UNIVERSITÀ DEGLI STUDI DI NAPOLI

"FEDERICO II"

Scuola di Dottorato in Medicina Molecolare

Dottorato di Ricerca in Patologia e Fisiopatologia Molecolare

"The Neutrophil gelatinase-associated lipocalin (NGAL), a NF-κB regulated gene, is a survival factor for thyroid neoplastic cells"

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UNIVERSITA' DEGLI STUDI DI NAPOLI

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Dipartimento di Biologia e Patologia Cellulare e Molecolare "Luigi Califano"

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XXI ciclo

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Candidato: dott. Alessio Iannetti

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INTRODUCTION

NF-κB

Nuclear factor-κB (NF-κB) was identified twenty years ago as a regulator of expression of the κB light chain in B cells. NF-κB transcription factors are important in integrating multiple stress stimuli and regulating innate and adaptive immune responses seen in states of inflammation (1).

The NF-κB family of transcription factors consists of five members, p50, p52, p65 (RelA), c-Rel and RelB, which share an N-terminal Rel homology domain (RHD) responsible for DNA binding and homo- and heterodimerization (Figure 1). NF-κB dimers bind to κB sites within the promoters/enhancers of target genes and regulate transcription through the recruitment of coactivators and corepressors. The transcription activation domain (TAD), necessary for the positive regulation of gene expression, is present only in p65, c-Rel and RelB. As they lack TADs, p50 and p52 may repress transcription unless associated with a TAD-containing NF-κB family member or other proteins capable of coactivator recruitment.

Crystal structures of NF-κB dimers bound to κB sites reveal how the immunoglobulin-like domains that comprise the RHD contact DNA. The NH_2 – terminal Ig-like domain confers selectivity for certain types of κB sites, whereas the hydrophobic residues within the C-terminal domain provide the dimerization interface between NF-κB subunits. Although RHD and TAD function are typically considered independent, both domains undergo posttranslational modifications that can affect NF-κB transcriptional activity as well as DNA binding (Figure 1).

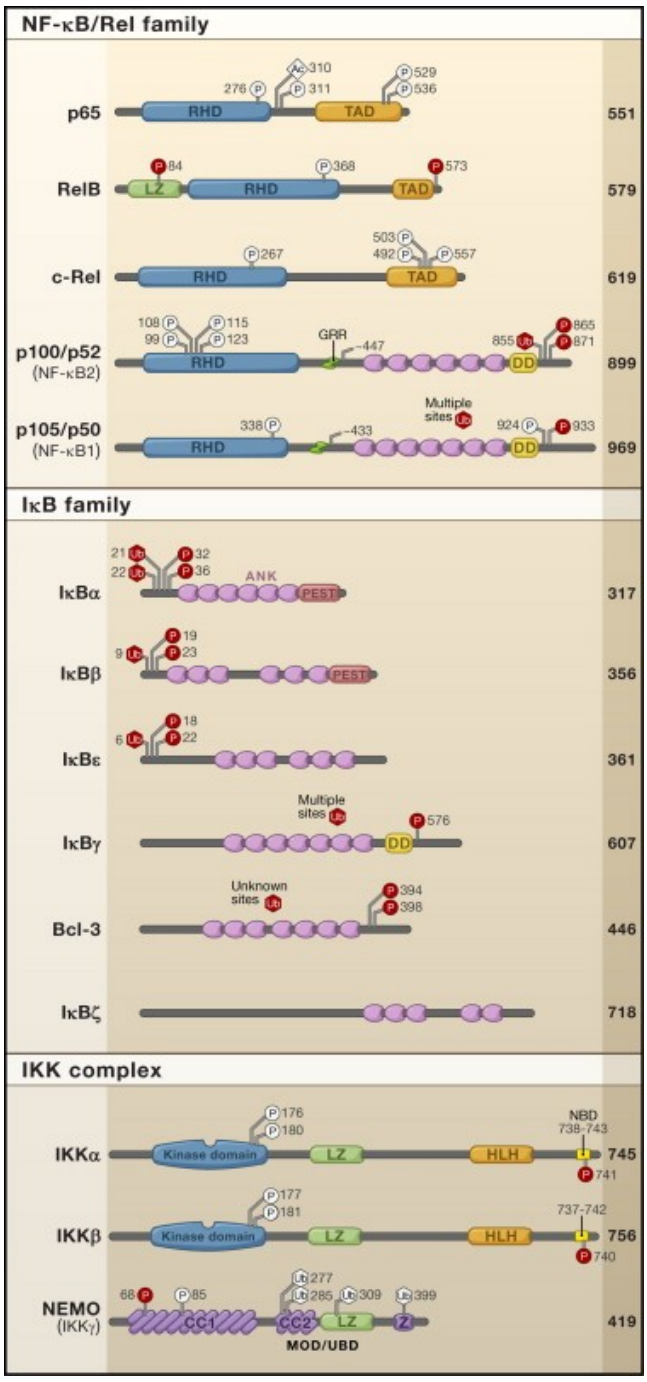


Figure 1. The NF-κB, IκB, and IKK Protein Families

In its inactive state, NF-κB dimers are associated with one of three typical IκB proteins, IκBα, IκBβ, or IκBε, or the precursor proteins p100 and p105. These IκBs maintain NF-κB dimers in the cytoplasm and are crucial for signal responsiveness. There are two inducibly expressed, atypical IκB proteins, Bcl-3 and IκBζ, that

function quite differently in the regulation of NF-κB. Lastly an alternative transcript of the p105 gene in mouse encodes an IκB molecule, IκBγ, whose biological role remains unclear. All IκB proteins are characterized by the presence of multiple ankyrin repeat domain (Figure 1). The prototypical and most extensively studied member of the family is IκBα. IκBα is rapidly degraded during activation of canonical NF-κB signaling pathways leading to the release of multiple NF-κB dimers, although the p65:p50 heterodimer is likely the primary target of IκBα. The established model of IκB function posits that IκBα retains NF-κB dimers in the cytoplasm, thereby preventing their nuclear translocation and subsequent DNA binding; however, the situation is actually more complex. The crystal structure of IκBα bound to the p65/p50 heterodimer reveals that the IκBα protein masks only the nuclear localization sequence (NLS) of p65, whereas the NLS of p50 remains exposed. The exposed NLS of p50 coupled with nuclear export sequences (NES) in IκBα and p65 leads to constant shuttling of IκBα/NF-κB complexes between the nucleus and the cytoplasm, despite steady-state localization that appears almost exclusively cytosolic (2). Degradation of IκBα drastically alters the dynamic balance between cytosolic and nuclear localization signals to favor nuclear localization of NF-κB. The noncanonical or alternative NF-κB pathway, however, proceeds through proteasomal processing, rather than degradation, of p100 to p52, thereby liberating p52 containing NF-κB dimers that drive a transcriptional response that is distinct from that induced by the canonical, IκBα-regulated pathway. In part because IκBα degradation and p100 processing regulate different populations of NF-κB dimers, canonical and noncanonical NF-κB pathways regulate distinct sets of target genes (Figure 2).

Degradation of IκB is a rapidly induced signaling event that is initiated upon specific phosphorylation of these molecules by activated IKK. The IKK complex contains two highly homologous kinase subunits, IKKα and IKKβ, and a regulatory

subunit NEMO (NF-κB essential modulator) (3). Although they are generally found in a heteromeric kinase complex, IKKα and IKKβ are somewhat selectively required for specific NF-κB signaling pathways. In most canonical NF-κB signaling, e.g., downstream of TNFR1, IKKβ is both necessary and sufficient for phosphorylation of IκBα on Ser32 and Ser36 and of IκBβ on Ser19 and Ser23. While not generally required for IκBα phosphorylation and degradation in canonical signaling pathways, IKKα can mediate IκBα phosphorylation and appears to play a critical role in canonical NF-κB-dependent transcriptional responses. The noncanonical pathway, conversely, depends only on the IKKα subunit, which functions by phosphorylating p100 and causing its inducible processing to p52. The noncanonical pathway is activated by a subset of TNFR superfamily members, while the canonical pathway is activated by a broader and overlapping array of receptors (Figure 2).

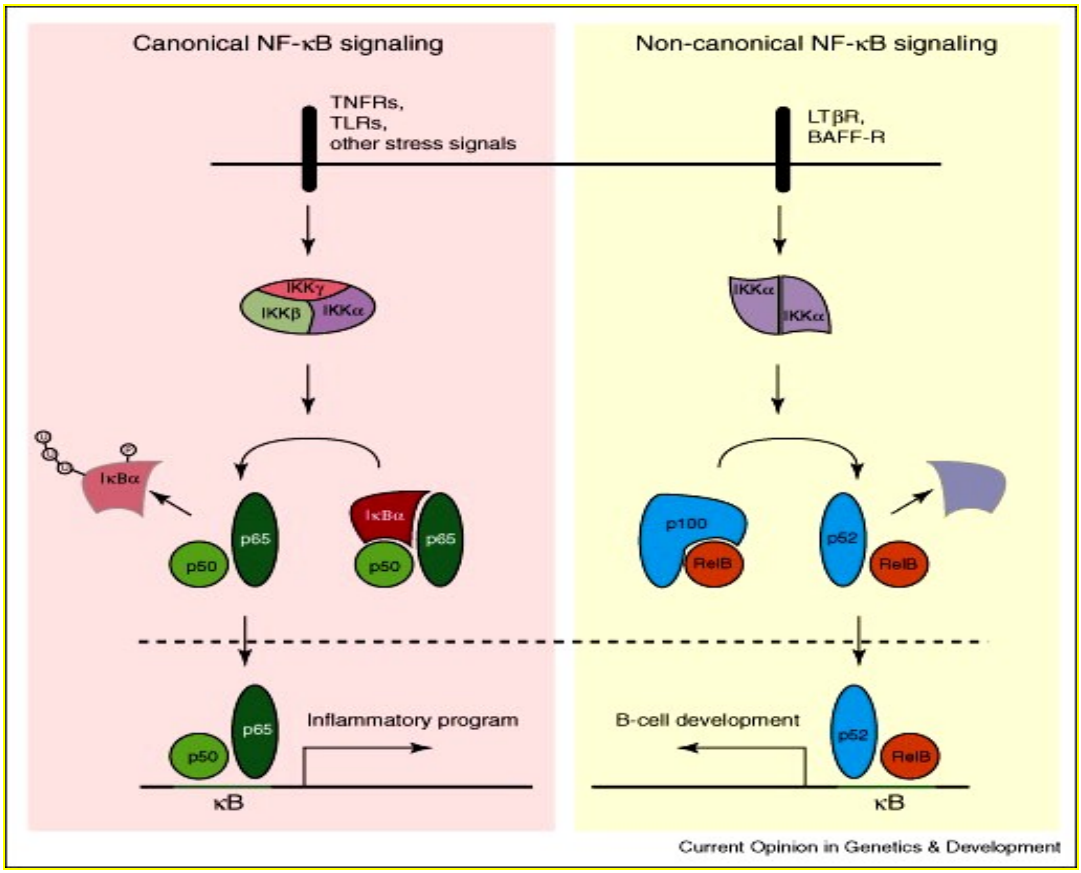


Figure 2. NF-κB signaling pathways

Phosphorylation of the conserved serine residues ($DS^{*}GXXS^{*}$) in IκB proteins results in their K48-linked polyubiquitination by βTrCP containing Skp1-Culin-Roc1/Rbx1/Hrt-1-F-box (SCF) E3 ubiquitin ligase complexes ($SCF^{\beta TrCP}$) coordinately with the E2 UbcH5 (4). The released NF-κB dimers bind promoter and enhancer regions containing κB consensus sequences 5′ GGGRNWYYCC 3′ (N—any base; R—purine; W—adenine or thymine; and Y—pyrimidine) (5). The degenerate nature of the κB site sequence, which shows far greater sequence variability than the consensus sequence given here, combined with the varied binding preferences of NF-κB dimers yields the large list of NF-κB-regulated genes (6). Transcription of target genes is further regulated through posttranslational modifications of NF-κB that affect the ability of NF-κB dimers to interact with transcriptional coactivators. NF-κB-dependent transcription of IκB proteins as well

as additional mechanisms targeting DNA-bound NF-κB dimers terminate the response (7).

NF-κB and cancer

Activation of NF-κB (usually assessed by the presence of nuclear RelA) has been observed in many cancers, including but not limited to breast cancer (8), melanoma (9), lung cancer (10), colon cancer (11), multiple myeloma (12), pancreatic cancer (13), esophageal adenocarcinoma (14), and various types of leukemias (15-17) and lymphomas (18-19). The presence of activated NF-κB in tumors does not, however, establish a causal link. Only with the advent of recent advances in experimental mouse models of cancer have investigators been able to tie specific functions of NF-κB activation to the carcinogenesis process, as well as tumor progression and metastatogenesis (20).

The role of NF-κB in solid tumors has been well documented in several studies performed on primary tumors and neoplastic cell lines derived from different human tissues. These studies show that the inhibition of constitutive NF-κB activity blocks the oncogenic potential of neoplastic cells by different ways: by sensitizing tumor cells to chemotherapeutic drug-induced apoptosis, by decreasing the highly proliferative rate which characterizes transformed cells, by inhibiting tissue invasiveness and metastatic potential of highly malignant cells (21). In addition, since it is now generally accepted that chronic inflammation contributes to the genesis of many solid tumors, such as gastric, colon or hepatic carcinomas, it has been recently shown that activation of NF-κB by the classical IKKβ-dependent pathway, is a crucial mediator of inflammation-induced tumor growth and progression in animal models of inflammation-associated cancer (22-23). Therefore, NF-κB is able to regulate the expression and the function of a wide

spectrum of genes involved in the control of cell cycle, apoptosis, cell growth, tissue invasiveness and inflammation. It is just this ability that makes NF-κB the crucial point of convergence of a number of stimuli that can influence different aspects of cellular homeostasis and, therefore, can lead to the onset of cancer.

According to Hanahan and Weinberg (24), six essential alterations in cell physiology characterize a tumor cell: self-sufficiency in growth, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Many of the genes able to mediate such effects are under transcriptional control of NF-κB (25) (Figure 3). In fact, the activity and the expression of Cyclin D1 (26), CDK2 kinase (27), c-myc (28), which are involved in the control of cell cycle and are altered in several types of cancer, such as breast, prostate and ovarian tumors, are regulated by NF-κB. The expression and the function of numerous cytokines, that are growth factors for tumor cells, are NF-κB-dependent. Among them are: IL-1β, a growth factor for acute myeloid leukemia (AML), TNF, a growth factor for Hodgkin's lymphoma, cutaneous T cell lymphoma and gliomas, interleukin (IL)-6, a growth factor for multiple myeloma (29). Some growth factors, such as epidermal growth factor (EGF), or receptors for growth factors, such as HER2, able to promote growth of solid tumors, activate NF-κB (30). Tissue invasion and metastasis, two crucial events of tumor progression, are regulated by NF-κB-dependent genes, including matrix metalloproteinases (MMPs) (31), urokinase type of plasminogen activator (uPA) (32), IL-8 (33), the adhesion molecules VCAM-1, ICAM-1 and ELAM-1 (34) and chemokine receptors such as CXCR4 (35). NF-κB activity is also involved in the regulation of angiogenesis, the process by which tumor cells promote neo-vascularization, an essential step for their growth and invasiveness. Vascular endothelial growth factor (VEGF), which is the main member of angiogenic factors family, is under transcriptional control of NF-Kb (36). Finally, altered expression

of genes involved in regulation of apoptosis, which is a feature of neoplastic cells, is often due to deregulated NF-κB activity. TNF receptor associated factor (TRAF)1/2 (37), IAP proteins (XIAP, cIAP-1 and -2) (37-38), members of Bcl-2 family (39), c-FLIP (40), GADD45β (41), Ferritin Heavy Chain (42) are all anti-apoptotic genes. By promoting cell survival, these genes lead to the maintaining of cell-transformed state and are responsible for the resistance to chemotherapeutic drugs. Therefore, it is not surprising that altered NF-κB promotes neoplastic transformation by controlling expression of these genes. In fact, inactivation of NF-κB in different cell lines derived from tumors displaying high constitutive NF-κB activity, leads to the loss of their tumorigenic potential due to either an increased susceptibility to apoptosis or a decrease of their uncontrolled proliferative rate. Moreover, in some cases, blocking NF-κB activity inhibits the metastatic potential of many cancer cell lines or reduces the tumor size (Figure 3).

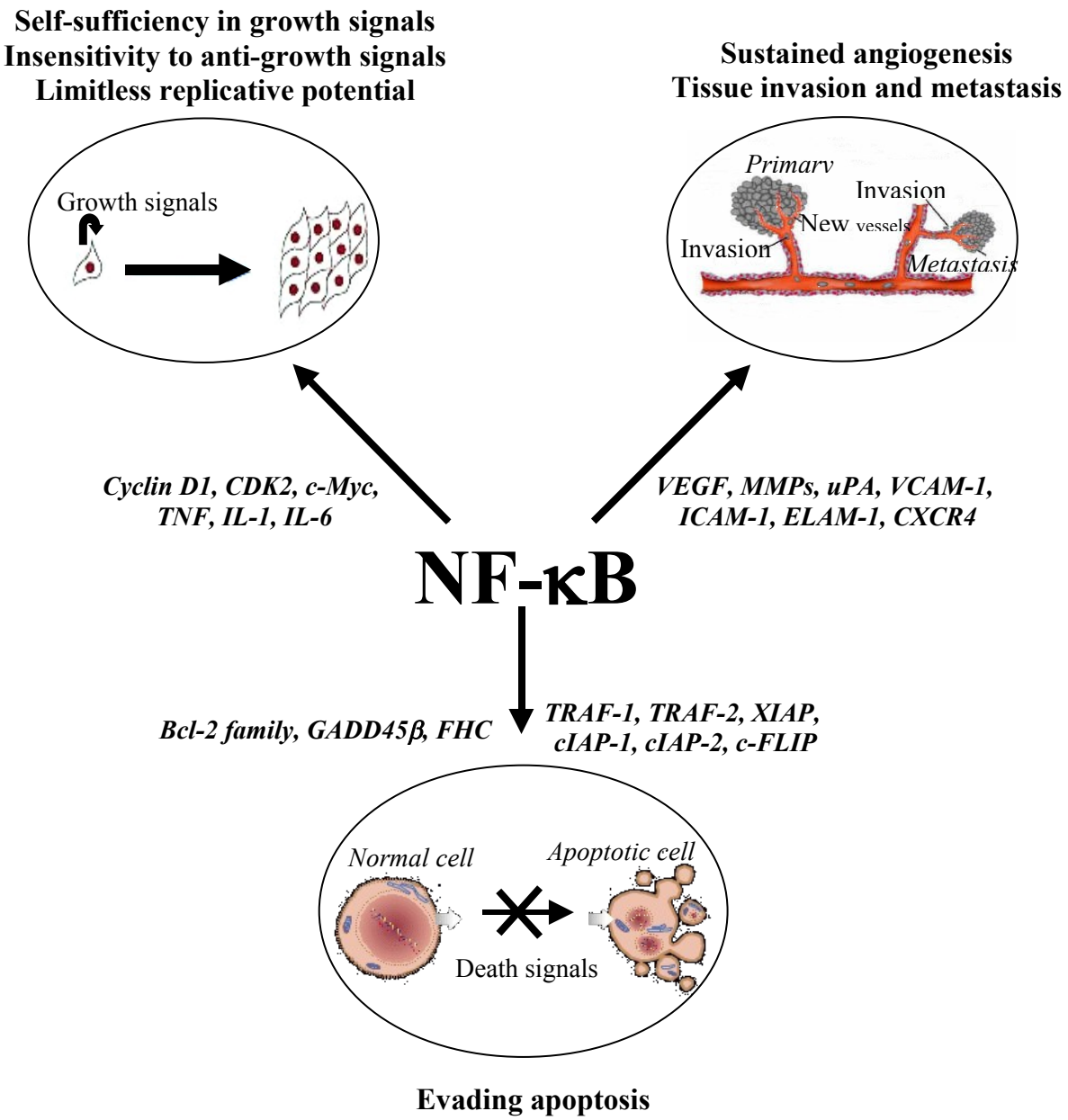


Fig. 3 NF-κB contributes to cancer development through regulation of different genes involved in oncogenesis. According to Hanahan and Weinberg, six essential alterations in cell physiology characterize a tumor cell: self-sufficiency in growth, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Many of the genes able to mediate such effects are under transcriptional control of NF-κB.

What is the cause of NF-κB constitutive activation in tumors? The mechanisms determining persistent and de-regulated NF-κB activity in cancer cells are not well understood. Since NF-κB signaling pathway is tightly controlled by several regulatory proteins, the constitutive activation of NF-κB in solid tumors has been mainly attributed to disruption of this process (i.e., defective IκBα activity, constitutive IKK activity, enhanced proteasomal activity), consistent with scenario observed in NF-κB-associated haematological malignancies. Another possibility is that constitutive NF-κB activation could be determined by prolonged and persistent stimulation of cancer cells by factors promoting induction of NF-κB signaling by autocrine or paracrine fashion. Thus, NF-κB activity is held at high levels until this autocrine or paracrine loop is not interrupted.

NF-κB links inflammation and cancer

For many years, a link between inflammation and cancer has been strongly suspected. The Greek doctor Galen two millenniums ago, noticed a link between inflammation and cancer. In the 19th century Wirkow suggested that tumors may arise in area of chronic inflammation. More recently, population based studies show that susceptibility to cancer increases when tissues are chronically inflamed, and long-term use of non-steroidal anti-inflammatory drugs reduces the risk of several cancer.

Inflammation is central to control our fight against pathogens, and to regulate wound healing. These are very intricate biological responses that involved complex interactions between different cell types that regulate the expression of biological mediators which promote cell chemotaxis, cell migration and cell proliferation. If inflammation is not ordered and timely, the resulting chronic inflammation can

contribute to a variety of diseases including cancer. Cells of the innate immune system neighbouring the tumor (inflamed area) secrete pro-inflammatory cytokines, such as TNFα, IL-1, IL-6 and IL-8, as well as extracellular matrix-degrading enzymes, growth factors and ROS. This microenvironment enhances cell proliferation, cell survival, cell migration and angiogenesis, thereby promoting tumor development. Chronic inflammation due to viruses or bacteria infections can directly induce mutations in tumor suppressors or oncogenes of epithelial cells, but can also stimulate the formation of carcinomas through an indirect mechanism involving activation of surrounding inflammatory cells. In addition, activated innate immune cells can interfere with T cell response to cancer. Macrophages can inhibit antigen presentation by dendritic cells, directly suppress T cell response, and indirectly down regulate immunity by inducing regulatory T cell through affects of IL-10 and TGFβ. Thus, the ongoing inflammatory response has the capacity to profoundly alter the ability of the host to mount adaptive immune response within the inflammatory stroma of tumors. The interplay between epithelial and inflammatory cells is thought to be crucial for the genesis and the establishment of carcinomas. One of the main actors in inflammatory process is NF-κB which, by regulating the expression and the function of different cytokines and chemokines in inflammatory cells, stimulates the growth and its own activity in epithelial cells. Thus, NF-κB establishes a network that, after prolonged time, can lead epithelial cells to undergo malignant transformation. For example, one of the main risk factors linked to gastric cancer is *Helicobacter pylori* (*H. pylori*) infection. *H. pylori* is a potent NF-κB activator (43): once NF-κB is activated in gastric cells, it induces transcription of IL-1, IL-6, IL-8, TNFα and other growth factors that can promote uncontrolled proliferation either by autocrine stimulation or by paracrine stimulation of surrounding inflammatory cells. On the other hand, *H. pylori* induces NF-κB-dependent production of COX2, ROS, iNOS that determine DNA damage,

thereby increasing the susceptibility to malignant transformation of gastric cells. Another example for the role played by NF-κB in controlling inflammation and cancer is represented by colitis-associated cancer. Ulcerative colitis is a chronic inflammatory bowel disease that, together to Crohn's disease, shows persistent NF-κB activation in tissue macrophages and epithelial cells of the colonic mucosa. By a mechanism similar to that of gastric cells, NF-κB is able to promote colorectal cancer. These two examples demonstrate that NF-κB activates a network between epithelial cells and inflammatory cells that not only leads to neoplastic transformation, but also sustains the malignant phenotype of epithelial cells. To analyze in more detail the molecular mechanisms regulating these phenomena, Karin (22) and Ben-Neriah (23) have independently developed two animal models of inflammation-associated cancer, a colitis-associated cancer (CAC) model and a genetic model ($MDR2^{-/-}$ mice) of cholangitis (bile-duct inflammation), which leads to hepatocarcinoma.

Karin and colleagues showed that the administration of dextran sulfate to mice caused chronic colitis which greatly enhanced the incidence of colon carcinomas following administration of the pro-carcinogen azoxymethane. Chronic colitis was determined by disruption of intestinal barrier and exposure of macrophages in the lamina propria to enteric bacteria. The exposure of macrophages to bacteria induced the activation of NF-κB in these cells, leading to the production and secretion of pro-inflammatory cytokines that activated NF-κB in intestinal epithelial cells. It is also possible that necrotic tumor cells may activate adjacent macrophages and/or other myeloid cells which than secrete growth factors and mediators of inflammation. The sustained activation of NF-κB in enterocytes increased the tumor susceptibility following azoxymethane stimulation. If IKKβ was deleted in enterocytes, tumor incidence but not tumor size strongly decreased, indicating that the IKKβ-dependent NF-κB-activation pathway operated during

early phases of tumorigenesis. However, deleting IKKβ in myeloid cells, which are important for the development of CAC, decreased not only tumor incidence but also tumor size, as a consequence of diminished proliferation of transformed enterocytes which requires growth factors produced by myeloid cells (22). Thus, in this model, NF-κB promotes survival of malignant cells either in early or late phases of tumorigenesis, depending on cell types (epithelial or inflammatory cells) in which it has been activated.

Similarly, Ben-Neriah and Co. demonstrated that the appearance of hepatocarcinoma in MDR2$^{-/-}$ mice, a genetic model of cholangitis, was due to prolonged TNF-dependent NF-κB activation of hepatocyte by inflammatory cells surrounding them in the liver. This caused increased survival of dysplastic hepatocyte by up-regulation of anti-apoptotic genes such as GADD45β and BFL1. The inactivation of NF-κB by hepatocyte-specific expression of IκBα super-repressor, or by suppressing TNF activity blocked tumor development. This effect was seen only when NF-κB activity was inhibited between 7 and 14 months after mice birth, during late phases of tumor promotion, while its inactivation during the first 7 months caused no beneficial effects (23). Therefore, in this mouse model, the role of NF-κB was prominent in maintaining rather than in promoting malignant transformation of hepatocyte.

The data obtained in these two cancer models, suggest that the NF-κB pathway does not affect initiation but has multiple actions in tumor promotion, by preventing apoptosis of cells with malignant potential, by stimulating the production of pro-inflammatory cytokines in the inflammatory cells infiltrating the tumor mass (Figure 4). This model is consistent with the observed correlation between the number of inflammatory cells, level of cytokines and tumor aggressiveness and prognosis in both human and mouse. However, the transformed cells themselves can contribute to the overall level of secreted pro-inflammatory

cytokines, and to maintain the NF-κB pathway activated. In fact, it is possible to keep malignant cells in culture without the support of the innate immunity and still detect constitutive activation of NF-κB. In addition, at least for some cell types, such as undifferentiated thyroid carcinomas, it is possible to detect an NF-κB modulating activity secreted by the transformed cells (44).

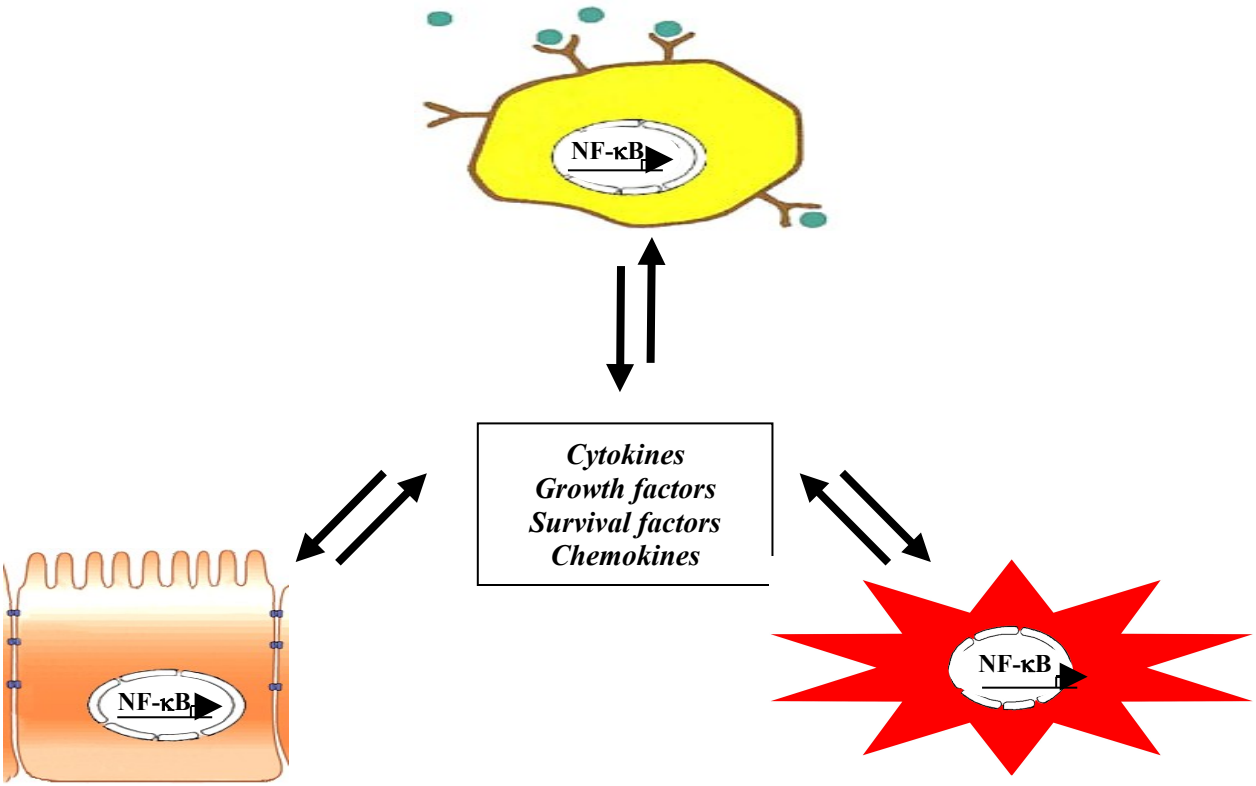


Fig. 4 Mechanisms by which NF-κB contributes to cancer development during inflammation. The interplay between epithelial and inflammatory cells is thought to be crucial for the genesis and the establishment of carcinomas. One of the main actors in inflammatory process is NF-κB which, by regulating the expression and the function of different cytokines and chemokines in inflammatory cells, stimulates the growth and its own activity in epithelial cells. Thus, NF-κB establishes a network that can sustain cell proliferation and resistance to apoptosis.

NGAL protein

The Neutrophil Gelatinase-Associated Lipocalin (NGAL), also known as lipocalin-2, is a member of the large family of lipocalins, a group of small extracellular proteins with great functional diversity (45). It is released from neutrophil granules as a 25 kDa monomer, a 46 kDa disulfide-linked homodimer and a 135 kDa disulfide-linked heterodimer with the matrix metalloproteinase-9 (MMP-9) (46).

NGAL is thought to be an acute phase protein (47), it is only expressed at the myelocyte/metamyelocyte stage in the course of neutrophil maturation. Once synthesized, the protein is stored in the azurophilic granules until it is released when the neutrophil is activated. As expected, based on its identification as an acute phase protein, elevated serum NGAL has been observed associated with several diseases with an inflammatory component, including atherosclerosis, ischemic cerebrovascular diseases and inflammatory bowel disease (48). Elevated NGAL expression has been also shown in different human tumors, including breast (49), lung (50), colon (48), ovary (51) and pancreas (52) carcinomas. However, the precise role of NGAL has not been well defined yet.

Several studies have suggested that NGAL is a potent bacteriostatic agent by a siderophore-mediated sequestering of iron (53). Lipocalin-2-deficient mice have normal litters and no apparent phenotype when housed in specific pathogen-free conditions. However, intraperitoneal challenge with a sub-lethal dose of a clinical strain of *Escherichia coli*, H9049, results in substantial increases in bacteraemia and bacterial burden in the liver and spleen.

The expression of lipocalin 2 in multiple tissues outside the setting of infection suggests that it may have other functions, such as have been postulated in

kidney development and the implantation and parturition stages of pregnancy. Although we cannot exclude a role for lipocalin 2 in these functions, the phenotype of lipocalin-2-deficient mice argues against an obligatory role in these biological processes (54).

Another function of NGAL is represented by its ability of binding Matrix metalloproteinase 9 (MMP-9). This protein has been shown able to promote tumor growth in a number of studies of tumor-induced and also to contribute to the metastatic and angiogenic phenotypes of human tumors. In engineered MCF-7 human breast cancer cell lines, to express elevated levels of NGAL, it is shown that NGAL overexpression leads to increased breast tumor growth that is accompanied by an increase in MMP-9 activity, tumor angiogenesis, and tumor cell proliferation. In addition, the enzymatic activity of urinary MMP-9/NGAL complex was detected in the urine of breast cancer patients but not in the age-matched, sex-matched, healthy control samples. Taken together, these findings suggest that NGAL may contribute to cancer disease progression, at least in part, by protecting MMP-9 from degradation, thereby enhancing its enzymatic activity and facilitating tumor progression. The appearance of MMP-9/NGAL complexes in the urine of breast cancer patients suggests that urinary MMP-9/NGAL detection may serve as an independent predictor of disease status, a noninvasive marker of cancer diagnostics and prognostics (55).

As a secreted binding protein, it has been reported that the murine ortholog, called 24p3, plays a crucial role in IL-3 deprivation-induced apoptosis by regulating intracellular iron delivery, very likely after interaction with a 24p3 receptor (56). Indeed, the human NGAL protects A459 and MCF7 cells from apoptosis induced by PDK1 inhibitors (57). Lastly, NGAL induces cell proliferation by promoting the iron dependent metabolism of nucleotides for DNA synthesis (58).

AIM OF THE WORK

NF-κB controls expression of a number of pro-inflammatory factors (cytokines, chemokines and growth factors), secreted by cancer cells in the tumor microenvironment, which substantially contribute to the tumor development (59-60). Understanding the molecular mechanism by which these factors play their role in cancer, could help in the comprehension of the role of NF-κB in inflammation-related cancer, and could open new perspectives in the treatment of tumors.

In the laboratory of Prof Formisano it was previously shown that NF-κB is strongly activated in primary human anaplastic thyroid carcinomas (ATC) (61). To study the role of NF-κB in thyroid cancer, it was inhibited its function by stably transfecting FRO cells (derived from a human ATC) with a super-repressor form of IκBα (IκBαM). As a result, FRO IκBαM cells lost their oncogenic potential mainly for an increased susceptibility to drug-induced apoptosis (61).

The cellular system FRO/FRO IκBαM represents an excellent model for the identification of NF-κB-regulated factors that, secreted in the extracellular milieu, could play a role in thyroid cancer. Thus, it was analyzed by a differential proteomic approach, the pattern of expression of secreted proteins from conditioned medium of parental FRO cells and FRO IκBαM clones. One of the proteins that showed a marked decrease of expression in FRO IκBαM cells was NGAL.

In the present PhD thesis I show that knocking down NGAL expression blocks the ability of FRO cells to form colonies in soft agar, to form tumor in nude mice, and increases their susceptibility to apoptosis. In addition, I show that the pro-survival activity of NGAL is mediated by its ability to bind and transport iron inside the cells.

EXPERIMENTAL PROCEDURES

Cell culture and biological reagents

FRO, FRO IκBαM and FRO siRNA NGAL were grown in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma) or in OPTIMEM (Invitrogen, Carlsbad, CA) supplemented or not with 10% fetal bovine serum (Sigma). Human NGAL was amplified by PCR from a human liver c-DNA library (Clontech, CA) and cloned into pcDNA3.1-HA, -FLAG vectors (Invitrogen, Carlsbad, CA) for expression in mammalian cells, and pET28 (Novagen) for expression in *e.coli*. Human Transferrin was from Kedrion (Naples, IT). Anti-NGAL (AF1757) and anti-actin (sc-8432) antibodies were purchased from R&D systems (Minneapolis, MN) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. A polyclonal rabbit anti-NGAL antibody was generated in rabbits by using human recombinant NGAL protein as antigen. IL1β and IL17 were from Peprotech (London, England) and Biosource (Nivelle, Belgium), respectively. Transient transfection of FRO cells was performed by using the NucleofectorTM (Amaxa Biosystem).

Processing of conditioned media

Conditioned media (1 liter) from FRO and FRO IκBαM cell lines, grown in OPTIMEM w/o serum, were collected and centrifuged at 3500 rpm for 10 min at 4°C to remove cells and cell debris. The samples were concentrated to 5 ml over a membrane pressure concentrator with a 50-kDa cut-off (Millipore, Billerica, MA)

and partially purified by using Mono-Q and Mono-S Econo-Pac column (Bio-Rad, Bedford, MA).

Two-dimensional gel electrophoresis (2-DE) analysis

The analysis was performed in triplicate on protein preparations from three different batches of conditioned media. Thyrty-fifty μg of proteins were loaded onto 13 cm, pH 3-10 L, 4-7 L or 6-11 L IPG strips (Amersham-Pharmacia Biosciences, Milan, Italy). IEF was performed using an IPGPhor II system (Amersham-Pharmacia Biosciences, Milan, Italy) according to the manufacturer's instructions. Focused strips were equilibrated with 6.0 M urea, 26 mM DTT, 4% (w/v) SDS, 30% (v/v) glycerol in 0.1 M Tris-HCl (pH 6.8) for 15 min, followed by 6.0 M urea, 0.38M iodoacetamide, 4% (w/v) SDS, 30% (v/v) glycerol, and a dash of bromophenol blue in 0.1 MTris-HCl (pH 6.8), for 10 min. The equilibrated strips were applied directly to SDS-10% (w/v) polyacrylamide gels and separated at 130 V. Gels were fixed and stained by ammoniacal silver (6). Gels were scanned with an Image Master 2-D apparatus and analysed by the Melanie 5 software (Amersham-Pharmacia Biosciences, Milan, Italy) that allowed estimating relative differences in spot intensities for each represented protein. Statistical analysis of differentially expressed proteins was performed as previously described (4-5).

Mass spectrometry analysis

Spots from 2-DE were excised from the gel, triturated and washed with water. Proteins were *in-gel* reduced, S-alkylated and digested with trypsin as previously reported (4-5). Digest aliquots were removed and subjected to a

desalting/concentration step on μZipTipC18 (Millipore, Bedford, MA, USA) using acetonitrile as eluent before MALDI-TOFS analysis. Peptide mixtures were loaded on the MALDI target, using the dried droplet technique and α-cyano-4-hydroxycinnamic acid as matrix, and analyzed by using Voyager-DE PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA). Internal mass calibration was performed with peptides deriving from trypsin autoproteolysis. PROWL software package was used to identify spots unambiguously (Est'd Z score > 2) from independent non-redundant sequence databases (7). Candidates from peptide matching analysis were further evaluated by the comparison with their calculated mass and pI using the experimental values obtained from 2-DE.

siRNA of NGAL

To knock-down NGAL expression, we designed double-stranded oligonucleotides containing sequences derived from the human NGAL (nucleotides 294-313 and 376-397) in forward and reverse orientation separated by a 7-bp spacer region (caagaga) to allow the formation of the hairpin structure in the expressed siRNAs. NGAL siRNA 220: sense strand, 5' -CCATCTATGAGCTGAAAGAcaagagaTCTTTCAGCTCATAGATGG; antisense strand, 5' –CCATCTATGAGCTGAAAGAtctcttgTCTTTCAGCTCATAGATGG. NGAL siRNA 2: sense strand, 5' –GGACTTTTGTTCCAGGTTGTTcaagagaAACAACCTGGAACAAAAGTCC; antisense strand, 5' –GGACTTTTGTTCCAGGTTGTTtctcttgAACAACCTGGAACAAAAGTCC. The resulting double-stranded oligonucleotides were cloned into the pcRNAi vector that we derived from the pcDNA3.1 vector (Invitrogen) by replacing the viral promoter-

cassette with the H1 promoter that is specifically recognized by RNA polymerase III (2).

Quantification of iron content by colorimetric assay

Cell cultures in 100-mm dishes were washed twice with PBS and then lysed in 400 µl of 50 mM NaOH for 2 h, on a shaker, in a humidified atmosphere. The lysates were incubated for 20 min at room temperature in 0.3 M HCl, TCA-precipitated and finally centrifuged at 1200 g for 15 min. The supernatants were mixed with chromogen (0.51 mM disulphonate bathophenantroline, 1.3 M sodium acetate, pH 4.6, 30 mM sodium pyrosulphite, 1.83 mM p-(N-methyl)aminophenol) and the absorbance was spectrophotometrically determined at the wavelength of 546 nm. This method was also used to confirm the presence of iron in both human transferrin and recombinant NGAL.

Immunoistochemical analysis

Specimens from normal and pathological human thyroid were isolated, rinsed with PBS, fixed in 4% buffered neutral formalin and embedded in paraffin. Then, 5-6 µm thick paraffin sections were deparaffinized and placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min, then washed in PBS before immunoperoxidase staining. Slides were then incubated overnight at 4°C in a humidified chamber with antibody anti-NGAL diluted 1:100 in PBS and subsequently incubated, first with biotinylated goat anti-rabbit IgG for 20 min (Vectostain ABC kits, Vector Laboratories), and then with pre-mixed reagent ABC (Vector) for 20 min. The antibody anti-NGAL used was a rabbit polyclonal raised against the recombinant NGAL protein. The immunostaining was performed by

incubating slides in diaminobenzidine (DAB-DAKO) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS, pH 7.6, for 5 min. After chromogen development, slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent mounting medium (Permount). As a control of the immune reaction, slides were incubated with an anti-NGAL Ab previously incubated with recombinant NGAL (1mg/ml).

Northern and Western blots

For Northern blot, 20 μg of total RNA from FRO and FRO IκBαM cell lines were analyzed by electrophoresis on a 1.2% formaldehyde agarose gel and blotted onto nitrocellulose membrane (Bio-Rad, Hercules CA). For Western blot, 20 μg of total proteins from cell lysates or supernatants were analyzed by 12% SDS-PAGE and blotted onto nitrocellulose membrane (Schleicher & Schuell, Whatman GmbH). Filters were blocked for 90 min at room temperature with 5% nonfat dry milk in TBST buffer (10mM Tris-HCl, pH 8.0, 0.1% Tween 20, 150 mM NaCl) and incubated with 1:2000 dilution of anti-NGAL or anti-actin antibodies 90 min. After TBST washing, blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, NXA931) diluted 1:5000 in TBST buffer and then revealed by ECL (Amersham Biosciences).

In vitro and *in vivo* tumorigenicity assays

To analyze the ability of the various FRO clones to form colonies in soft agar, 1 x 104 cells were seeded in 60-mm dishes onto 0.3% Noble Agar (Difco, Detroit, MI) on top of a 0.6% bottom layer. Colonies larger than 50 cells were scored after 2 weeks of incubation at 37°C. To analyze the ability of the various FRO clones to induce tumor growth in nude mice, 2 x 107 cells were injected s. c. on a flank of each 6-week-old nude mouse (Charles River, Lecco, Italy). Thirty days later, mice were killed and tumors were excised. Tumor weight was determined and their diameters were measured with caliper. Tumors volumes were calculated by the formula: *a2* x *b* x *0.4*, where *a* is the smallest diameter and *b* is the diameter perpendicular to *a*. No mouse showed signs of wasting or other visible indications of toxicity. The mice were maintained at the Dipartimento di Biologia e Patologia Cellulare e Molecolare animal facility and housed in barrier facilities on a 12 h light dark cycle with food and water available ad libitum. The animal experiments described here were conducted in accordance with accepted standards of animal care and in accordance with the Italian regulations for the welfare of animals used in studies of experimental neoplasia, and the study was approved by our institutional committee on animal care.

Measurement of apoptosis

2.5 x 105 cells/well were seeded in six-well culture plates and grown for 24 h at 37°C in the presence or in the absence of serum. In some cases cells were grown in serum-free conditioned medium from FRO cells or in serum-free medium containing human transferrin (10 μg/ml), recombinant NGAL (10 μg/ml), deferoxamine (200 μg/ml) or FeCl3 (50 μM). Cell death was assessed by Western

blot 2with anti-caspase 9 antibody (StressGen, Ann Arbor, MI) or by propidium iodide staining according to Nicoletti *et al* (3) In this case, samples were analyzed by flow cytometry using a CyAn cytofluoremeter (Dako) equipped with Summit Software. Results were mean ± S.D. of at least three separate experiments.

mRNA quantification by Real-time RT-PCR

Real-time RT-PCR was carried out with cDNAs reverse transcribed from total RNA using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostic) using ABI PRISM 7000 software (Applied Biosystems). The primer used were 5'-AAACAGAAGGCAGCTTTACGATG-3' and 5'-AAATGTTCTGATCCAGTAGCG-3' for NGAL, and 5'-TCCCAGAGCTGAACGG-3' and 5'-GAAGTCGCGGAGACA-3' for glyceraldehyde-3-phosphate dehydrogenase.

Statistics

Data were analyzed by using ANOVA test and student's t-test analysis. Data are presented as the means ±SD. P values less than 0.05 were considered significant.

Measurement of Mitochondrial Membrane Potential

The changes in mitochondrial membrane potential were quantified by staining cells with tetramethylrhodamine ethyl ester (TMRE) (Invitrogen). A TMRE stock was prepared at a concentration of 10 mg/ml in DMSO and stored at 20°C. Cells were loaded with TMRE by incubating cells in media containing 50 nM TMRE for 30 min at 37°C.

Flow cytometric analysis of Cytochrome C release

Detection of cyt C release was performed as described in reference 1.

References

1) Campos C. et al. Method for monitoring of mitochondrial cytochrome c release during cell death: Immunodetection of cytochrome c by flow cytometry after selective permeabilization of the plasma membrane. (2006) Cytometry A. 69 :515-23.
2) Mauro C. et al ABIN-1 binds to NEMO/IKKgamma and co-operates with A20 in inhibiting NFkappaB. (2006) J Biol Chem.281:18482-8.
3) Nicoletti I. et al A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. (1991) J Immunol Methods. 139:271-9.
4) Pacifico F. et al RbAp48 is a target of nuclear factor-kappaB activity in thyroid cancer. (2007) J Clin Endocrinol Metab. 92:1458-66.
5) Paron I et al. A differential proteomic approach to identify proteins associated with thyroid cell transformation. (2005) J Mol Endocrinol. 34(1):199-207.
6) Shevchenko A et al. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. (1996) Anal Chem. 68:850-8.
7) Zhang W., Chait BT. ProFound: an expert system for protein identification using mass spectrometric peptide mapping information. (2000) Anal Chem. 72:2482-9.

EXPERIMENTAL RESULTS

Differential proteomic analysis of conditioned media from FRO and FRO IκBαM cell lines

To identify factors secreted by thyroid cancer cells under transcriptional control of NF-κB, we analyzed the secretome of partially purified conditioned media from FRO and FRO IκBαM cell lines. As shown in Fig.1A, a number of proteins were differentially expressed between the two cell lines. Among the proteins whose expression decreased in the extracellular medium of FRO IκBαM cells as compared to FRO, at least three groups of them were examined because of their strong reduction (Fig. 1B, circled spots). The MALDI-TOF peptide mass fingerprint analysis identified all spots as Neutrophil Gelatinase-Associated Lipocalin (NGAL), whose post-translational modifications account for the multiple species showing variable molecular weight and pI values, in the 2-DE gel.

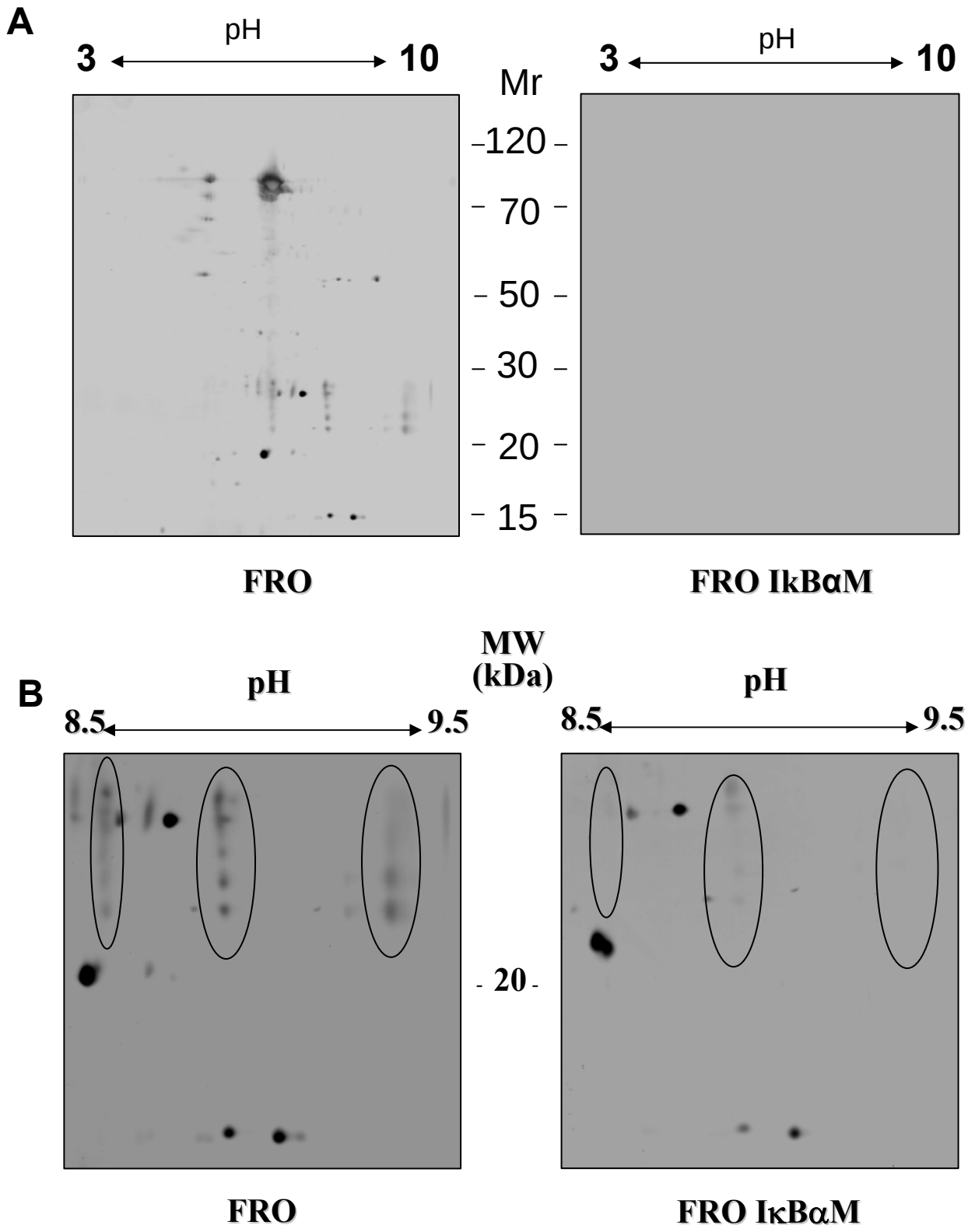


Fig.1A/B Differential proteomic analysis of conditioned media from FRO and FRO IκBαM cell lines. Two-dimensional gel electrophoresis showing expression of NGAL in the conditioned medium of FRO IκBαM cells, compared with parental counterpart FRO.

Immunoistochemical analysis of NGAL expression in normal and pathological human thyroid specimens

To verify the expression of NGAL in human primary thyroid cancer, we analyzed normal thyroid and specimens from different thyroid carcinomas by immunostaining. As shown in Table 1 and Fig. 2 A-D, although NGAL expression was not detected in normal thyroid, NGAL immunostaining was positive for papillary, follicular and anaplastic thyroid carcinomas. In Fig. 2 E-F is shown the lack of specific tissue staining when secondary antibodies (Fig. 2E) or blocked antibodies (Fig. 2F) were used.

Very interestingly, the levels of NGAL increased with the malignant phenotype of tumors, reaching the highest intensity in the anaplastic carcinomas (Table I and Fig. 2A-D), in parallel with NF-κB basal activity in the same types of thyroid tumors (61). Real time PCR analysis confirmed the increased expression on the NGAL mRNA in primary human thyroid cancer (Fig. 2G) and that this expression parallels the NF-κB activity.

Hystological type of thyroid samples	N° of total cases analyzed by immunohistochemistry	N°of positive cases/ N° of total cases analyzed by immunoistochemistry	NGAL staining score			
			0+	1+	2+	3+
Normal thyroid	3	0/3	3			
Papillary carcinoma	14	9/14	5	4	3	2
Follicular carcinoma	8	5/8	3		2	3
Anaplastic carcinoma	7	6/7	1		2	4

Table I. Immunohistochemical analysis of NGAL expression in normal and pathological human thyroid tissues.

Human specimens from normal thyroid, papillary, follicular and anaplastic thyroid carcinomas were collected and stained with anti-NGAL polyclonal antibodies. The percentage of malignant cells stained was scored from 0 to 3: 0, no positive cells; 1+, <10% of positive cells; 2+, 11-60% of positive cells; 3+, 61-100% of positive cells.

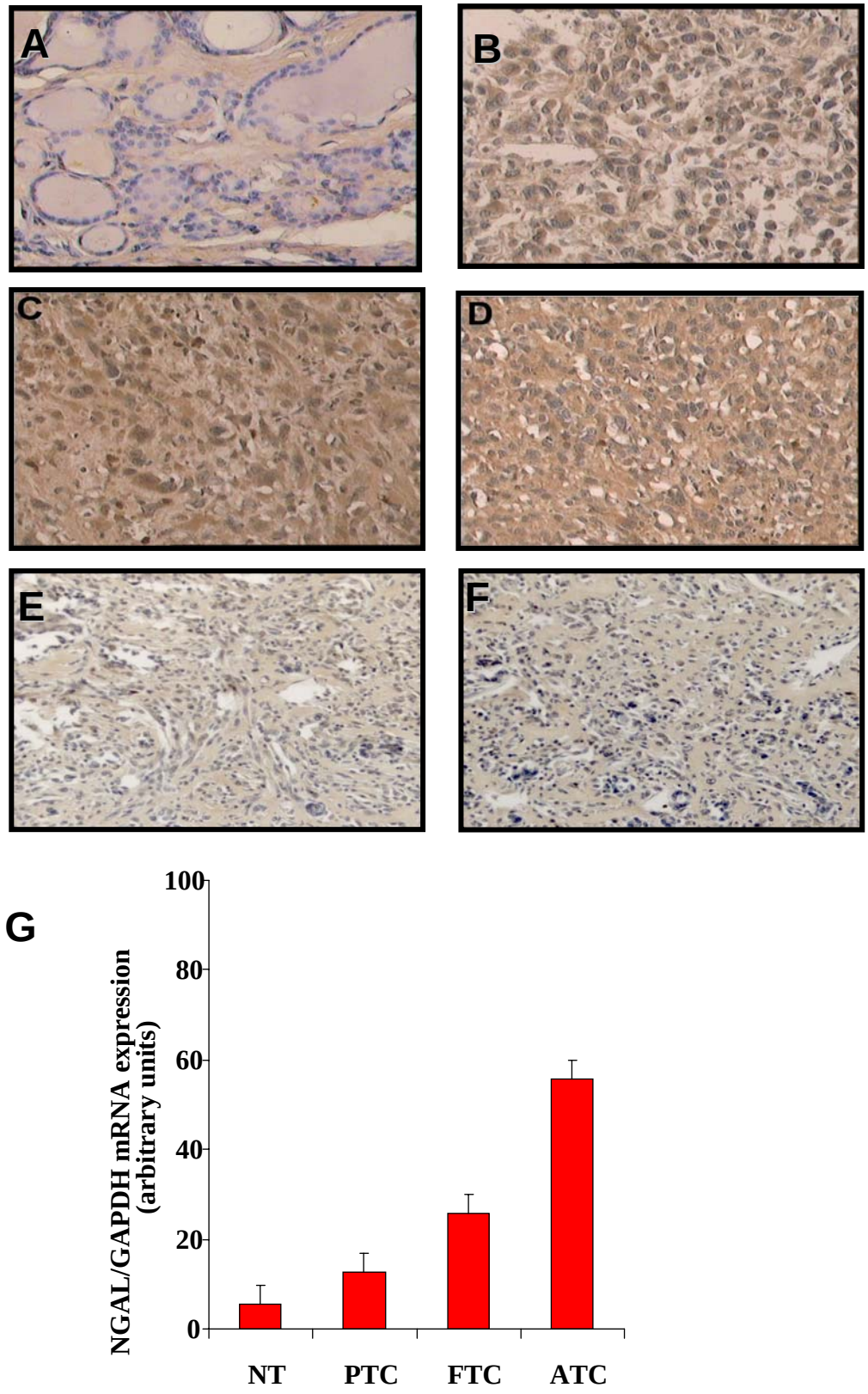


Fig. 2 Immunoistochemical analysis of NGAL expression in normal and pathological human thyroid specimens. Localization of NGAL in situ was determined by immunohistochemistry in sections from normal thyroid tissue (**A**) and three different anaplastic thyroid carcinomas (**BD**). Lack of specific tissue staining was seen when secondary Abs (**E**) or blocked Abs were used (**F**). Magnification, x400. **G)** qRT-PCR analysis of NGAL mRNA levels in primary human cancers. NT= normal thyroid (n=2); PTC=papillary thyroid carcinoma (n=3); FTC=follicular thyroid carcinoma (n=3); ATC=anaplastic thyroid carcinoma (n=3). * $P < 0.0005$.

NF-κB regulates NGAL expression

To confirm the differential NGAL expressione between FRO and FRO IκBαM cells we performed Northern and Western blot assays on total extracts from both FRO and FRO IκBαM cells. As shown in Fig. 3A-C, the expression of NGAL mRNA and of the corresponding protein are down-regulated in FRO IκBαM cells, compared to parental FRO cells, according to the data from proteomic analysis.

To confirm that NF-κB was controlling NGAL expression, we knocked down p65 expression in FRO cells by transfecting a siRNA (siRNA186) and a siRNA scrumble (siRNA1041) or the empty vector (pcRNAi) as control. We detected a decreased NGAL expression by Western blot (Fig. 3D) in the FRO cells transfected with siRNA186, while there is not reduction in the cells transfected with the control vectors.

We have also demonstrated the NF-κB dependent expression of NGAL in another system, by using wild type and p65-/- mouse embryonic fibroblast (MEF). These cells were treated with IL1β or IL17 that stimulate the NGAL expression via NF-κB, and the NGAL expression was detected by Northern and Western blot assays (Fig. 3E-F). No induction of NGAL was detected in absence of p65. In addition, we show that the re-expression of p65 in p65-/- MEF restored NGAL expression and that this re-expression was increased by stimulation with IL1β (Fig. 3G).

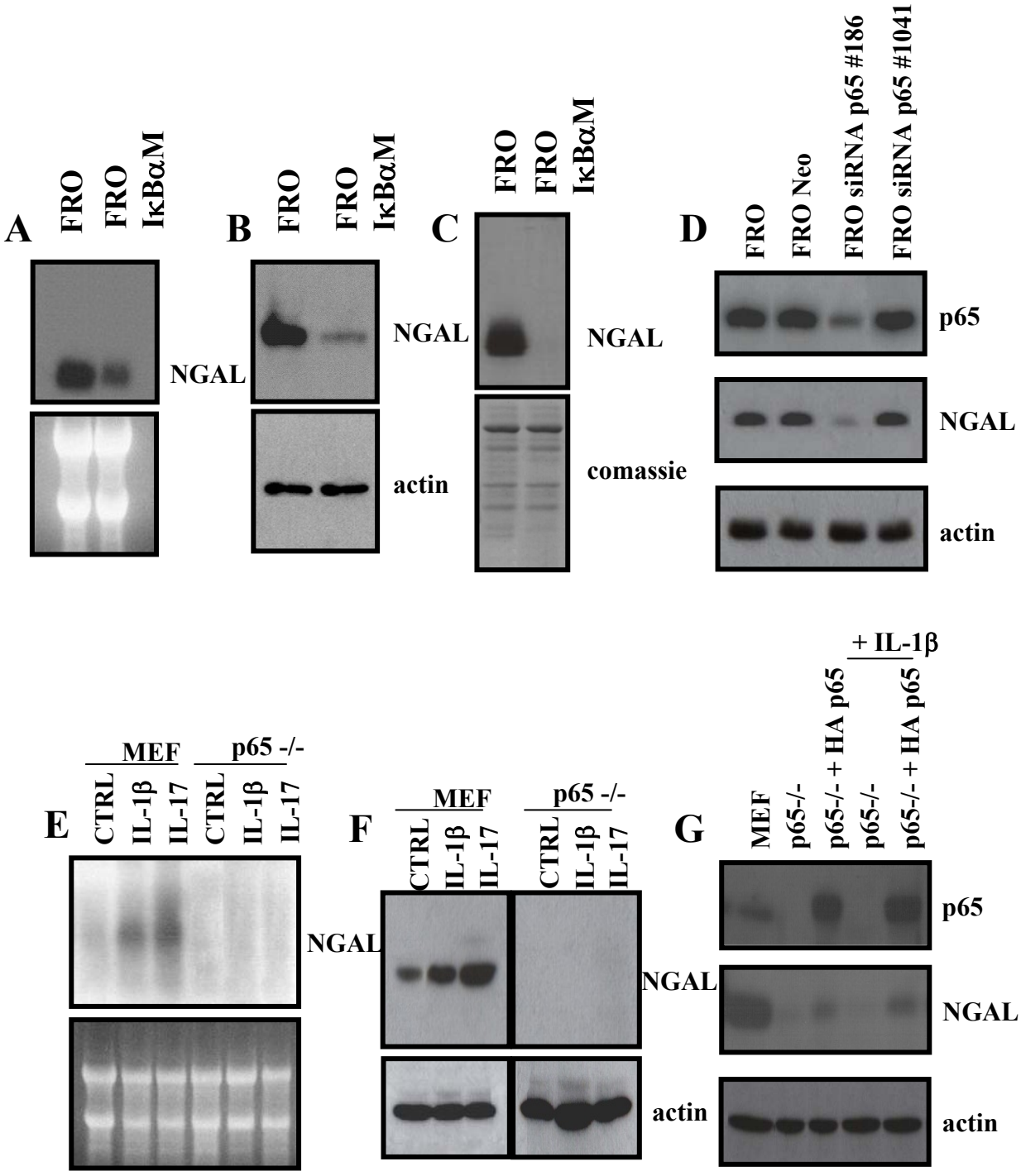


Fig.3 NF-κB regulates NGAL expression. The different levels of NGAL expression in the FRO and FRO IκBαM cell lines was analysed by Northern blot (**A**, top) and by Western blots on total cell lysates (**B**, top) as well as on conditioned media (**C**, top). The expression levels of NGAL mRNA were normalized to total RNA content (**A**, bottom), those of NGAL protein were normalized to actin expression in cell lysates (**B**, bottom) and to Coomassie staining of total protein in conditioned media (**C**, bottom). **D)** Expression of NGAL in FRO cells transiently transfected with a p65 siRNA (siRNAp65), or with the empty vector (pcRNAi), or with an unrelated control siRNA sequence (siRNA co) was determined by Western blot on total cell lysates. Induction of NGAL expression in p65-/- and wild type MEFs was analyzed by Northern blot (**E**) and Western blot (**F**). Induction of NGAL expression in p65-/- MEF reconstituted with an expression vector encoding HA-NGAL (**G**). p65-/- and wild type MEFs were treated with IL1β (20 ng/ml) or IL17 (200 ng/ml) for 6 hours

Inhibition of NGAL expression leads to the blockage of tumorigenicity in FRO cells

To study the role of NGAL in thyroid cancer we blocked its expression in FRO cells by stable transfection of a siRNA targeting NGAL. Figure 4A shows the levels of NGAL protein in two of the clones analyzed (indicated as siRNA NGAL 2/2 and 2/6), in FRO cells transfected with the empty vector (FRO Neo cells) or transfected with a control siRNA (indicated as siRNA NGAL 220/22). Figure 4B shows the levels of secreted NGAL. These clones were tested for their ability to grow in semi solid medium, and to form tumor in nude mice. In *in vitro* experiments, while FRO, FRO Neo and 220/22 cells gave rise to foci of transformation, 2/2 and 2/6 clones did not form any colonies (Fig. 4C). In *in vivo* assays, the injection of parental FRO or 220/22 cells in nude mice induced tumor formation in 12 out of 12 mice, while the injection of 2/2 or 2/6 cells induced tumor formation in 1 out of 6 and 4 out of 6 mice, respectively (Table II). The tumors developed from 2/2 and 2/6 cells were, 4-fold and 2-fold smaller than that formed after injection of parental cells, respectively (Table II). Of note, 2 out of 4 tumors from 2/6 cells showed NGAL staining after immunoistochemical analysis, indicating that the expression of the siRNA plasmid was lost. We also blocked NF-κB in FRO cells by transient overepression of IκBαM. In the absence of functional NF-κB, FRO cells did not form colonies in soft agar. Re-expression of NGAL partially rescued the ability of FRO cells to form colonies in soft agar (Fig. 6A-C).

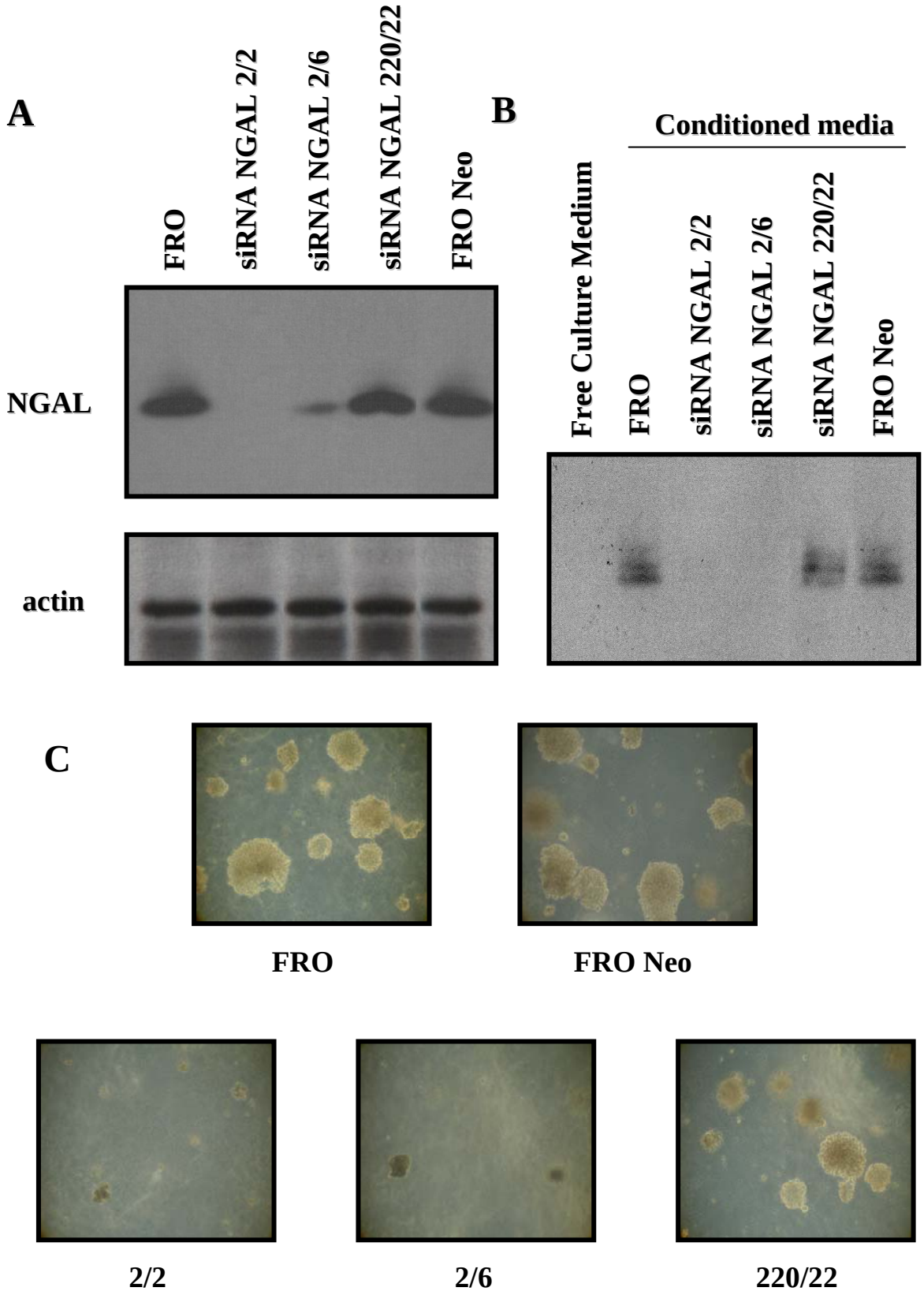


Fig.4 Inhibition of NGAL expression leads to the blockage of tumorigenicity in FRO cells. The expression of NGAL in FRO cells stably transfected with siRNA plasmids (2/2,2/6), control siRNA plasmid (220/22) or empty vector (Neo) was determined by Western blot on total cell lysates (**A**, *top*) and on conditioned media (**B**). **C**) Colony formation assay. Colonies larger than 50 cells were scored after 2 wk incubation at 37°C (**D**). Magnification, x200. *P<0.0001.

Cell type	Tumor incidence	Tumor volume average ($cm^{3)}$	Tumor weight average
FRO	6/6	0.37 ± 0.04	0.24 ± 0.05
FRO siRNA NGAL 2/2	1/6	0.01 ***	0.05 ***
FRO siRNA NGAL 2/6	4/6	0.16 ± 0.06 **	0.11 ± 0.02 **
FRO siRNA NGAL 220/22	6/6	0.33 ± 0.02	0.21 ± 0.03

Table II. In vivo tumor growth induced by FRO Neo cells and FRO siRNA NGAL clones. 2 x 10^7 cells were injected s. c. on a flank of each 6-week-old nude mouse. Tumor weight, diameter nd volume values were measured and determined as described in Supporting Information. Two out f four tumors developed by mice injected with FRO siRNA 2/6 cells showed partial NGAL taining after immunohistochemical analysis. ***FRO 2/2 vs. FRO: $p<0.0001$; **FRO 2/6 vs. FRO: < 0.001.

NGAL is a survival factor in FRO cells

We analyzed the response of parental FRO cells and NGAL siRNA clones to serum withdrawal-induced apoptosis. All clones, grown in the presence of serum, showed 2-3% of apoptosis, as assessed by propidium iodide staining (Fig. 5A). Serum deprivation did not affect the survival of FRO, FRO Neo and 220/22 clones, but induced a significant cell death (10-15%) in 2/2 and 2/6 clones (Fig. 5A). This effect was reverted by the addition of the conditioned medium of FRO cells (as well as of FRO Neo or 220/22 cells) to 2/2 and 2/6 clones. The same effect was also seen in the FRO cells transiently transfected with IκBαM (Fig. 6D). To confirm the activation of the apoptotic machinery, we analyzed by Western blot the caspase-9 activity in parental FRO cells and NGAL siRNA clones grown in the same experimental conditions used for propidium iodide assay (Fig. 5B). Serum deprivation induced cleavage of caspase-9 both in 2/2 and 2/6 clones, but not in parental FRO cells (Fig. 5B). Also in this case, incubation of NGAL siRNA clones with the conditioned medium of FRO cells (as well as of FRO Neo or 220/22 cells) restored their survival (Fig. 5A-B). These data strongly suggest that NGAL is a survival factor for thyroid carcinoma cells.

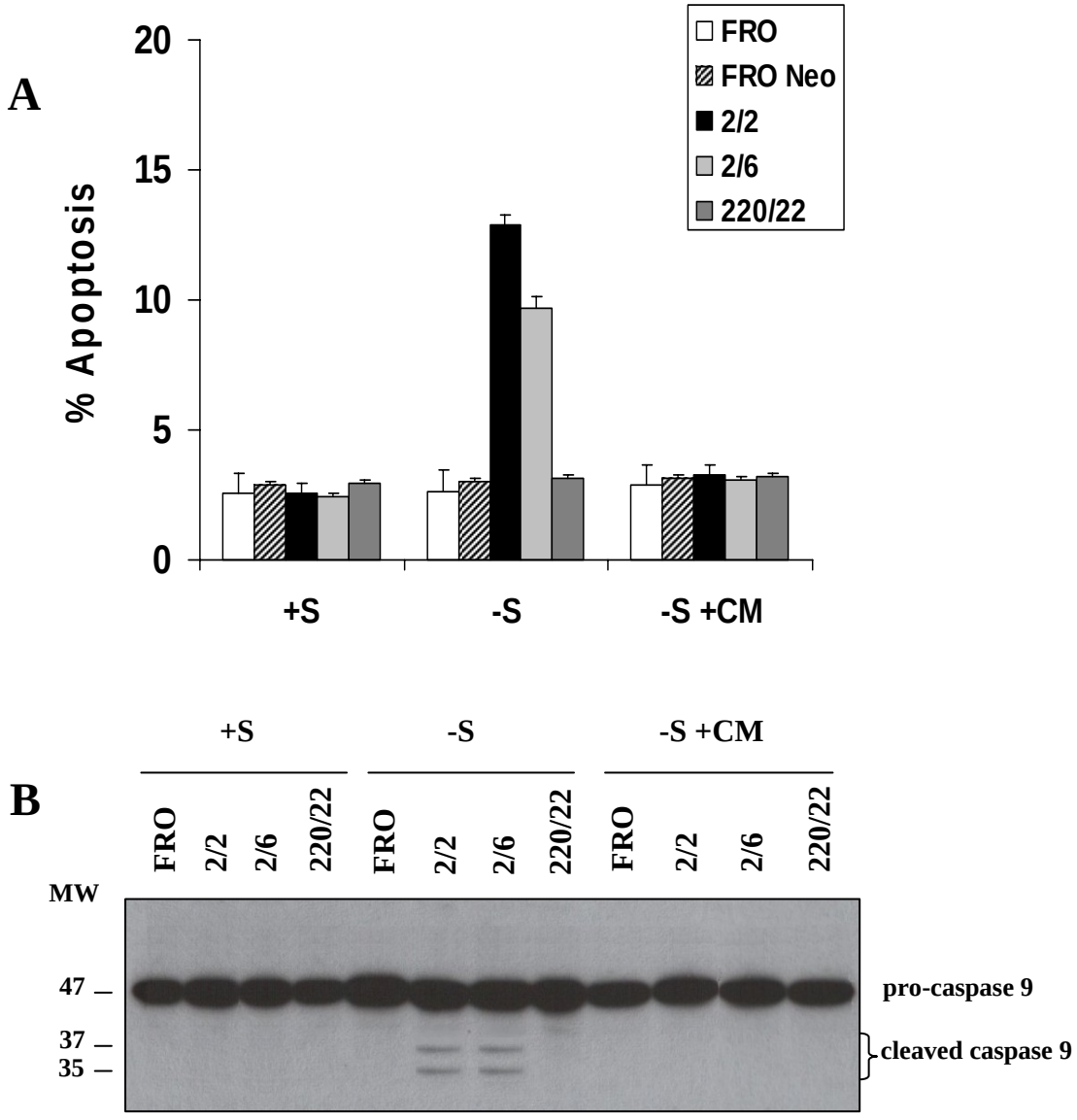


Fig. 5 NGAL is a survival factor in FRO cells. 2.5 x 10^5 cells/well were seeded in six-well culture plates and grown in the presence (+*S*), in the absence of serum (-*S*) or in serum-free conditioned medium from FRO cells (-*S*+*CM*), for 24 h, at 37°C. Cell death was assessed by propidium iodide staining (**A**) or by Western blot analysis (**B**). Results were mean ± S.D. of at least three separate experiments

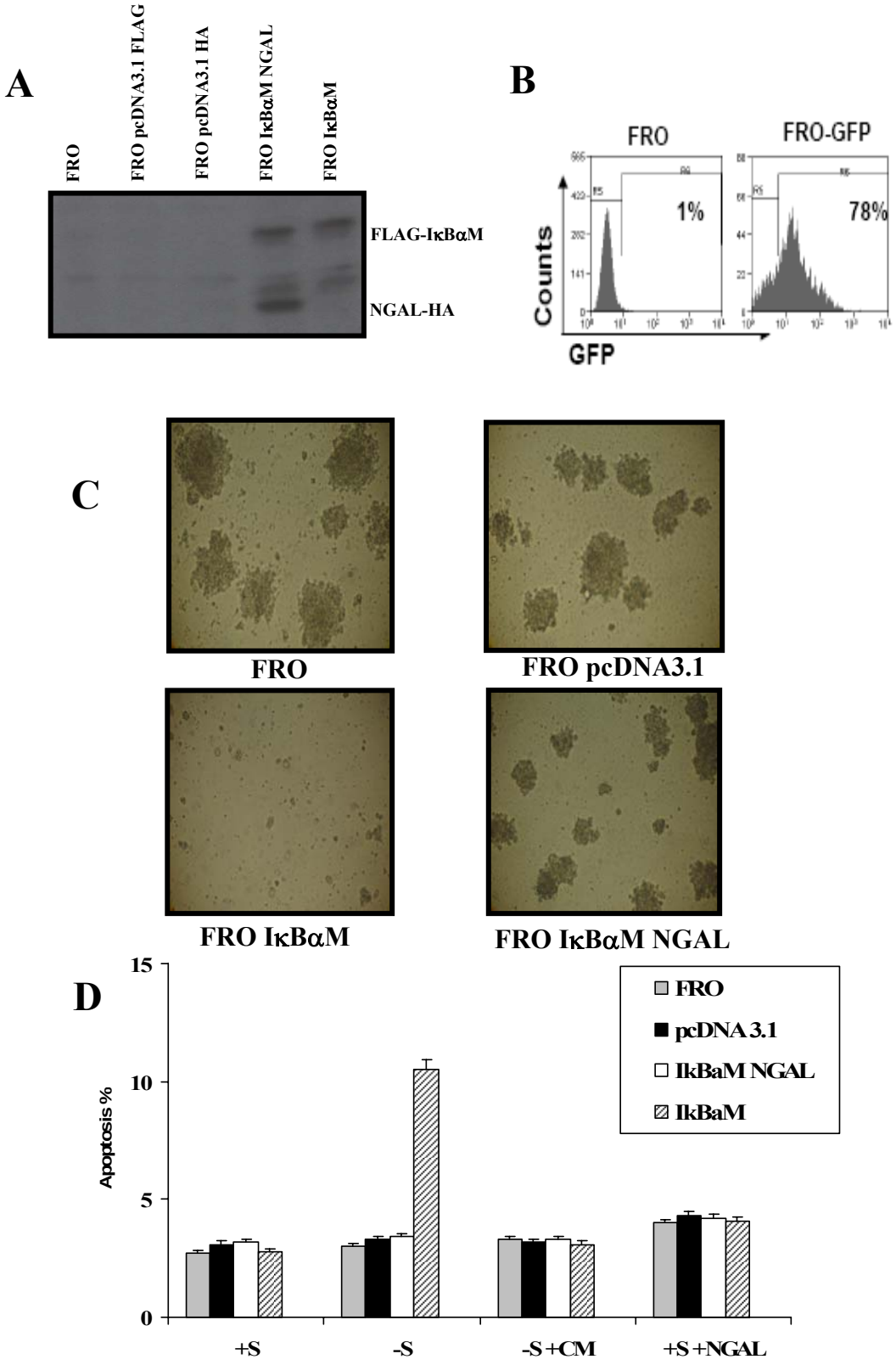


Fig.6 Effect of NGAL overexpression in FRO IκBαM cells. FRO cells were transiently transfected with an expression vector encoding FLAG tagged IκBαM and with an expression vector encoding HA-tagged NGAL. A vector encoding GFP was cotransfected to evaluate the transfection efficiency. **A**) Expression of FLAG-IκBαM and HANGAL in the whole cell lysate (both antibodies were used). **B**) Transfection efficiency was evaluated by FACS. **C**) Colony formation assay. Colonies larger than 50 cells were scored after 2 weeks of incubation at 37°C. Magnification, x200. **D**) 2.5 x 105 cells/well were seeded in six-well culture plates and grown in the presence (+*S*), in the absence of serum (-*S*) or in serum-free conditioned medium from FRO cells (-*S+CM*), or in the presence of recombinant NGAL (+NGAL) for 24 h, at 37°C. Cell death was assessed by TMRE staining on GFP positive cells.

NGAL mediated intake of extracellular iron accounts for the antiapoptotic activity of NGAL in FRO cells

One of the properties of NGAL is its ability to deliver iron from the extracellular milieu into the cells. Since it has been described that iron could play an important role in cancer and apoptosis (62), we tested if the increased susceptibility of siRNA NGAL clones to cell death was due to the decreased NGAL-mediated delivery of iron. To this purpose, we first analyzed the ability of iron-loaded transferrin and NGAL to rescue siRNA NGAL clones from serum deprivation-induced apoptosis. As reported in Figure 7, both propidium iodide staining (A) and Western blot anticaspase-9 (B) showed that addition of transferrin or recombinant NGAL to the serumfree medium, restored the survival of siRNA clones. To further confirm that NGAL acts as survival factor for cancer cells by delivering iron, we used DFO, an iron chelator, and ferric chloride ($FeCl_3$), an iron source, to evaluate the survival of the siRNA clones. When cells were grown in complete medium in presence of DFO, both parental FRO cells and derived siRNA clones underwent apoptosis (Fig. 7C-D). On the other hand, FeCl3 produced the opposite effects: it restored the resistance of siRNA NGAL clones to serum deprivation-induced apoptosis (Fig. 7E-F). Since these results indicated that the intracellular iron content could be relevant for the survival of FRO cells, we analyzed the intracellular iron concentration of parental FRO cells and NGAL siRNA clones (Fig. 8). The colorimetric assay showed that 2/2 and 2/6 clones lacked at least 20-30% of iron content ($p<0.05$) compared to FRO, FRO Neo and 220/22 cells, thus suggesting that the absence of NGAL determined a decrease of iron uptake in the clones.

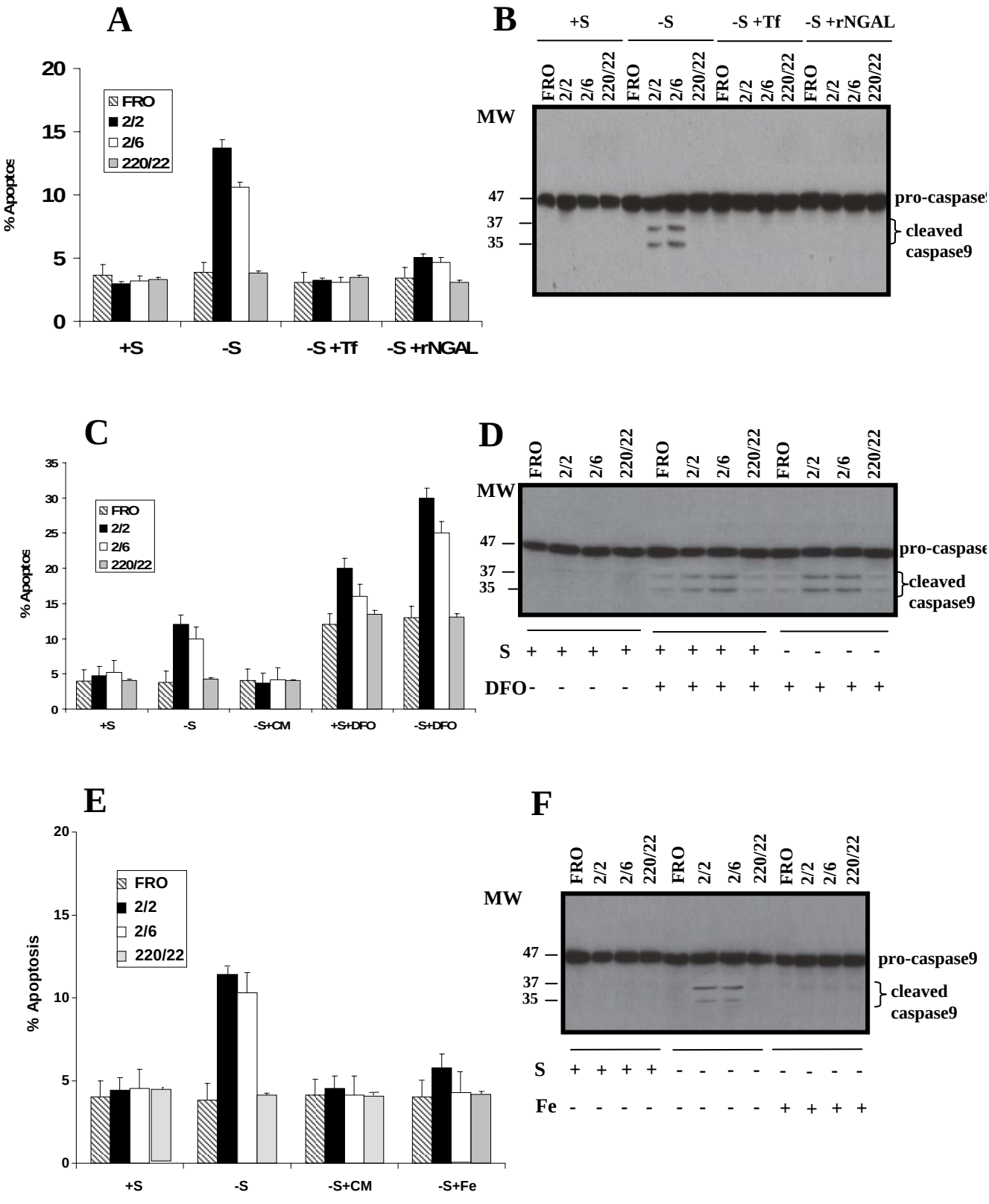
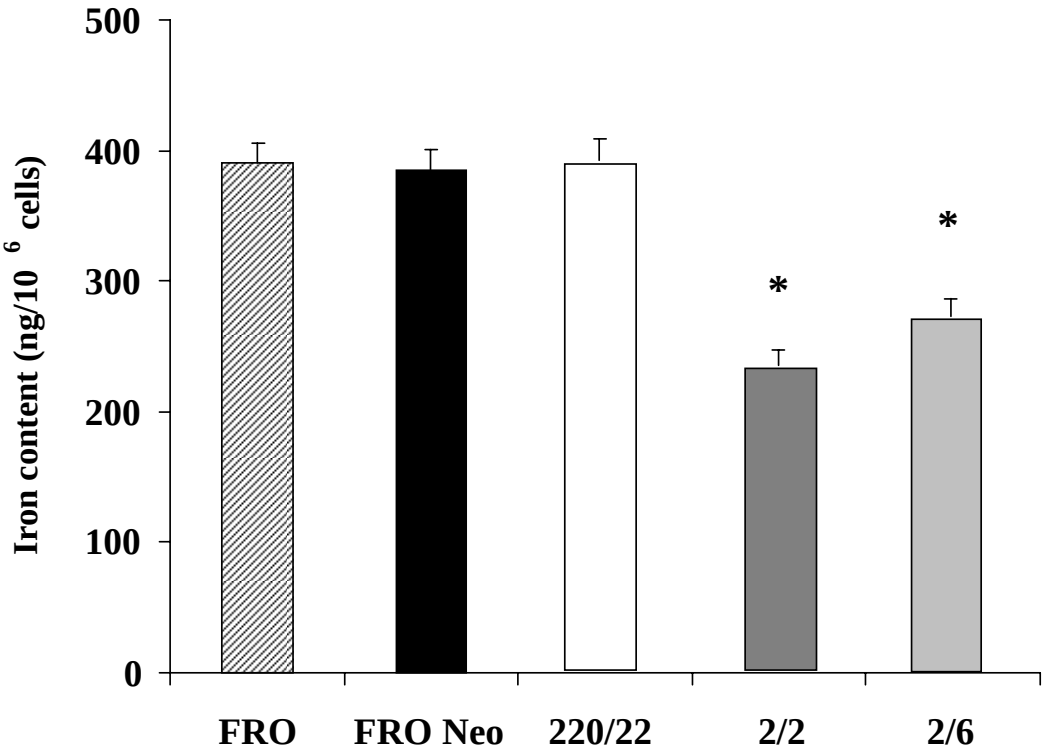


Figure 7. Iron mimicks the role of NGAL-mediated survival of FRO cells. 2.5 x 10^5 cells/well were seeded in six-well culture plates and grown in different experimental conditions, as indicated. Cell death was assessed by propidium iodide staining (**A, C, E**) or by Western blot analysis (**B, D, F**). Results were mean ± S.D. of at least three separate experiments. *S* = serum; *CM* = conditioned medium; *Tf* = transferrin; *rNGAL* = recombinant NGAL; *DFO* = deferoxamine; *Fe* = iron. **P<0.0005; *** P<0.0001



*** = p<0.05**

Fig. 8 Intracellular iron content of the different FRO cell lines. Colorimetric analysis of intracellular iron concentration. Results were mean ± S.D. ofat least three separate experiments, performed in triplicate. Significantly differentfrom controls: * $P < 0.05$.

Analysis of the apoptosis pathways

Apoptosis signalings converge in the activation of intracellular caspases. Caspases are cysteine proteases produced as inactive zymogens that cleave their substrates at aspartic acid residues contained within a tetrapeptide recognition motif. Activation of initiator caspases (procaspase-8, -9, -10) leads to the proteolytic activation of downstream effector caspases (caspase-3, -6, -7) that cleave specific substrates. We can distinguish two apoptosis pathways: the death receptor and the mitochondrial pathway. In the death receptor pathway, the death receptor pathway is triggered by members of the death receptor superfamily such as CD95. Binding of CD95-L to its receptor induces trimerization of CD95 and formation of a death-inducing complex. This complex recruits, via the adaptor molecule FADD, multiple procaspase-8 molecules, resulting in caspase-8 activation. Caspase-8 activation can be blocked by recruitment of c-FLIP. The mitochondrial death pathway is controlled by members of the Bcl-2 family, including the proapoptotic Bax and Bid proteins and the antiapoptotic Bcl-2 and Bcl-X_L proteins. Death stimuli induce the release of cytochrome c, AIF, Apaf-1, Smac/DIABLO, and possibly other factors from mitochondria. Cytochrome c associates with Apaf-1 and caspase-9 to form the apoptosome. The death receptor and mitochondrial pathways converge at the level of caspase-3 activation.

A dissection of the apoptosis pathways is illustrated in Fig. 9. In the NGAL siRNA clones grown in absence of serum, the initiating caspases-2 and -9 and the execution caspases-3/7 are activated, while the activity of other caspases, such as the initiating-, receptor-associated caspase-8, are not. This effect is reverted by the addition of recombinant NGAL (Fig. 9A). A very similar pattern of caspases activation is detected in FRO or 220/22 cells incubated with deferoxamine (DFO) (Fig.9B). Iron deprivation also resulted in the decrease of mitochondrial membrane

potential (Fig. 10A) and in the release of cytochrome C (Fig. 10B). These data suggest that iron deprivation induces apoptosis via collapse of the mitochondrial membrane potential, release of cytochrome C from mitochondria, and activation of execution caspases.

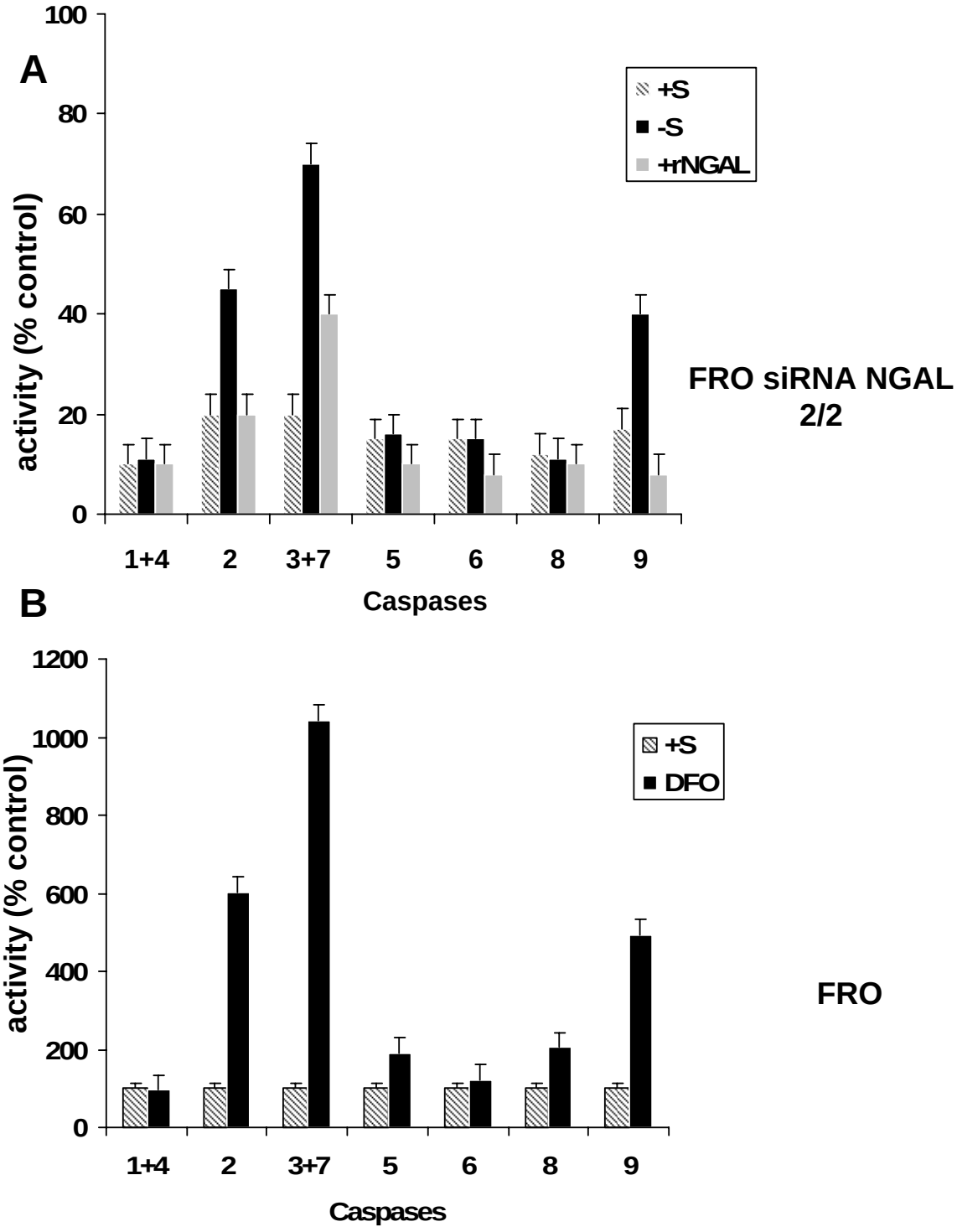


Fig.9 Analysis of the apoptosis pathway. 2.0 x 10^6 cells/well were grown in the presence (+*S*), of serum (+*S*) or in serum-free medium containing recombinant NGAL (*rNGAL*), or in presence of deferoxamine (*DFO*) for 24 h, at 37°C. Caspases activity was assessed by "Caspase Fluorometric Substrate Set Plus" (Alexis)

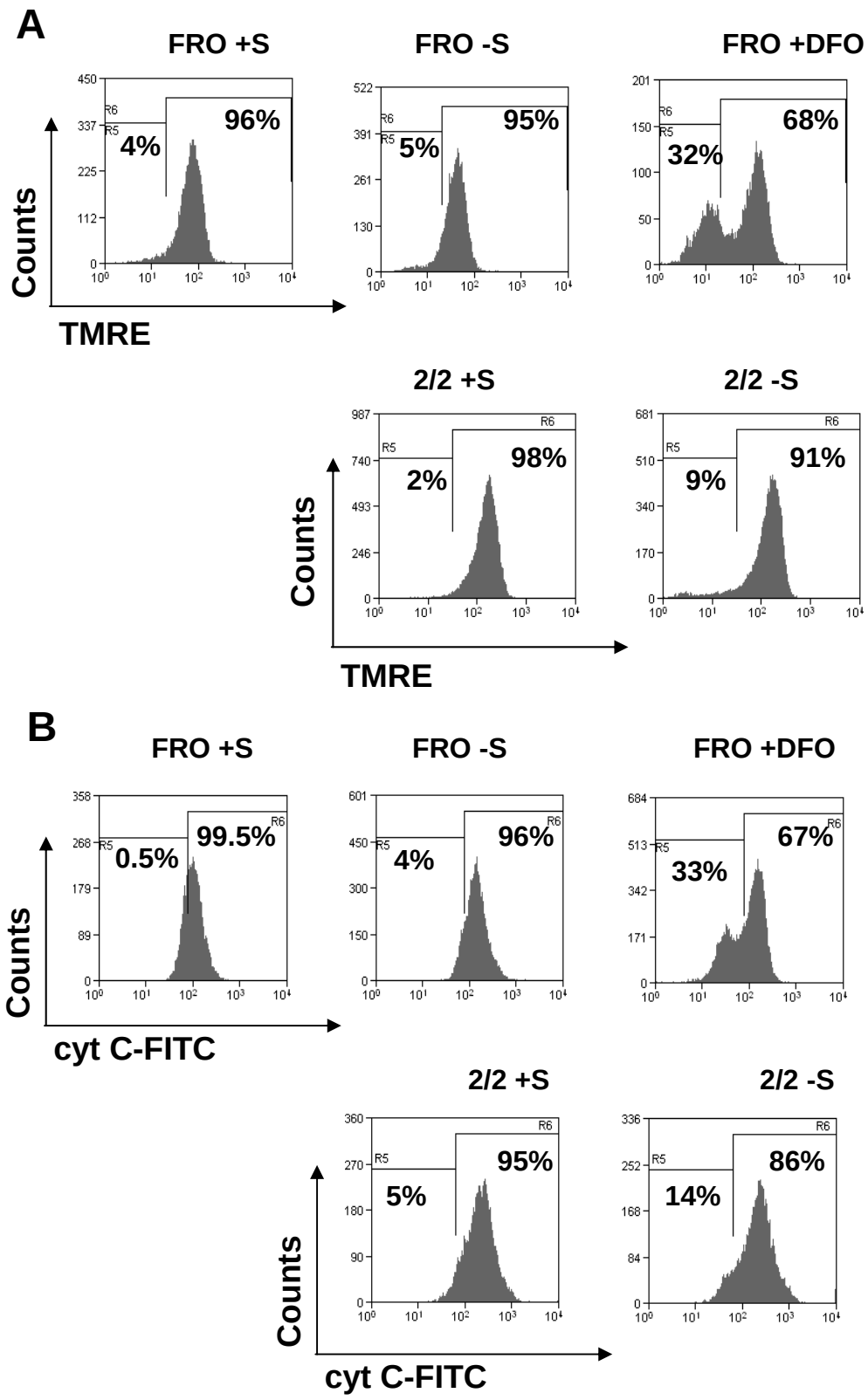


Fig.10 Analysis of the apoptosis pathway. 2.0 x 10^6 cells/well were grown in the presence (+*S*), in the absence of serum (-*S*) or in serum-free medium containing recombinant NGAL (*rNGAL*), or in presence of deferoxamine (*DFO*) for 24 h, at 37°C. (**A**).Mitochondrial membrane potential was assessed by TMRE staining (**B**). Cytocrome C release was assessed by flow cytometry (**C**). *P<0.005; **P<0.0005; *** P<0.0001.

Discussion

We have identified NGAL as a mediator of the NF-κB oncogenic activity in thyroid cancer. NGAL is highly expressed in the human thyroid carcinoma cell line FRO, and also other poorly differentiated thyroid cancer cell lines, (Fig. 1A and data not shown), is highly expressed in primary human anaplastic thyroid carcinomas (Fig. 1B-D and Table I) and acts as a survival factor for thyroid cancer cells (Fig. 5, 6, 7). The pro-survival activity of NGAL is mediated by its ability to bind iron and to transport it inside the cells (Fig. 7-8). These findings identify NGAL as a critical effector of the NF-κB-mediated oncogenic activity, define a pro-survival function for NGAL, and highlight iron as a central controller of cell survival. Various types of cancers express high levels of NGAL, including colon, pancreas, breast, bladder and liver (48-52). NGAL represents the human homolog of the rat neu-related lipocalin (NRL), a gene which was shown to be overexpressed in HER-2/neu oncogene induced rat mammary tumors (63). Whether NGAL is causal or contributory to cancer is unknown. It has been reported that NGAL is a surviving factor for cancer cells. Ectopic expression of NGAL in lung and breast cancer cell lines reduced apoptosis induced by a PDK1 inhibitor, and decreased expression of NGAL by siRNA had opposite effects (57). Our results confirm that NGAL is a surviving factor for cancer cells, and extend these findings by demonstrating that the protective effect of NGAL is mediated by its ability to bind and transport iron. Our results are in agreement with the model proposed by Devireddy et al. (56). In this model, it is proposed that internalization of the apo form of NGAL leads to iron loss and apoptosis. Conversely, internalization of iron-loaded NGAL might prevent apoptosis. Iron contributes to enzyme activity in DNA synthesis, metabolism and oxygen response, and its acquisition plays a critical role in development, cell growth and survival (62). Cancer cells have a higher

requirement for iron than normal cells, as they rapidly proliferate. This is reflected by the evidence that tumor cells have higher numbers of transferrin receptors on their surface, mediating a high rate of iron uptake (64). The importance of keeping constant the levels of iron uptake is further suggested by the evidence that multiple and redundant systems for iron uptake and transport exist. Most cells acquire iron by capturing iron loaded transferrin. However, 10 hypotransferrinemic mice (65-66) and humans (67) have defects in central nervous system development and hematopoiesis, but most epithelial organs are normal. Likewise, the mice lacking the transferrin receptor 1 initiate organogenesis but succumb to the effects of anemia (68). Given that iron is necessary for all the cells, there must be other pathways for iron acquisition in epithelial cells. One of such pathway is mediated by NGAL. In our experimental model, we observed that cells knocked-down for NGAL expression showed a decrease of about 20-30% in iron content; this decrease still allowed cell surviving. However, if the iron delivery was further decreased, or cells were exposed to an appropriate stress, such as serum deprivation, cells underwent apoptosis. Indeed, administration of iron, either as iron salt, transferrin or iron-loaded NGAL, restored the ability of knocked-down cells to survive in the absence of serum. Similarly, knock out mice for NGAL are vital and do not show gross developmental defects, although they succumb to bacterial infection (69). Many studies have demonstrated that iron chelators, such as deferoxamine (DFO), have effective anticancer activity (70). Iron is essential for the catalytic activity of ribonucleotide reductase, an enzyme mediating the conversion of all four ribonucleotides to their deoxyribonucleotide counterparts, the rate-limiting step of DNA synthesis (71). However, in our experimental system, the proliferation rate of the cells knocked-down for NGAL expression is not affected (data not shown). Iron levels strictly control the activity of specific prolyl hydroxylase-domain enzymes (PHDs), which, in turn, promote functional

activation of transcription factors involved in tumor development, as is the case of the HIF-1, which controls genes involved in energy metabolism and angiogenesis (72). HIF-1 is primarily regulated by specific PHDs that initiate its degradation via the von Hippel-Lindau tumour suppressor protein (VHL). The oxygen and iron dependency of PHD activity accounts for regulation of the pathway by both cellular oxygen and iron status. We have evidence that in our experimental system the protein level of HIF-1α is increased in the NGAL knockdown clones, and is down-regulated by the addition of iron. Conversely, in control cells, the level of HIF-1α protein is low and is up-regulated by the addition of DFO. Moreover, the activity of a HIF1-responsive promoter parallels the level of HIF-1 protein (Data not shown). Since NF- κB is a central component in the hypoxic response in that it positively regulates HIF1 1α expression (73), it is tempting to speculate that the involvement of NGAL as an iron transporter, as well as its opposing effect on HIF-1α expression, could be a part of an auto-regulatory loop with inhibitory function between NF-κB and HIF-1 that could control tumor progression. Modulation of cell survival may not be the only way NGAL influence the behaviour of a cancer cell. NGAL has been demonstrated to form a complex with MMP-9, playing a role in the maintenance of an extracellular pool of a potentially active form of the protease, whose activity is associated with angiogenesis and tumor growth (55). This is supported by the evidence that the level of NGAL expression correlates with the clinical outcome of the patients and also with the depth of the tumor invasion (56). We have evidence that a complex between NGAL and MMP9 also exists in transformed thyroid cells, although nor the exact role that such as complex plays in thyroid cancer is known, neither if the activity of MMP9 is affected by the presence of NGAL. In other experimental systems NGAL has been shown to induce expression of E-cadherin, to promote formation of polarized epithelia and to diminish invasiveness of Ras-transformed cell lines (74). In our experimental

system, NGAL seems to have a different role. In fact, ectopic expression of NGAL in normal thyroid cell line, or its knock-down in transformed cells, does not alter the expression of E-cadherin nor vimentin (data not shown). Our results, in addition to identify NGAL as a potential target for therapeutic intervention, also strengthens the rationale for the use of iron chelators in the treatment of cancer. Depleting iron from a rapidly dividing cancer cell, through the implementation of iron chelators, or decreasing NGAL expression, deprives it of a component critical for various cellular processes and induces apoptosis.

References

1) Karin M, Lawrence T, Nizet V: Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell*, **124** (2006), 823-835
2) Ghosh S and Karin M: Missing pieces in the NF-κB puzzle. *Cell*, **109** (2002), 81-S96
3) Hacker H and Karin M: Regulation and function of IKK and IKK related kinases. *Science*, STKE **13** (2006)
4) Perkins ND: Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene*, **25** (2006), 6717-6730
5) Hoffman A, Natoli G, Ghosh G: Transcriptional regulation via the NF-kappaB signaling module. *Oncogene,* **25** (2006) 6706-6716
6) Gilmore TD: *www.NF-κB.org* (2008)
7) Hayden MS and Ghosh S: Shared principles in NF-κB signaling. *Cell*, **132** (2008), 344-361
8) Chua HL, Bhat-Nakshatri, Clare SE, Morimiya A, Badve S, Nakshatri H: NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene,* **26**(2007), 711-714
9) Yang J, Pan WH, Clawson GA, Richmond A: Systemic targeting inhibitor of kappaB kinase inhibits melanoma tumor growth. *Cancer Research* **6**7(2007), 3127-3134
10) Tew GW, Lorimer EL, Berg TJ, Zhi H, Li R, Williams CL: SmgGDS regulates cell proliferation, migration, and NF-kappaB transcriptional activity in non-small cell lung carcinoma. *J Biol Chem* (2007).

11) Scartozzi M, Bearzi I, Pierantoni C et al: Nuclear factor-kB tumor expression predicts response and survival in irinotecan-refrectory metastatic colorectal cancer treated with cetuximab-irinotecan therapy. *J Clin Oncol*, **25**(2007), 3930-3935

12) Annunziata GM, Davis RE, Demchenko Y et al : Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. *Cancer Cell*,**12** (2007), 115-130

13) Weichert W, Boehm M, Gekeler V et al: High expression of REL A/P65 is associated with activation of nuclear factor-kappaB-dependent signaling in pancreatic cancer and marks a patient population with poor prognosis. *Br J Cancer*, **97**(2007), 523-530

14) Izzo JG, Malhotra U, Wu TT et al: Clinical biology of esophageal adenocarcinoma after surgery is influenced by nuclear factor-kappa expression. *Cancer Epidemiol Biomarkers Prev,* **16**(2007), 1200-1205

15) Rae C, Langa S, Tucker SJ, MacEwan DJ: Elevated NF-kappaB responses and FLIP levels in leukemic but not normal lymphocytes: reduction by salicylate allows TNF-induced apoptosis. *Proc Natl Acad Sci USA*, **104** (2007), 12790-12795

16) Vilimas T, Mascarenhas J, Palomero T et al: Targeting The NF-kappaB signaling pathway in Notch1-induced T-cell leukemia. *Nat Med*, **13**(2007):70-77

17) Fabre C, Carvalho G, Tasdemir E et al: NF-kappaB inhibition sensitizes to starvation-induced cell death in high risk myelodisplastic syndrome and acute myeloid leukemia. *Oncogene*, **26** (2007), 4071-4083

18) Zou P, Kawada J, Pesniack L, Cohen JI: Bortezomib induces apoptosis of Epstein-Barr virus (EBV)-transformed B cells and prolongs survival of

mice inoculated with EBV-transformed B cells. *J Virol*,**81**(2007), 10029-10036

19) Zhang B, Wang Z, Li T, Tsitsikov EN, Ding HF: NF-kappaB2 mutation targets TRAF1 to induce lymphomagenesis. *Blood*, **110** (2007), 743-751

20) Naugler WE and Karin M: NF-κB and cancer – identifying targets and mechanisms. *Current Opinion in Genetics & Development* **18** (2008), 19-26

21) M. Karin, Y. Cao, F.R. Greten and Z.W. Li, NF-κB in cancer: from innocent bystander to major culprit: *Nat Rev Cancer* **2** (2002), 301–310.

22) F.R. Greten, L. Eckmann, F.T. Greten, J.M. Park, Z.W. Li and L.J. Egan et al*l*: IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer: *Cell* **118** (2004), 285–296.

23) E. Pikarsky, R.M. Porat, I. Stein, R. Abramovitch, S. Amit and S. Kasem et al: NF-κB functions as a tumour promoter in inflammation-associated cancer, *Nature* **431** (2004), 461–466.

24) D. Hanahan and R.A. Weinberg: The hallmarks of cancer, *Cell* **100** (2000), 57–70.

25) B.B. Aggarwal, Nuclear factor-κB: the enemy within, *Cancer Cell* **6** (2004), 203–208.

26) D.C. Guttridge, C. Albanese, J.Y. Reuther, R.G. Pestell and A.S. Baldwin Jr: NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1, *Mol Cell Biol* **19** (1999), 5785–5799.

27) N.D. Perkins, L.K. Felzien, J.C. Betts, K. Leung, D.H. Beach and G.J. Nabel: Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator, *Science* **275** (1997), 523–527

28) D.W. Kim, L. Gazourian, S.A. Quadri, R. Romieu-Mourez, D.H. Sherr and G.E. Sonenshein: The RelA NF-kappaB subunit and the aryl hydrocarbon receptor (AhR) cooperate to transactivate the c-myc promoter in mammary cells, *Oncogene* **19** (2000), 5498–5506.

29) H.L. Pahl, Activators and target genes of Rel/NF-κB transcription factors: *Oncogene* **18** (1999), 6853–6866

30) D.K. Biswas, A.P. Cruz, E. Gansberger and A.B. Pardee, Epidermal growth factor-induced nuclear factor kappa B activation: a major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells: *Proc Natl Acad Sci USA* **97** (2000), 8542–8547.

31) A.R. Farina, A. Tacconelli, A. Vacca, M. Maroder, A. Gulino and A.R. Mackay: Transcriptional up-regulation of matrix metalloproteinase-9 expression during spontaneous epithelial to neuroblast phenotype conversion by SK-N-SH neuroblastoma cells, involved in enhanced invasivity, depends upon GT-box and nuclear factor kappaB elements, *Cell Growth Differ* **10** (1999), 353–367.

32) U. Novak, B.G. Cocks and J.A. Hamilton: A labile repressor acts through the NF-κB-like binding sites of the human urokinase gene, *Nucleic Acids Res* **19** (1991), 3389–3393.

33) C. Kunsch, C.A. Rosen et al : NF-kappaB subunit-specific regulation of the interleukin-8 promoter, *Mol Cell Biol* **13** (1993), 6137–6146

34) A. van de Stolpe, E. Caldenhoven, B.G. Stade, L. Koenderman, J.A. Raaijmakers and J.P. Johnson et al: 12-*O*-tetradecanoylphorbol-13-acetate- and tumor necrosis factor alpha-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. Functional analysis of the human intercellular adhesion molecular-1 promoter, *J Biol Chem* **269** (1994), 6185–6192.

35) G. Helbig, K.W. Christopherson 2nd, P. Bhat-Nakshatri, S. Kumar, H. Kishimoto and K.D. Miller et al: NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4, *J Biol Chem* **278** (2003), 21631–21638

36) S. Kiriakidis, E. Andreakos, C. Monaco, B. Foxwell, M. Feldmann and E. Paleolog: VEGF expression in human macrophages is NF-kappaB-dependent: studies using adenoviruses expressing the endogenous NF-kappaB inhibitor IkappaBalpha and a kinase-defective form of the IkappaB kinase 2, *J Cell Sci* **116** (2003), 665–674.

37) C.Y. Wang, M.W. Mayo, R.G. Korneluk, D.V. Goeddel and A.S. Baldwin Jr: NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation, *Science* **281** (1998), 1680–1683.

38) Q.L. Deveraux, N. Roy, H.R. Stennicke, T. Van Arsdale, Q. Zhou and S.M. Srinivasula et al: IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases, *Eur Mol Biol Org J* **17** (1998), 2215–2223.

39) L.H. Boise, M. Gonzalez-Garcia, C.E. Postema, L. Ding, T. Lindsten and L.A. Turka et al: bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death, *Cell* **74** (1993), 597–608

40) W.C. Yeh, A. Itie, A.J. Elia, M. Ng, H.B. Shu and A. Wakeham et al: Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development, *Immunity* **12** (2000), 633–642.

41) E. De Smaele, F. Zazzeroni, S. Papa, D.U. Nguyen, R. Jin and J. Jones et al: Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling, *Nature* **414** (2001), 308–313.

42) C.G. Pham, C. Bubici, F. Zazzeroni, S. Papa, J. Jones and K. Alvarez et al: Ferritin heavy chain upregulation by NF-kappaB inhibits TNFalpha-induced apoptosis by suppressing reactive oxygen species, *Cell* **119** (2004), 529–542.

43) S. Keates, Y.S. Hitti, M. Upton and C.P. Kelly: Helicobacter pylori infection activates NF-kappa B in gastric epithelial cells, *Gastroenterology* **113** (1997), 1099–1109

44) Pacifico F and Leonardi A: NF-κB in solid tumors, *Biochemical Pharmacology* **72** (2006), 1142-1152

45) Flower DR, North AC, Sansom CE. The lipocalin protein family: structural and sequence overview. *Biochim Biophys Acta* **1482** (2000): 9-24

46) Kjeldsen L, Johnsen AH, Sengeløv H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem.* **268** (1993): 10425-32

47) Nilsen-Hamilton M, Liu Q, Ryon J, Bendickson L, Lepont P, Chang Q. Tissue involution and the acute phase response. *Ann N Y Acad Sci* **995** (2003), 94-108.

48) Nielsen BS, Borregaard N, Bundgaard JR, Timshel S, Sehested M, Kjeldsen L. Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases. *Gut* **38** (1996), 414-20

49) Stoesz SP, Friedl A, Haag JD, Lindstrom MJ, Clark GM, Gould MN. Heterogeneous expression of the lipocalin NGAL in primary breast cancers. *Int J Cancer*. **79** (1998), 565-72

50) Friedl A, Stoesz SP, Buckley P, Gould MN. Neutrophil gelatinase-associated lipocalin in normal and neoplastic human tissues. Cell type-specific pattern of expression. *Histochem J*. **31** (1999), 433-41.

51) Bartsch S, Tschesche K. Cloning and expression of human neutrophil lipocalin cDNA derived from bone marrow and ovarian cancer cells. *FEBS Lett*. **357** (1995), 255-9.

52) Furutani M, Arii S, Mizumoto M, Kato M, Imamura M. Identification of a neutrophil gelatinase-associated lipocalin mRNA in human pancreatic cancers using a modified signal sequence trap method. *Cancer Lett*. **122 (**1998), 209-214

53) Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol Cell*. **10** (2002), 1033-43.

54) Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, Akira S, Aderem A. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature*. **432** (2004**)**, 917-21.

55) Fernández CA, Yan L, Louis G, Yang J, Kutok JL, Moses MA. The matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients. *Clin Cancer Res.* **11** (2005), 5390-5.

56) Devireddy LR, Gazin C, Zhu X, Green MR. A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. *Cell* **123** (2005), 1293-305.

57) Tong Z, Wu X, Ovcharenko D, Zhu J, Chen CS, Kehrer JP. Neutrophil gelatinase-associated lipocalin as a survival factor. *Biochem J*. **391** (2005), 441-8.

58) Le NT, Richardson DR. The role of iron in cell cycle progression and the proliferation of neoplastic cells. *Biochim Biophys Acta* **1603 (**2002), 31-46.

59) Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* **441** (2006), 431-446

60) Lin WW, Karin M. A citokine-mediated link between innate immunity, inflammation and cancer. *J Clin Invest* **117 (**2007), 1175-1183

61) Pacifico F et al. Oncogenic and anti-apoptotic activity of NF-κB in human thyroid carcinomas. *J Biol Chem* **279** (2004), 54610-54619

62) Dunn LL, Rahmanto YS, Richardson DR. *Trends cell bio.* **17** (2007), 93-100

63) Stoesz SP, Gould MN. Overexpression of neu-related lipocalin (NRL) in neu-initiated but not ras or chemically initiated rat mammary carcinomas. *Oncogene* **11** (1995), 2233-2241

64) Richardson DR, Ponka P. The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. *Biochim Biophys Acta* **1331 (**1997), 1-40

65) Huggenvik JI, Craven CM, Idzerda RL, Bernstein S, Kaplan J, McKnight GS. A splicing defect in the mouse transferrin gene leads to congenital atransferrinemia. *Blood* **74** (1989), 482-6.

66) Trenor CC 3rd, Campagna DR, Sellers VM, Andrews NC, Fleming MD. The molecular defect in hypotransferrinemic mice. *Blood*. **96** (2000), 1113-8.

67) Hayashi A, Wada Y, Suzuki T, Shimizu A. Studies on familial hypotransferrinemia: unique clinical course and molecular pathology. *Am J Hum Genet.* **53** (1993), 201-13.

68) Levy JE, Jin O, Fujiwara Y, Kuo F, Andrews NC. Transferrin receptor is necessary for development of erythrocytes and the nervous system. *Nat Genet.* **21** (1999), 396-9.

69) Berger T, Togawa A, Duncan GS, Elia AJ, You-Ten A, Wakeham A, Fong HE, Cheung CC, Mak TW. Lipocalin 2-deficient mice exhibit increased sensitivity to Escherichia coli infection but not to ischemia-reperfusion injury. *Proc Natl Acad Sci U S A*. **103** (2006), 1834-9.

70) Buss JL, Torti FM, Torti SV. The role of iron chelation in cancer therapy. *Curr Med Chem* **10 (**2003), 1021-1034.

71) Richardson DR. Molecular mechanisms of iron up-take by cells and the use of iron chelators for the treatment of cancer. *Curr Med Chem* **12** (2005), 2711-2729

72) Semenza G. HIF-1, 02, and the 3 PHDs: How animal cells signal hypoxia to the nucleus. *Cell* **107** (2001), 1-3

73) Rius J et al. NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. *Nature* **453** (2008), 807-811

74) Hanai J et al. Lipocalin 2 diminishes invasiveness and metastasis of Ras-transformed cells. *J Biol Chem* **280** (2005), 13641-13647

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