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## THE MECHANISM OF TUMORIGENESIS IN THE IMMORTALIZED HUMAN PANCREATIC CELL LINES: CELL CULTURE MODELS OF HUMAN PANCREATIC CANCER

Zhe Chang

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**THE MECHANISM OF TUMORIGENESIS IN THE IMMORTALIZED  
HUMAN PANCREATIC CELL LINES: CELL CULTURE MODELS OF  
HUMAN PANCREATIC CANCER**

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**THE MECHANISM OF TUMORIGENESIS IN THE IMMORTALIZED  
HUMAN PANCREATIC CELL LINES: CELL CULTURE MODELS OF  
HUMAN PANCREATIC CANCER**

**A  
DISSERTATION**

**Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M.D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment**

**Of the Requirements**

**For the Degree of**

**DOCTOR OF PHILOSOPHY**

**By**

**ZHE CHANG, M.S.**

**Houston, Texas**

**August, 2011**

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# **The mechanism of tumorigenesis in the immortalized human pancreatic cell lines: cell culture models of human pancreatic cancer**

Publication No. \_\_\_\_\_

Zhe Chang, Ph.D.

Supervisory Professor: Paul J. Chiao, Ph.D.

Pancreatic ductal adenocarcinoma (PDAC) is the most lethal cancer in the world. The most common genetic lesions identified in PDAC include activation of K-ras (90%) and Her2 (70%), loss of p16 (95%) and p14 (40%), inactivation p53 (50-75%) and Smad4 (55%). However, the role of these signature gene alterations in PDAC is still not well understood, especially, how these genetic lesions individually or in combination contribute mechanistically to human pancreatic oncogenesis is still elusive. Moreover, a cell culture transformation model with sequential accumulation of signature genetic alterations in human pancreatic ductal cells that resembles the multiple-step human pancreatic carcinogenesis is still not established.

In the present study, through the stepwise introduction of the signature genetic alterations in PDAC into the HPV16-E6E7 immortalized human pancreatic duct epithelial (HPDE) cell line and the hTERT immortalized human pancreatic ductal HPNE cell line, we developed the novel experimental cell culture transformation models with the most frequent gene alterations in PDAC and further dissected the molecular mechanism of transformation. We demonstrated that the combination of activation of K-ras and Her2, inactivation of p16/p14 and Smad4, or K-ras mutation plus p16 inactivation, was sufficient for the tumorigenic transformation of HPDE or HPNE cells respectively. We found that these transformed cells exhibited enhanced cell proliferation, anchorage-independent growth in soft agar, and grew tumors with PDAC histopathological features in orthotopic mouse model. Molecular analysis showed that the activation of K-ras and Her2 downstream effector pathways –MAPK, RalA, FAK, together with upregulation of cyclins and c-myc were involved in the malignant transformation. We discovered that MDM2, BMP7 and Bmi-1 were overexpressed in the tumorigenic HPDE cells, and that Smad4 played important roles in regulation of BMP7 and Bmi-1 gene expression and the tumorigenic transformation of HPDE cells. IPA signaling pathway analysis of microarray data revealed that abnormal signaling pathways are involved in transformation. This study is the first complete transformation model of human

pancreatic ductal cells with the most common gene alterations in PDAC. Altogether, these novel transformation models more closely recapitulate the human pancreatic carcinogenesis from the cell origin, gene lesion, and activation of specific signaling pathway and histopathological features.

## Table of Contents

Approval Page	i
Title Page	ii
Acknowledgements	iii
Abstract	iv
Table of Contents	vi
List of Figures	vii
List of Tables	ix
List of Abbreviations	x
Introduction	1
Materials and Methods	19
Results	33
Part I	33
Part II	60
Discussion	74
References	93
Vita	116

## List of Figures

1. Figure1. Genetic progression model of pancreatic ductal adenocarcinoma.	3
2. Figure 2. The Her2 and Ras signaling pathway.	5
3. Figure3. The transforming growth factor-beta (TGF $\beta$ ) signaling pathway.	10
4. Figure 4. Stable expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA in E6E7 immortalized human pancreatic ductal epithelial (HPDE) cells.	34
5. Figure 5. Cell growth properties of HPDE cell lines with stable expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA.	36
6. Figure 6. The abilities of cell migration of HPDE cells with expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA.	38
7. Figure 7. The abilities of cell invasion of HPDE cells with expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA	39
8. Figure 8. Anchorage-independent growth of HPDE cell lines with expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA in soft agar.	41
9. Figure 9. Activation of K-ras and Her2, inactivation p16/p14 and Smad4 in HPDE cell line induced tumorigenesis in the orthotopic mouse model.	43
10. Figure 10. Confirmation of tumorigenesis in mouse and the alterations of gene expression in the HPDE tumor cell lines isolated from mouse tumors.	45
11. Figure 11. Activation of signaling pathway downstream of K-ras and Her2 in HPDE cells.	47
12. Figure 12. The expression of cell cycle protein and cell proliferation related genes in HPDE cell lines.	48
13. Figure 13. The expression of EMT marker proteins in HPDE cell lines.	49
14. Figure 14. Confirmation of gene expression change in microarray analysis.	51
15. Figure 15. Confirmation of the changes of CDH2 and GRB10 gene expression in cDNA microarray by Real-time PCR analysis.	53
16. Figure 16. The activation of FAK in HPDE cell lines.	53
17. Figure 17. IPA biological function analysis of microarray data.	55
18. Figure 18. IPA canonical signaling pathway analysis of microarray data.	56
19. Figure 19. Changes of molecular mechanism of cancer signaling in IPA signaling pathway analysis of microarray data.	57
20. Figure 20. Stable expression of mutant K-ras and p16shRNA in hTERT-immortalized HPNE cells.	61
21. Figure 21. Cell growth of HPNE cell lines with stable expression of mutant K-ras and	63

p16shRNA.

22. Figure 22. Activation K-ras and inactivation p16 in HPNE cell line induced anchorage-independent cell growth in soft agar assay.	65
23. Figure 23. Activation of K-ras and inactivation of p16 in HPNE cell lines induced tumorigenesis and metastasis in the orthotopic mouse model.	66
24. Figure 24. Activation of signaling pathway downstream of K-ras in HPNE cell lines.	67
25. Figure 25. Increased expression of cell proliferation related proteins by mutant K-ras in HPNE cell lines.	69
26. Figure 26. The expression of EMT markers, and invasion-related proteins in HPNE cell lines.	70
27. Figure 27. The alterations of signaling pathways for malignant transformation in microarray analysis of the HPNE cell lines.	72
28. Figure 28. The proposed model for transformation of HPNE cell line.	76
29. Figure 29. The proposed model for transformation of HPDE cell line.	77
30. Figure 30. Overview of the interplay of the major genetic alterations identified in PDAC and gene alterations used in our transformation models.	78

## List of Tables

- |  |    |
|--|----|
| 1. Table 1. The most common gene alterations in human PDAC.  | 4  |
| 2. Table 2. The top most significant changes of gene expression for cell line<br>HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA versus HPDE/K-ras/Her2/p16p14shRNA cell<br>line in cDNA microarray analysis. | 50 |
| 3. Table 3. The top most significant molecular change for HPNE/K-ras versus HPNE and<br>HPNE/K-ras/p16shRNA versus HPNE/K-ras in the microarray analysis.  | 71 |

## List of Abbreviations

BMP	Bone morphogenic protein
CDK	Cyclin-dependent kinase
CK-19	Cytokeratin-19
DMEM	Dulbecco's modified Eagle medium
DPC4	Pancreatic carcinoma locus 4
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GFP	Green fluorescent protein
HE	Hematoxylin and eosin
HPDE	Human pancreatic ductal epithelial cell
HPNE	Human pancreatic nestin-expressing cell
HPV	Human papillomavirus
hTERT	Human telomerase reverse transcriptase
ILK	Integrin-linked kinase
IPA	Ingenuity pathway analysis
JNK	c-Jun N-terminal kinase
KSFM	Keratinocyte serum-free medium
LT	Large T
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
OIS	Oncogene-induced senescence
PanIN	Pancreatic intraepithelial neoplasia
PDX1	Pancreatic and duodenal homeobox gene 1
PBS	Phosphate buffered saline
Ptf1a	Pancreatic transcription factor 1a
RT-PCR	Reverse transcriptase - polymerase chain reaction

PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphoinositide-3-kinase
PP2A	Protein phosphatase 2A
Rb	Retinoblastoma
RIPA	Radioimmunoprecipitation assay protein lysis buffer
SA- $\beta$ -gal	Senescence-associated- $\beta$ -galactosidase activity
shRNA	small hairpin RNA
st	small t
SV40	Simian virus 40
TGF $\beta$	Transforming growth factor $\beta$
T $\beta$ RI	TGF $\beta$ type I receptor
T $\beta$ RII	TGF $\beta$ type II receptor
TSP-1	Thrombospondin-1



# Introduction

## Overview of pancreatic cancer

About 95% of pancreatic cancer originates from the exocrine pancreas. Pancreatic ductal adenocarcinoma (PDAC) accounts for > 85% of pancreatic cancer. PDAC ranks as the fourth leading cause of cancer-related death among both males and females in the U.S.A with a median survival of less than 6 months and a 5-year survival rate of less than 5% (1, 2). Pancreatic cancer has one of the highest fatality rates of all cancers. Each year in the United States more than 43,000 individuals are diagnosed with pancreatic cancer, and 36,800 individuals die from this disease. The high mortality rate of PDAC is due to the early and aggressive growth characteristics of this cancer leading to local invasion and distant metastasis, the late clinical presentation and lack of early detection strategy causing late diagnosis, and lack effective therapeutic strategy. At the time of diagnosis, only 10%-20% patients have tumors localized within pancreas, 40% of patients have locally advanced disease with tumors invasion to adjacent organs and tissues, and about 40% to 50% of patients have distant metastatic PDAC (3). Thus, at the time of diagnosis, about 80% of patients with pancreatic cancer present with distant or locally metastasis. Furthermore, PDAC show profound resistance to chemotherapy or irradiation. Although the overall pancreatic cancer incidence has not changed dramatically over the past 25 years, the total number of pancreatic cancer cases and deaths has increased each year since 2004. Therefore, PDAC is one of the most lethal human diseases, and carries one of the worst prognoses among all cancers. In the United States, pancreatic cancer incidence and mortality rates are higher in blacks than in whites, and it is also higher in men than in women. Pancreatic cancer is mainly a disease of the elderly; the median age at diagnosis is 72 years (<http://seer.cancer.gov/>).

The risk factors for the development of PDAC include chronic pancreatitis, older age, diabetes mellitus, hereditary pancreatitis, strong family history of pancreatic cancer, and environmental factors such as cigarette smoking and a high-fat low in fruits and vegetables diet, obesity, alcohol, occupational exposure to certain chemicals, radiation exposure (4-6).

Despite the advance in the molecular pathogenesis, clinical diagnosis and treatment, the prognosis of PDAC has not improved significantly over the past decades; the overall mortality rates for pancreatic cancer have been relatively stable over the past three decades. Pancreatic cancer still remains an important unresolved health problem and one of the greatest challenges in cancer research. Therefore, new diagnosis and more efficient therapies to target altered molecular and signaling pathways in PDAC are needed. The greatest hope for improving patient outcome is through a better understanding of the molecular and genetic basis for the development and progression of

PDAC to direct the development of new diagnosis and effective treatment strategy and thus ultimately lead to improved survival rates.

### **Structure and function of the pancreas**

Pancreas is derived from endoderm and consists of three major compartments –acinar, islet, and ductal. The pancreas is a glandular organ, and is comprised of two different functional units – endocrine and exocrine glands to regulate two major physiological processes: protein and carbohydrate digestion and glucose metabolism homeostasis.

The exocrine pancreas consists of acinar and duct cells; it secretes digestive enzymes into the duodenum and constitutes the majority of the pancreatic tissue. The acinar cells comprise over 80% of pancreatic mass, and are lobular units that secrete digestive enzymes into ducts, which are released into the duodenum. Ductal cells which constitute about 10% of the pancreas in number and 4% in volume form the epithelial lining of the branched tubes and the network of tubules that deliver enzymes produced by acinar cells into the duodenum. In addition, ductal cells secrete bicarbonate that regulates small intestine acidity and mucins to the enzyme mixture. The adult ductal cells have been proposed as pancreatic stem cells on the basis of pancreas regeneration experiments. These cells may have the ability to produce endocrine cells in the adult. Their mitotic activity increases when the pancreas is injured (7). Ductal differentiation includes tubular structures, cysts, papillae, and mucin formation.

The endocrine pancreas consists of the islets of Langerhans embedded within acinar tissue, comprising 1-2% of the organ mass. It contains four specialized cell types and secretes hormones into the bloodstream. The  $\alpha$ - and  $\beta$ -cells produce glucagon and insulin, respectively, to regulate the metabolism of glucose. PP and  $\delta$ -cells secrete pancreatic polypeptide and somatostatin that modulate the secretory properties of the other pancreatic cell types.

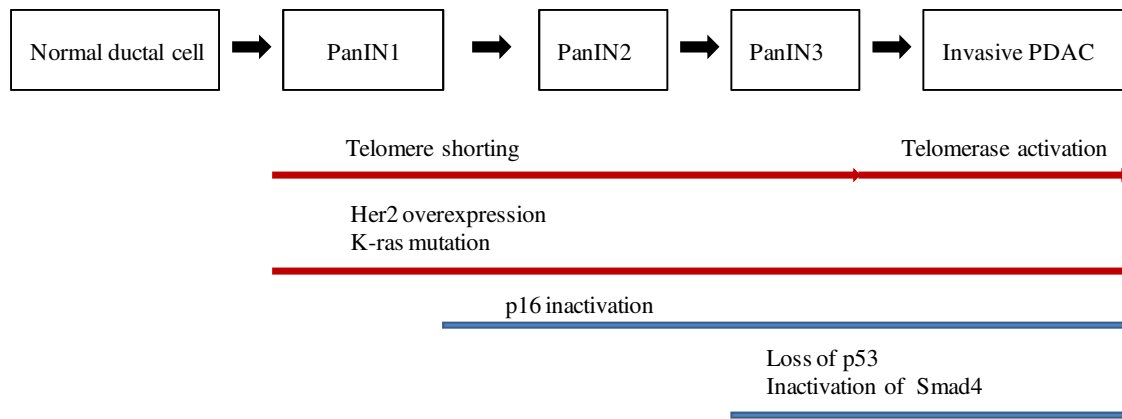
### **Histological feature of PDAC**

Pancreatic cancer is grossly a firm, highly sclerotic mass with poorly defined edges. Under microscopy, pancreatic cancer displays ductal morphology with duct-like structures–gland formation of neoplastic epithelium and dense desmoplastic stroma–hallmark feature of PDAC. PDAC exhibits varying degrees of cellular atypia and differentiation; it may display well to poorly differentiated histological feature with abnormal gland formation, and desmoplastic stroma. The cancer cells may contain abnormal mitotic figures; the nuclei may display marked pleomorphism and hyperchromasia. Less common histologic variants/subtypes include colloid carcinoma, medullary carcinoma, signet-ring cell carcinoma, undifferentiated carcinoma (including sarcomatoid carcinoma, undifferentiated

carcinoma with osteoclast-like giant cells), and adenosquamous carcinoma. Some subtypes such as adenosquamous and undifferentiated carcinoma are more aggressive and have worse prognosis than ductal adenocarcinoma (8). Most PDAC arise in the head of the pancreas with invasion to surrounding organs and tissues including lymphatics, spleen, and peritoneal cavity and with metastasis to regional lymph nodes, liver and lung, and distant sites (1, 9).

### Tumor progression model and gene alterations in pancreatic cancer

PDAC is generally believed to arise from pancreatic ductal cells. The development of pancreatic cancer is a multi-step process resulting from the sequential accumulation of genetic lesions from normal cells during the progression of pancreatic cancer (10, 11). A commonly accepted PDAC progression model by Hruban (10) proposed that normal ductal cells progress from normal ductal cells to increased grades of pancreatic intraepithelial neoplasia (PanIN) –through flat (PanIN-1A) and papillary lesions (PanIN -1B) to atypical papillary lesions (PanIN-2) to carcinoma in situ (PanIN 3) and finally to invasive PDAC (Fig. 1). There are now sufficient evidences from clinical, genetic, histopathological and mouse model data to support the tumor progression model of PDAC in pancreatic carcinogenesis.



**Figure1. Genetic progression model of pancreatic ductal adenocarcinoma.** The histopathological progression from normal pancreatic ductal cell to low-grade pancreatic intraepithelial neoplasia (PanIN1), through middle and high-grade PanINs, to invasive PDAC (left to right) is associated with the sequential accumulation of specific genetic alterations. The histological features of PanINs display flat or papillary duct lesion in PanIN1, atypical papillary duct lesion in PanIN2, carcinoma in situ in PanIN 3. Pancreatic ductal cells acquire successive gene lesions during the progression of PDAC, developing from low-grade PanINs to high-grade PanINs to invasive PDAC. The most common gene alterations are at early stage: K-ras mutation, telomere shortening and overexpression of Her2, at intermediate stage: p16 inactivation, at late stage: inactivation of Smad4 and p53 (8, 11).

Histologic progression from low-grade PanINs through intermediate-grade PanINs to high-grade PanINs and to invasive carcinoma is associated with the accumulation of specific genetic alterations and an increasing number of gene lesions in higher grade PanINs (Fig.1) (9, 10, 12-14). Genetic lesions are also frequently associated with the different histopathological stages. The accumulation of gene alterations correlates with increasing degree of cytological and architectural atypia or increasing grades of dysplasia during the process of pancreatic carcinogenesis (10).

PDAC is basically a genetic disease, caused by inherited germline and acquired somatic alterations in cancer-associated genes. PDAC is characterized by a specific pattern of gene lesions. The most common genetic alterations identified in PDAC at different stages include K-ras mutation (90%) and Her2 overexpression (70% ) at early stage, loss of p16 function (95%) at middle stage, inactivation p53 (50-75%) and Smad4 (55%) in the late stage, and p14 deletion (40%) (12, 15, 16) (Table 1).

**Table 1. The most common gene alterations in human PDAC**

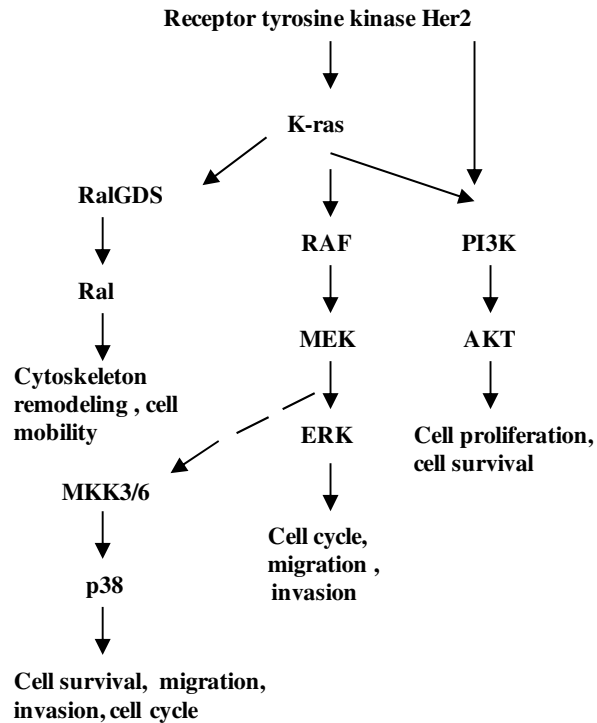
Gene	Mechanism of alteration	Frequency %	Chromosome location	Stage of occurrence
Oncogenes				
K-ras	Point mutation	>90	12p12	Early
Her2	Overexpression	70	17q	Early/Middle
Tumor suppressor genes				
p16	Inactivation	95	9p21	Middle
	HD	40		
	LOH and IM	40		
	Promoter hypermethylation	15		
p14	HD	40	9p21	
p53	LOH and IM	50-75	17p31	Late
Smad4	Inactivation	55	18q21	Late
	HD	35		
	LOH and IM	20		

HD, homozygous deletion; IM, Intragenic mutation; LOH, loss of heterozygosity

### **K-ras oncogene**

K-ras encodes a small GTPase, and functions downstream of mitogenic growth factor receptors-mediated signal transduction pathways. Frequent mutation sites involve codons 12, 13 and 61, but in PDAC the most common mutation occurs in codon 12. Point mutation of K-ras impairs the intrinsic

GTPase activity and results in constitutively active GTP-bound form. Activation of K-ras induces a variety of cellular responses including cell proliferation, cell survival, motility, invasion and cytoskeletal remodeling (17-19). The most common identified Ras effector pathways involved in Ras-induced transformation are Raf/MEK/ERK, PI3K/AKT and RalGDS/Ral pathways (17-19) (Fig. 2).



**Figure 2. The Her2 and Ras signaling pathway.** K-ras protein is transiently activated by a various extracellular signals, such as binding of growth factor ligands to the cognate growth factor receptor. In its active state, Ras is bound to GTP. Oncogenic mutation of K-ras impairs the intrinsic GTPase activity and results in constitutively active form of K-ras. Overexpression of Her2 and mutation of K-ras render the downstream pathway constitutively active in pancreatic cancer. K-ras and Her2 proteins induce several of downstream effector pathways, including mitogen activated protein kinase (MAPK)-RAF/MEK/ERK and p38MAPK, phosphoinositide-3-kinase (PI3K)/AKT, and RalGDS/Ral pathways. Activation of these pathways induces a variety of cellular responses such as proliferation, cell survival, and cell motility, cell invasion.

K-ras mutation occurred in the early stage, and is one of earliest genetic lesions identified in human PanIN lesion (20, 21). The frequency of K-ras mutation is increased with the disease progression (20, 22-25). K-ras mutations are detected in approximately 30% of early neoplastic

lesions with the frequency rising nearly to 100% in PDAC (22, 23), and are believed to be a critical initiating event in the development of PDAC (1, 8, 23, 26).

Mouse models have demonstrated that pancreas specific activation of K-ras induced the development of PanIN lesion which slowly progressed to invasive PDAC, whereas mouse models of pancreas-specific deletion of either *p16INK4A/p14ARF* or *p53*, or *Smad4* in the absence of K-ras mutation do not demonstrate any obvious pancreatic phenotype or induce PDAC. Additional inactivation of *p16/p14*, or *p53* or *Smad4* was found to dramatically accelerate the progression of K-ras initiated PDAC (27-32). These results suggested that K-ras mutation plays a critical initiating role in the development of PDAC and the additional genetic hits are required for the cancer progression (26).

### **Her2 oncogene**

Human Epidermal growth factor Receptor 2 (EGFR2)/ Her2 is a 185-kDa transmembrane glycoprotein receptor tyrosine kinase. Expression of Her2 induces activation of different downstream signalings (Fig. 2). Her2 overexpression promotes tumorigenesis by stimulating cell proliferation, survival and invasion. Overexpression of Her2 occurred in the early stage of PDAC and is found in about 70% of cases (33-35).

There was a significant correlation between tumors with well-differentiated histology and Her2 expression. Her2 is frequently overexpressed in well-differentiated of PDAC as well as in the early-stage precancerous (PanIN) lesions (35, 36). Her2 expression appears to correlate with the severity of the dysplasia in the PanIN precursor lesions (33, 36).

Overexpression of Her2 correlates with shorter survival, and is proved to be an independent factor for a worse prognosis in PDAC. PDAC patients with Her2 overexpression tumors had significantly shorter survival times than those with Her2 normal expression tumors (median survival time, 14.7 vs 20.7 months, respectively) (37, 38).

### **CDKN2A gene locus-p16 and p14 tumor suppressor genes**

CDKN2A gene located in chromosome 9q21 locus encodes two tumor suppressor genes- *p16INK4A* and *p14ARF* via different first exon and alternative reading frames in shared common downstream the second and third exons (39). As a consequence, p16INK4A and p14ARF are not isoforms and do not share any amino acid sequence homology.

p16 belongs to the cyclin-dependent kinase (CDK) inhibitor family and regulates cell cycle progression in G1 phase by binding to cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and inhibiting CDK4/6 –mediated phosphorylation of retinoblastoma protein (Rb), leading to G1 arrest

(40, 41). Expression of p16 is enhanced in replicative arrested cells or cellular senescence, and play an important role in cellular senescence. Elevated p16 activity plays a major role in the resistance to transformation of cells.

Loss of *p16* tumor suppressor gene function in PDAC occurs through homozygous deletion (40%), an intragenic mutation coupled with loss of the second allele (40%), and promoter hypermethylation (15%) (23, 42-45). p16 is the most frequently inactivated gene in pancreatic cancer. Inactivation of p16 occurs in the intermediate stage, and is found in almost all the PDAC (95%) (23, 42, 45). Thus, the p16/Rb pathway is inactivated in nearly all pancreatic cancers, resulting in phosphorylation of Rb and thereby leading to an inappropriate progression of the cell cycle through the G1/S transition. p16 may contribute to pancreatic carcinogenesis by additional mechanisms, distinct from its role in regulation of CDK activity.

p14 tumor suppressor stabilizes p53 protein by binding to MDM2 and promoting its degradation, thus inhibiting MDM2-mediated proteasomal degradation of p53 protein (41, 46, 47). Inactivation of p14 via homozygous deletion of the locus occurs in ~50% of the tumors (48).

Physiological level of p14 in most normal tissues is very low and its expression is not detectable in most normal tissues. The expression of p14 is induced by mitogenic stimulation such as c-myc and Ras. p14ARF response is complex, p14 functions to restrain abnormal cell growth and maintain genomic stability through p53-dependent or -independent pathways (49-52). p14 inhibits aberrant cell growth and induces cell cycle arrest or apoptosis through activation of p53. p14 plays an important protective role in oncogenic transformation and tumorigenicity (53-55). The expression of *p16INK4A/p14ARF* is increased at the early stage of tumorigenesis (39), p14ARF is thus a critical element of tumor surveillance mechanism, and its expression is decreased in human cancer.

p14 also has many p53 independent functions. For example, p14 suppresses cell growth by interacting with E2F1, myc, HIF-1 $\alpha$  and attenuating the transacting activity of growth promoting genes such as E2F1 and c-myc (49, 51, 52, 56-59). p14 decreased ribosomal RNA transcription and processing (60, 61). Moreover, p14 is also involved in DNA damage response by activation of p53 pathway (62) or by activation of ATM/ATR/CHK pathway (63), or by maintenance of chromosomal stability or activation DNA repair pathways (64). p14 may negatively regulate angiogenesis through a p53-independent pathway. p14ARF null mice become blind soon after birth indicating that p14 expression is required to induce vascular regression in the developing eye (65). Loss of p14ARF function promotes tumorigenesis via facilitating angiogenesis, p14ARF suppresses tumor angiogenesis through a p53-independent mechanisms via post-transcriptional control of VEGF-A expression (66, 67).

Mouse models showed that activation of *K-ras* induced PanIN lesions, whereas the inactivation of *p16INK4A/p14ARF* alone failed to generate any neoplastic lesions in the pancreas. Deletion of *p16INK4A/p14ARF* greatly accelerates the malignant progression of mutant K-ras triggered PanIN lesions into highly invasive or metastatic PDAC (27, 28, 30). The histology of these tumors resembles human PDAC, displaying a proliferative desmoplastic stroma and ductal structure with a propensity to develop to a poorly differentiated neoplasm with sarcomatoid feature –an uncommon subtype of human PDAC (28, 30). These findings from mouse models provide experimental evidence to support the widely accepted model of human PDAC in which activation of K-ras serves to initiate premalignant ductal PanIN lesions, and the p16 INK4A/p14ARF tumor suppressors normally function to inhibit the malignant transformation potential of mutant K-ras and thus to restrain the malignant progression of these PanIN lesions triggered by mutant K-ras into invasive PDAC. p14ARF null mouse tend to develop sarcomas and other cancers (49, 68).

Deletion of p14 and p53 mutation coexists in ~40% of pancreatic cancer (1, 23, 45, 69), indicating there is non-overlapping functions for p14ARF and p53 in tumor suppression of PDAC. Expression of p14 inhibited PDAC cell growth through upregulation of p53 (70). p14 expression also suppressed the migration and invasion in PDAC cell lines *in vitro*, and reduced tumor formation and metastasis *in vivo* via p53 independent pathway (71). The results suggest that p14ARF have both p53 dependent and independent functions in PDAC development. However, how inactivation of p16/p14 contributes to the tumorigenesis of human pancreatic ductal cells is still elusive.

### **p53 tumor suppressor gene**

The *p53* gene is located on chromosome 17p. p53 not only regulates genes in apoptosis, and cell cycle –G1/S cell cycle arrest and G2/M cell cycle checkpoint but also functions to maintain genomic stability (72, 73). Inactivation of p53 is generally through missense mutation of DNA-binding domain. It is inactivated almost always by an intragenic mutation in one allele coupled with loss of the second allele (74). Loss of *p53* function during carcinogenesis can lead to aberrant cell growth, increased cell survival, and genetic instability (75) .

In pancreatic cancer, inactivation of p53 tumor suppressor gene occurs at late stage and is inactivated in 50%–75% of the cases (15, 76). The loss of *p53* function results in dysregulation of two critical controls of cell number –cell division and cell death in PDAC. Loss of p53 may contribute to the extensive genetic instability in PDAC. p53 plays an important role in constraining malignant progression of PDAC, as demonstrated in recent mouse models of pancreatic cancer, loss of p53 function accelerated the progression of K-ras initiated pancreatic neoplasia (29, 30). Inactivation of p53 is correlated with shorter survival in PDAC patients (77).

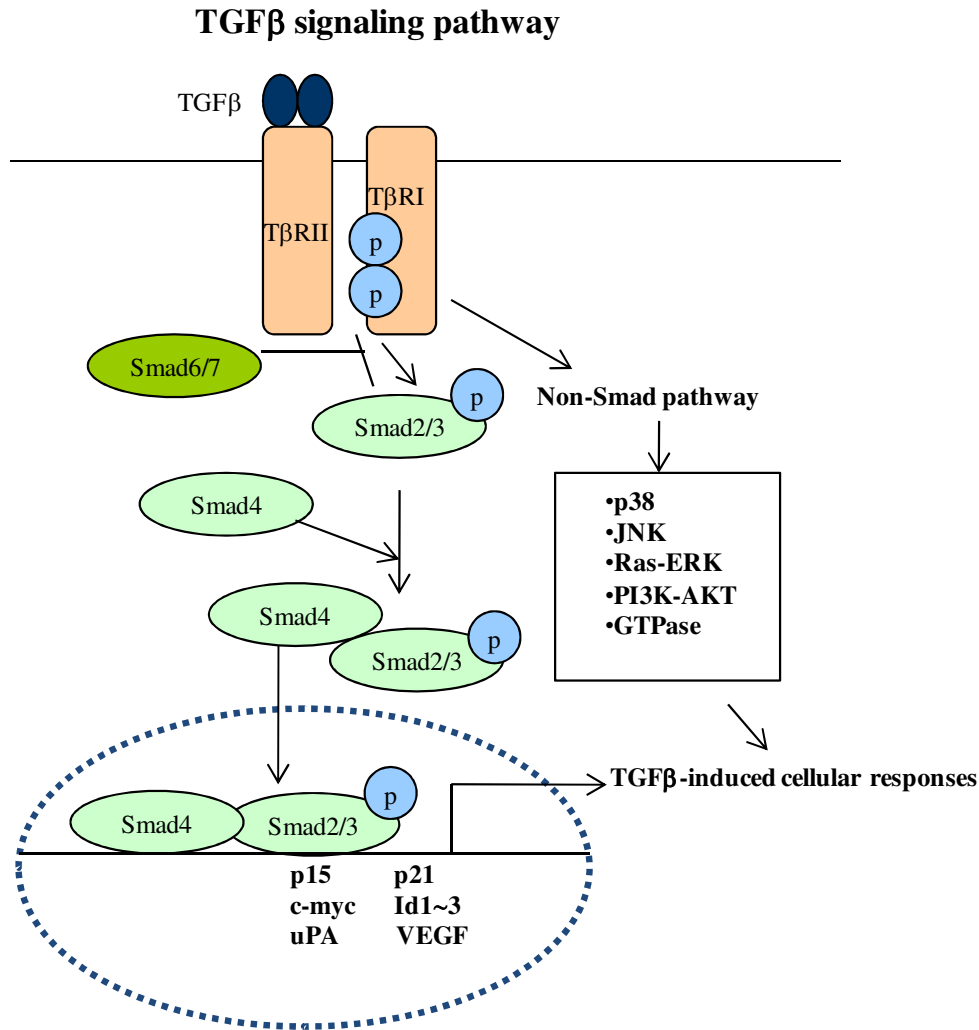


### **Smad4/DPC4 tumor suppressor gene**

Smad4/*DPC4* tumor suppressor gene, which is first identified as deleted in pancreatic carcinoma locus4 (DPC4) on chromosome 18q21.1 (78), is a transcription factor and a central mediator of transforming growth factor  $\beta$  (TGF  $\beta$ ), bone morphogenic protein (BMP), and activin signaling pathway. It belongs to the Smad gene family.

TGF  $\beta$ , BMP, activin signaling pathway transductions are through TGF  $\beta$ , BMP, or activin family ligands binding to their cognate receptor serine/threonine kinases that phosphorylate receptor Smads –Smad1, Smad2, Smad3, Smad5, and Smad8. The activated receptor Smads bind to Smad4 and translocate to the nucleus, where the transcriptional complexes regulate the expression of various genes. TGF  $\beta$  signaling plays important roles in regulating cell proliferation, differentiation, apoptosis, migration, invasion, metastasis and immunosurveillance (79-82). Upon TGF  $\beta$  dimers binding to cell surface receptors TGF  $\beta$  Type II receptors (T $\beta$ RII), the type II receptors associate with Type I receptors (T $\beta$ RI), conformational change of Type I receptors induces phosphorylation of the GS domain of the type I receptor and the activity of its serine/threonine kinase. After receptor activation, cytoplasmic Smad2/3 proteins are phosphorylated by an activated Type I receptor, the activated Smad2/3 binds to Smad4 and form heteromeric complexes. Heteromeric complexes of Smad2/3-Smad4 then translocate to the nucleus and bind to the promoters of target genes to regulate the expression of multiple TGF  $\beta$ -responsive Smad4 target genes. The pathway is inhibited by Smad6/7. TGF  $\beta$  signaling can also activate Smad4-independent pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen activated protein kinase (MAPK), PI3K/AKT and small GTPases such as RHOA and CDC42 pathway.

TGF  $\beta$  signaling plays a dual role in tumorigenesis and tumor progression. During the tumor initiation or early stages of carcinogenesis, TGF  $\beta$  signaling functions as tumor suppressor to inhibit epithelial cell growth and cell proliferation, induce differentiation and apoptosis. In late stages of tumor progression, due to loss or mutation of the members of the TGF  $\beta$  signaling pathway, TGF  $\beta$  signaling loses its growth inhibitory effects response and becomes an oncogenic factor, acts as a tumor promoter stimulating tumor cell proliferation, angiogenesis, migration, invasion, epithelial to mesenchymal transition (EMT), metastasis and immunosuppression (83). During carcinogenesis, TGF  $\beta$  signaling loses its cytostatic response by regulation of cell growth genes of p21, p15, c-myc,



**Figure 3. The transforming growth factor-beta (TGF $\beta$ ) signaling pathway.** The TGF  $\beta$  signaling pathway is activated by binding of the ligand TGF  $\beta$  to TGF  $\beta$  type II receptor (T $\beta$ RII) at the cell surface, TGF  $\beta$  type II receptor associates with type I TGF  $\beta$  receptor (T $\beta$ RI) leading to conformational changes, phosphorylation and activation of type I receptors. Upon receptor activation, cytoplasmic molecules Smad2/3 are phosphorylated by an active form of type I receptor, activated Smad2/3 associates with Smad4 and hetero-oligomeric complex of Smad2/3-Smad4 translocates to the nucleus and binds to specific DNA sequence in the promoter of target genes, where they regulate the expression of a variety of Smad4 target genes. TGF  $\beta$  signaling can also function via Smad-independent mechanisms of non-Smad pathway to activate p38, JNK, Ras-ERK, PI3K/AKT, and small GTPases such as RHOA and CDC42.

Id1-3, and promotes angiogenesis and tumorigenesis by expression of VEGF, MMP2, 9 (83).

Homozygous deletion of *Smad4* in mice was embryonically lethal at embryonic day 6.5 -8.5. These mice showed defective gastrulation and abnormal visceral endoderm development and the absence of the mesoderm and extraembryonic ectoderm because of impaired extraembryonic membrane formation and decreased epiblast proliferation (84, 85); whereas *Smad4* heterozygous mice developed gastrointestinal polyps and invasive gastric cancer (86-88). The germline mutation of *Smad4* gene causes familial juvenile polyposis (89, 90). Homozygous deletion or intragenic mutation of somatic *Smad4* gene is frequently found in the carcinomas of the pancreas (55%), gastrointestinal (15%), and skin.

Inactivation of *Smad4* tumor suppressor gene by homozygous deletion (35%) or by an intragenic mutation of one allele coupled with loss of the remaining second allele (20%) has been found in approximately 55% of PDAC cases (15). Loss of *Smad4* function occurs in the late stage of PDAC, its loss is detected only in later-stage PanINs and PDAC, indicating inactivation of *Smad4* is a late genetic event in PDAC progression (14) .

The role of *Smad4* in tumorigenesis and metastasis of PDAC has been demonstrated in previous studies. Previous research showed that *Smad4* regulates the TGF  $\beta$ -inducible expression of p21*WAF1* and p15*INK4B* (91-94). *Smad4* expression in *Smad4*-null pancreatic cancer cell lines reduced anchorage-independent growth and suppressed xenograft tumor growth *in vivo* in nude mice through downregulation of angiogenesis by decreasing expression of VEGF and increasing expression of thrombospondin-1 (TSP-1) –an angiogenesis inhibitor, as well as by downregulation of MMP2 and MMP9 (93, 95-97). Another study reported that restoration of *Smad4* expression in *Smad4*-deficient pancreatic cancer cells inhibits TGF  $\beta$ -mediated motility and invasion *in vitro* and reduces metastasis *in vivo* (98, 99).

Recent reports demonstrated that inactivation of *Smad4* is significantly associated with shorter survival, poor prognosis and metastatic propensity of pancreatic cancer (100-104). The reports from Tascilar and others showed that inactivation of *Smad4* gene in pancreatic cancer is significantly correlated with a higher possibility of metastasis and significantly shorter overall survival and poorer prognosis in PDAC patients with surgical resection of pancreatic cancer than those with intact *Smad4* gene (100). Among patients with surgical resection of their pancreatic adenocarcinoma, survival of patients with *Smad4* protein expression was significantly longer compared with those patients with *Smad4* gene inactivation (100, 103). The clinical findings have displayed that *Smad4* is also associated with metastasis. Loss of *Smad4* is correlated with increased metastatic progression in PDAC patients (14, 102). Loss of *Smad4* results in a biologically more aggressive form of pancreatic cancer when compared with tumors with intact *Smad4*. *Smad4* gene status was highly correlated with the presence of widespread metastasis but not with locally destructive tumors.

Widespread metastatic PDAC was found in patients whose tumors have mutation of Smad4 compared to the more locally destructive disease of PDAC observed in patients whose tumors express wild-type Smad4 (104).

Mouse models (31, 32, 105) have showed that *Smad4* is dispensable for normal pancreas development, Smad4 deletion alone does not notably affect pancreatic development or physiology and does not play a role in the initiation of pancreatic cancer, but acts as a tumor promoter to accelerate mutant K-ras triggered pancreatic cancer development and progression, the combination of activated K-ras with Smad4 deficiency results in rapid progression of K-ras-initiated neoplasms. These results suggest that Smad4 is a PDAC tumor suppressor, functioning to block the progression of mutant K-ras-driven neoplasms to PDAC.

Smad4 has been demonstrated to be associated with histological differentiation of PDAC. Mouse models showed Smad4 deficiency affected the tumor histology phenotype. Inactivation of Smad4 in mouse models developed a differentiated histopathology with increased expression of epithelial markers such as cytokeratin 19 and E-cadherin. In the *K-ras<sup>G12D</sup>/p16INK4A/p14ARF* heterozygous mouse model, Smad4 deficiency not only accelerated PDAC development but also promoted well differentiated PDAC. Tumors with intact Smad4 frequently demonstrated poorly differentiated carcinoma and exhibited EMT, while tumor with Smad4 deletion typically developed well to moderately differentiated adenocarcinomas (31, 32). The results from clinical studies demonstrated that the expression of Smad4 is inversely associated with histopathological grades of pancreatic cancers; in clinical samples tumors with loss of Smad4 may have a higher propensity for invasive histopathological feature (14, 106).

The function of Smad4 in tumorigenesis is still not well-understood. There are different contradictory reports regarding the function of Smad4 in the regulation of proliferation and EMT. The role of inactivation of Smad4 in tumorigenesis may be involved in its central role in TGF  $\beta$ -mediated inhibition of cell growth and cell proliferation, EMT and angiogenesis (107). The results from Smad4 silencing in keratinocyte and pancreatic cell lines indicated that TGF  $\beta$  induced growth inhibition might be dependent on Smad4. Inactivation of Smad4 which disrupting TGF  $\beta$  signaling pathways may abrogate the effect of TGF $\beta$ - mediated growth inhibition, and induce proliferation in tumor cells by TGF  $\beta$ . Levy (108) showed that that Smad4 is necessary for TGF $\beta$ -induced cell cycle arrest and migration, but is not involved in the TGF $\beta$ -induced EMT as TGF  $\beta$ -induced cell cycle arrest and migration, but not EMT, are eliminated by Smad4 silencing. The results from mouse model demonstrated that Smad4 is essential for TGF  $\beta$ -regulated growth inhibition; *Smad4* deficiency is associated with increased proliferation of both the neoplastic epithelium as well as the stromal tissue in mutant *K-ras/Smad4* deletion model (31, 32). Intact Smad4 signaling is required for

induction of EMT and enhanced cell migration. PDAC cell lines with wild type *Smad4* display TGF  $\beta$ -induced migration, loss of *Smad4* is associated with decreased EMT in the murine pancreatic cancers (31, 32). These results suggest that loss of Smad4 function resulting in disruption of TGF  $\beta$  signaling might switch the TGF  $\beta$  response into an oncogenic one, thus might promote TGF $\beta$ -mediated tumorigenesis by abrogating tumor-suppressive functions of TGF  $\beta$ -induced growth inhibition while stimulating tumor-promoting functions of TGF  $\beta$ -induced EMT response.

The results from these studies suggest that Smad4 plays important roles in regulating cell proliferation, EMT and tumorigenesis in PDAC, and that inactivation of Smad4 tumor suppressor gene thus disrupting TGF  $\beta$  signaling, is another key step in the progression of pancreatic cancer. However, it is still unclear whether inactivation of Smad4 plays a role in the switching of the TGF  $\beta$  tumor suppressive response into an oncogenic one, thus to promote cell proliferation, survival and EMT, thereby contributing to pancreatic carcinogenesis. Furthermore, how inactivation of Smad4 contributes to the progression of PDAC is still not well understood.

### **Human cancer cell lines**

Human cancer cell lines are the most common tool used to dissect the molecular mechanism of tumorigenesis and metastasis of human cancer, and to test new chemotherapy drug. However, human cancer cell lines derived from tumor specimens harbor a vast unknown number of genetic lesions, which make it difficult to identify the lesions that initiated transformation and to define the role of specific gene alteration contributing to the specific cancer phenotypes.

### **Mouse model of human carcinogenesis and species difference**

Mouse models have recapitulated some genetic and histopathological features of the human cancer, thus providing important insights into the pathogenesis of human cancer and a powerful tool to study human cancer development and progression. However, these murine systems do not faithfully recapitulate the process of tumor development in humans, as human cancers are different in multiple aspects from murine cancer (109). Telomeres of mice are substantially longer than those of humans and telomerase is functionally active in most cells of the mouse. The length of mouse telomeres are 3-10 times greater than those in human cells, telomerase activity can be detected in most cultured rodent cells (110), whereas many human somatic cells lack readily detectable levels of telomerase activity (111). Mice tend to develop cancers from mesenchymal tissues resulting in lymphomas and sarcomas, whereas most human cancers arise in epithelial cell and lead to carcinomas (109). Furthermore, spontaneous immortalization *in vitro* is a common event in mouse cells, while human cells are more resistant to both immortalization and malignant transformation

than rodent cells, which require only two genetic changes for transformation (112). Human cells are more difficult for malignant transformation than rodent cells, and require more numbers of genetic alterations than rodent cells for tumorigenic transformation (112). Studies in recent years have demonstrated that alterations in a defined number of genes are sufficient to transform normal human cells to cancer cells. At least 4-6 major gene alterations including disruption of Rb, p53, telomerase, as well as mitogenic stimulation such as Ras and angiogenesis, are required to transform human cells (113, 114). These results suggest that important differences exist between human and murine cancer biology and that there are fundamental differences in the requirements for transformation of human versus rodent cells (109).

### **Cell culture model of human cancer**

Cell culture model of malignant transformation has provided an important tool to study the cancer development with defined gene alterations in human. Recent advance in experimental cell culture models of immortalization and malignant transformation of human cells suggest that a limited number of gene alterations are sufficient to induce a tumorigenic phenotype in a wide variety of normal cells. These studies demonstrated the serial introduction of a defined number of genetic elements have successfully transformed different human cells, including human fibroblasts, kidney epithelial, human mammary epithelial cells, keratinocytes, airway epithelial cells, endothelial cells, mesothelial, and glial cells and ovarian epithelial cells (115-124). Many *in vitro* transformation systems to date have been successfully used to transform different human cells with different combinations of introduced genes. The most commonly used methods for transformation of normal human cells is comprised of the combination of the human telomerase reverse transcriptase (hTERT), the simian virus 40 (SV40) early region encoding both the large T (LT) and small t (st) antigens, and H-ras<sup>V12</sup> (115-117, 125). These introduced gene alterations involve the maintenance of telomeres by telomerase, the inactivation of the retinoblastoma (Rb) and p53 tumor suppressor by LT, the acquisition of mitogenic stimulation by oncogenic H-ras, and the disruption of the function of protein phosphatase 2A (PP2A) by st antigen.

To date many of these *in vitro* transformation models depended on the expression of DNA tumor viral oncogenes such as the SV40 early region including both the LT and st antigens, and human papillomavirus (HPV) E6E7 genes to induce malignant transformation (115-119, 121, 125). However, these DNA tumor viral oncogenes are not associated with most of human cancers, subsequently, these introduced gene elements in many transformation studies did not correspond to those altered in the respective human cancer. Moreover, the cellular target and function of these viral genes in transformation remain poorly defined, which makes the study of mechanism of

transformation more complicated. SV40 LT binds and inactivates Rb and p53 tumor suppressors, E6 targets the degradation of p53 through polyubiquitination and E7 protein targets the Rb family proteins.

Previous studies reported that expression of the HPV E6E7 proteins which also inactivate Rb and p53 together with hTERT and Ras failed to transform human fibroblasts, indicating that E6E7 combination is not functionally equivalent to the SV40 T-antigen (115, 126). A recent study showed that loss of p53 and p16 function is not equivalent to expression of LT. The deletion of p53 together with abolition of p16/*INK4A* function did not mimic the expression of SV40 LT in transformation of human fibroblast cells elicited by the combination of Ras and st (127). These results suggest that SV40 early region may have additional cellular targets other than p53 and Rb pathways to induce transformation.

Until recently the cellular target and function of SV40 st antigen in transformation are largely unknown. Previous study found that SV40 st disrupts the functions of the serine threonine protein phosphatase 2A (PP2A) by interacting with PP2A (125, 128, 129). Recent findings demonstrated that SV40 st can activate the PI3K pathway as a downstream target for the transformation of human mammary epithelial cells, and that constitutive PI3K signaling can substitute for st in transformation (130). Recent other studies (131) showed that SV40 st antigen also has many functions in transformation to increase cell proliferation, survival, and cytoskeletal changes. These studies showed that st activated the transcription of cyclin D and cyclin A, downregulated the cyclin-dependent kinase (CDK) inhibitor p27, stabilized c-myc, activated AKT and maintained energy homeostasis during glucose deprivation by activating AMPK, inhibiting mTOR, and inducing autophagy as an alternate energy source in cancers (131-133).

Several groups have recently described sequential introduction of a limited number of genetic alterations frequently observed in the corresponding human cancer in the absence of viral oncogenes, to transform different normal human cells into cancer cells (120-124), thus closely recapitulating the development of the corresponding human cancer. One study showed that coexpression of CDK4 and Ras induced malignant transformation of epithelial cells (122). Another report demonstrated that sequential introduction of hTERT, dominant negative p53 (p53DD), cyclin D1, CDK4<sup>R24C</sup>, c-myc, and H-ras into mammary epithelial cells lead to tumorigenic transformation without viral oncogenes (123). Serial introduction of the most frequent gene alterations in human oral squamous cancer including cyclin D1, p53 mutant, EGFR, c-myc resulted in the transformation of oral epithelial cells (120). Expression N-ras<sup>G12V</sup>, CDK4<sup>R24C</sup>, dominant negative p53<sup>R248W</sup> and hTERT in human melanocytes induced invasive human melanoma (124). These results suggest that there are cell type-specific differences in the requirements for tumorigenic transformation. Although cell transformation

requires a combination of similar gene alterations in most cells, specific gene combinations are required for the transformation of specific types of human cells. The different requirements for transformation of different human cell types –fibroblasts, embryonic kidney cells, and mammary epithelial cell suggest their intrinsic biological differences (134).

### **Mouse model of human pancreatic cancer**

Mouse models have provided important insights into the biology of pancreatic cancer and a powerful tool to study the development and progression of pancreatic cancer (27-32, 105). In recent years, different mouse models recapitulating the critical gene lesions involved in the different stages of the human pancreatic cancers have been generated (27-32) (105). The results from mouse models revealed the role of the most common gene lesions in PDAC, p16INK4A/p14ARF, p53, and Smad4 do not play a primary role in the initiation of PDAC, but play a critical role in blocking the progression of K-ras-initiated neoplasm. Mice with a pancreas-specific deletion of either *p16INK4A/p14ARF* or *p53* or *Smad4* do not induce the development of pancreatic cancer, pancreas-specific K-ras activation alone elicited premalignant PanIN lesions that progressed slowly to PDAC, additional inactivation of either *p16INKA/p14ARF*, or *p53* or *Smad4* was found to dramatically accelerate the progression of K-ras initiated PDAC (27-32) (105). The results from mouse models suggest that mutated K-ras plays a critical role in initiation of pancreatic cancer and that additional gene alterations are required for human PDAC progression. However, it is obvious that these murine systems do not accurately recapitulate human carcinogenesis because the cancer biology of the murine and human is clearly different, and human cells are more resistant to transformation than rodent cells. Many researches succeed in mouse model, but failed in human cell models. Therefore, it is more important to study human cancer using human cell experiment model and to compare observations in both of these types of experimental models with human cancer specimens. Therefore, cell culture model remains an important complement to mouse models and is an important tool to study human cancer.

### **Cell culture model of human pancreatic cancer**

Few human pancreatic cell culture model systems for studying human pancreatic tumorigenic transformation have been reported to date. Tao's group first used the cultured human pancreatic ductal epithelial cells as model system to study PDAC progression. Tsao's group (135) demonstrated that expression of mutant K-Ras<sup>G12V</sup> in the E6E7 immortalized human pancreatic ductal epithelial (HPDE) cell line only induced weak tumorigenesis in orthotopic mouse model with poorly differentiated tumor formation in 2 of 5 SCID mice after six months. Expressing K-ras<sup>G12V</sup>



also induced subcutaneous tumors in 4/7 (50%) SCID mice with an average latency of 2 months, and histological analysis revealed poorly differentiated carcinoma with focal glandular and epidermoid differentiation. But, interestingly, mutant K-ras in HPDE cells did not induce *in vitro* phenotypes of malignant transformation as cell growth rate is not increased compared with control cells and anchorage-independent colony formation in soft agar is not induced. The study in our lab showed that mutant K-ras failed to induce transformation of HPDE cell line as these HPDE cells failed to grow tumor in SCID mice (Niu J, unpublished data from our lab). These results indicated that K-ras alone are not sufficient for the development of human PDAC, and that additional genetic alterations are required to induce fully malignant transformation of the E6E7 immortalized HPDE cell line. These results again suggest that K-ras mutation is a critical tumor-initiating event in pancreatic carcinogenesis and that specific accelerating events are required for human PDAC progression as indicated in mouse model. Another recent study (136) described a complete malignant transformation cell model using an hTERT-immortalized normal human pancreatic ductal nestin-expressing progenitor cell line (Human Pancreatic Nestin-Expressing cells, HPNE) (137), sequential introduction of a combination of HPV-16 E6E7, K-ras<sup>G12D</sup>, and the SV40 st antigen into HPNE cell line have successfully transformed HPNE cell line. The transformed cell lines formed colonies in soft agar, grew tumor in nude mice subcutaneously. However, this model did not recapitulate molecular carcinogenesis in the human pancreas because the viral oncogenes used in this study are not associated with human PDAC development. Therefore, a human cell culture model system that recapitulates the various steps of PDAC and the molecular carcinogenesis in pancreas is lacking, which constitutes a major obstacle for rapid progress in PDAC research.

### **Gaps and study goal**

Several experimental animal models have been established recently to determine the functions of mutated K-ras, inactivated p16/p14 and Smad4 in pancreatic tumorigenesis. However, the role of activation of K-ras and Her2, inactivation of p16/p14 and Smad4 in human pancreatic carcinogenesis is still not well understood, especially, how these genetic lesions individually or in combination contribute mechanistically to human pancreatic oncogenesis is still elusive. Moreover, a cell culture transformation model with sequential acquisition of signature genetic alterations in human pancreatic ductal cells, resembling the process of multiple-step human pancreatic carcinogenesis, is still not established. The progress in understanding how these genetic lesions influence pancreatic cancer biology is needed and essential for the development of new methods for diagnosis and treatment of PDAC.

Currently, there are two immortalized human pancreatic cell lines: HPDE/E6E7 and HPNE/hTERT that were established by other investigators (137-139), thus, providing useful tools for studying molecular basis of pancreatic oncogenesis in cell culture. HPDE/E6E7 cell line is a mature, well-differentiated ductal epithelial cell line and immortalized by HPV E6E7 oncogenes; whereas HPNE/hTERT cell line is a progenitor ductal cell line and immortalized by hTERT. We used these two immortalized human pancreatic ductal cell lines as our cell culture system to establish an experimental cell culture model with the most frequent gene alterations in PDAC such as activation of K-ras and Her2, inactivation of p16/p14 and Smad4 to study the role of these signature molecular alterations in the development of PDAC, to determine which genes alterations are required to transform these cell lines, and to further dissect the molecular mechanism of transformation.

## Materials and Methods

### Cell lines and cell culture

Human pancreatic ductal epithelial cell line (HPDE/E6E7) was obtained from Dr. Ming-Sound Tsao (The Campbell Family Institute for Cancer Research, Ontario Cancer Institute at Princess Margaret Hospital, University Health Network, Toronto, ON, Canada M5G 2M9; Departments of Medical Biophysics, and Laboratory of Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada M5A 2N4). HPDE/E6E7 cells were cultured at 37°C and 5% CO<sub>2</sub> in keratinocyte serum-free (KSF) medium, (KSFM, Invitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 50 µg/mL bovine pituitary extract (Invitrogen), 5.0 ng/mL recombinant human EGF (Invitrogen). K-ras expressing HPDE cell lines were cultured in 1:1 mixture of complete keratinocyte serum-free medium (KSFM) and complete Dulbecco's modified Eagle medium (DMEM) (Hyclone Laboratories Inc., Logan, Utah) with 5% fetal bovine serum (FBS).

Human pancreatic duct cell line HPNE/hTERT was obtained from Dr. James W. Freeman at The University of Texas Health Science Center at San Antonio, Texas (140). HPNE cells were cultured in Medium D with mixtures of M3 and DMEM medium containing one volume of medium M3<sup>TM</sup> Base F culture media (InCell Corp., San Antonio, TX, USA), three volumes of glucose-free DMEM, 5% FBS, 5.5mM glucose, 10 ng/ml EGF, and 50 µg/ml gentamycin. After the transfection of altered genes, we cultured HPNE cells in complete DMEM medium with 10% FBS for the remaining experiments. The established HPNE cell lines described here were verified by DNA fingerprinting at the Characterized Cell Line Core of The University of Texas MD Anderson Cancer Center in Houston, Texas.

The 293T cell line and human pancreatic cancer cell lines were grown in DMEM supplemented with 10% FBS at 37°C in 5% CO<sub>2</sub>.

### Antibodies and reagents

The following antibodies and reagents were used in this study: Control β-actin antibody (A5316 Clone, AC-74) was purchased from Sigma-Aldrich Chemical Co (St. Louis, MO); Antibodies to E-cadherin (ab40772), N-cadherin (ab18203), and Vimentin (ab8978) were purchased from Abcam Co (Cambridge, MA); The antibodies to cyclin D1 (RM-9104-S1), Ki-67 (SP6) were purchased from NeoMarkers Co (Fremont, CA); Antibodies to phosphor-MEK1/2 (Ser221, 166F8 #2338), MEK1/2 (L38C12 mouse #4694), Smad2/3 (#3102), phosphor-c-Raf (Ser259, #9421S), phospho-p38 MAPK (Thr180/Tyr182, #9211S), p38 MAPK kinase (#9212), phosphor-JNK (Thr183/Tyr185, G9, mouse mAb, #9255), p14ARF (4C6/4, mouse, #2407), and p15 (rabbit #4822)

were purchased from Cell Signaling Technology Inc (Beverly, MA). Antibodies to c-K-ras (Ab-1, mouse ), Pan-ras Val-12 (Ab-1, OP38), Pan-ras (Ab3, Ras 10), and p21WAF1 (OP64, mouse Ab) were purchased from Calbiochem, EMD Chemicals (Gibbstown, NJ). Anti-human c-erbB2 (Her2) oncoprotein (Rabbit, A0485) was obtained from Dako Corporation (Carpinteria, CA); Antibodies to p16 (C-20, SC-468, rabbit), Pan-cytokeratin (C-11,SC8018), Smad4 (B-8, SC-7966, mouse), Id2(C-20,SC-489, rabbit), cytokeratin-19 (A53-B/A2,SC-6278, mouse), Erk (sc-135900), p-Erk (E-4, SC-7383), cyclin B1 (GNS1, SC-245, mouse), cyclin E (H-145,SC-20684), c-Myc (9E10, SC-40, mouse), MDM2(SMP14, SC-965, mouse), MMP2 (H-76, SC-10736, rabbit), uPA (H-140, SC-14019, rabbit), and Bcl-2 (N-19, SC-492, rabbit) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); Anti-p53 (Ab-6, mouse, OP43) was obtained from Oncogene Research Products (Cambridge, MA); Ras assay reagent (Raf-RBD,# 30867) and anti-human p27 (rabbit #06-445) were obtained from Upstate Inc. (Lake Placid, NY). Ral assay reagent (RalBP1, agarose #14-415), anti-Ral A (#07-2123, rabbit) and Anti-Bmi-1 (Clone F6, #05-637) were purchased from Millipore Inc. (Billerica, Massachusetts); Anti-human p-FAK (pY397, mouse, BD Bioscience) and FAK (c-20, sc-558, rabbit) were gifts from Dr. Zhimin Lu (MD Anderson Cancer Center, University of Texas). Propidine iodide (P-4170) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). D-Luciferin, (#luck-1G) was obtained from GoldBio Technology Inc (St. Louis, MO); Growth factor-reduced BD matrigel<sup>TM</sup> matrix (01730#356231) was purchased from BD Biosciences (Bedford, MA). Seaken LE agarose (# 50004) was obtained from BioWhittaker Molecular Applications (Rockland, Maine).

### **Plasmid construction, transfection, retroviral or lentiviral production, infection and establishment of stable cell lines**

Human mutant K-ras<sup>G12V</sup>containing the entire coding sequence was cloned into retrovirus vector PLHCX (Clontech Laboratories Inc., Mountain View, CA). Retroviral vectors K-ras<sup>G12V</sup>/PLHCX and Her2/PLPCX (a gift of Dr. Dihua Yu, MD Anderson Cancer Center, University of Texas) or the corresponding empty vector for generation of control cells together with amphotropic packaging vector pCL-Ampho were transfected into 293T cells. 48-72h after transfection of retroviral vectors, viral culture supernatants from 293-T cells were collected, filtered through a 0.45-um filter and used to infect the exponentially growing target cells HPDE or HPNE or HPDE/K-ras with 30-50% confluence in the presence of 8 µg/mL polybrene (Sigma-Aldrich Co., St. Louis, MO). Cells were then passaged after 48h -72h infection. Retroviral infections were performed serially with drug selection used to purify polyclonal-infected populations after each infection. Drug selection of infected HPDE or HPNE cells was performed with 300µg/mL hygromycin B (Roche Diagnostics,

Indianapolis, IN), 500ng/mL puromycin (Sigma-Aldrich Co., St. Louis, MO) to purify polyclonal-infected populations. The antibiotics-resistant clones of HPDE or HPNE cells from each infection were pooled to establish HPDE/K-ras, HPDE/K-ras/Her2 and HPNE/K-ras stable cell lines and their respective vector control stable cell lines.

Human lentiviral shRNA against human p16 or p16/p14 were designed. The sequence of p16shRNAs is: 288TAGAGGAGGTGCGGGCGCTGC, 329AACGCACCGAATAGTTACGGT, 338AATAGTTACGGTCGGAGGCCG. The sequence of p16/p14shRNA is: 907AACCATGCCCCGCATAGATGCC, 1250AAGCGCACATTCATGTGGGCA, 957AAAGAACCAGAGAGGCTCTGA. The shRNA oligos were purified and reannealed, then cloned into lentiviral vector pLKO.1-TRC cloning vector (Addgene Inc, Cambridge, MA). The puromycin cassette in p16/p14shRNA/pLKO.1 was replaced with a zeocin resistance gene. Lentiviruses were generated by transfecting lentiviral expressing vectors p16shRNA/pLKO.1, or p16/p14shRNA/pLKO.1 or empty vector control together with packaging vector psPAX2 and envelope plasmid pMD2.G (gifts from Dr. Dihua Yu, MD Anderson Cancer Center, University of Texas) into 293T cells. The following lentivirus production and infection were performed as described above as those of retrovirus: 48-72h after transfection of lentiviral vectors, the lentiviral supernatant were collected, filtered and used to infect target cells HPDE/K-ras and HPDE/K-ras/Her2 or HPNE/K-ras cells with 50 % confluence in the presence of 8 µg/mL polybrene. After 48-72h infection, drug selection of infected HPDE or HPNE cells was performed with 500ng/mL puromycin and 400 µg/mL zeocin (Invitrogen Life Technologies, Inc., Carlsbad, CA) to purify polyclonal-infected populations of p16shRNA or p16/p14shRNA expressing cells. Antibiotics-resistant clones were pooled to establish p16shRNA or p16/p14shRNA expressing and their respective vector control stable cell lines. The effectiveness of shRNA knockdown was confirmed by Western blot analysis with specific antibodies anti-p16 or anti-p14.

The sequence for Smad4shRNA is 318GGTGGAGAGAGTGAAACAT, 1389GGTGTGCAGTTGGAATGTA. Smad4shRNA oligos were purified, reannealed, and then cloned into lentiviral vector FG12 which contained a green fluorescent protein (GFP) gene. The luciferase sequence then was cloned into FG12 and Smad4shRNA/FG12 vector respectively for *in vivo* bioluminescence imaging of xenografted tumors. Recombinant lentivirus was generated by transient transfection of the lentiviral vectors together with packaging plasmids pMLg/pRRE, pRSV.rev and pHCMV-G into 293T cells. Virus-containing supernatants were collected at 48-72 hours after transfection to infect target cells according to the described as above. For each infection, parallel cultures were infected with control lentivirus containing only empty vector as controls. The infected cells were purified by GFP fluorescence-activated cell sorting (FACS) sorting. The

effectiveness of shRNA knockdown was confirmed by Western blotting analysis with specific antibody anti-Smad4.

For in vivo bioluminescence imaging of xenografted tumors, recombinant lentivirus was generated by transient transfection of the lentiviral vectors FG12 which contained a GFP gene and luciferase gene together with packaging plasmids pMLg/pRRE, pRSV.rev and pHCMV-G into 293T cells. The following lentiviral infection of target cells was performed according to the described method above. The infected cells were purified by GFP FACS sorting.

Retroviral and lentiviral were introduced serially. Drug selection was used to purify cell populations between infection, cells were selected with hygromycin 300 $\mu$ g/ml, puromycin 500ng/ml or zeocin 400 $\mu$ g/ml respectively. Empty retroviral or lentiviral vector were used to establish control cell lines.

### **Western blot analysis**

HPDE or HPNE cells were harvested from 10 cm dish. Cells were lysed in radioimmunoprecipitation assay protein lysis buffer (RIPA) (50mM Tris HCl at pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM EDTA, 1mM sodium orthovanadate, 1Mm NaF, 1 $\times$  protease inhibitor mixture). Proteins then were quantified spectrophotometrically by the Bio Rad protein assay (Bio-Rad, Hercules, CA). 50  $\mu$ g of protein extracts were run on 8-15% SDS-PAGE gel (Acrylamide: bis 30%, 29:1, Gendepot, Barker, TX), then transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA), Membranes were blocked in 5% nonfat milk (Bio-Rad) or 5% BSA in TBS-T (50mM Tris, 150mM NaCl at pH 7.6, 0.05% Tween 20) for 2 h at room temperature. Membranes were then probed with primary antibody diluted in 5% milk or 5% bovine serum albumin (BSA) in TBS-T overnight at 4°C, washed with TBS-T, incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Jackson Immo research Laboratories Inc.; 1:3000 ) in 5% non-fat milk in TBS-T for 2 h at room temperature, and washed in TBS-T. The signal was visualized with Lumi-light western blotting substrate (Roche Applied science, Indianapolis, IN) by exposing the membrane to radiographic film (Hyblot CL<sup>TM</sup>, Denville Scientific Inc., Metuchen, NJ).

### **Ras activity assay**

The activity of Ras protein was assayed using the Ras assay reagent (Upstate, Lake Placid, NY) according to the manufacturer's instruction. HPDE or HPNE cells were harvested at 80% confluence and lysed with Mg-containing lysis buffer (MLB). The cell lysates were diluted at about 1 $\mu$ g/ $\mu$ l total cell protein and precleared with glutathione agarose. Active Ras-GTP protein in the cell lysate was

precipitated by 10 $\mu$ g Raf-1 RBD agarose at 4°C for 1 hour. The agarose beads were washed 3 times with MLB and resuspended in 2  $\times$  sampling buffer and boiled for 5 minutes. The supernatant containing active Ras protein was detected with a Pan-ras antibody (Ab3, Ras10, Calbiochem, EMD Chemicals, Gibbstown, NJ) by Western blot analysis as described above. The same antibody was used to determine total Ras amount in cell lysate.

### **MTT assay**

1,000 cells per well were plated on 96 well plate in triplicate. At day1, day 3, day 5, day 7, the MTT assay was performed by adding 10  $\mu$ l of a 5 mg/ml solution of MTT (Sigma Chemical Co., St. Louis, MO) to wells and incubating for 4 hours at 37°C. The supernatant was removed and the blue MTT formazan precipitates was then dissolved in 100  $\mu$ l/well of dimethyl sulfoxide. The plates were placed on a shaker for 10 min at room temperature in the dark and the absorbance was measured on a plate reader at 570nm. Data were shown as the mean value or the mean value/day 1 mean value  $\pm$  standard error of the mean of three independent experiments. Growth curves were generated by microsoft Excel software.

### **Cell growth curve assay**

HPNE cells were plated in 6-well plates in triplicate at a concentration of 20,000 cells/well in growth medium. Numbers of cells were counted at day 1, 3, 5, and 7. Results were expressed as the means of cell number  $\pm$  standard error of the mean from three independent experiments.

### **Flow cytometry cell cycle analysis**

HPDE or HPNE cells were cultured in complete growth medium overnight, after starvation for 24h in serum-free medium, then stimulated with complete growth medium with serum for 12 h or 24h. Cells were harvested from the plate with 0.5% trypsin/EDTA.  $2 \times 10^6$  cells were fixed overnight in phosphate buffered saline (PBS) with 75% ethanol, then cells were washed 2 times with PBS, treated with 100 $\mu$ g/ml Dnase-free Rnase (Roche Applied Science, Indianapolis, IN) and stained with 50  $\mu$ g/ml of propidium iodide (Sigma Chemical Co., St. Louis, MO) for 30 minutes at room temperature. Cell cycle profiles of HPNE or HPDE cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA). Cell cycle status was then analyzed with FlowJo software. Each experiment was performed independently three times with similar results each time. Results were expressed as the means of percentage of cells in each phase  $\pm$  standard error of three independent experiments.

### **Senescence-associated $\beta$ -galactosidase activity (SA- $\beta$ -gal)**

HPNE cells were fixed with 2% formaldehyde/0.2% glutaraldehyde in phosphate buffered saline (PBS) for 5 min, washed with PBS, stained with X-gal staining solution (1 mg/ml X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl<sub>2</sub> at pH 6.0) at 37°C for 12-16 h. Cells were examined and photographed in 15× magnification under the Olympus IMT-2 phase contrast Microscope. A representative field of each experiment was shown. Results were showed as percentage of SA- $\beta$ -gal staining positive cells from three independent experiments.

### **Reverse transcription-PCR (RT-PCR)**

Total RNA was extracted from monolayer cultures with 80% confluent HPDE or HPNE cells using TRIzol reagent according to the protocol of the manufacturer (Invitrogen Life Technology, Carlsbad, CA). The reverse transcription (RT) reaction with oligo (dT) primers was performed according to the protocol of the manufacturer. Briefly, cDNA was synthesized by random priming from 1  $\mu$ g of total RNA using a Superscript first-strand synthesis system (Invitrogen Life Technology, Carlsbad, CA), 1 $\mu$ g total RNA was heated at 65°C for 3 minutes, and then put on ice for 3 minutes. Following that, RNA were mixed with RNase inhibitor, AMV reverse-transcriptase, 5× First strand buffer, 5mM dNTP, oligo dT, 0.1MDTT and dH<sub>2</sub>O in total 20 $\mu$ l reaction volume, and were incubated at 42°C for 1 hour, then 95°C for 3 minutes, and were put on ice.

1 $\mu$ l of the generated cDNA was used for PCR in a total 20 $\mu$ l of reaction volume, the primer concentration is 5pmol, 2 × PCR master mixes (Promega Corporation, Madison, WI) was used. The PCR condition was as follows: 95°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min. Analysis of PCR products was performed by electrophoresis on a 2% agarose gel.

The following primers were used in the PCR. The primer sequences were: human BMP7 forward primer 5'aatccgcggcacaacctggg3', reverse primer 5'ttgagcgggtctggctgcg3'; K-ras forward primer: 5'-agagaggcctgctgaaaatg-3', reverse primer: 5'-agtctcatggagcaggaaa-3'. Human p14 primer 1 forward primer: 5'-tcgtgctgatgctactgagg-3', reverse primer: 5'-ttcttcaatcggggatgctc 3'; p14 primer 2 forward primers: 5'-gaacatgggtgcgcaggttct-3', reverse primer: 5'-cctcagccagggtccacggg-3'. Human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as the internal control, forward primer: 5'-acggatttggtcgtattggg-3', reverse primer: 5'-tgattttgagggatctcgc-3'.

### **Migration assay and matrigel invasion assay**



The migration of cells was analyzed through a transwell chamber assay. Migration assay was performed in 24-well plate in triplicate by using Falcon cell culture inserts, which have a PET membrane with  $1 \times 10^5$  8.0- $\mu\text{m}$  pores per  $\text{cm}^2$  (BD Biosciences Labware, Bedford, MA). 700  $\mu\text{l}$  of complete growth medium was added to the lower well of the plate. The cells ( $5 \times 10^4$  HPDE cells/0.4 ml) were seeded to the insert in 1:1 mixture of serum free KSFM and DMEM medium. Cells were incubated at 37 °C and 5%  $\text{CO}_2$  for 24 h.

Invasion assay was performed in 24-well plate in triplicate by using a Matrigel-coated invasion chamber (BD Biosciences, Bedford, MA) with an 8.0- $\mu\text{m}$  pore size positron emission tomography (PET) membrane. Each membrane had a thin layer of matrigel basement membrane matrix, which serves as a reconstituted basement membrane *in vitro*. The inserts were rehydrated by adding 0.5 ml of warm culture medium at 37 °C to the inserts for 2 h. The  $5 \times 10^4$  HPDE cells were seeded in 0.5 ml of serum free medium to the invasion chambers, and 750  $\mu\text{l}$  of complete growth medium was added to the lower well of the invasion plate. Cells were incubated at 37 °C and 5%  $\text{CO}_2$  for 28 h.

The following procedures for staining, counting, and photographing were the same for both the migration and invasion assay. After incubation, the cells that did not migrate or invade were removed from the upper side of the inserts using cotton swabs, and the cells that migrated to or invaded the lower side of the inserts were fixed in 10% formalin for 10 minutes and stained with 1% crystal violet. Migrating or invading cells were observed under the microscope with 15 $\times$  magnification and the numbers of cells /per field were quantified using IMT-2 Olympus phase contrast microscope (Olympus Optical Co. Japan). The representative fields of each experiment were photographed with 15 $\times$  magnification and shown. Results were expressed as the means of number of cells migrated or invaded/per field/means of control cells  $\pm$  the standard error of the mean from three independent experiments.

#### **Anchorage-independent growth assay in soft agar**

To assess anchorage-independent growth in soft agar, colony formation assay in soft agar was performed. HPDE or HPNE cells were trypsinized and counted.  $5 \times 10^4$  cells were suspended in the complete growth media containing 0.3% agarose (Seakem LE agarose, Cambrex Bio Science, Rockland, ME) and seeded into 12 -well plates on top layer over bottom layer of solidified 0.6% low melting point agarose/growth media. Then cultures were fed with 1 ml of complete growth medium. The complete growth medium was replaced weekly, and cells were allowed to grow colonies for 3 weeks at 37°C under 5%  $\text{CO}_2$ . Only colonies  $\geq 0.2$  mm in diameter were counted under the IMT-2 Olympus phase contrast microscope (Olympus Optical Co. Japan). The representative fields of each experiment were photographed and shown in 15 $\times$  magnification. At least three

independent assays were performed in triplicate. Data were shown as the mean value of the number of colonies/per field  $\pm$  the standard error of the mean from three independent experiments.

### **Orthotopic tumorigenicity assay in NOD/SCID mice**

The animal protocol used for animal studies was approved by the M. D. Anderson Institutional Animal Care and Use Committee. 4 to 6-week-old female immunocompromised non-obese diabetic/severe combined immunodeficient (NOD/SCID-lack T and B-cell lymphocyte) mice (National Cancer Institute at Frederick, Frederick, MD) were purchased from National Cancer Institute at Frederick. Immunodeficient NOD/SCID mice were maintained in pathogen-free conditions, and were used at 8 weeks old at the time of orthotopic implantation. The various HPDE or HPNE cell lines were harvested from subconfluent cultures with 0.5% trypsin/ EDTA, and resuspended in the complete growth medium. The trypan blue exclusion assay was used to ensure >90% cell viability. A total of  $3 \times 10^6$  viable HPDE cells or  $2 \times 10^6$  viable HPNE cells were resuspended in 50  $\mu$ l of complete growth medium with 50% growth factor reduced matrigel (BD Biosciences, Bedford, MA) were injected into the pancreas of 8-week-old mice. At least twice the amount of cells needed for the experiments was prepared. A total of five mice were used for each cell line. Mice were anesthetized with inhalant isoflurane (Baxter Healthcare Corporation, Deerfield, IL). Once anesthetized, the left abdominal region of the mouse was shaved using electric clippers. The incision region was sterilized with 70% ethanol solution. A small left abdominal flank incision is made, and the pancreas is exposed. Then cells suspensions were carefully injected into the pancreatic parenchyma of each mouse. A cotton swab is held for 1 min over the site of injection to prevent cell leakage. A successful intrapancreatic injection of cells was confirmed by the appearance of a fluid bleb without leakage of cells into the peritoneal cavity. One layer of the abdominal wound was closed with wound clips. After cell implantation, mice were monitored daily for signs of illness and surgical wounds infection for one week. Mice were also monitored for the signs of animal sickness such as ruffled fur, lethargy and tachypnea.

The condition of mice was monitored three times weekly over a period of 6 months. For *in vivo* imaging, an enhanced green fluorescent protein (GFP)/firefly luciferase double-expressing cassette FG12 was introduced into HPNE or HPDE cells by lentiviral infection. The *in vivo* tumor growth was monitored for Bioluminescence measurement in real time using the Xenogen *In Vivo* Imaging System (IVIS) (Xenogen, Alameda, CA).

When tumor burden of mouse increased, moribund mice were sacrificed immediately upon any noticed decrease in activity according to the requirements of our institutional guidelines. The tumor was removed and then processed for isolation of tumor cell lines and routine histological analysis.

The remainder of the tumor was frozen in liquid nitrogen. The pancreas, liver, and spleen were removed and a small segment of organs was fixed in 10% buffered formalin and processed for paraffin embedding and histological analysis. The remainder of the organs was stored in liquid nitrogen.

### **In vivo bioluminescence imaging of xenografted tumors**

For *in vivo* imaging, an enhanced green fluorescent protein (GFP)/firefly luciferase double-expressing cassette FG12 was introduced into HPDE or HPNE cells by lentiviral infection. The *in vivo* tumor growth was monitored in real time using the Xenogen *In vivo* Imaging System (IVIS) (Xenogen, Alameda, CA). D-Luciferin 1g (#luck-1G, GoldBio Technology Inc. St. Louis, MO) was dissolved in 33ml sterile PBS and stored at -20°C. 100µl D-luciferin (100 mg/kg of body weight) was administered by intraperitoneal injection (i.p.) under anesthesia with inspiration of anesthetic isoflurane (Baxter Healthcare Corporation, Deerfield, IL). 5 to 10 minutes after injection, *in vivo* bioluminescent images were acquired.

### **Histopathological analysis of tumors –HE staining and immunohistochemistry analysis**

Mouse pancreas, liver, spleen, and tumors were fixed in 10% formalin, and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) according to standard procedures. The HE slides from mouse xenografts were reviewed by a pathologist (Dr. Huamin Wang, MD Anderson Cancer Center, University of Texas), who classified the tumor type and evaluated the tumor necrosis and metastasis to other organs. A representative field of each histological type was photographed and shown using an Olympus BX-51TF microscope.

For immunohistochemistry analysis of Ki-67 and cytokeratin -19, tumors were fixed and embedded as previously described. Briefly, the sections were de-waxed and rehydrated. After being retrieved antigen via unmasking solution (Antigen unmasking solutions, H3300- Vector Laboratories Inc., Burlingame, CA) in a steamer, slides were blocked with biotin blocking solution (Avidin /Biotin blocking kit, SP2001, Vector Laboratories Inc., Burlingame, CA) and 1% bovine serum albumin (BSA) 30 min, respectively; and then incubated with an anti-Ki-67 and cytokeratin -19 antibodies at 4°C overnight. Next day, all these slides were processed by ABC method (Vector Laboratories) as described in the manufacturer's instruction. Slides were subsequently incubated with biotinylated secondary antibody (Vectastain ABC-peroxidase kit, Vector Laboratories Inc., Burlingame, CA) and ABC reagent at room temperature for 30 min, respectively. 3,3'-Diaminobenzidine (DAB, #D4168, Sigma) was then incubated as the final chromogen, the staining time were 2 to 5 minutes to see brown color under the microscope. Finally slides were

counterstained with hematoxylin, and then serially dehydrated, and sealed with permount medium after air drying. Negative controls for each tissue section were prepared by substituting the primary antiserum with the isotype-matched non-immune mouse IgG. All samples were processed under the same conditions.

### **Isolation of tumor cells**

After the mice were sacrificed, the pancreatic tumors from orthotopic injections with HPDE/K-ras/Her2/p16p14sh/Smad4shRNA cell line or with HPNE/K-ras/p16p14shRNA cell line were collected and transferred into serum free medium. The tumors were sliced with sterile scalpels, reduced into the smallest segments, and washed twice with PBS. Then tumor segments were plated on culture dishes, growth medium was added to the dish to allow cells to grow at 37°C under 5% CO<sub>2</sub>. We also used another method to isolate tumor cells. Tumor was cut into small fragments, removed into the cell strainer, and mashed through the cell strainer (100µm, BD Biosciences Labware, Bedford, MA) into the petri dish using the plunger end of the syringe. After the cell strainer was rinsed with DMEM, the tumor cell suspension was centrifuged and washed with DMEM. Afterwards, tumor cells were cultured in the complete growth medium on plastic dishes. Hygromycin or puromycin, or zeocin treatment was used to select the antibiotics-resistant tumor cells.

$2 \times 10^6$  cells of tumor cell line of HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA T isolated from orthotopic mouse tumor, were orthotopically reinjected into pancreas of NOD/SCID mice using the same conditions as described above. *In Vivo* Bioluminescence imaging was performed at 40 days.

### **Ral activity assay**

The Ral activity was examined by precipitating the Ral-GTP protein using the Ral effector protein RalBP1. The assay was performed according to the manufacturer's instruction (Millipore, Temecula, CA). Briefly, HPNE cells were collected at 70% to 80% confluence, stored at -80°C, and lysed with 1× Ral activation assay buffer (RAB). The cell lysates were diluted at 1µg/µl total cell protein in 1 ml of volume and precleared with glutathione agarose. Ral-GTP was pull down by adding 10µg RalBP1 agarose for 1 ml of cell lysate at 4°C for 1 h. The agarose beads were washed three times with RAB and resuspended in 2× sampling buffer and boiled for 5 minutes. The active Ral protein was detected with an anti-RalA antibody (Millipore) by Western blot analysis as described above. The same antibody was used to determine total amounts of RalA in the cell lysates.

### **Short tandem repeat DNA fingerprinting**

Short tandem repeat (STR) DNA fingerprinting was performed in the Characterized Cell Line Core at The University of Texas MD Anderson Cancer Center. HPNE cell lines were validated by STR DNA fingerprinting using the AmpF $\ell$ STR Identifier kit according to manufacturer's instructions (Applied Biosystems, Cat 4322288). The STR profiles were compared to known ATCC fingerprints (ATCC.org), to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (<http://bioinformatics.istge.it/clima/>) (Nucleic Acids Research 37:D925-D932 PMID: PMC2686526), and to the MD Anderson Cancer Center fingerprint database. We also compared the STR profiles of HPNE/K-ras/p16shRNA cell line with the original HPNE cell line from the source.

### **Microarray analysis**

Total RNA was extracted from the exponentially growing cells of 50-60% confluent HPDE or HPNE cell lines. The culture medium was removed from the 10-cm plastic dishes, and washed two times with PBS. 2ml of TRIzol reagent was added to the dishes. Cell lysates were collected, then immediately frozen in -80°C. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA).

DNA microarray experiments and statistical analysis were performed by the Cancer Genomics Core Laboratory at The University of Texas MD Anderson Cancer Center with the Whole Human Genome 2 color gene expression Oligo Microarray from Agilent Technologies (Santa Clara, CA) according to the manufacturer's instructions. Agilent's two-color microarray-based gene expression analysis uses either cyanine 3-CTP or cyanine 5-CTP labeled two RNA samples targets to measure gene expression in experimental and control samples. Microarray experiments were carried out using the "Whole Human Genome Oligo Microarray Kit". This microarray contains 44,000 distinct biological 60-mer oligonucleotide probes representing the whole genome (44K Agilent Human Genome, Agilent Technologies, Santa Clara, CA). It includes one probe per gene for reference sequence and gene bank known genes and three probes for each of approximately 1100 known cancer genes of importance. The manufacturer's protocol was followed. Detailed protocol can be found at Agilent website: [www.Agilent.com](http://www.Agilent.com). Briefly, 500 ng of total RNA from each sample was used and labeled with either Cy3- or Cy5-CTP respectively. After 17h hybridization at 65°C, the arrays were washed and scanned with Agilent's dual-laser based scanner. Then, the feature extraction software GE2-v5\_95 (Agilent Technologies) was used to link a feature to a design file and determine the relative fluorescence intensity between the two samples. Captured microarray images

were transformed to data using default settings; background subtracted and normalized using the standard procedures of Agilent feature extraction software.

The Microarray data sorting, filtering and statistical analysis and generation of common differentially expressed gene lists was performed in the Cancer Genomics Core Laboratory at The University of Texas MD Anderson Cancer Center. Briefly, Dave's protocol for just the "significant" genes was used to perform basic analysis on gene expression tables generated by the Agilent scanner. Excel was used to sort the gene lists. The method uses the error-model (p-value) built into the output of Agilent arrays. Agilent has redundant spots on the arrays, and the error-model correlates the signals seen at the various replicate positions to predict the error of the other (non-repeated) probes. This gives an estimate of the p-values for any probe on the array. Gene lists were generated using p-value information from the internal replicates within the microarray. The data with insignificant p-value larger than 0.0001 were considered as insignificant and was deleted. The significant differential expression was defined as p-values <0.0001 as measured with the random variance t-test.

The Agilent log ratio (ALR) was used to obtain the relative expression level of the two samples hybridized on each array. The Log ratio values were calculated from the processed signals by Agilent's Feature Extraction software. ALRs were converted to log base 2 and then scale normalized. Microarray data are displayed on a log ratio scale where each unit represents a 2-fold change in expression level and the absolute fold change.

The generation of a filtered gene list for Agilent two-color data was performed as follows: (i) Agilent flagging rules were used, setting all absent and marginal features to missing. (ii) To obtain a reliable common gene set across two-color data, features with fewer than 50% present genes across all microarrays were filtered. (iii) Features with fewer than five present calls from each sample group across sites for two-color were filtered. The details for generating the Agilent two-color output gene lists can be found in the Agilent G2567AA FE8.5 Software Reference Guide (<http://www.chem.agilent.com/scripts/LiteraturePDF.asp?iWHID=41954>). Data used for the generation of the common differentially expressed gene lists were from the genes that passed data filtering criteria of Agilent platform. Significant differentially expressed genes were assessed with a one sample *t*-test of log<sub>2</sub> (B/A) ratio of five replicates that differ from 0. For two-color data, the dye swap result was averaged before conducting the *t*-test.

### **SYBR green quantitative real-time PCR**

PCR primers were designed using the primer 3 program software and were synthesized by Sigma. The primer sequences were: Human Bmi-1: forward primer 5'tcatccttctgctgatgctg3', reverse primer 5'gcatacacgtcattgctgct 3'; Human CDH2: forward primer 5'acagtggccacctacaaagg3, reverse

primer 5' tgatccctcaggaactgtcc3'; Human GRB10: forward primer 5'atgaatgcatccctggagag3', reverse primer 5'actgtgtgtcttctctga3'; Human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as the internal control, forward primer: 5'acggatttggtcgtattggg3', reverse primer: 5'tgattttggaggatctcgc3'.

The SYBR green real-time PCR was performed using Stratagene Mx4000<sup>TM</sup> multiplex quantitative PCR system (La Jolla, CA) with the Brilliant SYBR green QPCR Master mix (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Real-time PCR was performed in a total volume of 25 µl using 1µl of the first-strand cDNA synthesis mixture as a template, the primer concentration is 5pmol. The PCR conditions were as follows: 94 °C for 5 min, then 35 cycles at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1min, and finally extension at 72 °C for 7 min. GAPDH was used as the internal control. The values of GAPDH were used to normalize the expression data. The relative quantification of gene expression was calculated using the comparative CT method ( $\Delta\Delta CT$  method).

### **IPA signaling pathway analysis**

The changes of signaling pathways and biological functions were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)) (Ingenuity System Inc, Redwood City, CA). We performed two types of analysis –core analysis and comparison analysis to identify canonical signaling pathways and biological functions and their related group comparisons. In core analyses, the genes were categorized based on biological functions and canonical signaling pathways. IPA only differentiated whether a gene was significantly altered and did not differentiate the directional change of expression. The significance of the biological functions and the canonical pathways were tested by the Fisher Exact test based on the number of genes analyzed that mapped to a biological function and pathway in the IPA knowledge base. Comparison analysis was used to compare the difference in function and pathways side-by-side between two groups. An Excel spreadsheet file dataset containing gene identifiers ID, gene accession numbers, and their corresponding expression value's log ratio was uploaded into the IPA to create core analyses. The following general settings and filters for analysis were used: the Ingenuity knowledge base (genes and endogenous chemicals) were used as a reference set, and indirect and direct relationships were included, All data sources, human species, and all tissues and cell lines were used for the analysis under the stringent filter; log ratios for both up- and down-regulated genes were used as expression value parameters, and duplicates were resolved by using the averages of experimental log ratios. Next, the IPA software identified associated genes that were eligible for generating biological functions and pathways. p-value was calculated by Fisher's exact test to determine the probability

that the association between the genes in the dataset and the biological function or canonical pathway is due to chance alone. The results were shown by the  $-\log$  (p-value) of each pathway or biological function mapping to a biological function and pathway in the IPA knowledge base.

### **Statistical analysis**

Statistical analyses were performed with SPSS or Excel software. The significance of the data was determined by using the Student's t test and Fisher's exact test.  $p < 0.05$  was considered significant. For error bars in all experiments, standard deviation (s.d.) was calculated from three independent experiments and values represent mean  $\pm$  s.d.



## Results

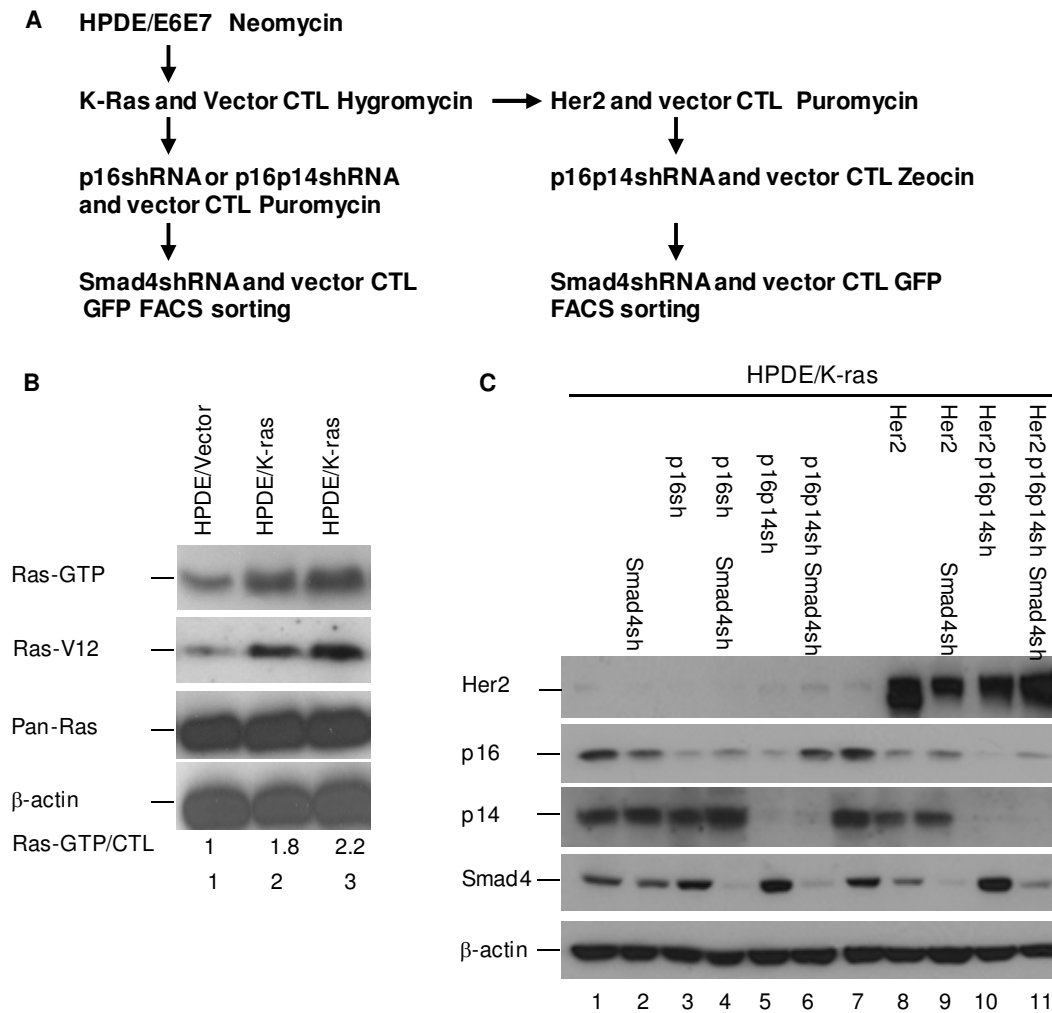
### Part I Transformation of E6E7 immortalized human pancreatic ductal epithelial (HPDE) cell line

#### Stable expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA in E6E7 immortalized human pancreatic ductal epithelial (HPDE) cell line

Human pancreatic ductal epithelial cell (HPDE) (138, 139) is a human papilloma virus (HPV)-16 E6/E7 gene immortalized primary human pancreatic ductal epithelial cell line originally derived from a 63-year-old female normal pancreas portion. This cell line shows many features of normal pancreatic ductal epithelial cells –near-diploid, expression of carbonic anhydrase II mRNA, expression of epithelial marker-cytokeratin 7, 8, 18, 19 and mucin 1; activation of telomerase; expression of wild type K-ras, p16 and c-myc; induction of growth inhibition by TGF  $\beta$ ; inactivation of p53 and Rb by E6 and E7 respectively, lack of functional p53 pathway as  $\gamma$ -irradiation can not upregulate p53 and p21 expression (138, 139).

Previous study showed that expression of mutant K-ras in HPDE cells induced only weak tumorigenesis, so the question is whether additional genetic alterations can fully transform the HPDE/K-ras cells into highly malignant cancer cells through sequential introduction of gene alterations. Therefore, we hypothesized that activation of K-ras, Her2, inactivation of p16/p14 cooperated with inactivated Smad4 contribute to the transformation of immortalized human pancreatic ductal epithelial cells. The goal of this study was to establish an experimental cell culture model with activation of K-ras and Her2, inactivation of p16/p14 and Smad4 to study the role of these signature molecular lesions in the development of PDAC, to determine which genetic alterations are required for transformation using the immortalized human pancreatic ductal epithelial cell line, and to further dissect the molecular mechanism of transformation.

To test our hypothesis and study the mechanisms of tumorigenic transformation in human pancreatic ductal epithelial cells, we sequentially introduced the most common gene alterations identified in PDAC –activation of K-ras and Her2, inactivation of p16 or p16/p14 and Smad4 by shRNA knockdown into HPDE cells via retroviral or lentiviral transduction using different drug selection or GFP FACS sorting to purify the antibiotics-resistant or GFP-positive cell population (Fig.4A). Mutant K-ras and Her2 were transduced via retroviral transduction, expression K-ras<sup>G12V</sup> in HPDE cell lines induced increased Ras activity 1.8-2.2 folds compared with vector control cells



**Figure 4. Stable expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA in E6E7 immortalized human pancreatic ductal epithelial (HPDE) cells.** (A) The strategy for introducing gene alterations into HPDE cell lines. The diagram showed sequential introduction of mutant K-ras, p16shRNA or p16/p14shRNA and Smad4shRNA into HPDE cells, or introduction of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA into HPDE cells using different antibiotics selections or GFP FACS sorting for purifying the cell population. (B) Stable expression of mutant K-Ras<sup>G12V</sup> in the HPDE cell line. Stable K-ras<sup>G12V</sup> expression in the HPDE cell line was identified by Ras activity assay, Western blot analysis of the expression of RasV12 and total Ras. (C) Stable expression p16shRNA, p16/p14shRNA, Her2, and Smad4shRNA in HPDE/K-ras cell lines. The stable cell lines were identified by Western blot analysis of the expression of Her2, p16, p14, and Smad4.

using RasGTP-Raf affinity precipitation in Ras activity assay (Fig. 4B). Total RasV12 level was also increased in K-ras<sup>G12V</sup> expressing cell line compared with control cells in Western blot analysis using

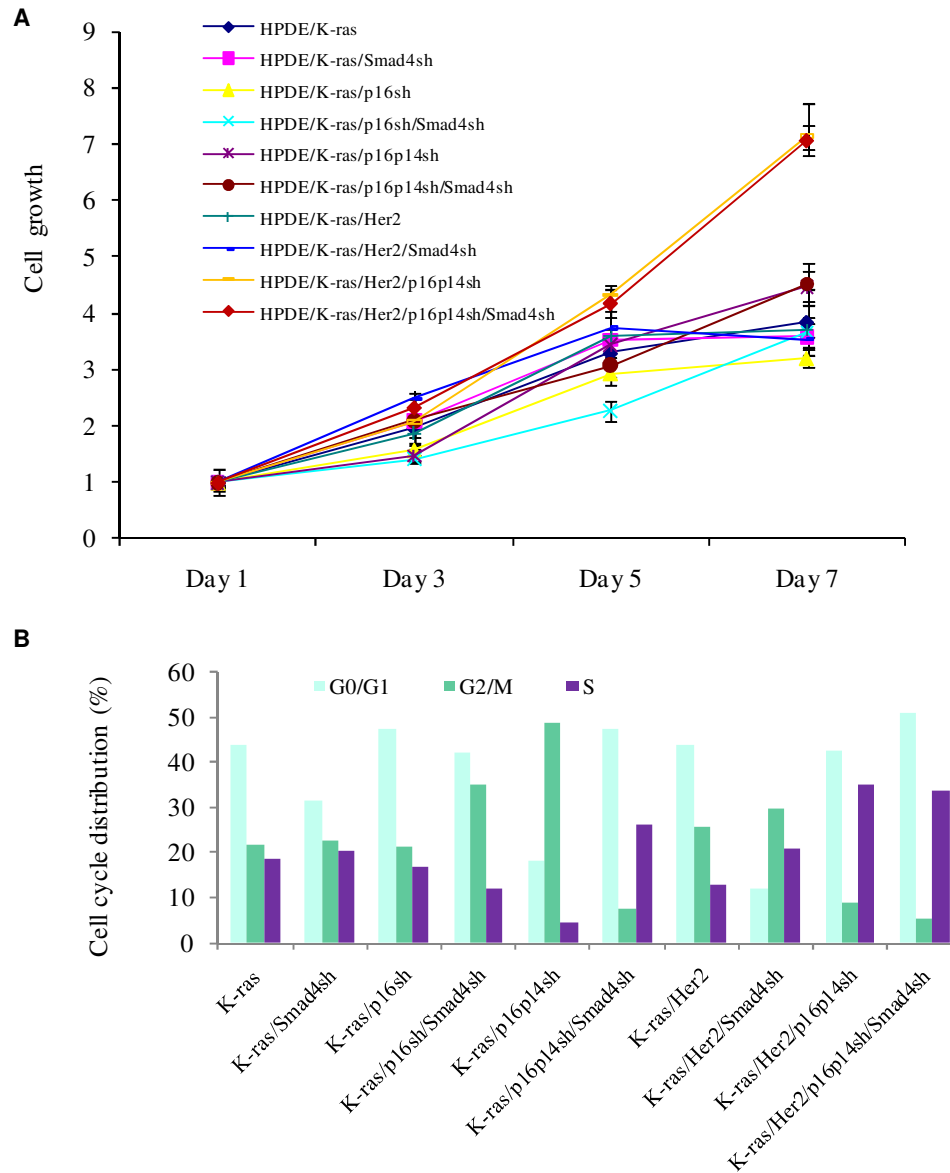
special antibody which can recognize RasV12. Stable expression of Her2, p16/p14shRNA and Smad4shRNA in HPDE cells was detected by Western blot analysis; these transduced cells demonstrated overexpression of Her2, decreased expression of p16, p14, and Smad4 as confirmed by Western blot analysis (Fig. 4C). We thus successfully established the stable cell lines with expression of mutant K-ras, Her2, and knockdown of p16INK4A/p14ARF and Smad4 tumor suppressor genes in immortalized human pancreatic ductal epithelial cells.

### **Cell growth properties of HPDE cell lines with stable expression of mutant K-ras, Her2, p16/p14shRNA, and Smad4shRNA**

The HPDE cells were grown in keratinocyte serum free medium (KSFM) with 5.0ng/mL recombinant EGF and 50 µg/mL bovine pituitary extract. After transfection of those gene alterations, we cultured HPDE cells in 5% serum-containing medium with 1:1 mixture of complete DMEM and KSFM growth medium. These cells appeared well adapted in new medium. Interestingly, we found that those cells displayed enhanced cell proliferation rate compared with cells grown in complete KSFM medium. We therefore grew K-ras expressing HPDE cells in 5% serum-containing 1:1 mixture of complete DMEM and KSFM growth medium for the remaining experiments.

Unchecked cell growth is a hallmark of cancer. Therefore, we first examined the growth characteristics of HPDE cells with expression of K-ras, Her2, knockdown of p16 or p16/p14 and Smad4 by cell morphology and cell proliferation to determine whether HPDEs acquire increased cell proliferation ability. These HPDE cells maintained epithelial-like appearance morphology on tissue culture plates, but did not show significant morphologic changes compared with control cells. Interestingly, HPDE/K-ras/p16shRNA cell line exhibited fibroblast-like morphology in culture dish. The morphology of the cell lines HPDE/K-ras/Her2/p16p14shRNA and HPDE/K-ras/Her2/p16p14shRNA/ Smad4shRNA changed to slightly small and round shapes, whereas other control cell lines retained the original morphology of the parental cells.

Among the HPDE cell lines, HPDE/K-ras/Her2/p16p14shRNA and HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA cell line demonstrated significantly elevated cell growth rate in MTT assay and a higher percentage of cells in S phase in cell cycle analysis compared with other control cell lines (Fig. 5). MTT assay revealed that the expression of a combination of mutant K-ras, Her2, and p16/p14shRNA, or plus Smad4shRNA, induced significantly increased cell proliferation in HPDE cells compared with control cells (Fig. 5A). These two cell lines also had higher percentage of cells in S phase than those of control cells in cell cycle analysis; the percentage of cells in S phase

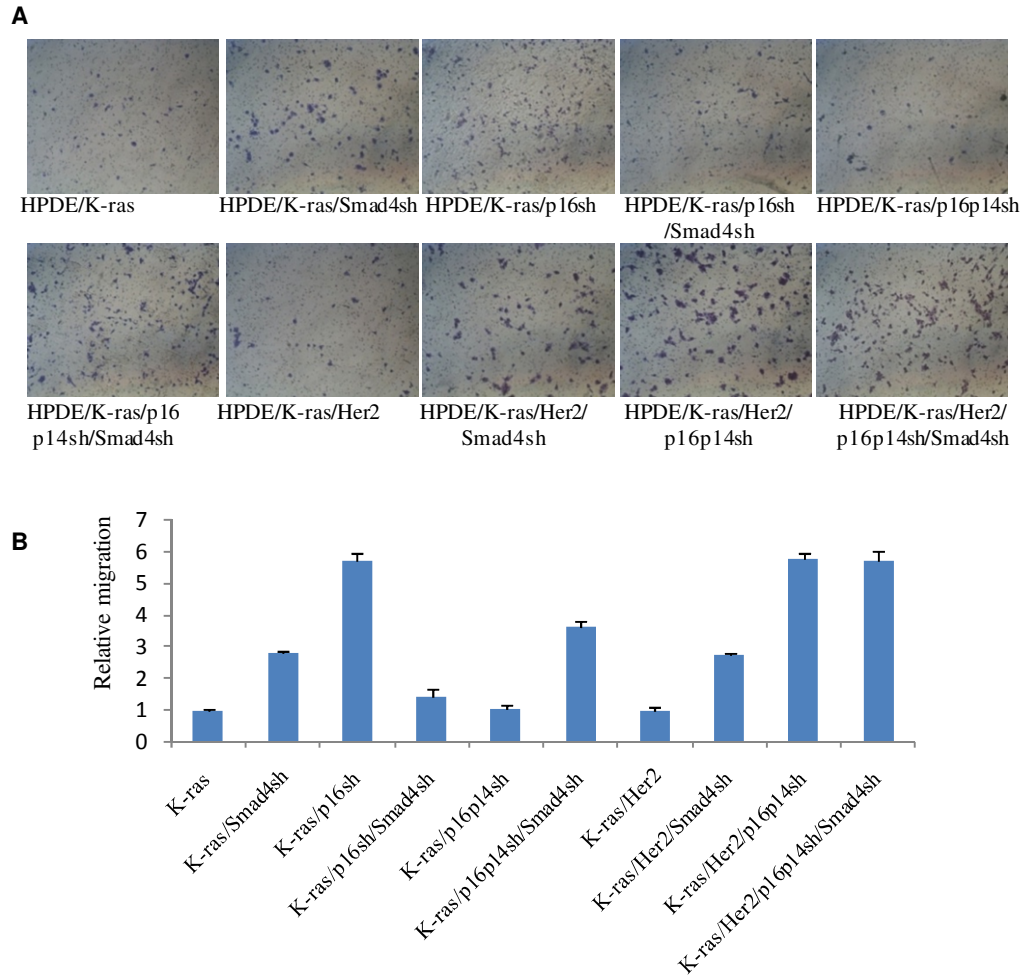


**Figure 5. Cell growth properties of HPDE cell lines with stable expression of mutant K-ras, Her2, p16/p14shRNA, and Smad4shRNA.** (A) The cell growth of HPDE cell lines in MTT assay.  $p < 0.05$  for HPDE/K-ras/Her2/p16p14sh and HPDE/K-ras/Her2/p16p14sh/Smad4sh versus all other control HPDE cell lines at Day 5 and Day 7.  $p < 0.05$  for cell line HPDE/K-ras/p16p14sh versus HPDE/K-ras/p16sh cell line and cell line HPDE/K-ras/p16p14sh/Smad4sh versus HPDE/K-ras/p16sh/Smad4sh cell line at Day 5 and Day 7. (B) Cell cycle analysis of HPDE cell lines. The percentage of cells in each phase of cell cycle was shown from a representative experiment.

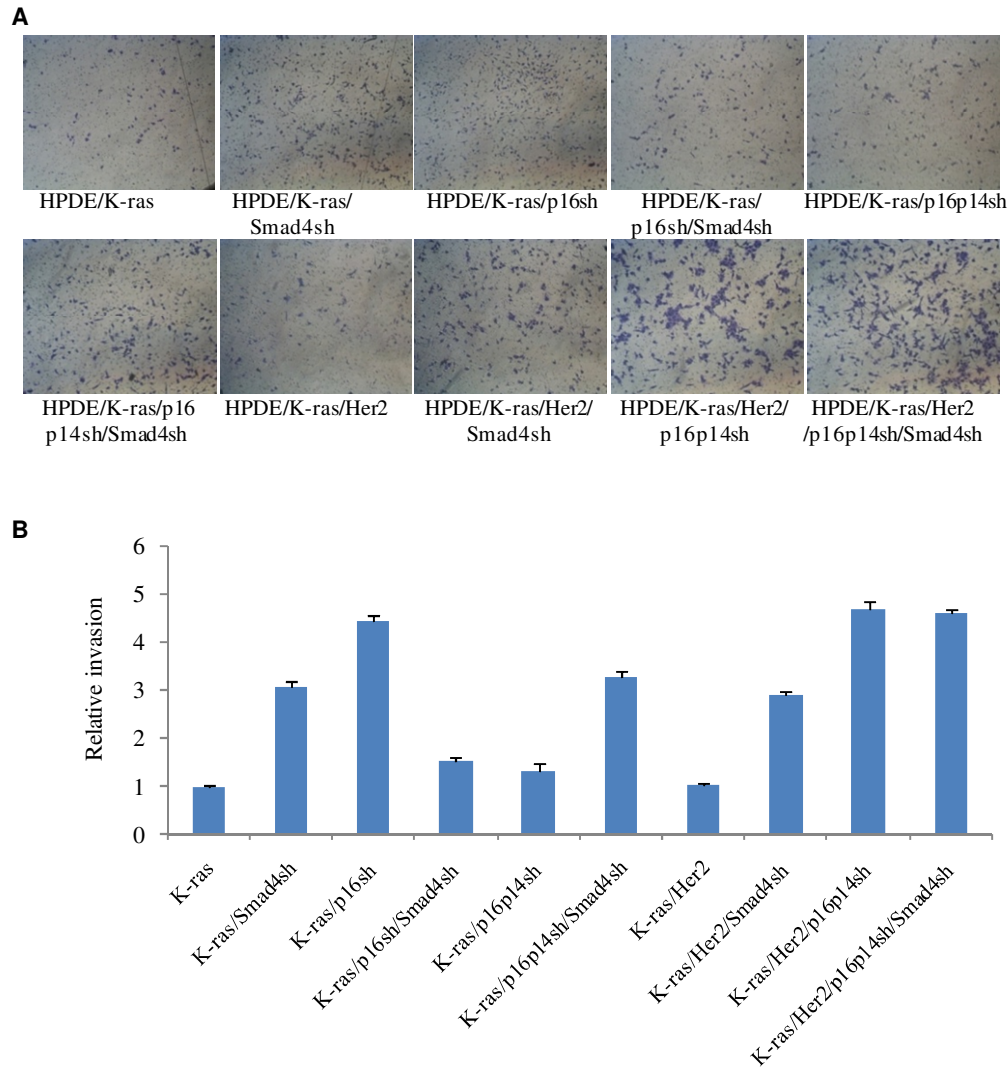
was 35.03%, 33.82% in the cell line HPDE/K-ras/Her2/p16p14shRNA and HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA, respectively (Fig. 5B). HPDE/K-ras/p16p14shRNA/Smad4shRNA cell line also displayed higher percentage of cells in S phase (26.3%) compared with its control cell lines (Fig. 5B). Knockdown p16 and p14 simultaneously induced higher cell proliferation rate than knockdown p16 alone as showed in MTT assay when compared HPDE/K-ras/p16p14shRNA cell line with HPDE/K-ras/p16shRNA cell line, or compared HPDE/K-ras/p16p14shRNA/Smad4shRNA cell line with HPDE/K-ras/p16shRNA/Smad4shRNA cell line (Fig. 5A). Our results showed that silencing both p16 and p14 further enhanced cell growth compared with knockdown p16 alone in HPDE/K-ras cells, indicating that the loss of p14 and p16 simultaneously gives cells additional growth advantage compared with that loss of p16 alone. Altogether, our results suggested that HPDE cell lines with expression of mutant K-ras, Her2, and knockdown p16/p14 or plus knockdown Smad4 acquired increased cell proliferation ability.

#### **The expression of mutant K-ras, Her2, and inactivation of p16/p14 and Smad4 tumor suppressor genes induced increased cell migration and invasion**

Acquiring the increased ability of cell migration and invasion is another hallmark of cancer. To determine whether expression of mutant K-ras, Her2, and inactivation of p16/p14 and Smad4 induce cell migration and invasion *in vitro*, Chamber migration and Matrigel invasion assay was performed. The results from chamber migration and matrigel invasion assays showed that cell lines HPDE/K-ras/Her2/ p16p14shRNA and HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA exhibited increased cell migration and invasion ability compared with other control cells. The number of cells that migrated or invaded through chamber or matrigel was significantly higher than that of control cells (Fig. 6 & Fig. 7). We also found that silencing Smad4 enhanced cell migration and invasion in most of HPDE cell lines. Surprisingly, HPDE/K-ras/p16shRNA cell line exhibited increased cell migration and invasion compared with control HPDE/K-ras cell line and HPDE/K-ras/p16sh/Smad4sh cell lines (Fig. 6 & Fig. 7). Our results suggest that activation of K-ras and Her2, inactivation of p16/p14 or plus Smad4 inactivation induced enhanced cell migration and invasion in HPDE cell lines. Thus these results indicated that HPDE cells with activation of K-ras and Her2, inactivation of p16/p14 or plus inactivation of Smad4 have acquired migration and invasion potential.



**Figure 6. The abilities of cell migration of HPDE cells with expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA.** The Chamber migration assay was performed to determine the abilities of cell migration. (A) The representative field of cell migration for each cell line is shown. (B) The quantification of cell migration. The relative migration was expressed by the migrated cells per field/control cell migration.  $p < 0.05$  for cell line HPDE/K-ras/Her2/p16p14sh, HPDE/K-ras/Her2/p16p14sh/Smad4sh and HPDE/K-ras/p16sh versus all other cell lines.  $p < 0.05$  for each group cell lines with or without Smad4shRNA expression except for group HPDE/K-ras/Her2/p16p14sh and HPDE/K-ras/Her2/p16p14sh/Smad4sh cell lines.

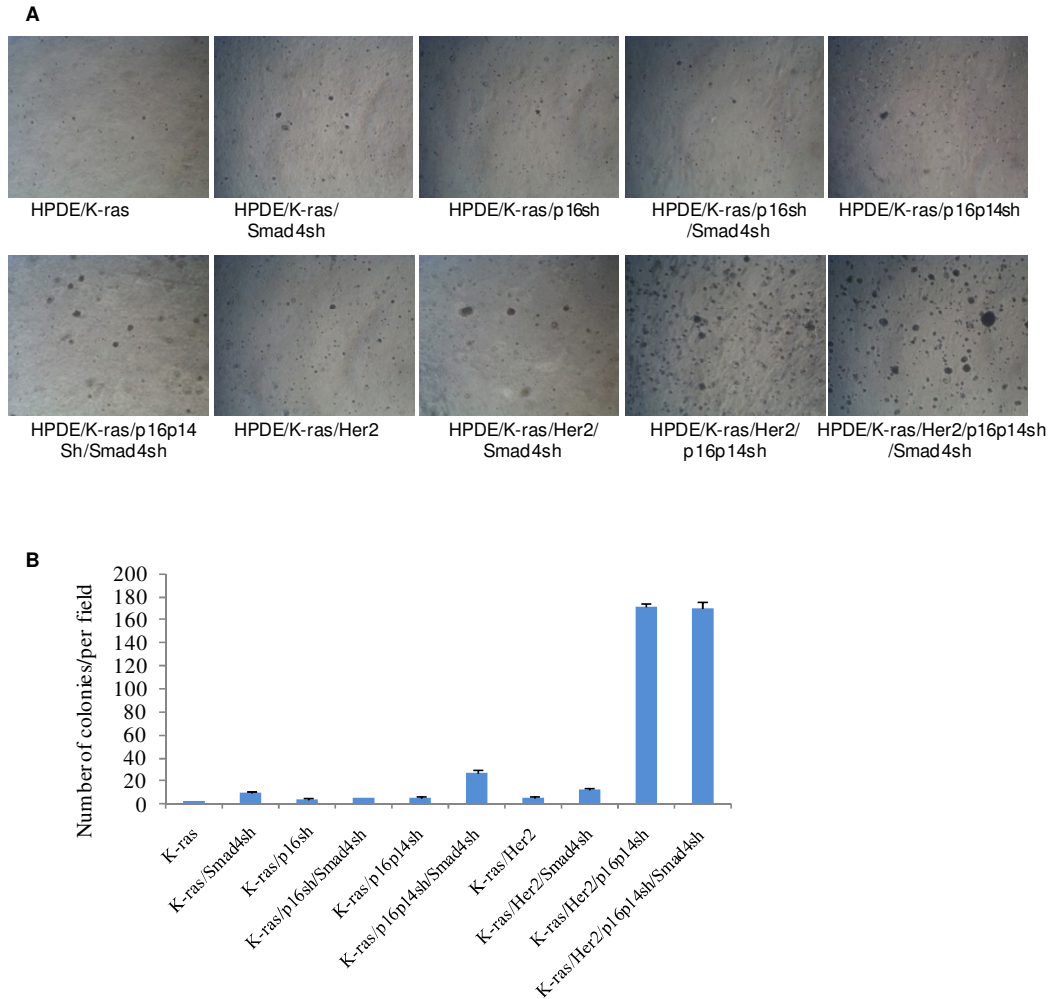


**Figure 7. The abilities of cell invasion of HPDE cells with expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA.** The Matrigel invasion assay was performed to determine the abilities of cell invasion. (A) The representative field of cell invasion for each cell line is shown. (B) The quantification of cell invasion, the relative invasion was expressed by the invaded cells per field/control cell invasion.  $p < 0.05$  for cell line HPDE/K-ras/Her2/p16p14sh, HPDE/K-ras/Her2/p16p14sh/Smad4sh and HPDE/K-ras/p16sh versus all other cell lines.  $p < 0.05$  for each group cell lines with or without Smad4shRNA expression except for group HPDE/K-ras/Her2/p16p14sh and HPDE/K-ras/Her2/p16p14sh/Smad4sh cell lines.

### **Anchorage-independent cell growth of HPDE cells**

To investigate whether the HPDE cells have acquired the ability of anchorage-independent growth, one of hallmarks of *in vitro* cell transformation, we performed soft agar assays to determine the transformation potential of mutant K-ras, Her2, knockdown of p16/p14 and Smad4 in HPDE cells. Expression of mutant K-ras, Her2, and inactivation of p16/p14 or plus inactivation of Smad4 tumor suppressor genes induced anchorage-independent growth in soft agar assay (Fig. 8). Soft agar assays demonstrated that these two cell lines HPDE/K-ras/Her2/p16p14shRNA and HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA grew significantly more number and bigger size of colonies compared with all other control cell lines with different gene expression (Fig. 8). We also found the cell line HPDE/K-ras/p16p14shRNA/Smad4shRNA grew more number of colonies than that of other control cell lines (Fig. 8). Although a few colonies were seen with other control cells, these were both significantly less numerous as well as markedly smaller in size. These results suggest that the specific combination of expression of mutant K-ras, Her2, knockdown of p16/p14 or plus Smad4 knockdown are sufficient to transform immortalized human ductal epithelial cells *in vitro*.





**Figure 8. Anchorage-independent growth of HPDE cell lines with expression of mutant K-ras, Her2, p16/p14shRNA and Smad4shRNA in soft agar.** (A)The representative field of soft agar for each cell line is shown.(B)The quantification of colonies per field in soft agar is shown.  $p < 0.05$  for cell line HPDE/K-ras/Her2/p16p14sh and HPDE/K-ras/Her2/p16p14sh/Smad4sh versus all other cell lines.  $p < 0.05$  for cell line HPDE/K-ras/p16p14sh/Smad4sh versus cell lines HPDE/K-ras, HPDE/K-ras/Smad4sh, and HPDE/K-ras/p16shRNA, HPDE/K-ras/p16shRNA/Smad4sh and HPDE/K-ras/p16p14shRNA.

#### Activation of K-ras and Her2, inactivation p16/p14 and Smad4 in HPDE cell line induced tumorigenesis in orthotopic mouse model

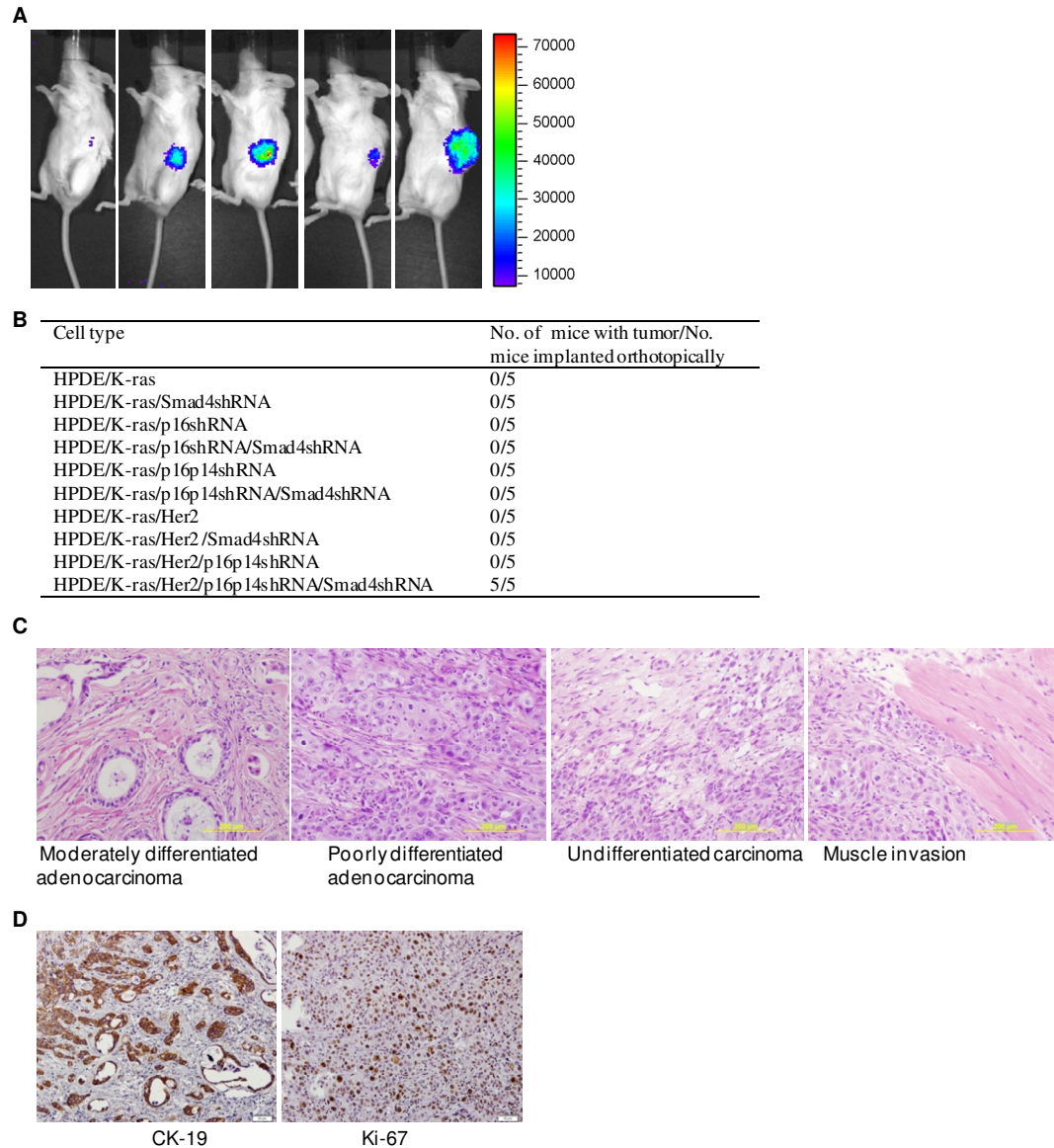
The tumorigenic potential of these HPDE cells *in vivo* was assessed using orthotopic tumorigenesis assay in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice.  $3 \times 10^6$  cells of each of these HPDE cell lines were implanted into the pancreas of NOD/SCID mice.

Tumor growth was monitored by *in vivo* bioluminescence measurements. Expression of mutant K-ras, Her2, and inactivation of p16/p14 and Smad4 tumor suppressor genes induced tumor growth in NOD/SCID mice. Only cell line HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA formed tumors in 5/5 of SCID mice as shown *in vivo* bioluminescence imaging (Fig. 9A & 9B), whereas when other control cell lines were injected into mice, no tumors were found even after 6 months observation. At 8 weeks' implantation of HPDE cells, mice started growing tumors and were sacrificed at 12 weeks to 20 weeks. Necropsy showed the tumors were very firm and had irregular shape.

Histological analysis of tumors formed *in vivo* by HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA cell line revealed a pancreatic ductal adenocarcinoma phenotype. Tumors formed moderately to poorly differentiated adenocarcinoma with large area of undifferentiated carcinoma (Fig. 9C). Metastases to other organs such as liver or spleen were not observed in mice either by gross inspection of mice organ or by hematoxylin and eosin (HE) staining of sections of the liver, spleen and abdominal cavity, but local invasion to adjacent wall of abdominal cavity of skeletal muscle was found in HE staining (Fig. 9C). The tumors had large pleomorphic nuclei and prominent nucleoli, and displayed several (3-7) mitotic figures per high-powered field. Stromal fibroblast was presented in some areas of the tumor masses indicating a tumor stroma interaction.

Immunohistochemistry analysis revealed that the tumors expressed the ductal cell marker cytokeratin-19 (CK-19) (Fig. 9D). To assess the tumor cell proliferation, Ki-67 proliferation marker was examined in tumor sections by immunostaining with the Ki-67 antibody. Ki-67 is a nuclear antigen associated with cell proliferation and is present throughout the active cell cycle (G1, S, G2 and M phases), but absent in resting cells (G0). Ki-67-positive cells were present in all five mouse tumors (Fig. 9D). These results indicated that expression of K-ras, Her2 and knockdown of p16/p14 and Smad4 is sufficient and essential to tumorigenically transform immortalized HPDE cells and induced tumor growth in orthotopic mouse model with PDAC histological features.

The expression of mutant K-ras, knockdown p16/p14 and Smad4 or the expression K-ras, Her2 and knockdown p16/p14 were not sufficient for oncogenic transformation of immortalized HPDE cells. However, only the combination of overexpression K-ras and Her2, and knockdown of p16/p14 and Smad4 simultaneously were able to transform HPDE *in vivo* in tumorigenesis assay. The other two non-tumorigenic cell lines HPDE/K-ras/p1614shRNA/Smad4shRNA and HPDE/K-ras/Her2/p1614shRNA have only one gene difference from the transformed HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA cell line. These results suggest that inactivation of Smad4

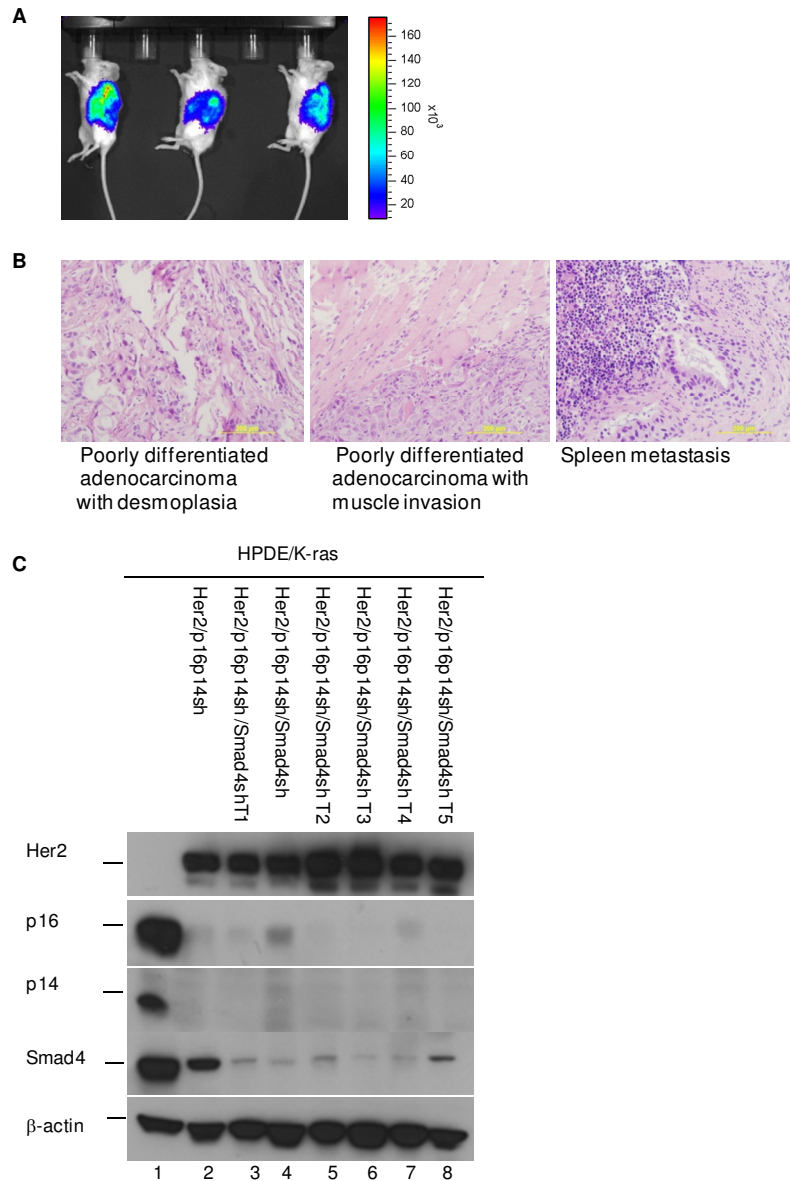


**Figure 9. Activation of K-ras and Her2, inactivation of p16/p14 and Smad4 in HPDE cell line induced tumorigenesis in the orthotopic mouse model.** Activation of K-ras and Her2, and inactivation of p16/p14 and Smad4 in HPDE cell line induced tumorigenesis as shown in orthotopic tumorigenesis assays in NOD/SCID mice. (A) *In vivo* bioluminescence imaging of tumor growth for HPDE/K-ras/p16p14shRNA/Smad4shRNA cell line at 12 weeks' postinjection is shown. (B) Tumor formation in NOD/SCID mice with injection of different HPDE cell lines is shown. (C) Representative micrographs showing the histopathology of the orthotopic tumors formed by HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA cells by HE staining. Different histological features of tumors such as moderately to poorly differentiated adenocarcinoma, undifferentiated ductal carcinoma, and muscle invasion were shown. Scale bar: 100  $\mu$ m. (D) The expression of cytokeratin-19 (CK-19) and Ki-67 in orthotopic tumors of HPDE/K-ras/Her2/p16p14sh/Smad4sh cell line by immunohistochemistry analysis. The representative field of immunostaining is shown. Scale bar: 100  $\mu$ m.

plays an important role in the tumorigenic transformation of HPDE cells, and that strong mitogenic stimulation of Her2 is required for tumorigenic transformation.

We successfully isolated and cultured the tumor cell line HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA T from the orthotopically growing tumors. In tissue culture, the tumor cells retained their epithelial morphology and showed comparable growth rate as the parental cell line HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA. When reimplanted orthotopically into the pancreas of NOD/SCID mice, the HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA T formed tumors in all animals (3/3) (Fig.10A & 10B) and the latency of tumor development was also markedly reduced. In 40 days huge tumors were observed in all mice. Histopathological analysis also exhibited that the tumor was pancreatic ductal adenocarcinoma with regions ranging from moderate to poor differentiation; Invasion to spleen was found in one mouse tumor by HE staining (Fig. 10B). The histological feature of these tumors resembled human PDAC. Our results suggested that our model faithfully mimic the human PDAC using human ductal epithelial cells.

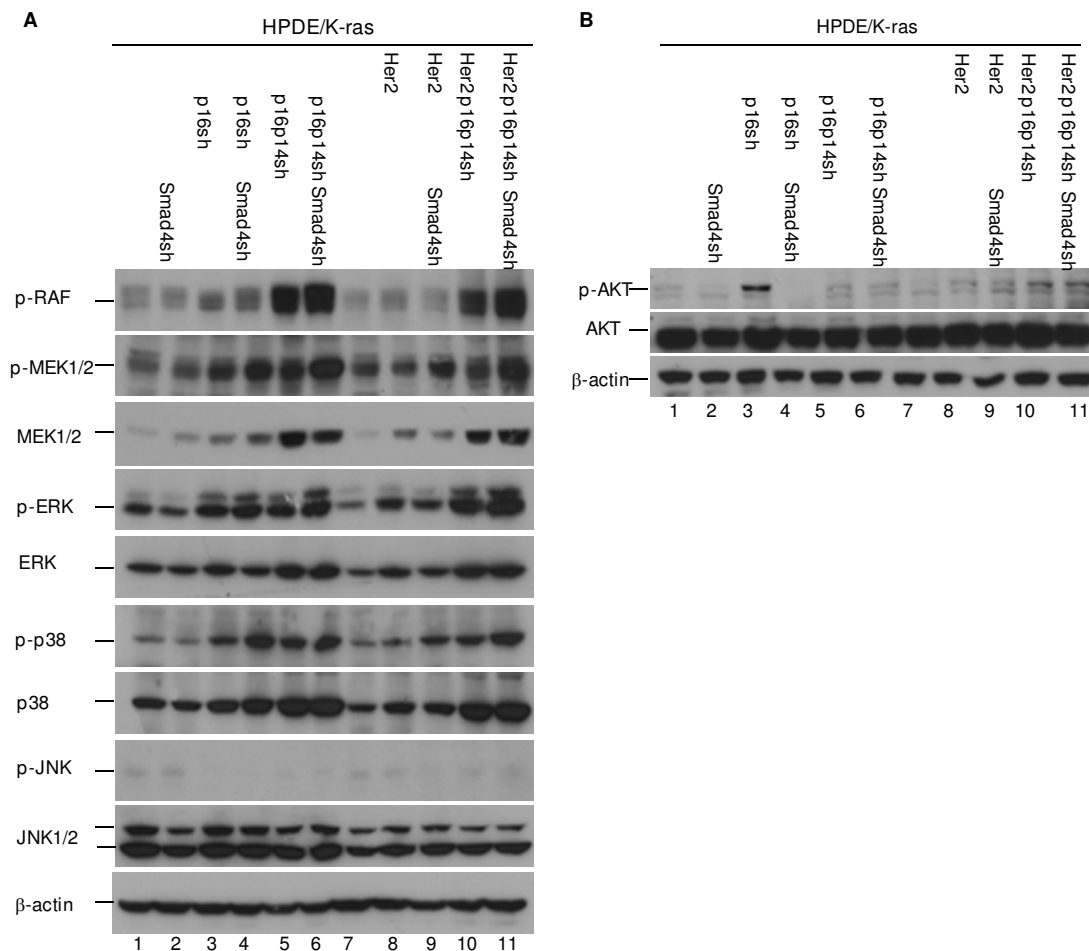
To validate the gene expression in HPDE tumor cell lines, we did Western blot analysis and found that Her2 was overexpressed, and that the expression of p16 and p14, Smad4 was decreased in tumor cell lines (Fig. 10C). Thus our results confirmed the alterations of gene expression in the tumors.



**Figure 10. Confirmation of tumorigenesis in mouse and the alterations of gene expression in the HPDE tumor cell lines isolated from mouse tumors.** (A) *In vivo* bioluminescence imaging of tumor growth of tumor cell line HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA T at 40 days postinjection is shown. Mouse formed tumors with tumor cell line of HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA T isolated from orthotopic growing tumors. (B) Representative micrographs showing the histopathology of the orthotopic tumors formed by tumor cell line HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA T by HE staining. Histology of desmoplasia, muscle invasion and spleen invasion were shown. (C) Confirmation of gene expression alterations in the different tumor cell lines of HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA T isolated from orthotopic mouse tumors by Western blot analysis.

## Activation of signaling pathway downstream of K-ras and Her2

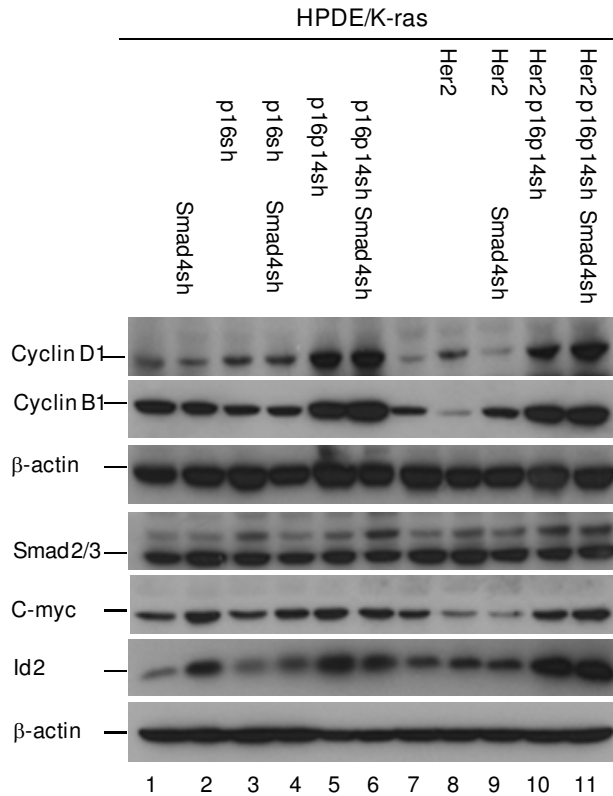
To identify the mechanism of transformation of HPDE cells and explore the downstream signals critical for the transformation, we first investigated the most common and well-characterized three downstream signaling pathways of K-ras and Her2: RAF/MEK/ERK, PI3K/AKT, and RalGDS/Ral. Previous studies have demonstrated that RAF/MEK/ERK, PI3K/AKT, and RalGDS/Ral were activated in pancreatic cancer. ERK activation results in cell cycle progression/cell division and a proliferation phenotype. Activation of p38 induced increased cell growth, cell migration and invasion phenotype. PI3K/AKT pathway regulates cell growth and apoptosis. To investigate whether mutant K-ras and Her2 induced activation of downstream pathways such as RAF/MEK/ERK, PI3K-AKT, and RalGDS-Ral, we examined the expression of phosphorylated protein level and total protein level in these cell lines by Western blot analysis. We found that the mitogen-activated kinase (MAPK) pathway was activated, the expression of phosphorylated protein of RAF, MEK, ERK, p38, and their respective total proteins level of MEK, ERK and p38 were enhanced in the cell lines of HPDE/K-ras/p16p14shRNA and HPDE/K-ras/p16p14shRNA/Smad4shRNA, and HPDE/K-ras/Her2/p16p14shRNA and HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA compared with their other control cell lines, but we did not found JNK activation in HPDE cell lines ( Fig. 11A). These results indicate that activation of K-ras and Her2 induced activation of MAPK-ERK and p38 pathway. We found weak AKT activity in the cell lines HPDE/K-ras/Her2/p16p14shRNA and HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA as the low level of phosphorylation of AKT was detected in the Western blot analysis (Fig. 11B). Interestingly, we found higher AKT activity in HPDE/K-ras/p16shRNA cell line than in all other cell lines (Fig. 11B). To determine whether RalGDS-Ral is activated in transformed cell lines, we examined the RalA and RalB activity. Our results demonstrated that there was no difference for RalA and RalB activity among different cell lines (data not shown). The activation of MAPK pathways ERK and p38 in transformed HPDE cells suggest that activation of K-ras and Her2 in HPDE cells led to the activation of its downstream effectors MAPK pathway activation, and activation of these pathways may play roles in the malignant transformation of HPDE cells.



**Figure 11. Activation of signaling pathway downstream of K-ras and Her2 in HPDE cells.** (A) Activation of MAPK pathways in HPDE/K-ras cell lines. The expression of phosphorylated and total proteins of RAF, MEK, ERK, p38 and JNK was detected in HPDE/K-ras cell lines by Western blot analysis. (B) Activation of AKT in HPDE/K-ras cell lines. The expression of phosphorylated AKT and total AKT level was detected in HPDE/K-ras cell lines by Western blot analysis.

### The expression of cell cycle proteins and cell proliferation related genes

As transformed cells exhibited increased cell proliferation, we therefore examined whether the expression of cell proliferation related genes such as cell cycle protein-cyclins was changed in the transformed cells. We found that the expression of cyclin D1, cyclin B1 was markedly elevated in HPDE/K-ras/p16p14sh, HPDE/K-ras/p16p14sh/Smad4sh cell line, and HPDE/K-ras/Her2/p16p14sh,

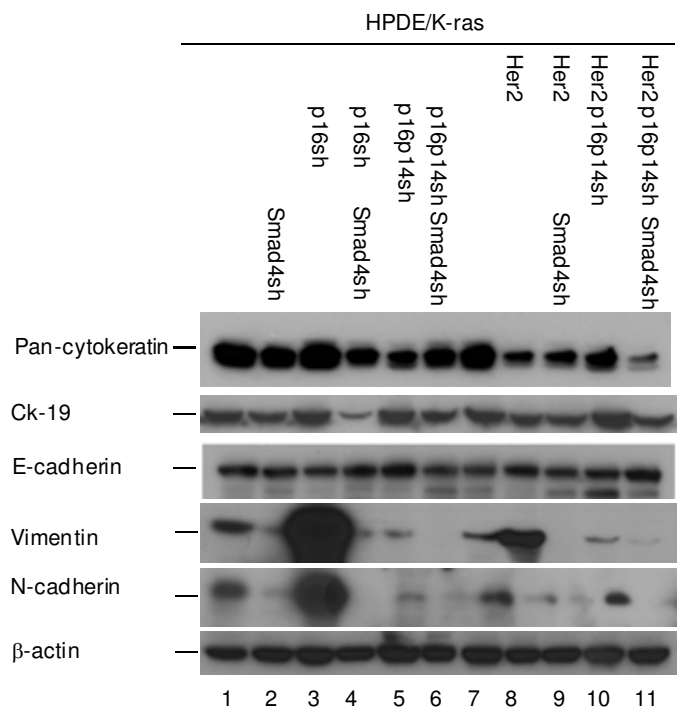


**Figure 12. The expression of cell cycle protein and cell proliferation related genes in HPDE cell lines.** The expression of cyclinD1, cyclinB, Smad2/3, c-Myc and Id2 in HPDE cell lines were detected by Western blot analysis.

HPDE/K-ras/Her2/p16p14sh/Smad4sh cell line compared with other control cell lines (Fig. 12). As Smad4 regulates the growth inhibition of TGF  $\beta$  and our results showed that Smad4 plays an important role in tumorigenic transformation. To determine the role of Smad4 in HPDE cell transformation, we therefore examined whether the expression of c-myc and Id2 –Smad4 downstream genes related with cell proliferation was changed in those transformed cells. Our results revealed that the expression of c-myc and Id2 was significantly increased in HPDE/K-ras/p16p14shRNA, HPDE/K-ras/p16p14shRNA/Smad4shRNA cell line, and HPDE/K-ras/Her2/p16p14shRNA, HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA cell line compared with their other control cell lines (Fig. 12). The protein level of c-myc and Id2 was also significantly enhanced in HPDE/K-ras/Smad4shRNA cell line compared with HPDE/K-ras cell line in the Western blot analysis (Fig. 12), indicating that Smad4 plays a role in regulation of c-myc and Id2, the knockdown of Smad4 increased the expression of c-myc and Id2 in HPDE/K-ras cell line.



However, in other HPDE cell lines, the increased c-myc and Id2 expression is not solely regulated by Smad4. c-myc and Id2 play roles in the regulation of cell proliferation and cell growth. These results suggest that increased expression of cell proliferation related genes cyclins, c-myc and Id2 may be involved in the enhanced cell proliferation and the transformation of HPDE cells.



**Figure 13. The expression of EMT marker proteins in HPDE cell lines.** The expression of pan-cytokeratin, cytokeratin-19, E-cadherin, vimentin and N-cadherin was detected by Western blot analysis.

### The expression of EMT marker in HPDE cell lines

As shown above, the transformed cells had elevated ability of migration and invasion, and tumor transformation always accompany with the epithelial to mesenchymal transition (EMT). We therefore determined the expression of EMT marker proteins' changes in the transformed cell line. We examined the expression of several EMT markers in HPDE cells by Western blot analysis (Fig. 13). We found that the expression of the epithelial marker pan-cytokeratin is decreased by knockdown Smad4 in some HPDE cell lines indicating its expression was not solely regulated by Smad4 and its expression was further decreased by expression of Her2. The epithelial marker cytokeratin-19 was also mediated by Smad4, as its expression was reduced by knockdown Smad4.

The expression of E-cadherin was not changed in those cells. In contrast, the expression of mesenchymal marker vimentin and N-cadherin was regulated by Smad4 as knockdown of Smad4 reduced the expression of those proteins in Western blot analysis (Fig.13). Taken together, our results indicated that overexpression of Her2 and inactivations of Smad4 were involved in the regulation of EMT marker protein expression.

### **The alterations of gene expression for transformation in microarray analysis**

In order to better understand the molecular mechanisms of human pancreatic cell transformation and to gain further insight into the detailed molecular alterations involved in the transformation, we performed microarray gene expression analysis. The top 10 most significantly upregulated and downregulated genes for cell line HPDE/K-ras/Her2/p16p14sh/Smad4sh versus cell line HPDE/K-ras/Her2/p16p14sh in microarray analysis were shown in Table 2. Some significantly altered expression of genes was the following: genes of up-regulation: GJA1, BMP7, THBS2, TGFB2, MDM2, TTC3 and BMI-1; genes of down-regulation: SPOCK1, ADAM19, CDH2, NLRP3, KRT34.

**Table 2. The top most significant changes of gene expression for cell line HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA versus HPDE/K-ras/Her2/p16p14shRNA cell line in cDNA microarray analysis.**

Top Molecules up-regulated	Fold change	Top Molecules down-regulated	Fold change
GJA1	41.30	SPOCK1	20.85
BMP7	18.66	ADAM19	16.26
TGFB2	12.50	COL22A1	12.62
THBS2	11.22	CDH2	11.99
SULT1E1	10.86	SYTL4	10.52
C12ORF48	8.71	CNTN3	10.33
C6ORF170	8.38	COL13A1	8.87
MDM2	8.26	NLRP3	8.57
TTC3	7.46	KRT34	7.69
BMI-1	2.13	GRB10	3.73

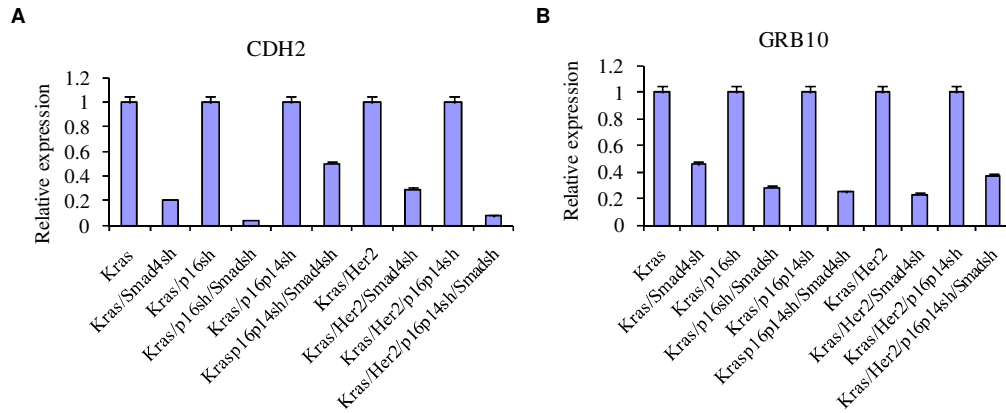
To validate the microarray results, we selected some upregulated and downregulated genes in transformation for confirmation of differential expression by Real-time-PCR and Western blot analysis. We first confirmed the higher expression of oncogene MDM2 in transformed cell line HPDE/K-ras/p16p14sh/Smad4sh in cDNA microarray data by Western blot analysis (Fig. 14A). The results demonstrated that MDM2 protein was highly expressed in the cell lines with knockdown



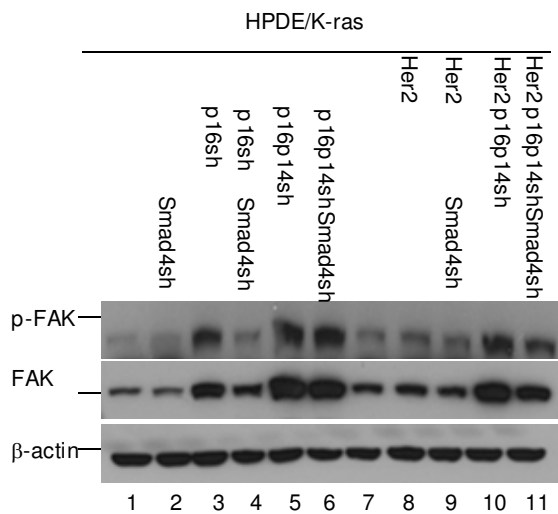
of p14 in Western blot analysis, thus confirming the previous finding of regulation of MDM2 by p14. The cell line HPDE/K-ras/p16shRNA/Smad4shRNA also had increased MDM2 expression (Fig. 14A), but the mechanism of elevated MDM2 expression is not clear and need to be further investigated. To further explore the role of MDM2 in transformation, we examined the expression of MDM2 in PDAC cell lines. The results revealed that MDM2 was overexpressed in 9 of 12 (75%) pancreatic cancer cell lines compared with immortalized pancreatic cell lines (Fig. 14B). These results suggest that MDM2 may play roles in the pancreatic cell transformation and tumorigenesis.

We then validated the increased expression of bone morphogenetic protein 7 (BMP7) mRNA in the transformed HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA cell line (Fig. 14C). In K-ras and Her2 expressing cell line, further knockdown Smad4 increased the expression of BMP7 (Fig. 14C), indicating Smad4 may play a role in regulating BMP7 expression. The expression of BMP7 was also elevated in cell line HPDE/K-ras/Smad4shRNA, HPDE/K-ras/p1614shRNA and HPDE/K-ras/p16p14shRNA/Smad4shRNA. Because the role of BMP7 in human pancreatic cancer is unknown, we then examined the expression of BMP7 mRNA level in human pancreatic cell lines, and found that BMP7 mRNA was highly overexpressed in 9 of 14 (64%) pancreatic cancer cell lines compared with the lower level in immortalized human pancreatic cell lines (Fig. 14D). These results suggest that BMP7 may play a role in the pancreatic cell transformation.

As our results showed that Smad4 plays an important role in the transformation, we are very interested in finding Smad4 dependent target genes which were changed during the transformation. Very interestingly, we found that the mRNA level of Bmi-1, a putative polycomb oncogene was highly overexpressed in the transformed cell line HPDE/K-ras/Her2/p16p14sh/Smad4sh compared with other control cell lines by real-time PCR analysis (Fig.14E). To further verify the expression of Bmi-1 in HPDE cells, we performed Western blot analysis, and found that the expression of Bmi-1 protein was regulated by Smad4; knockdown Smad4 enhanced the expression of Bmi-1 protein in HPDE cells, the highest expression level was found in the transformed cell line (Fig. 14F). To determine the role Bmi-1 in human pancreatic cancer, we examined the Bmi-1 expression in PDAC cell lines. The results showed that Bmi-1 was markedly overexpressed in 11 of 13 (85%) PDAC cell lines, but very low level was found in immortalized human pancreatic cell lines (Fig. 14G). We further examined the Bmi-1 expression in HPDE tumor cell lines, and found enhanced level of Bmi-1 in HPDE tumor cell lines compared with HPDE/K-ras cells (Fig. 14H). Altogether, these results suggest that Smad4-regulated Bmi-1 plays an important role in the transformation of HPDE cells and pancreatic cell oncogenesis.



**Figure 15. Confirmation of the changes of CDH2 and GRB10 gene expression in cDNA microarray by Real-time PCR analysis.** The expression of CDH 2 and GRB10 mRNA was compared with their control cell without Smad4 knockdown.



**Figure 16. The activation of FAK in HPDE cell lines.** The expression of phosphorylated and total FAK was detected by Western blot analysis in HPDE cell lines.

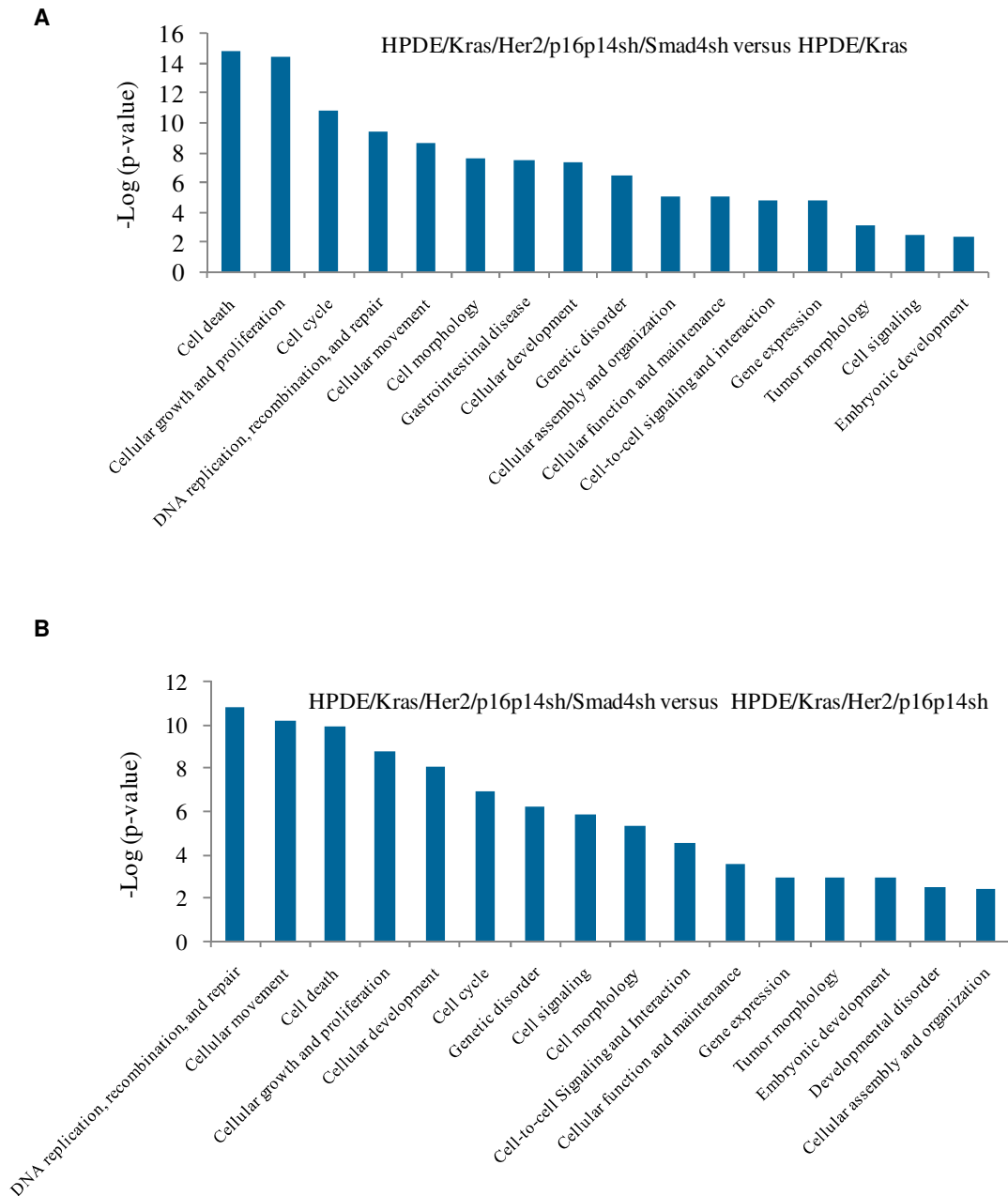
The other Smad4 regulated genes we found and validated, were CDH2 and GRB10. The results showed that knockdown Smad4 reduced the mRNA expression levels of those genes in real-time PCR analysis (Fig. 15). CDH2 encodes N-cadherin protein that mediates calcium-ion-dependent adhesion, and is a mesenchymal marker involved in tumor invasion. GRB10 is an adapter protein that interacts with several mitogenic receptor tyrosine kinases including the insulin and insulin-like

growth factor-I receptor (141). Overexpression of some isoforms of GRB10 inhibits tyrosine kinase activity and induces growth suppression (141). We also validated the increased expression of FAK in microarray data. We found FAK was activated in transformed cell line, the phosphorylated FAK and total FAK level was increased in transformed cell lines in Western blot analysis (Fig. 16). The FAK expression level was also elevated in the cell lines of HPDE/K-ras/p16sh, HPDE/K-ras/p16p14sh, HPDE/K-ras/p16p14sh/Smad4sh, and HPDE/K-ras/Her2/p16p14sh (Fig. 16).

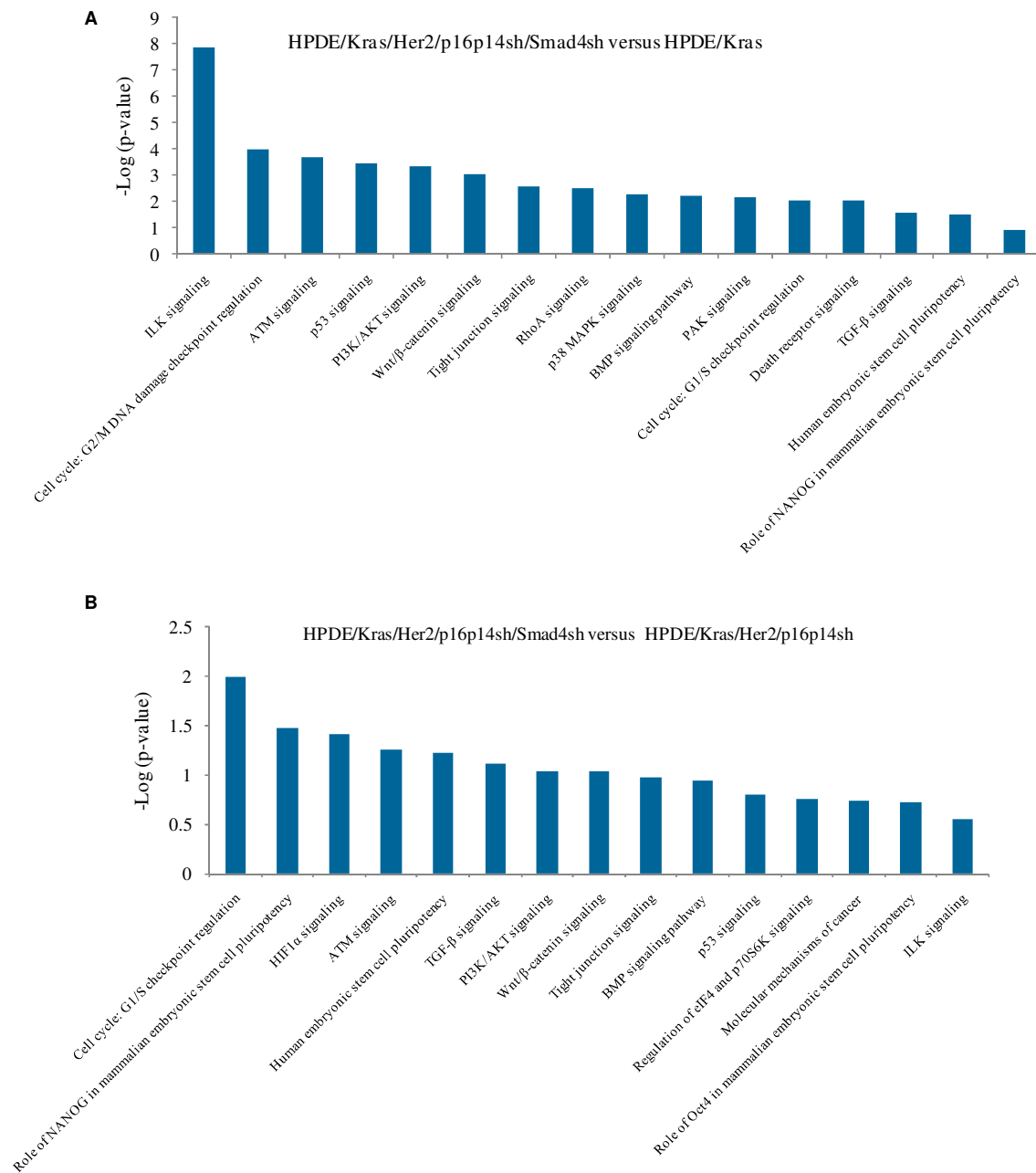
We confirmed the differentially expressed genes in the transformed cell lines compared with control cells. The results of real-time PCR, Western blot analysis and microarray methods are in good agreement. These results indicated that the microarray data accurately reflect the transcriptional difference between different cell lines.

### **The alterations of signaling pathway for transformation**

To explore the signaling pathway alterations critical for the transformation of pancreatic ductal cells, we performed Ingenuity Pathway Analysis (IPA) signaling pathway analysis of microarray data. The top biological function alterations which significantly associated with transformation for HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA cell line versus HPDE/K-ras cell line were: cell death, cellular growth and proliferation, cell cycle (Fig. 17A). The most significant alterations of biological functions for HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA cell line versus HPDE/K-ras/Her2/p16p14shRNA cell line were: DNA replication, recombination and repair, cellular movement, cell death, and cellular growth and proliferation (Fig. 17B). The results showed that the most significant signaling pathway alterations for cell line HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA versus HPDE/K-ras were ILK signaling, cell cycle: G2/M DNA damage checkpoint (Fig. 18A). The significantly changed pathways for cell line HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA versus HPDE/K-ras/Her2/p16p14shRNA were cell cycle: G1/S checkpoint regulation (Fig. 18B). We found that ILK signaling pathway was highly enriched in the transformed cell lines. ILK signaling is the integrin-linked kinase (ILK) signaling pathway which regulates a variety of cellular reactions including cell growth and proliferation, cellular adhesion, migration, differentiation, survival, invasion and angiogenesis (142). From microarray data signaling pathways analysis, we found several components of ILK signaling were altered, the expression of FAK, cyclin D1, IRS1,  $\beta$ -Catenin and c-Myc were up-regulated, keratin 18 and vimentin were reduced. The significantly altered genes in the molecular mechanism of cancer pathway compared

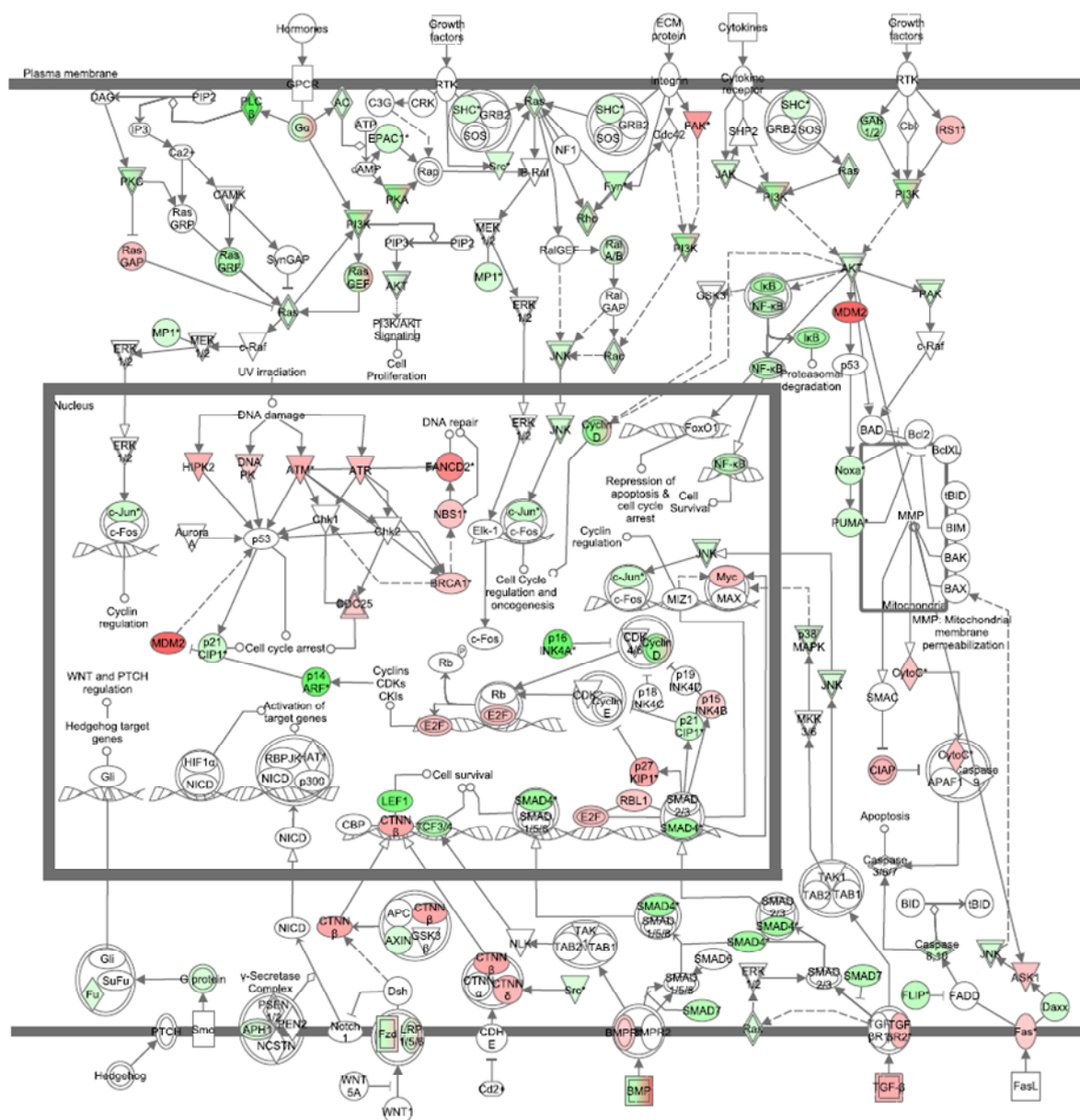


**Figure 17. IPA biological function analysis of microarray data.** Bar indicates significance; the significance of an altered biological function is expressed by  $-\log(p\text{-value})$  mapping to a biological function in the IPA knowledge base (A) IPA biological function analysis for HPDE/K-ras/Her2/p16p14sh/Smad4sh versus HPDE/K-ras cell line. (B) IPA biological function analysis for HPDE/K-ras/Her2/p16p14sh/Smad4sh versus HPDE/K-ras Her2/p16p14sh cell line.



**Figure 18. IPA canonical signaling pathway analysis of microarray data.** Bar indicates significance; the significance of an altered signaling pathway is expressed by  $-\log(p\text{-value})$  mapping to a signaling pathway in the IPA knowledge base; the length of the bar only indicates that the differentially expressed proteins are related to this pathway but does not indicate upregulation or downregulation of the pathway. (A) IPA canonical signaling pathway analysis for HPDE/K-ras/Her2/p16p14sh/Smad4sh versus HPDE/K-ras cell line. (B) IPA canonical signaling pathway analysis for HPDE/K-ras/Her2/p16p14sh/Smad4sh versus HPDE/K-ras Her2/p16p14sh cell line.





**Figure 19. Changes of molecular mechanism of cancer signaling in IPA signaling pathway analysis of microarray data.** Molecular mechanism of cancer signaling in IPA signaling pathway analysis for cell line HPDE/K-ras/Her2/p16p14sh/Smad4sh versus HPDE/K-ras cell line was shown. Red color and green color indicate upregulated and downregulated expression of genes respectively. The depth of color indicates the level of increase or decrease of a gene expression.

between HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA cell line with HPDE/K-ras cell line were: the upregulated genes: FAK, IRS1, MDM2, c-myc, E2F, TGF  $\beta$ , Her2,  $\beta$ -catenin, the downregulated genes: Smad4, p16, p14 (Fig. 19). The alterations of gene expression involved in the regulation of cell cycle G2/M and G1 checkpoints were: increased level of genes of ATM, ATR, MDM2, cyclin B, decreased level of genes: p14, MDM2, p21.

In conclusion, the results from IPA signaling pathways analysis suggested that abnormal ILK signaling, cell cycle dysregulation were significantly associated with transformation of human pancreatic ductal epithelial cells.

## Summary

In the present study, we established a novel experimental cell culture model of human pancreatic ductal epithelial cells carcinogenesis, in which defined genetic elements were introduced into HPV16-E6E7 immortalized human pancreatic ductal epithelial cells (HPDE) to create the tumorigenic PDAC cells. We found that through the stepwise introduction of the most frequently occurred genetic alterations in PDAC -mutant K-ras, Her2, inactivation of p16/p14 and Smad4 results in tumorigenic transformation of this cell line. *In vitro* the transformed cell line showed increased cell proliferation, migration and invasion, and displayed an anchorage-independent growth in soft agar. *In vivo*, the transformed cell line formed tumors in orthotopic mouse model, histopathological analysis of the tumors revealed PDAC features. These results indicated that the combination of activation of K-ras and Her2, inactivation p16/p14 and Smad4 are sufficient and essential for tumorigenic transformation of immortalized HPDEs into PDAC cells in this novel *in vitro* experimental cell culture model system. To identify the alterations of molecular and signaling pathways involved in the transformation of human pancreatic cells, we performed molecular and signaling pathway analysis of those cell lines. We found that the activation of K-ras and Her2 downstream signaling pathway MAPK signaling together with the upregulation of the expression of cell proliferation related genes cyclins, c-myc and Id2 were involved in the transformation. We then found that MDM2, BMP7 and Bmi-1 were overexpressed in the tumorigenic HPDE cells. Our results indicated that Smad4 plays important roles in the regulation of BMP7 and Bmi-1 gene expression and the tumorigenic transformation. IPA signaling pathway analysis of microarray data found that the most significant changes of signaling pathways involved in the transformation of HPDE cells were abnormal ILK signaling, cell cycle dysregulation. This study is the first fully transformation model of human pancreatic ductal epithelial cells using the most common gene alterations in human PDAC, thus our models not only more truly recapitulate the development of human PDAC from cell origin and gene lesion, activation of specific signaling pathways that occur in human PDAC, but also faithfully reproduce the histopathological characteristics of PDAC. This study provides a better understanding of the molecular mechanism of PDAC.

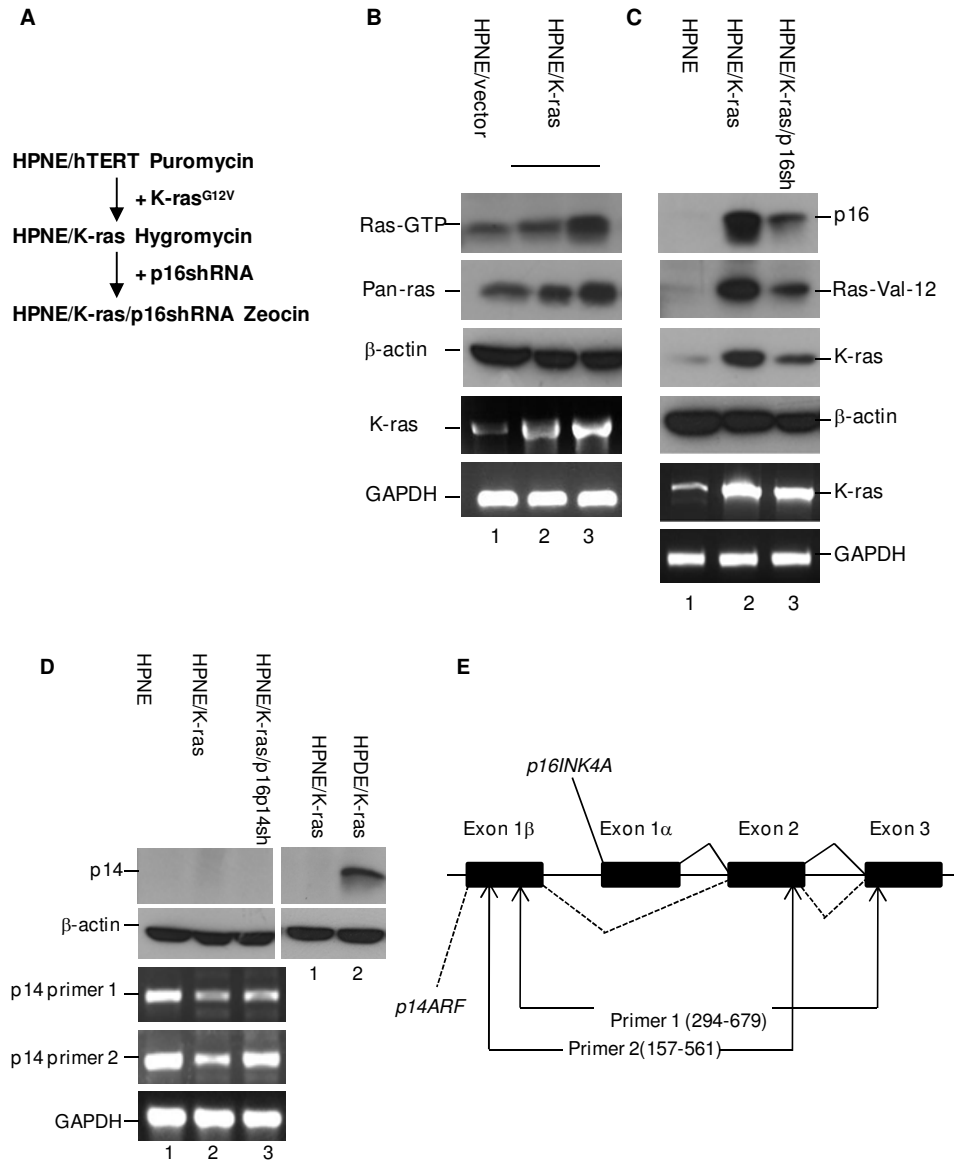
## **Part II    Tumorigenic transformation of hTERT immortalized human pancreatic ductal (HPNE) cell line**

### **Stable expression of mutant K-ras and p16shRNA in hTERT immortalized human pancreatic ductal nestin expressing cell line (HPNE)**

HPNE cell line is an hTERT gene immortalized normal human pancreatic ductal nestin-expressing cell line (termed HPNE, Human pancreatic nestin-expressing cells) isolated from the pancreatic ducts of a 52 year-old male's organ donor after death in an accident (137, 143, 144). This cell line is an exocrine pancreas progenitor cell, is diploid, expresses wild type K-ras, p16 and p53, and displays normal growth controls and does not exhibit any cancer-associated changes (137, 143).

The role and function of mutated K-ras and inactivated p16/p14 in pancreatic tumorigenesis have been identified in mouse model of PDAC. However, it is still unclear whether and how activation of K-ras in combination with loss of p16 induces tumorigenic transformation in human pancreatic ductal cells. Therefore, we hypothesized that activation of K-ras cooperated with inactivation of p16 contribute to the transformation of immortalized HPNE cells. The goal of this study is to establish an experimental cell transformation model with activation of K-ras and inactivation of p16 to study the function of these gene alterations in the process of human pancreatic carcinogenesis and to investigate the molecular mechanism of transformation.

To test our hypothesis and to study the role of mutation of K-ras and inactivation of p16, the most frequent gene lesions identified in PDAC, we serially introduced mutant K-ras, inactivation of p16 by small hairpin RNA (shRNA) into an hTERT immortalized human pancreatic ductal cell line (HPNE) cells via retroviral or lentiviral transduction using different antibiotics selection ( Fig.20A). We infected HPNE cells by retrovirus and lentivirus expressing mutated K-ras (K-ras<sup>G12V</sup>) and p16shRNA, respectively, and established pooled antibiotics-resistant clones as cell lines (Fig. 20A). Mutant K-ras was first introduced into HPNE cells via retroviral transduction. The expression of K-ras<sup>G12V</sup> in HPNE cell line induced enhanced Ras activity 2-3 folds higher compared with vector control cells in a Ras activity assay (Fig. 20B). Total Ras level was also enhanced in K-ras expressing cell line compared with control cell line (Fig. 20B). We also found that the total K-ras mRNA level was dramatically increased in K-ras expressing cell line compared with control cell line in RT-PCR analysis (Fig. 20B). As *p14ARF* gene is inactivated in 40% of PDAC, to study the role of CDKN2A locus p16 and p14 gene, we designed shRNA targeting their common third exon of



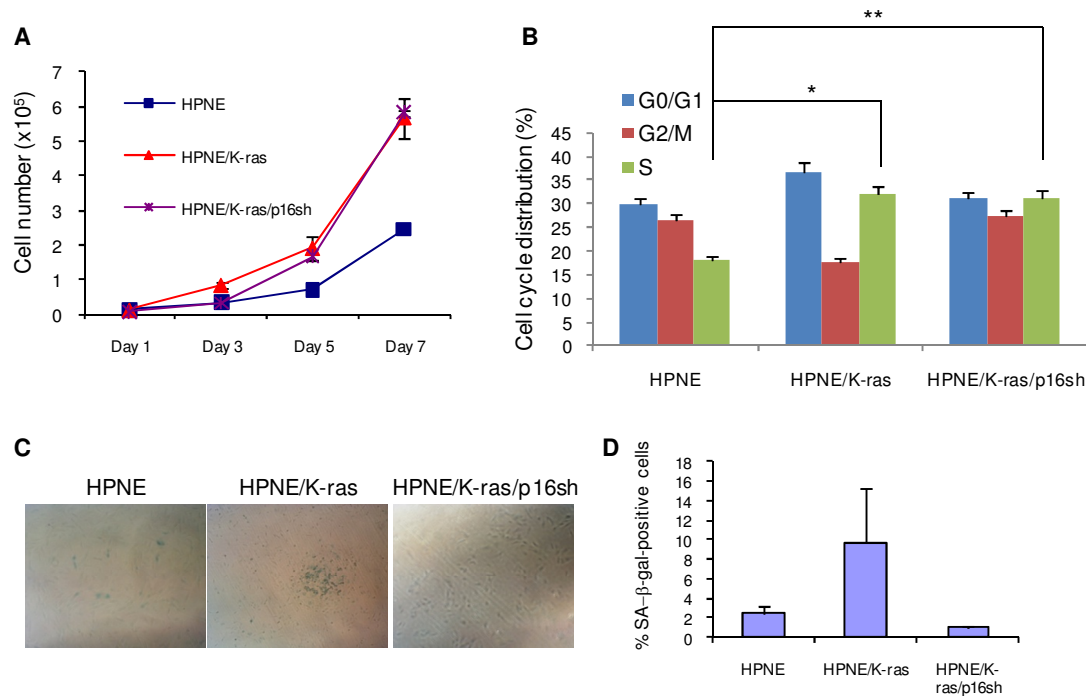
**Figure 20. Stable expression of mutant K-ras and p16shRNA in hTERT-immortalized HPNE cells.** (A) The strategy for introducing gene alterations into HPNE cell lines. The mutant K-ras and p16shRNA was introduced into HPNE cells by retrovirus or lentivirus via different antibiotics selection. (B) Stable expression of mutant K-Ras<sup>G12V</sup> in the HPNE cell line. The stable K-ras<sup>G12V</sup> expression in the HPNE cell line was identified by Ras activity assay, Western blot analysis of total Ras expression, and RT-PCR analysis of K-ras mRNA. (C) Stable expression of mutant K-ras and p16shRNA in HPNE cell lines. The stable cell lines were identified by Western blot analysis of the expression of p16, RasVal12, and K-ras, and RT-PCR analysis of K-ras mRNA. (D) The expression of p14 in HPNE cell lines. The Western blot and RT-PCR analysis of p14 expression in HPNE cell lines. The HPDE/K-ras cell line was used as a p14 positive control in Western blot analysis. Two pairs of primers for p14 were used in RT-PCR analysis. (E) Structure of the *p16INK4A/p14ARF* gene exon locus and p14 primer design position.

*CDKN2A* locus to silence the expression of both p16 and p14 as both genes share their exon 2 and 3. Stable expression of K-ras and p16shRNA in HPNE cells was identified by Western blot and RT-PCR analysis (Fig. 20C). These transduced cells demonstrated the overexpression of K-ras, decreased expression of p16 as confirmed by Western blot analysis (Fig. 20C). Increased level of RasV12 compared with control cells was also observed in Western blot analysis using a special antibody which recognizes RasV12. The total level of K-ras mRNA was dramatically increased in HPNE/K-ras and HPNE/K-ras/p16shRNA cell lines compared with control cell line in RT-PCR analysis (Fig. 20C). Interestingly, we found that p16 expression was markedly induced by mutant K-ras in HPNE/K-ras cells and the K-ras-upregulated p16 expression was greatly reduced by p16shRNA in HPNE/K-ras/p16shRNA cells (Fig. 20C). The level of p14 mRNA in both HPNE/K-ras and HPNE/K-ras/p16shRNA cells was similar as shown by RT-PCR analysis, but p14 protein expression was too low to be detectable in Western blot analysis (Fig. 20D). We thus confirmed the unchanged level of expression of p14 mRNA in HPNE/K-ras/p16shRNA cells (Fig. 20D), these results indicated that shRNA specially target p16 expression.

To rule out the possibility of cell cross-contamination, we performed DNA fingerprinting and found that the DNA fingerprint profile of the HPNE/K-ras/p16shRNA cell line did not match any known DNA fingerprints, but exactly matched that of the original source HPNE cell line (137, 140) (Data not shown). We thus successfully established the stable HPNE cell lines with expression of the mutant K-ras and inactivated p16 in immortalized HPNE cells.

### **The activation of K-ras and inactivation of p16 in HPNE cell line induced increased cell proliferation and cell growth**

HPNE cells are cultured in Medium D with mixtures of M3 and DMEM medium. After transfection of these gene alterations, we checked whether HPNE cells could adapt to grow in DMEM growth medium as most cancer cells. When HPNE cells were cultured in 10% serum-containing complete DMEM medium, those cells exhibited comparable cell proliferation rate compared with cells grown in Medium D. We therefore cultured HPNE cells in 10% serum-containing DMEM medium for the remaining experiments.



**Figure 21. Cell growths of HPNE cell lines with stable expression of mutant K-ras and p16shRNA.** Mutant K-ras and inactivation of p16 in HPNE cell lines induced increased cell proliferation. (A) The cell growth curve of HPNE cell lines is shown,  $p < 0.05$  for HPNE/K-ras versus HPNE and HPNE/K-ras/p16shRNA versus HPNE in day 5 and day 7,  $p > 0.05$  for HPNE/K-ras/p16shRNA versus HPNE/K-ras in day 5 and day 7. (B) Cell cycle analysis of HPNE cell lines. The percentage of cells in each phase of cell cycle was shown. \* $p < 0.05$ ; \*\*  $p < 0.05$ . (C) Representative of field of senescence-associated  $\beta$ -galactosidase activity (SA- $\beta$ -gal) in HPNE cell lines. (D) Quantification of SA- $\beta$ -gal staining in HPNE cells. Data are shown as percentage of positive cells representative of three independent experiments, error bar indicates s.e..  $p < 0.05$  for HPNE versus HPNE/K-ras, HPNE versus HPNE/K-ras/p16sh, and HPNE/K-ras/p16sh versus HPNE/K-ras.

As uncontrolled cell growth is a hallmark of cancer, we first examined the growth features of HPNE cells with expression of mutant K-ras and knockdown of p16 by cell morphology and cell proliferation. Neither HPNE/K-ras cells nor HPNE/K-ras/p16shRNA cells showed any significant morphological changes compared with control cells and original parental cells on tissue culture plates.

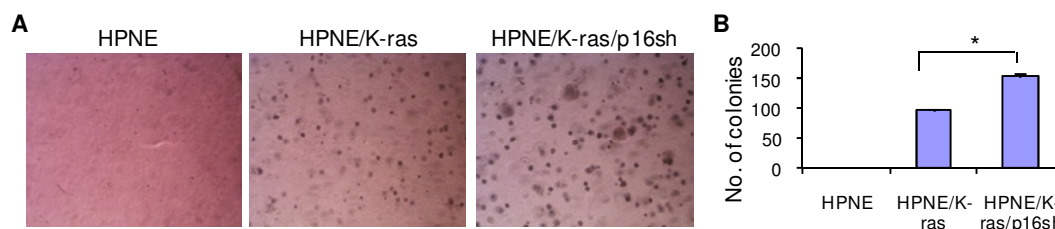
We determined the cell proliferation and growth by cell cycle analysis and cell growth curve. Expression of mutant K-ras in HPNE cells caused a marked increase in growth in HPNE/K-ras and HPNE/K-ras/p16shRNA cell lines compared with control cell lines as determined by cell growth

curve, and cell cycle analysis (Fig. 21A & 21B). HPNE/K-ras and HPNE/K-ras/p16shRNA cell lines showed significantly increased cell proliferation and cell growth rate compared with their empty vector control cell line in cell growth curve (Fig. 21A). Expression of mutant K-ras in HPNE caused a markedly increase in the cell growth; however, silencing p16 in K-ras expressing cell line did not further enhance the cell growth. In cell cycle analysis, the percentages of cells in S phase for HPNE/K-ras and HPNE/K-ras/p16shRNA cell lines were 32.05% and 31.26%, respectively, and thus both were higher than that of the control cells (18.2%) (Fig. 21B). HPNE/K-ras cells with enhanced p16 expression displayed higher senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity than did control cells (Fig. 21C & 21D), the percentage of cells with SA- $\beta$ -gal activity in HPNE/K-ras cells was higher than that of control HPNE cells. These results suggested that the activation of K-ras increased senescence in small fractions of HPNE/K-ras clones. K-ras-induced senescence was p16-dependent, as the silencing of K-ras-upregulated p16 expression by p16shRNA abrogated the senescence in HPNE/K-ras/p16shRNA cells (Fig. 21C & 21D). These results indicated that p16 knockdown in HPNE cells expressing mutant K-ras did not further enhance cellular growth, but enabled HPNE cells to overcome senescence. These results indicated that HPNE cell lines with expression of mutant K-ras and knockdown p16 had acquired increased cell proliferation ability.

#### **The activation of K-ras and inactivation of p16 in HPNE cell line induced anchorage-independent cell growth in soft agar**

To examine whether HPNE cells had acquired the ability of anchorage-independent growth, a hallmark feature of *in vitro* cell transformation, we conducted soft agar assays to determine the transformation potential of mutant K-ras and knockdown of p16 in HPNE cells. HPNE cell lines did not form colonies in soft agar, whereas mutant K-ras induced colony formation. Furthermore, a combination of expression of mutant K-ras and p16 knockdown induced even more marked increases in the number and size of colonies (Fig. 22), indicating that inactivation of p16 increased the transformation potential of mutant K-ras. These results suggest that the specific combination of expression of activated K-ras and knockdown of p16 are sufficient to transform immortalized human pancreatic ductal cells *in vitro*.

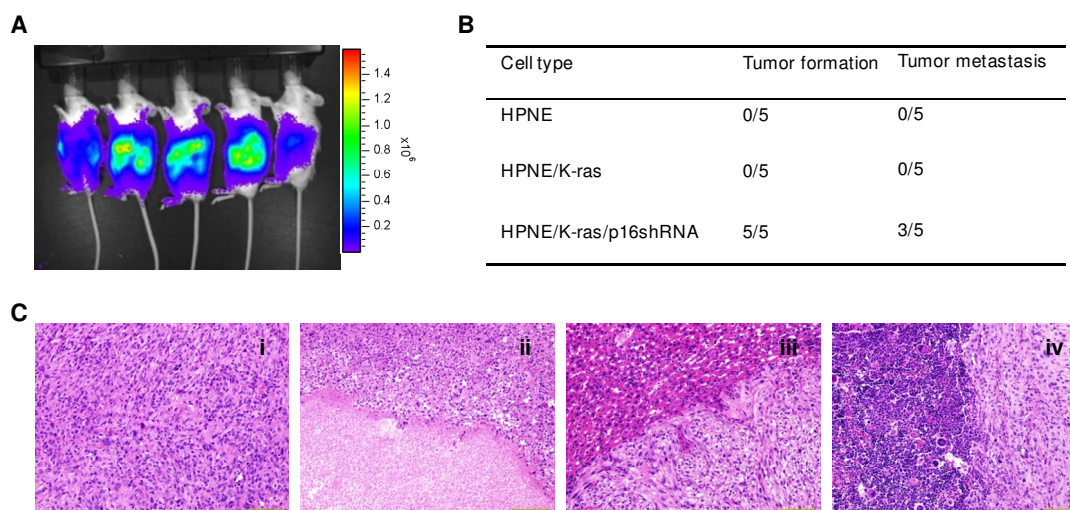




**Figure 22. Activation of K-ras and inactivation of p16 in HPNE cell line induced anchorage-independent cell growth in soft agar assay.** (A) The representative field of soft agar for each cell line is shown; (B) The quantification of colonies per field in soft agar is shown. \*:  $p < 0.05$ .

### **The activation of K-ras and inactivation of p16 in HPNE cell line induced tumorigenesis and metastasis by orthotopic tumorigenicity assay in NOD/SCID mice**

The tumorigenic potential of HPNE, HPNE/K-ras, and HPNE/K-ras/p16shRNA cells *in vivo* was examined by orthotopic tumorigenicity assays in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice.  $2 \times 10^6$  cells of each of these HPNE cell lines were injected into pancreas of mice. Tumor growth was monitored by *in vivo* bioluminescence imaging. HPNE and HPNE/K-ras cell lines did not grow tumors in mice even after 6 months' observation (Fig. 23A & 23B), indicating that activation of K-ras alone in HPNE cells was not sufficient for tumorigenic transformation. However, tumor formation was observed in 5/5 mice injected with HPNE/K-ras/p16shRNA cells (Fig. 23A & 23B). At 8 weeks' postinjection of HPNE/K-ras/p16shRNA cells, all mice had grown large tumors, became moribund, and were sacrificed. Subsequent necropsy revealed their tumors to have irregular shapes and multiple nodules. The tumors were highly aggressive, with metastases to the liver or spleen in 3/5 (60%) of mice identified by either gross examination of mice organs or hematoxylin and eosin (HE) staining sections of the liver and spleen (Fig. 23B & 23C). Histological analyses by HE staining showed that the tumors formed *in vivo* by HPNE/K-ras/p16shRNA cell lines were undifferentiated ductal carcinoma with sarcomatoid features (Fig. 23C). Tumors also displayed necrosis in many areas of tumor and many mitotic figures. The tumor cells had large pleomorphic nuclei and prominent nucleoli. The combination of K-ras activation and the loss of p16 in HPNE cells induced tumorigenesis in NOD/SCID mice, thus these results indicated that K-ras activation and the loss of p16 was sufficient to tumorigenically transform immortalized HPNE cells *in vivo*. These results suggest that activated K-ras and inactivated p16 play important roles in the development of human PDAC. Our results implied that our model using human pancreatic ductal cells faithfully mimics human pancreatic carcinogenesis.

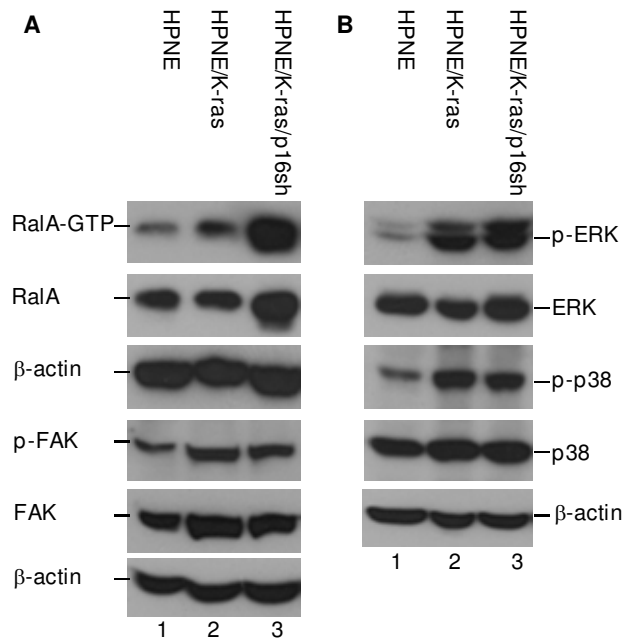


**Figure 23. Activation of K-ras and inactivation of p16 in HPNE cell line induced tumorigenesis and metastasis in the orthotopic mouse model.** (A) *In vivo* bioluminescence imaging of tumor growth for HPNE/K-ras/p16shRNA cell lines at 8 weeks' postinjection is shown. (B) The rate of tumor formation and metastasis in NOD/SCID mice is shown. (C) Representative micrographs showing the histopathology of the orthotopic tumors formed by HPNE/K-ras/p16shRNA cell lines by HE staining. (i) Undifferentiated ductal carcinoma with sarcomatoid feature, (ii) necrosis, (iii) liver metastasis, and (iv) spleen metastasis. Scale bar: 100  $\mu$ m.

We succeeded in isolating and culturing the tumor cell line HPNE/K-ras/p16p14shRNA T from the orthotopically growing tumors. In tissue culture plate, the tumor cells maintained their parental cell morphology and displayed comparable growth rate as the parental cell line HPNE/K-ras/p16shRNA.

#### **Aactivation of signaling pathway downstream of K-ras**

To dissect the molecular mechanisms of malignant transformation of HPNE cells, we first examined the three most common downstream signaling pathways of K-ras: RalGDS/Ral, RAF/MEK/ERK, and PI3K/AKT. To determine whether RalGDS-Ral is activated in transformed cell lines, we examined the RalA and RalB activity by precipitating Ral-GTP protein using Ral effector protein RalBP1. Very interestingly, we found that RalA activity was dramatically increased in transformed cell line HPNE/K-ras/p16shRNA cells and slightly increased in HPNE/K-ras cells compared with control cells (Fig. 24A). Immunoblot analysis revealed that the total level of RalA was also higher in HPNE/K-ras/p16shRNA cell line than in HPNE/K-ras and HPNE cell lines



**Figure 24. Activation of signaling pathways downstream of K-ras in HPNE cell lines.** (A) Activation of RalA and FAK in HPNE cell lines. RalA activity was detected in HPNE cell lines by Ral activity assay. The expression of total RalA, phosphorylated FAK, and total FAK was detected in HPNE cell lines by Western blot analysis. (B) Activation of MAPK signaling pathway by mutant K-ras in HPNE cell lines. The expression of phosphorylated ERK and phosphorylated p38 and their total protein levels was detected by Western blot analysis.

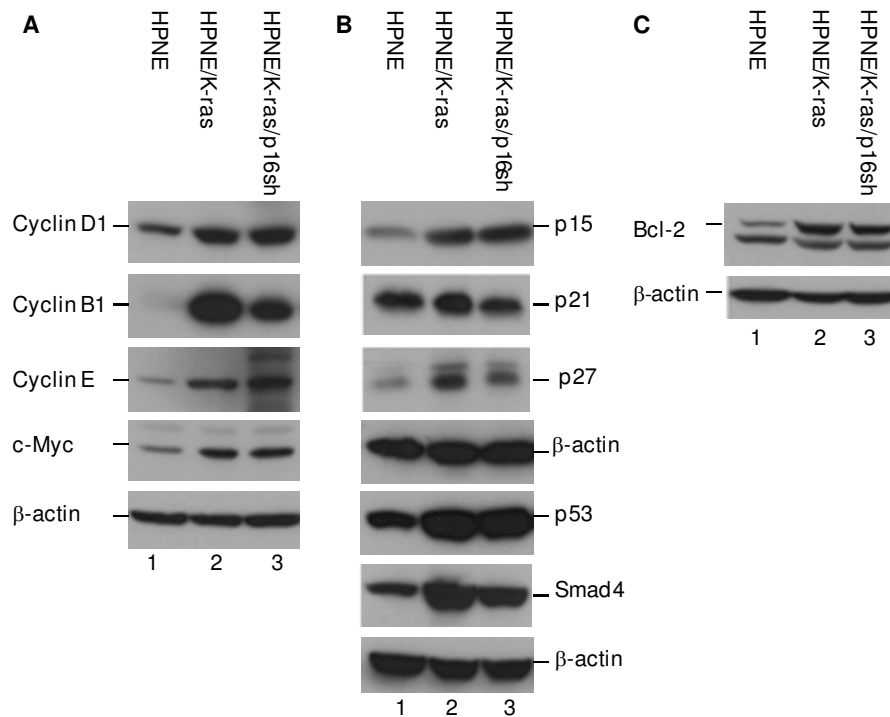
(Fig. 24A). However, there was no difference in activation of RalB among different cell lines (data not shown). We did not find activation of PI3K/AKT pathway in HPNE cell lines in Western blot analysis (data not shown). We also found the expression and activation of focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase regulating integrin and growth factor signaling, was induced by mutant K-ras in the HPNE/K-ras and HPNE/K-ras/p16shRNA cell lines (Fig. 24A). The total level and phosphorylated form of FAK (Tyr397) was remarkably enhanced compared with control cell line in Western blot analysis (Fig. 24A).

To explore whether mutant K-ras induced activation of downstream pathway MAPK ERK and p38, we examined the expression of phosphorylated protein level and total protein level in these cell lines by Western blot analysis. The mitogen-activated protein kinase (MAPK) pathway ERK and p38 were activated by activated K-ras in the HPNE/K-ras and HPNE/K-ras/p16shRNA cell lines (Fig. 24B). We found that the phosphorylated ERK, phosphorylated p38 were enhanced in the cell lines of HPNE/K-ras/p16shRNA and HPNE/K-ras compared with the control cell line, but their respective

total proteins levels were at comparable level (Fig. 24B). These results indicate that MAPK pathways ERK and p38 were activated in transformed HPNE cells. Taken together, these results suggested that mutant K-ras activated its downstream effectors–RalA, MAPK and FAK pathways, and that activation of these pathways may play critical roles in the transformation of HPNE cells.

### **Increased expression of cell proliferation related proteins by mutant K-ras**

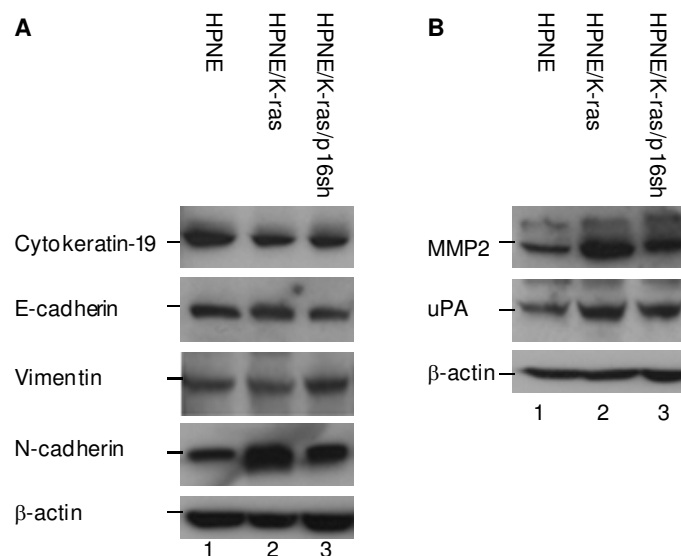
As Ras increased proliferation is due to induction of cell cycle regulators such as cyclins, and our results showed that mutant K-ras induced increased cell proliferation, we therefore examined whether the expression of cell proliferation-related genes cyclins and c-myc were altered in the transformed cells. We found that the expression of cyclin D1, cyclin E, cyclin B1, and c-Myc, which are involved in the regulation of cell cycle progression in the G1/S transition and the G2/M phases, as well as cell proliferation and growth, were significantly induced by mutant K-ras in HPNE/K-ras and HPNE/K-ras/p16shRNA cell lines (Fig. 25A). The expressions of cyclinD1, cyclinB1 and cyclin E was dramatically elevated in HPNE/K-ras and HPNE/K-ras/p16psh cell lines in Western blot analysis (Fig. 25A). The expression of c-myc was significantly enhanced in HPNE/K-ras and HPNE/K-ras/p16psh cell lines in Western blot analysis (Fig. 25A). However, we also found that K-ras upregulated the expression of cyclin-dependent kinase inhibitors- p15, p21, and p27 and apoptosis inhibitor Bcl-2 in HPNE cell lines (Fig. 25B & Fig. 25C), but the expression of p21 and p27 in the transformed cell line HPNE/K-ras/p16sh was lower than that of the HPNE/K-ras cell line (Fig. 25B). Hyperactivation of mitogenic stimulation may induce cyclin-dependent kinase inhibitors (CKI) such as p15, p16, p21. CKIs are strong inhibitors of cell proliferation and transformation. Overexpressions of c-myc and cyclins may abrogate cellular responses to a variety of growth-inhibitory signals, block the effect of CDK inhibitors, and thus induce increased cell proliferation in transformed cells. These results suggest that upregulation of cyclins and c-Myc by mutant K-ras may play a role in increased cell proliferation and transformation. As the late stage of PDAC always is accompanied by inactivation of p53 and Smad4, we examined the expression of p53 and Smad4 tumor suppressor genes in HPNE cell lines. We found that mutant K-ras also induced increased levels of p53 and Smad4 expression, but the Smad4 level was decreased in HPNE/K-ras/p16shRNA cells compared with HPNE/K-ras cells (Fig. 25B).



**Figure 25. Increased expression of cell proliferation related proteins by mutant K-ras in HPNE cell lines.** (A) The expression of cell cycle protein cyclins and c-Myc were increased by mutant K-ras in HPNE cell lines. Western blot analysis of the expression of cyclin D1, cyclin B1, and cyclin E, and c-Myc in HPNE cell lines. (B) The expression of cyclin-dependent kinase inhibitors, p53 and Smad4 in HPNE cell lines. The expression of cyclin-dependent kinase inhibitors p15, p21, p27, p53 and Smad4 was analyzed by Western blot in HPNE cell lines. (C) The expression of Bcl-2 in HPNE cell lines was detected by Western Blot analysis.

#### The expression of EMT marker and invasion related genes in HPNE cell lines

As epithelial to mesenchymal transition (EMT) plays a role in the tumor progression, and as our results above showed that the transformed cells were invasive, and formed metastasis to liver and spleen, we therefore determined whether activated K-ras and loss of p16 could induce changes of expression of EMT marker and other invasion related genes in the transformed cell line. We examined the expression of several EMT markers, uPA and MMP2 in HPNE cells by Western blot analysis. We found that the epithelial markers cytokeratin-19 and E-cadherin were expressed in HPNE cells and that the expression of cytokeratin-19 was slightly reduced by mutant K-ras. The expression of E-cadherin, and vimentin were not significantly changed (Fig. 26A). However, the expression of N-cadherin, matrix metalloproteinase 2 (MMP2), and urokinase plasminogen activator (uPA) were induced by activation of K-ras and were markedly increased in HPNE/K-ras and



**Figure 26. The expression of EMT markers and invasion-related proteins in HPNE cell lines.** (A) The expression of EMT markers in HPNE cell lines. The expression of cytokeratin-19, E-cadherin, vimentin, and N-cadherin was detected in HPNE cell lines by Western blot analysis. (B) The expression of invasion-related proteins MMP2 and uPA in HPNE cell lines was detected by Western blot analysis.

HPNE/K-ras/p16shRNA cell lines (Fig. 26A & 26B). Our results suggest that upregulation in the expression of N-cadherin, MMP2, and uPA may contribute to tumorigenic transformation and metastasis of HPNE cells.

### **The alterations of gene expression and signaling pathway for transformation in microarray analysis**

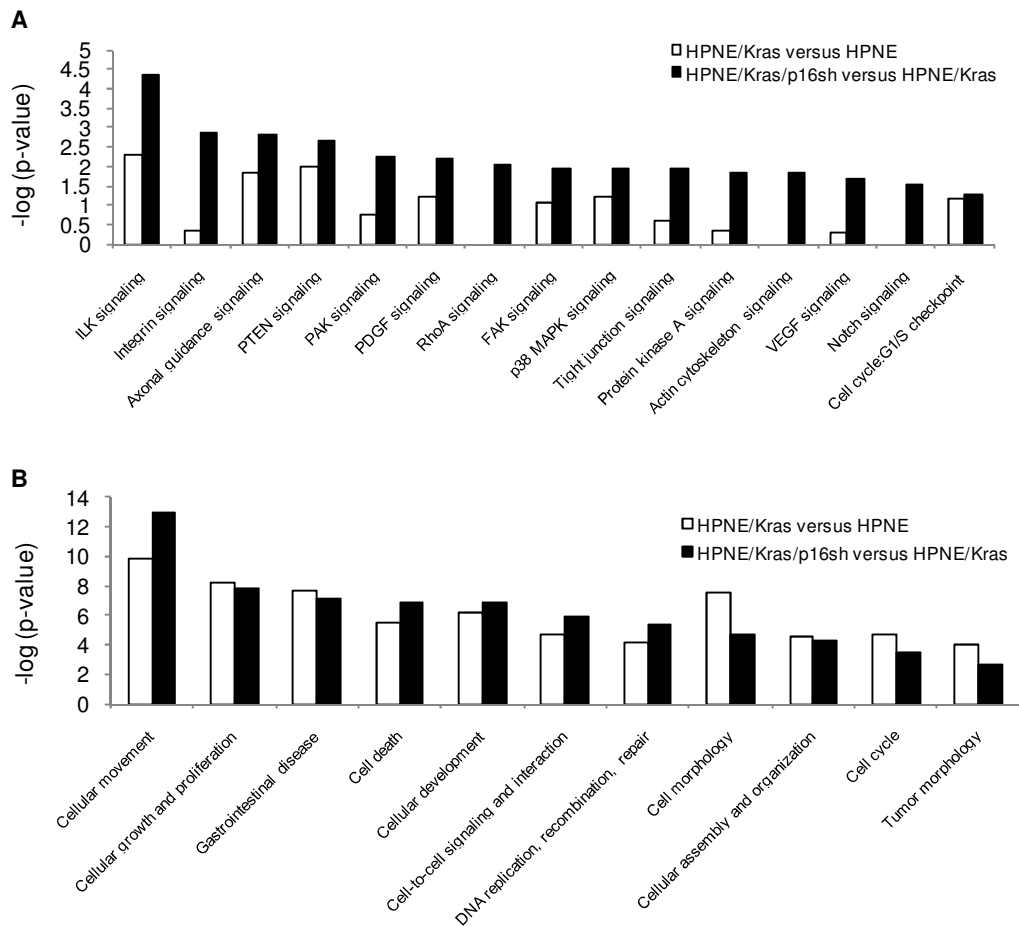
To explore the molecular mechanism of transformation and obtain further view into the detailed molecular and signaling pathway alterations involved in the transformation of pancreatic cells, we performed microarray analysis and Ingenuity Pathway Analysis (IPA) signaling pathway analysis with cDNA microarray data from HPNE, HPNE/K-ras, and HPNE/K-ras/p16shRNA cell lines. The top most significant gene alterations between HPNE/K-ras and HPNE, and between HPNE/K-ras/p16shRNA and HPNE/K-ras, were shown in Table 3. Some significantly altered expressions of

genes were the following: genes of up-regulation: IL13RA2, NOX4, CRK, PGR, ROBO1; genes of down-regulation: NEFL, MTSS1, PLXDC2, SEPP1.

**Table 3. The top most significant molecular changes for HPNE/K-ras versus HPNE and HPNE/K-ras/p16shRNA versus HPNE/K-ras in the microarray analysis.**

HPNE/K-ras versus HPNE				HPNE/K-ras/p16sh versus HPNE/K-ras			
Top molecules upregulated	Fold change	Top molecules downregulated	Fold change	Top molecules upregulated	Fold change	Top Molecules downregulated	Fold change
PITX2	304.09	PDPN	422.67	IL13RA2	176.20	SH3GL3	423.64
MEST	297.85	SOX17	353.18	TCF7L1	129.42	NEFL	358.92
POSTN	282.49	SH3GL3	334.97	NOX4	125.89	WWC1	331.89
TACSTD2	274.79	ARMC4	316.96	CRK	81.47	PDZD2	291.07
PCDH9	160.69	PDZD2	275.42	PGR	80.91	C21ORF129	138.68
GABRB1	159.22	CLMN	247.17	CNTN6	61.80	DAZL	137.40
FAM198B	140.28	CELF2	208.45	CHSY3	61.24	MTSS1	133.66
CRK	140.28	SLC35F3	180.30	MPDZ	54.95	PLXDC2	129.42
RNF150	136.14	CPPED1	143.55	FAM101B	53.83	RUNDC3B	119.12
MFAP5	135.52	NEFL	97.27	ROBO1	51.17	SEPP1	116.95

IPA canonical pathway analysis of the gene expression profiles between these cell lines demonstrated that the most significant signaling changes for transformation in HPNE/K-ras/p16shRNA versus HPDE/K-ras cell line were ILK, integrin, axonal guidance signaling, RhoA, actin cytoskeleton, and Notch signaling (Fig. 27A). The most significant alterations of biological functions associated with transformation were cellular movement, cellular growth and proliferation, gastrointestinal disease, and cell death (Fig. 27B). In conclusion, the IPA pathway analysis demonstrated that abnormal ILK signaling, integrin signaling and axonal guidance signaling were strongly associated with tumorigenic transformation of human pancreatic ductal cells.



**Figure 27. The alterations of signaling pathways for malignant transformation in the microarray analysis of the HPNE cell lines.** (A) IPA canonical signaling pathway analysis of microarray data. Bar indicates significance; the significance of an altered signaling pathway is expressed by  $-\log(p\text{-value})$  mapping to a signaling pathway in the IPA knowledge base; the length of the bar only indicates that the differentially expressed proteins are related to this pathway but does not indicate upregulation or downregulation of the pathway. (B) IPA biological function analysis of microarray data. Bar indicates significance; the significance of an altered biological function is expressed by  $-\log(p\text{-value})$  mapping to a biological function in the IPA knowledge base.



## Summary

In the present study, through the stepwise introduction of mutant K-ras and inactivation of p16 genes into the hTERT immortalized human pancreatic ductal HPNE cells, we developed a novel experimental cell culture model to recapitulate human pancreatic carcinogenesis. We demonstrated that the expression of mutant K-ras and the silencing of K-ras-upregulated p16 expression in hTERT-immortalized HPNE cells induced tumorigenic transformation. We found that the transformed cells exhibited increased cell proliferation and anchorage-independent growth in soft agar *in vitro* and tumor growth in an orthotopic mouse model *in vivo*. Histological analysis of the tumors formed by the HPNE/K-ras/p16shRNA cell line demonstrated an undifferentiated ductal carcinoma with sarcomatoid features and with metastasis to liver and spleen. Molecular analysis revealed that activation of K-ras downstream signaling pathways -RafA, FAK and MAPK signaling and upregulation of cyclins and c-myc were involved in the transformation. The IPA signaling pathway analysis from microarray data showed that abnormal ILK, integrin, RhoA, Notch, and actin cytoskeleton signalings were the most significant signaling alterations involved in the transformation of human pancreatic cells. These results suggest that expression of mutant K-ras and inactivation of p16 were sufficient for tumorigenic transformation of immortalized human pancreatic cells into pancreatic cancer cells and that mutation of K-ras and loss of p16 play important roles in the tumorigenic transformation. This novel transformation model is the first one in achieving malignant transformation of human pancreatic ductal cells without using any DNA tumor viral oncogenes, thus more closely recapitulates the development of PDAC from gene lesion, activation of specific signaling pathway. These findings enhance our understanding of the signaling pathways important for malignant transformation in PDAC.

## Discussion

### Conclusion

PDAC is the most lethal cancer in the world. The activation of K-ras, Her2, and inactivation of p16/p14 and Smad4 are the most common gene alterations in PDAC. We used two immortalized human pancreatic ductal cell lines as our in vitro system to establish experimental cell culture models with the most frequent gene alterations such as activation of K-ras and Her2, inactivation of p16/p14 and Smad4 in PDAC, to recapitulate human pancreatic carcinogenesis, to study the role of these signature molecular alterations in the development of PDAC, to determine which genes alterations are required to transform these cell lines, and to further dissect the molecular mechanism of transformation.

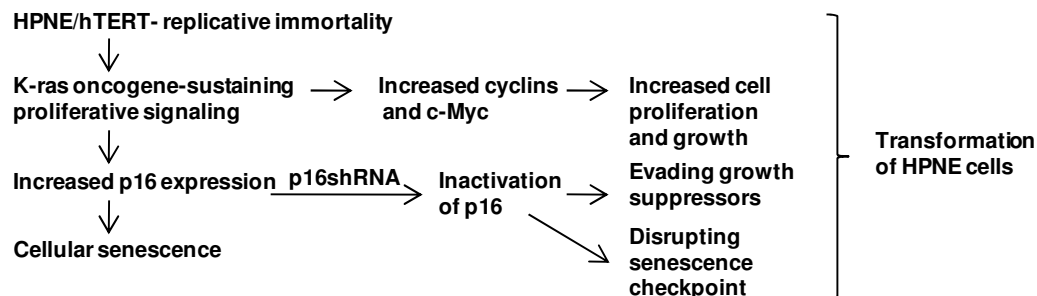
In the HPDE transformation study, we established a novel cell culture transformation model of human pancreatic ductal epithelial using E6E7 gene immortalized human pancreatic duct epithelial (HPDE) cell line. We demonstrated that sequential introduction of defined gene lesions frequently found in human PDAC including activation of K-ras, Her2, inactivation of p16/p14 and Smad4 genes, led to tumorigenic transformation of HPDE cells. We found that these transformed cells displayed increased cell proliferation, migration and invasion, formed colony in soft agar, and grew tumors with PDAC histopathological features in mice orthotopically. These results suggested that the combination of activation of K-ras and Her2, inactivation of p16/p14 and Smad4 was sufficient and essential to transform immortalized human pancreatic ductal epithelial into PDAC cells. Molecular analysis showed that the activation of K-ras and Her2 downstream signaling pathway MAPK signaling, upregulation of cell proliferation related genes such as cyclins, c-myc and Id2 were involved in the transformation. We then discovered that MDM2, BMP7 and Bmi-1 were overexpressed in the tumorigenic HPDE cells, our results implied that Smad4 plays important roles in the regulation of gene expression of BMP7 and Bmi-1 and the tumorigenic transformation of HPDE cells. IPA signaling pathway analysis from microarray data revealed that abnormal ILK signaling, and cell cycle dysregulation are the most significant changes of signaling pathways involved in the transformation of human pancreatic epithelial cells. This is the first report that a combination of the most common gene alterations in human PDAC induced completely tumorigenic transformation of human pancreatic ductal epithelial cells. This novel transformation model more accurately recapitulates the process of human pancreatic carcinogenesis in cell origin, gene lesion, activation of specific signaling pathway and histopathological features. This study largely enhances our understanding of the molecular mechanism of PDAC.

In the HPNE cell transformation study, we described the establishment of a relevant experimental cell culture model of human pancreatic cell carcinogenesis for studying altered signaling pathways driven by the two PDAC signature mutations –oncogenic K-ras and the loss of p16 expression. We found that the expression of p16 was induced by oncogenic K-ras in hTERT-immortalized HPNE cells and that further silencing mutant K-ras–upregulated p16 expression in HPNE cells resulted in tumorigenic transformation. The transformed cells displayed enhanced cell proliferation and an anchorage-independent growth in soft agar *in vitro* and formed tumors *in vivo* in an orthotopic xenograft mouse model, histological analysis revealed an undifferentiated ductal carcinoma with sarcomatoid features and with metastases. Molecular analysis showed that activation of K-ras downstream effectors –RafA, FAK and MAPK signaling together with upregulation of cyclins and c-myc were involved in the malignant transformation. Gene expression analysis and IPA signaling pathway analysis revealed that ILK, integrin, RhoA, Notch, and actin cytoskeleton signaling pathways are significantly involved in the transformation. This model is the first one for malignant transformation of human pancreatic ductal cells with the most common gene alterations in PDAC without using any DNA tumor viral oncogenes, thus more faithfully recapitulates the human pancreatic carcinogenesis.

### **The gene requirements for transformation of human pancreatic ductal cells HPDE and HPNE cells**

Cancer arises from normal cells through stepwise accumulation of genetic lesions. Tumorigenic transformation is a multi-step process. During the multistep development of human tumors, the cell acquires six biological capabilities of cancer hallmarks including sustaining proliferating signaling, evading growth suppressors, resisting cell death, acquiring replicative immortality, inducing angiogenesis, invasion and metastasis (145). Recent research in human cell culture transformation models have shown the malignant transformation of a variety of different normal human cells following introduction of a limited number of various combinations of genetic alterations (115-124). However, the many previous studies relied on the expression of DNA tumor viral oncogenes such as the SV40 early region encoding both the large T and small t antigens for malignant transformation (115-119, 125). In addition, these viral genes are not correlated with most of these human cancers. Furthermore, the role and cellular target of these viral genes in human carcinogenesis are still not well-understood which render the study of the molecular mechanisms of transformation more complicated. One striking difference for our models compared to other models is that our transformation models used the most frequent gene alterations in pancreatic cancer. In the HPNE

transformation study only the three most common gene alterations seen in PDAC: hTERT, mutant K-ras, and inactivation of p16, without using any DNA viral oncogene element, enabled

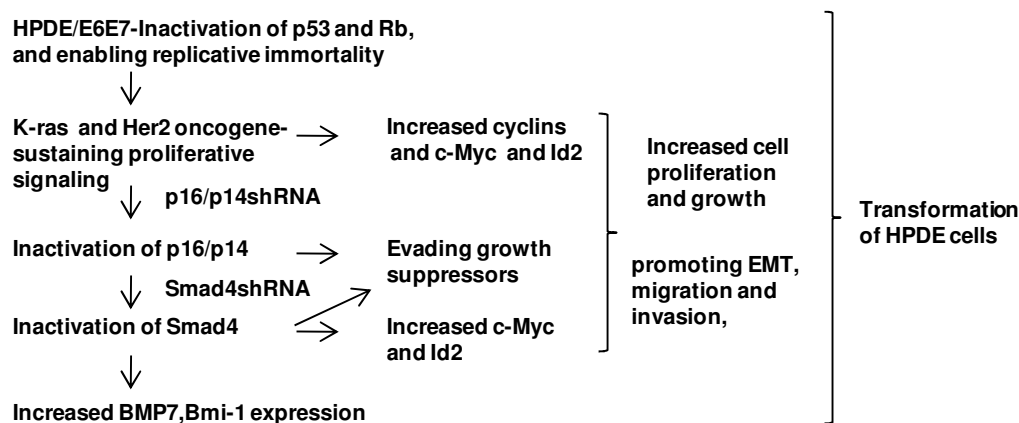


**Figure 28.**The proposed model for transformation of HPNE cell line. hTERT enabled cell to acquire replicating immortality, oncogenic K-ras rendered HPNE cells to obtain sustaining proliferative signaling, and thus the ability of increased cell proliferation and cell growth, inactivation of p16 by shRNA silencing enabled HPNE cells to evade growth suppressors and disrupt senescence checkpoint, and ultimately induce transformation of HPNE cells.

HPNE cells to acquire immortality, sustained proliferative signaling, evasion of growth suppressors, disruption of senescence checkpoint and ultimately attain tumorigenesis (Fig. 28). This is also the first study that mutated K-ras in combination with loss of p16 function induced tumorigenic transformation of human pancreatic ductal cells. We found that introduction of the mutant K-ras, inactivation of p16 into HPNE cells led to the rapid acquisition of a transformed phenotype as indicated by the ability of HPNE cells to form colonies in soft agar, exhibit increased cell proliferation and tumor formation in orthotopic mouse model. These results suggest that mutation of K-ras and inactivation of p16 play critical and cooperative role in PDAC progression. The present model thus more faithfully recapitulates the molecular mechanisms of human pancreatic carcinogenesis than previous models.

In the HPDE transformation model study, we show that sequential introduction of a limited set of genetic alterations frequently found in human PDAC sufficed to convert human pancreatic ductal epithelial cells into pancreatic cancer cells. This is the first study that K-ras and Her2 oncogene in conjunction with inactivation of p16/p14 and Smad4 function induced tumorigenic transformation in HPDE cells. The transformed HPDE cells exhibited increased cell proliferation, migration and invasiveness, anchorage-independent growth in soft agar and grew tumors in orthotopic mouse

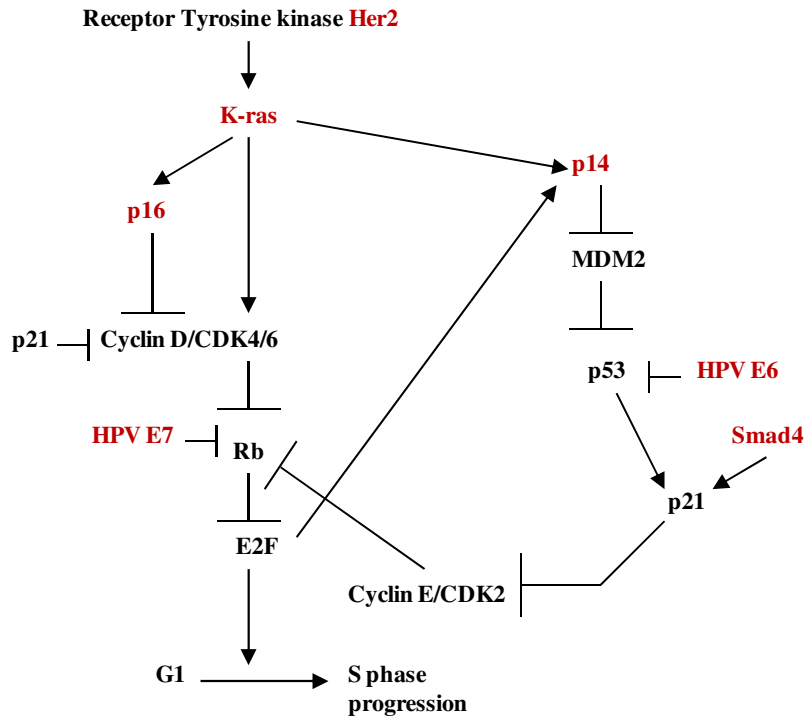
model, histopathological analysis displayed pancreatic ductal adenocarcinoma. This is the first report to achieve complete malignant transformation of human pancreatic ductal epithelial cells with defined gene alterations. One remarkable difference for this model from other models is that genetic elements used in this experimental transformation model are the most frequent gene alterations in PDAC: activation of K-ras and Her2, inactivation of p16/p14 and Smad4. These most common gene alterations enabled HPDE cells to acquire sustaining proliferative signaling, evade growth suppressors, and finally led to tumorigenic transformation (Fig.29). Therefore, this model more closely mimics the development of PDAC from molecular lesions and the histological characteristics of human PDAC than previous studies.



**Figure 29. The proposed model for transformation of HPDE cell line.** E6E7 inactivated p53 and Rb tumor suppressors, and also immortalized HPDE cell. Oncogenic K-ras and Her2 rendered HPDE cells to obtain sustaining proliferative signaling, and thus the ability of increased cell proliferation and cell growth. Inactivation of p16/p14 and Smad4 by shRNA silencing enabled HPDE cells to evade growth suppressors. Inactivation of Smad4 upregulated c-myc and Id2, and increased BMP7 and Bmi-1 expression. These alterations together induced increased cell proliferation and growth, prompted EMT, migration and invasion, and ultimately induced transformation of HPDE cells.

Our studies showed that different human pancreatic cell types had different requirements for malignant transformation. In the HPNE experimental model system, only three of the most common gene alterations in human pancreatic cancer –hTERT, mutant K-ras and inactivation of p16 are sufficient to transform the HPNE cell line. Whereas, in HPDE transformation model, disruption of six pathways including K-ras, Her2, p16/Rb, p53, p14 and Smad4 are required for tumorigenic

transformation of human pancreatic ductal epithelial cells (Fig. 30). The fact that more gene alterations are required for transformation of HPDE cells than HPNE cells indicates that more gene lesions hits are necessary for mature ductal cell transformation than progenitor HPNE cells. These results are similar with previous findings that different cell types have different specific requirements of gene alterations for transformation, which may be related with their intrinsic biological differences (134, 146). The difference in requirements of gene lesions for transformation may reflect



**Figure 30. Overview of the interplay of the major genetic alterations identified in PDAC and gene alterations used in our transformation models.** All major genetic alterations including K-ras, Her2, p16, p14, p53 and Smad4 directly or indirectly regulates the G1/S phase cell cycle progression. Genes used in our model are indicated in Red.

the nature of the different cells. The less number of gene alterations required for HPNE transformation than HPDE cell may be explained by the fact that HPNE cell is a progenitor, undeveloped cell, which is easier to transform than mature and differentiated HPDE cell. Similar findings were reported from mouse model. Mouse model of expressing mutant K-ras in differentiated pancreatic cells (acinar or ductal) do not progress to PanINs or PDAC. The expression of K-ras in embryonic pancreatic cells induced PDAC, while adult pancreatic cells are refractory to

transformation by mutant K-ras and require the induction of chronic pancreatitis to induce PDAC (147). Expression of K-ras under the control of cytokeratin-19 promoter target to mature ductal epithelial cells failed to induce PanINs or PDAC. These results suggested that mature ductal cells are resistant to K-ras induced transformation (148, 149).

One limitation in our models is the strategy with the shRNA experiment, knockdown is unlikely to be complete, because basal levels of p16 and Smad4 still can be detected compared with knock out experiments. Thereby, the effects of knockdown could be affected by the basal levels of p16 and Smad4 and thus may not be as prominent as knock out in mouse model. In HPDE model, the limitation of this model is that viral gene E6E7 was used to immortalize the HPDE cell line. Therefore, future study would investigate the transformation potential of the human pancreatic epithelial cell without using any viral gene.

### **Ras level and transformation**

There are different reports regarding the requirement of Ras expression level for transformation (116). Elenbaas showed that the tumorigenicity of the transformed human mammary epithelial cells by SV40 large T antigen, the telomerase catalytic subunit, and oncogenic H-ras, was dependent on the levels of Ras oncogene expression. High-level of oncogenic Ras expression was required for transformation (116). In contrast, other studies demonstrated that overexpression of mutant K-ras protein was not required for transformation. High levels of Ras expression may result in senescence (150). There may exist a selection against cells with overexpression of K-ras, leaving a population with moderate expression of activated K-ras to undergo transformation. In fact, lower levels of oncogenic Ras more closely approximate those observed in human tumors. In the HPNE cell transformation model, the Ras expression in transformed cells with mutant K-ras and knockdown p16 was decreased compared with the K-ras expressing cell line, indicating that high level of Ras expression is not required for transformation of HPNE cells.

### **The role of p16 and p14 in proliferation**

Both p16 and p14 are involved in the regulation of cell cycle and cell proliferation, p14 regulates cell cycle progression by inducing p53-dependent cell cycle arrest. Our results showed that knockdown of p14 and p16 further enhanced cell growth compared with knockdown of p16 alone in HPDE/K-ras cells, indicating that the loss of p14 and p16 simultaneously gives cells additional growth advantage compared with the loss of p16 alone. These results suggest that the p16 levels in these cells were not growth limiting, the loss of p14 increases the cell growth potential but did not change the tumorigenic effect between HPDE/K-ras /p16sh and HPDE/K-ras/p16p14sh cell line.

This result is consistent with the previous studies in human fibroblasts. Knockdown of p14 or p53 had a much more potent growth accelerating effects in human fibroblast cells that had reduced p16 protein levels compared with wild type cells. Suppression of p16 expression by knock down does not affect cellular proliferation, but synergizes with p53 loss to accelerate growth and cause transformation. Loss of p14 and p16 simultaneously caused a dramatic increase in growth speed compared to cells that have lost p16 expression alone (151, 152). Our results indicate that p16/p14 functions to suppress mitogenic stimulation K-ras and Her2 induced cell proliferation, loss of p16/p14 release the limitation for proliferation, thus induce increased cell proliferation in HPDE cells.

### **The role of p16 in oncogene-induced senescence, proliferation and transformation**

Oncogene-induced senescence (OIS) in cells harboring oncogenes such as myc, or Ras is thought to form an important barrier to cancer (150). Senescence is characterized by a flattened and enlarged cellular morphology, a large nucleus with a prominent nucleolus, chromatin reorganization, and activation of the p16 and p53 pathways, expression of senescence-associated  $\beta$ -galactosidase activity and senescence-associated heterochromatic foci (150).

In response to activation of K-ras, normal cells undergo growth arrest or cellular senescence to protect against transformation (153, 154). The different responses to activation of Ras are dependent on the cellular contexts (150, 154-158). Oncogenic K-ras-induced proliferation or senescence is dependent on its levels of expression (157, 159). Ras-induced senescence is also dependent on signal intensity. Overexpression of oncogenic Ras at super physiological level induces senescence; whereas it's normal levels or expression at physiological level does not activate tumor suppressor pathways and trigger growth arrest and senescence (150, 157, 158, 160-162). Our results showed that high levels of mutant K-ras expression in HPNE cells induced senescence in a small percentage of cells. These K-ras expressing cells also had enhanced proliferation rate. A recent study showed that expression of mutant-K-ras also inhibits senescence in pancreatic ductal cells (163). These results suggest that high level of K-ras in a small of percentage of cells triggered senescence, whereas expression of K-ras in most of these cells induced proliferation in HPNE cells.

Oncogenic Ras-induced senescence in primary human and rodent cells is associated with accumulation of p53 and p16. In response to Ras activation, cells undergo senescence which is abrogated by disruption of p53 or p16/Rb pathways (150, 159). Expression of mutant K-ras induces high level of p16 expression and senescence, the loss of p16 in cancer could be one key mechanism required to overcome growth arrest and senescence in mutated cells, thereby to abrogate senescence and allow tumor progression to full malignancy (162, 164). p16 is a potent inducer of senescence in



many cell types (165, 166). p16 level determines how a cell will respond to Ras, some cell types with lower p16 level are resistant to Ras-induced arrest (150, 153, 167-169).

OIS occurs during the early stages of tumorigenesis. Senescent cells are abundant in premalignant lesions or benign lesions which harbor activated oncogenes. In contrast, senescent cells were rare in malignant tumors due to loss of senescence effectors p16 or p53 (156, 160, 170-174). OIS has recently been described *in vivo* in preneoplastic tissues of both the mouse and human (160, 170, 172, 175). K-ras trigger senescence during early stage of tumorigenesis in the lung and pancreas, mutant K-ras mouse model developed PanIN preneoplastic lesions in the pancreas and lung with expression of p16 and displayed senescence; whereas senescence was negative in PDAC (170, 173, 176). Inactivation of senescence program results in the progression of the precursor lesions to malignant cancer in mouse model (160, 162, 176, 177). OIS acts as an important tumor suppressive mechanism and a barrier to cell transformation and tumorigenesis by restricting unchecked proliferation of cells with oncogenic mutations (178). The ability to escape senescence is a necessary step for cell tumorigenic transformation and one of the hallmarks of cancer cells (179). Therefore, disruption of senescence checkpoint by oncogenic activation or inactivation of tumor suppressor such as p53, p16 not only results in escaping from Ras-induced senescence, but also greatly promotes oncogenic transformation and causes the progression of tumors to the malignant stage (162, 164).

p16 is not normally expressed or typically difficult to detect in normal adult tissues(180). However, it is induced in various situations of stress and is highly expressed in senescent cells (166, 181). The level of p16 expression is upregulated by overexpression of K-ras in normal human and mouse cells (150). The level of p16 is low in normal HPNE cells, the marked increase in expression of p16 induced by mutant K-ras in HPNE cells is consistent with those earlier reports in which p16 expression in primary human fibroblasts was stimulated by Ras<sup>V12</sup> (150, 152). The upregulation of p16 expression in cells with mutant Ras is regarded as an important barrier to transformation. In fact, p16 plays a critical role in blocking the progression of K-ras-initiated pancreatic neoplasms into invasive or metastatic PDAC in mouse models (28, 30). Our results demonstrated that mutant K-ras induced increased level of p16 and silencing enhanced p16 caused tumorigenic transformation of HPNE cells. Upregulation of p16 by Ras mutation may restrain the proliferation signals provided by mutant K-ras thus blocking proliferation; loss of p16 release the inhibition on proliferation, thus promoting cell proliferation and cell transformation. In our model, mutant K-ras promoted cell proliferation, but loss of p16 did not further increase growth in K-ras-expressing HPNE cells, indicating that p16 may have other roles in promoting transformation beyond its role in cell cycle regulation. However, we found that silencing K-ras-upregulated p16 expression abrogated oncogenic

K-ras–induced senescence in HPNE cells. Induction of senescence is also one of roles of p16 tumor suppression (182). These results suggest that inactivation of p16 is one key mechanism required to overcome growth arrest, disrupt senescence checkpoint, and thus trigger cell transformation in mutated K-ras cells.

### **Role of Smad4 in proliferation and EMT regulation and transformation**

The role of Smad4 in tumor-promoting effects of TGF  $\beta$  signaling has been studied for years; however, the molecular mechanism of how inactivation of Smad4 contributes to the tumorigenesis and progression of PDAC is still not well understood. The function of inactivation of Smad4 in tumorigenesis may be involved in its central role in TGF  $\beta$ -mediated growth inhibition, cell proliferation and EMT. Inactivation of Smad4 leads to disruption of TGF  $\beta$  signaling in the regulation of cell cycle and cell proliferation by dysregulating the expression of Smad4 downstream genes such as p21, p15, p27, c-myc, Ids (91, 93, 94, 107).

Loss of Smad4 plays an important role in the transformation of HPDE cells. Our study demonstrated that there is not only no significant difference of *in vitro* growth rate in cell culture and soft agar but also no significant difference in the ability of *in vitro* cell migration and invasion between the two cell lines HPDE/K-ras/Her2/p16p14shRNA with or without Smad4shRNA cell line. Only HPDE/K-ras/Her2/p16p14shRNA/Smad4shRNA grew tumors in SCID mice, this result suggest that Smad4 plays an important role in tumorigenic transformation. This result is consistent with previous findings from mouse and human studies. The results from mouse model showed that inactivation of Smad4 accelerates the progression of K-ras-initiated pancreatic cancer (31, 32, 105). Restoration of Smad4 in human pancreatic cancer cells suppressed tumor growth *in vivo* (92, 93, 95-97).

Previous studies showed that Smad4 plays an important role in the regulation of cytostatic response of TGF  $\beta$  signaling. We found that Smad4 inactivation was not significantly associated with cell proliferation of HPDE cells. To investigate the role of Smad4 in transformation, we examined the expression of EMT marker proteins in HPDE cells. We found that inactivation of Smad4 reduced the expression of mesenchymal marker N-cadherin and vimentin, and epithelial marker cytokeratin simultaneously in transformed cell line. Our results showed that inactivation of Smad4 reduced the expression of epithelial marker pancytokeratin and cytokeratin-19. Cytokeratins are components of intermediate filament system and regulate the tissue homeostasis of high-turnover epithelia, downregulation of cytokeratin was found in tumor tissues. The pattern of expression of cytokeratin is a marker of cell differentiation and transformation (183). Decreased expression of cytokeratins in Smad4-inactivated cells suggests a more mesenchymal phenotype in Smad4

knockdown cells. We found that inactivation of Smad4 increased the ability of cell migration and invasion in some HPDE cell lines, knockdown Smad4 increased cell invasion *in vivo* for HPDE/Kras/Her2/p16p14sRNAh/Smad4shRNA tumorigenic cell line, tumor invaded locally. Therefore, the overall effect of Smad4 inactivation may promote EMT in HPDE cells. These results are consistent with the findings from previous studies in human PDAC cells, which showed that expression of Smad4 in Smad4-deficiency PDAC cell line suppressed TGF  $\beta$ -induced cell motility and invasion, and inactivation of Smad4 by knockdown in Smad4 intact PDAC cell line significantly enhanced TGF $\beta$ -induced invasion (98, 99). There are contradictory reports about the role of Smad4 in regulation of EMT. Mouse model showed that Smad4 is required for TGF  $\beta$ -induced EMT, loss of Smad4 results in decreased EMT in the murine pancreatic cancer (31). In contrast, another study described that Smad4 is required for TGF $\beta$ -induced cell cycle arrest and migration, but not EMT in keratinocyte and human pancreatic cancer cell line (108). The role of Smad4 in EMT regulation is still elusive and need to be further explored.

Our results showed that transformed HPDE cells with Smad4 inactivation formed differentiated PDAC histology with expression of epithelial marker cytokeratin-19 and E-cadherin. This result is similar with the findings from mouse model. Smad4 deletion induced well-differentiated PDAC histopathology in the K-ras/p16INK4A/p14ARF deletion model compared with mice with intact Smad4 (31). This result also is consistent with the study of human PDAC cell lines, Smad4 expression was associated with poor cell differentiation in human PDAC cell lines, poor differentiation was observed in 4/6 Smad4 wild-type compared with 0/5 Smad4 mutant cell lines (184). Furthermore, in a study of xenografts from 16 human PDAC cell lines, undifferentiated histology was observed only in tumors derived from Smad4 wild-type cell lines (31). Loss of Smad4 results in a well-differentiated tumor histopathology, suggesting disruption of TGF  $\beta$ -driven EMT.

### **Cyclins, c-myc and Id2 in cell proliferation of transformed cell lines**

One hallmark characteristic of cancer is enhanced proliferation and growth which is controlled by the cell cycle. We found that both the transformed HPNE cell line with expression of mutant K-ras and knockdown of p16 and the transformed HPDE cell line with expression of mutant K-ras, Her2, and knockdown of p16/p14 and Smad4 displayed increased cell proliferation rate. The expression of cell cycle proteins cyclin B1, cyclin D1 and cyclin E in the transformed HPNE cell line, and the levels of cyclin B1 and cyclin D1 in the transformed HPDE cell line were elevated. As cyclin D1 and cyclin E are involved in the regulation of cell cycle progression of G1/S transition and cyclin B1 is associated with transition G2/M phase. These results suggested that dysregulation of cell cycle is responsible for the increased cell proliferation and transformation.

Our results showed that c-myc was overexpressed in both of the transformed HPDE and HPNE cell lines. c-myc is an important transcription inducer of cell division, and is involved in regulation of cell cycle progression, cell growth and cell proliferation, stem cell self-renewal and transformation (185, 186). The upregulation of c-myc in transformed cell lines indicate that c-myc is correlated with increased cell proliferation and transformation.

We found that the expression of Id2 was increased in the transformed HPDE cell line. Id2 is a nuclear factor that functions as inhibitor of differentiation and negative regulator of bHLH transcription factors, Id2 is implicated in the regulation of cell cycle and cell proliferation (187-190). It promotes cell proliferation by interacting with and inactivating the tumor suppressor Rb (191) and interfering with proliferation bHLH transcription factors (192). Id2 is frequently overexpressed in pancreatic cancer samples and pancreatic cancer cell lines, increased Id2 expression may be associated with enhanced proliferative potential of pancreatic cancer cells (193, 194). The enhanced Id2 expression in the transformed HPDE suggested that Id2 may play a role in increased cell proliferation and transformation.

We found CDK inhibitors p15, p21, p27 were induced by mitogenic stimulation K-ras in HPNE cell lines, and thus may block K-ras induced transformation. Overexpression of c-myc, Id2 and cyclins may blunt the effect of CDK inhibitors, thus may induce increased cell proliferation in transformed cells (195).

### **EMT regulation and transformation**

Epithelial-mesenchymal transition (EMT) is a biological process by which an epithelial cell loses features of epithelial-cell and acquires properties of mesenchymal cell and becomes more motile (196-198). During EMT, cell-cell junctions are altered; epithelial cells lose their polarity and cell to cell contact, reorganize the actin cytoskeleton, and acquire a fibroblastoid morphology, and migration and invasive phenotype. Cells undergoing EMT display downregulation of epithelial marker proteins (E-cadherin, cytokeratin) and other components of epithelial cell junctions, and upregulation of mesenchymal marker proteins (vimentin,  $\alpha$ -smooth muscle actin and N-cadherin). EMT is associated with the acquisition of invasive potential, elevated cell migration and invasion is a hallmark of cancer, and is believed to be involved in carcinogenesis and plays an important role in tumor progression by stimulating invasiveness and metastasis (196, 197).

Local invasion and distant metastasis are hallmark of pancreatic cancer; our results showed that transformed HPNE cells exhibit invasion and metastasis. We therefore examined the EMT marker changes in HPNE cells. Our results showed that K-ras induced increased N-cadherin and slight decreased cytokeratin-19 expression in HPNE cells. These results suggest that EMT is involved in

transformation of HPNE cells. Lee (144) showed that HPNE cells have the ability to differentiate to pancreatic ductal cells. Treatment of HPNE with inhibitors of histone deacetylases (sodium butyrate) and DNA methyltransferases (5-aza-2'-deoxycytidine) lead to the formation of pancreatic ductal cells marked by the expression of p-glycoprotein 9, multidrug resistance MDR-1, carbonic anhydrase II, cytokeratin 7, 8 and 19. The expression of E-cadherin and cytokeratin 19 in HPNE cell line indicates that these HPNE cells have bi-potential or multipotential ductal stem cell characteristics.

Our results showed that uPA and MMP2 were overexpressed in tumorigenic HPNE cells. uPA plays a crucial role in pericellular proteolysis during cell migration and tissue remodelling. uPA convert plasminogen to plasmin, which degrades fibrin, type IV collagen, fibronectin, and laminin and activates latent collagenases. Overexpression of uPA is found in pancreatic cancer (199). The matrix metalloproteinase 2 (MMP2) is a type IV collagenase, a zinc-containing proteolytic enzyme that break down extracellular matrix proteins and plays an important role in cancer invasion. The level of MMP enzyme activity has been demonstrated to be correlated with tumor grade, regional lymph node metastases, and distant metastases. MMP-2 is overexpressed in pancreatic cancer (200, 201). The expression of these MMPs has been shown to directly correlate with invasion and metastasis in pancreatic cancer. Overexpression of uPA and MMP2 in the transformed and metastatic HPNE cells suggest that uPA and MMP2 play roles in transformation and metastasis of HPNE cells.

### **p16 and Her2 in EMT regulation**

Our results showed that different cell lines have different growth rate and migration, invasion. HPDE/K-ras/p16shRNA cell line display many EMT characteristics, showed fibroblast-like morphology in culture dish, increased cell migration and invasion, markedly increased EMT protein expression such as vimentin and N-cadherin. However, this cell line is not tumorigenic in mice. It seems that p16 is also involved in the regulation of EMT and EMT protein expression, inactivation of p16 promoted EMT in HPDE-K-ras cell as it increased mesenchymal marker expression and induced migration and invasion. A recent report showed that p16 is involved in the regulation of myocardin expression and the differentiation process which is at least partially responsible for p16 tumor-suppressor function (202). The role of inactivation of p16 in EMT regulation need to be further investigated.

Our results showed that Her2 is involved in regulation of EMT; it reduced the expression of pancytokeratin in HPDE cell line. This result is consistent with the report which showed that Her2 expression induced EMT, increased vimentin and N-cadherin expression in mammary epithelial cells (203).

### **Histopathological characteristics recapitulate PDAC**

Histological analysis of the tumors formed by transformed HPDE/-K-ras/Her2/p16p14shRNA/Smad4shRNA cell line revealed pancreatic ductal adenocarcinoma feature, and the tumors were locally invasive, as characterized by infiltration into the neighboring muscle tissue. This result is different from the tumor formed by HPDE/K-ras cell line reported from Tsdao's group. The tumor was a poorly differentiated carcinoma. This result is also distinct from K-ras mouse model that showed a sarcomatoid or a poorly differentiated histology (27, 28, 30). One of the remarkable features of PDAC is that it almost always elicits an intense desmoplastic host reaction. Histology analysis showed that stromal fibroblasts were found in HPDE tumors. These results suggest that this model more closely recapitulated histological characteristic of human PDAC. In addition, we did not observe metastasis to other organs in mice, suggesting that additional genetic or epigenic changes may be necessary to acquire a metastasis phenotype.

Histological analysis of the tumors formed by the transformed HPNE/-K-ras/p16p14shRNA cell line revealed undifferentiated ductal carcinoma with sarcomatoid feature, a poorly differentiated histology and the tumors were invasive and metastatic to liver and spleen. The histological features of these tumors are consistent with the results found in a previous mouse model of mutant K-ras and deletion of p16INK4A/p14ARF (28, 30) with a higher propensity for poorly differentiated PDAC histology, undifferentiated sarcomatoid features. In human, ductal adenocarcinoma histology predominates, sarcomatoid subtypes are uncommon variants of PDAC with more aggressive clinical behavior (1, 8, 204). Undifferentiated carcinomas are among the most aggressive of all the carcinoma of pancreas, the median survival is only 5 months (204, 205). The different cell lines form different histological subtypes, suggesting that cell origin also affects the histological differentiation of tumors.

### **Cell origin of PDAC**

The cell origin of PDAC has remained elusive; it is unclear whether PDAC arises from duct cells, acinar cell, islet cells, stem cell or progenitor cell in the pancreas. Ductal cells have been widely believed as the cell origin since PDAC cells morphologically resemble pancreatic ductal cells at the histological levels, displaying cuboidal shape, formation of tubular structures, and expression of ductal markers (7, 10). There are many evidences to support that PDAC arise from ductal cells. Precursor PanIN lesions are located in ducts. Studies from mouse model showed that PDAC developed from PanIN lesions to invasive cancer (28, 206).

Although the histologic appearance of PDAC suggests a ductal origin, stem cells and other differentiated cells such as acinar cells have been suggested as potential origin. Mouse model showed that endogenous expression of mutated K-ras in the nestin-expressing pancreatic exocrine progenitor cells induced pancreatic intraepithelial neoplasms (PanINs) lesions as in a Pdx1-Cre/LSL-K-ras model with K-ras<sup>G12D</sup> targeting to the whole pancreas, this result suggested that the adult nestin-expressing cells are the cells origin of PDAC, a progenitor cell origin for PDAC (207, 208). The frequent observations of premalignant acinar-to-ductal metaplasia in humans and mouse models suggest an acinar origin (208, 209). Acinar cells are capable of giving rise to PanIN-like lesions and PDAC in the mouse model, expressing oncogene K-ras in acinar cells of mouse pancreas showed that precursor PanIN lesions transdifferentiation of acinar cells led to mPanIN and PDAC (147, 210, 211).

Mouse models can't address the cellular origin of PDAC as mouse models using PDX1 (pancreatic and duodenal homeobox gene 1) or Ptf1a (pancreatic transcription factor 1a) promoter target mutant K-ras in all pancreatic progenitor cells including endocrine and exocrine pancreas, also including both acinar and duct cells, making it impossible to determine the cell origin of PDAC (28). A recent study showed that targeting expression of K-ras<sup>G12D</sup> mutation to pancreatic ducts using the CK19CreERT allele induced small numbers of mucinous metaplastic lesions with the morphological and molecular characteristics of early PanINs, but not PDAC. These results indicated that pancreatic ducts may have the potential to give rise to pancreatic cancer (212). Therefore, a defined cell culture based system or more differentiated pancreatic cell lineage will be a better model to address the question of the cell origin of PDAC and allow us to test the hypothesis that ducts could be the origin of PDAC. Our results demonstrated that human pancreatic ductal cells gave rise to PDAC, thus providing support for the hypothesis that ductal cells are the cell origin of PDAC. PDAC arise from progenitor HPNE cells suggesting a progenitor origin of PDAC. Two different pancreatic ductal cell lines gave rise to PDAC but formed different histology in our cell culture models, suggesting that cell origin influences tumor pathological feature of PDAC. Understanding how PDAC arises is important for finding new biomarkers and developing new targets for treatment of PDAC.

### **Activation of signaling pathway downstream of K-ras in transformation**

Dissecting the underlying mechanism of transformation, especially dissecting which K-ras effector pathways are critical in tumorigenic transformation would lead to the finding novel therapeutic targets for human PDAC. The three principal Ras downstream effector pathways RAF/MEK/ERK, RalGDS/Ral, PI3K/AKT play pivotal roles in Ras-triggered transformation and tumorigenesis (17, 18). Our results showed that mitogen-activated kinase (MAPK) –ERK and p38

were activated in both the transformed HPDE and HPNE cells. Activation of ERK MAPK signaling cascade leads to cell proliferation, resistance to apoptosis, and cell migration. Activation of MAPK pathway is found in a variety of transformed cells and mediates the transformation induced by Ras, RAF and other oncogenes, and is recognized as one of the hallmarks in cancer models. Therefore, inhibition of MEK/ERK signaling pathway is a promising therapeutic target in pancreatic cancer, and results in decreased cell proliferation and cell growth (213, 214). In our two models, we found ERK MAPK was activated by K-ras in transformed cell line, suggesting that K-ras-induced ERK MAPK activation may play a role in the malignant transformation of human pancreatic ductal cells.

p38 MAPK pathway is involved in regulation of cell cycle and cytoskeleton remodeling, apoptosis, and differentiation. The role of p38 in cancer is complex. The function of p38 in cancer may depend on the cellular context, tumor cell type and tumor stage. There are many evidences to support its role as a tumor suppressor to inhibit cell growth and induce apoptosis (215-217). p38 may function as an oncogene to promote cancer progression such as invasion and migration (218). Increased level of p38 phosphorylation has been associated with malignancy in various cancers. Several studies showed that p38 MAP kinase pathway is required for TGF  $\beta$  induced EMT and cell migration in different cells (219-221). Our results showed that p38 MAPK are activated in both transformed HPDE and HPNE cells, indicating that activation of p38MAPK pathway may play an important role in the tumorigenic transformation of human pancreatic ductal cells. However, the role of p38 activation in pancreatic cancer remains to be further investigated. The activation of MAPK-ERK and p38 in transformed cell line suggest that these pathways may play an important role in the tumorigenic transformation of human pancreatic ductal cells.

RalA is a small GTPase. It mediates diverse cellular effects including cellular motility, proliferation, cytoskeletal organization, and transformation. Previous studies showed that activation of RalA signaling is a crucial effector involved in Ras-induced transformation and tumorigenesis of human cells (222). Recent studies have demonstrated that elevated levels of active RalA were frequently detected in human PDAC tumor tissues and cell lines (222, 223). Recently, Lim showed that RalA not RalB is critical for PDAC cell line anchorage-independent growth *in vitro* and tumorigenic growth *in vivo* (222, 223). Inhibition of RalA by siRNA suppresses anchorage-independent growth *in vitro* and tumorigenic growth *in vivo* of human PDAC cell lines (223, 224). Knockdown of RalA also suppressed tumorigenicity of Ras-transformed human cells. Our results showed that RalA is highly activated in transformed HPNE/K-ras/p16shRNA cell line, indicating that RalA may play a critical role in Ras-driven tumorigenic transformation of HPNE cells. As the strategy for targeting oncogenic K-ras such as K-ras inhibitor always failed, and as RalA is critical



for Ras-mediated tumor growth of human PDAC cells, targeting specific Ras downstream effector pathway such as inhibiting RalA function may be an effective anti-Ras strategy for cancer treatment.

The results of different Ras effectors activation in tumorigenic HPDE and HPNE cells is similar to previous findings that different human cell types have distinct requirements of Ras effector pathways for transformation (17, 134, 225). Ras effector pathway may be modulated by distinct cell-type specific signaling pathways, pancreatic cancer cell lines harboring with mutated K-ras differ in other genetic lesions, Ras can have vastly different effects dependent on cellular gene lesions context (17).

Focal adhesion kinase (FAK) is non-receptor cytoplasmic protein tyrosine kinase, localized in cellular focal adhesion. FAK is associated with integrin and growth factor signaling and regulates cell proliferation, cell growth, survival, cell adhesion, angiogenesis migration and invasion of tumor cells (226, 227). Enhanced expression of FAK was found in human PDAC (227, 228); FAK expression is correlated with the grade of tumor differentiation, tumor size, and the presence of distant metastasis (229, 230). Activation of FAK is involved with the aggressive capability of PDAC through enhancing cell adhesion and invasion of PDAC cells (231). Inhibition of FAK by siRNA and FAK kinase inhibitor has anti-tumor activity; silencing FAK has been shown to inhibit cell survival, mobility, invasions and metastasis of PDAC cell lines in mouse model (230, 232-236). The expression and activation of FAK is increased in transformed HPNE and HPDE cells, suggesting FAK may play a role in transformation.

### **Molecular alterations in transformation**

In an attempt to better understand the molecular mechanisms that are involved in pancreatic cancer transformation, we performed microarray gene expression analysis. Because Smad4 plays an important role in the transformation, we are interested in finding which Smad4 downstream genes are changed during the transformation. One interesting finding is that the expression of BMP7 mRNA level was significantly increased in transformed cell lines. The studies from other cancers have showed that BMP7 plays important role in cancer development and metastasis. BMP7 expression was increased in melanoma and bone metastatic prostate cancer, colorectal cancer and breast cancer and was correlated with metastasis and poor prognosis (237-241). The role of BMP7 in human PDAC is unknown. We found that enhanced expression of BMP7 in 9 of 14 (64%) human pancreatic cancer cell lines. These results indicate that BMP7 may be involved in the transformation of HPDE cells and play a role in tumorigenesis of PDAC. As Smad4 is also a central signal mediator of bone morphogenetic protein (BMP) and activin signaling pathways (242, 243). BMP7 has been shown to inhibit Smad4-mediated TGF  $\beta$  signaling such as inhibition of EMT (244, 245).

Inactivation of Smad4 thus disrupting both BMP signaling and TGF  $\beta$  signaling might contribute to the development of human PDAC.

Another very significant finding we discovered is that Bmi-1 was involved in the transformation of human pancreatic cells. We first found that the mRNA level of Bmi-1 was enhanced in transformed HPDE cell line, and that overexpression of Bmi-1 protein was found in transformed HPDE cell line and HPDE tumor cell lines isolated from mouse tumor. Very interestingly, we further found that Bmi-1 was regulated by Smad4 in HPDE cells; inactivation of Smad4 enhanced the expression of Bmi-1 protein in Western blot analysis. Bmi-1 was also overexpressed in 85% (11/13) pancreatic cancer cell lines compared with very low level of expression in immortalized human pancreatic cell lines. Bmi-1 is a putative polycomb ring finger oncogene; it is involved in regulation of cell proliferation and senescence, growth and survival, EMT, invasion and metastasis (246-255). Bmi-1 is also a stem cell marker, plays a key role in regulating the proliferation and self-renewal of normal stem and progenitor cells (251, 252, 256-259). Overexpression of Bmi-1 is found in diverse cancers (247, 253, 255, 260, 261). Bmi-1 expression has been reported to be correlated with aggressiveness of the cancer, metastatic propensity, poor prognosis, and shorter survival time (262-264). Bmi-1 has been demonstrated to play important roles in malignant transformation and tumorigenesis of several cancers such as prostate cancer, breast cancer and skin cancer (258, 265, 266).

Previous studies have shown that Bmi-1 plays a role in PDAC. Mouse model displayed that Bmi-1 expression was upregulated in premalignant PanIN lesions and PDAC (267). Bmi-1 was significantly elevated in pancreatic cancer stem cells, suggesting it may play a role in the self renewal capacity of these cells (268). Bmi-1 expression also was found in human PanIN lesions (268). Overexpression of Bmi-1 was found in both PDAC cell lines and patient tissues; level of Bmi-1 expression is correlated with lymph node metastasis, patient's survival and poor prognosis (267, 269). Bmi-1 has been demonstrated to play roles in regulation of cell cycle, cell proliferation and survival of pancreatic cancer cells; silencing of Bmi-1 suppressed proliferation and growth, survival of PDAC cells and reduced anchorage-independent colony growth *in vitro* and xenograft tumor growth *in vivo* in animal models (269). These results indicated that Bmi-1 plays important roles in the progression of PDAC. Altogether, these results suggest that Smad4 regulated Bmi-1 expression and that increased Bmi-1 may play an important role in transformation of human pancreatic cells. Bmi-1 may represent a novel target for treatment of PDAC.

Taken together, these results indicate that inactivation of Smad4 results in dysregulation of Smad4 downstream genes and thereby might contribute to transformation and tumorigenesis of human pancreatic cells.

### **Alterations of IPA signaling pathways in transformation**

PDAC is a complex genetic disease resulting from sequential accumulation of multiple gene lesions that function through a relatively small number of pathways, biological and disease processes. To better understand the molecular mechanisms that involved in pancreatic cancer transformation and pathogenesis lies in the identification of disease-associated signaling pathways and biological process. Dissecting the signaling pathway critical for tumorigenic transformation and understanding biological process responsible for these cancer phenotypes will be useful for the discovery of potential tumor biomarkers and for the development of efficacious therapeutic agents that target the altered signaling pathways and process.

IPA signaling pathway analysis is a more comprehensive form of analysis compared with the analysis of individual gene expression. Our gene expression profiling and IPA signaling pathway analysis made it possible to identify the crucial signaling pathways involved in the transformation and tumorigenesis of pancreatic ductal cells. Our results from IPA signaling pathways analysis demonstrated that ILK signaling and cell cycle dysregulation are the most significant changes involved in transformation of HPDE cells. The top alterations in molecular biological functions in HPDE cell transformation were cell death; cellular growth and proliferation; cell cycle; DNA replication, recombination and repair; and cellular movement. In the HPNE cell transformation study, our bioinformatics pathway analysis revealed that a number of the signaling pathways such as ILK, integrin signaling, RhoA, and Notch are significantly associated with the malignant transformation of HPNE cells. The present studies, for the first time, used bioinformatics pathway analysis to reveal the significant signaling pathways that are associated with tumorigenic transformation of pancreatic ductal cells. These findings contribute to a better understanding of the molecular mechanisms of tumorigenic transformation in PDAC.

### **Significance and future direction**

In the present study through the sequential introduction of the most common genetic alterations in PDAC, we developed a novel experimental cell culture model system to transform the immortalized human pancreatic ductal cells, to study the biology of the human pancreatic cancer and to further dissect the molecular basis of pancreatic cancer transformation.

These models are the first in vitro model systems more faithfully recapitulating the human pancreatic carcinogenesis. The HPDE cell model is the first fully transformation model of human pancreatic ductal epithelial cells using the most common gene alterations in human PDAC. The HPNE cell transformation model is the first experimental cell culture system for studying mechanism

of tumorigenic transformation of human pancreatic ductal cells by utilizing the signature lesions found in PDAC without using any DNA tumor viral oncogenes. Taken together, these novel cell transformation model systems more accurately recapitulate human pancreatic ductal cell carcinogenesis from the cell origin, genetic lesions, activation of specific signaling pathways, and histopathological characteristics of PDAC.

The present study used bioinformatics pathway analysis for the first time to analyze differential signaling pathways that are involved in tumorigenic transformation of human pancreatic ductal cells. These findings would substantially enhance our understanding of the molecular mechanisms of transformation, especially, to better understand the signaling pathways and processes critical for transformation and tumorigenesis of PDAC. These novel *in vitro* experimental cell transformation model systems will be useful in several respects. First, these experimental cell culture systems would provide a necessary tool for studying the molecular mechanism of PDAC development. These models can be used to study the function and role of new genes in the progression or metastasis of PDAC by introducing the suspected genes into these cells and analyzing their effects. Second, these studies can serve as a guide to direct the development of new detection strategy and more effective therapeutic agents to target the specific altered oncogenic signaling pathways in human PDAC. These cell model systems with genetically defined nature could be used as an *in vivo* cancer model to assess the efficacy of new therapeutic agents on tumors with various gene or signaling pathway alterations in PDAC without the confounding interference by other unknown genetic alterations. For example, the HPNE model will be useful for testing new therapeutic agents targeting K-ras-mediated oncogenic signaling pathways.

Therefore, future study will first focus on identifying the critical molecular and signaling pathway alterations in transformation and metastasis; importantly, determining Smad4 regulated downstream gene's function in transformation and tumorigenesis. Second, future research will use these models to develop new detection strategies and therapeutic agents to target the specific altered oncogenic signaling pathways, such as testing the new therapeutic agents targeted RalA or Bmi-1.

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