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The Roles of Putative Tumor Suppressors Ink4a and Ink4b in Response to Oncogenic Ras and Bladder Specific Carcinogen BBN

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Abstract

Putative tumor suppressor genes located on 9p21 locus including Ink4a and Ink4b have been found to be deleted in many human cancers. Ink4a gene is one of key cell cycle inhibitors and has been intensively studied, however, little is known about Ink4b and no study compares the importance of Ink4a and Ink4b in suppressing tumor development. Although it has been established that there are some synergetic effects between deficiency of tumor suppressors and oncogene activation in triggering tumorigenesis, our previous study showed that Ink4a deficiency had no effect on tumor latency of Ras transgenic mice. In this thesis study, we showed that Ink4b deficiency accelerated bladder tumor development in Ras transgenic mice, which is in contrast with Ink4a deficiency. Elevated level of MAPK activation and CDKs were related with the cooperative effects. We further investigated the effects of overexpression of Ink4a and Ink4b *in vitro* and found that Ink4b suppressed cell growth more significantly than Ink4a by arresting more cells in G1 phase. Nevertheless, Ink4a knockout mice showed much higher susceptibility to bladder specific carcinogen BBN than wild type, Ink4b knockout and heterozygous Ink4a and Ink4b knockout counterparts. All Ink4a knockout mice developed invasive bladder tumors and loss of E-Cadherin was involved in this process. This is the first study that compares the importance of Ink4a and Ink4b. Ink4b is more important than Ink4a in terms of suppressing cell growth and low-grade bladder tumor development, while Ink4a is more potent than Ink4b in suppressing high-grade invasive bladder tumor development.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

1. Zhang Feng-Xia, Shao Hong-Lian, Wang Jin-Xing, Zhao Xiao-Fan, 2011, β-thymosin is upregulated by the steroid hormone 20 hydroxyecdysone and microorganisms. Insect Molecular Biology. Aug. 20, 519–527.

2. Liu Wen, Zhang Feng-Xia, Cai Mei-Juan, Zhao WL, Li XR, Wang Jin-Xing, Zhao Xiao-Fan, 2013, The hormone-dependent function of Hsp90 in the crosstalk between 20-hydroxyecdysone and juvenile hormone signaling pathways in insects is determined by differential phosphorylation and protein interactions. Biochim Biophys Acta. Nov;1830(11):5184-92

3. Gu Wen-yi, Prasadam Indira, Yu Mei-hua, Zhang Feng-xia, Ling Patrick, Xiao Yin and Yu Cheng-Zhong. Gamma Tocotrienol Targets Both Tyrosine Phosphatase SHPI and SHP2 in Mamma-spheres resulting in Cell Death through RAS/ERK pathway. Breast Cancer Research. 2014 (Under review)

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Dr. Lijie Ma contributed to a part of tumor-free rate

Statement of parts of the thesis submitted to qualify for the award of another degree

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Keywords

Ink4a, Ink4b, Ras, bladder cancer, transgenic, knockout

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Abbreviations

ATF	activating transcription factor
BBN	N-butyl-N-(4-hydroxybutyl) nitrosamine
BCPN	N-butyl-N-(3-carboxypropyl) nitrosamine
COX2	cyclooxygenase 2
CDK	cyclin-dependent kinase
CIS	carcinoma in situ
DMEM	dulbecco's Modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FGFR3	fibroblast growth factor receptor 3
GAPs	GTPase-activating proteins
GDP	guanosine diphosphate
GEFs	guanine nucleotide exchange factors
GTP	guanosine triphosphate
HBEGF	hepairin-binding epidermal growth factor-like growth factor
IACUC	Institutional Animal Care and Use Committee
LOH	loss of heterozygous
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
MEFs	mouse embryo fibroblasts
MMPs	Matrix metalloproteinases
PBS	Phosphate buffered saline
PI3K	phosphatidylinositide 3-kinase
Rb	retinoblastoma protein
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRF	serum response factor
VEGF	Vascular endothelial growth factor
WST-1	water soluble tetrazolium salt

Chapter 1: Introduction

1.1 9p21 loss in human tumors

The loss of 9p21 locus has been reported to happen in many human tumors (table 1), and it includes both LOH (loss of heterozygous) and homozygous deletion (Cairns, Polascik et al. 1995). In bladder cancer, the percentage of 9p21 homozygous deletion is pretty high. Apart from the tumors listed in table 1, 9p21 loss also exist in 57 % of melanoma cases (Holland, Beaton et al. 1994). Allelic loss of 9p21 happens in about 50% of hepatocellular carcinoma (Wang, Huang et al. 2000, Wang, Zhao et al. 2001). This phenomenon reveals that there must be some important genes located at this locus, and they are probably potential tumor suppressors.

1.2 Negative cell cycle regulators Ink4a and Ink4b

1.2.1 Gene structure of Ink4a and Ink4b

Ink4a and Ink4b are closely linked genes at the chromosome 9p21 locus in human, and are homologous to a region on mouse chromosome 4 (Kamb 1995). These two genes belongs to INK4 (inhibitor of cyclin-dependent kinase 4) family (Harper and Elledge 1996). The protein product of Ink4b is p15, while the Ink4a gene encodes two proteins $p16^{Ink4a}$ and $p14^{Arf}$ (p19 in mouse) by alternative splicing (Fig.1). The sequences between p16 and ARF have low similarity although they share the exons of Ink4a gene. The reason is the two variants use different reading frames. However, p15 and p16 share 83% identity (Sharpless 2005).

1.2.2 The tumor suppressor role of $p16^{INK4a}$

$p16^{Ink4a}$ activates retinoblastoma tumor suppressor pRb, which controls cell growth by transcriptional repression of genes required for transition from G1 phase to S phase of cell cycle. The activation is mediated by Cyclin-dependent kinases CDK4 and CDK6. They play an important role in phosphorylation of Rb (Fig.2) (Serrano, Lee et al. 1996). Loss of $p16^{Ink4a}$ function causes

pRb hyper-phosphorylation through CDK4/6 activation and eventually leads to uncontrolled transition to S phase.

1.2.3 The tumor suppressive role of $p19^{Arf}$

With regard to $p19^{Arf}$, its tumor suppressor function is evident by its ability to induce cell cycle arrest and prevent oncogenic transformation (Chin, Pomerantz et al. 1997, Pomerantz, Schreiber-Agus et al. 1998). $p19^{Arf}$ activities requires p53 (Kamijo, Zindy et al. 1997). The biochemical basis of p53-dependent $p19^{Arf}$ function is that $p19^{Arf}$ interacts with MDM2 and prevents MDM2 mediated p53 degradation (Kamijo, Zindy et al. 1997). In p53-deficient cells, $p19^{Arf}$ are upregulated and it is downregulated by p53, which suggest that there is a feedback loop between p53 and $p19^{Arf}$ (Stott, Bates et al. 1998).

1.2.4 The tumor suppressive role of $p15^{Ink4b}$

Compared with abundant evidence for tumor suppressive role of p16, there is limited data showing the role of $p15^{Ink4b}$ in tumor suppression. Both Ink4a and Ink4b loci or Ink4a alone are homozygous deleted in most tumors (Ruas and Peters 1998). Specific deletion of Ink4b occurs in only a few cases. However, inactivation of p15 by hypermethylation seems to be frequent in leukemias and lymphomas. This inactivation is p16 independent (Malumbres, Perez de Castro et al. 1997). As a cell cycle inhibitor, p15 is able to produce cell cycle arrest and suppress Ras-activated transformation in mouse embryo fibroblast (Malumbres, Perez De Castro et al. 2000). In this case, the roles of $p15^{Ink4b}$ in specific tissue deserve further investigation.

To address whether Ink4a and Ink4b loss are correlated with tumor development, I used bladder cancer as the cancer model in this thesis project.

1.3 Bladder cancer

1.3.1 Urothelium

All bladder tumors are originated from urothelium, which is a specialized epithelium. It covers the inner surface of bladder, renal pelvis, ureter and prostatic urethra. These urothelia have different embryonic origin and cellular differentiation although they have the same morphology (Wu, Kong et al. 2009). Urothelium serves as a barrier between urine and blood and it also has secretory activity (Negrete, Lavelle et al. 1996, Lewis 2000). Under normal condition, urothelium renew very slowly in order to keep stability. The tritium-thymidine labeling index is less than 0.01% (Walker 1960, Hicks 1975).

Urothelium cells present different phenotypes between different layers (Fig 1). The least differentiated basal cells with smallest size reside in the basal layer, where urothelial stem cells are supposed to be (Kurzrock, Lieu et al. 2008). The intermediate cells, differentiated from the basal cells, reside just on top of basal cells (Negrete, Lavelle et al. 1996). The superficial urothelium cells, also named umbrella cells face to bladder lumen. They are quite distinguishable as they are large, polyhedral, and bi-nucleated, which is probably formed by cell-cell fusion of intermediate cells (Koss 1969). Unlike other cells, they form the apical surface of the urothelium (Hicks 1965). The umbrella cells are terminally differentiated and unable to undergo cell division (Cheng, Huang et al. 2002).

1.3.2 Bladder cancer and subtypes

Urothelial tumor is one of the most common cancers in the Unites States and around the globe. The number of new cases annually is 336,000 and this number is estimated to be 800,000 within next 5 years due to the aging and accumulated exposure to carcinogens (Parkin, Bray et al. 2001). Etiological factors associated with the development of bladder cancer include cigarette smoking, occupational exposure to specific chemicals, treatment with cytostatic drug, arsenic and Schistosoma infection (Johansson and Cohen 1997).

These carcinomas are mainly composed of two variants with different phenotypes and molecular genetic markers (Koss 1992). The low-grade non-invasive papillary tumor progressed from urothelial hyperplasia accounts for about 80 % of urothelial carcinomas. About 70% of these tumors will recur, but only 15% have the potential to progress into muscle invasive stage (Miyamoto, Miller et al. 2010). The 5-year survival rate is about 90%. High grade invasive tumor is the second main variant which accounts for about 20% of the urothelial carcinomas. These tumors occur as invasive tumors or arise from flat, high-grade carcinoma *in situ* (CIS) (Steinberg, Trump et al. 1992, Liebert and Seigne 1996). The 5-year survival rate for highly invasive bladder cancer is about 6%. Over 50% of these invasive tumors develop to metastasis.

1.3.3 Genetic factors underlie bladder tumorigenesis

Accumulated evidence suggests that there are two different pathways underlying non-invasive and invasive bladder tumorigenesis (Fig.4)(Wu 2005). In low grade papillary bladder tumor, about 46% of urothelial tumors have H-ras mutation and about 70% harbor mutation of fibroblast growth factor receptor 3 (FGFR3) (Cappellen, De Oliveira et al. 1999, Zhu, Xing et al. 2004). Deletions of the arms of chromosomes including 8p, 11p, 13q and 14q are involved in the progression to invasive stage (Knowles, Shaw et al. 1993, Cordon-Cardo, Cote et al. 2000). Chromosome 9p and 9q loss are present in both low grade non-invasive and invasive bladder tumor at early stage (Spruck, Ohneseit et al. 1994). In invasive bladder tumor and its precursor flat CIS, over 50% have p53 mutation. Retinoblastoma gene (RB1) dysfunction, including lack of RB expression and hyper-phosphorylated RB overexpression, is also prevalent in human invasive bladder tumor (Cairns, Proctor et al. 1991, Chatterjee, George et al. 2004). Increased N-cadherin, which promotes cell invasion, and Reduced E-cadherin expression caused by promoter hyper-methylation happen mainly in invasive tumor (Ramos, Palacios et al. 2000, Ribeiro-Filho, Franks et al. 2002). Matrix metalloproteinases (MMPs), which degrade extracellular matrix and base membranes, are frequently up-regulated in invasive tumor particularly (Kanayama 2001). Vascular endothelial

growth factor (VEGF) which is an angiogenesis inducer, and cyclooxygenase 2 (COX2) which catalyzes the synthesis of prostaglandins are both over-expressed in bladder cancer, especially in invasive cancer (Campbell, Volpert et al. 1998, Komhoff, Guan et al. 2000) .

These genetic alterations do not happen alone, there must be synergisms. In this thesis, I focus on oncogenic Ras and two genes Ink4a and Ink4b at 9p21 locus.

1.4 Ras and its oncogenic effects

1.4.1 Ras

Ras was first identified in T24 and EJ human bladder cancer cell lines in the early 1980s and it was the first named human oncogene (Parada, Tabin et al. 1982, Reddy, Reynolds et al. 1982). There are three Ras genes in humans, which encode four different Ras proteins with high homology including H-ras, N-ras, K-ras4a and K-ras4b. Ras is a GTPase and activated by binding with GTP while inactivated by binding with GDP. The transition between active and inactive status is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs promote Ras activation by stimulating GDP for GTP exchange and GAPs inactivate Ras by promoting Ras mediated GTP hydrolysis.

Residue mutations Q61, G12 and G13 impair GTP hydrolysis and result in persistent GTP-binding state of Ras. As a result, Ras-dependent downstream effectors are constantly activated (Scheffzek, Ahmadian et al. 1997, Scheidig, Burmester et al. 1999). The activated Ras harbors transforming properties by gain-of-function mutation, which leads to successive accumulation of genetic alterations and then confer cancer cells the capability to evade homeostatic barriers. These capabilities including self-sufficiency in growth signals, insensitivity to growth inhibitors, angiogenesis, metastasis, and escape from programmed cell death (Hanahan and Weinberg 2000).

The oncogenic outputs of mutation in different Ras isoforms are tissue dependent. K-ras mutations are mostly detected in biliary cancer, lung cancer and pancreatic carcinomas. H-ras mutations are more linked with endometrial cancer, tumors of skin, and salivary gland. N-Ras mutations are common in hematopoietic cancers (Bos 1989, Karnoub and Weinberg 2008). In bladder cancer, the most frequently mutated ras is H-ras (Knowles and Williamson 1993).

1.4.2 Oncogenic Ras promotes proliferation

Studies revealed that overexpression of constitutively active H-ras was sufficient for driving the G0 phase-arrested cells into the cell cycle without growth factor (Feramisco, Gross et al. 1984, Stacey and Kung 1984). Active Ras upregulates the transcription of growth factors such as hepairin-binding epidermal growth factor-like growth factor (HBEGF) and transforming growth factor-α (TGFα) (Mccarthy, Samuels et al. 1995) . To tip the proliferation balance, oncogenic Ras can inhibit TGFβ signal and thus interfere with anti-proliferative signals (Filmus, Zhao et al. 1992). The proliferative signals resulting from oncogenic Ras culminate with upregulation of several transcription factors that are required for cell cycle entry and progression, including FOS, serum response factor (SRF), the leucine zipper protein JUN, the ETS domain-containing transcription factor ELK1, activating transcription factor (ATF2) and nuclear factor-κB. These transcription factors in turn trigger the expression of G1 cyclin, cyclin D1 (Filmus, Robles et al. 1994, Winston, Coats et al. 1996). To stimulate the transcription of cyclin D1, oncogenic Ras also regulates its stability via PI3K-dependent inhibition of glycogen synthase kinase 3β (GSK3β). GSK3β is responsible for the phosphorylation and consequent ubiquitylation and proteasomal degradation of cyclin D1 (Diehl, Cheng et al. 1998). Cyclin D1 is a crucial determinant of oncogenic effects of Ras mutation as revealed by Cyclin D1 knockout mice studies (Robles, Rodriguez-Puebla et al. 1998).

Excessive proliferation leads to DNA replicative stress, which ultimately results in DNA damage and the activation of DNA damage response (Bartkova, Horejsi et al. 2005, Gorgoulis, Vassiliou et

al. 2005). In a normal cell that has functional DNA damage checkpoint, DNA damage response induced by oncogenic Ras leads to senescence (Di Micco, Fumagalli et al. 2006). However, this response creates selective pressure in favor of cells that are key check-point deficiency, which contribute to tumorigenic process (Halazonetis, Gorgoulis et al. 2008).

1.4.3 Oncogenic Ras inhibits apoptosis

Apoptosis is a defense mechanism against malignancy. It can be initiated intrinsically through mitochondria-mediated pathway when activated by DNA damage or nutrient deprivation. It can also be initiated extrinsically through extracellular cues such as growth factor withdrawal and matrix detachment. Pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family act on mitochondria-mediated pathway while FLIP acts on extrinsic pathway. Inhibitors of apoptosis (IAPs) act on both pathways (Cox and Der 2003).

The PI3K pathways activated by oncogenic Ras leads to downregulation of pro-apoptotic protein BCL-2-homologous antagonist/killer 1 (BAK1), and enhanced IAP level (Rosen, Rak et al. 1998, Mayo and Baldwin 2000). In addition, RAF pathway activated by oncogenic Ras upregulates the anti-apoptotic proteins BCL-2 (Kinoshita, Yokota et al. 1995, Fang, Yu et al. 1999). Both pathways have been demonstrated to mediate the phosphorylation of BCL-2-associated agonist of cell death (BAD), which eventually inactivate BCL-2 (Fang, Yu et al. 1999). Recent studies also showed that oncogenic Ras can induce epigenetic silencing of pro-apoptotic CD95 (Gazin, Wajapeyee et al. 2007). However, the precise role of Ras in tumorigenesis need to be further elucidated.

1.5 Transgenic mouse study on active Ras

Dr. Wu's lab has been studying the *in vivo* role of Ras activation in bladder urothelium for over ten years. Mutant H-Ras (G12V), which is constitutively active, is expressed under the control of a mouse uroplakin II promoter, which makes the expression of Ras urothelial specific (Zhang, Pak et

al. 2001). The latency of tumor development is dose-dependent. Hyper-activation of Ras (Ras homozygous transgenic) leads to about 50% urothelium transformation in 3 months. Low-level expression of activated H-ras (Ras heterozygous transgenic) leads to simple hyperplasia. After 10-month latency, about 60% of Ras heterozygous transgenic mice develop low-grade papillary tumor (Mo, Zheng et al. 2007). Such a long latency suggests that tumor suppressors impede the tumor development.

It has been reported that oncogenic Ras activity induces up-regulation of negative cell cycle regulators $p15^{Ink4b}$ and $p16^{Ink4a}$ in cultured cells (Malumbres, Perez De Castro et al. 2000). In mouse urothelial tumor induced by transgenic active Ras, there is no change for ARF while both $p15^{Ink4b}$ and $p16^{Ink4a}$ are up-regulated (Mo, Zheng et al. 2007). The induction of tumor suppressor $p15^{Ink4b}$ and $p16^{Ink4a}$ may represent host defense mechanism for controlling cell proliferation. It would be interesting to find out whether it is $p15^{Ink4b}$ or $p16^{Ink4a}$ that suppresses tumor development induced by Ras activation. Dr. Wu's group found that mice with transgenic Ras and Ink4a deficiency did not show accelerated bladder tumor development (Mo, Zheng et al. 2007). However, another transgenic animal study revealed that Ink4a deficiency cooperated with Ras activation in the pathogenesis of melanoma (Chin, Pomerantz et al. 1997). Thus, the synergism of genes in tumorigenesis may be tissue specific.

Whether $p15^{Ink4b}$ induction suppresses bladder tumor development is unknown. In this project, we aim to find out whether Ink4b deficiency in combination with oncogenic Ras can trigger bladder tumorigenesis. I will show that mice with transgenic Ras and Ink4b deficiency develop low grade bladder tumor in a significant contrast with Ras transgenic and Ink4a knockout mice. Cell cycle regulators including CDK2, CDK4, CDK6 and Cyclin D1 are up-regulated. MAPK is also strongly activated in urothelium of Ras transgenic and Ink4b knockout mice.

What we find *in vivo* suggests that Ink4b is more important than Ink4a in suppressing urothelial tumorigenesis. Up to date, no studies yet have compared Ink4a and Ink4b and their difference in function is not clear. We aim to find how Ink4b is different from Ink4a *in vitro*. I will show that p14 overexpression does not inhibit cell growth while both p15 and p16 overexpression suppress cell growth in human bladder cancer cell line UM-UC-3 cells, which do not express p15, p16 and p14. In addition, both p15 and p16 overexpression cause cell cycle arrest in G1 phase, however the amount of suppression is greater with p15 overexpression.

1.6 BBN as an urothelial specific carcinogen

BBN (N-butyl-N-(4-hydroxybutyl) nitrosamine) is extensively used to create experimental models for chemical urothelial carcinogenesis in mouse and rat (Cohen 1998, Yamamoto, Nakata et al. 1999). Bladder tumors induced by BBN in rat are non –invasive papillary tumors (Fukushima, Hirose et al. 1976). In contrast, mouse bladder tumors induced by BBN are invasive tumors (Tamano, Hagiwara et al. 1991). BBN targets bladder with remarkable specificity. The reason underlying this specificity is that bladder urothelium is the major targeted tissue for BBN induced mutagenesis (He, Kosinska et al. 2012). BBN is primarily metabolized in the liver and its genotoxic effects are mediated by its metabolite N-butyl-N-(3-carboxypropyl) nitrosamine (BCPN) (Bonfanti, Magagnotti et al. 1988). BCPN is a direct bladder carcinogen. It reaches the bladder through urine and initiates the carcinogenic process (Cohen, Ohnishi et al. 2007).

p53 and H-ras mutations have been detected in murine bladder tumors induced by BBN, which resemble what happen in human bladder cancer (Masui, Dong et al. 1996). Loss of chromosome 4 is also detected, which is similar to 9p21 loss in human bladder cancer (Ogawa, Uzvolgyi et al. 1998). These human relevant evidences suggest that this model is useful for studying the pathogenesis of human bladder cancer, particularly invasive bladder cancer.

Transgenic rats with human oncogene H-ras are more susceptible to BBN-induced carcinogenesis, and H-ras overexpression is correlated with malignant progression (Ota, Asamoto et al. 2000). p53 heterozygous knockout mice also show high susceptibility, which may be caused by increased cell proliferation rate (Ozaki, Sukata et al. 1998). This study further addresses the importance of p53 in suppressing tumor development. So far, there is no study on how Ink4a and Ink4b, which are located at 9p21, are involved in pathogenesis of BBN-induced bladder tumor. In this thesis project, I aim to find out whether Ink4b is also more important than Ink4a in BBN induced carcinogenesis. I will show mice with Ink4a deficiency are more susceptible to BBN treatment while Ink4b deficiency is not important.

Table 1-1: Allelic status in primary human tumors (Cairns, Polascik et al. 1995). SCLC stands for small cell lung cancer. NSCLC stands for non-small cell lung cancer.

Table 1-1

Tumour source	Total	Any Loss of of 9p (%)	Total LOH only	Total homozygous deletion (%)
Bladder	285	177 (62)	51	126 (71)
Head and Neck	65	49 (75)	33	16 (33)
Renal Cell	45	18 (40)	11	7 (39)
SCLC	39	30 (77)	28	2 (7)
NSCLC	39	31 (79)	26	5 (16)
Breast	20	13 (65)	11	2(15)
Prostate	15	8 (53)	5	3(40)
Endometrial	18	5 (28)[f]	5	0 (0)
Cervical	10	2 (30)	2	0 (0)
Colon	9	3 (33)	3	0 (0)

Figure 1-1: The Ink4a/Ink4b locus (Gillespie, Markerink-van Ittersum et al. 2006). The exons are represented as boxes. The sequences encoding p15 are marked with light blue color, and sequences for p16 is marked with green color while those encoding $p14^{Arf}$ are showed with yellow color.

Figure 1-1

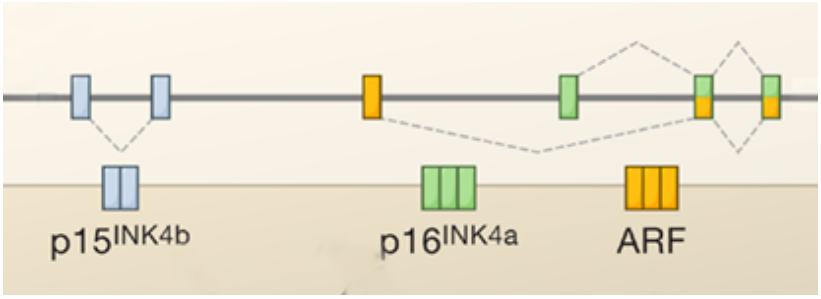


Figure 1-2: The regulation of G1/S transition by pRB (Chin, Merlino et al. 1998). In G1, hypo-phosphorylated pRB binds to transcription factor E2F and therefore represses the transcription of E2F responsive genes. Upon receiving growth signaling, CDK4/Cyclin D complex phosphorylates and releases pRB from E2F. E2F responsive genes are activated subsequently and cells enter into S phase. The activity of CDK4 is negatively regulated by $p16^{INK4a}$.

Figure 1-2

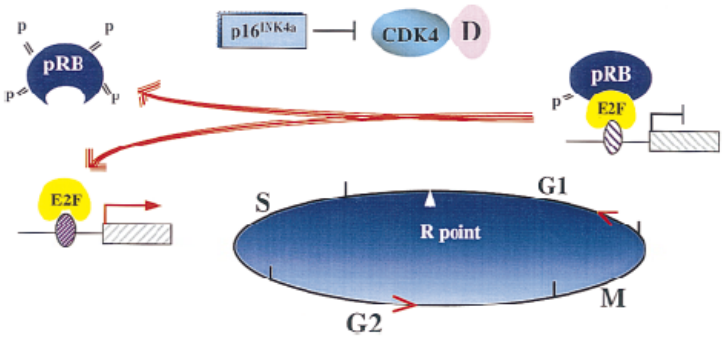


Figure 1-3: Representation of urothelium cell types modified from Gillespie et al., 2006 (Gillespie, Markerink-van Ittersum et al. 2006). The orange cells with red nuclear at the bottom are basal cells. The white cells with purple nuclear stand for intermediate cells. Blue cells with pink nuclear present as umbrella cells.

Figure 1-3

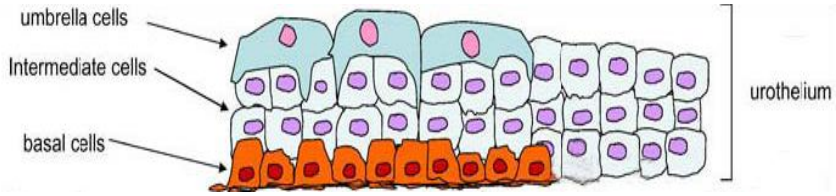
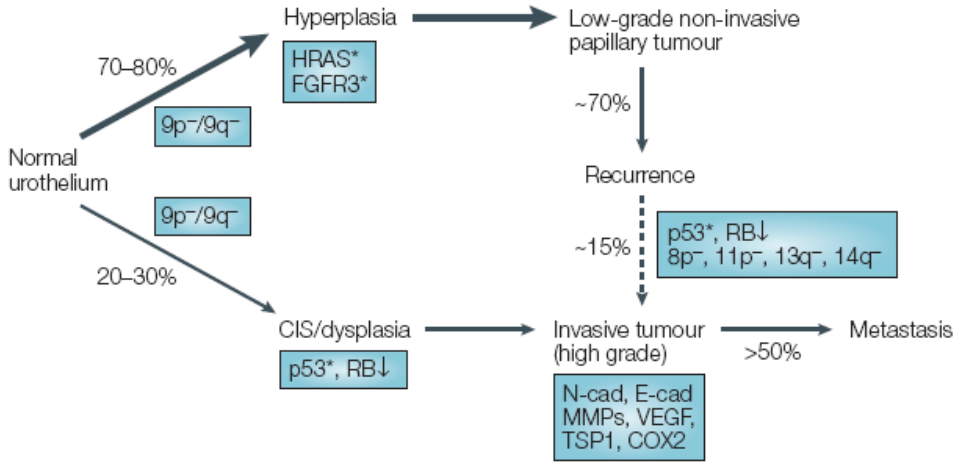

umbrella cells
Intermediate cells
basal cells
urothelium

Figure 1-4: Genetic alterations underlie two pathways of urothelial tumorigenesis (Wu 2005). About 70-80% of bladder cancer is low-grade non-invasive papillary tumor which develops from hyperplasia. The genetic alterations underlie this pathway include 9p/9q loss, HRAS and FGFR3 mutation. About 70% of low-grade tumor recurs after surgical removal. 15% of the recurred low-grade tumor progresses to invasive tumor with p53 mutation, RB down-regulation and loss of 8p, 11p, 13q and 14q. The other pathway which accounts for 20-30% of bladder cancer is high-grade invasive tumor arising from CIS/dysplasia which harbors p53 mutation and RB down-regulation. The genetic alterations underlie invasive tumor include N-cadherin, E-cadherin, MMPs, VEGF, TSP1 and COX2. Over 50% of invasive tumor becomes metastasis.

Figure 1-4


Hyperplasia
Low-grade non-invasive papillary tumour
HRAS*
FGFR3*
70–80%
9p⁻/9q⁻
Normal urothelium
~70%
Recurrence
9p⁻/9q⁻
~15%
p53*, RB↓
8p⁻, 11p⁻, 13q⁻, 14q⁻
20–30%
CIS/dysplasia
Invasive tumour (high grade)
>50%
Metastasis
p53*, RB↓
N-cad, E-cad
MMPs, VEGF,
TSP1, COX2

Chapter 2 Methods

2.1 Methods for *in vivo* study

2.1.1 Transgenic and knockout mice

Transgenic mice expressing constitutively active Ha-Ras in urothelium cells under the control of UPII promoter were generated previously (Zhang, Pak et al. 2001). P15 knockout mice were donated by Angel Pellicer (Department of Pathology, New York University Langone Medical Center). The heterozygous p15 knock out mice were inbred to obtain homozygous mice. Ras transgenic and Ink4b knockout mice were crossed and then inbred to produce compound mice. Ink4a knockout mice, which have deletion of both p16Ink4a and p19Arf, were obtained from Dr. Ron DePinho (Harvard Medical School, Boston, Massachusetts, USA) (Serrano, Lee et al. 1996). To obtain Ras transgenic and Ink4a homozygous knockout mice, Ras heterozygous transgenic mice were crossed to Ink4a homozygous knockout mice. The Ras heterozygous transgenic and Ink4a heterozygous knockout mice from the offspring were backcrossed to Ink4a homozygous knockout mice.

For BBN treatment, mice were housed in a special room used for carcinogen and toxicity treatment. They were fed with 0.05% BBN (TCI America) in drinking water, which was refreshed twice a week.

All these mouse strains were maintained in pathogen-free facility. Mice are housed in 12-h light/dark cycle with free access to food and drink at constant 22 ℃. All animal studies were conducted in accordance with government guidelines and under an active protocol approved by the Institutional Animal Care and Use Committee (IACUC).

2.1.2 Genotyping

2.1.2.1 Genomic DNA extraction

About 1 cm tail per mouse was cut and digested overnight with 500 µl extraction buffer (50 Mm Tris-Hcl pH7.4, 100 mM EDTA, 100 mM NaCl) supplemented with 12.5µl 20% SDS (sodium dodecyl sulfate) and 10 µl protease K at 50 ℃. Then 135µl 5M NaCl was added to the mixture and the tube was centrifuged at 14,000 rpm for 20 min after shaking vigorously. The supernatant was then transferred to a new tube and added with 1 ml 100% ethanol. The DNA will precipitate after centrifuging at 14,000 rpm for 10 min. The pellet was washed with 1 ml 70% ethanol and air dried before dissolved in 200 µl distilled water. 60 µl sodium acetic acid and 520 µl 100% ethanol were added to the solution and the mixture was centrifuged at 14,000 rpm for 10 min. The pellet was washed with 1 ml 70% ethanol and air dried before dissolved in 50 µl (for southern blot)/150 µl (for PCR) water.

2.1.2.2 PCR for Ink4a and Ink4b genotyping

Ink4a wt F and Ink4a wt R primers were used for wild type Ink4a allele, yielding a 278 bp product. Ink4a ko F and Ink4a ko R primers were used for knockout Ink4a allele, yielding a 313 bp product. The PCR procedures for both Ink4a wild type allele and knockout allele were as follow: 94 ℃ 3 min; 94 ℃ 30 sec, 60 ℃ 30 sec, 72 ℃ 30 sec, 40 cycles; 72 ℃ 5 min; 4 ℃, hold. The primer pairs (Ink4b wt F and Ink4b wt R) for wild type Ink4b allele are from second exon, yielding a 273 bp product. The PCR procedures were as follow: 94 ℃ 3 min; 94 ℃ 30 sec, 54 ℃ 30 sec, 72 ℃ 30 sec, 40 cycles; 72 ℃ 5 min; 4 ℃, hold. The primer pairs (Ink4b ko F and Ink4b ko R) for Ink4b knockout allele are from first intron and *neo* gene, yielding a 500 bp product. The PCR procedures were as follow: 94 ℃ 3 min; 94 ℃ 30 sec, 57 ℃ 30 sec, 72 ℃ 30 sec, 40 cycles; 72 ℃ 5 min; 4 ℃, hold. All primer sequences are listed in table 2.

2.1.2.3 Southern Blotting for Ras genotyping

10 μg gDNA per mouse was digested with NcoI overnight at 37 ℃. Then the DNA will be applied to eletrophorysis on 0.8% agarose gel at low voltage (3 V/cm). Then the gel was soaked in 0.25 M HCl for 20 min with gentle agitation followed by rinsing with deionized water for 5 min. After that, the gel was soaked twice in base solution composed of 1.5 M NaCl and 0.5 M NaOH for 20 min with shaking. Then the gel went through wet-transfer overnight. The next day, Nylon membrane was rinsed twice in 2×SSC with agitation for 5 min, and crosslinked in Stratalinker. Then the membrane was incubated in hybridization buffer at 60 ℃ in hybridization oven for 1 h. The probe was 3' end of UPII promoter, which could identify both endogenous (1.4 kb) and transgenic (1.7 kb) UPII promoter (Zhang, Pak et al. 2001). The hybridization and detection steps were done following manufacture's instruction (Amersham Gene Images AlkPhos Direct Labelling and Detection System, GE Healthcare).

2.1.3 Histopathology and Immunohistochemistry

Mouse bladders were histopathologically analyzed. Bladders were fixed in 10% formalin overnight and then embedded in paraffin, cut to 4 μm sections, and stained with H&E for histopathological examination. Briefly, deparaffinized sections were stained with hematoxylin for 2-5 min and then washed with running water for 10 min, followed by staining with eosin for 30 sec- 1min. The sections were then dehydrated and mounted with mounting medium. For immunohistochemistry, sections were deparaffinized, soaked in antigen unmasking solution (Vector laboratories) and microwaved at the 9^{th} power level (Sensor Microwave Oven, GE) for 20 min, followed by washing with PBS for 3 times. Slides were then soaked in 3% H_2O_2 for 10 min and washed with H_2O for 3 times. After blocking with 3% BSA for 30 min, the sections were incubated with primary antibodies diluted in 3% BSA overnight at 4 ℃. The next day, the sections were washed with PBS for 3 times, and then incubated with HRP conjugated secondary antibody for 1 h at room temperature. After washing with PBS (Phosphate buffered saline) for 3 times, the sections were developed with DAB Peroxidase (HRP) Substrate Kit, 3,3'-diaminobenzidine for 5-10 min at room temperature

according to manufacturer's instruction (Vector Laboratories). The reaction was stopped by soaking slides in running water for 10 min. The slides were then dehydrated and mounted with mounting medium. The primary antibodies used in immunohistochemistry were as follow: anti-Cyclin D1, anti-phospho-MAPK (Cell Signaling Technology), anti-E-cadherin (Santa Cruz Biotechnology), anti-Zeb1, anti-Zeb2, anti-Twist, anti-cytokeratin 5 (Abcam).

2.1.4 Double Immunofluorescence staining

The procedures on the first day were the same as immunohistochemistry. The two primary antibodies (rabbit anti-E-cadherin and goat anti-cytokeratin 14 (Santa Cruz)) were mixed together. The next day, the sections were washed with PBS for three times and then incubated with a mixture of Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 594 anti-goat IgG for 1 h at room temperature. After washing with PBS, the sections were counterstained with DAPI for 10 min, followed by being mounted with aqueous mounting medium (DAKO). Pictures were taken under Immunofluorescence microscopy (Nikon).

2.1.5 Western Blotting analysis

Total proteins were prepared in a lysis buffer containing 20 mM Tris-HCl buffer (pH 7.5), 10% SDS, 5 mM beta-mercaptoethanol, 50 mM NaCl and cocktail of protease inhibitors. After determination of protein concentration, 50 mg of proteins per lane were resolved by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and electrophoretically transferred onto polyvinylidene fluoride membrane. After incubated with primary antibodies at 4 ℃ overnight, the membrane was washed 3 times with TBST and then incubated with secondary antibodies for 1 h at room temperature. After another round of washing with TBST, the membrane was developed using enhanced chemiluminescent method (Amersham Biosciences). The primary antibodies adopted in western immunoblotting were as follow: anti-CDK2, anti-CDK4, anti-CDK6, anti-Cyclin D1, anti-MAPK, anti-phospho-MAPK, anti-p16, anti-p14, anti-p19 (Cell Signaling

Technology); anti-p15 (Abcam), Antibody against mouse β-actin (Sigma) was used as an internal loading control.

2.1.6 Cell cycle analysis

The bladders from 8 month old mice were taken out and turned inside out. Then the whole bladders were incubated with 1 mg/ml dispase at 4℃ overnight. The next day, the urothelial cells were gently scraped off and washed with PBS. The cells were digested with 0.25% Trypsin-EDTA (ethylenediaminetetraacetic acid) solution at 37℃ for 30 min. The digestion was stopped by adding fetal bovine serum to a final concentration of 20%. The cells were collected by centrifugation at 800 g for 5 min and washed twice with PBS. After filtered through 100 μm pore-size filter, the cells were fixed with pre-cooled 70% ethanol at 4℃ overnight. On third day, the cells were washed with PBS for 3 times, and then stained with 40 μg/ml propidium iodide (Roche) containing 100 μg/ml RNase (Thermo Scientific). These cells were then sorted with Facscan (Beckman). The data were analyzed using ModiFit 3.2 (Verity Software House).

2.2 Methods for *in vitro* study

2.2.1 Cell culture

RT4 and T24 cells were cultured in Myco5A medium containing 10% fetal bovine serum (FBS). RT112 and 5637 cells were cultured in RPMI1640 medium supplemented with 10% FBS. UM-UC-3, J82, and HT1376 were cultured in DMEM (dulbecco's Modified Eagle's medium, high glucose) medium with 10% FBS. T24T and Urosta cells were cultured in DMEM/F12 (1:1) medium with 10% FBS.

2.2.2 RT-PCR

Total RNA was isolated from cultured cells using RNeasy Micro Kit according to manufacturer's instruction (Qiagen). 2 μg total RNA per each sample was used as template for cDNA synthesis using High Capacity cDNA Reverse Transcription Kit following manufacturer's protocol (Applied Biosystem). 0.5 μl cDNA was used as template for RT-PCR procedures. The primer pairs and sequences are listed in table 3. PCR procedures are as follow: 94 ℃ 3min; 94 ℃ 30 s, 60 ℃ 30 s, 72 ℃ 30 s, 30 cycles; 72 ℃, 5 min; 4 ℃ hold.

2.2.3 Plasimid construction

Human p15, p16 and p14 ORF clones were purchased (OriGene). Each ORF was inserted into pTRE-Tight vector at EcoR1 and EcoRV restriction enzyme sites. The sequences were confirmed by sequencing service (Genewiz).

2.2.4 Transfection

UM-UC-3 cells were seeded to 30–40% confluence in 6-well plates 24 h prior to transfection. The vacant vector was used as control. 4 μg plasimid and 10 μl Lipofectamine 2000 were diluted with 100 μl Opti-MEM respectively and incubated for 5 min. The diluted lipofectamine 2000 was added to the diluted plasimid and the mixture was incubated for 20 min at room temperature to allow the formation of DNA-lipofectamine complex, which was added to the well after the growth medium without antibiotics was refreshed. The medium containing the complex was removed and replaced the transfection medium with fresh growth medium 6 h later. To make a stable transfected cell line, the cells were then cultured in the medium containing 800 μg/ml G418 (Life Technology) 48 h after transfection. A single cell which survived the antibiotic selection was picked and propagated under G418 selection for one month. The stably transfected cells were maintained in complete growth medium containing 200 μg/ml G418.

2.2.5 Inducible stable transfected cells

Firstly, UM-UC-3 cells were transfected with pTet-On Advanced and stable transfected cell line was made by selecting with 800 μg/ml G418 for one month. The induction capability was tested by transfecting this cell line with pTRE-Tight-BI-ZsGreen1. The cells were then incubated with 0.1, 0.5 and 1 μg/ml doxycycline for 48 h respectively. The expression of ZsGreen was checked under fluorescence microscope. Secondly, the stable pTet-On Advanced transfected UM-UC-3 cells were co-transfected with pTRE-Tight-p15 and puromycin linear DNA, or pTRE-Tight-p16 and puromycin linear DNA (20:1) respectively. 24 h after transfection, cells were split into two wells. Cells in one well were incubated with 1 μg/ml doxycycline for 48 h. P15 and p16 expression were confirmed by western blotting. Cells in the other well were applied to 2 μg/ml puromycin selection. Three days later, the cells were trypsinized and seeded into 96-well plates. Only one cell was seeded in each well. Specific primers for RT-PCR were used to identify which clone expressed the inducible p15 and p16 respectively. The positive clones were further confirmed by western blotting. The double stable transfected cell lines were maintained in complete growth medium containing 200 μg/ml G418 and 0.5 μg/ml puromycin.

2.2.6 Cell proliferation assay

1×10^4 cells in 100 μl growth medium were seeded in each well of 96-well plates. 10 μl WST-1 (water soluble tetrazolium salt) reagent (Roche) was added to each well and incubated at 37 ℃ for 1 h. The OD value was measured at 450 nm. The measurement was taken at 24 h, 48 h, 72 h and 96 h respectively. For the starting point, the measurement was taken 2 h after cells seeded. There were 5 repeats at each time point for each sample.

2.2.7 Cell cycle analysis

Cells were incubated with 1μg/ml doxycycline for 72 h prior to harvest. After washing with PBS, the cells were fixed with pre-cold absolute ethanol overnight. Then the cells were washed with PBS

and incubated with 40 μg/ml propidium iodide and 0.1 mg/ml DNAse-free RNase A for 15 min at 37. Finally, the stained cells were subjected to FACS (fluorescence-activated cell sorting) analysis using a FACScan (Beckman). 10,000 cells per each sample were analyzed. The data were analyzed by ModFit LT 3.2 software.

Table 2-1: primers and sequences

Ink4a wt F	GTG ATCCCTCTACTTTTTCTTCTGACTT
Ink4a wt R	CGGAACGCAAATATCGCAC
Ink4a ko F	GTG ATCCCTCTACTTTTTCTTCTGACTT
Ink4a ko R	GAGACTAGTGAGACGTGCTACTTCCA
Ink4b wt F	GTCATGATGATGGGCAGCG
Ink4b wt R	CCGGAATTCGCGTGCAGATACCTCGC
Ink4b ko F	ATCCGAGTGCCTACACCTCCA
Ink4b ko R	GCTCCCGATTCGCAGCGAT
h-p14 F	GGTTTTCGTGGTTCACATCCC
h-p14 R	CCCATCATCATGACCTGGTCTT
h-p16 F	CATGGAGCCTTCGGCTGACT
h-p16 R	CCATCATCATGACCTGGATCG
h-p15 F	GAATGCGCGAGGAGAACAAG
h-p15 R	CCATCATCATGACCTGGATCG

Chapter 3: Ink4b deficiency cooperates with oncogenic Ras to trigger low grade bladder tumor development

Introduction

Activated oncogene Ras has been found to cooperate with Ink4a deficiency to accelerate the melanoma development (Chin, Pomerantz et al. 1997). However, this cooperative event does not exist in bladder urothelial tumorigenesis. From the previous microarray data, we know that both Ink4a and Ink4b are up-regulated in urothelium of active Ras transgenic mice. Thus, it would be interesting to find out whether Ink4b deficiency synergies with activated Ras to trigger tumorigenesis in bladder.

3.1 $p15^{Ink4b}$ expression was induced by Ras activation

Upregulation of tumor suppressors is one of the cellular responses to oncogenic Ras activation. P16Ink4a has been reported to be upregulated by oncogenic Ras both *in vitro* and *in vivo*. As revealed by microarray data, RNA level of Ink4b is upregulated in low grade bladder tumor. To further confirm it, we checked the p15 protein expression and we found that $p15^{Ink4b}$ was significantly induced in bladder urothelium of acitive Ras transgenic mice compared to wild type mice (Figure 3-1).

3.2 Ras heterozygous transgenic and Ink4b homozygous knockout mice were generated

Since induction of $p15^{Ink4b}$ in response to Ras activation may be a tumor suppression strategy, we hypothesized that oncogenic Ras activation needed to cooperate with Ink4b deficiency in order to trigger tumor development. To test this hypothesis, we generated heterozygous Ras transgenic and homozygous Ink4b knockout mice ($Ras^{*/wt}$ $Ink4b^{-/-}$) by cross breeding and backcross breeding. In the meantime, we got heterozygous Ink4b knockout mice ($Ink4b^{+/-}$), homozygous Ink4b knockout mice $Ink4b^{-/-}$, heterozygous Ras transgenic only mice ($Ras^{*/wt}$), and heterozygous Ras transgenic

heterozygous Ink4b knockout mice ($Ras^{*/wt}$ $Ink4b^{+/-}$), which were used as controls (Figure 3-2).

3.3 Ink4b deficiency accelerates Ras-triggered bladder tumorigenesis

As blood in urine is an important symptom of bladder cancer, we did dipstick urinalysis to check whether mice might have developed bladder tumor. The dipstick was used to check 10 parameters in the urine including leukocytes, nitrite, urobilinogen, protein, pH, haemoglobin (blood), specific gravity, ketone, bilirubin and glucose. From urinalysis of 8-month-old mice of different genotypes, we found that there was no difference in all the parameters except blood. No blood was detected in control groups while it was detected in the urine of $Ras^{*/wt}$ $Ink4b^{-/-}$ counterparts (Figure 3-3A), which suggested that this group of mice probably developed bladder cancer.

Subsequently, each mouse was sacrificed and dissected. The $Ras^{*/wt}$ $Ink4b^{-/-}$ mice had much larger bladders, which were about 65 times heavier than control groups although there was no much difference in the body weight (Figure 3-3B upper panel and C). All bladders from control groups showed almost the same size as that from wild type mice (Figure 3-3B upper panel). The hydronephrosis, which occurred when urinary path was obstructed, happened in $Ras^{*/wt}$ $Ink4b^{-/-}$ mice (Figure 3-3B lower panel).

Histopathology analysis of 8-month old mice bladder of each group revealed that $Ink4b^{+/-}$ and $Ink4b^{-/-}$ mice had normal bladder urothelium phenotype. Constant with what was found in Dr. Mo's paper, the bladder urothelium of transgenic Ras mice developed simple hyperplasia. The bladder urothelium of $Ras^{*/wt}$ $Ink4b^{+/-}$ mice, which represented majority of this group, also developed simple hyperplasia. Those from $Ras^{*/wt}$ $Ink4b^{-/-}$ mice were cellular (Figure 3-4). All tumors found in this study were low grade.

Following up these mice by pathology analysis every two months, we found that $Ras^{*/wt}$ $Ink4b^{-/-}$

mice developed low grade bladder tumor by as early as two months. By 8 months, all these mice developed low grade bladder tumors. This was in striking contrast to what happened in the $Ras^{*/wt}$ $Ink4b^{+/-}$ mice, 10% of which developed bladder tumor in 4 months and the percentage rose to 30% by 6 months. For all other control groups, including wild type mice, $Ink4b^{+/-}$ and $Ink4b^{-/-}$ mice and $Ras^{*/wt}$ mice, no tumor was found within 10 months (Figure 3-5). This result revealed that Ink4b deficiency accelerated tumor development in heterozygous Ras transgenic mice.

3.4 Cell cycle was disturbed due to up-regulation of CDKs and p-MAPK

Tumor development results from the break of tight control of cell proliferation. In order to check how the proliferation of urothelial cells got out of control, we analyzed the cell cycle distributions of each group. Over 90% of the normal urothelial cells (wild type) were in G1 phase, which suggested that the proliferation rate of normal urothelial cells was extremely low. Ink4b knockout (both heterozygous and homozygous) slightly decreased the proportion of cells in G1 phase and increased the proportion of cells in S phase by about 10% (Figure 3-6, Table 3-1). These changes suggested that G1/S transition was enhanced. There were no aneuploid cells in wild type mice, Ink4b knockout (both heterozygous and homozygous) and $Ras^{*/wt}$ mice, while they were present in $Ras^{*/wt}$ $Ink4b^{+/-}$ and $Ras^{*/wt}$ $Ink4b^{-/-}$ mice (Figure 3-6, Table 3-1).

Cyclin-dependent kinase2, 4 and 6 (CDK2, CDK4 and CDK6), which are the positive regulator of G1/S transition, were found to be up-regulated in mouse urothelium with transgenic Ras, especially in $Ras^{*/wt}$ $Ink4b^{-/-}$ mice (Figure 3-7A). Both western blotting and immunohistochemistry showed that MAPK was activated in urothelium with transgenic Ras, and it was more activated in $Ras^{*/wt}$ $Ink4b^{-/-}$ mouse urothelim (Figure 3-7A and 3-7B).

Discussion

The transformation capability of oncogenes has been intensively studied since last century. At least two oncogenes are required to work cooperatively in order to transform primary cells (Land, Parada et al. 1983). The oncogenic activity of single mutant H-Ras allele is very weak. For instance, melanocyte-specific expression of mutant H-Ras resulted in hyperplasia and no melanoma was observed in transgenic mice (Powell, Hyman et al. 1995). Another example is urothelial-specific expression of H-Ras also resulted in hyperplasia in the time frame when all mice with double alleles of mutant H-Ras developed bladder tumors (Mo, Zheng et al. 2007).

The reason why primary cells need long latency to be transformed by single oncogene is the induction of tumor suppressor genes (Weinberg 1997). It has been reported that oncogenic Ras induces the expression of p16, p19, p21 and p53 in MEFs and immortal rat fibroblast REF52 cells (Serrano, Lin et al. 1997, Palmero, Pantoja et al. 1998). Angel Pellicer group later reported that oncogenic Ras also induced p15 in mouse embryo fibroblasts (MEFs) and p15 inhibited transformation of NIH 3T3 cells by Ras (Malumbres, Perez De Castro et al. 2000). As each cell type is different, whether p15 can be induced by oncogenic Ras in bladder urothelium is worthy of investigation. We previously discovered that both Ink4a and Ink4b were upregulated in activated H-Ras-induced urothelial tumors by microarray analysis (Mo, Zheng et al. 2007). In this study, we found that p15 protein expression could be significantly induced by oncogenic Ras in bladder urothelium (Fig.1). The induction from the protein level is consistent with the induction from RNA level.

The logic basis of this study is that transgenic mice with one mutant H-Ras allele might have short tumor latency when the induction of one tumor suppressor gene is blocked. Our results indicates that Ink4b deficiency significantly shortened the tumor latency of oncogenic Ras (Figure 3-5), suggesting that Ink4b cooperates with mutant H-Ras to trigger low-grade bladder tumor. Chin *et al*

reported that melanocyte-specific expression of activated Ras cooperated with Ink4a deficiency to trigger melanoma with high penetration after a short latency (Chin, Pomerantz et al. 1997). Nevertheless, urothelial-specific expression of constantly active Ras in combination with Ink4a deficiency did not lead to the development of bladder cancer after 10-month latency (Mo, Zheng et al. 2007). It reveals that the cooperative effects between active Ras and Ink4a deficiency in tumorigenesis is tissue specific. Thus, we conclude that Ink4b instead of Ink4a synergizes with mutant H-Ras to accelerate tumor development in bladder urothelium.

It has been well established that there are two types of bladder tumors. One is low-grade, non-invasive papillary bladder tumor and the other one is muscle invasive bladder tumor (Wolff, Liang et al. 2005, Wu 2005). About 70-80% of clinical cases on bladder cancer are low-grade, and they tend to recur after surgical removal. However, they rarely progress to invasive stage. Unlike other human epithelial tumors, the invasive bladder tumors do not progressed from low-grade bladder tumors. This pathology analysis in this study reveals that the cooperative effects between oncogenic Ras and Ink4b deficiency are responsible for the development of low-grade bladder tumor (Figure 3-4). This transgenic and knockout mice study contributes to our understanding of the genetic defects underlying the pathogenesis of low-grade non-invasive bladder cancer.

The signal pathways of Ras-induced low-grade bladder tumorigenesis have been investigated. P42/44 MAPK is the best characterized Ras/Raf-MEK-ERK signal pathway, which is activated in both Ras-induced hyperplasia lesions and tumors. Compared with hyperplasia lesions, the two kinases are activated more in bladder tumors (Mo, Zheng et al. 2007, Zhou, Huang et al. 2012). The activation of p42/44 MAPK pathway is responsible for cell cycle progression and cell proliferation by regulating the Cyclin D-CDK4/CDK6 complex and Cyclin A/E-CDK2 complex respectively (Blanchard, Mouhamad et al. 2000, Ewen 2000). The administration of MAPK inhibitor UO126 strongly reduced the tumor size in transgenic Ras and SV40T mice (Mo, Zheng et al. 2007, Zhou,

Huang et al. 2012). In this study, p42/44 MAPK was also found to be activated in the urothelium of transgenic H-Ras mice, and the level was elevated in the bladder tumors of transgenic H-Ras and Ink4b knockout mice (Figure 3-7). Here we make two points: 1) p42/44 MAPK signal pathway is crucial for tumor growth although MAPK activation is not the key event that initiated the tumorigenesis. MAPK activation is involved in urothelial hyperplasia in Ras transgenic mice. To drive tumor development, other kinases like Stat 3 and Akt are required to be activated. 2) It is Ink4b deficiency rather than Ink4a deficiency that cooperates with active Ras to drive cell cycle progression and proliferation, thus accelerates the tumor development.

By searching Gene Expression Omnibus (GEO) datasets, we found overexpression of CDK2 and CDK4 were involved in tumorigenesis in laryngeal squamous cell carcionoma (LSCC), and the CDK2 was indicated to be therapeutic target gene by Drug association analysis database (Lian, Fang et al. 2013). In both mouse and human T-cell acute lymphoblastic leukemias (T-ALL) models, CDK4/6 activities were required for tumor maintenance, and their pharmacology inhibition represents a strategy for anti-cancer therapy (Choi, Li et al. 2012). In our bladder cancer model, CDK2/4/6 were also found to be over-expressed (Figure 3-7). Therefore, restoration of Ink4b function or inhibition of CDK2/4/6 activities might be a therapy strategy for bladder cancer.

In summary, we discovered the synergy between activated H-Ras and Ink4b deficiecy in bladder tumorigenesis for the first time in history. This effect is due to enhanced cell proliferation and cell cycle progression as revealed by elevated expression of phosphorylated MAPK and cell cycle regulators CDK2/4/6. The fact that Ink4b instead of Ink4a cooperates with active H-Ras to trigger bladder tumorigenesis reveals that Ink4b is more potent than Ink4a as a tumor suppressor. The mechanisms underlying why Ink4b is more potent need further investigation.

Figure 3-1: Induction of $p15^{Ink4b}$ in bladder urothelium of Ras transgenic mice. The wild type mice (left three lanes) and Ras heterozygous transgenic mice (right three lanes) were compared by western blotting. p15 in heterozygous Ras transgenic mice was significantly induced.

Figure 3-1

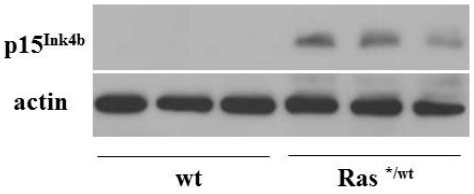


Figure 3-2: Genotyping of mice with Ras transgene and knockout Ink4b. (A) Representative Southern blotting showing wild-type mice with endogenous UPII fragment (Lane 3 and 7), heterozygous mice with transgenic/endogenous ratio of about 1:2 (lane 4, 5 and 6), and homozygous mice with transgenic/endogenous ratio of about 1:1 (lane 1, 2 and 8). (B) Regular PCR showing mice with wild type Ink4b (lanes 2 and 6), mice with heterozygous knockout p15 (lanes 1, 5, 7 and 8), and mice with homozygous knockout Ink4b (lanes 3 and 4).

Figure 3-2

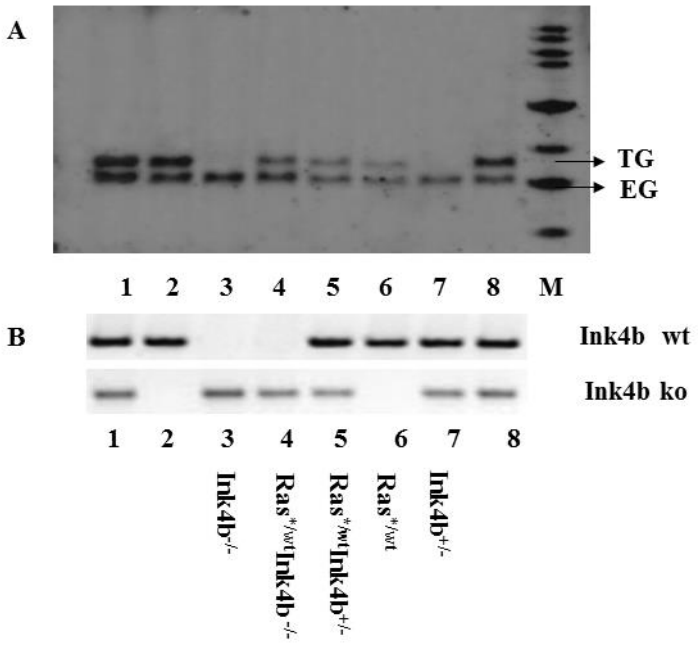

A
TG
EG
1 2 3 4 5 6 7 8 M
B
Ink4b wt
Ink4b ko
1 2 3 4 5 6 7 8
Ink4b-/-
Ras*/wtInk4b-/-
Ras*/wtInk4b+/-
Ras*/wt
Ink4b+/-

Figure 3-3: Bladder tumor formation in 8-month old heterozygous Ras transgenic and Ink4b knockout mice. (A) Dipstick urinalysis. From the bottom to the top, the parameters were leukocytes, nitrite, urobilinogen, protein, pH, haemoglobin (blood), specific gravity, ketone, bilirubin and glucose. Blood was detected in the urine from heterozygous Ras transgenic and Ink4b knockout mice. (B) Representative bladder phenotypes (upper panel) and corresponding kidney phenotypes (lower panel) of wild type mice (wt), Ink4b knockout mice (heterozygous $Ink4b^{+/-}$, homozygous $Ink4b^{-/-}$), heterozygous Ras transgenic mice ($Ras^{*/wt}$), heterozygous Ras transgenic and p15 knockout mice ($Ras^{*/wt}$ $Ink4b^{+/-}$), and heterozygous Ras transgenic and homozygous Ink4b knockout mice ($Ras^{*/wt}$ $Ink4b^{-/-}$). The bladder size and kidneys of wt, $p15^{+/-}$, $p15^{-/-}$, $Ras^{*/wt}$ and $Ras^{*/wt}$ $Ink4b^{+/-}$ mice showed no much difference, Bladder size of $Ras^{*/wt}$ $Ink4b^{-/-}$ mice was much bigger and the corresponding kidney showed hydronephrosis. (C) Body weight and bladder weight of different mice groups. There was no difference in the body weight among different genotypes while the bladder weight of $Ras^{*/wt}$ $Ink4b^{-/-}$ mice was about 60 times more than other groups.

Figure 3-3

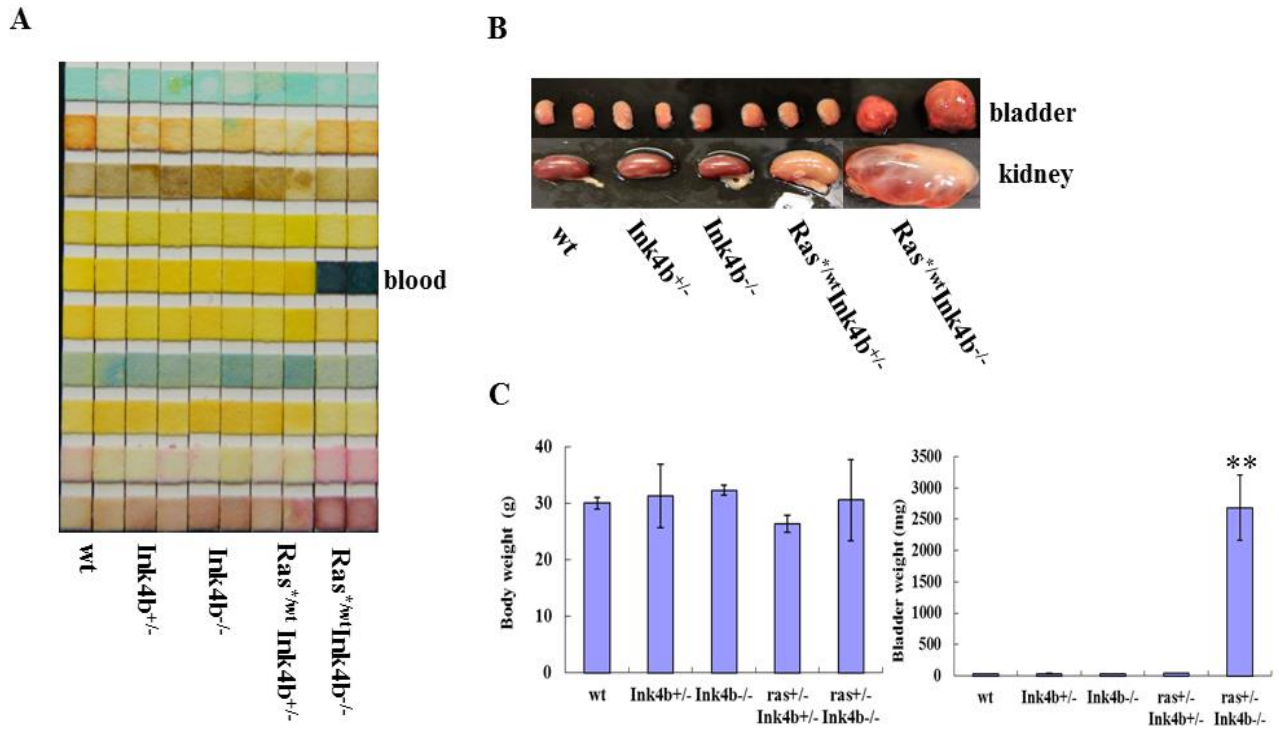

A
blood
wt
Ink4b+/-
Ink4b-/-
Ras*/wt Ink4b+/-
Ras*/wtInk4b-/-
B
bladder
kidney
wt
Ink4b+/-
Ink4b-/-
Ras*/wtInk4b+/-
Ras*/wtInk4b-/-
C
Body weight (g)
40
30
20
10
0
wt
Ink4b+/-
Ink4b-/-
ras+/- Ink4b+/-
ras+/- Ink4b-/-
Bladder weight (mg)
3500
3000
2500
2000
1500
1000
500
0
**
wt
Ink4b+/-
Ink4b-/-
ras+/- Ink4b+/-
ras+/- Ink4b-/-

Figure 3-4: Histopathology analysis of bladder urothelium of wild type, $p15^{+/-}$, $p15^{-/-}$, Ras $^{*/wt}$, Ras $^{*/wt}$ $p15^{+/-}$ and Ras $^{*/wt}$ $p15^{-/-}$ mice by HE staining. The wild type, $p15^{+/-}$ and $p15^{-/-}$ mice showed normal morphology. Ras $^{*/wt}$ and Ras $^{*/wt}$ $p15^{+/-}$ mice showed hyperplasia. The urothelium cells in Ras $^{*/wt}$ $p15^{-/-}$ mice were much more cellular. All the images were 400 times magnification.

Figure 3-4

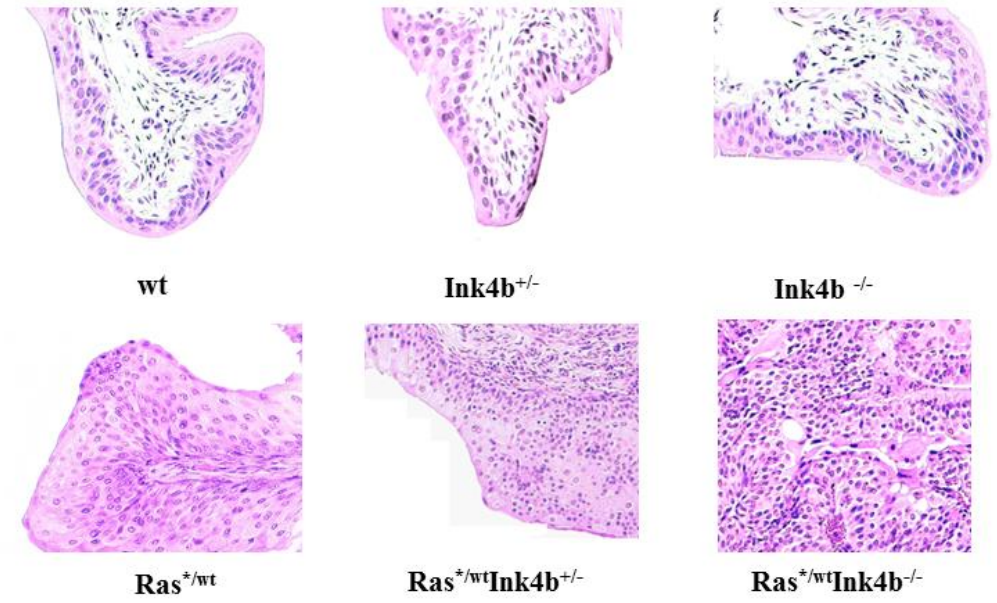


Figure 3-5: Cohort of the tumor-free rates among different genotypes. No tumor was found in wild type, $p15^{+/-}$, $p15^{-/-}$ and $Ras^{*/wt}$ mice within 10 months. Their curves were overlapped as a line. The purple curve represents the tumor-free rate of $Ras^{*/wt}p15^{+/-}$ mice while the blue curve represents that of $Ras^{*/wt}p15^{-/-}$ mice.

Figure 3-5

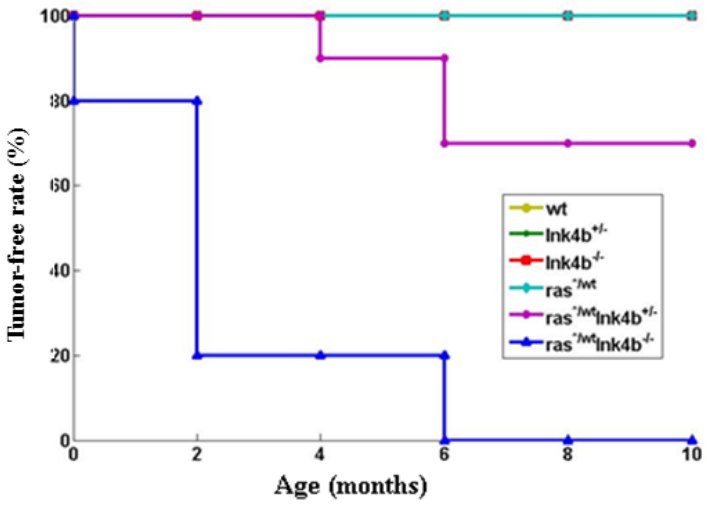

Tumor-free rate (%)
100
80
60
40
20
0
0
2
4
6
8
10
Age (months)
wt
Ink4b+/-
Ink4b-/-
ras+/wt
ras+/wtInk4b+/-
ras+/wtInk4b-/-

Figure 3-6: Cell cycle analysis by fluorescein-activated cell sorting (FACS). The urothelium cells from each group were applied to FACS analysis. The urotheium cells from $Ras^{*/wt}$ $p15^{-/-}$ mice showed marked increase in the proportion of aneuploidy cells. The first red area represents diploid G1 phase, the second red area represents diploid G2 phase, and the area with slash blue line between thses two red areas represents S phase. The first yellow area represent aneuploid G1 phase, the second yellow area stands for aneuploid G2 phase, and the area between these two yellow areas stands for aneuploid S phase.

Figure 3-6

A

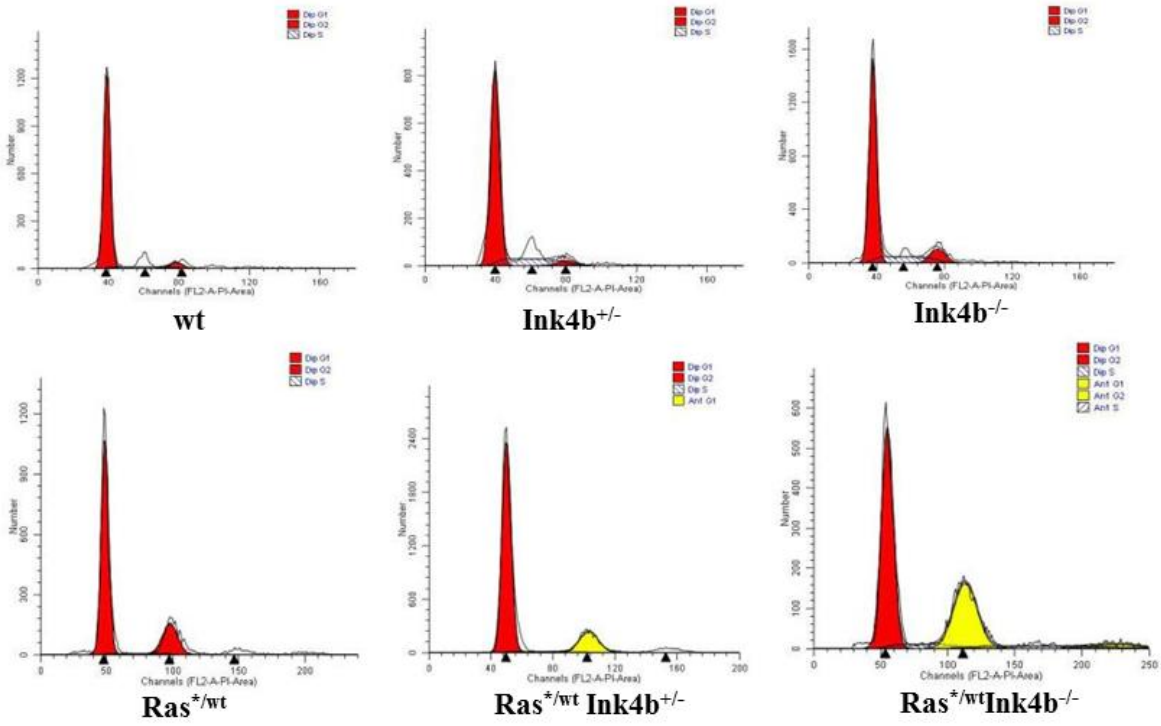


Table 3-1: Cell cycle distribution of urothelium cells from each group.

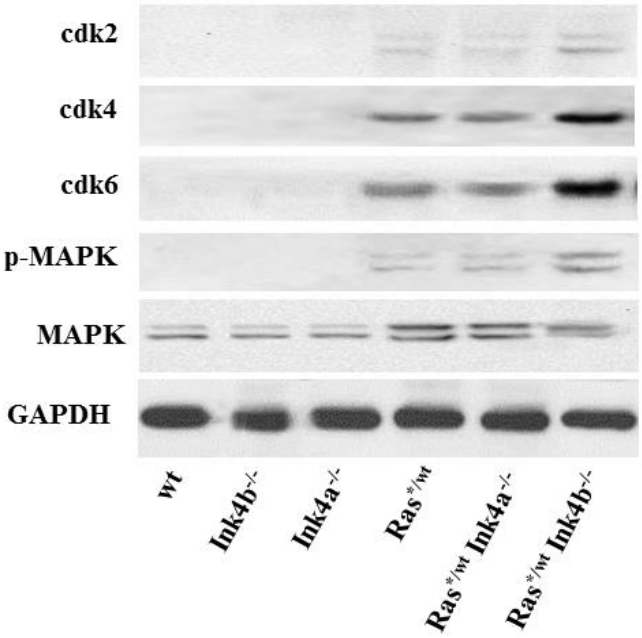
Table 3-1

Phase		wt	Ink4b$^{+/-}$	Ink4b$^{-/-}$	ras$^{*/wt}$	ras$^{*/wt}$ Ink4b$^{+/-}$	ras$^{*/wt}$ Ink4b$^{-/-}$
Diploid	G1	90.09	78.98	74.91	76.34	81.39	54.95
	S	4.93	16.44	14.91	1.71	7.3	4.62
	G2	4.97	4.58	10.18	21.95	2.57	5.18
Tetraploid	G1	0	0	0	0	8.74	26.84
	S					0	5.06
	G2					0	3.33

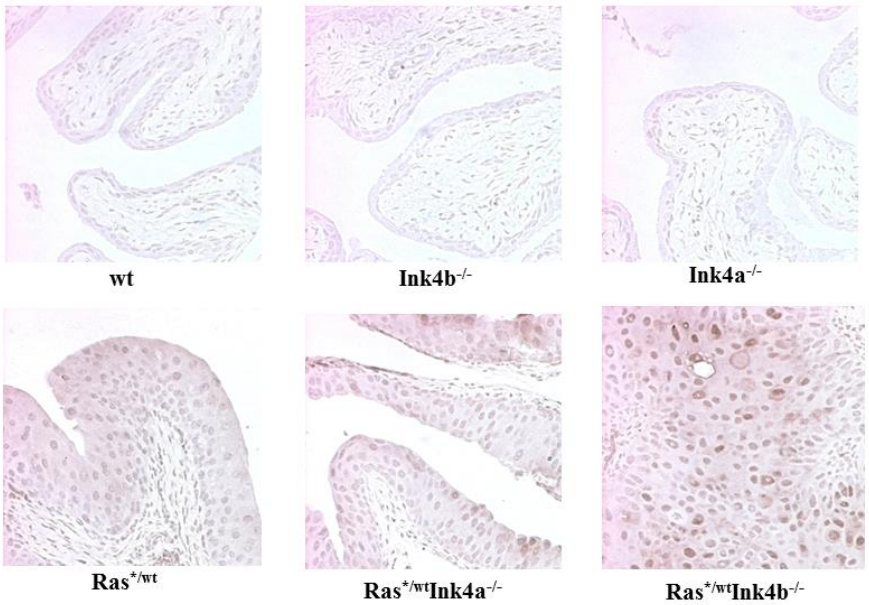
Figure 3-7: Increased level of positive cell cycle regulators and phosphor-MAPK in Ras$^{*/wt}$ p15$^{-/-}$ mouse urothelium. (A) The expression of CDK2, CDK4, CDK6, total MAPK and phosphor-MAPK were detected by western blotting. CDK2, CDK4, CDK6, and phosphor-MAPK were not detectable in wild type, p15$^{+/-}$ and p15$^{-/-}$ mice. All proteins were up-regulated in the urothelium of Ras$^{*/wt}$p15$^{-/-}$ mouse. (B) Immunohistochemistry of phosphor-MAPK. The urothelium in Ras$^{*/wt}$p15$^{-/-}$ mice showed very strong staining of phosphor-MAPK. The expression pattern was constant with western result.

Figure 3-7

A


cdk2
cdk4
cdk6
p-MAPK
MAPK
GAPDH
wt
Ink4b-/-
Ink4a-/-
Ras*/wt
Ras*/wt Ink4a-/-
Ras*/wt Ink4b-/-

B


wt
Ink4b-/-
Ink4a-/-
Ras*/wt
Ras*/wtInk4a-/-
Ras*/wtInk4b-/-

Chapter 4: Ink4b is more important than Ink4a in suppressing cell growth

Introduction

Transgenic and knockout mice studies showed that Ink4b deficiency had cooperative effects with activated Ras in accelerating bladder tumorigenesis while Ink4a deficiency did not. We hypothesized that Ink4b was more important than Ink4a. To test this hypothesis, we checked the functions of Ink4a and Ink4b *in vitro* respectively.

4.1 UM-UC-3 cell line is selected

The first step was to select a cell line expressing neither Ink4a nor Ink4b. From the literature, we know that RT4, RT112 and UM-UC-3 cell lines are negative for Ink4b. By screening 9 urothelial cell lines in the lab including RT4, RT112, UM-UC-3, J82, HT-1376, 5637, T24, T24T and Urosta, we found that Ink4a was negative in RT4, RT112 and UM-UC-3 from genomic DNA level (Figure 4-1A), p16Ink4a was negative in RT4, RT112, UM-UC-3, T24 and T24T cell lines from mRNA level (Figure 4-1B), and the proteins p14 and p16 were both negative in RT4, RT112, UM-UC-3, T24 and T24T cell lines, which was constant with RT-PCR result (Figure 4-1C). T24T cell line was derived from T24 cell line (Gildea, Golden et al. 2000). The promoter of Ink4a in T24 was found to be hypermethylated, so there was no mRNA transcription and protein translation in both T24 and T24T cell lines (Nguyen, Gonzales et al. 2001). Because of low transfection efficiency of RT4 and RT112 cell lines, we chose UM-UC-3 cell line for the following experiments.

4.2 p14 overexpression doesn't inhibit cell growth

Next, we tried to make stable transfected cell lines and then test the effect of Ink4a and Ink4b on the cell growth. 24 h after transfection, each deducted protein was detectable (Figure 4-2A). However, after one-month selection with G418, cells transfected with p14-pCDNA3.1 still express p14 while cells transfected with either p15-pCDN3.1 or p16-pCDNA3.1 do not express p15 or p16 (Figure 4-

2B). We proposed that UM-UC-3 cells could not survive with p15 and p16 overexpression. Cells could survive p14 overexpression suggested that the effect of p14 on cell growth inhibition was not as strong as p15 and p16. It was demonstrated by proliferation assay which showed that p14 overexpression had no effect on cell growth (Figure 4-2C).

4.3 p15 overexpression suppresses cell growth more than p16

To find out whether p15 suppressed cell growth more than p16, we used Tet-On inducible expression system to make stable transfected cell lines. Both p15 and p16 had remarkable expression by doxycycline induction for 24 h, and their expression increased as the doxycycline incubation time reached extended to 48 h and 72 h (Figure 4-3). The proliferation assay showed that the proliferation rate of UM-UC-3 cell line with p15 overexpression was significantly slower than that with p16 overexpression (Figure 4-4). It advises that p15 is more important in cell growth inhibition.

4.4 p15 overexpression arrests cells in G1more than p16

To determine the changes of cell cycle progression, we analyzed the cell cycle distribution. Both p15 and p16 overexpression increased the proportion of the cells in G1 phase and reduced the number of cells in S phase. However, the changes made by p15 overexpression were more significant (Figure 4-5). This result explained why cells with p15 overexpression grew slower than those with p16 overexpression, and it reveals again that p15 is more important than p16.

Discussion

Loss of Ink4a and/or Ink4b has been reported in human bladder tumors and derivative bladder cancer cell lines (Orlow, Lacombe et al. 1995, Le Frere-Belda, Cappellen et al. 2001). Ink4a and Ink4b are well-known cyclin-dependent kinase inhibitors (Harper and Elledge 1996). The roles of Ink4a have been intensively studied both *in vitro* and *in vivo*. It has been well-established that overexpression of p16 which is encoded by Ink4a results in G1 arrest in both normal and tumor cell lines (Koh, Enders et al. 1995, Lukas, Parry et al. 1995). Compared with abundant reports of in *vitro* work on p16, there is limited information about p15. Angel Pellicer group found that retroviral-mediated p15 overexpression alone was sufficient to induce cell cycle arrest and senescence in early passage MEFs (Malumbres, Perez De Castro et al. 2000).

In agreement with the published studies, our results indicated that both Ink4a and Ink4b overexpression leaded to decreased cell growth rate and cell cycle arrest in UM-UC-3 cells (Figure 4-4 and 4-5). When comparing p15 with p16, p15 overexpression inhibited cell growth and G1/S transition more effectively than p16 overexpression. In fact, it has been reported that p15 can suppress cell growth and induce cell senescence much more efficiently than p16, p21 and p27 in TIG-3 cells (McConnell, Starborg et al. 1998). Among p16, p21 and p27, p16 is the most effective protein. The reason why Ink4 proteins have more pronounced effects than CIP1 proteins p21 and p27 could be explained that p16 binds much more CDK4/6 than p21 (Alcorta, Xiong et al. 1996). In other words, the activities of CDK4/6 could be mostly inhibited by p16. Thus, we predict that p15 may be able to bind more CDK4/6 than p16.

Regarding the roles of p14, we did not observe any obvious inhibition effects on cell growth and cell cycle arrest (Figure 4-2). The stably transfected cells with constitutive p14 expression could be established while that with p15 and p16 expression could not. This fact is indirect evidence that continuous p14 expression is not toxic for UM-UC-3 cell growth. However, there are other studies

showing p19 (mouse) can induce cell cycle arrest in mouse cell lines including MEFs and NIH 3T3 cells (Quelle, Zindy et al. 1995, Kamijo, Zindy et al. 1997). In human breast cancer cells and mesothelioma cells, p14 overexpression also induces cell cycle arrest (Yang, You et al. 2000, Deng, Kim et al. 2002). Our explanation is urothelial cells are different from other cell types and the inhibition effects of p14 are likely to be weak in fast-growing UM-UC-3 cells if it really can inhibit cell growth. Our previous microarray data showing p19 level is not affected in both hyperplasia lesions and tumors induced by transgenic mutant H-Ras indicates that p19 is not as important as p15 and p16 (Mo, Zheng et al. 2007).

In summary, the results from present study show that p15 is more important than p16 and p14 in suppressing cell growth and cell cycle progression. These results are supportive to our finding that Ink4b instead of Ink4a cooperates with mutant H-Ras to accelerate tumor development. Finding out the mechanism why p15 is more effective than p16 will be worthy of exploration.

Table 4-1: p15 in human bladder cancer cell lines (Frere-Belda et al, 2001)

	RT4	RT112	UM-UC-3	J82	HT1376	T24
Ink4b homozygous deletion	+	+	+	-	-	-
Ink4b mRNA level	0	0	0	0.4	0.7	0.9

Figure 4-1: Ink4a status in human bladder urothelial cell lines. (A) Southern blotting detection of Ink4a genomic DNA. Ink4a genomic DNA was not detected in RT4, RT112 and UM-UC-3 cells, but it was present in J82, HT1376, 5637, T24, T24T and Urosta cells. Genomic DNA of GAPDH was used as loading control. (B) RT-PCR detection of p16 mRNA. P16 mRNA was not detectable in RT4, RT112, UM-UC-3, T24 and T24T cell lines, while it was present in J82, HT1376, 5637 and Urosta cell lines. β-actin was used as control. (C) Western blotting detection of p14 and p16 protein. P16 protein expression pattern was constant with mRNA. however, p14 protein in HT1376 cell line was not detected. β-actin was used as control.

Figure 4-1

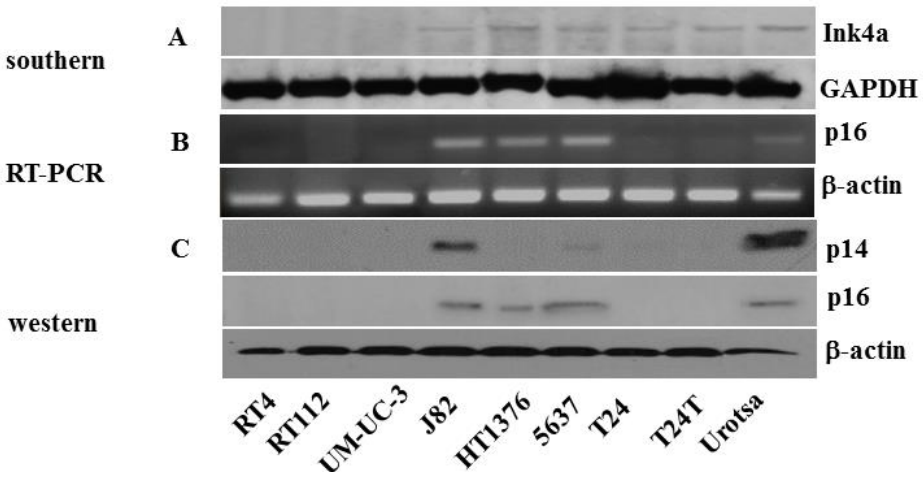

southern
A
Ink4a
GAPDH
RT-PCR
B
p16
β-actin
C
p14
western
p16
β-actin
RT4
RT112
UM-UC-3
J82
HT1376
5637
T24
T24T
Urotsa

Figure 4-2: No effect of p14 overexpression on cell proliferation. (A) P14, p16 and p15 proteins were detected by western blotting 24 h after transfection. (B) Detection of p14, p16 and p15 proteins after 1 month G418 selection. Only p14 was detected. β-actin was used as control. (C) Comparison of cell proliferation by WST-1 assy. Cells stably transfected with vacant plasimid pCDNA3.1 was used as control. The OD value was measured at 450 nm.

Figure 4-2

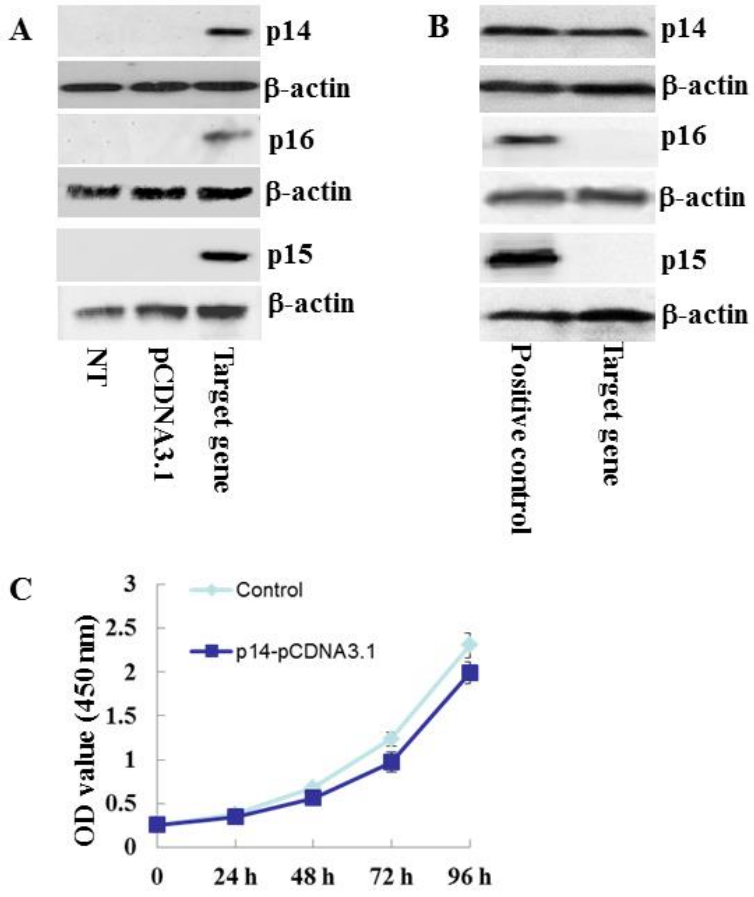

A
p14
β-actin
p16
β-actin
p15
β-actin
NT
pCDNA3.1
Target gene
B
p14
β-actin
p16
β-actin
p15
β-actin
Positive control
Target gene
C
Control
p14-pCDNA3.1
OD value (450 nm)
3
2.5
2
1.5
1
0.5
0
0
24 h
48 h
72 h
96 h

Figure 4-3: Establishment of stable inducible p15 and p16 expression cell lines respectively. (A) western blotting of p15 induction by doxycycline for 0 h, 24 h, 48 h and 72 h. (B) western blotting of p16 induction by doxycycline for 0 h, 24 h, 48 h and 72 h. Dox: doxycycline.

Figure 4-3

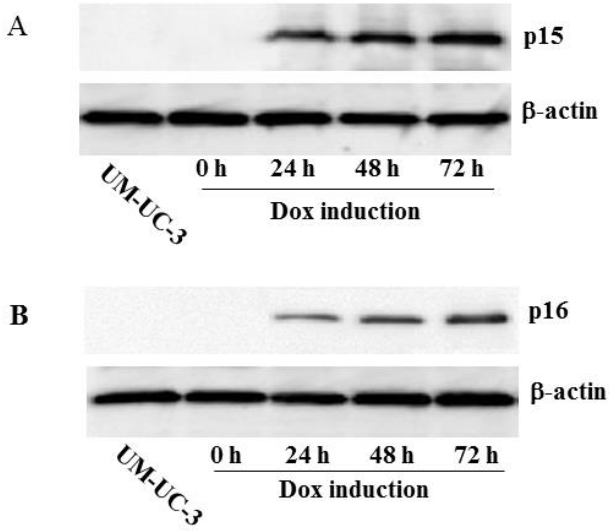

A
p15
β-actin
0 h
24 h
48 h
72 h
UM-UC-3
Dox induction
B
p16
β-actin
0 h
24 h
48 h
72 h
UM-UC-3
Dox induction

Figure 4-4: Comparison of cell proliferation after p15 and p16 induction. Red curve represents the growth curve of UM-UC-3 cells with p15 induction, and blue curve represents that with p16 induction. The cell line which was stably transfected with the same vacant plasimid was used as control.

Figure 4-4

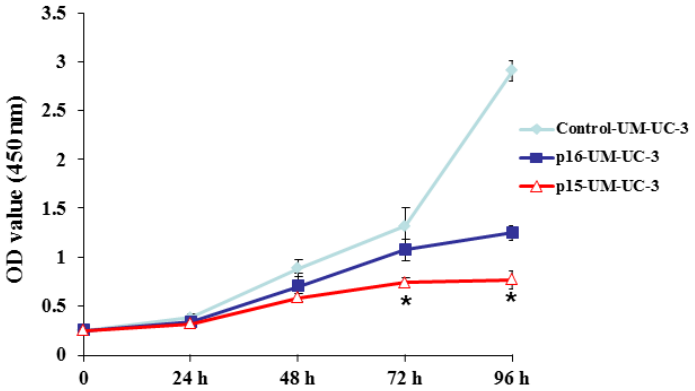
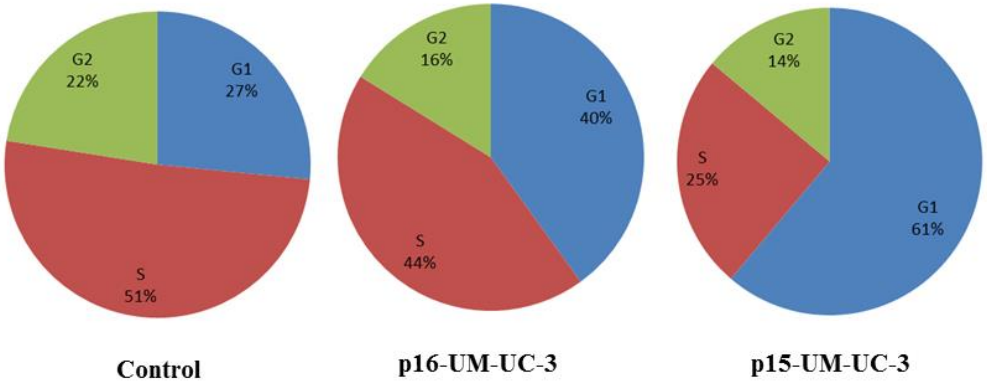

OD value (450 nm)
3.5
3
2.5
2
1.5
1
0.5
0
0
24 h
48 h
72 h
96 h
*
*
Control-UM-UC-3
p16-UM-UC-3
p15-UM-UC-3

Figure 4-5: Alterations of cell cycle distribution after p15 and p16 induction respectively. Each cell line was treated with 1μg/ml doxycycline for 72 h and then subjected to FACS analysis. The proportion of cells in G1 phase was presented as blue area, and that in S phase was presented as red area, and that in G2 phase was presented as green area.

Figure 4-5


G2
22%
G1
27%
S
51%
Control
G2
16%
G1
40%
S
44%
p16-UM-UC-3
G2
14%
S
25%
G1
61%
p15-UM-UC-3

Chapter 5: Ink4a deficiency accelerates the development of invasive bladder tumor in mice treated with BBN

Introduction

We found that Ink4b was a stronger tumor suppressor than Ink4a as shown in chapter 3, and we also found that Ink4b inhibited cell cycle progression more than Ink4a, Thus we further proposed that mice with Ink4b deficiency were more susceptible to bladder specific carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN). To test this proposal, we treated Ink4a knockout mice, Ink4b knockout mice with BBN respectively, wild type mice were used as a control.

5.1 All Ink4a knockout mice developed invasive tumor

In order to clarify whether Ink4b provided more protection to the urothelium than Ink4a against carcionogenesis induced by BBN, we examined the susceptibility of Ink4a knockout mice and Ink4b knockout mice to BBN-induced bladder carcinogenesis respectively. The incidence of invasive tumor in wild type mice, $Ink4b^{-/-}$ mice and $Ink4a^{+/-}Ink4b^{+/-}$ mice was the same: 10%. The other mice in these groups showed hyperplasia. In striking contrast, the incidence increased to 100% in $Ink4a^{-/-}$ mice (Table 5-1). To our surprise, these results indicate that loss of both alleles of Ink4a causes much higher susceptibility to BBN-induced carcinogenesis.

5.2 All invaded tumor cells are originated from Urothelium

To confirm whether the phenotype was hyperplasia or Invasive tumor, we further performed cytokeratin 5 staining. For the representative hyperplasia induced by BBN, both basal cells and intermediate cells were positive for cytokeratin 5 staining, which indicated that the proliferation rate of urothelial cells increased dramatically upon BBN treatment (Figure 5-3, upper panel). For the invasive tumor, all invasion cells were positive for cytokeratin 5 staining (Figure 5-3, lower panel), which suggested that these cells were originated from urothelial cells.

5.3 Loss of E-cadherin is involved in the development of invasive tumor

To acquire invasive capacity is the pre-requisite of invasive cancer development. It has been reported that E-Cadherin, which is important for epithelium integrity, is strongly related with invasive capability when its expression is reduced. To find out whether E-Cadherin was involved in the development of BBN-induced invasive bladder cancer, we checked whether E-Cadherin was differently expressed in bladder urothelium of different mouse groups. In wild type mice, $Ink4b^{-/-}$ mice and $Ink4a^{+/-}Ink4b^{+/-}$ mice with hyperplasia, all the urothelium cells were positive for E-Cadherin, while urothelium cells in $Ink4a^{-/-}$ mice were negative for E-Cadherin (Figure 5-4). It suggests that loss of E-Cadherin is associated with bladder invasive tumor development.

Discussion

Ink4a and Ink4b are closely linked genes located at 9p21 locus, which is frequently found to be deleted in primary human tumors (Cairns, Polascik et al. 1995). Ink4a gene has been intensively studied as a tumor suppressor while Ink4b gene receives much less attention. The studies on comparison of the two genes are even less. Testing their susceptibility to carcinogen treatment is a good way To compare the roles of these two tumor suppressors. Here in this study we found that Ink4a knockout mice were more prone to invasive bladder tumor formation induced by BBN (Table 5-1), which suggests that Ink4a is more important than Ink4b in suppressing invasive bladder tumor development.

Looking into each gene individually, Ink4a interruption occurs in a wide variety of human tumors covering both hematological malignancies and solid tumors such as melanoma (Ruas and Peters 1998). Individuals with germ-line mutations of Ink4a are predisposed familial melanoma while that with somatic mutations are prone to other cancers. Most mice with Ink4a deficiency developed sarcomas and lymphomas which are also human cancers associated with Ink4a deletions, and showed susceptibility to carcinogen treatment (Serrano, Lee et al. 1996). However, no bladder cancer was observed within the same time frame. In our previous study, we did not find bladder tumor in Ink4a deficiency mice either (Mo, Zheng et al. 2007). The specific p16 and p19 knockout only mice have been generated to study their roles respectively. P16 knockout mice developed soft tissue sarcoma, splenic lymphoma and melanoma 7 months later (Sharpless, Bardeesy et al. 2001). Most p19 knockout mice developed sarcomas and T-cell lymphomas in 6 months (Kamijo, Zindy et al. 1997, Kamijo, Bodner et al. 1999). According to their tumor spectrums, it is hard to tell their exact contributions to tumor suppressor role of Ink4a gene.

In contrast to Ink4a knockout mice, only 8.2% Ink4b knockout mice were observed to develop tumors, most of which were originated from connective tissue (Latres, Malumbres et al. 2000). The same as Ink4a knockout mice, no bladder tumor was observed. Exposure to 9,10-dimethyl-1,2-benzanthrancene (DMBA) followed by exposure to ultraviolet B rays (UVB) did not lead to any tumor development in 12 months, which is quite different from Ink4a knockout mice. These studies may serve as indirect evidences that Ink4b alone has limited roles in suppressing tumor development induced by carcinogen compared with Ink4a, which support our finding in this study. Nevertheless, we can't rule out Ink4b may have important tumor suppressing role in a specific organ when it synergies with certain oncogene.

Previous studies have shown that homozygous deletion of Ink4a is the main mechanism underlying the inactivation of this tumor suppressive gene. The deletion occurs in both low grade tumor and high grade tumor, and there is no correlation between Ink4a homozygous deletion and the stage of tumor. When taking FGFR3 mutation into consideration, Ink4a homozygous deletion is strongly correlated with more aggressive bladder tumor (Rebouissou, Herault et al. 2012). Besides deletion, hypermethylation is also an important mechanism to inactivate Ink4a. It has been reported that the frequency of promoter hypermethylation of both p14 and p16 increase in muscle invasive bladder tumor (Ali Hosseini, Sobti et al. 2010). These evidences reveal that Ink4a inactivation is associated with bladder tumor progression in invasive tumorigenesis. In this study, we found that Ink4a deficiency accelerates BBN-induced invasive bladder tumor development. We predict Ink4a deletion is involved in the progression of BBN-induced invasive bladder tumor. The incidence of invasive tumor in $Ink4a^{+/-}Ink4b^{+/-}$ mice indicates that Ink4a gene is haplo-sufficient for BBN-induced tumor suppression because their phenotype is close to wild type mice.

Ink4a plays its roles through both Rb pathway and p53 pathway via its two proteins p16 and p14 (p19). P16 inhibits CDK4 and CDK6, thus inhibits the inactivation of Rb, which leads to cell cycle

arrest (Serrano, Lee et al. 1996). P14 (p19) inhibits the E3 activity of MDM2, thus prevents the degradation of p53, which facilitate p53-mediated cell cycle arrest and apoptosis (Kamijo, Zindy et al. 1997). In clinical study, reduced or loss of Rb expression and p53 mutation are found to co-exist in 40-50% of muscle invasive bladder cancer. Urothelial specific knockout mouse study reveals that Rb deficiency collaborates with p53 deficiency to promote invasive tumor development when treated with BBN (He, Mo et al. 2009). The incidence of invasive tumor in these mice is 50%, which is very similar to the situation in humans. When Ink4a is absent, the functions of both Rb and p53 are attenuated. This may explain why Ink4a knockout mice developed invasive tumor while other groups with identical treatment did not.

There is a doubt that Ink4b is functional in Ink4a knockout mouse, and it keeps Rb active. We proposed two possibilities. One is that p15 and p16 work together to maintain the activity of Rb. Once p16 is not functional, Rb is partially inactivated, which contributes to the promotion of invasive tumorigenesis together with deficiency of p53 pathway. Another one is that p15 alone can maintain Rb fully functional, and p14 deficiency plays crucial role via p53 pathway in promoting invasive tumor development. To find out which possibility is true in future, we need to look into p16Ink4a knockout only and p19Arf knockout only mice respectively.

The inconsistency in terms of importance between Ink4a and Ink4b is noticeable. As described in chapter 1, the development of low-grade and high-grade bladder tumors follows different signal pathways. Low-grade bladder tumors were caused by active oncogenic Ras and Ink4b deficiency. It was mutant Ras that initiated the low-grade tumor development. The effect of Ink4b deficiency was the promotion of the low-grade tumor development. However, the high-grade tumors were initiated by urothelial specific carcinogen BBN. The role of Ink4a deficiency was acceleration of high-grade tumor development. In one words, the inconsistency is due to the difference of oncogenic events.

In conclusion, Ink4a deficiency accelerates invasive bladder tumor formation in response to carcinogen treatment and thus Ink4a gene plays an essential role as an invasive tumor suppressor. In terms of ability to suppress invasive tumor development, Ink4a is more important than Ink4b. Whether p16 and/or p19 contribute to the suppressor capability needs further investigation.

Table 5-1: The incidence of hyperplasia and invasive tumor of different genotypes.

Urothelial phenotype	Genotype			
	wt (n=10)	Ink4b $^{-/-}$ (n=10)	Ink4a $^{+/-}$ Ink4b $^{+/-}$ (n=10)	Ink4a $^{-/-}$ (n=10)
Normal	0	0	0	0
Hyperplasia	9	9	9	0
Invasive tumor	1	1	1	10

Figure 5-1: Representative appearance of BBN treated urinary bladders, lymph nodes, spleens and kidneys. (A) Anatomy of bladders. The surface of bladders with hyperplasia in wild type, $Ink4b^{-/-}$, $Ink4a^{+/-}Ink4b^{+/-}$ mice was smooth, while that of the bladder with invasive tumor in $Ink4a^{-/-}$ mice was rough. (B) Anatomy of lymph nodes, spleens and kidneys in wild type and $Ink4a^{-/-}$ mice.

Figure 5-1

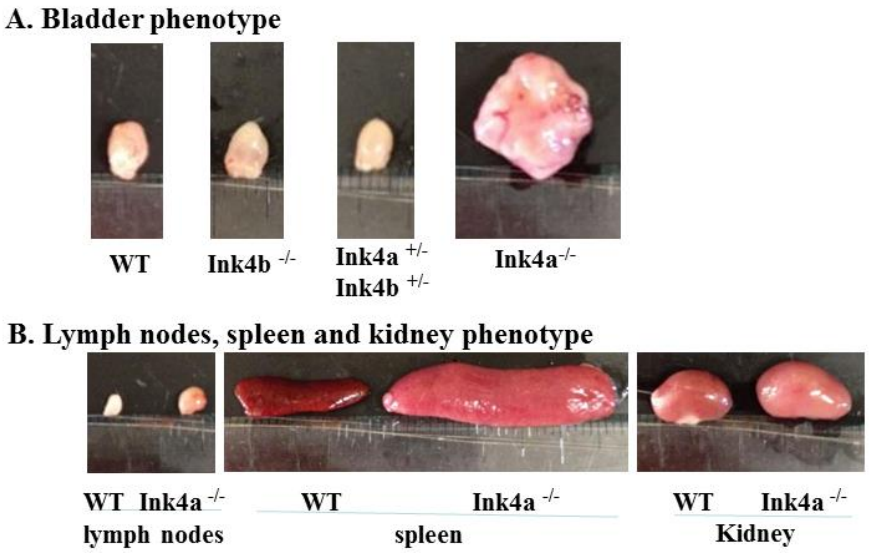

A. Bladder phenotype
WT
Ink4b -/-
Ink4a +/-
Ink4b +/-
Ink4a-/-
B. Lymph nodes, spleen and kidney phenotype
WT Ink4a -/-
lymph nodes
WT
Ink4a -/-
spleen
WT
Ink4a -/-
Kidney

Figure 5-2: Histopathology analysis by H&E staining. Bladder urothelium in wild type, Ink4b$^{-/-}$, Ink4a$^{+/-}$Ink4b$^{+/-}$ mice showed hyperplasia. Bladder urothelium in Ink4a$^{-/-}$ mice showed invasive tumor.

Figure 5-2

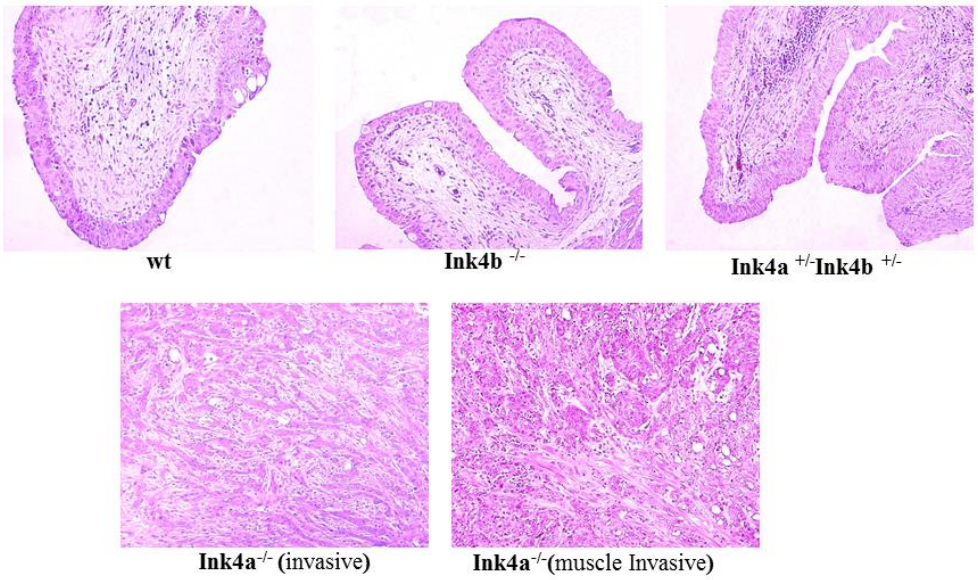


Figure 5-3: Confirmation of the phenotype by cytokeratin 5 staining. Both basal and intermediate cells were positive for cytokeratin 5 staining in bladder urothelium in wild type, Ink4b$^{-/-}$, and Ink4a$^{+/-}$Ink4b$^{+/-}$ mice. All epithelium cells invading in muscle and connective tissue were cytokeratin 5 positive.

Figure 5-3

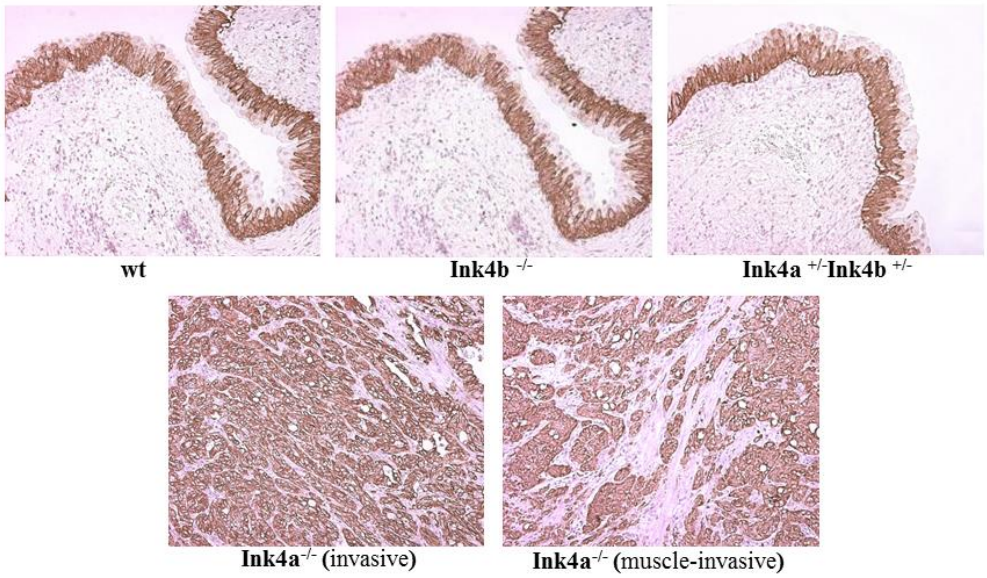
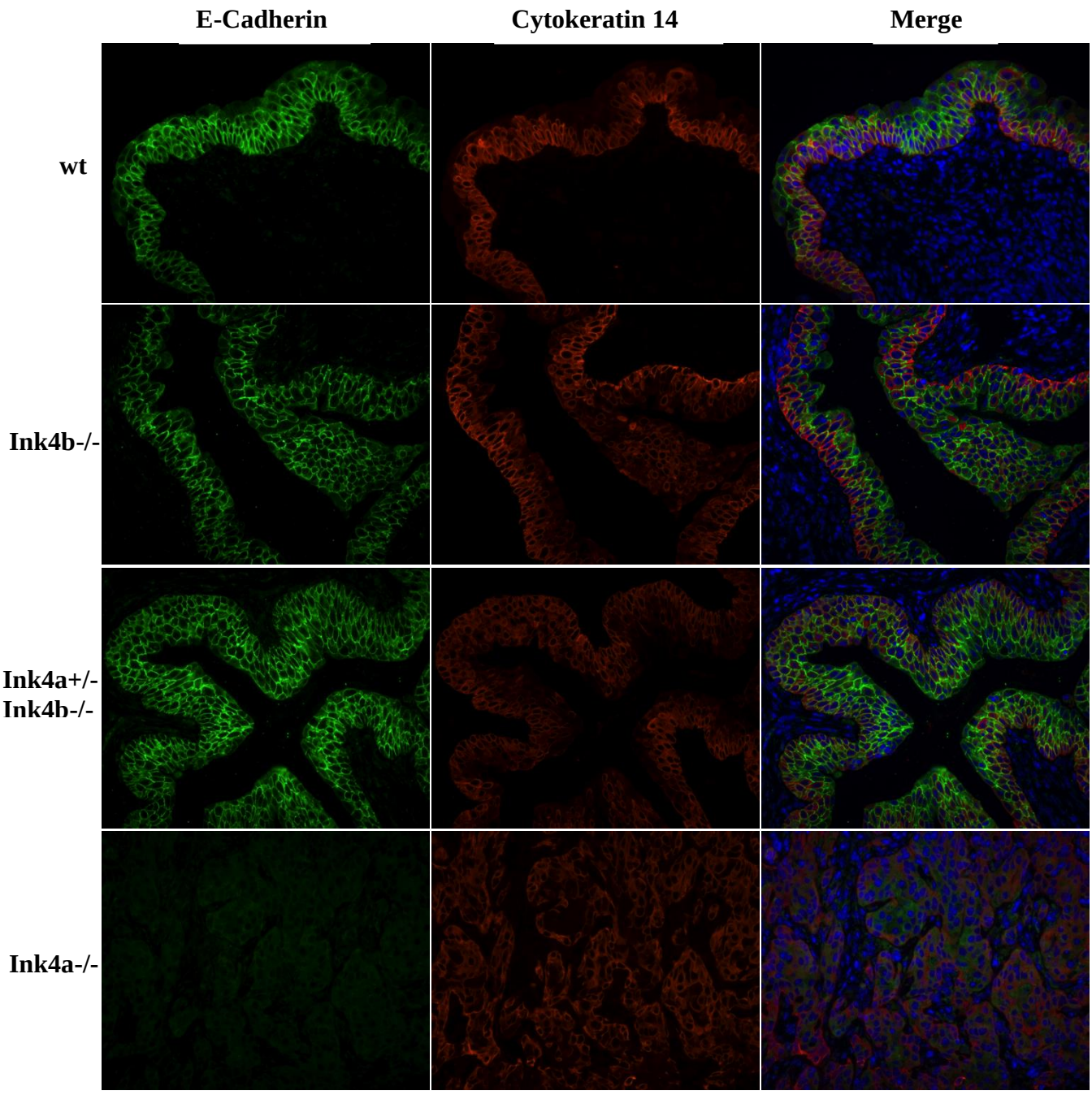


Figure 5-4: Involvement of E-Cadherin in the development of invasive tumor. E-Cadherin expression was analyzed by immunofluorescence.

Figure 5-4



Chapter 6: Conclusions and future directions

Conclusions

This study reveals for the first time that Ink4b is more important than Ink4a in suppressing oncogenic Ras induced low-grade tumor development. Ink4b deficiency significantly shortened the tumor latency in transgenic mutant H-Ras mice while Ink4a deficiency did not. The synergistic effects between Ink4b and mutant H-Ras was associated with accelerated cell cycle progression and proliferation, which was evidenced by elevated level of CDKs and MAPK activation.

We further discovered that p15 overexpression inhibited cell growth and cell cycle progression more than p16 overexpression. P14 overexpression had no effect on cell growth. These results support the hypothesis that Ink4b is more important than Ink4a.

Finally, Ink4a is more important than Ink4b in suppressing invasive bladder tumor induced by BBN. We found out that Ink4a deficiency promotes invasive tumor development in mice treated with BBN for the first time.

Taken all the results together, we conclude that Ink4b is more crucial for cell growth and proliferation while Ink4a is more important for cell invasion. This study addresses the importance of both Ink4a and Ink4b at 9p21 locus (human) in suppressing the development of two types of tumors

Future direction

For the mechanism why Ink4b is more important than Ink4a in cooperating with active H-Ras to trigger bladder tumorigenesis, there is still much to be understood. I have shown that p15 overexpression suppresses cell growth, and more cells are arrested in G1 phase more than p16

overexpression. It would be of great interest to find out why inhibition effect of G1/S transition by p15 is stronger than p16, whether p15 can bind more CDK4/6 both *in vivo* and *in vitro*, and whether p15 and p16 have different partners in suppressing tumor development.

Another interesting future direction is to find out whether transgenic active H-Ras mice in both Ink4a and Ink4b deficiency background could develop bladder tumor in shorter latency than that in Ink4b deficiency background alone. The reason why it would be interesting to find out is that p16 also suppresses cell growth and arrests cells in G1 phase in our *in vitro* experiments.

Finally, it would be of great interest to determine the susceptibility of p16 knockout only and p19 knockout only mice to urothelium specific carcinogen BBN respectively. Then we will be able to elucidate which protein contributes to the invasive tumor suppressor role of Ink4a gene.

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