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The function of Krüppel-like factor 4 in esophageal squamous cell carcinoma

Geerte Struik
S1978594

Supervisor: Prof. dr. Frank Kruyt
Department of Medical Oncology, UMCG

TABLE OF CONTENTS

The function of Krüppel-like factor 4 in esophageal squamous cell carcinoma	1
Abstract	1
Introduction.....	1
KLF Family.....	3
KLF4	4
Name and Expression	4
Major Functions of KLF4	5
Structure and mechanism of action of KLF4	6
Role and regulation of KLF4 in cancer	8
KLF-4 in esophageal squamous cell carcinoma	9
Tumor suppressor function of KLF 4 in esophageal sqamous cell carcinoma	9
KLF4 as an oncogene in esophageal squamous cell carcinoma.....	11
Discussion	13

Krüppel-like factor 4 is a tumor suppressor in
gastro-intestinal squamous cell carcinoma

The function of Krüppel-like factor 4 in esophageal squamous cell carcinoma

Abstract

Krüppel-like factor 4 (KLF4) is one of the best-described members of the KLF family and has a differential expression in several tissues. Krüppel-like factors are transcription factors that contain 3 C-terminal C2H2-type DNA binding zinc fingers and are involved in development, proliferation, differentiation, and apoptosis. Further, Krüppel-like factors have also been implicated in the establishment and maintenance of pluripotency and stem cell properties and also important tumor suppressive and oncogenic functions have been discovered in cancer.

KLF4 is highly expressed in differentiating cells of the gastrointestinal tract, as well as the skin. Recently, seemingly contradictory findings showed that Krüppel-like factor 4 can act as an oncogene and as a tumor suppressor in esophageal squamous cell carcinoma. A question that consequently arises is how KLF4 can switch between these modes in esophageal epithelial tissue.

Tissue specific KLF4 knockout in ED/L2 mice lead to the development of epithelial hypertrophy and subsequent dysplasia by six months of age, which confirms that KLF4 might act as a tumor-suppressor and plays an important role in the maintenance of normal homeostasis in esophageal epithelia. Interaction of KLF4 with KLF5, envoplakin and Slurp1 might be involved in this tumor-suppressive mechanism of KLF4. Further, downregulation of KLF4 lead to reduced expression of adhesion molecules like laminin. Reduced adhesion molecule expression was shown to be involved in the metastasis process of cancer cells. The known mechanisms involved in the oncogenic function of KLF4 are interaction of KLF4 with p21^{waf1/cip1} and BAX, leading to reduced apoptosis. Further, KLF4-induced activation of the IKK/NF- κ B pathway in esophageal squamous cell carcinoma may contribute to carcinogenesis. Recent findings have shown that oncogenic and tumor-suppressive function of KLF4 might coexist within the esophageal squamous cell carcinoma. KLF4 overexpression might promote proliferation through cytokine activation within esophageal epithelial cells with subsequent recruitment of inflammatory cells and likely disruption of the epithelial barrier, which promotes pro-carcinogenic inflammatory milieu, whereas interactions of KLF4 with other mechanisms might represent its tumor suppressive functions. Thus, the role of KLF4 may depend on the genetic context and its interaction with tissue-specific proliferation/differentiation pathways.

Introduction

The Krüppel-like family consists of 17 members with functions that are in some cases overlapping and in other cases widely divergent.¹ Krüppel-like factors are transcription factors that play an important role in many fundamental biologic processes including development, proliferation, differentiation, and apoptosis.² Along with these roles, Krüppel-like factors have also been implicated in the establishment

and maintenance of pluripotency and stem cell properties.^{3,4}

Krüppel-like factors contain 3 C-terminal C2H2-type zinc fingers that bind DNA, and were named 'Krüppel-like' due to strong homology in this region with the *Drosophila Melanogaster* gene product Krüppel, a member of the Gap class of segmentation gene products that regulates body segmentation in the thorax and anterior abdomen of the *Drosophila* embryo.⁵

Beside the role for Krüppel-like factors in normal cells and tissues, important tumor suppressive and oncogenic functions have been discovered in cancer.¹ Over the past few years, the number of publications regarding the identification of functions of Krüppel-like factors in cancer has hugely increased, indicating that the study of the functions of KLFs in human cancers is a rapidly emerging field.

Krüppel-like factor 4 (KLF4) is one of the best-described members of the KLF family and has a differential expression in several tissues. KLF4 is highly expressed in differentiating cells of the gastrointestinal tract, including the suprabasal and superficial layers of the esophagus, as well as the skin.⁶ This distribution suggests that KLF4 may function in the switch from proliferation to differentiation in stratified squamous epithelia. In vitro, KLF4 overexpression inhibits proliferation and promotes differentiation of esophageal keratinocytes by keratin 4 activation.^{7,8}

In a number of human epithelial cancers, including esophageal, colorectal, gastric, and bladder carcinomas, KLF4 reportedly is down-regulated.^{9,10,11,12} However, in other contexts, KLF4 may promote carcinogenesis.¹³

Recently, KLF4 loss in esophageal epithelial cells was linked to hyperplasia and squamous cell dysplasia in vivo.¹⁴ Esophageal cancer is the sixth leading cause of cancer death in the world, and worldwide more than 90% of esophageal cancers are squamous cell cancers.^{15,16,17} This form of cancer arises from the uncontrolled proliferation of cells of epithelium lining the esophagus, or cells showing particular characteristics of squamous cell differentiation, such as the presence of keratin, tonofilament bundles, or desmosomes, which are structures involved in cell-to-cell adhesion.¹⁸ Ingestion of alcohol or use of tobacco, chronic irritants responsible for up to 90% of esophageal squamous cell cancers, as well as other dietary risk factors, give rise to a process that generally takes decades before becoming apparent.²³ Because most patients do not show symptoms of disease before the development of later stages of cancer, the early phenotypic changes and molecular events preceding the development of cancer are not well known.^{23, 19}

Alterations in a number of genes have been linked to human esophageal squamous cell cancer, but these genetic alterations were identified by examining existing esophageal squamous cell cancers.²³ Thus, little direct evidence has linked genetic alterations functionally to the development of esophageal squamous cell cancer.

In this essay, the role and functions of KLF4 in esophageal squamous cell carcinoma is described. In addition, key pathways involved are highlighted and further areas of investigation are proposed, which

may lead to new insights and discoveries for cancer diagnosis and treatment.

KLF Family

As mentioned before, the KLF family consists of 17 members, which can be grouped on the basis of structural or functional relationships. Structural homologies of Krüppel-like factors (KLFs) correlate with functional similarities; this connection is likely due to homologous protein interaction motifs in amino-terminal domains.⁴ Besides, some members of the family are expressed throughout the whole body, whereas others are tissue restricted, leading to the possibility of exclusive or spare functions for each KLF. Deletion of Klf2, Klf5 or Klf6, for example, is lethal in mice, which is indicative of non-redundant functions during development. Several other KLF-knockout mice are viable though, which suggests functional compensation by other factors.²⁰ Constitutive Klf4 knockout is also lethal in the early postnatal phase.²¹ Structurally, all members of the KLF family have a triple zinc-finger DNA-binding domain at the carboxyl terminus, but other regions can be highly divergent. An activation or repression domain is typically located at the amino terminus, and alternative splicing of some KLFs can lead to additional alterations in protein structure.^{22,23} This also leads to the binding of different co-activators, co-repressors or other cofactors, including histone-modifying enzymes, resulting in additional functional diversity.³ Thus, another potential grouping on functional characteristics arises, dividing KLFs that are mostly trans-activators from those that are predominantly repressors.³

KLF expression and activity were found to be altered in human cancers, and individual KLFs can be tumor suppressors or oncogenes, often with context-dependent functions depending on target gene, tissue, tumor type or cancer stage. Cancer-related target genes of the different KLFs are displayed in Table 1. These context-dependent functions of KLFs may be mediated by so-called molecular switches, such as p53, p21 (also known as WAF1 and CIP1; encoded by the CDKN1A gene) or SIN3 transcription regulator homologue A (SIN3A), through alternative splicing, or by post-translational modifications.^{24,25,26,27,28} One of the pathways that might be involved in these processes, is canonical Wnt signaling, which is mediated by β -catenin. For example, KLF4 physically interacts with both β -catenin and transcription factor TCF4 to antagonize β -catenin-TCF binding and inhibit WNT signaling. Other pathways that might be involved are the Ras signaling pathway, signaling via the estrogen receptor, transforming growth factor beta (TGF- β) signaling, NOTCH signaling, since different KLFs normally interact with these pathways.¹

Table 1 Gene targets of KLFs in cancer

KLF	Target
KLF2	CXCR4
KLF4	BAX, BCL2, BIRC5, BMI1, CCNB1, CCND1, CCNE1, CDH1, CDK1, CDKN1A, CDKN1B, CTNNB1, ESR1, FOXM1, KLF5, MCL1, MMP2, NOTCH1, NOXA, ODC1, PUMA, RELA, SNAIL, SLUG, TERT, TP53
KLF5	AKT1, ASK1, BAD, BAX, BIRC5, CCNA2, CCND1, CDH1, CDKN1A, CDKN1B, CDKN2B, CDT1, CTNNB1, E2F3, ESR1, FOXO1, MAPK1, MAPK3, MKK4, MYC, NOTCH1, PDGFA and PIM1
KLF6	ATF3, BAX, BCL2, BCLXL, CCND1, CDH1, CDK4, CDKN1A, CDKN1B, CTNNB1, MAPK1, MAPK3, MCL1, MDM2, MMP9, NOXA, PTTG1, SRC, TP53, TWIST and VEGF
KLF8	CCND1, CDH1, CTNNB1, MMP9, USP44
KLF9	ESR1, FOS, KLF4, MYC, NOTCH1, NOXA, PRA, PRB and TERT
KLF10	BI1, CDKN1A and STMN1
KLF11	MYC, SIN3A, SMAD3 and SMAD7
KLF13	PRB
KLF17	ID1

ASK1, apoptosis signal regulating kinase 1; ATF3, activating transcription factor 3; BI1, BAX inhibitor 1; CCN, cyclin; CDK, cyclin-dependent kinase; CDKN, CDK inhibitor; CDT1, chromatin licensing and DNA replication factor 1; CTNNB1, β -catenin; CXCR4, chemokine (C-X-C motif) receptor 4; ESR1, oestrogen receptor- α ; FOX, forkhead box; ID1, inhibitor of DNA binding 1; KLF, Krüppel-like factor; MKK4, MAPK kinase 4; MMP, matrix metalloproteinase; ODC1, ornithine decarboxylase 1; PDGFA, platelet-derived growth factor- α ; PR, progesterone receptor; PTTG1, pituitary tumour-transforming 1; PUMA, p53-upregulated modulator of apoptosis; SIN3A, SIN3 transcription regulator homologue A; STMN1, stathmin 1; TERT, telomerase reverse transcriptase; USP44, ubiquitin specific peptidase 44; VEGF, vascular endothelial growth factor. (based on: Tetreault et al, 2013)

KLF4

Name and Expression

KLF4 was given two names since it was discovered by 2 different laboratories: gut-enriched Krüppel-like factor (GKLF) due to the fact that it was found to be highly expressed in the intestine and epithelial zinc finger (EZF) due to its high expression in the skin epithelium.^{29,30} GKLF/EZF was later renamed KLF4 to avoid confusion, as expression of KLF4 is also detectable in many other tissues. In addition, KLF4 is

Krüppel-like factor 4 is a tumor suppressor in gastro-intestinal squamous cell carcinoma

important in development, as it is detectable and imperative in the mouse embryo, with the highest expression occurring in the later stages.^{22, 31}

Major Functions of KLF4

Inhibition of cell proliferation

KLF4 is known to induce growth arrest, while it inhibits cell proliferation by regulating the expression of key cell cycle genes.¹² In actively proliferating NIH3T3 mouse fibroblast cells, KLF4 expression is low. When these cells are subjected to serum starvation, KLF4 levels were significantly higher expressed and the expression of KLF4 in NIH3T3 cells resulted in inhibition of DNA synthesis.²⁹

Progression through cell cycle is driven by cyclins and cyclin dependent kinases (Cdks) which phosphorylate and inactivate cell cycle inhibitors like p16 and p21 and allow the cells to go through the cell cycle.^{32,33} KLF4 is known to activate a number of genes, which function as negative regulators of cell cycle as well as suppresses genes that promote cell cycle progression. Further, KLF4 has been shown to inhibit cell proliferation by blocking G1/S progression in cell cycle and to mediate p53 dependent G1/S cell cycle arrest in response to DNA damage.^{34,35,36} Other studies suggest that KLF4 is a critical factor in regulating entry of the cells into the mitotic phase.³⁶ The CDKN1A gene encoding the cyclin dependent kinase inhibitor p21 is a transcriptional target for tumor suppressor signalling pathways, which include p53, transforming growth factor beta (TGF- β) and activated protein C (APC). A lot of evidence supports the finding that KLF4 plays a vital role in regulating p21 gene expression. This process is further described below.

Activation of p21 leads to downregulation of the expression of cyclin D and cyclin B, thereby restricting the entry of the cells from G1 to S and from G2 to M.^{37,38} KLF4 mediates p53 transactivation activity on the p21 promoter upon DNA damage (e.g. γ radiation induced damage) and in turn p53 upregulates KLF4 promoter activity. Also, the levels of KLF4 mRNA increase in a p53 manner, which coincides with the increased expression of p21 when the cells are exposed to γ radiation.⁹⁴

Promotion of cell differentiation

As previously discussed, KLF4 plays a vital role in goblet cell differentiation in the intestine, conjunctiva and also in the formation of the epithelial barrier of the skin.^{74,108} Its has been shown to be higher expressed in well-differentiated cells than in actively proliferating cells. Microarray analysis showed that many keratin genes were upregulated on KLF4 induction, which reflects its role in epithelial differentiation. Besides, KLF4 has been reported to transactivate promoters of epithelial genes like CYP1A1, laminin α 3A, laminin 1, keratin 4 in the esophagus, keratin 19 in the pancreas and keratin 12 and Aqp5 in the cornea.^{39,40,41, 54,91}

An interesting and at the same time somewhat contradictive finding was that KLF4 has been shown to repress TGF β -dependent increase of smooth muscle cell differentiation marker genes, which includes α -

smooth actin and SM22 α .⁴² Another recent study has shown that in response to vascular injury, KLF4 was rapidly upregulated.⁴³ KLF4 is normally not expressed in differentiated smooth muscle cells in vivo. These results indicated that KLF4 represses smooth muscle cell genes, and suggested that KLF4 may be a key effector of platelet derived growth factor- (PDGF) BB and injury-induced phenotypic switching of smooth muscle cells.⁴⁴ Even though these findings might contradict an earlier reported function of KLF4 as a promoter of cell differentiation, the main activity of KLF4 leads to differentiation.

Structure and mechanism of action of KLF4

Human and mouse KLF4 are 470 and 483 amino acids in length, respectively, and produce a 55kDa protein. KLF4 can be divided into three separate domains: an N-terminal activation domain, a central repressive domain and a C-terminal DNA binding domain.^{45,46} The DNA binding domain consists of three successive zinc fingers (Figure 1). Each Zinc finger contains an anti-parallel β -sheet, followed by a short loop and an α -helix. Within the β -sheet, two cysteines and within the α -helix two histidines work together to coordinate a single zinc-ion, which stabilizes the fold. Each zinc finger interacts with three consecutive nucleotides on a target DNA sequence. Adding additional zinc fingers can increase the sequence specificity of a zinc finger protein.⁴⁷



Figure 1. Subdomains for KLF4. ZN² indicates Zn²⁺, Yet et al,

In general, KLF4 interacts with GT-rich or CACCC elements on target genes.^{48,49} KLF4 is exclusively nuclear and appears to contain two discrete nuclear localization sequences (NLS), which is an amino acid sequence that tags a protein for import into the nucleus by nuclear transport. The first is a basic hexapeptide sequence just N-terminal to the three C-terminal zinc fingers and the second is placed within the first two zinc fingers themselves.⁵⁰

Mechanism of activation

Activation of transcription of target genes is major function of KLF4. Consistent with this function, the N-terminus of KLF4 contains a strong transactivation domain.^{51,52,53} The transactivation domain alone, when directly fused to its three C-terminal zinc fingers, is sufficient to activate a synthetic reporter construct.³² In addition, the N-terminal domain interacts with the transcriptional co-activators p300 and CBP, which is required for its function. Point mutations that block interactions of the N-terminal domain with CBP also completely blocks its ability to activate transcription.^{2, 32} p300/CBP are histone acetyltransferase (HAT) proteins, and recruitment of p300/CBP results in an increase in localized histone acetylation at the promoter. Acetylation of histones leads to the recruitment of other transcription factors as well as the basal transcriptional machinery. Further, KLF4 itself is acetylated by p300/CBP at

lysine residues 225 and 229. Acetylation of KLF4 appeared to be important for its function, since mutation of the two lysines to arginine, significantly decreases the ability of KLF4 to transactivate target genes, as well as its ability to inhibit proliferation.²

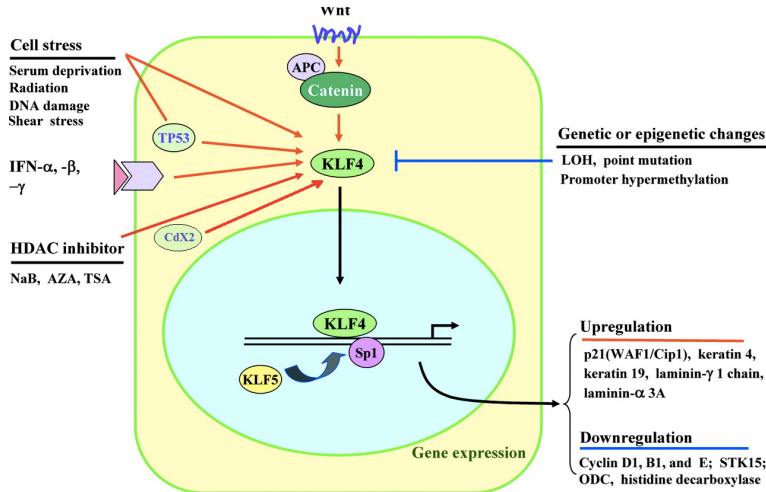


Figure 2 Diagram of the major pathways of KLF4. KLF4 expression can be upregulated by cell stress signal and interferon (IFN)- α , - β , and - γ . KLF4 expression is also regulated by the Wnt/APC signal. LOH, point mutations in the coding region and promoter hypermethylation can cause KLF4 gene silencing. KLF4 enters the nucleus and binds to the gene promoter region to regulate cell proliferation or differentiation-related gene expression. (Wei et al, 2005)

One report stated that KLF4 could interact with Tip60, a bi-functional cofactor that contains intrinsic histone acetyltransferase (HAT) activity, but can also recruit histone deacetylase 7 (HDAC7).⁵⁴ Tip60 is a co-activator for several nuclear hormone receptors as well as amyloid precursor protein (APP) but appears to function as a co-repressor for transcription factor STAT3 by recruiting HDAC7.^{55,56,57} Further, another zinc finger protein Krox20, can directly interact with KLF4 and together they activate the CCAAT/Enhancer-binding protein beta (*CEBPB*) gene in 3T3-L1 cells.⁵⁸ Besides, KLF4 interacts with the NF- κ B subunit p65/RelA which leads to synergistical activation of the expression of the enzyme iNOS, which on its turn catalyses the production of signalling molecule NO.⁵⁹ These findings indicate that the mechanisms of

transactivation mediated by KLF4 may be gene-dependent.

Mechanism of repression

One of the (passive) mechanisms for repression by a transcription factor is to simply compete with an activator for binding to a target DNA sequence. KLF4 binds to a sequence that overlaps a sequence on the CYP1A1, HDC, and SP1 genes, which is recognized by the activator Sp1, displacing Sp1 from the promoter and resulting in repression of the target gene.^{16,60,61} Since Sp1 is ubiquitously expressed and positively regulates many genes, it is likely this mechanism is used by KLF4 to repress many of its target genes.⁶² Further, instead of or in addition to passive repression via competition with a transcriptional activator, the presence of the central repressive domain in KLF4 suggests that KLF might actively repress the expression of some genes.

KLF4 recruits and interacts with HDAC1 and HDAC2 to repress the CD11d gene, whereas it represses cyclin B₁ via specifically recruiting of HDAC3.^{16,63} On the TP53 gene, cell surface associated Mucine 1 (MUC1-C) recruits KLF4, as well as HDAC1 and HDAC3, to mediate repression.⁶⁴ KLF4 inhibits Smad3-mediated activation of PAI-1 by directly competing with Smad3 for p300 binding.³⁸ Smad3 are intracellular proteins that transduce extracellular signals from TGF β ligands to the nucleus where they

activate downstream gene transcription. Finally, KLF4 represses transcriptional targets of Wnt signaling by directly interacting with β -catenin/TCF4.³⁹ These data also strongly suggest that KLF4-mediated activation and repression is complex and gene-dependent (Figure 2).

Given the large number of genes regulated by KLF4, it is not unexpected that expression of KLF4 itself should be highly regulated. A report has shown that KLF4 has a half-life of only 2 hours in the colon cancer cell line HCT116 and is quickly degraded by the proteasome.⁶⁵ However, a variety of stimuli can stimulate KLF4 expression including serum starvation, contact inhibition,⁶⁶ interferon-^{67,68}, sodium butyrate,⁶⁹ cAMP,⁷⁰ gastrin,³³ DNA damage,³⁹ and oxidative stress (figure 2).^{71,72}

The precise mechanism of how the majority of these stimuli increase the expression of KLF4 is unclear, although possibilities include increased transcription of the KLF4 gene, increased mRNA stability and increased protein stability.

Role and regulation of KLF4 in cancer

It appears that KLF4 is a complex transcription factor that can act as a transcriptional activator and as a transcriptional repressor. As mentioned before, KLF4 can, like many KLFs, also act as an oncogene or a tumor suppressor, depending on the cellular context. KLF4 plays a critical role as transcription factor in regulating cell proliferation. Since cancers display uncontrolled cell growth, KLF4 is thought to play a key role in cancer progression and development. KLF4 is proved to induce growth arrest, so it can be assumed to have anti-cancerous activity. KLF4 expression is indeed shown to be downregulated in a number of cancers and it is reported to display tumor suppressive activity. Mechanisms that are believed to be involved in the tumor-suppressive function of KLF4 are the Wnt/B-catenin - and Notch signalling pathways.^{73,74}

One of the mechanisms via which KLF4 is believed to exert its oncogenic function is repression of Ras^{V12} - induced senescence in primary fibroblasts. KLF4 also has the ability to repress the tumor suppressor p53, which leads to inhibition of apoptosis and eventually to transformation.⁷⁵ Further, KLF4 inhibits differentiation of embryonic stem cells and, in combination with other factors, de-differentiates adult cells into cells with a stem-like character.⁷⁶ This leads to the suggestion that KLF4 may contribute to self-renewal activity of cancer stem cells.⁷⁷

Consistent with this, seemingly contradictory findings showed that KLF4 can indeed act as an oncogene and as a tumor suppressor in esophageal squamous cell carcinoma. A question that consequently arises is how KLF4 can switch between these modes in esophageal epithelial tissue. Here, the possible underlying mechanisms of this contradictive functioning of KLF4 will be addressed.

KLF-4 in esophageal squamous cell carcinoma

The development of human esophageal squamous cell cancer is a multi-step process starting with esophageal basal cell hyperplasia, dysplasia, carcinoma in situ, and eventually progression towards advanced carcinoma.⁷⁸

The molecular mechanisms underlying this progression are related to a series of errors in cell polarity, proliferation, differentiation and apoptosis. Further, as mentioned before, genetic alterations of cell adhesion molecules, growth factors, cell cycle regulators, and pro- and anti-apoptotic factors also contribute to esophageal carcinogenesis.⁷⁹

Nonetheless, the specific epithelial transcription factors that regulate these processes in esophageal carcinogenesis are not fully established. The effect of KLF4 on epithelial proliferation and differentiation suggest that these factors may also play key roles in esophageal squamous cell carcinogenesis.

The first reports of KLF4 in relation to esophageal squamous cell carcinogenesis mostly focussed on downregulation of KLF4 and consequently possible tumor suppressive activity of KLF4.⁸⁰

Tumor suppressor function of KLF 4 in esophageal squamous cell carcinoma

KLF4, which has been shown to be down-regulated in esophageal squamous cell carcinoma in human and mice, is likely to have tumor-suppressive properties in esophageal keratinocytes, as suggested in many studies.^{81,82,83} This loss of KLF4 seems to occur due to hypermethylation or loss-of-heterozygosity,⁸⁴ which is the case for many KLFs, and produces epithelial hypertrophy, increased proliferation, altered cell morphology with evidence of delayed cellular maturation, and eventually esophageal epithelial dysplasia in mice by 6 months of age.⁸⁵

Tissue-specific KLF4 knockout and effect on differentiation/proliferation switch

In order to knockout KLF4 tissue specifically, Cre-Lox recombination technology was used, known as a site-specific recombinase technology. Cre-Lox recombination is widely used to carry out deletions, insertions, translocations and inversions at specific sites in the DNA of cells. This allows the DNA modification to be targeted to a specific tissue or to be triggered by a specific external factor.⁸⁶ In a study by Tetreault et al, 2010, the ED-L2 promoter of Epstein-Barr virus was used to drive *Cre*, which created the possibility to induce tissue specific knockout of KLF4 in the esophagus.⁸⁷ This tissue-specific KLF4 knockout led to increased basal cell proliferation and a delay in cellular maturation in mice. Consequently, these mice developed epithelial hypertrophy and subsequent dysplasia by six months of age, which again indicates that KLF4 might act as a tumor-suppressor and plays an important role in the maintenance of normal homeostasis in esophageal epithelia.⁶⁷

Even though tissue specific downregulation of KLF4 and subsequent esophageal squamous cell carcinogenesis suggest that KLF4 might be a tumor suppressor, the exact regulation of this process is not

fully established. Recently though, possible underlying mechanisms have been proposed.⁶⁷ In the esophageal epithelia of the mice used in the study by Tetreault et al, 2010, a 1.9-fold increase in proliferation was registered. Esophageal epithelia proliferation has been shown to be regulated by KLF5 in vitro and in vivo, and mice with transgenic expression of KLF5 in the esophagus, display increased proliferation confined to the basal layer of the esophagus.^{14,88} A suggested explanation for this finding is the normally repressive function of KLF4 towards KLF5 in transit-amplifying cells as they exit the esophageal basal layer. Besides, KLF4 may compete with KLF5 for binding to target genes. Overall, KLF4 expression seems to be turned on in the suprabasal layer of the esophagus, KLF4 represses KLF5 both transcriptionally and post transcriptionally and at the same time competes with KLF5 for binding to the promoters of key regulatory genes, thus switching cells from the proliferation to differentiation program.

A potential KLF4 target that may be critical for KLF4 function in squamous cell epithelia is *envoplakin*, a cytoskeletal linker protein and a component of the epidermal cornified envelope.^{67,89} The epidermis of *envoplakin* knockout mice has a higher proportion of immature cornified envelopes than that of control mice.⁶⁷ Among others, this major cornified envelope component is induced during keratinocyte terminal differentiation.⁹⁰

Another identified gene that was differentially expressed was *Slurp1*, encoding a secreted member of the LY6/PLAUR protein family, which was decreased between 7-9-fold in ED-L2/Cre KLF4^{LoxP/LoxP} mice.⁶⁷ SLURP1 is a marker of late keratinocyte differentiation expressed in the granular layer of the skin and, as a key ligand for the $\alpha 7$ nicotinic acetylcholine receptor, is important for terminal differentiation of epidermal keratinocytes, for homeostasis and for the formation of the skin barrier.^{91,92}

All these recent findings are intriguing but the exact roles of the genes in oesophageal epithelial homeostasis, hyperproliferation and carcinogenesis remain to be elucidated and are currently under investigation.⁶⁷

KLF4 downregulation and metastasis

Another finding that subscribes the role of KLF4 as a tumor-suppressor was the observation that downregulation of KLF4 expression in an esophageal squamous cell carcinoma EC9706 cell line decreased its adhesion, implicating that KLF4 is involved in the metastasis of esophageal cancer. The basic mechanism of tumour cell metastasis is reduced expression of adhesion molecules, which results in an increased migratory ability of the cancer cells.⁹³ Abnormal expression of such cell adhesion molecules as cadherin, integrin and mucin have been proven to have relationship with cancer metastasis.^{94,95} It is known that KLF4 could regulate mucin 5B (MUC5B) gene transcription directly.⁹⁶ KLF4 also regulates laminin expression while laminin is a component of extracellular matrix, which plays a key role in cell adhesion and migration.⁹⁷ More frequent allelic loss at 9q region where the KLF4 gene

located was reported in esophageal squamous cell carcinoma patients with metastasis, suggesting a clinically significant role of KLF4 gene in esophageal cancer.⁹⁸

These results, as mentioned before, suggest that down-regulation of KLF4 may contribute to malignant phenotype of esophageal cancer.⁹⁹

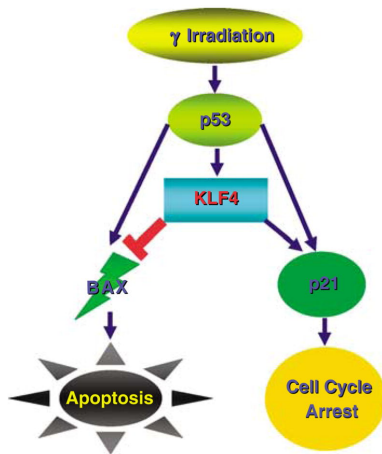
Beside its role as a tumor-suppressor, recent findings indicated that KLF4 might also act as an oncogene in esophageal squamous cell carcinoma.

In this process, the ability of KLF4 to affect the levels of expression of the cell-cycle regulator p21 seems to be involved. Another possible oncogenic function of KLF4 might be caused by its interaction with pro-inflammatory pathways.

KLF4 as an oncogene in esophageal squamous cell carcinoma

Anti-apoptotic functions of KLF4 in ESC

KLF4 transcriptionally regulates a number of genes critical for gastrointestinal and esophageal tumor formation, including ornithine decarboxylase, p21^{WAF1/CIP1}, the cyclin D1 oncogene, and keratin 19, which are linked to tumor progression in the esophagus and pancreas.¹⁰⁰



An explanation for these findings is primarily the effect of KLF4 on p21^{waf1/cip1}, also known as cyclin-dependent kinase inhibitor 1, and BAX. The protein p21^{waf1/cip1} usually inhibits the activity of several cyclin/cyclin-dependent kinase complexes and blocks cell-cycle progression, induces growth arrest and repair mechanisms in response to DNA damage.^{101,102} The expression of BAX, a bcl-2-like protein, promotes apoptosis in response to DNA damage. Notably, p21^{waf1/cip1} polymorphisms and downregulation of BAX have been described in esophageal cancer.^{103,104,105}

Both p21^{waf1/cip1} and BAX are regulated by p53, whereas KLF4 is a crucial mediator for the checkpoint functions of p53 after γ -irradiation induced DNA damage.¹⁰⁶ KLF4 does so by inhibiting the transition from the G₁ to S and G₂ to M phases of the cell cycle. Recent findings have shown that γ -irradiated cells underwent apoptosis if KLF4 was absent in three independent cell systems including colorectal cancer cells and mouse embryo fibroblasts in which expression of KLF4 could be manipulated. In

Figure 3 A model for the role of KLF4 in suppressing apoptosis after γ -irradiation. Expression of KLF4 is activated in a p53-dependent manner after γ -irradiation. The increased KLF4 leads to increased p21^{WAF1/CIP1} and decreased BAX expression. Apoptosis is blocked and cell cycle arrest induced. (Ghaleb et al, 2007)

the presence of KLF4, the degree of apoptosis was significantly reduced and cells resorted to checkpoint arrest. The mechanism by which KLF4 accomplished this anti-apoptotic effect is by activating expression of the cell cycle arrest gene p21^{WAF1/CIP1}, and by inhibiting the ability of p53 to trans-activate expression of the pro-apoptotic gene, BAX.³⁵ This illustrates an unexpected anti-apoptotic function of KLF4, and

suggest that KLF4 may be an important determinant of cell fate following γ -radiation-induced DNA damage.

Thus, expression of KLF4 may determine the response of the cell to DNA damage, either growth arrest and repair or programmed cell death.⁸¹

Decrease in apoptosis is one of the hallmarks of cancer and if the repair mechanism of the cell is defect and the cell cycle continues after its arrest, the proliferation of malign cells continues.¹⁰⁷

Pro-inflammatory activity of KLF4 via IKK/NF- κ B pathway

A novel pro-inflammatory role for KLF4 via activation of the IKK/NF- κ B pathway within esophageal epithelial cells was presented recently.²⁰ Interestingly, recruitment of inflammatory cells to the site of inflammation in the esophagus is preceded by increased nuclear localization of NF- κ B and increased levels of the cytokines TNF α and CXCL5.^{108,109} This initial activation of the NF- κ B pathway is KLF4-dependent and does not require bacterial infection or injury.²⁰ The arising question is: how does KLF4 activate NF- κ B? Preliminary studies do not suggest a direct effect of KLF4 on NF- κ B transcription, and, given the diversity of stimuli leading to NF- κ B activation, the specific targets of KLF4 may be difficult to appoint.¹¹⁰ Deletion of p120 catenin, a protein found at cell-cell junctions, was previously shown to similarly initiate inflammation in skin keratinocytes through indirect activation of NF- κ B by a complex mechanism, possibly involving induction of RhoA guanosine triphosphatase activity.¹¹¹ In macrophages, KLF4 interacts with the p65 subunit of NF- κ B to cooperatively induce the inducible nitric oxide synthase promoter, but no evidence is found yet confirming that KLF4-p65 binding in keratinocytes was altered by increased KLF4 expression.⁴² Thus, precisely how KLF4 influences NF- κ B activity remains to be determined.

Since inflammation is linked to carcinogenesis in a number of epithelial tissues, and various cytokines and other inflammatory agents can act as tumor promoters in the context of chronic inflammation, the involvement of KLF4 is an interesting finding.¹¹² For example, TNF- α is an important regulator of the early stages of tumor promotion in the skin. CXCL5 is overexpressed in head and neck squamous cell carcinoma and its downregulation inhibits squamous carcinogenesis by decreasing invasion and cell proliferation. NF- κ B signalling is involved in epidermal development, squamous cell homeostasis, chronic inflammatory diseases and cancers, including esophageal squamous cell carcinoma. However, in some mouse models, NF- κ B inhibition in epithelial cells results in the spontaneous development of severe inflammation. Thus a careful balance of NF- κ B activation is required for the maintenance of normal epithelial and immune homeostasis.

Discussion

Krüppel-like factor 4 (KLF4) is one of the best-described members of the Krüppel-like family and is differentially expressed in several tissues. KLF4 is highly expressed in differentiating cells of the gastrointestinal tract, including the suprabasal and superficial layers of the esophagus, as well as the skin. This suggests that KLF4 may function in the switch from proliferation to differentiation in stratified squamous epithelia.¹² In vitro, KLF4 overexpression inhibits proliferation and promotes differentiation of esophageal keratinocytes by keratin 4 activation, supporting the theory that KLF4 acts as a tumor suppressor.⁸³ Indeed, KLF4 reportedly is down-regulated in a number of human epithelial cancers. Yet in other contexts, KLF4 may promote carcinogenesis.

A recent finding linked loss of KLF4 in esophageal epithelial cells to hyperplasia and squamous cell dysplasia in vivo, which might implicate a tumor suppressive function of KLF4.⁸⁷ Another recent report though, established oncogenic activity of KLF4 upon overexpression within esophageal squamous cells.¹⁴ The mechanism of KLF4 loss in esophageal cancers is not known but may be similar to loss in other gastrointestinal cancers, in which hypermethylation and hemizygous deletion have been implicated.⁸⁴

Further, alterations in a number of genes that are known to interact with KLF4, for example the Ras gene and TP53 gene, have been linked to human esophageal squamous cell cancer, but little direct evidence has linked genetic alterations functionally to the development of esophageal squamous cell cancer.¹¹³

KLF4 might exert its tumor suppressor function via repression of KLF5 both transcriptionally and post transcriptionally and via competition with KLF5 for binding to the promoters of key regulatory genes, thus switching cells from the proliferation to differentiation program.⁸³ Further, KLF4 might interact with the protein envoplakin and the SLURP1 gene to induce keratinocyte terminal differentiation. KLF4 downregulation also decreases the expression of cell adhesion molecules, e.g. laminin, implicating that KLF4 normally regulates this process and downregulation is involved in the metastasis of esophageal cancer.¹⁴

Reported oncogenic functions of KLF4 in esophageal squamous cell cancer are anti-apoptotic and pro-inflammatory activity via the p53/BAX pathway and NF- κ B respectively.¹⁰¹

At first, the development of squamous cell dysplasia with both loss and higher expression of KLF4 might seem to be confusing and contradictive. Nonetheless, recent findings have shown it might be possible that the critical event leading to dysplasia and squamous cell carcinoma is inflammation.¹⁴ As mentioned before, inflammation is linked to carcinogenesis in a number of epithelial tissues, and various cytokines and other inflammatory agents can act as tumor promoters in the context of

chronic inflammation. Disruption of the epithelial barrier can induce an inflammatory response, and observations have shown structural changes in esophageal epithelia of ED-L2/KLF4 mice, with dilated paracellular spaces and spongiosis, which is intercellular edema characteristic for epidermal inflammation. Spongiosis occurs in patients with gastro-esophageal reflux disease and in animals exposed to various damaging agents. Since KLF4 induces a pro-inflammatory response in a sterile environment *in vitro*, activation of the NF- κ B pathway might be the initial event after KLF4 overexpression *in vivo*. This might subsequently induce dilatation of paracellular spaces. So in sum, KLF4 overexpression might promote proliferation through cytokine activation within esophageal epithelial cells with subsequent recruitment of inflammatory cells and likely disruption of the epithelial barrier, which promotes a pro-proliferative, pro-carcinogenic inflammatory milieu. Since KLF4 was absent in the tumor cells of the ED-L2/KLF4 model, used by Tetreault et al 2010, KLF4 might in fact function as a tumor suppressor in esophageal squamous cells, as previously proposed.⁸⁷

However, since contradictory reports on functioning of KLF4 within different models exist, it is clear that KLF4 expression must be tightly regulated in normal esophageal epithelia. KLF4 mediates the proliferation-differentiation switch in esophageal keratinocytes and defects in this switch are often associated with cancer. Therefore accumulating evidence suggests that the role of KLF4 may depend on the genetic context and its interaction with tissue-specific proliferation/differentiation pathways, including p53/p21^{WAF1/CIP1}/BAX signalling, IKK/NF- κ B signalling and many other pathways.

Future perspectives

Further research needs to be conducted on the interaction of KLF4 with the NF- κ B processes or other inflammatory pathways. This could possibly be considered in the chemoprevention of esophageal squamous cell cancer.

The reported interactions of KLF4 with the protein envoplakin and the SLURP1 gene, which induce keratinocyte terminal differentiation, are interesting findings and these interactions might be involved in the tumorsuppressive functioning of KLF4. This is also the case for the finding that KLF4 downregulation decreases the expression of cell adhesion molecules like laminin. Downregulation of cell adhesion molecules is, as previously mentioned, involved in the metastasis of esophageal cancer. These processes need to be further investigated in the future and might be important markers or possibly give rise to targeted therapeutic options.

Further, the interaction of KLF4 with An p21^{waf1/cip1} and BAX, leading to reduced apoptosis, needs to be elucidated since targeting of this pathway may lead to increased apoptosis in esophageal squamous cell carcinoma.

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