Dicer expression is negatively associated with AJCC stage of melanoma cases (*P* = 0.001).

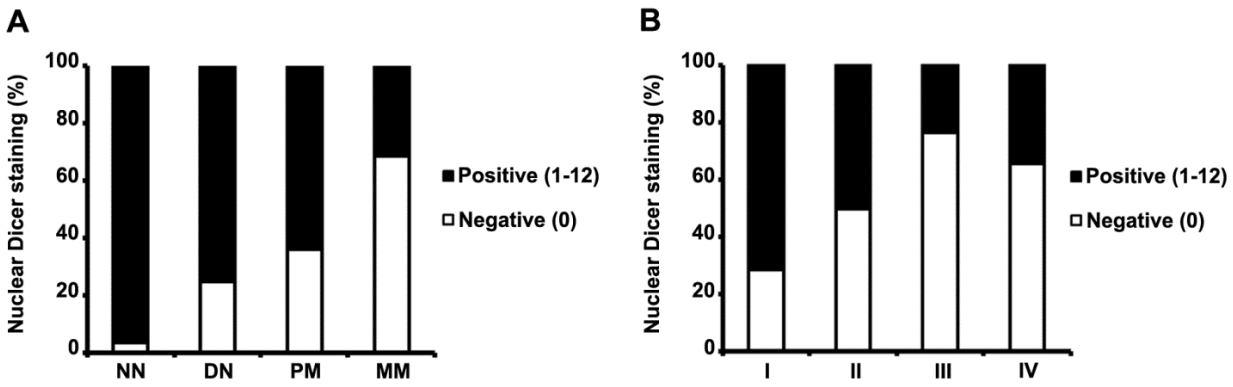


Table 5.1. Nuclear Dicer staining and clinicopathologic characteristics of 262 primary melanomas

	Nuclear Dicer staining			
	Negative	Positive	Total	*p* value*
Age				
≤62	43 (32.3%)	90 (67.7%)	133	0.48
>62		72 (55.8%)	129	
Sex				
Male	62(44.6%)	77 (55.2%)	139	0.023
Female	38 (30.9%)	85 (69.1%)	123	
Tumor thickness (mm)				
≤2	37 (27.9%)	106 (72.1%)	143	0.0001
>2	59 (51.3%)	56 (48.7%)	115	
Ulceration				
Present	28 (51.9%)	26 (48.1%)	54	0.02
Absent	72 (34.6%)	136 (65.3%)	208	
Lymphocytic response				
Present	40 (42.6%)	54 (57.4%)	94	0.274
Absent	60 (35.7%)	108 (64.3%)	168	
Tumor subtype				
Lentigo maligna	14 (29.8%)	33 (70.2%)	47	0.338
Superficial spreading	34 (37.4%)	57 (62.6%)	91	
Other†	52 (41.9%)	72 (58.1%)	124	
Site‡				
Sun-exposed	20 (30.8%)	45 (69.2%)	65	0.157
Sun-protected	80 (40.6%)	117 (59.4%)	197	

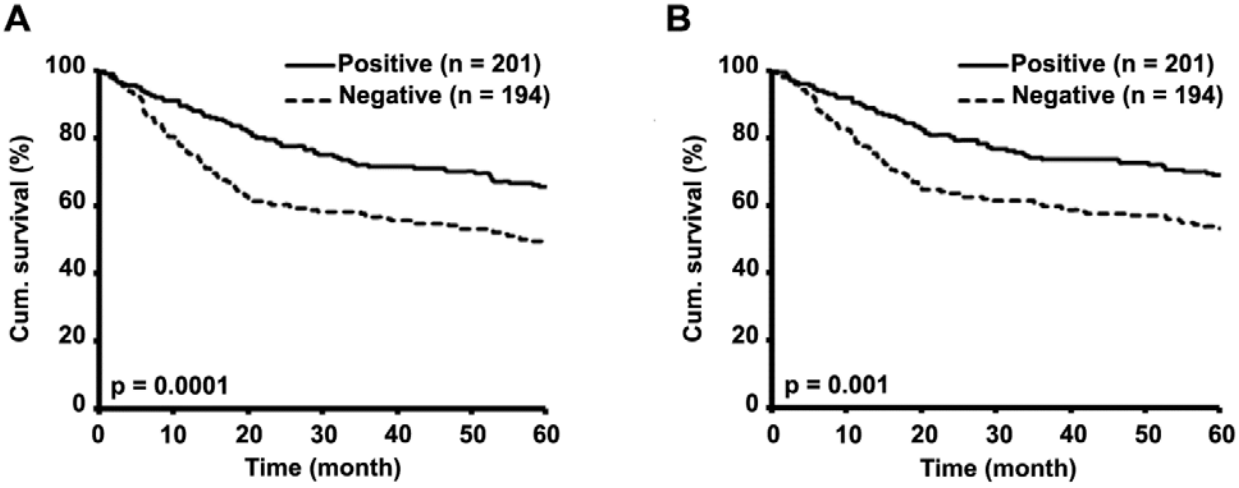
* χ^2 test.
† Other: unspecified subtype.
‡ Sun-protected sites: trunk, arm, leg and feet; Sun-exposed sites: head and neck.

5.2.4. Correlation between nuclear Dicer staining and melanoma patient survival

We found that similar to cytoplasmic Dicer (Figure 4.6), nuclear Dicer expression is also correlated with better overall ($P = 0.0001$; Figure 5.3A) and disease-specific 5-year survival ($P = 0.001$; Figure 5.3B) of primary and metastatic melanoma patients. Accordingly, patients with positive staining of nuclear Dicer had overall and disease-specific survival of 65.6% and 69.6%, respectively, compared with 48.9% and 54.6% for those with negative staining. This positive

correlation between nuclear Dicer expression and patient survival may be explained by the inverse correlation between its expression and tumor thickness and ulceration status (Table 5.1), two pathologic variables that are believed to have prominent prognostic significance for melanoma (Barnhill et al, 1996).

Figure 5.3. Nuclear Dicer expression positively correlates with 5-year survival of melanoma patients. (**A**) Overall and (**B**) disease-specific 5-year survival of all primary and metastatic melanoma patients.



5.2.5. A monoclonal anti-Dicer antibody confirms the reduced nuclear Dicer expression during melanoma progression and its correlation with patient survival

To rule out the possible epitope-related non-specific signals using a polyclonal antibody and to further validate our results, we also obtained the immunoreactivity scores from a smaller tissue microarray construct, containing 148 melanocytic lesions including 31 dysplastic nevi, 71 cases of primary and 46 cases of metastatic melanomas stained with a monoclonal anti-Dicer antibody (Clonegene) (Figure 5.4A). Consistent with our initial observation with the first anti-Dicer

antibody, we found a significant reduction in expression of nuclear Dicer during melanoma progression (P = 0.0001 for Kruskal-Wallis test, and P = 0.0001 for χ^2 test; Figure 5.4B and C). Interestingly, the staining with this antibody also confirmed a positive correlation between nuclear Dicer expression and overall and disease-specific survival of melanoma patients (*P* = 0.001). Patients with positive staining for nuclear Dicer had overall and disease-specific survival of 52.5% and 63.6% respectively, versus 16.6% and 27.7% for those with negative staining (Figure 5.5A and B).

Figure 5.4. TMA staining using a second monoclonal anti-Dicer antibody (Clonegene). (**A**) Representative images of DN with strong nuclear Dicer staining, PM with weak staining, and MM with negative nuclear Dicer staining. Scale bar = 50 µm. (**B**) Significant different was found in nuclear Dicer expression between PM and MM (Mann-Whitney $P = 0.0001$). (**C**) Chi-square test also showed Significant difference between PM and MM ($P = 0.0001$).

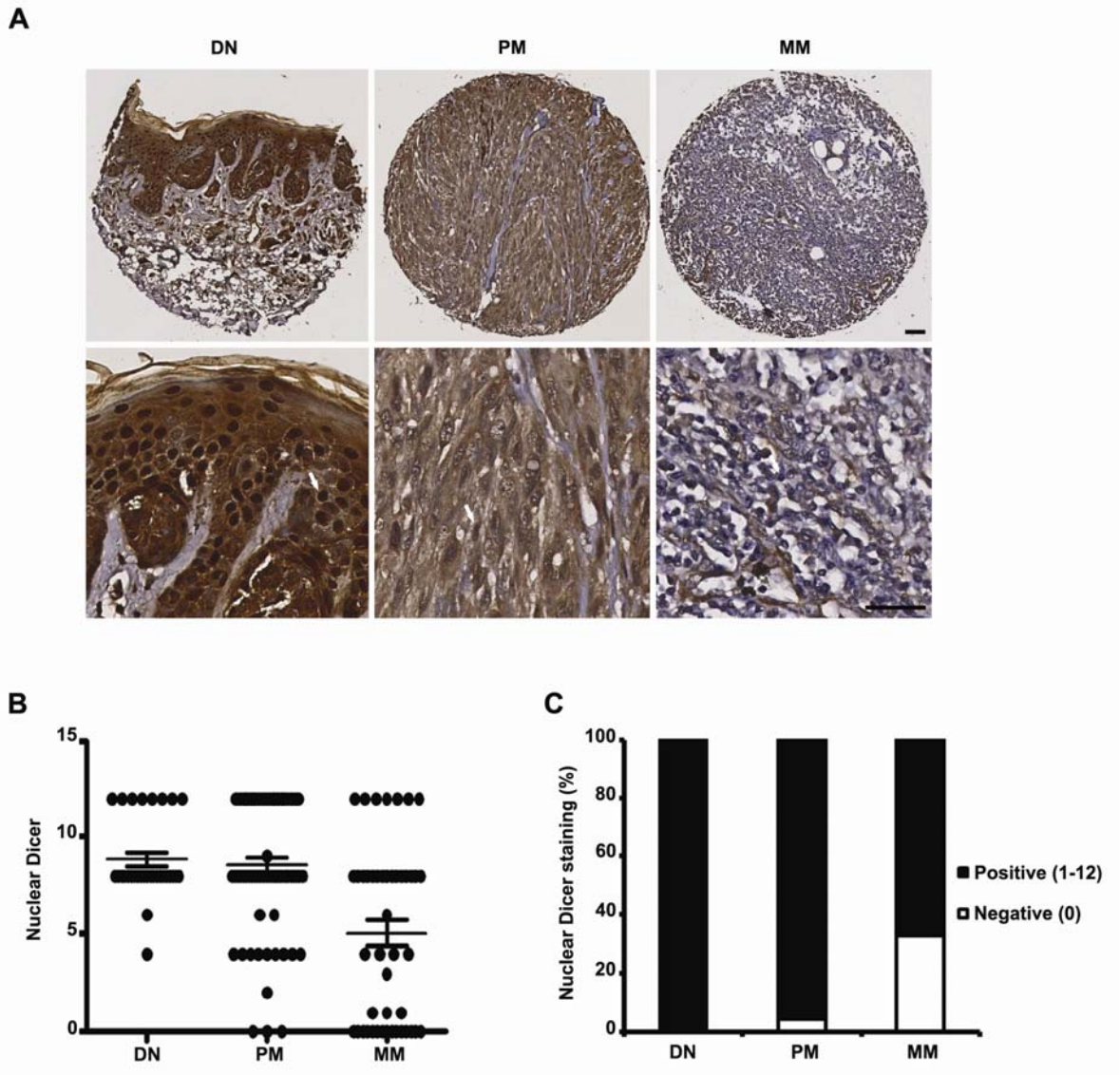
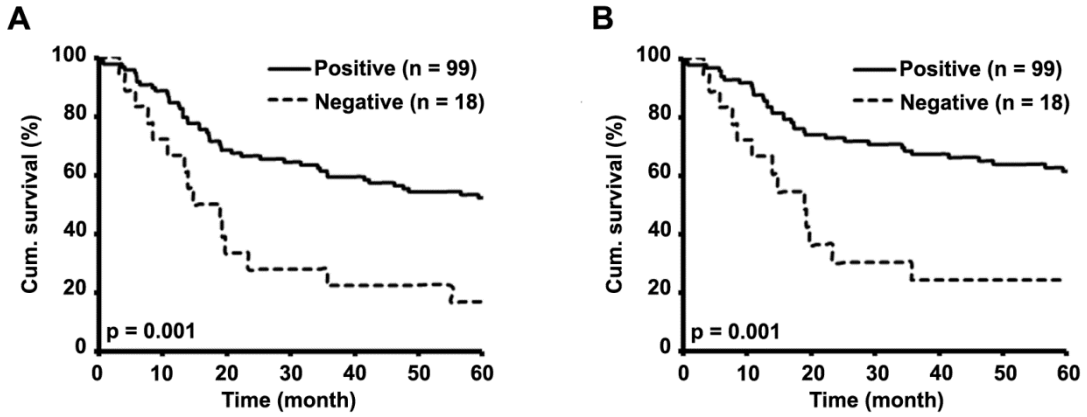


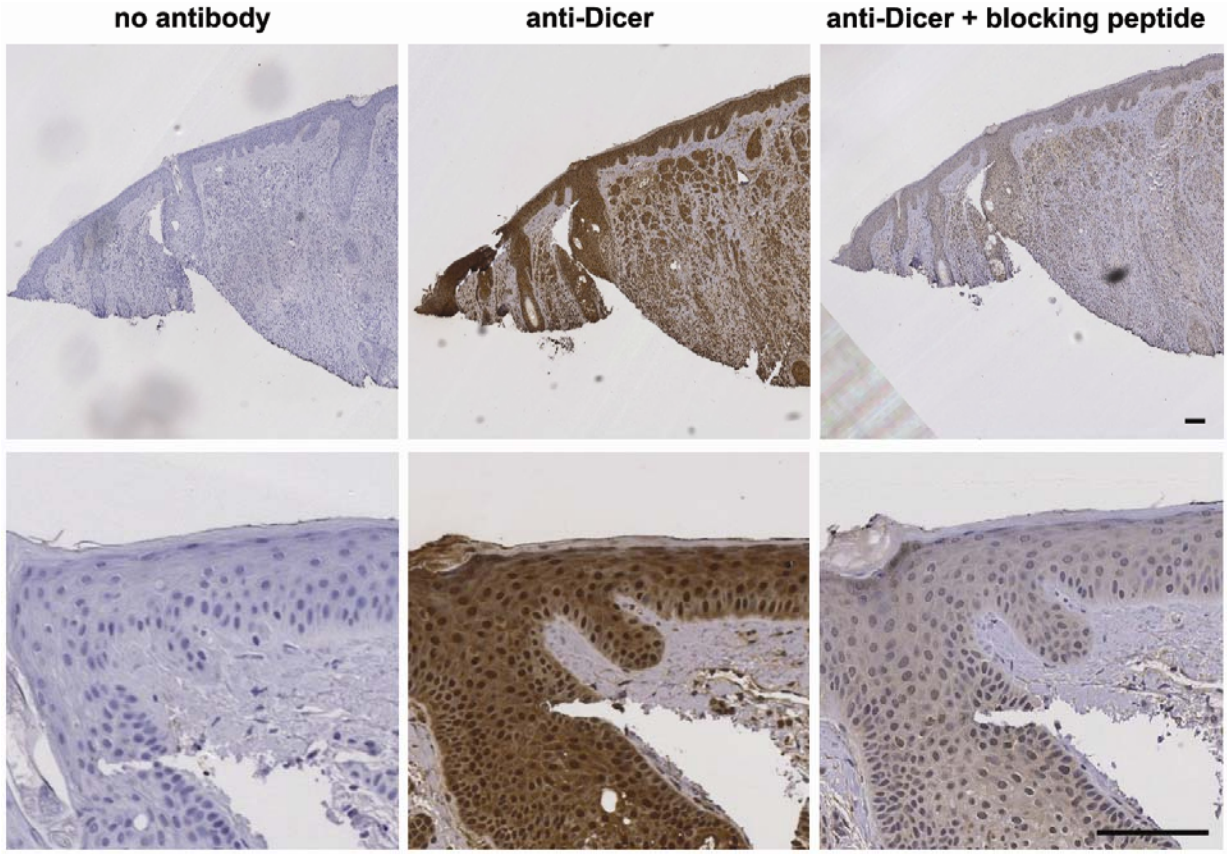
Figure 5.5. Monoclonal antibody against Dicer (Clonegene) confirms the positive correlation between nuclear Dicer expression and melanoma patient survival. Kaplan-Meier survival curves of (**A**) overall and (**B**) disease-specific 5-year survival of melanoma patients.



5.2.6. Blocking the cytoplasmic and nuclear signals of the monoclonal anti-Dicer antibody with a synthetic immunogenic peptide

In order to further verify the specificity of the antibody and exclude the possibility of interference from non-specific background signals, we used the synthetic immunogenic peptide against the monoclonal anti-Dicer antibody (Clonegene) and found that this peptide substantially blockes both cytoplasmic and nuclear Dicer staining (Figure 5.6), indicating that the observed cytoplasmic and nuclear signals for Dicer by using this antibody was specific and not due to random binding of the antibody to other intracellular components.

Figure 5.6. Blocking the cytoplasmic and nuclear signals of monoclonal anti-Dicer antibody with synthetic immunogenic peptide. Application of the synthetic immunogenic peptide impeded both cytoplasmic and nuclear Dicer staining. Scale bar = 100 μm.



5.3. Discussion

Existence of components of the miRNA biogenesis pathway such as Ago2, in the nucleus and the ability of RNAi machinery to modulate post-transcriptional gene expression in the nucleus has been previously reported (Robb et al, 2005). Other groups also reported the presence of Dicer protein in mammalian cells' nucleoplasm and its interaction with nuclear proteins or chromatin (Ando et al, 2011; Ohrt et al, 2012; Sinkkonen et al, 2010). As a direct evidence toward the subcellular localization of Dicer in the nuclear compartment in human cells, a recent immunohistochemistry study in triple-negative breast cancer samples and breast cancer cell lines revealed that in normal breast tissues Dicer is detectable predominantly in the cytoplasm of basal/myoepithelial cells only, whereas in the majority of triple-negative breast cancers, intense Dicer staining was detectable also in the nuclear compartment with the fraction of positive nuclei ranging from 5% to 70% (Passon et al, 2012). Nevertheless, no functional or clinical significance has been yet attributed to the nuclear Dicer in human cells as opposed to its well-known function of miRNA maturation in cytoplasm. In chapter 4 we showed that Dicer is required for suppression of melanoma invasion. However, whether this function is exerted solely by the cytoplasmic form of Dicer or whether the nuclear Dicer also contributes to this process is not known. dcr-1, the C. elegans homologue of Dicer, was shown to be cleaved by the CED-3 caspase to generate a C-terminal fragment with DNase activity, which promoted apoptosis by fragmentation of chromatin (Nakagawa et al, 2010). Nonetheless, it is not known if mammalian caspases can cleave Dicer or whether the mammalian Dicer would acquire DNase ability upon cleavage. New findings have highlighted the involvement of Dicer in exogenous DNA damage and oncogene-induced genotoxic stress response, independent of its miRNA-maturation function through production of certain types of RNA which are localized to the site of DNA damage

(Francia et al, 2012), although it is not known whether nuclear localization of Dicer is required for this process. The possibility that the observed nuclear Dicer in melanocytic cells could contribute to this function would be an interesting subject for future studies.

It should be noted that at least in some types of human tissues such as breast, *Dicer* gene encodes several different mRNA variants through the processes of alternative initiation and splicing (Grelier et al, 2009). These variants can produce multiple isoforms of Dicer protein. At least three different isoforms of Dicer protein have been detected in breast cancer cells corresponding to the full-length variants (218 kDa) and two alternatively spliced variants (113 and 93 kDa) (Hinkal et al, 2011). Interestingly, expression of these different isoform seemed to be differentially regulated in breast cancer (Hinkal et al, 2011). Similarly, two Dicer protein isoforms were discovered in neuroblastoma cells, the long isoform Dicer, and a truncated isoform called t-Dicer that lacked the dsRNA-binding domain and is defective in one of the two RNase III catalytic centers (Potenza et al, 2010). Although the functional significance of these protein isoforms is not fully understood, it is plausible that they can contribute to the observed staining for Dicer in the immunohistochemistry studies. In addition, it would be interesting to elucidate the subcellular localization of these isoforms and its variation during tumorigenesis in different types of malignancy such as melanoma.

The presence of Dicer in both cytoplasmic and nuclear compartments also points towards the existence of mechanisms that regulates the cytoplasmic-nuclear shuttling of the Dicer protein. Future studies to identify the mechanisms responsible for regulation of subcellular localization of Dicer, the biological functions of human Dicer protein in the nucleus and its significance in regulation of tumorigenesis may further contribute to our understanding of the pathogenesis of melanoma.

Chapter 6. Expression of the RNase III enzyme Drosha is reduced during progression of human cutaneous melanoma

6.1. Background and rationale

Aberrant expression or function of miRNA biogenesis factors could potentially alter the miRNAome of the cells; therefore contribute to the tumorigenesis process. Widespread deregulated expression of miRNAs in melanoma has been observed in different studies (Caramuta et al, 2010; Xu et al, 2012) accompanied by revelation of involvement of several individual miRNAs in regulation of different features of melanoma such as invasion, proliferation and evasion of apoptosis (Felicetti et al, 2008; Schultz et al, 2008; Xu et al, 2012). In chapter 4 we showed that expression of Dicer is reduced in metastatic melanoma which was inversely correlated with survival of melanoma patients. Nevertheless, so far very little is known about the expression pattern of other miRNA biogenesis factors in melanoma.

Drosha is another crucial RNAse in the canonical miRNA biogenesis pathway which is responsible for the processing of pri-miRNAs into pre-miRNAs. Therefore, it's expression is required for proper biogenesis of the majority of miRNAs. Several lines of evidence indicate that expression of Drosha is deregulated in malignancies and may influence clinical outcomes (Guo et al, 2012; Merritt et al, 2008; Papachristou et al, 2012; Shu et al, 2012). We hypothesized that similar to Dicer, expression of Drosha is deregulated in melanoma.

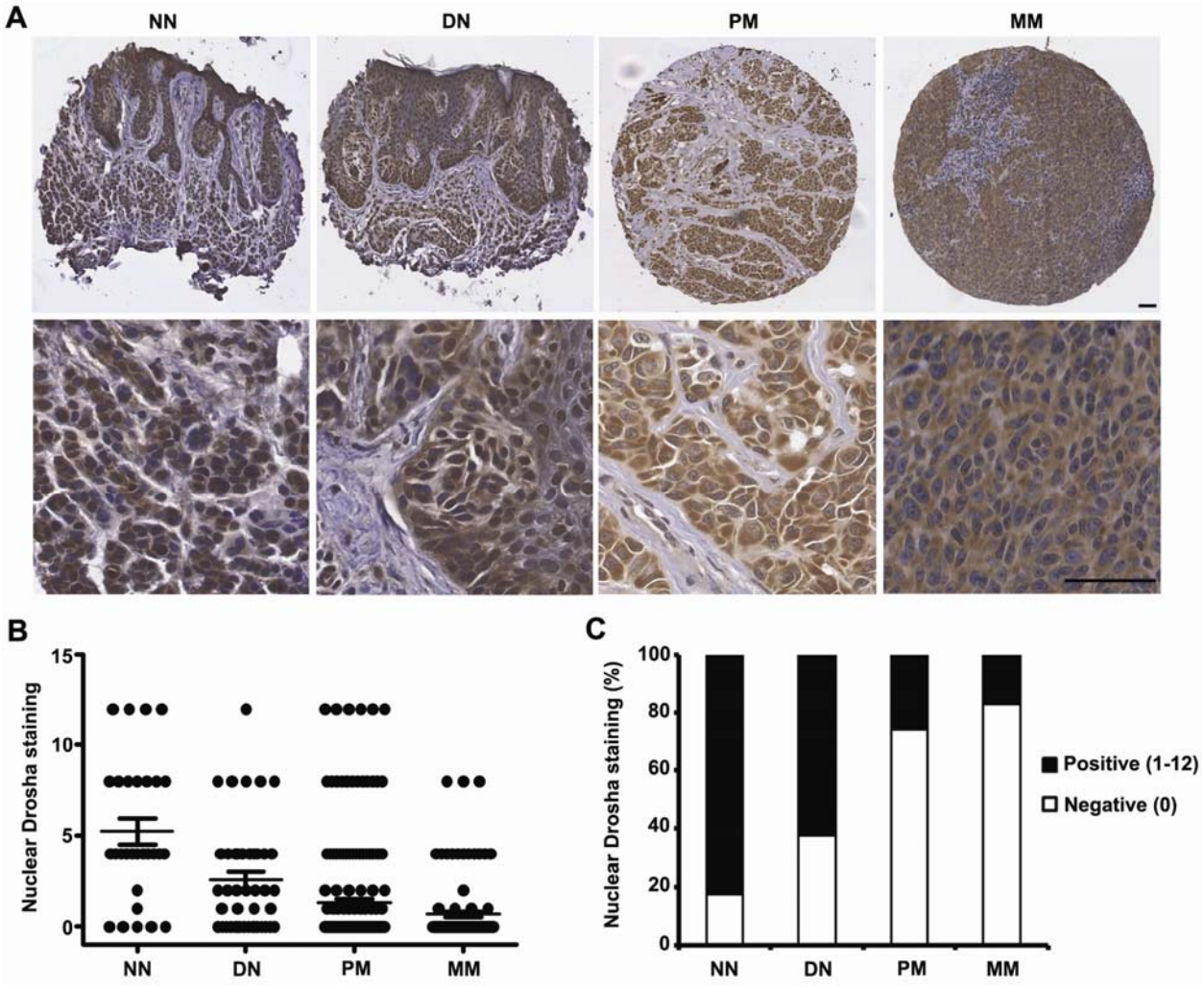
In this study, we investigated the expression of Drosha in a large set of melanocytic lesions by immunohistochemistry to identify the expression pattern of Drosha in melanoma.

6.2. Results

6.2.1. Reduced nuclear Drosha staining correlates with melanoma progression

We used a polyclonal rabbit antibody raised against the N-terminal (residues 1-100) of human Drosha protein to investigate its expression pattern in 409 melanocytic lesions (29 normal nevi, 43 dysplastic nevi, 226 primary melanomas, and 111 metastatic melanomas). We observed a predominant nuclear Drosha staining in different samples (Figure 6.1A). We also detected some cytoplasmic signal with this antibody in melanocytic lesions in all stages. A significant difference in nuclear Drosha staining was observed between different stages of melanoma. Kruskal-Wallis test revealed a clear reduction in expression of nuclear Drosha during melanoma progression ($P = 0.0001$; Figure 6.1B). We found significant reduction in expression of nuclear Drosha from normal nevi to dysplastic nevi ($P = 0.002$) and from dysplastic nevi to primary melanomas ($P = 0.0001$) but not between primary melanomas and metastatic melanomas ($P = 0.052$). Similarly, when we divided the samples from each stage into two groups based on expression of nuclear Drosha, we observed an increase in percentage of samples with no nuclear Drosha staining during melanoma progression ($P = 0.0001$; Figure 6.1C). Accordingly, while 83% of normal nevi and 63% of dysplastic nevi had positive nuclear staining for Drosha, only 26% of primary melanomas and 17% of metastatic melanomas stained positive for nuclear Drosha.

Figure 6.1. Reduced expression of nuclear Drosha correlates with melanoma progression. (**A**) Representative images of NN and DN with strong nuclear Drosha staining, PM with weak and MM with negative nuclear Drosha staining. Scale bar = 50 µm. (**B**) Kruskal-Wallis test for differences in nuclear Drosha staining among NN, DN, PM, and MM. Median is depicted as a horizontal line in each group ($P = 0.0001$). (**C**) Chi-square test for differences in nuclear Drosha staining in NN, DN, PM, and MM. Significant difference was found between DN and PM ($P = 0.0001$) but not NN and DN ($P = 0.068$) or PM and MM ($P = 0.066$).



6.2.2. Inverse correlation between nuclear Drosha staining and tumor thickness, AJCC staging and ulceration status

To assess whether reduced nuclear Drosha staining correlates with clinicopathologic variables of the patients, we examined the expression pattern of nuclear Drosha in 226 primary melanoma samples (Table 6.1). Although Drosha staining did not have a significant correlation with patients' age or sex, location of tumor and lymphocytic response, it showed a significant inverse correlation with tumor thickness (Breslow's depth of invasion). Accordingly, percentage of samples with positive staining for nuclear Drosha reduced from 37% in tumors ≤ 2mm thick to 11% in tumors thicker than 2 mm ($P = 0.0001$, χ^2 test; Figure 6.2A). We also observed an inverse correlation between expression of nuclear Drosha and ulceration status of the melanoma patients. While 30.6% of the samples without ulceration stained positive for nuclear Drosha, only 7% of those with ulceration had positive nuclear Drosha staining ($P = 0.002$, χ^2 test; Table 6.1). In addition, when compared the nuclear Drosha staining between different subtypes of melanoma, we found that lentigo maligna and superficial spreading subtypes express less Drosha than other subtypes ($P = 0.016$, Table 6.1).

Importantly, our data also demonstrated that nuclear Drosha expression is inversely correlated with American Joint Committee on Cancer (AJCC) stages of melanoma ($P = 0.0001$, χ^2 test; Figure 6.2B). Interestingly, we found that the main and only significant difference in nuclear Drosha staining exist between stage I and II ($P = 0.0001$, χ^2 test) but not the other stages ($P > 0.05$, χ^2 test).

Figure 6.2. Negative correlation between nuclear Drosha expression, thickness and AJCC stage. (**A**) Tumor thickness and (**B**) AJCC stage of melanoma (P = 0.0001, χ^2 test).

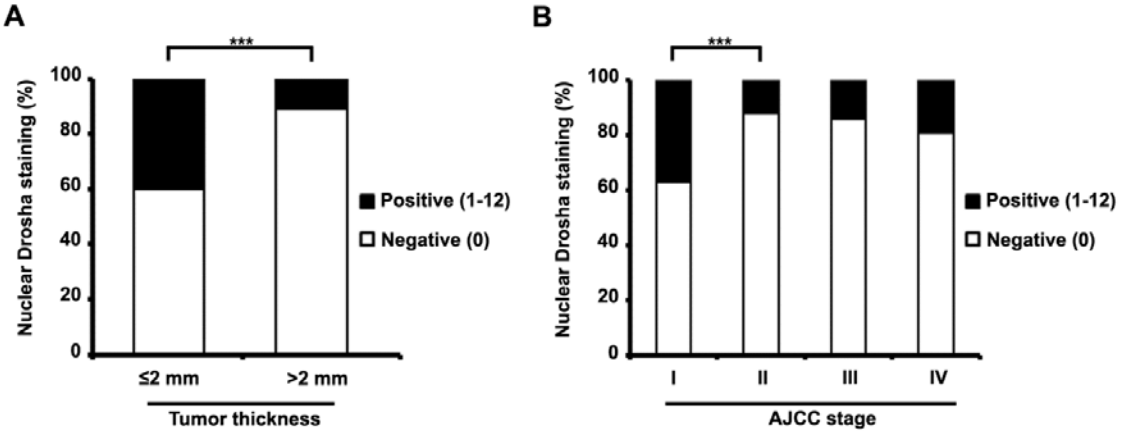


Table 6.1. Nuclear Drosha staining and clinicopathologic features of 226 primary melanomas.

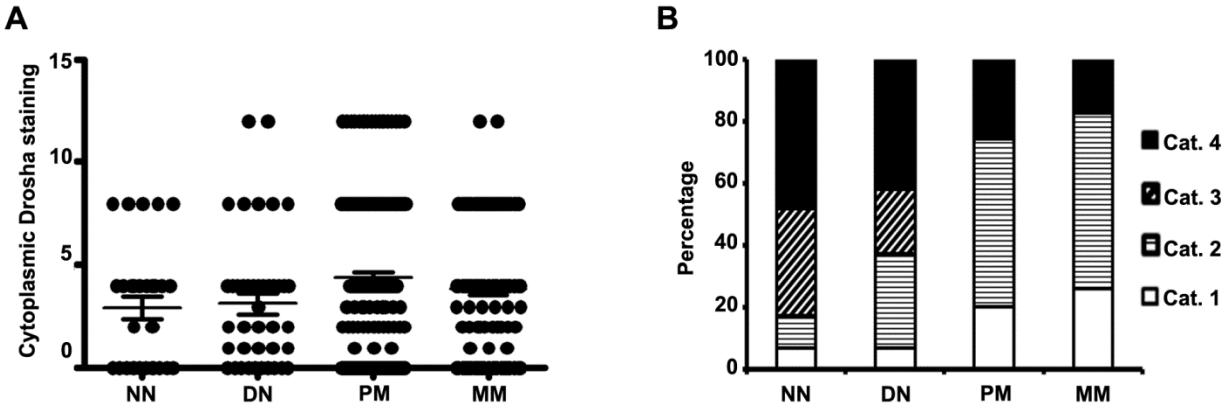
	Nuclear Drosha staining			
Variables	Negative	Positive	Total	*P* value*
Age				
≤63	81 (71.1%)	33 (28.9%)	114	0.327
>63	86 (76.8%)	26 (23.2%)	112	
Sex				
Male	91 (71.6%)	36 (28.4%)	127	0.385
Female	76 (76.8%)	23 (23.2%)	99	
Ulceration				
Present	40 (93%)	3 (7%)	43	0.002
Absent	127 (69.4%)	56 (30.6%)	183	
Lymphocytic response				
Present	56 (75.7%)	18 (24.3%)	74	0.670
Absent	111 (73%)	41 (27%)	152	
Tumor subtype				
Lentigo maligna	36 (65%)	19 (35%)	55	0.016
Superficial spreading	56 (68.3%)	26 (31.7%)	82	
Other†	75 (84.3%)	14 (15.7%)	89	
Site‡				
Sun-exposed	44 (74.6%)	15 (25.4%)	59	0.890
Sun-protected	123 (73.7%)	44 (26.3%)	127	

* χ^2 test.
† Other: unspecified subtype.
‡ Sun-protected sites: trunk, arm, leg and feet; Sun-exposed sites: head and neck.

6.2.3. Subcellular shift of Drosha to the cytoplasm during melanoma progression

As mentioned earlier, in addition to the nuclear staining for Drosha we also observed cytoplasmic staining for this protein in all stages of melanocytic lesions. To investigate if the cytoplasmic nuclear staining has any significance in melanoma progression, we evaluated Drosha staining in cytoplasm. Kruskal-Wallis test revealed a marked increase in cytoplasmic expression of Drosha during melanoma progression (P = 0.026; Figure 6.3A). Significant difference for the cytoplasmic Drosha staining was observed between dysplastic nevi and primary melanomas (P = 0.016), but not between normal nevi and dysplastic nevi or between primary melanomas and metastatic melanomas ($P > 0.05$). Furthermore, to examine whether the reduced nuclear Drosha staining is due to a subcellular shift we divided the samples in each stage of melanoma progression into four categories based on their nuclear and cytoplasmic Drosha expression profile: 1) negative nuclei and cytoplasm; 2) negative nuclei but positive cytoplasm; 3) positive nuclei but negative cytoplasm; 4) positive nuclei and cytoplasm. Interestingly, we observed a considerable shift in Drosha expression from nuclei to cytoplasm during melanoma progression (P = 0.0001, χ^2 test; Figure 6.3B). Accordingly, category 2 which represents samples with negative nuclear but positive cytoplasmic staining for Drosha increased from 10.3% in normal nevi to 30.2% in dysplastic nevi, 54.2% in primary melanomas and 56.8% in metastatic melanomas, whereas category 3 which represents samples with positive nuclear but negative cytoplasmic staining for Drosha decreased from 34.5% in normal nevi to 20.9% in dysplastic nevi, and further to 1.3% in primary melanomas and 0.9% in metastatic melanomas.

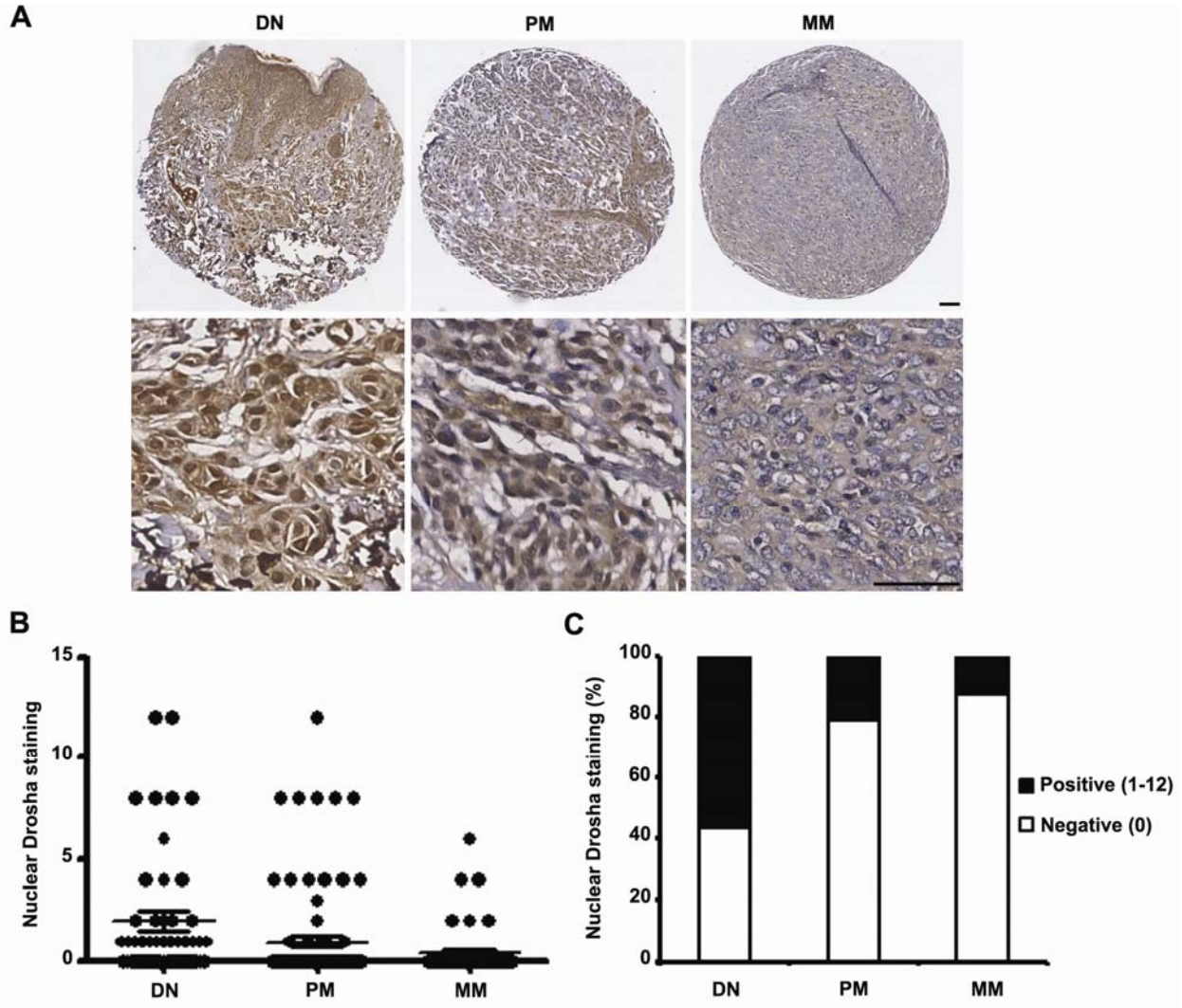
Figure 6.3. Cytoplasmic expression of Drosha is increased during melanoma progression. (**A**) Kruskal-Wallis test for differences in cytoplasmic Drosha expression ($P = 0.026$). Significant difference was found between DN and PM ($P = 0.016$), but not NN and DN or PM and MM ($P > 0.05$). (**B**) Combined analysis of nuclear and cytoplasmic Drosha staining. Each sample was categorized as following: 1) negative nuclei and cytoplasm; 2) negative nuclei but positive cytoplasm; 3) positive nuclei but negative cytoplasm; and 4) positive nuclei and cytoplasm.



6.2.4. Validation of Drosha staining with a second antibody

To further validate our observation that expression of nuclear Drosha is reduced in melanoma we used a second rabbit polyclonal antibody against the C-terminal (amino acids 1071-1370) to probe for expression of Drosha in a smaller TMA construct, containing 48 dysplastic nevi, 86 primary melanomas, and 49 metastatic melanomas (Figure 6.4). We observed an expression pattern of nuclear Drosha very similar to the first antibody by using this antibody (Figure 6.4A). Kruskal-Wallis test revealed a reduced expression of nuclear Drosha during melanoma progression (P = 0.0001, Figure 6.4B). Furthermore, when we divided the samples from each stage into two groups (positive and negative) based on expression of nuclear Drosha, we observed an increase in percentage of samples with no nuclear Drosha staining during melanoma progression (P = 0.0001; Figure 6.4C). Accordingly, while 56% of dysplastic nevi had positive nuclear staining for Drosha, only 21% of primary melanomas and 12% of metastatic melanomas stained positive for nuclear Drosha.

Figure 6.4. Tissue microarray using a second specific anti-Drosha antibody. (**A**) Representative images of DN with strong nuclear Drosha staining, PM with weak and MM with negative nuclear Drosha staining. (**B**) Kruskal-Wallis test (P = 0.0001). Significant difference was found between DN and PM (Mann-Whitney P = 0.001) but not PM and MM. (**C**) Chi-square test for differences in nuclear Drosha staining in DN, PM, and MM. Significant difference was found between DN and PM (P = 0.001) but not PM and MM (P = 0.204).



6.2.5. Concomitant loss of Drosha and Dicer expression renders worse prognosis for melanoma progression

To evaluate whether the reduced nuclear Drosha staining in human melanomas correlates with patient survival, we constructed Kaplan-Meier survival curves using overall or disease-specific 5-year survival to compare biopsies with positive nuclear Drosha staining to those with negative nuclear Drosha staining. Our results revealed that despite a trend toward higher survival rate in samples with positive nuclear Drosha, this correlation is not significant for either overall (P = 0.073) or disease-specific (P = 0.097) survival of the patients (Figure 6.5 A and B).

We previously showed that expression of cytoplasmic Dicer is reduced during melanoma progression towards metastasis. Since Drosha and Dicer are two main players in miRNA biogenesis pathway, we investigated the potential correlation in the expression of these two proteins in melanocytic lesions and the correlation between concomitant expression of these two factors and melanoma patients' survival. We found that despite reduced expression of cytoplasmic Dicer and nuclear Drosha during melanoma progression, there is no significant correlation between their expression patterns in the single sample level (Spearman r value = 0.124, χ^2 *P value* = 0.07; Figure 6.6 A and B). However, when we divided the samples into three groups; positive Drosha-positive Dicer (category 1; n = 49), positive Dicer-negative Drosha or negative Dicer-positive Drosha (category 2; n = 192) and negative Dicer-negative Drosha (category 3; n = 33), we found a significant correlation between their concomitant expression and patient survival (P = 0.019 for overall and 0.001 for disease-specific survival; Figure 6.7A and B). We observed that samples in category 3 represented the worst survival outcome (36.7% for both overall and disease-specific survival; Figure 6.7A and B), whereas category 1 represented the best outcome (65.3% for overall and 75.5% for disease-specific survival;

Figure6.7 A and B). Interestingly, survival rate for samples in category 2 was between the other two categories (54.7% for overall and 59.4% for disease-specific survival; Figure 6.7A and B).

Figure 6.5. Correlation between nuclear Drosha expression and 5-year survival of melanoma patients. (**A**) Overall and (**B**) disease-specific 5-year survival of all primary and metastatic melanoma patients.

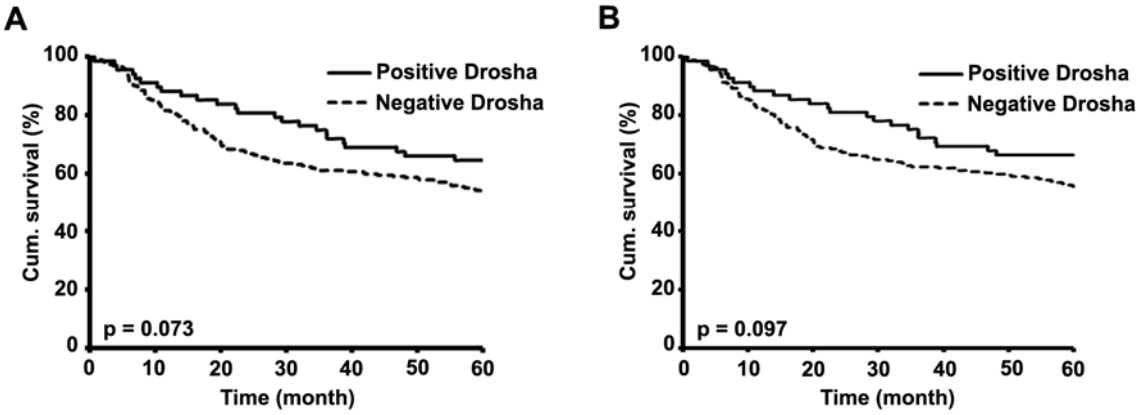


Figure 6.6. No significant correlation between expression of cytoplasmic Dicer and nuclear Drosha. (**A**) Spearman test; r = 0.124, *P* = 0.25. (**B**) Chi-square test; *P* = 0.07.

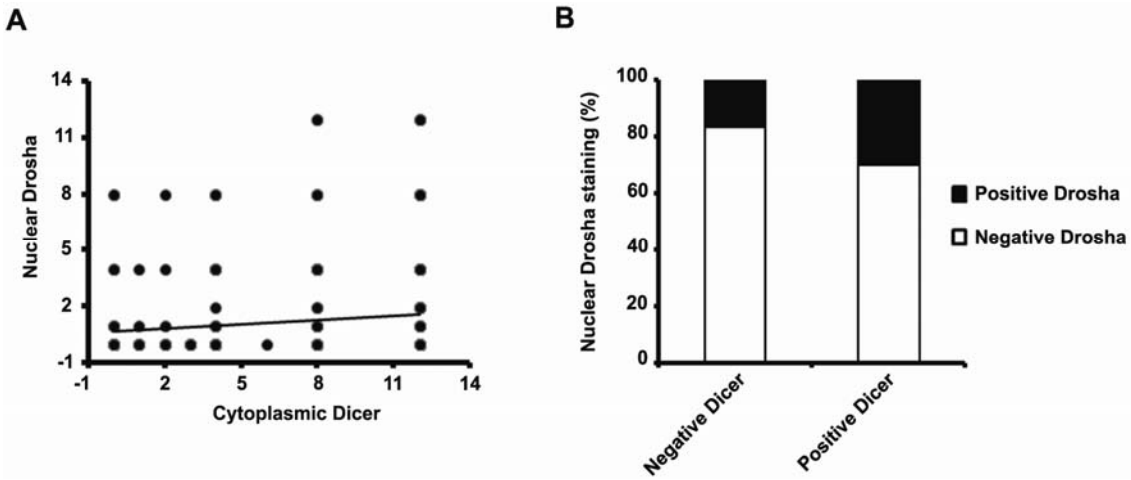
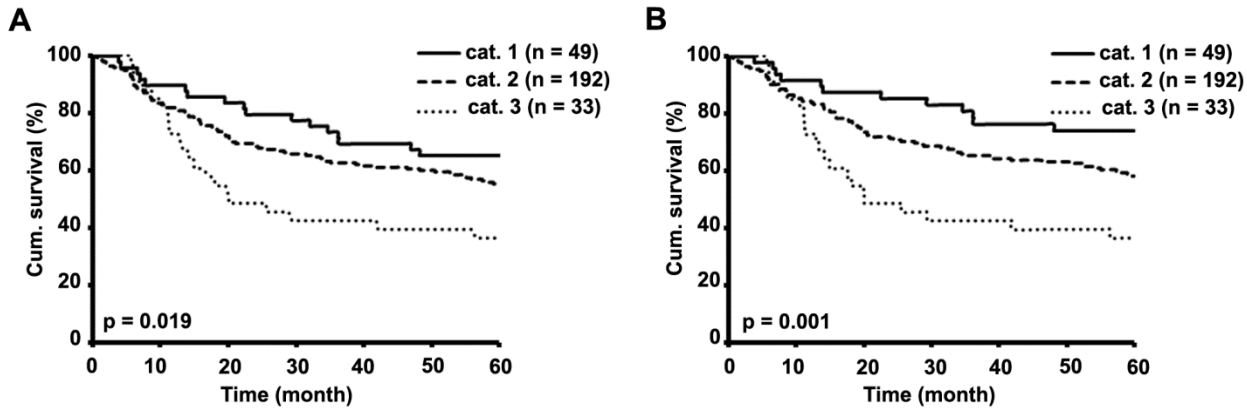


Figure 6.7. Concomitant loss of nuclear Drosha and cytoplasmic Dicer confers worse survival. Correlation between concomitant expression of nuclear Drosha and cytoplasmic Dicer and (**A**) Overall and (**B**) disease-specific 5-year survival of all primary and metastatic melanoma patients.



6.3. Discussion

Expression pattern of Drosha has been investigated in regards to tumor progression and survival prediction in a number of malignancies. In breast cancer, downregulation of Drosha expression at mRNA level was observed in patients receiving adjuvant anthracycline-based chemotherapy and was associated with high grade, high Ki-67, lack of Bcl2 expression, HER2 over-expression and gene amplification and TOPO2A gene amplification (Dedes et al, 2011). Reduced expression of Drosha was also reported in endometrial carcinoma, (Torres et al, 2011) gallbladder cancer (Shu et al, 2012), and high-risk neuroblastoma tumors (Landi et al, 2010). Reduced expression of Drosha has also been reported in ovarian cancers, in which higher Drosha expression was significantly correlated with better patient survival (Merritt et al, 2008). Although, a recent report showed an increased expression of Drosha in serous ovarian carcinoma

(Vaksman et al, 2012). On the other hand, Drosha expression levels were enhanced in a fraction of esophageal cancers (Sugito et al, 2006). However, to our knowledge, this is the first study to investigate the Drosha protein expressions in human cutaneous melanoma. Using two different antibodies raised against different regions of the Drosha protein, we observed a marked downregulation of nuclear Drosha expression during melanoma progression (Figure 6.1 and 6.4). We also observed an inverse correlation between nuclear expression of Drosha and tumor thickness, AJCC stage, and ulceration status (Figure 6.2 and Table 6.1). These data indicate a possible tumor suppressor function for Drosha in melanoma.

Interestingly, we also observed an increased cytoplasmic staining for Drosha along with the reduced nuclear Drosha expression during melanoma progression (Figure 6.3). This observation suggests that the nuclear to cytoplasm translocation of Drosha protein may be a relevant event in melanomagenesis. Detection of Drosha in cytoplasm is not unprecedented in human cells. Indeed, in esophageal cancer samples, Drosha was shown by immunohistochemistry to be strongly expressed in both nuclei and cytoplasm (Sugito et al, 2006). Cytoplasmic localization of Drosha was also recently reported in colorectal carcinoma cells by immunofluorescence analyses (Papachristou et al, 2011). Similarly, cytoplasmic Drosha expression was detected in MCF-7 breast cancer cell line (Passon et al, 2012). Phosphorylation of Drosha at two serine residues (S300 and S302) has proven to be critical for its nuclear localization (Tang et al, 2010). Consistently, mutation of these two residues abrogates the nuclear localization and resulted in cytoplasmic accumulation of the Drosha protein (Tang et al, 2010). Although it is not clear which signaling pathways are responsible for regulation of phosphorylation of these residues, it would be interesting to identify the phosphorylation status of Drosha in melanocytic lesions, possible mutation in these residues as well as the activity of

the putative signaling pathway(s) responsible for regulation of its phosphorylation or dephosphorylation. Noteworthy, the cytoplasmic Drosha has also been suggested to participate in processing of virus-derived cytoplasmic pri-miRNAs. Accordingly, the involvement of Drosha was mediated by a dramatic relocalization to the cytoplasm following virus infection (Shapiro et al, 2012). Nevertheless, it is not known whether the cytoplasmic Drosha has any other function in the cytoplasm, other than that of processing the viral miRNAs, and whether this re-localization has any role in tumorigenesis.

It should be noted that alternatively spliced transcripts, encoding C-terminally truncated Drosha proteins lacking part of the RIIIDb and the entire dsRBD have been detected in human melanoma cell lines. Proteins generated from these alternative splice variants fail to bind to DGCR8 and form the microprocessor complex (Grund et al, 2012). These splice variants are deficient in pri-miRNA processing. However, these aberrant transcripts in melanoma cells do not hamper miRNA biogenesis process (Grund et al, 2012). In our study, it is possible that these splice isoforms contribute to the signals detected by the antibody that targets the N-terminal of Drosha protein; nevertheless, we believe this possibility is remote since the second antibody that targets the C-terminal of the Drosha protein (therefore, unable to detect these isoforms) shows a pattern of Drosha expression in melanocytic lesions very similar to the other antibody.

Previous reports demonstrated various degrees of correlation between Drosha expression and survival. For instance, in gallbladder and ovarian cancers loss of Drosha expression was associated with decreased survival (Merritt et al, 2008; Shu et al, 2012). Although, another study showed that overexpression of Drosha in serous ovarian carcinoma was not significantly correlated with overall or progression-free survival of the patients (Vaksman et al, 2012). Low expression of Drosha protein was also significantly correlated with shorter progression-free

survival and overall survival of nasopharyngeal carcinoma patients (Guo et al, 2012). In colorectal cancer, the expression level of Drosha was reported to not correlate with overall survival (Papachristou et al, 2011). In breast cancer patients receiving adjuvant anthracycline-based chemotherapy, despite a reduced expression of Drosha no significant associations between expression of Drosha and outcome was observed (Dedes et al, 2011). On the other hand, in esophageal cancer patients the probability of survival was significantly lower for patients with high levels of Drosha expression (Sugito et al, 2006). Nevertheless, despite a clear trend, our Kaplan–Meier analysis did not reveal a significant correlation between nuclear Drosha expression and patients' overall or disease-specific survival. This lack of significant correlation may be explained by the notion that reduction of nuclear Drosha expression mainly happens at earlier stages of melanomagenesis (from normal nevi to dysplastic nevi and further from dysplastic nevi to primary melanomas; Figure 6.1 and 6.4).

A previous report on breast cancers showed that Drosha and Dicer were concurrently down-regulated in 15% of cases (Dedes et al, 2011). Down-regulation of Drosha in endometrial cancer was also shown to be significantly correlated with decreased expression of Dicer (Torres et al, 2011). Nonetheless, we did not observe any correlation between nuclear Drosha and cytoplasmic Dicer expression in melanocytic lesions. In addition, when we compared the expression pattern of nuclear Drosha with that of cytoplasmic Dicer during melanoma progression, we observed that while the expression of Dicer reduces mainly in the latest stage of melanoma progression (metastatic melanoma), nuclear Drosha expression is progressively reduced in almost all stages of melanoma progression. These data indicate that expression patterns of these two factors are distinct in different stages of melanoma progression, suggesting the existence of distinct mechanisms responsible for differential regulation of their expression.

However, despite this lack of correlation at the expression level, concomitant expression of these two factors confers the best survival outcome for the patients (Figure 6.7). In line with our observation in melanoma, studies in ovarian cancer showed that cases with both high Dicer expression and high Drosha expression were associated with increased survival (Merritt et al, 2008). Altogether, our data suggest that integrity of the miRNA biogenesis pathway is perturbed in a considerable subset of melanomas and that those cases whose expression pattern of miRNA biogenesis factors (and probably expression pattern of miRNAs) resembles that of normal tissues represent a better rate of survival.

Loss of expression of Drosha and Dicer has been shown before to cause different phenotypes. For instance, it was reported that Dicer knockdown significantly decreases migration of endothelial cells, whereas Drosha knockdown has no such effect (Kuehbacher et al, 2007). Similarly, silencing of Dicer but not Drosha reduces angiogenesis in vivo (Kuehbacher et al, 2007). Also, Chong et al revealed that in early-stage thymocytes, deficiency in Drosha or Dicer does not always result in identical phenotypes and using the mouse embryonic fibroblasts (MEFs) they showed that loss of Drosha expression results in a pattern of gene expression different than loss of Dicer (Chong et al, 2010). Interestingly, they showed that Drosha also recognizes and directly cleaves many mRNAs with secondary stem–loop structures. In addition, a subset of miRNAs was found to be generated by a Dicer-dependent but Drosha-independent mechanism, explaining the distinct and non-overlapping phenotypes caused by loss of Drosha and Dicer (Chong et al, 2010). Reduced Drosha, but not Dicer, expression in human mesenchymal stem cells (hMSCs) was demonstrated to significantly reduce proliferation rate (Oskowitz et al, 2011). In addition, unlike Dicer knockdown, Drosha-KD hMSCs contained an increased number of G1 phase cells, with a reduced level of cells in S phase, accompanied by

decreased pRB, 28S and 18S rRNA expression and increased p16 and p15 (two key regulators of the G1/S phase transition) expression, indicating that Drosha modifies hMSCs proliferation through a miRNA independent mechanism (Oskowitz et al, 2011). Our data demonstrating further reduction in survival rate of the melanoma cases with negative Dicer and negative Drosha expression compared with that of the cases with loss of either Dicer or Drosha (Figure 6.7) also indicate the possible existence of at least some non-overlapping functions of these two factors. However, beside its direct role in biogenesis of miRNAs, so far very little is known of putative functions of Drosha in melanocytic cells. Future studies will delineate these functions of Drosha and its possible contribution to suppression of melanomagenesis.

In conclusion, our data revealed a decreased nuclear expression of Drosha during melanoma progression along with subcellular compartment shift of its expression toward the cytoplasm. We observed that expression of Drosha inversely correlates with tumor thickness, AJCC stage and ulceration status. In addition, we revealed that concurrent loss of expression of Drosha and Dicer confers an adverse affect on melanoma patient survival, worse than singular loss of each of these factors. Our data suggest a possible tumor suppressor function for Drosha in melanoma and highlights the requirement for future studies on its role in this type of malignancy and it possible miRNA-independent functions.

Chapter 7. General conclusions

7.1. Summary of the findings and implications

Melanoma is an aggressive disease due to its notorious potential to invade and metastasize to other organs as well as its resistance to chemotherapy (Gray-Schopfer et al, 2007). Similar to other types of malignancies, frequent genetic and epigenetic abnormalities in the form of chromosomal deletion or gain and altered genes expression is observed in melanoma (Curtin et al, 2005; Hodis et al, 2012; Muthusamy et al, 2006a). Some of these changes are required for melanocytes to adapt to the environments other than epidermis which host the secondary tumors, while many other factors are involved in tumor cell invasion, motility, angiogenesis, proliferation, and evasion of apoptosis (Chin et al, 2006). Despite past progress many aspects of melanoma biology, especially the mechanisms responsible for its progression toward metastasis, have remained to be discovered. Understanding these mechanisms is of crucial importance for development of novel targets for treatment of melanoma.

In recent years, several studies investigated the expression pattern of Sox4 in different types of cancers and described some possible downstream mechanisms required for exerting its role in tumorigenesis or tumor suppression (Aaboe et al, 2006; Bangur et al, 2002; de Bont et al, 2008; Frierson et al, 2002; Huang et al, 2009; Liu et al, 2006; Pramoonjago et al, 2006). In this study, we revealed a reduced expression of Sox4 in metastatic melanoma. Our data also demonstrated that the Sox4 expression is correlated with improved melanoma patient survival (Figure 3.2). These data suggest a possible tumor suppressor role for Sox4 in melanoma, and its possible implication for melanoma prognosis.

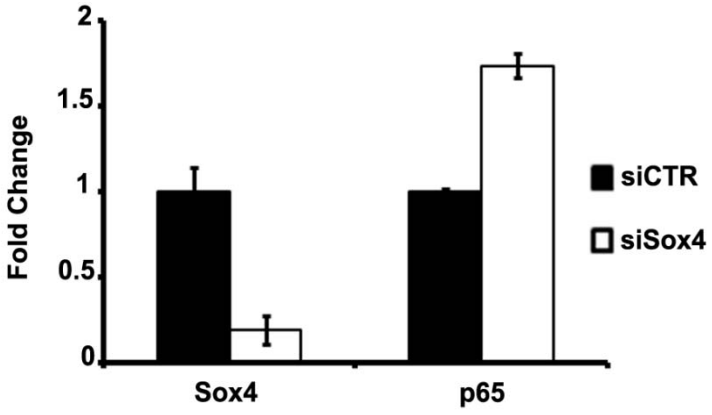
It has been previously noted that increased migration and invasion ability of melanoma cells results in higher metastatic potential and shorter survival of patients (Condeelis et al, 2005;

Friedl and Wolf, 2003). Therefore, the observed positive correlation between expression of Sox4 and better survival of melanoma patient may be explained by suppression of melanoma cells invasion and migration by Sox4. This may also explain the observed reduced expression of Sox4 in metastatic melanoma as an escape route for tumor cells to relieve themselves from an endogenous mechanism responsible for suppression of invasion and migration.

We further demonstrated that Sox4 may suppress the *in vitro* migration and invasion of melanoma cells through at least two different mechanisms. First, we showed that Sox4 is able to bind to the regulatory sequences of the NF-κB p50 gene and suppress its expression, therefore inhibit the downstream pathway which promotes melanoma migration and invasion (Amiri and Richmond, 2005; Gao et al, 2006; van de Stolpe et al, 1994). We confirmed the NF-κB p50-dependent mechanism of Sox4 by co-knockdown of the two genes which resulted in abrogation of the enhanced cell migration and invasion in the absence of Sox4 (Figure 3.3). In addition, we showed that suppression of ROCK, a downstream factor of the NF-κB pathway also reverts the Sox4-KD phenotypes (Figure 3.6), which further confirms the suggestion that Sox4-mediated suppression of melanoma invasion and migration is at least partially through inhibition of the NF-κB pathway. Next, we observed that Sox4 positively regulates expression of Dicer by binding to the regulatory regions of its gene and enhances its transcription. We further demonstrated that expression of Sox4 is required for regulation of expression of mature miRNAs in melanoma cells, mainly in a Dicer-dependent manner. This observation implicates the miRNA pathway as a means by which Sox4 can regulate melanoma progression. We also found that expression of Dicer (therefore, probably biogenesis of miRNAs) is required for suppression of melanoma invasion by Sox4. It is worth mentioning that Sox4 may also regulate the NF-κB pathway through controlling expression of other components of this large signaling cascade such

as NF-κB p65. Indeed our preliminary results showed that Sox4-KD may enhance expression of NF-κB p65 mRNA (Figure 7.1). However, whether Sox4 regulates expression of this gene in a similar manner to that of NF-κB p50 and the functional significance of this phenomenon in melanoma biology in general and Sox4-mediated suppression of melanoma migration and invasion should be revealed by future studies.

Figure 7. 1. Expression of p65 mRNA in MMRU cells after Sox4 knockdown. Quantitative RT-PCR results show that expression NF-κB p65 mRNA is increased after Sox4-KD.



It is possible that in addition to the transcription level, Sox4 can also regulate expression of these genes in post-transcriptional and/or post-translational levels. For instance, our miRNA-array after Sox4-KD showed a 5-fold reduction in expression of miR-9 (GEO database; accession number: GSE36715). miR-9 has been reported to target the NF-κB p50 mRNA and suppress its expression (Guo et al, 2009). Although we are not aware of the possible suppression of NF-κB p50 expression by miR-9 in melanocytic cells, it is possible that decreased expression

of miR-9 due its reduced biogenesis by Dicer in cells with low Sox4 expression is another mechanisms used by cancer cells to enhance the expression of p50 at post-transcriptional level.

Our miRNA expression profile also revealed a decreased expression of the let-7 family of miRNAs after Sox4 knockdown. Let-7 miRNas are prominent tumor suppressor that target several important oncogenic factors such as Ras (Johnson et al, 2005). Since the Ras/Raf regulated MAPK pathway has a very important role in melanomagenesis (discussed in more detail in section 1.1.4) it is possible that reduced let-7 expression in the absence of Sox4 may contribute to the activation of this pathway in melanoma cells.

Overall, it seems that Sox4 can either upregulate or repress expression of its target genes. For example, Sox4 inhibits NF-κB p50 expression as opposed to the enhanced expression of Dicer by this protein. These observations imply that Sox4 can simultaneously act as a suppressor or enhancer of transcription in the same cells depending on the nature of its regulatory sequences. This phenomenon may be explained by the inherent property of the Sox proteins which require specific binding partners to regulate expression of their target genes (Kamachi et al, 1999). It is worth mentioning that in addition to the role of binding partners and their specificity and distribution in different types of cells, regulation of miRNA biogenesis by Sox4 may also, at least partially, explain the pleiotropic function of Sox4 in different types of cancers. miRNAs' functions in any given cell depend on the pool of existing target mRNAs. Therefore, expression pattern of the target genes of any miRNA in a cell can determine the outcome of that particular miRNA. Similarly, the outcome of global change in the expression of miRNAs, as a result of change in the expression of their biogenesis factors, will entirely depend on the mRNA profile of the host cell. Therefore, the effect of reduced or increased expression of Sox4 (hence miRNA biogenesis factors that are regulated by Sox4) may not be similar in different cell types.

So far several studies investigated the aberrant expression of one or a small group of miRNAs in melanoma and their implication in regulation of different steps of tumorigenesis such as cell proliferation, migration and invasion as well as their prognostic value in predicting disease outcome (Segura et al, 2012). Nevertheless, the expression pattern of miRNA biogenesis factors and their functional significance in melanoma has remained largely unknown. In this study we showed that expression of two prominent miRNA biogenesis factors, Dicer and Drosha, is reduced during melanoma progression. It appeared that loss or reduction of Drosha expression happens at different stages than that of Dicer. In fact, we observed that while loss of expression of Drosha starts at the dysplastic nevi (Figure 6.1), Dicer expression persists at a level more or less similar to the normal nevi in dysplastic nevi and primary melanomas but dramatically reduces in metastatic melanoma (Figure 4.4). These observations suggest that the melanocytic cells probably use different regulatory mechanism to control expression of these two factors. Also, it can be implied that these factors may have non-overlapping functions other than the common role in biogenesis of miRNAs which may be significant in the process of melanomagenesis.

We further revealed that the subcellular localization of Drosha and Dicer is likely deregulated in melanoma. Accordingly, we observed that while nuclear expression of Drosha decreases during disease progression (Figure 6.1), its cytoplasmic expression increases (Figure 6.3). This event may signify a tendency of tumor cells to apply multiple approaches in inactivating a tumor suppressor by either reducing its expression or changing its subcellular localization to separate it from its substrate. A similar phenomenon has been reported for several other tumor suppressors (Chen et al, 1995; Sgambato et al, 1999). In addition, we observed a reduced expression of nuclear Dicer (Figure 5.1) along with the decreased cytoplasmic Dicer

expression (Figure 4.4). It is noteworthy that the reduced expression of nuclear Dicer shows a distinct pattern from its cytoplasmic expression. Accordingly, cytoplasmic Dicer only reduces in the metastatic phase while the nuclear Dicer decrease is more gradual and evident in all stages of melanocytic lesions. These observations indicate that although the general reduction in Dicer expression may play an important part in the reduced nuclear Dicer expression, deregulated nuclear localization may also contribute to this phenomenon.

7.2. Limitations of this study and future directions

In this study, we discovered a reduced expression of Sox4 in metastatic melanoma and its role in suppression of melanoma cell migration and invasion *in vitro*. However, it is not clear whether or not the expression of Sox4 is required for suppression of melanoma metastasis *in vivo*. We believe xenograft models or tail vain injection tumor metastasis assays would be powerful tools for addressing this question.

The NF-κB pathway is a complex network of a large number of proteins that regulate multiple aspects of cell biology (Ghosh et al, 1998; Perkins, 2000). In this study, we found that Sox4 inhibits the transcription of NF-κB p50 through binding to its regulatory sequences. However, it is not clear whether Sox4 can also influence the expression of this gene at other levels such as mRNA stability, translation efficiency or protein stability. In addition, Sox4 may also affect the nuclear localization and dimerization of the p50 protein which are essential for its proper function. Furthermore, it is not understood if Sox4 can, either directly or indirectly, affect the expression or activity of other components of this pathway such as IKK, IκB, etc. Our preliminary result showed that Sox4 may regulate expression of NF-κB p65 mRNA (Figure 7.1). However, further investigation is required to delineate the full scale of this phenomenon and its

significance in biology of melanoma. Also, it is not clear whether the activation of NF-κB pathway upon Sox4-KD is relevant in the *in vivo* setting. We believe that *in vivo* metastasis assays with melanoma cells that are concomitantly overexpressing both Sox4 and p50 and/or p65 or otherwise deficient in Sox4 and p50/p65 may address this question.

We further showed that regulation of expression of Dicer is another mechanism by which Sox4 can suppress melanoma invasion *in vitro*. It would be very helpful in understanding the biology of melanoma to use the metastasis assay to investigate whether this phenomenon is also relevant in the *in vivo* setting. For this purpose, stable knockdown of Sox4 and Dicer may be useful.

It is pertinent that Sox4 can also regulate the expression of miRNA biogenesis factors other than Dicer in melanoma cells. As a matter of fact, genome-wide promoter analysis in prostate cancer cells has shown binding of Sox4 to the promoter sequences of Argonaute 1, and RNA Helicase A (Scharer et al, 2009). Nevertheless it is not clear if a similar mechanism functions in melanoma cells.

Expression of Dicer in melanocytes is also regulated by the Microphthalmia-Associated Transcription Factor (MITF). In fact expression of Dicer protein and RNA is upregulated during melanocyte differentiation in a MITF-dependent manner (Levy et al, 2010b). Our data showed a marked reduction in Dicer expression in metastatic melanomas which may be consistent with the suggested reversal of the cells to an undifferentiated stage during tumorigenesis (Ben-Porath et al, 2008). It is notable that expression of MITF in melanocytes is regulated by Sox9 and Sox10 (Passeron et al, 2007; Potterf et al, 2000). Although so far there is no report of a possible regulation of MITF expression or activity by Sox4, expression of MITF was shown to be reduced

in metastatic melanoms {Salti, 2000 #6}. Therefore, it would be interesting to investigate the possibility of their cooperation in regulating Dicer expression in melanocytic cells.

We also showed that similar to Dicer, expression of Drosha markedly reduces in melanoma albeit in a different pattern than Dicer or Sox4. Although the functional significance of the downregulated Drosha in melanoma cells has remained to be identified, this observation implies existence of multiple mechanisms responsible for regulation of expression (or possibly activity) of these factors which deserve further investigation. In addition, it indicates that restoration of miRNA biogenesis pathway in advanced melanoma stages may not be feasible by rescued expression of one (or perhaps even several) factors. Indeed, a thorough study of the expression and activity of all known factors involved in the biogenesis and function of miRNAs may be required to fully understand the extend of aberration in its activity and necessary steps for its complete restoration as a possible treatment for melanoma.

The aberrant localization of Drosha and possible differential regulation of Dicer expression in the nucleus and cytoplasm indicate the existence of pathways which can regulate these events. It is as well possible that these putative pathways are malfunctional in melanoma. We believe identification of these putative pathways and understanding their functional significance in melanoma may further help in development of the treatment strategies.

Finally, we investigated the downstream mechanisms by which Sox4 exerts its function in melanoma including control of expression of NF-κB p50 and Dicer. However, very little is known about the mechanisms that regulate the expression of Sox4 in melanocytic cells. Prostaglandin A2/delta12-PGJ2 was shown to induce Sox4 mRNA expression in hepatocarcinoma cells (Ahn et al, 1999). Interestingly, administration of prostaglandin A, D, and

J analogues to established human melanoma tumors growing in athymic nude mice was shown to cause a significant growth inhibition. Nevertheless, it is not clear whether Sox4 plays a role in this process.

As mentioned earlier, members of Sox family such as Sox9 and Sox10 play important role in development and normal functions of melanocytes and are implicated in melanomagenesis. In addition, it is known that some members of the Sox family can regulate the expression of their other siblings. In breast cancer cells, transfection of Sox7 resulted in activation of the Sox4 promoter, leading to the increased expression at both mRNA and protein levels (Saegusa et al, 2012). It would be interesting to investigate whether any other member of this family can also regulate Sox4 expression and if this regulation has any relevance to melanomagenesis.

Tavazoie et al. demonstrated that expression of Sox4 can also be negatively regulated at post-transcriptional level by miR-335 in breast cancer and this event is crucial for suppression of breast cancer metastasis (Tavazoie et al, 2008). The reports on the oncogenic and tumor suppressor functions of miR-335 have been controversial in different types of cancer (Lynch et al, 2012; Ronchetti et al, 2008; Shu et al, 2011; Tavazoie et al, 2008). Nonetheless, to our knowledge there is no report on differential expression of miR-335 in metastatic melanoma tissues. Examining the expression pattern of this miRNA in melanoma tissues and its possible contribution to progression of melanoma remains to be done in future studies. At the protein level, Sox4 was shown to have a very short half-life which was prolonged by Syntenin through inhibition of proteasomal degradation and extension of its half-life (Beekman et al, 2012a). Nevertheless, Syntenin had previously been demonstrated to promote metastasis in human melanoma cells by various mechanisms such as activating c-Src (Boukerche et al, 2008) and NF-

κB (Boukerche et al, 2007). It is not clear whether interaction between Syntenin and Sox4 is relevant to the differential regulation of NF-κB by these two proteins in melanocytic cells.

Finally, genetic abnormalities such as chromosomal gain and deletion has been reported for the chromosomal regions encoding Sox4 and miRNA biogenesis factors such as Dicer (Grasemann et al, 2005; Hurst et al, 2004; Zhang et al, 2006). Nonetheless, the genomic and mutational status of these genes in melanoma remains uncharacterized. We believe a comprehensive analysis of the corresponding genomic sequences may add to our knowledge of the mechanisms responsible for the deregulated expression of these genes in melanoma.

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