The role of Krüppel-Like Factor 4 in Human Gastric Cancer Development and Progression and Comparison of the context dependent role of KLF4 and KLF5 in carcinogenesis

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Dekan: Professor Dr. I. B. Autenrieth

1. Berichterstatter: Professor Dr. L. Kanz
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To my parents Peter und Dorothee Schlunk, my wife Elke
and my children Lilli, Elias, Raphael and Kolja

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Introduction

Epidemiology and risk factors in gastric cancer

Despite a steady decline in incidence and mortality over several decades[1], gastric cancer accounted for 7,8% of new cancer cases in 2008, and is the fourth most common malignancy in the world. It is also still the second most common cause of death from cancer[2]. There is a wide variety in the distribution of gastric cancer with two-thirds of the cases occurring in developing countries and 42% in China alone[3]. About 90% of stomach tumors are adenocarcinomas and are histological subdivided into well-differentiated or intestinal type, and undifferentiated or diffuse type. The two main tumor sites of gastric adenocarcinoma are proximal (cardia) and distal (noncardia). The observed differences between gastric cancers by anatomic site suggest that they are distinct diseases with different etiologies[4].

The prognosis of gastric cancer remains poor. Survival rates vary, with better survival rates in countries with higher incidence rates of gastric cancer[5]. The prognosis varies due to the location of tumors, with lower 5-year survival and higher operative mortality in tumors located in the gastric cardia, compared to those in the pyloric antrum[6].

In countries with well-established screening programs like in Japan, mortality rates have more than halved since the early 1970s[7]. In the US and in European countries, the 5-year relative survival rates are less than 20%[8] and 10% to 20%[9] respectively. There is strong evidence that environmental factors play an important role as risk factors. Migration from high-risk areas in the world for gastric cancer to lower risk areas, is associated with marked diminution in risk for gastric cancer[10]. This data also fits with the observation concerning the importance of location at birth in determining risk[11].

Another well-established risk factor is infection with Helicobacter pylori classifying the bacterium as carcinogenic by the IARC. Its action is probably indirect by provoking gastritis, a precursor of gastric atrophy, metaplasia, and dysplasia[12, 13].

Different nutrition has certainly an important impact. Consumption of considerable quantities of salted foods, especially meats and pickle, and salt per se is associated

with increased risk. High intake of fruits and vegetables is negatively related to risk of gastric cancer, which may be in part related to a protective anti-oxidant effect and inhibition of endogenous nitrosation through their vitamin C content[14, 15, 16, 17]. Tobacco smoking has also been clearly accepted as increasing the risk of stomach cancer[18, 19]. Further risk factors are obesity[20], pernicious anemia[21], blood type A[22], prior gastric surgery for benign conditions[23] and Epstein-Barr virus[24]. In addition, a positive family history is a significant risk factor, particularly with genetic syndromes[25, 26, 27].

Molecular aspects of gastric cancer

Gastric cancer carcinogenesis is a multistep process involving genetic instability and numerous genetic and epigenetic changes in oncogenes, tumor-suppressor genes, cell-cycle regulators, cell adhesion molecules and DNA repair genes. The two different histological types of cancer: gastric and diffuse, arise from different combinations of these changes and steps.
Oncogenes including c-Met[28] and K-sam[29] are more often expressed in diffuse-type cancer, whereas c-erbB2[30] and K-ras[31, 32, 33] are preferentially amplified in intestinal-type gastric cancer. Further well-characterized oncogenes include c-myc, ErBB2 and *cyclin* D[34, 35]. Distinct patterns of c-myc alterations have been shown between intestinal and diffuse-type cancers, and c-myc locus amplification has been implicated as a predictor of aggressiveness in intestinal-type gastric cancer[36, 37].

Tumor suppressor genes including p53, which is usually inactivated through the classic two-hit mechanism, that is loss of heterozygosity and mutation and frame shift deletions of the remaining allele[38, 39, 40, 41]. Further examples of inactivation of tumor suppressor genes, include loss of heterozygosity of p73[42] and bcl-2 gene[43], mutation of APC[44,45] and epigenetic gene silencing via hypermethylation of runt-related genes as RUNX3[46, 47] and nuclear retinoic acid receptor beta (RARbeta)[48].

Cell-adhesion molecules like E-cadherin may act as tumor suppressors[49, 50] and have been reported to be inactivated via the classic two-hit mechanism in diffuse type gastric cancer. Moreover, epigenetic inactivation of E-cadherin via promoter methylation has been described in diffuse type cancer[51]. E-cadherin plays an important role in the Wnt-signaling pathway[52, 53] and builds the cadherin-catenin complex, which is necessary for sequestering β-catenin.

Cell-cycle regulators as *cyclin* E is amplified in 15-20% of gastric carcinomas. And the CDK inhibitor p27 is reduced in advanced gastric carcinoma[54, 55].

Another mechanism of gastric carcinogenesis is microsatellite instability, a hallmark of the DNA mismatch repair deficiency. A potent trigger of microsatellite instability, especially high-frequency microsatellite instability is epigenetic methylation of hMLH1 promoter region CpG island. Microsatellite instability due to epigenetic inactivation of hMLH1 is found in 15%-39% of intestinal-type cancer, 70% of which are associated with loss of hMLH1 by hypermethylation of the promoter[56, 57].

Expression of growth factors and cytokines play an important role in gastric cancer. Intestinal-type gastric carcinomas show an overexpression of EGF, TGF-α, IGF II and bFGF, meanwhile TGF-β, IGF II and bFGF are predominantly overexpressed in the diffuse subtype of gastric carcinoma[58].
Emerging evidence also underscores the importance of signaling pathways involved in the developmental process in gastric cancer, including transforming growth factor-beta/bone morphogenetic protein signaling, Wnt/beta-catenin signaling, Hedgehog signaling and Notch signaling[59].

A schematic representation of the main genetic and epigenetic changes involved in the development of intestinal-type and diffuse-type gastric cancer is shown in Figure 1 and 2 respectively[60].

Figure 1

APC mutation
P53 mutation

DS191 instability
hypermethylation

Intestinal
metaplasia

Adenoma

p53 mutation/LOH
reduced p27 expression

C-erb B2 amplification

Normal
mucosa

H pylori
infection

pS2 reduction
P16 loss
hMLH1 loss

Early
intestinal
type
cancer

Advanced
cancer

Invasion
metastasis

Chr 7q LOH
RUNX3 loss
Cyclin E amplification
Reduced p27 expression
Reduced nm23 expression
GF overexpression
CD46 aberrant transcription

Telomere reduction
Genetic instability

pS2 expression
P73 LOH

RUNX3 loss
CD44 aberrant transcripts
Cyclin E overexpression
CDC25B overexpression

Figure 1. Intestinal type gastric cancer: Genetic and epigenetic alterations during carcinogenesis. Slightly modified from Smith et al.[60]

Figure 2

E-caderin mutation
CDH1 loss
RAR$_\beta$APC loss

K-sam amplification
c-met amplification

Normal
mucosa

H pylori
infection

Early
diffuse
type
cancer

Advanced
cancer

Invasion
metastasis

Chr 7q LOH
RUNX3 loss
Cyclin E gene amplification
Reduced p27 expression
Reduced nm23 expression
GF overexpression
CD46 aberrant transcription

Genetic instability
17 q21 LOH

p53 mutation/LOH
Histone deacetylation
H TERT expression
With telomerase activation

RUNX3 loss
CD44 aberrant transcripts
Cyclin E overexpression
CDC25B overexpression

Figure 2. Diffuse type gastric cancer: Genetic and epigenetic alterations during carcinogenesis. Slightly modified from Smith et al.[60]

Specificity Protein/Krüppel-like factor (SP/KLF) transcription factor family

Increasing evidence has indicated that Krüppel-like factor 4 (KLF4) appears to be a putative tumor suppressor in both gastric cancer and colorectal cancer. Krüppel-like factor 4 belongs to the Specificity Protein/Krüppel-like factor (SP/KLF) transcription factor family. The nomenclature of Krüppel-like factors is based on the homology of its founding member KLF1, to the Drosophila Krüppel protein[61]. At present, 17 Krüppel-like factors have been identified. The array of three Cys_2/His_2 zinc fingers close to the C-termini, which serve as DNA binding domain, is the most outstanding feature of the SP/KLF family members[62]. Without exception, the finger domain of mammalian SP/KLFs consists of 81 amino acids; also the interfinger domains are highly conserved[63]. KLFs bind very similar "GT- box" or "CACCC element" consensus sequences. Thus, the specificity of their activities is determined by differing amino termini and/or by tissue-specific expression[64]. KLFs exert important regulatory functions on many biological processes, such as embryogenesis[65], growth, development, differentiation and apoptosis[66]. Some members of KLFs are potentially novel oncogenes or tumor suppressors.

Krüppel-like factor 4

Krüppel-like factor 4 (KLF4), also known as gut-enriched Krüppel-like factor (GKLF) or epithelial zinc finger (EZF), is known to play important roles in myriads of physiological processes such as cell cycle control, transcriptional regulation, DNA repair, apoptosis, differentiation, and determination of cell fate[67,68,69,70,71,72,73,74,75,76,77,78,79,80].

Using the zinc finger region from KLF1 as a probe to screen a human endothelial cell cDNA library, KLF4 was first identified in 1998. KLF4 is mapped to chromosome 9q31, contains five exons and the RNA-transcript in aortic endothelial cells and human umbilical vein endothelial is about 3,5 kb[81].

Human KLF4 encodes a polypeptide of 470 amino acids with a molecular mass of 50 kDa and contains the typical three zinc fingers in the very C-terminal end. The region immediately N terminal to the three zinc fingers is a 20 amino acid peptide containing the nuclear localization signal (NLS) of the protein[82]. Gene fusion experiments

revealed a repression domain mapped to 181-388 amino acids, as well as an activation domain between amino acid residues 91-117[81], which also interacts with the co-activator, p300/CBP[83]. Moreover a potential "PEST" sequence, which is found in proteins with intracellular half-lives of <2 h, is present between amino acid residues 113 and 152[84, 85].

The structural organization of the Klf4 gene and the corresponding protein is shown in Figure 3.
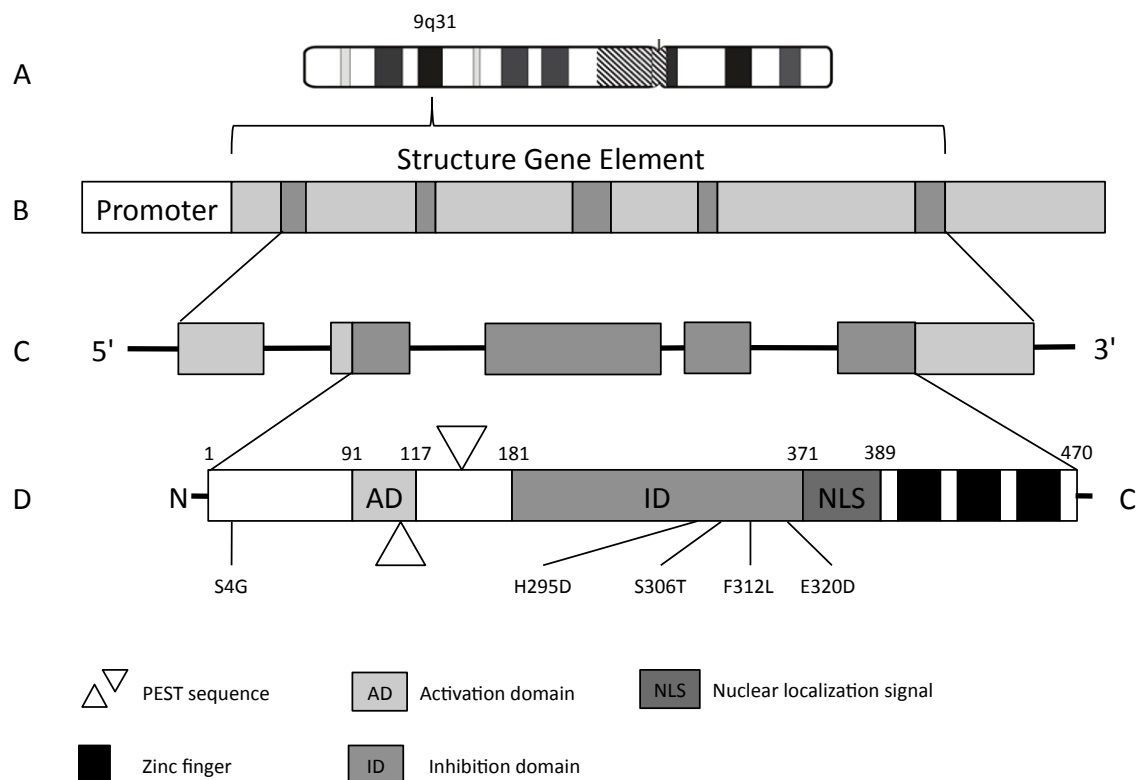
Figure 3. (A) The Klf4 gene is located on chromosome 9q31, which covers a 6.3 kb region. (B) The dark grey bars mark the locations of the five identified exons of the Klf4 gene. (C) Below the genomic map is the Klf4 RNA transcript. The five boxes represent corresponding exons, whereas the dark grey boxes show the Klf4 open reading frame within the 2639 bp cDNA. (D) The Klf4 open reading frame encodes a protein of 470 amino acids with several functional domains, including the transcriptional activation domain (AD), transcriptional inhibitory domain (ID), zinc finger DNA-binding domain, nuclear localization signal (NLS) and potential PEST sequence. Several point mutations have been identified in tumor cells, which are shown at the bottom. Slightly modified from Wei *et al.* 2006 [86].

KLF4 is usually expressed in tissues that have a high rate of cell turnover. KLF4 is highly expressed in epithelial tissues including gut and skin[85, 87]. In addition, KLF4 is found in other tissues including the lung, testis, thymus, cornea, lymphocytes, vascular endothelial cells and cardiac myocytes[85, 87, 88, 89, 90, 91, 92, 93].

Molecular mechanisms of KLF4: DNA-binding, activation and repression

KLF4 interacts with GT-rich or CACCC elements segments on target genes, also an empirically determined 5'-RRGGYGY-3' sequence (where R=A/G and Y=C/T) has been reported to be a target[94]. In addition, KLF4 interacts with the basic transcription element.

KLF4 functions as a transcription activator or repressor. Examples of activation include p21$^{WAF1/CIP1}$ [95], keratin and alkaline phosphatase[69, 96] and its own gene[97]. In addition, the N-terminal domain interacts with the transcriptional co-activators p300 and CBP, and this interaction is required for its function[83, 98].

Examples of repression include the cytochrome p450 1A1 (CYP1A1), which is repressed in a BTE-dependent manner[99] and several cyclin genes such as *cyclin* D[112], *cyclin* B1[73] and *cyclin* E[72].

KLF4 in differentiation and development

KLF4 is primarily localized to the mitotically inactive (post-mitotic) population of cells as in the post-mitotic villus epithelial cells of the intestine[85, 100], suprabasal layer of the epidermis[87, 101] and the quiescent cortical cells of the thymus epithelium[89]. These studies indicate that KLF4 expression is temporally associated with terminal differentiation of epithelial cells.

Most of the evidence for the role of KLF4 in differentiation and development has been derived from knockout or overexpression transgenic mouse studies. Klf4_/_ mice die soon after birth of dehydration due to defects in the epidermal barrier of the skin[101]. Conversely, targeted overexpression of KLF4 results in early formation of the epithelial permeability barrier[102]. The ability to affect barrier function is likely due to its ability to regulate gene clusters of the Sprr[103] and keratin families[69], which are key

components in maintaining epithelial barrier integrity.

KLF4 plays a critical role in differentiation in the intestinal epithelium. Klf4_/_ mice have a selective loss of goblet cells from the colon, suggesting that KLF4 is a specific goblet cell differentiation factor[104].

Moreover, *Lama1*, which encodes the basement membrane component Laminin-1, and the gene encoding intestinal alkaline phosphatase, an enterocyte differentiation marker are transcriptional targets of KLF4[105, 106].

Using tissue-specific gene ablation, lacking Klf4 in gastric epithelia, cornea or smooth muscle cells respectively, KLF mutant mice showed altered proliferation and differentiation of the gastric epithelium[107], corneal epithelial fragility, stromal edema of the cornea, loss of conjunctiva goblet cells[108] and altered proliferation and differentiation of vascular smooth muscle cells[109].

In addition, Klf4 appears to be important for both resident and inflammatory monocyte differentiation[74, 76]. Furthermore, in a cell culture model of adipocyte differentiation, knockdown of KLF4 via siRNA completely blocked expression of several phenotypic markers of differentiated adipocytes[77].

Collectively, these data strongly implicate KLF4 as a factor involved in the differentiation of many tissues.

The role of KLF4 in proliferation and cell cycle control

Since KLF4 is highly expressed in terminally differentiated postmitotic intestinal cells, suggesting a link to growth arrest, further investigations have been performed to emblaze the role of KLF4 in cell cycle progression. Growth arrest of NIH3T3 cells by either serum starvation or contact inhibition increased KLF4 mRNA levels. Conversely, proliferating NIH3T3 cells showed decreased levels of KLF4 mRNA. Furthermore ectopic expression of KLF4 in these cells resulted in inhibition of DNA synthesis[85].

Using oligonucleotides microarray analysis, expression profiling of KLF4 revealed that KLF4 activates numerous genes that encode negative regulators of the cell cycle, as well as suppresses expression of genes that promote cell cycle progression[69].

In addition another study revealed a global inhibitory function for KLF4 in regulating

the expression of groups of genes involved in macromolecular synthesis[110].

The biochemical mechanism by which KLF4 inhibits cell proliferation was elucidated from studies examining the role of KLF4 in DNA-damage-induced cell-cycle arrest[111]: During serum deprivation and DNA damage, KLF4 is induced in a p53-dependent manner[111]. KLF4 itself activates p21$^{WAF1/CIP1}$ through a specific Sp1-like *cis*-element in the p21$^{WAF1/Cip1}$ proximal promoter. This same *cis*-element is also needed for p53 to activate *p21$^{WAF1/Cip1}$* transcription, although p53 does not directly bind to it. Instead, p53 and KLF4 physically interact with each other. A result of this complex relationship is the synergistic induction of the activity of the p21$^{WAF1/Cip1}$ proximal promoter[111] (Figure 4).

Activation of p21$^{WAF1/Cip1}$ expression results in cell cycle arrest at both the G1–S and G2–M transition points. KLF4 is necessary in mediating the checkpoint function of p53 at both of these transition points [70, 72]. In addition to its activation of p21$^{WAF1/Cip1}$, KLF4 suppresses *cyclin* D1[112] and *cyclin* B1[73], which are required for the G1/S and G2/M transitions, respectively. Consistent with its inhibitory effect on cell cycle, KLF4 prevents chromosomal amplification by suppression of transcription of *cyclin* E[72]. Furthermore KLF4 blocks mRNA expression of ornithine decarboxylase, an essential enzyme for cell growth[113] (Figure 5).
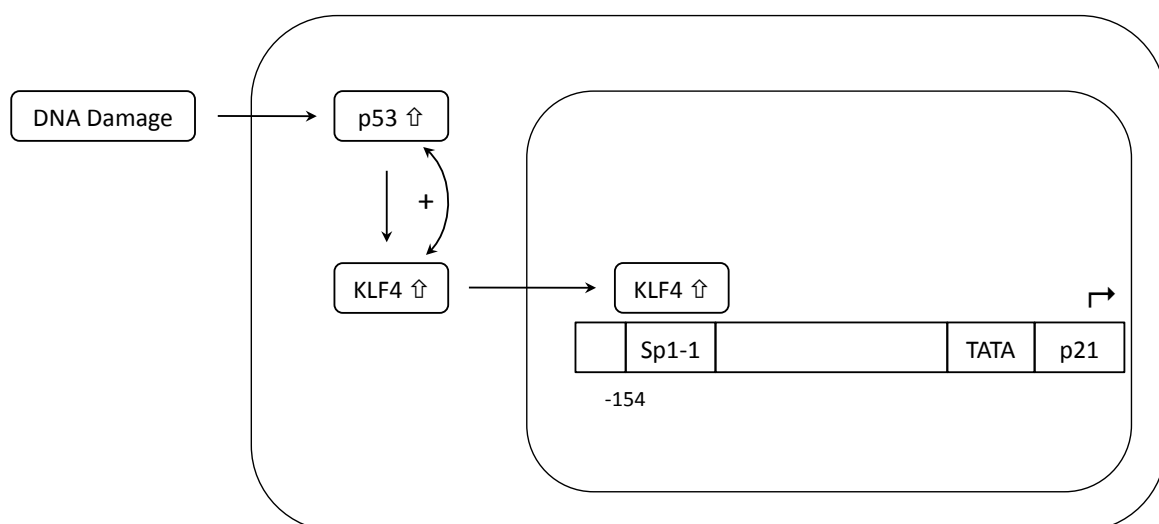
Figure 4. Regulation of the p21*WAF1/Cip1*** proximal promoter by p53 and KLF4:** activation of p53 by DNA damage leads to both an increase in KLF4 synthesis and an interaction between p53 and KLF4 (*double arrow*), which cumulates in the binding of KLF4 to the Sp1-1 element of the p21*WAF1/Cip1* promoter. Modificated from Zhang et al.[111]
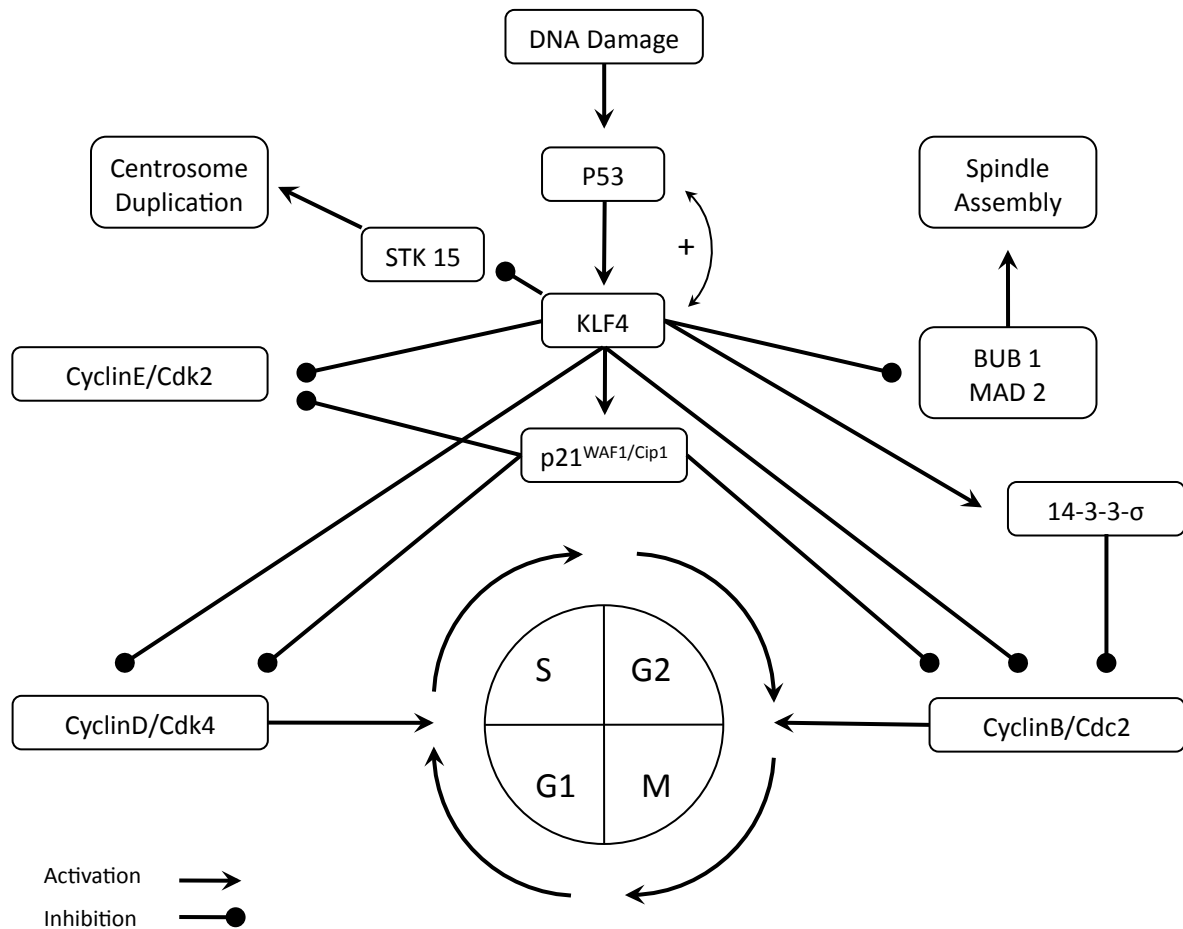
Figure 5. Schematic overview by which KLF4 mediates the cell cycle checkpoint functions of p53 in response to DNA damage. The tumor suppressor p53 is activated in response to DNA damage, which in turn activates KLF4. KLF4 then exerts a multitude of effects on expression of downstream genes by activating (−>) or inhibiting (−•) their expression: p53 −> KLF4 −> p21[111]; KLF4 −• *cyclin* D/Cdk4[112]; KLF4 −• *cyclin* B/Cdc2[73]; KLF4 −• *cyclin* E/Cdk2[72]; KLF4 −• STK15[69]; KLF4 −> 14-3-3 σ; KLF4 −• BUB1/MAD2[69]. Modification from Ghaleb et al.[114] and McConnell et al.[100].

KLF as antiapoptotic factor

In response to γ-radiation-induced DNA damage, organisms either activate cell cycle checkpoint and repair machinery, or undergo apoptosis to eliminate damaged cells. p53 is involved in both mechanisms by expressing either proapoptotic genes like *BAX*, *PUMA*, *FAS* and *NOXA*[115, 116, 117, 118,] or cell cycle checkpoint target genes like p21[WAF1/Cip1 119] and 14-3-3σ[120].

KLF4 has been shown to play a crucial role as an antiapoptotic factor, in part by suppressing *BAX* expression following γ-radiation. KLF 4 accomplishes this

antiapoptotic effect by activating p21$^{WAF1/Cip1}$, and by inhibiting the ability of p53 to transactivate the proapoptotic gene *BAX* [121]. A net effect is to steer cells away from apoptosis and toward cell cycle arrest (Figure 6).
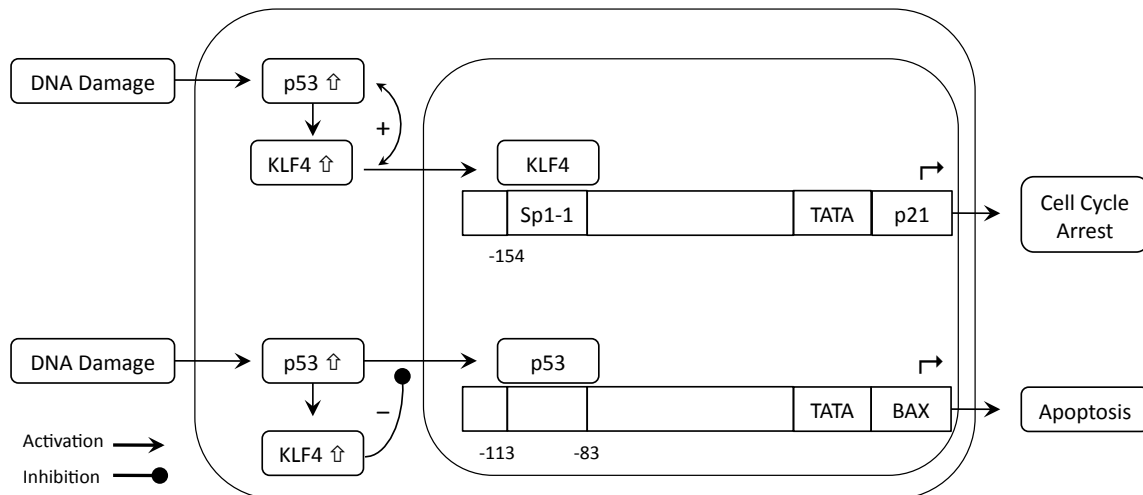
Figure 6. A model for the role of KLF4 in suppressing apoptosis after γ-irradiation. Following γ-radiation, p53 then activates KLF, p21$^{WAF1/Cip1}$ and *BAX*. KLF4 has now dual functions to either synergistically induce p21$^{WAF1/CIP1}$ leading to cell cycle arrest or to suppress *BAX* expression, both directly and indirectly by inhibiting activation of *BAX* by p53. A net effect is to steer cells away from apoptosis and toward cell cycle arrest. Modification from Ghaleb et al.[121]

KLF as a Tumor Suppressor Gene

The observation, that KLF4 plays a critical role in differentiation and has an outstanding role in mediating the checkpoint function of cell cycle, suggests that KLF4 may act as a tumor suppressor. Indeed, many human tumors including colorectal, gastric, esophageal and bladder cancers show loss of KLF4 expression[122, 123, 124, 125, 126, 127, 128, 129, 130, 131 and 132].

Particularly in colorectal cancer, KLF4 has been thoroughly investigated and there is both, *in vivo* and *in vitro* evidence that KLF4 acts as a tumor suppressor. Thus, overexpression of KLF4 in human cancer line RKO reduces tumorigenecity in vivo and cell migration/invasion in vivo[133]. KLF4 mRNA levels are reduced in intestinal adenomas from *ApcMin/+* mice as well as in colonic adenomas derived from patients with familial adenomatous polyposis[113]. Finally, haploinsufficiency of Klf4 promotes the development of intestinal adenomas in *Apc*$^{Min/+}$ mice[134]. In addition, KLF4-positive colorectal cancer patients with lymph node metastasis showed better overall survival than KLF4-negative patients[135]. Molecular mechanisms leading to loss of expression

of KLF4 in colorectal cancer include deletion, mutation and methylation silencing[127]. In the stomach, using conditional KLF4-knockout mouse, loss of KLF4 results in precancerous changes[136].

In pancreatic cancer ectopic overexpression of KLF4 suppressed pancreatic cancer cell growth *in vitro* and *in vivo* via induction of p27^{Kip1} expression and cell cycle arrest, suggesting that KLF4 has tumor suppressor functions in pancreatic cancer[137].

KLF4 has also been shown to play a role in different pathways of tumorigenesis. As APC, a tumor suppressor gene and a major component of the Wnt/ß-catenin pathway of tumorigenesis, KLF4 counters the Wnt/ß-catenin signaling by blocking the recruitment of the transcriptional co-activator p300/CBP, and thus suppresses transformation of cancer cells both *in vitro* and *in vivo*[138, 139]. Furthermore KLF4 is involved in inhibiting the Notch signaling pathway, which is often up-regulated in intestinal tumors and colorectal cancer cells[78]. Consistent with this fact, KLF4 expression and goblet cell differentiation in the intestines of wild type and *Apc*$^{Min/+}$ mice, is enhanced with inhibition of Notch, as well as proliferation and tumor formation is reduced in the intestines of *Apc*$^{Min/+}$ mice[78].

KLF as an Oncogene

Analysis of expression in dysplastic cells and tumor cells in vivo provided independent evidence that KLF4 exhibits also properties expected of an oncogene. First evidence was provided from investigations using E1A-immortalized rat kidney epithelial cells (RK3E) to screen for factors that could induce transformation. There, KLF4 was identified. In addition, KLF4-transformed RK3E cells are able to produce tumors in xenografted mice[140].

Furthermore, KLF4 mRNA and protein levels are also increased in ductal carcinoma of the breast[141] and increased nuclear staining of KLF4 is associated with a more aggressive phenotype and poorer prognosis in early-stage breast cancer[142]. Similar observations have been made, connecting nuclear expression of KLF4 in squamous epithelial dysplasia and squamous cell carcinoma of the skin[130, 143, 144]. One mechanism, by which KLF4 allows bypass of oncogenic RAS$_{v12}$-induced senescence, is by suppressing p53 expression via inhibiting directly the p53 promoter. Thus, allowing for RAS$_{v12}$-mediated transformation and providing

resistance to DNA-damage-induced apoptosis. Consistently, KLF4 depletion from breast cancer cells restored p53 levels and caused p53-dependent apoptosis[145]. Moreover, Ras$_{V12}$ targets *cyclin D1*, which then neutralizes the cyclin inhibitor p21$^{WAF1/CIP1}$, conferring KLF4 the ability to transform cells[146]. These results unmask KLF4 as a regulator of p53 that oncogenically transforms cells as a function of p21$^{WAF1/CIP1}$ status.

Another mechanism demonstrates an anti-apoptotic effect of KLF4 following DNA damage, due to the inhibitory effect on p53's ability to activate the *BAX* promoter by sequestering p53 from the BAX promoter, or to direct repress the *BAX* promoter[121]. Thus, in the absence of p21$^{WAF1/CIP1}$, KLF4's anti-apoptotic effect dominates its cytostatic effect, rendering KLF4 oncogenic. These findings provide a mechanistic explanation for the context-dependent oncogenic or tumor-suppressor functions of KLF4 (Figure 7).
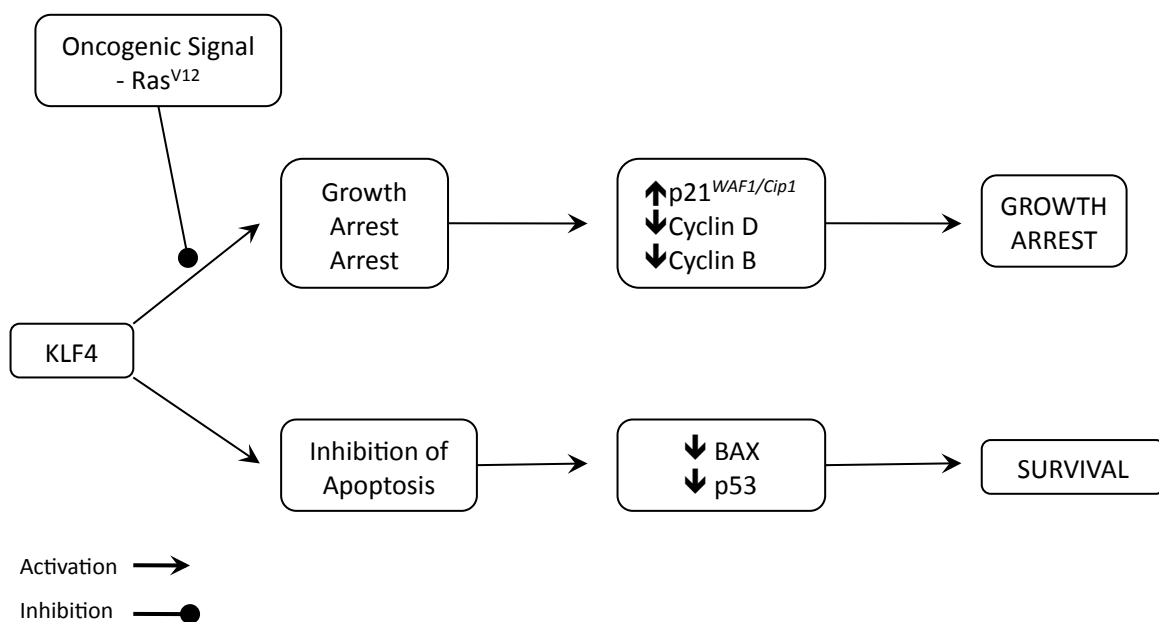
Figure 7. This model may explain the context-dependent nature by which KLF4 functions as either a tumor suppressor or an oncogene. Inactivation of p21WAF1/CIP1 by oncogenic RASV12 neutralizes the cytostatic action of KLF4, converting the latter to a transforming protein owing to its antiapoptotic activity. Slightly modified from McConnell et al.[100]

KLF4 and induced pluripotent stem cells

Consistently with its pleotropic function is the recent discovery that KLF4, in combination with Oct3/4, Sox2 and c-Myc, three other transcription factors exerts a crucial role in somatic cell reprogramming and maintenance of embryonic stem cell self-renewal.

Mechanistically, KLF4 may suppress apoptosis induced by c-Myc, and c-Myc neutralizes KLF4's cytostatic effect by suppressing p21$^{WAF1/CIP1}$ [147]. In this manner, the balance between KLF4 and c-Myc might play a critical role in the establishment of an immortalized state of inducible pluripotent stem cells cells. Reprogramming of somatic cells to pluripotent stem cells appears to be an alternative to circumvent the ethical and moral issues problems arisen from ES cell research.

Objective and background

KLF4 can be added to a growing list of genes that have multiple, context-dependent roles in cancer. In the present study, the goal was to investigate the role of Krüppel-like factor 4 in human gastric cancer *in vivo* and *in vitro*. First, KLF4 expression in primary tumors and lymph node metastasis from human gastric cancer tissues were measured, and multivariate analysis was generated to look for independent prognostic marker. To study the function of KLF4 in gastric cancer cell cultures, and in an orthotopic animal model, an adenovirus vector system containing KLF 4 was constructed. Enforced restoration of KLF4 expression was carried out, to investigate the impact on cell growth inhibition *in vitro* and tumor growth of metastasis in an orthotopic animal model of gastric cancer. Finally, mechanism studies were carried out, to provide information on how KLF4 is deactivated and thus contributes to gastric cancer development and progression.

In 2003 I had the possibility to do research for my dissertation in the laboratory of Professor Xie at the MD Anderson Cancer Center in Houston, USA. In 2005 we published a paper in *Cancer Research* named "Drastic Down-regulation of Krüppel-Like Factor 4 Expression Is Critical in Human Gastric Cancer Development and Progression". This paper is the basis of the present dissertation and originated from beneficial collaboration of coworkers, including Assistant Professor Daoyan Wei (project manager), Dr. Weida Gong, Dr. Masashi Kanai, Dr. Liwei Wang, Dr. James C. Yao, Dr. Tsung-Teh Wu, and Professor Keping Xie. My part in this work was the construction of the Ad-KLF4 virus including PCR, sequencing, expression of KFL4 in gastric cancer cell lines via Western blot, investigation of the infection efficiency of the adenovirus vector system via green fluorescence protein and cell culture experiments with enforced restoration of KLF4. In addition, I was involved in the animal experiment.

In order to picture the whole context of the work, also experiments of coworkers who were involved are shown in this thesis. These parts are labeled with a * and are written in a smaller text size to clearly distinguish them from the personally conducted experiments.

Materials and Methods

Chemicals

Ampicillin	USB Cleveland, Ohio, USA
APS (Ammonium Persulfate)	Acros Organics - Fisher Scientific, USA
BenchMark Pre Stained Protein Ladder	Cat. No. 10748-010 Invitrogen
BSA (acetylated bovine serum albumin)	New England Biolabs Ipswich, MA
Buffer B (10x)	Roche Diagnostics GmbH, Mannheim, Germany
Buffer1, Buffer 4	New England Biolabs Ipswich, MA
Dephosphorylation buffer	Roche Diagnostics GmbH, Mannheim, Germany
1 kb DNA Ladder	Promega Corporation, WI, USA and Invitrogen Cat. No. 15615-016
EDTA	Fisher Scientific Inc., USA
Ethanol	AAPER Alcohol and Chemical Company Shelbyville, KY, USA
Ethidium bromide	Sigma Aldrich Co., USA
Fat dry milk	Nestlé
Kanamycin	Alexis Biochemical's, San Diego, CA, USA
LB Agar, Lennox	Talon Scientific & Medical Products Ltd.
LB Broth, Lennox	Talron Scientific & Medical Products Ltd.
Lipofectamine	Invitrogen Life Technologies, Inc., Rockville, MD, USA

MEM (minimal essential medium) supplemented with 10% fetal bovine serum sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution	CMEM, Flow Laboratories, Rockville, MD
PCR Master Mix	Promega Corporation, Madison, WI, USA (Cat. No. M7502)
Phosphatase, alkaline	Roche Diagnostics GmbH, Mannheim, Germany (Cat. No. 713 023)
2X Quick Ligation Buffer	New England Biolabs, Inc.
Quick T4 DNA Ligase	New England Biolabs, Inc.
Sodium Acetate (NaAc)	Sigma Aldrich Co., USA
Sodium Chloride	Sigma Aldrich Co., USA
Sodium dodecyl sulfate	Fisher Scientific Inc., USA
TEMED	Bio Rad
Tris (hydroxymethyl) aminomethane	Fisher Scientific Inc., USA
Triton X-100	Curtis Matheson Scientific, USA
Trypan blue	Invitrogen Corporation - USA (Cat. No.15250-061)
Tween 20 (Polyoxethylene sorbitan monolaurate)	Sigma Aldrich Co., USA

Buffers

Western Blot

Lysis Buffer	20mM Tris- HCl (pH 8.0)
	137 mM Sodium Chloride
	10% (weight/ volume) Triton X-100;
	2mM EDTA

Running Buffer	25mM Tries-HCL (pH 8.3)
	192mM Glycine
	0.1% SDS
Transfer Buffer	25mM Tris-HCL
	192mM Glycine
	20% methanol
Sample buffer for the WB	50 ml 4X Tris/HCl/SDS (pH6.8)
	40 ml Glycerol
	6.2 g 2-ME (or DTT)
	2 mg Bromophenol Blue
	100 ml ddH$_2$O
Separation buffer:	375mM Tris-HCl (pH 8.8)
	0.1% SDS
Stacking buffer:	125mM Tris-HCl (pH 8.8)
	0.1% SDS
TBS (20X)	Tris-HCl – 24.6g, NaCl – 176g, in 1L ddH$_2$O
	pH adjusted to 8.0, diluted with ddH$_2$O to 1X
TTBS	TBS (1X) – 99.9%, Tween 20 – 0.1%
TAE Buffer (50X)	Tris base – 242mg, Acetic acid 57.1 ml,
	0.5M EDTA – 100 ml
Milk preparation	2.5g Fat dry milk in 50 ml TTBS
Ab + milk + TTBS	2 ml Fat dry milk,
	8 ml TTBS and
	Antibody in specific concentration

Electrophoresis

Gel Loading Buffer (10X)	250 mM Tris-HCl (pH 7.05)
	0,2% Bromophenol Blue
	40% Glycerol
TAE Buffer (50X)	Tris base – 242mg, Acetic acid 57.1 ml,
	0.5M EDTA – 100 ml in 1L ddH$_2$0 (pH 8.5)
Passive Lysis Buffer	Roche Diagnostics GmbH, Mannheim, Germany

LB Broth

1g of LB Broth was suspended in ddH$_2$O and then autoclaved at 121°C for 15 min. After cooling down to 45-50°C in a water bath ampicillin or kanamycin was added (conc. 1 mg/1 ml).

LB Agar

4.25g LB Agar was suspended in 125 ml of ddH$_2$O and then autoclaved at 121°C for 15 min. After cooling down to 45-50°C in a water bath ampicillin or kanamycin was added. The sterile LB Agar was dispensed into a sterile Petri dish. The hardened plates were stored at 4°C.

Antibodies

First Antibody

Anti-FLAG Ab (1:1000)	Sigma Aldrich Co., USA
Anti-KLF4 Ab (1:600)	H-180 polyclonal rabbit antibody, Santa Cruz Biotechnology, USA

Second Antibody

Anti-mouse Ab (1:2000)	Amersham Biotechnology, UK
Anti-goat Ab (1:3000)	Santa Cruz Biotechnology, USA

Accessories

Film for WB	Hyperfilm MP - Amersham Biosciences, UK
Membrane for the WB	Trans-Blot Transfer Medium Pure
Nitrocellulose Membrane (0.45µm)	Bio-Rad Laboratories, USA
3MM paper	Whatman Chromatography Paper, Fisher Scientific

Enzymes

Taq Polymerase (PCR Master Mix)	Promega Corporation, Madison, WI, USA (Cat. No. M7502)

Restriction Enzymes

Asp718	Roche Diagnostics GmbH

Asp 718 recognizes the same sequence as *Kpn 1*, but generates fragments with 5'-cohesive ends instead of the 3'-ends produced by *Kpn 1*.

```
5'...  G  G T A C  C  ...3'
3'...  C  C A T G  G  ...5'
```
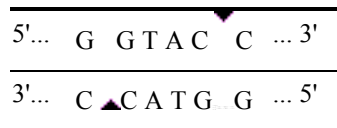
Not 1	Roche Diagnostics GmbH

Not 1 recognizes the sequence GC/GGCC/GC and generates fragments with 5'-cohesive termini. *Not 1* belongs to the class of "rare-cutter" enzymes. It is one of the two known enzymes recognizing an octameric sequence comprised solely of G and C residues.

```
5'...  G C  G G C C G C  ...3'
3'...  C G C C G G  C G  ...5'
```

Pme 1   New England Biolabs Ipswich, MA

```
5'...  G T T T   A A A C  ...3'

3'...  C A A A   T T T G  ...5'
```

Pac 1   New England Biolabs Ipswich, MA

```
5'...  T T A A T   T A A  ...3'

3'...  A A T   T A A T T  ...5'
```

Vectors

<u>pcDNA3.1</u>   Invitrogen Corporation – USA

pcDNA3.1 contain a human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells, a multiple cloning site and a neomycin and ampicillin resistance gene for selection of stable cell lines[148].

<u>pShuttle-CMV</u>   Stratagene – USA

The vector pShuttle-CMV contains am multiple cloning site sandwiched between the CMV promoter and the SV40 polyadenylation signal. It contains two stretches of sequence homology indicated as left arm/right arm homology and these are responsible for homologous recombination with pAdEasy[149].

<u>pAdEasy</u>   Stratagene – USA

The plasmid pAdEasy, containing most of the human adenovirus serotype 5 (Ad5) genome, is deleted for the genes E1 and E3. The removal of these two viral genes creates space for foreign DNA and eliminates self-replication capabilities. The E1 deletion renders the viruses defective for replication and incapable of producing

infectious viral particles in target cells (provided there is no complementation by the host cell); the E3 region encodes proteins involved in evading host immunity and is dispensable. The E1 gene, which is necessary for production of viral particles, is provided in *trans* by AD-293 cells. pAdEasy carries the ampicillin resistance gene, which is lost after recombination with a shuttle vector[149].

KLF4-pcDNA3.1	gift by Chichuan Tseng at Boston Medical College
KLF4-FLAG-pcDNA3.1	
KLF4-FLAG-pShuttle-CMV	
KLF4-FLAG-pAdEasy	
GFP-pcDNA3.1	

Adenovirus

Ad-EGFP	Adenovirus vector encoding enhanced green fluorescent protein were generated with the use of the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA)
Ad-KLF4-FLAG	Adenovirus vector encoding FLAG tagged KLF4

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Bacteria (Competent Cells)

DH5 alpha   Invitrogen Corporation - USA

E. Coli DH5 alpha cells are used for amplifying the amount of plasmids. DH5 alpha have been made deficient in some genes, which protects foreign DNA. The endA1 mutation inactivates an intracellular endonuclease that degrades plasmid DNA in many miniprep methods. recA eliminates homologous recombination. This makes the strain somewhat sickly, but reduces deletion formation and plasmid multimerization[150].

BJ5183   Stratagene (Cat.No.200154)

BJ5183 electroporation competent cells are a recombination proficient bacterial strain. These cells supply the components necessary to execute the recombination event between a transfer vector containing the gene of interest and a vector containing the adenoviral genome, provided that appropriate regions of homology are shared between the two vectors.

Animals

Female athymic BALB/c nude mice were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH.

Kits

Apopladder Ex kit*	Takara Biochemicals, Shiga, Japan
Cell Death Detection Kit*	Roche Applied Science, Indianapolis, USA
ECL western blotting kit	Amersham
High Pure PCR Product Purification Kit	Roche Applied Science (Cat. No.1 732 668)
Plasmid Maxi Kit	QIAGEN Inc. – USA
Rapid DNA Ligation Kit	Boehringer, Mannheim, Germany
RC DC Protein Assay Kit 2 (Reagent A, B, S)	Bio Rad
Wizard plus minipreps	Promega Corporation, Madison, WI, USA
DNA Purification System	

Equipment

Balance Model AB104, Mettler-Toledo Inc., USA

Developer Kodak X-OMAT M35 Processor, Kodak, USA

Eppendorf centrifuge, Model 5415C

Microscope, Nikon

Optima XL-80K Ultracentrifuge, Beckman-Coulter, Inc., USA

Packard Spectra Count, Packard Bioscience Company USA

Power station for electrophoresis and blotting Bio-Rad Model 200/2.0 Power Supply

Shaker at R.T. Model 260300 F, Boekel Scientific, Feasterville, PA, USA

Shaker 4° C CMS, Fisher Scientific, USA

Shaker 37° C for amplifying the bacteria, Forma Scientific – Orbital Shaker

Cell Line and Culture Conditions

Human gastric adenocarcinoma cell lines AGS, HTB103, HTB135, N87, SNU-1, and TMK1 were purchased from the American Type Culture Collection (Manassas, VA) and SK-GT-5 was obtained from Dr. Gary K. Schwartz (Memorial Sloan-Kettering Cancer Center, New York, NY). FG cells and 293 cells were used for confirmation of protein expression and adenovirus construction respectively.
All cell lines were maintained in plastic flasks as adherent monolayer in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids, L-glutamine, and vitamin solution in a humidified atmosphere of 95% air- 5% CO_2 at 37°C.

AGS is a poorly differentiated gastric adenocarcinoma cell line from fragments of a tumor resected from the stomach of a patient who had received no prior therapy. The cell line is 54 years old and the cell line is tumorigenic in athymic BALB/c mice. Karyotype: modal number = 47; range = 39 to 92.

NCI-N87 is a gastric carcinoma cell line derived in 1976 by A. Gazdar and associates at the National Cancer Institute from a liver metastasis of a well-differentiated carcinoma of the stomach taken prior to cytotoxic therapy. The tumor was passaged as a xenograft in athymic nude mice for three passages before the cell line was established. The cell line derives from a female organism and is tumorigenic in athymic nude mice. Karyotype: near diploid; DM were present in 64% of cells examined. BCI- N87 cells were minimally positive for vasoactive intestinal peptide (VIP) receptors and lacked gastrin receptors. They expressed receptors for muscarinic cholinergic agents.
No evidence of amplification or rearrangements was noted with the N-Myc, L-myc, d-

cis, IGF-2, or gastrin releasing peptide, they grow as an adherent monolayer of tightly knit epithelial cells[151].

The **SK-GT5** cell line was established from a primary adenocarcinoma of the gastro esophageal junction[152]. The cell line derives from a 67-year-old male. Karyotype: modal number 63. The doubling time averages (amounts) 41h. The cell line is proved to be tumorigenic[153].

AD-293 cell line (Stratagene) is a derivative of the commonly used HEK293 cell line, with improved cell adherence and plaque formation properties. HEK293 cells are human embryonic kidney cells transformed by sheared adenovirus type 5 DNA. AD-293 cells, like HEK293 cells, produce the adenovirus E1 gene in *trans,* allowing the production of infectious virus particles when cells are transfected with E1-deleted adenovirus vectors such as the pAdEasy-1 vector. The Ad5 insert was cloned and sequenced, and it was determined that a collinear segment from nucleotide 1 to 4344 is integrated into chromosome 19 (19q13.2)[154]. AD-293 is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. Cells have been passaged when the culture was at ≤50% confluency.

Subculturing Protocol/Hemocytometer Counting

<u>Subculturing Protocol</u> (splitting cells to lower confluency, cell counting, transfer into Petri dishes):
Old media was removed from the flask and washed with 5 ml cold PBS. PBS was removed and 3 ml of trypsin was added. The flask was placed back into the incubator until cells were detached. Then 3 ml of MEM was added to the cell suspension.
A few drops of the cell suspension were aseptically removed and were loaded in both chambers of a Neubauer type hemocytometer. Cells were counted with a compound microscope with a 10x ocular and 10x objective (total magnification 100x), so that one large square of the hemocytometer (1 mm x 1 mm) would fill the field. Each of the four large corner squares of a Neubauer type hemocytometer was counted. Each large square measured 1 x 1 mm and was 0.1 mm deep. Hence, each large square

had a volume of 0.1 mm^3 or 0.001 cm^3 or 0.0001 ml (10^{-4} ml). Hence the number of cells/ml of sample was calculated as follows:

$$\text{Conc. Cells/ml} = \frac{\text{total \# of cells counted} \times \text{dilution} \times 10^4}{\text{number of squares counted}}$$

DNA Precipitation

The DNA was precipitated to remove enzymes and buffer, kill bacteria and enhance transfection efficiency.

20 μl of KLF4-FLAG-pAdEasy digested with Pac 1 was combined with 150μl of ddH2O. 17μl 3M NaAc (10% of the total volume) was added. Then the DNA was precipitated with ethanol (100%) (2 x of total volume). The tube was stored for 1h at –70°C and was thereupon centrifuged for 5 min. Subsequently the salt was washed out with ethanol (70%). After the second centrifugation the supernatant was sucked off and adding 50μl ddH2O diluted the DNA.

PCR
Introduction:
The polymerase chain reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA. A PCR cycle consists of three steps:

1) Strand separation (Denaturation). The two strands of the parent DNA molecule are separated by heating the solution to 95°C for 15 seconds
2) Hybridization of primers (Annealing). The solution is then abruptly cooled down to 54°C to allow each primer to hybridize to a DNA strand. One primer hybridizes to the 3'-end of the target on one strand, and the other primer hybridizes to the 3'-end on the complementary target strand.
3) DNA synthesis (Extension). The solution is then heated to 72°C, the optimal temperature for Taq DNA polymerase. This heat-stable polymerase comes from Thermus aquaticus, a thermophilic bacterium that lives in hot springs. The

polymerase elongates both primers in the direction of the target sequence because DNA synthesis is in the 5'- to 3' direction. Subsequent cycles will amplify the target sequence exponentially while the template DNA is amplified only arithmetically. Ideally, after n cycles, this sequence is amplified 2^{n-fold}. Normally 20 to 30 cycles are carried out. The amplification is a billion fold after 30 cycles.

To construct the FLAG-tagged KLF4 expression vector PCR was carried out under following conditions (Figure 8)

	KLF4 wild type	Control
ddH$_2$O 22.5µl	x	23ul
PCR reaction mix 25µl	x	x
PCR primer 2.0µl	x	x
Template DNA 0.5µl	x	-

Figure 8

The reaction was overlaid with mineral oil to prevent evaporation. PCR was carried out in an automated thermal cycler for 25 cycles under following conditions (Figure9):

Initial Denaturation	94°C	4 min.	

Denaturation	94°C	45 sec.	
Annealing	62°C	45 sec.	CYCLE 25x
Extension	72°C	45 sec.	

Extension	72°C	7 min.
Store	4°C	∞

Figure 9

Subcloning/Ligation/Transformation and Cotransformation

The sense human FLAG tagged KLF4 (cDNA; consisting of the full-length coding sequence) was subcloned into pcDNA3.1 and pShuttle-CMV in frame. In a last step cotransformation of KLF4-FLAG-pShuttleCMV and pAdEasy was performed to allow recombination (Figure 10).

Enzymatic digestion via restriction endonuclease

	PCR-product	KLF4-FLAG-pcDNA3.1 (Fig. 16/18)	KLF4-FLAG-pshuttle-CMV (Fig. 19)	KLF4-FLAG-pshuttle-CMV (Fig. 22)	KLF4-FLAG-pAdEasy (Fig. 24)	KLF4-FLAG-pAdEasy (Fig. 25)	KLF4-FLAG-pAdEasy (Fig. 27)
DNA	20µl	1,5µl/10µl	3µl	3µg (20,56µl)	4µl	1,5µl	6µg (24,69µl)
Buffer B (10x)	4µl	3µl	3µl	—	—	3µl	—
Buffer 4	—	—	—	4µl	—	—	—
Buffer 1	—	—	—	—	2µl	—	4 µl
BSA	—	—	—	0,4µl	—	—	—
Asp718	1,5µl	1,0µl	0,5µl	—	—	0,5µl	—
Not1	1,0µl	1,5µl	0,75µl	—	—	0,75µl	—
Pme 1	—	—	—	1.5µl	—	—	—
Pac 1	—	—	—	—	0,5µl	—	2,0µl
ddH₂0	13,5µl	23µl/14,5µl	22.75µl	13,56µl	13,5µl	24,25µl	9,3 µl
Phosphatase	—	—	—	2µl	—	—	—
Buffer (10X)	—	—	—	4,2µl	—	—	—

Figure 10. Digestion was carried out at 37°C water bath for 1h.

Agarose gel electrophoresis

Agarose gel electrophoresis was employed to check the progression of restriction enzyme digestion, to quickly determine the yield and purity of DNA isolation or PCR reaction, and to size fractionate DNA molecules, which then could be eluted from the gel using a DNA Purification System as to manufacture's instructions. After electrophoresis, the gel was placed on a UV light box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern was taken.

Gel preparation (1% Agarose Gel)

Agarose, ddH$_2$0 and 10x TAE buffer was combined in a Erlenmeyer flask, and heated in a microwave for 2 min. until the agarose was dissolved. Ethidium bromide was added and the Gel was poured in a form with casting combs in place. After solidification of the gel 1x TAE electrophoresis buffer

Agarose	1.5 g
10X TAE	15 ml
ddH$_2$O	142.5 ml
EtBr (5 mg/ml)	25 µl
Total volume	150 ml

was added to the reservoir. Each DNA sample was combined with loading dye. A 1 kb DNA ladder (5µl/lane) was added to determine the size of the specific DNA bands.

Electrophoresis was carried out at 50 V until the required separation has been achieved. The band of interest was excised with a sterile razor blade and then eluted from agarose using the High Pure PCR Product Purification Kit according to the manufacturer's instructions. Pre-weighing a sterile centrifuge tube, then re-weighing with gel piece inside determined the excised gel piece weight. 300 ml of binding buffer was added for each 100 mg of agarose gel slice. DNA was released from the gel by placing the centrifuge tube into a 56 °C water bath interrupted by brief vortexing every 2 minutes. After the gel was completely dissolved, 150 ml isopropanol was added for every 100 mg of agarose gel previously calculated. After vortexing the solution was transferred into a High Pure filter tube and inserted into a Collection tube. The sample was centrifuged for one minute at maximum speed and room temperature. The flow through was discarded and 500 ml of wash buffer added above the filter. Once again the sample was centrifuged at maximum speed for one minute before repeating the wash with 200 ml of wash buffer. The filter was then

transferred to a new sterile micro centrifuge tube and 30 ml of Elution Buffer was added above the filter. The flow through was the resulting purified DNA.

Ligation

Ligation was performed using the Rapid DNA Ligation Kit. pcDNA3.1-/pshuttle-CMV-vector was combined with a 3-fold molar excess of insert and the volume was adjusted to 10µl with ddH$_2$O. 10µl of 2x Quick Ligation Buffer and 1µl of T4 DNA Ligase was added. Following briefly centrifugation the reaction mix was incubated at room temperature for 5 minutes. Until the transformation was carried out the reaction mix was chilled on ice (Figure 11).

	KLF4-FLAG-pcDNA3.1	KLF4-FLAG-pShuttle-CMV
pcDNA3.1	3µl	—
pShuttle-CMV	—	3µl
KLF4-FLAG	7µl	7µl
Ligase	1µl	1µl
2X Buffer	10µl	10µl

Figure 11

Transformation/Cotransformation

The newly ligated plasmids were then transformed into DH5alpha competent cells by gently mixing the plasmid and thawed competent cells and placing them on ice for 30 minutes.

For cotransformation pAdEasy-1 vector and the linearized pShuttle-CMV-vector containing the gene of interest were gently mixed with BJ5183 cells.

Subsequently the solution was heat shocked for 90 seconds at 42°C and directly placed back on ice for 3min. 900 ml of LB Broth (not containing antibiotics) was then added to the mixture and put into a shaker at 37°C for 45 minutes. The solution was then centrifuged at maximum speed for 1min and pipetted onto an LB Agar plate containing ampicillin (pcDNA3.1) or kanamycin (pShuttle-CMV/cotransformation). A sterile loop was then used to equally distribute the cells on the plate before being placed in an incubator for 12-16 hours. Colonies were then plucked off the plate and grown up in 5 ml LB broth with ampicillin/kanamycin respectively for an additional 12-16 hours. The plasmid from these bacteria were then isolated and purified using the Wizard Plus Minipreps DNA purification system:

Centrifuging at 1,400-x g for 10 minutes pelleted cells. Following aspiration of the supernatant, the cells were resuspended in 400 ml of the Cell Resuspension Solution. 400 ml of Cell Lysis Solution was added and the tube was inverted four times to mix. 400 ml of Neutralization Solution was then added and the tubes centrifuged for 5 min at 10,000 x g. The supernatant was then suspended in 1 ml of resin and placed in a vacuum column with filter at the bottom. The filter was then washed twice by filling the column with Column Wash Solution. The filter was transferred to a sterile microcentrifuge tube and 50 ml of nuclease-Free water was added to the top of the filter and the apparatus was centrifuged for 20 sec at 10,000 x g to elute the DNA. The resulting DNA was cut with restriction enzymes and separated on an agarose gel to check for the proper clone.

The proper bacterial strain containing the proper clone was stored in 50% Glycerol at –80°C. Then the recombinant plasmid was amplified in a larger scale and purified using the QIAGEN Plasmid Maxi Kit according to the manufacturer's instructions.

Sequencing

Material for Sequencing was send to SeqWright, Inc. Houston, TX, USA
Following vectors containing the KLF4-FLAG gene were send for sequencing:

- KLF4-FLAG-pcDNA3.1
- KLF4-FLAG-pAdEasy

Primer:

pcDNA3.1:

- T7 forward primer: 5'-TAA-TAC-GAC-TCA-CTA-TAG-GG-3'
- BGH reverse primer: 5'-TAGAAGGCACAGTCGAGG-3'

pAdEasy:
- CMV forward: 5'-CGCAAATGGGCGGTAGGCGTG-3'
- LF down reverse primer: 5'-GAGGTAGGGGCGCCAGGTTG-3'

Alignment with the cDNA sequence (AF105036) using BLAST was carried out to confirm the right clone.

Transfection

Plasmids: pcDNA3.1, pShuttle-CMV, KLF4-pcDNA3.1, KLF4-FLAG-pcDNA3.1, KLF4-FLAG-pShuttle-CMV, KLF4-FLAG-pAdEasy, GFP-pcDNA3.1 were transfected at 80% confluency of the tumor cells using Lipofectamine as to manufacture's instructions (Figure 12).

Adenovirus: GT5, N87, and AGS cells were grown up until reaching 80% of confluency.
To determine the transfection efficiency cells were transfected with Ad-GFP in different multiple of infection (MOI's).

To investigate the influence of KLF4 on the tumor cells GT5, N87, and AGS were transfected with Ad-GFP or Ad-KLF4 respectively with a multiple of infection (MOI) of 40. For the Animal experiment GT5 cells were grown up in flasks up until reaching 80% confluency. The cells were transfected with Ad-GFP or Ad-KLF4 respectively with a multiple of infection (MOI) of 40. 12h after transfection $1x\ 10^6$ GT5 cells were injected in the subcutaneous fat tissue of the mouse (Figure 12).

	transfected cell line	amount of DNA/well	amount of Lipofectamine/ well	MOI (multiple of infection)	plate
KLF4-FLAG-pcDNA3.1	COS-1	10µl	5µl	—	6-well plate
KLF4-FLAG-pShuttle CMV	COS1	2µg	6µg	—	6-well plate
KLF4-FLAG-pAdEasy	293	1,5µg	4,5µl	—	6-well plate
Ad-GFP	AGS N87 GT5	—	—	30-60	6-well plate Petri dish
Ad-KLF4-FLAG	AGS N87 GT5 AD-293	—	—	30-60	6-well plate Petri dish

Figure 12

Determination of Transfection efficiency

Transfection efficiency was determined transfecting the cells with Ad-GFP in different MOI's. Cells were then inspected under UV- and bright light under the microscope. Hence it was possible to calculate the percentage of infected cells by forming the fraction of cells counted under UV light to cells counted under bright light.

GT5, N87 and AGS cell lines were plated due to their different growth in different concentrations:

GT 5: 0,5 x 10^6/well

N87:   0,6 x 10^6/well

AGS: 0,4 x 10^6/well

After 24 h of incubation at 37°C the cells were transfected with Ad-GFP (4 x 10^7 pfu/µl) in different MOI (multiple of infection) reaching from 30 to 60.

24h later the cells were counted under UV- and bright light.

Cell proliferation assay

AGS, N87 and SK-GT5 cells were seeded at 4 x 10^5 cells per well in 6-well culture plates. Twelve hours later, the cells were incubated for 2 hours at 37°C in serum-free medium or serum-free medium with Ad-KLF4 or Ad-EGFP at a multiplicity of infection (MOI) of 20. After being washed with serum-free medium, the transduced cells were replenished with DMEM and incubated for 1 to 4 days. The cell numbers were counted daily via the trypan blue exclusion method with a hemocytometer. Trypan blue is the most common stain used to distinguish viable cells from nonviable cells. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Only non-viable cells absorb the dye and appear blue and may also appear asymmetrical. Conversely, live, healthy cells appear round and refractile without absorbing the blue-colored dye.

Tumor growth and metastasis*

To prepare tumor cells for inoculation, cells in the exponential growth phase were harvested via brief exposure to a 0.25% trypsin/0.02% EDTA solution (w/v). Cell viability was determined using trypan blue exclusion, and only single-cell suspensions that were >95% viable were used. Tumor cells (1 x 10^6 cells per mouse) were then injected into either subcutis or stomach wall of nude mice. The animals were killed 60 days after the tumor cell injection or when they had become moribund. Next, the primary gastric tumors were harvested and weighed. Regional lymph nodes (at least five for each

mouse) were collected and examined for tumor metastasis by histopathology. Metastasis was expressed as percentage incidence using the following formula: metastasis incidence (%) = [mice with metastasis / total mice used] x 100; where metastasis were regional and/or distant. In addition, each mouse's liver was fixed in Bouin's solution for 24 hours to differentiate the neoplastic lesions from the organ parenchyma; metastases on the surface of liver were counted (double blinded) with the aid of a dissecting microscope[155].

Western Blot

Preparing the cell lysate

Protein isolates were prepared from human gastric cancer cell lines. After transfection with the proper plasmid cell lines were cultured for ca 18h to 24h in 6-well plates at 37°C upon reaching > 85% confluency, cells were washed twice with cold PBS. 250µl – 300µl of Lysis Buffer was added and the cells were left on the shaker for 20 minutes at 4°C. The lysate was then transferred into a sterile eppendorf tube and centrifuged at maximum speed (14000 rpm) for 20 minutes at 4 °C. Supernatant was then aliquoted and frozen for further use.

Determination of Protein concentration

Four dilutions of a protein standard containing from 0,125 mg/ml to about 1.0 mg/ml protein were prepared. Reagent A and S were mixed in a 100:2 ratio (stock solution). 25 µl of the stock solution was added with 5 µl of the standard/sample and 200 µl of Reagent B into one well of a 96 well plate. After 15 minutes absorbencies were read at 750 nm with a spectrophotometer. A standard curve was prepared and concentrations of the samples were calculated using Excel.

SDS- polyacrylamide gel electrophoresis of protein

10% SDS- polyacrylamide gel (SDS-PAGE)

Separation Gel:	8.3 ml	ddH$_2$O
	5.0 ml	4x separation buffer
	6,7 ml	30% acrylamid
	240 µl	APS
	24 µl	TEMED
Stacking Gel	6.5 ml	ddH$_2$O
	2.5 ml	4x stacking buffer
	1 ml	30% acrylamid
	240 µl	APS
	24 µl	TEMED

25 µg of the peptide sample was mixed with sample buffer and heated at 100°C for 5 minutes. After sitting on ice for 5 minutes the sample was centrifuged for 1 minute and then loaded into the well. A protein ladder was added to determine the size of the specific protein. Protein samples were separated with running buffer on a 10% SDS gel at a constant voltage of 75 V through the stacking gel and 125 V through the remainder of the gel at 4°C. Followed by electro transfer of peptide from SDS-PAGE to nitrocellulose induced with a constant voltage of 50 V at 4°C for 1.5 h with transfer buffer. The membrane was blocked by incubating with TTBS containing 5% non-fat dry milk for 1h, then incubated with first antibody (Anti- FLAG (conc. 1:1000)/ Anti- KLF4 (conc. 1:500)) for 1h at 4°C then for half an hour at R.T. on a shaker. After incubation with the first antibody the membrane was washed with TTBS (5 times short wash followed by 3 times for 10 minutes on a shaker at room temperature). Second anti-mouse (conc. 1:2000)/ anti-gout (conc. 1:3000) antibody was added overnight. After washing a second time with TTBS (5 times short washing then 3 times for 10 minutes) and in a final step for 10 minutes with TBS on a shaker at room temperature the probe proteins were detected using the Amersham enhanced chemiluminescence system and mixing equal parts of both solutions before passing the membrane through the combined solution for one minute (ECL Western Blotting Detecting Agents). Film development was carried out using UV film, fixer and

developer. The film was exposed to the membrane for increments of time 30s, 1min, 5min, and 10min.

*Fresh gastric cancer and corresponding noncancerous gastric tissues were obtained from patients who underwent gastrectomy at M.D. Anderson Cancer Center. The cancerous and noncancerous portions were macroscopically identified and excised by experienced pathologists and further confirmed by histopathologic examination. Additionally, whole cell lysates were prepared from human normal and gastric and gastric tumor tissue specimens or cell cultures. Four paired normal gastric and gastric tumor tissue specimens were selected from the patients with known expression levels of KLF4 as confirmed by immunostaining as well as a similar percentage of tumor epithelial cells relative to stromal cells.

Protein sample loading was monitored by incubating the same membrane filter with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody[25].

Human tissue specimens and immunohistochemistry*

86 human gastric cancer tissue specimens were used and patient characteristics are summarized in Figure 13. Also 51 lymph node metastasis specimens and 60 normal gastric tissue specimens obtained from patients without gastric cancer have been included in this study[156]. Sections (5-µm thick) of formalin-fixed, paraffin-embedded tumor specimens were prepared and processed[156]. Standard immunostaining procedure was done using a rabbit polyclonal antibody against human KLF4 (clone H180, 1:200 dilution). Reddish-brown precipitate in the nuclei and cytoplasm indicated a positive reaction. Depending on the percentage of positive cells and staining intensity, KLF4 staining was classified into three groups: negative, weak, and strong expression[156].

Patient characteristics and KLF4 expression*

Characteristic	Total (n = 86)	KLF4 staining			P
		Negative (%), n = 27	Weak (%), n = 47	Strong (%), n = 12	
Sex					
Men	56	20 (35.7)	29 (51.7)	7 (12.5)	0.487
Women	30	7 (23.3)	18 (60.0)	5 (16.7)	
Age (years)					
Mean (SD)	61.8 (14.0)	57.4 (15.0)	62.6 (13.5)	68.4 (11.3)	0.463
Pathology type					
Papillary	12	1 (8.3)	9 (75.0)	2 (16.7)	0.358
Tubular	28	7 (25.0)	15 (53.6)	6 (21.4)	
Diffuse	8	2 (25.0)	4 (50.0)	2 (25.0)	
Mucinous	5	2 (40.0)	2 (40.0)	1 (20.0)	
Signet ring	21	9 (42.9)	11 (52.3)	1 (4.8)	
Mixed	12	6 (50.0)	6 (50.0)	0 (0.0)	
Stage					
I	14	0 (0.0)	12 (85.7)	2 (14.3)	0.005
II	28	5 (17.9)	17 (60.7)	6 (21.4)	
III	30	15 (50.0)	11 (36.7)	4 (13.3)	
IV	14	7 (50.0)	7 (50.0)	0 (0.0)	
Residual disease					
R0	69	16 (23.2)	41 (59.4)	12 (17.4)	0.003
R1, R2	17	11 (64.7)	6 (35.3)	0 (0.0)	
Lauren's classification					
Intestinal	53	14 (26.4)	30 (56.6)	9 (16.9)	0.351
Diffuse	33	13 (39.4)	17 (51.5)	3 (9.1)	

Figure 13

Pearson's χ^2 test was done to determine the statistical significance of the relationship of KLF4 expression with various variables.

DNA ladder assay*

N87 and SK-GT-5 cells were seeded at 1 x 10^6 cells per well in 100-mm culture plates. Twelve hours later, the cells were incubated for 2 hours at 37°C in serum-free medium with Ad-KLF4 at MOI of 0, 10, or 20, and the total MOI in each group was adjusted with Ad-EGFP to equal of 20. After being washed with a serum-free medium, the transduced cells were replenished with complete minimal essential medium and incubated for 36 hours. Next, DNA was isolated and a DNA ladder assay was done with ApopLadder Ex kit according to the manufacturer's instructions. Extracted DNA was then subjected to electrophoresis on a 1,5% agarose gel and detected by SYBR Green staining.

Detection of apoptosis *in situ*

N87 and SK-GT5 cells were seeded at 0,5 to 1 x 10^5 cells per well in eight- chamber culture slides. Twelve hours later, the cells were infected with Ad-KLF4 at a MOI o 20. Thirty-six hours after infection, the cells were processed and apoptotic cells were detected *in situ* with the *In situ* Cell Death Detection Kit according to the manufacturer's instructions. A positive control slide known to express the target antigen and a negative control slide without the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) reaction mixture added were included for each staining procedure. Brown staining of nuclei was interpreted as positive immunoreactivity.

Northern blot analysis*

Total RNA was extracted using TRIzol reagent (Invitrogen, San Diego, CA), and standard Northern blotting was performed[155]. For detecting KLF4, [^{32}P]-dCTP-labeled *KLF4* cDNA probe was applied, and [^{32}P]-dCTP-labeled *GAPDH* cDNA probe was used to monitor RNA sample loading.

Southern blot analysis*

DNA samples were isolated using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Extraction of genomic DNA from cell lines was conducted according to standard procedures. Southern blot analysis was done using 8 μg of genomic DNA digested with *Eco*RI or *Eco*RI plus *Nco* I. A full-length KLF4 cDNA was used as the probe.

Methylation-specific PCR*

Methylation-specific PCR was done using genomic DNA, which was modified with bisulfite according to manufacturer's instruction (EN DNA Methylation Kit, Orange, CA). For detecting unmethylated DNA, the forward primer was 5'-gtttttatattaatgaggtaggtgaggtg-3' and the reverse primer was 5'-aaacaaaaacaaaaaatcaaaaaca-3', which were designed to amplify a 118-bp sequence between nucleotides 156 and 39 relative to the translation initiation site of the human KLF4 exon 1 region. For detecting methylated DNA, the forward primer was 5'-tttatattaatgaggtaggtgaggc-3' and the reverse primer was 5'-gaaaacaaaaaaatcaaaaacgac-3V, which were designed to amplify a 111-bp sequence between nucleotides 153 and 43 relative to the translation initiation site of the human KLF4 exon 1 region. The negative control was water. The positive control for the methylation-specific reaction was normal human genomic DNA from Promega (Madison, WI), which was treated *in vitro* with *SssI* methylase (New England Biolabs, Beverly, MA). Each PCR reaction of 50 μL consisted of 40 ng DNA and 200 nmol/L each of the forward and reverse primers and 0.5 μL HotStart Taq enzyme (Qiagen). Each PCR reaction was hot started at 95jC for 15 minutes and then amplified for 35 cycles (94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds). PCR products were visualized on a 2% agarose gel stained with ethidium bromide. The products were also directly sequenced to determine the locations of CpG methylation.

Statistics*

The two-tailed χ2 test was done to determine the significance of the difference between the covariates. The Kaplan-Meier method was used to calculate survival durations, and the log-rank test was used to compare the cumulative survival durations in the patient groups. Furthermore, the Cox proportional hazards model was used to compute multivariate hazards ratios for the study variables; the level of KLF4 expression, age, sex, Lauren's histology type, stage (American Joint Committee on Cancer), and completeness of surgical resection (R0 versus R1 and R2) were included in the model. The SPSS software program (version 11.05; SPSS, Inc., Chicago, IL) was used for the analyses. For *in vitro* and *in vivo* studies, each experiment was done independently at least twice with similar results; one representative experiment was presented. The significance of the *in vitro* data was determined using Student's *t* test (two tailed), whereas that of the *in vivo* data was determined using the two-tailed Mann-Whitney *U* test. In all of the tests, a *P* < 0.05 was defined as statistically significant.

Construction of Ad-KLF4-FLAG

Construction of FLAG-tagged KLF4 expression vector

The FLAG-tagged KLF4 vector was constructed by PCR amplification of the DNA using the pcDNA3.1 vector containing the full-length KLF4 cDNA as template. The FLAG epitope was included on the N-terminus of KLF to generate a FLAG fusion Protein. To subclone the KLF-FLAG gene into vectors containing compatible restriction sites synthetic oligonucleotides (primers) incorporating new unique restriction sites for *Asp718* and *Not1* were used to amplify the target gene. Following primers were used:

Up stream primer: 5' – Gat aa**g gta cc** ATG GAT TAC AAG GAT GAC GAC GAT AAG ggg gct gtc agc gac gcg ctg ctc – 3' (61bp; in capital letters: FLAG sequence; *Asp718/Kpn1* restriction site **underlined**)

```
5'···  G G T A C  C  ··· 3'
3'···  C ▲C A T G G ··· 5'
```

Down stream primer: 5' – TCAAT **gcggccgc** ttaaaaatgcctcttcatgtgtaagg –3' (39bp; *Not1* restriction site **underlined**)

```
5'···  G C   G G C C G C  ··· 3'
3'···  C G C C G G ▲C G  ··· 5'
```
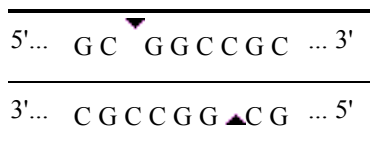
The PCR product was analysed by agarose gel electrophoresis to verify that the amplification has yielded the expected product (Figure 14).
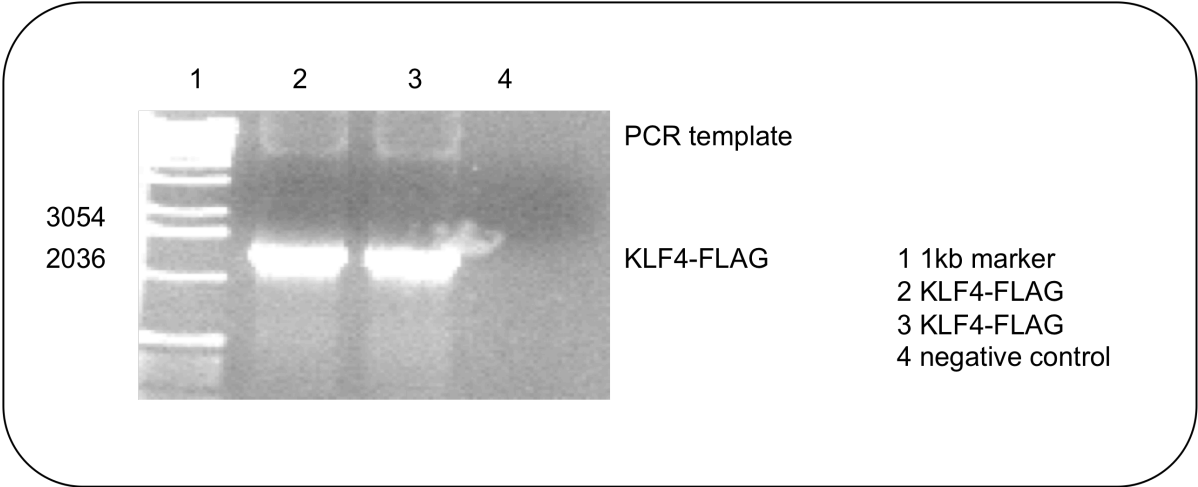
Figure 14

Subcloning FLAG-tagged KLF4 into pcDNA3.1

The amplified DNA fragment is purified out of the Gel using the DNA High Purification Kit and was subjected to enzymatic digestion at the new restriction sites for Asp718 and Not1 (Figure 15).
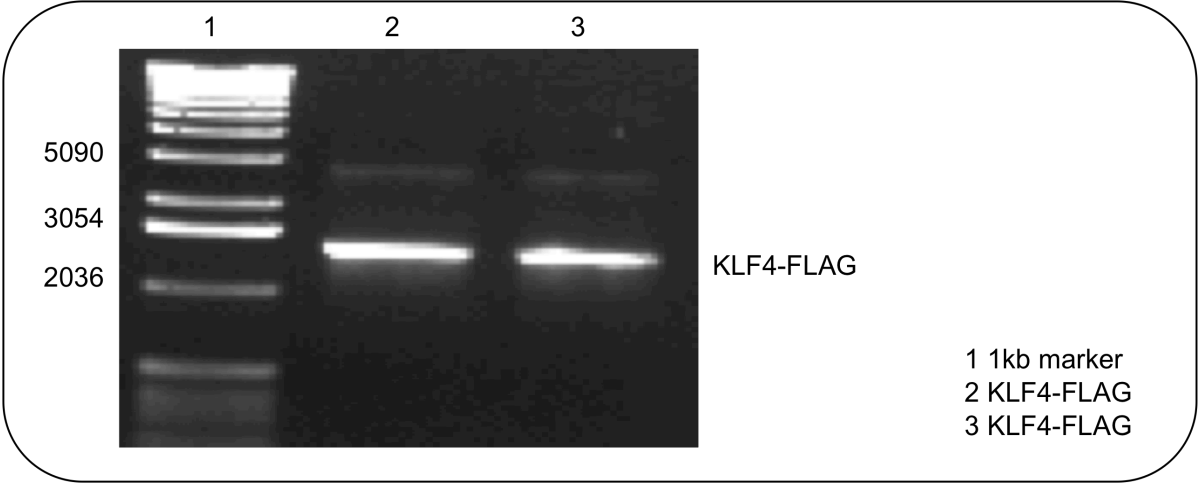
Figure 15

After re-purification, the insert was then ligated into the pcDNA3.1 vector and transformed in DH5alpha competent cells. The individual subclones were analysed by restriction endonuclease digestion (Figure 16).
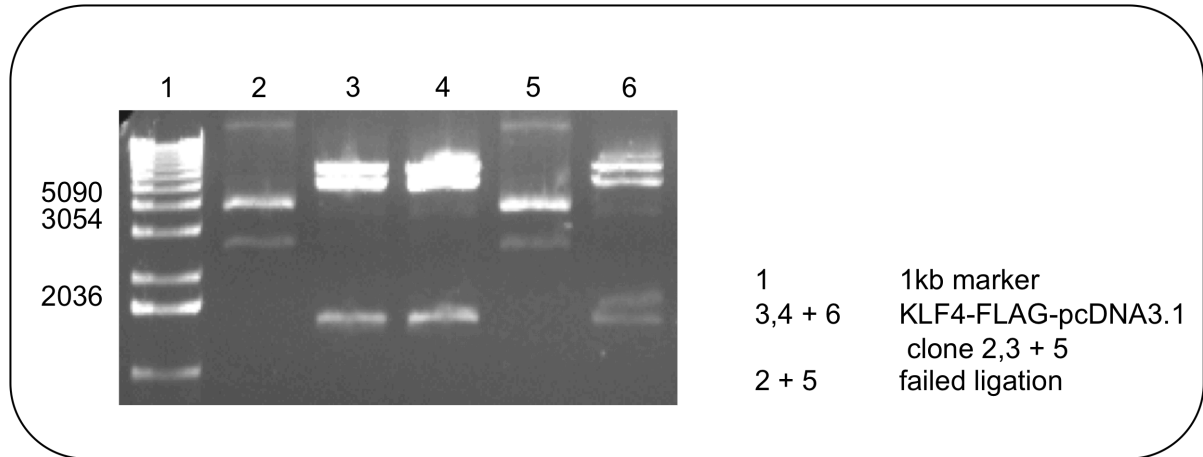
Figure 16

Western Blot analysis transfecting COS-1 cells with the KLF4-FLAG-pcDNA3.1 vector using Lipofectamine confirmed gene expression. Protein was extracted after 24h incubation time at 37°C. Protein concentration was measured and a standard WB was carried out using Anti-FLAG as 1st antibody and Anti-mouse as 2nd antibody (Figure 17).
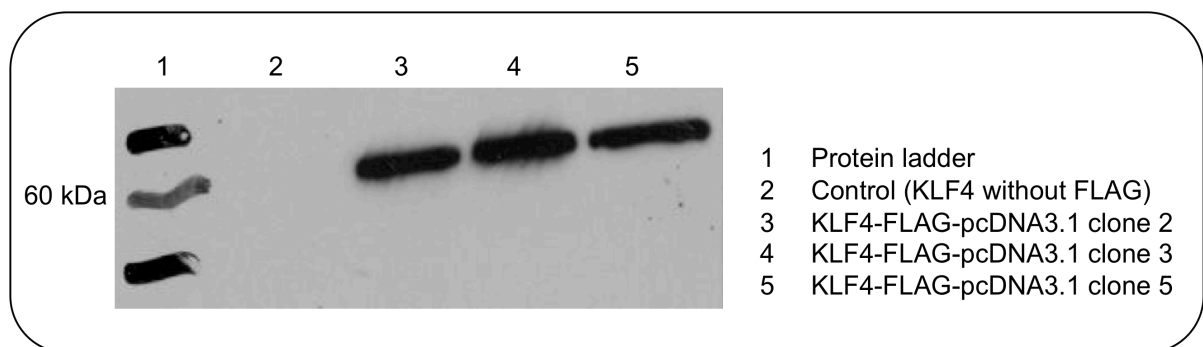
Figure 17

Clone 2, 3 and 5 were expressed in COS-1 cell line.

Sequencing using the T7 forward and BGH reverse primer was performed next.
Alignment with the cDNA sequence (AF 105036) using BLAST was carried out to
confirm the proper sequence.

Sequencing revealed for KLF4-FLAG-pcDNA3.1 clone 5 the right sequence; hence
clone 5 was amplified on a large scale using Plasmid Maxi Kit from QUIAGEN.

Subcloning of KLF4-FLAG into pShuttle-CMV

To subclone the KLF4-FLAG gene in pShuttle-CMV, KLF4-FLAG-pcDNA3.1 was
subjected to enzymatic digestion at the restriction sites for *Asp718* and *Not1* (Figure
18). The KLF4-FLAG band was excised and then eluted from agarose gel using the
High Pure PCR Product Purification Kit.

Figure 18

46
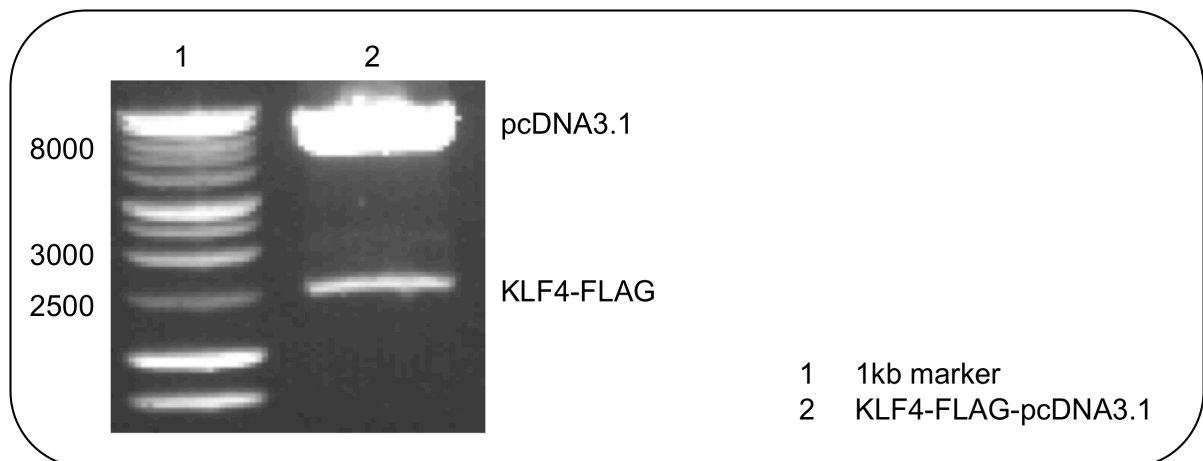
The purified DNA was then ligated into the pShuttle-CMV vector. After transformation in DH5alpha competent cells, individual subclones were subjected to enzymatic digestion at the restriction sites for Asp718 and Not1 (Figure 19).

1	1kb marker
2	KLF4-FLAG-pShuttle-CMV clone 1
3	KLF4-FLAG-pShuttle-CMV clone 2
4	KLF4-FLAG-pShuttle-CMV clone 3
5	KLF4-FLAG-pShuttle-CMV clone 4
6	KLF4-FLAG-pShuttle-CMV clone 5
7	KLF4-FLAG-pShuttle-CMV clone 6
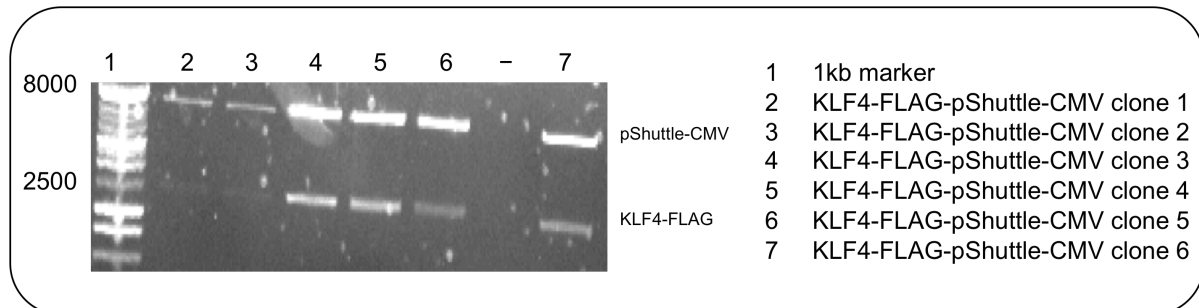
Figure 19

KLF4-FLAG-pShuttle-CMV clone 3 was chosen for further procedure. WB analysis confirmed the expression of KLF4 protein (Figure 20).

1   Protein ladder
2   KLF4-FLAG-pShuttle-CMV
3   (+) control KLF4-FLAG-pcDNA3.1
4   (−) control KLF4 without FLAG)
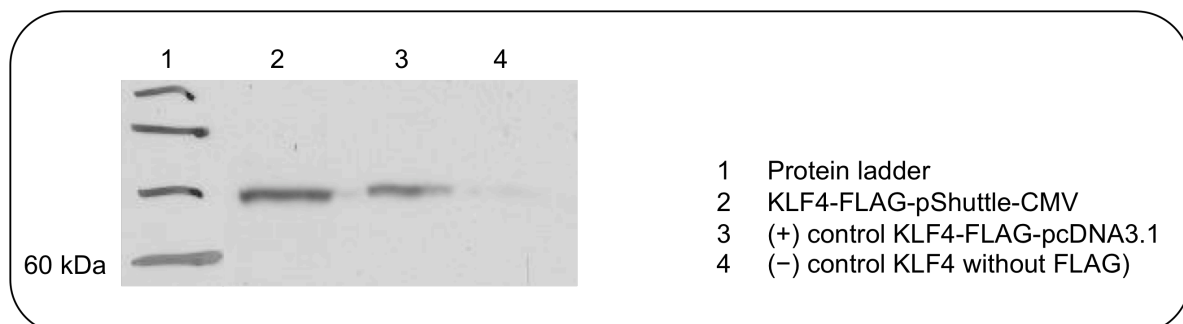
Figure 20

Cotransfection and homologous recombination of KLF4-FLAG-pshuttleCMV and pAdEasy

A schematic overview of the adenoviral vector system is shown in Figure 21 (Stratagene)

Cloning Gene of Interest
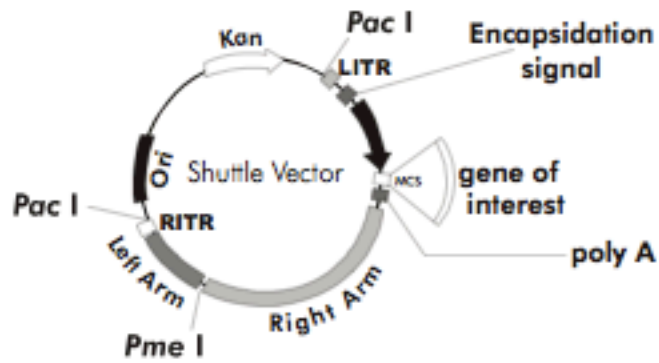
Homologous Recombination In vivo in Bacteria
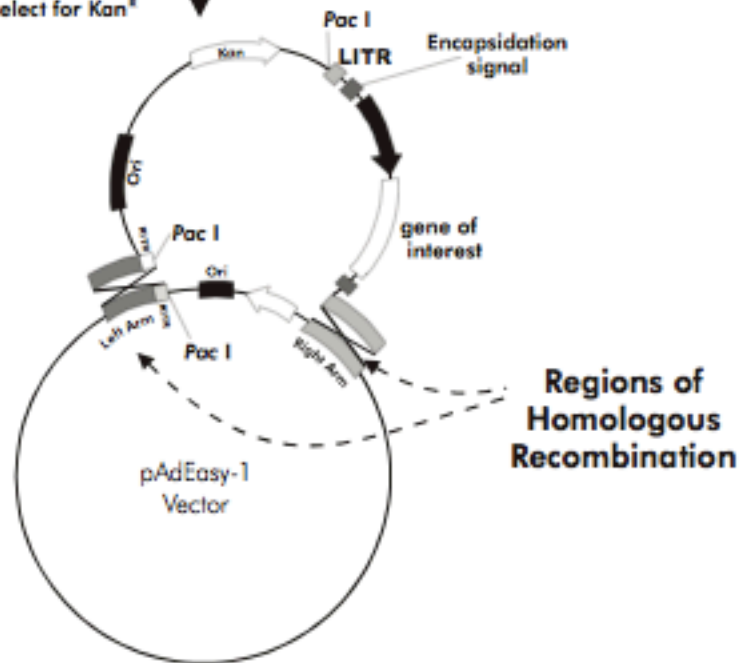
Virus Production in AD-293 Cells

Figure 21

To allow recombination between KLF4-FLAG-pShuttle-CMV vector and pAdEasy vector, KLF4-FLAG-pshuttle-CMV was linearized with *Pme1* for 1,5 h at 37°C (Figure 22). Once completed digestion with *Pme1* was confirmed, the enzyme was deactivated by heating the sample for 20 minutes at 65°C. Subsequently the DNA was treated with alkaline phosphatase for 1h at 37°C. Enzyme and buffer was removed using the High Pure PCR Product Purification Kit.

1 1kb marker
2 control: KLF4-FLAG-pShuttle-CMV uncut
3 KLF4-FLAG-pShuttle-CMV cut with *Pme1*
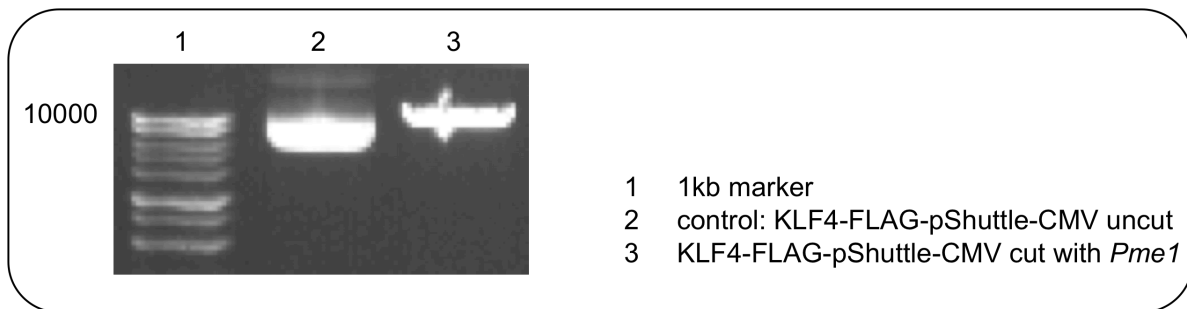
Figure 22

BJ5183 cells were cotransfected with the linearized shuttle vector containing the KLF4-FLAG gene (1µg) and the pAdEasy-1 vector (0,1µg). Like mentioned above only the BJ 5183 competent cells have the cellular components necessary to carry out recombination.

The transformants on the plates containing pAdEasy recombinants appeared as two populations: normal size and tiny size at an approximate ratio of 3:1. The tiny colonies were the potential recombinants and the normal-sized colonies were background from the shuttle vector.

20 clones of the smallest, well-isolated colonies were cultured in LB-broth containing kanamycin.

A miniprep DNA from the culture was prepared and examined for the right clone with following methods:

1) Electrophoresis with normal pAdEasy as a control
2) Enzymatic digestion with *Pac 1*
3) Enzymatic digestion with *Not1* and *Asp718*
4) Sequencing
5) WB analysis

1) Electrophoresis with normal pAdEasy as a control (Figure 23).
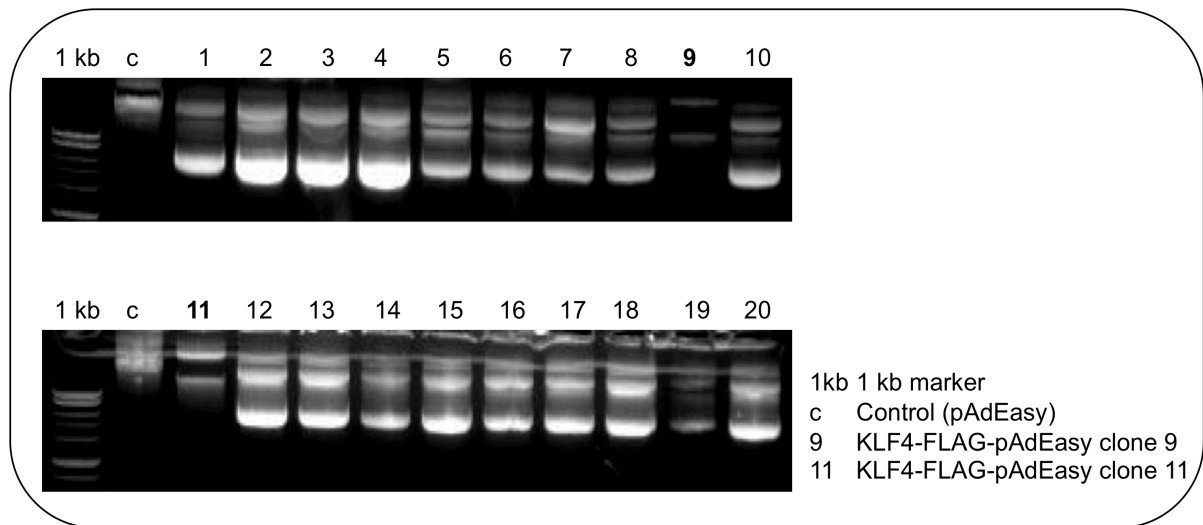
Figure 23

2) Enzymatic digestion with *Pac 1*

Restriction of recombinant KLF4-FLAG-pAdEasy with *Pac 1* yielded a large fragment of ≈ 30 kb, and a smaller fragment of either 3.0 kb, if recombination took place between the left arms or 4,5 kb, if recombination took place at the origins of replication (Figure 24).
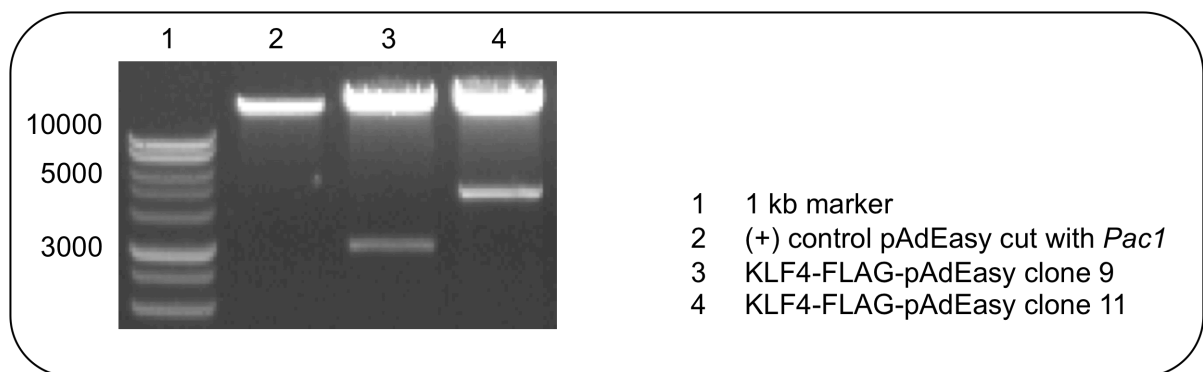

Figure 24

KLF4-FLAG-pAdEasy clone 9: recombination took place between the left arms

KLF4-FLAG-pAdEasy clone 11: recombination took place between the origins of replication.

3) Enzymatic digestion of KLF4-FLAG-pAdEasy with *Not 1* and *Asp 718* (Figure 25)

KLF4-FLAG

1	1 kb marker
2	(+) control KLF4-FLAG-pcDNA3.1
3	KLF4-FLAG-pAdEasy clone 9
4	KLF4-FLAG-pAdEasy clone 11
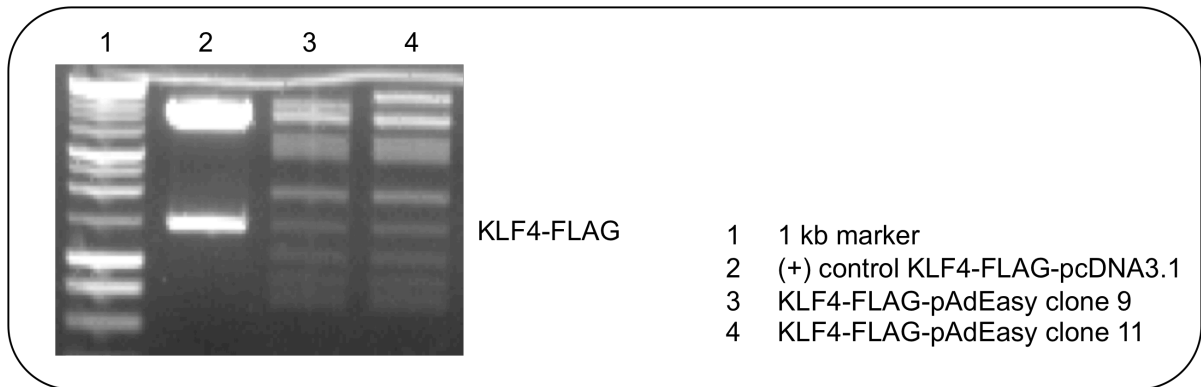
Figure 25

4) Sequencing:

Sequencing using the CMV forward and LF down primer (M.D. Anderson, Houston, USA) and alignment with the cDNA sequence (AF103056) using BLAST were carried out. Sequencing revealed for KLF4-FLAG-pAdEasy clone 9 the right sequence.

5) Western Blot was performed to confirm expression of the recombinant KLF-FLAG-pAdEasy (Figure 26)

60 kDa

1	Protein ladder
2	(+) control KLF4-FLAG-pcDNA3.1
3	KLF4-pAdEasy
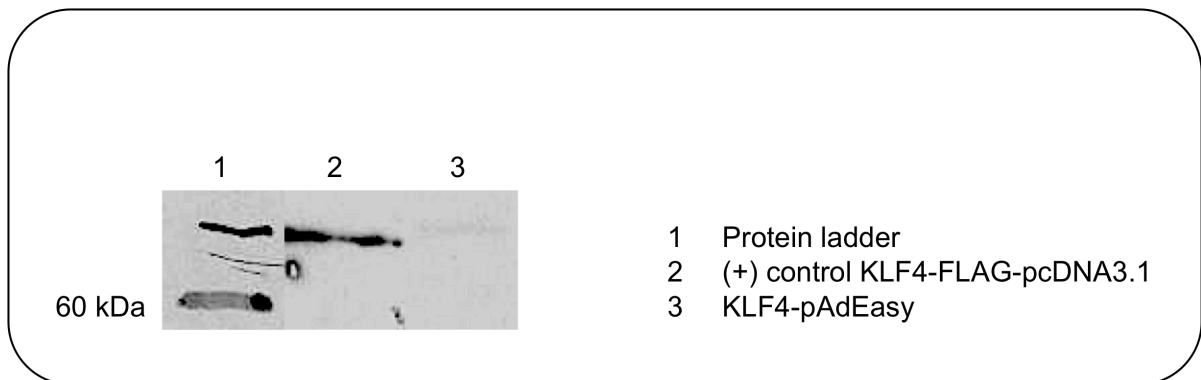
Figure 26

KLF4-FLAG-pAdEasy clone 9 was then transformed into DH5alpha competent cells to amplify the plasmid and yield higher concentrated DNA.

For confirmation KLF4-FLAG-pAdEasy clone 9 was subjected to enzymatic digestion with *Pac 1* (Figure 27).
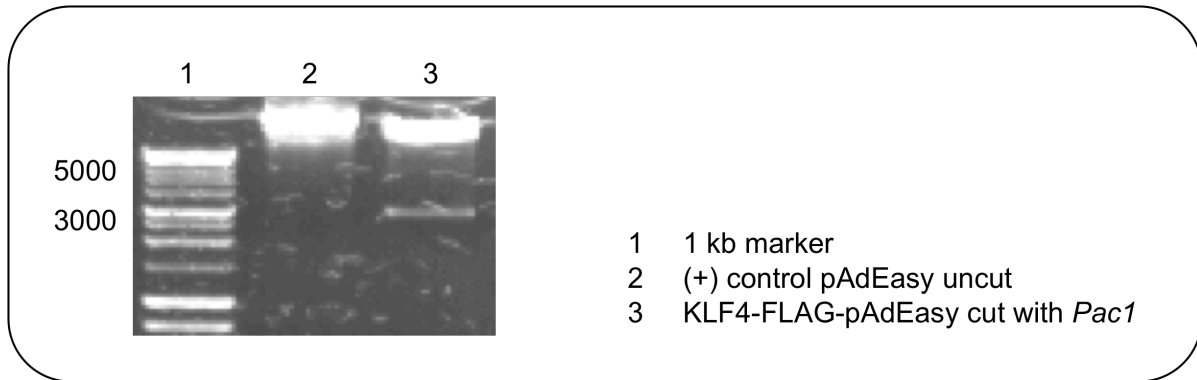
Figure 27

To infect 293 cells 6µg of KLF4-FLAG-pAdEasy clone 9 was linearized with *Pac 1* (Figure 28).
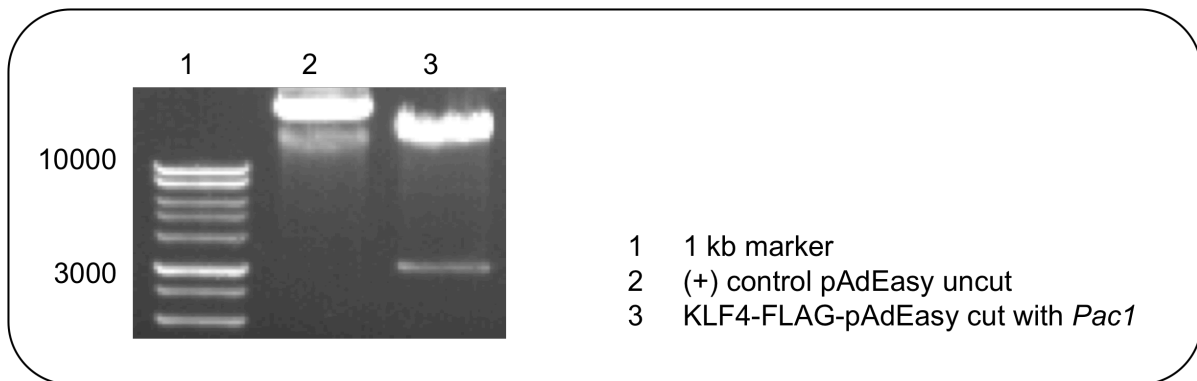
Figure 28

To remove buffer and ensure good transfection conditions the digested DNA was precipitated with ethanol.

A 6-well plate of 293 cells was transfected at 70% confluency using Lipofectamine. The transfected cells were incubated at 37°C.

To monitor the progress of adenoviral infection the phenotypic changes to infected cells were observed under a microscope. After around 9-10 days of continuously incubating the cells at 37°C the cells showed evidence of a cytopathic effect (CPE):

the cells rounded up and detached from the plate, and the nucleus occupied a major part of the cell due to the high level of virus production.

Three colonies in the 6-well plate could be located and were used to prepare the primary viral stocks. The growth medium (MEM) was carefully removed from the plates and 2ml of PBS was added in each of the 3 wells. The cells were mixed by gently pipetting until all cells were detached. The cell suspension was transferred to a 15 ml screw cap centrifuge tube and the cells were pelleted by low speed centrifugation for 5 min. at 2000 rpm. The cell lysate was stored at – 80°C. Western Blot was performed to confirm expression of the recombinant virus (clone #1, #2, #3) (Figure 29).
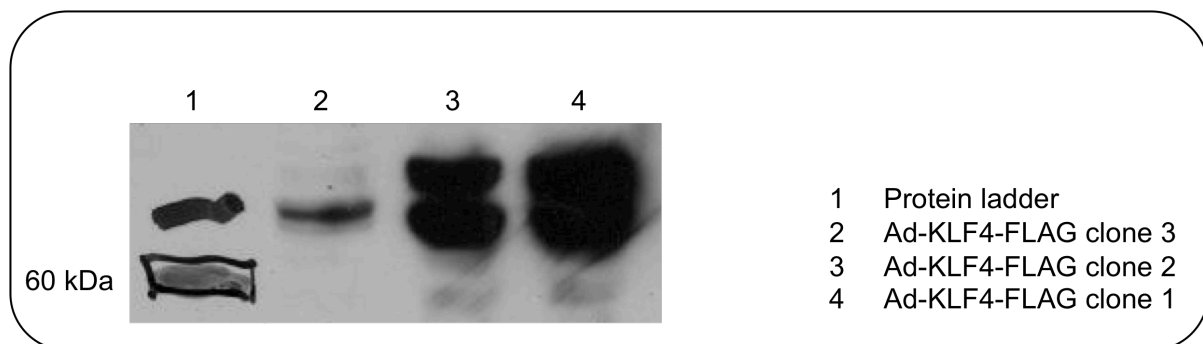
Figure 29

As reported by Chen et al.[188], two bands are observed. The predominant, higher molecular weight band presented KLF4.

All three recombinant viruses expressed the KLF4-FLAG gene. For the following experiments recombinant Ad KLF4-FLAG clone #3 was used.

Virus Amplification

To amplify the virus 293 cells were plated in 20 150mm dishes and were grown until reaching 70-80% confluency. Next 30mL of purified virus and 40mL of virus specific supernatant combined with 360mL complete media was added to the plates. The infected 293 cells were incubated at 37°C for 48h or until 90% of the cells were rounded but not detached. The supernatant was collected in a 50ml centrifuge tube and placed at –80°C (virus specific supernatant).

The remaining cells were collected centrifuged (at 2000 rpm for 5 min) and finally resuspended in 5ml of cold PBS and stored at –80°C. Once frozen the virus stock was subjected to 2 thaw and freeze cycles (-80°C to 37°C). Once thaw 10ml PBS was added and the virus stocks were again subjected a last thaw and freeze cycle.

Virus Purification

The thawed virus stock was centrifuged at 8000 rpm for 15 min. at 4°C.

Following caesium chloride solutions in different concentrations were prepared:

1,45g/ml (61g CsCl/100ml PBS)

1,35g/ml (48,6g CsCl/100ml PBS)

1,25g/ml (35g CsCl/100ml PBS)

Preparation Dialysis buffer (in1L of ddH$_2$O)

1M Tris-HCL – 10mL

Sucrose – 40g

0.5M EDTA – 2mL

Solution needed to be pre-cooled

Caesium chloride gradient centrifugation

	1,45g/ml CsCl	1,35g/ml CsCl	1,25g/ml CsCl	Virus solution	Centrifugation speed/time
1st gradient	3ml	—	3ml	5ml of virus stock	42000rpm 2h 4°C
2nd gradient	—	3ml	—	Virus layer from first gradient	62000rpm 16h 4°C

Figure 30

After collecting the virus layer from the second gradient the virus layer was placed in a membrane. The membrane containing the purified virus was slided into an Erlenmeyer flask containing 1l of dialysis buffer. The dialysis buffer was changed every 2 hours for 6 hours. The purified virus was aliquoted into tubes and stored at – 80°C.

<u>Virus Titration</u>

To determine the virus titer 293 cells were plated a 12-well plate (4x 10^5 cells/well).
The cells were incubated at 37°C for 24h. 30µl of the purified virus stock solution was
added to 270µl serum free media (10^{-1}) subsequently the sample was diluted to 10^{-2},
2 x 10^{-2}, 4 x 10^{-2}, 6 x 10^{-2}, 8 x 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}.
100µl of diluted virus solution was added to each well. The infected 293 cells were
cultured for 48h. They were then observed for cytopathic effect (CPE) under the
microscope. The virus titer was calculated using the dilution factor of the well in
which almost 100% of the 293 cells had apparent cytopathic effect.

$$\text{Virus titer} = \frac{(4\text{x}10^5)(+\text{dilution factor})(10\ \text{virus/cell})}{0.1\text{ml}}$$

It was assumed that one cell is infected by 10 viruses.

Ad-GFP: 4,0 x 10^7 pfu (plaque formation unit)/µl

Ad-KLF-FLAG: 2,0 x 10^6 pfu/µl

Determination of transfection efficiency

Transfection efficiency was determined transfecting the cells with Ad-EGFP (4 x 10^7
pfu/µl) in different MOI's reaching from 30 to 60. GT5, N87 and AGS cell lines were
plated due to their different growth in different 24h after transfection the cells were
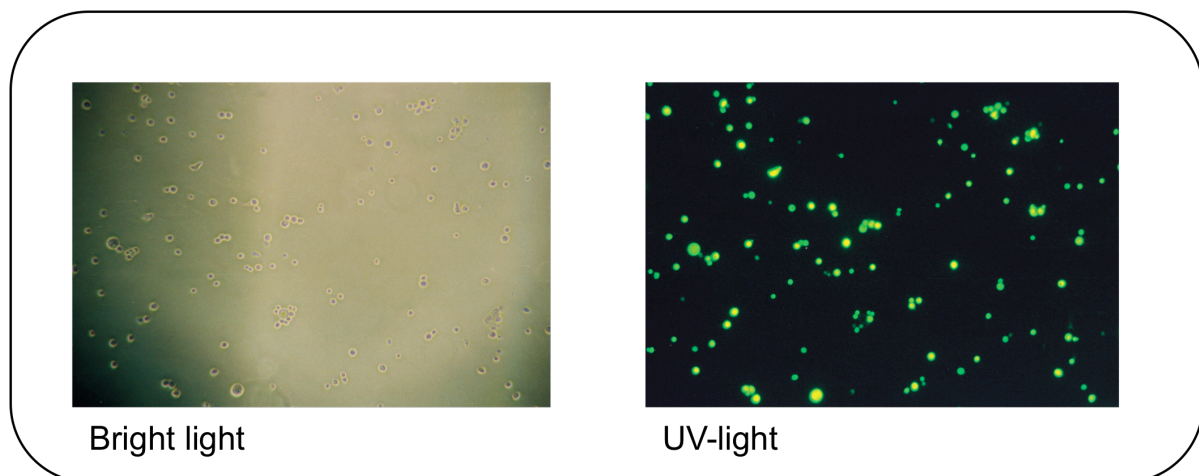counted under UV- and bright light (Figure 31-33; Picture 1-3).

<u>AGS cell line</u>

MOI	Bright light x 10000 cells/ml	Average	Fluorescent light x 10000 cells/ml	Average	Transfection efficiency
30	55 97	76	45 92	68,5	**90%**
40	55 63	59	49 56	52,5	**89%**
50	149 121	135	143 120	131,5	**97,5%**
60	113 121	117	112 101	106,5	**91%**

Figure 31

Bright light   UV-light

Picture 1

<u>N87 cell line</u>

MOI	Bright light x 10⁴ cells/ml	Average	Fluorescent light x 10000 cells/ml	Average	Transfection efficiency
30	133 251	192	91 189	140	**73%**
40	71 174	122,5	64 150	107	**87%**
50	39 40	39,5	34 37	35,5	**90%**
60	77 52	64,5	75 44	59,5	**92%**

Figure 32

Bright light   UV-light

Picture 2

GT5 cell line

MOI	Bright light x 10^4 cells/ml	Average	Fluorescent light x 10000 cells/ml	Average	Transfection efficiency
30	55 97	76	45 92	68,5	**90%**
40	55 63	59	49 56	52,5	**89%**
50	149 121	135	143 120	131,5	**97,5%**
60	113 121	117	112 101	106,5	**91%**

Figure 33

Bright light   UV-light

Picture 3

Under the MOI of 40 the best result between virus toxicity and transfection efficiency was achieved.

Results

Distinct Krüppel-like factor 4 Expression in human normal gastric and gastric tumor tissue*

To determine the effect of KLF4 expression on gastric cancer development and progression, immunohistochemical staining of paraffin-embedded normal gastric tissues and gastric cancer tissue specimens with an antibody against KLF4 protein was carried out. KLF4 expression was found in the cytoplasm and nuclei in most of the cells in the specimens obtained from patients who did not have cancer. Strong positive staining was observed in the cytoplasm and nuclei of cells localized predominantly in the glandular epithelium (glandular differentiation; Figure 34A) but decreased KLF4 expression in the cells located near the neck region of gastric mucous and near the gastric pit (foveolar differentiation). In sharp contrast, KLF4 expression was significantly decreased or lost in the cytoplasm and nuclei of various types of gastric cancer cells (Figure 34B).
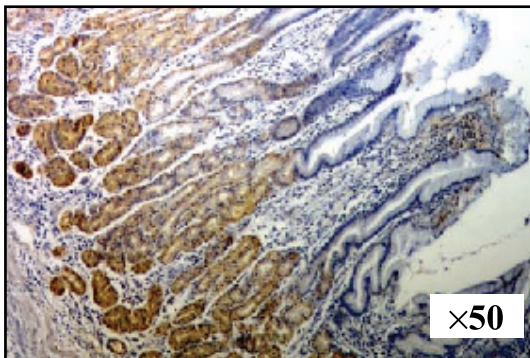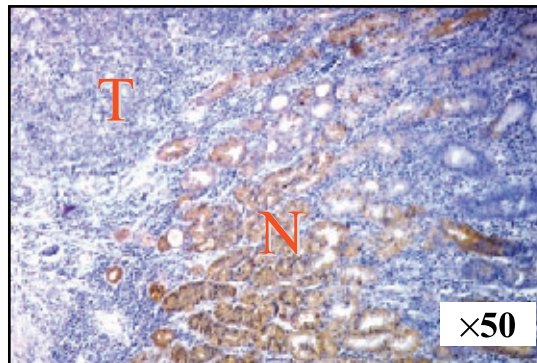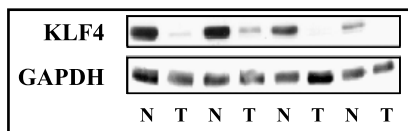

Figure 34A   **Figure 34B**

The majority of the tumor cells (T) exhibited a loss of KLF4 expression in sharp contrast to the residual normal glandular epithelial cells (N).

Western blot analysis was used to further examine the expression of KLF4 in four paired human normal gastric (N) and tumor tissue (T) specimens (Figure 35A and B). Two bands for KLF4 in Western blot analysis were observed, and the band shown (Figure 35F) was the predominant and higher molecular weight band. Consistent with the level of KLF4 protein expression determined via immunostaining, Western blot analysis showed that normal gastric tissue specimens had a significantly higher level of KLF4 expression than did gastric tumor tissue specimens. These results indicated that KLF4 is commonly expressed in human normal gastric cells but rarely expressed in human gastric cancer.
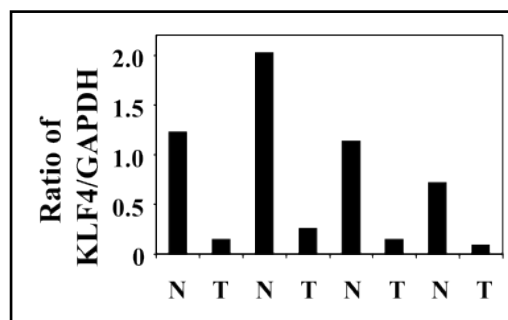
Figure 35A   **Figure 35B**

Equal protein sample loading was monitored by hybridizing the same membrane filter with an anti-GAPDH-antibody (N −> normal gastric tissue; T −> gastric tumor tissue)

Krüppel-like factor 4 expression in normal human gastric mucosa, gastric tumor tissue, and metastatic lymph nodes*

KLF4 expression was systematically analyzed in 86 primary gastric tumor, 51 lymph node metastasis, and 60 normal human gastric tissue specimens. In the primary tumor tissue specimens, the level of KLF4 expression was strong, weak, and negative in 12 (14%), 47 (54.7%), and 27 (31.4%) of the cases, respectively (Figure 36).
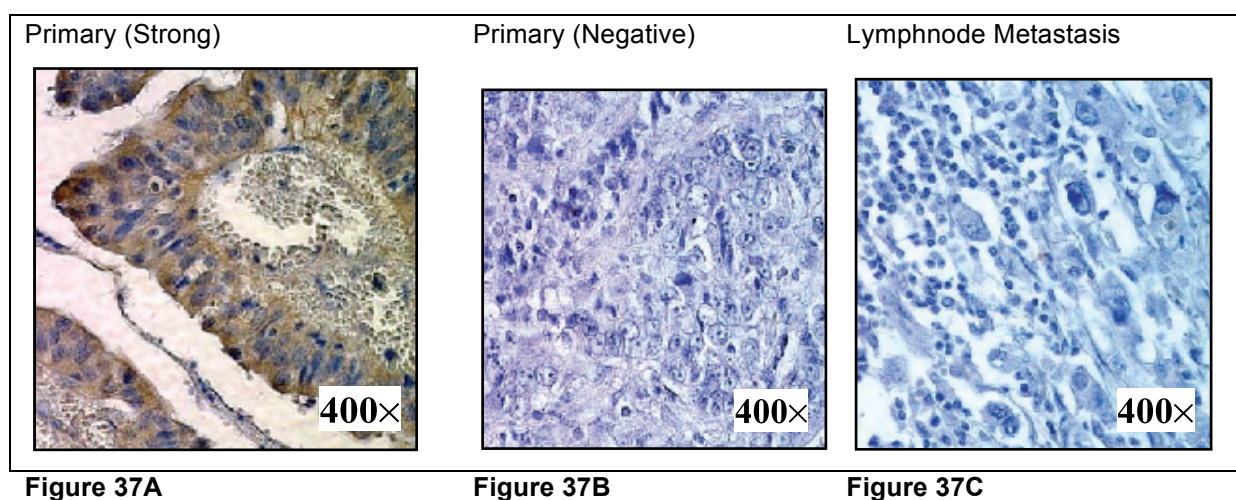
Characteristic	Total (n = 86)	KLF4 staining			P
		Negative (%), n = 27	Weak (%), n = 47	Strong (%), n = 12	
Sex					
Men	56	20 (35.7)	29 (51.7)	7 (12.5)	0.487
Women	30	7 (23.3)	18 (60.0)	5 (16.7)	
Age (years)					
Mean (SD)	61.8 (14.0)	57.4 (15.0)	62.6 (13.5)	68.4 (11.3)	0.463
Pathology type					
Papillary	12	1 (8.3)	9 (75.0)	2 (16.7)	0.358
Tubular	28	7 (25.0)	15 (53.6)	6 (21.4)	
Diffuse	8	2 (25.0)	4 (50.0)	2 (25.0)	
Mucinous	5	2 (40.0)	2 (40.0)	1 (20.0)	
Signet ring	21	9 (42.9)	11 (52.3)	1 (4.8)	
Mixed	12	6 (50.0)	6 (50.0)	0 (0.0)	
Stage					
I	14	0 (0.0)	12 (85.7)	2 (14.3)	0.005
II	28	5 (17.9)	17 (60.7)	6 (21.4)	
III	30	15 (50.0)	11 (36.7)	4 (13.3)	
IV	14	7 (50.0)	7 (50.0)	0 (0.0)	
Residual disease					
R0	69	16 (23.2)	41 (59.4)	12 (17.4)	0.003
R1, R2	17	11 (64.7)	6 (35.3)	0 (0.0)	
Lauren's classification					
Intestinal	53	14 (26.4)	30 (56.6)	9 (16.9)	0.351
Diffuse	33	13 (39.4)	17 (51.5)	3 (9.1)	

Figure 36

Among the three KLF4 expression categories, there were no significant differences in distribution according to sex, tumor pathologic types, and Lauren's histology classification. However, there was a significant difference in the distribution of the patients according to residual disease status (P = 0.003), and significantly, patients showed a clearly progressive loss of KLF4 expression from stage I to stage IV according to the American Joint Committee on Cancer staging (P = 0.005), suggesting that loss of KLF4 expression contributed to gastric cancer progression.

Comparison of KLF4 expression in normal gastric mucosa, primary tumors, and metastatic lymph nodes, revealed significantly lower expression in both the primary tumors and metastatic lymph nodes than in the normal mucosa (P < 0.0001). Moreover, the expression of KLF4 was even lower in the metastatic lymph nodes than in the primary tumors (P < 0.05; Figure 37A, B, and C; Figure 38), suggesting that loss of KLF4 expression may also contribute to gastric cancer metastasis. Overall, there was a decrease in or loss of KLF4 expression, which correlated with tumor development and progression.

Primary (Strong)   Primary (Negative)   Lymphnode Metastasis

400×   400×   400×

Figure 37A   **Figure 37B**   **Figure 37C**

Expression levels in different tissue specimens

Specimens	Total	KLF4 staining			P
		Negative (%)	Weak (%)	Strong (%)	
Normal	60	0 (0)	12 (20.0)	48 (80.0)	
Primary tumor	86	27 (31.4)	47 (54.7)	12 (14.0)	<0.0001*
Lymph node metastases	51	26 (51.0)	24 (47.1)	1 (2.0)	<0.0001[†], 0.015[‡]

NOTE: Pearson's two-tailed χ^2 test was done with the SPSS software program to determine the statistical significance of the level of expression of KLF4 in different tissue specimens.
*Primary tumor versus noncancerous tissue.
[†]Lymph node metastasis versus noncancerous tissue.
[‡]Primary tumor versus lymph node metastasis.

Figure 38

Effect of Krüppel-like factor 4 expression on patient survival*

The median survival duration in patients, who had a tumor with negative, weak, or strong KLF4 expression was 378, 1242, and 2489 days, respectively. Thus, decreased KLF4 expression was associated with an inferior survival duration (P = 0.0002). According to Kaplan-Meier plots of overall survival in patients with gastric cancers, the survival for 12 patients who had a tumor with strong KLF4 expression was significantly longer than that for the 47 patients with weak KLF4 expression and the 27 patients with negative KLF4 expression (P < 0.001; Figure 39).
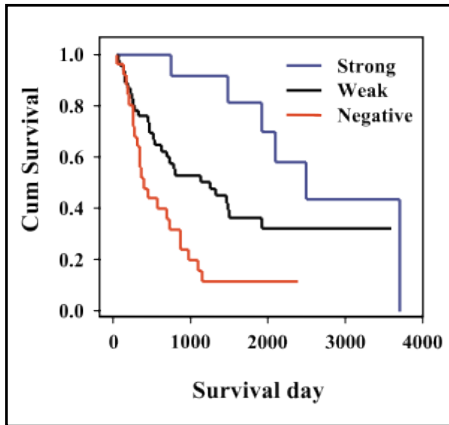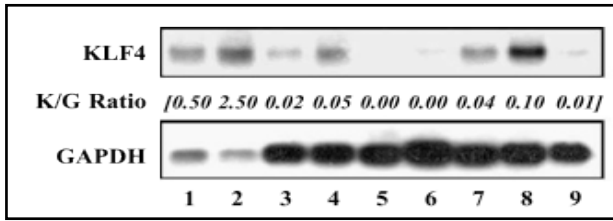
Figure 39

Other variables that affected survival in univariate analyses included disease stages (P < 0.001) and completeness of resection (P = 0.0002). The patients' age at diagnosis (as a continuous variable in Cox proportional hazards analysis), sex, and Lauren's classification did not have a statistically significant effect on survival.

Next, the patients' level of KLF4 expression, disease stage, completeness of resection, Lauren's histology type, age, and sex were entered in a Cox proportional hazards model for multivariate analysis. When the effect of covariates was adjusted, the loss of KLF4 expression was an independent predictor of poor survival (P = 0.017). The odds ratio in the group with negative KLF4 expression (4.86; 95% confidence interval, 1.614-14.640) and weak KLF4 expression (3.83; 95% confidence interval, 1.38-10.640) were statistically significantly higher (P < 0.01) than that in the group with strong KLF4 expression (reference). In addition, the advanced stage (P < 0.01) was also independent predictors of poor survival in this Cox proportional hazards model of multivariate survival analysis. However, patients' gender or age at diagnosis, completeness of resection, and Lauren's histology type had no statistically significant effect on survival in the multivariate analyses.

In vitro cell growth suppression by restoration of Krüppel-like factor 4 Expression*

To examine the biological activities of the KLF4 gene in gastric cancer cells, the expression of KLF4 in various human gastric cancer cell lines at the mRNA level via Northern blot analysis (Figure 40A) and protein level via Western blot analysis was examined (Figure 40B). Normal gastric mucosa specimens were included as references for KLF4 expression.

Figure 40A (Northern blot*)

Lane 1 and 2: normal human gastric mucosa
Lane 3:   AGS
Lane 4:   HTB103
Lane 5:   HTB135
Lane 6:   SK-GT5   Tumor cell lines
Lane 7:   N87
Lane 8:   SNU-1
Lane 9:   TMK-1
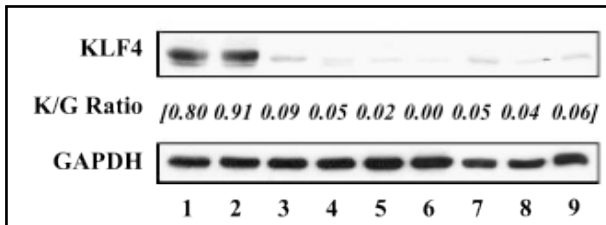
Figure 40B (Western blot*)

The relative KLF4 expression was expressed as K/G ratio (ratio between KLF4 and GAPDH). Both KLF4 RNA and protein expression were decreased in all human gastric cancer cell lines
The expression of KLF4 was substantially decreased in all seven gastric cancer cell lines compared with that in normal gastric mucosa cells.

For restoration of KLF4 expression and suppression of tumor growth in vitro, N87 and SK-GT5, which express KLF4 at low levels were chosen for restoration of KLF4 expression via transduction of adenoviral KLF4. The cells were incubated for 18 hours in medium alone (lane 1) or with control Ad-EGFP (lane 2) at an MOI of 30 or Ad-KLF4 (lanes 3-5) at an MOI of 10, 20, and 30. Ad-EGFP was used to adjust the total MOI equal to 30 MOI (Figure 41A/B).

Cellular RNA and total protein lysates were harvested from the cell cultures for KLF4 expression analysis for Northern blot analysis (Figure 41A) and Western blot analysis (Figure 41B).
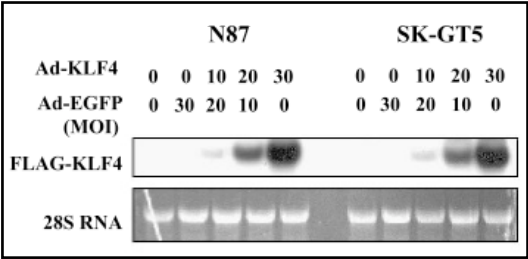

Figure 41A (Northern blot)   **Figure 41B** (Western blot)

At both mRNA (Figure 41A) and protein levels (Figure 41B), KLF4 was dose-dependently expressed in the tumor cells.

The increased KLF4 expression was consistent with cell growth suppression in vitro as determined via cell counting.

AGS, N87 and SK-GT5 cells were seeded at $0,34 \times 10^6$, $1,17 \times 10^6$ and $0,343 \times 10^6$ respectively cells per well in 6-well culture plates. Twelve hours later, the cells were incubated Ad-KLF4 or Ad-EGFP at a multiplicity of infection (MOI) of 40. The transduced cells were incubated for 1 to 3 days. The cell numbers were counted daily via the trypan blue exclusion method with a hemocytometer (Figure 42-44).
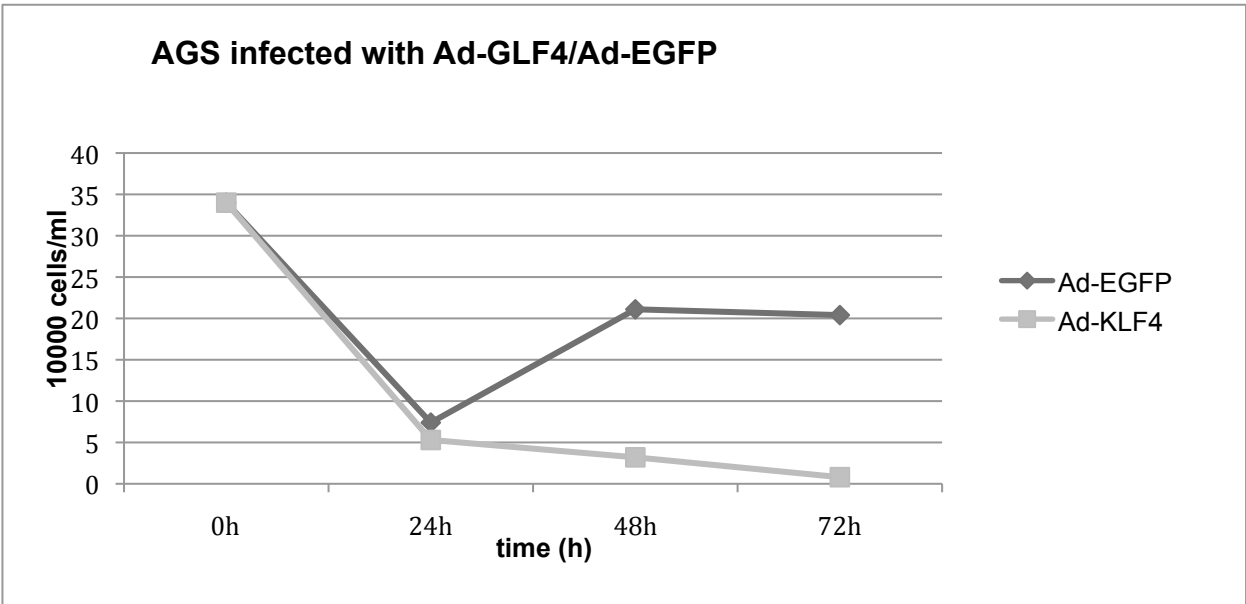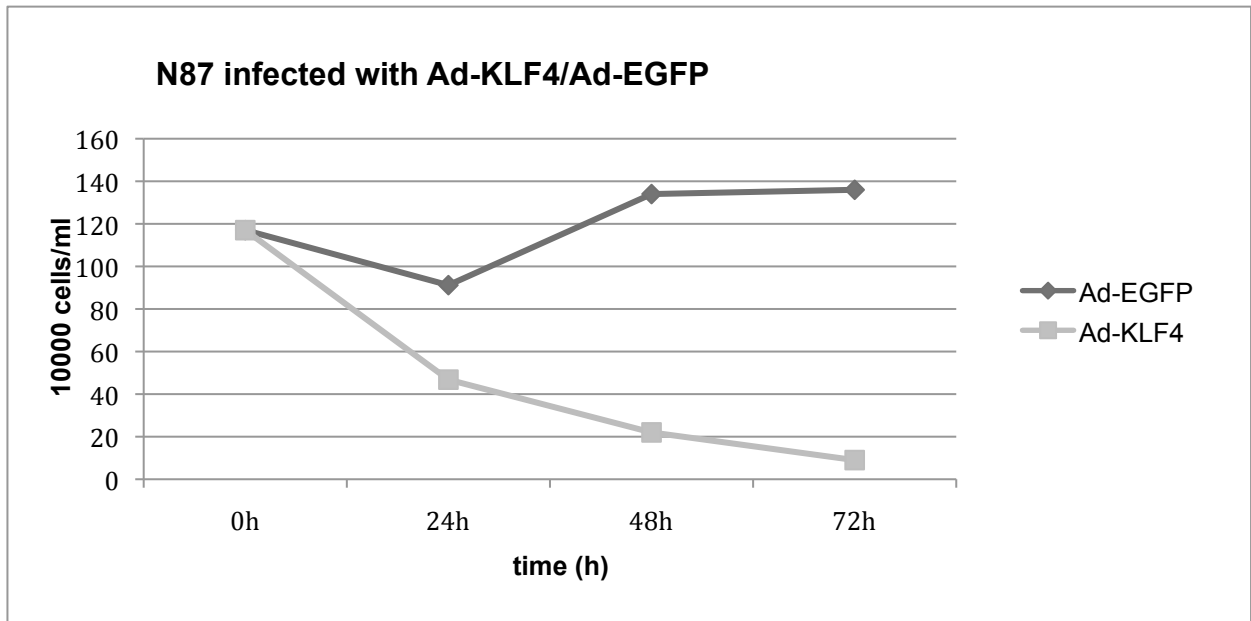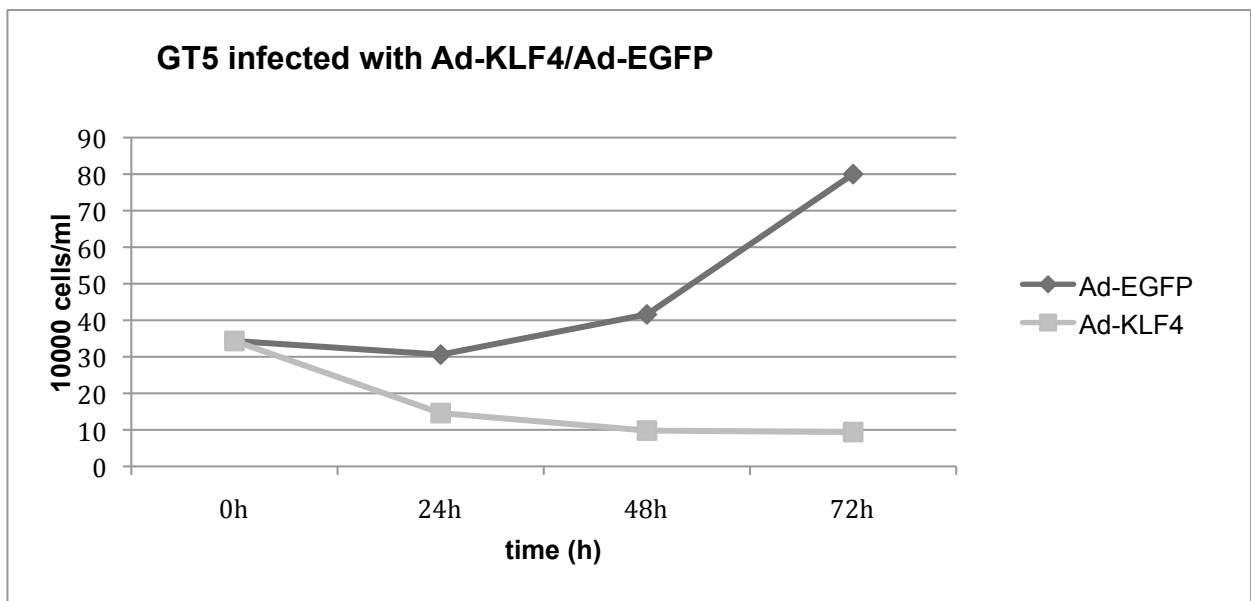

Figure 42

Figure 43

Figure 44

These experiments were repeated for N87 and SK-GT5 with a lower MOI to reduce virus toxicity. Tumor cells (Figure 45A for N87 and Figure 45B for SK-GT5) were plated into 60-mm dishes for 18 hours and incubated with an adenovirus for 2 hours. The cell numbers were determined by cell counting 1, 2, 3, and 4 days after adenoviral transduction at an MOI of 20.
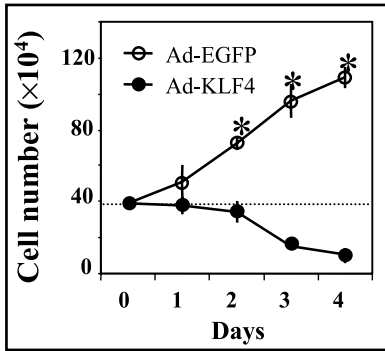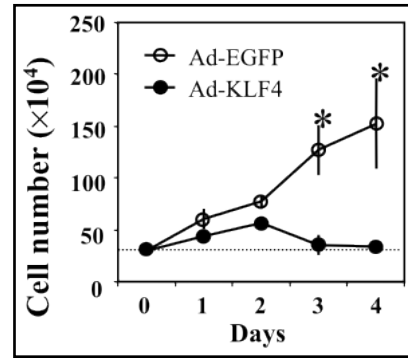
Figure 45A

Figure 45B

These data clearly showed that restoration of KLF4 expression led to suppression of tumor cell growth.

Inhibition of human gastric cancer growth and metastasis by Krüppel-like factor 4 in vivo

To determine the effect of KLF4 expression on tumor growth kinetics, N87 and SK-GT5 cells were injected s.c. into nude mice (1 x 10^6 cells/mouse). As shown in Figure 46 A and 46 B. N87 and SK-GT5 cells transduced with control Ad-EGFP grew progressively, whereas N87 and SK-GT5 cells transduced with KLF4 only grew slowly.
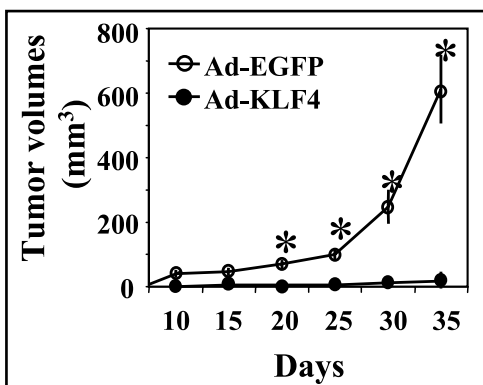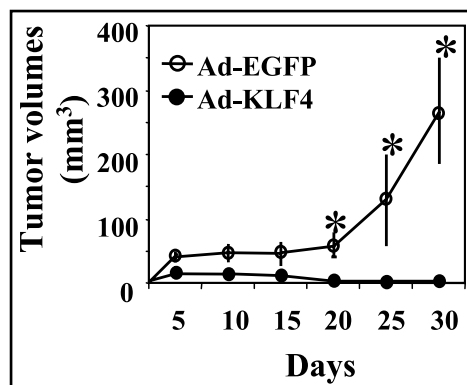
N87 cells

Figure 46 A

SK-GT5 cells

Figure 46 B

To make them more biologically relevant, N87 and SK-GT5 cells were injected into the stomach wall of mice (1 x 10^6 cells/mouse) in groups of 10 (an orthotopic animal model of gastric cancer).
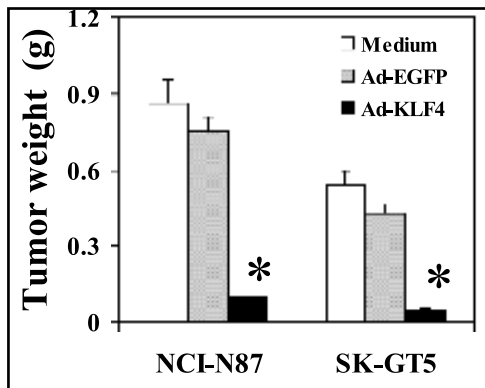
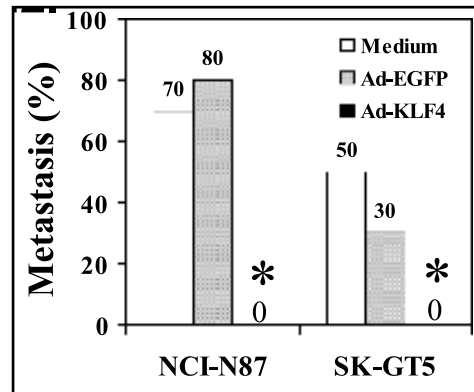Tumor weight

Figure 47 A

Metastasis

Figure 47 B

The control N87 and SK-GT5 cells and N87 and SK-GT5 cells transduced with control Ad-EGFP produced larger tumors and metastasized to regional lymph nodes and the liver, whereas N87 and SK-GT5 cells transduced with KLF4 only produced localized small tumors (Figure 46 A/47 A and 46 B/47B). In a comparison between the treated and respective untreated groups statistical significance with P < 0.01 was achieved.

Therefore, enforced restoration of KLF4 expression suppressed human gastric cancer growth and metastasis.

Induction of apoptosis by restored Krüppel-like factor 4 expression in gastric cancer cells*

To further investigate the mechanism by which KLF4 inhibited gastric cancer cell growth, the effects of KLF4 expression on apoptosis and the cell cycle via fluorescence-activated cell sorting (FACS) analysis were studied. There was increased expression of KLF4 induced apoptosis of both N87 and SK-GT5 cells in a dose-dependent manner by FACS analysis (data not shown). These findings were confirmed using two additional assays for apoptosis: genomic DNA laddering (Figure 48A) and TUNEL assays (Figure 48B and C).

N87 and SK-GT5 cells were incubated for 36 hours with Ad-KLF4 at MOI of 0, 10, or 20. Ad-EGFP was used to adjust the total MOI equal to 20. Genomic DNA laddering was determined (Figure 48A). Percentage apoptosis was determined by TUNEL assay (Figure 48B). Representative apoptotic morphology of SK-GT5 cells was photographed after TUNEL assay (Figure 48C).
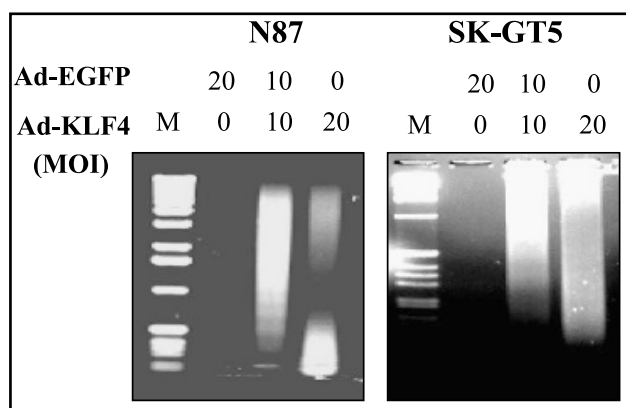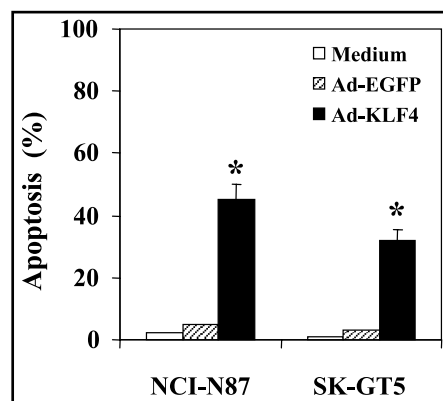

Figure 48 A

Figure 48 B

*P < 0.01, statistical significance in comparison between the treated and respective untreated groups.
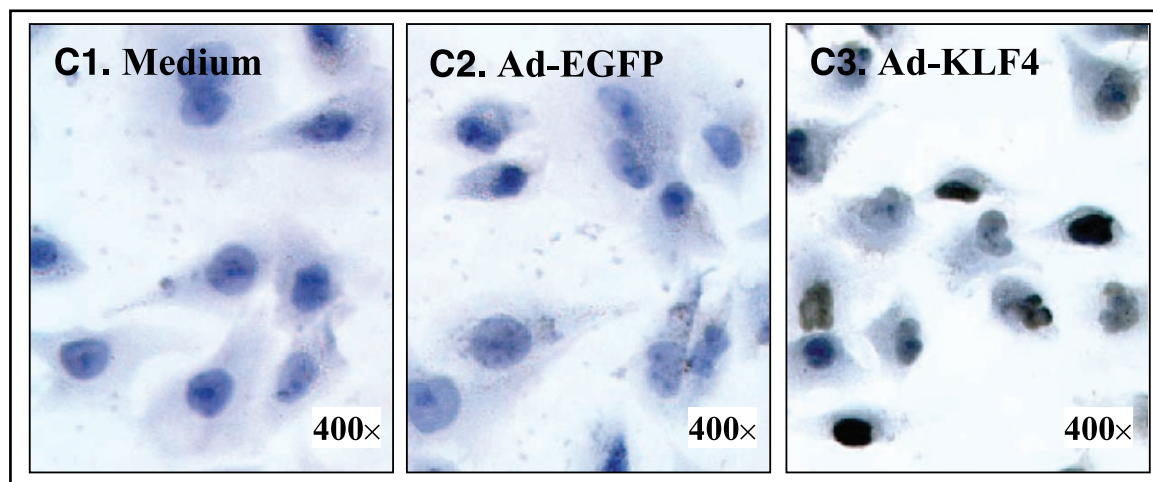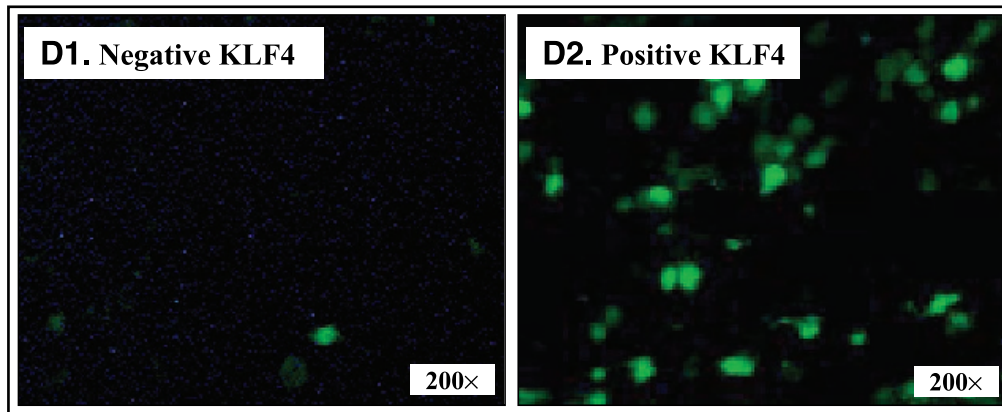

Figure 48 C1-3

These results were consistent with those of our in vitro cell counting assay, which showed that the cell number progressively decreased upon KLF4 transduction. Lastly, TUNEL assay on gastric cancer tissues with known negative or positive KLF4 expression was performed. Significantly decreased apoptosis was detected in tumor tissues with decreased or lost KLF4 expression (Figure 48 D1) compared with that in tumor tissues with positive KLF4 expression (Figure 48 D2).

Figure 48 D1/2

Hemizygous deletion and DNA methylation of exon 1 region of Krüppel-like factor 4*

To explore mechanisms for the decrease or lost KLF4 expression in gastric cancer, Southern blot analysis of genomic DNA extracted from eight gastric cancer cell lines were performed. Based on the genomic structure of wild-type KLF4 locus, digestion of genomic DNA by *Eco RI* or *Eco RI* plus *Nco I* and then probe with full-length KLF4 cDNA will generate 11- or 3-kb band, respectively. All cell lines exhibited a single 11-kb band when the genomic DNA was digested with Eco RI and probed with full-length KLF4 cDNA (Figure 49 A1).

In a next step the same membrane was striped and hybridized with GAPDH probe. The intensities of KLF4 band signals from SK-GT5 and SNU-16 were reduced by 50% compared with those of other cell lines, after normalization of DNA loading by calculating the ratio between KLF4 and GAPDH (K/G ratio, Figure 49 A2).
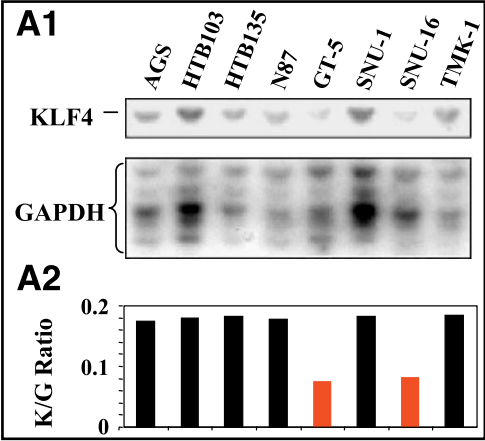
Figure 49 A1/A2

Digestion with EcoRI plus NcoI produced a similar result (data not shown). These data suggested that there were hemizygous deletions of the KLF4 gene in SK-GT5 and SNU-16 cells.

The promoter region of KLF4 contains typical CpG islands (Figure 49 B1):
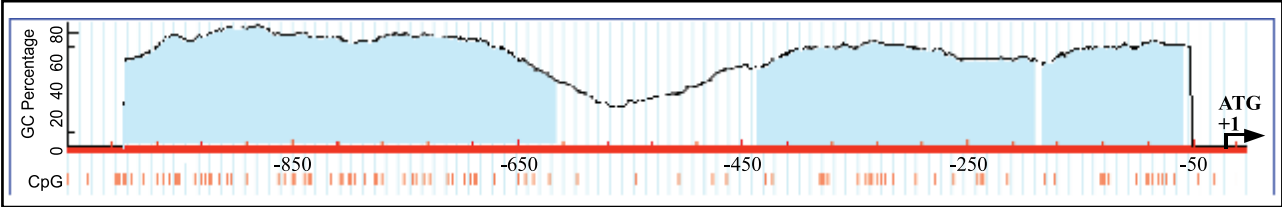
Figure 49 B1

The DNA methylation status was determined by methylation-specific PCR using genomic DNA extracted from surgically resected gastric cancer specimens and matched normal gastric mucosa tissues as well as from the gastric cancer cell lines. The primers were specific for the unmethylated (U) or methylated (M) KLF4 exon 1 region in the genomic DNA derived from gastric cell lines (Figure 49 B2) or gastric tumor tissue (T) and the matched normal gastric tissues (N, Figure 49 B3)

Five gastric cancer cell lines (Figure 49 B2) and four of the five gastric tumors (Figure 49B3) exhibited hypermethylation in the exon 1 region of KLF4, whereas none of the matched adjacent normal tissues had hypermethylation in the same region (Figure 49 B3). In vitro, SssI methylase-treated genomic DNA was used as a positive control and H_2O was used as a negative control.
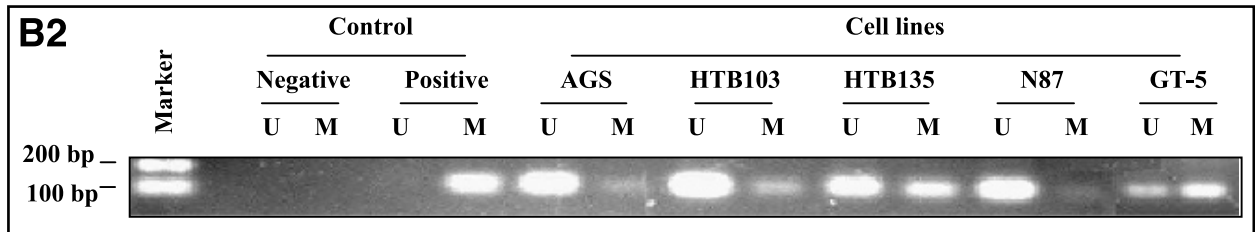
Figure 49 B2
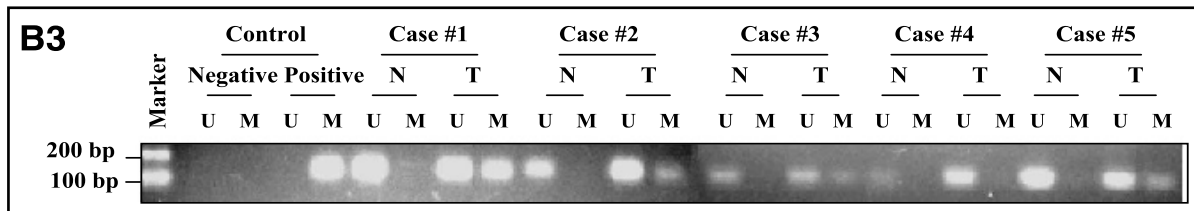
Figure 49 B3

These results were further confirmed by direct DNA sequencing of the PCR products using methylation-specific primers.

Finally, it was determined whether blockade of gene hypermethylation reactivates KLF4 expression in human gastric cancer cells. AGS, HTB103, N87, and SK-GT5 cell lines were incubated (for 20 hours) in medium or medium containing 5-aza-2-deoxycytidine, an inhibitor of DNA methyltransferase, sodium butyrate (NaB), an inhibitor of histone deacetylase, or both. Total RNA was extracted and KLF4 expression was measured by Northern blot using KLF4 cDNA as a probe. The same membrane was striped and hybridized with GAPDH probe (Figure 49 C1). The relative KLF4 signal was normalized by GAPDH (Figure 49 C2)

The treatments increased KLF4 expression in all four cell lines compared with their controls (Figure 49 C1/2).

Figure 49 C1/C2

Therefore, promoter hypermethylation may contribute to the reduced KLF4 expression in a subset of gastric cancer tissues and gastric cancer cell lines.

Discussion

The mammalian gut epithelium is a highly organized and dynamic system that requires continuous, controlled proliferation and differentiation throughout life. Gastric tissue consists of at least five different mature cell types:

- pit or surface mucous cells, which produce mucins and other factors involved in mucosal protection;
- parietal or oxyntic cells, which secrete acid;
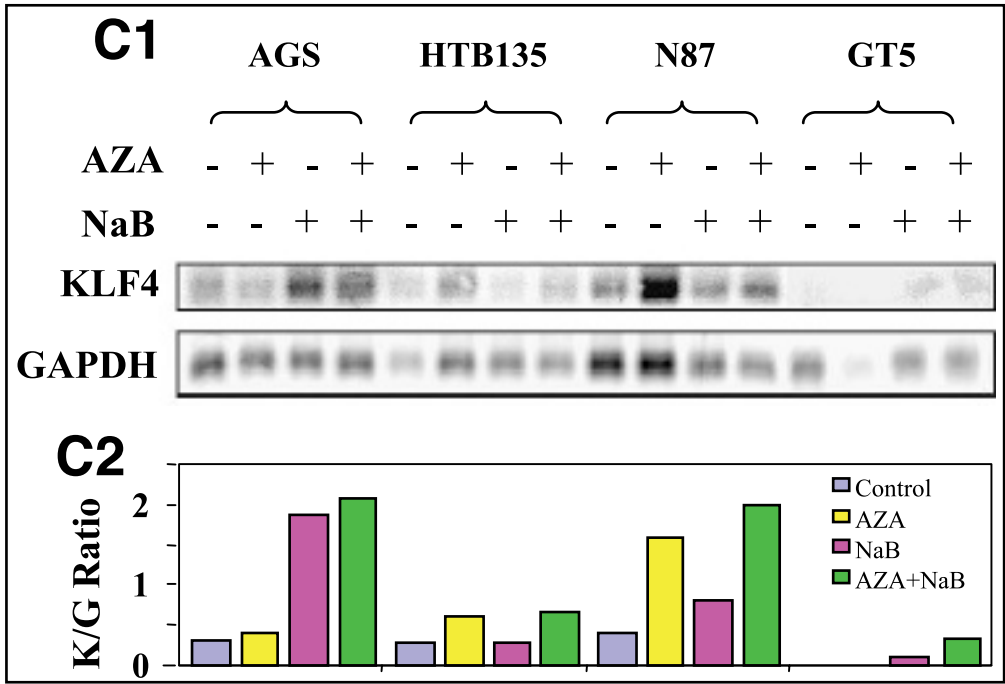- zymogenic or chief cells, which secrete pepsin;
- enteroendocrine cells providing a number of gastric hormones including gastrin;
- caveolated or brush cells.

The basic unit of proliferation and differentiation in the gastric epithelium is a tubular invagination of the mucosa called the pit-gland unit, which consists of the pit, isthmus, neck, and base. Multipotent stem cells in the isthmus give rise to daughter cells, which proliferate and differentiate while migrating either to the surface or to the base of the gland[157]. Perturbation of this complex homeostasis may lead to the development of gastric cancer.

The development and progression of gastric cancer involves a number of genetic and epigenetic alterations of tumor suppressor genes, oncogenes, cell cycle and mismatch repair genes and tumor-related genes. Molecular mechanisms include inactivation of tumor suppressor genes via loss of heterozygosity and mutation of the remaining allele (classical two-hit mechanism), hypermethylation of promoter CpG islands, mutations and microsatellite instability.

Multiple well-established oncogenic pathways, such as those mediated by cell cycle regulators, nuclear factor-κB, cyclooxygenase-2 and epidermal growth factor receptor are implicated in gastric carcinogenesis. Emerging evidence also emphasizes the importance of signaling pathways involved in the developmental process, including transforming growth factor-β/bone morphogenetic protein signaling, Wnt/β-catenin signaling, Hedgehog signaling and Notch signaling[158].

KLF4 and KLF5 belong both to the Specificity Protein/Krüppel-like factor transcription factor family. They are evolutionarily conserved zinc finger-containing transcription factors with diverse regulatory functions in cell growth, proliferation, differentiation, apoptosis, and embryogenesis. KLF4 and KLF5 show similar tissue distribution in embryos and adults. However, despite binding to the same, *cis*-acting DNA sequence, KLF4 and KLF5 often exhibit opposite effects on regulation of gene transcription. Moreover, the two KLFs exert contrasting effects on cell proliferation in many instances. While KLF4 is an inhibitor of cell growth, KLF5 stimulates proliferation. Both, KLF4 and KLF5 have been implicated in the Wnt/β-catenin pathway and possess context-dependent tumor suppressor or oncogenic functions.

One of the most striking features of the KLF-family is the involvement in the reprogramming of somatic cells to induced pluripotent stem cells (iPS cells). In this context, researchers in[159, 160, 161 and 162] showed that KLF4, together with Oct4, Sox2 and c-Myc, has the capacity to reprogram fetal as well as adult fibroblast into pluripotent embryonic stem cells. Further KLF4 and KLF5 together with KLF2 have been shown to regulate the self-renewal of embryonic stem cells[163]. This is one example that both KLF4 and KLF5 also can perform similar functions.

In the following part the results of this study will be discussed in the context of the unity and diversity of KLF4 and KLF5, respectively.

According to its crucial role in cell differentiation, KLF4 showed a specific expression pattern in normal gastric tissue specimens. In the present study, KLF4 was expressed both in the cytoplasm and nuclei of cells localized predominantly in the glandular epithelium but the cells located near the neck region of gastric mucous and close to the gastric pit showed decreased KLF4 expression (Figure 34 A).

In accordance with these results, Foxa3-Cre/Klf4[loxP/loxP] mice, lacking KLF4 exclusively in the stomach, showed a twofold increase in the number of pit cells and a fourfold increase in the number of mucus neck cells. The number of parietal and zymogenic cells on the other hand was reduced to half. From these findings, it was concluded that KLF4 is required for directing the cell-fate decisions of the gastric epithelial precursor cells[107]. Moreover, the expression pattern of KLF4 in the small and large bowel is similar to the observations made in the gastric mucosa. This means that KLF4 is present in the villi and upper crypts of terminally differentiated epithelial cells[85].

In contrast, KLF5, which often plays an antagonistic role functioning as a pro-proliferative factor, showed a mutually exclusive expression pattern in the intestine, being expressed in the proliferative compartment at the base of the crypts[100]. Comparisons of expression of KLF4 and KLF5 also revealed temporal differences during development in mice. Thus, in the embryonic day 10,5 mice embryos showed absent KLF4 expression, whereas high-level expression of KLF5 was detected. At embryonic day 15,5 both KLF4 and KLF5 were abundant in the gut. At embryonic day 17,5 KLF4 transcripts were nearly absent while KLF5 expression remained high in the intestine as development progressed[164]. On a molecular level, both KLF4 and KLF5 regulate the KLF4 promoter by competing directly for binding to their cognate DNA sequence[165]. These findings indicate that KLF4 plays an important role in homeostasis and maintenance in gastric mucosa.

Compared to normal gastric tissue, gastric cancer tissue specimens showed considerably decreased or lost KLF4 expression (Figure 34 B). Western blot analysis confirmed these results determined by immunostaining, showing significantly higher level of KLF4 protein expression in normal gastric tissue than in gastric cancer tissue in four paired normal and cancer specimens respectively (Figures 35 A and 35 B).

In addition, examination of the biological activity of the KLF4 gene in various gastric cancer cells lines yielded similar results, showing decreased KLF4 expression at both m-RNA and protein level (Figures 40 A and 40 B).

Katz et al. yielded similar results, finding a 96% decrease in KLF4 mRNA expression detected via quantitative PCR in both intestinal and diffuse-type human gastric cancer compared to normal controls. By using conditional ablation of KLF4 in the stomach via Foxa3-Cre, the same group showed that KLF4 mutant mice had precancerous changes in the gastric epithelia including gastric hypertrophy and mucus cell hyperplasia starting at two weeks of age and the Foxa3-Cre/Klf4*loxP/loxP* mice had a 4-fold increased proliferation by six months of age. KLF4 mutants also showed an aberrant expression of acidic mucins and TFF2/SP-positive cells. Such findings are characteristic for premalignant conditions[107].

The results described in this thesis emphasize these findings: the restoration of KLF4 by an adenovirus vector system in N87 and SK-GT5 (i.e. two cancer cell lines), which express KLF4 at low levels, resulted in distinct suppression of tumor cell growth, determined by the trypan blue exclusion method (Figures 45 A and 45 B). To determine the effect of KLF4 expression on tumor growth kinetics *in vivo*, N87 and SK-GT5 cells were injected s.c. into nude mice. As shown in Figures 46A and 46B cells transduced with control Ad-EGFP grew exponentially, whereas cells transduced with KLF4 only grew slowly.

In an orthotopic animal model of gastric cancer, N87 and SK-GT5 were injected into the stomach wall of mice. The control group produced larger tumors and metastasized to regional lymph nodes and the liver, whereas N87 and SK-GT5 transduced with KLF4 only produced localized small tumors and did not show any metastasis (Figures 47 A and 47 B). Hence, enforced restoration of KLF4 expression suppressed human gastric cancer growth and metastasis *in vitro* and *in vivo*.

Furthermore, KLF4 has been reported to be downregulated in numerous human cancers including colorectal cancer[127, 130, 135], esophageal cancer[129], bladder cancer[123], non-small-cell lung carcinoma[166] and leukemia[167, 168]. In pancreatic cancer cell lines, KLF4 expression is associated with increased doubling time, resulting in slower growth[169].

In adenomas of the *Apc*[min/+] mouse, a model of colorectal cancer, KLF4 is as well down-regulated[122,124]. Crossing the *Apc*[min/+] mice with KLF4^{+/−} heterozygote mice resulted in significantly more adenomas compared to *Apc*[min/+] mice only[134].
In this context, these observations clearly define KLF4 as a tumor suppressor.

In contrast to KLF4, KLF5 expression particularly showed a positive effect on cell cycle progression and proliferation. Because of the pro-proliferative function, KLF5 has been suspected in mediating oncogenic events in different tissues.
In gastric cancer, KLF5 was significantly high expressed in early-staged cancer, in gastric cancer without lymph node metastasis, and in small gastric cancer tissues[170].
In human cancer, KLF5 has been reported as a downstream target of oncogenic H-Ras in vitro, where it mediated pro-proliferative and transforming activities of H-Ras[171]. In addition, KLF5 is increased in human colorectal cancer cell lines as well as in primary tumors that possess oncogenic mutation of K-Ras. Overexpression of oncogenic K-Ras in mice resulted in increased KLF5[172]. Furthermore, in the mouse model of intestinal tumorigenesis, haploinsufficiency of KLF5 rescued the tumor initiating effect of the *Apc*[Min] mutation in the intestine[173].
Another report supporting the oncogenic potential of KLF5 showed that higher KLF5 expression in sporadic breast cancer is correlated with shorter disease-free survival and poorer overall survival[174]. In the TSU-Pr1 human bladder cancer cell line, KLF5 provided further evidence of its oncogenic potential promoting cell proliferation and tumorigenesis *via* increased G_1 to S phase transition, up-regulation of *cyclin* D1 expression, phosphorylation of MAPK and Akt proteins, and inhibition of p27 and p15 expression[175]. In addition, amplification of the KLF5 locus has been reported in salivary gland tumors[176].
In this context, these findings support a pro-proliferative and oncogenic function for KLF5 at least in a subset of these tumors.

On a molecular level, KLF4 and KLF5 seem to play different roles, too. KLF4 showed clearly antiproliferative and tumor suppressive function: as mentioned in the introduction part, activation of p21[WAF1/Cip1] by KLF4 expression resulted in cell cycle arrest at both the G1–S and G2–M transition points[70, 72]. In addition, KLF4 suppressed *cyclin* D1[112], *cyclin* B1[73] and *cyclin* E[72] (Figure 6).

In contrast to KLF4, KLF5 has a positive effect on cell cycle progression and proliferation. KLF5 proved as a mediator of the transforming effect of oncogenic H-Ras via MAPK signaling. Upon activation of MAPK by H-Ras, KLF5 expression is induced, and KLF5 activated several cell cycle promoting factors, including *cyclin* D1, *cyclin* B1 and Cdk1/Cdc2[177, 178]. Using mouse primary esophageal epithelial cells, it has been demonstrated that KLF5 increases cell proliferation and upregulates the epidermal growth factor receptor (EGFR) expression, binding to the 5'-regulatory region of EGFR. EGFR signaling, in turn, activated MEK/ERK, which further induces KLF5 expression, creating a positive feedback loop[179].

The Wnt signal transduction pathway is involved in many differentiation events during embryonic development and may result in tumor formation after aberrant activation of its components, including in intestinal and gastric cancer.

In this pathway, β-catenin plays a crucial role as an effector, translocating to the nucleus and subsequent activating the β-catenin/TCF4 complex with resultant increased proliferation[180, 181, 182]. In the absence of Wnts, β-catenin is constitutively degraded in proteasomes with the help of APC (adenomatous polyposis coli protein), promoting the phosphorylation by glycogen kinase 3. In tumors, β-catenin degradation is blocked by mutations of the tumor suppressor gene APC, or of β-catenin itself. As a consequence, constitutive TCF/β-catenin complexes are formed and activate oncogenic target genes.

KLF4 exhibits an expression pattern in the intestinal epithelium that is similar to APC, a known negative regulator of Wnt signaling. Indeed, KLF4 counters the Wnt/ß-catenin signaling by direct interaction with the C-terminal transactivation domain of β-catenin inhibiting p300/CBP recruitment by β-catenin. Furthermore, KLF4 inhibits p300/CBP-mediated β-catenin acetylation as well as histone acetylation on Wnt target genes and directly interacts with TCF4 independent of β-catenin. Thus, KLF4 suppresses transformation of cancer cells both in vitro and in vivo[138, 139].

Moreover, Dang et al. showed in the RKO human colon cancer cell line that APC activates Cdx2, a member of the caudal- related homeobox gene family, with sequence homology to the caudal gene of Drosophila melanogaster. Cdx2 is specifically expressed in the intestine and is important for regulation of intestinal epithelial cell development and maintenance. Cdx2 in turn transactivates the KLF4 promoter resulting in expression of KLF4 and subsequent growth arrest [183].

The Wnt pathway has been examined thoroughly in colorectal cancer. There is now evidence that the APC/β-catenin pathway also plays a significant role in gastric carcinogenesis. Using the $Apc^{Min/+}$ mouse, which is a model for intestinal tumorigenesis, aged $Apc^{Min/+}$ mice developed multiple adenomas in the stomach. These adenomas carried loss of heterozygosity of APC and showed strong nuclear accumulation of β-catenin, suggesting that APC/β-catenin pathway is an initiating event in gastric tumorigenesis in the $Apc^{Min/+}$ mouse. In addition, the adenomatous lesions displayed increased Myc and *cyclin D1* expressions, which are transcriptional targets of β-catenin/TCF[184]. Moreover, Foxl1, a winged helix transcription factor expressed in the gastrointestinal tract, plays an important role in regulating epithelial cell proliferation and differentiation. Foxl1^{−/−} mice displayed severe structural defects in the epithelia of the stomach, which are a result of an increase in the number of proliferating cells. The level of nuclear β-catenin has been shown two times greater in Foxl1^{−/−} mice than in Foxl^{+/+} mice. These findings further evidence that the Wnt/APC/β-catenin pathway is activated in the gastrointestinal tract of Foxl1^{−/−} mice[185].

In contrast, the expression pattern of KLF5 in the intestine is similar to several Wnt signaling pathway components localized to the proliferating zones of the crypt. Indeed, KLF5 has been reported to be a downstream target of Wnt signaling[186], being an important component of cell proliferation regulated by Wnt. $Apc^{Min/+}$KLF5^{+/−} mice, which are haploinsufficient for KLF5, showed a 96% reduction in the number of intestinal adenomas. This reduced tumorigenicity correlated with reduced levels and nuclear localization of β-catenin as well as reduced expression of two β-catenin targets: *cyclin D1* and c-Myc. *In vitro* studies revealed a physical interaction between KLF5 and β-catenin that enhanced the nuclear localization and transcriptional activity of β-catenin[167]. In addition, investigations of KLF5 heterozygosity on the propensity of Apc^{Min}/KRAS[V12] double transgenic mice revealed a drastic decrease in the number of tumors. The levels of β-catenin *cyclin D1* and Ki-67, all known markers of proliferation and transformation, were also reduced in the intestinal mucosa of Apc^{Min}/KRAS[V12]/KLF5^{+/−} mice compared to Apc^{Min}/KRAS[V12] mice[187]. Figure 50 provides an overview of the different roles of KLF4 and KLF5.
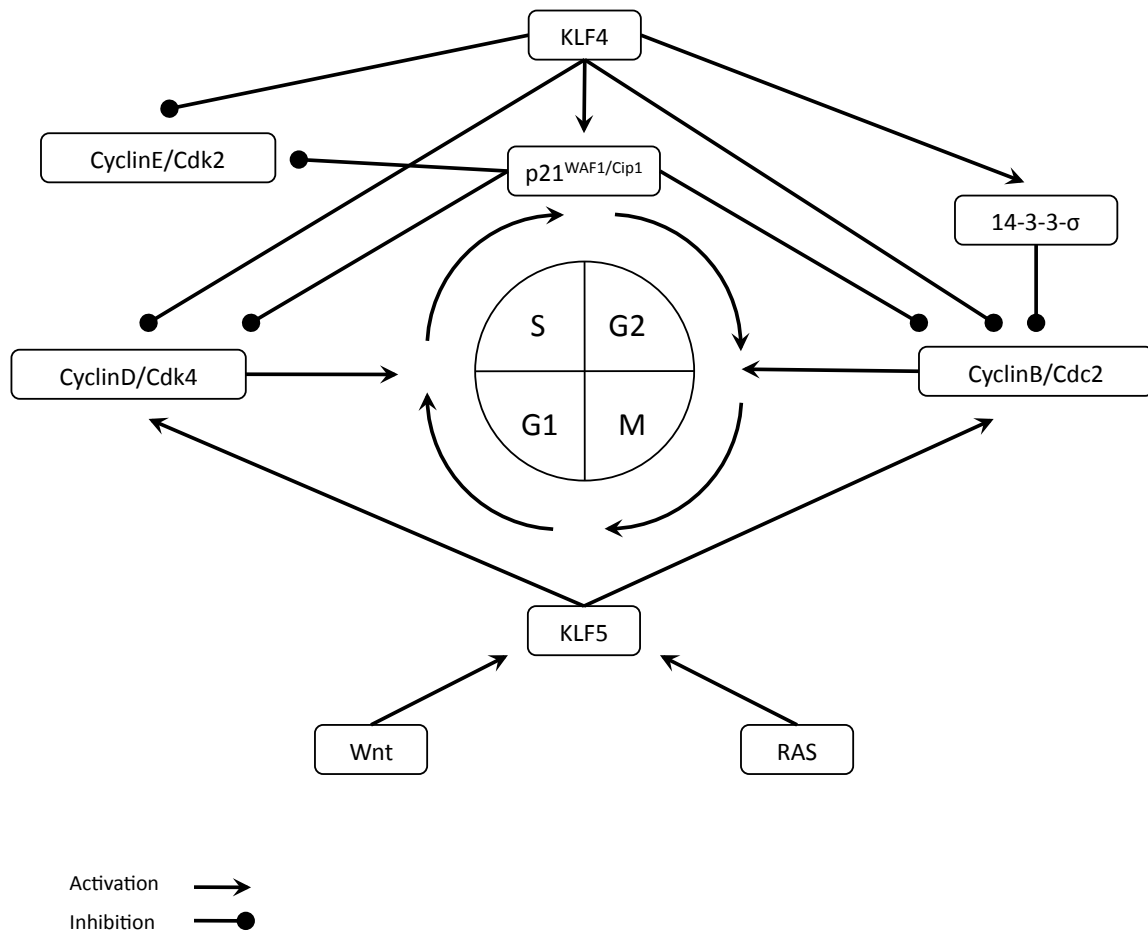
Figure 50. KLF4 has a growth inhibitory role by regulating expression of key cell cycle genes, including the cell cycle inhibitor, p21$^{Waf1/Cip1}$. In contrast, KLF5 plays a growth-promoting role in response to mitogenic signals through its activation of *cyclin D*, *cyclin B*, and the *cyclin B* kinase, Cdc2. Slightly modificated from McConnell et al.[100]

As described above, there are a few lines of evidence indicating that altered KLF4 expression affects cell cycle via $^{p21WAF1/Cip1}$ activation. This leads to subsequent cell cycle arrest (Figures 5 and 50). But there is still little known about the mechanisms by which KLF4 may influence cancer development and progression.

In this work, there was increased expression of KLF4 induced apoptosis of both N87 and SK-GT5 cells in a dose-dependent manner by FACS analysis. These findings were confirmed using two additional assays for apoptosis: genomic DNA laddering (Figure 48A) and TUNEL assays (Figures 48B and 48C). Moreover, these results are consistent with the in vitro cell counting assay, resulting in progressively decreased cell number upon KLF4 transduction.

These results are consistent with those of previous studies on bladder cancer cells[123], colon cancer[188], and leukemia cells[189]. However, the mechanisms by which KLF4 induces apoptosis are not yet completely understood.

In classical Hodgkin, lymphoma KLF4 overexpression induced massive cell death. A quantitative RT-PCR gene expression array revealed KLF4 target genes including the proapoptotic gene BAK1. Using a small hairpin RNA-mediated knockdown approach, BAK1 has been found to be largely responsible for KLF4-induced apoptosis[190]. Another report showed that the level of KLF4 expression is inversely correlated with the extent of DNA damage. There, KLF4 is activated by p53 following cytostatic, mild DNA damage, whereas it is strongly repressed via enhanced turnover of mRNA on severe DNA damage that irreversibly drives cells to apoptosis[191].

In prostate cancer, KLF4 knockdown weakened the effects of selenium on DNA synthesis inhibition, apoptosis induction, and the expression of three KLF4 target genes, *cyclin* D1, p21$^{WAF1/CIP1}$, and p27^{Kip1}. In addition, the overexpression of KLF4 not only led to an induction of apoptosis in the control cells, but also enhanced the DNA synthesis-suppressive and-proapoptotic activities of selenium[192].

As described in the introduction part, KLF4 also has an important role as an antiapoptotic factor, in parts by suppressing *BAX* expression following γ-radiation. KLF4 accomplishes this antiapoptotic effect by activating p21$^{WAF1/Cip1}$, and by inhibiting the ability of p53 to transactivate the proapoptotic gene *BAX*[121]. A net effect is to steer cells away from apoptosis and toward cell cycle arrest (Figure 6). In the absence of p21$^{WAF1/CIP1}$ due to an oncogenic signal (Ras^{V12}), KLF4's anti-apoptotic effect dominates its cytostatic effect, rendering KLF4 oncogenic. These findings provide a mechanistic explanation for the context-dependent oncogenic or tumor-suppressor functions of KLF4 (Figure 7).

Another mechanism by which KLF4 allows bypass of oncogenic RAS$_{v12}$-induced senescence is by suppressing p53 expression via inhibiting directly the p53 promoter. Thus, allowing for RAS$_{v12}$-mediated transformation and providing resistance to DNA-damage-induced apoptosis. In this setting p21$^{WAF1/Cip1}$ was induced by KLF4. But unlike in normal cells, in RAS$_{v12}$ expressing cells the increased *cyclin D1* level neutralized the p21$^{WAF1/Cip1}$ function. This left the inhibition of p53 by KLF4 as the dominant function, thereby, promoting cellular proliferation, loss of contact inhibition, an ability to grow in an anchorage-independent manner and

resistance to cisplatin-induced apoptosis[145, 146].

The possibility that KLF5 expression is induced by DNA-damaging agents brought up the question whether or not KLF5 functions in an alternative capacity in damage-response and/or apoptotic pathways. In this context, Zhao et al. showed that KLF5 modulated apoptosis secondary to DNA damage in a p53-independent manner. It is hypothesized that in response to DNA damage, KLF5 is induced, and in turn functions as a transcriptional activator of Pim1 expression, a survival kinases that functions downstream of JAK/STAT activation, and promotes inactivation of the pro-apoptotic Bad protein by phosphorylation. Conversely, the loss of KLF5 resulted in a failure to transactivate Pim1, reduced Bad phosphorylation and increased apoptosis[193].

A different role for KLF5 has been found in TE2 esophageal cancer cell line. Ectopic expression of either KLF5 or KLF4 enhanced detachment-induced apoptosis (anoikis). In addition, expression of KLF5 in these cells reduced cell viability through the activation of *BAX* expression to promote apoptosis[194].

Disparate roles for KLF4 and KLF5 may be accounted for by various genetic backgrounds in the cancer cell lines and/or by tissue-specific differences[100].

In this work, the precede experiments evidenced that KLF4 expression in gastric cancer tissue was lost or significantly decreased when compared to normal gastric tissue. To explore the mechanisms for the drastic altered KLF4 expression in gastric cancer, Southern blot analysis of genomic DNA extracted from eight cancer cell lines was performed. Two of eight gastric cancer cell lines showed hemizygous deletions of KLF4 (Figure 49 A2).

Aberrant DNA methylation of CpG islands located within gene promoters has been identified as a mechanism for transcriptional inactivation of tumor suppressor genes[195]. The fact that the promoter region of KLF4 contains typical CpG islands (Figure 49 B1) prompted to question if KLF4 is silenced by hypermethylation of its promoter. Therefore, DNA methylation status was determined by methylation-specific PCR using surgically resected gastric cancer specimens and matched normal gastric mucosa tissues as well as gastric cancer cell lines. All five gastric cancer cell lines (Figure 49 B2) and four of five gastric tumors (Figure 49 B3) exhibited hypermethylation in the exon 1 region of KLF4, whereas none of the matched adjacent normal tissues had hypermethylation in the same region (Figure 49 B3).

Finally, it was determined whether a blockade of gene hypermethylation reactivates KLF4 expression in human AGS, HTB103, N87, and SK-GT5 gastric cancer cells cell lines. Incubating the cell lines in 5-aza-2-deoxycytidine, an inhibitor of DNA methyltransferase, sodium butyrate, an inhibitor of histone deacetylase, or both the treatments increased KLF4 mRNA expression in all four cell lines compared with their controls determined via Northern blot (Figure 49 C1/2). Therefore, promoter hypermethylation may contribute to the reduced KLF4 expression in a subset of gastric cancer tissues and gastric cancer cell lines.

This observation is also supported by other reports. In differentiated carcinomas of the stomach loss of heterozygosity has been reported for several chromosomal arms, including 2q, 4p, 5q, 6p, 7q, 11q, 17q, 18q 21q[196]. For undifferentiated carcinomas, frequent loss of heterozygosity is found at 5q[197]. More important, the loss of heterozygosity has been found in human gastric cancer on chromosome 9q31.1 where KLF4 is located. In addition, the loss of heterozygosity at 9q31.1 did not show any preference to either histologic types of gastric cancer, thus is common in both differentiated-type and undifferentiated-type carcinomas[198]. Molecular mechanisms leading to loss of expression of KLF4 in colorectal cancer included deletion, mutation and hypermethylation silencing in the 5'-untranslated region of KLF4[127] and loss of heterozygosity in KLF4 has been found in 83% of CRC-derived cell lines, and in 20% of CRC surgically resected specimens[127].

In contrast, KLF4 DNA was not methylated in either normal or tumor tissues from adenomas of Apc^{Min} mice using methylation-specific PCR[122]. However, KLF4 expression was stimulated by treating colorectal cancer cell lines with 5-Aza-2-deoxycytidine, an inhibitor of DNA methyltransferase[135]. Apart from loss of heterozygosity and promoter hypermethylation, the loss of APC expression or β-catenin activation may also contribute to the down-regulation of KLF4 in gastric cancer[199, 200, 201], because KLF4 is a downstream target of the Wnt/APC/β-catenin pathway[202].

In contrast to its overexpression in various cancer tissues, tumor analyses have demonstrated the loss of KLF5 expression in numerous cancers, either through hemizygous deletion and gene silencing or through activation of ubiquitination–proteosome pathways[70, 203, 204, 205]. Because of this selective loss, KLF5 has been implicated as a tumor suppressor in esophageal, breast, prostate and intestinal

cancers[194, 203, 206,207, 204, 208]. Like KLF4, KLF5 seems to have a context-dependent role, acting either as oncogene or as tumor suppressor depending on the genetic context of diverse cancer tissues. It is hypothesized that KLF5 can activate expression of growth-suppressive or pro-apoptotic factors that can override KLF5's growth-promoting activity. This was best illustrated in a study, in which KLF5 was required – as expected – for proliferation of the spontaneously immortalized keratinocyte cell line HaCat. Surprisingly, it was also required for TGF-β-mediated growth inhibition. The knockdown of KLF5 in HaCat cells resulted in an inhibition of cell proliferation. These cells showed reduced proliferation compared to the control, but failed to undergo growth inhibition upon TGF-β stimulation[209]. Mechanistically, the TGF-β mediated acetylation of KLF5 altered its protein interactions and promoter binding, thereby, switching from a transcriptional repressor to an activator on the p15 promoter[210].

In addition, KLF5 potentially mediates pro-apoptotic or anti-proliferative effects through the induction of *BAX* and p21$^{Cip1/Waf1}$ [194]. Therefore, the timing of changes in KLF5 expression may explain this apparent discrepancy, where increased expression early in tumor formation is growth-promoting, and the loss of KLF5 expression in later stages of cancer aids tumor cell survival. One study, which examined KLF5 expression by stage support this theory, reflecting high KLF5 expression in early stages and lower KLF5 expression in later stages of gastric carcinoma[170].

As described above, KLF4 and KLF5 exhibit both context dependent natures, by which they are able to perform opposite functions. A model for the dual roles of KLF4 and KLF5 in tumor suppression and oncogenesis shows Figure 51.
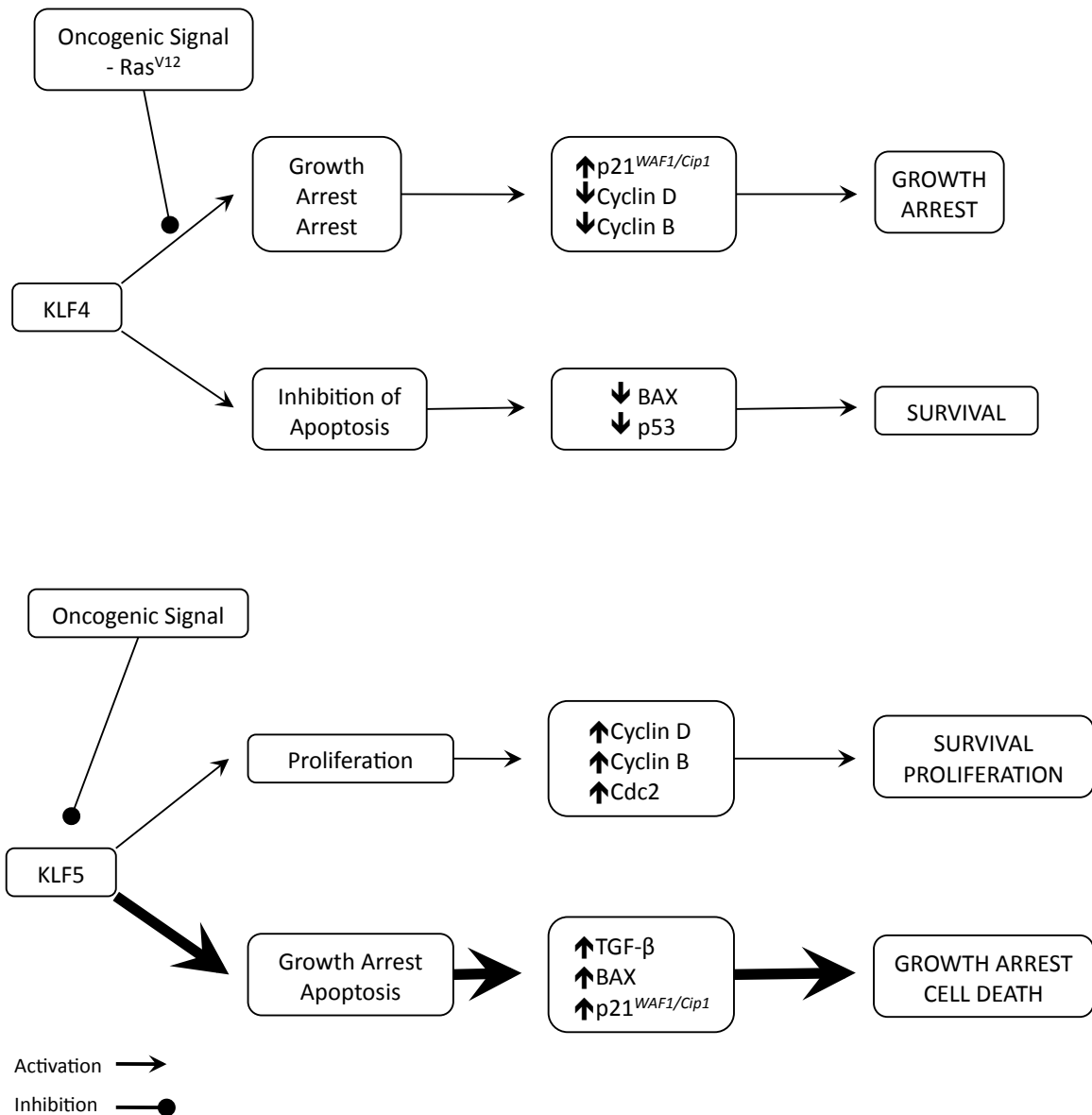
Figure 51. Models for the dual roles of KLF4 and KLF5 in tumor suppression and oncogenesis. KLF4 normally acts as a tumor suppressor through p21$^{Cip1/Waf1}$-dependent cell cycle arrest. In the presence of an oncogenic signal the p21$^{Cip1/Waf1}$ pathway is inactivated, providing a survival advantage, allowing for cellular transformation.

The pro-proliferative activity of KLF5 contributes to transformation in response to oncogenic signals, such as H- or K-Ras. However, in certain contexts, KLF5 possess growth inhibitory activities in the presence of an oncogenic signal, acting as a tumor suppressor, overriding its growth-promoting effects. Slightly modificated from McConnell et al.[100]

Finally, analyzing 86 primary gastric tumor, 51 lymph node metastasis, and 60 normal human gastric tissue specimens supported the tumor suppression role of KLF4 in gastric cancer. KLF4 expression in the primary tumor tissue specimens was significantly decreased or lost when compared with the KLF4 expression in the normal gastric tissue (Figure 36). Moreover, weak or negative staining of KLF4 was associated with higher grade of residual disease status (P = 0.003) and progression from stage I to stage IV disease according to the American Joint Committee on Cancer staging, was associated with a progredient decrease of KLF4 expression (P = 0.005) (Figure 36), suggesting that the loss of KLF4 expression contributed to gastric cancer progression.

Comparisons of KLF4 expression in normal gastric mucosa, primary tumors, and metastatic lymph nodes, revealed the lowest KLF4 expression in metastatic lymph nodes, suggesting that loss of KLF4 may also contribute to gastric cancer metastasis (Figures 37A, 37B, 37C, and Figure 38).

It is important to mention that this loss of KLF4 expression was associated with poor survival, according to Kaplan-Meier plots of overall survival (P < 0.001; Figure 39). Moreover, using multivariate survival analysis KLF4 was an independent prognostic factor to predict the outcome of patients with gastric cancer (P = 0.017), whereas patients' gender or age at diagnosis, completeness of resection, and Lauren's histology type had no statistically significant effect on survival in the multivariate analyses.

All of this clinical and experimental evidence strongly suggests that KLF4 functions as a tumor suppressor gene in human gastric cancer. Furthermore, its alteration of expression seems to play an important role in gastric cancer development and progression. Therefore, KLF4 pathway may be a potential target for the treatment of gastric cancer.

Conclusion

The work presented in this thesis, which is based on our publication in *Cancer Research* in 2005: "Drastic Down-regulation of Krüppel-Like Factor 4 Expression Is Critical in Human Gastric Cancer Development and Progression" provides new insights of the role of KLF4 in human gastric cancer.

First, we discovered the distinct KLF4 expression patterns in normal gastric and gastric tumor tissues. Specifically, we found that KLF4 protein was expressed in the cytoplasm and nuclei of cells localized predominantly in the glandular epithelium (glandular differentiation), suggesting that KLF4 plays an important role in the homeostasis and maintenance of gastric mucosa. In contrast, we observed a substantially decreased or lost KLF4 expression in both gastric tumor specimens and tumor cell lines.

Second, restored expression of KLF4 significantly inhibited gastric cancer growth in vitro and tumorigenicity in animal models.

Third, mechanism study showed that promoter hypermethylation and hemizygous deletion were found in a subset of gastric cancer tissues and cell lines and restoration of KLF4 expression induced typical apoptosis in gastric cancer cells.

Finally, we observed an inverse correlation between decreased KLF4 expression and survival, and the expression of KLF4 was an independent prognostic factor to predict the outcome of patients.

These results show that KLF4 plays an important role in the regulation of homeostasis and maintenance of gastric mucosa and functions as a tumor suppressor in gastric carcinogenesis and progression and that KLF4 pathway is both prognostic marker and potential therapeutic target for human gastric cancer treatment.

Comparison with KLF5 revealed that both, KLF4 and KLF5 can act as either tumor suppressor or as promoter of tumorigenesis, depending on the cellular, tissue and genetic context. Further investigations of KLF4 and KLF5 may advance the understanding of the physiological and pathophysiological roles of KLF4 and KLF5 in regulating cellular proliferation and tumor formation in diverse tissues.

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