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High Mobility Group A1 (HMGA1): A Novel Prognostic Indicator and Therapeutic Target in Pancreatic Adenocarcinoma

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MD Degree

The University of Edinburgh

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High Mobility Group A1 (HMGA1): A Novel Prognostic Indicator and Therapeutic Target in Pancreatic Adenocarcinoma

A dissertation submitted for the degree of
Doctor of Medicine
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To my wife, Hilary

Abstract

Introduction: High mobility group A1 (HMGA1) proteins are architectural transcriptional factors that are overexpressed in a range of human malignancies. However, their roles in the pathogenesis of pancreatic cancer are largely unknown. Pancreatic cancer is the deadliest of human malignancies with its aggressive locoregional invasion, such that the majority of patients have locally advanced cancer not amenable to surgical therapy at the time of diagnosis. In patients with advanced disease, the only therapeutic option is chemotherapy with nucleoside analogue gemcitabine. Further, the clinical impact of gemcitabine is only modest due to the high degree of chemoresistance. There is an urgent need to understand the biology of this cancer.

Hypotheses and aims: We hypothesised that *HMGA1* is overexpressed in pancreatic cancers and plays a role in the malignant phenotype of pancreatic cancer cells. There are two broad components to this work. The aims of the first component were to examine the expression of HMGA1 in a large sample of pancreatic cancer tissues from patients who underwent resection and to assess if HMGA1 expression could be used as a prognostic biomarker. In the second component, we addressed the roles of *HMGA1* in the pancreatic cancer progression, and assessed the effects of therapeutic targeting of *HMGA1* in experimental pancreatic cancer models.

Studies: Five complementary studies were performed. 1) Firstly, analysis of HMGA1 expression in pancreatic cancer tissues was carried out using a constructed tissue microarray consisting of tumours from 89 consecutive patients who underwent pancreatic resection over a 12-year period from 1991 to 2002 in a single institute. HMGA1 expression was correlated with postoperative survival and clinicopathological features. 2) Secondly, a study was performed to assess the effects of HMGA1 on cellular invasiveness using loss-of-function (RNA interference, RNAi) and gain-of-function (ectopic

HMGA1 overexpression) experiments. *In vivo* experiments were performed to assess the effects of targeted silencing of HMGA1 on the process of metastasis. 3) Thirdly, the roles of *HMGA1* in regulating anoikis resistance were studied. 4) In the fourth study, the effects of HMGA1 on anchorage-independent proliferation and *in vivo* tumour growth were assessed. 5) Lastly, experiments were performed to assess the ability of HMGA1 to modulate chemoresistance to gemcitabine in pancreatic cancer cells. This study assessed the feasibility of RNAi of *HMGA1* as a potential chemosensitising strategy using *in vivo* model. In each of the above studies, downstream molecular mediators of *HMGA1* were identified and experiments were performed to assess the mechanistic dependence of HMGA1-induced phenotype on these pathways.

Results: We showed that HMGA1 is overexpressed in more than 90% of pancreatic cancers and is associated with poorer postoperative survival. Further, tumoural HMGA1 expression was found to be an independent prognostic indicator in patients who underwent resection. Our *in vitro* studies suggest that HMGA1 promotes cellular invasiveness in pancreatic cancer cells through PI3-K/Akt-dependent MMP9 expression. Post-transcriptional silencing of HMGA1 suppressed metastasis *in vivo*. Overexpression of HMGA1 promotes PI3-K/Akt-dependent anoikis resistance and anchorage independent proliferation. The regulatory roles of HMGA1 on the PI3-K/Akt and MMP9 pathways are novel and previously undescribed. Although HMGA1 also regulates ERK activity, the contribution of this pathway is less important in controlling the HMGA1-induced anchorage-independent growth. *In vivo* silencing of HMGA1 resulted in attenuation of tumour growth in xenograft mouse model. Lastly, we found that HMGA1 represents a novel molecular determinant of chemoresistance to gemcitabine in pancreatic cancer cells. Targeted silencing of *HMGA1* chemosensitised pancreatic cancer cells to gemcitabine both *in vitro* and *in vivo*.

Conclusions: HMGA1 represents a novel prognostic biomarker in pancreatic cancer. Targeting of HMGA1 may be a novel therapeutic (anti-metastatic, anti-proliferative and chemosensitising) strategy to ameliorate the aggressive phenotype of pancreatic cancer cells. PI3-K/Akt, ERK and MMP9 are novel downstream mediators of HMGA1.

Contents

Preface

The following work was carried out at the Pancreatic Cancer Research Laboratory, Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, USA during the years of 2004–2006. This research was performed as part of my appointment as Specialist Registrar in General Surgery to the East Anglia Higher Surgical Training Programme, which has a linked research training with Professor Stanley Ashley's laboratory at the Brigham and Women's Hospital. This experience has proved to be immensely satisfying. During the course of this work, I have learnt and performed a comprehensive number of complex techniques in molecular biology, which I am certain will provide the foundation to be an independent investigator.

The introduction of this thesis outlines the research motivation for this work, a brief history of pancreatic cancer and describes the epidemiology, risk factors, pathology, molecular biology, clinical features and current evidence-based management of this deadly disease. This is followed by a review of the current understanding of the biology of HMGA1 and its potential roles in oncology. A background of the emerging field of RNA interference as a molecular tool and therapeutic strategy is also provided.

In Chapter 2, the materials and methods of all studies are consolidated. A detailed description of methodology is provided to ensure reproducibility of results. Chapter 3 describes the results of the clinical study involving the use of tissue microarrray to look at tumoural HMGA1 expression and its potential correlations with patient clinicopathological characteristics. Chapter 4 documents the results of *in vitro* and *in vivo* experiments investigating the roles of HMGA1 in regulating cellular invasiveness and metastasis. Chapter 5 deals with HMGA1 and anoikis-resistance in pancreatic cancer cells. Chapter 6 describes the roles of HMGA1 in anchorage-independent cellular proliferation and *in vivo* tumour growth. Lastly, roles of HMGA1 in

chemoresistance to gemcitabine were investigated and the results provided in Chapter 7. The concluding part of the thesis is in Chapter 8 where the research strategy is described, all the findings from this thesis are consolidated and discussed, and ideas for future work to translate this work into clinical applications are given.

I hereby declare that I have not submitted this dissertation, in whole or in part, for any other degree, diploma or qualification at any University. This dissertation is the result of my own work and I have attempted to reference appropriately findings that are not my own.

Mr Siong-Seng Liau
Cambridge, UK
January 2008

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I would like to thank Professor Stanley Ashley and Dr Edward Whang for the opportunity to work in their laboratory and for their supervision of this work.

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- Thorn building animal facility, Brigham and Women's Hospital – for care and maintenance of research mice

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Last but not least, I would like to express my gratitude to my internal supervisors, Professor James Ross and Professor Stephen Wigmore for their help in reviewing this thesis.

Abbreviations

ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
bps	Base pairs
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
COX2	Cyclooxygenase 2
CP	Chronic pancreatitis
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ERCP	Endoscopic retrograde cholangiopancreatogram
EST	Expressed sequence tag
EUS	Endoscopic ultrasound
FAMM	Familial atypical multiple mole melanoma
FBS	Foetal bovine serum
GAP	GTPase-activating protein
HA	Haemagglutinin
HIPK2	Homeodomain interacting protein kinase 2
HMGA1	High mobility group A1 proteins
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IL	Interleukin
i.p.	Intraperitoneal
IPMT	Intraductal papillary mucinous tumor of pancreas
IRES	Internal ribosome entry site
MAPK	Mitogen activated protein kinase
MDM2	Human homologue of murine double minute 2 (mdm2)
MMP	Matrix metalloproteinase

MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
NCBI	National Centre for Biotechnology Information
PanIN	Pancreatic intraepithelial neoplasia
PCR	Polymerase chain reaction
PDAC	Pancreatic adenocarcinoma
PDGF	Platelet-derived growth factor
Pfu	Plaque-forming unit
PI3-K	Phosphoinositide-3-kinase
RCT	Randomised controlled trial
RNAi	Ribonucleic acid (RNA) interference
RT-PCR	Reverse transcriptase polymerase chain reaction
SAR	Scaffold attachment regions
SCID	Severe combined immunodeficiency
shRNA	Short-hairpin RNA
siRNA	Short-interfering RNA
SSH	Suppression substractive hybridisation

List of Tables

List of Figures

Chapter One: Introduction

1.1 RESEARCH MOTIVATION: THE CLINICAL PROBLEM

Pancreatic cancer has a very poor prognosis. The aggressive biology of the tumour and the late presentation of this disease accounts for the overall low median survival of less than 6 months and a 5-year survival rate of 0.4-5 per cent (Bramhall et al., 1995; Hedberg et al., 1998). In UK, USA and Europe, the incidence of pancreatic adenocarcinoma (PDAC) almost equals the mortality, indicating a universally lethal disease. Although it is a relatively rare disease, it stands as one of the leading causes of cancer deaths. Worldwide, the survival rates of this disease have not improved during the last 25 years despite medical advancement.

Surgery is the only curative therapy for this cancer. Unfortunately, only a small minority of patients (10-20%) present early enough with potentially resectable disease at the time of diagnosis. Even if a curative resection has been performed, many patients still succumb to local or metastatic recurrence following surgery. The implication of this is that PDAC must have metastasised before clinical presentation in most cases. Some improvements in surgical outcome occur in patients who receive adjuvant chemotherapy, though the impact on 5-year survival has been minimal. In patients with advanced disease, chemotherapy remains the only option. Due to the highly chemoresistant nature of this cancer, only a small number of agents have been shown to confer modest benefit in terms of survival and clinical response. Although surgery as a mainstay of curative treatment has made significant progress over the years, it is the consensus that further improvement in surgical technique is unlikely to impact on survival of this deadly disease.

To improve the prognosis of this cancer, better understanding of this cancer at the molecular level is urgently required (Kern et al., 2001). The work

described in this thesis identified *HMGA1* gene as a novel molecular determinant of malignant phenotype in this cancer and its potential role as a prognostic indicator and therapeutic target in this deadly disease.

1.2 PANCREATIC CANCER: A BRIEF HISTORICAL ACCOUNT

Early description of pancreatic carcinoma was provided by Giovanni Battista Morgagni (1682-1771), the father of 'pathological anatomy', in his famous work entitled 'The Seats and Causes of Diseases' (Morgagni, 1960; Sedivy & Patzak, 2002). As he astutely noted, Morgagni described the difficulty of clinically diagnosing pancreatic tumours due to the inaccessible anatomical location of this organ. With this, it is not surprising that few cases were reported in the eighteenth and nineteenth centuries. In 1890, Ludwig Georg Courvoisier (1843-1918) published on his experience with the classic sign of a palpable distended gallbladder from which the Courvoisier's law originated. Courvoisier's law states that obstruction of the common bile duct due to stone rarely results in dilatation of the gallbladder whilst if the obstruction is due to other causes, dilatation of gallbladder is common. These causes other than stone imply neoplastic causes including pancreatic cancers. In fact, Louis-Félix Terrier, a French surgeon, described a case of gallbladder dilatation associated with pancreatic cancer in 1889 which gave rise to the term 'Courvoisier-Terrier's law' (McClusky et al., 2002).

The first surgical treatment for malignant pancreatic lesions was perhaps a total pancreatectomy performed by Billroth in 1884 (Howard, 1999). Walter Kausch first described the technique of pancreaticoduodenectomy in 1912. The modern era of pancreatic surgery started when Whipple, Parsons and Mullins presented their paper on pancreaticoduodenectomy to the American Surgical Association in 1935. Whipple and colleagues initially performed a two-stage pancreaticoduodenectomy which consisted of biliary diversion and gastrojejunostomy during the initial operation followed by the resection of pancreatic head and duodenum up to 3 weeks later. In 1941, Whipple

modified the procedure to a one-stage pancreaticoduodenectomy with a pancreaticojejunostomy (Whipple, 1949). It is perhaps to Allen O. Whipple, more than any other individual, who is due the recognition as being the 'Father of Pancreatic Surgery'.

1.3 PANCREATIC CANCER: EPIDEMIOLOGY AND ITS DISMAL PROGNOSIS

Currently, there is no effective treatment for this universally lethal disease. This is in part due to the difficulty of diagnosing this disease at an early stage given the few early indicators of disease and lack of screening tests. Prevention of this cancer is difficult as little is known about its aetiology. Although pancreatic cancer is relatively less common than cancers of lung, breast, large bowel or prostate, its high mortality rate has ranked it as the eighth most common cause of cancer mortality worldwide, accounting for nearly quarter of a million deaths yearly (Parkin et al., 2005). The m ajority (61%) of pancreatic cancers occur in the developed countries.

Pancreatic cancer is the sixth most common cause of cancer death in the UK (Cancer Research UK, 2007). In the USA, pancreatic cancer is the fourth leading cause of cancer-related mortality (Jemal et al., 2006). In 2005, pancreatic cancer resulted in 3455 male and 3783 female deaths in the UK (Cancer Research UK, 2007). In USA, the American Cancer Society estimates that there will be 37170 new cases of pancreatic cancer (18,830 men and 18,340 women) with 33370 deaths from this disease in 2007 (American Cancer Society, 2007).

In the UK, there has been little improvement in survival over the last 3 decades (Figure 1.1). Survival rates in pancreatic cancer are amongst the lowest of all cancers (Figure 1.2). Effective treatments for this cancer have not been forthcoming. Surgical resection remains the only curative treatment option for this group of patients. The technical advances in pancreatic surgery together with improvement of surgical outcome due to regionalisation of pancreatic surgeries in high patient load centres have not impact on the survival of patients with PDAC. The addition of chemo-, radiotherapy or a combination of these makes little difference to the survival outcome over the last 30 years (Jemal et al., 2006). This is in large part due to locoregional disease recurrence in 50-80% of patients who underwent resection, and a similar figure will develop liver metastases (Staley et al., 1996).

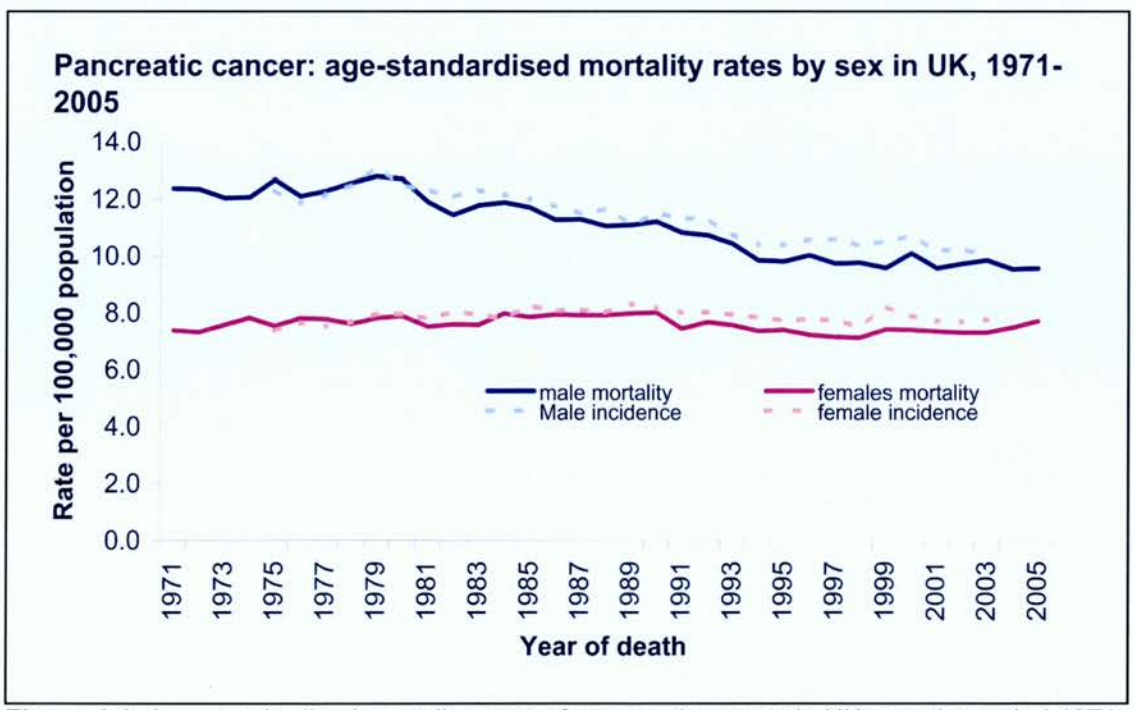


Figure 1.1. Age-standardised mortality rates of pancreatic cancer in UK over the period 1971-2005 (Cancer Research UK, 2007). Mortality rates in female patients have remained static over the last 30 years, with only a slight decline in male patients.

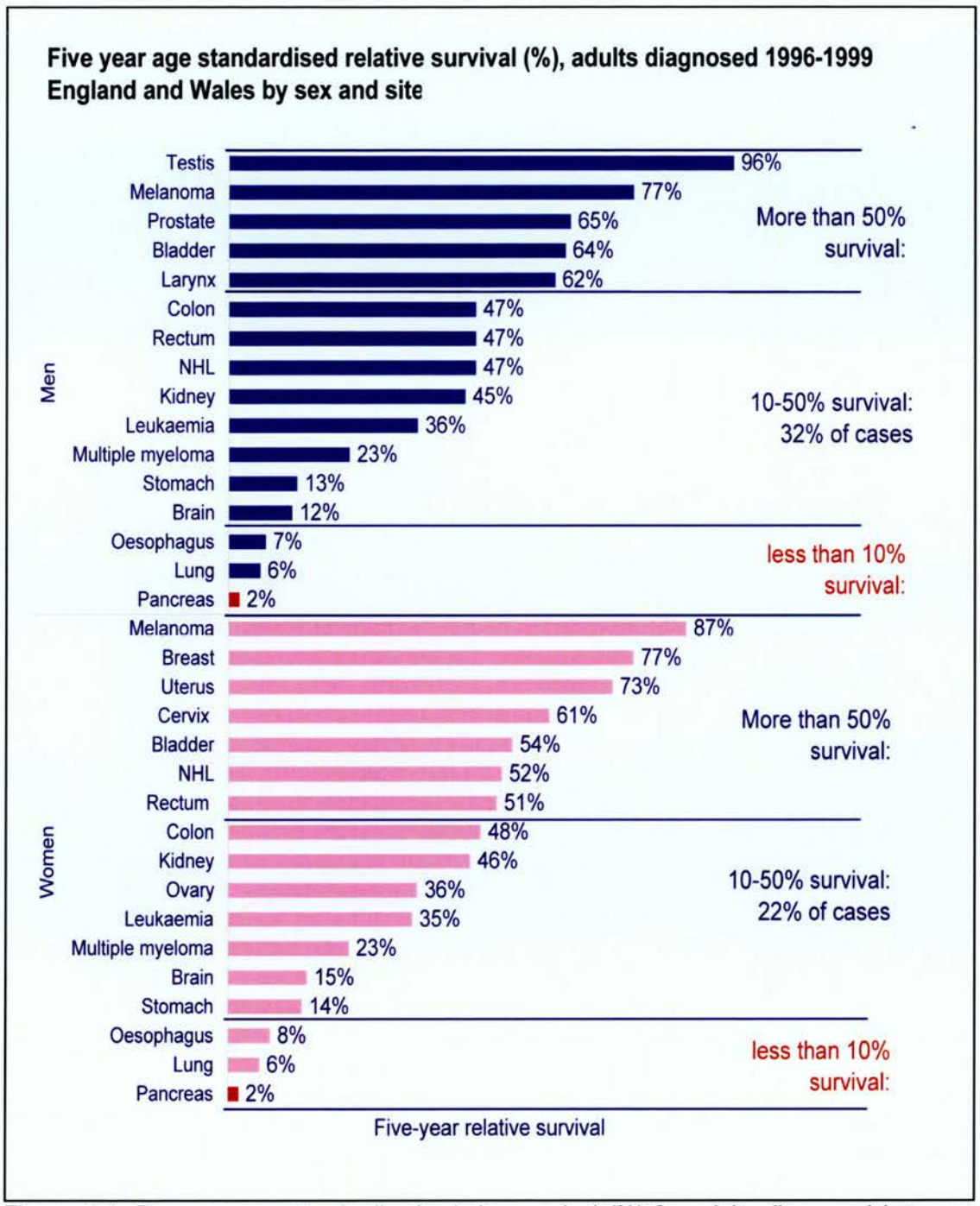


Figure 1.2. Five-year age-standardised relative survival (%) for adults diagnosed between 1996 and 1999 in England and Wales by sex and cancer site (Cancer Research UK, 2007). Pancreatic cancer (red bar) represents one of the cancers with the lowest five-year relative survival.

1.4 AETIOLOGY AND RISK FACTORS FOR PANCREATIC CANCER

The only established risk factors for pancreatic cancer are age and smoking status. In the following sections, the evidence implicating each of these risk factors in pancreatic cancer is discussed.

1.4.1 Age

The incidence of pancreatic cancer increases exponentially with age and peaks in the seventh and eighth decades (Figure 1.3). As the populations of the developed countries age, one can predict that there would be an increasing incidence of pancreatic cancer.

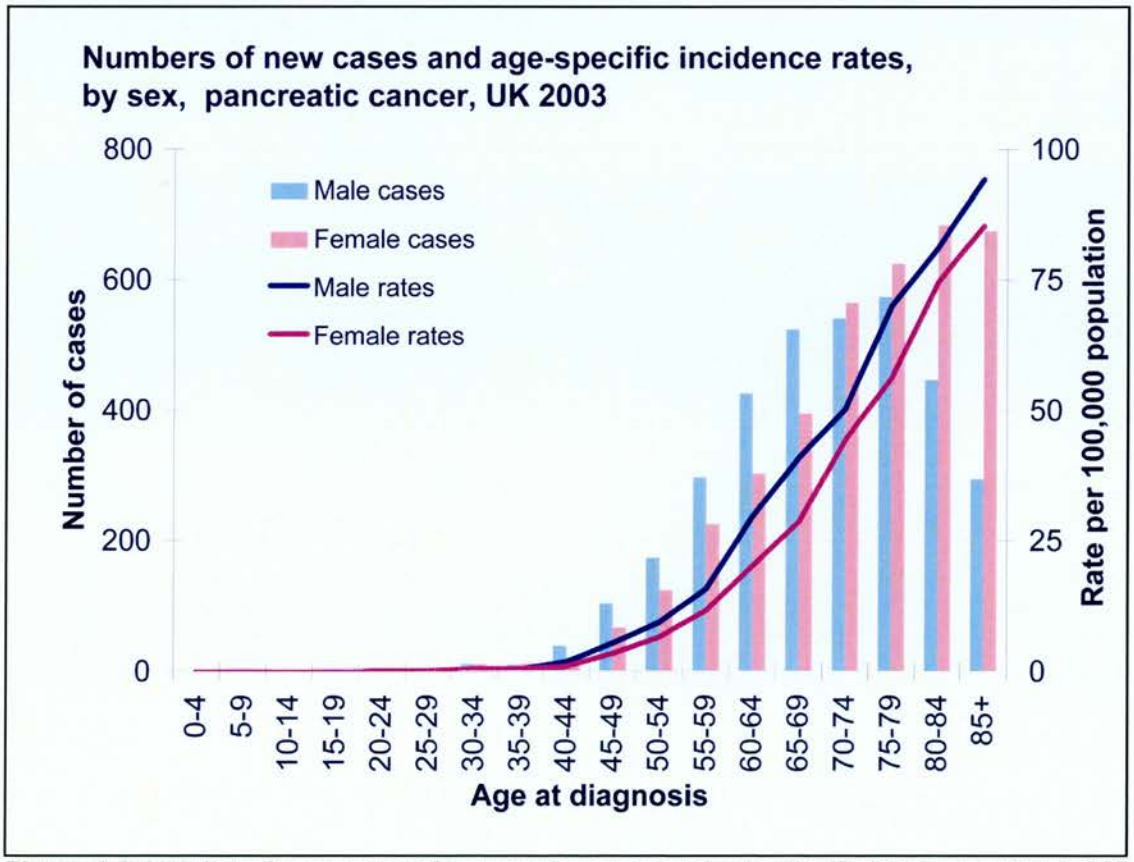


Figure 1.3. Number of new cases of pancreatic cancer and age-specific incidence rates in UK for year 2003. Approximately 10% of pancreatic cancers occur prior to age 50. Figure was adapted from Cancer Research UK Cancerstats (Cancer Research UK, 2007).

1.4.2 Tobacco use

Cigarette smoking shows the strongest association with the risk of pancreatic cancer, accounting for an estimated 25% of all cases (Lowenfels & Maisonneuve, 2006). The risk of pancreatic cancer is proportional to the duration and intensity of cigarette smoking. Doll and colleagues confirmed these findings by showing that ex-smokers were 1.4 times more likely to die from pancreatic cancer compared to men who had never smoked (Doll et al., 1994). Further, the risk of pancreatic cancer increases with the number of cigarettes smoked. The risk of pancreatic cancer increases from 1.8 times in current smokers of less than 25 cigarettes a day to 3.1 times in those who smoke 25 or more cigarettes a day. The risk remains high in the heavy smokers and only returns to a level compatible with never-smokers after 15 years of cessation (Boyle et al., 1996). For light smokers, it takes approximately 5 years to return to baseline risk after smoking cessation.

It has also been reported that cigarette smoking shows a positive interaction with family history of pancreatic cancer (Schenk et al., 2001). In relatives who smoke and had a family history of pancreatic cancer, the risk is significantly increased at 6.02-fold elevation (95% CI: 1.98-18.29) compared to relatives who did not smoke and who had no family history of pancreatic cancer. First degree relatives who did not smoke but had a family history of pancreatic cancer had an intermediate risk (RR = 3.64, 95% CI: 1.12-11.80). The risk is even higher in relatives who smoke and had a first degree relatives who had pancreatic cancer diagnosed before age of 60 (RR = 8.23, 95% CI=2.18-31.07). The above findings provide evidence for a potential gene-environment interaction in the aetiology of pancreatic cancer.

Although the exact chemical component of tobacco that leads to cancer is unknown, it has been postulated that N-nitroso compounds in tobacco, which are known to promote pancreatic carcinogenesis in experimental models,

could potentially be carried to the pancreas via the bloodstream and exerts their tumourigenic effects there (Risch, 2003).

1.4.3 Chronic pancreatitis

Chronic pancreatitis (CP) develops following recurrent episodes of acute pancreatitis. The commonest cause in Western countries is heavy alcohol consumption. Other causes of CP include hereditary pancreatitis and tropical pancreatitis. Hereditary pancreatitis is an autosomal dominant disease with a high penetrance level of 80% and the implicated genetic defect being located on chromosomal locus of 7q35 which codes for cationic trypsinogen gene, PRSS1 (Whitcomb et al., 1996). Tropical pancreatitis is a form of idiopathic pancreatitis, primarily found in southern India and in parts of sub-Saharan Africa. In all forms of CP, the risk of pancreatic cancer is elevated. Cohort and case-control studies have provided risk estimate ratios for pancreatic cancer in patients suffering from CP, ranging from a 2- to 27-fold increase in risk (Bansal & Sonnenberg, 1995; Ekbom et al., 1994; Malka et al., 2002). For patients with hereditary pancreatitis, the lifetime risk is approximately 30-40%. Similarly, the risk of pancreatic cancer in patients with tropical pancreatitis is also elevated (Chari et al., 1994).

The link between CP and pancreatic cancer seems clear. However, the molecular mechanism of this association remains poorly understood. One can postulate that chronic inflammation induces epithelial damage and reactive cellular turnover. This inflammatory cycle provides selection pressure for cells harbouring pro-oncogenic mutations. Supporting evidence for this principle can be found in analysis of tissues from CP patients which indicates the presence of p16 inactivation and K-ras mutations which are frequent mutations found in pancreatic cancers (Lohr et al., 2005; Rosty et al., 2003).

1.4.4 Diabetes

The problem with diabetes as a risk factor for pancreatic cancer concerns the possibility of diabetes as an early manifestation of pancreatic cancer, rather than a true risk factor. Everhart et al reported a meta-analysis in 1995 providing a definite association between pre-existing type II diabetes and pancreatic cancer. The pooled relative risk of pancreatic cancer for diabetics compared to non-diabetics was 2.1 (Everhart & Wright, 1995). More recently, Huxley and colleagues reported a further meta-analysis including 9200 pancreatic cancer patients which concluded that there is a modest causal relationship between type II diabetes and pancreatic cancer. Patients who had been diagnosed to have type II diabetes for more than 5 years had an odds ratio of 1.5 (95% CI: 1.3-1.8) for developing pancreatic cancer (Huxley et al., 2005). The risk was even higher for patients who had diabetes for less than 5 years at odds ratio of 2.1 (95% CI: 1.9-2.3).

1.4.5 Body weight

The link between pancreatic cancer and obesity is less well-defined. Two large prospective cohort studies have shown that obesity (as defined by body mass index of at least 30 kg/m^2) does increase the risk of pancreatic cancer (Calle et al., 2003; Michaud et al., 2001). A recent meta-analysis showed that there is a small positive increase in the risk of pancreatic cancer with increasing weight. The calculated relative risk of pancreatic cancer per unit increase in body mass index was 1.02 (95% CI: 1.01-1.03) (Berrington de Gonzalez et al., 2003). This calculated increase in relative risk translates into a 19% higher risk of pancreatic cancer for obese people (body mass index >30 kg/m^2) compared to those of normal body weight (22 kg/m^2).

1.4.6 Genetic background

Approximately 5-10% of pancreatic cancers have a familial component (Lynch et al., 1996). In some instances, this is associated with a general familial

cancer syndrome such as Peutz-Jeghers syndrome, familial atypical multiple mole melanoma (FAMM) syndrome, hereditary breast ovarian cancer syndrome, hereditary non-polyposis colorectal cancer (Lynch syndrome) and familial adenomatous polyposis syndromes (Giardiello et al., 2000; Giardiello et al., 1993; Lynch et al., 2002).

Familial pancreatic cancer is a rare syndrome with an apparent autosomal dominant inheritance although the candidate gene or genes responsible have not been identified yet (Klein et al., 2004). Approximately 19% of the familial pancreatic cancer families have been found to harbour germline mutations in the breast cancer susceptibility gene, BRCA2 (Hahn et al., 2003). In one family with familial pancreatic cancer, the chromosomal locus 4q32-34 has been found to be a pancreatic cancer susceptibility locus (Eberle et al., 2002). In those with a family history of pancreatic cancer, the risk of developing pancreatic cancer in first-degree relatives of an affected individual is estimated to be 18-fold if there are two affected family members and as high as 57-fold if there are three or more affected family members (Tersmette et al., 2001).

1.4.7 Diet and alcohol intake

Over the last two decades, many epidemiological studies have been performed to elucidate the potential link between pancreatic cancer and various dietary items. This effort has been largely inconclusive. Most of these studies have been plagued by methodological issues that led to inconsistent findings. For instance, many of these reports are case-control studies that are susceptible to recall bias.

Consumption of a high fibre diet has been proposed to reduce the risk of cancer. Michaud and colleagues recently reported results from two large cohort studies comprising of approximately 125,000 subjects, specifically assessing if the consumption of a 'prudent' diet (high fruit and vegetable diet)

and a 'Western' diet (high meat and fat intake) have a link to risk of pancreatic cancer (Michaud et al., 2005). No associations were found between the two dietary patterns to the risk of developing pancreatic cancer.

1.4.8 Other risk factors

There is evidence to suggest that a small proportion of pancreatic cancers are due to occupational exposure (Lowenfels & Maisonneuve, 2004). Occupational exposure to pesticides, chlorinated hydrocarbon solvents (e.g. dry cleaners) (Ojajarvi et al., 2000) and paint (Brown et al., 2002) or textile (Zhang et al., 2005) manufacturing process may increase the risk of developing cancer of the pancreas.

1.5 PATHOLOGY OF PANCREATIC ADENOCARCINOMA

Pancreatic ductal adenocarcinoma is the most common exocrine pancreatic tumour, accounting for 85% of all malignant pancreatic tumours. Histologically, PDAC is defined as a malignant epithelial neoplasm of pancreas in which the neoplastic epithelial cells form glands (Hruban et al., 2006). One of the characteristic features of PDAC is the frequent occurrence of an intense surrounding desmoplastic host reaction. Approximately 80-90% of tumours are located in the head of pancreas with the remaining tumours located in body and tail of pancreas (Alexakis et al., 2004).

Even a small primary lesion will commonly exhibit perineural and lymphovascular invasion, suggesting a propensity for aggressive invasion and early metastasis. PDACs frequently metastasize to multiple lymph node groups, and lymph node metastases are found in 20-77% of the resection specimens of patients with pancreatic head adenocarcinomas (Alexakis et al., 2004). Many patients despite curative resection will develop locoregional recurrence and metastasis, suggesting the presence of micrometastases at the time of diagnosis. The most common sites for metastasis are liver and

peritoneum whilst the most common extraperitoneal metastatic site is the lung.

1.5.1 Cell of origin of PDAC

The issue of cells of origin of pancreatic cancer is controversial and remains an area of ongoing study. Traditionally, solid tumours are thought to arise from multi-step mutational events in differentiated epithelial cells, leading to carcinogenesis. PDAC is generally thought to arise from pancreatic ductal cells. The landmark study by Cubilla and Fitzgerald provided the histological evidence for a ductal cell origin for PDAC based on the observations that there is an increased incidence of abnormal ductal structures (now designated pancreatic intraepithelial neoplasia, PanIN), spatially related to malignant cells which could represent incipient PDAC (Cubilla & Fitzgerald, 1976). It has also been postulated that PDAC can be derived from acinar or islet cells. The plasticity of each cell type of adult pancreas and the ability to 'transdifferentiate' from one cell type to another make the identification of tumour cell origin more complex (Pour et al., 2003).

More recently, the emerging concept of stem cell origin of cancer provides an alternative model for cancer development. It emphasises the roles of stem cells as the initiating structure for cancer development, rather than a particular differentiated cell type. Recent study has identified a subset of cancer cells in pancreatic tumours that retain the stem cell characteristics of having the capacity to self-renew, generate and recapitulate the phenotype of the tumour from which they are derived (Li et al., 2007). These cells have been labelled cancer stem cells. The existence of cancer stem cells have also been previously proven in leukemia (Pour et al., 2003), breast (Al-Hajj et al., 2003), brain (Singh et al., 2004), ovarian (Szotek et al., 2006) and prostate cancers (Patrawala et al., 2006). At present, we do not have sufficient evidence to conclude if these pancreatic cancer stem cells arise from a mutated stem cell

or a downstream progenitor cell that has regained stem cell-like characteristics through cumulative genetic mutations.

A practical consequence of this model is that strategies aimed at treating this cancer should take into account the unique characteristics of cancer stem cells. It has been proposed that failure of conventional cancer treatments is the result of failure to kill cancer stem cells which have been shown to be more resistant to standard chemotherapy (Costello et al., 2000; Guzman et al., 2002). Therapy that misses the stem cells will allow their regeneration and hence, cancer recurrence. Clearly, treatment that specifically targets cancer stem cells may prove to be more effective.

1.6 MOLECULAR GENETICS AND ADENOMA-CARCINOMA SEQUENCE OF PANCREATIC ADENOCARCINOMA

Over the last decade, significant advances have been made in our understanding of the biology of pancreatic cancer. It is now considered one of the better characterized tumours at the molecular and genetic levels. Pancreatic cancer probably represents a genetically-driven disease, characterized by sequential and cumulative genetic perturbations that dictate subsequent aberrant molecular signalling and uncontrolled proliferation (Hruban et al., 2000). Consequently, our line of investigations of pancreatic cancer has shifted from the histopathological to the molecular and genetic levels. In the subsequent subsections, a summary of the signature mutations, their linked pathways and biological significance is provided.

1.6.1 PanINs and genetic progression model

The common view is that pancreatic cancer evolves through a multistep process with cells gaining cumulative genetic and phenotypic alterations resulting in pathological progression from normal pancreas to precursor lesions known as pancreatic intraepithelial neoplasia (PanIN), through to

locally invasive pancreatic adenocarcinoma, and finally metastasis (Hruban et al., 2000). There is much evidence to support the theory that PDAC develops from precursor lesions into malignant tumours, similar to the adenoma-carcinoma sequence described in colorectal adenocarcinoma (Vogelstein et al., 1988). The nomenclature of PanIN was first proposed in 1994 (Klimstra & Longnecker, 1994), and further developed by the Pancreatic Cancer Think Tank sponsored by the National Cancer Institute, USA (Hruban et al., 2001). Based on the degree of cytological and architectural atypia, the lesions have been classified into PanIN-1A, PanIN-1B, PanIN-2 and PanIN-3 (Figure 1.4).

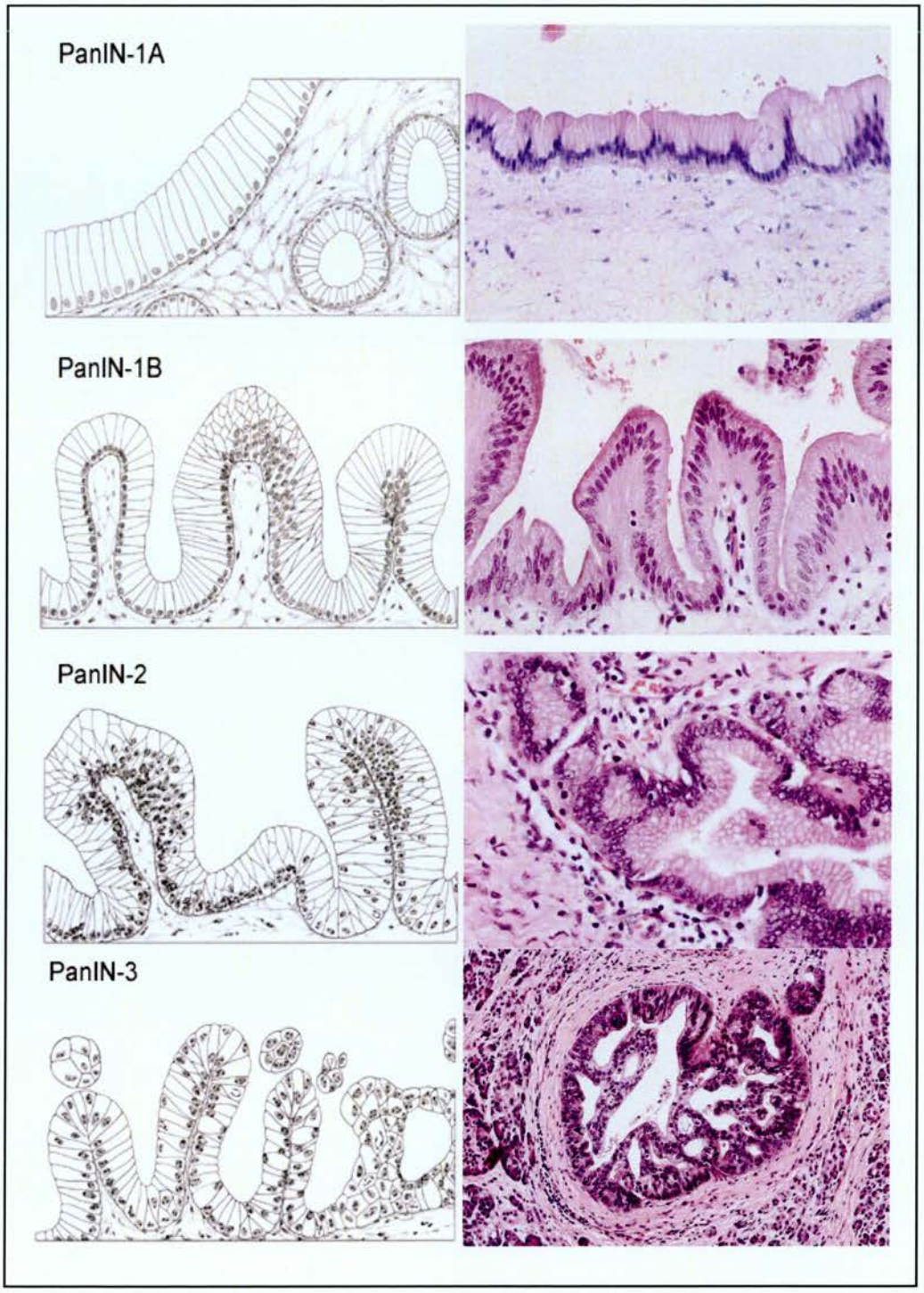


Figure 1.4. Pancreatic intraepithelial neoplasia (PanIN) lesions with schematic diagrams and histological features being illustrated. PanIN-1A lesions consist of tall columnar mucin-containing cells showing slight or no atypia. PanIN-1B lesions present epithelial lesions that have a papillary, micropapillary or basally pseudostratified architecture but otherwise identical to PanIN-1A. PanIN-2 lesions show moderate atypia including loss of polarity, nuclear crowding, pseudo-stratification, nuclear enlargement and nuclear hyperchromatism. PanIN-3

lesions are usually papillary or micropapillary with severe atypia. Diagrams and images were kindly provided by Department of Pathology, John Hopkins Hospital, Baltimore, USA (http://pathology.jhu.edu/pancreas/panin/) (Hruban et al., 2001).

1.6.2 Genetic signature of PanIN lesions

It is interesting to note that there seems to be an ordered series of mutational events associated with specific neoplastic stages in the development of PDAC (Jaffee et al., 2002). Of note, these genetic alterations have not been correlated with the acquisition of specific histopathologic features.

There is a temporal sequence to the occurrence of these mutations in preinvasive pancreatic lesions with K-ras mutations occurring early, followed by p16 and lastly p53 and DPC4/Smad4 mutations (Jaffee et al., 2002; Wilentz et al., 2000). K-ras mutations are present in 36%, 44% and 87% of PanIN-1A, PanIN-1B and PanIN2-3 lesions respectively (Lohr et al., 2005). Loss of p16 expression is seen in 30% of PanIN-1A and -1B, 55% of PanIN-2 and 71% of PanIN-3 lesions (Wilentz et al., 1998). The p53 gene is usually inactivated in only PanIN3 lesions (Feldmann et al., 2007). On the other hand, SMAD4/DPC4 is intact in PanIN-1 and PanIN-2 lesions but it is lost in 31%-41% of PanIN-3 lesions (Wilentz et al., 2000).

1.6.3 Genetic signature of PDAC

Nearly all PDACs have mutation in K-ras (95%) and inactivation of $p16^{1NK4a}$ (80-95%). Other high frequency mutations in spontaneous pancreatic cancer include p53 (50-75%) and DPC4/Smad4 (55%) genes (Jaffee et al., 2002). As such, cumulative genetic aberrations probably drive the malignant phenotype of pancreatic cancer cells (Hanahan & Weinberg, 2000).

1.6.3.1 *K-ras* gene

K-ras is a member of the RAS family of GTP-binding proteins. The Ras protein coded by wild-type K-ras binds to GTPase-activating protein (GAP) and regulates cell cycle progression. Activated K-ras results in a constitutively activated molecule that engages downstream effector pathways, notably the RAF-mitogen activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3-K) pathways. These effector pathways mediate a wide variety of cellular functions including proliferation, differentiation and survival (Malumbres & Barbacid, 2003).

K-ras mutations occur relatively early during the development of pancreatic neoplasia, occurring in approximately 30% of lesions with earliest histological disturbance (Klimstra & Longnecker, 1994). This is important in that tests to detect mutant K-ras have the potential to detect early, curable non-invasive pancreatic lesions, before they progress to incurable invasive cancers. Further, activating point mutations in the *K-ras* gene are largely restricted to a single codon in the gene (codon 12). This clearly facilitates the development of a screening test for mutant K-ras. Such a test is currently being developed and employed in the research setting for screening programmes for PDAC in high risk groups as discussed in the Section 1.10.8 (Screening for pancreatic adenocarcinoma).

The importance of *K-ras* mutations in the development of PDAC was emphasized by the elegant study by Hingorani and co-workers, who established a mouse model in which mutated *K-ras* gene was directed to progenitor cells of the pancreas (Hingorani et al., 2003b). In this model, histological progression was observed to develop progressively through stages of PanIN leading to invasive and metastatic tumours. The results of this study have several important implications. Firstly, this model provides evidence that supports the PanIN/Adenoma-carcinoma sequence model. Secondly, it shows that *K-ras* mutation alone is capable of inducing the

development of PDAC, clearly indicating that *K-ras* mutations occur early during tumour development. Further, it supports the emerging theory that pancreatic progenitor or stem cells could be the cells of origin for pancreatic adenocarcinoma.

1.6.3.2 *p16/CDKN2A* tumour suppressor gene

The *p16/CDKN2A* gene, located at 9p21, encodes two tumour suppressors – INK4A and ARF – through distinct first exons and alternative reading frames of shared downstream exons. Loss of function of *INK4A* is brought about by intragenic mutation with loss of second allele, homozygous deletion or promoter hypermethylation that results in gene silencing. Germline mutations in *INK4A* are associated with the familial atypical multiple mole melanoma (FAMM) syndrome and increase the risk of pancreatic cancer by 13-fold (Goldstein et al., 1995) (as described in Section 1.4.6: Genetic background as risk factor). Mutations in *INK4A* occur in moderately advanced PanIN lesions showing features of dysplasia (see Figure 1.4). INK4A inhibits CDK4/6-mediated phosphorylation of retinoblastoma protein, thereby blocking entry into the S-phase of cell cycle.

Given the physical juxtaposition of *INK4A* and *ARF* and frequent homozygous deletion of 9p21 (approximately 40% of PDACs), many PDACs sustain loss of both INK4A and ARF. Given that ARF stabilises p53 by inhibiting its MDM2-mediated proteasomal degradation, loss of ARF will lead to degradation of tumour suppressor p53 and hence, promotes tumourigenesis.

1.6.3.3 *p53* tumour suppressor gene

The p53 gene is located on chromosome 17p and is inactivated in PDAC almost always through a combination of intragenic mutation and loss of the second allele (Yan et al., 2005). The inactivating mutations usually affect the DNA binding domain of p53. Mutations in p53 arise late during the PanIN progression when significant dysplasia has been acquired, indicating its

function in preventing malignant progression (Feldmann et al., 2007). p53 protein regulates the G_1/S cell cycle checkpoint, maintenance of G_2/M arrest and induction of apoptosis. Given that p53 functions as the 'guardian of the genome', its mutations will allow cells to bypass DNA damage checkpoints and apoptotic signals. Loss of p53 functions also contribute to the genomic instability observed frequently in PDAC as evident by the high degree of complex cytogenetic rearrangements (Feldmann et al., 2007).

1.6.3.4 *SMAD4/DPC4* tumour suppressor gene

The *SMAD4/Deleted in Pancreatic Carcinoma 4 (DPC4)* gene maps to chromosome 18q21 and is targeted for deletion or intragenic mutation in late stages of PanIN lesions (Wilentz et al., 2000). Inactivation of *SMAD4* impairs the intracellular signalling cascades downstream of transforming growth factor β (TGF- β) family of cell surface receptors. The TGF-β pathway is activated when the TGF- β protein binds to its cognate receptors, triggering an intracellular cascade that results in nuclear localisation of SMAD4 protein. Once in the nucleus, SMAD4 exerts its effects on target genes involved in growth control. As such, inactivating mutations of *SMAD4* lead to decreased growth inhibition and uncontrolled proliferation.

1.6.3.5 Telomeric stability and genomic instability

Telomeres are specialised nucleoprotein complexes that cap chromosomal ends and confer chromosomal stability during cell division by preventing the ends from becoming 'sticky'. At the DNA level, telomeres are composed of hexameric repeats of the sequence TTAGGG at the ends of chromosome arms. Telomeric shortening is one of the earliest demonstrable genetic aberrations in PDAC, with >90% of the lowest grade PanIN harbouring this abnormality (van Heek et al., 2002). Telomeric attrition predispose to chromosomal rearrangements through breakage-fusion-bridge cycles (Artandi et al., 2000). This allows for continued acquisition of oncogenic chromosomal alterations.

1.6.3.6 BRCA2

Mutations in *BRCA2* have been observed in approximately 17% of PDAC patients with a family history (Goggins et al., 2000). Normally BRCA2 protein is responsible for homologous recombination-based DNA repair and hence, maintenance of the genomic stability. Loss of *BRCA2* gene occurs late and is restricted to severely dysplastic PanINs and small proportions of PDACs (Goggins et al., 2000).

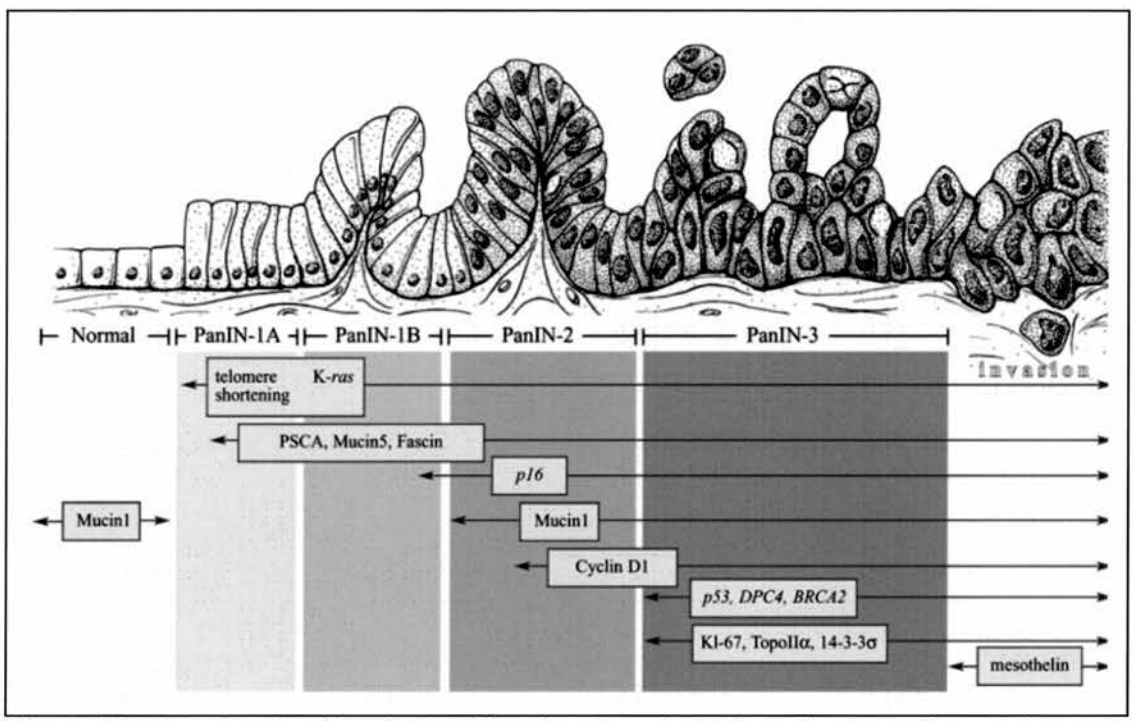


Figure 1.5. Genetic alterations in PanIN lesions were investigated using a tissue microarray technique by Maitra et al, demonstrating progressive accumulation of genetic mutations, leading to invasive cancers (Maitra et al., 2003).

1.7 CLINICAL FEATURES

The clinical features of pancreatic cancer depend on the size and location of the tumour. Early symptoms can be non-specific. The classic symptom of painless jaundice is associated with pancreatic head tumours and is present in 50-60% of patients at diagnosis. The presence of jaundice is generally indicative of less advanced disease and hence, a higher chance of being surgically resectable. The more common symptoms include epigastric

abdominal pain (often radiating to the back), weight loss, fatigue and anorexia. Back pain can be a premontory symptom of locally invasive disease whilst systemic symptoms of weight loss and fatigue can be indicative of metastatic disease. Steatorrhoea and malabsorption can be due to biliary and pancreatic duct obstruction. The recent onset of diabetes is a common finding in newly diagnosed pancreatic cancer. As such, this differential diagnosis should be considered in any patients who develop diabetes late in life.

Apart from jaundice, there are rarely any early clinical signs of pancreatic cancer. In some patients, there is a palpable distended gallbladder as noted in the Courvoisier-Terrier's law. Other late signs include ascites, a palpable mass secondary to peritoneal metastases, left-sided supraclavicular lymphadenopathy (Virchow's node) and recurring superficial thrombophlebitis (Trousseau's sign). More importantly, the clinical assessment of the patient's performance status should be performed to determine patient's suitability for aggressive surgical and non-surgical therapy.

1.8 CLINICAL AND PATHOLOGIC STAGING

Accurate pathological staging is only possible in patients who have undergone surgical resection, whereas clinical staging in all other patients is based on diagnostic imaging. The Union International contre la Cancer (UICC) tumour node metastasis (TNM) classification is the widely accepted tumour staging system and is shown in Table 1.1 (Sobin & Wittekind, 2002). In the USA, the American Joint Committee on Cancer staging system, which was developed in cooperation with the TNM committee of the UICC, is used and depicted in Table 1.2. Although the TNM and AJCC staging systems confer prognostic value for overall survival, they are not particularly useful in guiding treatment. Most clinicians generally stratify pancreatic cancer patients into 'resectable' (UICC stage I or II), 'locally advanced' (UICC stage III) or 'metastatic' (UICC stage IV) disease, based on findings of diagnostic imaging studies. Such

classification is useful in guiding treatment for patients as well as stratifying patients for prospective clinical trials.

Table 1.1. Union International contre la Cancer (UICC) TNM classification

Definition of tumour	Regional lymph nodes	Distant metastasis	UICC stage
Tx: primary tumour cannot be assessed	Nx: regional lymph nodes cannot be assessed	Mx: distant metastasis cannot be assessed	0: Tis, N0, M0
T0: no evidence of primary tumour	N0: no regional lymph node metastasis	M0: no distant metastasis	IA:T1, N0, M0
Tis: in situ carcinoma	N1: regional lymph node metastasis	M1: distant metastasis	IB: T2, N0, M0
T1: tumour limited to the pancreas, ≤2 cm in greatest dimension	pN1a: metastasis in a single regional lymph node		IIA: T3, N0, M0
T2: tumour limited to the pancreas, >2 cm in greatest dimension	pN1b: metastasis in multiple regional lymph nodes		IIB: T1-3, N1, M0
T3: tumour extends into duodenum, bile duct or peripancreatic tissues			III: T4, N *Any*, M0
T4: tumour extends into stomach, spleen, colon or coeliac axis vessels			IV: T *Any*, N *Any*, M1

Table 1.2. American Joint Committee on Cancer (AJCC) staging of pancreatic cancer

Stage	Tumour	Regional lymph nodes	Distant metastases
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage III	T4	N any	M0
Stage IV	T any	N any	M1

1.9 DIAGNOSIS AND ASSESSMENT OF SURGICAL RESECTABILITY

The principal goal of staging in pancreatic cancer is the determination of resectability. In patients who undergo pancreatic resection surgery, the critical importance is to achieve microscopically negative surgical margin (i.e. R0 resection). Clearly this will have impact on survival outcome as numerous studies have shown that patients with microscopic or macroscopic positive resection margins have survival rates as poor as those treated non-operatively (Klempnauer et al., 1995; Nitecki et al., 1995; Willett et al., 1993). Significant morbidity from laparotomising patients who are subsequently found to be irresectable, combined with the crucial importance of complete surgical resection, make it critically important to accurately define the anatomical involvement of the primary tumour with its surrounding structures and also to detect the presence of metastasis, both of which will impact on the likelihood of potentially curative surgery.

1.9.1 CT and MRI scans

The current standard for pancreatic cancer staging is the use of high-quality thin-section computerized tomography (CT) scans. CT is accurate in defining the relationship of the primary tumour to the superior mesenteric vein (SMV)/portal vein (PV) confluence, superior mesenteric artery (SMA) and coeliac axis. Based on the CT appearance, a patient can be considered resectable if there is no evidence of extrapancreatic or distant metastatic disease, whilst the involvement of regional vessels (e.g. PV, SMV or PV/SMV confluence) may still be resectable if vascular resection and reconstruction is deemed feasible. Magnetic resonance imaging (MRI) can also be used to stage pancreatic cancer, though it offers no major advantages over CT and its use is currently limited to patients with allergies to CT contrast agent.

1.9.2 Endoscopic ultrasound

In cases where the tumours are small or poorly defined on CT scan, endoscopic ultrasound provides a highly sensitive, non-invasive method of assessing the extent of primary tumour/vessel relationships and evaluating surrounding lymph nodes. EUS is currently the method of choice for obtaining a fine needle aspiration for pathologic diagnosis. In patients who are deemed resectable by CT criteria, a pretreatment histological diagnosis is unnecessary. However, pathological confirmation of malignancy is critical for patients with locally advanced or metastatic disease who will be treated non-operatively.

1.9.3 Staging laparoscopy

Laparoscopy, including laparoscopic ultrasonography, is sensitive for detecting small-volume (<1 cm) peritoneal and liver metastases. It remains controversial if laparoscopy should be used for all patients or applied selectively. With the use of high-quality CT scan, the yield of laparoscopy in detecting occult metastatic disease is decreasing. As such, the trend is now to

use laparoscopy selectively in patients who are deemed resectable on initial CT scan and at high risk of metastasis (e.g. large tumour > 3cm, tumour in pancreatic body or tail, high CA 19-9 levels or equivocal findings of locally advanced or metastatic disease on CT scan). The additional advantage of laparoscopy is the possibility of obtaining tissue diagnosis through peritoneal biopsy or fine needle aspiration of liver metastasis especially in cases where EUS has failed to obtain a sample (Butturini et al., 2007). Additionally, laparoscopic ultrasound may help to identify vascular invasion and hepatic metastases.

1.9.4 Endoscopic retrograde cholangiopancreatography

ERCP currently has no role in the primary staging process of pancreatic cancer. The role of ERCP is confined to the palliative stenting of obstructive jaundice due to irresectable disease. Pre-operative biliary decompression by ERCP stenting in patients with resectable pancreatic cancer does not confer any advantage in terms of surgical outcomes (State-of-the-science panel, 2002). Although ERCP can provide cytological brushings to confirm diagnosis, its use is now limited in view of the availability of less invasive endoscopic ultrasound. Brushings during ERCP have high specificity (98%) but sensitivity is relatively poor.

1.9.5 Percutaneous biopsy

There is a widely held view that transperitoneal biopsy in patients thought to be potentially resectable should be avoided due to the possibility of peritoneal tumour seeding. To support this, a recent study has shown that the incidence of peritoneal carcinomatosis is significantly higher in pancreatic cancer patients who underwent percutaneous biopsy as compared to EUS-guided fine needle aspiration (16.3% versus 2.2%) (Micames et al., 2003). Further, if EUS-guided biopsy is performed in a patient with potentially resectable disease, there is the theoretical advantage that the needle tract is contained in

the eventual surgical specimen. Therefore, if tissue diagnosis is required, EUS-guided biopsy is the method of choice.

1.9.6 Tumour marker

CA19-9 is a tumour associated antigen that is frequently elevated in pancreatic cancer (Ritts & Pitt, 1998). It remains uncertain regarding the correct cutoff level required to diagnose malignancy. Studies have found accuracy rates of 95% in the diagnosis of pancreatic malignancy when levels were >200 U/mL (Ritts & Pitt, 1998). When used in combination with CT, a positive predictive value of 99% can be achieved with levels >120 U/mL. Currently, CA19-9 can be helpful as supportive evidence of malignancy in equivocal cases. This tumour marker can also be used as a surrogate for therapeutic response, recurrence and prognosis. In some centres, abnormally high CA19-9 levels may also be used to indicate a high risk for occult metastases and hence, is deemed an indication for additional staging modalities such as staging laparoscopy.

1.10 MANAGEMENT

It is now commonly accepted that the centralisation of surgery for pancreatic cancers in high-volume centres improves the results of surgery for all outcome measures including long-term survival (Birkmeyer et al., 2002; Birkmeyer et al., 1999; Neoptolemos et al., 1997). Currently, only a small percentage of patients with pancreatic cancer are diagnosed early enough to have a resectable disease, with 2.6-9% of patients undergoing pancreatic resection (Bramhall et al., 1995; Hedberg et al., 1998; Sener et al., 1999). The current management of pancreatic cancer should be multidisciplinary, involving the pancreatic surgeon, gasteroenterologists, medical and radiation oncologists and dedicated radiologists.

1.10.1 Surgical resection

1.10.1.1 Preoperative biliary drainage

Preoperative biliary drainage was previously performed routinely due to concerns that surgery in jaundiced patient carried an increased risk of postoperative complications (Blamey et al., 1983). However, this has been proven to be unfounded with current evidence. Recent meta-analysis of available randomised controlled trials has shown no benefit from preoperative biliary drainage (Sewnath et al., 2002). In fact, some series have shown an increase in wound infection rates with no impact on clinical outcome (Pisters et al., 2001; Povoski et al., 1999; Sohn et al., 2000). Currently, the practice of biliary drainage is performed in settings when the surgery will be delayed for logistic reasons or if patients are entered into neoadjuvant trials.

1.10.1.2 Kausch-Whipple resection

The principle aim of the Kausch-Whipple operation is to remove all gross and microscopic disease within the pancreas and draining lymph nodes to achieve a margin negative (R0) resection. The anatomic location of the primary tumour dictates the type of resection. Pancreatic cancer in the head and uncinate process will require a Kausch-Whipple procedure or pancreaticoduodenectomy which is the most common operation for pancreatic cancer given the predominance of tumours in the head of pancreas. Tumours in the body and tail of pancreas may be amenable to distal pancreatectomy. However, tumours of the body and tail are notorious for late diagnosis and hence, often present at a locally advanced stage or with distant metastasis, precluding surgical treatment. If a central tumour at the neck or body of the pancreas is resectable, the type of resection will depend on its exact anatomic location. If the tumour is nearer to the head of pancreas, an extended pancreaticoduodenectomy will be appropriate. However, if the tumour is nearer to the tail, a distal subtotal pancreatectomy should be performed. Given the increased risk of morbidity, a total pancreatectomy is only indicated

if there is involvement of the pancreas beyond the division margins of a partial pancreaticoduodenectomy (Launois et al., 1993). The involvement of regional vessels is no longer an absolute contraindication to resection. Vascular resection and reconstruction is now feasible and is only supported if an R0 resection can be obtained (Nakao et al., 2006). In particular, portal venous resection is safe and does not increase the operative morbidity or mortality (Bachellier et al., 2001; Nakagohri et al., 2003). Arterial resection has been described but the data on its benefit are limited (Kondo et al., 2003; Nakano et al., 2002; Sasson et al., 2002).

The pylorus-preserving pancreaticoduodenectomy was first popularized in the 1980s by Traverso and Longmire (Traverso & Longmire, 1978). The initial concerns regarding the surgery were whether the preservation of pylorus would prevent adequate clearance of the suprapyloric and infrapyloric perigastric nodes, and adversely affect the clinical outcome. However, this has not been proven to be the case. So far, three prospective randomised trials have compared the outcomes of standard and pylorus-preserving Kausch-Whipple procedures and found no significant difference in long term survival and perioperative morbidity between the two groups (Lin & Lin, 1999; Seiler et al., 2005; Tran et al., 2004). The general consensus now is that both operations are equally effective for the treatment of pancreatic and periampullary carcinoma. Thus, a surgeon's preference and experience should dictate the type of pancreaticoduodenectomy performed.

Although extended lymph node dissection has been suggested to improve survival in a previous retrospective study (Ishikawa et al., 1988), randomised controlled trials have not supported this (Pedrazzoli et al., 1998; Yeo et al., 2002). Further, a recent meta-analysis of randomised controlled trials has not shown any survival advantage with extended lymphadenectomy compared to standard lymphadenectomy following pancreatic resection for pancreatic cancers (Michalski et al., 2007).

1.10.2 Adjuvant chemo- and radiotherapy

The high incidence of locoregional recurrence following surgical resection for PDAC has prompted investigations into the possible roles of adjuvant chemoradiotherapy. Currently, there is no clear consensus on adjuvant therapy for PDAC. In the UK and Europe, adjuvant chemotherapy is favoured based on the results of European Study Group for Pancreatic Cancer (ESPAC)-1 (Neoptolemos et al., 2004) and CONKO studies (Oettle et al., 2007). However, in North America, the standard adjuvant regime includes chemo-radiotherapy followed by chemotherapy, based on the results of the Gastrointestinal Tumour Study Group (GITSG) study (1987). The role of radiotherapy is controversial as addition of radiotherapy has been shown to have a negative effect on survival in ESPAC-1 study. Further, the EORTC study did not find a significant survival advantage with chemoradiation in PDAC patients (Klinkenbijl et al., 1999). More recently, meta-analyses of randomised control trials of adjuvant therapy in PDAC patients have shown chemoradiation to be ineffective (Stocken et al., 2005; Yip et al., 2006).

The ESPAC-1 study is the largest randomised trial of adjuvant chemoradiation and chemotherapy. In this study, adjuvant chemotherapy (5-fluorouracil and folinic acid) has been demonstrated to confer significant survival benefit (median and 5-year survival rates) in patients following curative resection of PDAC (Neoptolemos et al., 2004). As mentioned earlier, this study found that chemoradiation had a negative impact on survival when compared to chemotherapy alone. Criticisms of this study included its complex 2X2 factorial design and concerns regarding the quality assurance and execution of radiotherapy. On the other hand, the GITSG showed that adjuvant chemoradiotherapy in PDAC patients conferred significant survival advantage compared to no adjuvant therapy (Gastrointestinal Tumor Study Group, 1987). This study was criticised for its small sample size. A summary of the randomised trials performed so far to address the issue of adjuvant therapy for pancreatic cancer is provided in Table 1.3.

The development of gemcitabine is considered a major advance in the treatment of pancreatic cancer. In a landmark phase III study by Burris and colleagues, single agent gemcitabine was shown to provide significant improvements both in survival and clinical benefit (weight loss and tumour-associated pain) when compared to single agent 5-FU as first line chemotherapy for advanced pancreatic cancer (Burris et al., 1997). A recent RCT has shown that adjuvant chemotherapy based on gemcitabine significantly prolonged disease-free survival and delayed the development of recurrent disease after curative resection of PDAC (Oettle et al., 2007). It is now commonly accepted that significant antitumour activity in advanced disease is a prerequisite for a cytotoxic agent to be effective in the adjuvant setting for early disease. Given the superiority of gemcitabine over 5-FU, gemcitabine is now widely accepted to be the treatment standard for irresectable PDAC. Further, given its efficacy, gemcitabine is now the agent of choice in the adjuvant setting. However, the ongoing ESPAC-3 study on completion will provide definitive data on efficacy of gemcitabine versus 5-FU in the adjuvant setting.

Adjuvant regional chemotherapy has also been explored. The rationale behind this mode of therapy is to deliver high concentrations of active agent to the major sites of potential recurrence while limiting systemic toxicity. The chemotherapeutic agent is usually given through catheters placed in the coeliac/hepatic artery or portal vein. Currently, there are only a few small, non-randomised studies on regional chemotherapy. Ishikawa and colleagues have provided promising data on regional 5-FU therapy through catheters placed in coeliac trunk and portal vein for infusion in the adjuvant setting (Ishikawa et al., 1997; Ishikawa et al., 1994). The long term follow-up of these patients gave a 5-year survival of 39%. The ESPAC-2 study is currently evaluating the prospect of this form of therapy in a randomised controlled trial.

Table 1.3. Randomised controlled trials of adjuvant treatment for pancreatic adenocarcinoma

Trial (year), author	Comparison	Adjuvant regime	Number of patients	Median survival (months)	Conclusions
GITSG (1987), (Gastrointestinal Tumor Study Group, 1987)	Chemoradiation Observation	40 Gy + 5-FU	43	20 11	Significant survival benefit in the treatment arm (p=0.035)
Norway (1993), (Bakkevold et al., 1993)	Chemotherapy Observation	5-FU +doxorubicin +mitomycin C	61 (47 pancreatic, 14 ampullary), 46 additional patients, non-randomised	23 11	Significant survival benefit in the treatment arm (p=0.02)
EORTC (1999), (Klinkenbijl et al., 1999)	Chemoradiation Observation	40 Gy + 5-FU	218 (120 pancreatic, 98 ampullary)	17.1 12.6	No significant survival difference (p=0.099)
Japan (2002), (Takada et al., 2002)	Chemotherapy Observation	5-FU+mitomycin C	508 (173 pancreatic, 335 bile duct/gallbladder/ampullary)	5-YS: 11.5% 5-YS: 18%	No significant survival difference
ESPAC1 (2004), (Neoptolemos et al., 2004)	Chemotherapy No chemotherapy	5-FU	289	20.1 15.5	Chemotherapy significantly superior to observation (p=0.009)
	Chemoradiation No chemoradiation	40 Gy + 5-FU		15.9 14.8	Chemoradiation is not different from observation, and is inferior to chemotherapy
CONKO-001 (2005), (Oettle et al., 2007)	Chemotherapy Observation	Gemcitabine	368	DFS: 13.4 DFS: 6.9	Significant increase in median DFS

DFS: disease-free survival, 5-YS: 5-year survival, 5-FU: 5-fluorouracil

1.10.3 Targeted therapy

The interest in novel targeted therapy in pancreatic cancer is immense given the dismal prognosis of this disease and the general consensus that survival benefits from conventional therapy have nearly been maximised. Truly transforming results will need to come from continuing development of novel molecular therapies which are more targeted and have high efficacy without undue toxicity. Investigators are now focusing on new strategies that target cancer cells at the molecular level. Currently, phase III trials using molecular agents are being targeted at patients with advanced pancreatic cancers in whom any advance in therapy that improves survival is urgently needed. Results of published phase III trials using molecular targeted therapy are summarised in Table 1.4.

1.10.3.1 Epidermal growth factor inhibition

The most exciting results relate to the use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, Erlotinib (Tarceva®, Genentech, USA). In a recent placebo-controlled randomised phase III trial, for the first time, superiority of a gemcitabine combination with an EGFR-targeted agent (erlotinib) over single agent gemcitabine in advanced pancreatic cancer has been shown (Moore et al., 2005). Moore et al showed that combination of gemcitabine and erlotinib conferred a modest but statistically significant survival benefit compared with gemcitabine and placebo (1 year survival rates: 23.2% vs. 19.4%, p=0.025). Correspondingly, the progression free survival (PFS) times were also in favour of the erlotinib-treated group (median PFS: 3.75 vs. 3.55 months, p=0.003). Given these results, the Food and Drug Administration in USA has approved erlotinib for use in advanced pancreatic cancer patients.

Cetuximab is an anti-EGFR monoclonal antibody (Erbitux®, ImClone Systems, USA). Preliminary findings of a Phase II trial testing the combination of cetuximab and gemcitabine in advanced pancreatic cancer has shown a

one-year survival rate of 31.7%, median survival of 7.1 months and median progression free survival time of 3.8 months (Xiong et al., 2004). This combination is currently being investigated in a Southwest Oncology Group (SWOG S0205) phase III trial.

1.10.3.2 Angiogenesis inhibition

Targeting the vascular endothelial growth factor (VEGF) signalling pathway has also been investigated. Bevacizumab (Avastin®, Genentech, USA) is a recombinant humanised monoclonal antibody to VEGF with an antiangiogenic action. In preclinical models, bevacizumab has been shown to suppress the growth of pancreatic tumours. A recent phase II trial testing the use of bevacizumab and gemcitabine combination in advanced pancreatic cancer has shown some promising results; a one-year survival rate of 29% with median survival of 8.8 months (Kindler et al., 2005). These results had led to a phase III trial by the Cancer and Leukemia Group B (CALGB 80303 trial). The preliminary results of this trial has been recently reported at the 2007 American Society of Clinical Oncology meeting (Saif, 2007). Unfortunately, the trial did not show any survival benefit of adding bevacizumab to gemcitabine.

1.10.3.3 Matrix metalloproteinase inhibition

Matrix metalloproteinases (MMPs) are a group of proteases that are involved in tissue remodelling involved in cellular invasion, metastasis, growth and angiogenesis. The MMPs are frequently overexpressed in pancreatic cancers. The rationale of MMP inhibitor is to impair the process of local invasion and early metastasis which is characteristic of pancreatic cancer. Marimastat is an inhibitor of MMPs. A large phase III trial previously assessed the feasibility of using marimastat as the first-line therapy for advanced pancreatic cancer. However, this trial showed that marimastat was inferior to gemcitabine in terms of overall survival (median survival: 2 vs. 3.8 months, p=0.001) (Bramhall et al., 2001). In a further phase III placebo controlled trial,

combination of marimastat and gemcitabine did not have a survival impact when compared to single agent gemcitabine (Bramhall et al., 2002). As such, there is no evidence to support the use of marimastat in advanced pancreatic cancer.

Table 1.4. Phase III clinical trials of molecular targeted therapies in advanced pancreatic cancer

Trial (Author)	Comparison	Mechanism	No. of patients	Response	PFS (months)	Median survival (months)	One-year survival
(Moore et al., 2005)	Erlotinib/gemcitabine	EGFR tyrosine kinase inhibitor	569	8.6%	3.75*	6.37*	23.2%*
	Gemcitabine			8%	3.55	5.91	19.4%
(Bramhall et al., 2002)	Marimastat/gemcitabine	MMP inhibitor	239	11%	3.56	5.5	18%
	Gemcitabine			16%	2.96	5.4	17%
(Moore et al., 2003)	BAY 12-9566	MMP inhibitor	277	0.9%	1.68	3.74	10%
	Gemcitabine			5%	3.5	6.59*	25%*
(Van Cutsem et al., 2004)	Tipifarnib/gemcitabine	Ras farnesylation inhibitor	688	6%	3.7	6.4	27%
	Gemcitabine			8%	3.6	6.0	24%

* indicates statistical significance. EGFR: epidermal growth factor receptor, MMP: matrix metalloproteinase, PFS: progression free survival

1.10.4 Treatment of locally advanced or metastatic disease

Given the poor outcome of advanced pancreatic cancer, the main consideration in management is ultimately if a patient is a candidate for aggressive therapy. This decision should be based on the patient's performance status, extent and intensity of symptoms, nutritional status and potential toxicity of the proposed therapy and more importantly, its impact on the quality of life.

Single-agent gemcitabine is the standard therapy for advanced or metastatic pancreatic cancer with improved overall survival and offering significant clinical benefit compared with 5-fluorouracil (Burris et al., 1997). Encouraging interim results were reported by the UK GemCap phase III trial comparing gemcitabine and capecitabine (GemCap) combination with single agent gemcitabine in the treatment of advanced pancreatic cancer. The median overall survival was superior with GemCap compared to gemcitabine alone (7.4 vs. 6 months, p=0.001) and the one-year survival rates were 26% and 19% respectively (Cunningham et al., 2005). On the other hand, the Swiss Group for Clinical Cancer Research and the Central European Cooperative Oncology Group has published their phase III trial results showing no statistically significant improvement in overall survival with GemCap compared to standard gemcitabine. However, in a subgroup of patients with good performance status, median survival was improved significantly with GemCap (Herrmann et al., 2007).

Apart from the UK GemCap Trial, many other phase III trials have investigated the efficacy of double chemotherapeutic agents combining gemcitabine and 5-FU (Berlin et al., 2002), irinotecan (Rocha Lima et al., 2004), pemetrexed (Oettle et al., 2005), cisplatin (Heinemann et al., 2006) or oxaliplatin (Louvet et al., 2005) and found no definite benefit in overall survival.

1.10.5 Neoadjuvant therapy

There is currently no randomised clinical trial assessing the potential benefit of neoadjuvant therapy and surgery over surgery alone in pancreatic cancer. As such, the practice of neoadjuvant therapy is evolving and remains at the clinical trial phase (Desai et al., 2007; Heinrich et al., 2008). The underlying rationale for neoadjuvant therapy in pancreatic cancer is logical and particularly attractive (Quiros et al., 2007). These principles include: 1) neoadjuvant therapy may downstage the tumour and increase the likelihood of R0 resection, 2) treatment of micrometastases prior to surgery, 3) possibility of delivering neoadjuvant therapy without the potential delays caused by prolonged recovery or postoperative complications, and 4) avoidance of surgery in patients with metastatic progression found on restaging following neoadjuvant therapy. Clearly, the downside of this novel concept is that ineffective neoadjuvant therapy may risk disease progression during therapy and may compromise the chance of definitive surgical resection.

1.10.6 Palliative therapy

The majority of patients with pancreatic cancer have irresectable disease at the time of diagnosis. The major symptom requiring palliation in this group of patients is malignant obstructive jaundice. In most units, the strategy is to place an expandable, metal biliary stent endoscopically in patients who have locally advanced and irresectable disease. However, if the patients are found to be irresectable at the time of laparotomy, then consideration is given to surgical biliary (Roux-en-Y hepatojejunostomy) and gastric bypass depending on the extent of local disease and patient's performance status. Increasingly, laparoscopic biliary and gastric bypass is being adopted and is a safe and effective technique. Pain can be controlled by neurolytic coeliac plexus block administered intraoperatively or under CT/EUS guidance. This has been shown to be effective in more than 70% of patients with irresectable disease (Freelove & Walling, 2006).

1.10.7 Outcome following surgery

The current perioperative mortality following pancreatic resection in high-volume centres stands at 1-4%. In patients who undergo surgical resection for an early stage pancreatic cancer, the 5-year survival rates range from 7-25%, with a median survival of 11-20 months (Alexakis et al., 2004). Patients with irresectable locally advanced, non-metastatic disease have a median survival of 6-11 months whilst those with metastatic disease have a median survival of 2-6 months. Patients who develop recurrent disease usually do so after a mean duration of 9-12 months following surgery.

1.10.8 Screening for pancreatic adenocarcinoma

Given the generally dismal prognosis of PDAC, screening for this cancer may be a way forward to improve the prognosis. However, the prevalence of pancreatic cancer remains too low to permit screening of the general population. Further, the limited sensitivity of current diagnostic techniques renders screening of the asymptomatic population not feasible. In a small high-risk subgroup, the prevalence of pancreatic cancer may reach a level high enough to permit secondary screening. For instance, the lifetime risk of developing pancreatic cancer for patients with hereditary pancreatitis is extremely high with a risk of 20% at age of 60 years and up to 40% by the age of 70 years (Lowenfels et al., 1997).

The primary screening process is the identification of people with high risk of developing pancreatic cancer whilst secondary screening identifies patients with early asymptomatic cancer amenable to curative surgical resection. Secondary screening programme such as the European registry of hereditary pancreatitis and familial pancreatic cancer (EUROPAC) programme based in Liverpool, UK is currently under investigation to assess its feasibility in identifying and screening these high-risk groups. Clearly, the challenge of such a programme is to have a diagnostic strategy that provides a high positive predictive value to prevent unnecessary surgery (i.e total

pancreatectomy) and a high negative predictive value to avoid missing any resectable cancers. In patients with hereditary pancreatitis, the gross features of chronic pancreatitis can seriously undermine the diagnostic accuracy of conventional imaging techniques. As such, any diagnostic strategy will need to be multimodal including the use of molecular screening to identify a genuinely high-risk group.

Under the EUROPAC secondary screening programme, patients who are over 40 years of age with hereditary pancreatitis or a significant family history of familial pancreatic cancer are screened for early pancreatic cancer using multimodality imaging (CT and/or endoscopic ultrasound) and molecular analysis of pancreatic juice obtained at ERCP (for K-ras, p53 and p16 mutations) (Yan et al., 2005). The application of molecular screening is potentially the most powerful method and adds to the specificity and sensitivity of imaging techniques. Although K-ras mutation can be detected in the pancreatic juice of most patients with pancreatic cancer, it is also detectable in patients with benign conditions (e.g. non-inherited chronic pancreatitis) and older populations with no pancreatic disease. Bearing this in mind, K-ras mutational status remains useful to risk stratify patients to focus additional imaging and molecular diagnostics in a genuinely high-risk group. The screening protocol that is currently under investigation is illustrated in Figure 1.6.

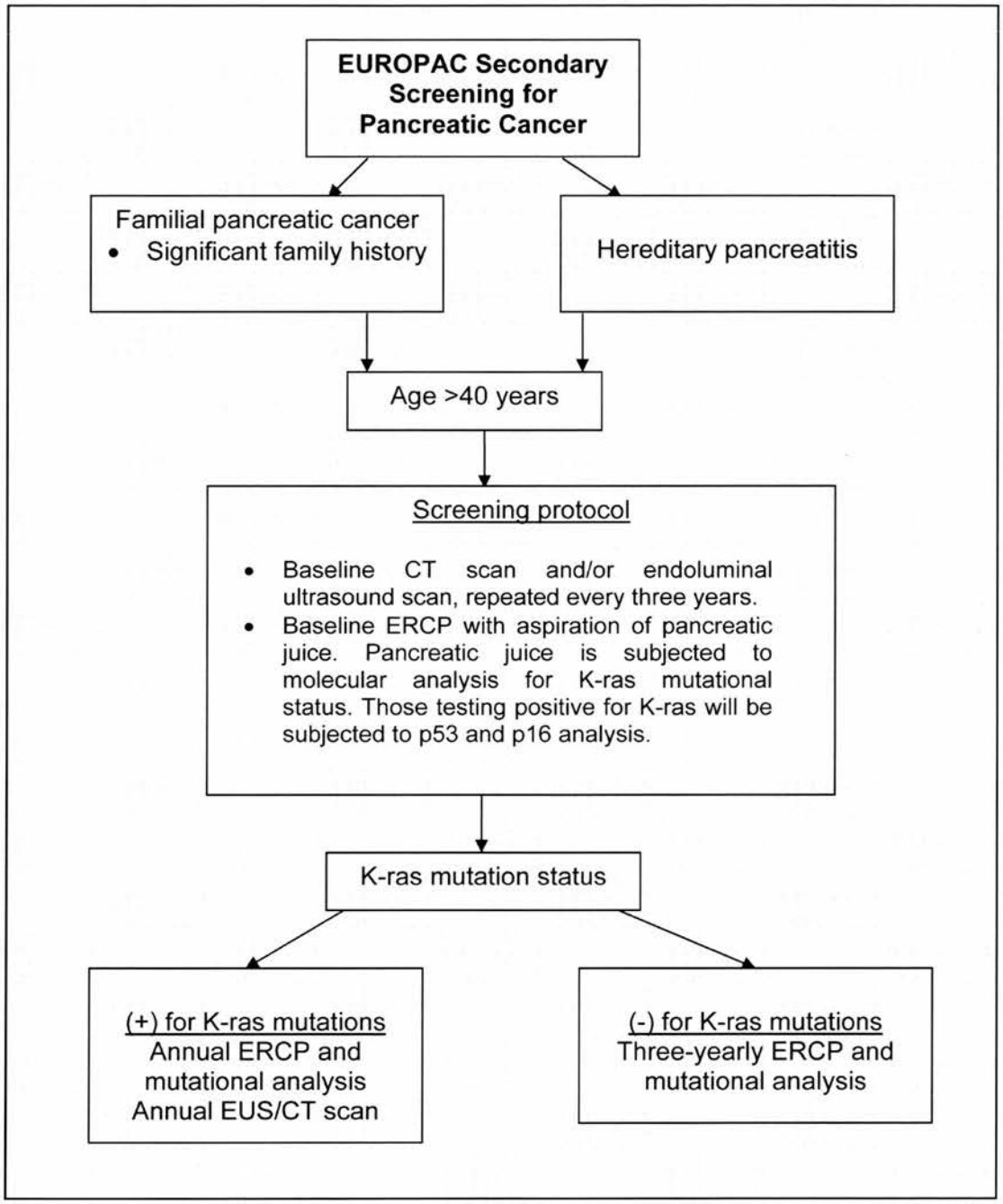


Figure 1.6. The screening protocol of the European registry of hereditary pancreatitis and familial pancreatic cancer (EUROPAC) programme for pancreatic cancer developed in Liverpool, UK. Figure adapted from online resource by University of Liverpool (EUROPAC Project, 2007).

1.11 HMGA1: AN ARCHITECTURAL TRANSCRIPTIONAL FACTOR AND A PUTATIVE ONCOGENE

1.11.1 In search of a novel therapeutic target: HMGA1 identified from global transcriptomic profiling of pancreatic cancers

The genetic abnormalities described in PDAC (in Section 1.6: Molecular genetics and adeno-carcinoma sequence of PDAC) exert their effects by altering gene expression and production of specific proteins. These proteins can serve as the crucial driving forces behind the development of pancreatic cancer and the maintenance of its malignant phenotype. The gene expression patterns in PDAC have been studied using state-of-the-art technologies that profile the expression of multiple genes simultaneously. Such global gene expression profiling technologies that have been applied to PDAC include the serial analyses of gene expression (SAGE), cDNA arrays and oligonucleotide arrays (i.e. 'gene chips'). The application of such technologies allowed the discovery of a comprehensive list of genes which are either selectively overexpressed or suppressed in PDAC compared to normal pancreas. Such technologies have led to discovery of novel molecular targets with considerable potential for developing rational early detection and therapeutic strategies for PDAC.

Several large-scale analyses of human PDAC tissues and cell lines have identified a number of differentially overexpressed genes (Crnogorac-Jurcevic et al., 2003; Friess et al., 2003; Logsdon et al., 2003). Using a cDNA microarray format, Iacobuzio-Donahue and colleagues identified *HMGA1* as highly overexpressed in both pancreatic cancer tissues and cell lines when compared to normal pancreatic tissue (Iacobuzio-Donahue et al., 2003). Similarly, Han and co-workers showed that *HMGA1* is significantly overexpressed (between 4 to 14-fold higher) in PDAC cell lines compared to normal pancreas (Han et al., 2002). At a smaller scale (about 15 PDAC tissue specimens), Abe and colleagues studied the protein expression of HMGA1 in

PDAC tissues using immunohistochemistry. This analysis revealed that HMGA1 is overexpressed in the sample of PDAC tissues and there is a strong correlation between HMGA1 expression and a diagnosis of carcinoma (Abe et al., 2000).

Using the orthotopic SCID mouse model for pancreatic cancer, Tarbe et al set out to identify genes associated with metastasis of PDAC cells (Tarbe et al., 2001). They demonstrated that cell lines derived from metastatic lesions showed remarkable upregulation of the HMGA1 expression when compared to cell lines derived from primary tumours using the oligonucleotide array technology. In studies by Bussemakers and colleagues, the Dunning R-3327 benign rat prostatic tumour cell line was compared with the highly metastatic MatLyLu cells derived from this cell line using the technique of differential hybridisation (Bussemakers et al., 1991). They found that HMGA1 was overexpressed in metastatic cell lines and its expression correlated with metastatic ability, rather than with proliferation rate. As such, it was suggested that *HMGA1* may represent a gene of functional significance in the context of metastasis.

Based on the observations from gene expression profiling, HMGA1 has been identified as a novel candidate gene to be differentially overexpressed in PDACs and may have functional roles in cancer biology. In this thesis, we sought to establish the clinical relevance of HMGA1 in patients with PDAC and to characterise the roles of HMGA1 in the malignant phenotype of PDAC cells and in particular, to identify its downstream molecular mediators, which hitherto have not been described. There is accumulating evidence that HMGA1 is functionally involved in the pathobiology of cancer cells. In the following sections, the current evidence supporting the roles of HMGA1 in tumourigenesis will be discussed.

1.11.2 HMGA1 proteins: nomenclature and molecular structure

Thirty years ago, the high mobility group (HMG) proteins were originally discovered and isolated from mammalian cells (Goodwin et al., 1973). They were named according to their high electrophoretic mobility in polyacrylamide gels and were characterised by their solubility in 2-5% trichloroacetic acid (Lund et al., 1983). Despite early purification and biochemical characterisation, their functions remained elusive for many years. Following the cloning of cDNAs encoding HMG proteins, it became apparent that this group of heterogenous proteins could be classified into several families (Mouse Gene Nomenclature Committee, 2007)(see this reference for full classification of HMG proteins). One of these families is the high mobility group A (HMGA) protein family which consists of HMGA1a, HMGA1b and HMGA2 proteins. The first two members (HMGA1a and HMGA1b) are alternatively-spliced proteins which are identical except for internal deletion of 11 amino acids in HMGA1b and are encoded by the same gene (i.e. HMGA1). The HMGA2 protein is the product of a different gene located at 12q14-15 (Chau et al., 1995).

In this thesis, the focus will be on the roles of HMGA1 proteins which have been found to be upregulated in PDAC cells. In human, the HMGA1 gene is located on short arm of chromosome 6 (6p21) (Friedmann et al., 1993). The HMGA1 proteins are small non-histone chromatin-associated proteins that bind DNA with little or no sequence specificity (Bustin et al., 1990). HMGA1 proteins have a low molecular weight (10.6-12kDa), but their molecular weight *in vivo* can be higher due to post-translational modifications. HMGA1 proteins have little, if any, secondary structure when free in solution but adopt a disordered-to-ordered structured conformation when bound to DNA or other proteins (Huth et al., 1997). It has been proposed that the high degree of intrinsic flexibility due to a lack of secondary structure enables the HMGA1 proteins to participate in a wide range of biological processes (Reeves, 2001).

Each HMGA1 protein has three DNA-binding motifs called the 'AT-hooks' that preferentially bind to adenine and thymine (AT)-rich DNA sequences in the minor groove of DNA by recognising structure rather than nucleotide sequence (Reeves & Nissen, 1990) (Figure 1.7). The AT-hook domain consists of the palindromic pentapeptide sequence of Pro-Arg-Gly-Arg-Pro (PRGRP) which is highly conserved in the HMGA family of proteins (Figure 1.8).

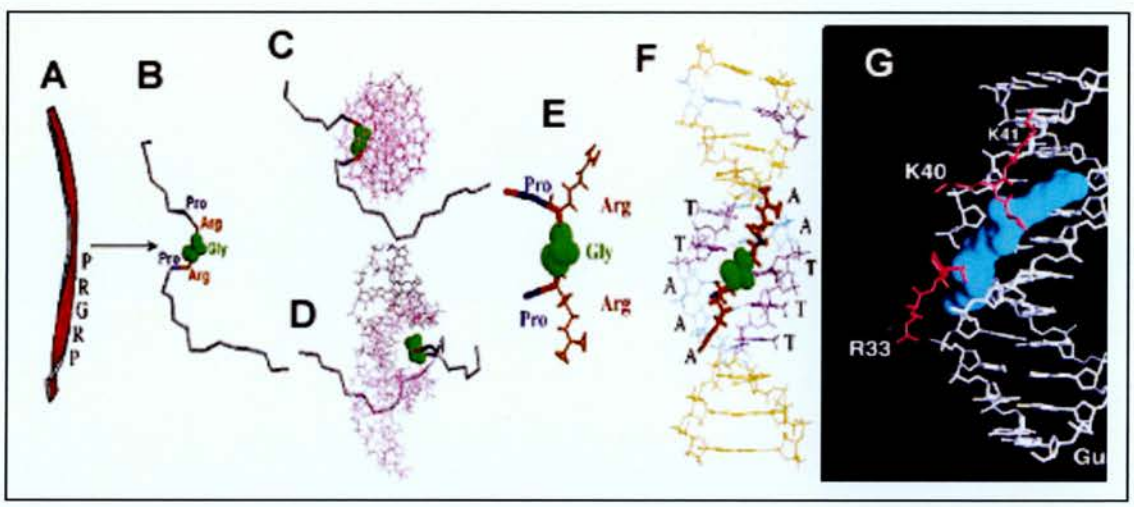


Figure 1.7. Schematic diagrams showing the binding of second AT-hook motif (PRGRP) (A and B) of the human HMGA1a protein to minor groove of an AT-rich segment of DNA. C and D, Various projection views of the peptide bound to DNA (side and along the long axis) are shown. E-G, Three dimensional reconstruction of the HMGA1 AT-hook bound to the minor groove of DNA. Adapted from (Reeves, 2000) and (Huth et al., 1997)

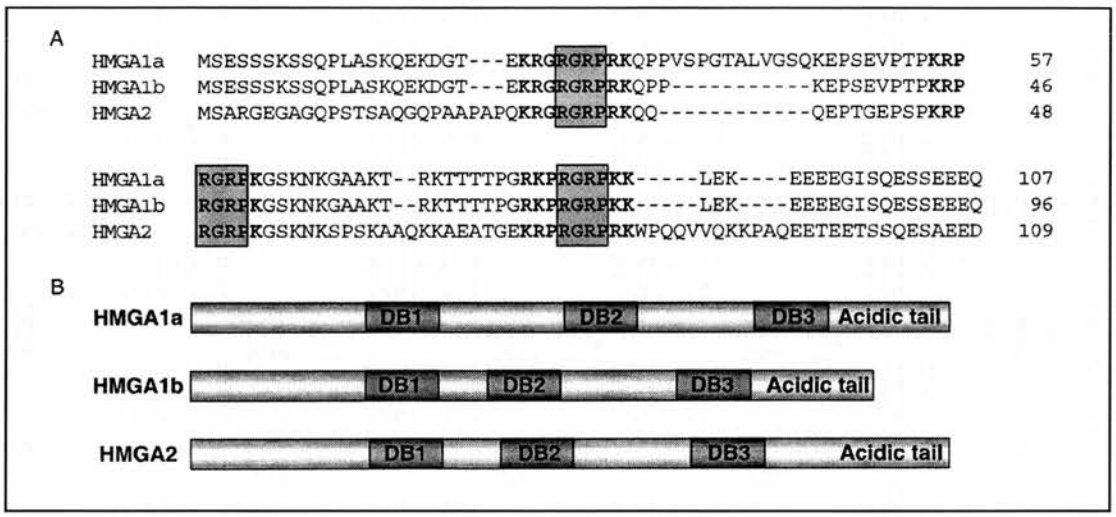


Figure 1.8. Aligned amino acid sequences of the members of HMGA family with the position of the DNA binding domains (DB1-3) shown. In HMGA1, the DBs are known as the AT-hooks. Figure adapted from (Giannini et al., 2005).

1.11.3 Mechanisms of actions of HMGA1

HMGA1 proteins are known as 'architectural transcriptional factors'. Although they do not directly act as transcription factors, they are able to induce allosteric changes in DNA that promote gene regulation. There are several mechanisms proposed for their actions. These mechanisms are not necessarily mutually exclusive.

Firstly, HMGA1 is capable of binding to AT-rich promoter regions of genes. When bound to AT-rich promoter elements, HMGA1 proteins induce a conformational change in DNA to promote binding of transcriptional factors. Further, they form and stabilise multiprotein complexes together with other sequence-specific transcriptional factors through direct protein-protein interaction. These complexes are known as 'enhanceosomes' which act as transcription activating complexes (Figure 1.9). HMGA1 proteins are known to physically interact with transcriptional factors such as Oct 2, ATF-2/c-Jun, AP-1, NFκB, Elf-1, SRF, PU-1, RAR, NFAT, Sp1 and CAAT/enhancer binding protein-β to modulate gene activation (Reeves, 2001). Their multiple protein partners provide HMGA1 proteins with considerable flexibility in modulating

transcriptional activity of a large number of genes. Of note, HMGA1 is not only capable of positively regulating gene expression, it can also negatively regulate human interleukin 4 (Chuvpilo et al., 1993) and GP91-PHOX (a respiratory burst gene expressed in phagocytic cells) genes (Skalnik & Neufeld, 1992). However, the mechanisms underlying gene expression inhibition are largely unknown.

Secondly, HMGA1 proteins can enhance the binding of transcriptional factors to DNA. This function is performed through direct protein-protein interactions with transcriptional factors and hence, increasing their affinity for DNA binding. The enhancement of serum-response factor by HMGA1 is a classic example of this mechanism (Chin et al., 1998).

Finally, HMGA1 proteins can act as 'antirepressor' molecules by competing out histones (known transcriptional repressor) in binding to scaffold attachment regions (SAR) of specific DNA segments and thus, induce an open chromatin conformation that promotes binding of transcriptional factors and transcriptional activation (Zhao et al., 1993).

Post-translational modifications such as phosphorylation, acetylation and methylation can also influence the functions of HMGA1 proteins (Sgarra et al., 2006). Interestingly, upon inspection of the peptide sequence of the AT-hook region of HMGA1, it matches the consensus sequence for phosphorylation by cdc2 kinase. With this, it was further demonstrated that HMGA1 is phosphorylated in a cell cycle-dependent manner by cdc2 kinase *in vitro*, and this modification reduces the binding affinity of HMGA1 proteins for DNA (Nissen et al., 1991; Reeves et al., 1991).

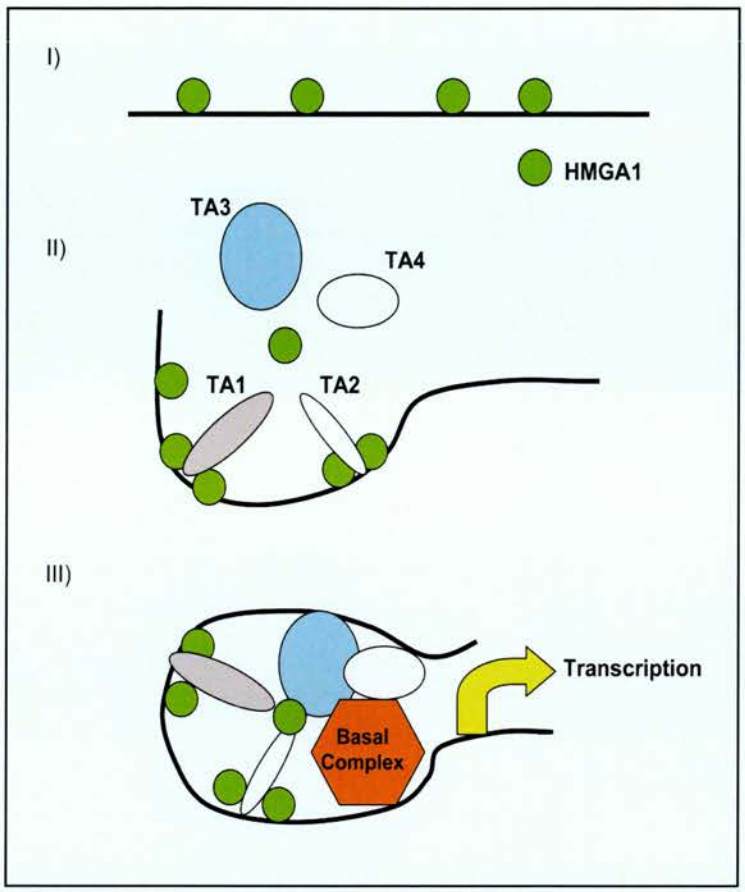


Figure 1.9. HMGA1 facilitates formation of enhanceosomes. I) HMGA1 binds to AT-rich sequences of enhancing/promoter element of genes, II) Through binding to these sequences, HMGA1 proteins are capable of 'bending' the DNA and reduces the free energy needed for subsequent binding of transcriptional activators (TA) (which include transcriptional factors), III) A further mode of action is through direct protein-protein interaction with TAs, resulting in the formation of a very stable nucleoprotein complex known as enhanceosome which strongly activates transcription of the downstream gene. Figure redrawn based on (Cerignoli et al., 2004).

1.11.4 HMGA1 is a bona fide oncogene

HMGA1 proteins are present at high levels during embryogenesis while their expression is low or completely absent in adult differentiated tissues (Chiappetta et al., 1996). In the mouse, HMGA1 proteins are detected at a very high level in all embryonic tissues up to 8.5 days of gestation. This represents the period critical for organogenesis. Following this, the HMGA1 expression remains at a low level until the end of gestation. Collectively, these observations suggest the roles of HMGA1 proteins in cellular differentiation and proliferation. Further, these proteins are undetectable or present only at very low levels in normal adult tissues in both rodents and humans.

In queiescent, non-transformed cells, HMGA1 expression is rapidly induced following treatment with phorbol esters or growth factors such as EGF and PDGF (Friedmann et al., 1993; Ogram & Reeves, 1995). Moreover, transformation with viral oncogenes such as v-ras, v-mos and polyoma middle T antigen results in increased expression of HMGA1 proteins in the transformed cells (Giancotti et al., 1987). Similar effects are also seen in tumour cell lines when exposed to growth stimuli. In highly metastatic breast cancer cell lines, there was a dramatic induction of HMGA1 mRNA expression when the cells were treated with EGF or 17β-estradiol (Holth et al., 1997).

In an elegant study by Ram and colleagues, they followed the expression of HMGA1 during the process of transformation of mouse mammary epithelial cells (Ram et al., 1993). Expression of HMGA1 was examined in cell lines derived from spontaneously arising mammary epithelial hyperplasia in BALB/C mice. Three cell lines (CL-S1, +SA and –SA) were derived from the same hyperplastic cell line, called the D1 parent line. The CL-S1 cell line is an immortalised cell line that will not form colonies in soft agarose or tumour *in vivo*. The +SA and –SA cell lines were derived from an adenocarcinoma which developed spontaneously from the original D1 line when the cells were retransplanted *in vivo*. +SA and –SA cells were differentiated by their ability (+SA) or inability (-SA) to grow in soft agar. Notably, the expression of HMGA1 was highest in the highly tumourigenic +SA line which formed poorly differentiated adenocarcinomas that metastasized early. Intermediate levels of HMGA1 were found in the –SA line which formed well-differentiated adenocarcinomas with low propensity for metastasis. The lowest levels were found in the non-tumourigenic CL-S1 line. One of the important observations from this study was that the elevated expression of HMGA1 was probably related to the stage of neoplastic transformation rather than the rate of cellular proliferation.

More convincing evidence supporting the roles of HMGA1 in tumourigenesis came from a mouse model developed by Xu and co-workers. Transgenic mice

expressing HMGA1 under control of the murine H-2K promoter and immunoglobulin μ enhancer drove the overexpression of HMGA1 transgene in B and T-cells. The mice spontaneously developed aggressive lymphoma consistent with a mature T-cell phenotype, leading to death between 1 and 8.5 months of age (Xu et al., 2004). More recently, it was shown that transgenic mice with HMGA1 overexpression targeted to uterine tissues developed adenocarcinomas by 9 months of age (Tesfaye et al., 2007).

1.11.5 Roles of HMGA1 in malignant tumours

Although HMGA1 overexpression is a phenomenon observed in several cancer types (Section 1.11.7), the basis for the elevated expression remains mostly unknown. So far, HMGA1 has been shown to be a c-Myc target gene indicating that binding of c-Myc oncoprotein to HMGA1 promoter induces its transcription (Wood et al., 2000b). More recently, it was shown that HMGA1 promoter is strongly regulated by oncogenic Ras (Cleynen et al., 2007). This suggests that in tumours with a mutated Ras gene, the Ras GTPase signalling could be a mechanism through which the tumours overexpress HMGA1. This could be particularly important in PDAC cells which have a high incidence of K-ras mutations.

In Burkitt's lymphoma cell lines which are known to overexpress c-Myc protein, abrogation of HMGA1 expression through antisense ribozyme approach prevents their transformation (Wood et al., 2000b). Similarly, *HMGA1* gene suppression using antisense approach inhibited the transformation of human breast cancer cells (Reeves et al., 2001). In a study by Reeves and co-workers, the human breast epithelial cells harbouring the tetracycline-regulated *HMGA1* transgene developed primary and metastatic tumours in nude mice only when the transgene was actively expressed, implicating the roles of *HMGA1* in carcinogenesis (Reeves et al., 2001). Overexpression of HMGA1 proteins induces transformation of Rat-1 fibroblast cells and CB33 human lymphoblastoid cells (Wood et al., 2000a; Wood et al.,

2000b). Studies using Rat-1 cells have indicated that HMGA1 is required for c-Jun/Ap-1 induced transformation (Hommura et al., 2004). Antisense-mediated targeting of HMGA1 prevents or inhibits retrovirus-induced transformation of rat thyroid cell line (Berlingieri et al., 2002) and induces apoptosis in two human thyroid anaplastic carcinoma cell lines (Scala et al., 2000). Of note, the suppression of HMGA1 expression did not have an impact on normal thyroid cells.

Interestingly, HMGA1 has been found to bind to the BRCA1 gene promoter and downregulates its expression in breast cancer cells (Baldassarre et al., 2003). Given that BRCA1 functions as a tumour suppressor gene involved in DNA damage response, the ability of HMGA1 to suppress its expression will render HMGA1 a pro-oncogenic protein. Moreover, it was speculated that the downregulation of BRCA1 commonly found in aggressive breast tumours could be due to the inhibitory effect of HMGA1 overexpression in these tumours. More recently, HMGA1 has been found to be capable of interfering with p53 tumour suppressor functions, in particular cellular apoptosis (Pierantoni et al., 2006; Pierantoni et al., 2007). Pierantoni and colleagues found that HMGA1 binds p53 *in vitro* and *in vivo* and interferes with p53-mediated transcription of p53 effectors (i.e. BCL2-associated X protein and cyclin-dependent kinase inhibitor 1A). Further, HMGA1 is able to cooperate with p53 to activate transcription of p53 inhibitor MDM2. The HMGA1 has also been found to interfere with functions of p53 by another mechanism: HMGA1 promotes HIPK2 (homeodomain-interacting protein kinase 2) relocalisation in cytoplasm which inhibits p53 functions as HIPK2 nuclear localisation is required for p53 activity (Pierantoni et al., 2007). Taking the above together, HMGA1 is able to inhibit p53-mediated apoptosis by modulating p53 target genes and cytoplasmic relocalisation of HIPK2.

Interestingly, Takaha and colleagues demonstrated that HMGA1 overexpression may predispose tumour cells to accumulate unbalanced translocation leading to cumulative mutations, and potentially more

aggressive tumour phenotype (Takaha et al., 2002). Using cDNA array analyses, Reeves and co-workers demonstrated that HMGA1 overexpression in a breast epithelial cell line resulted in a dramatic elevation of integrin expression and their potential signalling pathways (Reeves et al., 2001). Though the array used was only limited in the number of genes investigated, it was hypothesized that HMGA1 may execute its pro-tumourigenic functions by enhancing the integrin and its related downstream pathways. This 'model' was based purely on 'piecing' together a list of potentially related genes whose expression was elevated by HMGA1 overexpression. To date, there have not been any functional studies demonstrating the dependence of HMGA1-induced tumourigenesis on integrin-related signalling pathways.

HMGA1 is also known to regulate several genes involved in inflammation. It is now commonly accepted that inflammation represents a risk factor for carcinogenesis. It is possible that HMGA1 is capable of stimulating the inflammatory pathways which in turn promote cancer progression. Inflammatory genes regulated by HMGA1 include cyclooxygenase 2 (COX2) (Ji et al., 1998), inducible nitric oxide (NO) synthase (iNOS) (Perrella et al., 1999), E-selectin (Whitley et al., 1994), IL2 (Kim et al., 2001b) and IL4 (Himes et al., 1996). Some of these genes have been implicated in different stages of cancer development. For instance, COX2 has been implicated in growth and progression of several human cancers (Harris, 2007).

HMGA1 proteins are multifunctional and probably play a complex role in cellular functions. HMGA1 has been shown to modulate the expression of a large constellation of genes involved in tumourigenesis. These included genes associated with cell signalling, proliferation, migration, tissue invasion, angiogenesis and metastatic progression (Reeves, 2001). Further, Reeves and co-workers have shown a potential link between HMGA1 expression and metastatic progression (Reeves et al., 2001). The basis for the roles of HMGA1 in the metastatic process remains unclear. Athough there are some functional data suggesting the roles of HMGA1 in tumourigenesis, the

mechanisms through which these roles are achieved have so far been relatively unstudied. The molecular events induced by HMGA1 overexpression to execute its functions are largely unknown.

1.11.6 Chromosomal translocations in benign tumours

HMGA1 gene at 6p21 locus has been shown to be rearranged in lipomas, uterine leiomyomas, endometrial polyps and pulmonary chondroid hamartomas (Williams et al., 1997; Xiao et al., 1997). In tumours with epithelial and mesenchymal components such as pulmonary hamartomas and endometrial polyps, the aberrant chromosomal rearragements involving the 6p21 locus are interestingly only seen in the stromal component, suggesting that only the mesenchymal component is neoplastic or undergoing aberrant growth. Xiao et al described an occurrence of pericentric inversion in chromosome 6 with inversion breakpoints at 6p21 and 6q21 (Xiao et al., 1997). This created an intragenic fusion between the HMGA1 and the laminin α4 (LAMA4) genes. The resultant chimeric protein fused the AT hook region of HMGA1 to a cysteine-rich EGF-like domain of the LAMA4 protein. The discovery that the genes encoding HMGA1 proteins undergo aberrant translocations in benign mesenchymal tumours raises many interesting questions. The reason behind the propensity of the HMGA1 gene for chromosomal rearrangements is unknown. Further, it is unknown if any of the chimeric fusion proteins formed as a result of translocations are oncogenic. The frequency and consistency in the occurrence of chromosomal rearrangements involving HMGA1 gene may suggest, though unproven, that HMGA1 may have a role in the formation and aberrant growth of these benign tumours.

1.11.7 Extensive correlative evidence for HMGA1 in neoplastic transformation and metastatic progression

Many studies have shown that HMGA1 gene is up-regulated in many types of cancer. More specifically, HMGA1 overexpression seems to correlate with neoplastic transformation and metastatic progression. Current evidence has been summarised in Table 1.5. There is also some evidence to suggest that HMGA1 protein expression correlates with presence of metastasis and with a reduced patient survival (as evident in Table 1.5).

Colon carcinoma is one of the cancers in which HMGA1 is best studied. In one study, the majority of colorectal carcinoma samples were HMGA1-positive with no expression being detected in normal colon mucosa tissue or in most hyperproliferative lesions (Fedele et al., 1996). In colonic adenomas, HMGA1 expression was high and correlated closely with increasing cellular atypia. It is thought that HMGA1 expression is induced during the early stages of colonic neoplastic transformation. Finally, HMGA1 expression was significantly correlated with presence of lymph node metastasis and advanced Duke's staging, indicative of poor prognosis for patients with HMGA1-positive colorectal cancers.

Table 1.5. Correlative evidence suggesting a role for HMGA1 in tumourigenesis and metastasis.

Tumour type	Author (Year)	Experimental method	Key results	Weakness
Breast	(Ram et al., 1993)	Murine cell lines, HMGA1 mRNA levels	↑HMGA1 expression correlates with neoplastic transformation	Correlative
	(Nestl et al., 2001)*	Rat cell lines, SSH	HMGA1 expression correlates with metastatic potential	
	(Liu et al., 1999)	Human cell lines, HMGA1 mRNA and protein levels	↑HMGA1 expression correlates with metastatic potential HMGA1 expression induced by heregulin	Correlative
	(Baldassarre et al., 2003)	Human cell lines and tissue samples, HMGA1 mRNA and protein levels	↑HMGA1 expression in highly tumourigenic cell lines and tumour tissues HMGA1 binds and inhibits BRCA1 gene promoter ↑HMGA1 -> ↑growth in vitro	Correlative with small functional component
	(Dolde et al., 2002)	Human cell lines, HMGA1 protein levels, antisense approach	↑HMGA1 expression correlates with metastatic potential ↓HMGA1 by antisense -> ↓transformation but no effect on proliferation	Correlative with small functional component
Colorectal	(Fedele et al., 1996)	Human cell lines and tissue samples, HMGA1 mRNA and protein levels	↑HMGA1 expression in cell lines and tumour tissues No expression in normal epithelium	Small number of tissue samples
	(Abe et al., 1999)	Human tissue samples, HMGA1 mRNA and protein levels	↑HMGA1 expression in adenocarcinoma and adenoma with severe atypia	Small number of tissue samples Correlative
	(Kim et al., 1999)	Human tissue samples, HMGA1 mRNA and protein levels	↑HMGA1 expression in adenocarcinoma No expression in normal epithelium No correlation with clinicopathological variables	Correlative

	(Chiappetta et al., 2001)	Human tissue samples, HMGA1 protein levels	↑HMGA1 expression in adenocarcinoma and adenoma with severe atypia HMGA1 expression associated with early stages of neoplastic transformation	Small number of tissue samples Correlative
Prostate	(Bussemakers et al., 1991)	Rat cell lines, differential hybridization analysis	HMGA1 expression correlates with metastatic potential	Correlative
	(Nestl et al., 2001)	See above *	See above *	See above *
	(Tamimi et al., 1996)	Human tissue samples, HMGA1 mRNA levels	↑HMGA1 expression in high grade tumours	
	(Leman et al., 2003)	Transgenic mice, HMGA1 protein levels	HMGA1 expression correlates with neoplastic transformation	Correlative
Neuroblastoma	(Giannini et al., 2000)	Human tissue samples, HMGA1 protein levels	↑HMGA1 expression correlates with poor tumour differentiation	Correlative
Pancreatic	Nestl et al, 2001	See above*	See above*	See above*
	(Tarbe et al., 2001)	Human cell lines, cDNA microarray analysis	↑HMGA1 associated with metastasis	Correlative
	(Abe et al., 2000)	Human tissue samples, HMGA1 protein levels	↑HMGA1 is diagnostic of carcinoma ↑HMGA1 in benign tumours with atypia	Small sample Correlative
	(Abe et al., 2003)	Human tissue samples, HMGA1 protein levels	↑HMGA1 in liver metastases from pancreatic cancer	Very small sample
	(Abe et al., 2002)	Human tissue samples, HMGA1 protein levels	↑HMGA1 in IPMT tumours with dysplasia	Small sample Correlative
Thyroid	(Chiappetta et al., 1995)	Human cell lines and tissue samples,	↑HMGA1 in cell lines and tumour tissues	Correlative

		HMGA1 protein levels		
	(Chiappetta et al., 1998)	Human tissue samples, HMGA1 mRNA and protein levels	↑HMGA1 is highly diagnostic of carcinoma	
	(Kim et al., 1989)	Human tissue samples, HMGA1 mRNA levels	↑HMGA1 correlates with diagnosis of carcinoma	Correlative Small sample
Cervical	(Bandiera et al., 1998)	Human tissue samples, HMGA1 protein levels	↑HMGA1 correlates with tumour grade	Correlative Small sample
Gastric	(Xiang et al., 1997)	Human tissue samples, HMGA1 mRNA levels	↑HMGA1 correlates with tumour grade	Correlative
Hepatocellular	(Chang et al., 2005)	Human tissue samples, HMGA1 mRNA and protein levels	↑HMGA1 correlates with disease recurrence and poor prognosis	Correlative Small sample
	(Abe et al., 2003)	Human tissue samples, HMGA1 protein levels	HMGA1 is not overexpressed in hepatocellular carcinoma	
Leukemia	(Pierantoni et al., 2003)	Human cell lines and and tissue samples, HMGA1 mRNA and protein levels	↑HMGA1 in most leukemia tissues and all cell lines studied	Small sample
	(Xu et al., 2004)	Transgenic mice, human tissue samples, HMGA1 mRNA and protein levels	↑HMGA1 results in development of aggressive lymphoma in mice ↑HMGA1 found in leukemia tissues	Functional data

1.12 LIMITATIONS OF CURRENT RESEARCH

1.12.1 Relatively few studies of HMGA1 in pancreatic cancer

As shown in Table 1.5, overexpression of HMGA1 proteins has been correlated with appearance of highly malignant phenotype in colon, prostate, thyroid and cervical carcinomas. Clinically, high tumour expression of HMGA1 has been correlated with poor prognosis in patients with several cancers. Of note, most of these studies suggest that HMGA1 is overexpressed in many tumour types and provide only correlative evidence with little direct evidence for the role of HMGA1 in tumourigenesis. Although there are some data suggesting that HMGA1 is overexpressed in pancreatic cancer (Section 1.11.1), the clinical relevance of HMGA1 in this cancer type remains largely unknown. In a small study using immunohistochemistry by Abe and co-workers, high levels of HMGA1 expression were found PDAC tissues (Abe et al., 2000). Moreover, HMGA1 expression seems to correlate with cellular atypia in cases of pancreatic cystic neoplasm.

1.12.2 Relatively small number of cancer specimens

In the majority of studies correlating the expression of HMGA1 to malignant tumours, the number of cancer tissue samples investigated is small, ranging from 10 – 20 samples. This obviously does not provide an overall profile of the expression of HMGA1 in malignant tumours and certainly, it will be difficult to substantiate the possible use of HMGA1 expression in tissue samples as a diagnostic or prognostic marker. Studies assessing the value of HMGA1 expression as a prognostic marker will need far larger number of tissue samples. To date, no study has investigated the expression of HMGA1 in more than 50 tumour samples.

1.12.3 Functional roles of HMGA1 in pancreatic cancer remain unknown

Few studies have investigated the role of HMGA1 in pancreatic cancer cells in terms of hallmark features of cancer cell such as proliferation, invasion, metastasis, anoikis and chemoresistance (Kolb et al., 2007). Although we can postulate the roles of HMGA1 in pancreatic cancer through the limited functional studies available on HMGA1 and tumourigenesis in other cancer types, specific investigations to study the roles of HMGA1 in pancreatic cancer cells are needed. This is especially important as accumulating evidence suggests that HMGA1 overexpression in different cellular context might account for different effects (Fedele et al., 2001).

One important issue that has not been addressed so far is if HMGA1 overexpression in tumours is an epiphenomenon related to the rapid cell growth (i.e. HMGA1 overexpression is a surrogate marker of rapid cell cycle progression) or if HMGA1 overexpression is truly an oncogenic event responsible for neoplastic progression. Although accumulating evidence suggests that HMGA1 has an pro-oncogenic property, few studies have elucidated an oncogenic downstream pathway or target gene of HMGA1 that mediates its oncogenic property. To date, the oncogenic MAPK/ERK pathway has been described as a downstream mediator pathway of HMGA1 (Treff et al., 2004). No study has so far demonstrated if the malignant phenotype induced by HMGA1 is dependent on any of its downstream target pathways. Although we have some evidence from gene array studies which identified genes potentially regulated by HMGA1, there has been limited functional data supporting the roles of these genes in mediating the pro-oncogenic properties of HMGA1 (Reeves, 2001).

1.12.4 Limitations of antisense approach for functional studies

As mentioned in Section 1.11.5, earlier *in vitro* studies using the 'loss-of-function' approach to investigate the roles of HMGA1 in tumourigenesis were largely based on antisense gene suppression techniques. Interpretation of the

results of such experiments was hampered by the potential non-specificity of this technique and the significant off-target effects (Lebedeva & Stein, 2001). With the advent of RNA interference, a major step forward has been with the use of this technique in 'loss-of-function' experiments. RNA interference is highly efficient and specific. Cellular toxicity is low and reliable experimental results can be achieved if the experiments are conducted with appropriate controls. The use of RNA interference as a novel molecular strategy is discussed in the following sections.

1.13 RNA INTERFERENCE AS A NOVEL MOLECULAR AND THERAPEUTIC STRATEGY

1.13.1 Discovery of RNA interference

Andrew Fire and Craig Mello first described RNA interference (RNAi) in nematode worms Caenorhabditis elegans as a phenomenon whereby double-stranded RNA molecules are capable of inducing post-transcriptional gene silencing (Fire et al., 1998). Introduction of double-stranded RNA into these worms resulted in silencing of a specific gene with a sequence complementary to that of the double-stranded RNA. Furthermore, it was noted that only a few RNA molecules were required to reduce the population of target mRNA, implying an extremely potent mechanism of action. These findings proved crucial for the discovery of the RNAi phenomenon in mammalian cells. For this, Andrew Fire and Craig Mello were awarded the ***2006 Nobel Prize for Physiology or Medicine***.

The RNAi phenomenon is primarily mediated by short interfering RNA (siRNA) which can be generated from long double-stranded RNA derived endogenously or exogenously (Zamore et al., 2000). After intracellular introduction, the long double-stranded RNA is cleaved by ribonuclease III (RNAse III)-type protein called Dicer. Long double-stranded RNA is cleaved into RNA duplexes with 21-23 nucleotides. These RNA duplexes are known

as short interfering RNA (siRNA). The siRNA then gets incorporated into a protein complex, known as the RNA induced silencing complex (RISC), and loses the sense strand of the duplex by helicase activity. The antisense strand bound to RISC interacts with the complimentary target sequence of the mRNA resulting in site-specific cleavage of the mRNA, thus inhibiting protein synthesis (Zamore et al., 2000).

Other emerging mechanisms of siRNA have also been described. This includes inhibition of mRNA transcription (Verdel et al., 2004) and translation (Hannon, 2002).

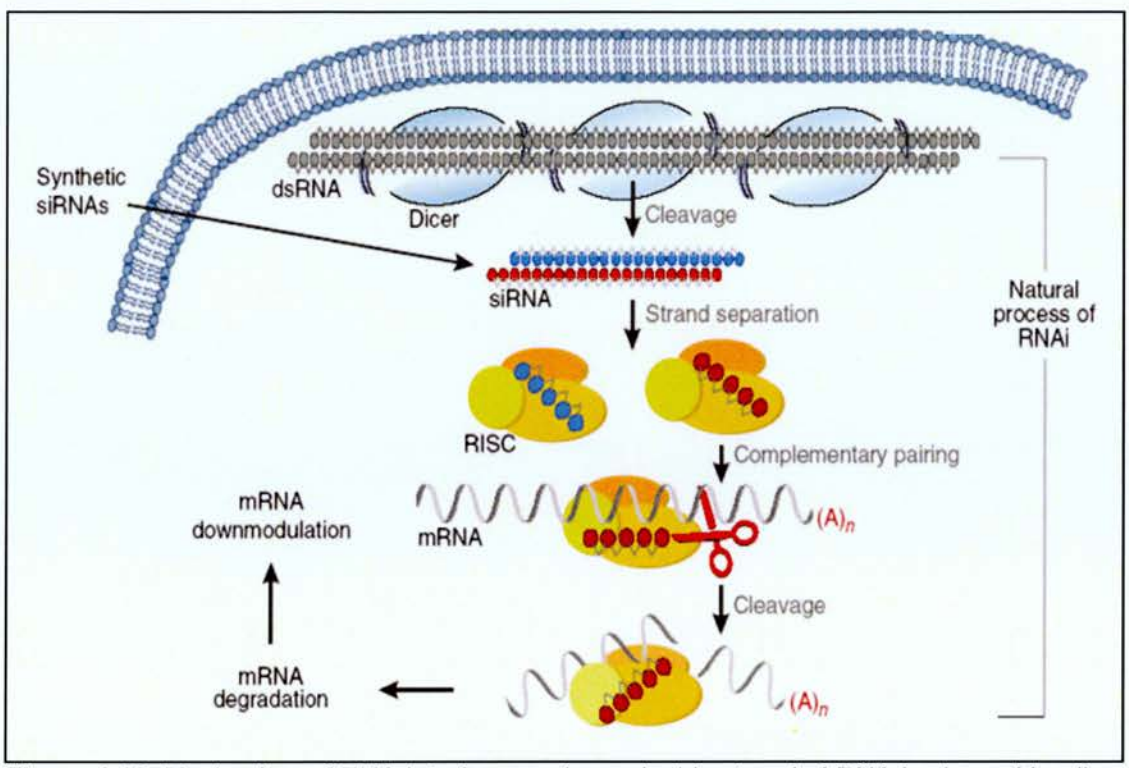


Figure 1.10. Mechanism of RNA interference. Long double-stranded RNA is cleaved by dicer into short interfering RNAs (siRNAs) which associate with RNA-induced silencing complex (RISC) to lead to mRNA degradation. Figure adapted from (Bumcrot et al., 2006).

1.13.2 RNAi as a potent gene knockdown tool and potential therapeutic strategy in oncology

In 2001, Elbashir, Tuschl and colleagues in the Max Planck Institute published their observations that synthetic siRNA, consisting of approximately 21-23

nucleotides, can silence target genes in mammalian cells (Elbashir et al., 2001a). In mammalian cells, the administration of long double-stranded RNA led to an interferon response and subsequently cellular toxicity. However, the discovery that 21- to 23-nucleotide long RNA sequences can effectively silence gene expression in many mammalian cells without triggering the interferon response was a major step forward (Elbashir et al., 2001b).

The relatively few years since have seen an expansion on the applications to harness the potential of RNAi both as a molecular tool to elucidate gene function as well as a potential therapeutic strategy. With RNAi, targeting of specific genes involved in cancer development is now a reality. This has been facilitated by the completion of the human genome project which provided us with the genomic information required to identify genes that are dysregulated in cancer cells.

1.13.3 Molecular strategies to achieve RNA interference

RNAi can be achieved in mammalian cells by introduction of synthetic double-stranded short interfering RNA (siRNA) of 21-23 base pairs in length or by plasmid or viral vector systems that express double-stranded short hairpin RNAs (shRNAs) that are subsequently processed to siRNAs by the cellular machinery. Gene silencing using synthetic siRNAs is only transient in mammalian cells and the duration of activity depends on the proliferative status of the cells, such that siRNA activity lasts for 3-7 days in actively proliferating cells, but can persist for up to 3 weeks in terminally differentiated cells such as neurones (Omi et al., 2004). To get around this problem, vector-based systems for induction of RNAi have been developed. These vectors express shRNAs and once stably transfected through selection for a drug resistance marker can achieve efficient and stable knockdown of target genes. Recent work has suggested that the shRNA approach is a more effective inducer of RNAi than the siRNA approach (Siolas et al., 2005).

In our laboratory, we have identified vector- and lentivirus-based shRNA strategies to silence HMGA1 gene. We have identified effective shRNA sequences that are capable of inducing profound silencing of HMGA1 expression. Our preliminary studies using multiple sequences of synthetic siRNA could achieve only a modest degree of silencing of HMGA1 expression at the protein levels. As such, subsequent in vitro experiments focused on the use of the shRNA-based silencing strategy. This strategy has proven to be a potent tool to study the roles of HMGA1 in PDAC cells.

1.13.4 Clinical applications of RNA interference

Due to the exquisite specificity and efficiency of RNA interference, it has drawn much attention as a therapeutic strategy, especially in oncology. Proof-of-principle experiments have demonstrated the therapeutic potential of RNAi in many disease models such as HIV infection (Novina et al., 2002), hepatitis (Giladi et al., 2003), inflammatory bowel disease (Zhang et al., 2006a), neurological disorders (Harper et al., 2005; Xia et al., 2004), respiratory infectious diseases (Bitko et al., 2005; Li et al., 2005) and ocular neovascularisation causing macular degeneration (Shen et al., 2006).

In oncology, RNA interference has been used to suppress tumour growth by targeting oncogenes such as K-ras, HER2 and VEGF genes. siRNA targeting K-ras transcripts carrying the valine-12 oncogenic mutation has been used as a therapeutic strategy in pancreatic and colon cancer with constitutively active K-ras mutation (Zhang et al., 2006b; Zhu et al., 2006). Suppression of HER2 expression in HER2-positive tumour cells results in growth inhibition and apoptosis (Yang et al., 2004). In addition, silencing of VEGF, a known potent stimulator of tumour angiogenesis, has been used to promote anti-angiogenesis in cancer cells (Kerbel & Folkman, 2002). In a number of cancer types, failure of chemotherapy is accounted by overexpression of MDR-1 gene which codes for P-glycoprotein, a membrane-bound efflux pump that actively eliminates a diverse range of anticancer drugs from cells (Gottesman

et al., 2002). As such, siRNA targeting MDR-1 gene is a highly promising application of RNAi in cancer therapy. Downregulation of P-glycoprotein by silencing MDR-1 enhances intracellular accumulation of chemotherapeutic agents (e.g doxorubicin, paclitaxel) and reinstates sensitivity to such agents.

1.14 AIMS OF STUDIES, RESEARCH QUESTIONS AND HYPOTHESIS

It is now widely accepted that the prognosis for patients with pancreatic cancer will only be improved by our understanding of the biology of this cancer. Subtle changes in surgical techniques will not improve the prognosis further given that the majority of local failure is due to the aggressive nature of the cancer rather than a failure of surgical techniques. The general consensus is that surgery has reached its limits. Major break-through therapies will probably come from the advancement of our understanding the basic biology of this cancer. This thesis was aimed at characterising the roles of a novel mediator of malignant phenotype in pancreatic adenocarcinoma cells: the *HMGA1* gene. This research work was designed initially to examine the clinical relevance of *HMGA1* in patients with PDAC. This was followed by *in vitro* and *in vivo* studies to examine the specific roles of HMGA1 in the malignant phenotype of PDAC cells. We utilised three investigative methodologies to achieve our aims. The first was the use of human pancreatic cancer specimens; the second was the human PDAC cell lines and finally, the xenograft mouse models of PDAC.

Study 1 (Chapter 3): Clinical relevance of HMGA1 in PDAC and its potential role as a prognostic indicator in patients with PDAC

Hypothesis:

We hypothesised that HMGA1 is overexpressed in tissues of patients with PDAC and its expression could be an indicator of the degree of

aggressiveness of the tumour and hence, may have a role as a prognostic indicator in these patients.

Aims:

1. To determine the prevalence of HMGA1 overexpression in tumour tissues from patients with PDAC using a constructed tissue microarray
2. To correlate the expression of HMGA1 to clinicopathological features of patients with PDAC
3. Using univariate and multivariate analyses, to assess if HMGA1 expression could represent a prognostic indicator in PDAC patients

Study 2 (Chapter 4): Roles of HMGA1 in cellular invasiveness and metastatic potential of PDAC cells

Hypothesis:

Given the extensive evidence that HMGA1 expression may be correlated with metastastic phenotype, we tested if HMGA1 expression would have a functional role in the cellular invasive capacity of pancreatic adenocarcinoma cells. Given the previous evidence suggesting that HMGA1 regulates the activity of the MAPK/ERK oncogenic pathway (Treff et al., 2004) and that PI3-K/Akt pathway is intrinsically linked to MAPK/ERK activity (Rodriguez-Viciana et al., 1994), we hypothesised that modulating the level of HMGA1 expression would have an impact on the pro-survival PI3-K/Akt pathway. If PI3-K/Akt was indeed a downstream pathway of HMGA1, we would further assess if the cellular invasiveness induced by HMGA1 was dependent on this pathway.

Aims:

1. To assess the effects of modulating HMGA1 expression by RNA interference and ectopic overexpression on cellular invasiveness
2. To identify the downstream pathways (e.g. MAPK/ERK, PI3-K/Akt and matrix metalloproteinase 9) that mediate the actions of HMGA1 in the context of cellular invasiveness and metastasis

3. To assess the effects of *in vivo* HMGA1 gene silencing on the metastatic process using an orthotopic nude mouse model

Study 3 (Chapter 5): Effects of HMGA1 modulation on anoikis resistance in PDAC cells

Hypothesis:

The phenomenon of apoptosis when cells lose their attachment to substratum is known as anoikis. Cancer cells are inherently anoikis-resistant and the degree of such resistance is a major determinant of metastatic potential *in vivo* (Zhu et al., 2001). We hypothesised that targeting HMGA1 may impair anoikis resistance in PDAC cells.

Aims

1. To assess the effects of ectopic overexpression and silencing of HMGA1 on anoikis resistance in PDAC cells using flow cytometric experiments
2. To assess the potential involvement of PI3-K/Akt signalling pathway in the HMGA1-mediated anoikis resistance using specific small molecule inhibitors
3. To investigate the effects of HMGA1 modulation on anoikis-induced caspase activation

Study 4 (Chapter 6): Roles of HMGA1 in cellular proliferation *in vitro* under anchorage-independent and –dependent conditions and tumour growth *in vivo*

Hypothesis:

Previous study has shown that HMGA1 can have an impact on cellular transformation in breast and haematological cancers (Reeves et al., 2001; Wood et al., 2000b). It remains unknown if these effects of HMGA1 are cell type-specific and if HMGA1 expression would influence cellular transformation

and proliferation in PDAC cells. Given that PI3-K/Akt has previously been shown to be crucial in anchorage-independent growth (Nakanishi et al., 2002; Yao et al., 2002), we hypothesised that modulating HMGA1 expression may have an impact on this signalling pathway and HMGA1-induced growth may be dependent on this pathway.

Aims

1. To investigate the effects of targeted silencing of HMGA1 expression on anchorage-independent and -dependent growth in PDAC cell lines
2. To assess if ectopic overexpression of HMGA1 promotes anchorage-independent and –dependent growth
3. To determine if anchorage-independent growth mediated by HMGA1 is dependent on the PI3-K/Akt pathway
4. To assess the effects of targeting HMGA1 on tumour growth *in vivo* and to examine the effects of *in vivo* HMGA1 silencing on apoptosis and proliferation using immunohistochemical analyses

Study 5 (Chapter 7): Roles of HMGA1 in chemoresistance to gemcitabine in PDAC cells

Hypothesis:

One of the characteristic features of PDAC cells is the extreme chemoresistance. To date, only one chemotherapeutic agent (i.e. gemcitabine) has been proven to have a modest impact on survivals in advanced pancreatic cancer. We hypothesised that HMGA1 modulation may also have an effect on chemosensitivity to gemcitabine in PDAC cells.

Aims

1. To characterise the roles of HMGA1 in chemoresistance to gemcitabine in PDAC cells
2. To explore if targeted silencing of HMGA1 using a lentiviral RNAi vector could be a potential chemosensitising strategy in PDAC cells

3. To assess if HMGA1 modulation has an impact on gemcitabine-induced apoptotic process (i.e. caspase cascade activation)
4. To assess if HMGA1 silencing could chemosensitise tumours *in vivo* using a subcutaneous xenograft mouse model

Chapter Two: Materials and methods

2.1 TISSUE MICROARRAY

Permission to use archival specimens of pancreatic adenocarcinomas was granted by the Institutional Review Board for Medical Research (Brigham and Women's Hospital, Boston, USA; Protocol No: 03328; Molecular analysis of metastatic pancreatic cancer).

2.1.1 Clinical outcomes database

The work in this thesis involved the use of an existing clinical outcomes database for patients having undergone surgical resection for pancreatic cancer at the Brigham and Women's Hospital. During the construction of this database, pathology reports were searched to identify patients who underwent curative surgical resection for pancreatic adenocarcinoma between the years 1991 and 2002, at the Brigham and Women's Hospital. The exclusion criteria were the presence of metastases or local invasion precluding surgical resection of the pancreatic cancer. The database contained demographic information including the results of diagnostic tests, operative and pathologic findings (i.e. tumour size, stage, lymphovascular invasion (LVI), perineural invasion (PNI), differentiation, surgical resection margin status, and lymph node status), survival and recurrence data, and information of adjuvant therapies administered. Pathologic staging was updated according to current American Joint Committee on Cancer guidelines (see Table 1.2). All data was saved in the form of a Microsoft Access database. The final database consisted of 89 patients (42 males, 47 females) with a median age of 63 years. The mean follow-up period was 19.5 months.

2.1.2 Construction of tissue microarray

Under an IRB-approved protocol, our laboratory has also previously assembled a corresponding tissue bank of paraffin-embedded pancreatic

cancer specimens for the cohort of patients within our clinical outcomes database. Formalin-fixed, paraffin-embedded specimens were used to construct the pancreatic adenocarcinoma tissue microarray (TMA). Standard H&E-stained slides from each tumour specimen were reviewed by a single pathologist (Dr. Mark Redston, Consultant Pathologist, Brigham and Women's Hospital). Representative tumour regions were selected from each tissue block and two tissue cores (0.6mm diameter) were taken from each region using an automated tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). In addition, cores were also taken from normal adjacent pancreas for use as internal controls. Cores were transferred to recipient blocks. Five μm sections were cut from each recipient block to make the TMA slides.

2.1.3 Immunohistochemistry of tissue microarray

Expression of HMGA1 in pancreatic cancers and adjacent non-cancerous tissues was assessed using standard immunohistochemical techniques. TMA slides were deparaffinized, rehydrated through graded alcohol, washed with Tris-buffered saline, and processed using a streptavidin–biotin–peroxidase complex method. Antigen retrieval was performed by microwaveheating sections in 10 mM sodium citrate buffer (pH 6) for 10 minutes. After quenching of endogenous peroxidase activity and blocking of nonspecific binding, anti-HMGA1 antibody (Santa Cruz) was added at a 1:50 dilution, after which slides were incubated at 4°C overnight. The secondary biotinylated rabbit anti-goat antibody (DAKO, Carpinteria, CA) was used at a dilution of 1:200 for 30 minutes at 37°C. After further washing with Tris-buffered saline, sections were incubated with StrepABComplex/horse radish peroxidase (1: 100, DAKO) for 30 minutes at 37°C. Chromogenic immunolocalization was performed by exposure to 0.05% 3,3-diaminobenzidine tetrahydrochloride. Cores containing pancreatic adenocarcinoma served as positive controls for HMGA1 expression. Normal serum was used in the place of primary antibody as a negative control. Slides were counterstained with hematoxylin before dehydration and mounting.

2.1.4 TMA analysis

Slides were reviewed by 2 independent observers (Siong-Seng Liau, Flavio Rocha) blinded to clinical and pathologic data. HMGA1 expression was scored according to nuclear staining intensity as follows: 0, no staining or weak intensity staining in less than 5% of cells; 1, weak intensity; 2, moderate intensity; 3, strong intensity. For statistical analyses, expression was dichotomized into a HMGA1-negative group (score 0) and a HMGA1-positive group (scores 1, 2, or 3). In cases of disagreement, a consensus was reached by joint review.

2.1.5 Data analysis

Each patient was represented by staining of 3 tumour cores and 3 cores of normal adjacent pancreas (internal controls) in the tissue microarray. The median staining intensity of tumour sections for each patient was used for the final data analysis. The univariate associations between HMGA1 expression and each of the pathologic variables were assessed using the chi-square or Fisher's exact test as appropriate. Overall survival was calculated from the date of surgical resection of the pancreatic cancer to the date of death or last follow-up. Deaths within 30 days of surgery were excluded from analysis. For disease specific survival, data for patients who died from other causes other than pancreatic cancer were censored at the time of death. Overall disease specific survival curves were calculated using the Kaplan-Meier method and compared using the log-rank test.

2.2 CELLS AND CELL CULTURE

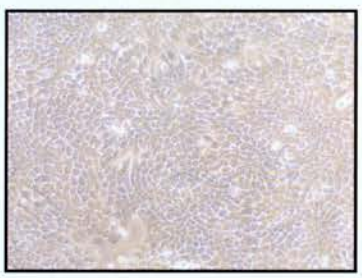
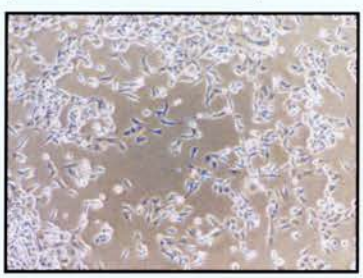
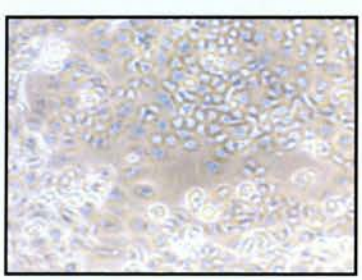
2.2.1 Cell lines

BxPC3, MiaPaCa2 and PANC1 human pancreatic ductal adenocarcinoma cells were obtained from American Type Culture Collection, ATCC (Manassas, VA). Cells were tested negative for mycoplasma contamination at six monthly intervals by Yale University Cell Culture Service (New Haven, Connecticut, USA).

2.2.2 General technique

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Life Technologies Inc., Gaithersburg, MD, USA) and incubated in a humidified (37°C, 5% CO2) incubator, grown in 75-cm^2 culture flasks, and passaged on reaching 80% confluence and reseeded at the density shown in Table 2.1.

Table 2.1. Cell lines used in this project. Each cell line was used in experiments to a maximum passage number of 10 before returning to lower passage cells for further experiments.

Cell line	Source of tumour cells	Lineage	Protocol for splitting	Established by
BxPC3 (ATCC ID: CRL-1687) 	Primary tumour	Moderately differentiated PDAC	Split 1:5 Every 7 days	(Tan et al., 1986)
MiaPaCa2 (ATCC ID: CRL-1420) 	Primary tumour	Poorly differentiated PDAC	Split 1:10 Every 4 days	(Yunis et al., 1977)
PANC1 (ATCC ID: CRL-1469) 	Primary tumour	Poorly differentiated PDAC	Split 1:10 Every 5 days	(Lieber et al., 1975)

2.2.3 Cryopreservation

Cell lines obtained from ATCC were expanded at passages 1-3 and frozen in cryovials using a freezing container (Mr Frosty, Nalgene, Rochester, NY, USA) at a cooling rate of 1˚C per min in -80˚C overnight followed by long term storage in liquid nitrogen. Freezing medium consisted of DMEM with 5% DMSO and 10% FBS.

2.3 TRANSFECTION OF CULTURED CELLS

2.3.1 Transfection with siRNA duplexes

HMGA1-targeting short interfering RNA (siRNA) and control siRNA, bearing no homology to human genes, were used in experiment to achieve transient gene silencing. Cells ($5X10^5$) were plated onto wells of 35mm six-well culture plates and allowed to adhere for 24 hours. Five microlitres of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) per well were diluted into 120 µL of OptiMEM I media (Invitrogen). The siRNAs diluted in 120 µL Opti-MEM media to a final concentration of 100nM were added to the Lipofectamine 2000 transfection mix, gently mixed and incubated at room temperature for 20 min. The transfection agent/siRNA complex was added into the wells containing 800 µl DMEM and incubated under normal cell culture conditions for 12 hrs, after which the media was changed to DMEM with 10% FBS. Antibiotics were omitted from all media used during transfection. All assays were performed 48 h post-treatment.

2.3.2 Transfection with plasmid DNA

Transient transfection of plasmid DNA was performed using Lipofectamine 2000 transfection reagent (Invitrogen). Cells ($1x10^6$) were plated onto wells of 35mm six-well culture plates and allowed to adhere for 24 hours. This should achieve a confluency of 70% on the day of transfection. Five micrograms of plasmid DNA per well were diluted in 500 µL of OptiMEM I (Invitrogen) media.

Five microlitres of Lipofectamine 2000 per well were diluted in 500 µL of OptiMEM I media. Diluted plasmid DNA and Lipofectamine were left to stand for 10min and subsequently mixed gently to generate the transfection agent/plasmid DNA complex mixture which was incubated at room temperature for 20 min. Following aspiration of culture medium from cells, one millilitre of transfection agent/plasmid DNA complex mixture was added to each well. Cells were incubated for 8 hours and the medium was replaced with fresh DMEM containing serum. Again, antibiotics were omitted from media used during transfection. Experiments were performed on the cells at 48 hours following transfection.

To generate stable transfectants, cells were subjected to puromycin-containing medium starting at 1µg/mL, with puromycin concentration being increased at 1µg/mL every 3 days to a final concentration of 3 µg/mL. Final stable transfectants were maintained in medium with 3 µg/mL of puromycin.

2.4 DNA ANALYSIS

2.4.1 General techniques

All solutions used in experiments involving nucleic acids were made with DNAse- and RNAse-free water (Sigma, St Louis, MO, USA). Tubes and disposable tips were autoclaved before use. Disposable rubber gloves were worn when handling nucleic acids. TE buffer (Sigma) was used to suspend DNA. All suspended DNA solutions were stored at -20˚C.

2.4.2 Quantification of nucleic acids

DNA concentrations were determined by measuring the UV absorbance at 260nm of 100x diluted samples using a SpectraMax M5 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). DNA concentration was calculated using the formula: concentration (µg/mL) = Absorbance at 260 nm (A260nm) x 50 x dilution factor. This is based on the assumption that the

molecular weight of a nucleotide pair is 600Da, giving rise to the formula: 1 unit of A260nm is equivalent to 50 µg/mL of double stranded DNA, 33 µg/mL of single stranded DNA and 40 µg/mL of RNA.

The purity of DNA was estimated by the ratio of A260nm/A280nm. DNA absorbs light at 260 nm, and protein absorbs UV light at 280 nm; a pure sample of DNA has the A260nm/A280nm ratio at 1.8 or higher. A DNA preparation that is contaminated with protein will have an A260nm/A280nm ratio lower than 1.8.

2.4.3 Digestion of DNA with restriction endonucleases

Restriction endonucleases were obtained from New England Biolabs (Ipswich, MA, USA). DNA was digested using the manufacturer's recommended 10x restriction buffers and digestion temperatures. A standard protocol involved digestion of 1-5µg DNA overnight at 37˚C in a total volume of 20-30µL using 5-10 units of restriction endonucleases.

2.4.4 DNA gel electrophoresis

Separation of DNA fragments was performed using the E-gel electrophoresis system (Invitrogen) which utilised pre-cast agarose gels with ethidium bromide. Agarose concentrations of 0.8 to 2% were used depending on the size of fragments to be separated. DNA solutions were mixed with a tenth volume of 10x DNA loading buffer and loaded into wells of the gel. The samples were electrophoresed at 100V for 30 minutes in parallel with a DNA size marker (New England Biolabs). DNA fragments were visualised under UV illumination and gels were photographed using a conventional gel camera (Kodak, Rochester, NY, USA).

2.4.5 Purification of DNA samples

DNA was extracted and purified from agarose gel slices using the QIAquick kit (Qiagen, Valencia, CA, USA). Briefly, agarose was dissolved in 3 volumes of buffer QG (assuming 100µg agarose = 100µL) at 50˚C for 10 minutes. If the DNA fragment size was less than 500bp, 1 gel volume of isopropanol was added. The mixture was added to a silica-gel spin column to bind the DNA. The flow-through following centrifugation at 15000 x g for one minute was discarded. The silica-gel-DNA complex was washed with buffer PE. DNA was finally eluted by addition of TE buffer to the spin column and centrifuged for 1 minute at 15000 x g. The purified DNA was analysed by gel electrophoresis to confirm the approximate DNA size and concentration.

2.4.6 Transformation of E.coli

Plasmid DNA was expanded in chemically competent *E coli* (TOP10 cells, Invitrogen, Carlsbad, CA, USA). Generally, either 5 µL of ligation reaction or plasmid DNA solution was mixed with 50 µL of competent bacteria. The bacteria were chilled on ice for 30 minutes and heat shocked at 42˚C for 30 seconds and replaced on ice for 5 minutes. The bacteria were then mixed with 100 µL of LB medium (Appendix) and incubated for 1 hour at 37˚C in a shaking incubator. Bacteria were then plated on LB agar plate containing 100 µg/mL of ampicillin and incubated overnight at 37˚C.

2.4.7 Preparation of plasmid DNA

A single bacterial colony was picked from the selection plate and inoculated into 10mL of LB medium with 100 µg/mL of ampicillin. This was incubated for 8 hours as a starter culture. Following this, 1 mL of the starter culture was added into 200 mL of LB with ampicillin and incubated in a shaking incubator overnight at 37˚C. The use of starter culture will ensure a healthy exponential growth of bacteria without nutrient exhaustion.

2.4.8 Extraction and purification of plasmid DNA

Plasmid DNA was purified using the GenElute Maxiprep kit (Sigma Aldrich). Bacterial cells grown, as described above, were harvested by centrifugation at 5000 x g for 10 min at room temperature. The pellet was resuspended in 5 ml of resuspension buffer with RNAse A. Resuspended cells were lysed with addition of 5 mL of lysis buffer and mixed gently by inverting 4-6 times. The mixture was incubated at room temperature for not more than 5 minutes. The cellular debris was precipitated by addition of 4 mL of neutralisation solution. The precipitated material formed contained genomic DNA, proteins, cell debris and SDS. The cellular debris was pelleted by centrifugation at 5000 x g for 20 minutes at 4˚C. The supernatant containing plasmid DNA was removed and mixed with 4 mL of DNA bind solution. The DNA binding column was prepared by adding 12 mL of Column preparation solution and centrifuging at 5000 x g for 5 minutes. The supernatant was loaded into the prepared column seated in a collection tube and centrifuged at 3000 x g for 3 minutes. Following this, the column was washed with 2 x 10 mL of Wash buffer before eluting the DNA with 5 mL of endotoxin-free water. The DNA was precipitated by adding 10 mL of isopropanol and then, centrifuged at 15 000 x g for 30 minutes at 4˚C. The DNA pellet was washed with room-temperature 70% ethanol and centrifuged at 15 000 x g for 10 minutes. The pellet was air-dried for 5-10 minutes before being re-dissolved in a suitable volume of TE buffer.

2.4.9 Polymerase chain reaction (PCR)

Typically, each reaction consisted of a final volume of 100 µL. The following components were mixed on ice: template DNA (1 µg), 0.2pmol/µL primers and 50 µL of Taq PCR master mix (5 units of Taq DNA polymerase, 2 x PCR buffer, 400 µM dNTP, 3 mM $MgCl_2$) (QIAgen). The reactions were performed using a pre-programmed thermal cycler (GeneAmp PCR systems, Applied Biosystems, Foster City, CA, USA). PCR reactions were started with an initial denaturation step of 95˚C for 3 minutes followed by 35 cycles at 95˚C for 30 seconds (denaturation), 50-68˚C for 30 seconds (annealing) and 72˚C for 60

seconds (elongation). The reactions were ended with a final extension step at 72°C for 10 minutes. Gel electophoresis of 5 µL of the PCR reaction was performed to confirm the size and yield of the product.

2.5 RNA ANALYSIS

2.5.1 General techniques

Dedicated work areas for RNA analysis was designated and cleaned with RNAse decontamination solution (RNAse*Zap*, Ambion, Austin, TX, USA). Gloves were worn at all times when handing RNA samples. All tips and containers used were RNAse-free.

2.5.2 RNA isolation

Total RNA was extracted from cells using the mirVana® RNA isolation kit according to the manufacturer's recommendation (Ambion). Typically, 1 x 10^6 cultured cells were used to harvest RNA. Cells were lysed with 600 µL of Lysis/Binding Buffer and vortexed vigorously to completely disrupt the cells. This was followed by addition of 60 µL of RNA Homogenate Additive and mixed thoroughly. Further addition of an equal volume of Acid Phenol: Chloroform solution resulted in separation into two phases: aqueous and organic. The upper aqueous phase which contains RNA was carefully removed without disrupting the lower organic phase consisting of protein and DNA. The aqueous phase was mixed with 1.25 volumes of 100% ethanol and the mixture was loaded into the filter cartridges and centrifuged at 15 000 g for 15 seconds. The filters were washed twice with each of the Wash solutions provided. The total RNA was subsequently eluted from the filter using 100 µL of nuclease-free water containing 0.1mM EDTA. The amount of total RNA isolated was quantified using the method described in Section 2.3.2.

2.5.3 Reverse transcription

First-strand cDNA was synthesized from 5 µg of total RNA using the SuperScript III First-Strand Synthesis System, according to manufacturer's instructions (Invitrogen). The system, containing random primers, allowed reverse transcription of all RNA present into cDNA. The initial reaction mixture contained 5 µg of RNA, 1 µL of random primers (250 ng), 1 µL 10mM dNTP mix and RNAse-free water made up to 13 µL. This mixture was heated to 65˚C for 5 minutes and chilled on ice for 1 minute. This was followed by addition of 4 µL of 5X First-Strand Buffer, 1 µL of 0.1 M DTT, 1 µL of RNaseOUT Recombinant RNase Inhibitor (40 units/µl) and 1 µl of SuperScript™ III Reverse Transcriptase (200 units/µl). The final reaction volume of 20 µL was mixed thoroughly and incubated for 5 minutes at 25˚C. This was followed by incubation at 50˚C for 60min and final inactivation step of 70˚C for 15 minutes. Samples containing cDNA were stored at -20˚C.

2.5.4 Quantitative Real-time PCR

Transcript analysis by quantitative real-time PCR was performed using the Taqman assay, based on the manufacturer's instructions (Applied Biosystems, Foster City, CA). Hybridization probe and primer sets for human MMP-9 gene (Hs00234579_m1 MMP9) and β-actin endogenous control were obtained from Applied Biosystems. PCR reactions were performed in triplicates and data acquired using the GeneAmp Sequence Detection System (Applied Biosystems). Each reaction was performed in 25 µL and contained 5 ng of cDNA, 12.5 µL Taqman 2x PCR Master Mix (Applied Biosystems) and 1.25 µL of the probe/primer set. Typically, thermal cycling was initiated with a denaturation step for 10 minutes at 95°C followed by 40 cycles done in two steps: for 15 seconds at 95°C and for 1 minute at 60°C. During each PCR cycle, the Taqman probe is digested by nuclease activity and generates a fluorescent signal proportional to the amount of cDNA amplified. The detection hardware quantifies the fluorescence level and constructs a kinetic profile of DNA amplification over the 40-cycle PCR reaction. The cycle

threshold (C_T) is defined as the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. The sequence detection software converts the measured cycle thresholds into relative copy numbers using primer-specific standard curves by interpolation. Results are expressed as the ratio of MMP9 to β-actin.

2.6 RNA INTERFERENCE OF HMGA1 GENE

HMGA1 gene silencing was achieved using RNA interference (Section 1.13). RNA interference in mammalian cells can be achieved through several methods. Firstly, transient silencing can be achieved using chemical transfection of synthesised short double stranded RNA known as short interfering RNA (siRNA). Plasmid DNA expressing short hairpin RNA can be transfected into cells to utilise the cell's endogenous machinery to generate hairpin RNA (shRNA) which is then processed into siRNA within the cells, hence achieving gene silencing. Further, virus that encodes hairpin RNA can be generated and is capable of integrating into the cellular chromosome upon transduction. Viral integration will result in endogenous production of shRNA that can effect gene silencing. In this thesis, we explored the utility of all three methods of effecting gene silencing in pancreatic adenocarcinoma cell lines.

2.6.1 Short-interfering RNA (siRNA) sequences

Early on during this project, we utilised a panel of pre-designed, commercially available siRNA sequences to attempt to silence the HMGA1 gene (Table 2.2). The sequences of siRNA were pre-designed using conventional algorithms with proprietary modifications. The use of commercially available pre-designed sequences increases the likelihood of finding an effective sequence. The siRNA duplexes were synthesized by the various companies and were supplied as HPLC-purified, lyophilised pellets. The pellets were resuspended in nuclease-free water.

Table 2.2. Sequences of siRNA used in pilot experiments to silence *HMGA1* gene. For control sequences (in italic), scr denotes scramble sequence and non-t denotes non-targeting sequence with no homology to any human gene.

Duplex	Source	Design algorithm	Region of HMGA1 mRNA homology	HMGA1 transcript variant targeted	Level of HMGA1 knockdown (protein level)
SC-37115	Santa Cruz	Proprietary	Proprietary sequence	All 7 variants	30-50%
Control duplex SC-37007	Santa Cruz	Proprietary	Scr	-	-
Pre-designed #217139	Ambion	Proprietary	Proprietary sequence	All 7 variants	30%
Pre-designed # 38139	Ambion	Proprietary	Proprietary sequence	All 7 variants	Minimal
Pre-designed # 38308	Ambion	Proprietary	Proprietary sequence	All 7 variants	Minimal
Negative control	Ambion	Proprietary	Non-t	-	-

We found that transient silencing with the chemical transfection of siRNA was only modestly effective in one out of the 4 sequences of siRNA used. Level of HMGA1 knockdown was determined using Western blotting. There are several reasons that could potentially render transient silencing by siRNA ineffective. Firstly, transient siRNA transfection may not be effective as HMGA1 protein has a long half-life intracellularly (Holth et al., 1997), as such, persistence of intracellular HMGA1 store may render transient RNA interference (i.e. siRNA) ineffective in reducing the overall HMGA1 protein levels. Secondly, pancreatic adenocarcinoma cell lines are inherently difficult to transfect using chemical transfection. We achieved approximately 30-50% transfection efficiency in the cell lines used (Figure 2.1). Transfection efficiency was monitored using fluorescein-labelled siRNA (Invitrogen). As such, the low transfection efficiency may render siRNA ineffective. Fluorescence microscopy was performed using Olympus BX6 fluorescence microscope (Olympus America, Center Valley, PA, USA) equipped with

appropriate filter cube (Exciter BP460-490, Dichroic DM500, Emitter BA515). Cells were visualised directly from tissue culture plates.

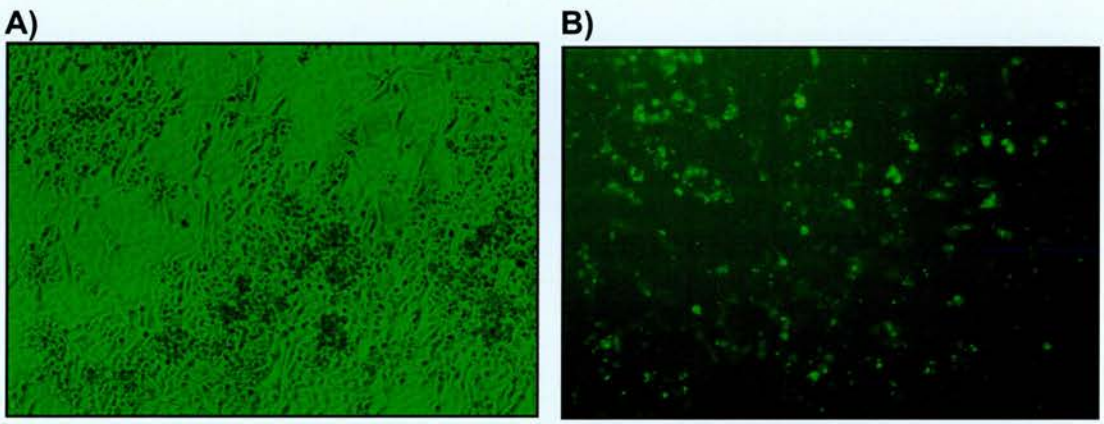


Figure 2.1. Transfection efficiency for siRNA was monitored in MiaPaCa2 cells by transfection of fluorescein-labelled siRNA (BLOCK-IT Oligo, Invitrogen). Confocal microscopy image (x20 magnification) of MiaPaCa2 cells (A) and the corresponding fluorescence microscopy image of the same cells (B), indicating an approximate transfection efficiency of 30-50%.

2.6.2 Short haipin RNA (shRNA) design

A brief summary of the hairpin RNA (shRNA) design process has been described (Broad Institute at Massachusetts Institute of Technology, 2007). All sequences used for RNAi experiments were chosen using guidelines described by Elbashir et al (Elbashir et al., 2001a). Despite the rules for shRNA design, shRNA sequences still have to be experimentally assessed for knockdown efficiency, as not all sequences matching the design criteria are potent. The sequence of each shRNA was subjected to a BLAST-search against the expressed-sequence-tagged libraries (EST) on the NCBI database to ensure that the RNAi sequence would only target the HMGA1 transcripts. The control sequences were also BLAST-searched to verify that they were non-targeting and would not affect expression of any genes.

2.6.3 Plasmid-based shRNA sequences

Hairpin RNA interference plasmids (pLKO.1-HMGA1, TRCN0000018949), constructed as described previously (Stewart et al., 2003), were obtained from The RNAi Consortium (Mission TRC Hs. 1.0, Sigma Aldrich, St Louis, MO) (see Figure 2.2). shRNA sequences were designed to target all transcript variants of *HMGA1* gene to ensure effective silencing. The sequences of short hairpin RNA targeting the human HMGA1 gene (GenBank accession no. NM_002131) are given in Table 2.3. Each of these vectors had been sequence-verified.

Pooled stable transfectants were established using puromycin selection (3μg/ml of puromycin) for each of the shRNA plasmids and the silencing efficiency was determined by Western blotting. As shown, shHMGA1-1 and shHMGA1-2 plasmids most effectively silenced the HMGA1 expression and hence, were selected for subsequent *in vitro* and *in vivo* experiments. Of note, the use of at least two independent RNAi target sequences helped to control for potential off-target effects of RNAi.

In subsequent experiments, we used the plasmid- and virus-based shRNA approaches to assess the effects of HMGA1 silencing as our experience suggested that these approaches allowed stable RNA interference and were more effective than transient transfection of siRNA duplexes.

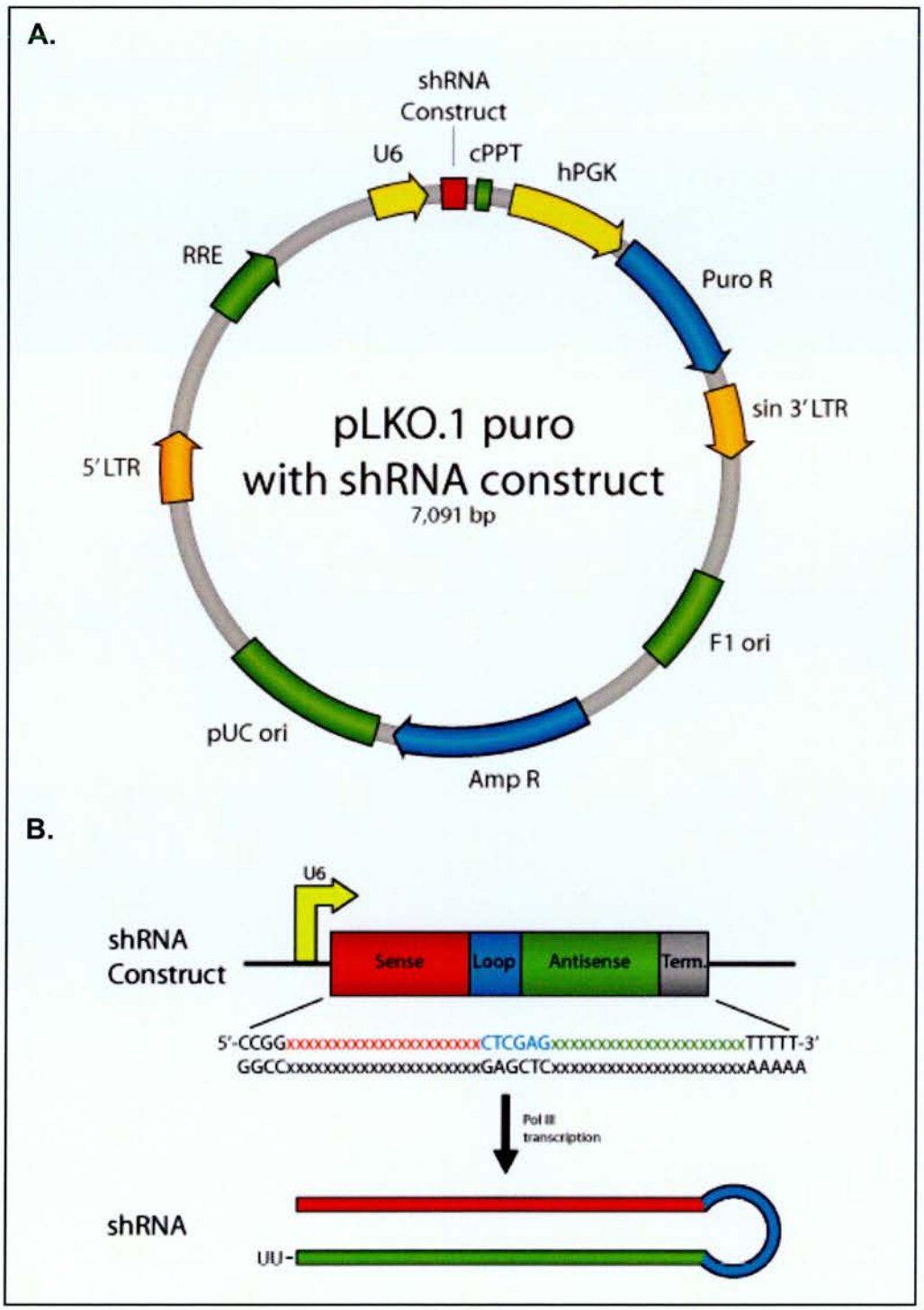


Figure 2.2. A, Map of pLKO.1-puro plasmid containing an shRNA insert and a puromycin resistance gene (Puro R) for selection in mammalian cells. B, The human U6 promoter allows transcription of the shRNA insert (i.e shHMGA1-1, 1-2 etc.). The shRNA contains a 21-base 'sense' sequence that is identical to the target region of the HMGA1 gene (see Table 2.3), a 6-base 'loop' sequence (forming the 'head' of hairpin RNA), and a 21-base 'antisense' sequence that is complementary to the 'sense' sequence.

Table 2.3. Targeting sequences of shRNA plasmids used in pilot experiments to silence *HMGA1* gene. For control sequences (in italic), scr denotes scramble sequence and non-t denotes non-targeting sequence with no homology to any human gene.

shRNA	Source	Region of HMGA1 mRNA homology	Coding region of HMGA1 transcript variant 2 targeted	Level of HMGA1 knockdown (protein level)
shHMGA1-1	Sigma Aldrich	5'–CAACTCCAGGAAGGAAACCAA–3	446-466	90-100%
shHMGA1-2	Sigma Aldrich	5'–CCTTGGCCTCCAAGCAGGAAA–3'	281-301	80-90%
shHMGA1-3	Sigma Aldrich	5'-CCAGCGAAGTGCCAACACCTA-3'	359-379	30%
shHMGA1-4	Sigma Aldrich	5'-GAAGGAGGAAGAGGCGGGCAC-3'	489-509	Minimal
Negative control (shControl)	Sigma Aldrich	Scr, Non-t	-	-

2.6.4 Small-scale generation of lentiviral particles expressing hairpin RNA

To generate lentiviral particles, human embryonic kidney 293 cells (ATCC) were co-transfected with the lentiviral RNAi vector (shHMGA1-1, shHMGA1-2 or shControl plasmids) and compatible packaging plasmid mixture (Virapower lentiviral packaging system, Invitrogen) using Lipofectamine 2000 (Invitrogen). The co-transfection ratio was lentiviral RNAi vector:packaging plasmid mixture = 1μg:3μg. Virus supernatant was harvested at 72 hours post-transfection. Pancreatic adenocarcinoma cells were exposed to lentivirus-containing supernatant for 16 hours in the presence of 6μg/ml polybrene (Sigma). Pooled stable transfectants were established using puromycin selection as described above (Section 2.2.2).

2.6.5 High-titre lentiviral particles production

High-titre lentivirus expressing shHMGA1 (shHMGA1-1) and control shRNA were generated from five-plasmid transient transfection into 293T cells. Helper plasmids including pHDM-Hgpm2, pMD-tat, pRC/CMV-rev and pCMV-VSV-G were obtained from Harvard Gene Therapy Initiative (Harvard Medical School, Boston, MA). The lentiviral transfer vectors were either shControl (non-targeting shRNA sequence) or shHMGA1-1 plasmids as described above. shHMGA1-1 plasmid was chosen for generation of lentivirus, as it induces the greatest degree of HMGA1 silencing (Table 2.3). Virus preparations were concentrated by ultracentrifuge and titred by Southern blotting. We achieved titre of $7.2X10^8$/mL for control shRNA lentivirus and $2.5X10^8$/mL for shHMGA1 lentivirus. All virus preparations were made in affiliation with Harvard Gene Therapy Initiative. Pooled stable transfectants were developed following infection of lentivirus at MOI of 10 for 48 hours, and stable selection in puromycin was achieved as described above.

2.7 CLONING OF *HMGA1* GENE AND GENERATION OF HMGA1 GENE EXPRESSION VECTOR

2.7.1 Generation of HMGA1 expression vector

To construct the HMGA1 expression vector, HMGA1 coding sequence was PCR amplified (see Section 2.3.9) from the IMAGE clone 5399570 (GenBank accession no. BC063434) plasmid purchased from Open Biosystems (Huntsville, AL, USA). The gene-specific primers were modified to include the appropriate restriction sites at their 5' ends. The primers used were: forward, 5'– TTTTGATATCATGAGTGAGTCGAGCTCGAAG–3' and backward, 5'-TTTTGAATTCTCACTGCTCCTCCTCCGAGGA-3'. These primers included the EcoRV and EcoRI restriction enzyme sites respectively. Purified PCR products were digested with EcoRV and EcoRI before ligation into the corresponding sites in pIRES-puro3 vector (Clontech, Palo Alto, CA). The expression plasmids were named pIRES-HMGA1, after sequence verification.

The pIRES-puro3 vector permits two genes of interest (i.e. two open reading frames for HMGA1 and puromycin resistance [puromycin-N-acetyl-transferase] genes) to be translated from a single mRNA transcript, using the internal ribosome entry site (IRES) of the encephalomyocarditis virus (Jang et al., 1988; Rees et al., 1996) (Figure 2.3). The vector utilises the strong constitutive cytomegalovirus (CMV) promoter to drive gene expression.

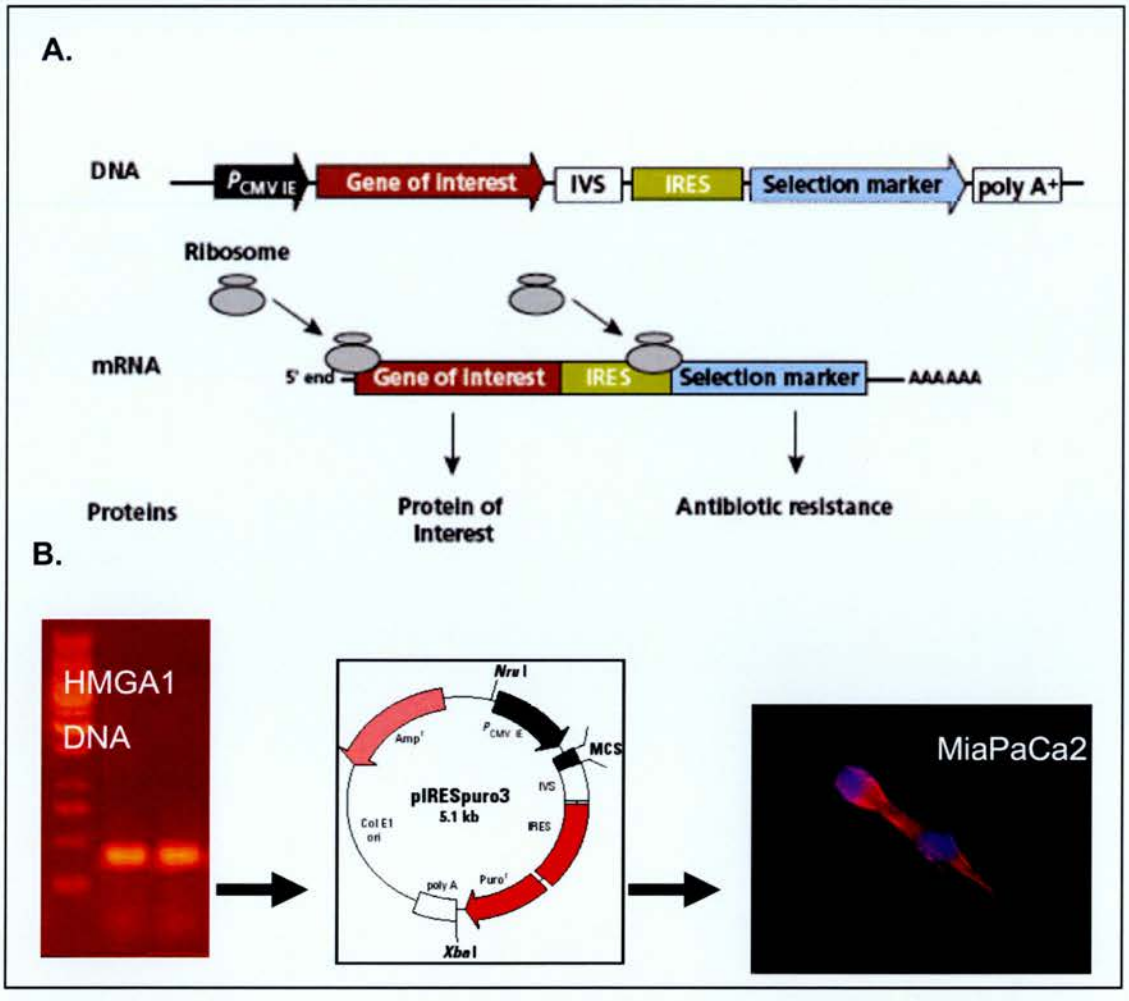


Figure 2.3. A, pIRES-puro3 (Clontech) vector incorporates an internal ribosomal entry stie (IRES) which allows transcription of one mRNA from two genes of interest (i.e. gene of interest and a selectable marker e.g puromycin resistance gene). B, Generation of HMGA1 DNA by PCR followed by ligation into the pIRES-puro3 expression vector which has a puromycin resistance selection marker gene. The HMGA1-pIRES-puro3 vector was transfected into MiaPaCa2 cells, followed by selection of stable clones.

2.7.2 Creation of HMGA1-overexpressing stable transfectants

MiaPaCa2 cells were transfected with pIRES-HMGA1 or empty pIRES-puro3, which acted as a control, using Lipofectamine 2000 (Invitrogen). Stable clones were selected by exposure to incrementally increasing concentrations of puromycin (Invivogen, San Diego, CA, USA), isolated using cloning cylinders, and maintained in medium containing 3µg/ml puromycin (Invivogen). Clones pIRES-HMGA1.1 and pIRES-HMGA1.2, which expressed the highest levels of HMGA1 as confirmed on Western blotting, were used for further studies.

2.8 PROTEIN ANALYSIS

2.8.1 Harvesting of monolayer culture and total lysate extraction

Cells in monolayer were disaggregated, harvested and rinsed twice with ice-cold PBS. Cell extracts were prepared with lysis buffer containing 20mM Tris, 0.1% Triton X, 0.5% deoxycholate, 1mM PMSF, 10 mg/ml, aprotinin, 10 mg/ml leupeptin. Lysis was completed by incubation on ice for 20 minutes and sonication of sample for 10 seconds on ice. Protein was cleared by centrifugation at 15000 x g, 4°C. Protein samples were stored at -80°C.

2.8.2 Nuclear protein extraction

Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents based on the manufacturer's instruction (Pierce, Rockford, IL, USA). Nuclear extracts were used for the analysis of HMGA1 expression. Briefly, $5X10^6$ cells were harvested and centrifuged at 15 000 x g, 4 °C. The cell pellet was resuspended in 200 µL of CERI Buffer and allowed to incubate on ice for 10 minutes. This was followed by addition of 11 µL of CERII Buffer and intermittent vortexing at high speed for 1 minute. Addition of CERI and II buffers will result in the disruption of cytoplasmic membrane and release of cytoplasmic contents. The intact nuclei were then isolated by centrifugation at 15 000 x g, 4 °C for 5 minutes. The isolated nuclei were

further lysed by addition of 50 µL of ice-cold NER Buffer. This was vortexed at high speed for 15 seconds every 10 minutes for at least 40 minutes in total. Final centrifugation at 16 000 x g at 4 °C for 10 minutes gave rise to the nuclear protein extract in the supernatant which was immediately stored at -80°C.

2.8.3 Protein quantification

Total protein concentration was measured using the bicinchoninic acid (BCA) assay kit (Sigma) with bovine serum albumin as a standard, according to the manufacturer's instructions. The BCA assay utilises the well-known reduction of Cu2+ to Cu1+ by protein in an alkaline medium with the sensitive colorimetric detection of the cuprous cation (Cu1+) by bicinchoninic acid. The BCA/copper complex exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. In short, 50 parts of reagent A were mixed with 1 part of reagent B and 200 µL was added to 10 µL of protein (including protein standards) in each well of a 96-well plate. Protein standards range from 200-1,000 µg/mL of bovine serum albumin (BSA). Following 20 minutes of incubation at 37 °C, the protein concentration was analysed using a SpectraMax M5 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 562 nm. The protein concentration was intrapolated from a standard curve generated using BSA standards.

2.8.4 Polyacrylamide gel electrophoresis (PAGE) and Western transfer

Electrophoresis was applied to separate proteins according to molecular weight. Cell lysates were mixed with a quarter volume of 4X LDS sample buffer (Invitrogen) and denatured at 95°C for 5 minutes followed by centrifugation at 5000 x g for 30 seconds to collect condensation. Cell extracts containing 50 µg total protein or 10 µg nuclear protein were loaded into each well of the pre-cast Bis-Tris-HCl buffered (pH 6.4) 10% PAGE minigel (NuPAGE Novex Bis-Tris minigel system, Invitrogen). Upon loading into the wells, the protein samples were subjected to a constant running voltage of

120V for 45 minutes in parallel with molecular weight marker (Kaleidoscope Protein Standard, Biorad, Hercules, CA, USA). The running buffer used was 1 x MES SDS buffer (Invitrogen). Following PAGE, the resolved proteins on the gel were transferred electrophoretically to PVDF membranes (Invitrogen) using the XCell II Blot Module and 1 x NuPAGE transfer buffer (Invitrogen) at a constant transfer voltage of 45 V for 2 hours. Prior to the removal of minigel from its casing, the PVDF membrane was activated in 100% ethanol for 2 seconds. Two similarly sized filter papers, 6 pieces of sponge pads and the activated PVDF membrane were soaked in the transfer buffer. The PVDF membrane was laid gently on the gel, ensuring no air bubbles were caught in between. This was then sandwiched between two filter papers. The stack was compressed to remove any air bubbles that might interfere with uniform transfer. The stack was then laid between 3 pieces of sponge pads on each side and placed within the transfer unit with the PVDF membrane closer to the cathode side. A constant voltage of 45V was applied for 2 hours to achieve transfer of proteins to the PDVF membrane. Equal protein loading and transfer was confirmed by Coomassie (BioRad) staining of the gel.

2.8.5 Western immunoblotting

Once the proteins were transferred, the PVDF membrane was blocked in PBS containing 0.1% Tween 20 (PBST) and 3% bovine serum albumin (BSA) for 1 h at room temperature to prevent non-specific antibody binding. Membranes were blocked for 1 hour at room temperature or overnight at 4˚C on a shaking platform. The membranes were cut in half horizontally based on the molecular weight marker band if more than one protein was to be probed on the same membrane. For example, to assess HMGA1 expression and lamin B1 levels, the membrane was cut at 40 kDA marker. The top half (>40 kDa) was incubated with primary antibody for lamin B1 (molecular weight of 60kDa) and the bottom half (<40 kDa) was probed for HMGA1 (molecular weight of ~ 20 kDa). The membranes were incubated with the protein side down to ensure uniform exposure to the antibody. The optimal duration and temperature of

incubation depended on the specific antibody. Blocking membrane with BSA reduced background non-specific binding especially when using phosphor-specific antibodies.

Following incubation with primary antibody, the membrane was washed 3 x with PBST and incubated with secondary antibody, diluted 1: 1000 to 1:10000 in PBST. Antibodies used in this project are listed in Table 2.4. The secondary antibody was either goat anti-rabbit or goat anti-mouse conjugated with horse-radish peroxidase (HRP) (Vector lab). Membranes were washed as before and the detected bands were visualised with ECL system (Amersham Biosciences, NJ, USA). This method used HRP-induced catalytic oxidation of luminol in ECL detection reagent in the presence of hydrogen peroxide. The light emitted as luminol decayed from its excited state was visualised on X-ray (Biomax MR film, Kodak). Radiographs were scanned and imported into Photoshop software (Adobe Systems Inc., USA). Faint exposures were used to quantitate to minimise error due to overexposure or signal saturation. Band intensity was quantified and typically normalised to loading controls such as β-actin or laminin, using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Table 2.4. Primary antibodies used for Western blotting.

Protein	Phospho-epitope	Supplier/Cat Number	Dilution	Animal source	Incubation time
HMGA1	-	Santa Cruz Sc-1564	1:200	Goat	2 hrs
p-Akt	Ser473	Santa Cruz Sc-7985	1: 200	Rabbit	2 hrs
p-Akt	Ser473	Cell Signaling 587F11-#4051	1: 200	Mouse	2 hrs
Akt1	-	Santa Cruz Sc-7126	1:200	Goat	2 hrs
p-ERK1/2	Thr177 and Thr160	Santa Cruz Sc-23759	1:200	Rabbit	2 hrs
ERK1/2	-	Santa Cruz Sc-93	1:200	Rabbit	Overnight
p-mTOR	Ser2448	Cell Signalling #2971	1:1000	Rabbit	Overnight
mTOR	-	Cell Signalling #2972	1:1000	Rabbit	Overnight
MMP9	-	Santa Cruz sc-12759	1:100	Mouse	Overnight
MMP2	-	Santa Cruz Sc-13594	1:100	Mouse	Overnight
MMP2	-	Lab Vision MS-805	1:100	Mouse	Overnight
β-Actin	-	Lab Vision RB-9421	1:500	Rabbit	2 hrs
Lamin B	-	Santa Cruz Sc-6216	1:200	Goat	2 hrs
Hemagglutinin (HA)	-	Santa Cruz Sc-57592	1:200	Mouse	2 hrs

2.9 IN VITRO CELLULAR INVASION ASSAY

Cellular invasion was quantified using a modified Matrigel Boyden chamber assay. The BD BioCoat Matrigel invasion chambers (BD Bioscience, Bedford, MA, USA) were used according to the manufacturer's instructions. The upper surface of the invasion chamber was coated with 250µg/ml growth factor-reduced Matrigel matrix. In principle, the Matrigel matrix mimics the basement membrane through which tumour cells will invade. The chambers were rehydrated once with 0.1% BSA in DMEM for 1 hour at room termperature and then placed in 24-well tissue culture plates. Following this, cells were removed from tissue culture plates by trypsinisation, washed once in PBS and counted. Then, $2.5X10^4$ pancreatic cancer cells in serum-free DMEM were seeded onto Matrigel-coated chambers. In the lower chambers, 5% FBS was added as a chemoattractant. After 12-24 hours' incubation, the cells from the upper surface of the membrane were removed by wiping with a cotton swab. The membranes were stained using the Diff-Quik kit (BD Biosciences), and the number of cells that had invaded through the filter was counted under X400 magnification (randomly selected high-power fields). The counting was performed for twenty fields in each sample, and mean values from three independent experiments were calculated. The number of invading cells from the control group was assigned a value of 1.0 in each experiment to facilitate comparison. Each determination represents a mean of three separate experiments.

In additional studies, the effect of matrix metalloproteinase-9 (MMP-9) antagonism was determined by performing invasion assays in the presence of 10 mg/ml anti-MMP-9 neutralizing antibody (Santa Cruz) or isotype-matched control (irrelevant) immunoglobulin (IgG) (Santa Cruz).

2.10 FLUOROMETRIC MMP-9 ACTIVITY ASSAY

Matrix metalloproteinase-9 activities of cell or tumour lysates were assessed using the Enzolyte Plus™ 520 Enhanced Selectivity MMP-9 activity assay, in accordance with the manufacturer's instructions (AnaSpec, San Jose, CA, USA). This assay was highly specific for human MMP-9 without any cross-reactions with other MMPs. The assay utilised 96-well microplate coated with anti-human MMP9 monoclonal antibody. The monoclonal antibody was therefore used to pull down MMP-9 from biological samples. The proteolytic activity of the MMP-9 was then quantitated using a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET) peptide. The fluorescence of 5-FAM (fluorophore) was quenched by QXL™520 (quencher) in the intact FRET peptide. However, in the presence of MMP-9, the FRET peptide was cleaved by MMP-9 to release 5-FAM which when not linked to its quencher, released a fluorescent signal.

The standard protocol for the assay included preparation of cell or tumour lysates. Cells or tumour xenografts were lysed in assay buffer G containing 0.1% Triton-X100. Protein content was normalised between samples using the BCA assay. Six serial dilutions of the MMP-9 standard (recombinant human MMP-9 protein) were performed. In a microplate, 100 µl of MMP-9 standards, blank controls and samples to be tested were added to their respective wells. Plates were incubated at 4˚C overnight on a microplate shaker. Following this, the wells were washed 4 times with wash buffer. The immobilised MMP-9 was then activated by adding 100 µl of 1mM APMA solution to each well. Again, the plate was washed 4 times. The final step included addition of the FRET peptide substrate to each well and incubation at room temperature in the dark for 1 hour. Fluorescence intensities were then quantified using SpectraMax M5 microplate spectrophotometer (Molecular Devices) at excitation = 490 nm and emission = 520 nm. Total MMP-9 activity was quantitated by interpolation using the constructed standard curve.

2.11 ASSESSMENT OF *MATRIX METALLOPROTEINASE-9* (*MMP-9*) GENE PROMOTER ACTIVITY

2.11.1 Construction of MMP-9 gene promoter reporter plasmid

The MMP-9 promoter reporter construct (pGL4-MMP9) was custom-synthesized by Aviva Systems Biology (San Diego, CA, USA). Cloning of the MMP-9 promoter sequence into the pGL4.12 vector upstream of the firefly luciferase (*Photinus pyralis*) gene allows the assessment of MMP-9 promoter activity using the surrogate expression of firefly luciferase protein. Briefly, the full-length human MMP-9 promoter region ranging from −992 to+304 bps, relative to the transcription initiation site, was amplified by PCR from human placental DNA, using primers designed according to the MMP-9 gene sequence. The primers were: forward, 5'–GGTACCTCTTTCTGGGCTCAAGCAATC–3' and backward, 5'–CTCGAGCTAACCCTGGACACCTCTGTTC–3'. These primers included the XhoI and KpnI restriction sites respectively. The amplified promoter fragment was cloned into the pGL4.12 vector (Promega, Madison, WI, USA) at the XhoI/KpnI site upstream of the firefly luciferase gene. By direct sequencing, the sequence of the cloned promoter region was confirmed. The empty pGL4.12 vector (pGL4e) was used as control.

2.11.2 Quantitation of MMP-9 promoter activity using luciferase assay

Cells were plated onto 6-well cell culture plates at a concentration of $5X10^5$ cells per well and allowed to adhere for 12 hours in serum-containing medium. To assess the effects of modulating HMGA1 expression on MMP-9 promoter activity, transient transfection experiments were performed on MiaPaCa2 cells. For HMGA1 suppression experiments, 5 µg of shHMGA1 or control shRNA plasmid was co-transfected with 5 µg of pGL4-MMP9 or pGL4e and 0.5µg of pRLCMV vector (Promega), which contains a cytomegalovirus promoter upstream of a renilla luciferase (*Renilla reniformis*) gene. For

HMGA1 overexpression experiments, either pIRES-HMGA1 or control vector, pIRES-puro3 was used in co-transfection.

Luciferase activity in lysate of the transfected cells was measured using the dual-luciferase reporter assay, according to the manufacturer's recommended protocol (Promega). This assay allowed sequential measurement of the firefly and renilla luciferase activities. After 48 hours, cells were lysed directly on the cell culture plate using 500µL of PLB reagent. Using luminometer tube, 20 µL of each cell lysate was mixed thoroughly with 100 µL LARII reagent. The firefly luciferase activity was measured for 10 seconds using the SpectraMax M5 luminometer (Molecular Devices). This was followed by addition of 100 µL Stop and Glow reagent. A second reading for the renilla luciferase activity was performed for 10 seconds. Relative luciferase activity was calculated as fold-induction of luciferase activity above the background (taken as activity associated with promoterless vector, pGL4.12). The activity of renilla luciferase was used to normalise any variation in transfection efficiencies.

2.12 PROLIFERATION ASSAY

Cell proliferation was quantified using the MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium) assay (Cell Titer 96 Aqueous One Solution Assay, Promega, Madison, WI, USA) where the viable cells convert the tetrazolium dye into a chromogenic product. The absorbance values of the chromogen were correlated with the number of viable, proliferating cells. The results of the MTS assay was confirmed by cell counting using the Neubauer haemocytometer (Fisher Scientific, Orlando, FL, USA) in each experiment.

Logarithmically-growing cells were seeded into 96-well plates at $5X10^3$cells per well and allowed to adhere overnight in medium containing 10% or 2% FBS. Cellular proliferation was determined after 48 hours. Plates were read with the use of the SpectraMax M5 microplate spectrophotometer (Molecular

Devices, Sunnyvale, CA, USA) at a wavelength of 490nm. Ten samples were used for each experimental condition, and experiments were performed in triplicates. At identical time points, cell counting was performed and the results concurred with MTS assay.

2.13 ANCHORAGE-INDEPENDENT GROWTH

Soft agar assays were performed using the cell transformation detection assay, according to the manufacturer's instruction (Chemicon, Temecula, CA, USA). Briefly, assays were performed in 6-well plates with $5X10^3$ cells, resuspended as a single cell suspension, in 0.4% agar and layered on top of 0.8% agar. Plates were incubated for 10-12 days. Colonies were stained and counted manually at high power (X400) magnification. The counting was performed for ten fields in each well, and at least 6 wells per condition were performed in each experiment. Average values from three independent experiments were calculated. The relative number of colonies was calculated by dividing the average number of colonies for each group with that of the control group such that the ratio between the groups could be more easily interpreted.

2.14 ASSESSMENT OF CHEMOSENSITIVITY TO GEMCITABINE

2.14.1 Cytotoxicity assay

Gemcitabine-induced cytotoxicity was quantified by the MTS as described above (Section 2.12). Cells were seeded into 96-well plates at $5X10^3$ cells per well and allowed to adhere overnight in medium containing 10% FBS. Cell viability was determined after 48-72 hours in the presence of 0-10 μM gemcitabine. Six samples were used for each experimental condition. Each independent experiment was performed three times. The concentration of gemcitabine required to inhibit proliferation by 50% (IC50) was calculated from these results.

2.14.2 Apoptosis assay

After exposure to gemcitabine (1 μM) for 48 hours, $1X10^6$ cells were washed, trypsinized and resuspended in 0.5 ml of PBS containing 2% FBS and 0.1μM EDTA. Apoptosis staining was performed using 1μl/ml YO-PRO-1 and propidium iodide (Vybrant Apoptosis Assay Kit #4; Molecular Probes, Eugene, OR, USA). Cells were incubated for 30 min on ice. If there was a delay in analysis, the cells were centrifuged at 500 g for 5 min and fixed in 300μL of PBS containing 3.7% paraformaldehyde. Samples were analyzed by FACScan (Becton Dickinson, Franklin Lakes, NJ) two-colour flow cytometer (FL1=530/30, FL2=610/20) equipped with a single argon laser (488nm). Cells were gated to exclude debris. Cells stained with the green fluorescent dye YO-PRO-1 were counted as apoptotic; necrotic cells were stained with propidium iodide. The number of apoptotic cells was divided by the total number of cells (minimum of 10^4 cells) to calculate the apoptotic fraction. Data were analyzed using CellQuest software (Becton Dickinson). All assays were reproduced at least three times in independent experiments.

2.14.3 Fluorometric caspase profiling assay

Whole cell lysates were assayed for caspase 2, 3, 8 and 9 activities using the BD ApoAlert Caspase Assay Plate (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. This assay utilised caspase-specific fluorogenic substrates immobilised in the wells of a 96-well microplate. Upon addition of a cell lysate containing activated caspase, the immobilised fluorogenic substrate was cleaved by the corresponding activated caspase to release a fluorescent product that could be detected.

In short, following treatment with gemcitabine (1 μM) for 48 hours, $1X10^6$ cells were harvested and lysed in 100 μL of lysis buffer provided. Fifty microlitres of lysates (equivalent to $5X10^5$ cells) were transferred into each well of the microplate and incubated for 2 hours at 37˚C. Plates were then read (excitation, 360 nm; emission, 480 nm) using SpectraMax M5 microplate

reader in fluorescence mode (Molecular Devices). The levels of fluorescence by the various activated caspases were determined and expressed as the number of fold increase relative to control cells. Independent experiments were performed at least three times, with three replicate samples in each experiment.

2.15 ANOIKIS ASSAY

2.15.1 Anoikis induction

Anoikis was assayed by plating cells on polyHEMA-coated plates. The polyHEMA coat created a non-adherent surface which prevented the cells from attaching. Cells which normally grew adherently would undergo apoptosis with the loss of attachment. To make the polyHEMA-coated plate, a solution of 120 mg/ml polyHEMA (Sigma, St Louis, MI, USA) in 100% ethanol was made and diluted 1 : 10 in 95% ethanol. This solution (0.95 ml per mm^2) was overlaid onto 35mm 6-well plates and left to dry in a heated dryer system (Fisher Scientific, Waltham, MA, USA) for 12 hours. Prior to use, wells were washed twice with PBS and once with DMEM.

In all experiments, $1x10^6$ cells suspended in 2ml DMEM with 10% FBS were incubated in the polyHEMA-coated wells for 12-18 hours in a humidified (37˚C, 5% CO2) incubator. Cells were harvested and resuspended in 0.3 ml of PBS containing 2% FBS and 0.1μM EDTA before apoptosis staining.

2.15.2 Flow cytometric analysis

Following induction of anoikis, apoptosis staining was performed using 1μl/ml YO-PRO-1 and propidium iodide (Vybrant® Apoptosis Assay Kit #4; Molecular Probes, Eugene, OR). The methods for staining apoptotic cells have already been described in Section 2.14.2. Each experiment was reproduced three times with at least three replicate samples in each condition.

2.16 FLUOROMETRIC REAL-TIME AKT KINASE ASSAY

Assay was performed using the Omnia® Lysate Akt kinase assay (Biosource-Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. This assay uses the chelation-enhanced fluorophore 8-hydroxy-5-(N, N-dimethysulfonamido)-2-methylquinoline (referred to as Sox). The Sox peptide is linked to an Akt substrate peptide using standard solid-phase peptide chemistry. With phosphorylation of the Akt substrate, Mg^{2+} is chelated to form a bridge between the Sox moiety and phosphate group added by Akt to the substrate. Formation of this bridge results in an increase in fluorescence signal which can be quantitated.

Briefly, cell lysate was prepared using the Omnia cell extraction buffer. Total cell lysate equivalent to 5µg per µL was loaded into each well of a 96-well microplate. Following the addition of 45µL of a master mix (10µM Akt substrate-Sox peptide, 1X kinase buffer, 1mM ATP, 1mM DTT, 2µM GF109203X, 2µM Calmidazolium, 4µM PKC and 0.4µM PKA inhibitor peptides), the fluorescent signal was acquired real-time (excitation, 360nm; emission, 485) for 60 minutes using the SpectraMax M5 microplate reader (Molecular Devices). The Akt kinase activity was calculated from the slope of the activity curve. Experiments were performed in triplicates on at least three occasions.

2.17 MOLECULAR REAGENTS USED FOR PATHWAY DISSECTION

2.17.1 Specific small molecule inhibitors

PI3-kinase inhibitor LY294002 and MEK inhibitor PD98059 were purchased from Calbiochem (San Diego, CA, USA). LY294002 is a selective and reversible inhibitor of PI3-kinase (Vlahos et al., 1994). PD 98059 is a selective inhibitor of MAPK/ERK kinase or MEK.

2.17.2 Adenovirus expressing dominant negative or active Akt constructs

Adenovirus carrying HA-tagged dominant negative (Ad-DN-Akt), dominant active (Ad-myr-Akt) Akt1 and control virus (Ad-CMV-null) (all titred at $1X10^{10}$ PFU per mL) were purchased from Vector Biolabs (Philadelphia, PA). Adenoviral infection was performed at multiplicity of infection of 10 in the presence of 6 µg/ml polybrene for 8 hours. Experiments were performed on cells 48 hours following infection. Infection efficiency was confirmed by performing Western blotting on cell lysates using anti-HA antibody.

2.18 XENOGRAFT MOUSE MODELS OF PANCREATIC CANCER

Male athymic nu/nu mice 5 weeks of age, weighing 20–22 g and specific pathogen-free were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). Nude mice were chosen as human cancer cells are tumourigenic in these mice. Human cancer cells do not form tumour in immunocompetent mice.

Mice were housed in microisolator cages in a specific pathogen-free facility with 12-hr light–dark cycles. They received water and food *ad libitum*. Animals were observed for signs of tumour growth, activity, feeding and pain in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals.

2.18.1 Nude mouse orthotopic xenograft model

To determine the effects of HMGA1 gene silencing on *in vivo* metastasis, MiaPaCa2 cells stably expressing the control or HMGA1 shRNA (shHMGA1-1) constructs were orthotopically implanted into the pancreata of nude mice. The use of this model offers a more 'physiological' microenvironment for evaluating tumour progression, especially the metastatic process.

2.18.1.1 Surgical procedure

Mice were anesthetized with intraperitoneal ketamine (200 mg/kg) and xylazine (10 mg/kg). In a laminar flow hood, the abdomen was cleaned with isopropyl alcohol, and a left upper transverse incision was made. The pancreas was exposed and $1X10^6$ cells suspended in 75 µl of PBS were slowly injected into the body of the pancreas. The pancreas was returned and the abdomen closed with 5-0 Vicryl (Ethicon, Somerville, NJ, USA). Ten mice for each cell line were observed over 4 weeks and killed by overdose of ketamine (400 mg/kg) and xylazine (50 mg/kg). The liver of each animal was harvested, and metastatic foci were counted under a dissecting microscope (Gorelik et al., 1993). The primary tumours were excised and the tumour lysates were assayed for HMGA1 expression and MMP-9 activity using Western blotting and MMP-9 activity assay (Section 2.9), respectively.

2.18.1.2 Postoperative care

Following orthotopic implantation, mice received postoperative analgesia in the form of buprenorphine 0.05-0.1mg/kg subcutaneously every 12 hours for at least 48 hours. The mice receiving subcutaneous implantation typically did not require analgesia. Animals were checked at least once a day for the duration of the study with records kept on each animal with regards to feeding habits, pain and level of activity.

2.18.2 Nude mouse subcutaneous xenograft model

To determine the effects of HMGA1 gene silencing on *in vivo* growth, the subcutaneous xenograft model was used.

2.18.2.1 Tumour implantation

The mice were restrained in a head down position in a sterile laminar flow hood. The injection site was cleaned with alcohol swabs. Injection site was on

the flank well away from the limbs so that the tumour would not impede mobility. Two million cells (MiaPaCa2 and PANC1 stable transfectants expressing the control or HMGA1 shRNA i.e shHMGA1.1 sequence) in 100 µL PBS were subcutaneously implanted using a 30G needle. Tumour dimensions were measured weekly using micrometer calipers. Tumour volumes were calculated using the following formula: Volume = $1/2\ a \times b^2$, where *a* and *b* represent the larger and smaller tumour diameters, respectively. Eight weeks following implantation, the primary tumour was excised, formalin-fixed and paraffin-embedded.

2.19 XENOGRAFT TUMOUR ANALYSIS

2.19.1 Harvesting protein from xenograft tumours

At the end of the experimental period, the xenograft tumours were harvested from the mice and snap-frozen in liquid nitrogen. To extract proteins from tumours for Western blot analysis, a modified protocol for the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) was used (Section 2.7.2).

The harvested tumour was pulverised in 500-1000 µL of CERI Buffer using a tissue homogeniser (Omni International, Marietta, GA, USA) and allowed to incubate on ice for 30 minutes. This was followed by addition of 100-200 µL of CERII Buffer and intermittent vortexing at high speed for a further 20 minutes. Addition of CERI and II buffers will result in the disruption of cytoplasmic membrane and release of cytoplasmic contents. The cytoplasmic extract and intact nuclei were separated by centrifugation at 15 000 x g, 4 ˚C for 5 minutes. The cytoplasmic extract was stored at -80˚C until further analysis. The isolated nuclei were further lysed by addition of 100-200 µL of ice-cold NER Buffer. This was vortexed at high speed for 15 seconds every 10 minutes for at least 40 minutes in total. Final centrifugation at 16 000 x g at 4 ˚C for 10 minutes gave rise to the nuclear protein extract in the supernatant

which was immediately stored at -80°C. Nuclear extracts from tumour xenografts were used for the analysis of HMGA1 expression.

2.19.2 Ki-67 and HMGA1 immunohistochemistry

Tumour sections (5 µm) were cut using a microtome (Olympus America Inc, Melville, NY, USA) and mounted onto polylysine-coated slides (Vector Laboratories, Burlingame, CA, USA). Slides were deparaffinised and processed using a streptavidin-biotin-peroxidase complex method (Section 2.1.3). Following quenching of endogenous peroxidase activity and blocking of nonspecific binding, sections were incubated with anti-Ki-67 (DAKO, Carpinteria, CA) or anti-HMGA1 (Santa Cruz) at 4°C overnight at a 1:200 or 1:50 dilution respectively. The secondary antibody was biotinylated rabbit antimouse or rabbit anti-goat antibody (DAKO) used at a dilution of 1:200 for 30 minutes at 37°C. Tumour cells were considered positive for the Ki67 antigen or HMGA1 expression if there was intranuclear staining. Images of the slides were acquired at X40 magnification with a Nikon microscope using ImagePro Plus software (Mediacybernetics, Bethesda, MD, USA). The cells with positively stained nuclei were counted in four to five random fields from each section.

2.19.3 Apoptosis staining

Following preparation of 5 µm tumour sections, apoptosis was quantified using a commercially available terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) kit, in accordance with the manufacturer's protocol (Apoptag Peroxidase *In Situ* Apoptosis Detection Kit, Chemicon, Temecula, CA, USA). The TUNEL assay detects single or double stranded DNA breaks associated with apoptosis. Digoxigenin-conjugated nucleotides are added to the DNA breaks by terminal deoxynucleotidyl transferase (TdT). The digoxigenin-labelled DNA breaks are then detected by antibody conjugated to a peroxidase reporter molecule which gives an intense colour from chromogenic substrate. The intense staining identifies apoptotic cells in the sections.

Briefly, the slides were pre-treated with Proteinase K (20µg/mL) for 15 minutes after deparaffinising the section. This was followed by application of 75µL/5cm^2 of equilibration buffer and 55 µL/5 cm^2 of TdT enzyme. The final step included staining with anti-digoxigenin conjugate followed by peroxidase colour development. The number of apoptotic cells in four to five random fields from each section was counted.

2.20 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS Ver 12 (SPSS Inc., USA) and Statistica 5.5 (Stat Soft Inc., Tulsa, OK, USA) softwares. Differences between the groups were analyzed using Student's t-test, ANOVA or Mann–Whitney U-test as appropriate. The unpaired Student's t-test was utilised to compare two independent groups with the assumption that the variable has a normal distribution. The One-Way Analysis of Variance (ANOVA) was used to compare three or more groups if the data sets had a normal distribution. However, it the data was non-parametric, the Mann-Whitney test was used to compare two independent groups. In cases in which averages were normalised to controls, the standard deviations of each nominator and denominator were taken into account in calculating the final standard deviation. When the data was nominal in nature, the chi-square test was used to determine the presence of association between two qualitative variables. In cases with a 2x2 table, Fisher's exact test was used instead of chi-square test. $P<0.05$ was considered statistically significant.

Chapter Three: Evidence for the clinical relevance of HMGA1 expression in pancreatic cancer

3.1 ABSTRACT

Background: HMGA1 proteins are architectural transcription factors that are overexpressed in a range of human malignancies. However, limited data is available on the expression of HMGA1 in pancreatic adenocarcinomas.

Aims: To establish the clinical relevance of HMGA1, we evaluated tumoural HMGA1 expression status and investigated if HMGA1 expression could be a prognostic biomarker in pancreatic adenocarcinomas.

Methods and results: Using a tissue microarray format, tumoural HMGA1 expression was examined by immunohistochemical analysis of tissues from 89 consecutive patients undergoing resection for pancreatic adenocarcinoma. Tumoural HMGA1 expression was detected in 83 (93%) cases of pancreatic adenocarcinoma. Little or absent expression of HMGA1 was present in normal pancreas. Based on Kaplan-Meier analysis, patients with HMGA1-negative cancers had a significantly longer median survival (3-fold longer; 4.3 years versus 1.3 years; $p<0.05$) than patients with HMGA1-expressing cancers. HMGA1 expression represents a novel prognostic factor in univariate ($p=0.0028$) and multivariate ($p<0.05$) analyses. However, HMGA1 expression was not correlated with any of the clinico-pathological features in this patient cohort.

Conclusions: Our findings suggest HMGA1 is an independent prognostic indicator in pancreatic adenocarcinoma patients. Given these promising clinical data, we embarked on the study of biology of *HMGA1* in pancreatic cancer cells (see Chapters 4-7).

3.2 INTRODUCTION

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States. Overall prognosis for patients diagnosed with this malignancy remains dismal, with 5-year survival rates averaging less than 5% (Jemal et al., 2006). Rational identification of molecular targets, based on an understanding of their clinical relevance and biological roles in the aggressive behaviour of pancreatic cancer is a high-priority goal. Dividends from this approach include identification of novel therapeutic target and biomarkers that facilitate early detection, prognostication and monitoring of response to therapy.

HMGA1 proteins are overexpressed in a range of human cancers (Section 1.11.7, Table 1.5). Further, the degree of tumoural HMGA1 expression has been reported to be inversely correlated with patient survivals for a range of human cancers (Chang et al., 2005; Sarhadi et al., 2006). In Chapter 1, we have previously discussed the rationale behind the study of the *HMGA1* gene in pancreatic adenocarcinoma. Based on gene profiling studies, *HMGA1* has been identified to be overexpressed in PDAC tissues and cell lines (see Section 1.11.1). These studies assessed only a small number of tissue specimens (up to 20 specimens). Using immunohistochemistry, Abe and co-workers demonstrated that HMGA1 is overexpressed in a small series of 15 PDAC specimens (Abe et al., 2000).The clinical relevance of HMGA1 expression in PDAC tissues has been relatively unstudied. Further, expression of HMGA1 in PDAC has not been correlated with clinical outcomes.

In this study, we hypothesised that HMGA1 is overexpressed in the majority of pancreatic cancer tissues and that expression of HMGA1 is indicative of adverse clinical prognosis. Firstly, we investigated the prevalence of HMGA1 overexpression in pancreatic cancer tissues using a tissue microarray format. As pancreatic cancer is a relatively rare disease, the cohort of patients in our

study represents one of the largest series with full clinicopathological correlates/information. Secondly, we evaluated the correlation of HMGA1 expression with clinicopathological characteristics of pancreatic cancer patients. Lastly, we assessed if tumoural HMGA1 expression status could be used as a novel biomarker that can be used to predict postoperative survival in patients having undergone surgical resection for pancreatic adenocarcinoma.

3.3 RESULTS

3.3.1 Characteristics of patients included in pancreatic adenocarcinoma tissue microarray

Using a constructed tissue microarray (TMA), we performed immunohistochemical analysis of tumoural HMGA1 expression in pancreatic resection specimens in a cohort of 89 patients with pathologically-proven pancreatic adenocarcinoma (42 men and 47 women) treated at the Brigham and Women's Hospital in Boston, USA over the period between 1991 and 2002. A summary of the clinicopathological characteristics of the cohort is provided in Table 3.1. The mean age at diagnosis was 63 years (median 63 years; range 34-84 years). Median survival was 16.6 months (range 91-3462 days). The actuarial 1-year survival rate was 70.3%, with a 5-year survival rate of 8.1%. The median tumour size was 2.7 cm with a range of 0.1-8.4 cm. Majority (55/89, 61.8%) of tumours were positive for lymph node metastasis. Most of the tumours were moderately differentiated (47/89, 53%), 9% (8/89) were well differentiated and 38% (34/89) were poorly differentiated. The staging of disease was performed using the American Joint Committee on Cancer (AJCC) classification system (see Table 1.2). Thirteen patients were staged as AJCC stage I, 74 as stage II, none as stage III, and 2 as stage IV.

Table 3.1. Clinicopathological characteristics of pancreatic adenocarcinoma cohort

Characteristics	
Age (years)	
Median	63
Range	34–84
Gender	
Male	42
Female	47
Overall stage	
I	13
II	74
III	0
IV	2
Lymph node status	
Negative	34
Positive	55
Tumour size, pathologic	
Median (cm)	2.70
Range (cm)	0.10–8.40
Histopathologic differentiation	
Well	8 (9%)
Moderate	47 (53%)
Poor	34 (38%)

3.3.2 Analysis of tissue microarray

Paraffin-embedded specimens were used to construct the pancreatic adenocarcinoma tissue microarray (Figure 3.1A). For each patient within the TMA, cores were also taken from adjacent normal pancreas to act as internal controls as well as to assess the expression of HMGA1 in normal pancreas. Following immunostaining with anti-HMGA1 antibody, HMGA1 expression was scored according to nuclear intensity (see Section 2.1). HMGA1 expression was scored according to nuclear staining intensity as follows: 0, no staining or weak intensity staining in less than 5% of cells; 1, weak intensity;

2, moderate intensity; 3, strong intensity. Expression was dichotomised into a HMGA1 negative group (Score 0) and HMGA1 positive group (Score ≥ 1).

3.3.3 HMGA1 expression in normal tissue and pancreatic adenocarcinoma specimens.

We detected the presence of nuclear HMGA1 expression in 93% (83/89) of pancreatic adenocarcinomas in our cohort. In the majority (52%) of tumour specimens, the degree of HMGA1 staining was graded with score ≥ 2 [score 1=42% (37/89), score 2=35% (31/89), score 3=17% (15/89)]. HMGA1 staining was predominanty localised to the nucleus of cancerous cells, with some staining of the cytoplasm (Figure 3.1F). The majority of normal pancreatic ducts had no detectable nuclear HMGA1 expression, while some had very weak expression (Figure 3.1C). Detailed pathology case review of the 6 specimens with absent tumoural HMGA1 expression demonstrated no atypical features for pancreatic adenocarcinoma.

3.3.4 Association of HMGA1 expression with clinicopathological variables

To identify associations of HMGA1 expression (HMGA1–negative versus – positive) with clinicopathological variables, the variables were dichotomised as shown in Table 3.2. Comparing patients with positive or negative tumoural HMGA1 expression, there were no significant differences in the distribution of patient age, gender, tumour size, differentiation, lymphovascular involvement (LVI), perineural involvement (PNI), margin status, lymph node involvement, disease stage and receipt of chemotherapy (Fisher exact test, $p>0.05$) (Table 3.2).

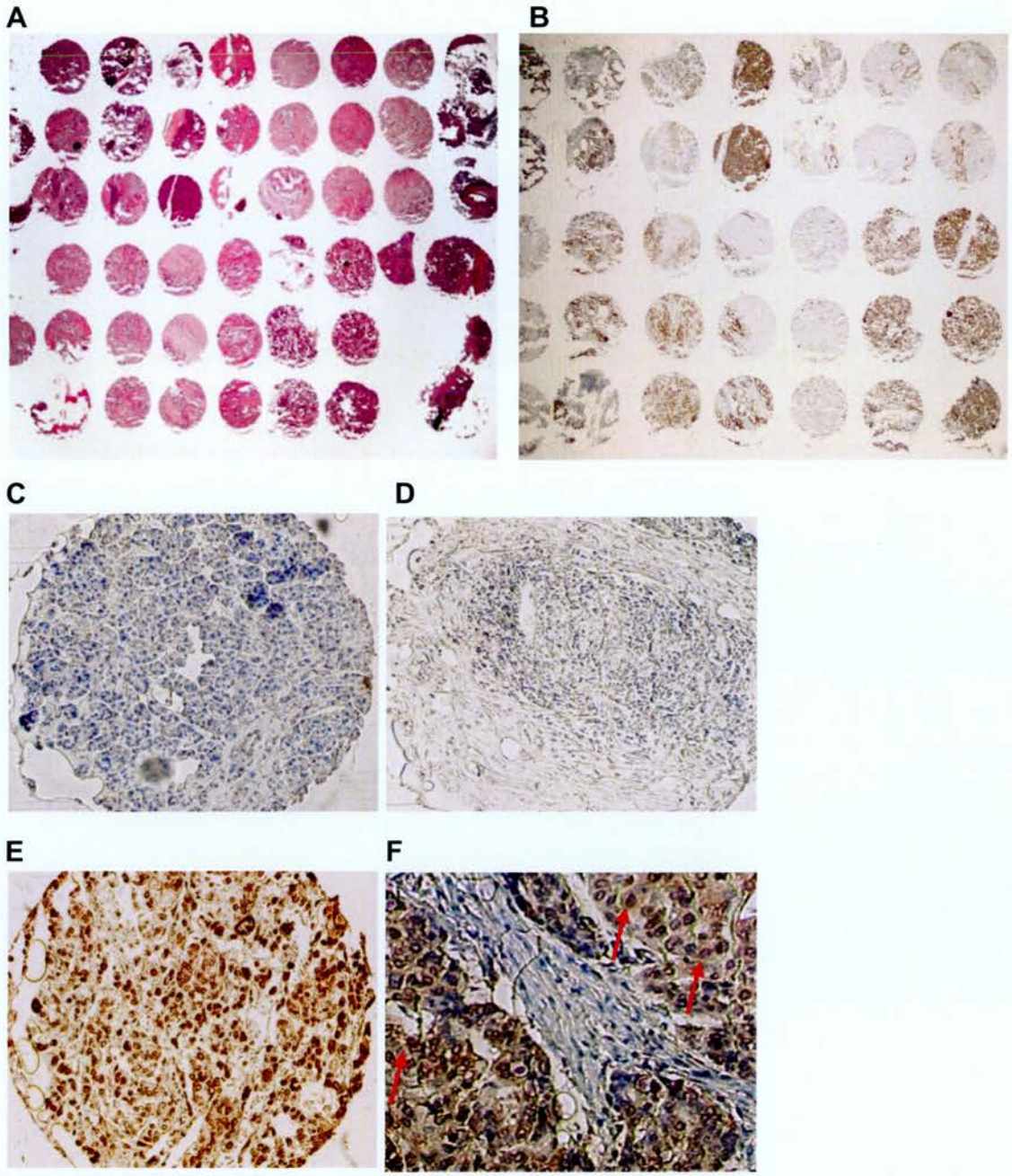


Figure 3.1. Overview of the pancreatic adenocarcinoma tissue microarray stained with H&E (A) and HMGA1 immunostaining (B). Magnified view (Magnification X100) of HMGA1 immunostaining in normal pancreas and pancreatic adenocarcinoma specimens of the tissue microarray: (C) Normal pancreas (HMGA1-negative), (D) Pancreatic adenocarcinoma (HMGA1-negative), (E) Pancreatic adenocarcinoma (HMGA1-positive). F) A high power magnification of HMGA1-positive section showing intense nuclear staining for HMGA1 (Magnification X400) as indicated by red arrows.

Table 3.2. Associations of HMGA1 expression with clinicopathological features

Variable	HMGA1 Status		P value
	HMGA1 negative N=6	HMGA1 positive N=83	
Age			
≤64	3	44	1.000
>64	3	39	
Gender			
Male	2	40	0.680
Female	4	43	
Tumour differentiation			
1	1	6	0.713
2	3	44	
3	2	32	
Size			
<2.5	3	38	0.416
>2.5	3	55	
Nodal metastasis			
No	4	30	0.197
Yes	2	53	
LVI			
No	5	49	0.397
Yes	1	34	
PNI			
No	3	40	1.000
Yes	3	43	
Microscopic margin			
Negative	3	49	0.690
Positive	3	34	
Tumour location			
Head	6	77	1.000
Tail	0	6	
T stage			
T1/2	2	11	0.210
T3/4	4	72	
Chemo			
No	1	4	0.272
Yes	3	59	

LVI: lymphovascular invasion, PNI: perineural invasion. Fisher's exact and Chi-square tests were used as appropriate to compare variables.

3.3.5 Absence of HMGA1 expression predicts favourable clinical outcome

Survival analysis was performed using the Kaplan-Meier method. Patients with absent HMGA1 expression (HMGA1 –ve) had significantly longer overall postoperative survival (mean 5.7 years, median 4.3 years), compared to those with HMGA1-positive tumours (mean 1.6 years, median 1.3 years; log-rank test, P=0.0028; Figure 3.2).

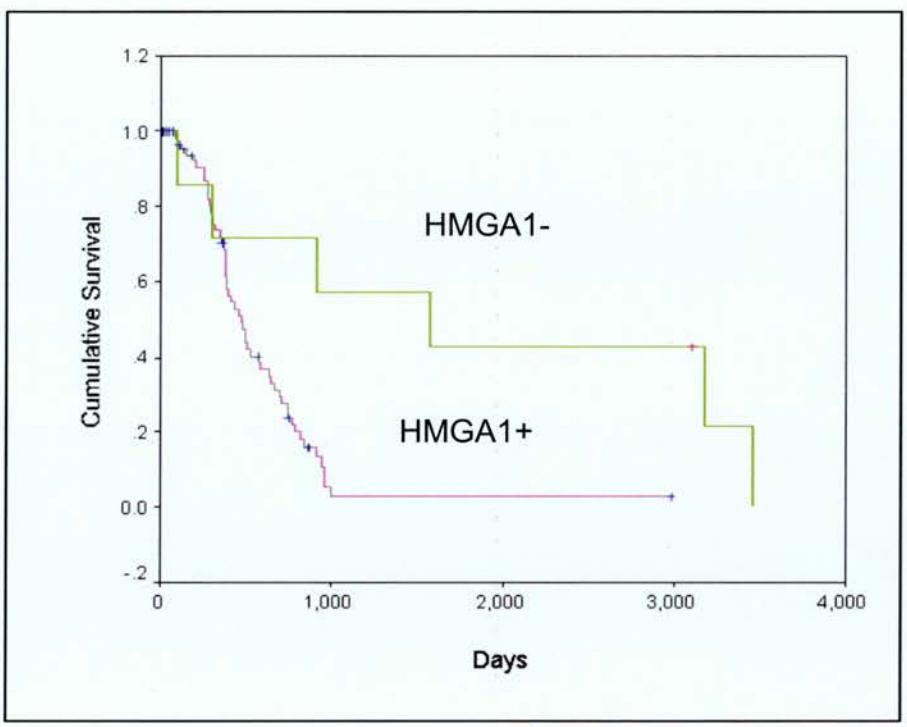


Figure 3.2. Kaplan-Meier analysis for overall survival for pancreatic adenocarcinoma patients based on HMGA1 expression. Survival of immunohistochemically HMGA1-negative patients were compared with HMGA1 positive patients using log-rank test (P=0.0028).

3.3.6 HMGA1 represents an independent prognostic indicator in pancreatic adenocarcinoma

To assess if HMGA1 expression was an independent predictor of overall postoperative survival, a Cox proportional hazards model was created in a forward fashion including only those covariates that showed a statistically significant (inclusion threshold of $p \leq 0.05$) relation with postoperative survival.

Univariate analysis showed that increasing tumour size, poor tumour differentiation and HMGA1 positivity were predictors of poorer survival (Table 3.3). Further, multivariate analysis demonstrated that after correction for confounding variables, HMGA1 expression remained a significant independent prognosticator for postoperative survival. HMGA1 positivity resulted in a hazard ratio of 10.87 (95% CI: 2.65-45.45, p=0.001) for poorer survival when compared to HMGA1-negative group. As expected, tumour size, tumour differentiation and HMGA1 expression were also independent prognostic markers.

Table 3.3. Predictors of poor postoperative survival

	Univariate			Multivariate		
Risk factors	**Hazard**	**95% CI**	**P value**	**Hazard**	**95% CI**	**P value**
Increasing age	1.015	0.994-1.037	0.171	1.008	0.983-1.033	0.545
Female gender	0.742	0.442-1.244	0.258	1.056	0.576-1.936	0.861
Increasing tumor size	1.217	1.022-1.450	0.028*	1.398	1.130-1.730	0.002*
Presence of lymph node metastasis	1.733	0.995-3.019	0.052	1.551	0.781-3.082	0.210
Advanced tumor stage (T3/4 vs. T1/2)	2.034	0.906-4.567	0.085			
Local invasion	1.078	0.612-1.897	0.796			
Poor tumor differentiation	1.859	1.183-2.921	0.007*	3.203	1.746-5.877	0.000*
Presence of PNI	1.088	0.651-1.819	0.746			
Presence of LVI	1.704	0.982-2.958	0.058	1.710	0.944-3.098	0.077
Presence of tumor at microscopic margin	1.490	0.886-2.505	0.133			
Chemotherapy treatment	0.440	0.132-1.474	0.183			
HMGA1 expression	5.473	1.614-18.557	0.006*	12.474	2.705-57.520	0.001*

CI: confidence interval, LVI: lymphovascular invasion, PNI: perineural invasion . * indicates $p<0.05$. Data were analysed using Cox proportional hazards model.

3.4 DISCUSSION

The primary aim of this study was to examine HMGA1 expression in pancreatic adenocarcinomas. HMGA1 expression was observed in more than 90% of PDAC specimens. Although HMGA1 expression was not significantly associated with any of the clinicopathological variables, HMGA1 negativity predicted improved survival in patients with PDAC and this relationship persists even after adjusting for other confounding variables. More importantly, our study demonstrates that HMGA1 expression may help to identify subsets of cancers with distinct clinical outcomes despite similar pathological characteristics. The median survival time was significantly longer (up to 3-fold) in HMGA1-negative patients than that of HMGA1-positive patients. Although HMGA1 has been shown to be indicative of poor prognosis in patients with other cancers, our current study is novel in that it is the first study to demonstrate that HMGA1 is an independent prognostic indicator in pancreatic adenocarcinoma patients.

Although our current study represents one of the largest immunohistochemical studies of pancreatic adenocarcinoma, our cohort size is relatively small and this limits interpretation beyond the results presented. Given that the current study had only 6 HMGA1-negative patients, further conclusion will require a study involving a greater cohort with greater number in each subgroup. However, we believe the current results are promising and highlight the clinical importance of *HMGA1*. Clearly, identification of HMGA1 as a prognostic indicator could be potentially useful in that it allows identification of patients who would benefit from more aggressive treatment of their disease. Our data regarding the role of HMGA1 as a prognostic indicator in PDAC is consistent with studies by other groups investigating other cancers. In colorectal cancers, HMGA1 expression has been correlated with development of distant metastases (Balcerczak et al., 2003). HMGA1-positivity in hepatocellular carcinoma has also been shown to predict disease recurrence and metastases (Chang et al., 2005). Further, in breast cancer,

HMGA1 expression correlates closely with the expression of Her2/neu, indicating its prognostic significance (Chiappetta et al., 2004).

In addition, the vast majority of pancreatic cancers in our study were positive for HMGA1 whilst the normal pancreatic tissues had little or no expression of HMGA1. Potentially, HMGA1-positivity can be used as a diagnostic marker for pancreatic cancer. This had been previously suggested by Abe and colleagues when they found HMGA1 overexpression correlated strongly with diagnosis of pancreatic adenocarcinoma (Abe et al., 2000). Our current study was not designed to address this issue as the TMA did not include benign tumours or non-neoplastic conditions that would allow assessment if HMGA1 differentiated a cancer from other benign conditions. In our TMA, the internal controls were normal adjacent pancreatic tissue. One may argue that the adjacent tissue surrounding a pancreatic cancer is often not 'normal' and may have features of fibrosis or pre-malignant ductal changes. To address this issue during the TMA construction, we selected only areas of pancreas surrounding the tumour which are morphologically normal (with no evidence of desmoplastic or pre-malignant features) as internal controls. This was verified by our collaborator, Dr Mark Redston who is a Consultant Pathologist at the Department of Pathology, Brigham and Women's Hospital (Boston, MA, USA).

Taken together, our results indicate that HMGA1 expression is associated with adverse prognosis in patients undergoing resection for PDAC. This study is the first to demonstrate that HMGA1 may represent a novel prognosticator in PDAC patients. Beyond its prognostic value, it is possible that HMGA1 may have a role in the pathogenesis of PDAC and may show promise as a therapeutic target in this deadly disease. HMGA1 would be an ideal target as it has little or absent expression in the surrounding normal pancreas, and as such, theoretically should not affect normal cells when HMGA1 is targeted. The fact that HMGA1 expression predicts poor prognosis supports its roles in the malignant phenotype of PDAC cells.

Having established the clinical relevance of HMGA1 in patients with PDAC, we embarked on further studies to elucidate the roles of HMGA1 in the aggressive phenotype of PDAC cells using a series of *in vitro* and *in vivo* experiments (see Chapters 4-7).

Chapter Four: *HMGA1* promotes cellular invasiveness and in vivo metastatic potential in pancreatic adenocarcinoma

4.1 ABSTRACT

Background: HMGA1 proteins are architectural transcription factors that are overexpressed in a range of human malignancies, including pancreatic adenocarcinoma. We hypothesized that HMGA1 expression is a determinant of cellular invasiveness and metastasis in pancreatic cancer.

Methods and results: Stable silencing of HMGA1 in MiaPaCa2 and PANC1 pancreatic adenocarcinoma cells was achieved by transfection of short hairpin RNA-generating vectors. Additionally, stable overexpression of HMGA1 in MiaPaCa2 cells (characterized by low levels of inherent HMGA1 expression) was achieved. HMGA1 silencing resulted in significant reductions in cellular invasiveness through Matrigel, in cellular MMP-9 activity, mRNA levels and gene promoter activity, and in Akt phosphorylation at Ser473. Conversely, forced HMGA1 overexpression resulted in significant increases in cellular invasiveness, in cellular MMP-9 activity, mRNA levels and promoter activity, and in Akt phosphorylation at Ser473. HMGA1 overexpression-induced increases in invasiveness were MMP-9-dependent. The role of PI3-K/Akt in mediating HMGA1-dependent invasiveness was elucidated by specific PI3-K inhibitor (LY294002), constitutively active and dominant negative Akt adenoviral constructs. Akt-dependent modulation of MMP-9 activity contributed significantly to HMGA1 overexpression-induced increases in invasive capacity. Furthermore, HMGA1 silencing resulted in reductions in metastatic potential and tumour growth *in vivo* and in tumoural MMP-9 activity.

Conclusion: Our findings suggest that HMGA1 may be a novel molecular determinant of invasiveness and metastasis, as well as a potential therapeutic target, in pancreatic adenocarcinoma.

4.2 INTRODUCTION

Pancreatic adenocarcinoma is the fourth and sixth leading cause of cancer-related deaths in the United Kingdom and United States respectively (Cancer Research UK, 2007; Jemal et al., 2006). Its biology is characterized by the propensity for early and aggressive invasion and metastasis, such that less than 10% of patients have surgically resectable disease at the time of diagnosis (Sener et al., 1999). Even among patients able to undergo resection of all apparent disease, most are destined to succumb to locally-recurrent and metastatic cancer. Hence, further understanding of the molecular mechanisms underlying pancreatic adenocarcinoma cellular invasion and metastasis is needed, as this information may facilitate the identification of novel molecular targets for the rational therapy of this deadly disease.

In Chapter 3, we have shown that HMGA1 overexpression is highly prevalent in resected pancreata from patients with pancreatic adecarcinoma. Further, expression of HMGA1 in tumours of these patients predicts an adverse clinical outcome. Hence, HMGA1 may be both biologically and clinically relevant in this deadly disease entity. The human *HMGA1* gene, located on chromosomal locus 6p21, encodes two HMGA1 splice variants (HMGA1a and HMGA1b) (Friedmann et al., 1993). These HMGA1 proteins are architectural transcription factors that play a role in both positive and negative transcriptional regulation of human gene expression (Du et al., 1993; Thanos & Maniatis, 1995) (see Section 1.11.3). HMGA1 protein overexpression has been reported to be associated with metastasis in hepatocellular carcinoma (Chuma et al., 2004) and breast cancer (Liu et al., 1999). In addition, HMGA1 has been shown to promote transcriptional up-regulation of genes implicated in promoting metastasis (Reeves et al., 2001). However, the molecular mechanisms through which HMGA1 mediates the metastatic process remain largely unknown.

The purpose of this study was to test the hypothesis that HMGA1 is a molecular regulator of cellular invasiveness and metastasis in pancreatic adenocarcinoma. Using the RNA interference methodology, we sought to examine the roles of HMGA1 in pancreatic adenocarcinoma cellular invasiveness *in vitro* and metastasis *in vivo*. Ectopic overexpression of HMGA1 using HMGA1 cDNA construct was used to elucidate further the effects of modulating HMGA1 expression on cellular invasiveness. We also sought to investigate the mechanism through which HMGA1 mediates this process. Finally, we sought to prove that HMGA1 overexpression mechanistically promoted metastasis in PDAC, and not merely associated with this phenomenon.

4.3 RESULTS

4.3.1 Effect of HMGA1 Gene Silencing on Cellular Invasiveness

Experiments were performed on MiaPaCa2 and PANC1 pancreatic adenocarcinoma cells which are highly invasive cell lines. We analyzed HMGA1 gene expression using Western blot analysis. Cellular invasiveness was quantified using Matrigel-coated Boyden chambers. We used two short-hairpin RNA (shRNA) expressing plasmids with different target sequences to induce HMGA1 gene silencing. MiaPaCa2 and PANC1 cell lines were stably transfected with each of the HMGA1-targeting shRNA expression vectors (shHMGA1-1 and shHMGA1-2). The use of two independent target sequences for RNAi helps to control for any non-specific off-target effects. The shRNA approach was associated with high efficacies in HMGA1 silencing (as confirmed on Western blot analysis, Fig 4.1A). Greater suppression of HMGA1 expression was achieved in MiaPaCa2 cells (up to 90%) compared to PANC1 cells, which have HMGA1 silenced to a lesser degree. To assess cellular invasiveness, we used a modified Boyden chamber assay with 5% FBS as a chemoattractant (see Section 2.8). Silencing of HMGA1 significantly attenuated cellular invasiveness in both MiaPaCa2 and PANC1 cells (Fig 4.1B). For these experiments, controls were cells stably transfected with a vector encoding a non-targeting shRNA.

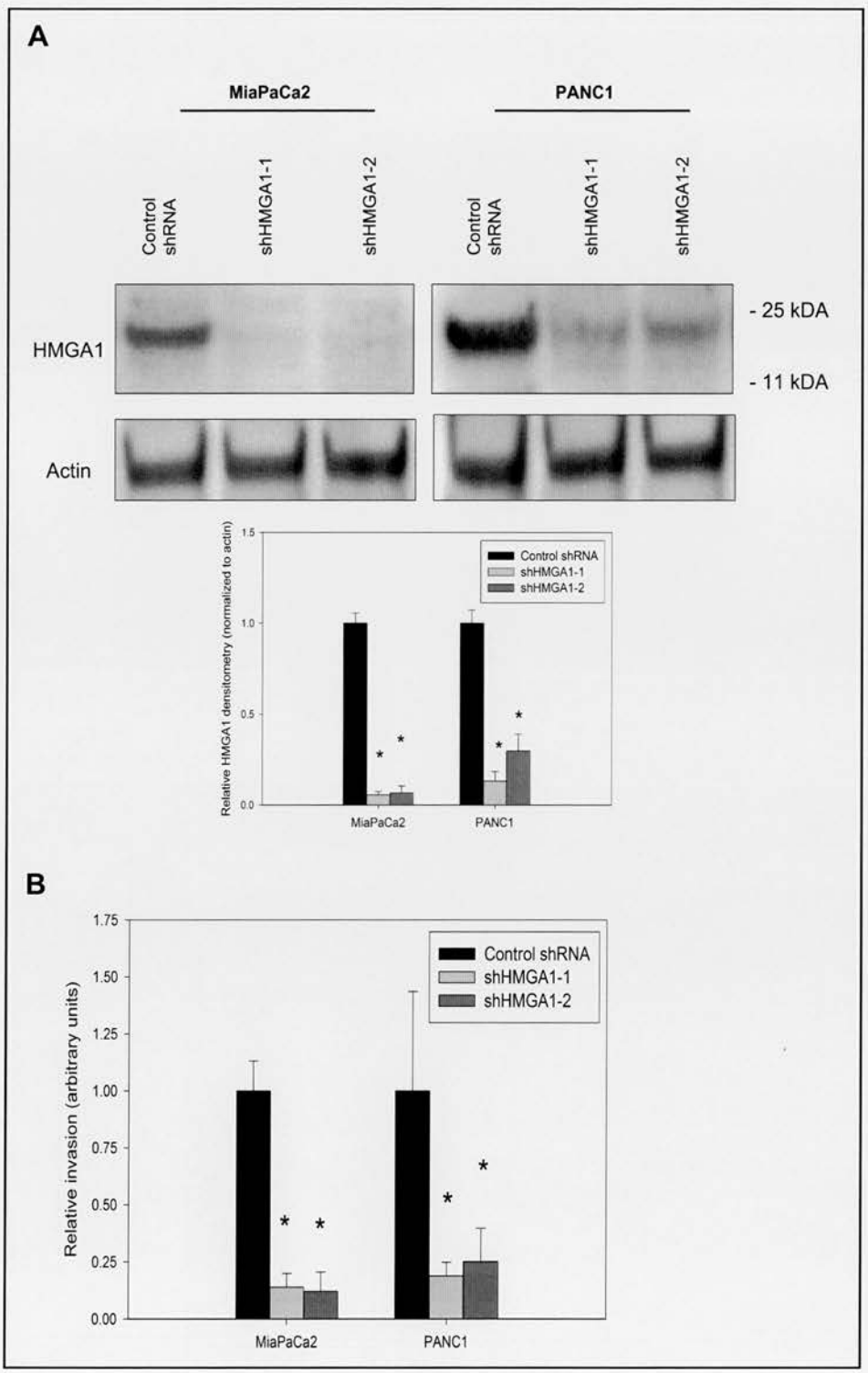


Figure 4.1. *A*, Stable silencing of HMGA1 expression using short hairpin RNA (shRNA) expression vectors with two independent shRNA target sequences (shHMGA1-1 and shHMGA1-2) was confirmed on Western blot analysis. Controls were cells stably transfected with non-targeting, scrambled shRNA. Greater suppression of HMGA1 expression was achieved in MiaPaCa2 cells (up to 90%) compared to PANC1 cells, which have HMGA1 silenced to a lesser degree. * $P<0.05$ versus control shRNA. *B,* Cellular invasiveness was

determined using Matrigel-coated Boyden chamber assays. Stable suppression of HMGA1 expression resulted in significant reductions in cellular invasiveness in both MiaPaCa2 and PANC1 cells. * $P<0.05$ versus control shRNA.

4.3.2 Effect of HMGA1 Overexpression on Cellular Invasiveness

Next, we sought to determine the impact of HMGA1 overexpression on cellular invasiveness. HMGA1 cDNA was cloned into pIRES-puro3 vector as described in Section 2.6. This expression vector was named pIRES-HMGA1. Using MiaPaCa2 cells, which have relatively low levels of inherent HMGA1 expression, we developed the clones pIRES-HMGA1.1 and pIRES-HMGA1.2 which stably overexpress HMGA1 following transfection of pIRES-HMGA1 vector. Methods used in development of HMGA1-overexpressing clones were provided in Section 2.6.2. Overexpression of HMGA1 was confirmed on Western blot analysis (Figure 4.2A). MiaPaCa2 cells stably transfected with empty pIRES-puro3 vector served as controls. HMGA1 expression in parental MiaPaCa2 cells and empty pIRES–puro3 transfectants did not differ (Figure 4.2A). pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited approximately 3- to 3.5 fold increases in cellular invasiveness, compared to control cells (Figure 4.2B).

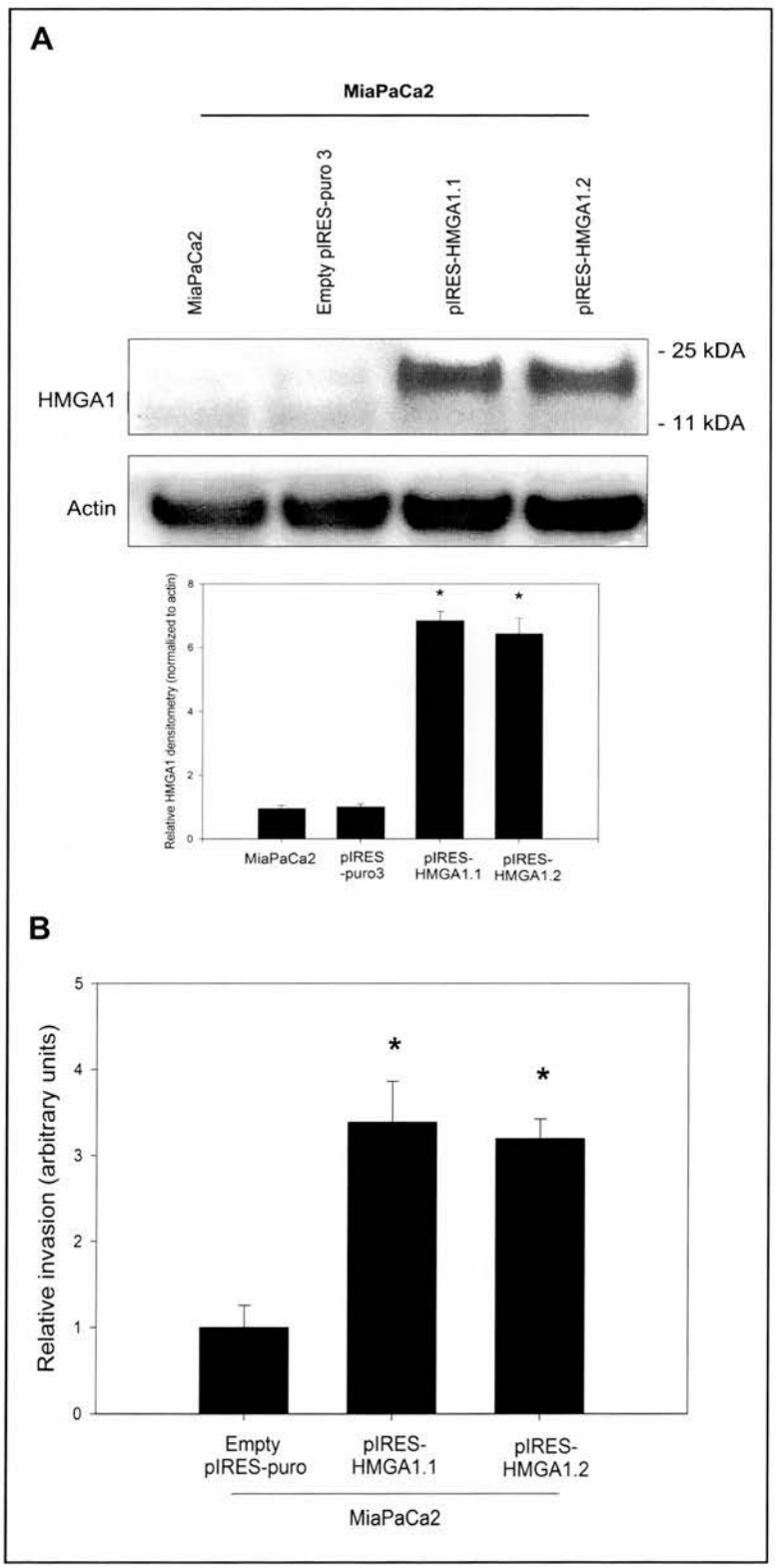


Figure 4.2. *A,* Two clones of MiaPaCa2 cells were confirmed to stably overexpress HMGA1 (pIRES-HMGA1.1 and 1.2) on Western blot analysis. Controls were cells stably transfected with empty pIRES-puro3 vector. * P<0.05 versus empty pIRES-puro3 vector and parental MiaPaCa2 cells. Blots shown are representative of three independent experiments.

Densitometry values are mean (± s.d.). B, Cellular invasiveness was determined using Matrigel-coated Boyden chamber assays. Forced overexpression of HMGA1 in MiaPaCa2 cells (pIRES-HMGA1.1 and 1.2) resulted in approximately three-fold increases in cellular invasive capacity when compared to empty pIRES-puro3 transfectants. * $P<0.05$ versus empty pIRES-puro3 vector. Invasion assays were performed in triplicate. Mean values (± s.d.) from 20 randomly selected fields are shown.

4.3.3 Effects of Modulating HMGA1 Expression on Matrix Metalloproteinase 9 (MMP-9) Activity, mRNA Expression and Promoter Activity

Given the importance of MMP-9 as a mediator of malignant cellular invasiveness and metastasis (Itoh et al., 1999; MacDougall et al., 1999; Sehgal et al., 1998), we hypothesized that MMP-9 is a downstream effector that plays a critical role in HMGA1-dependent cellular invasiveness. Using a sensitive fluorometric MMP-9 activity assay, the total MMP-9 activities from cellular lysates were determined (described in Section 2.9). HMGA1 silencing in MiaPaCa2 cells was found to be associated with a reduction in total MMP-9 activities (Figure 4.3A). In addition, HMGA1 silencing led to reductions in MMP-9 mRNA levels as quantified by real-time quantitative RT-PCR (Figure 4.3B). Conversely, MMP-9 activities and mRNA levels were significantly higher in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones than in controls (Fig 4.3A and B). We further constructed the MMP-9 promoter reporter construct to assess the transcriptional activity of *MMP-9* gene (see Section 2.10 for methods used to construct reporter plasmid). To assess the effect of modulating HMGA1 expression on MMP-9 promoter activity, we performed co-transfection experiments in which MiaPaCa2 cells were transiently transfected with either shHMGA1 or pIRES-HMGA1 vector together with MMP-9 promoter reporter vector. MMP-9 promoter activities were significantly lower with HMGA1 silencing, whereas MMP-9 promoter activity levels were markedly higher with HMGA1 overexpression, compared to controls (Figure 4.3C).

4.3.4 Matrix Metalloproteinase 9 is a Mediator of HMGA1-dependent Increases in Cellular Invasiveness

The contribution of MMP-9 to the increased cellular invasiveness induced by HMGA1 overexpression was determined by performing the invasion assay in the presence of anti-MMP-9 neutralizing antibody. Isotype matched control IgG was used in control experiments. MMP-9 immunoneutralization reduced the cellular invasiveness of MiaPaCa2 pIRES-HMGA1.1 and pIRES-HMGA1.2 clones by approximately 70% (Figure 4.3D). This suggests that HMGA1-induced increases in cellular invasiveness are dependent on MMP-9 expression and activity.

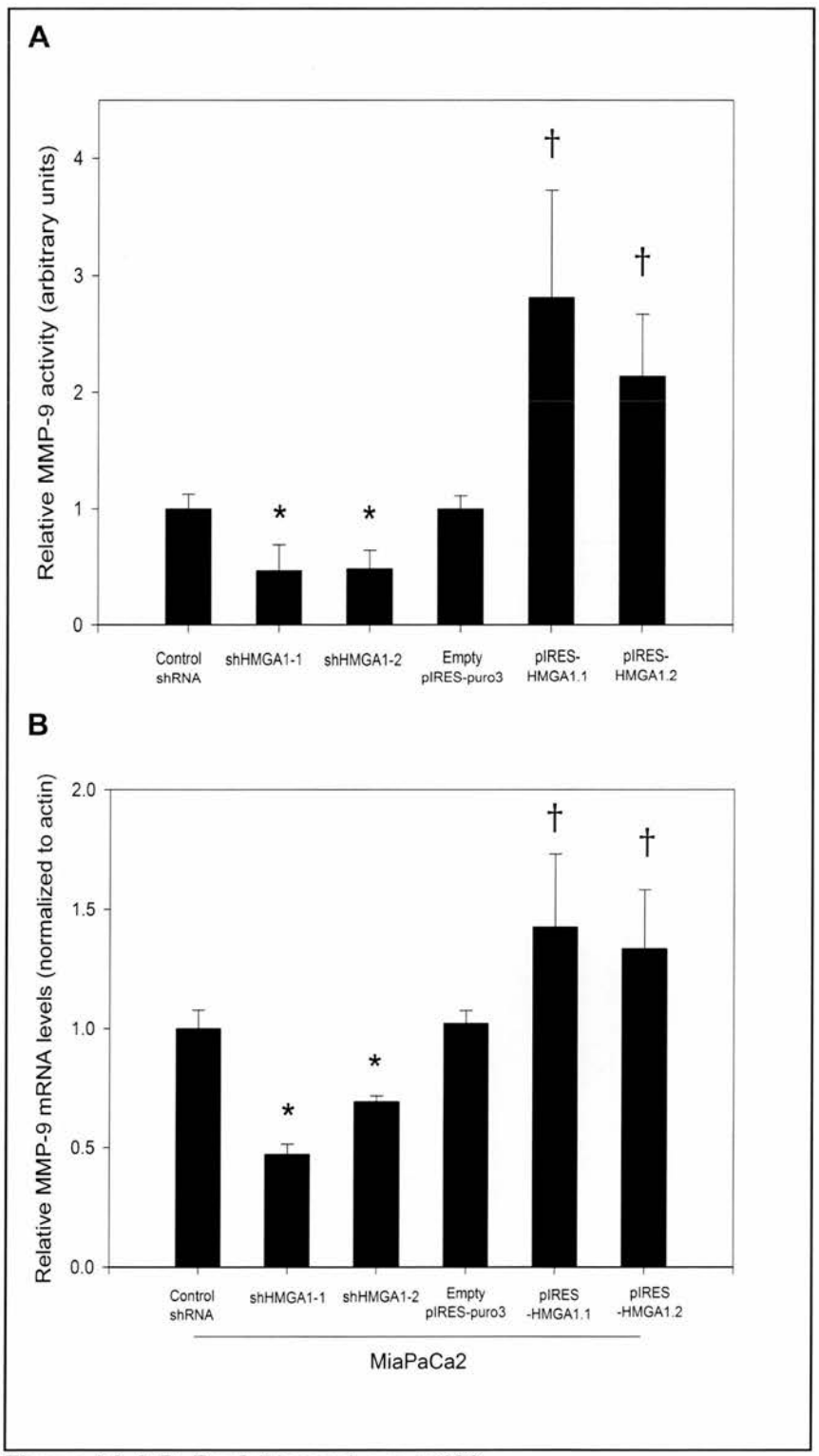


Figure 4.3 A-B. For full legend, see p 133.

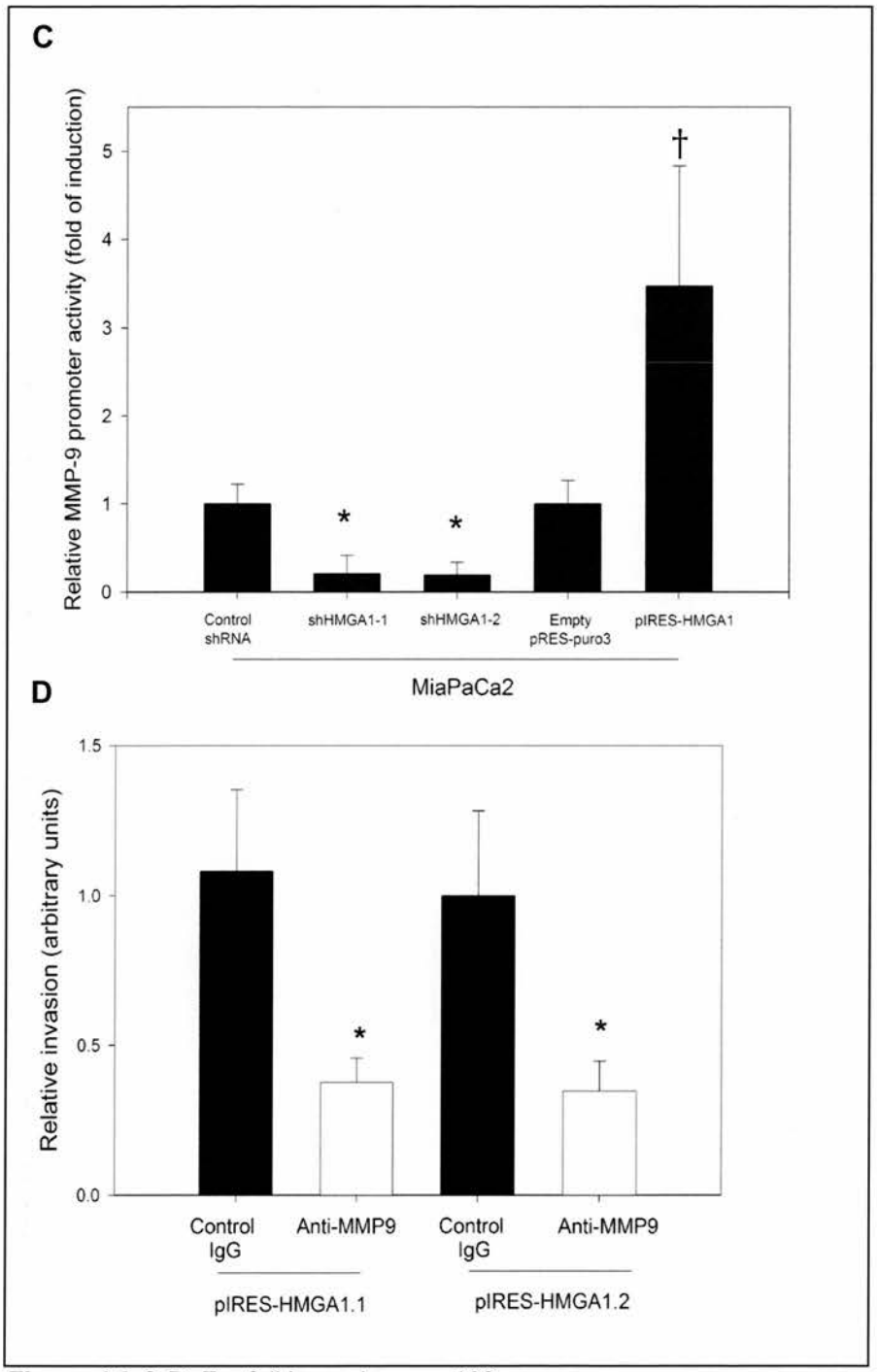


Figure 4.3 C-D. For full legend, see p 133.

Legend

Figure 4.3. HMGA1 expression positively regulates matrix metalloproteinase-9 (MMP-9) activities, mRNA levels and promoter activities. ***A***, Cellular MMP-9 activities were quantitated using a fluorometric MMP-9 activity assay. Targeted suppression of HMGA1 expression (shHMGA1-1 and shHMGA1-2) resulted in significant reductions in MMP-9 activity in MiaPaCa2 cells while forced overexpression of HMGA1 led to increased cellular MMP-9 activity. Both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited approximately 2- to 2.5-fold increases in MMP-9 activity. * $P<0.05$ versus control shRNA and † $P<0.05$ versus empty pIRES-puro3 transfectants. ***B***, HMGA1 modulates MMP-9 mRNA levels. On quantitative real-time RT-PCR, MMP-9 levels were significantly lower in shHMGA1-1 and shHMGA1-2 transfectants compared to control shRNA transfectants. Overexpression of HMGA1 led to increases in MMP-9 mRNA levels. * $P<0.05$ versus control shRNA and † $P<0.05$ versus empty pIRES-puro3 transfectants. **C**, The effects of HMGA1 silencing and overexpression on MMP-9 gene promoter activities in MiaPaCa2 cells were determined using transient transfection and luciferase reporter assays. Co-transfection experiments were performed. MiaPaCa2 cells were transiently transfected with shRNA (shHMGA1-1, shHMGA1-2 or control shRNA) or overexpression vectors (pIRES-HMGA1 or empty pIRES-puro3 control) together with the reporter plasmids. Reporter plasmids included pGL4e (empty firefly luciferase vector) or pGL4-MMP9, containing the full-length MMP9 promoter and pRL vector containing a renilla luciferase gene. Forty-eight hours following transfection, luciferase activity in cell lysates was assayed. Renilla luciferase activities were used to normalise results for transfection efficiencies. Consistent with MMP-9 activity and mRNA studies, targeted suppression of HMGA1 expression using shRNA resulted in a significant reduction in MMP-9 promoter activities while forced overexpression of HMGA1 in MiaPaCa2 cells led to significant increases in MMP-9 promoter activities. * $P<0.05$ versus control shRNA and † $P<0.05$ versus empty pIRES-puro3 vectors. ***D***, The contribution of MMP-9 to the increased cellular invasiveness induced by HMGA1 overexpression was determined by performing the invasion assay in the presence of anti-MMP-9 neutralising antibody. MMP-9 immunoneutralisation reduced the cellular invasiveness of pIRES-HMGA1.1 and pIRES-HMGA1.2 clones by approximately 70%.

4.3.5 Effects of HMGA1 Modulation on Akt Activation

Activation of the serine/threonine kinase Akt is common in pancreatic adenocarcinoma (Semba et al., 2003) and is an important determinant of malignant cellular invasiveness (Kim et al., 2001a). The phosphatidylinositol-3 kinase (PI-3K)/Akt signaling pathway has previously been reported to be

important in the regulation of MMP-9 expression (Ellerbroek et al., 2001; Lu & Wahl, 2005; O. Charoenrat et al., 2004). Given our observations that HMGA1 expression regulates MMP-9 activity via transcriptional activation, we hypothesized that HMGA1 modulates Akt activation. We found that HMGA1 silencing results in a reduction in Akt phosphorylation at Serine 473 (Figure 4.4A), whereas HMGA1 overexpression results in increased levels of phospho-Akt (Ser473) (Figure 4.4B). The antibody used for this study was the anti-phospho Akt (ser473) purchased from San Cruz Biotechnology (San Cruz, CA, USA). Of note, the upper band corresponds to a non-specific band as the phospho-Akt band (lower band) remained constant when immunoblotted with a different phospho-specific Akt antibody (Cell Signaling Technology, Danvers, MA, USA) (see Figure 5.3). For further confirmation, phospho-Akt (ser 473) was immunoprecipitated using anti-phospho-Akt antibody and this yielded a same band as the lower band (data not shown). Modulating HMGA1 expression had no effect on total Akt levels.

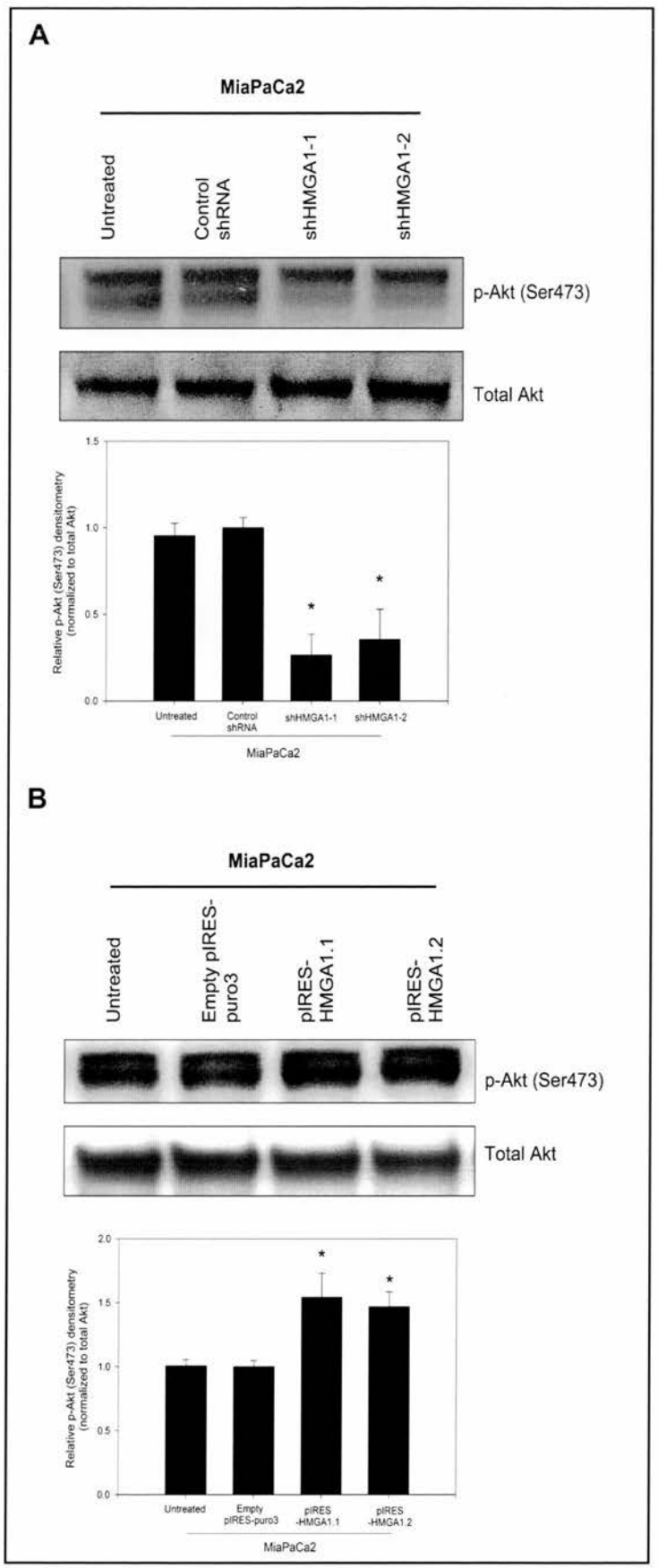


Figure 4.4. The effects of HMGA1 modulation on Akt phosphorylation in MiaPaCa2 cells were confirmed on Western blot analysis using a phosphospecific antibody. *A*, In MiaPaCa2 cells, stable silencing of HMGA1 resulted in a decreased level of phosphorylation of Akt at Ser 473, a marker of Akt activation. * $P<0.05$ versus MiaPaCa2 cells and control shRNA transfectants. *B*, Overexpression of HMGA1 led to increased activation of Akt, as evident from increased levels of phospho-Akt. There was no difference in the level of expression of total Akt with suppression or overexpression of HMGA1. * $P<0.05$ versus MiaPaCa2 cells and empty pIRES-puro3 transfectants. Blots shown are representative of three independent experiments. Densitometry values are mean (± s.d.).

4.3.6 HMGA1-induced Cellular Invasiveness and MMP-9 activity is PI-3K/Akt-dependent

Given our findings that HMGA1 modulates Akt activation, we tested whether cellular invasiveness mediated by HMGA1 is dependent on PI-3K/Akt signaling. First, we determined if the invasive phenotype in MiaPaCa2 cells in which HMGA1 had been stably silenced could be rescued using constitutively active Akt. MiaPaCa2 stable transfectants shHMGA1-1 and shHMGA1-2 were infected with adenovirus expressing myristoylated Akt (Ad-myr-Akt) and infection efficiency was monitored by immunoblotting for HA (hemagglutinin)-tagged Akt. Constitutively active Akt was able to rescue the invasive phenotype in shHMGA1-1 and shHMGA1-2 transfectants resulting in an increased invasiveness compared to cells infected with control adenovirus (Figure 4.5A). Next, given that PI-3K is an upstream regulator of Akt, we assessed the effects of PI-3K inhibition on cellular invasiveness in MiaPaCa2 cells overexpressing HMGA1 (pIRES-HMGA1.1 and pIRES-HMGA1.2). Inhibition of PI-3K activity using the PI-3K inhibitor LY294002 resulted in dose-dependent reductions in cellular invasiveness in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Figure 4.5B). Consistent with this finding, infection with adenovirus expressing dominant negative Akt resulted in attenuation of HMGA1-induced invasiveness in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Figure 4.6A). Importantly, dominant negative Akt also induced reductions in cellular MMP-9 activity in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones, indicating that HMGA1-induced MMP-9-dependent cellular invasiveness is PI-3K/Akt-dependent (Figure 4.6B).

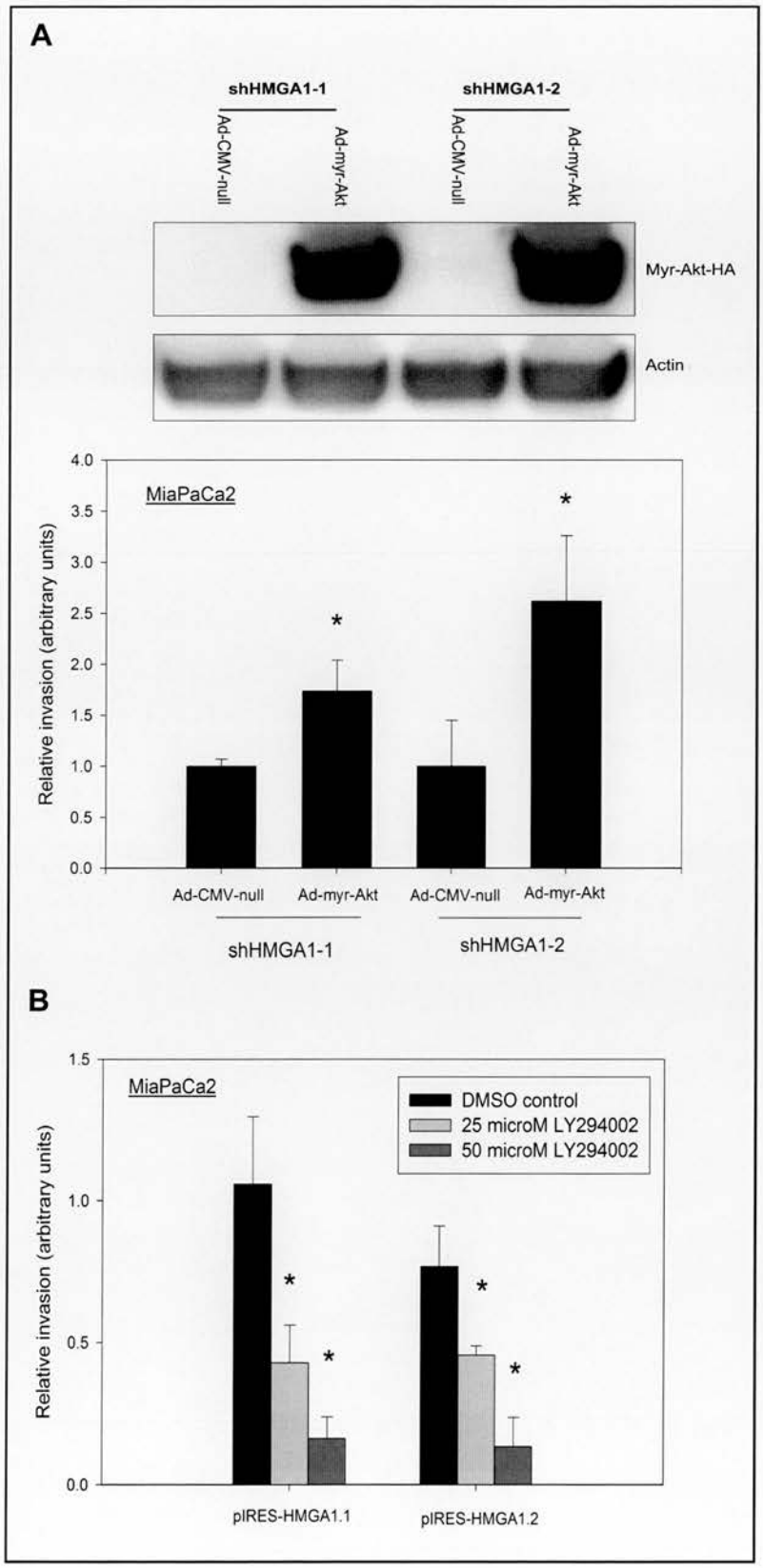


Figure 4.5. A, Contribution of Akt to HMGA1-mediated invasiveness was assessed by transductions of MiaPaCa2 shHMGA1-1 and shHMGA1-2 transfectants with adenovirus expressing constitutively active Akt (Ad-myr-Akt). Constitutively active Akt was able to rescue the invasive phenotype in the MiaPaCa2 cells with HMGA1 silencing. Cell lysates were immunoblotted with anti-HA to detect expression of HA-tagged myristoylated Akt. * $P<0.05$ versus MiaPaCa2 transduced with control adenovirus (Ad-CMV-null). *B*, Inhibition of PI-3K with LY294002 attenuated the HMGA1 induced increases in invasiveness in pIRES-HMGA1.1 and 1.2 clones. The effects of PI3-K were dose-dependent (25μM and 50μM). * $P<0.05$ versus DMSO controls.

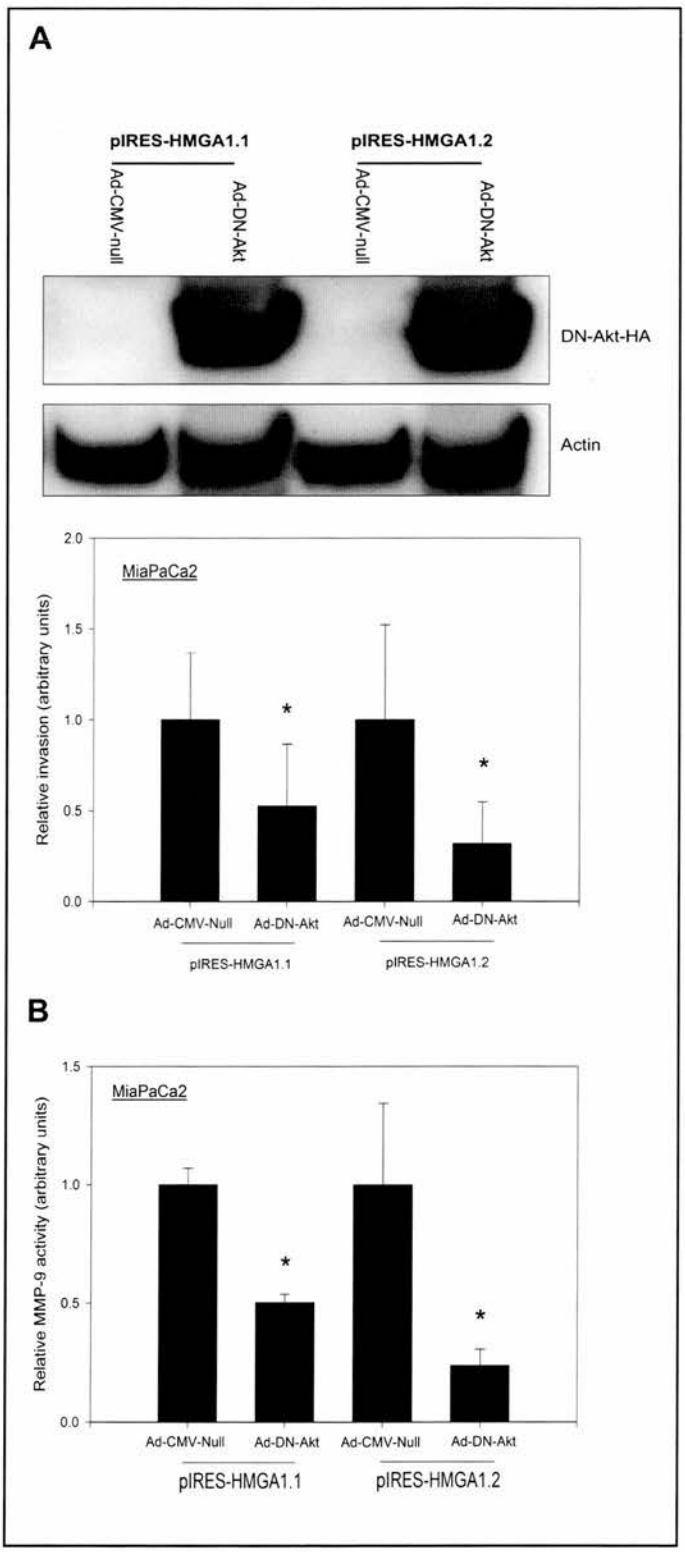


Figure 4.6. *A*, Dominant negative Akt attenuates HMGA1-induced invasiveness and MMP-9 activity. MiaPaCa2 pIRES-HMGA1.1 and 1.2 clones were transduced with adenovirus expressing dominant negative Akt (Ad-DN-Akt). *B*, Dominant negative Akt resulted in reductions in invasiveness and in MMP-9 activities in both HMGA1-overexpresing clones, compared to control adenovirus. Cell lysates were immunoblotted with anti-HA to detect expression of HA-tagged DN Akt. * $P<0.05$ versus control adenovirus (Ad-CMV-null).

4.3.7 Modulation of HMGA1 Expression Has No Impact on Cellular Proliferation in Monolayer Culture

The effects of modulating HMGA1 expression on cellular proliferation in monolayer culture were determined. Using the MTS assay, we observed no impact on cellular proliferation in low (2% FBS) and high (10% FBS) serum conditions with either HMGA1 gene silencing or overexpression (Figure 4.7).

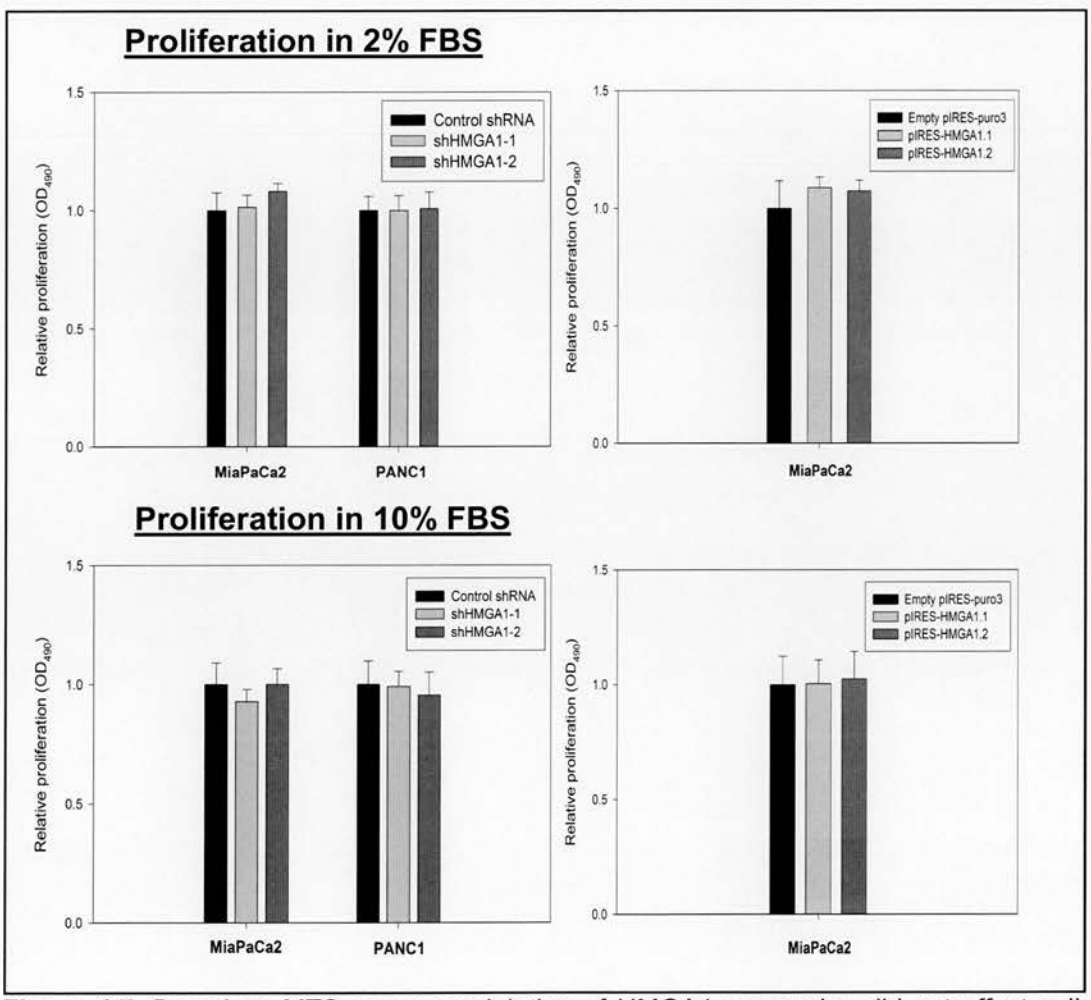


Figure 4.7. Based on MTS assay, modulation of HMGA1 expression did not affect cellular proliferation in monolayer culture. Experiments were performed at low (2% FBS) and high (10%) conditions.

4.3.8 Effects of HMGA1 Modulation on Phosphorylation of ERK and mTOR

Previous studies have shown that HMGA1 modulates ERK activation (Treff et al., 2004). In our study, HMGA1 silencing had no impact on ERK phosphorylation, whereas HMGA1 overexpression resulted in increased ERK phosphorylation with no effects on the total ERK levels (Figures 4.8A and B). Given that mTOR is a well-known downstream mediator of the PI-3K/Akt pathway (Nave et al., 1999; Peterson et al., 2000), we sought to determine the effects of HMGA1 modulation of mTOR phosphorylation. HMGA1 silencing resulted in reductions in mTOR phosphorylation at Ser2448, while HMGA1 overexpresssion led to increases in mTOR phosphorylation (Figures 4.8C and D). Modulation of HMGA1 expression had no impact on levels of total mTOR expression.

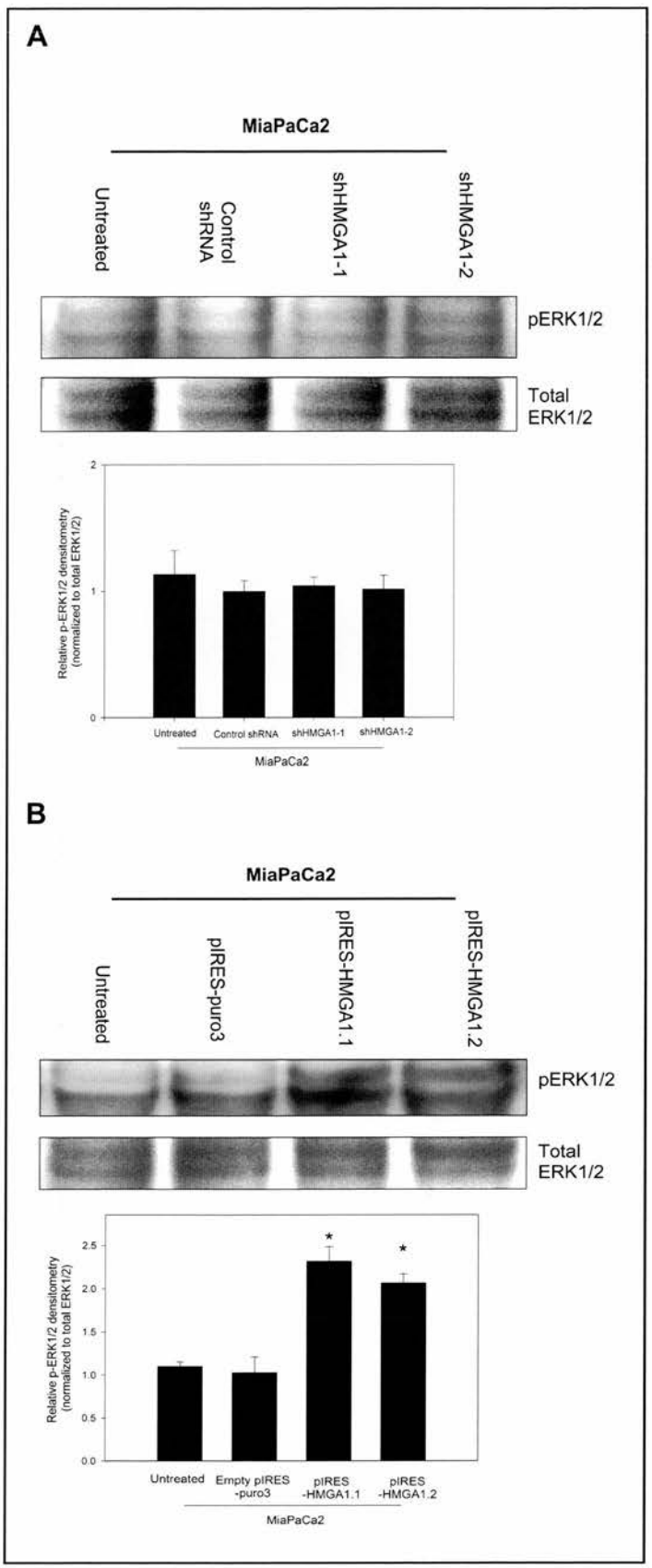


Figure 4.8. The effects of HMGA1 modulation on ERK phosphorylation in MiaPaCa2 cells confirmed on Western blot analysis using a phosphospecific antibody. *A-B*, Although HMGA1 silencing did not alter the levels of ERK phosphorylation, overexpresssion of HMGA1 increased ERK phosphorylation. There were no differences in the levels of total ERK.

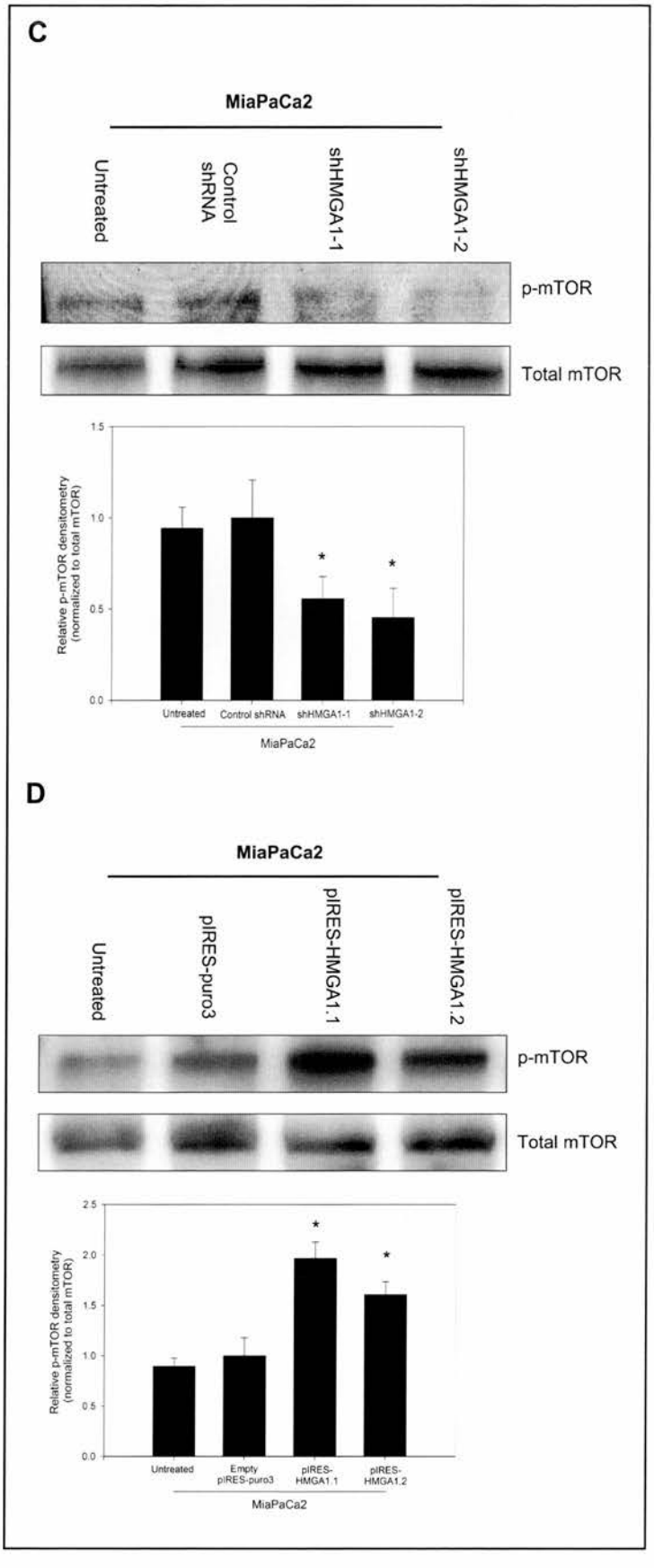


Figure 4.8. *C-D*, Targeted suppression of HMGA1 expression resulted in reductions in phosphorylation of mTOR at Ser2448 in both shHMGA1-1 and shHMGA1-2 transfectants while overexpression of HMGA1 led to increases in phospho-mTOR levels. There was no difference in total mTOR levels among groups. * $P<0.05$ versus control shRNA or empty pIRES-puro3 transfectants, as appropriate.

4.3.9 HMGA1 Silencing Suppresses In Vivo Metastatic Potential of Pancreatic Cancer Cells and Reduces Tumoural Growth and MMP-9 Activity

Given these *in vitro* findings, we sought to determine the impact of modulating HMGA1 expression on metastatic potential *in vivo*. Four weeks following surgical orthotopic implantation of MiaPaCa2 stable transfectants expressing control shRNA (n=10) or shHMGA1 (n=10), necropsy was performed. Methodology of orthotopic pancreatic adenocarcinoma mouse model is provided in Section 2.17.1 and xenograft immunohistochemistry is described in Section 2.18. All mice in the control shRNA group were found to have histologically-confirmed liver metastases (Figure 4.9), and six of these mice were found to have gross ascites. In the shHMGA1 group, only one mouse developed metastases (Table 4.1, $P<0.05$), and none of them had ascites. No lung metastases were detected in either group. There were no apparent differences in the histological appearance of haematoxylin and eosin-stained sections of xenografts harvested from animals in the shHMGA1 and control groups (Figure 4.10A). Stable silencing of tumoural HMGA1 expression in the shHMGA1 group *in vivo* was confirmed by immunohistochemistry (Figure 4.9A) and Western blotting (Figure 4.10B). Additionally, HMGA1 silencing was associated with significant reductions in tumour growth in the explanted tumours compared to the control group (Figure 4.10C). To confirm our in vitro results, lysates from explanted tumours were analysed for total MMP-9 activity using the fluorometric MMP-9 activity assay. We found that there were significant reductions in MMP-9 activity in tumours with HMGA1 knockdown, implying that HMGA1 silencing could alter tumour behaviour *in vivo* (Fig 4.10C and D). We speculated that *in vivo* HMGA1 silencing reduced the MMP-9 dependent cellular invasiveness and finally, metastasis.

Figure 4.9. Macroscopic evidence of liver metastases at laparotomy of a mouse four weeks following orthotopic implantation of MiaPaCa2 cells with control shRNA. Liver metastases were shown in the red square.

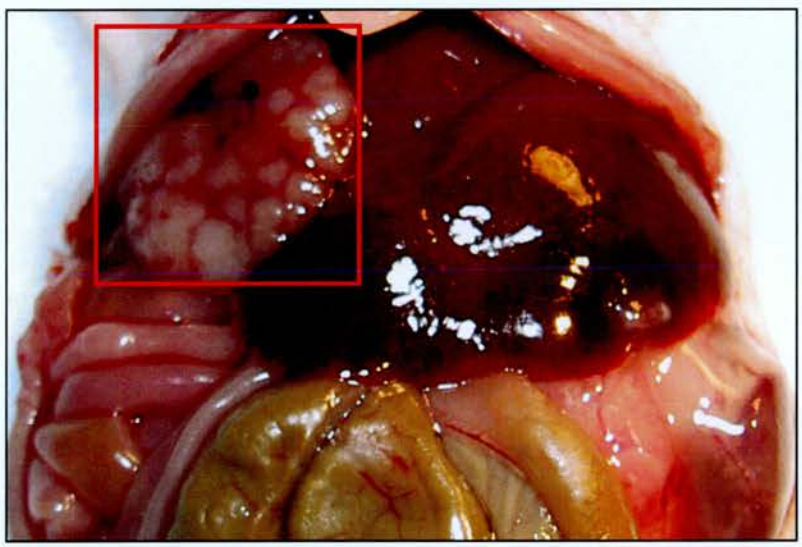


Table 4.1. HMGA1 gene silencing by RNA interference suppresses the metastatic potential of pancreatic adenocarcinoma cells *in vivo*

	Control shRNA (n = 10)	shHMGA1 (n=10)
Median no. of metastases (range)	6 (1-20)	**0* (0-3)**
Mice with metastases	100%	**10%***

At 4 weeks following orthotopic implantation of $1X10^6$ MiaPaCa2 cells stably expressing control or shHMGA1 constructs, necropsy was performed. Liver metastases were counted and confirmed histologically. HMGA1 silencing significantly inhibited metastasis in this nude mouse model. *P<0.05 vs. control shRNA group.

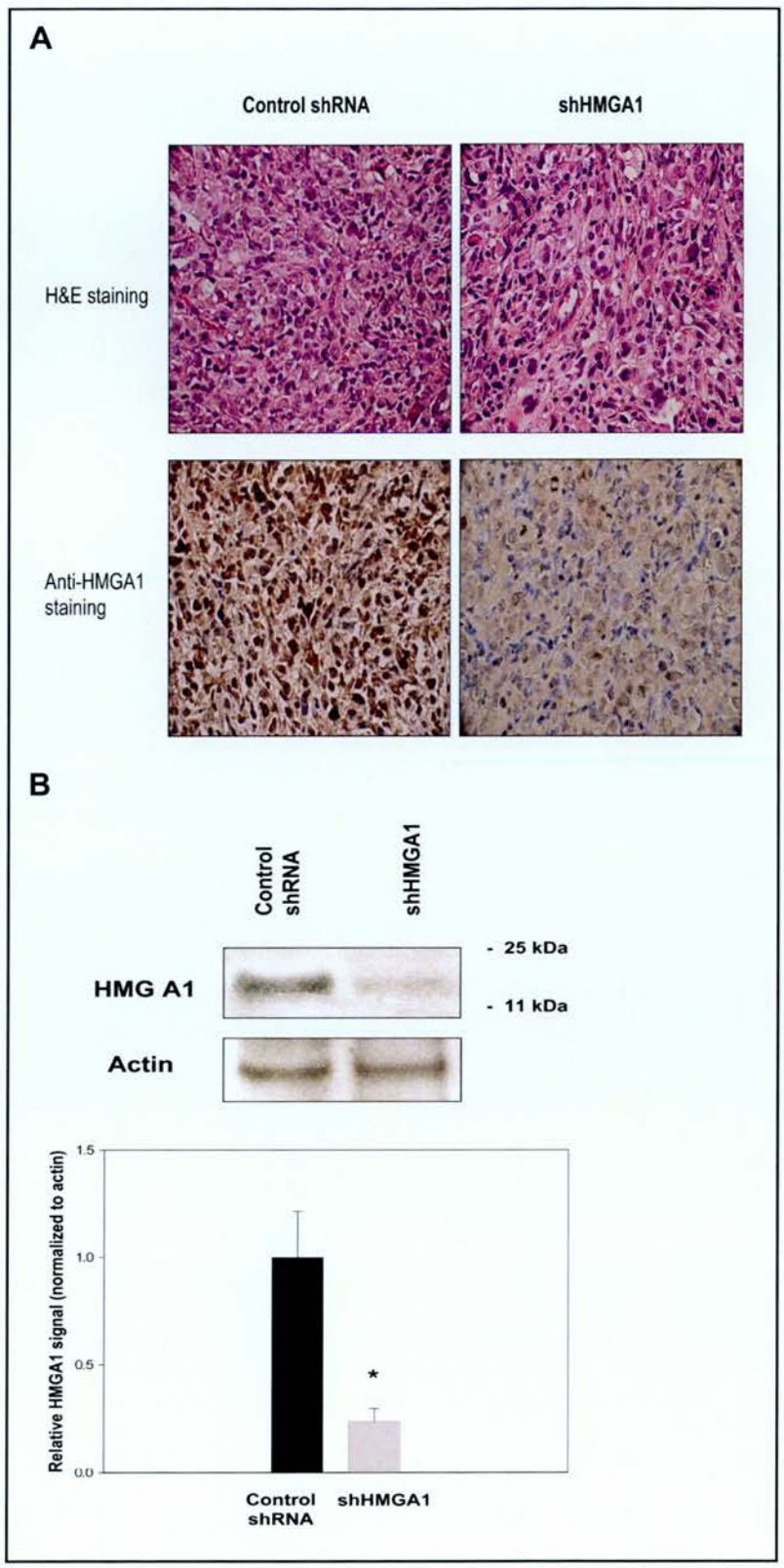


Figure 4.10. MiaPaCa2 stable transfectant cells expressing the control shRNA or shHMGA1 (shHMGA1-1) were orthotopically implanted into the pancreata of nude mice. Four weeks following implantation, necropsy was performed and the primary tumours were explanted. *A*, Histological appearance of primary tumours was examined at 400X magnification following H&E staining of tumour sections. There was no apparent morphological difference in tumours harvested from the two groups of animals. However, sections stained with anti-HMGA1 antibody revealed that tumours from shHMGA1 group exhibited reductions in nuclear staining for HMGA1. *B*, Stable HMGA1 suppression in lysates of shHMGA1- derived tumours was further confirmed by Western blot.

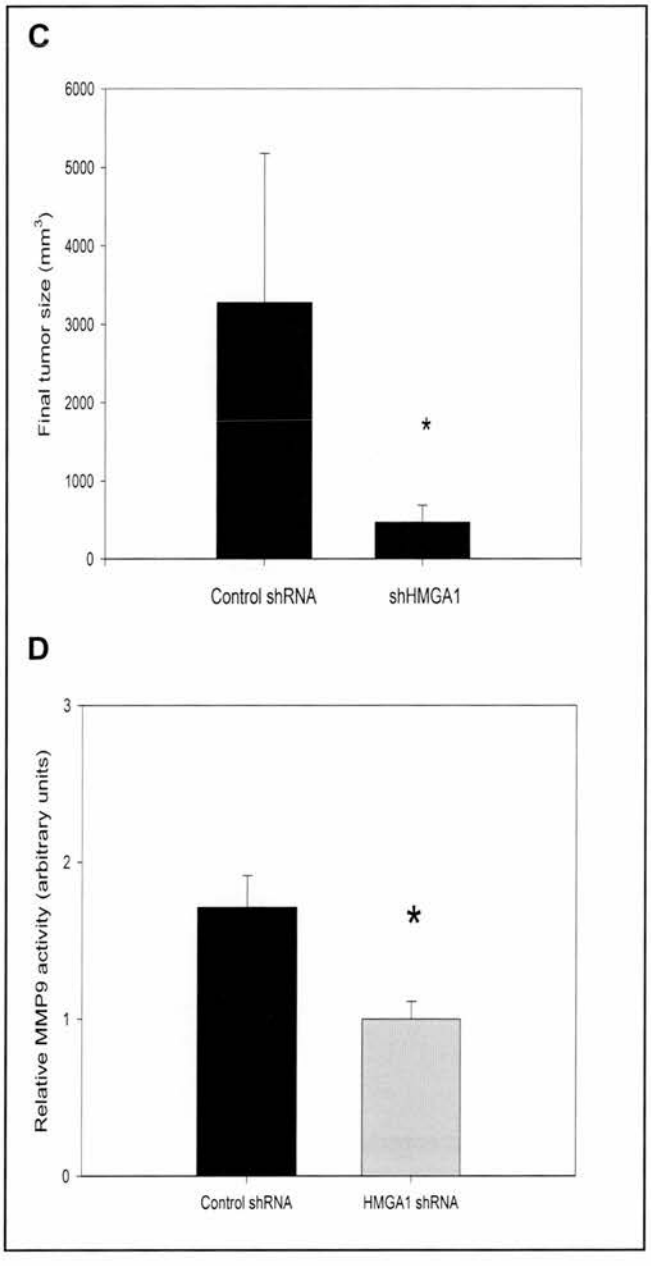


Figure 4.10. *C*, Final volume of primary tumours harvested from shHMGA1 group was markedly reduced compared to those of the control shRNA group. *D*, MMP-9 activities in lysates obtained from HMGA1 shRNA transfectant-derived tumours were significantly lower than those of control shRNA transfectant-derived tumours. Mean values (± s.d.). * P <0.05 vs. control shRNA transfectant-derived tumours.

4.4 DISCUSSION

Pancreatic adenocarcinoma is among the most aggressive of human malignancies. The prognosis associated with this cancer remains dismal, despite the considerable advances in medical and surgical management of patients diagnosed with pancreatic cancer. There is an urgent need to identify new therapeutic approaches. This study was designed to determine the role of HMGA1 in pancreatic adenocarcinoma cellular invasiveness and metastasis. We have demonstrated that HMGA1 overexpression promotes cellular invasiveness and that specific suppression of HMGA1 expression inhibits cellular invasiveness *in vitro* and metastasis *in vivo*. HMGA1-induced cellular invasiveness is in part due to PI3-K/Akt-dependent modulation of MMP-9 activity. Together, these findings suggest that HMGA1 architectural transcriptional factors represent a molecular determinant of cellular invasiveness and a potential therapeutic target in pancreatic adenocarcinoma.

HMGA1 previously has been reported to be associated with malignant cellular behaviour in a range of human cancers. HMGA1 has been shown to confer the ability of non-tumourigenic breast epithelial cells to grow under anchorage-independent conditions (Reeves et al., 2001). Antisense oligonucleotide-mediated suppression of HMGA1 expression has been reported to inhibit neoplastic transformation in breast cancer (Dolde et al., 2002) and in Burkitt's lymphoma cells (Wood et al., 2000b). Although there is extensive correlative evidence suggesting a role for HMGA1 in tumour metastasis (Abe et al., 2003; Balcerczak et al., 2003; Chang et al., 2005), there have been few studies showing a direct functional link between HMGA1 expression and invasion and metastasis (Liu et al., 1999; Reeves et al., 2001; Wood et al., 2000a).

Our study is the first to demonstrate a role for HMGA1 in pancreatic adenocarcinoma cellular invasiveness and metastasis, and provides evidence that HMGA1 expression mediates cellular invasiveness through a PI3-K/Akt-

MMP-9-dependent pathway. While MMP-9 is unlikely to be the sole effector of HMGA1-dependent invasiveness, this pathway represents a potential mechanism that may contribute to the decrease in cellular invasiveness induced by HMGA1 gene silencing. Our finding that PI-3K/Akt signaling mediates regulatory actions of HMGA1 is novel. Our finding that HMGA1 promotes mTOR activation provides further support for the relationship between HMGA1 and PI-3K/Akt signaling, as mTOR is a downstream target of this pathway (Nave et al., 1999; Peterson et al., 2000).

Our finding that HMGA1 overexpression results in increased ERK phosphorylation is consistent with findings of previously reported studies suggesting that HMGA1 is able to positively regulate the Ras-extracellular signal-related kinase (Ras/ERK) mitogenic signaling pathway (Treff et al., 2004). The Ras/ERK signaling pathway is intrinsically linked to PI-3K/Akt pathways. PI-3K/Akt has been shown to be downstream of Ras/ERK signaling pathway (Gupta et al., 2001; McKenna et al., 2003), and Ras can also directly activate the PI-3K/Akt signaling pathway (Rodriguez-Viciana et al., 1994). Alternatively, HMGA1 has been shown to transcriptionally regulate the human insulin receptor gene (Brunetti et al., 2001; Foti et al., 2005), and given that PI-3K/Akt are downstream mediators of insulin signaling (Hara et al., 1994), it is not surprising that HMGA1 expression affects PI-3K/Akt signalling. Both Ras/ERK and PI-3K/Akt signaling pathways have been found to be critical in mediating cellular invasion in pancreatic cancer cells (Veit et al., 2004).

Our observation that HMGA1 silencing suppresses Akt activity is significant, as Akt is now recognized as an important mediator of malignant cellular behaviour, including the capacity for resisting apoptotic stimuli, in pancreatic adenocarcinoma (Maitra & Hruban, 2005; Shah et al., 2001; Takeda et al., 2004). Trapasso and colleagues have reported that antisense-mediated suppression of HMGA1 expression results in an apoptotic response in three pancreatic cancer cell lines (Trapasso et al., 2004). Our findings that HMGA1 positively regulates PI3-K/Akt signaling provides a possible mechanism

through which HMGA1 suppression promotes apoptosis. Unlike Trapasso *et al*, we found that modulating HMGA1 expression has no impact on cellular proliferation in 2-dimensional monolayer culture. However, our data indicate that HMGA1 silencing does inhibit tumour growth *in vivo*.

Our study provides support to the hypothesis that the HMGA1 proteins are potential therapeutic targets for inhibiting the activation of the PI-3K/Akt pathway in cancer cells. From a therapeutic standpoint, targeting HMGA1 is attractive in that it is overexpressed in a range of human malignancies. HMGA1 expression is absent or present at only very low levels in normal adult tissues (Chiappetta et al., 1996). As such, targeting HMGA1 may have little or no effect on non-cancerous tissues. Given our finding that RNA interference-mediated HMGA1 silencing inhibits invasive and metastatic potential, HMGA1 represents a rational molecular therapeutic target. The feasibility of *in vivo* gene silencing induced by the delivery of therapeutic siRNA has already been demonstrated (Zimmermann et al., 2006). Technological advances, such as the development of improved delivery systems for siRNAs, will facilitate this approach.

In summary, our findings suggest that HMGA1 promotes pancreatic cancer cellular invasive and metastatic potential. Our findings also indicate that HMGA1 represents a potential therapeutic target for strategies designed to inhibit the progression of pancreatic cancer.

Chapter Five: Overexpression of HMGA1 promotes anoikis resistance through constitutive Akt activation

5.1 ABSTRACT

Background: HMGA1 proteins are architectural transcription factors that are overexpressed by pancreatic adenocarcinomas. Roles of HMGA1 in mediating the malignant phenotype of this cancer are poorly understood. We tested the hypothesis that overexpression of HMGA1 promotes resistance to anoikis (apoptosis induced by anchorage deprivation) in pancreatic cancer cells.

Methods and results: HMGA1 cDNA was stably transfected into MiaPaCa2 human pancreatic adenocarcinoma cells (which have low baseline expression levels of HMGA1). Cells were grown in suspension on PolyHEMA-coated plates, and their susceptibility to anoikis was assayed using flow cytometry. Overexpression of HMGA1 was associated with marked reductions in susceptibility to anoikis in concert with increases in Akt phosphorylation (Ser473) and in Akt kinase activity and with reductions in caspase 3 activation. Inhibition of PI3-kinase/Akt pathway with either the small molecule inhibitor LY294002 or dominant negative Akt resulted in reversal of anoikis resistance induced by HMGA1 overexpression. Further, RNA interference-mediated HMGA1 silencing in MiaPaCa2 and BxPC3 (a human pancreatic adenocarcinoma cell line with high baseline levels of HMGA1 expression) cells resulted in significant increases in susceptibility to anoikis.

Conclusions: Our findings suggest HMGA1 promotes anoikis resistance through a PI3-kinase/Akt-dependent mechanism. Given the putative associations between anoikis resistance and metastatic potential, HMGA1 represents a potential therapeutic target in pancreatic adenocarcinoma.

5.2 BACKGROUND

Pancreatic adenocarcinoma is among the deadliest of all human cancers, with 5-year survival rates averaging less than 5% (Jemal et al., 2006). The propensity for pancreatic adenocarcinoma cells to metastasize early in the course of disease progression makes this cancer particularly refractory to standard therapies. Characterizing the underlying mechanisms mediating metastatic dissemination in pancreatic adenocarcinoma may reveal novel targets for inhibiting this process, which is so aggressive in this cancer. In Chapter 4, we have demonstrated that HMGA1 is a molecular determinant of cellular invasiveness and metastatic potential in PDAC cells. However, cellular invasion is only a part of the complex and inter-related processes involved in metastasis. For cancer cells to transfer from a primary tumour focus to distant sites will involve invasion of normal surrounding tissues, penetration of lymphatic and vascular channels, survival of cancer cells within the lymphatic or circulatory systems followed by extravasation from the walls of these lymphovascular channels in distant organs and establishment of a viable metastatic focus.

Anoikis, derived from a Greek word meaning 'homelessness', was used in describing the observation that depriving cells from attachment to matrix triggers apoptosis in these cells (Frisch & Francis, 1994). Increasingly, metastasis is conceptualised to be a multi-step process facilitated by the evolution of anoikis resistant subsets of cancer cells that are capable of surviving in the blood stream or lymphatic system during dissemination after they detach from the primary tumour and its stroma. Indeed, numerous studies suggest a close correlation between signaling events mediating cellular anoikis resistance *in vitro* and those mediating metastatic potential *in vivo* (Berezovskaya et al., 2005; Douma et al., 2004; Yawata et al., 1998; Zhu et al., 2001).

Given that we have previously shown that HMGA1 plays an important role in the metastatic potential of PDAC cells, we examined in this study if HMGA1 had an impact on the process of anoikis resistance in cancer cells. This line of investigation will help to clarify the potential role and underlying biology for HMGA1 in the context of cancer progression, especially in the metastatic process, which hitherto remains poorly understood. The purpose of this study was to test the hypothesis that HMGA1 promoted anoikis resistance in pancreatic adenocarcinoma. We further assessed the roles of PI3-K/Akt pathway and caspase activation as possible mediators of this process.

5.3 RESULTS

5.3.1 HMGA1 overexpression promotes anoikis resistance in MiaPaCa2 pancreatic adenocarcinoma cells

Stable overexpression of HMGA1 was achieved in MiaPaCa2 cells, which have low inherent expression levels of HMGA1. Two stable HMGA1-overexpressing clones were selected and named pIRES-HMGA1.1 and pIRES-HMGA1.2. HMGA1 overexpression was confirmed on Western blot analysis (Figure 5.1). Levels of HMGA1 overexpression in pIRES-HMGA1.1 and pIRES-HMGA1.2 were 4- and 3.5-fold higher than in control cells, respectively. There was no difference in the levels of HMGA1 expression between the empty pIRES-puro3 transfectants and parental MiaPaCa2 cells.

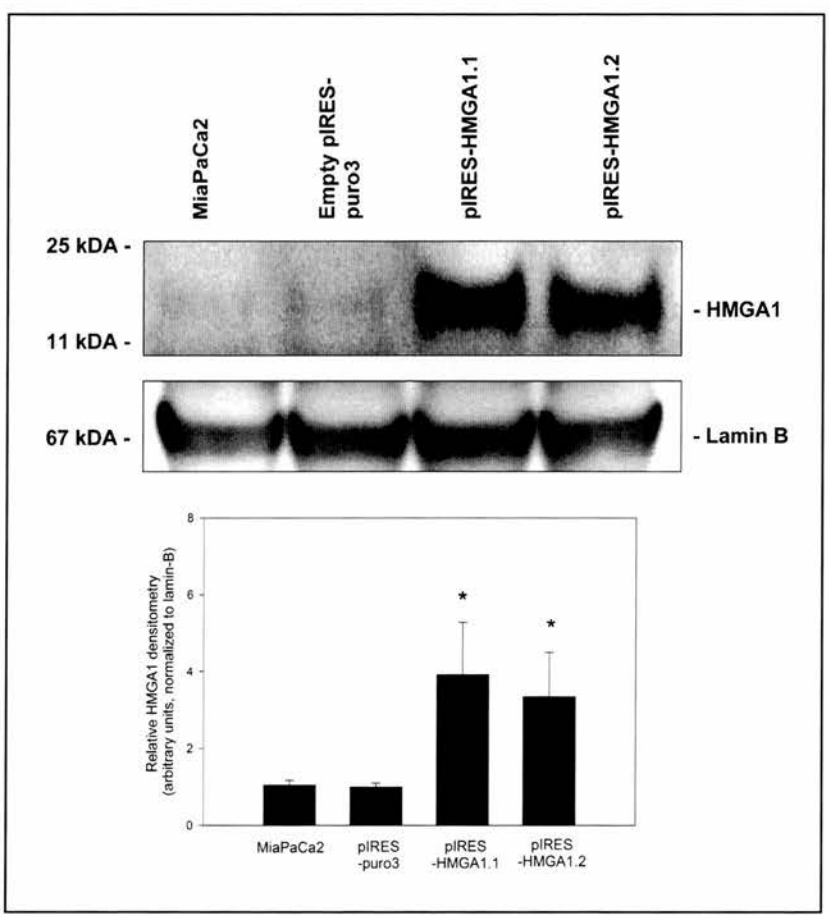


Figure 5.1. Two stable transfectant clones derived from MiaPaCa2 cells were confirmed to overexpress HMGA1 (pIRES-HMGA1.1 and 1.2) on Western blot analysis of nuclear extracts. Lamin B was used as a loading control. Controls were cells stably transfected with empty pIRES-puro3 vector. *Blots shown are representative of three independent experiments. Densitometry values, normalised to Lamin B signals, are mean (± s.d.). $P<0.05$ versus empty pIRES-puro3 vector and parental MiaPaCa2 cells.

The anoikis assay was performed by culturing cells on poly-2-hydroxyethylmethacrylate (polyHEMA)-coated plates which prevent cellular attachment, allowing cells to grow or survive in suspension (see Section 2.14). This technique was originally described by Folkman and Moscona in 1978 and has since been used extensively to induce this subset of apoptosis by depriving cells of its normal substratum attachment (Folkman & Moscona, 1978). Following 18 hours of anchorage-deprivation (on polyHEMA plates), anoikis (or apoptosis with deprivation of attachment) was quantified using flow cytometric assessment of YO-PRO-1- and propidium iodide-stained cells. In short, the green-fluorescent dye YO-PRO-1 identifies apoptotic cells and propidium iodide stains necrotic cells. The anoikis fraction was calculated from the ratio of apoptotic cells to total cells counted. pIRES-HMGA1.1 and pIRES-HMGA1.2 clones showed significantly increased anoikis resistance (mean anoikis fractions of 11% and 13% respectively) when compared to controls (mean anoikis fractions for parental MiaPaCa2 and empty pIRES-puro3 transfectants were 26% and 27% respectively, Figure 5.2A and B). Interestingly, pIRES-HMGA1.1 and pIRES-HMGA1.2 clones adopt a rounder morphology and tend to grow in clumped colonies even when grown in standard adherent culture conditions.

5.3.2 HMGA1 overexpression results in protection from caspase-mediated apoptosis

Given that disruption of cell-matrix interactions can trigger anoikis via caspase-dependent apoptosis, we examined the effects of HMGA1 overexpression on caspase 3 activity (a central mediator of apoptosis) in the context of anchorage deprivation. During induction of anoikis on polyHEMA plates, HMGA1-overexpressing clones demonstrated markedly reduced levels of caspase 3 activity compared to parental MiaPaCa2 or pIRES-puro3 controls (Fig 5.2C).

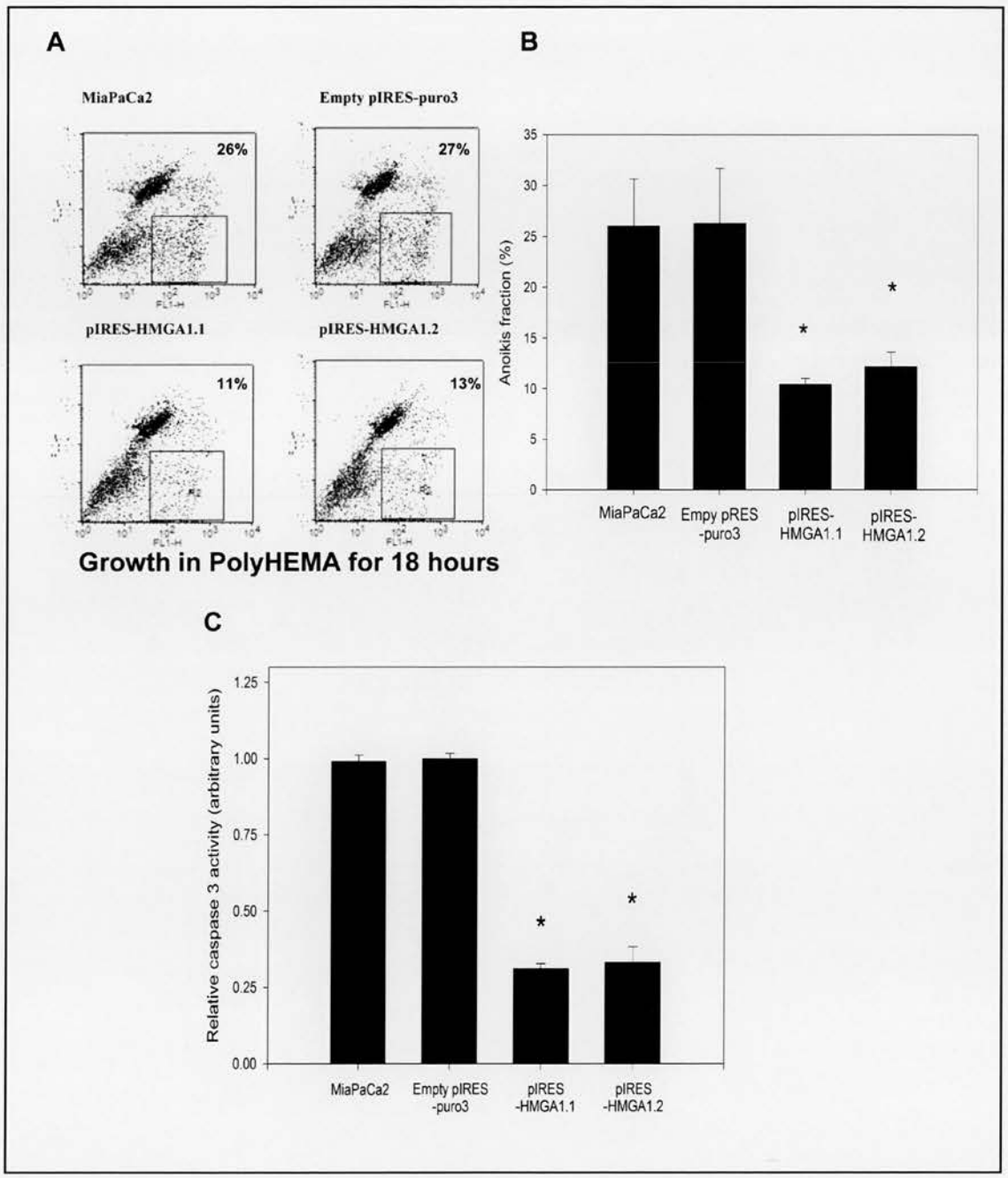


Figure 5.2. *A-B*, Forced HMGA1 overexpression protected MiaPaCa2 cells, which have low inherent expression of HMGA1, from anoikis. Representative flow cytometric images are shown with anoikis fractions highlighted in the inserted square (Fig 5.2A). Clones pIRES-HMGA1.1 and pIRES-HMGA1.2 exhibited 2- to 2.5-fold reductions in anoikis fraction compared to parental MiaPaCa2 and pIRES-puro3 control stable transfectants (Fig 5.2B). * $P<0.05$ versus empty pIRES-puro3 vector. *C*, HMGA1 overexpression protected MiaPaCa2 cells from caspase-mediated anoikis. pIRES-HMGA1.1 and pIRES-HMGA1.2 clones showed significant reductions in caspase 3 activity, compared to parental MiaPaCa2 cells and empty

pIRES-puro3 transfectants, following induction of anoikis on polyHEMA plates for 18 hours. *P<0.05 versus empty pIRES-puro3 vector.

5.3.3 Overexpression of HMGA1 increases levels of Akt phosphorylation and Akt kinase activity

Phosphoinositidyl-3 kinase/Akt signaling pathway is of critical importance in mediating anoikis resistance and enhancing anchorage-independent cell cycle progression (Moore et al., 1998; Nguyen et al., 2002) . Using a different phospho-specific antibody (Cell Signaling Technology, 587F11-#4051) to that used in Chapter 3, we sought to confirm if HMGA1 overexpression would modulate Akt phosphorylation at Ser473, a marker of Akt activation. HMGA1 overexpression resulted in elevated Akt (Ser473) phosphorylation (Figure 5.3A). Consistent with this finding, HMGA1 overexpression was found to be associated with increases in Akt kinase activity (Figure 5.3B), as assessed using a fluorometric real-time Akt kinase assay.

5.3.4 Inhibition of phosphoinositidyl-3 kinase/Akt signaling reverses HMGA1 overexpression-induced anoikis resistance.

Having demonstrated that HMGA1 overexpression induces constitutive activation of PI3-K/Akt pathway, we next examined the effects of inhibiting this pathway in cells overexpressing HMGA1. First, we used a specific small molecule inhibitor of PI3-K, LY294002, to assess the effects of inhibiting PI3-K on anoikis resistance of these cells. Addition of 25μM of LY294002 significantly increased anoikis fractions in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Figure 5.3C). Treatment of parental MiaPaCa2 and empty pIRES-puro3 controls with LY294002 also inhibited anoikis resistance, although to lesser extent than for pIRES-HMGA1.1 and pIRES-HMGA1.2 clones. Second, infection of pIRES-HMGA1.1 and pIRES-HMGA1.2 clones with adenovirus expressing a dominant negative Akt construct also significantly increased anoikis fractions in both HMGA1-overexpressing clones (Figure 5.3D). Parental MiaPaCa2 and empty pIRES-puro3 controls

also exhibited increased anoikis fractions with infection of adenovirus expressing dominant negative Akt construct but to a lesser extent than the HMGA1 overexpressing clones. Taken together, these results suggest that HMGA1 overexpression-induced anoikis resistance is dependent on PI3-K/Akt signaling.

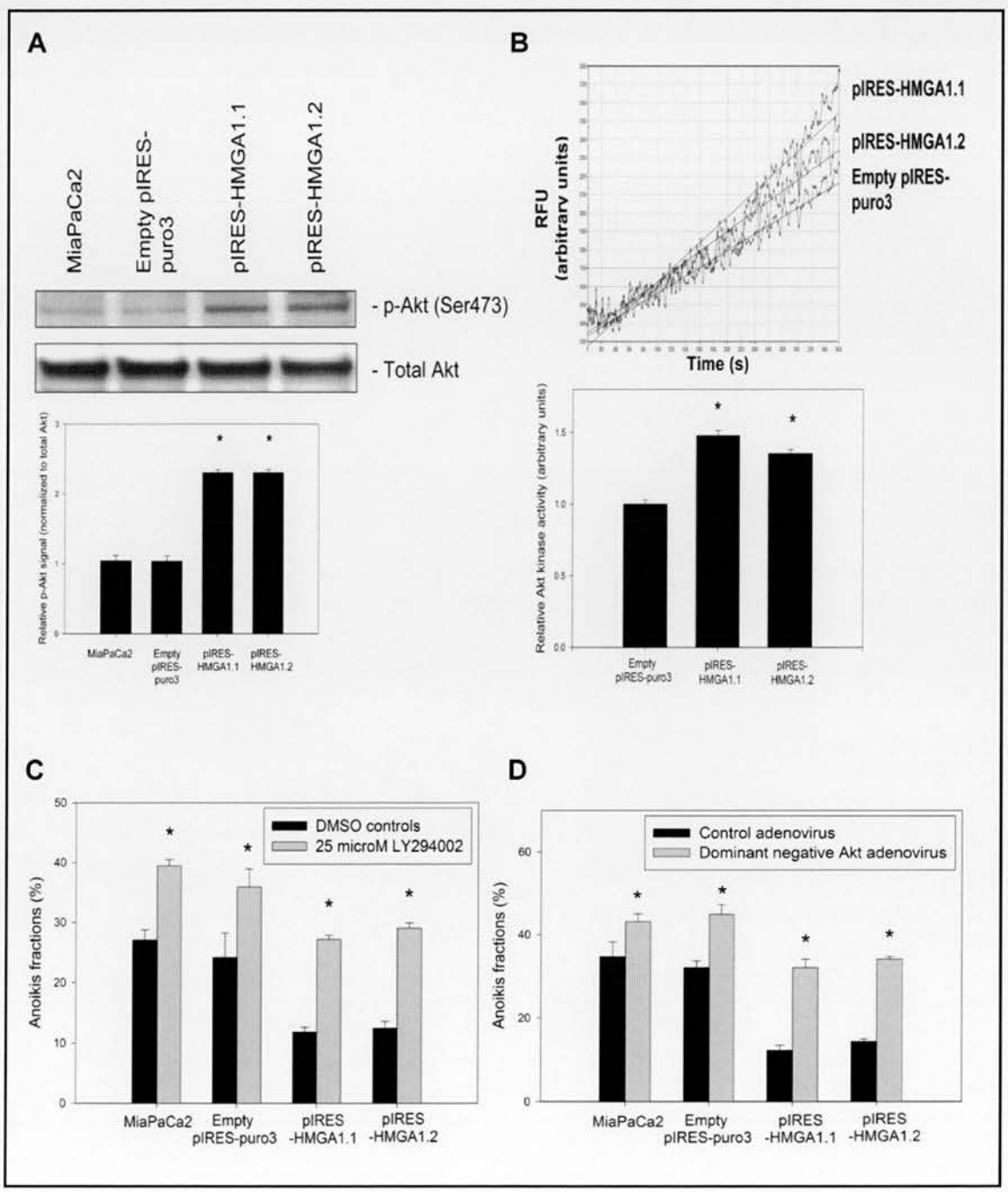


Figure 5.3. *A,* Overexpression of HMGA1 led to increased activation of Akt, as evident from increased levels of phospho-Akt (Ser473), a marker of Akt activation. Both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited a higher degree of Akt phosphorylation compared to control cells. There were no differences in the level of expression of total Akt with overexpression of HMGA1. * P<0.05 versus parental MiaPaCa2 cells or empty pIRES-puro3 transfectants. Blot shown is representative of three independent experiments. *B,* Correspondingly, overexpression of HMGA1 results in increased Akt kinase activities as determined by fluorometric real-time Akt kinase assays. Slope of Akt kinase activity curves indicates the levels of Akt kinase activity. Representative results of Akt kinase activity assay from three independent experiments are shown. pIRES-HMGA1.1 and pIRES-HMGA1.2 clones showed steeper activity curve slopes and hence Akt kinase activities, when compared to empty pIRES-puro3 controls. * P<0.05 versus empty pIRES-puro3 vector transfectants. *C,* Anoikis resistance induced by HMGA1 overexpression in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones was reversed by pre-incubating cells in 25μM LY294002, a specific inhibitor of PI-3K. Inhibiting PI-3K resulted in increases in anoikis fractions in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones, to levels similar to parental MiaPaCa2 and empty pIRES-puro3 controls. Controls were cells treated with the DMSO vehicle. *p<0.05 versus DMSO controls. *D,* Similarly, infection of pIRES-HMGA1.1 and pIRES-HMGA1.2 clones with adenovirus carrying dominant negative Akt1 resulted in reversal of anoikis resistance with increases in anoikis fractions in both HMGA1 overexpressing clones. Controls were cells infected with adenovirus expressing an empty CMV promoter (Ad-CMV-null). *p<0.05 versus control adenovirus (Ad-CMV-Null).

5.3.5 Targeted RNA interference of HMGA1 in MiaPaCa2 and BxPC3 pancreatic adenocarcinoma cells increases susceptibility to anoikis.

Given the effects of HMGA1 overexpression on anoikis resistance in MiaPaCa2 cells, we asked if silencing of HMGA1 in the same cell line will have the reverse effects. In addition, we also selected BxPC3 pancreatic adenocarcinoma cell line for RNA interference experiments as these cells have high levels of HMGA1 expression at baseline. Generation of lentivirus capable of silencing HMGA1 expression is provided in Section 2.5.4. In these lentivirus-mediated RNAi experiments, we developed pooled stable transfectants. Stable transfectants derived from infection with lentivirus developed from empty PLKO.1 and scramble shRNA transfer plasmids served as controls. We achieved at least 90% silencing of HMGA1 expression in

both MiaPaCa2 and BxPC3 cells (Figure 5.4A). Of note, neither HMGA1 overexpression nor silencing had any impact on Lamin B expression (used to normalise for protein loading, Figures 5.1 and 5.4A). HMGA1 silencing was associated with significant increases in anoikis fractions in both cell lines (Figure 5.4B).

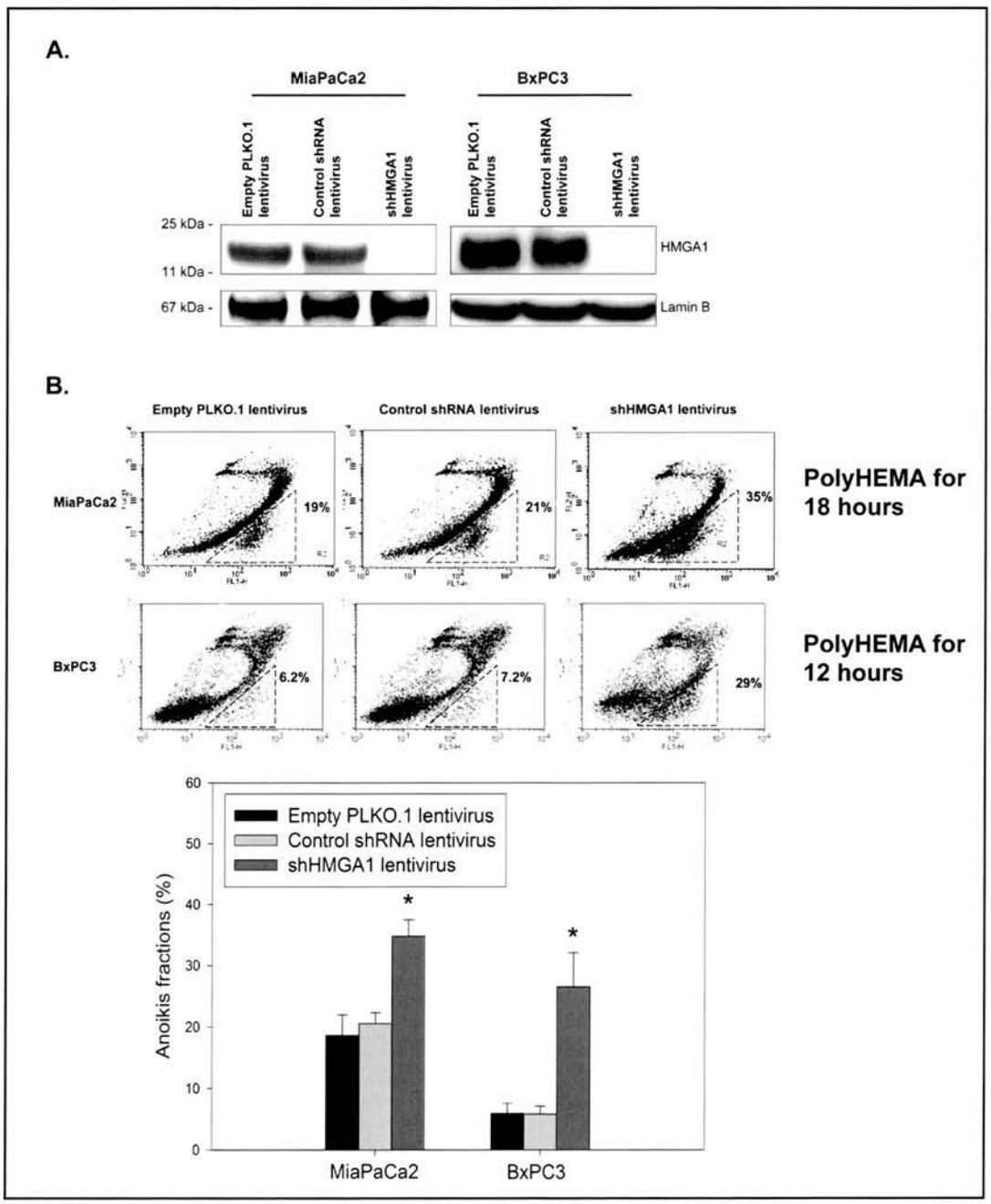


Figure 5.4. *A,* Using lentivirus-mediated RNA interference (RNAi) of HMGA1, we achieved up to 90% silencing of HMGA1 expression in both MiaPaCa2 and BxPC3 cell lines. A

representative Western blot performed on nuclear extracts showing the degree of HMGA1 silencing is shown. Controls were stable transfectants developed following infection with lentivirus expressing a scramble RNAi or empty PLKO.1 sequence. *B,* Silencing of HMGA1 promoted anoikis in both cell lines following culture in polyHEMA plates for 18 hours (for MiaPaCa2) and 12 hours (for BxPC3). Representative images of flow cytometric analyses of anoikis fractions with apoptotic populations highlighted in triangles drawn are shown. Targeted suppression of HMGA1 resulted in significant increases in anoikis fractions in both MiaPaCa2 and BxPC3 cells, compared to empty PLKO.1 and scramble RNAi stable transfectants. * $p<0.05$ vs. empty PLKO.1 or scramble RNAi transfectants.

5.4 DISCUSSION

Pancreatic adenocarcinoma is associated with dismal prognosis, in large part resulting from metastatic disease, even in patients initially deemed to be surgically resectable. We have focused our efforts in studying the mechanisms underlying metastasis in pancreatic adenocarcinoma, with the goal of rationally identifying molecular targets that can be exploited to inhibit this process. In our work, we have used cellular invasiveness (Chapter 4) and anoikis resistance as an *in vitro* correlates of metastatic potential. In this study, we have shown that HMGA1 overexpression confers anoikis resistance to pancreatic adenocarcinoma cells by activating the pro-survival PI3-K/Akt signaling pathway. This is the first report describing a regulatory role of HMGA1 on anoikis resistance in a cancer model.

Recently, data suggesting mechanisms by which HMGA1 may mediate cancer progression have begun to emerge. For example, HMGA1 proteins have been demonstrated to promote tumour progression and epithelial-mesenchymal transition in human breast epithelial cells (Reeves et al., 2001). In immortalized rat embryonic fibroblast Rat1a cells, HMGA1 was found to be a c-Jun target gene and its suppression by antisense methodology was found to reduce the ability of c-Jun overexpressing cells to grow under anchorage-independent conditions (Hommura et al., 2004).

Although previous reports have implicated HMGA1 as having the capacity to promote tumourigenesis and anchorage independent growth in normal epithelial cells (Reeves & Beckerbauer, 2001), no studies reported to date have examined specifically the role of HMGA1 in mediating anoikis resistance in the context of cancer cells. Our findings suggest that HMGA1 overexpression represents a molecular determinant of anoikis resistance in pancreatic adenocarcinoma cells. The biological significance of this finding relates to emerging understanding that anoikis resistance is a phenotypic hallmark of metastatic cancer cells (Douma et al., 2004). Although the underlying mechanisms rendering anoikis resistance in cancer cells are incompletely understood, our findings provide evidence for a novel role for HMGA1 in mediating this process. Further, our findings provide a potential mechanism by which HMGA1 overexpression induces anoikis resistance: activation of the anti-apoptotic PI3-K/Akt signaling pathway.

Description of regulatory roles for HMGA1 in the context of apoptosis is not without precedent. HMGA1 has been found to associate *in vivo* with p53 family members and inactivate their functions. As such, overexpression of HMGA1 may lead to suppression of p53-induced apoptosis and tumour suppressor activity (Frasca et al., 2006). In addition, antisense-mediated suppression of HMGA1 expression in thyroid carcinoma cell lines has been reported to induce an apoptotic response (Scala et al., 2000). Our findings that HMGA1 overexpression results in the constitutive activation of PI3-K/Akt pathway provide another mechanism through which HMGA1 mediates its anti-apoptotic functions. HMGA1-dependent activation of Akt signaling is likely to protect cancer cells from a broad array of pro-apoptotic stimuli, not just those related to anchorage deprivation. Indeed, we have begun work investigating if HMGA1 protects pancreatic adenocarcinoma cells from gemcitibine-induced apoptosis and in this context, may represent a potential molecular determinant of chemoresistance in pancreatic cancer (Chapter 7).

Our findings, taken together with those previously reported, provide a mechanistic basis for conceptualizing the known correlations between HMGA1 expression and poor prognosis in cancer patients (Chapters 1 and 3). These findings also have obvious therapeutic implications. Future studies will need to address whether targeted therapies directed against HMGA1 will inhibit other features of malignant phenotype that make pancreatic cancer so lethal.

Chapter Six: HMGA1 promotes anchorage-independent growth in pancreatic adenocarcinoma

6.1 ABSTRACT

Background: We previously showed that HMGA1 proteins are architectural transcription factors that are overexpressed by pancreatic adenocarcinomas. We tested the hypothesis that HMGA1 promotes anchorage-independent cellular proliferation and *in vivo* tumourigenicity. Having identified PI3-K/Akt pathway as a downstream mediator of HMGA1, we examined if this aspect of malignant phenotype was also dependent of this pathway.

Methods: shRNA-mediated RNAi was used to silence HMGA1 expression in MiaPaCa2 and PANC1 pancreatic cancer cells. Anchorage-independent proliferation was assessed using the soft agar assays. The roles of PI3-K/Akt and extracellular signal-regulated kinase (ERK) signaling were investigated using specific inhibitors and adenoviral dominant negative/active Akt constructs. *In vivo* tumourigenicity was assessed using a nude mouse subcutaneous xenograft model.

Results: shRNA-mediated HMGA1 silencing resulted in significant reductions in anchorage-independent colony formation in soft agar, tumourigenicity *in vivo*, and Akt phosphorylation (Ser473), without changes in ERK1/2 phosphorylation. Forced HMGA1 overexpression promoted colony formation in soft agar, Akt phosphorylation (Ser473), and ERK 1/2 phosphorylation. HMGA1 overexpression-induced increases in anchorage-independent growth were dependent on PI3-K/Akt- but MEK/ERK signaling. Further, HMGA1 silencing *in vivo* resulted in reductions in cellular proliferation (Ki-67 index) and increases in apoptosis (TUNEL staining).

Conclusions: Our findings suggest HMGA1 promotes tumourigenicity through a PI3-K/Akt-dependent mechanism in pancreatic adenocarcinoma cells. HMGA1 warrants further evaluation, both as a therapeutic target and a prognostic marker in pancreatic cancer.

6.2 INTRODUCTION

Our group has previously shown that HMGA1 overexpression is a common occurrence in pancreatic adenocarcinomas and its tumoural expression indicates a poor clinical outcome in patients with this deadly disease (Chapter 3). Dysregulated overexpression of HMGA1 has been reported in numerous human cancers (reviewed in Section 1.11.7). HMGA1 proteins are architectural transcription factors that were once thought to be merely 'associated' with tumourigenesis (Evans et al., 2004). However, evidence is accumulating that HMGA1 has a functional importance in tumourigenesis. Known functions of HMGA1 proteins include forming stereo-specific, multiprotein complexes termed "enhanceosomes" on the promoter/enhancer regions of genes, where they bind to the minor groove of AT-rich DNA sequences (Reeves & Nissen, 1990; Thanos & Maniatis, 1995). This mode of action seems to transcriptionally regulate the expression of many genes. However, the mechanism through which HMGA1 mediates its pro-tumourigenic effects remains unclear.

In breast cancer, HMGA1 has been shown to promote malignant transformation of breast epithelial cell lines (Reeves et al., 2001). It was suggested that HMGA1 overexpression promotes epithelial-mesenchymal transition during the process of breast tumourigenesis. In leukemia, unequivocal evidence exist whereby transgenic mice with HMGA1 overexpression targeted to lymphoid tissue developed spontaneous aggressive lymphomas (Xu et al., 2004). In pancreatic cancer, there is a paucity of data on the roles of HMGA1 in pancreatic tumourigenesis. Although we have previously described evidence for the roles of HMGA1 in mediating invasion, anoikis resistance and ultimately, metastasis, one other important determinant of biological aggressiveness that has yet to be studied is the tumourigenic potential of the cells. Traditionally, one of the most stringent tests for tumourigenicity of malignant cells is the anchorage-independent growth assay in soft agar. The ability to form tumours *in vivo* requires the cells

to have two sets of capabilities: firstly, to be able resist apoptosis under anchorage-independent conditions, and secondly, to be able to proliferate under similar conditions. Apoptosis under conditions of inappropriate or inadequate contact with substratum is termed anoikis (see Chapter Five). If a particular subset of tumour cells is more biologically aggressive and malignant, they are generally less susceptible to anoikis and proliferate more readily in soft agar. Hence, the soft agar assay is a stringent assay that closely corresponds with *in vivo* tumourigenicity.

To further evaluate the potential roles of HMGA1 in mediating the malignant phenotype, we tested the hypotheses that HMGA1 promotes anchorage-independent cell proliferation in pancreatic adenocarcinoma and that suppression of HMGA1 expression would impair anchorage-independent colony formation *in vitro* and tumour growth *in vivo*. We also hypothesised that effects of HMGA1 silencing on *in vivo* tumour growth will be mediated by a reduction in cellular proliferation and an increase in apoptosis. Our observations indicate that HMGA1 indeed promotes pancreatic adenocarcinoma tumourigenesis and that a key effector of HMGA1-induced anchorage-independent growth is the anti-apoptotic phosphoinositidyl-3 kinase/Akt pathway.

6.3 RESULTS

6.3.1 Stable RNAi-mediated suppression of HMGA1 expression inhibits anchorage-independent growth

Both MiaPaCa2 and PANC1 pancreatic adenocarcinoma cell lines express HMGA1, with MiaPaCa2 cells having a lower expression level of HMGA1 at baseline. We used two independent shRNA target sequences (named shHMGA1-1 and shHMGA1-2) to suppress HMGA1 expression. Each of these shRNA sequences was associated with an 80% reduction in HMGA1 expression in MiaPaCa2 cells (Figure 6.1), as confirmed by Western blot analyses of nuclear extracts. These same shRNA sequences were associated with 61% (shHMGA1-1) and 56% (shHMGA1-2) reductions in HMGA1 expression in PANC1 cells.

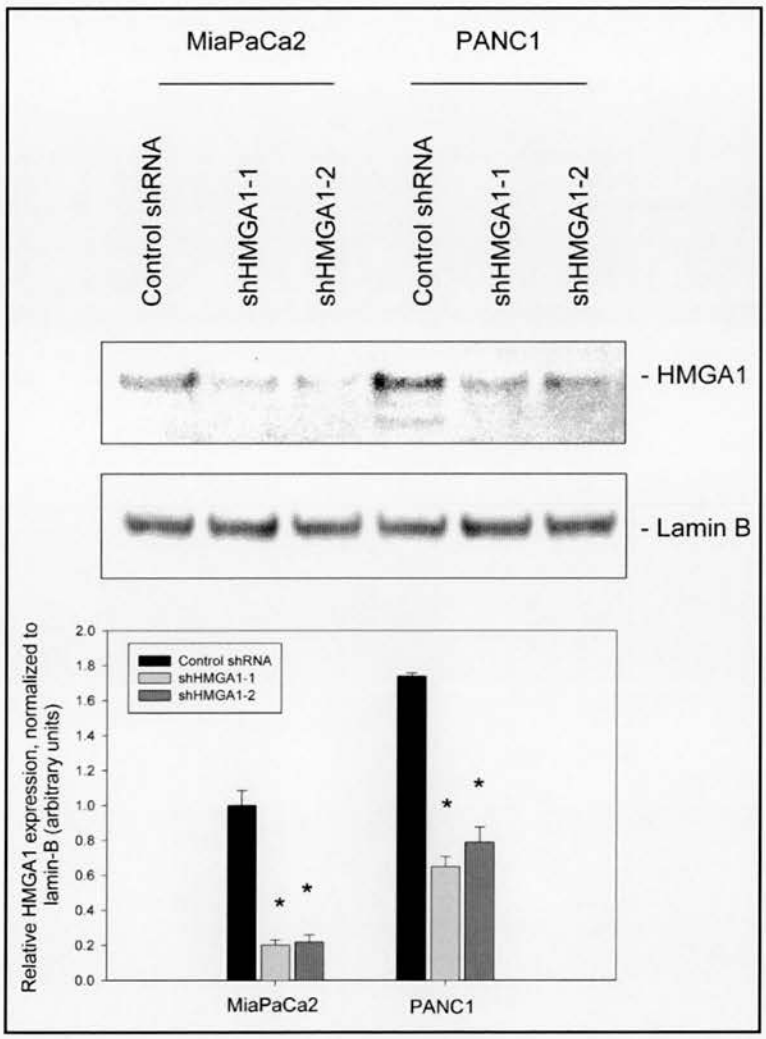


Figure 6.1. Stable silencing of HMGA1 expression using two short interfering RNA hairpin (shRNA) expression vectors with independent target sequences (shHMGA1-1 and shHMGA1-2) was confirmed on Western blot analysis of nuclear extracts. Controls were shRNA expression vectors with a scrambled, non-targeting sequence. Greater suppression of HMGA1 expression was achieved in MiaPaCa2 cells, with approximately 80% silencing with shRNA sequences. In PANC1 cells, there was 55-60% suppression of HMGA1 expression with each shRNA sequence.

In MiaPaCa2 cells, the high degree of HMGA1 silencing induced by each of the shRNA sequences was associated with marked reductions in growth in soft agar (Figure 6.2A). Similar, although less marked, reductions in growth in soft agar were observed for PANC1 cells (Figure 6.2B), corresponding to lower degrees of HMGA1 silencing in this cell line.

MiaPaCa2 cells

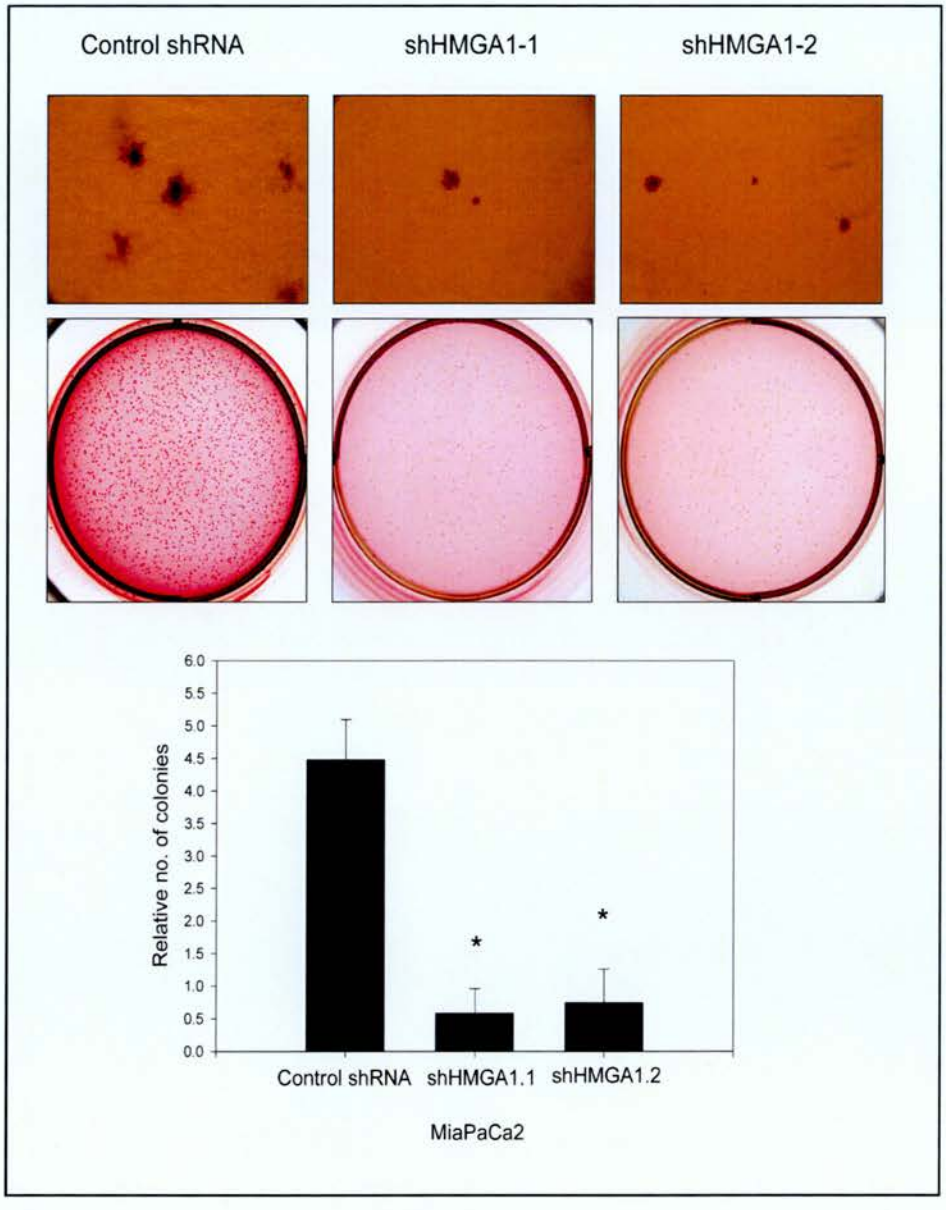


Figure 6.2A. For full legend, see pg 170

PANC1 cells

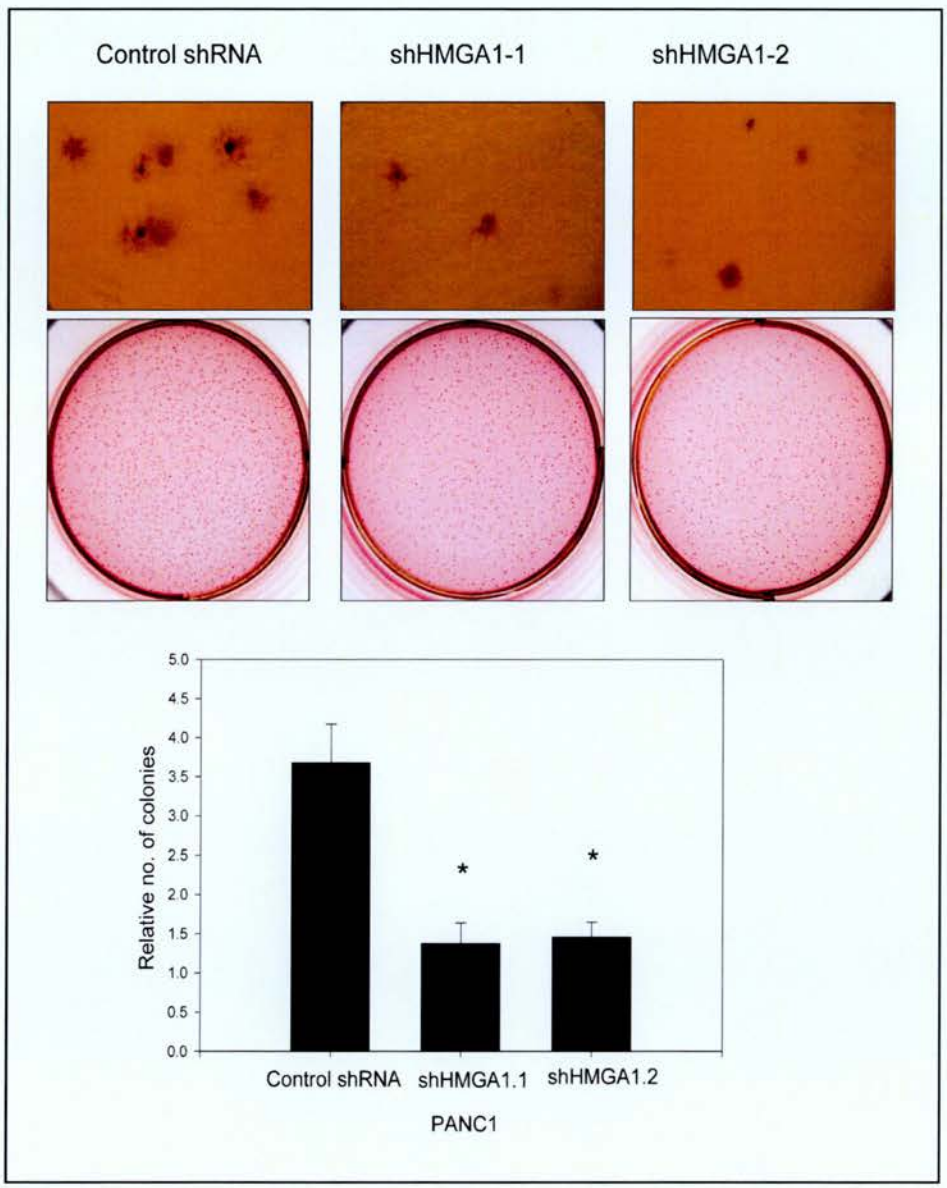


Figure 6.2B. For full legend, see below.

Legend

Figure 6.2. The effects of HMGA1 silencing on anchorage-independent growth was assessed using soft agar assays. Stable HMGA1 silencing using each of two independent shRNA sequences (shHMGA1-1 and shHMGA1-2) resulted in reductions in soft agar colony formation in both MiaPaCa2 (A) and PANC1 cells (B) when compared to the scrambled control shRNA-transfected cells. Effects on soft agar growth were greater in MiaPaCa2 cells, corresponding to greater silencing of HMGA1 in these cells. *P<0.05 versus control shRNA.

6.3.2 Forced HMGA1 overexpression promotes anchorage-independent growth.

Given the relatively low baseline expression level of HMGA1 in MiaPaCa2 cells, we chose this cell line to test the effects of forced HMGA1 overexpression. We transfected this cell line with an overexpression vector carrying the full-length HMGA1 cDNA. Two clones stably overexpressing HMGA1 were selected and named (pIRES-HMGA1.1 and pIRES-HMGA1.2). The degree of HMGA1 overexpression (2.5-fold and 3-fold overexpression, respectively, over controls) was documented on Western blot analyses of nuclear extracts (Figure 6.3A). HMGA1 overexpression was associated with increased colony formation in soft agar (Figure 6.3B).

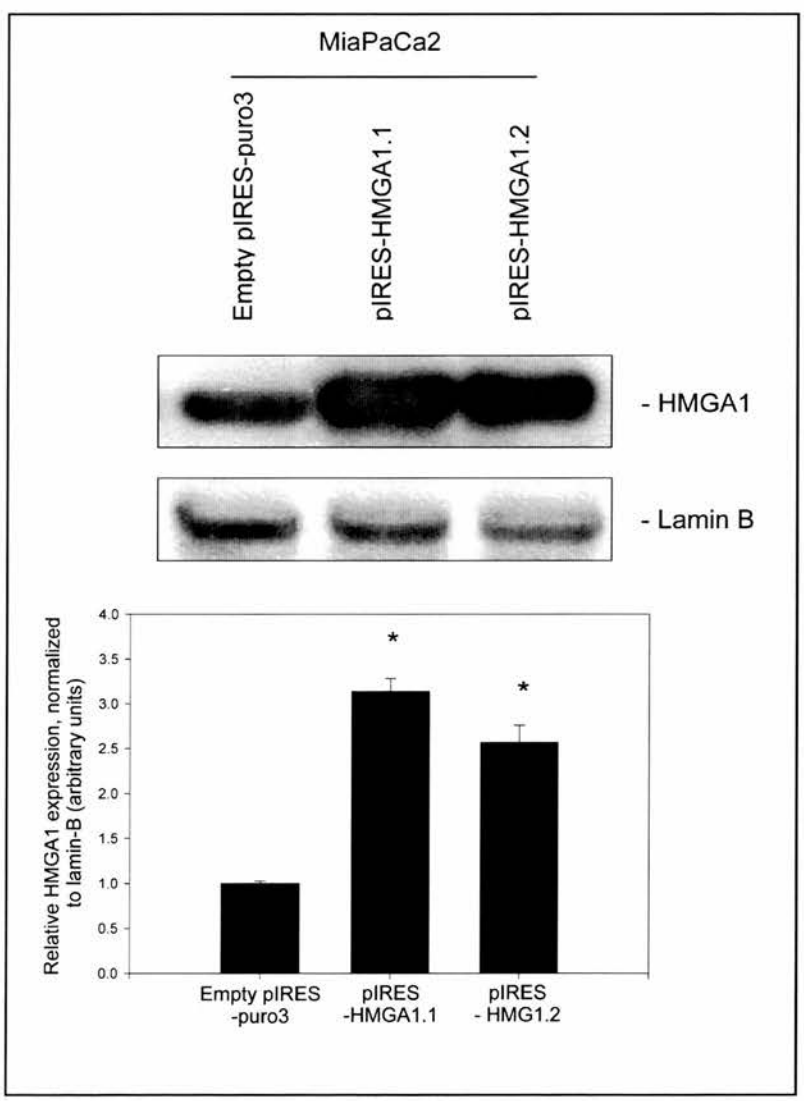


Figure 6.3A. Two clones of MiaPaCa2 cells were confirmed to stably overexpress HMGA1 (pIRES-HMGA1.1 and 1.2) on Western blot analysis of nuclear extracts. Blots shown are representative of three independent experiments. Densitometry values are mean (± SD).

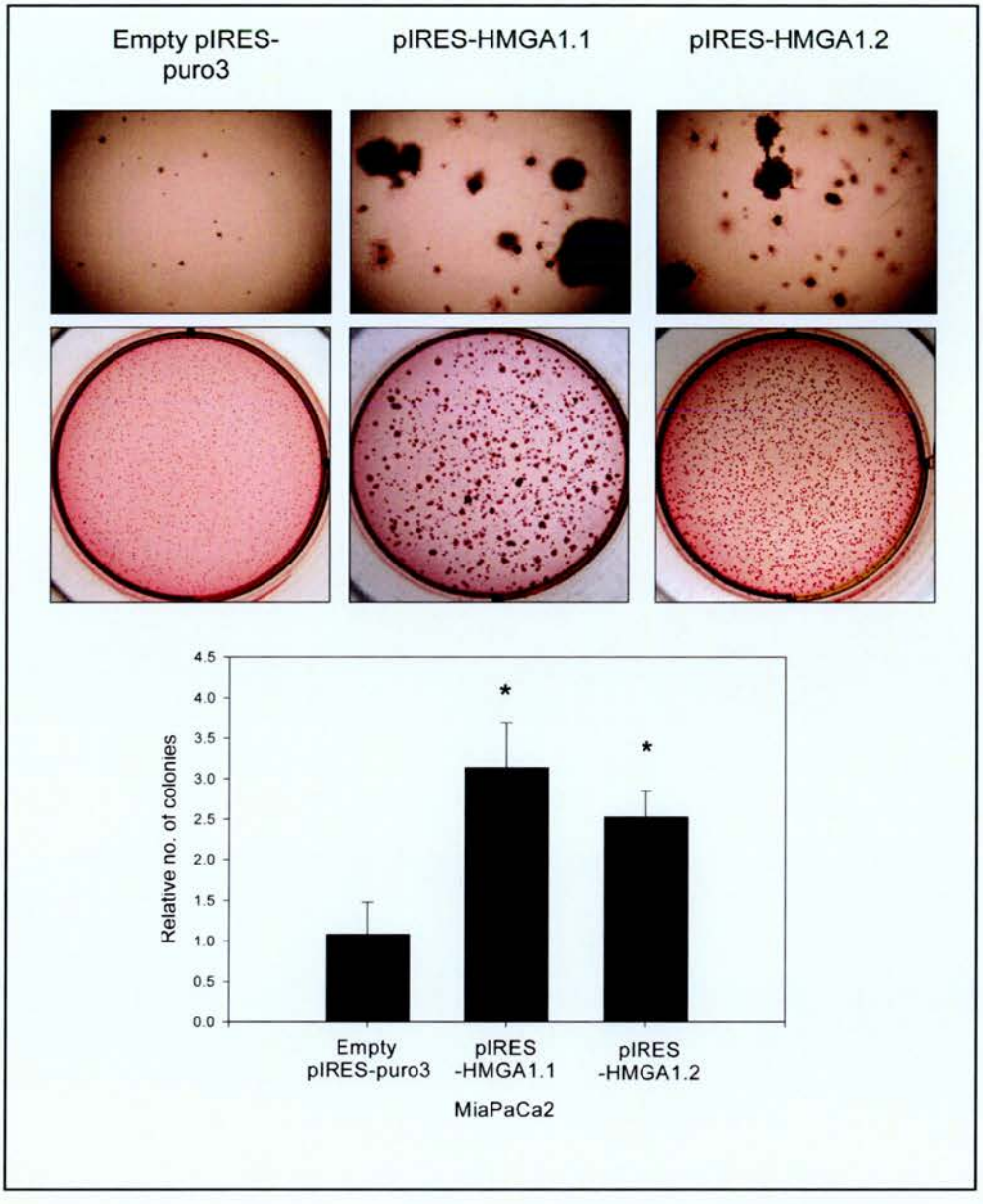


Figure 6.3B. Overexpression of HMGA1 in MiaPaCa2 clones (pIRES-HMGA1.1 and pIRES-HMGA1.2) resulted in unequivocal increases in anchorage-independent growth in soft agar. The number and size of colonies in soft agar were clearly larger with overexpression of HMGA1. *P<0.05 versus empty pIRES-puro3 transfected cells. Values are means (± SD).

6.3.3 HMGA1-induced increases in anchorage-independent growth are PI-3K/Akt- but not MEK/ERK-dependent.

We have previously shown in Chapters 4 and 5 that HMGA1 is able to positively regulate Akt and ERK activation. To assess if the effects of HMGA1 were dependent on Akt activation, we performed soft agar assays in the presence of PI-3K inhibitor LY294002. LY294002 (at concentrations of either 25μM or 50 μM) was associated with significant reductions in soft agar growth by pIRES-HMGA1.1 and pIRES-HMGA1.2 clones to levels similar to those exhibited by parental MiaPaCa2 cells and MiaPaCa2 cells stably transfected with empty pIRES-puro3 vector (Figure 6.4A). In contrast, the MEK/ERK inhibitor PD98059 (at concentrations of either 50 μM and 100 μM) had no impact on soft agar growth by pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Figure 6.4B). In addition, infection of adenovirus carrying dominant negative (Ad-DN-Akt) abrogated soft agar growth in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Figure 6.5A). Further, infection of dominant active Akt adenovirus (Ad-myr-Akt) rescued the ability to grow in soft agar of MiaPaCa2 cells with HMGA1 silencing (i.e. shHMGA1-1 and shHMGA1-2) (Figure 6.5B).

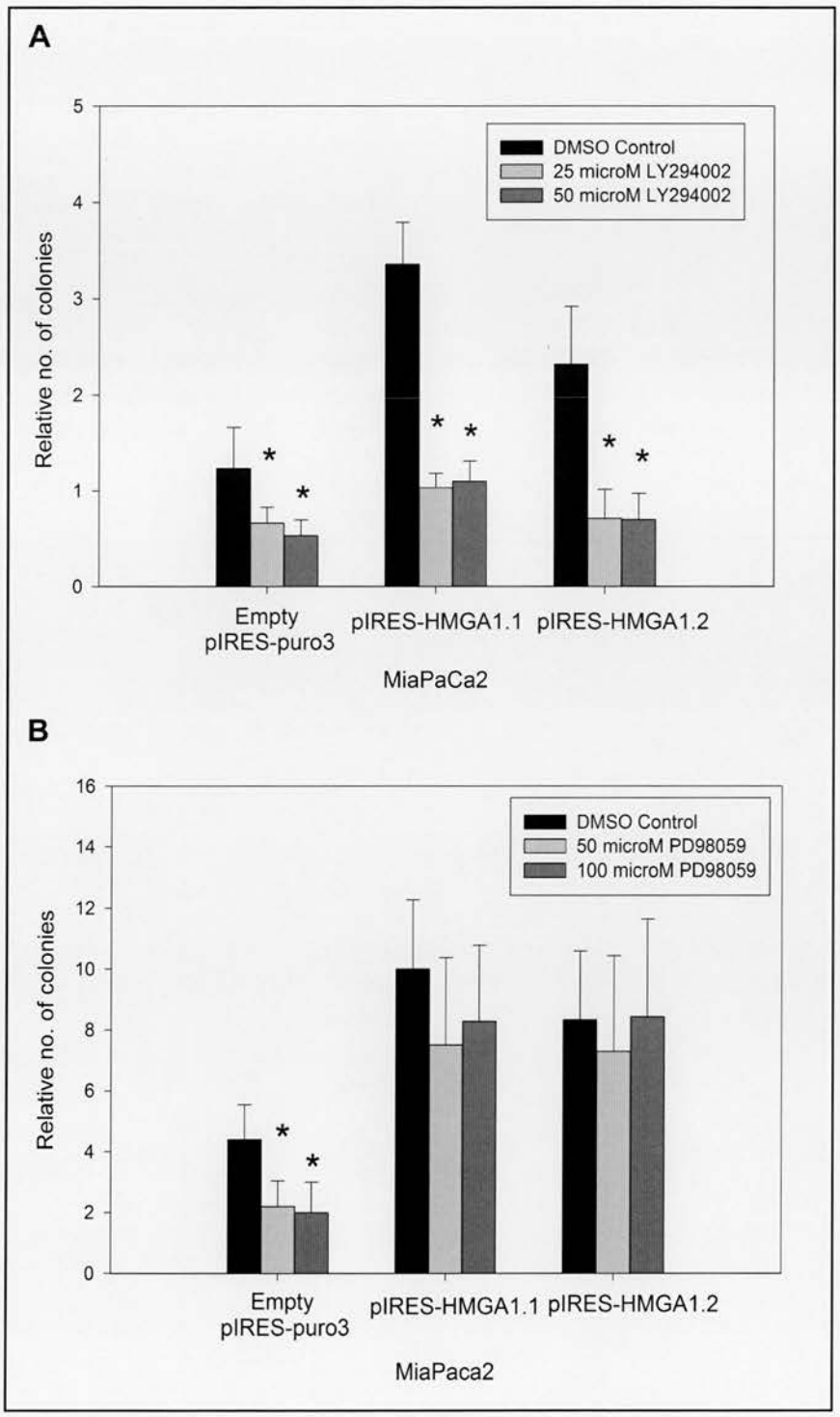


Figure 6.4. A, Soft agar assays were performed in the presence of 25μM and 50μM of LY294002 (a specific PI3-K inhibitor). Clones pIRES-HMGA1.1 and pIRES-HMGA1.2 exhibited significant inhibition of soft agar growth in the presence of LY294002 at either concentration when compared to the DMSO-treated controls. Although LY294002 also had an effect on soft agar growth in pIRES-empty puro3 controls, the degree of inhibition was clearly less marked compared to that of in pIRES-HMGA1.1 and 1.2 clones. *P<0.05 versus DMSO-treated controls. B, MEK/ERK inhibitor PD98059 had no effects on HMGA1 overexpression-induced increases in soft agar growth, as both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited no significant reductions in growth even in high 50μM and 100 μM concentrations of PD98059. This is in contrast to the significant inhibition of soft agar growth when the pIRES-puro3 controls were exposed to PD98059. *P<0.05 versus DMSO-treated controls.

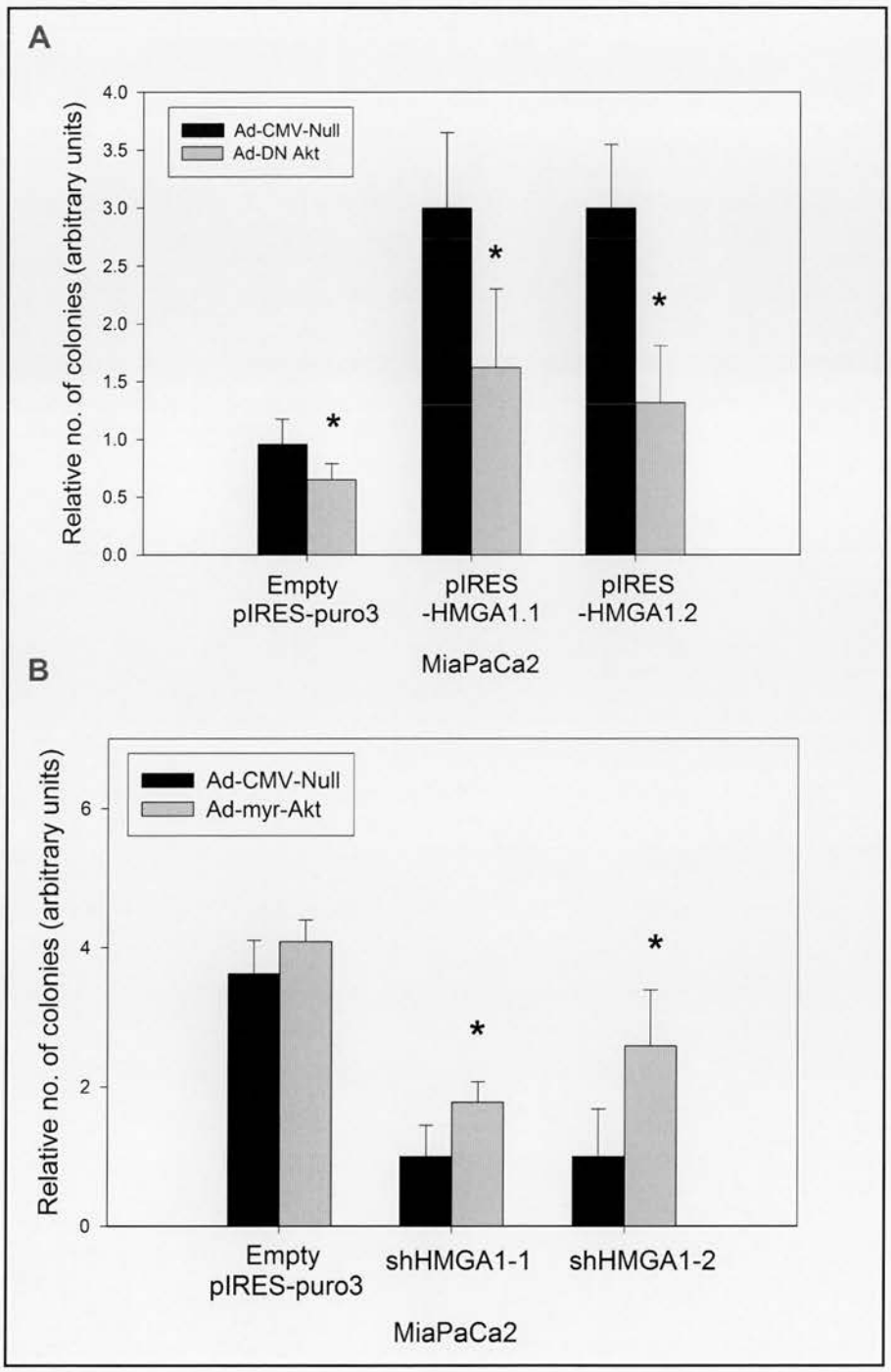


Figure 6.5. A, Infection of pIRES-HMGA1.1 and 1.2 clones with dominant negative Akt adenovirus resulted in significant reductions in HMGA1 overexpression-induced increase in soft agar growth. Dominant negative Akt adenovirus resulted in a small effect in soft agar growth in the empty pIRES-puro3 control cells. *P<0.05 versus control adenovirus (Ad-CMV-Null). B, Conversely, infection of adenovirus expressing constitutively active Akt (Ad-myr-Akt) rescued the ability to grow under anchorage-independent conditions in shHMGA1-1 and shHMGA1-2 stable transfectants. No effects were seen when empty pIRES-puro3 controls

were infected with constitutively active Akt adenovirus. *P<0.05 versus control adenovirus (Ad-CMV-Null). Values are means (± SD).

6.3.4 HMGA1 silencing resulted in significant inhibition of tumour growth in vivo.

Tumours derived from subcutaneous implantation of MiaPaCa2 and PANC1 cells stably transfected with HMGA1 shRNA vectors exhibited reduced growth rates in nude mice compared to corresponding controls (tumours derived from MiaPaCa2 and PANC1 cells stably transfected with control shRNA vectors) during the 8-week period following implantation (Figure 6.6A and B). Stable knockdown of HMGA1 was confirmed by performing Western blot analysis on nuclear extracts of tumour xenografts (Figure 6.7A and B).

Immunohistochemical analysis of tumours harvested at the end of this observation period suggests that HMGA1 silencing is associated with an inhibition in tumoural cell proliferation (Ki-67 reactivity) and an increase in tumoural apoptosis (TUNEL-staining) (Figure 6A and B).

Modulation of HMGA1 expression had no impact on cellular proliferation in monolayer culture. Neither HMGA1 silencing nor overexpression had a significant effect on the cellular proliferation in standard monolayer culture as determined by MTS assay (see Chapter 4, Figure 4.7).

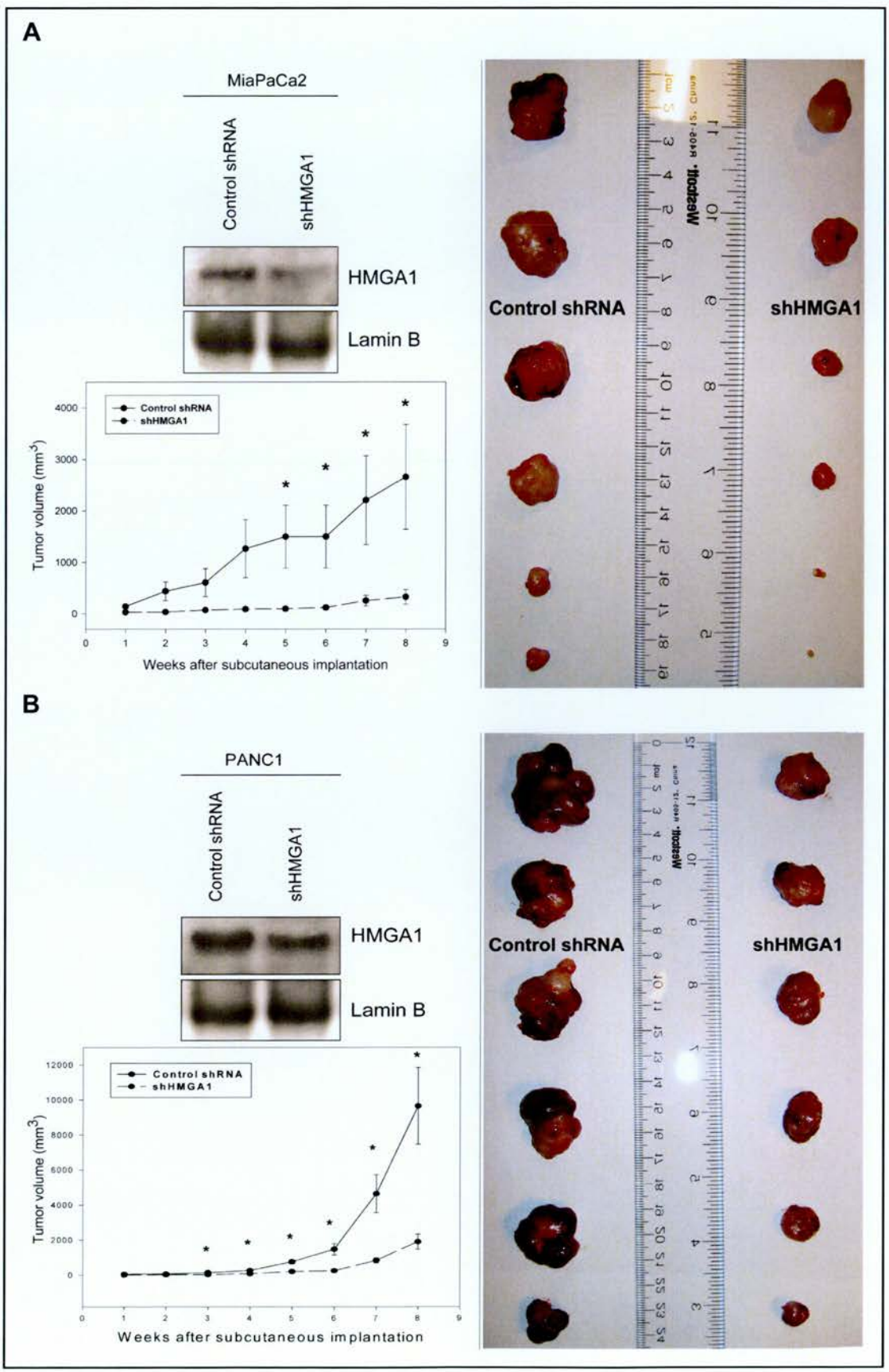


Figure 6.6. For full legend, see pg 178.

Legend

Figure 6.6. *A,* Stable silencing of HMGA1 resulted in significant attenuation in growth of tumours derived from subcutaneous implantation of MiaPaCa2 and PANC1 cells in nude mice. Mice (n=6 per group) were subcutaneously implanted with stable transfectant cells (either scrambled shRNA control or shHMGA1-1 plasmid). Subcutaneous tumour size was monitored weekly for 8 weeks. Stable HMGA1 silencing was confirmed by Western blot analysis of nuclear extracts from explanted xenograft tumours. Values are means (± SEM). *P<0.05 versus control shRNA xenografts.

A. *In vivo* proliferation (Ki-67 reactivity)

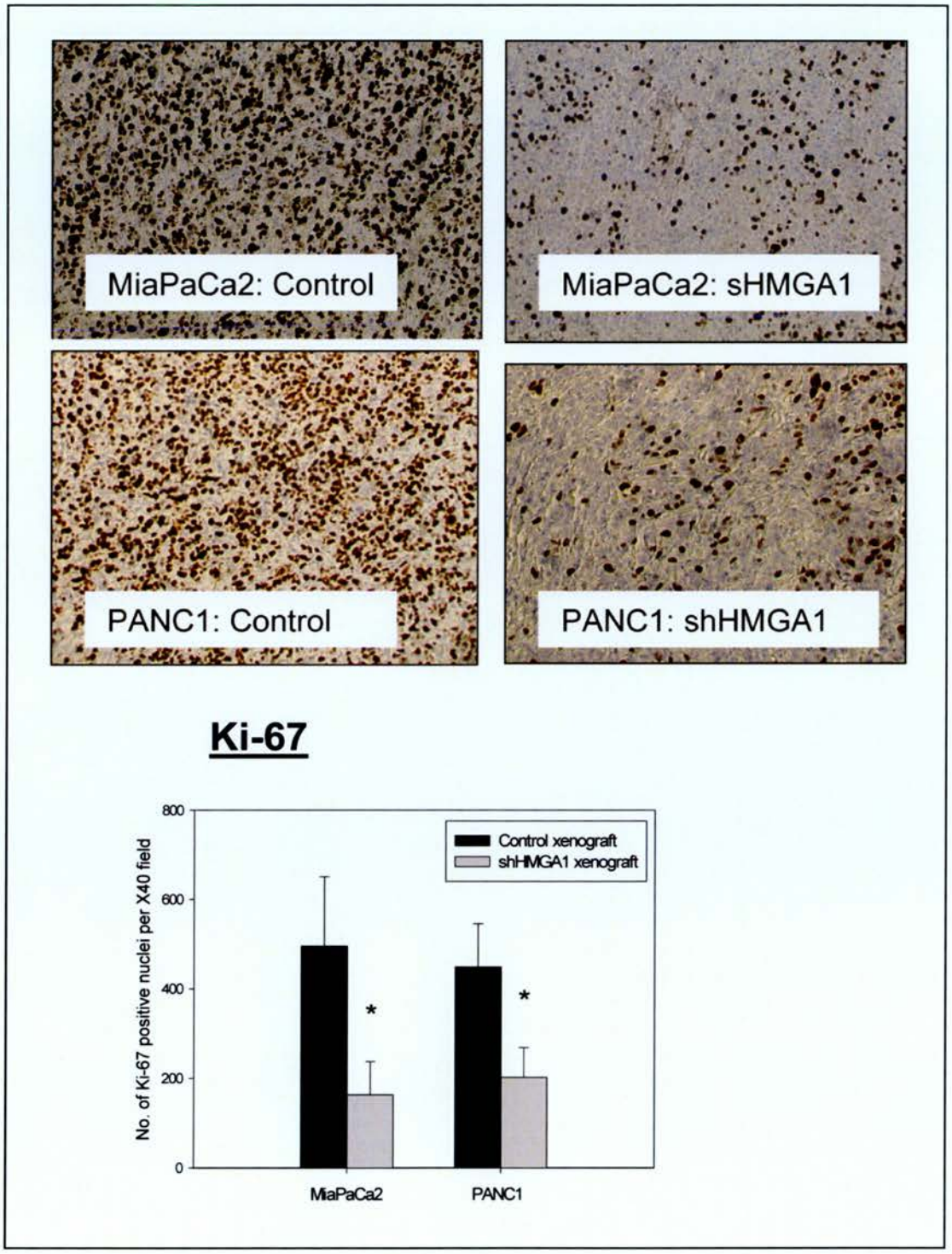


Figure 6.7A. For full legend, see pg 181.

B. *In vivo* apoptosis (TUNEL staining)

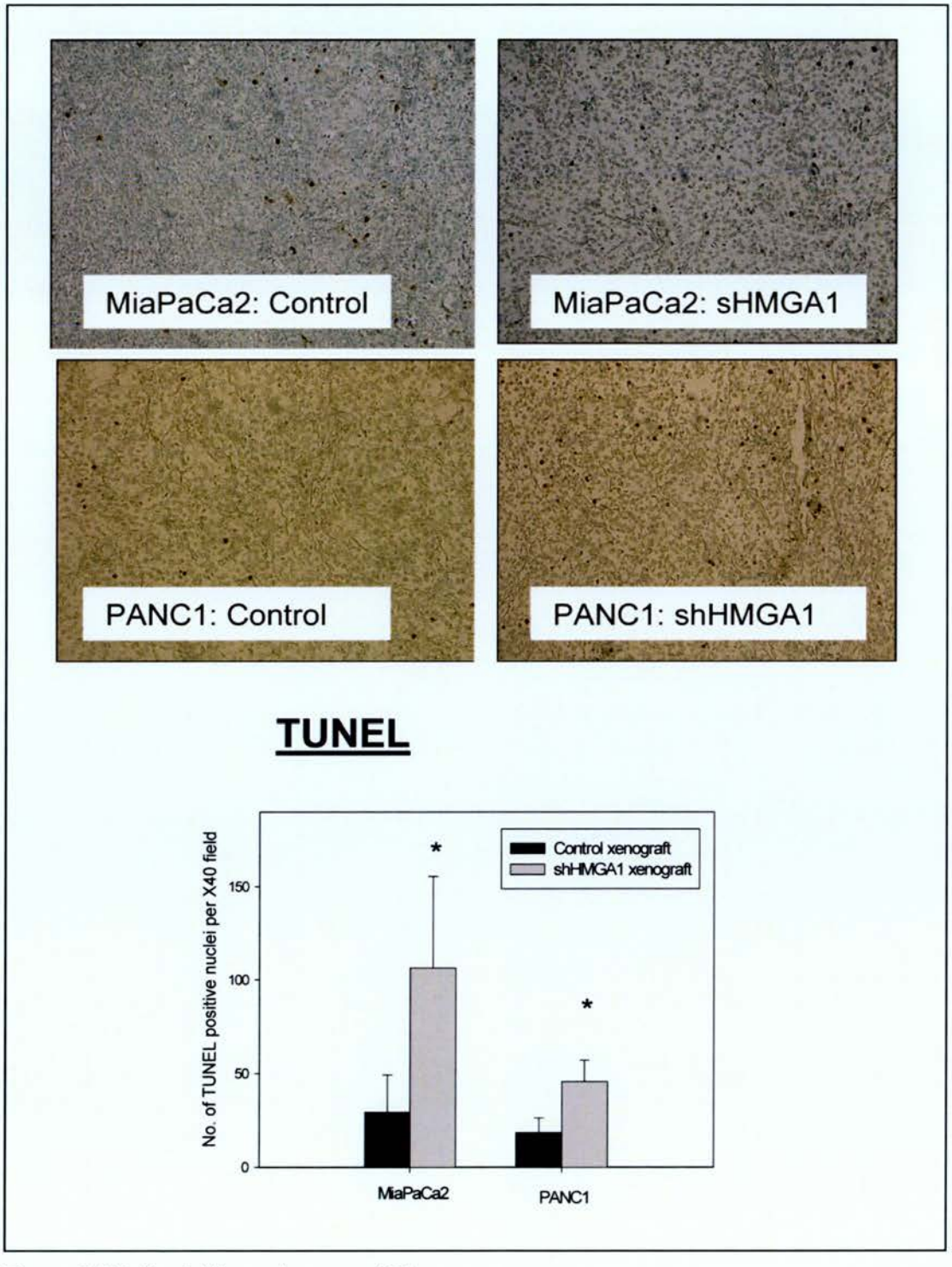


Figure 6.7B. For full legend, see pg 181.

Legend

Figure 6.7. *A*, Suppression of HMGA1 resulted in reduction of Ki-67 immunoreactivity *in vivo*. Each tumour slide was stained for Ki-67 and the number of Ki-67 positive cells were counted in at least 5 randomly selected fields at X40 magnification. Representative tumour sections stained for Ki-67 immunoreactivity in MiaPaCa2 and PANC1 tumour xenografts are shown. *P<0.05 versus control shRNA transfectant-derived xenografts. *B*, HMGA1 silencing led to increased apoptosis in tumour xenografts as demonstrated on TUNEL staining. TUNEL-positive cells were counted in at least 5 randomly selected fields at X40 magnification in each xenograft slide. Representative tumour sections stained for TUNEL in MiaPaCa2 and PANC1 tumour xenografts are shown. *P<0.05 versus control shRNA transfectant-derived xenografts. Values are means (± SD).

6.4 DISCUSSION

This study was designed to investigate the role of HMGA1 in a further aspect of the malignant phenotype: anchorage-independent growth. We have shown that HMGA1 overexpression promotes pancreatic adenocarcinoma tumourigenesis via a PI3-K/Akt-dependent mechanism. Taken together, this study suggests that HMGA1 may be a mechanism by which pancreatic tumour cells acquire their biological aggressiveness which is so evident clinically. Clearly, HMGA1 may represent a novel therapeutic target in pancreatic cancer.

Our findings indicate that HMGA1 promotes colony formation by pancreatic cancer cells under anchorage-independent conditions *in vitro* and tumourigenesis *in vivo*. In contrast, modulating HMGA1 expression in pancreatic cancer cells has no impact on their proliferation under standard monolayer culture conditions, an observation suggesting that HMGA1 does not act simply as a mitogenic stimulus. This implies that HMGA1 assumes a greater functional importance under anchorage-independent conditions, suggesting HMGA1 may influence important cancer survival pathways. In addition, these findings are not surprising, given that kinetics of three-dimensional colony formation *in vitro* more closely approximates those of *in vivo* tumour growth than cells in monolayer culture (Demicheli et al., 1989). The normal cellular response to deprivation from appropriate contact with substratum is to undergo apoptosis which, in this context, is termed anoikis (Frisch & Francis, 1994). A defining feature of transformed cells is resistance to anoikis and the ability to proliferate under anchorage-independent conditions (e.g., in soft agar). This feature of the malignant phenotype is conceptualized to contribute to tumourigenesis and metastasis in a wide range of cancers (Douma et al., 2004; Takaoka et al., 1997; Yawata et al., 1998). Anchorage independent growth is probably a result of the ability to proliferate in the absence of substratum and to resist apoptosis due to loss of substratum. To address the question of which aspect is modulated by

HMGA1, our group previously investigated the specific roles of HMGA1 in anoikis by investigating the apoptotic status of pancreatic adenocarcinoma cells grown in polyHEMA-coated plates (Chapter 5) (Liau et al., 2007). We found that HMGA1 overexpression promotes anoikis resistance in pancreatic adenocarcinoma cells. Potentially, HMGA1 mediates its function in tumourigenesis by enhancing resistance to apoptosis and allowing continued three-dimensional growth.

Our findings are consistent with those of a previously reported study in which HMGA1 overexpression was shown to induce anchorage-independent growth in breast epithelial cells (Reeves et al., 2001). Our study builds on this observation by defining a novel mechanism through which HMGA1 mediates anchorage-independent cellular proliferation: PI-3K/Akt signaling. Our data clearly demonstrate that intact PI-3K/Akt signaling is necessary for HMGA1 overexpression to promote colony formation in soft agar. They also demonstrate that constitutively active PI-3K/Akt signaling is sufficient to maintain the capacity for colony formation in soft agar in the context of HMGA1 silencing. In Chapter 4, we confirmed the effects of modulating HMGA1 expression on the functional status of Akt-dependent pathways by assessing its effects of mTOR phosphorylation, a well-known downstream target of Akt (Liau et al., 2006). We found that modulation of HMGA1 expression had a direct effect on mTOR phosphorylation, indicating that HMGA1 does have a functional effect on the PI3-K/Akt/mTOR pathway. Although the mechanism through which HMGA1 modulates the activity of PI3-K/Akt pathway remains unknown, clues can be obtained from a previous study which identified genes regulated by HMGA1 using cDNA microarray analysis (Reeves et al., 2001). Among the list of genes, multiple fibroblast growth factor (FGF) pathway components (e.g. FGFR1, FGF2b, FGF6, FGF7, FGF9) seem to be positively regulated by HMGA1. It is plausible that induction of the FGF pathway, by binding of FGF to its receptors could result in downstream stimulation of survival signaling pathways such PI3-K/Akt as demonstrated in this study (Wente et al., 2006). Further, our study adds to the

growing body of evidence suggesting that Akt represents a molecular hub for signaling events mediating cellular proliferation and resistance to apoptosis under conditions in which cells are deprived of contact with matrix (Nakanishi et al., 2002). Our *in vivo* data provide corroborating findings: HMGA1 silencing was associated with reductions in cellular proliferation and increases in apoptosis, corresponding to overall reductions in tumour growth. These findings are important as they demonstrate that HMGA1 silencing alters the tumour behaviour *in vivo* and confirms our hypothesis that HMGA1 suppression promotes tumour apoptosis and reduces tumour proliferation *in vivo*. This corroborates with our in vitro findings that the PI3-K/Akt survival pathway is downstream of HMGA1 and may mediate the *in vitro* and *in vivo* apoptotic and anti-growth effects of HMGA1 suppression.

It is now clear that HMGA1 exerts its effects not only by altering the conformational structure of DNA. More importantly, accumulating evidence suggests that HMGA1 exerts its functions by other mechanisms. Recent studies have suggested that HMGA1 is capable of inhibiting the functions of p53, a well-known oncosuppressor gene, by cytoplasmic relocalization of its proapoptotic activator HIPK2 (Frasca et al., 2006; Pierantoni et al., 2007). Nuclear HMGA1 has also been described to directly influence mitochondrial functions (Dement et al., 2006). Further study is likely to reveal increasing complexity in the mechanisms mediating the biological actions of HMGA1. HMGA1 is already known to modulate the expression of a wide array of genes, many of which are implicated in mediating the malignant phenotype (reviewed by Reeves et al) (Reeves, 2001). Which of these genes represent critical downstream effectors of HMGA1 signaling remain to be defined. Given a previous report suggesting a role for HMGA1-dependent MEK/ERK signalling (Treff et al., 2004), we tested the effects of inhibiting this pathway. In our study, although overexpression of HMGA1 resulted in ERK activation, inhibition of this pathway with the small molecule inhibitor PD98059 had no impact on proliferation in soft agar by cells with ectopic HMGA1 overexpression. Further, HMGA1 silencing clearly resulted in reductions in

cellular proliferation in soft agar but had no impact on ERK phosphorylation. Taken together, these findings suggest that HMGA1-induced cellular proliferation in soft agar growth is independent of MEK/ERK signaling.

In summary, HMGA1 promotes anchorage-independent growth by pancreatic cancer cells through a PI-3K/Akt-dependent mechanism. Given the minimal or absent expression of HMGA1 in normal adult tissues, HMGA1 represents a potential therapeutic target in pancreatic cancer.

Chapter Seven: HMGA1 is a novel molecular determinant of chemoresistance to gemcitabine

7.1 ABSTRACT

Background: HMGA1 proteins are architectural transcription factors that are overexpressed by pancreatic adenocarcinomas. In this study, we tested the hypothesis that HMGA1 promotes chemoresistance to gemcitabine in pancreatic cancer cells.

Methods and results: Stable shRNA-mediated HMGA1 silencing in BxPC3, MiaPaCa2 and PANC1 cells promoted chemosensitivity to gemcitabine, with reductions in gemcitabine IC50 and increases in gemcitabine-induced apoptosis and caspase activation. In contrast, forced HMGA1 overexpression in MiaPaCa2 cells promoted chemoresistance to gemcitabine, with increases in gemcitabine IC50 and reductions in gemcitabine-induced apoptosis and caspase activation. Given that HMGA1 modulates Akt activation, dominant negative Akt abrogated HMGA1 overexpression-induced increases in chemoresistance to gemcitabine. Finally, HMGA1 silencing promoted chemosensitivity to gemcitabine *in vivo* in a nude mouse xenograft model of pancreatic adenocarcinoma.

Conclusions: Our findings suggest that HMGA1 promotes chemoresistance to gemcitabine through an Akt-dependent mechanism. Targeted therapies directed at HMGA1 represents a potential strategy for ameliorating chemoresistance in pancreatic adenocarcinoma.

7.2 INTRODUCTION

Overall prognosis for patients diagnosed with pancreatic adenocarcinoma remains dismal. At the time of diagnosis, most patients have locally-advanced or metastatic disease precluding surgical resection (Sener et al., 1999). First line therapy for most patients with advanced pancreatic cancer is based on the nucleoside analogue gemcitabine. However, the clinical response rate to gemcitabine remains modest, in large part due to the profound chemoresistance inherent in pancreatic cancer cells. Therefore, characterization of molecular mechanisms mediating chemoresistance in pancreatic adenocarcinoma is an important priority. Furthermore, chemosensitizing strategy through the use of a novel molecular therapy is an appealing concept as it allows maximum tumour cell kill with the low serum chemotherapy levels achieved, to allow for tolerable toxicity.

In Chapter 4, we have previously reported the roles of HMGA1 in mediating cellular invasiveness and metastatic potential of pancreatic adenocarcinoma cells (Liau et al., 2006). Overexpression of HMGA1 promotes cellular invasion *in vitro* whilst post-transcriptional silencing of HMGA1 inhibits the ability of pancreatic adenocarcinoma cells to form metastases *in vivo*. Furthermore, we demonstrated that overexpression of HMGA1 is associated with increased soft agar growth (see Chapter 6) and resistance to apoptosis under anchorage-independent culture ('anoikis resistance') (see Chapter 5) (Liau et al., 2007). In the above studies, we have reported that Akt is a key downstream effector of HMGA1-dependent signaling in pancreatic cancer. Given the central role of Akt in mediating chemoresistance to gemcitabine (Duxbury et al., 2004; Ng et al., 2000; Ng et al., 2001), we hypothesized that HMGA1 would promote chemoresistance to this agent through an Akt-dependent mechanism. In this study, we have found that overexpression of HMGA1 promotes chemoresistance to gemcitabine through an Akt-dependent process. Lastly, we have demonstrated that targeted post-transcriptional silencing of HMGA1 promotes chemosensitivity *in vivo*, further confirming our

in vitro findings. Our findings are of particular clinical importance as they suggest that targeted therapies directed against HMGA1 may ameliorate chemoresistance to gemcitabine.

7.3 RESULTS

7.3.1 Targeted silencing of HMGA1 promotes chemosensitivity to gemcitabine in pancreatic adenocarcinoma cells

To begin to test our hypothesis that HMGA1 expression is a determinant of chemoresistance to gemcitabine, we used stable shRNA-mediated gene silencing to suppress HMGA1 expression in MiaPaCa2 cells. To help control for potential off-target effects of RNA interference, we transfected cells with one of two shRNA-expressing plasmid vectors, each carrying one of two independent shRNA target sequences (shHMGA1-1 and shHMGA1-2). HMGA1 silencing was confirmed on Western analysis; shHMGA1-1 was associated with a greater degree of HMA1 silencing than shHMGA1-2 (Figure 7.1). Suppression of HMGA1 expression using either shRNA target sequence resulted in significant reductions in IC50 to gemcitabine, as compared with control cells stably expressing non-targeting shRNA (Figure 7.1).

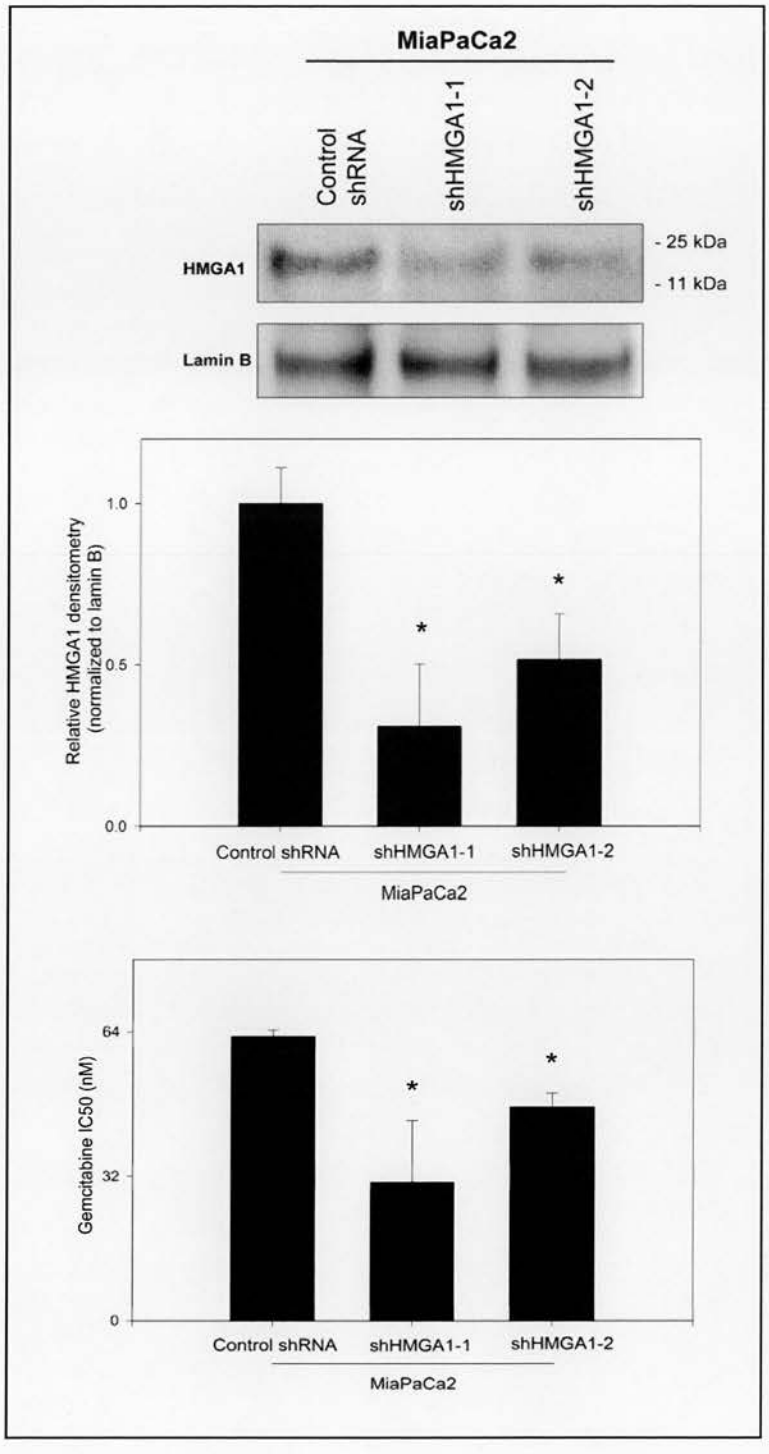


Figure 7.1. Plasmid-mediated stable silencing of HMGA1 expression in MiaPaCa2 cells using two independent short hairpin RNA (shRNA) target sequences (shHMGA1-1 and shHMGA1-2) was confirmed on Western blot analysis of nuclear extracts. Control cells were transfected with shRNA expression vectors carrying a scrambled, non-targeting sequence. Greater suppression of HMGA1 expression was achieved using shHMGA1-1 as compared to shHMGA1-2 sequence, with approximately 70% and 50% silencing respectively. The relative IC50 to gemcitabine was determined using survival curves generated from the MTS assay following 48-72 hours of treatment with 0 to 10μM gemcitabine. Silencing of HMGA1 using either shHMGA1-1 or shHMGA-2 sequence significantly reduced the relative IC50 to gemcitabine compared to controls, indicating an increased in chemosensitivity. The use of two independent shRNA sequences helped to control for any off-target effects of shRNA. *P<0.05 versus control shRNA.

7.3.2 Generation of high-titer lentiviral particles expressing shHMGA1 and lentivirus-mediated HMGA1 silencing

Having shown that HMGA1 silencing promotes chemosensitivity to gemcitabine using two independent shRNA target sequences, we generated high-titre lentivirus particles (see Section 2.6.5) expressing shHMGA1-1, the sequence which was consistently associated with greater silencing efficacy than shHMGA1-2. In the following experiments, lentivirus-mediated HMGA1 silencing with the shHMGA1-1 target sequence was used. Lentivirus-mediated silencing of HMGA1 is highly consistent and reproducible in the three pancreatic adenocarcinoma cell lines used, BxPC3, PANC1 and MiaPaCa2. Following lentiviral transduction (MOI of 10) and selection in puromycin, we achieved almost complete silencing of HMGA1 (Figure 7.2).

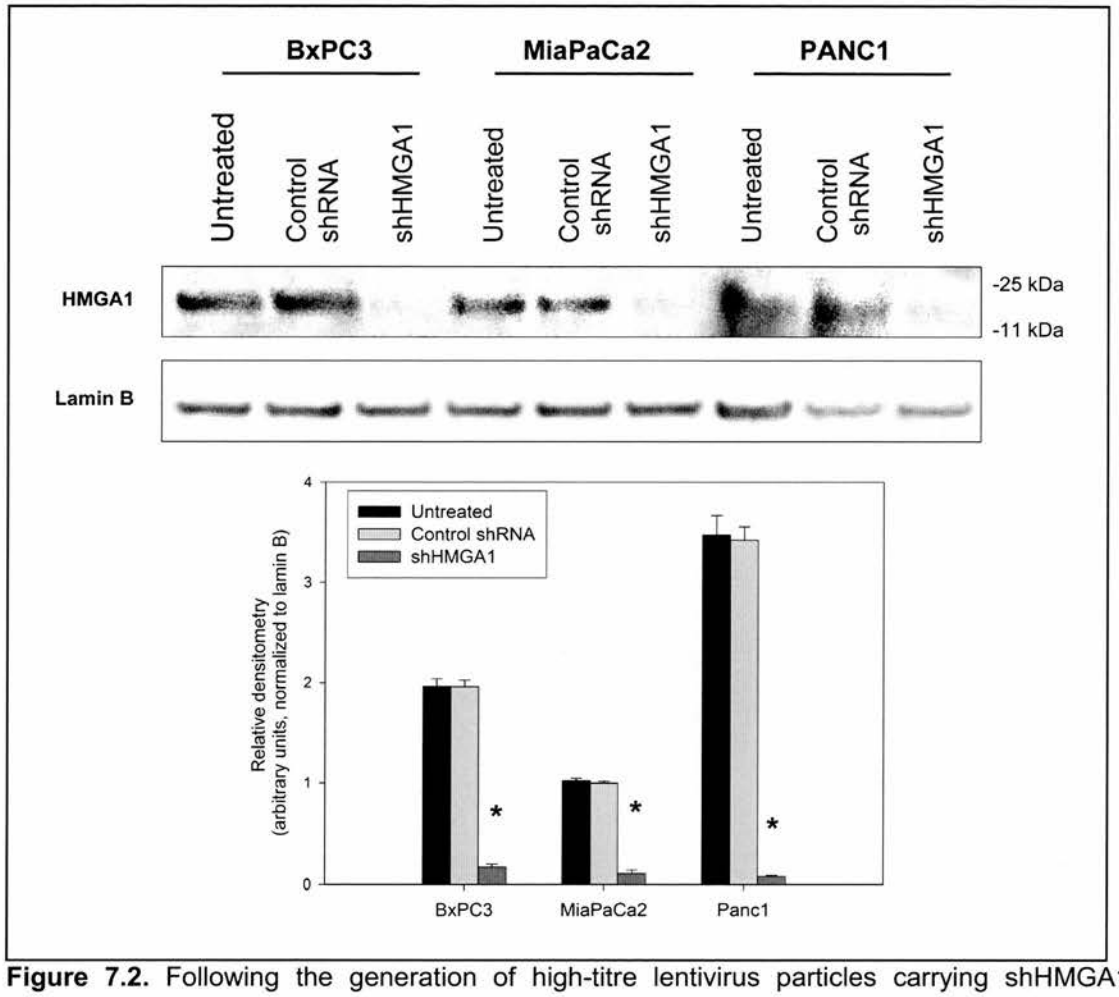


Figure 7.2. Following the generation of high-titre lentivirus particles carrying shHMGA1 (shHMGA1-1 sequence), BxPC3, MiaPaCa2 and PANC1 cells were transduced with lentivirus at MOI of 10 and stable transfectants were developed following selection with puromycin. Robust suppression of HMGA1 was achieved using lentivirus with a high degree of silencing of HMGA1 as compared to plasmid-mediated shRNA. In all three cell lines, lentivirus-mediated shHMGA1 achieved almost complete silencing of HMGA1. Controls were stable transfectants developed using lentivirus carrying scrambled, non-targeting shRNA. *P<0.05 versus control shRNA.

7.3.3 HMGA1 silencing promotes chemosensitivity to gemcitabine

Lentivirus-mediated HMGA1 silencing resulted in a marked increase in chemosensitivity to gemcitabine in BxPC3 cells, with approximately four-fold reductions in IC50 to gemcitabine (mean IC50: control shRNA versus. shHMGA1; 50nM versus 12 nM, p=0.001) (Figure 7.3A, B and C). Interestingly, BxPC3 cells in which HMGA1 had been silenced developed

spiculated morphology on exposure to 1μM gemcitabine for 48 hours, whereas control cells did not (Figure 7.3A). There were no morphological changes in PANC1 cells when exposed to gemcitabine apart from reductions in viability with HMGA1 silencing (Figure 7.3A). Lentivirus-mediated HMGA1 silencing had similar effects on PANC1 (Figure 7.3D and E) and MiaPaCa2 (Figure 7.4A and B) cells, with shifting of the gemcitabine IC50 curves to the left and approximately two-fold reductions of IC50. In MiaPaCa2 cells, the mean gemcitabine IC50 reduced from 60 nM (control shRNA) to 30nM (shHMGA1) with silencing of HMGA1 ($p=0.001$). In PANC1 cells, suppression of HMGA1 led to reduction of mean gemcitabine IC50 from 128 nM (control shRNA) to 65 nM (shHMGA1) ($p=0.001$).

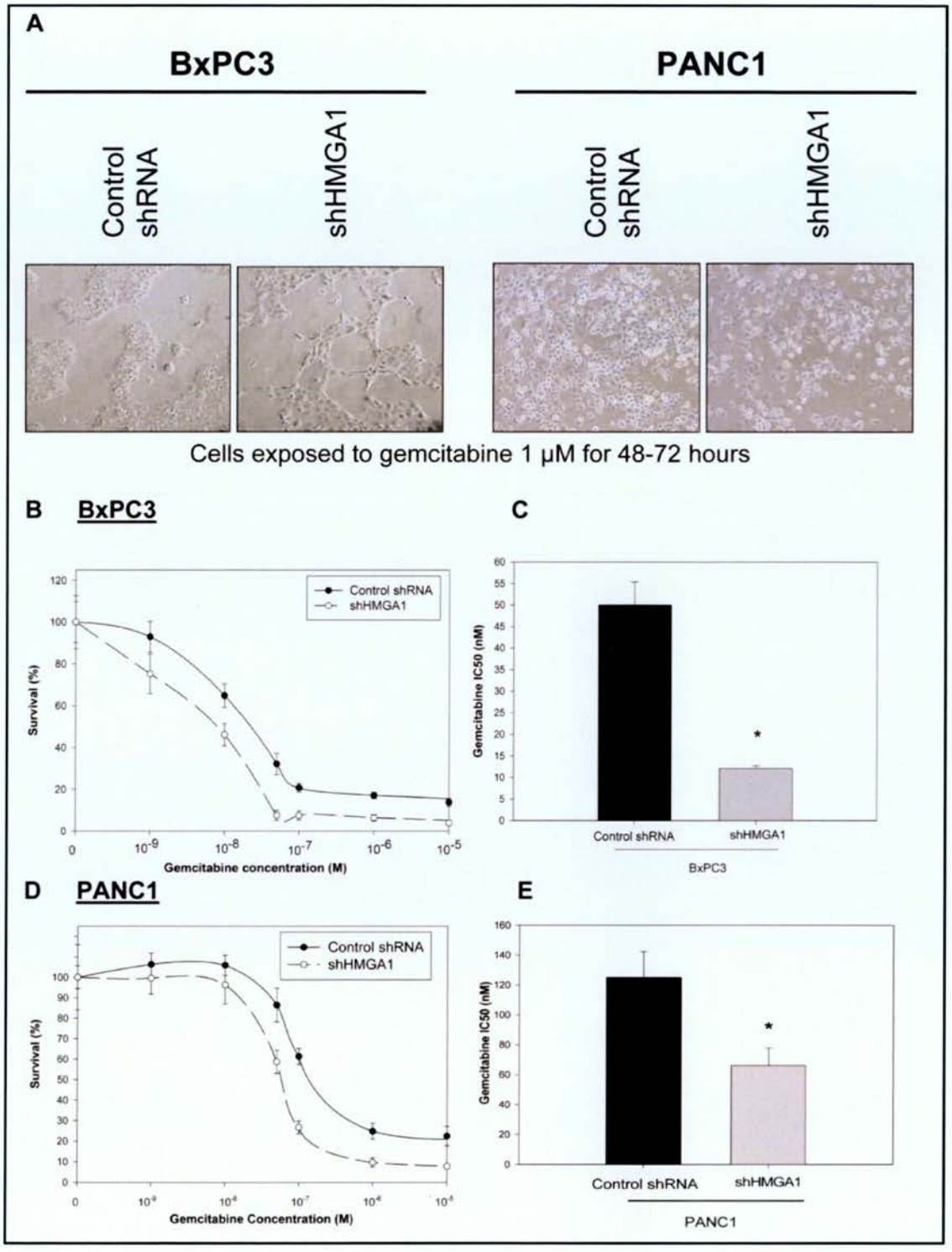


Figure 7.3. *A*, The effects of lentivirus-mediated HMGA1 silencing on chemosensitivity to gemcitabine were assessed. When BxPC3 cells in which HMGA1 had been silenced were exposed to 1μM gemcitabine for 48 hours, they adopted a less healthy, spiculated morphology as compared to control cells. Photomicrographs were taken using an inverted microscope at 40X magnification. In PANC1, there were no morphological changes apart from reductions in viability on exposure to gemcitabine, with HMGA1 silencing. *B-E*, Survival

curves following exposure to 0-10μM gemcitabine were analyzed following MTS assay. Lentivirus-mediated stable HMGA1 silencing in BxPC3 cells shifted the survival curve to the left indicating an increased in chemosensitivity to gemcitabine, when compared to the controls. Correspondingly, there was 4-fold reductions in the mean IC50 to gemcitabine with silencing of HMGA1 when compared to the controls. In PANC1 cells, there was similar effect with two-fold reduction in mean IC50. *P<0.05 versus control shRNA.

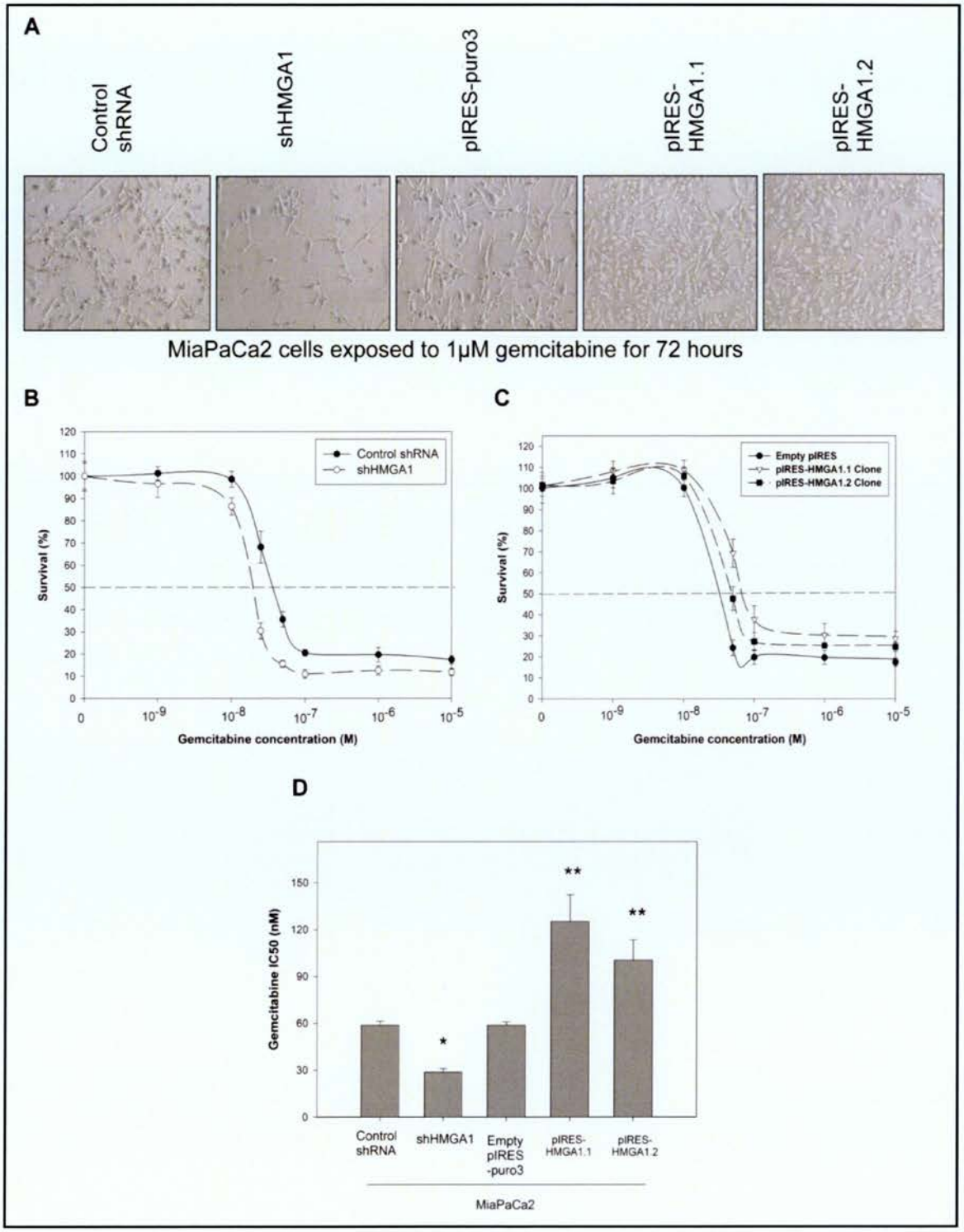


Figure 7.4. *A*, The effects of modulating HMGA1 expression on chemosensitivity to gemcitabine in MiaPaCa2 cells were assessed. In MiaPaCa2 cells, lentivirus-mediated silencing of HMGA1 resulted in marked reductions in cellular viability as compared to controls when exposed to 1μM gemcitabine for 72 hours. In contrast, MiaPaCa2 cells with HMGA1 overexpression (pIRES-HMGA1.1 and 1.2) demonstrated increased viability following

exposure to 1μM gemcitabine, when compared to empty pIRES-puro3 transfectants. Photomicrographs were taken using an inverted microscope at 40X magnification. *B-C*, Lentivirus-mediated RNA interference of HMGA1 in MiaPaCa2 cells resulted in increases in chemosensitivity to gemcitabine with shifting of survical curves to the left while overexpression of HMGA1 led to increases in chemoresistance to gemcitabine with shifting of the survival curves to the right in both pIRES-HMGA1.1 and 1.2 clones, compared to their respective controls. *D*, Targeted suppression of HMGA1 using lentivirus-mediated shHMGA1 resulted in 2-fold reductions in IC50 compared to control, while overexpression of HMGA1 in pIRES-HMGA1.1 and 1.2 clones resulted in approximately 2.2- and 1.7-fold increases in IC50 to gemcitabine respectively. *P<0.05 versus control shRNA transfectants. **P<0.05 versus empty pIRES-puro3 transfectants.

7.3.4 HMGA1 is a molecular determinant of chemoresistance to gemcitabine in pancreatic adenocarcinoma cells.

We then tested the impact of forced overexpression of HMGA1 on cellular chemoresistance to gemcitabine. MiaPaCa2 cells (which have low inherent expression of HMGA1) were stably transfected with the pIRES-HMGA1 vector, as described in Section 2.6. We selected two transfectant clones with highest expression levels of HMGA1 (pIRES-HMGA1.1 and pIRES-HMGA1.2). In our previous studies (Chapters 4, 5 and 6), we have characterized the pIRES-HMGA1.1 and pIRES-HMGA1.2 clones and verified their overexpression of HMGA1 by Western analysis. pIRES-HMGA1.1 and pIRES-HMGA1.2 clones overexpress HMGA1 by 4-fold and 3.5-fold respectively, when compared with empty pIRES-puro3 transfectants. Overexpression of HMGA1 resulted in significant increases in chemoresistance to gemcitabine, with increases in IC50 to gemcitabine (Fig 7.4D) and shifting of the IC50 curves to the right for both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Fig 7.4C). The mean IC50s for pIRES-HMGA1.1 and pIRES-HMGA1.2 were 130nM and 100nM respectively, compared to 60nm of empty pIRES-puro3 controls (p=0.003 and p=0.006 versus empty pIRES-puro3 controls). Of note, pIRES-HMGA1.1 consistently overexpressed HMGA1 at a greater level than pIRES-HMGA1.2 and consequently, the IC50 for pIRES-HMGA1.1 was also greater. This implies

that the level of HMGA1 expression does correspond closely with degree of chemoresistance. Forced HMGA1 overexpression enhanced the viability of cells exposed to 1μM of gemcitabine for 72 hours, whereas HMGA1 silencing was associated with the opposite effect (Figure 7.4A).

7.3.5 HMGA1 expression status modulates gemcitabine-induced apoptosis and caspase 3 activation

Following exposure to 1 μM gemcitabine for 24 hours, cells were subjected to flow cytometric quantitation of apoptosis and fluorometric caspase 3 profiling. Lentivirus-mediated HMGA1 silencing was associated with increases in gemcitabine-induced apoptosis (Figure 7.5A) and caspase 3 activation (Figure 7.6A) in BxPC3, MiaPaca2 and PANC1 cells. In contrast, HMGA1 overexpression was associated with reductions in gemcitabine-induced apoptosis (Figure 7.5B) and in caspase 3 activation (Figure 7.6B).

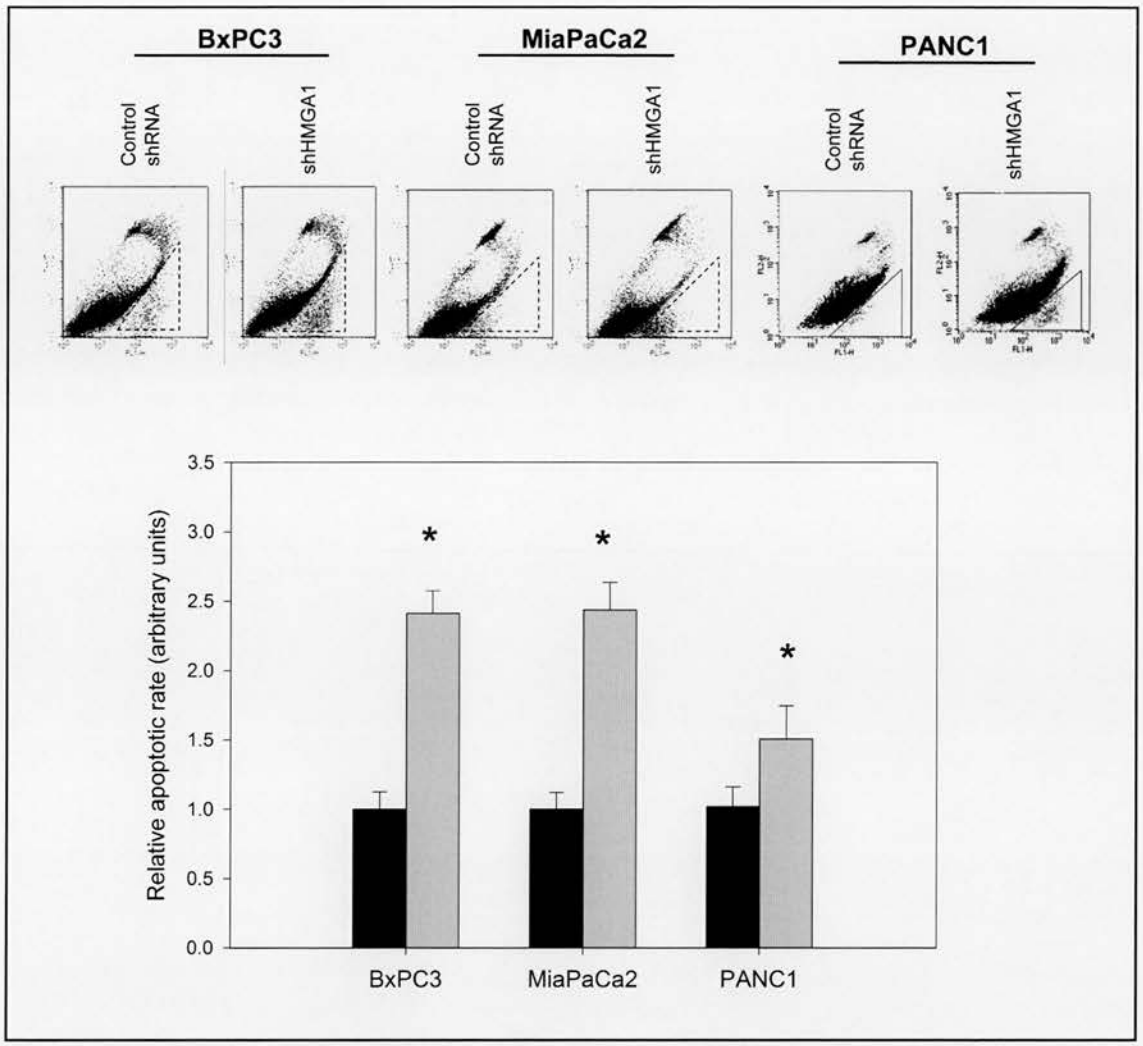


Figure 7.5A. For full legend, see pg 199.

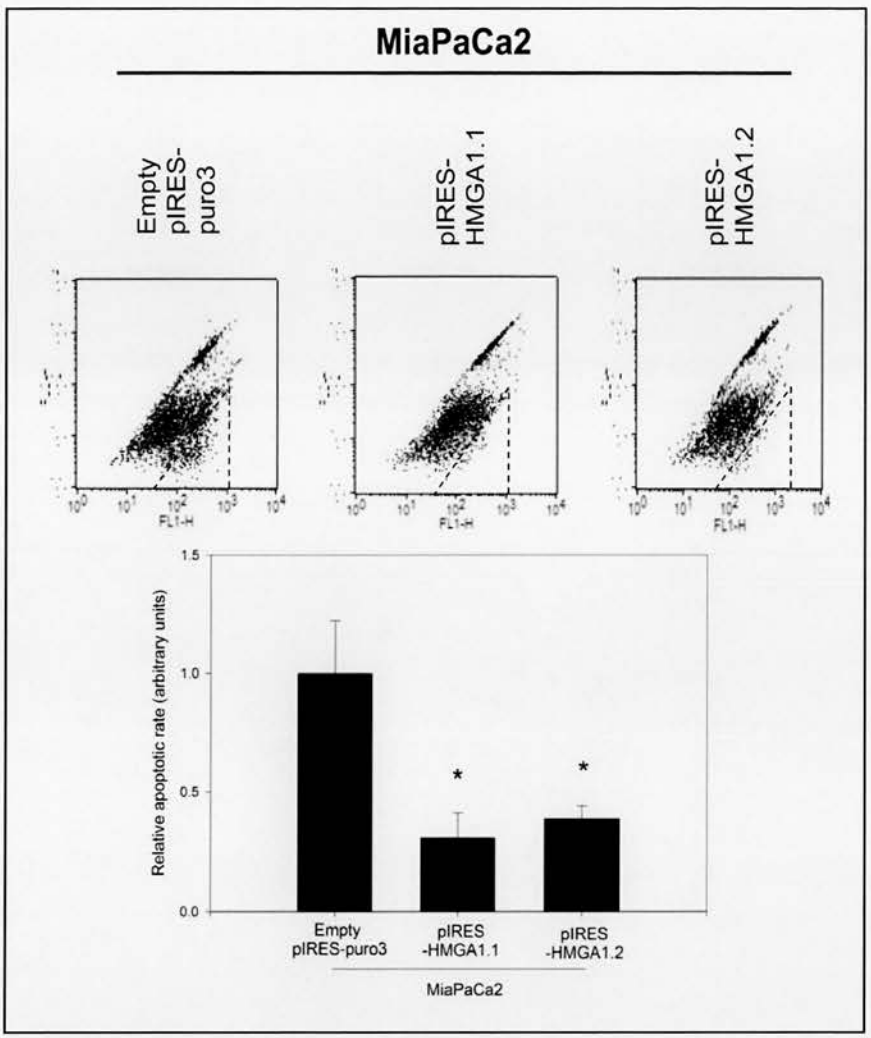


Figure 7.5B. For full legend, see below.

Legend

Figure 7.5. *A*, Lentivirus-mediated silencing of HMGA1 promoted gemcitabine-induced apoptosis as assessed by flow cytometric analyses of YO-PRO-1/Propidium iodide-stained cells. Silencing of HMGA1 resulted in approximately 2-fold increases in the relative apoptotic rates in both BxPC3, MiaPaCa2 and PANC1 cells following exposure to 1μM gemcitabine for 24 hours. *P<0.05 versus control shRNA. Representative flow cytometric images of three experiments are shown, with the apoptotic fractions being highlighted in triangles drawn. *B*, Forced overexpression of HMGA1 in pIRES-HMGA1.1 and 1.2 clones protected the cells from gemcitabine-induced apoptosis with approximately 70-80% reductions in relative apoptotic rates, as assessed by flow cytometry.* indicates p<0.05 versus empty pIRES-puro3 control.

7.3.6 HMGA1-specific silencing enhances gemcitabine-induced activation of caspases 3, 8, 9 and 2

Caspase activation is required for gemcitabine-induced cytotoxicity in cancer cells (Nabhan et al., 2002). Having shown that HMGA1 modulation affects gemcitabine-induced caspase 3 activation, we sought to profile the effect of HMGA1 silencing on the caspase cascade after exposure to gemcitabine for 24 hours. Gemcitabine-induced activation of caspases 3, 8, 9 and 2 was markedly increased with targeted suppression of HMGA1 in MiaPaCa2 cells, compared to controls (Figure 7.6C).

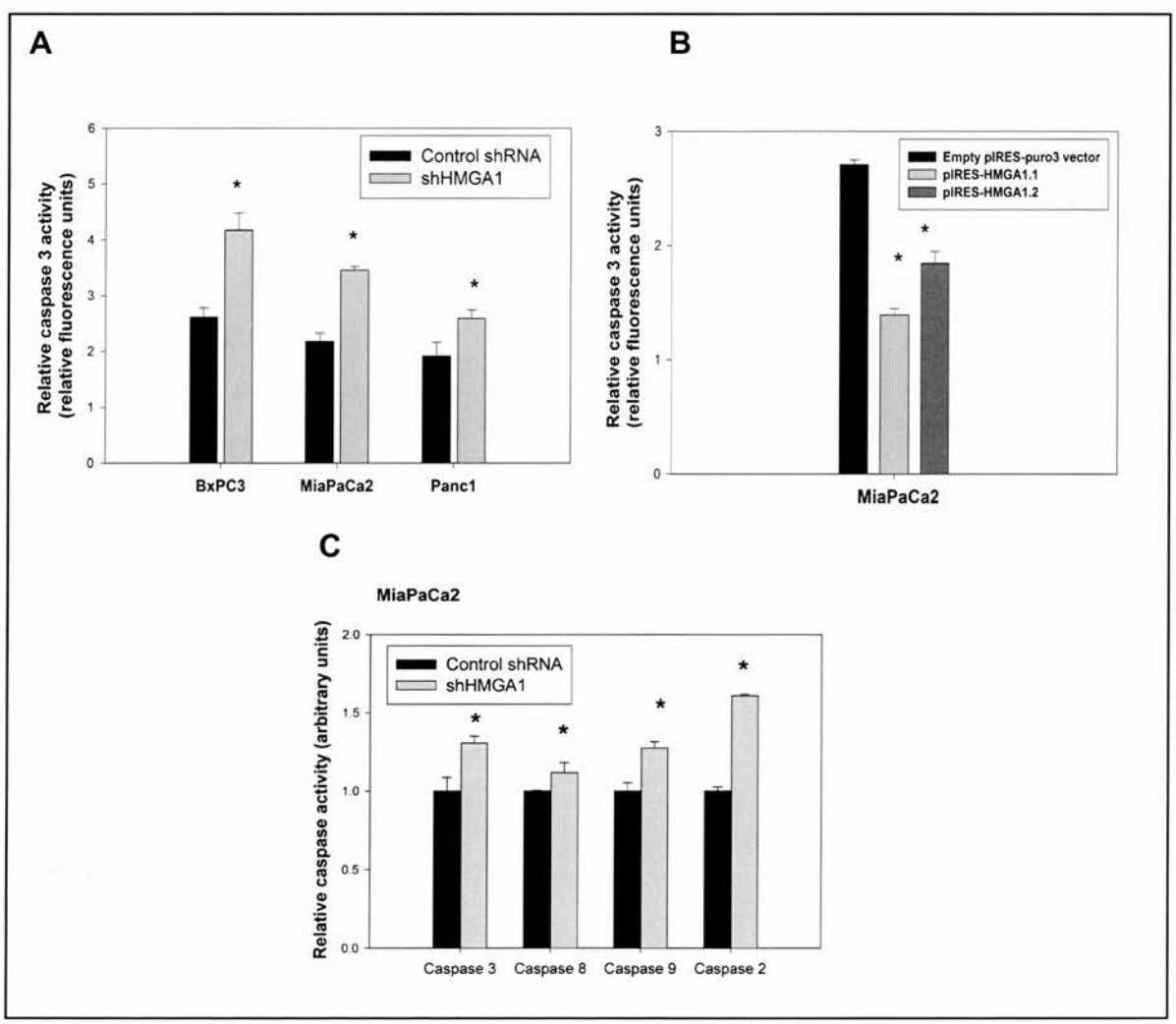


Figure 7.6. *A*, Relative caspase 3 activities were determined using a fluorometric caspase 3 substrate assay following exposure of cells to 1μM gemcitabine for 24 hours. Lentivirus-mediated silencing of HMGA1 promoted gemcitabine-induced caspase 3 activities. *P<0.05

versus control shRNA transfectants. *B*, As expected, overexpression of HMGA1 in pIRES-HMGA1.1 and 1.2 clones resulted in reductions in caspase 3 activation following exposure to 1μM gemcitabine for 24 hours, indicating protection from gemcitabine-induced caspase-mediated apoptosis. *P<0.05 versus empty pIRES-puro3 transfectants. *C*, Activities of caspases 3, 8, 9 and 2 were quantified using a fluorometric profiling assay after exposure to 1μM gemcitabine for 24 hours. Activities of each of the four caspases profiled exhibited a significant increase in MiaPaCa2 cells with lentiviral-mediated HMGA1 silencing, compared to controls. * P<0.05 versus control shRNA transfectants.

7.3.7 HMGA1-induced chemoresistance to gemcitabine is dependent on Akt signalling

We have previously reported that the Akt signaling pathway is a downstream mediator of HMGA1 (see Chapters 4, 5 and 6). In our previous reports, silencing of HMGA1 reduces Akt phosphorylation (a marker of Akt activation) whilst ectopic overexpression of HMGA1 results in an increase in Akt kinase activity through an increase in Akt phosphorylation (see Chapter 5). Given the importance of the PI3-K/Akt pathway in anti-apoptotic signaling, particularly in the context of chemoresistance, we sought to determine if chemoresistance to gemcitabine induced HMGA1 overexpression is Akt-dependent. Each of pIRES-HMGA1.1 and 1.2 clones were transduced with dominant negative Akt adenovirus at MOI of 10. The efficiency of transduction and expression of dominant negative Akt were confirmed by immunoblotting for the HA tag of the dominant negative Akt construct (Fig 7.7). We next assessed the effects of dominant negative Akt on chemosensitivity to gemcitabine in each HMGA1 overexpressing clone. Dominant negative Akt was found to reverse the chemoresistance induced by HMGA1 overexpression, with reductions of IC50 to gemcitabine in both pIRES-HMGA1.1 and 1.2 clones to levels similar to control and untreated cells. As such, HMGA1 overexpression-induced chemoresistance is dependent on Akt signaling.

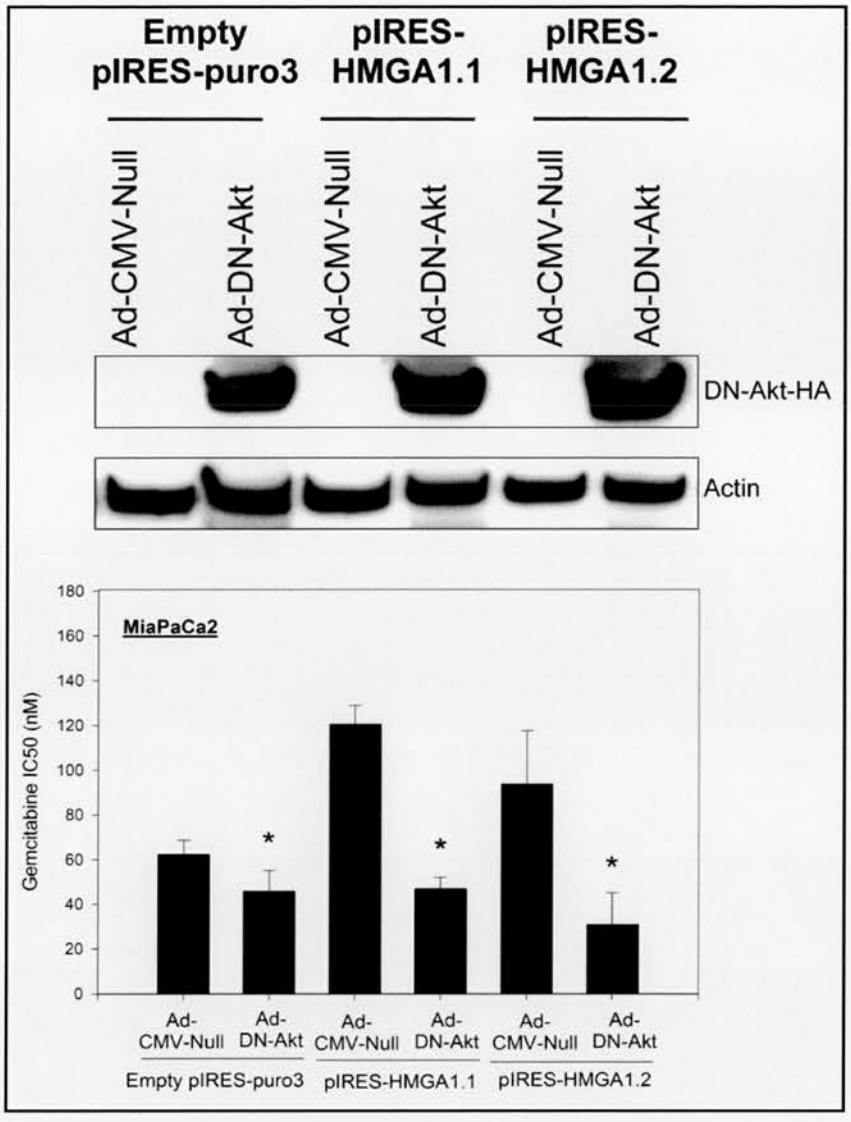


Figure 7.7. *A*, HMGA1-induced increases in chemoresistance to gemcitabine were dependent on the Akt signaling pathway. The roles of Akt in mediating the HMGA1-induced chemoresistance was assessed by transducing pIRES-HMGA1.1 and 1.2 clones with adenovirus carrying HA-tagged dominant negative Akt. Transduction efficiency and expression of dominant negative Akt were assessed by Western blotting for hemagglutinin (HA). Infection of pIRES-HMGA1.1 and 1.2 clones with adenovirus carrying dominant negative Akt (Ad-DN-Akt) resulted in significant reductions in IC50 to gemcitabine when compared to cells infected with control adenovirus (Ad-CMV-Null). Dominant negative Akt resulted in reductions in IC50 to gemcitabine in pIRES-HMGA1.1 and 1.2 clones to levels similar to parental MiaPaCa2 cells or empty pIRES-puro3 transfectants, indicating abrogation of the increased chemoresistance associated with HMGA1 overexpression. *P<0.05 versus control adenovirus (Ad-CMV-Null).

7.3.8 HMGA1 silencing promotes chemosensitivity to gemcitabine in vivo

BxPC3 cells in which HMGA1 had been silenced through stable lentiviral shRNA-mediated RNAi or control BxPC3 cells transduced with lentivirus carrying non-targeting shRNA were subcutaneously implanted into nude mice. Once the resulting xenograft tumours had grown to reach approximately $50mm^3$ in diameter, a 6-week course of gemcitabine administration was initiated. Tumours derived from BxPC3 cells in which HMGA1 had been silenced (n=8 animals) regressed during the treatment period while tumours derived from control cells (n=8 animals) continued to grow during the treatment period (Fig 7.8A-C). Stable suppression of HMGA1 expression in tumours derived from shHMGA1 transfectants was confirmed on Western blotting of nuclear extracts of tumour homogenates (Fig 7.9A) and on immunohistochemical analysis of xenografts harvested at the end of the study period (Fig 7.9B). TUNEL staining revealed a significantly higher apoptotic index in shHMGA1 transfectant-derived tumours than in control cell-derived tumours (Fig 7.9B and C).

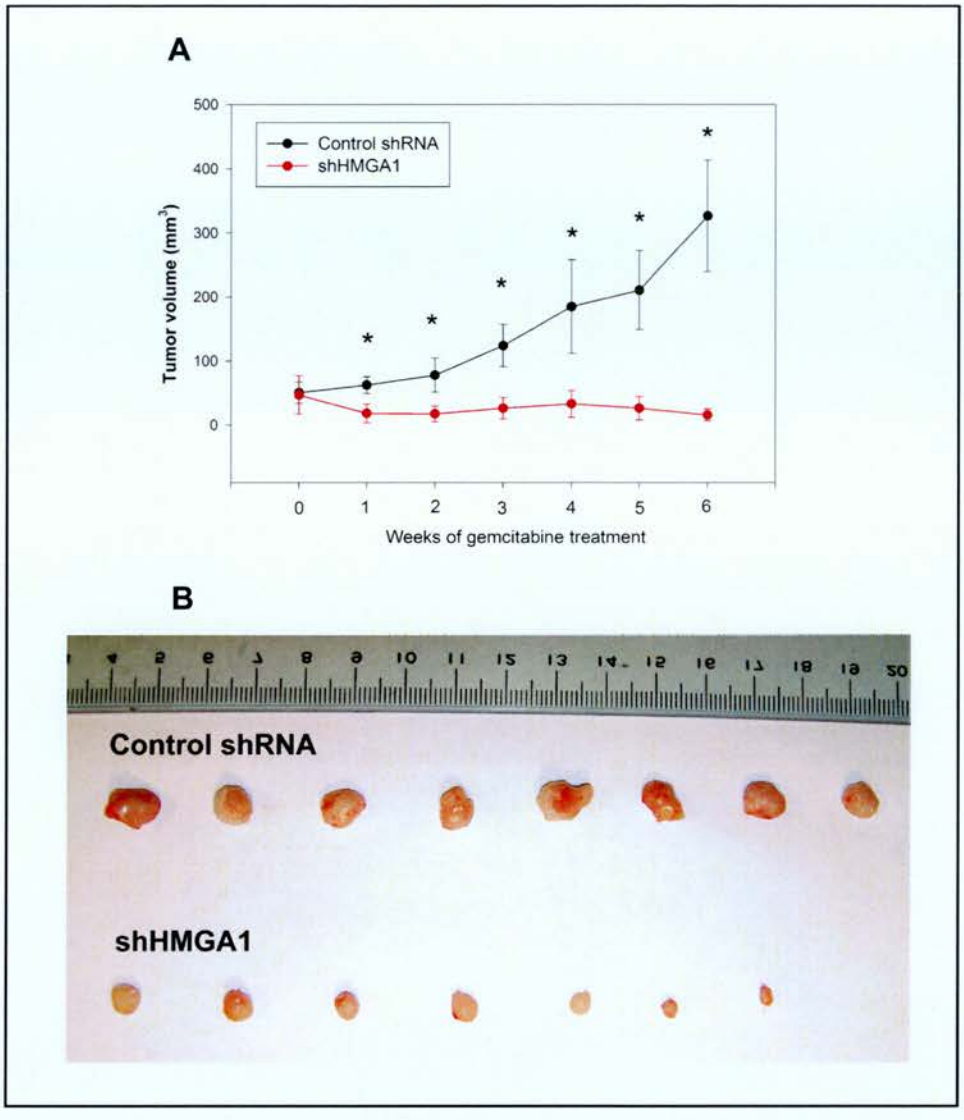


Figure 7.8. *A*, Stable silencing of HMGA1 promoted chemosensitivity to gemcitabine *in vivo* with evidence of tumour regression in nude mouse subcutaneous model. Mice (n=8 per group) were subcutaneously implanted with $2X10^6$ lentivirus-mediated stable transfectant BxPC3 cells (either shHMGA1 or control shRNA). Gemcitabine treatment was commenced in each group 14 days after implantation when the tumours were approximately $50mm^3$ in volume. Mice received gemcitabine (150 mg/kg) in 100 µL of PBS vehicle by twice-weekly i.p. injection. Subcutaneous tumour size was monitored weekly during the 6 weeks of treatment. Tumours with HMGA1 silencing showed evidence of regression in size during the treatment period while tumours in the control group continued to grow with time. Values are means (± SD). *P<0.05 versus control shRNA xenografts. *B*, The size of the explanted tumours at the end of the 6-week treatment period was shown. Of note, one of the mice in the shHMGA1 had the tumour completely regressed following 6 weeks of gemcitabine treatment.

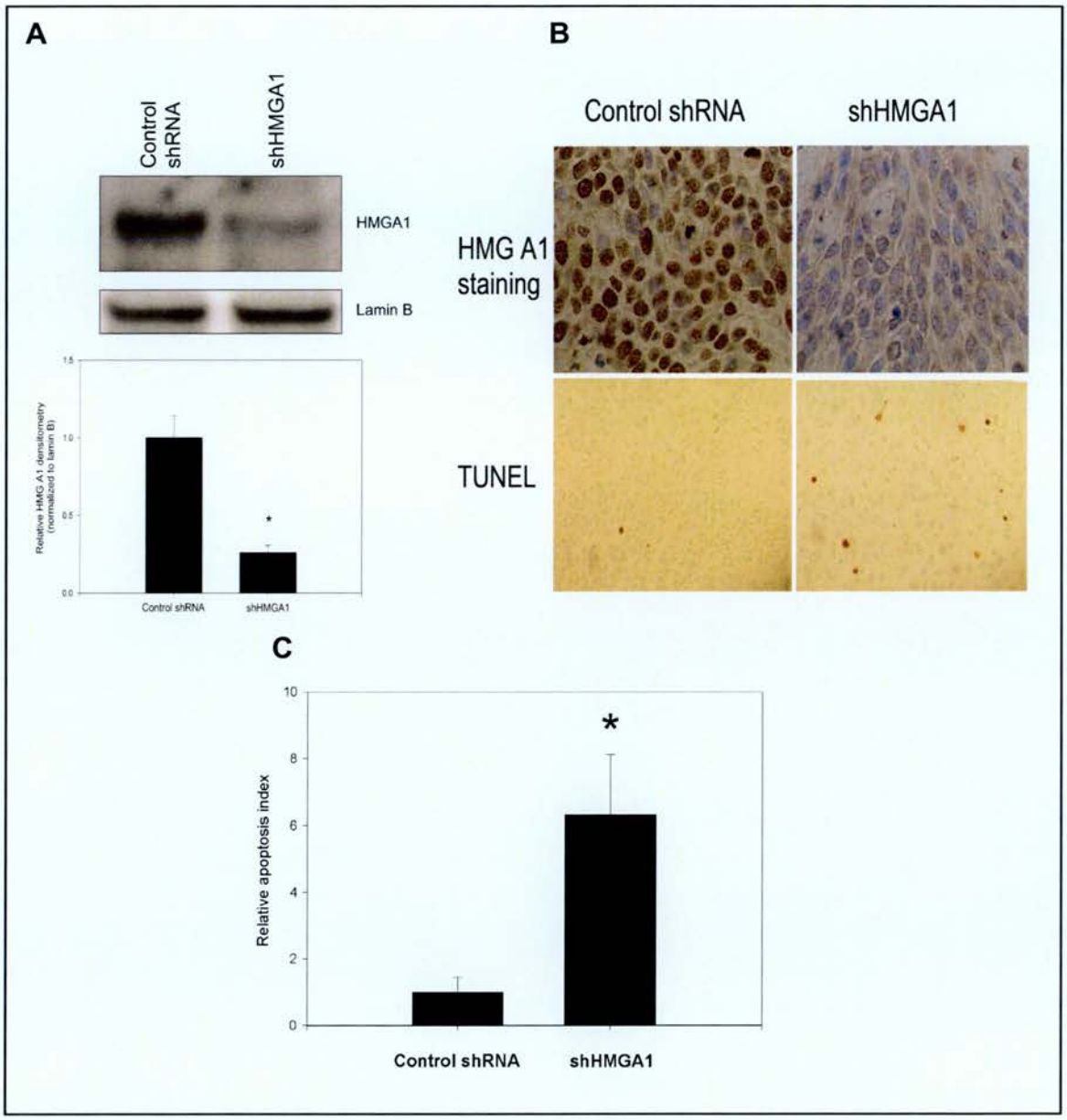


Figure 7.9. *A*, *In vivo* HMGA1 silencing was confirmed by Western blot analysis of nuclear extracts from explanted xenograft tumours. Densitometric values are means (± SD). *P=0.001 versus control shRNA xenografts. *B-C*, Immunohistochemistry of xenograft sections demonstrated little or absent staining for HMGA1 in the shHMGA1 xenografts, when compared to the control shRNA xenografts which showed intense staining for HMGA1. Photomicrograph of HMGA1 staining was obtained at 40X magnification. In the same sections, TUNEL staining was performed. In each tumour slide stained with TUNEL, the number of TUNEL-positive cells were counted in at least 5 randomly selected fields at 40X magnification. HMGA1 silencing led to significant increases in relative apoptotic index when compared to control shRNA xenografts. Representative tumour sections stained for TUNEL

photographed at X20 magnification are shown. *P<0.001 versus control shRNA xenografts. Values are means (± SD).

7.4 DISCUSSION

HMGA1 proteins are overexpressed in a wide range of human cancers, including pancreatic adenocarcinoma (see Chapter 3). Experimental data implicating biologically important roles for HMGA1 in cancer pathogenesis are rapidly accumulating (Reeves & Beckerbauer, 2001; Wood et al., 2000a). Our study provides the first data suggesting that HMGA1 may mediate a critical feature of malignant phenotype: chemoresistance. First, our findings demonstrate that forced HMGA1 overexpression promotes chemoresistance to gemcitabine in pancreatic cancer cells *in vitro*, while HMGA1 silencing promotes gemcitabine-induced cytotoxicity and therefore abrogates chemoresistance to gemcitabine. Second, we have confirmed that HMGA1 silencing promotes gemcitabine-induced cytotoxicity and increases chemosensitivity *in vivo* in a nude mouse xenograft model of pancreatic cancer. Finally, our findings suggest a plausible mechanism by which HMGA1 promotes chemoresistance to gemcitabine: activation of Akt signaling. These findings imply that targeted suppression or inactivation of HMGA1 could be a potential therapeutic manoeuvre to induce chemosensitivity to gemcitabine in this highly-chemoresistant cancer.

The pro-survival phosphatidylinositol 3-kinase (PI-3K)/Akt pathway is frequently activated in pancreatic cancer (Asano et al., 2004). The chemoresistant phenotype in pancreatic cancer cell lines has been associated with overexpression of genes involved in the PI-3K/Akt pathway (Akada et al., 2005). A previous study has highlighted the importance of PI-3K/Akt pathway in mediating the chemoresistance to gemcitabine in pancreatic cancer cells (Ng et al., 2000). The inhibition of PI-3K pathway has been shown to improve the therapeutic index of gemcitabine in pancreatic cancer *in vivo* (Ng et al., 2001). The exact mechanism through which PI-3K/Akt pathway induces

chemoresistance remains largely unknown. The primary mode of action of gemcitabine is to inhibit DNA synthesis by competing for incorporation into DNA. Gemcitabine is a nucleoside analogue (diflourodeoxycytidine) that is phosphorylated by deoxycytidine kinase. Gemcitabine triphosphate is incorporated into nascent DNA to inhibit DNA synthesis (Shore et al., 2003). Although we speculate that the primary action of HMGA1 in chemoresistance to gemcitabine is mediated through the PI3-K/Akt survival pathway, it is likely that HMGA1 may have some actions on the gemcitabine metabolism pathway. This will require further elucidation in future studies.

Mechanisms of HMGA1 action has been shown to be largely due to its ability to alter DNA conformation. Proposed mechanisms for gene regulation include derepression of gene promoter by displacement of histone H1 nucleoproteins, a strong repressor of gene transcription, from scaffold attachment regions (SAR), allowing for a more open chromatin structure that facilitates transcriptional activation (Zhao et al., 1993). Further, HMGA1 is able to bind to AT-rich promoter regions, and modify DNA conformation to encourage attachment of other transcriptional factors that promote gene transcription (Yie et al., 1999). By binding to promoter regions, HMGA1 is also able to form multiprotein complexes, the "enhanceosomes", that serve as transcription activating complexes (Yie et al., 1999). Given these putative functions, it is not surprising that HMGA1 is involved in the regulation of a large number of target genes. Previous studies have shown that HMGA1 overexpression is associated with increased expression of growth factors/cytokines (e.g. fibroblast growth factors (FGF), interferons α and β, interleukins 10-14 and 17), growth factor receptors (e.g. FGFR, EGFR, ERBB3 and 4) and multiple integrins (α1, α6, α9, αE, β1, β3, β8) (Reeves, 2001). Clearly, it is not surprising that through induction of these growth factor-related signaling pathways, HMGA1 could have an impact on the pro-survival PI3-K/Akt pathways. It is also plausible that by increasing integrin expression, HMGA1 may stimulate integrin-linked kinase, which is known to directly interact with

integrins and phosphorylate Akt in a PI3-K-dependent manner (Tabe et al., 2007).

In the current study, we have examined the effects of silencing HMGA1 on chemoresistance in BxPC3 and MiaPaCa2 pancreatic adenocarcinoma cells. BxPC3 cells are known to inherently overexpress HMGA1 whilst MiaPaCa2 cells have relatively lower inherent HMGA1 expression. Interestingly, although BxPC3 cells have a higher HMGA1 expression, their IC50 values to gemcitabine is comparable to MiaPaCa2 cells. On the other hand, PANC1 cells consistently have the highest level of HMGA1 expression and are the most chemoresistant to gemcitabine (see Figure 7.3). Interestingly, BxPC3 cells are known to have wild type K-ras whilst MiaPaCa2 and PANC1 cells are K-ras mutants (Aoki et al., 1997). This implies that there are other factors involved in chemoresistance such as differences in genetic alterations between cell lines. To examine the effects of modulating HMGA1 expression in a single cell line system, we performed both loss-of-function (HMGA1 silencing) and gain-of-function (HMGA1 overexpression) experiments on MiaPaCa2 cells. As such, by using the same cell line and theoretically controlling for other genetic alterations, we were able to demonstrate that HMGA1 silencing reduces chemoresistance whilst HMGA1 overexpression results in the reverse effects on chemoresistance.

The efficacy of gemcitabine-based regimens, although currently standard of care for the treatment of advanced pancreatic cancer, is limited by profound chemoresistance. Recently, the combination of gemcitabine and the human epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib has been reported, for the first time, to provide improved survival over single agent gemcitabine in patients with advanced pancreatic cancer in a phase III clinical trial (Moore et al., 2005). Although these results are encouraging, the benefits provided by the addition of erlotinib are only incremental. Truly transformative increases in efficacy are likely to arise only through incorporation of targeted therapies selected on the basis of rational understanding of mechanisms

mediating chemoresistance in pancreatic cancer. In this context, our findings suggest that HMGA1 warrants further investigation as a novel therapeutic target in this deadly cancer.

Chapter Eight: Conclusions

8.1 ROLES OF HMGA1 IN PANCREATIC CANCER

8.1.1 HMGA1 is a clinically relevant therapeutic target

The first aim of this research was to address the clinical relevance of *HMGA1* in pancreatic cancer. To do this, we determined the prevalence of HMGA1 overexpression in tumours of patients with pancreatic cancer. We found that more than 90% of pancreatic cancers have HMGA1 overexpression as determined using our tissue microarray analysis. Importantly, we also found that HMGA1 expression level is low or absent in normal pancreatic tissues. It is interesting that although HMGA1 expression is not correlated to any of the adverse clinicopathological characteristics (e.g. lymphovascular or perineural involvement), its expression was predictive of poorer patient survival and remained as an independent prognostic indicator on multivariate analysis. With these encouraging results, we moved to 'the bench' to perform *in vitro* experiments to elucidate the roles of HMGA1 in pancreatic cancer biology. Pancreatic adenocarcinoma cell lines with differing degrees of HMGA1 overexpression were used. Experiments were performed using the loss-of-function (RNA interference) and gain-of-function (ectopic overexpression) approaches to analyse the effects of modulating HMGA1 expression on cancer phenotype. We demonstrated that targeted suppression of HMGA1 using RNAi significantly attenuated pancreatic cancer progression by inhibiting cellular invasiveness and anchorage-independent proliferation. Further, HMGA1 overexpression promoted anoikis resistance, which is a phenotypic determinant of metastasis. We also showed that HMGA1 is an important regulator of chemoresistance in PDAC cells. More importantly, we translated the *in vitro* findings to the *in vivo* settings, as we demonstrated that targeted silencing of HMGA1 significantly attenuated metastasis, tumour growth and promoted chemosensitivity in xenograft mouse models. Our research provided compelling data that HMGA1 plays a major role in the

biology of pancreatic cancer and its expression is not just a phenomenon associated with malignancy with no functional significance, as previously speculated (Evans et al., 2004). Following on from this, it is not surprising that patients with HMGA1 overexpressing pancreatic cancers will have a more aggressive disease and hence, poorer prognosis.

8.1.2 Pro-oncogenic downstream pathways

The strength of this work lies in the mechanistic dissection of the downstream molecular pathways of HMGA1 which are relatively unstudied so far. Our preliminary results (see Chapter 4) suggest that HMGA1 is able to regulate MMP-9 expression through its effects on the transcriptional activity of *MMP-9* gene promoter. Given that the PI3-K/Akt is a well-known pathway that regulates MMP-9 expression (Ellerbroek et al., 2001; Lu & Wahl, 2005; O. Charoenrat et al., 2004), we speculated that HMGA1 would modulate PI3-K/Akt activity. Further clues on the nature of downstream molecular mediators of HMGA1 were gleaned from studies of HMGA1 in non-cancer models. Foti and colleagues reported a genetic flaw in four diabetic patients that markedly reduced their HMGA1 expression leading to decreased insulin receptor expression in their tissues (Foti et al., 2005). They went on to demonstrate that HMGA1-knockout mice have decreased insulin receptor expression and impaired insulin signaling leading to phenotype characteristic of type II diabetes. Given that PI3-K/Akt is a crucial downstream mediator of insulin receptor, they demonstrated that muscle tissues from HMGA1-knockout mice have significantly lower PI3-kinase activities and hence lower levels of Akt activation. Based on the above data, we explored if modulating HMGA1 expression in pancreatic cancer cells would have an effect on PI3-K/Akt signaling pathway, which is a well-known pro-oncogenic pathway.

We found that HMGA1 modulation directly regulates Akt activation. In particular, we described a novel pathway of HMGA1/Akt/MMP9 which controls cellular invasiveness in PDAC cells. Although we showed that HMGA1 is able

to regulate the *MMP-9* gene promoter transcriptional activity which is dependent on PI3-K/Akt activity, our study did not address if HMGA1 is able to bind to the MMP-9 promoter and directly activate the promoter activity as an alternative mechanism. This mechanism is possible as the analysis of *MMP-9* gene promoter did reveal multiple segments of AT-rich DNA which are classic positions for HMGA1 binding (personal observation). To study this aspect, further work will need to utilise chromatin immunoprecipitation (ChIP) to confirm the binding of HMGA1 to the *MMP-9* gene promoter. Then, formal gene promoter analysis, using vectors cloned with varying lengths of *MMP-9* gene promoter containing potential regions for HMGA1 binding, will need to be performed to ascertain the exact binding site of HMGA1.

To verify that the effects of HMGA1 on Akt activation were functional, we further demonstrated that HMGA1 expression also directly regulated the phosphorylation of mammalian target of rapamycin (mTOR) which is a known downstream target of Akt. As such, HMGA1 functionally regulates the PI3-K/Akt/mTOR pathway. Interestingly, the HMGA1-induced malignant cellular characteristics (such as cellular invasiveness, anoikis resistance, soft agar growth and chemoresistance) were all dependent on the PI3-K/Akt pathway. In addition to Akt, we also found that ERK and caspase pathways are downstream mediators of HMGA1. Our findings on the effects of HMGA1 on ERK activation concur with previous studies by Treff and co-workers who found that HMGA1 regulates ras/ERK signaling (Treff et al., 2004).

Given that activated Akt provides protection from apoptosis (Peruzzi et al., 1999) and conventional chemotherapy kills by apoptosis (Arlt et al., 2003; Blanco-Aparicio et al., 2005; Fahy et al., 2003; Ng et al., 2000), we postulated that HMGA1 will have an effect on chemoresistance in pancreatic cancer cells. We found that HMGA1 silencing markedly chemosensitise pancreatic cancer cells to gemcitabine. This probably represents one of our most important findings and highlights the potential role for HMGA1 to be a therapeutic target in pancreatic cancer. Clearly, the ability to increase the

sensitivity of pancreatic cancer cells to gemcitabine is a major advance given the high degree of chemoresistance inherent in this cancer type.

Recently, another group has described that HMGA1 positively regulates insulin receptor expression in pancreatic cancer cells (Kolb et al., 2007). As such, this provides a possible mechanism through which HMGA1 is able to regulate the PI3-K/Akt pathway by its regulation of insulin receptor expression. However, future studies will need to be performed to assess if regulation of Akt by HMGA1 is dependent on insulin receptor expression. To examine this dependence, cells overexpressing HMGA1 with overactivity of the PI3-K/Akt pathway need to be assessed for their insulin receptor expression. Further, experiments will need to be performed with silencing of insulin receptors in these cells to examine if PI3-K/Akt pathway activity is dependent on insulin receptor expression. Alternatively, experiments could be conducted in cells with stable silencing of HMGA1 by transfecting these cells with expression vectors with cloned insulin receptor cDNA to investigate if this can rescue the activity of PI3-K/Akt in these cells. This study would help to establish if there is a HMGA1-insulin receptor-PI-3K/Akt link.

The findings of this thesis are summarized in Figure 8.1. Taking the findings together, this thesis has established the clinical relevance of HMGA1 in pancreatic cancer, and characterised the roles of HMGA1 as a novel mediator of malignant phenotype in this cancer.

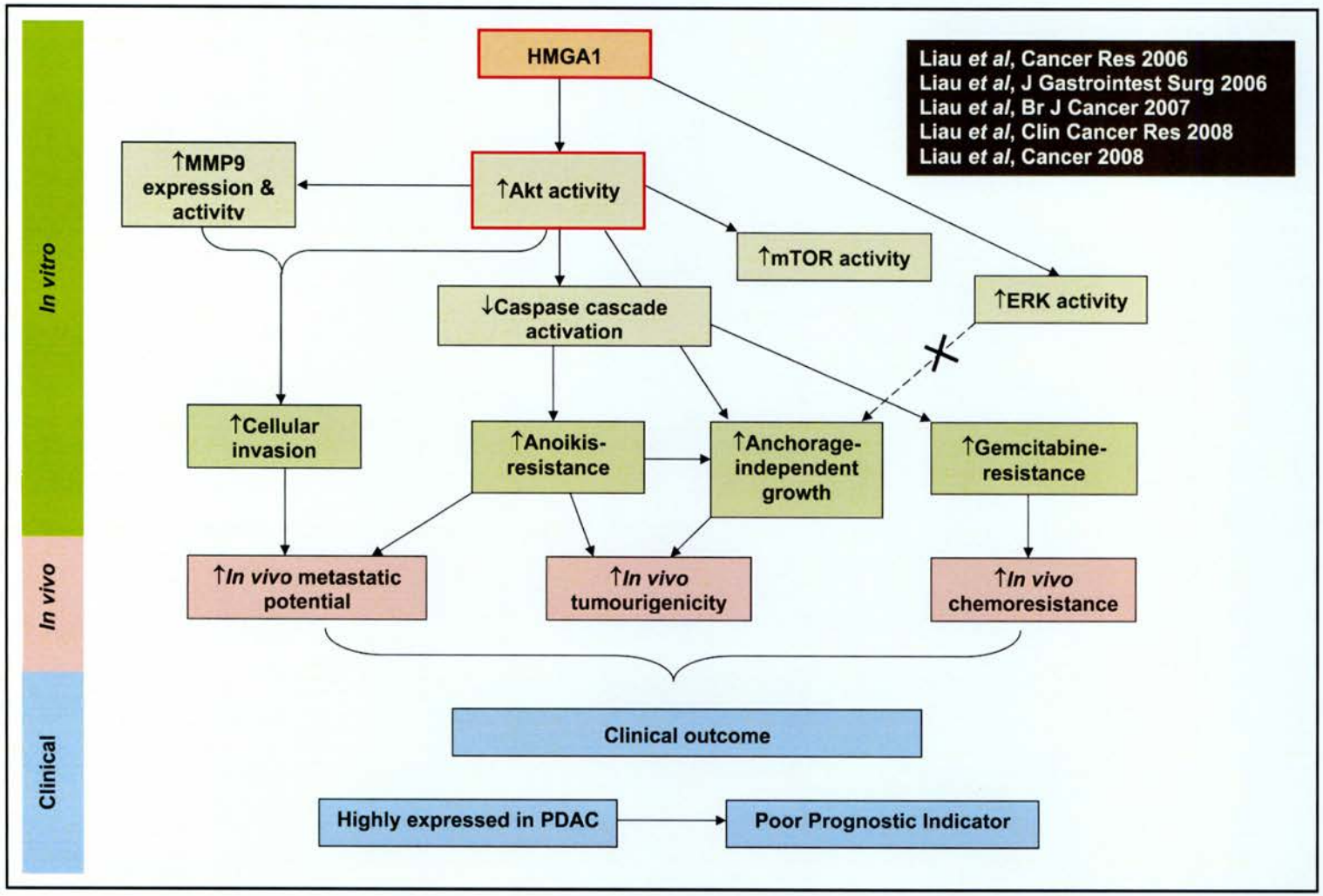


Figure 8.1. Schematic diagram summarising the roles and mediators of HMGA1 in pancreatic adenocarcinoma, as described in this thesis.

8.2 TARGETING HMGA1 IN PANCREATIC ADENOCARCINOMA

The aggressive nature of pancreatic cancer and failure of conventional therapy in improving outcome of patients with this cancer type calls for an urgent need for novel therapeutic approaches. Accumulating evidence suggests that targeting HMGA1 in tumour cells may represent a novel and attractive strategy to treat cancers.

8.2.1 Advantages of targeting HMGA1

Targeting HMGA1 in cancer has several advantages. HMGA1 is expressed at high levels during embryogenesis, and its expression reduces to almost absent levels during adulthood (Chiappetta et al., 1996). As such, targeting HMGA1 would theoretically affect only tumour cells which overexpress HMGA1, leaving the normal tissues unaffected or with minimal collateral damage (Chiappetta et al., 1996). Given HMGA1 is overexpressed in a large number of malignancies, its potential as a therapeutic target in oncology is immense.

Based on our findings in Chapters 4 and 6, HMGA1 silencing had no effect on the anchorage-dependent growth (i.e standard monolayer culture) whilst there was a significant inhibition of anchorage-independent growth. Normal tissue grows under anchorage-dependent conditions. As such, one could speculate that targeted suppression of HMGA1 may have a lesser effect on this mode of growth and hence, with little effects on normal tissue. It is plausible that suppression of HMGA1 may have preferential effects on malignant tissues.

Given that HMGA1 probably regulates a large number of genes involved in cancer progression (Reeves, 2001), inhibition of HMGA1 function may inactivate a range of pro-oncogenic pathways and serve as an effective target for oncology.

8.2.2 Developing HMGA1-specific therapeutics

Molecular approaches such as antisense oligonucleotides and ribozymes have been previously used to knockdown HMGA1 expression (Trapasso et al., 2004). Trapasso and colleagues have shown that suppression of HMGA1 expression may result in an apoptotic response in pancreatic cancer cells. However, previous experience with these approaches has shown low efficiency *in vitro* and *in vivo* due to premature degradation and significant, non-specific cellular toxicity (Lebedeva & Stein, 2001). Unlike cell surface receptors (e.g. Her2/neu), HMGA1 is predominantly localised to the nucleus. As such, it will not be amenable to monoclonal antibody targeting due to its inaccessibility *in vivo*. Development of novel small molecule inhibitors of HMGA1 will move the field closer to clinical therapy. Development of small molecule inhibitors of HMGA1 will need a detailed study of the chemical structure of HMGA1 molecule to identify the potential sites (e.g. AT-hooks) on the HMGA1 molecule that can be inhibited by designed small molecule inhibitors. Distamycin A is an antibiotic that has been used as a small molecule inhibitor of HMGA1 by competing with HMGA1 for binding to AT-rich minor grooves of DNA. Clearly, this mode of action of Distamycin A will not be specific for HMGA1 and will affect other nuclear proteins that bind to DNA minor grooves (Ghersa et al., 1997; Massaad-Massade et al., 2002). Other means of targeting HMGA1 include cross-linking of HMGA1 molecules to DNA and hence, limiting its availability for intranuclear actions such as binding to promoter regions of DNA and forming complexes with other transcriptional factors. This mechanism was described for mitomycin C which has been reported to cross-link HMGA1 molecules to DNA (Reeves & Beckerbauer, 2003). Of note, this is not the main mechanism through which mitomycin C mediates its anti-tumour actions.

In this thesis, we described a highly effective and specific molecular approach to HMGA1 silencing: the RNA interference. Worldwide, pharmaceutical companies are actively pursuing RNAi-based therapeutics, confident that

harnessing the naturally occurring RNAi mechanism will lead to transfer of pre-clinical success to clinical application. However, there are several concerns that need to be addressed before RNAi can be a real therapeutic modality. Firstly, the issue of toxicity and off-target effects of RNAi will need to be addressed and warrants intense investigation (Grimm et al., 2006).

Secondly, delivery of RNAi to specific tissue sites remains a major stumbling block. The pace of RNAi-based drug development has been rapid. Clinical trials using RNAi therapeutics are currently underway for age-related macular degeneration (e.g. Cand5 is in Phase II trials by Acuity Pharmaceuticals, Philadelphia, PA, USA) (Behlke, 2006). This disease is chosen for early trials as it is amenable to local delivery of RNAi therapeutics (i.e. directly to sites of disease in the eye). However, for other diseases requiring systemic administration, the clinical utility of RNAi-based therapeutics will depend on the development of safe and efficacious delivery systems. Cancer can be considered a systemic disease due to metastatic distribution of disseminated cells, and thus requires systemic therapy. So far, delivering RNA interference to target cells remains the major stumbling block. Delivery of siRNA is complicated by the serum instability of siRNA, low cellular uptake efficiency and lack of understanding on its pharmacokinetics and biodistribution (Behlke, 2006). The siRNA duplexes are rapidly degraded in serum by naturally occurring RNAses, and are cleared quickly by the kidneys (Aagaard & Rossi, 2007). However, new molecular innovations are being developed. Chemical modifications of siRNA oligonucleotides such as cholesterol conjugation (Soutschek et al., 2004) or phosphorothioate linkage (Braasch et al., 2004) have been shown to enhance the serum stability and resistance to nuclease or RNAse digestion. To reduce renal filtration, siRNAs have been complexed with larger molecules such as encapsulation with liposomes or nanoparticles (Aagaard & Rossi, 2007). Targeted delivery to specific tissues can be made by linking siRNA to ligand, antibody or aptamer (Pirollo et al., 2006). Alternatively, as demonstrated by this thesis, short hairpin RNA as transcribed from RNA polymerase promoters from plasmid- and virus-based vectors

provide effective strategies for RNA interference. Established gene therapy techniques could be utilised to deliver vectors encoding short hairpin RNA. The disadvantages of traditional gene therapy techniques include insertional mutagenesis, variable expression efficiency and tissue specific delivery. However, future advances such as the use of tissue-specific or cell-specific promoter vectors and development of effective vector delivery systems that target cancer cells will move vector-based RNAi closer to clinical application.

8.2.3 Potential adverse effects of therapeutic targeting of HMGA1

Although HMGA1 is absent or present at very low levels in adult tissues, it remains uncertain to what extent HMGA1 expression is required in adult tissues. The loss of HMGA1 function in transgenic mice has led to the development of diabetes through the decreased expression of insulin receptors and impaired insulin signalling pathways (Foti et al., 2005). As such, any targeting of HMGA1 can be predicted to lead to glucose intolerance and perhaps, clinical diabetes. Further, in the HMGA1-knockout mouse model, disruption of HMGA1 resulted in cardiac hypertrophy and development of haematologic malignancies (Fedele et al., 2006). Fedele and co-workers found that surprisingly, $Hmga1^{-/-}$ and $Hmga1^{+/-}$ mice developed B-cell expansion, resembling human B-cell lymphomas. This revealed an unsuspected haploinsufficient tumour suppressor role of HMGA1. More recently, HMGA1 has been shown to be an essential component of the senescence machinery (Narita et al., 2006). Given that senescence is a barrier to malignant transformation, this implies that HMGA1 also acts in the tumour suppressor network. The above tumour suppressor roles of HMGA1 are in contrast to its pro-oncogenic properties. It is possible that the dual effect of HMGA1 might depend on cellular context. Clearly, any future clinical trials involving therapeutic targeting of HMGA1 will require close monitoring for possible cardiac, haematological and endocrine complications.

8.3 OTHER POTENTIAL CLINICAL APPLICATIONS OF *HMGA1*

In Chapter 3, we showed that tumoural HMGA1 expression is an indicator of poor prognosis in pancreatic cancer patients. HMGA1 protein detection could be introduced in tumour analysis for patients who underwent pancreatic resection to help identify patients who would benefit from aggressive management of their disease.

Future development of techniques for detection of HMGA1 proteins or mRNA in clinical samples (e.g. blood samples of pancreatic cancer patients) will be useful to monitor response to chemotherapy and allow early diagnosis of recurrence following surgical resection. Given our findings that HMGA1 expression is low or absent in normal pancreas, there may be a role for HMGA1 as a diagnostic marker for pancreatic cancer. The ability to detect HMGA1 protein or mRNA in pancreatic juice sampled during ERCP could be used as part of a panel of markers to screen for early pancreatic cancer in secondary screening programme such as the EUROPAC programme (see Section 1.10.8).

In addition, the expression of HMGA1 may be an important factor in the choice of therapy. We have shown in Chapter 7 that HMGA1 expression correlates with resistance to gemcitabine. It may be that detection of high HMGA1 expression in tumour samples may indicate a high degree of gemcitabine chemoresistance, and may affect the clinician's choice of chemotherapeutic agents.

8.4 FUTURE WORK

The development of cancer is a multistep process including initiation, growth, invasion and ultimately establishment of metastatic disease. In this thesis, we have addressed the roles of HMGA1 in pancreatic cancer progression in particular with respect to growth, invasion and metastasis. However, we have

not addressed the roles of HMGA1 in tumour initiation. There are three main studies that will help our understanding of the roles of HMGA1 in pancreatic cancer development. These include: 1) study examining the expression of HMGA1 in the pre-malignant PanIN lesions, 2) development of a mouse model with HMGA1 overexpression targeted to the pancreas and 3) study investigating the roles of HMGA1 in pancreatic cancer stem cells.

Although we have shown that HMGA1 overexpression is a common phenomenon in pancreatic cancers, our study did not address the issue whether HMGA1 overexpression is an 'early' or 'late' event in pancreatic carcinogenesis. To address this, future studies would need to examine the expression of HMGA1 in early and late stages of PanIN lesions, possibly through the construction of a PanIN tissue microarray. This will allow the characterisation of HMGA1 expression in pancreatic cancer precursor lesions, giving evidence for the roles of HMGA1 in early stages of cancer development.

Further, the creation of a transgenic mouse model with HMGA1 overexpression specifically targeted to the developing pancreas using techniques previously described (Hingorani et al., 2003a) will help to address the question if HMGA1 has a role in the development of pancreatic cancer. Recent evidence suggests that cancer development could be initiated in a cellular entity known as the 'cancer stem cells' (see Section 1.5.1). It is interesting that HMGA1 is overexpressed during embryonic development but its expression then subsides to very low or absent levels in adult tissues. We speculate that HMGA1 may have a role in cancer stem cells. We propose that future studies should examine the expression of HMGA1 in isolated pancreatic cancer stem cells (Li et al., 2007). Interestingly, cancer stem cells have been shown to be more chemoresistant (Wicha et al., 2006) and more invasive (Hermann et al., 2007) than other cancer cells. Given that HMGA1 promotes a malignant phenotype in pancreatic cancer cells, it is possible that HMGA1 may promote the expansion of the stem cell compartment in the

cancer cell population and hence, lead to more invasive and chemoresistant cancer as described by this thesis.

Lastly, gene array analysis can also be used to identify the downstream molecular mediators of HMGA1 in pancreatic cancer cells. Although we have identified PI3-K/Akt is an important downstream mediator of HMGA1, it is more likely that the effects of HMGA1 are mediated by a range of other pro-oncogenic proteins that have yet to be identified. Investigations along this line will be useful to identify other novel therapeutic targets and biomarkers that will help to progress our understanding, treatment and detection of pancreatic cancers.

8.5 CONCLUDING REMARK

In this thesis, we have dissected out the roles of HMGA1 in each of the aspects of a malignant phenotype. We have provided compeling evidence to support the potential use of HMGA1 as a prognostic biomarker and therapeutic target in pancreatic cancer.

Research supervision and ethics

The work presented in this thesis was conducted between 30 September 2004 and 30 September 2006 in the Pancreatic Cancer Research Laboratory, Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA.

Details of supervisors

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Ethical approval

Ethical approval for the use of archival specimens of pancreatic cancer tissue was granted by the Institutional Review Board, Brigham and Women's Hospital (Study title: Molecular analysis of pancreatic cancer).

Ethical approval for animal studies using xenograft models were given by Institutional Review Board and Harvard Standing Committee on Animal Research. Procedures were undertaken in the Animal Facility, Thorn Building, Room 1603, Brigham and Women's Hospital.

All work presented in this studies was done personally apart from the construction of tissue microarray which was performed by the TMA core at the Brigham and Women's Hospital.

Research dividends

The following list contains achievements resulting from this research project. Papers, either published or in press, directly relating to this thesis are provided in Appendix section.

AWARDS & PRIZES

1. **Society for Surgery of the Alimentary Tract (SSAT, USA) Resident Research Award**, awarded annually for best research by surgical residents (2006).
2. **Patey Prize from Society of Academic Research and Surgery (SARS, UK)** for best research paper presented, SARS 2006 meeting, Edinburgh (2006).
3. **IHPBA Kenneth W. Warren Fellowship** from the International Hepato-Pancreato-Biliary Association, worth USD 20K towards research fellowship at Boston, USA (2005-6)
4. **Aid for Cancer Research (USA) Grant** to sponsor research equipment requirements, worth USD 25K (2005)
5. **Department of Surgery Research Grant, Brigham and Women's Hospital**, worth USD 25K (2005)
6. **American Association for Cancer Research Trainee Scholarship** to attend the AACR Molecular Biology for Clinical Oncology Course, Aspen, Colorado, USA.
7. **British Pancreatic Society Travelling Fellowship** from the Pancreatic Society of Great Britain and Ireland (2005)

PAPERS

1. **S-S. Liau**, S.W Ashley, E.E. Whang (2006). Lentivirus-mediated RNA interference of HMGA1 promotes chemosensitivity to gemcitabine in pancreatic adenocarcinoma. *Journal of Gastrointestinal Surgery 2006, 10(9): 1254-63.* (2006 Impact factor: 2.265). *Paper highlighted in **S.B. Hanauer** (2007). HMGA1 identified as a therapeutic target in pancreatic adenocarcinoma.. *Nature Clinical Practice Gastroenterology & Hepatology 2007* ***4****, 126-127*
2. **S-S. Liau**, A. Jazag, E.E. Whang (2006). HMGA1 is a determinant of cellular invasiveness and in vivo metastatic potential in pancreatic adenocarcinoma. *Cancer Res 2006; 66: 24-34* (2006 Impact factor: 7.656)

3. **S-S. Liau**, A. Jazag, E. E. Whang (2007). Overexpression of HMGA1 promotes anoikis resistance and constitutive Akt activation in pancreatic adenocarcinoma cells. *Br J Cancer. 2007; 96(6):993-1000* (2006 Impact factor: 4.459)

4. **S-S. Liau**, E. E. Whang (2007). HMGA1 is a molecular determinant of chemoresistance to gemcitabine in pancreatic cancer cells. *Clin Cancer Res 2008; 14(5):1470-7*, (2006 Impact factor: 6.177)

5. **S-S. Liau**, F. Rocha, E. Matros, M. Redston, E.E. Whang (2008). HMGA1 promotes anchorage-independent growth and is an independent prognostic factor in pancreatic adenocarcinoma. *Cancer 2008; 113(2):302-14* (2006 Impact factor: 4)

6. J.R Benson, **S-S. Liau** (2008). Cancer genetics: a primer for surgeons. *Surgical Clinics of North America 2008; 88(4):681-704* (2006 Impact factor: 1.656)

BOOK CHAPTERS

1. **S-S. Liau,** E. E Whang: Acute cholangitis, Chapter in Current Surgical Therapy, 9th Edition, (John L. Cameron, ed.), Chicago: Mosby 2007

2. J. R Benson, **S-S. Liau**: Nature and development of cancer. Chapter in Oxford Textbook of Medicine, 5th Edition, (David A. Warrell, Timothy M. Cox, John D. Firth, eds), Oxford: Oxford University Press 2009 (In press)

PUBLISHED ABSTRACTS

1. **S-S. Liau**, E. Benoit, S. Ashley, E.E Whang (2005). HMG I(Y) is a determinant of pancreatic adenocarcinoma cellular invasiveness. *Journal of American College of Surgeons 2005, 201 (3S): S81*

2. **S-S. Liau**, M.S Duxbury, S. Ashley, E. E Whang (2005). RRM2 overexpression induces induces pancreatic adenocarcinoma cellular invasiveness via Raf-1 dependent NF-kB activation. *Pancreas 2005, 31 (4): 452*

3. **S-S. Liau**, F. Rocha, S. Ahsley, E. E Whang (2006). Short-hairpin RNA interference targeting HMGA1 gene attenuates pancreatic adenocarcinoma cellular invasiveness. *Journal of Surgical Research 2006, 130 (2)*

4. **S-S. Liau,** S. Ashley, E. E. Whang (2006). HMGA1: a novel therapeutic target in pancreatic adenocarcinoma. *Gastroenterology 2006, 130 (4), Supplement 2: A677*

5. **S-S. Liau,** S. Ashley, E. E. Whang (2006). Lentivirus-mediated silencing of HMGA1 gene promotes chemosensitivity to gemcitabine in pancreatic adenocarcinoma. *Gastroenterology 2006, 130 (4), Supplement 2: A854*

6. **S-S. Liau**, F. Rocha, S. Ashley, E. E. Whang (2006). Characterization of a novel mediator of malignant phenotype in pancreatic adenocarcinoma. *British Journal of Surgery 2006, 93 (7): 899*

7. **S-S. Liau**, F. Rocha, E. Matros, S. Ashley, E. E. Whang (2006). HMGA1: a mediator of metastasis and a novel prognostic marker in pancreatic adenocarcinoma. *Journal of American College of Surgeons 2006, 3 (3S): S45*

INVITED PRESENTATIONS

1. Characterization of a novel mediator of malignant phenotype in pancreatic adenocarcinoma (Patey Prize Presentation)
S-S. Liau, F. Rocha, S. Ahsley, E. E Whang

- Prize winner session, First Academic Surgical Congress, Society of University Surgeons (SUS), San Diego, USA, February 2006
- Walter Brendel Session, European Society of Surgical Research (ESSR), Rostock-Warnemunde, Germany, May 2006. http://www.surgicalresearch.org.uk/patey.htm

2. Lentivirus-mediated silencing of HMGA1 promotes chemosensitivity to gemcitabine in pancreatic cancer (Resident Research Award)
S-S. Liau, S. W. Ashley, E.E. Whang

- 21st Residents & Fellows Research Conference, Society for Surgery of the Alimentary Tract, Los Angeles, May 2006. Top 5% of abstracts submitted.

3. Kenneth W. Warren fellowship report: HMGA1 as a novel therapeutic target in pancreatic adenocarcinoma
S-S. Liau, S.W. Ashley, E.E Whang

- General Assembly, International Hepato-pancreato-biliary Association (IHPBA) 7th World Congress, Edinburgh, Sept 2006

PRESENTATIONS AT NATIONAL & INTERNATIONAL MEETINGS

1. Roles of HMGA1 in pancreatic adenocarcinoma tumorigenesis
S. S. Liau, A. Jazag, S. Ashley, E. E Whang
Poster presentation – Brigham & Women's Hospital-Brigham Research Institute Cancer Research Center Retreat, Boston, October 2006

2. HMGA1: a mediator of metastasis and a novel prognostic marker in pancreatic adenocarcinoma
S.S. Liau, F. Rocha, E. Matros, E. E Whang
Oral presentation – Surgical Forum, 92nd Annual Clinical Congress of the American College of Surgeons,Chicago, October 2006

3. HMGA1: a novel mediator of malignant phenotype in pancreatic adenocarcinoma
S.S. Liau, F. Rocha, S. Ahsley, E. E Whang
Poster presentation – Pancreas Club, DDW, Los Angeles, May 2006

4. HMGA1 gene: a novel therapeutic target for pancreatic adenocarcinoma
S-S. Liau, S.W. Ashley, E.E. Whang
Poster presentation – American Gasteroenterological Association, DDW, Los Angeles, May 2006

5. Lentivirus-mediated RNA interference of HMGA1 gene promotes chemosensitivity to gemcitabine in pancreatic adenocarcinoma
S-S.Liau, S.W. Ashley, E.E. Whang
Plenary presentation – Society for Surgery of the Alimentary Tract, DDW, Los Angeles, May 2006
Paper given highest rating at the abstract selection process. Selected for resident research conference, SSAT.

6. Characterization of a novel mediator of malignant phenotype in pancreaticadenocarcinoma
S.S. Liau, F. Rocha, S. Ahsley, E. E Whang
Plenary presentation – Society of Academic Research and Surgery, Edinburgh, January 2006 (Awarded the 2006 Patey Prize)

7. Hairpin RNA interference targeting HMG A1 gene attenuates pancreatic adenocarcinoma cellular invasiveness
S.S. Liau, S. Ashley, E.E Whang
Oral poster presentation – Society of University Surgeons, San Diego, February 2006.

8. RRM2 overexpression induces pancreatic adenocarcinoma cellular invasiveness via Raf-1-dependent NF-kB activation
S.S.Liau, M.S Duxbury, J. Irani, S. Ashley, E.E Whang
Poster presentation – American Pancreatic Association, Chicago, November 2005

9. HMG I(Y) is a determinant of pancreatic adenocarcinoma cellular invasiveness
S.S.Liau, E. Benoit, S. Ashley, E.E Whang
Oral presentation - Surgical Forum, 91st Annual Clinical Congress of the American College of Surgeons, San Francisco, October 2005

MAJOR RESEARCH GRANT APPLICATIONS

National Institute of Health R01 Oncology Application (Role of HMGA1 as a therapeutic target in pancreatic cancer) (USD 150K per year, 5 years)

Role: Significant Contributor

Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

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APPENDIX

PUBLISHED ARTICLES DIRECTLY RELATED TO THIS THESIS

HMGA1 Is a Determinant of Cellular Invasiveness and *In vivo* Metastatic Potential in Pancreatic Adenocarcinoma

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Abstract

HMGA1 proteins are architectural transcription factors that are overexpressed in a range of human malignancies, including pancreatic adenocarcinoma. We hypothesized that HMGA1 expression is a determinant of cellular invasiveness and metastasis in pancreatic cancer. Stable silencing of HMGA1 in MiaPaCa2 and PANC1 pancreatic adenocarcinoma cells was achieved by transfection of short hairpin RNA–generating vectors. Additionally, stable overexpression of HMGA1 in MiaPaCa2 cells (characterized by low levels of inherent HMGA1 expression) was achieved. HMGA1 silencing resulted in significant reductions in cellular invasiveness through Matrigel; in cellular matrix metalloproteinase-9 (MMP-9) activity, mRNA levels, and gene promoter activity; and in Akt phosphorylation at Ser^{473}. Conversely, forced HMGA1 overexpression resulted in significant increases in cellular invasiveness; in cellular MMP-9 activity, mRNA levels, and promoter activity; and in Akt phosphorylation at Ser^{473}. HMGA1 overexpression–induced increases in invasiveness were MMP-9 dependent. The role of phosphatidylinositol-3 kinase (PI3K)/Akt in mediating HMGA1-dependent invasiveness was elucidated by a specific PI3K inhibitor (LY294002) and constitutively active and dominant-negative Akt adenoviral constructs. Akt-dependent modulation of MMP-9 activity contributed significantly to HMGA1 overexpression–induced increases in invasive capacity. Furthermore, HMGA1 silencing resulted in reductions in metastatic potential and tumor growth *in vivo* and in tumoral MMP-9 activity. Our findings suggest that HMGA1 may be a novel molecular determinant of invasiveness and metastasis, as well as a potential therapeutic target, in pancreatic adenocarcinoma. (Cancer Res 2006; 66(24): 11613-22)

Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States (1). Its biology is characterized by the propensity for early and aggressive invasion and metastasis, such that <10% of patients have surgically resectable disease at the time of diagnosis (2). Even among patients able to undergo resection of all apparent disease, most are destined to succumb to locally recurrent and metastatic cancer. Hence, further understanding of the molecular mechanisms underlying pancreatic adenocarcinoma cellular invasion and metastasis is needed, as this information may facilitate the identification of novel molecular targets for the rational therapy of this deadly disease.

The human *HMGA1* gene, located on chromosomal locus 6p21, encodes two HMGA1 splice variants (HMGA1a and HMGA1b; ref. 3). These HMGA1 proteins are architectural transcription factors that play a role in both positive and negative transcriptional regulation of human gene expression *in vivo* (4, 5). They form stereo-specific, multiprotein complexes termed "enhanceosomes" on the promoter/enhancer regions of genes, where they are able to bind to the minor groove of AT-rich DNA sequences to induce DNA helix bending (4, 6). HMGA1 proteins are overexpressed in a range of human cancers, including pancreatic adenocarcinoma (7–11). Furthermore, HMGA1 protein overexpression has been reported to be associated with cancer metastasis (12–14) and transcriptional up-regulation of genes implicated in promoting metastasis (15).

The purpose of this study was to test the hypothesis that HMGA1 is a molecular determinant of cellular invasiveness and metastasis in pancreatic adenocarcinoma. Our findings indicate that HMGA1 promotes cellular invasiveness through phosphatidylinositol-3 kinase (PI3K)/Akt–dependent modulation of matrix metalloproteinase-9 (MMP-9) activity.

Materials and Methods

Cells and cell culture. MiaPaCa2 and PANC1 human pancreatic ductal adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM containing 10% fetal bovine serum (FBS; Life Technologies, Inc., Gaithersburg, MD) and incubated in a humidified (37°C, 5% CO_2) incubator, grown in 75-cm^2 culture flasks, and passaged on reaching 80% confluence.

Reagents. The PI3K inhibitor LY294002 was purchased from Calbiochem (San Diego, CA). Anti-HMGA1, anti-actin, anti–phospho-Akt (Ser^{473}), anti-Akt1, anti–phospho-extracellular signal-regulated kinase 1/2 (anti–phospho-ERK1/2), anti-ERK1/2, and anti-hemagglutinin (anti-HA) tag antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–phospho-mammalian target of rapamycin (anti–phospho-mTOR; Ser^{2448}) and anti-mTOR antibodies were obtained from Cell Signaling Technology (Danvers, MA).

Dominant-negative and dominant-active Akt constructs. Adenovirus carrying HA-tagged dominant-negative (Ad-DN-Akt), dominant-active (Ad-myr-Akt) murine Akt1, and control virus (Ad-CMV-null; all tittered at 1×10^{10} plaque-forming units/mL) were purchased from Vector Biolabs (Philadelphia, PA). Adenoviral infection was done at multiplicity of infection of 10 in the presence of 6 μg/mL polybrene for 8 hours. Experiments were done on cells 48 hours following infection.

Stable HMGA1 RNA interference. Hairpin RNA interference plasmids (pLKO.1-HMGA1, TRCN0000018949), constructed as described previously (16), were obtained from The RNAi Consortium (Mission TRC Hs. 1.0, Sigma-Aldrich, St Louis, MO). The sequences of short hairpin RNA (shRNA) targeting the human *HMGA1* gene (Genbank accession no. NM_002131)

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were shHMGA1-1 plasmid, 5′-CAACTCCAGGAAGGAAACCAA-3′ (coding region positions 446–466 of HMGA1 mRNA transcript variant 2) and shHMGA1-2 plasmid, 5′-CCTTGGCCTCCAAGCAGGAAA-3′ (coding region positions 281–301 of HMGA1 mRNA transcript variant 2). The control plasmid, which has a scrambled non-targeting shRNA sequence, was obtained from The RNAi Consortium. Each of these vectors had been sequence verified. Pooled stable transfectants were established using puromycin selection. Stable transfectant cells were maintained in medium containing 3 μg/mL puromycin (Invivogen, San Diego, CA).

Expression vector and transfection. The HMGA1 coding sequence was PCR amplified from IMAGE clone 5399570 (Genbank accession no. BC063434) using gene-specific primers modified to include the appropriate restriction sites at their 5′ end. The primers used were forward, 5′-TTTTGATATCATGAGTGAGTCGAGCTCGAAG-3′ and backward, 5′-TTTTGAATTCTCACTGCTCCTCCTCCGAGGA-3′. Purified PCR products were digested with *Eco*RV and *Eco*RI before ligation into an *Eco*RV/*Eco*RI–digested pIRES-puro3 vector (Clontech, Palo Alto, CA). The expression plasmids were named pIRES-HMGA1. MiaPaCa2 cells were transfected with pIRES-HMGA1 or empty pIRES-puro3, which acted as a control, using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. Stable clones were selected by exposure to incrementally increasing concentrations of puromycin (Invivogen), isolated using cloning cylinders, and maintained in medium containing 3 μg/mL puromycin (Invivogen). Clones pIRES-HMGA1.1 and pIRES-HMGA1.2, which expressed the highest levels of HMGA1, were used for further studies.

MMP-9 gene promoter and luciferase assay. The MMP-9 promoter reporter construct was custom-synthesized by Aviva Systems Biology (San Diego, CA). Briefly, the full-length human MMP-9 promoter region ranging from −992 to +304 bp, relative to the transcription initiation site, was amplified by PCR from human placental DNA, using proper primers designed according to the *MMP-9* gene sequence. The primers were forward, 5′-GGTACCTCTTTCTGGGCTCAAGCAATC-3′ and backward, 5′-CTCGAGCTAACCCTGGACACCTCTGTTC-3′. The amplified promoter fragment with the addition of *Xho*I and *Kpn*I restriction sites at each end was cloned into the pGL4.12 vector (Promega, Madison, WI) at the *Xho*I/*Kpn*I site upstream of the firefly luciferase gene. By direct sequencing, the sequence of the cloned promoter region was confirmed. The empty pGL4.12 vector (pGL4e) was used as control.

Cells were plated onto a six-well cell culture plates at a concentration of 5×10^5 per well and allowed to adhere for 12 hours in serum-containing medium. To assess the effects of modulating HMGA1 expression on MMP-9 promoter activity, transient transfection experiments were done on MiaPaCa2 cells. For HMGA1 suppression experiments, 5 μg shHMGA1 or control shRNA vector was cotransfected with 5 μg pGL4-MMP9 or pGL4e and 0.5 μg pRLCMV vector (Promega), which contains a cytomegalovirus (CMV) promoter upstream of a *Renilla* luciferase gene. For HMGA1 overexpression experiments, either pIRES-HMGA1 or control vector pIRES-puro3 was used in cotransfection. After 48 hours, luciferase activity in lysates of the transfected cells was measured using the dual-luciferase reporter assay, according to the manufacturer's recommended protocol (Promega).

Relative luciferase activity was calculated as fold-induction of luciferase activity above the background (taken as activity associated with promoterless vector, pGL4.12). The activity of *Renilla* luciferase was used to normalize any variation in transfection efficiencies.

Invasion assay. Cellular invasion was quantified using a modified Matrigel Boyden chamber assay. The BD BioCoat Matrigel invasion chamber (BD Biosciences, Bedford, MA) was used according to the manufacturer's instructions. Pancreatic cancer cells (2.5×10^4) in serum-free media were seeded onto Matrigel-coated filters. In the lower chambers, 5% FBS was added as a chemoattractant. After 24 hours of incubation, the filters were stained using the Diff-Quik kit (BD Biosciences), and the number of cells that had invaded through the filter was counted under magnification (randomly selected high-power fields). The counting was done for 20 fields in each sample, and mean values from three independent experiments were calculated. In additional studies, invasion assays were done in the presence of 10 mg/mL anti–MMP-9-neutralizing antibody (Santa Cruz, San Ramon, CA) or isotype-matched control (irrelevant) immunoglobulin (Santa Cruz).

Proliferation assay. Cell proliferation was quantified using an 3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay (CellTiter 96 Aqueous One Solution Assay, Promega), in accordance to manufacturer's instructions and confirmed by cell counting. Logarithmically growing cells were seeded into 96-well plates at 5×10^3 per well and allowed to adhere overnight in medium containing 10% or 2% FBS. Cell proliferation was determined after 48 hours. Plates were read with the use of the SpectraMax M5 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 490 nm. Ten samples were used for each experimental condition, and experiments were done in triplicate. At identical time points, cell counting was done. Cells were trypsinized to form a single-cell suspension. Viable cells, determined by trypan blue exclusion, were counted with the use of a Neubauer hemocytometer (Hausser Scientific, Horsham, PA).

Fluorometric MMP-9 activity assay. MMP-9 activities of cell or tumor lysates were assessed using the Enzolyte Plus 520 Enhanced Selectivity MMP-9 activity, in accordance with the manufacturer's instructions (AnaSpec, San Jose, CA). Fluorescence intensities were quantified using SpectraMax M5 microplate spectrophotometer (Molecular Devices) at excitation of 490 nm and emission of 520 nm.

Quantitative real-time PCR. Total RNA was extracted from cells using mirVana RNA isolation kit according to the manufacturer's recommendation (Ambion, TX). First-strand cDNA was synthesized from 5 μg of total RNA using the SuperScript III First-Strand Synthesis System, according to manufacturer's instructions (Invitrogen). Transcript analysis was done by quantitative real-time PCR using the Taqman assay, based on manufacturer's instructions (Applied Biosystems, Foster City, CA). Hybridization probe and primer sets for human MMP-9 (Hs00234579_m1 MMP9) and β-actin were obtained from Applied Biosystems. Typically, thermal cycling was initiated with a denaturation step for 10 minutes at 95°C followed by 40 cycles done in two steps: for 15 seconds at 95°C and for 1 minute at 60°C. Reactions were done, and data were analyzed using the GeneAmp Sequence Detection System (Applied Biosystems). Results are expressed as the ratio of MMP9 to β-actin. Measured threshold cycles were converted to relative copy numbers using primer-specific standard curves.

Western blotting. Cells were harvested and rinsed twice with PBS. Cell extracts were prepared with lysis buffer [20 mmol/L Tris (pH 7.5), 0.1% Triton X-100, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 10 mg/mL leupeptin] and cleared by centrifugation at 15,000 × *g*, 4°C. Total protein concentration was measured using the bicinchoninic acid assay kit (Sigma, St Louis, MO) with bovine serum albumin as a standard, according to the manufacturer's instructions. Cell extracts containing 50 μg total protein were subjected to 10% SDS/PAGE, and the resolved proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Invitrogen). Equal protein loading was confirmed by Coomassie (Bio-Rad, Hercules, CA) staining of the gel. After blocking with PBS containing 3% bovine serum albumin for 1 hour at room temperature, membranes were incubated with 3 to 5 mg/mL antibody in PBS containing 0.1% Tween 20 overnight at 4°C. Chemiluminescent detection (Amersham Biosciences, Piscataway, NJ) was done in accordance with the manufacturer's instructions. The densitometric signal was quantified using ImagePro Plus software version 4.0 and normalized to that of actin. Blots were done in triplicate. Mean ± SD densitometric values are shown.

Nude mouse orthotopic xenograft model. Male athymic *nu/nu* mice 5 weeks of age, weighing 20 to 22 g, and specific pathogen-free were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed in microisolator cages in a specific pathogen-free facility with 12-hour light/dark cycles. They received water and food *ad libitum*. Animals were observed for signs of tumor growth, activity, feeding, and pain in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals.

To determine the effect of *HMGA1* gene silencing on *in vivo* metastasis, MiaPaCa2 cells stably expressing the control or HMGA1 shRNA

(shHMGA1-1) constructs were orthotopically implanted into the pancreata of nude mice. Mice were anesthetized with i.p. ketamine (200 mg/kg) and xylazine (10 mg/kg). In a laminar-flow hood, the abdomen was cleaned with isopropyl alcohol, and a left upper transverse incision was made. The pancreas was exposed, and 1×10^6 cells suspended in 75 μL PBS were slowly injected into the body of the pancreas. The pancreas was returned, and the abdomen was closed with 5-0 Vicryl (Ethicon, Somerville, NJ). Ten mice for each cell line were observed over 4 weeks and killed by overdose of ketamine (400 mg/kg) and xylazine (50 mg/kg). The liver of each animal was harvested, and metastatic foci were counted under a dissecting microscope (17). The primary tumors were excised, and the tumor lysates were assayed for HMGA1 expression and MMP-9 activity using Western blotting and MMP-9 activity assay, respectively.

Immunohistochemistry. Tumor sections (5 μm) were deparaffinized, rehydrated through graded alcohol, and processed using a streptavidin-biotin-peroxidase complex method. Antigen retrieval was done by microwave heating sections in 10 mmol/L sodium citrate buffer (pH 6) for 10 minutes. Following quenching of endogenous peroxidase activity and blocking of nonspecific binding, sections were incubated with anti-HMGA1 (Santa Cruz) at 4°C overnight at a 1:50 dilution. The secondary antibody was biotinylated rabbit anti-goat antibody (DAKO, Carpinteria, CA) used at a dilution of 1:200 for 30 minutes at 37°C. After further washing with TBS, sections were incubated with StrepABComplex/horseradish peroxidase (1:100; DAKO) for 30 minutes at 37°C. Immunolocalization was done by exposure to 0.05% 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. Normal serum was used in place of the primary antibody as a negative control. Slides were counterstained with hematoxylin before dehydration and mounting. For H&E staining, standard procedures were used to stain the tumor sections.

Statistical analysis. Differences between groups were analyzed using Student's *t* test, multifactorial ANOVA of initial measurements, and Mann-Whitney *U* test, for nonparametric data, as appropriate, using Statistica 5.5. software (StatSoft, Inc., Tulsa, OK). In cases in which averages were normalized to controls, the SDs of each nominator and denominator were taken into account in calculating the final SD. $P < 0.05$ was considered statistically significant.

Results

Effect of HMGA1 gene silencing on cellular invasiveness. We analyzed *HMGA1* gene expression using Western blot analysis. Cellular invasiveness was quantified using Matrigel-coated Boyden chambers. We used two shRNA-expressing plasmids with different target sequences to induce *HMGA1* gene silencing. MiaPaCa2 and PANC1 cell lines were stably transfected with each of the HMGA1-targeting shRNA expression vectors (shHMGA1-1 and shHMGA1-2). The shRNA approach was associated with high efficacies in HMGA1 silencing (up to 90% silencing, as confirmed on Western blot analysis; Fig. 1*A*). Silencing of HMGA1 significantly attenuated cellular invasiveness in both MiaPaCa2 and PANC1 cells (Fig. 1*C*). For these experiments, controls were cells stably transfected with a vector encoding a non-targeting shRNA.

Effect of HMGA1 overexpression on cellular invasiveness. Next, we sought to determine the effect of HMGA1 overexpression on cellular invasiveness. Using MiaPaCa2 cells, which have relatively low levels of inherent HMGA1 expression, we developed the clones pIRES-HMGA1.1 and pIRES-HMGA1.2, which stably overexpress HMGA1. Overexpression of HMGA1 was confirmed on

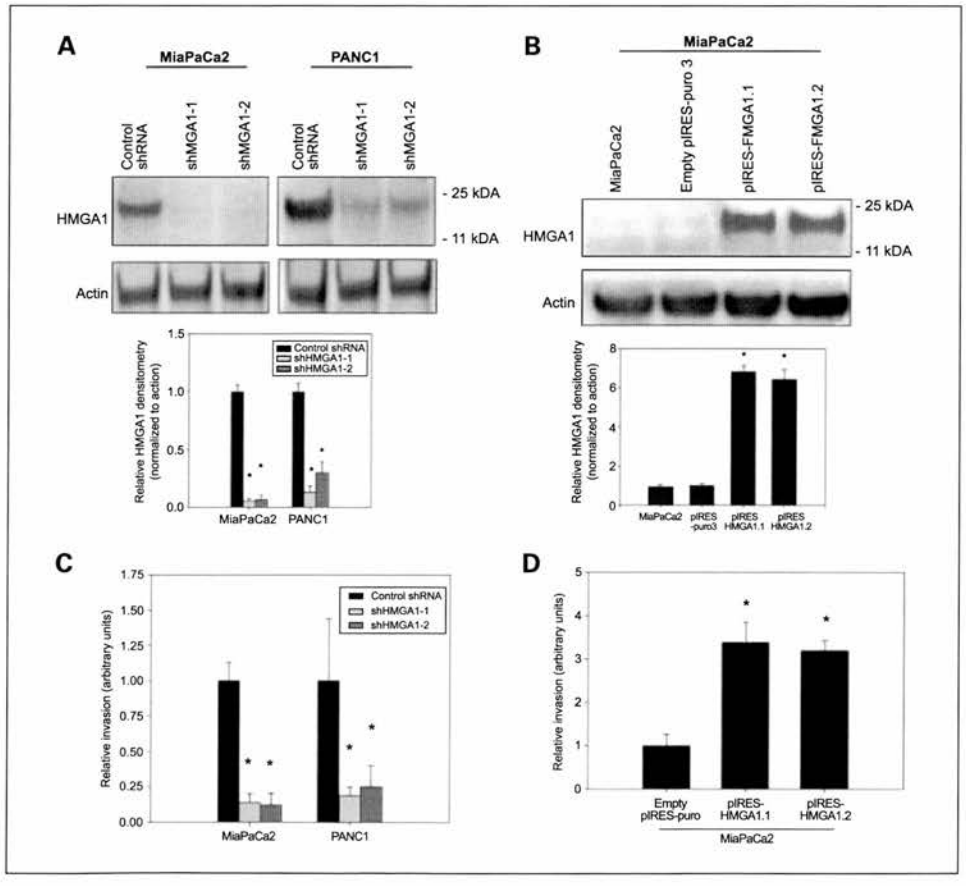

Figure 1. *A*, stable silencing of HMGA1 expression using shRNA expression vectors with two independent shRNA target sequences (shHMGA1-1 and shHMGA1-2) was confirmed on Western blot analysis. Controls were cells stably transfected with non-targeting, scrambled shRNA. Greater suppression of HMGA1 expression was achieved in MiaPaCa2 cells (up to 90%) compared with PANC1 cells, which have HMGA1 silenced to a lesser degree. *, $P < 0.05$ versus control shRNA. *B*, two clones of MiaPaCa2 cells were confirmed to stably overexpress HMGA1 (pIRES-HMGA1.1 and pIRES-HMGA1.2) on Western blot analysis. Controls were cells stably transfected with empty pIRES-puro3 vector. *, $P < 0.05$ versus empty pIRES-puro3 vector and parental MiaPaCa2 cells. Representative blots of three independent experiments. *Columns*, mean densitometry values; *bars*, SD. *C*, cellular invasiveness was determined using Matrigel-coated Boyden chamber assays. Stable suppression of HMGA1 expression resulted in significant reductions in cellular invasiveness in both MiaPaCa2 and PANC1 cells. *, $P < 0.05$ versus control shRNA. *D*, forced overexpression of HMGA1 in MiaPaCa2 cells (pIRES-HMGA1.1 and pIRES-HMGA1.2) resulted in ~3-fold increase in cellular invasive capacity when compared with empty pIRES-puro3 transfectants. *, $P < 0.05$ versus empty pIRES-puro3 vector. Invasion assays were done in triplicate. *Columns*, mean from 20 randomly selected fields; *bars*, SD.

Western blot analysis (Fig. 1*B*). MiaPaCa2 cells stably transfected with empty pIRES-puro3 vector served as controls. HMGA1 expression in parental MiaPaCa2 cells and empty pIRES-puro3 transfectants did not differ (Fig. 1*B*). pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited ~3- to 3.5-fold increases in cellular invasiveness, compared with control cells (Fig. 1*D*).

Effects of modulating HMGA1 expression on MMP-9 activity, mRNA expression, and promoter activity. Given the importance of MMP-9 as a mediator of malignant cellular invasiveness and metastasis (18–20), we hypothesized that MMP-9 is a downstream effector that plays a critical role in HMGA1-dependent cellular invasiveness. HMGA1 silencing in MiaPaCa2 cells was found to be associated with a reduction in total MMP-9 activities (Fig. 2*A*). In addition, HMGA1 silencing led to reductions in MMP-9 mRNA levels as quantified by real-time quantitative reverse transcription-PCR (Fig. 2*B*). Conversely, MMP-9 activities and mRNA levels were significantly higher in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones than in controls (Fig. 2*A* and *B*). To assess the effect of modulating HMGA1 expression on MMP-9 promoter activity, we did cotransfection experiments in which MiaPaCa2 cells were transiently transfected with either shHMGA1 or pIRES-HMGA1 vector together with MMP-9 promoter reporter vector. MMP-9 promoter activities were significantly lower with HMGA1 silencing, whereas MMP-9 promoter activity levels were markedly higher with HMGA1 overexpression, compared with controls (Fig. 2*C*).

MMP-9 is a mediator of HMGA1-dependent increases in cellular invasiveness. The contribution of MMP-9 to the increased cellular invasiveness induced by HMGA1 overexpression was determined by performing the invasion assay in the presence of anti–MMP-9-neutralizing antibody. MMP-9 immunoneutralization reduced the cellular invasiveness of MiaPaCa2 pIRES-HMGA1.1 and pIRES-HMGA1.2 clones by ~70% (Fig. 2*D*).

Effects of HMGA1 modulation on Akt activation. Activation of the serine/threonine kinase Akt is common in pancreatic adenocarcinoma (21) and is an important determinant of malignant cellular invasiveness (22). The PI3K/Akt signaling

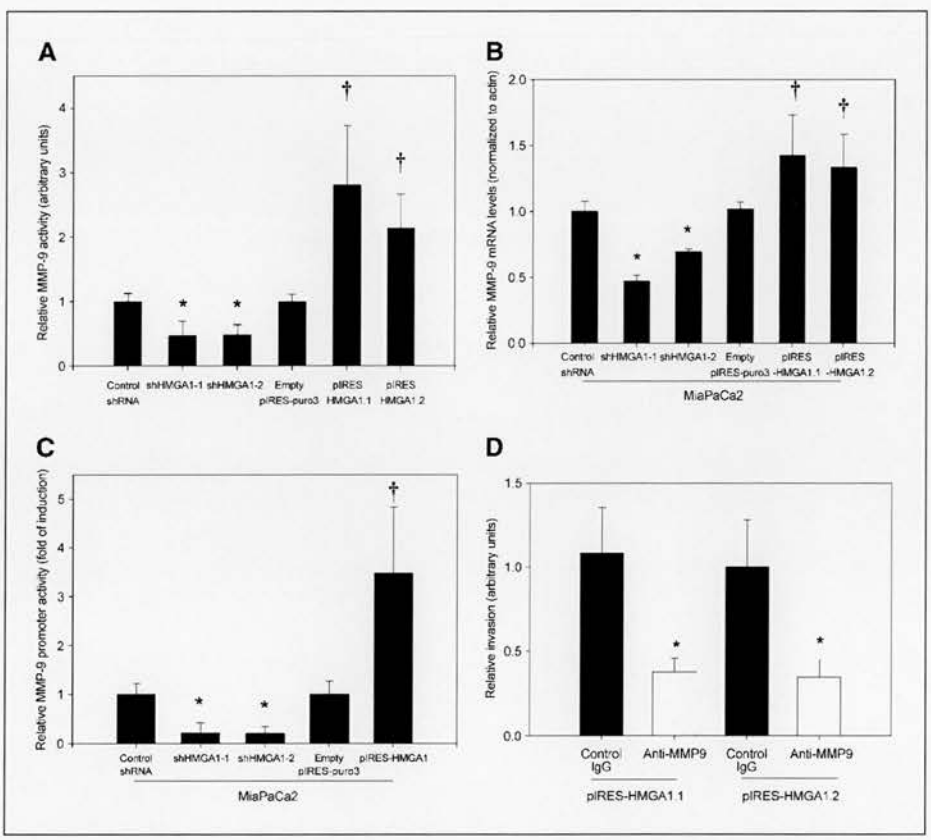

Figure 2. HMGA1 expression positively regulates MMP-9 activities, mRNA levels, and promoter activities. *A,* cellular MMP-9 activities were quantitated using a fluorometric MMP-9 activity assay. Targeted suppression of HMGA1 expression (shHMGA1-1 and shHMGA1-2) resulted in significant reductions in MMP-9 activity in MiaPaCa2 cells, whereas forced overexpression of HMGA1 led to increased cellular MMP-9 activity. Both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited ~2- to 2.5-fold increases in MMP-9 activity. *, $P < 0.05$ versus control shRNA; †, $P < 0.05$ versus empty pIRES-puro3 transfectants. *B,* HMGA1 modulates MMP-9 mRNA levels. On quantitative real-time PCR, MMP-9 levels were significantly lower in shHMGA1-1 and shHMGA1-2 transfectants compared with control shRNA transfectants. Overexpression of HMGA1 led to increases in MMP-9 mRNA levels. *, $P < 0.05$ versus control shRNA; †, $P < 0.05$ versus empty pIRES-puro3 transfectants. *C,* the effects of HMGA1 silencing and overexpression on MMP-9 promoter activities in MiaPaCa2 cells were determined using transient transfection and luciferase reporter assays. Cotransfection experiments were done. MiaPaCa2 cells were transiently transfected with shRNA (shHMGA1-1, shHMGA1-2, or control shRNA) or overexpression vectors (pIRES-HMGA1 or empty pIRES-puro3 control) together with the reporter plasmids. Reporter plasmids included pGL4e (empty firefly luciferase vector) or pGL4-MMP9, containing the full-length MMP9 promoter and pRL vector containing a *Renilla* luciferase gene. Forty-eight hours following transfection, luciferase activity in cell lysates was assayed. *Renilla* luciferase activities were used to normalize results for transfection efficiencies. Consistent with MMP-9 activity and mRNA studies, targeted suppression of HMGA1 expression using shRNA resulted in a significant reduction in MMP-9 promoter activities, whereas forced overexpression of HMGA1 in MiaPaCa2 cells led to significant increases in MMP-9 promoter activities. *, $P < 0.05$ versus control shRNA; †, $P < 0.05$ versus empty pIRES-puro3 vectors. *D,* the contribution of MMP-9 to the increased cellular invasiveness induced by HMGA1 overexpression was determined by performing the invasion assay in the presence of anti–MMP-9-neutralizing antibody. MMP-9 immunoneutralization reduced the cellular invasiveness of pIRES-HMGA1.1 and pIRES-HMGA1.2 clones by ~70%.

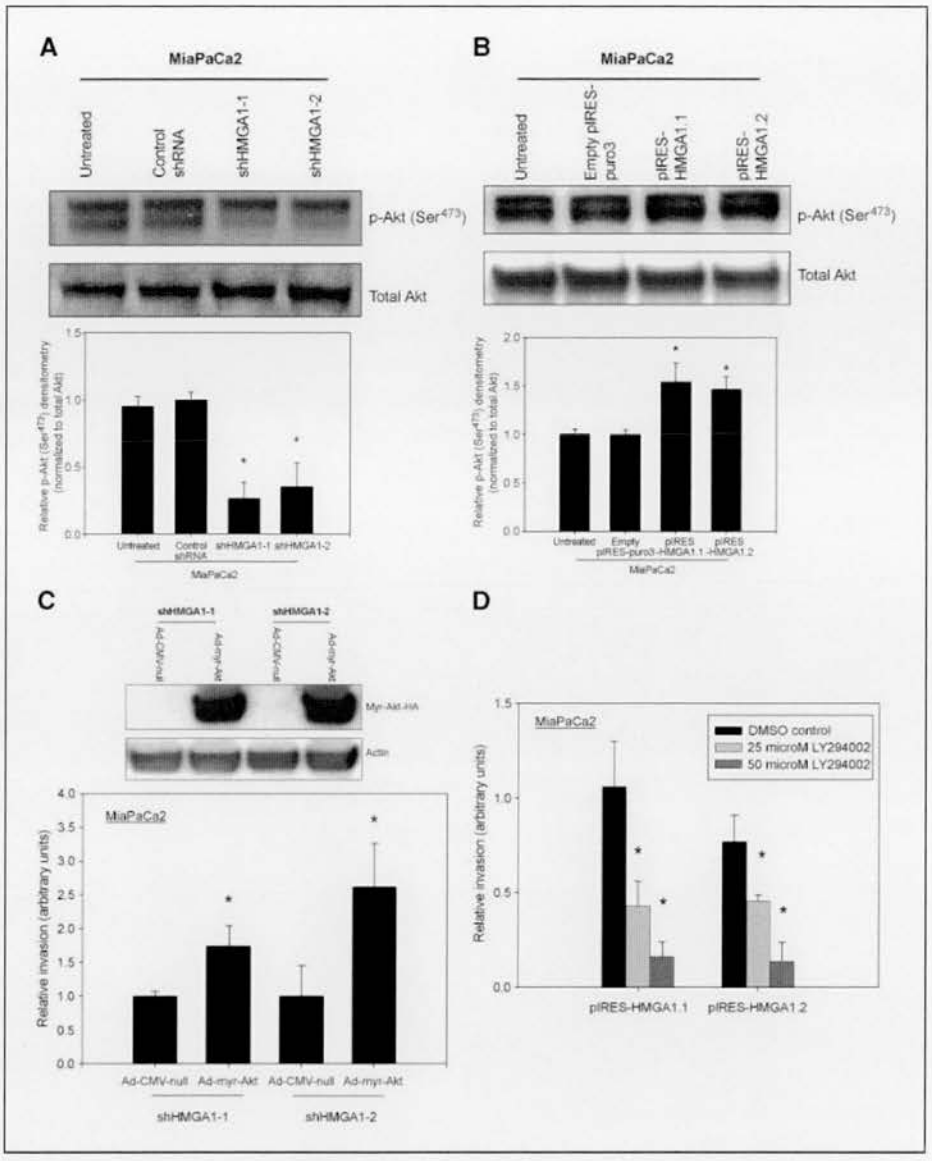

Figure 3. The effects of HMGA1 modulation on Akt phosphorylation in MiaPaCa2 cells were confirmed on Western blot analysis using a phospho-specific antibody. *A,* in MiaPaCa2 cells, stable silencing of HMGA1 resulted in a decreased level of phosphorylation of Akt at Ser^{473}, a marker of Akt activation. *, $P < 0.05$ versus MiaPaCa2 cells and control shRNA transfectants. *B,* overexpression of HMGA1 led to increased activation of Akt, as evident from increased levels of phospho-Akt (*p-Akt*). There was no difference in the level of expression of total Akt with suppression or overexpression of HMGA1. *, $P < 0.05$ versus MiaPaCa2 cells and empty pIRES-puro3 transfectants. Representative blots of three independent experiments. *Columns,* mean densitometry values; *bars,* SD. *C,* contribution of Akt to HMGA1-mediated invasiveness was assessed by transductions of MiaPaCa2 shHMGA1-1 and shHMGA1-2 transfectants with adenovirus expressing constitutively active Akt (Ad-myr-Akt). Constitutively active Akt was able to rescue the invasive phenotype in the MiaPaCa2 cells with HMGA1 silencing. Cell lysates were immunoblotted with anti-HA to detect expression of HA-tagged myristoylated Akt. *, $P < 0.05$ versus MiaPaCa2 transduced with control adenovirus (Ad-CMV-null). *D,* inhibition of PI3K with LY294002 attenuated the HMGA1-induced increases in invasiveness in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones. The effects of PI3K were dose dependent (25 and 50 μmol/L). *, $P < 0.05$ versus DMSO controls.

pathway has previously been reported to be important in the regulation of MMP-9 expression (23–25). Given our observations that HMGA1 expression regulates MMP-9 activity via transcriptional activation, we hypothesized that HMGA1 modulates Akt activation. We found that HMGA1 silencing results in a reduction in Akt phosphorylation at Ser^{473} (Fig. 3*A*), whereas HMGA1 overexpression results in increased levels of phospho-Akt (Ser^{473}; Fig. 3*B*). Modulating HMGA1 expression had no effect on total Akt levels.

HMGA1-induced cellular invasiveness and MMP-9 activity is PI3K/Akt dependent. Given our findings that HMGA1 modulates Akt activation, we tested whether cellular invasiveness mediated by HMGA1 is dependent on PI3K/Akt signaling. First, we determined if the invasive phenotype in MiaPaCa2 cells in which HMGA1 had been stably silenced could be rescued using constitutively active Akt. MiaPaCa2 stable transfectants shHMGA1-1 and shHMGA1-2 were infected with adenovirus expressing myristoylated Akt (Ad-myr-Akt). Constitutively active Akt was able to rescue the invasive phenotype in shHMGA1-1 and shHMGA1-2 transfectants resulting in an increased invasiveness compared with cells infected with control adenovirus (Fig. 3*C*). Next, given that PI3K is an upstream regulator of Akt, we assessed the effects of PI3K inhibition on cellular invasiveness in MiaPaCa2 cells overexpressing HMGA1 (pIRES-HMGA1.1 and pIRES-HMGA1.2). Inhibition of PI3K activity using the PI3K inhibitor LY294002 resulted in dose-dependent reductions in cellular invasiveness in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Fig. 3*D*). Consistent with this finding, infection with adenovirus expressing dominant-negative Akt resulted in attenuation of HMGA1-induced invasiveness in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones. Importantly, dominant-negative Akt also induced reductions in cellular MMP-9 activity in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones, indicating that HMGA1-induced MMP-9-dependent cellular invasiveness is PI3K/Akt dependent (Fig. 4*A*).

Modulation of HMGA1 expression has no effect on cellular proliferation in monolayer culture. The effects of modulating HMGA1 expression on cellular proliferation in monolayer culture were determined. Using the MTS assay, we observed no effect in

cellular proliferation in low (2% FBS) and high (10% FBS) serum conditions with either *HMGA1* gene silencing or overexpression (Fig. 4*B*).

Effects of HMGA1 modulation on phosphorylation of ERK and mTOR. Previous studies have shown that HMGA1 modulates ERK activation (26). In our study, HMGA1 silencing had no effect on ERK phosphorylation, whereas HMGA1 overexpression resulted in increased ERK phosphorylation with no effects on the total ERK levels (Fig. 5*A* and *B*). Given that mTOR is a well-known downstream mediator of the PI3K/Akt pathway (27, 28), we sought to determine the effects of HMGA1 modulation of mTOR phosphorylation. HMGA1 silencing resulted in reductions in mTOR phosphorylation at Ser^{2448}, whereas HMGA1 overexpression led to increases in mTOR phosphorylation (Fig. 5*C* and *D*). Modulation of HMGA1 expression had no effect on levels of total mTOR expression.

HMGA1 silencing suppresses *in vivo* metastatic potential of pancreatic cancer cells and reduces tumoral growth and MMP-9 activity. Given these *in vitro* findings, we sought to determine the effect of modulating HMGA1 expression on metastatic potential *in vivo*. Four weeks following surgical orthotopic implantation of MiaPaCa2 stable transfectants expressing control

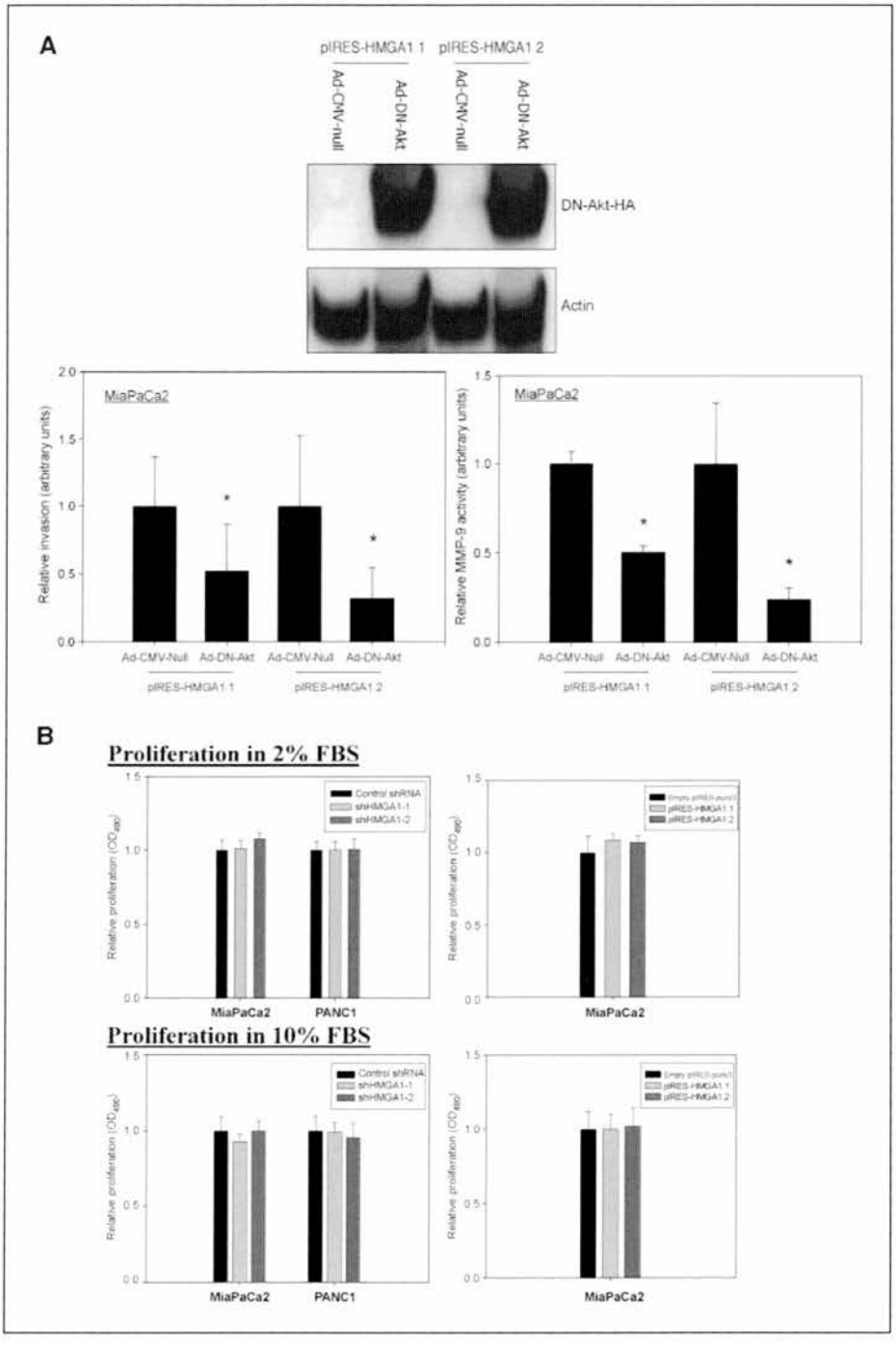

Figure 4. *A*, dominant-negative Akt attenuates HMGA1-induced invasiveness and MMP-9 activity. MiaPaCa2 pIRES-HMGA1.1 and pIRES-HMGA1.2 clones were transduced with adenovirus expressing dominant-negative Akt (Ad-DN-Akt). Dominant-negative Akt resulted in reductions in invasiveness and in MMP-9 activities in both HMGA1-overexpressing clones compared with control adenovirus. Cell lysates were immunoblotted with anti-HA to detect expression of HA-tagged DN Akt. *, $P < 0.05$ versus control adenovirus (Ad-CMV-null). *B*, based on MTS assay, modulation of HMGA1 expression did not affect cellular proliferation in monolayer culture. Experiments were done at low (2% FBS) and high (10%) conditions.

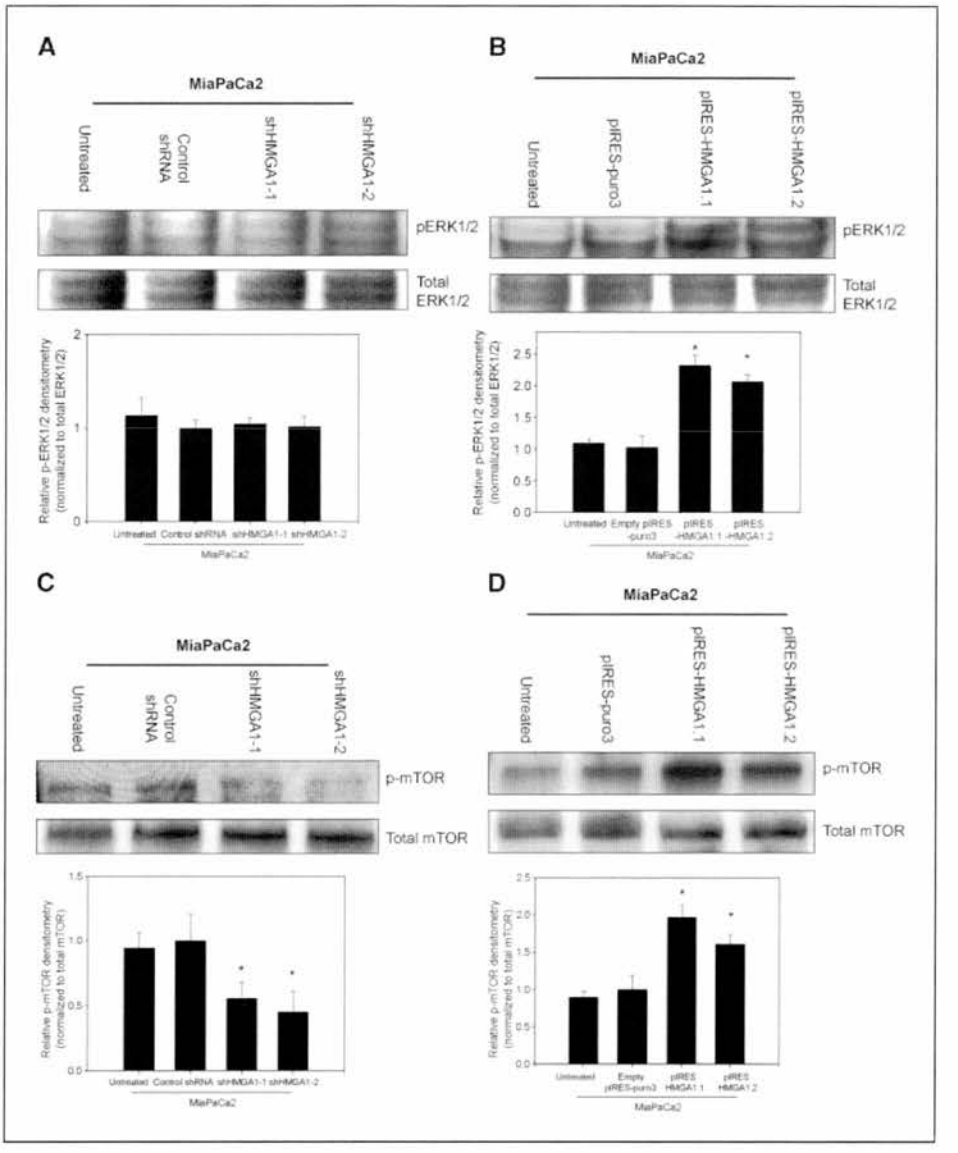

Figure 5. The effects of HMGA1 modulation on ERK and mTOR phosphorylation in MiaPaCa2 cells were confirmed on Western blot analysis using a phospho-specific antibody. *A* and *B*, although HMGA1 silencing did not alter the levels of ERK phosphorylation, overexpression of HMGA1 increased ERK phosphorylation. There were no differences in the levels of total ERK. *C* and *D*, targeted suppression of HMGA1 expression resulted in reductions in phosphorylation of mTOR at Ser^{2448} in both shHMGA1-1 and shHMGA1-2 transfectants, whereas overexpression of HMGA1 led to increases in phospho-mTOR levels. There was no difference in total mTOR levels among groups. *, $P < 0.05$ versus control shRNA or empty pIRES-puro3 transfectants, as appropriate.

shRNA ($n = 10$) or shHMGA1 ($n = 10$), necropsy was done. All mice in the control shRNA group were found to have histologically confirmed liver metastases, and six of these mice were found to have gross ascites. In the shHMGA1 group, only one mouse developed metastases (Table 1; $P < 0.05$), and none of them had ascites. No lung metastases were detected in either group. There were no apparent differences in the histologic appearance of H&E-stained sections of xenografts harvested from animals in the shHMGA1 and control groups (Fig. 6*A*). Stable silencing of tumoral HMGA1 expression in the shHMGA1 group *in vivo* was confirmed by immunohistochemistry (Fig. 6*A*) and Western blotting (Fig. 6*B*). Additionally, HMGA1 silencing was associated with significant reductions in tumor growth and MMP-9 activity (Fig. 6*C* and *D*).

Discussion

Pancreatic adenocarcinoma is among the most aggressive of human malignancies. The prognosis associated with this cancer remains dismal, despite the considerable advances in medical and surgical management of patients diagnosed with pancreatic cancer. There is an urgent need to identify new therapeutic approaches. This study was designed to determine the role of HMGA1 in pancreatic adenocarcinoma cellular invasiveness and metastasis. We have shown that HMGA1 overexpression promotes cellular invasiveness, and that specific suppression of HMGA1 expression inhibits cellular invasiveness *in vitro* and metastasis *in vivo*. HMGA1-induced cellular invasiveness is in part due to PI3K/Akt–dependent modulation of MMP-9 activity. Together, these findings suggest that HMGA1 architectural transcriptional factors represent a molecular determinant of cellular invasiveness and a potential therapeutic target in pancreatic adenocarcinoma.

HMGA1 previously has been reported to be associated with malignant cellular behavior in a range of human cancers (11, 29, 30). HMGA1 has been shown to confer the ability of non-tumorigenic breast epithelial cells to grow under anchorage-independent conditions (31). Antisense oligonucleotide-mediated suppression of HMGA1 expression has been reported to inhibit neoplastic transformation in breast cancer (32) and in Burkitt's

Table 1. HMGA1 gene silencing by RNA interference suppresses the metastatic potential of pancreatic adenocarcinoma cells *in vivo*

	Control shRNA (n = 10)	shHMGA1 (n = 10)
Median no. metastases (range)	6 (1–20)	0* (0–3)
Mice with metastases	100%	10%*

NOTE: At 4 weeks following orthotopic implantation of 1×10^6 MiaPaCa2 cells stably expressing control or shHMGA1 constructs, necropsy was done. Liver metastases were counted and confirmed histologically. HMGA1 silencing significantly inhibited metastasis in this nude mouse model.
*$P < 0.05$ versus control shRNA group.

lymphoma cells (33). Although there is extensive correlative evidence suggesting a role for HMGA1 in tumor metastasis (9, 34, 35), there have been few studies showing a direct functional link between HMGA1 expression and invasion and metastasis (14, 30, 31).

Our study is the first to show a role for HMGA1 in pancreatic adenocarcinoma cellular invasiveness and metastasis and provides evidence that HMGA1 expression mediates cellular invasiveness through a PI3K/Akt/MMP-9–dependent pathway. Although MMP-9 is unlikely to be the sole effector of HMGA1-dependent invasiveness, this pathway represents a potential mechanism that may contribute to the decrease in cellular invasiveness induced by *HMGA1* gene silencing. Our finding that PI3K/Akt signaling mediates regulatory actions of HMGA1 is novel. Our finding that HMGA1 promotes mTOR activation provides further support for the relationship between HMGA1 and PI3K/Akt signaling, as mTOR is a downstream target of this pathway (27, 28).

Our finding that HMGA1 overexpression results in increased ERK phosphorylation is consistent with findings of previously reported studies suggesting that HMGA1 is able to positively regulate the Ras/ERK mitogenic signaling pathway (26). The Ras/ERK signaling pathway is intrinsically linked to PI3K/Akt pathways. PI3K/Akt has been shown to be downstream of Ras/ERK signaling pathway (36, 37), and Ras can also directly activate the PI3K/Akt signaling pathway (38). Alternatively, HMGA1 has been shown to transcriptionally regulate the human insulin receptor gene (39, 40), and given that PI3K/Akt are downstream mediators of insulin signaling (41), it is not surprising that HMGA1 expression affects PI3K/Akt signaling. Both Ras/ERK and PI3K/Akt signaling pathways have been found to be critical in mediating cellular invasion in pancreatic cancer cells (42).

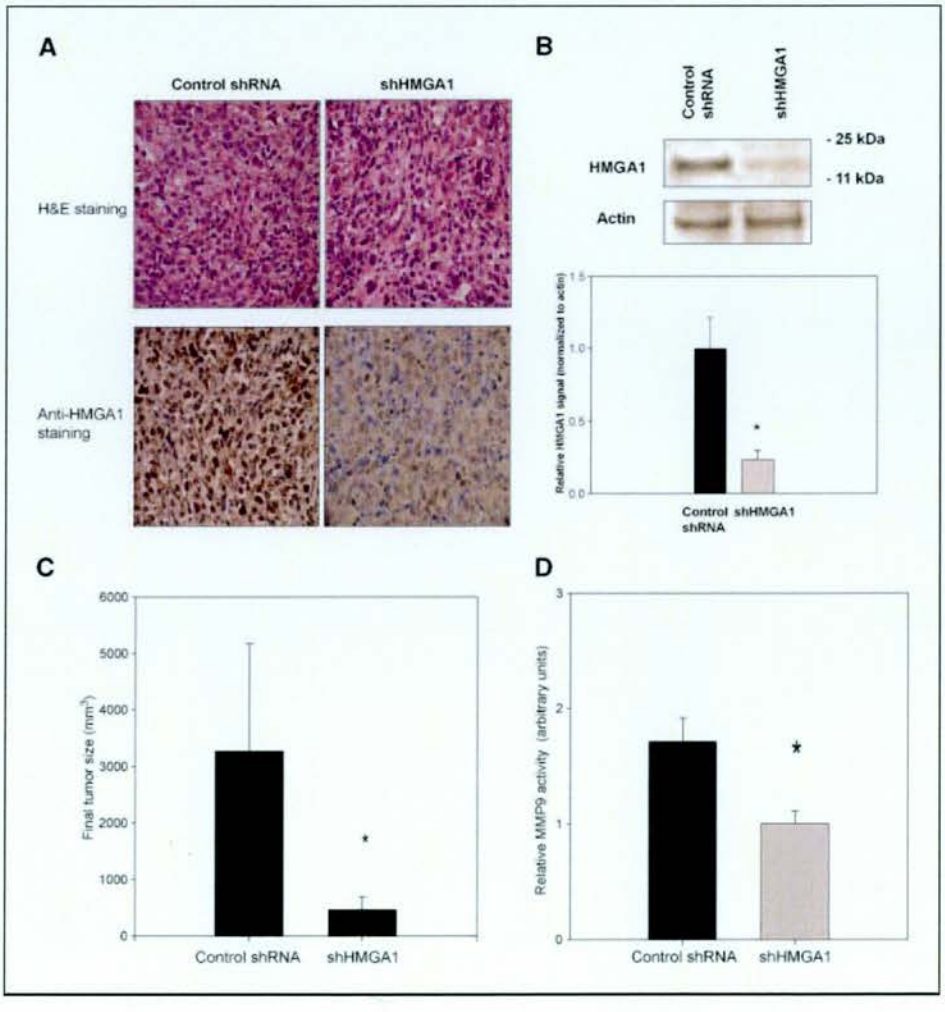

Figure 6. MiaPaCa2 stable transfectant cells expressing the control shRNA or shHMGA1 (shHMGA1-1) were orthotopically implanted into the pancreata of nude mice. Four weeks following implantation, necropsy was done, and the primary tumors were explanted. *A*, histologic appearance of primary tumors was examined at ×400 magnification following H&E staining of tumor sections. There was no apparent morphologic difference in tumors harvested from the two groups of animals. However, sections stained with anti-HMGA1 antibody revealed that tumors from shHMGA1 group exhibited reductions in nuclear staining for HMGA1. *B*, stable HMGA1 suppression in lysates of shHMGA1-derived tumors was further confirmed by Western blot. *C*, final volume of primary tumors harvested from shHMGA1 group was markedly reduced compared with those of the control shRNA group. *D*, MMP-9 activities in lysates obtained from HMGA1 shRNA transfectant–derived tumors were significantly lower than those of control shRNA transfectant–derived tumors. *Columns*, mean; *bars*, SD. *, $P < 0.05$ versus control shRNA transfectant–derived tumors.

Our observation that HMGA1 silencing suppresses Akt activity is significant, as Akt is now recognized as an important mediator of malignant cellular behavior, including the capacity for resisting apoptotic stimuli, in pancreatic adenocarcinoma (43–45). Trapasso et al. have reported that antisense-mediated suppression of HMGA1 expression results in an apoptotic response in three pancreatic cancer cell lines (46). Our findings that HMGA1 positively regulates PI3K/Akt signaling provides a possible mechanism through which HMGA1 suppression promotes apoptosis. Unlike Trapasso et al., we found that modulating HMGA1 expression has no effect on cellular proliferation in two-dimensional monolayer culture. However, our data indicate that HMA1 silencing does inhibit tumor growth *in vivo*.

Our study provides support to the hypothesis that the HMGA1 proteins are potential therapeutic targets for inhibiting the activation of the PI3K/Akt pathway in cancer cells. From a therapeutic standpoint, targeting HMGA1 is attractive in that it is overexpressed in a range of human malignancies. HMGA1 expression is absent or present at only very low levels in normal adult tissues (47). As such, targeting HMGA1 may have little or no effect on non-cancerous tissues. Given our finding that RNA interference–mediated HMGA1 silencing inhibits invasive and metastatic potential, HMGA1 represents a rational molecular therapeutic target. The feasibility of *in vivo* gene silencing induced by the delivery of therapeutic small interfering RNA (siRNA) has already been shown (48). Technological advances, such as the development of improved delivery systems for siRNAs, will facilitate this approach.

In summary, our findings suggest that HMGA1 promotes pancreatic cancer cellular invasive and metastatic potential. Our findings also indicate that HMGA1 represents a potential therapeutic target for strategies designed to inhibit the progression of pancreatic cancer.

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Overexpression of HMGA1 promotes anoikis resistance and constitutive Akt activation in pancreatic adenocarcinoma cells

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HMGA1 proteins are architectural transcription factors that are overexpressed by pancreatic adenocarcinomas. Roles of HMGA1 in mediating the malignant phenotype of this cancer are poorly understood. We tested the hypothesis that overexpression of HMGA1 promotes resistance to anoikis (apoptosis induced by anchorage deprivation) in pancreatic cancer cells. HMGA1 cDNA was stably transfected into MiaPaCa2 human pancreatic adenocarcinoma cells (which have low baseline expression levels of HMGA1). Cells were grown in suspension on PolyHEMA-coated plates and their susceptibility to anoikis was assayed using flow cytometry. Overexpression of HMGA1 was associated with marked reductions in susceptibility to anoikis in concert with increases in Akt phosphorylation (Ser473) and in Akt kinase activity and with reductions in caspase 3 activation. Inhibition of phosphoinositidyl-3 (PI3-K)/Akt pathway with either the small molecule inhibitor LY294002 or dominant-negative Akt resulted in reversal of anoikis resistance induced by HMGA1 overexpression. Further, RNA interference-mediated HMGA1 silencing in MiaPaCa2 and BxPC3 (a human pancreatic adenocarcinoma cell line with high baseline levels of HMGA1 expression) cells resulted in significant increases in susceptibility to anoikis. Our findings suggest HMGA1 promotes anoikis resistance through a PI3-K/Akt-dependent mechanism. Given the putative associations between anoikis resistance and metastatic potential, HMGA1 represents a potential therapeutic target in pancreatic adenocarcinoma.

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Pancreatic adenocarcinoma is among the deadliest of all human cancers, with 5-year survival rates averaging less than 5% (Jemal *et al*, 2006). The propensity for pancreatic adenocarcinoma cells to metastasise early in the course of disease progression makes this cancer particularly refractory to standard therapies. Characterising the underlying mechanisms mediating metastatic dissemination in pancreatic adenocarcinoma may reveal novel targets for inhibiting this process, which is so aggressive in this cancer.

Anoikis, derived from a Greek word meaning 'homelessness', was used in describing the observation that depriving cells from attachment to matrix triggers apoptosis in these cells (Frisch and Francis, 1994). Increasingly, metastasis is conceptualised to be a multistep process facilitated by the evolution of anoikis-resistant subsets of cancer cells that are capable of surviving in the blood stream during dissemination after they detach from the primary tumour and its stroma. Indeed, numerous studies suggest a close correlation between signalling events mediating cellular anoikis resistance *in vitro* and those mediating metastatic potential *in vivo* (Yawata *et al*, 1998; Zhu *et al*, 2001; Douma *et al*, 2004; Berezovskaya *et al*, 2005).

The human *HMGA1* gene, located on chromosomal locus 6p21, encodes two HMGA1 splice variants (HMGA1a and HMGA1b) (Friedmann *et al*, 1993). These HMGA1 proteins are architectural transcription factors that regulate gene expression *in vivo* by forming stereo-specific, multiprotein complexes termed 'enhanceosomes' on the promoter regions of genes (Reeves and Nissen, 1990; Thanos and Maniatis, 1995). HMGA1 proteins are overexpressed in a range of human cancers, notably including pancreatic adenocarcinoma (Abe *et al*, 2000; Balcerczak *et al*, 2003; Chiappetta *et al*, 2004; Czyz *et al*, 2004; Donato *et al*, 2004; Chang *et al*, 2005; Sarhadi *et al*, 2006; Xu *et al*, 2004). Moreover, high tumoural expression of HMGA1 has been reported to be predictive of poor prognosis among patients with cancer (Tamimi *et al*, 1996; Balcerczak *et al*, 2003; Chiappetta *et al*, 2004).

Although these correlative data suggest a potential role for HMGA1 in cancer pathogenesis, the underlying biology of HMGA1 in the context of cancer progression is poorly understood. The purpose of this study was to test the hypothesis that HMGA1 promotes anoikis resistance in pancreatic adenocarcinoma. Our observations indicate that HMGA1 promotes anoikis resistance through the phosphoinositidyl-3 kinase (PI3-K)/Akt pathway.

MATERIALS AND METHODS

Cells and cell culture

MiaPaCa2 and BxPC3 human pancreatic ductal adenocarcinoma cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in DMEM containing 10% fetal bovine serum (FBS) (Gibco Life Technologies Inc., Gaithersburg, MD, USA) and incubated in a humidified

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(37°C, 5% CO_2) incubator, grown in 75-cm^2 culture flasks, and passaged on reaching 80% confluence.

Reagents and dominant-negative Akt adenovirus

The PI3-K-inhibitor LY294002 was purchased from Calbiochem (San Diego, CA, USA). Anti-HMGA1, anti-lamin B and anti-Akt antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho Akt (ser473) antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Adenovirus expressing dominant-negative murine Akt1 and control virus (Ad-CMV-null) (both titred at 1×10^{10} PFU ml^{-1}) were purchased from Vector Biolabs (Philadelphia, PA, USA). Adenoviral infection was performed at multiplicity of infection of 10 in the presence of 6 $\mu g\,ml^{-1}$ polybrene for 12 h. Experiments were performed on cells 48 h following infection.

Expression vector and transfection

The *HMGA1*-coding sequence was PCR amplified from IMAGE clone 5399570 (GenBank accession no. BC063434) using gene-specific primers modified to include the appropriate restriction sites at their 5′ end. The primers used were: forward, 5′-TTTTGATATCATGAGTGAGTCGAGCTCGAAG-3′ and backward, 5′-TTTTGAATTCTCACTGCTCCTCCTCCGAGGA-3′. Purified PCR products were digested with *Eco*RV and *Eco*RI, before ligation into a *Eco*RV/*Eco*RI-digested pIRES-puro3 vector (Clontech, Palo Alto, CA, USA). The expression plasmid was named pIRES-HMGA1. MiaPaCa2 cells were transfected with pIRES-HMGA1 or empty pIRES-puro3, which acted as a control, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Stable clones were selected by exposure to incrementally increasing concentrations of puromycin (Invivogen, San Diego, CA, USA), isolated using cloning cylinders and maintained in medium containing 3 $\mu g\,ml^{-1}$ puromycin (Invivogen). Clones pIRES-HMGA1.1 and pIRES-HMGA1.2, which expressed the highest levels of HMGA1, were used for further studies.

Lentivirus-mediated *HMGA1* RNAi

Lentiviral hairpin RNA interference (RNAi) plasmids (pLKO.1-HMGA1, TRCN0000018949), constructed as described previously (Stewart *et al*, 2003), were obtained from the RNAi Consortium (Mission TRC Hs. 1.0, Sigma Aldrich, St Louis, MO, USA). The sequence of short hairpin RNA (shRNA) targeting the human *HMGA1* gene (GenBank accession no. NM_002131) was 5′-AACTCCAGGAAGGAAACCAA-3′, corresponding to the coding region positions 446–466. The controls were lentiviral particles produced with empty pLKO.1 and pLKO.1, which has a scramble nontargeting shRNA sequence obtained from Addgene (Cambridge, MA, USA), deposited by Dr David Sabatini (Sarbassov *et al*, 2005). Each of these vectors had been sequence-verified. Vectors were expanded in chemically competent *Escherichia coli* (TOP10 cells, Invitrogen) and purified using Genelute maxiprep kit (Sigma Aldrich). To generate lentiviral particles, human embryonic kidney 293 cells (ATCC) were cotransfected with the lentiviral vector and compatible packaging plasmid mixture (Virapower lentiviral packaging system, Invitrogen) using Lipofectamine 2000 (Invitrogen), in accordance to manufacturer's instruction. Pancreatic adenocarcinoma cells were exposed to lentivirus-containing supernatant for 16 h in the presence of 6 $\mu g\,ml^{-1}$ polybrene (Sigma, St Louis, MI, USA). Pooled stable transfectants were established using puromycin selection. Stable transfectant cells were maintained in medium containing 3 $\mu g\,ml^{-1}$ of puromycin (Invivogen).

Anoikis induction and flow cytometry

Anoikis was assayed by plating cells on polyHEMA-coated plates. A solution of 120 mg ml^{-1} polyHEMA (Sigma) in 100% ethanol was made and diluted 1:10 in 95% ethanol; 0.95 ml mm^{-2} of this solution was overlaid onto 35-mm wells and left to dry in a heated dryer system for 12 h. Before use, wells were washed twice with PBS and once with DMEM. In all, 1×10^6 cells of each line, suspended in 2 ml DMEM with 10% FBS were incubated in the polyHEMA-coated wells for 12–18 h in a humidified (37°C, 5% CO_2) incubator. Cells were harvested and resuspended in 0.3 ml of PBS containing 2% FBS and 0.1 μM EDTA. Apoptosis staining was performed using 1 $\mu l\,ml^{-1}$ YO-PRO-1 and propidium iodide (Vybrant Apoptosis Assay Kit #4; Molecular Probes, Eugene, OR, USA). Cells were incubated for 30 min on ice and then analysed by flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA), measuring fluorescence emission at 530 and 575 nm. Cells stained with the green fluorescent dye YO-PRO-1 were counted as apoptotic; necrotic cells were stained with propidium iodide. The number of apoptotic cells was divided by the total number of cells (minimum of 10^4 cells), resulting in the apoptotic fraction. Data were analysed using CellQuest software (Becton Dickinson). All assays were performed in triplicates.

Western blotting

Cells were harvested and rinsed twice with PBS. Total cell extracts were prepared with lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton X, 0.5% deoxycholate, 1 mM PMSF, 10 mg ml^{-1}, aprotinin, 10 mg ml^{-1} leupeptin) and cleared by centrifugation at 15 000 *g*, 4°C. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents based on the manufacturer's instruction (Pierce, Rockford, IL, USA). Total protein concentration was measured using the BCA assay kit (Sigma) with bovine serum albumin as a standard, according to the manufacturer's instructions. Total cell lysates containing 50 μg total protein or nuclear protein containing 10 μg total protein were subjected to 10% SDS/PAGE and the resolved proteins transferred electrophoretically to PVDF membranes (Invitrogen). Equal protein loading was confirmed by Coomassie (BioRad, Hercules, CA, USA) staining of the gel. After blocking with PBS containing 3% bovine serum albumin for 1 h at room temperature, membranes were incubated with 3–5 mg ml^{-1} antibody in PBS containing 0.1% Tween-20 overnight at 4°C. Chemoluminescensce detection (Amersham Biosciences, Piscataway, NJ, USA) was performed in accordance with the manufacturer's instructions. The densitometric signal was quantified using ImagePro Plus software version 4.0 (Media Cybernetics, Silver Spring, MD, USA) and normalised to that of loading control as appropriate. Blots were performed in triplicate in at least three independent experiments. Mean densitometric values ($\pm$standard Deviation (s.d.)) are shown.

Fluorometric real-time Akt kinase assay

Assay was performed using the Omnia Lysate Akt kinase assay (Biosource-Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, total cell lysate equivalent to 5 $\mu g\,\mu l^{-1}$ was loaded into each well. Following addition of fluorescent Akt substrates and kinase buffer, fluorescent signal was acquired real-time (excitation, 360 nm; emission, 485 nm) for 60 min using SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The Akt kinase activity was calculated from the slope of activity curve. Experiments were performed in triplicates on at least three occasions.

Fluorometric caspase profiling

Whole-cell lysates were assayed for caspases 3 activity using the BD ApoAlert Caspase Assay Plate (BD Biosciences Clontech, Palo

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Alto, CA, USA) according to the manufacturer's instructions. Plates were read (excitation, 360 nm; emission, 480 nm) using SpectraMax M5 microplate reader in fluorescence mode (Molecular Devices). All measurements were performed in triplicate, each with three determinations for each condition.

Proliferation assay

Cell proliferation was quantified using an MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium) assay (CellTiter 96 Aqueous One Solution Assay, Promega, Madison, WI, USA), in accordance to the manufacturer's instructions and confirmed by cell counting. Logarithmically growing cells were seeded into 96-well plates at 5×10^3 cells per well and allowed to adhere overnight in medium containing 10% FBS. Cell proliferation was determined after 48 h. Plates were read with the use of the SpectraMax M5 microplate spectrophotometer (Molecular Devices) at a wavelength of 490 nm. Ten samples were used for each experimental condition and experiments were performed in triplicate. At identical time points, cell counting was performed. Cells were trypsinised to form a single-cell suspension. Viable cells, determined by trypan blue exclusion, were counted with the use of a Neubauer hemocytometer (Hausser Scientific, Horsham, PA, USA).

Statistical analysis

Differences between groups were analysed using Student's *t*-test, multifactorial ANOVA of initial measurements and Mann–Whitney *U*-test for nonparametric data, as appropriate, using Statistica 5.5 software (StatSoft Inc., Tulsa, OK, USA). In cases in which averages were normalised to controls, the s.d. of each nominator and denominator were taken into account in calculating the final s.d.. $P<0.05$ was considered statistically significant.

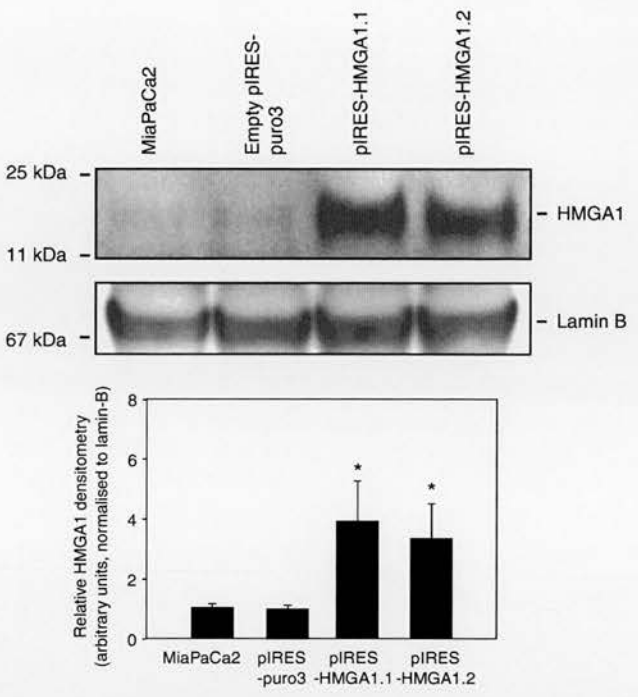

Figure 1 Two stable transfectant clones derived from MiaPaCa2 cells were confirmed to overexpress HMGA1 (pIRES-HMGA1.1 and 1.2) on Western blot analysis of nuclear extracts. Lamin B was used as a loading control. Controls were parental MiaPaCa2 cells or cells stably transfected with empty pIRES-puro3 vector. *Blots shown are representative of three independent experiments. Densitometry values, normalised to Lamin B signals, are mean ± s.d.. $P<0.05$ *vs* empty pIRES-puro3 vector and parental MiaPaCa2 cells.

RESULTS

HMGA1 overexpression promotes anoikis resistance in MiaPaCa2 pancreatic adenocarcinoma cells

Stable overexpression of HMGA1 was achieved in MiaPaCa2 cells, which have low inherent expression levels of HMGA1. Two stable HMGA1-overexpressing clones were selected and named pIRES-HMGA1.1 and pIRES-HMGA1.2. HMGA1 overexpression was confirmed on Western blot analysis (Figure 1). Levels of HMGA1 overexpression in pIRES-HMGA1.1 and pIRES-HMGA1.2 were four- and 3.5-fold higher than in control cells, respectively. There was no difference in the levels of HMGA1 expression between the empty pIRES-puro3 transfectants and parental MiaPaCa2 cells. Following 18 h of anchorage-deprivation (on polyHEMA plates), pIRES-HMGA1.1 and pIRES-HMGA1.2 clones showed significantly increased anoikis resistance (mean anoikis fractions of 11 and 13%, respectively) when compared to controls (mean anoikis fractions for parental MiaPaCa2 and empty pIRES-puro3 transfectants were 26 and 27%, respectively, Figure 2A and 2B).

HMGA1 overexpression results in protection from caspase-mediated apoptosis

Given that disruption of cell–matrix interactions can trigger anoikis via caspase-dependent apoptosis, we examined the effects of HMGA1 overexpression on caspase 3 activity (a central mediator of apoptosis) in the context of anchorage deprivation. During induction of anoikis on polyHEMA plates, HMGA1-overexpressing clones demonstrated markedly reduced levels of caspase 3 activity compared to parental MiaPaCa2 or pIRES-puro3 controls (Figure 2C).

Overexpression of HMGA1 increases levels of Akt phosphorylation and Akt kinase activity

PI3-K/Akt-signalling pathway is of critical importance in mediating anoikis resistance and enhancing anchorage-independent cell cycle progression (Moore *et al*, 1998; Nguyen *et al*, 2002). Given these observations, we sought to determine if HMGA1 overexpression would modulate Akt phosphorylation at Ser473, a marker of Akt activation. HMGA1 overexpression resulted in elevated Akt (Ser473) phosphorylation (Figure 3A). Consistent with this finding, HMGA1 overexpression was found to be associated with increases in Akt kinase activity (Figure 3B).

Inhibition of PI3-K/Akt signalling reverses HMGA1 overexpression-induced anoikis resistance

Having demonstrated that HMGA1 overexpression induces constitutive activation of PI3-K/Akt pathway, we next examined the effects of inhibiting this pathway in cells overexpressing HMGA1. First, we used a specific small molecule inhibitor of PI3-K, LY294002, to assess the effects of inhibiting PI3-K on anoikis resistance of these cells. Addition of 25 μM of LY294002 significantly increased anoikis fractions in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Figure 3C). Treatment of parental MiaPaCa2 and empty pIRES-puro3 controls with LY294002 also inhibited anoikis resistance, although to lesser extent than for pIRES-HMGA1.1 and pIRES-HMGA1.2 clones. Second, infection of pIRES-HMGA1.1 and pIRES-HMGA1.2 clones with adenovirus expressing a dominant-negative Akt construct also significantly increased anoikis fractions in both HMGA1-overexpressing clones

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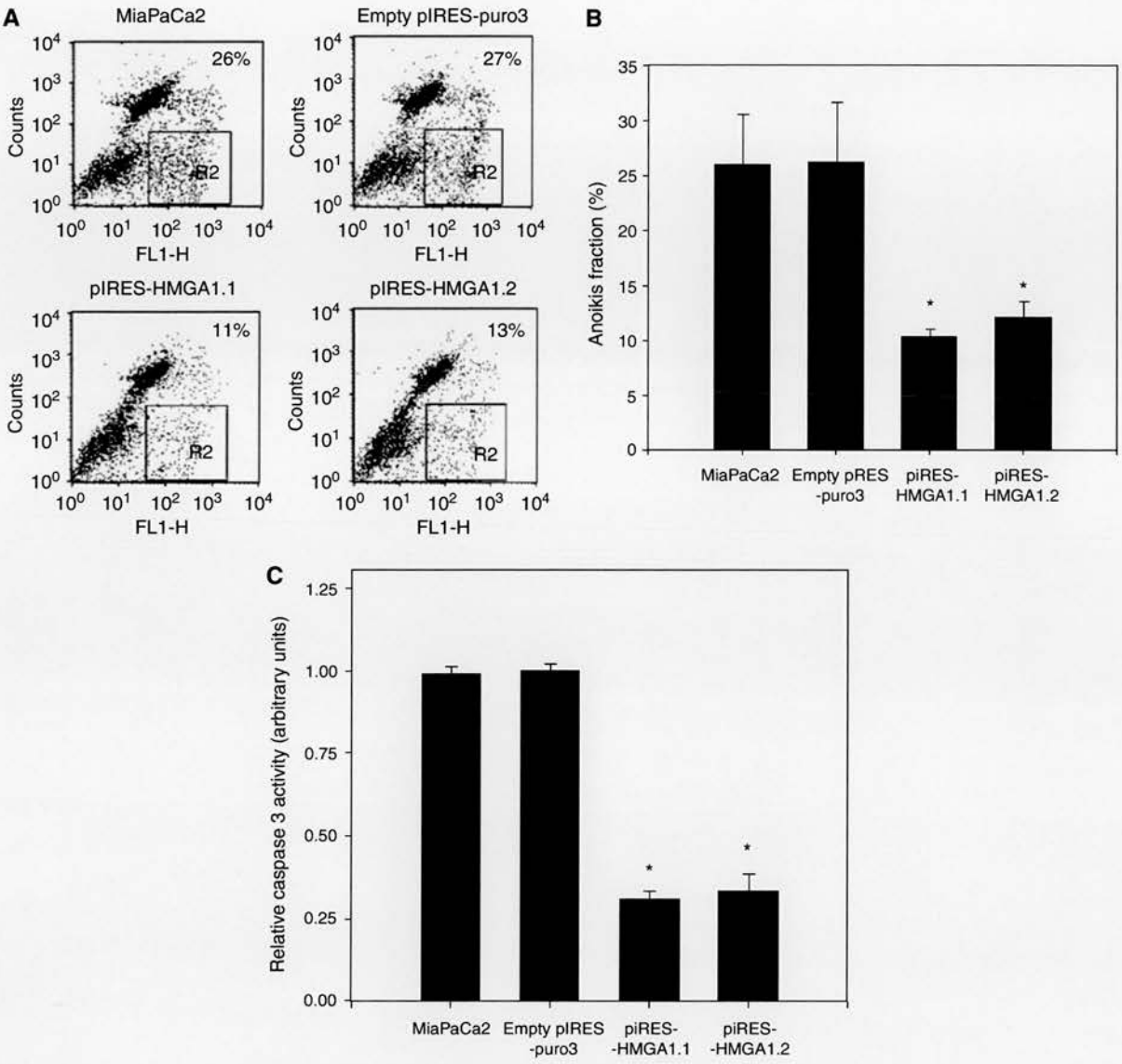

Figure 2 (**A**, **B**) Forced HMGA1 overexpression protects MiaPaCa2 cells, which have low inherent expression of HMGA1, from anoikis. Representative flow cytometric images are shown with anoikis fractions highlighted in the inserted square (Figure 2A). Clones pIRES-HMGA1.1 and pIRES-HMGA1.2 exhibited 2- to 2.5-fold reductions in anoikis fraction compared to parental MiaPaCa2 cells and pIRES-puro3 control stable transfectants (Figure 2B). $^*P<0.05$ *vs* parental MiaPaCa2 cells or empty pIRES-puro3 transfectants. (**C**) HMGA1 overexpression protects MiaPaCa2 cells from caspase-mediated anoikis. pIRES-HMGA1.1 and pIRES-HMGA1.2 clones showed significant reductions in caspase 3 activity, compared to parental MiaPaCa2 cells and empty pIRES-puro3 transfectants, following induction of anoikis on polyHEMA plates for 18 h. $^*P<0.05$ *vs* parental MiaPaCa2 cells or empty pIRES-puro3 transfectants.

(Figure 3D). Parental MiaPaCa2 and empty pIRES-puro3 controls also exhibited increased anoikis fractions with infection of adenovirus expressing dominant-negative Akt construct, but to a lesser extent than the HMGA1-overexpressing clones. Taken together, these results suggest that HMGA1 overexpression-induced anoikis resistance is dependent on PI3-K/Akt signalling.

Targeted RNAi of HMGA1 in MiaPaCa2 and BxPC3 pancreatic adenocarcinoma cells increases susceptibility to anoikis

Given the effects of HMGA1 overexpression on anoikis resistance in MiaPaCa2 cells, we asked if silencing of HMGA1 in the same cell line will have the reverse effects. In addition, we also selected BxPC3 pancreatic adenocarcinoma cell line for RNAi experiments as these cells have high levels of HMGA1 expression at baseline. In these lentivirus-mediated RNAi experiments, we developed pooled stable transfectants. Stable transfectants derived from infection with lentivirus developed from empty PLKO.1 and scramble shRNA transfer plasmids served as controls. We achieved at least 90% silencing of HMGA1 expression in both MiaPaCa2 and BxPC3 cells (Figure 4A). Of note, neither HMGA1 overexpression nor silencing had any impact on lamin B expression (used to normalise for protein loading, Figures 1 and 4A). HMGA1 silencing was associated with significant increases in anoikis fractions in both cell lines (Figure 4B).

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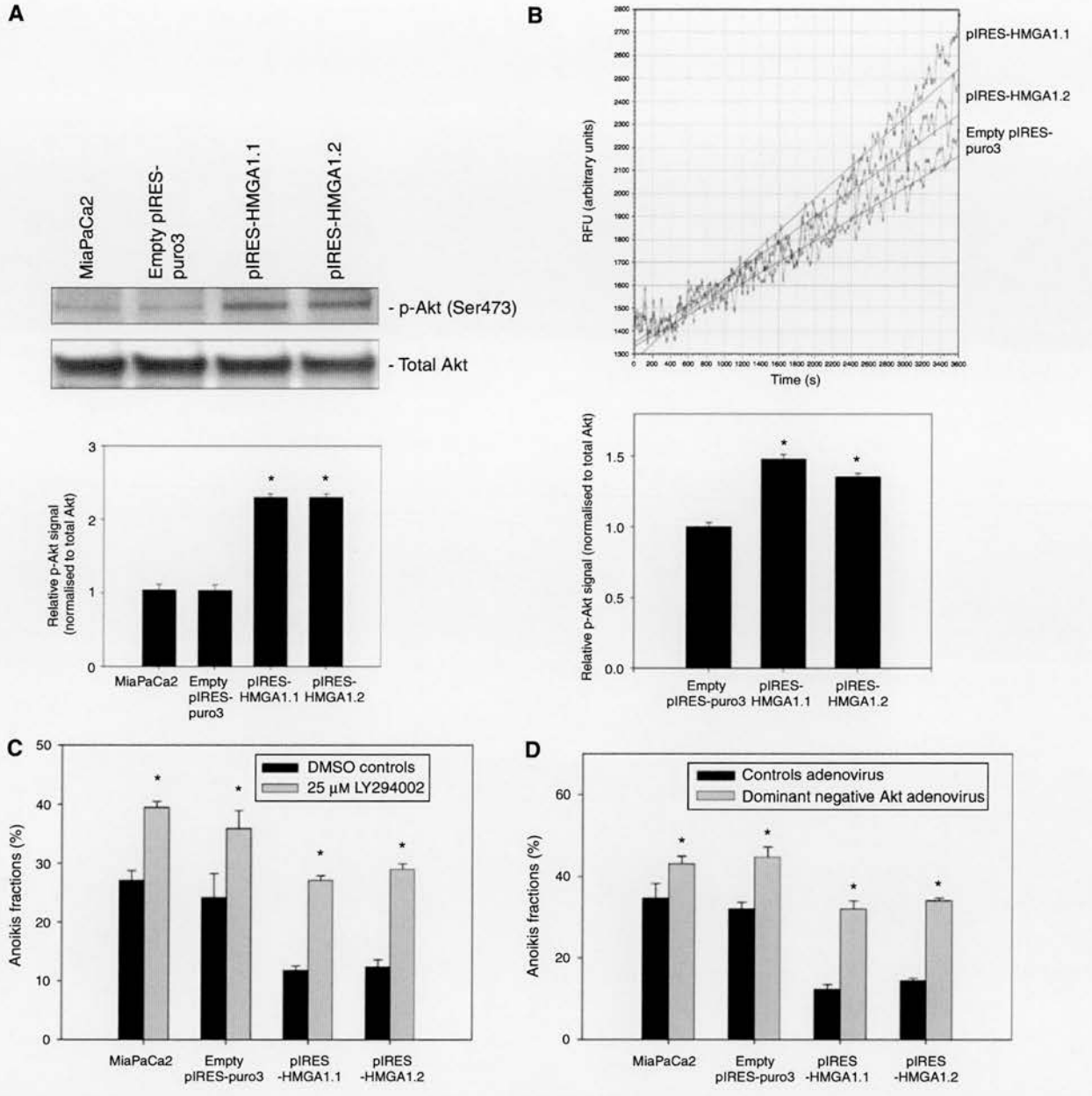

Figure 3 (**A**) Overexpression of HMGA1 led to increased activation of Akt, as evident from increased levels of phospho-Akt (Ser473), a marker of Akt activation. Both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited a higher degree of Akt phosphorylation compared to control cells. There were no differences in the level of expression of total Akt with overexpression of HMGA1. *$P<0.05$ *vs* parental MiaPaCa2 cells or empty pIRES-puro3 transfectants. Blot shown is representative of three independent experiments. (**B**) Correspondingly, overexpression of HMGA1 results in increased Akt kinase activities as determined by fluorometric real-time Akt kinase assays. Slope of Akt kinase activity curves indicates the levels of Akt kinase activity. Representative results of Akt kinase activity assay from three independent experiments are shown. pIRES-HMGA1.1 and pIRES-HMGA1.2 clones showed steeper activity curve slopes and hence Akt kinase activities, when compared to empty pIRES-puro3 controls. *$P<0.05$ *vs* empty pIRES-puro3 vector transfectants. (**C**) Anoikis resistance induced by HMGA1 overexpression in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones was reversed by preincubating cells in 25 μM LY294002, a specific inhibitor of PI3-K. Inhibiting PI3-K resulted in increases in anoikis fractions in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones to levels similar to parental MiaPaCa2 and empty pIRES-puro3 controls. Controls were cells treated with the DMSO vehicle. *$P<0.05$ *vs* DMSO controls. (**D**) Similarly, infection of pIRES-HMGA1.1 and pIRES-HMGA1.2 clones with adenovirus carrying dominant-negative Akt1 resulted in reversal of anoikis resistance with increases in anoikis fractions in both HMGA1-overexpressing clones. Controls were cells infected with adenovirus expressing an empty CMV promoter (Ad-CMV-null). *$P<0.05$ *vs* control adenovirus (Ad-CMV-null).

Modulation of HMGA1 expression did not have an impact on growth characteristics of pancreatic adenocarcinoma cells

Neither overexpression (Figure 5A) nor targeted suppression of HMGA1 expression (Figure 5B) had any impact on proliferation rates of pancreatic adenocarcinoma cells studied.

DISCUSSION

Pancreatic adenocarcinoma is associated with dismal prognosis, in large part resulting from metastatic disease, even in patients initially deemed to be surgically resectable. We have focused our efforts in studying the mechanisms underlying metastasis in

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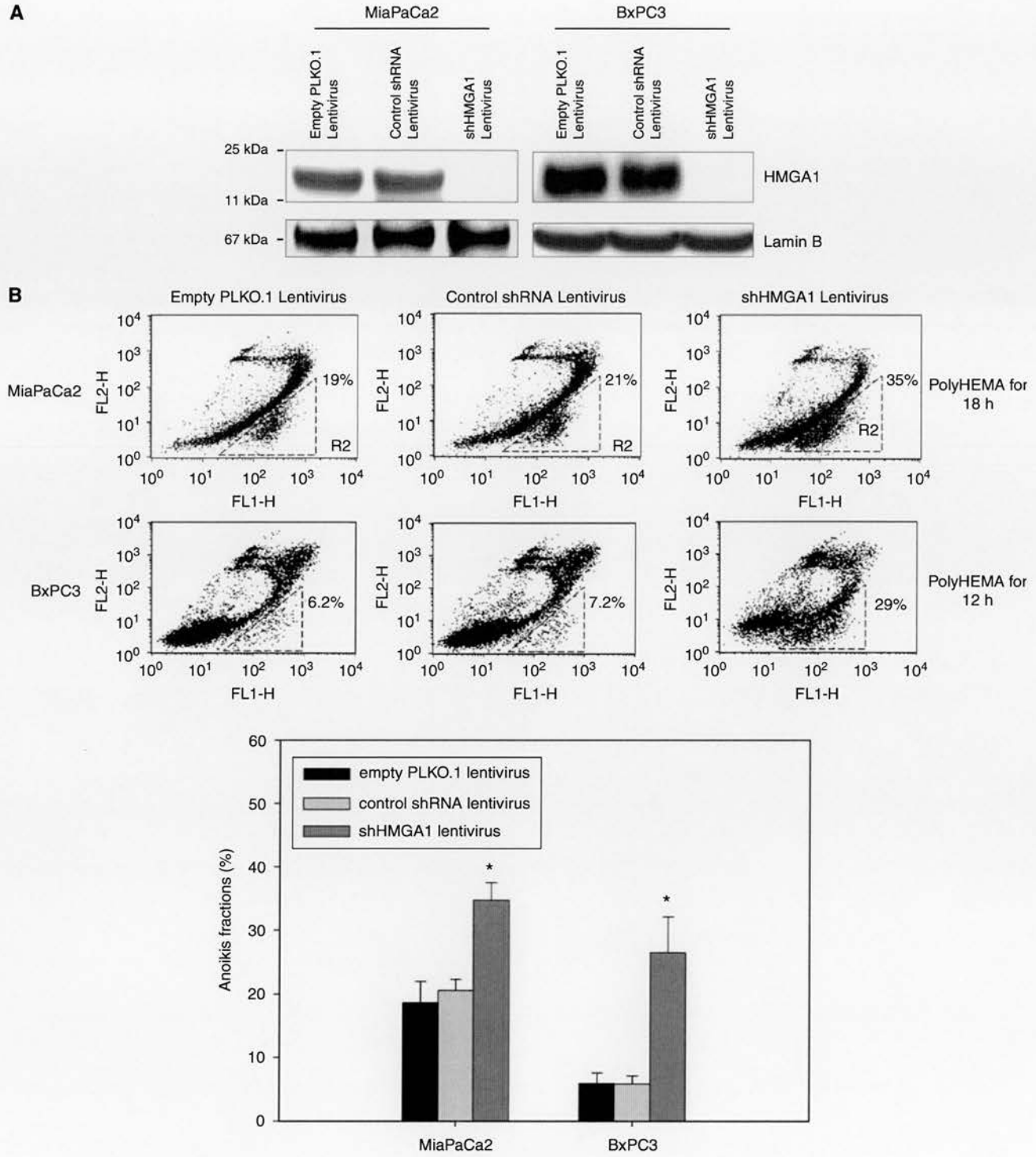

Figure 4 (**A**) Using lentivirus-mediated RNAi of HMGA1, we achieved up to 90% silencing of HMGA1 expression in both MiaPaCa2 and BxPC3 cell lines. A representative Western blot performed on nuclear extracts showing the degree of HMGA1 silencing is shown. Controls were stable transfectants developed following infection with lentivirus expressing empty PLKO.1 or scramble RNAi sequence. (**B**) Silencing of HMGA1 promoted anoikis in both cell lines following culture in polyHEMA plates for 18 h (for MiaPaCa2) and 12 h (for BxPC3). Representative images of flow cytometric analyses of anoikis fractions with apoptotic populations highlighted in triangles drawn are shown. Targeted suppression of HMGA1 resulted in significant increases in anoikis fractions in both MiaPaCa2 and BxPC3 cells compared to empty PLKO.1 and scramble RNAi-stable transfectants. *$P<0.05$ *vs* empty PLKO.1 or scramble RNAi transfectants.

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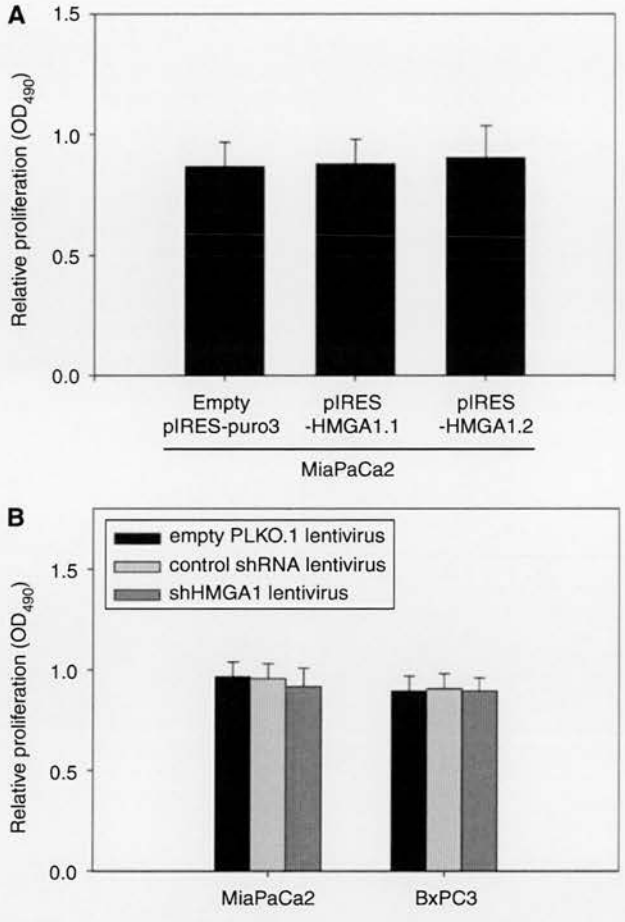

Figure 5 (**A** and **B**) Cellular proliferation was quantified by MTS assay 48 h following seeding wells with 5×10^3 cells. Modulation of HMGA1 expression did not affect cellular proliferation in monolayer culture. Values are mean ± s.d. from triplicate experiments with 10 determinations per condition.

pancreatic adenocarcinoma, with the goal of rationally identifying molecular targets that can be exploited to inhibit this process. In our work, we have used anoikis resistance as an *in vitro* correlate of metastatic potential. In this study, we have shown that HMGA1 overexpression confers anoikis resistance to pancreatic adenocarcinoma cells by activating the prosurvival PI3-K/Akt signalling pathway. This is the first report describing a regulatory role of HMGA1 on Akt signalling in a cancer model.

Numerous reports have described HMGA1 overexpression to be prevalent in a wide range of human malignancies. Increasingly, tumoural HMGA1 expression status is reported to have prognostic value among patients with cancer (Sarhadi *et al*, 2006). Recently, data suggesting mechanisms by which HMGA1 may mediate cancer progression have begun to emerge. For example, HMGA1 proteins have been demonstrated to promote tumour progression and epithelial–mesenchymal transition in human breast epithelial cells (Reeves *et al*, 2001). In immortalised rat embryonic fibroblast Rat1a cells, HMGA1 was found to be a c-Jun-target gene and its suppression by antisense methodology was found to reduce the ability of c-Jun-overexpressing cells to grow under anchorage-independent conditions (Hommura *et al*, 2004).

Although previous reports have implicated HMGA1 as having the capacity to promote tumourigenesis and anchorage-independent growth in normal epithelial cells (Reeves *et al*, 2001), no studies reported to date have examined specifically the role of HMGA1 in mediating anoikis resistance in the context of cancer cells. Our findings suggest that HMGA1 overexpression represents a molecular determinant of anoikis resistance in pancreatic adenocarcinoma cells. The biological significance of this finding relates to emerging understanding that anoikis resistance is a phenotypic hallmark of metastatic cancer cells (Chuma *et al*, 2004; Douma *et al*, 2004). Although the underlying mechanisms rendering anoikis resistance in cancer cells are incompletely understood, our findings provide evidence for a novel role for HMGA1 in mediating this process. Further, our findings provide a potential mechanism by which HMGA1 overexpression induces anoikis resistance: activation of the antiapoptotic PI3-K/Akt signalling pathway.

Description of regulatory roles for HMGA1 in the context of apoptosis is not without precedent. HMGA1 has been found to associate *in vivo* with p53 family members and inactivate their functions. As such, overexpression of HMGA1 may lead to suppression of p53-induced apoptosis and tumour-suppressing activity (Frasca *et al*, 2006). In addition, antisense-mediated suppression of HMGA1 expression in thyroid carcinoma and pancreatic adenocarcinoma cell lines has been reported to induce an apoptotic response (Scala *et al*, 2000; Trapasso *et al*, 2004). Our findings that HMGA1 overexpression results in constitutive activation of PI3-K/Akt pathway provide another mechanism through which HMGA1 mediates its antiapoptotic functions. HMGA1-dependent activation of Akt signalling is likely to protect cancer cells from broad array of proapoptotic stimuli, not just those related to anchorage deprivation. Indeed, we have recently reported that HMGA1 protects pancreatic adenocarcinoma cells from gemcitibine-induced apoptosis and in this context is a potential molecular determinant of chemoresistance in pancreatic cancer (Liau *et al*, 2006).

Our findings, taken together with those previously reported, provide a mechanistic basis for conceptualising the known correlations between HMGA1 expression and poor prognosis in cancer patients (Chiappetta *et al*, 2004). These findings also have obvious therapeutic implications. Future studies will need to address whether targeted therapies directed against HMGA1 will inhibit metastasis in pancreatic cancer.

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Lentivirus-Mediated RNA Interference of *HMGA1* Promotes Chemosensitivity to Gemcitabine in Pancreatic Adenocarcinoma

[Q1] *Siong-Seng Liau, Stanley W. Ashley, Edward E. Whang*

[Q3] The high mobility group A1 (HMGA1) proteins are overexpressed in pancreatic cancers. They are architectural nuclear proteins, which regulate expression of multiple genes implicated in the malignant phenotype. In this study, we hypothesized that HMG A1 silencing will promote chemosensitivity in pancreatic adenocarcinoma. We studied highly malignant pancreatic adenocarcinoma cell lines (MiaPaCa2 and PANC1). Lentiviral short-hairpin RNA (sh *HMGA1*) expression vectors targeting HMG A1 were used for generation of lentiviral particles. Stable transfectants were developed after lentiviral transduction. Nuclear expression of *HMGA1* was assayed using Western blot analysis. Chemosensitivity to gemcitabine was determined by IC50 analysis. Caspase activity was quantitated using fluorometric caspase profiling. Apoptosis was assessed by flow cytometric analysis. Lentivirus-mediated RNA interference resulted in 90% silencing of HMG A1 expression in each of MiaPaCa2 and PANC1 cell lines. *HMGA1* silencing enhanced chemosensitivity to gemcitabine with an approximately 50% reduction in IC50 in each cell line. Lentivirus-mediated *HMGA1* silencing promoted the activation of caspases 3, 2, 9, and 8, on exposure to gemcitabine. *HMGA1* silencing resulted in reduction in Akt kinase activity. Lentivirus-mediated RNA interference of *HMGA1* promoted chemosensitivity to gemcitabine in pancreatic adenocarcinoma. *HMGA1* may represent a novel therapeutic target in pancreatic cancer. (J GASTROINTEST SURG 2006;■:■) © 2006 The Society for Surgery of the Alimentary Tract

KEY WORDS: *HMGA1*, gemcitabine, chemotherapy, pancreatic adenocarcinoma

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States.[1] Its biology is characterized by the propensity for early and aggressive invasion and metastasis, such that less than 10% of patients have surgically resectable disease at the time of diagnosis.[2] Gemcitabine, a nucleoside analog, is generally considered to be first-line therapy for unresectable pancreatic cancer.[3,4] However, the impact of gemcitabine on overall survival and clinical outcomes remains modest, largely because of chemoresistance. Further understanding of the molecular mechanisms underlying pancreatic adenocarcinoma chemoresistance may facilitate the identification of novel strategies for increasing chemosensitivity in this deadly cancer.

The human *HMGA1* gene, located on chromosomal locus 6p21, encodes two high mobility group A1 (HMGA1) splice variants (HMGA1a and HMGA1b).[5] These HMGA1 proteins are architectural transcription factors that play a role in both positive and negative transcriptional regulation of human gene expression in vivo.[6–8] They form stereo-specific, multiprotein complexes termed "enhanceosomes" on the promoter/enhancer regions of genes, where they are able to bind to the minor groove of AT-rich DNA sequences to induce DNA helix bending.[6,9] HMGA1 proteins are overexpressed in a range of human cancers, including pancreatic adenocarcinoma.[10–17] HMGA1 proteins have been reported to regulate signaling pathways implicated in the malignant behavior of cancer cells, including KIT ligand expression[18] and Ras/ERK signaling.[19] Moreover, *HMGA1* is a c-Myc and AP-1 target gene and has been shown to play a role in malignant cellular transformation.[20–22] Recently, it has also been reported that HMGA1

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proteins bind to *p53* in vivo and inhibit their tumor suppressor activity in thyroid cancer cells.[23]

Although there is extensive evidence for the prooncogenic roles of *HMGA1*, little is known about its roles in chemoresistance. The purpose of this study was to test the hypothesis that *HMGA1* is a determinant of pancreatic adenocarcinoma chemoresistance and that suppression of *HMGA1* expression would enhance pancreatic adenocarcinoma chemosensitivity to gemcitabine. Using lentivirus-mediated RNA interference, we assessed the effect of suppressing *HMGA1* expression on pancreatic adenocarcinoma cell gemcitabine chemoresistance and apoptotic pathways. Our observations indicate that *HMGA1* represents a rational therapeutic target in pancreatic adenocarcinoma.

MATERIALS AND METHODS

Cells and Cell Culture

MiaPaCa2 and PANC1 human pancreatic ductal adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM containing 10% fetal bovine serum (Gibco Life Technologies Inc, Gaithersburg, MD) and incubated in a humidified (37°C, 5% CO_2) incubator, grown in 75-cm^2 culture flasks, and passaged on reaching 80% confluence.

Lentivirus-Mediated HMGA1 RNA Interference

Lentiviral hairpin RNA interference plasmids (pLKO.1-HMGA1, TRCN0000018949), constructed as described previously (24), were obtained from The RNAi Consortium (Mission TRC Hs. 1.0; Sigma Aldrich, St Louis, MO). The sequence of short hairpin RNA targeting the human *HMGA1* gene (GenBank accession no. NM_002131) was 5′–AACTCCAGGAAGGAAACCAA–3′, corresponding to the coding region positions 446–466. The control plasmid that has a scramble nontargeting short-hairpin RNA sequence was obtained from Addgene (Cambridge, MA), deposited by Dr. David Sabatini.[25] Each of these vectors had been sequence-verified. Vectors were expanded in chemically competent *Escherichia coli* (TOP10 cells; Invitrogen, Carlsbad, CA) and purified using Genelute maxiprep kit (Sigma Aldrich). To generate lentiviral particles, human embryonic kidney 293 cells (ATCC) were co-transfected with the lentiviral vector and compatible packaging plasmid mixture (Virapower lentiviral packaging system, Invitrogen) using LipofectAMINE 2000 (Invitrogen), in accordance to the manufacturer's instruction. Pancreatic adenocarcinoma cells were exposed to lentivirus-containing supernatant for 16 hours in the presence of 6 μg/ml Polybrene (Sigma). Pooled stable transfectants were established using puromycin selection. Stable transfectant cells were maintained in medium containing 3 μg/ml puromycin (Invitrogen).

Cytotoxicity Assay

Gemcitabine-induced cytotoxicity was quantified by an MTS [3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2*H*-tetrazolium] assay (CellTiter 96; Promega), in accordance with the manufacturer's instructions. Cells were seeded into 96-well plates at 5×10^3 cells per well and allowed to adhere overnight in medium containing 10% FBS. Cell viability was determined after 72 hours in presence or absence of 0–10 μM gemcitabine. Plates were read with the use of the SpectraMax M5 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 490 nm. Six samples were used for each experimental condition, and experiments were performed in triplicate. IC_{50} values were calculated using the SoftMax Pro software (Molecular Devices). At identical time points, cell counting was performed. Cells were trypsinized to form a single-cell suspension. Viable cells, determined by Trypan blue exclusion, were counted with the use of a Neubauer hemocytometer (Hausser Scientific, Horsham, PA). Cell counts were used to confirm MTS results.

Western Blotting

Cells were harvested and rinsed twice with PBS. Total cell extracts were prepared with lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton X, 0.5% deoxycholate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin) and cleared by centrifugation at 15,000*g*, 4°C. Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents based on the manufacturer's instruction (Pierce, Rockford, IL). Total protein concentration was measured using the BCA assay kit (Sigma) with bovine serum albumin as a standard, according to the manufacturer's instructions. Total cell lysates containing 50 μg of total protein or nuclear protein containing 10 μg of total protein was subjected to 10% SDS/PAGE, and the resolved proteins were transferred electrophoretically to PVDF membranes (Invitrogen). Equal protein loading was confirmed by Coomassie (BioRad, Hercules, CA) staining of the gel. After blocking with PBS containing 3% bovine serum albumin for 1 hour at room temperature, membranes were incubated with 3–5 mg/ml antibody in PBS containing 0.1% Tween 20 overnight

at 4°C. Anti- *HMGA1* and anti-lamin B1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Chemoluminescent detection (Amersham Biosciences, NJ) was performed in accordance with the manufacturer's instructions. The densitometric signal was quantified using ImagePro Plus software version 4.0 (Media Cybernetics, Silver Spring, MD) and normalized to that of actin. Blots were performed in triplicate in at least three independent experiments. Mean densitometric values (±SD) are shown.

Apoptosis Assay

After gemcitabine (1 μM) treatment for 48 hours, 1×10^6 cells were washed, trypsinized, and resuspended in 0.5 ml of PBS containing 2% FBS and 0.1 μM EDTA. Apoptosis staining was performed using 1 μl/ml YO-PRO-1 and propidium iodide (Vybrant Apoptosis Assay Kit #4; Molecular Probes, Eugene, OR). Cells were incubated for 30 minutes on ice and then analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ), measuring fluorescence emission at 530 and 575 nm. Cells stained with the green fluorescent dye YO-PRO-1 were counted as apoptotic; necrotic cells were stained with propidium iodide. The number of apoptotic cells was divided by the total number of cells (minimum of 10^4 cells), resulting in the apoptotic fraction. Data were analyzed using CellQuest software (Becton Dickinson). All assays were performed in triplicate.

Fluorometric Caspase Profiling

Whole cell lysates were assayed for caspase 2, 3, 8, and 9 activities using the BD ApoAlert fluorometric Caspase Assay Plate (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions. Plates were read (excitation, 360 nm; emission, 480 nm) using SpectraMax M5 microplate reader in fluorescence mode (Molecular Devices). All measurements were performed in triplicate, each with three determinations for each condition.

Akt Kinase Assay

Active Akt was immunoprecipitated from 1 mg of clarified total cell lysate using the catch and release reversible immunoprecipitation system (Upstate, Charlottesville, VA) according to the manufacturer's protocol. Four micrograms of mouse monoclonal anti-Akt (PH domain) antibody (Calbiochem, San Diego, CA) was used per 500 μg of cell lysate. Following immunoprecipitation, equivalent amounts of eluate were used for Akt kinase assay withg an ELISA-based Akt activity assay that uses a biotinylated peptide substrate that is phosphorylated by Akt kinase (K-LISA Akt activity assay; Calbiochem). Akt activity was quantified by reading the absorbance at 450 nm, with a reference wavelength set at 540 nm, using SpectraMax M5 microplate reader (Molecular Devices). All measurements were performed in triplicate, each with three determinations for each condition.

Statistical Analysis

Differences between groups were analyzed using Student's *t*-test, multifactorial ANOVA of initial measurements, and Mann–Whitney *U* test, for nonparametric data, as appropriate, using Statistica 5.5 software (StatSoft, Inc, Tulsa, OK). In cases in which averages were normalized to controls, the standard deviations of each nominator and denominator were taken into account in calculating the final standard deviation. $P < 0.05$ was considered statistically significant.

RESULTS

Lentivirus-Mediated RNA Interference of HMGA1

Cell lines stably expressing hairpin RNA were developed following lentiviral transduction and selection with puromycin. Lentivirus-mediated RNA interference of *HMGA1* (shHMGA1) resulted in up to 90% silencing of *HMGA1*, as confirmed by Western blot analysis (Fig. 1). Infection with control lentivirus encoding scramble hairpin RNA (shControl) had no effect on *HMGA1* expression.

Suppression of HMGA1 Expression Enhances Gemcitabine-Induced Cytotoxicity

The baseline level of HMGA1 protein expression was approximately 3-fold higher in PANC1 cells than in MiaPaCa2 cells (Fig. 1). PANC1 cells were found to be more resistant to gemcitabine-induced cytotoxicity than MiaPaCa2 cells, with the IC_{50} of PANC1 cells being 128 nM compared to 64 nM for MiaPaCa2 cells. To determine the IC_{50}, cells were exposed to 0 to 10 μM gemcitabine for 72 hours. The IC_{50} was calculated from MTS cytotoxicity assay data. Suppression of *HMGA1* expression resulted in reduction of the gemcitabine IC_{50} in both MiaPaCa2 and PANC1 cells (Fig. 2). The *HMGA1* silencing-induced increases in gemcitabine-induced cytotoxicity were accompanied by

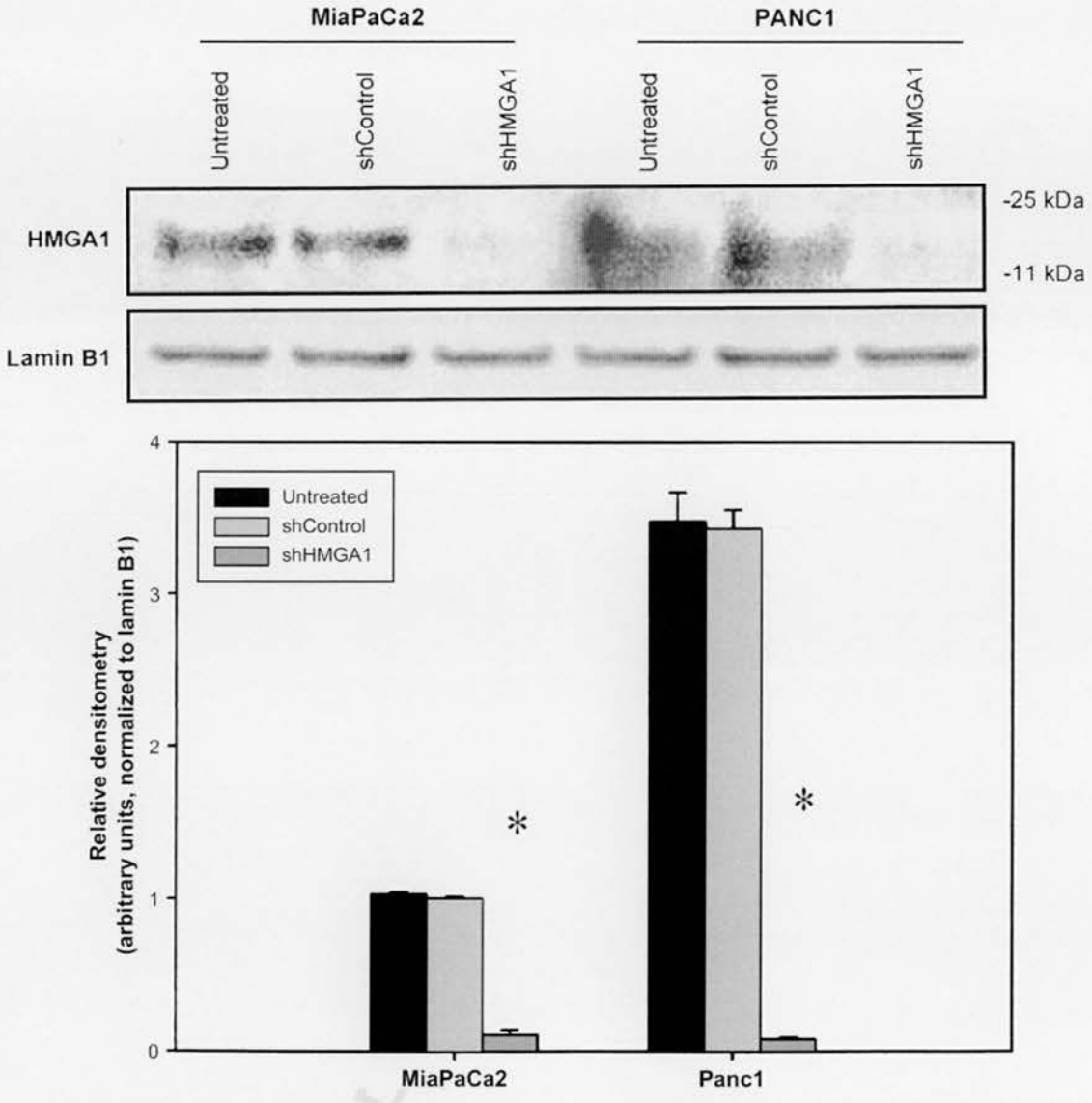

Fig. 1. Stable silencing of *HMGA1* expression using lentivirus encoding short-hairpin RNA (shRNA) was confirmed on Western blot analysis of nuclear extracts. Up to 90% silencing of *HMGA1* expression was achieved using the lentivirus-mediated shRNA approach. MiaPaCa2 and PANC1 cell lines differentially express *HMGA1*, with PANC1 cells having higher expression (up to 3-fold higher than MiaPaCa2 cells). In each experiment, controls were cells stably transfected with lentivirus encoding scramble shRNA (shControl). Densitometry values are mean (± SD). $^*P < 0.05$ versus shControl or untreated cell line.

significant increases in cellular apoptotic fractions (Fig. 3).

HMGA1-Specific Silencing Enhances Gemcitabine-Induced Activation of Caspases 3, 8, 9, and 2

Caspase activation is required for gemcitabine-induced cytotoxicity in cancer cells.[26] As such, we sought to determine the effect of *HMGA1* silencing on caspase activities after exposure to gemcitabine for 48 hours. Gemcitabine-induced activation of caspases 3, 8, 9, and 2 was markedly increased with targeted suppression of *HMGA1* in MiaPaCa2 cells, compared to controls (Fig. 4). On exposure to 1 μM gemcitabine for a similar duration of time, PANC1 cells exhibited modest but statistically significant elevations in activities of each of the caspases profiled with suppression of *HMGA1*.

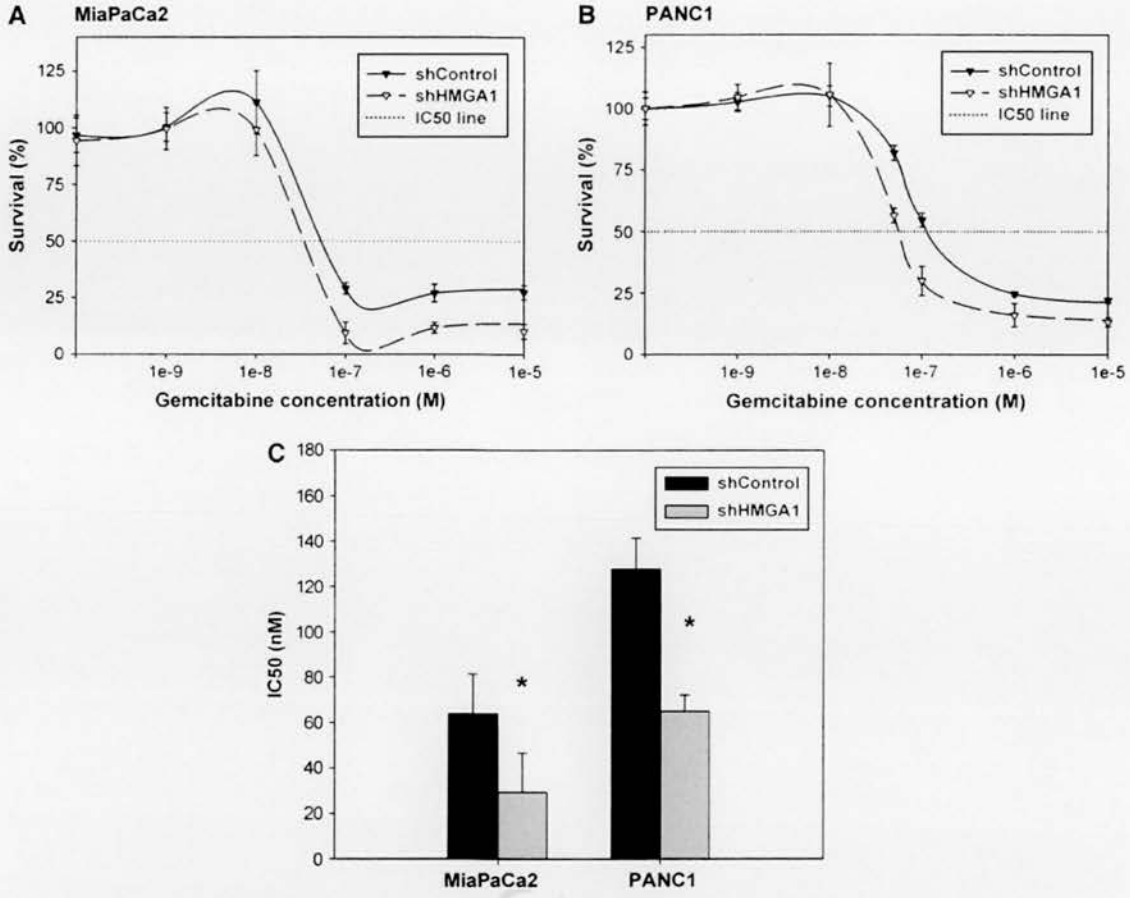

Fig. 2. Lentiviral-mediated RNA interference of *HMGA1* expression enhances gemcitabine-induced cytotoxicity in MiaPaCa2 and PANC1 cell lines. Growth curves of MiaPaCa2 (**A**) and PANC (**B**) cells show the effect of silencing *HMGA1* on chemosensitivity to gemcitabine, as determined using the 3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2*H*-tetrazolium) assay. Suppression of *HMGA1* expression shifts the growth curves to the left in both MiaPaCa2 and PANC1 cells, indicating an increased in chemosensitivity to gemcitabine. (**C**). The mean IC50 of gemcitabine was reduced by approximately 2-fold in both MiaPaCa2 (shHMGA1 versus shControl: 64 nM versus 29 nM) and PANC1 (128 nM versus 65 nM).

Akt Activity Is Inhibited by Suppression of HMGA1

Activation of the serine/threonine kinase Akt is common in pancreatic cancer.[27] Akt has been recognized as a determinant of pancreatic adenocarcinoma gemcitabine chemoresistance.[28–30] As such, we examined the effect of *HMGA1* silencing on Akt activity using an ELISA-based Akt kinase activity assay. Suppression of *HMGA1* expression resulted in significant reduction Akt kinase activity in MiaPaCa2 and PANC1 cell lines, with a greater effect seen in MiaPaCa2 cells (Fig. 5). The decrease in Akt activity we observed with targeted suppression of *HMGA1* expression may in part contribute to the increase in gemcitabine-induced, caspase-mediated cytotoxicity and apoptosis.

DISCUSSION

Pancreatic adenocarcinoma is among the most aggressive and chemoresistant of human malignancies. The prognosis associated with this cancer remains dismal, despite considerable advances in the medical and surgical management of this disease.[31] At the time of diagnosis, most patients will have unresectable disease. Although the nucleoside analog gemcitabine has proven efficacy against pancreatic cancer, it is associated with only modest improvement in clinical outcomes.[3,4] There is an urgent need to identify new therapeutic approaches.

Overexpression of *HMGA1* has previously been reported to be present in a range of human cancers, including pancreatic adenocarcinoma.[10–17] *HMGA1* overexpression is causally associated with both

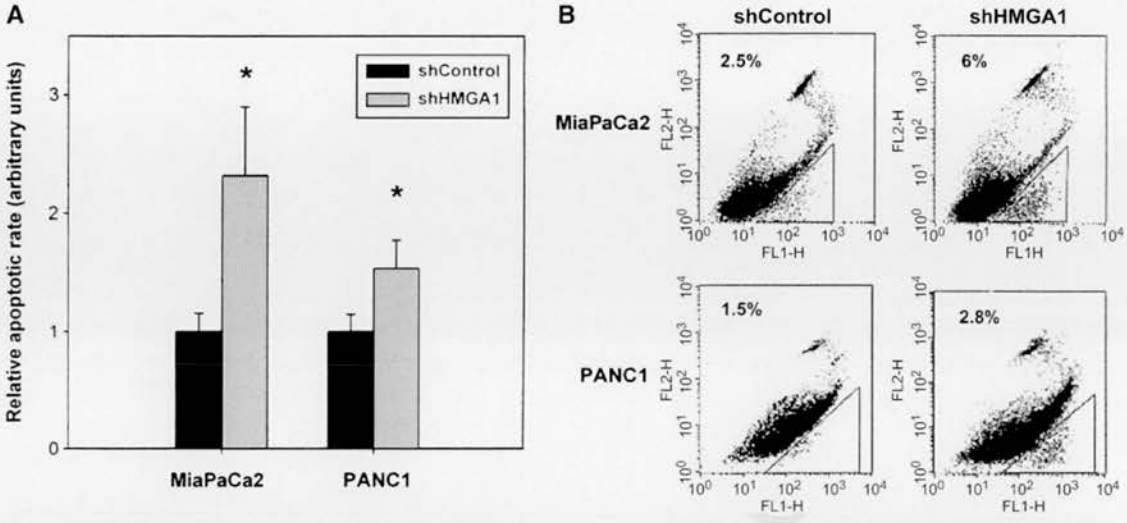

Fig. 3. Apoptotic fraction was quantitated using flow cytometry after staining of cells with Yo-Pro-1 and propidium iodide dyes. (**A**). Consistent with results of the cytotoxicity assay, suppression of *HMGA1* expression promotes apoptosis with increases in apoptotic fraction on exposure to gemcitabine 1 μM for 48 hours. Data are means of at least three determinations ±SD. $^*P < 0.05$ versus shControl transfectants. (**B**). Representative flow cytometric analyses of apoptotic fraction showing suppression of *HMGA1* expression led to increased apoptotic fractions on exposure to gemcitabine 1 μM for 48 hours. The apoptotic cell population is shown by the triangle drawn around the cell population in each analysis.

neoplastic transformation and metastatic progression in breast cancer.[32] Furthermore, *HMGA1* is a c-Myc and AP-1 target gene[20–22] and has been reported to regulate pro-oncogenic signaling pathways, including KIT ligand expression[18] and Ras/ERK signaling.[19] Recent reports also suggest that HMGA1 proteins bind to *p53* in vivo and inhibit their tumor suppressor activity in thyroid cancer cells.[23] Suppression of *HMGA1* expression by antisense oligonucleotides has been reported to inhibit pancreatic cell proliferation.[33] In addition, antisense-mediated suppression of *HMGA1* expression has been reported to inhibit the growth of experimental pancreatic cancers in vitro and in vivo.[33]

HMGA1 has received little attention in the context of chemoresistance. In our study, we have identified *HMGA1* as a potential target through which chemosensitivity to gemcitabine may be increased in pancreatic adenocarcinoma cells. From a therapeutic standpoint, targeting *HMGA1* is attractive in that although it is overexpressed in a range of human malignancies, *HMGA1* expression is absent or present at only very low levels in normal adult tissues.[34] As such, targeting *HMGA1* may have little or no effect on noncancerous tissues.[35] An important caveat is that the role of *HMGA1* in chemoresistance varies according to the chemotherapeutic agent used. For instance, overexpression rather than suppression of *HMGA1* has been shown to chemosensitize MCF-7 human breast adenocarcinoma cells to cisplatin.[36] In view of these findings, therapeutic applications of *HMGA1* silencing would need to be carefully evaluated in the context of cancer characteristics and the specific chemotherapeutic agents used.

Our observation that *HMGA1* silencing suppresses Akt activity is interesting for several reasons. Inhibition of the PI3K/Akt pathway is reported to induce chemosensitization in pancreatic cancer cells both in vitro[37] and in vivo.[38] Active Akt has been reported to protect cells from apoptotic stimuli by inhibiting activation of initiator caspase 9 and effector caspase 3 at a postmitochondrial level.[39] As such, we have identified the PI-3K/Akt kinase signaling pathway as one of the likely molecular mechanisms by which overexpression of HMGA1 proteins promotes chemoresistance to gemcitabine. However, in view of the modest effect of *HMGA1* suppression on Akt kinase activity, it is unlikely that PI-3K/Akt signaling is the sole effector of *HMGA1*-mediated chemoresistance to gemcitabine.

In this study, we have shown that lentivirus-mediated RNA interference of *HMGA1* promotes chemosensitivity to gemcitabine. As such, *HMGA1* represents a rational molecular therapeutic target. The feasibility of in vivo gene silencing using lentiviral vectors has already been demonstrated.[40] The lentivirus vector used in this study is derived from HIV-1 and is replication deficient on transducing the first cell with which it comes into contact. The ability of lentivirus to efficiently transduce cells,

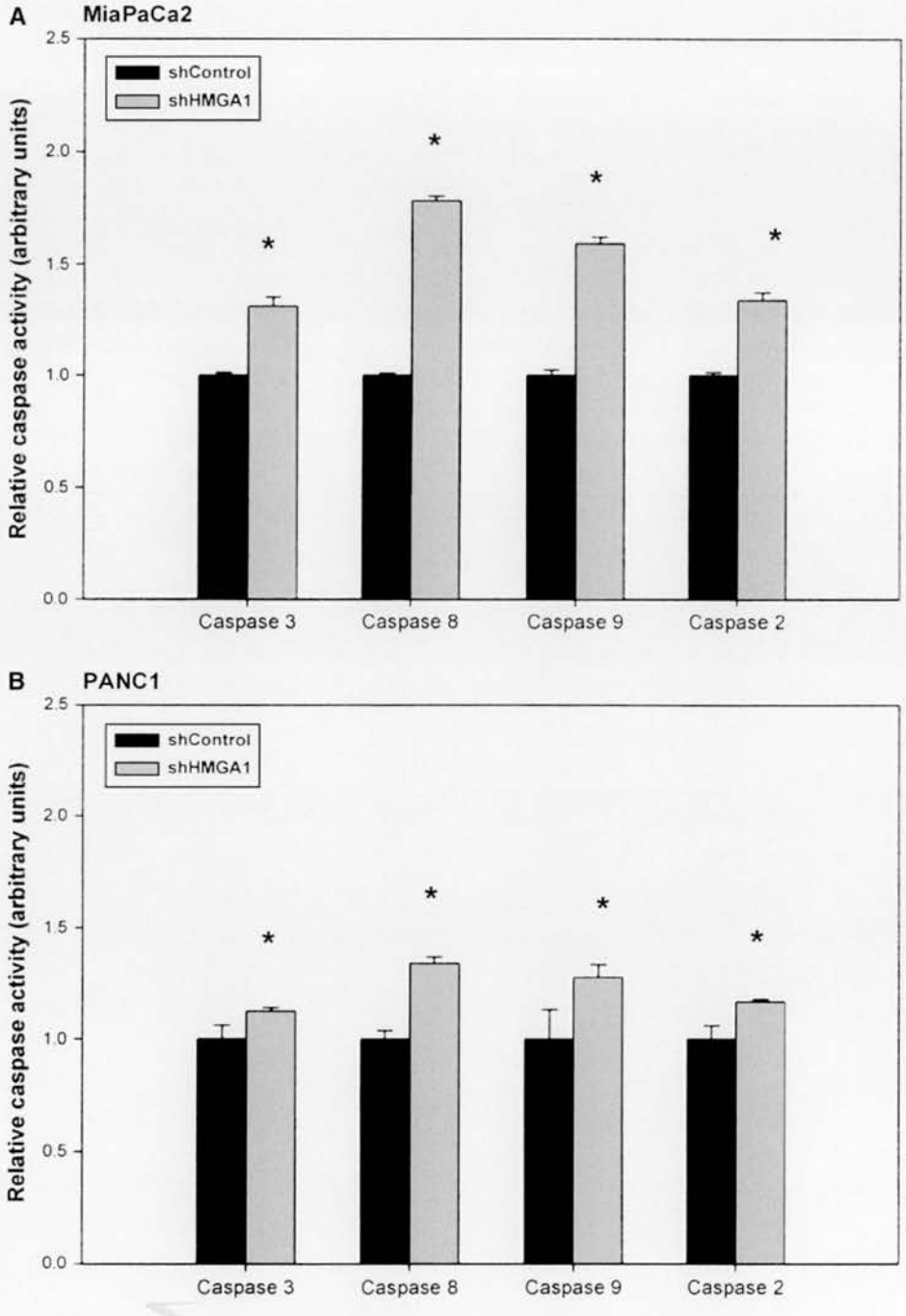

Fig. 4. The effect of targeted suppression of *HMGA1* expression resulted in increased caspase activation on exposure to gemcitabine. Activities of caspases 3, 8, 9, and 2 were quantified using a fluorometric assay after exposure to 1 μM gemcitabine for 48 hours. Activities of each of the four caspases profiled exhibited a significant increase in both MiaPaCa2 (**A**) and PANC1 (**B**) cell lines with lentiviral-mediated *HMGA1* silencing, compared to controls. Controls were cells stably transfected with lentivirus encoding scramble shRNA (shControl). Values are means (±SD) of three experiments with triplicate determinations. *$P < 0.05$ versus shControl transfectants.

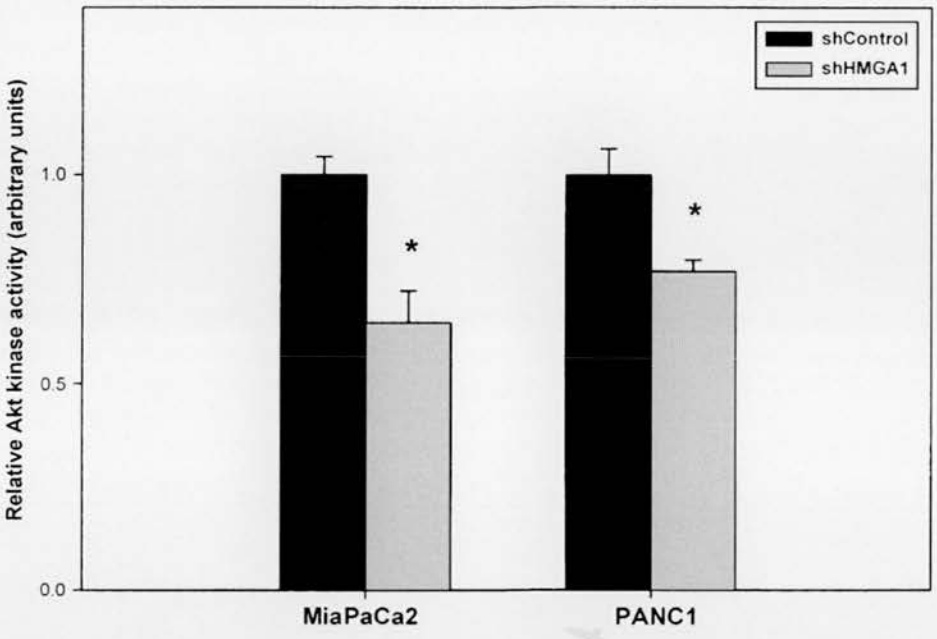

Fig. 5. Akt kinase activity was quantitated using an ELISA-based Akt activity assay following immunoprecipitation of active Akt from total cell lysates. Targeted suppression of *HMGA1* expression using lentivirus-mediated RNA interference resulted in approximately 30–40% reductions in Akt kinase activity in each of MiaPaCa2 and PANC1 cells, compared to controls. Silencing of *HMGA1* has no effect on the level of expression of total Akt, as determined on Western blot analysis (data not shown). Controls were cells stably transfected with lentivirus encoding scramble shRNA (shControl). Values are means (±SD) of three experiments with triplicate determinations. $^*P < 0.05$ versus shControl transfectants.

even nonproliferating ones, is a considerable advantage over other vectors. This feature in combination with the emerging power of RNA interference will facilitate development of viral RNA interference-based therapies in oncology. The first clinical trial involving a HIV-based lentiviral vector in AIDS patients has already been completed in the United States in 2005.[41]

In summary, our findings demonstrate for the first time that suppression of *HMGA1* expression by lentivirus-mediated RNA interference represents a novel strategy for chemosensitizing pancreatic adenocarcinoma to gemcitabine. As such, *HMGA1* warrants further investigation as a therapeutic target in pancreatic adenocarcinoma.

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Discussion

Mark P. Callery, M.D., Boston, Mass: Thank you. Dr. Liau, you have shown us that HMGA-1 knockdown chemosensitizes these pancreatic cancer cell lines to gemcitabine. You achieved this both in cultured cells and in xenografts generated by these HMGA-1 knockdown cells. You implicate a reduction in Akt kinase activity upon HMGA-1 knockdown, and because you could defeat this anti apoptotic survival pathway, you suggest we can use this molecule as a target for future therapy. Your hypothesis was tested with a logical series of experiments and presented to us quite nicely.

Do you have actual data for HMGA-1 overexpression in human pancreatic cancer specimens, and do you have any idea as to the mechanism of HMGA-1 overexpression? Does HMGA-1 correlate or better cause a particularly malignant phenotype, for example, cellular invasiveness or metastasis? Finally, how might you suggest targeting HMGA-1 for theraply? Are there any available drugs today?

Now, I asked you this on Saturday as well, but your xenografts were all made with customized knockdown cells, something that is just not possible in the clinical setting. Can you deliver somehow your silencer to native xenografts, prove that HMGA-1 knockdown occurs, and in fact sensitizes them to treatment with gemcitabine?

Congratulations to you and Stan Ashley, and particularly Ed Whang. You all can be justifiably proud of your contribution.

Siong-Seng Liau, M.D., Boston, Mass: Thank you very much, Dr. Callery. To answer these questions, we have embarked on looking at the expressin of HMGA-1 in pancreatic cancer tissues. We previously constructed a tissue microarray containing samples from 89 patients with pancreatic cancer. Of these 89 patients, 92% have tumoral HMGA-1 overexpression on immunohistochemistry, with little or no expression in normal pancreas.

In terms of the effect of HMGA-1 on the malignant phenotype of pancreatic cancers, we previously have shown that silencing of HMGA-1 results in significant reductions in cellular invasiveness and in in vivo metastasis. In addition, we have shown that overexpression of HMGA-1 allows these cells to grow under anchorage independent conditions, that is, in a soft agar colony formation assay. The reverse is also true; as we silence HMGA-1, there is a significant reduction in the ability of these cells to grow under anchorage independent conditions. HMGA-1 silencing is also associated with a reduction in tumor size in a nude mouse xenograft model of pancreatic cancer.

There is no drug that specifically targets HMGA-1. There is a family of drugs, related to mitomycin C, that crosslinks HMGA-1 to DNA. Howeer, these agents are by no means specific inhibitors of HMGA-1 activity.

It is true that the xenografts we implanted to demonstrate in vivo chemosensitivity were derived from stably transfected cells. We are currently embarking on a gene therapy approach to pancreatic cancer in which we generate a high titre lentivirus expressing hairpin RNA targeting HMGA-1. We hope to demonstrate that intratumoral injection of this lentivirus will chemosensitize pancreatic cancer xenografts.

In closing, I'd like to thank you for insightful questions and kind comments.

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HMGA1 Is a Molecular Determinant of Chemoresistance to Gemcitabine in Pancreatic Adenocarcinoma

Siong-Seng Liau and Edward Whang

Abstract **Purpose:** HMGA1 proteins are architectural transcription factors that are overexpressed by pancreatic adenocarcinomas. We previously have shown that RNA interference targeting the *HMGA1* gene may represent a potential chemosensitizing strategy in pancreatic adenocarcinoma cells. In this study, we tested the hypothesis that HMGA1 promotes chemoresistance to gemcitabine in pancreatic cancer cells.

Experimental Design and Results: Stable short hairpin RNA–mediated HMGA1 silencing in BxPC3 and MiaPaCa2 cells promoted chemosensitivity to gemcitabine, with reductions in gemcitabine IC_{50} and increases in gemcitabine-induced apoptosis and caspase-3 activation. In contrast, forced HMGA1 overexpression in MiaPaCa2 cells promoted chemoresistance to gemcitabine, with increases in gemcitabine IC_{50} and reductions in gemcitabine-induced apoptosis and caspase-3 activation. Dominant negative Akt abrogated HMGA1 overexpression–induced increases in chemoresistance to gemcitabine. Finally, HMGA1 silencing promoted chemosensitivity to gemcitabine *in vivo* in a nude mouse xenograft model of pancreatic adenocarcinoma.

Conclusion: Our findings suggest that HMGA1 promotes chemoresistance to gemcitabine through an Akt-dependent mechanism. Targeted therapies directed at HMGA1 represent a potential strategy for ameliorating chemoresistance in pancreatic adenocarcinoma.

Overall prognosis for patients diagnosed with pancreatic adenocarcinoma remains dismal, with 5-year survival rates averaging <5% (1). At the time of diagnosis, most patients have locally advanced or metastatic disease precluding surgical resection (2). First line therapy for most patients with advanced pancreatic cancer is based on the nucleoside analogue gemcitabine. However, the clinical response rate to gemcitabine remains modest, in large part due to the profound chemoresistance inherent in pancreatic cancer cells. Therefore, characterization of mechanisms mediating chemoresistance in pancreatic adenocarcinoma is an important priority.

The human *HMGA1* gene, located on chromosomal locus 6p21, encodes two HMGA1 splice variants (HMGA1a and HMGA1b; ref. 3). These HMGA1 proteins are architectural transcription factors that form stereospecific, multiprotein complexes termed "enhanceosomes" on the promoter/enhancer regions of genes they regulate (4–6). Each HMGA1 protein has three AT-hook domains that can bind to the minor groove of AT-rich DNA sequences (4, 7). HMGA1 proteins are overexpressed in a wide range of human cancers, including pancreatic adenocarcinoma (8–15). Further, tumoral HMGA1 overexpression has been reported to be associated with poor prognosis in cancer patients (9, 10, 16).

Our group has previously reported the important roles played by HMGA1 in mediating cellular invasiveness and metastatic potential of pancreatic adenocarcinoma cells (17). Overexpression of HMGA1 promotes cellular invasion *in vitro* whereas posttranscriptional silencing of HMGA1 inhibits the ability of pancreatic adenocarcinoma cells to form metastases *in vivo*. Furthermore, we showed that overexpression of HMGA1 is associated with increased resistance to apoptosis under anchorage-independent culture ("anoikis resistance"; ref. 18). In these studies, we have reported that Akt is a key downstream effector of HMGA1-dependent signaling in pancreatic cancer (17). Given the central role of Akt in mediating chemoresistance to gemcitabine (19–21), we hypothesized that HMGA1 would promote chemoresistance to this agent through an Akt-dependent mechanism. In this study, we have confirmed this hypothesis to be correct, and in addition, we have shown that targeted posttranscriptional silencing of HMGA1 promotes chemosensitivity *in vivo*. In conclusion, our findings are of particular clinical importance as they suggest that targeted therapies directed against HMGA1 may ameliorate chemoresistance to gemcitabine.

Materials and Methods

Cells and cell culture. MiaPaCa2 and PANC1 human pancreatic ductal adenocarcinoma cells were obtained from American Type Culture Collection. Cells were maintained in DMEM containing 10% fetal bovine serum (Life Technologies) and incubated in a humidified

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(37°C, 5% CO_2) incubator, grown in 75-cm^2 culture flasks, and passaged on reaching 80% confluence.

Reagents and dominant negative Akt constructs. Anti-HMGA1, anti-lamin B1, anti-phospho-Akt (Ser^{473}), anti-Akt1, and anti-hemagglutinin antibodies were obtained from Santa Cruz Biotechnology. Hemagglutinin-tagged dominant negative Akt adenovirus (Ad-DN-Akt) and its control adenovirus (Ad-CMV-Null), both titered at 1×10^{10} plaque-forming units per milliliter, were purchased from Vector Biolabs.

Lentivirus-mediated HMGA1 RNA interference. Hairpin RNA interference plasmids (pLKO.1-HMGA1, TRCN0000018949) were obtained from The RNAi Consortium (Sigma Aldrich). The sequences of short hairpin RNA targeting the human *HMGA1* gene (Genbank accession no. NM_002131) was 5′-CAACTCCAGGAAGGAAACCAA-3′ (shHMGA1 targets coding region positions 446-466 of HMGA1 mRNA transcript variant 2). The control PLKO.1 plasmid, which has a scrambled nontargeting short-hairpin RNA sequence, was obtained from Addgene (22). High-titer lentivirus expressing shHMGA1 and control short hairpin RNA (shRNA) were generated from five-plasmid transient transfection into 293T cells. Helper plasmids included pHDM-Hgpm2, pMD-tat, pRC/CMV-rev, and pCMV-VSV-G obtained from Harvard Gene Therapy Initiative (Harvard Medical School, Boston, MA). The lentiviral transfer vectors were either control PLKO.1 (nontargeting shRNA sequence) or shHMGA1 plasmids as described above. Virus preparations were concentrated by ultracentrifuge and titered by Southern blotting. We achieved titer of 7.2×10^8/mL for control shRNA lentivirus and 2.5×10^8/mL for shHMGA1 lentivirus. Pooled stable transfectants were developed following infection of lentivirus at multiplicity of infection of 10 for 48 hours, and stable selection in puromycin was achieved as described above.

Expression vector and transfection. The HMGA1 coding sequence was PCR amplified from IMAGE clone 5399570 (Genbank accession no. BC063434) using gene-specific primers modified to include the appropriate restriction sites at their 5′ end. The primers used were as follows: forward, 5′-TTTTGATATCATGAGTGAGTCGAGCTCGAAG-3′ and backward, 5′-TTTTGAATTCTCACTGCTCCTCCTCCGAGGA-3′. Purified PCR products were digested with *Eco*RV and *Eco*RI, before ligation into a *Eco*RV/*Eco*RI-digested pIRES-puro3 vector (Clontech). The expression plasmids were named pIRES-HMGA1. MiaPaCa2 cells were transfected with pIRES-HMGA1 or empty pIRES-puro3 (control), using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. Stable clones were selected by exposure to incrementally increasing concentrations of puromycin (Invivogen), isolated using cloning cylinders, and maintained in medium containing 3 μg/mL puromycin (Invivogen). Clones pIRES-HMGA1.1 and pIRES-HMGA1.2, which expressed the highest levels of HMGA1, were used for further studies.

Western blotting. Cells were harvested and rinsed twice with PBS. Total cell extracts were prepared with Phosphosafe lysis buffer (Novagen). Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents based on the manufacturer's instruction (Pierce). Protein concentration was measured using the BCA assay kit (Sigma). Cellular protein was subjected to 10% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Invitrogen). After blocking with PBS containing 3% bovine serum albumin for 1 hour at room temperature, membranes were incubated with 3 to 5 mg/mL antibody in PBS containing 0.1% Tween 20 overnight at 4°C. Chemoluminescence detection (Amersham Biosciences) was done in accordance with the manufacturer's instructions. The densitometric signal was quantified using ImagePro Plus software version 4.0 (Media Cybernetics).

Cytotoxicity assay. Gemcitabine-induced cytotoxicity was quantified by a MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay (CellTiter 96, Promega). Cells were seeded into 96-well plates at 5×10^3 per well and allowed to adhere overnight. Cell viability was determined after 48 to 72 hours in presence or absence of 0 to 10 μmol/L gemcitabine. Plates were read with the use of the SpectraMax M5 microplate spectrophotometer (Molecular Devices) at a wavelength of 490 nm. IC_{50} values were calculated. At identical time points, cell counting was done. Viable cells, identified by trypan blue exclusion, were counted using a Neubauer hemocytometer (Hausser Scientific). Cell counting confirmed MTS results.

Apoptosis assay. After exposure to gemcitabine (1 μmol/L) for up to 24 hours, 1×10^6 cells were washed, trypsinized, and resuspended in 0.5 mL of PBS containing 2% fetal bovine serum and 0.1 μmol/L EDTA. Apoptosis staining was done using 1 μL/mL YO-PRO-1 and propidium iodide (Vybrant Apoptosis Assay Kit 4; Molecular Probes). Cells were then analyzed by flow cytometry (FACScan; Becton Dickinson), measuring fluorescence emission at 530 and 575 nm. Cells stained with the green fluorescent dye YO-PRO-1 were counted as apoptotic; necrotic cells were stained with propidium iodide. The number of apoptotic cells was divided by the total number of cells (minimum of 10^4 cells) to calculate the apoptotic fraction.

Fluorometric caspase profiling. Whole-cell lysates were assayed for caspase-3 activity using the BD ApoAlert Caspase Assay Plate (BD Biosciences) according to the manufacturer's instructions. Plates were read (excitation, 360 nm; emission, 480 nm) using SpectraMax M5 microplate reader in fluorescence mode (Molecular Devices).

Nude mouse subcutaneous xenograft model. Male athymic nu/nu mice 5 weeks of age, weighing 20 to 22 g, and specific pathogen-free were obtained from Harlan Sprague-Dawley. Mice were housed in microisolator cages in a pathogen-free facility with 12 hours light-dark cycles. Animals were observed for signs of tumor growth, activity, feeding, and pain in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals. To determine the effect of *HMGA1* gene silencing on *in vivo* chemosensitivity, 2×10^6 BxPC3 cells stably expressing control or HMGA1 shRNA (shHMGA1.1 sequence, lentiviral transduction) were s.c. implanted in nude mice. Mice were anesthetized with i.p. ketamine (200 mg/kg) and xylazine (10 mg/kg) and inoculated with 2×10^6 cells in 100 μL of PBS s.c. Gemcitabine administration was commenced 14 days after implantation when tumors were ~50 mm^3 in volume. Mice received gemcitabine (150 mg/kg) in 100 μL of PBS vehicle by twice-weekly i.p. injection. Tumor dimensions were measured weekly using micrometer calipers. Tumor volumes were calculated using the following formula: volume = $1/2\ a \times b^2$, where a and b represent the larger and smaller tumor diameters, respectively. After 6 weeks of gemcitabine administration and 4 days after final gemcitabine injection, necropsy was done, and the primary tumor was excised, formalin-fixed, and paraffin embedded.

Immunohistochemistry. Tumor sections (5 μm) were deparaffinized, rehydrated through graded alcohol, and processed using a streptavidin-biotin-peroxidase complex method. Sections were incubated with anti-HMGA1 antibody (Santa Cruz Biotechnology) at 4°C overnight at a 1:50 dilution. The secondary antibody was biotinylated rabbit anti-goat antibody (DAKO) used at a dilution of 1:200 for 30 minutes at 37°C. Sections were then incubated with StrepABComplex/horseradish peroxidase (1:100; DAKO) for 30 minutes at 37°C. Immunolocalization was done by exposure to 0.05% 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. Normal serum was used in the place of primary antibody as a negative control.

Apoptosis staining. Following preparation of 5-μm tumor sections, apoptosis was quantified using a commercially available terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) kit, in accordance with the manufacturer's protocol (Chemicon). The number of apoptotic cells in at least five random fields from each section was counted. The apoptotic index was calculated by dividing the number of TUNEL-positive cells by the total number of nuclei counted in each field.

Statistical analysis. Differences between groups were analyzed using Student's t test, multifactorial ANOVA of initial measurements, and Mann-Whitney U test, for nonparametric data, as appropriate, using Statistica 5.5 software (StatSoft, Inc.). In cases in which averages were normalized to controls, the SDs of each nominator and denominator were taken into account in calculating the final SD. $P < 0.05$ was considered statistically significant.

Results

HMGA1 is a molecular determinant of chemoresistance to gemcitabine in pancreatic adenocarcinoma cells. Lentivirus-mediated HMGA1 silencing resulted in a marked increase in chemosensitivity to gemcitabine in BxPC3 cells (in which we achieved almost 90% silencing of HMGA1 protein expression; Fig. 1A), with ~4-fold reductions in IC_{50} to gemcitabine (mean IC_{50}, control shRNA versus shHMGA1; 50 versus 12 nmol/L, $P = 0.001$; Fig. 1C and D). Interestingly, BxPC3 cells in which HMGA1 had been silenced developed spiculated morphology on exposure to 1 μmol/L gemcitabine for 48 hours, whereas control cells did not (Fig. 1B). In our previous study, we achieved up to 90% silencing of HMGA1 expression in MiaPaCa2 cells using lentivirus-expressing shRNA with the same target sequence (23). In the current study, we achieved a similar degree of HMGA1 knockdown in MiaPaCa2 cells (data not shown). Lentivirus-mediated HMGA1 silencing had similar effects on MiaPaCa2 cells, with shifting of the gemcitabine IC_{50} curve to the left (Fig. 2B) and ~2-fold reductions on IC_{50} (mean IC_{50}, control shRNA versus shHMGA1; 60 versus 30 nmol/L, $P = 0.001$; Fig. 2D).

We then tested the effect of forced overexpression of HMGA1 on cellular chemoresistance to gemcitabine. MiaPaCa2 cells (which have low inherent expression of HMGA1) were stably transfected with the pIRES-HMGA1 vector, as described in Materials and Methods. We selected two transfectant clones with highest expression levels of HMGA1 (pIRES-HMGA1.1 and pIRES-HMGA1.2). In our previous studies, we have characterized the pIRES-HMGA1.1 and pIRES-HMGA1.2 clones and verified their overexpression of HMGA1 by Western analysis (17, 18). pIRES-HMGA1.1 and pIRES-HMGA1.2 clones overexpress HMGA1 by 4-fold and 3.5-fold, respectively, when compared with empty pIRES-puro3 transfectants (data not shown). Overexpression of HMGA1 resulted in significant increases in chemoresistance to gemcitabine, with increases in IC_{50} to gemcitabine (Fig. 2D) and shifting of the IC_{50} curves to the right for both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Fig. 2C). The mean IC_{50} for pIRES-HMGA1.1 and pIRES-HMGA1.2 were 130 and 100 nmol/L, respectively ($P = 0.003$ and $P = 0.006$, respectively, versus empty pIRES-puro3 controls). Notably, pIRES-HMGA1.1 consistently overexpressed HMGA1 to a greater degree than pIRES-HMGA1.2; this greater HMGA1 overexpression was associated with a higher mean IC_{50} value. Forced HMGA1 overexpression enhanced the viability of cells exposed to 1 μmol/L of gemcitabine for 72 hours, whereas HGMA1 silencing was associated with the opposite effect (Fig. 2A).

HMGA1 expression status modulates gemcitabine-induced apoptosis and caspase-3 activation. Following exposure to 1 μmol/L gemcitabine for 24 hours, cells were subjected to flow cytometric quantitation of apoptosis and fluorometric caspase-3 profiling. Lentivirus-mediated HMGA1 silencing was associated with increases in gemcitabine-induced apoptosis (Fig. 3A) and caspase-3 activation in both BxPC3 and MiaPaca2 cells (Fig. 3C). In contrast, HMGA1 overexpression was

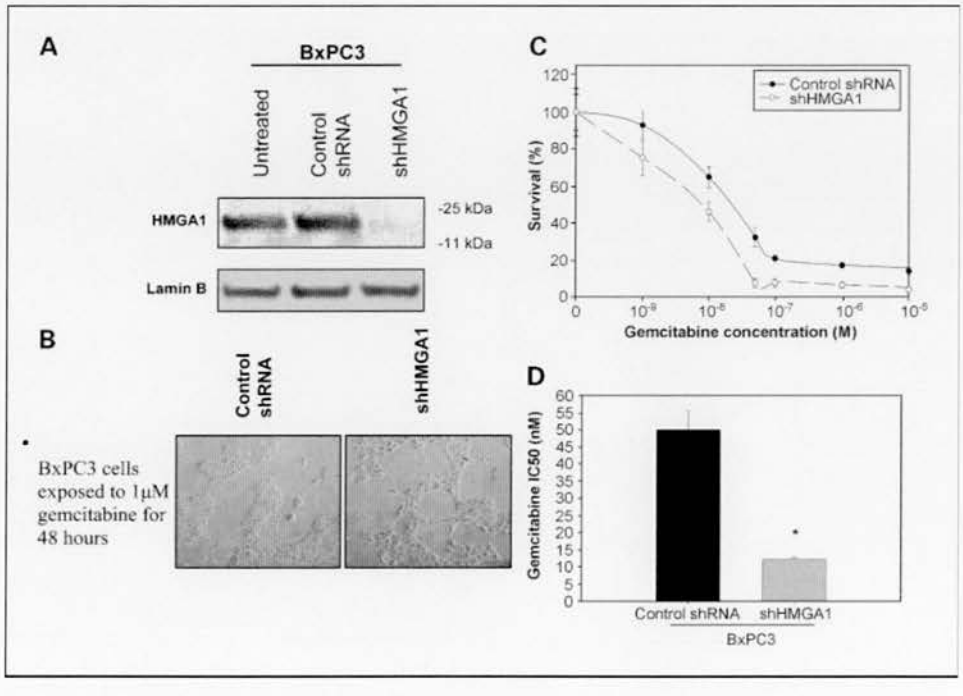

Fig. 1. *A,* following the generation of high-titer lentivirus particles carrying shHMGA1, BxPC3 was transduced with lentivirus at multiplicity of infection of 10 and stable transfectants were developed following selection with puromycin. Robust suppression of HMGA1 was achieved using lentivirus with a high degree of silencing of HMGA1. In BxPC3 cells, lentivirus-mediated shHMGA1 achieved almost complete silencing of HMGA1. Controls were stable transfectants developed using lentivirus carrying scrambled, nontargeting shRNA. *B,* the effects of lentivirus-mediated HMGA1 silencing on chemosensitivity to gemcitabine were assessed. When BxPC3 cells in which HMGA1 had been silenced were exposed to 1 μmol/L gemcitabine for 48 h, they adopted a less healthy, speculated morphology compared with control cells. Photomicrographs were taken using an inverted microscope at ×40 magnification. *C,* survival curves following exposure to 0 to 10 μmol/L gemcitabine were analyzed following MTS assay. Lentivirus-mediated stable HMGA1 silencing in BxPC3 cells shifted the survival curve to the left, indicating an increase in chemosensitivity to gemcitabine, when compared with the controls. Correspondingly, there was a 4-fold reduction in the IC_{50} to gemcitabine with silencing of HMGA1 when compared with the controls. *, $P = 0.001$ versus control shRNA.

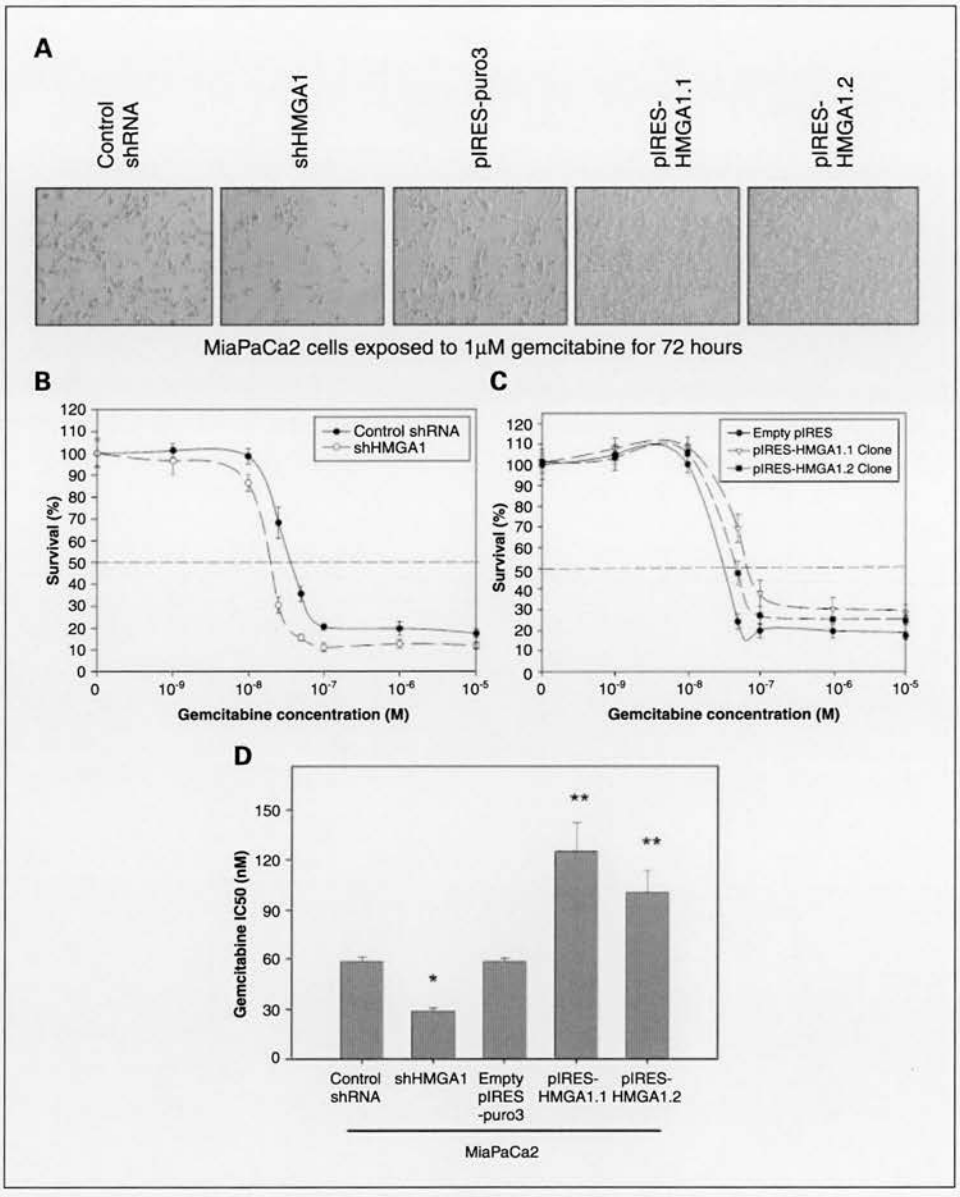

Fig. 2. MiaPaCa2 cells were transduced with shRNA lentivirus at multiplicity of infection of 10 and stable transfectants were developed following selection with puromycin. Stable silencing of HMGA1 was confirmed by Western analysis (data not shown). Two previously characterized clones (pIRES-HMGA1.1 and pIRES-HMGA1.2) of MiaPaCa2 cells that stably overexpress HMGA1 were used in this study (18). Controls were cells stably transfected with empty pIRES-puro3 vector. *A,* the effects of modulating HMGA1 expression on chemosensitivity to gemcitabine in MiaPaCa2 cells was assessed. In MiaPaCa2 cells, lentivirus-mediated silencing of HMGA1 resulted in marked reductions in cellular viability compared with controls when exposed to 1 μmol/L gemcitabine for 72 h. In contrast, MiaPaCa2 cells with HMGA1 overexpression (pIRES-HMGA1.1 and pIRES-HMGA1.2) showed increased viability following exposure to 1 μmol/L gemcitabine, when compared with empty pIRES-puro3 transfectants. Photomicrographs were taken using an inverted microscope at ×40 magnification. *B* and *C,* lentivirus-mediated RNA interference of HMGA1 in MiaPaCa2 cells resulted in increases in chemosensitivity to gemcitabine with shifting of survival curves to the left, whereas overexpression of HMGA1 led to increases in chemoresistance to gemcitabine with shifting of the survival curves to the right in both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones, compared with their respective controls. *D,* targeted suppression of HMGA1 using lentivirus-mediated shHMGA1 resulted in 2-fold reductions in IC_{50} compared with control ($P = 0.001$ versus control shRNA), whereas overexpression of HMGA1 in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones resulted in ~2.2- and 1.7-fold increases in IC_{50} to gemcitabine, respectively [$P = 0.003$ (pIRES-HMGA1.1) and $P = 0.006$ (pIRES-HMGA1.2) versus empty pIRES-puro3 control]. *, $P < 0.05$ versus control shRNA transfectants. **, $P < 0.05$ versus empty pIRES-puro3 transfectants.

associated with reductions in gemcitabine-induced apoptosis and in caspase-3 activation (Fig. 3B and D).

HMGA1-induced chemoresistance to gemcitabine is dependent on Akt signaling. We have previously reported that Akt is a downstream effector of HMGA1 (18, 23). We have found that HMGA1 silencing is associated with reductions in Akt phosphorylation (a marker of Akt activation), whereas forced HMGA1 overexpression is associated with increases in Akt kinase activity and in Akt phosphorylation (17, 18). Given the importance of the phosphatidylinositol 3-kinase/Akt pathway in antiapoptotic signaling, particularly in the context of chemoresistance, we sought to determine if chemoresistance to gemcitabine-induced HMGA1 overexpression is Akt dependent. Each of pIRES-HMGA1.1 and pIRES-HMGA1.2 clones were transduced with dominant negative Akt adenovirus at multiplicity of infection of 10. The efficiency of transduction and expression of dominant negative Akt were confirmed by immunoblotting for the hemagglutinin tag of the dominant negative Akt construct (Fig. 4). We next assessed the effects of dominant negative Akt on chemosensitivity to gemcitabine in each HMGA1-overexpressing clone. Dominant negative Akt was found to reverse the chemoresistance induced by HMGA1 overexpression, with reductions of IC_{50} to gemcitabine in both pIRES-HMGA1.1 and pIRES-HMGA1.2 clones. As such, HMGA1 overexpression–induced chemoresistance is dependent on Akt signaling.

HMGA1 silencing promotes chemosensitivity to gemcitabine in vivo. BxPC3 cells in which HMGA1 had been silenced through stable lentiviral shRNA-mediated RNAi or control BxPC3 cells transduced with lentivirus carrying nontargeting shRNA were s.c. implanted into nude mice. Once the resulting xenograft tumors had grown to reach ~50 mm^3 in diameter, a 6-week course of gemcitabine administration was initiated. Tumors derived from BxPC3 cells in which HMGA1 had been silenced ($n = 8$ animals) regressed during the treatment period, whereas tumors derived from control cells ($n = 8$ animals) continued to

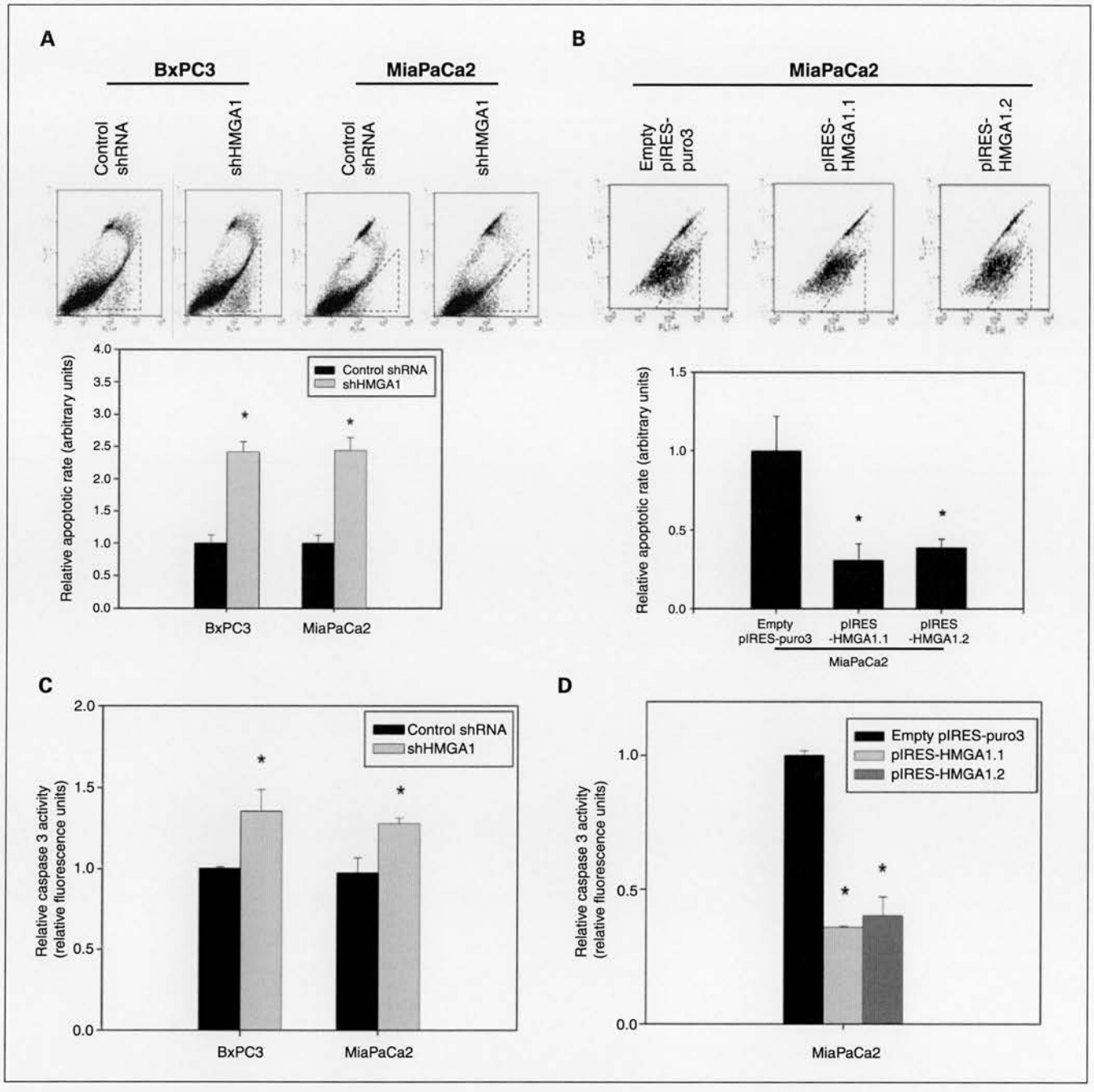

Fig. 3. *A,* lentivirus-mediated silencing of HMGA1 promoted gemcitabine-induced apoptosis as assessed by flow cytometric analyses of YO-PRO-1/propidium iodide – stained cells. Silencing of HMGA1 resulted in ~2-fold increases in the relative apoptotic rates in both BxPC3 and MiaPaCa2 cells following exposure to 1 μmol/L gemcitabine for 24 h. *, $P = 0.001$ versus control shRNA. Representative flow cytometric images of three experiments are shown, with the apoptotic fractions being highlighted in triangles drawn. *B,* forced overexpression of HMGA1 in pIRES-HMGA1.1 and HMGA1.2 clones protected the cells from gemcitabine-induced apoptosis with ~70% to 80% reductions in relative apoptotic rates, as assessed by flow cytometry [$P = 0.001$ (pIRES-HMGA1.1) and $P = 0.002$ (pIRES-HMGA1.2) versus empty pIRES-puro3 control]. *, $P < 0.05$ versus empty pIRES-puro3 control. Representative flow cytometric images of three independent experiments are shown, with the apoptotic fractions being highlighted in triangles drawn. *C,* relative caspase-3 activities were determined using a fluorometric caspase-3 substrate assay following exposure of cells to 1 μmol/L gemcitabine for 24 h. Lentivirus-mediated silencing of HMGA1 promoted gemcitabine-induced caspase-3 activities. *, $P = 0.008$ (BxPC3) and $P = 0.005$ (MiaPaCa2) versus control shRNA transfectants. *D,* as expected, overexpression of HMGA1 in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones resulted in reductions in caspase-3 activation following exposure to 1 μmol/L gemcitabine for 24 h, indicating protection from gemcitabine-induced caspase-mediated apoptosis. *, $P = 0.001$ versus empty pIRES-puro3 transfectants.

grow during the treatment period (Fig. 5A-C). Stable suppression of HMGA1 expression in tumors derived from shHMGA1 transfectants was confirmed on Western blotting of nuclear extracts of tumor homogenates (Fig. 6A) and on immunohistochemical analysis of xenografts harvested at the end of the study period (Fig. 6B). TUNEL staining revealed significantly higher apoptotic index in shHMGA1 transfectant–derived tumors than in control cell–derived tumors (Fig. 6B and C).

Discussion

HMGA1 proteins are overexpressed in a wide range of human cancers, including pancreatic adenocarcinoma (8). Experimental data implicating biologically important roles for HMGA1 in cancer pathogenesis are rapidly accumulating (24, 25). Our study provides the first data suggesting that HMGA1 may mediate a critical feature of malignant phenotype: chemoresistance. First, our findings show that forced HMGA1 overexpression promotes chemoresistance to gemcitabine in pancreatic cancer cells *in vitro*, whereas HMGA1 silencing promotes gemcitabine-induced cytotoxicity and therefore abrogates chemoresistance to gemcitabine. Second, we have confirmed that HMGA1 silencing promotes gemcitabine-induced cytotoxicity and reduces tumor growth *in vivo* in a nude mouse xenograft model of pancreatic cancer. Finally, our findings suggest a plausible mechanism by which HMGA1 promotes chemoresistance to gemcitabine: activation of Akt signaling. Phosphatidylinositol 3-kinase/Akt signaling is well described as a mediator of chemoresistance to gemcitabine in the context of pancreatic cancer (19, 26, 27). These findings imply that targeted suppression or inactivation of HMGA1 could be a potential therapeutic strategy for increasing chemosensitivity to gemcitabine in this highly chemoresistant cancer.

Proposed mechanisms by which HMGA1 regulates gene expression include derepression of gene promoters by displacement of histone H1 nucleoproteins, which are strong repressors of gene transcription, from scaffold attachment regions, thus

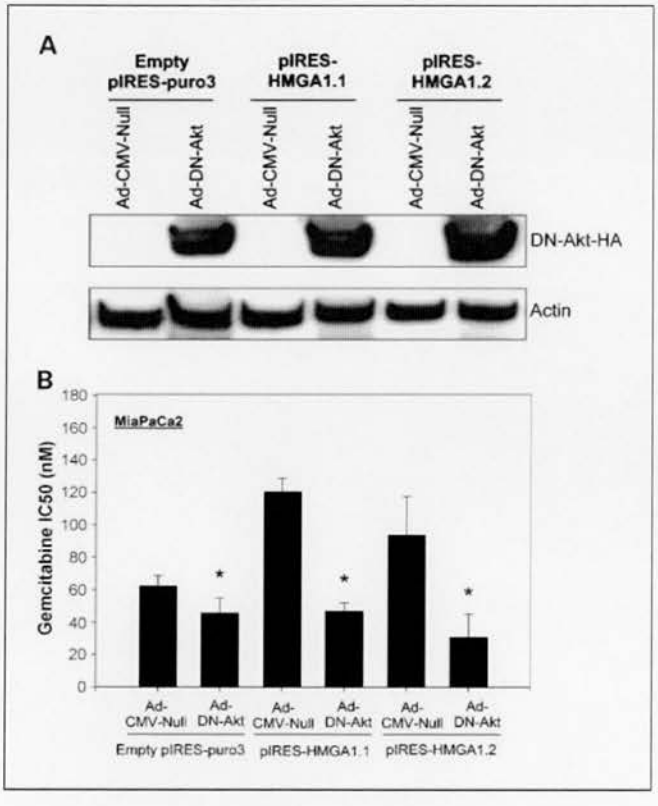

Fig. 4. We have previously reported that in MiaPaCa2 cells, HMGA1 silencing reduces Akt phosphorylation whereas HMGA1 overexpression promotes Akt phosphorylation, and neither HMGA1 silencing nor overexpression have any effect on the level of expression of total Akt. To assess the role of Akt in mediating the HMGA1-induced chemoresistance, we transduced pIRES-HMGA1.1 and pIRES-HMGA1.2 clones with adenovirus carrying hemagglutinin-tagged dominant negative Akt to examine its effects on chemoresistance. Transduction efficiency and expression of dominant negative Akt were assessed by Western blotting for hemagglutinin. Infection of pIRES-HMGA1.1 and pIRES-HMGA1.2 clones with adenovirus carrying dominant negative Akt (Ad-DN-Akt) resulted in significant reductions in IC_{50} to gemcitabine when compared with cells infected with control adenovirus (Ad-CMV-Null). Dominant negative Akt resulted in reductions in IC_{50} to gemcitabine in pIRES-HMGA1.1 and pIRES-HMGA1.2 clones to levels similar to parental MiaPaCa2 cells or empty pIRES-puro3 transfectants, indicating abrogation of the increased chemoresistance associated with HMGA1 overexpression. *, $P < 0.05$ versus control adenovirus (Ad-CMV-Null).

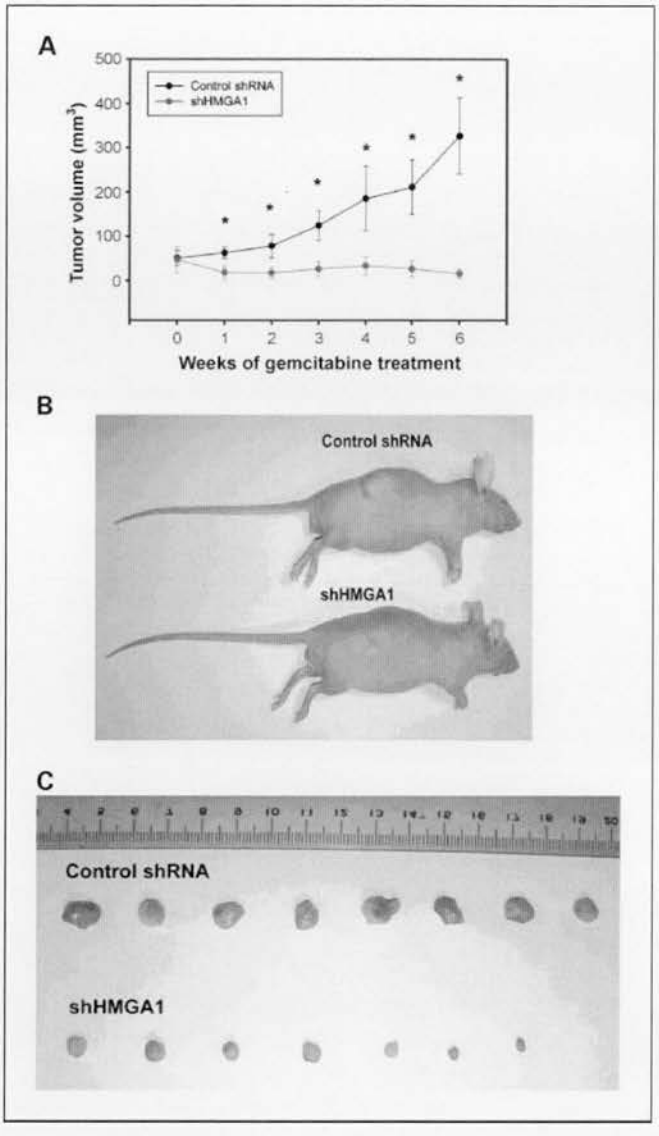

Fig. 5. *A,* stable silencing of HMGA1 promoted chemosensitivity to gemcitabine *in vivo* with evidence of tumor regression in nude mouse s.c. model. Mice ($n = 8$ per group) were s.c. implanted with 2×10^6 lentivirus-mediated stable transfectant BxPC3 cells (either shHMGA1 or control shRNA). Gemcitabine treatment was commenced in each group 14 d after implantation when the tumors were ~50 mm^3 in volume. Mice received gemcitabine (150 mg/kg) in 100 μL of PBS vehicle by twice-weekly i.p. injection. Subcutaneous tumor size was monitored weekly during the 6 wk of treatment. Tumors with HMGA1 silencing showed evidence of regression in size during the treatment period whereas tumors in the control group continued to grow with time. Points, mean; bars, SD. *, $P < 0.05$ versus control shRNA xenografts. *B,* representative photograph of one mouse from each group is shown, with tumors located in their flanks. *C,* the explanted tumors at the end of the 6-wk treatment period. Of note, one of the mice in the shHMGA1 group had its tumor completely regressed during the 6-wk gemcitabine treatment period.

allowing for a more open chromatin structure that facilitates transcriptional activation (28). Further, HMGA1 is able to bind to AT-rich promoter regions, where it modifies DNA conformation to facilitate binding of other transcriptional factors that promote gene transcription (29). By binding to promoter regions, HMGA1 is also able to form multiprotein complexes, the enhanceosomes, that serve as transcription-activating complexes (29). Given these putative functions, it is not surprising that HMGA1 is involved in the regulation of a large number of target genes. Previous studies have shown that HMGA1 overexpression is associated with increased expression of growth factors/cytokines (e.g., fibroblast growth factors, IFNs α and β, and interleukins 10-14 and 17), growth factor receptors (e.g., fibroblast growth factor receptor, epidermal growth factor receptor, ERBB3, and ERBB4), and multiple integrins (α_1, α_6, α_9, α_E, β_1, β_3, β_8; ref. 24). Clearly, it is not surprising that through induction of these growth factor–related signaling pathways, HMGA1 could have an effect on prosurvival phosphatidylinositol 3-kinase/Akt pathways, as shown in this study. It is also plausible that by increasing integrin expression, HMGA1 may stimulate integrin-linked kinase, which is known to directly interact with integrins and phosphorylate Akt in a phosphatidylinositol 3-kinase–dependent manner (30).

In the current study, we have examined the effects of silencing HMGA1 on chemoresistance in BxPC3 and MiaPaCa2 pancreatic adenocarcinoma cells. BxPC3 cells inherently express relatively high levels of HMGA1 whereas MiaPaCa2 cells inherently express relatively low levels of HMGA1 under baseline conditions. Because these cell lines differ in ways other than in HMGA1 expression levels alone, we examined the effects of modulating HMGA1 expression in a single cell line system. This approach allowed us to control for potential confounders, such as variable K-*ras* mutation status (31), inherent in comparisons involving multiple cell lines. We did both loss-of-function (HMGA1 silencing) and gain-of-function (HMGA1 overexpression) experiments on MiaPaCa2 cells. As such, by using the same cell line and theoretically controlling for variations in genetic background, we were able to show that HMGA1 silencing reduces chemoresistance whereas HMGA1 overexpression results in the reverse effects on chemoresistance.

Although gemcitabine-based regimens are currently standard of care for the treatment of advanced pancreatic cancer, their efficacy is limited by profound chemoresistance. Recently, the combination of gemcitabine and the human epidermal growth factor receptor tyrosine kinase inhibitor erlotinib has been reported, for the first time, to be associated with improved

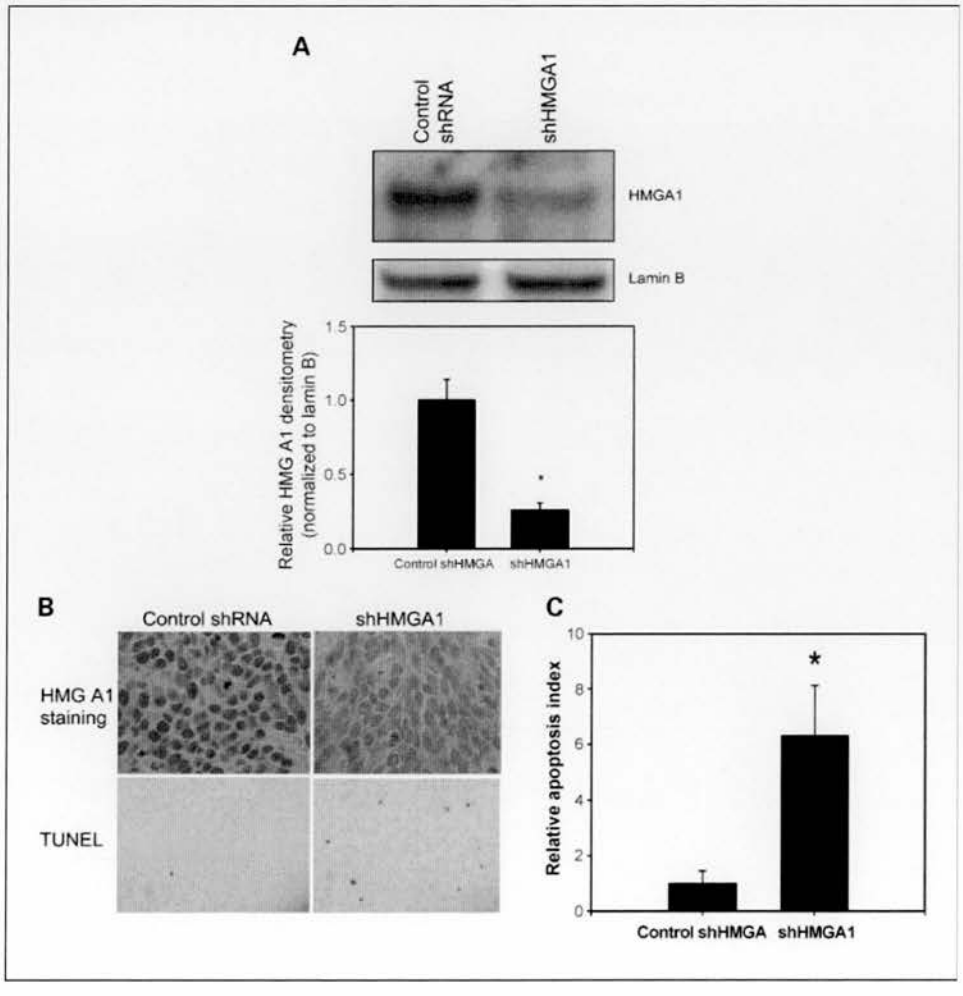

Fig. 6. *A, in vivo* HMGA1 silencing was confirmed by Western blot analysis of nuclear extracts from explanted xenograft tumors. Columns, mean; bars, SD. *, $P = 0.001$ versus control shRNA xenografts. *B* and *C*, immunohistochemistry of xenograft sections showed little or absent staining for HMGA1 in the shHMGA1 xenografts, when compared with the control shRNA xenografts that showed intense staining for HMGA1. Photomicrograph of HMGA1 staining was obtained at ×40 magnification. In the same sections, TUNEL staining was done. In each tumor slide stained with TUNEL, the number of TUNEL-positive cells was counted in at least five randomly selected fields at ×40 magnification. HMGA1 silencing led to significant increases in relative apoptotic index when compared with control shRNA xenografts. Representative tumor sections stained for TUNEL photographed at ×20 magnification. *, $P < 0.001$ versus control shRNA xenografts. Columns, mean; bars, SD.

survival over single-agent gemcitabine in patients with advanced pancreatic cancer in a phase III clinical trial (32). Although these results are encouraging, the benefits provided by the addition of erlotinib are only incremental. Truly transformative increases in efficacy are likely to arise only through incorporation of targeted therapies selected on the basis of rational understanding of mechanisms mediating chemoresistance in pancreatic cancer. In this context, our findings suggest that HMGA1 warrants further investigation as a novel therapeutic target in this deadly cancer.

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High Mobility Group AT-Hook 1 (HMGA1) Is an Independent Prognostic Factor and Novel Therapeutic Target in Pancreatic Adenocarcinoma

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BACKGROUND. High mobility group AT-hook 1 (HMGA1) proteins are architectural transcription factors that are overexpressed by pancreatic adenocarcinomas. The authors hypothesized that tumor HMGA1 status represents a novel prognostic marker in pancreatic adenocarcinoma. They also tested the hypothesis that HMGA1 promotes anchorage-independent cellular proliferation and in vivo tumorigenicity.

METHODS. Tumor HMGA1 expression was examined by immunohistochemical analysis of tissues from 89 consecutive patients who underwent resection for pancreatic adenocarcinoma. Short-hairpin RNA (shRNA)-mediated RNA interference was used to silence HMGA1 expression in MiaPaCa2 and PANC1 pancreatic cancer cells. Anchorage-independent proliferation was assessed by using soft agar assays. The roles of phosphatidylinositol 3-kinase (PI3-K)/Akt and extracellular signal-regulated kinase (ERK) signaling were investigated by using specific inhibitors and adenoviral dominant-negative/active Akt constructs. In vivo tumorigenicity was assessed by using a nude mouse xenograft model.

RESULTS. Tumor HMGA1 expression was detected in 93% of patients with pancreatic adenocarcinoma. Patients with HMGA1-negative tumors had a significantly longer median survival than patients with HMGA1-expressing cancers in univariate analysis ($P = .0028$) and in multivariate analysis ($P<05$). shRNA-mediated HMGA1 silencing resulted in significant reductions in anchorage-independent proliferation in soft agar. Forced HMGA1 overexpression promoted proliferation in soft agar through a process that was dependent on PI3-K/Akt-activited signaling, but not on mitogen-activated protein kinase (MEK)/ERK signaling. Targeted silencing of HMGA1 reduced tumor growth in vivo through reduced proliferation (Ki-67 index) and increased apoptosis (terminal deoxynucleotidyl transferase nick-end labeling).

CONCLUSIONS. The current findings suggested that HMGA1 is an independent predictor of poor postoperative survival in patients with pancreatic adenocarcinoma. Furthermore, HMGA1 promotes tumorigenicity through a PI3-K/Akt-dependent mechanism. HMGA1 warrants further evaluation as a prognostic marker and therapeutic target in pancreatic cancer. ***Cancer* 2008;113:302–14.** © *2008 American Cancer Society.*

KEYWORDS: high mobility group AT-hook 1, Akt, extracellular signal-regulated kinase, growth, survival, pancreatic adenocarcinoma.

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States. The overall prognosis for patients who are diagnosed with this malignancy remains dismal, with 5-year survival rates averaging <5%.[1] The rational identification of clinically relevant therapeutic targets based on an under-

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standing of the biologic mechanisms underlying the aggressive behavior of pancreatic cancer is a high-priority goal.

The human high mobility group AT-hook 1 gene *HMGA1*, which is located on chromosomal locus 6p21, encodes 2 HMGA1 splice variants (HMGA1a and HMGA1b).[2] These HMGA1 proteins are architectural transcription factors that regulate gene expression in vivo.[3,4] They form stereo-specific, multiprotein complexes termed "enhanceosomes" on the promoter/enhancer regions of genes, where they bind to the minor groove of AT-rich DNA sequences.[3,5] HMGA1 proteins are overexpressed in a range of human cancers.[6–9] By using a small sample of tumor tissues, Abe and colleagues previously demonstrated that HMGA1 is overexpressed in pancreatic cancers, although the clinical relevance of HMGA1 expression in this tumor type remains uncertain.[10] The degree of tumor HMGA1 expression reportedly is correlated inversely with patient survival duration in some human cancers.[6,7] These correlative data suggest that HMGA1 may play an important role in cancer progression; however, the mechanisms by which HMGA1 may mediate features of the malignant phenotype are poorly understood. We demonstrated previously that HMGA1 promotes pancreatic cancer cellular invasiveness and that the targeted suppression of HMGA1 reduces metastasis in vivo.[11] We also demonstrated that HMGA1 promotes resistance to anoikis (apoptosis caused by the loss of substratum attachment)[12] and chemoresistance to gemcitabine in pancreatic cancer cells.[13] Although we have demonstrated that HMGA1 affects the metastatic and apoptotic processes, its role in pancreatic tumor growth is unknown, and its potential as an antigrowth therapeutic target remains to be explored.

To establish the clinical relevance of tumor HMGA1 expression in patients with pancreatic cancer, we examined tumor HMGA1 expression in a large cohort of patients using a tissue microarray (TMA) with clinicopathologic correlates. Our findings suggest that tumor HMGA1 expression status represents a biomarker that can be used to predict postoperative survival in patients who have undergone surgical resection for pancreatic adenocarcinoma. In this study, to further evaluate the potential roles of HMGA1 in mediating the malignant phenotype, we tested the hypotheses that HMGA1 promotes anchorage-independent cellular proliferation in pancreatic adenocarcinoma cells and that suppression of HMGA1 expression would impair anchorage-independent proliferation in vitro and tumor growth in vivo. Our observations indicate that HMGA1 indeed promotes pancreatic adenocarcinoma tumorigenesis and that a key effector of HMGA1-induced, anchorage-independent growth is the phosphoinositidyl-3 kinase (PI3-K)/Akt pathway.

MATERIALS AND METHODS

Tissue Microarray Construction and Analysis

Under an Institutional Review Board-approved study protocol, pathology reports were searched to identify patients who underwent curative surgical resection for pancreatic adenocarcinoma between the years 1991 and 2002 at Brigham and Women's Hospital. A pancreatic adenocarcinoma TMA was constructed from 89 consecutive patients who underwent curative resection for pancreatic adenocarcinoma. Formalin-fixed, paraffin-embedded specimens were used to construct the pancreatic adenocarcinoma TMA. Representative tumor regions were selected from each tissue block, and 2 tissue cores (0.6 mm in greatest dimension) were taken from each region using an automated tissue arrayer (Beecher Instruments, Sun Prairie, Wis). Cores also were taken from normal adjacent pancreas for use as internal controls. To avoid areas of pancreas that may have harbored premalignant changes (eg, desmoplasia), we selected only cores that were normal morphologically as internal controls during the TMA construction. Standard hematoxylin and eosin-stained slides from each tumor and its surrounding normal pancreas were reviewed by a single pathologist (M.R.).

Five-micrometer sections were cut from each recipient block to make the TMA slides. Clinical information, including age, sex, and use of chemotherapy, was gathered retrospectively from patient records. Pathologic findings, including tumor size, stage, lymphovascular invasion (LVI), perineural invasion (PNI), differentiation, surgical resection margin status, and lymph node status, were obtained from original pathology reports. Pathologic staging was updated according to current American Joint Committee on Cancer guidelines.

TMA slides were deparaffinized and processed using a streptavidin-biotin-peroxidase complex method. Antigen retrieval was performed by microwave heating sections in 10 mM sodium citrate buffer, pH 6.0, for 10 minutes. After quenching of endogenous peroxidase activity and blocking nonspecific binding, anti-HMGA1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif) was added at a 1:50 dilution; then, slides were incubated at 4 °C overnight. The secondary biotinylated rabbit antigoat antibody (DAKO, Carpinteria, Calif) was used at a dilution of 1:200 for 30 minutes at 37 °C. Next, sec-

tions were incubated with streptavidin-biotin complex/horseradish peroxidase (1:100 dilution; DAKO) for 30 minutes at 37 °C. Chromogenic immunolocalization was determined by exposure to 0.05% 3,3-diaminobenzidine tetrahydrochloride. Normal serum was used in the place of primary antibody as a negative control. Slides were reviewed by 2 independent observers who were blinded to clinical and pathologic data. HMGA1 was scored according to nuclear staining intensity as follows: 0, no staining or weak-intensity staining in <5% of cells; 1, weak-intensity staining; 2, moderate-intensity staining; 3, strong-intensity staining. For statistical analyses, expression was dichotomized into an HMGA1-negative group (score 0) and an HMGA1-positive group (scores ≥1). In cases of disagreement, a consensus was reached by joint review.

Cells and Cell Culture

MiaPaCa2 and PANC1 human pancreatic ductal adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, Va). Cells were maintained in Dulbecco Modified Eagle Medium containing 10% fetal bovine serum (Gibco Life Technologies Inc., Gaithersburg, Md).

Reagents and dominant-negative Akt adenovirus

The PI3-K inhibitor LY294002 and mitogen-activated protein kinase 1/2 (MEK1/2) inhibitor PD98059 were purchased from Calbiochem (San Diego, Calif). Anti-HMGA1 and antilamin B1 antibodies were obtained from Santa Cruz Biotechnology. Adenovirus carrying dominant-negative (Ad-DN-Akt) and dominant-active (Ad-myr-Akt) Akt1 and control cytomegalovirus (Ad-CMV-null) were obtained from Vector BioLabs (Philadelphia, Pa). Adenoviral infection was performed at a multiplicity of infection of 10 in the presence of 6 μg/mL polybrene for 24 hours. Experiments were performed on cells 48 hours after infection.

Plasmid-mediated HMGA1 RNA interference

Hairpin RNA interference plasmids were obtained from The RNA-mediated Interference (RNAi) Consortium (Mission TRC-Hs 1.0; Sigma Aldrich, St. Louis, Mo). The sequences of short-hairpin RNA (shRNA) targeting the human *HMGA1* gene were as follows: shHMGA1-1 plasmid, 5′-CAACTCCAGGAAGGAAACC AA-3′; and shHMGA1-2 plasmid, 5′-CCTTGGCC TCCAAGCAGGAAA-3′. The control plasmid, which has a scrambled, nontargeting shRNA sequence, was obtained from Addgene (Cambridge, Mass). Pooled stable transfectants were established using puromycin (InvivoGen, San Diego, Calif) selection.

Expression vector and transfection

The *HMGA1* coding sequence was amplified by polymerase chain reaction (PCR) from IMAGE clone 5399570 by using gene-specific primers that were modified to include the appropriate restriction sites at their 5′ end. The following primers were used: forward, 5′-TTTTGATATCATGAGTGAGTCGAGCTCGAAG -3′ and backward, 5′-TTTTGAATTCTCACTGCTCCTCC TCCGAGGA-3′. Purified PCR products were digested with EcoRV and *Eco*RI before ligation into an EcoRV/*Eco*RI-digested pIRES-puro3 vector (Clontech, Palo Alto, Calif). The expression plasmids were named pIRES-HMGA1. MiaPaCa2 cells were transfected with pIRES-HMGA1 or with empty pIRES-puro3, which acted as a control, using Lipofectamine 2000 (Invitrogen). Clones pIRES-HMGA1.1 and pIRES-HMGA1.2 were selected by using puromycin (InvivoGen) were used for further studies, because they expressed the highest levels of HMGA.

Soft agar colony formation assay

Assays were performed by using a cell transformation detection assay according to the manufacturer's instructions (Chemicon, Temecula, Calif). Briefly, assays were performed in 6-well plates with 5×10^3 cells, resuspended as a single cell suspension in 0.4% agar, and layered on top of 0.8% agar. Plates were incubated for 10 to 12 days. Colonies were stained and counted manually at high-power magnification (×40). The counting was performed for 10 fields in each well, and at least 6 wells per condition were counted in each experiment. Average values from 3 independent experiments were calculated. The relative number of colonies was calculated by dividing each value by the mean value of the control group.

Western blot analysis

Total cell extracts were prepared with Phosphosafe lysis buffer (Novagen, San Diego, Calif). Nuclear extracts were prepared by using NE-PER Nuclear Extraction Reagents (Pierce, Rockford, Ill). Protein concentrations were measured by using a bicinchoninic acid assay kit (Sigma) with bovine serum albumin as a standard. Cell lysates that contained 50 μg protein or nuclear protein that contained 10 μg protein were subjected to 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis, as described previously.[11] Chemiluminescence detection (Amersham Biosciences, NJ) was performed in accordance with the manufacturer's instructions.

Nude mouse subcutaneous xenograft model

Male athymic nu/nu mice aged 5 weeks were obtained from Harlan Sprague-Dawley (Indianapolis, Ind). Mice

housed in a pathogen-free facility were observed for signs of tumor growth, activity, feeding, and pain in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals. To determine the effect of HMGA1 gene silencing on in vivo growth, 2×10^6 MiaPaCa2 cells and PANC1 stable transfectants that expressed the control or HMGA1 shRNA (shHMGA1.1 plasmid) were implanted subcutaneously in nude mice. Tumor dimensions were measured weekly by using micrometer calipers. Tumor volumes were calculated as follows: volume = $1/2\ a \times b^2$, where a and b represented the larger and smaller tumor dimensions, respectively. Eight weeks after implantation, the primary tumor was excised, fixed in formalin, and embedded in paraffin.

Immunohistochemistry

Xenograft tumor sections (5 μm) were deparaffinized and processed by using the streptavidin-biotin-peroxidase complex method described above. Sections were incubated with anti-Ki-67 (DAKO) at 4 °C overnight at 1:200 dilution. The secondary antibody was biotinylated rabbit-antimouse antibody (DAKO), which was used at 1:200 dilution for 30 minutes at 37 °C. Tumor cells were considered positive for the Ki-67 antigen if there was intranuclear staining. Cells with positively stained nuclei were counted at ×40 magnification in 5 random fields from each section.

Apoptosis staining

After preparation of 5-μm tumor sections, apoptosis was quantified by using a commercially available terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) kit (Chemicon). The number of apoptotic cells was counted in 5 random fields from each section.

Statistical Analysis

Differences between groups were analyzed using Student t tests, multifactorial analyses of variance of initial measurements, and Mann-Whitney U tests for nonparametric data, as appropriate, with Statistica software (version 5.5; StatSoft, Inc., Tulsa, Okla). In cases in which averages were normalized to controls, the standard deviations of each nominator and denominator were taken into account in calculating the final standard deviation. $P < .05$ was considered statistically significant.

RESULTS

Pancreatic Adenocarcinoma TMA: Patient Characteristics

The cohort consisted of 89 patients with pathologically proven pancreatic adenocarcinoma (42 men and 47 women). The mean age at diagnosis was 63 years (median age, 63 years; age range 34-84 years). The median survival was 16.6 months (range, 91-3462 days). The actuarial 1-year survival rate was 70.3%, and the 5-year survival rate was 8.1%. A summary of the clinicopathologic characteristics of the cohort is provided in Table 1.

TABLE 1
Clinicopathologic Characteristics of the Pancreatic Adenocarcinoma Cohort

Characteristics	No. of patients (%)
Age, y	
Median	63
Range	34–84
Sex	
Men	42
Women	47
Overall disease stage	
I	13 (15)
II	74 (83)
III	0 (0)
IV	2 (2)
Lymph node status	
Negative	34 (38)
Positive	55 (62)
Pathologic tumor size, cm	
Median	2.70
Range	0.10-8.40
Histopathologic differentiation	
Well	8 (9)
Moderate	47 (53)
Poor	34 (38)

HMGA1 Expression in Normal Tissue and Pancreatic Adenocarcinoma Specimens

Paraffin-embedded specimens were used to construct a pancreatic adenocarcinoma TMA. For each patient within the TMA, cores also were taken from adjacent normal pancreas to act as internal controls and to assess the expression of HMGA1 in normal pancreas. After immunostaining with anti-HMGA1 antibody, HMGA1 expression was scored according to nuclear intensity. Expression was dichotomized into an HMGA1-negative group (score 0) and an HMGA1-positive group (scores ≥1). On immunohistochemical analysis, we detected the presence of nuclear HMGA1 expression in 83 of 89 (93%) pancreatic adenocarcinoma specimens (Fig. 1C). The majority of normal pancreatic ducts had no detectable nuclear HMGA1 expression (Fig. 1A), whereas some normal pancreatic ducts had very weak expression. In the majority of tumor specimens (52%), the degree of HMGA1 staining was graded with a score ≥2 (score 1, 42% [37 of 89 tumors]; score 2, 35% [31 of

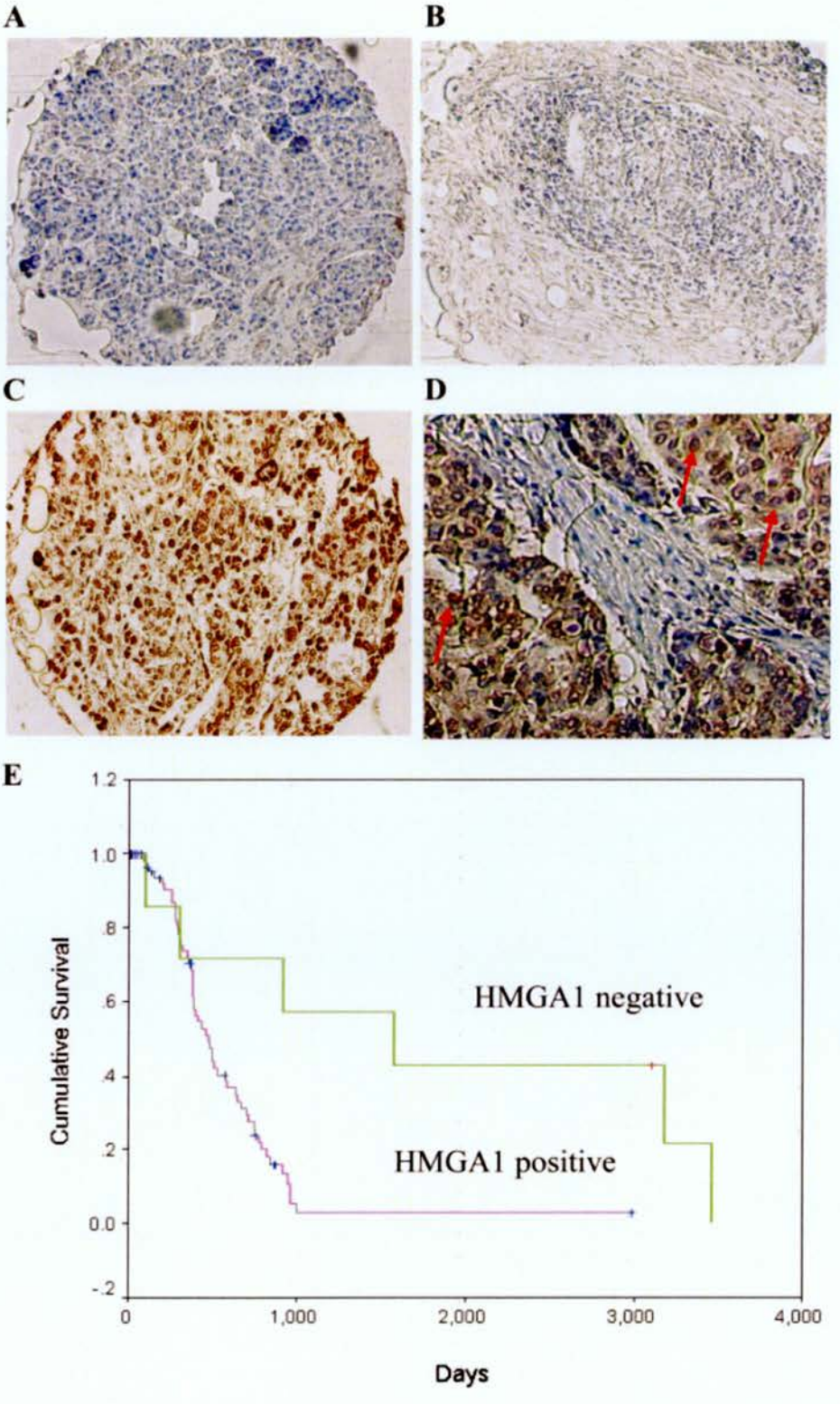

FIGURE 1. Immunohistochemical staining for high mobility group AT-hook 1 (HMGA1) in normal pancreas and pancreatic adenocarcinoma specimens using a tissue microarray. (A) Normal pancreas (HMGA1 negative). (B) Pancreatic adenocarcinoma (HMGA1 negative). (C) Pancreatic adenocarcinoma (HMGA1 positive). (D) High-power magnification (original magnification, ×400) of an HMGA1-positive section that has intense nuclear staining for HMGA1 (indicated by red arrows) with some staining of cytoplasm. (E) Kaplan-Meier analysis of overall survival for patients with pancreatic adenocarcinoma based on HMGA1 expression. Survival of immunohistochemically HMGA1-negative patients was compared with survival of HMGA1-positive patients by using the log-rank test ($P = .0028$).

89 tumors]; score 3, 17% [5 of 89 tumors]). HMGA1 staining predominantly was localized to the nucleus of cancerous cells with some staining of the cytoplasm (Fig. 1C). A detailed pathology case review of the 6 specimens with absent tumor HMGA1 expression (Fig. 1B) demonstrated no atypical features for pancreatic adenocarcinoma.

Tumor HMGA1 Expression Status: Association With Clinicopathologic Features

To identify associations of HMGA1 expression (HMGA1-negative expression vs HMGA1-positive-expression) with clinicopathologic variables, the variables were dichotomized as shown in Table 2. There were no significant differences patients with positive or negative tumor HMGA1 expression were compared with respect to patient age, sex, tumor size, differentiation, LVI, PNI, receipt of chemotherapy, margin status, lymph node involvement, and disease stage (Fisher exact test; $P > .05$) (Table 2). In a Kaplan-Meier analysis, patients who had no HMGA1 expression (HMGA1-negative tumors) had significantly longer overall postoperative survival (mean, 5.7 years; median, 4.3 years) compared with patients who had HMGA1-positive tumors (mean, 1.6 years; median, 1.3 years; $P = .0028$; log-rank test) (Fig. 1D). For the patients who had positive HMGA1 expression, increasing degree of HMGA1 expression was not associated with worse clinicopathologic features or shorter survival.

HMGA1 Represents an Independent Prognostic Indicator in Pancreatic Adenocarcinoma

To assess whether HMGA1 expression was an independent predictor of overall postoperative survival, a Cox proportional-hazards model was created in a forward fashion that included only the covariates that had a statistically significant correlation (inclusion threshold, $P \leq .05$) with postoperative survival. Univariate analysis demonstrated that increasing tumor size, poor tumor differentiation, and HMGA1-positive tumors were significant predictors of poorer survival ($P < .05$) (Table 3). Furthermore, multivariate analysis demonstrated that, after correction for confounding variables, HMGA1 expression remained a significant independent prognosticator for postoperative survival ($P = .001$) (Table 3).

Stable RNAi-mediated Suppression of HMGA1 Expression Inhibits Anchorage-independent Growth

Both MiaPaCa2 and PANC1 pancreatic adenocarcinoma cell lines expressed HMGA1, and MiaPaCa2 cells had a lower expression level of HMGA1 at baseline. We used 2 independent shRNA target sequences (called shHMGA1-1 and shHMGA1-2) to suppress HMGA1 expression. Each of these shRNA sequences was associated with an approximately 80% reduction in HMGA1 expression in MiaPaCa2 cells (Fig. 2A), as confirmed by Western blot analyses of nuclear extracts. These same shRNA sequences were associated with approximately 55% to 60% reductions in HMGA1 expression in PANC1 cells (Fig. 2A).

In MiaPaCa2 cells, the high degree of HMGA1 silencing induced by each of the shRNA sequences was associated with marked reductions in growth in soft agar (Fig. 2C). Similar but less marked reductions in growth in soft agar were observed for PANC1

TABLE 2
Associations of High Mobility Group AT-hook 1 Expression With Clinicopathologic Features

	HMGA1 status		
Variable	Negative (N = 6)	Positive (N = 83)	*P*
Age, y			
≤64	3	44	1.000
>64	3	39	
Sex			
Men	2	40	.680
Women	4	43	
Tumor grade (differentiation)			
1	1	6	.713
2	3	44	
3	2	32	
Tumor size, cm			
<2.5	3	38	.416
>2.5	3	55	
Lymph node metastasis			
No	4	30	.197
Yes	2	53	
Lymphovascular invasion			
No	5	49	.397
Yes	1	34	
Perineural invasion			
No	3	40	1.000
Yes	3	43	
Microscopic margin status			
Negative	3	49	.690
Positive	3	34	
Tumor location			
Head	6	77	1.000
Tail	0	6	
Tumor classification			
T1/T2	2	11	.210
T3/T4	4	72	
Chemotherapy			
No	1	4	.272
Yes	3	59	

HMGA1 indicates high mobility group AT-hook 1.

TABLE 3
Predictors of Postoperative Survival: High Mobility Group AT-hook 1 as an Independent Prognostic Indicator

	Univariate analysis			Multivariate analysis		
Risk factor	Hazard	95% CI	*P*	Hazard	95% CI	*P*
Increasing age	1.015	0.994–1.037	.171	1.008	0.983–1.033	.545
Women	0.742	0.442–1.244	.258	1.056	0.576–1.936	.861
Increasing tumor size	1.217	1.022–1.450	.028*	1.398	1.130–1.730	.002*
Presence of lymph node metastasis	1.733	0.995–3.019	.052	1.551	0.781–3.082	.210
Advanced tumor stage (T3/T4 vs T1T/2)	2.034	0.906–4.567	.085			
Local invasion	1.078	0.612–1.897	.796			
Poor tumor differentiation	1.859	1.183–2.921	.007*	3.203	1.746–5.877	.000*
Presence of PNI	1.088	0.651–1.819	.746			
Presence of LVI	1.704	0.982–2.958	.058	1.710	0.944–3.098	.077
Presence of tumor at microscopic margin	1.490	0.886–2.505	.133			
Chemotherapy treatment	0.440	0.132–1.474	.183			
HMGA1 expression	5.473	1.614–18.557	.006*	12.474	2.705–57.520	.001*

95% CI indicates 95% confidence interval; LVI, lymphovascular invasion; PNI, perineural invasion; HMGA1, high mobility group AT-hook 1.
* $P < .05$.

cells (Fig. 2D) corresponding to lower degrees of HMGA1 silencing in this cell line.

Forced HMGA1 Overexpression Promotes Anchorage-independent Growth

Given the relatively low baseline expression level of HMGA1 in MiaPaCa2 cells, we chose this cell line to test the effects of forced HMGA1 overexpression. We transfected this cell line with an overexpression vector that carried the full-length HMGA1 combinational DNA (cDNA). Two clones that stably overexpressed HMGA1 were selected and named pIRES-HMGA1.1 and pIRES-HMGA1.2. The degree of HMGA1 overexpression (2.5-fold and 3-fold overexpression, respectively, over controls) was documented on Western blot analyses of nuclear extracts (Fig. 2B). HMGA1 overexpression was associated with increased colony formation in soft agar (Fig. 2E).

HMGA1-induced Increases in Anchorage-independent Growth Are PI3-K/Akt-dependent but Not MEK/Extracellular Signal-regulated Kinase-dependent

The importance of the PI-3K/Akt and MEK/extracellular signal-regulated kinase (ERK) pathways in tumor growth has been described previously.[14,15] Our group also previously demonstrated that Akt and ERK activation depends on HMGA1 expression levels.[11,12] Therefore, we sought to determine the dependence of HMGA1-induced increase in soft agar growth on these pathways. Given the effects of HMGA1 on Akt activation, we performed soft agar assays in the presence of the PI-3K inhibitor LY294002. At concentrations of either 25 μM or 50 μM, LY294002 was associated with significant reductions in soft agar growth by the pIRES-HMGA1.1 and pIRES-HMGA1.2 clones at levels similar to those exhibited by parental MiaPaCa2 cells and MiaPaCa2 cells stably transfected with empty pIRES-puro3 vec-

FIGURE 2. (A) Stable silencing of high mobility group AT-hook 1 (HMGA1) expression using 2 short-hairpin RNA (shRNA) expression vectors with independent target sequences (shHMGA1-1 and shHMGA1-2) was confirmed on Western blot analysis of nuclear extracts. Controls were shRNA expression vectors with a scrambled, nontargeting sequence. Greater suppression of HMGA1 expression was achieved in MiaPaCa2 cells, in which there was approximately 80% silencing with shRNA sequences. In PANC1 cells, there was 55% to 60% suppression of HMGA1 expression with each shRNA sequence. (B) Results confirmed that 2 clones of MiaPaCa2 cells stably overexpressed HMGA1 (pIRES-HMGA1.1 and pIRES-HMGA1.2) on Western blot analysis of nuclear extracts. Blots shown are representative of 3 independent experiments. (C) The effects of modulating HMGA1 expression on anchorage-independent growth was assessed by using soft agar assays. Stable HMGA1 silencing using each of 2 independent shRNA sequences (shHMGA1-1 and shHMGA1-2) resulted in reductions in soft agar colony formation in both MiaPaCa2 cells and PANC1 cells compared with the scrambled control shRNA-transfected cells. Effects on soft agar growth were greater in MiaPaCa2 cells, corresponding to greater silencing of HMGA1 in these cells. An asterisk indicates $P < .05$ versus control shRNA. (D) Overexpression of HMGA1 in MiaPaCa2 clones (pIRES-HMGA1.1 and pIRES-HMGA1.2) resulted in unequivocal increases in anchorage-independent growth in soft agar. The number and size of colonies in soft agar clearly were larger with overexpression of HMGA1. An asterisk indicates $P < .05$ versus empty pIRES-puro3-transfected cells. Values are means (±standard deviation).

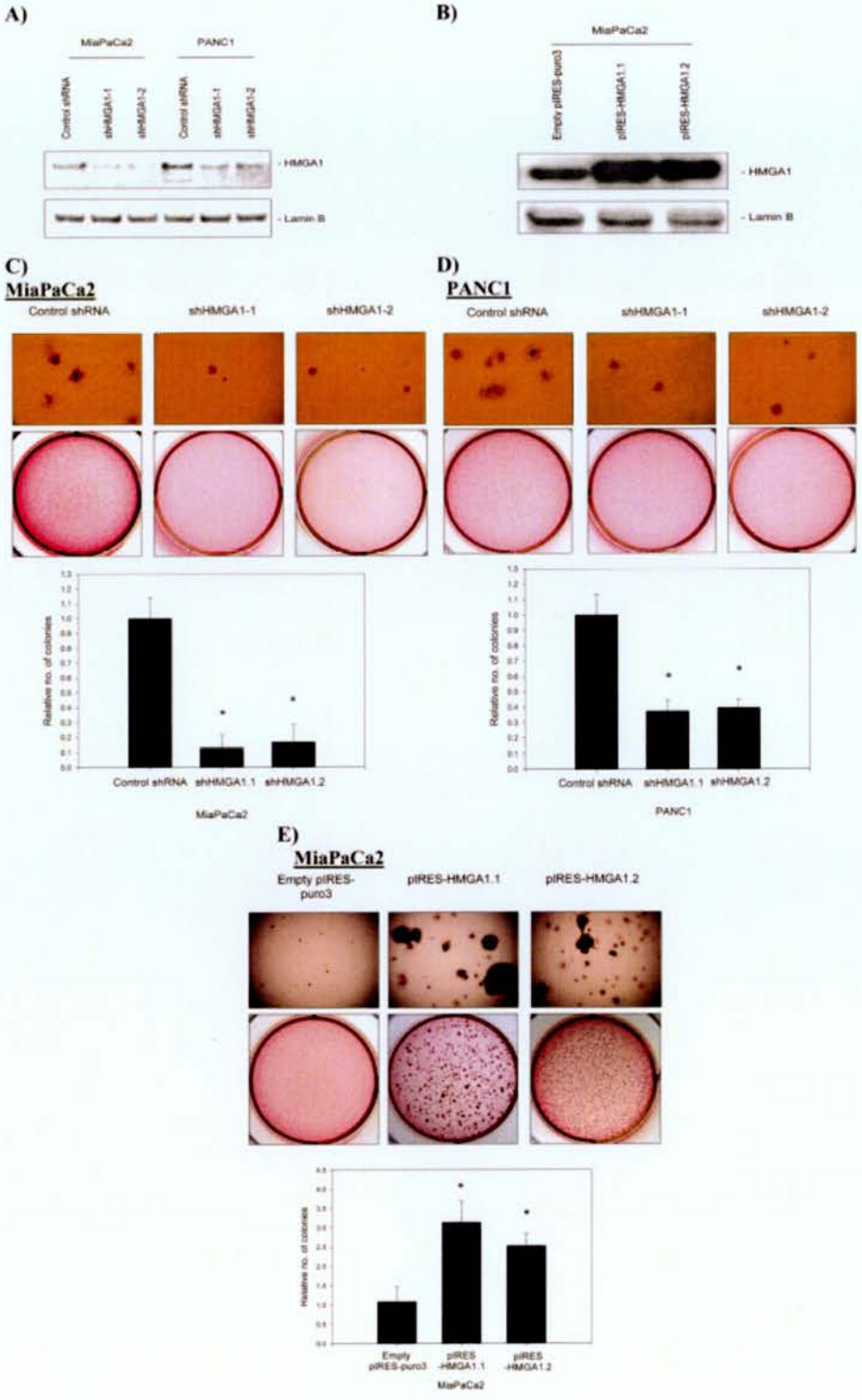
A)
MiaPaCa2
PANC1
Control shRNA
shHMGA1-1
shHMGA1-2
Control shRNA
shHMGA1-1
shHMGA1-2
- HMGA1
- Lamin B
B)
MiaPaCa2
Empty pIRES-puro3
pIRES-HMGA1.1
pIRES-HMGA1.2
- HMGA1
- Lamin B
C)
MiaPaCa2
Control shRNA
shHMGA1-1
shHMGA1-2
Relative no. of colonies
Control shRNA
shHMGA1.1
shHMGA1.2
MiaPaCa2
D)
PANC1
Control shRNA
shHMGA1-1
shHMGA1-2
Relative no. of colonies
Control shRNA
shHMGA1.1
shHMGA1.2
PANC1
E)
MiaPaCa2
Empty pIRES-puro3
pIRES-HMGA1.1
pIRES-HMGA1.2
Relative no. of colonies
Empty pIRES-puro3
pIRES -HMGA1.1
pIRES -HMGA1.2
MiaPaCa2

FIGURE 2.

tor (Fig. 3A). In contrast, the MEK/ERK inhibitor PD98059 (at concentrations of either 50 μM and 100 μM) had no impact on soft agar growth by the pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Fig. 3B). In addition, infection of Ad-DN-Akt abrogated soft agar growth in the pIRES-HMGA1.1 and pIRES-HMGA1.2 clones (Fig. 3C). Infection of Ad-myr-Akt significantly increased the soft agar growth in MiaPaCa2 cells stably transfected with shHMGA1-1 and shHMGA1-2 (Fig. 3D).

HMGA1 Silencing Resulted in Significant Inhibition of Tumor Growth in Vivo

Tumors derived from the subcutaneous implantation of MiaPaCa2 and PANC1 cells that were stably transfected with HMGA1 shRNA vectors exhibited reduced growth rates in nude mice compared with the growth in corresponding controls (tumors derived from MiaPaCa2 and PANC1 cells stably transfected with control shRNA vectors) during the 8-week period after implantation (Fig. 4A,B). Stable knockdown of HMGA1 was confirmed by performing Western blot analysis on nuclear extracts of tumor xenografts (Fig. 4A and B). Immunohistochemical analysis of tumors that were harvested at the end of this observation period suggested that HMGA1 silencing was associated with an inhibition of tumor cell proliferation (Ki-67 reactivity) (Fig. 4C) and an increase in tumor apoptosis (TUNEL staining) (Fig. 4D).

Modulation of HMGA1 expression had no impact on cellular proliferation in monolayer culture (data not shown). We previously demonstrated that modulation of HMGA1 expression did not affect the cellular proliferation in standard monolayer culture.[12]

DISCUSSION

At the time of diagnosis, most patients with pancreatic adenocarcinoma have metastatic or locally advanced disease that precludes surgical resection. Even among the few patients who are able to undergo successful resection, most are destined to die from recurrent cancer. Therefore, the identification of novel prognostic markers and molecular targets for this disease is of high priority. In this study, we focused on 1 such molecule: HMGA1. First, we examined the clinical relevance of HMGA1 expression in pancreatic adenocarcinomas. HMGA1 expression was observed in >90% of pancreatic adenocarcinoma specimens. Although HMGA1 expression was not associated significantly with any of the clinicopathologic variables that were studied, negative HMGA1 status predicted improved survival in patients with pancreatic cancer, and this correlation persisted even after adjusting for other confounding variables. More important, our study demonstrated that HMGA1 expression may help to identify subsets of patients with distinct clinical outcomes despite similar pathologic characteristics. The median survival was significantly longer (up to 3-fold) in HMGA1-negative patients than in HMGA1-positive patients. Although it has been demonstrated that HMGA1 is indicative of a poor prognosis in patients with other cancers, our current study is novel because, to our knowledge, it is the first study to demonstrate that HMGA1 is an independent prognostic indicator in patients with pancreatic adenocarcinoma. Clearly, the identification of HMGA1 as a prognostic indicator potentially may be useful, because it allows the identification of patients who would benefit from more aggressive treatment of their disease. Although our current study represents 1 of the largest immunohistochemical studies of pancreatic adenocarcinoma, our relatively modest sample size limits interpretation beyond the results presented. Our preliminary data on the value of HMGA1 as a prognostic marker is promising and warrants future study with a larger series of pancreatic cancer patients.

Having established the clinical relevance of HMGA1 in patients with pancreatic cancer, we embarked on studies to elucidate the roles of HMGA1 in the aggressive phenotype of pancreatic cancer cells by using in vitro and in vivo experiments. In this study, our findings indicate that HMGA1 promotes anchorage-independent proliferation by pancreatic cancer cells in vitro and tumorigenesis in vivo. In contrast, our previous observation suggested that modulating HMGA1 expression in pancreatic cancer cells had no impact on their proliferation under standard monolayer culture conditions.[12] These results suggest that HMGA1 does not act simply as a mitogenic stimulus. The findings are not surprising, because the kinetics of 3-dimensional colony formation in vitro more closely approximate those of in vivo tumor growth than cells in monolayer culture.[16] The normal cellular response to deprivation from appropriate contact with substratum is to undergo apoptosis, which, in this context, is termed anoikis.[17] A defining feature of transformed cells is resistance to anoikis and the ability to proliferate under anchorage-independent conditions (eg, in soft agar).[18–20] Anchorage-independent growth probably is a result of the ability to proliferate in absence of substratum and to resist apoptosis because of the loss of substratum. To address the question of which aspect is modulated by HMGA1, our group

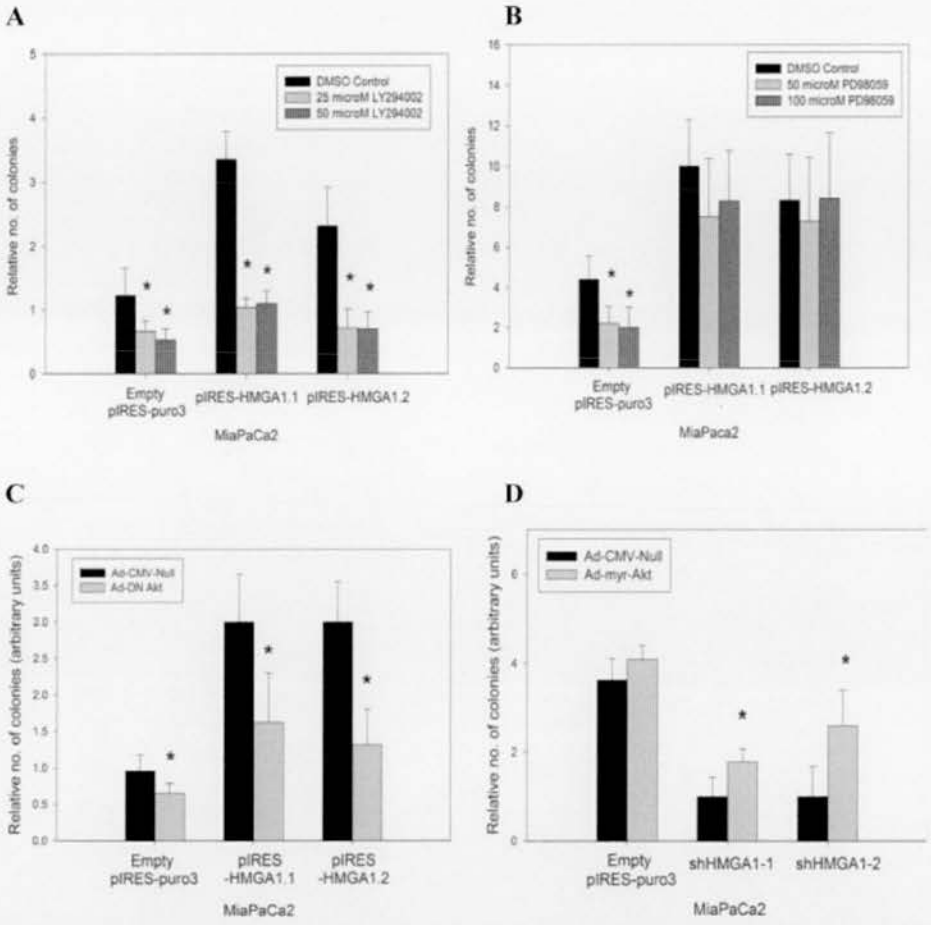

FIGURE 3. (A) Given the effect of high mobility group AT-hook 1 (HMGA1) on Akt activation, soft agar assays were performed in the presence of 25 μM and 50 μM of LY294002 (a specific phosphatidylinositol 3-kinase [PI3-K] inhibitor). Clones pIRES-HMGA1.1 and pIRES-HMGA1.2 exhibited significant inhibition of soft agar growth in the presence of LY294002 at either concentration compared with the dimethyl sulfoxide (DMSO)-treated controls. Although LY294002 also had an effect on soft agar growth in pIRES-empty puro3 controls, the degree of inhibition clearly was less marked compared with that in the pIRES-HMGA1.1 and pIRES-HMGA1.2 clones. An asterisk indicates $P < .05$ versus DMSO-treated controls. (B) Mitogen-activated protein kinase(MEK)/extracellular signal-regulated kinase (ERK) inhibitor PD98059 had no effects on HMGA1 overexpression-induced increases in soft agar growth, because the pIRES-HMGA1.1 and pIRES-HMGA1.2 clones exhibited no significant reductions in growth even in high concentrations (50 μM and 100 μM) of PD98059. This is in contrast to the significant inhibition of soft agar growth when the pIRES-puro3 controls were exposed to PD98059. An asterisk indicates $P < .05$ versus DMSO-treated controls. (C) Infection of the pIRES-HMGA1.1 and pIRES-HMGA1.2 clones with dominant-negative Akt adenovirus resulted in significant reductions in HMGA1 overexpression-induced increase in soft agar growth. Dominant-negative Akt adenovirus resulted in a small effect on soft agar growth in the empty pIRES-puro3 control cells. An asterisk indicates $P < .05$ versus control adenovirus-cytomegalovirus (Ad-CMV-Null). (D) Conversely, infection of adenovirus-expressing, constitutively active Akt (Ad-myr-Akt) rescued the ability to grow under anchorage-independent conditions in shHMGA1-1 and shHMGA1-2 stable transfectants. No effects were observed when empty pIRES-puro3 controls were infected with constitutively active Akt adenovirus. An asterisk indicates $P < .05$ versus control adenovirus (Ad-CMV-Null). Values are means (±standard deviations).

previously investigated the specific roles of HMGA1 in anoikis by investigating the apoptotic status of pancreatic adenocarcinoma cells grown in polyhydroxyethylmethacrylate-coated plates.[12] We observed that HMGA1 overexpression promoted anoikis resistance in pancreatic adenocarcinoma cells. Taken this together, the findings indicate that HMGA1 mediates its function in tumorigenesis through 2 aspects: 1) by enhancing resistance to anoikis, as demonstrated in our previous study,[12] and 2) by allowing continued 3-dimensional proliferation, as demonstrated in the current study. Our in vivo data provide corroborating findings: HMGA1 silencing was associated with reductions in cellular proliferation (Ki-67 index) and

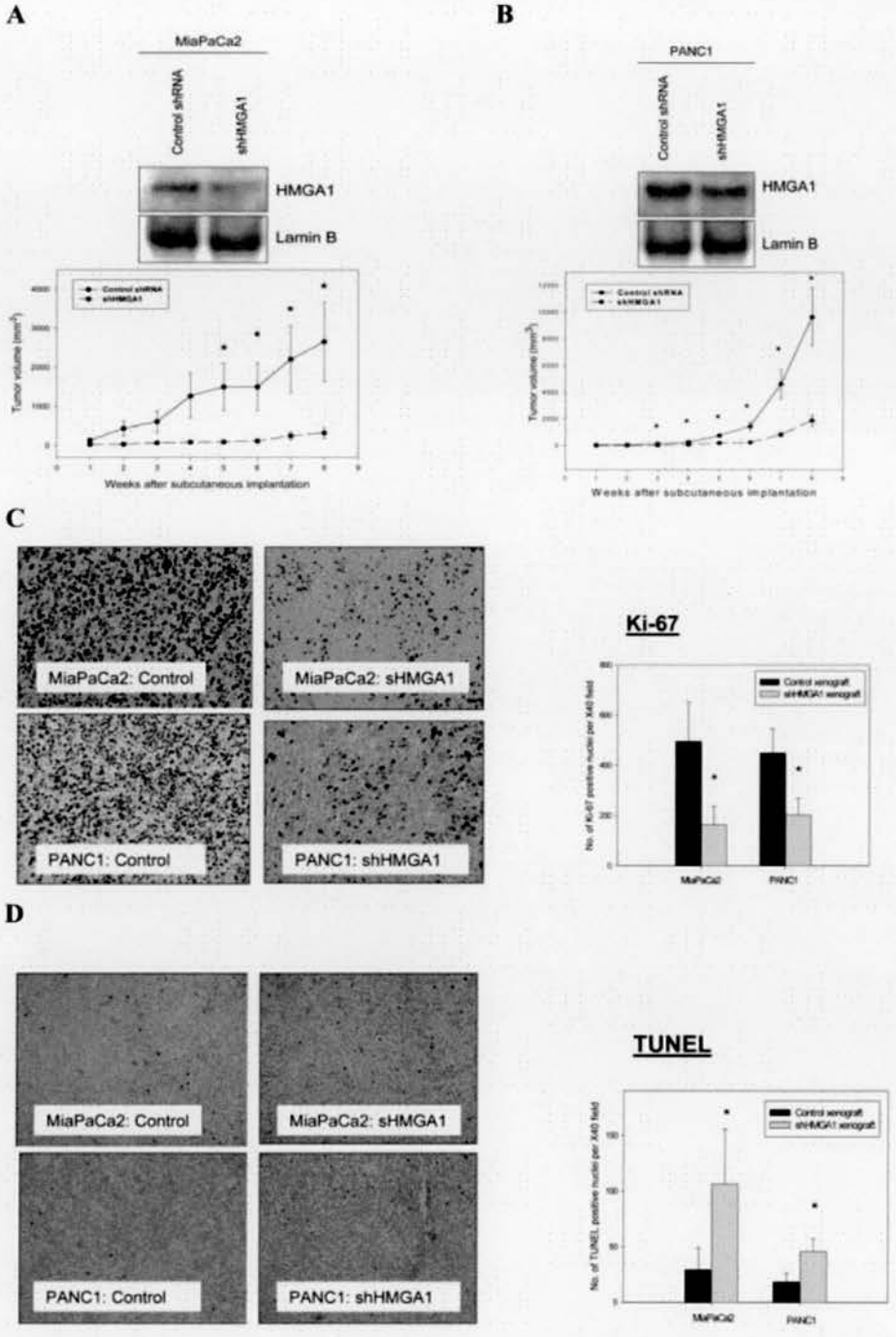

FIGURE 4. (A,B) Stable silencing of high mobility group AT-hook 1 (HMGA1) resulted in significant attenuation in the growth of tumors derived from subcutaneous implantation of MiaPaCa2 cells and PANC1 cells in nude mice. Mice (n = 6 per group) were implanted subcutaneously with stably transfected cells (either a scrambled short-hairpin RNA [shRNA] control or an shHMGA1-1 plasmid). Subcutaneous tumor size was monitored weekly for 8 weeks. Stable HMGA1 silencing was confirmed by Western blot analysis of nuclear extracts from explanted xenograft tumors. Values are means (±standard error of the means). An asterisk indicates $P < .05$ versus control shRNA xenografts. Suppression of HMGA1 resulted in reduction of Ki-67 immunoreactivity in vivo. Each tumor slide was stained for Ki-67, and the numbers of Ki-67–positive cells were counted in at least 5 randomly selected fields at ×40 magnification. Representative tumor sections stained for Ki-67 immunoreactivity in MiaPaCa2 and PANC1 tumor xenografts are shown. An asterisk indicates $P < .05$ versus control shRNA trasnfectant-derived xenografts. (D) HMGA1 silencing led to increased apoptosis in tumor xenografts, as demonstrated on terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) staining. TUNEL-positive cells were counted in at least 5 randomly selected fields at ×40 magnification in each xenograft slide. Representative tumor sections stained for TUNEL in MiaPaCa2 and PANC1 tumor xenografts are shown. An asterisk indicates $P < .05$ versus control shRNA transfectant-derived xenografts. Values are means (±standard deviation).

increases in apoptosis (TUNEL staining), corresponding to overall reductions in tumor growth.

Our study further builds on this observation by defining a novel mechanism through which HMGA1 mediates anchorage-independent cellular proliferation: PI-3K/Akt signaling. Previously, we demonstrated that HMGA1 silencing is associated with reductions in Akt phosphorylation at Ser473 (a marker of Akt activation), whereas forced HMGA1 overexpression is associated with increases in Akt kinase activity and in Akt phosphorylation. Neither HMGA1 silencing nor HMGA1 overexpression had any impact on levels of total Akt expression.[11] Our current data clearly demonstrate that intact PI-3K/Akt signaling is necessary for HMGA1 overexpression to promote colony formation in soft agar. The findings also demonstrate that constitutively active PI-3K/Akt signaling is sufficient to maintain the capacity for colony formation in soft agar in the context of HMGA1 silencing. In our previous study, we confirmed the effects of modulating HMGA1 expression on the functional status of Akt-dependent pathways by assessing its effects on mammalian target rapamycin (mTOR) phosphorylation, a well known downstream target of Akt.[11] We observed that modulation of HMGA1 expression has a direct effect on mTOR phosphorylation, indicating that HMGA1 does have a functional effect on the PI3-K/Akt/mTOR pathway. Although the mechanisms by which HMGA1 modulates the activity of PI3-K/Akt pathway remain unknown, clues can be obtained from a previous study that identified which genes are regulated by HMGA1 using cDNA microarray analysis.[21] Among the list of genes, multiple fibroblast growth factor (FGF) pathway components (eg, FGF receptor 1 [FGFR1], FGF2b, FGF6, FGF7, and FGF9) appear to be regulated positively by HMGA1. It is plausible that induction of the FGF pathway, by binding of FGF to its receptors, could result in downstream stimulation of survival signaling pathways like the PI3-K/Akt pathway, as demonstrated in this study.[22]

Our group[11] and others[23] previously described a role for HMGA1-dependent MEK/ERK signaling. Thus, in the current study, we tested the effects of inhibiting this pathway. We observed that the inhibition of this pathway with the small-molecule inhibitor PD98059 had no impact on the proliferation in soft agar of cells with ectopic HMGA1 overexpression. MEK/ERK inhibitor did not even have a basal effect (as demonstrated by control cells after inhibition of MEK/ERK) in cells that overexpressed HMGA1. These findings suggest that HMGA1 overexpression reduced the sensitivity of the cells to MEK/ERK inhibition. First, this implies that HMGA1-induced colony formation is not dependent on the ERK pathway and highlights the relative unimportance of this pathway in the context of HMGA1 overexpression. Second, HMGA1 overexpression likely has effects on several pro-oncogenic pathways (1 of which is described in this study: the PI3-K/Akt pathway). The effects on these other pathways may be more crucial in promoting soft agar growth and, hence, rendering the inhibition of a single pathway like the MEK/ERK pathway ineffective in reducing colony formation. In our previous study,[11] we demonstrated that HMGA1 silencing had no impact on ERK phosphorylation, although HMGA1 silencing clearly resulted in reductions in cellular proliferation in soft agar. Taken together, these findings suggest that HMGA1-induced cellular proliferation in soft agar growth is independent of MEK/ERK signaling.

It is clear now that HMGA1 exerts its effects not only by altering the conformational structure of DNA. More important, accumulating evidence suggests that HMGA1 exerts its functions by other mechanisms. Recent study has suggested that HMGA1 is capable of inhibiting the functions of *p53*, a well known oncosuppressor gene, by cytoplasmic relocalization of its proapoptotic activator HIPK2.[24,25] Nuclear HMGA1 also has been described to directly influence mitochondrial functions.[26] Further study is likely to reveal increasing complexity in the mechanisms that mediate the biologic actions of HMGA1.

In summary, HMGA1 represents a novel prognostic marker in pancreatic cancer. Functionally, HMGA1 mediates tumor progression by promoting anchorage-independent proliferation by pancreatic cancer cells through a PI-3K/Akt-dependent mechanism. Given the minimal or absent expression of HMGA1 in normal adult tissues, HMGA1 warrants further investigation as a tumor cell-specific therapeutic target.

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Content of supplementary CD-rom

Data presented in the CD-rom are stored in either 1) ***PDF format*** which can be opened using Adobe Acrobat Reader (download from http://www.adobe.com/products/acrobat/) **or** 2) ***SigmaPlot file format*** which can be opened using SigmaPlot software (download a trial version from http://www.systat.com/downloads/ to open the file).

Content of CD-rom is divided into two main folders: 1) Supplementary data folder, and 2) Archive of raw data.

Supplementary data (PDF file format)

1. Tissue microarray database (Chapter 3)
2. Descriptive analysis of survival data (Chapter 3)
3. Log rank analysis of survival data (Chapter 3)
4. Cox regression analyses (Chapter 3)
5. Western blot of HMGA1 silencing using chemically-synthesised siRNA (Chapter 2)
6. Western blot showing the molecular weight location of phospho-Akt (Chapter 4)
7. Western blot showing the molecular weight location of phospho-ERK (Chapter 4)
8. Macroscopic view of explanted livers with metastasis (orthotopic nude mouse model) (Chapter 4)
9. Supportive data on the effects of HMGA1 silencing on Akt kinase activity (using an alternative assay i.e. GSK-3-based assay) (Chapter 4-7)
10. Raw data of a representative gemcitabine IC50 curve in BxPC3 cells with HMGA1 silencing (Chapter 7)
11. Microscopic view of MiaPaCa2 cells with HMGA1 modulation following exposure to gemcitabine (Chapter 7)
12. Photograph of nude mice with subcutaneous xenografts following treatment with gemcitabine (Chapter 7)

Archive of raw data (SigmaPlot file format)

Archive of raw data for each chapter is provided. Each data file has been labelled and coded appropriately to reflect the nature of experiment (see *Notation* file for coding of data).

1. Notation file for data (PDF file)
2. Chapter 4 (Invasion study)(18 data files)
3. Chapter 5 (Anoikis study) (9 data files)
4. Chapter 6 (Soft agar growth study) (15 data files)
5. Chapter 7 (Chemosensitivity study) (16 data files)