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

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NF-κB is constitutively activated in primary human thyroid tumors, particularly in those of anaplastic type. The inhibition of NF-κB activity in the human anaplastic thyroid carcinoma cell line, FRO, leads to an increased susceptibility to chemotherapeutic drug-induced apoptosis and to the blockage of their ability to form tumors in nude mice. To identify NF-κB target genes involved in thyroid cancer, we analyzed the secretome of conditioned media from parental and NF-κB-null FRO cells. Proteomic analysis revealed that the neutrophil gelatinase-associated lipocalin (NGAL), a protein involved in inflammatory and immune responses, is secreted by FRO cells whereas its expression is strongly reduced in the NF-κB-null FRO cells. NGAL is highly expressed in human thyroid carcinomas, and knocking down its expression blocks the ability of FRO cells to grow in soft agar and form tumors in nude mice. These effects are reverted by the addition of either recombinant NGAL or FRO conditioned medium. In addition, we show that the prosurvival activity of NGAL is mediated by its ability to bind and transport iron inside the cells. Our data suggest that NF-κB contributes to thyroid tumor cell survival by controlling iron uptake via NGAL.

Cancer is a multistep process during which cells undergo alterations of their normal functions that progressively lead to the genesis of a tumor. Among the most significant changes, cancer cells begin to secrete, into the tumor microenvironment, a number of factors that stimulate their own growth/survival and mediate angiogenesis and metastasis. During the last years, the role of NF-κB in the pathogenesis of human cancer has strongly emerged. In particular, because 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 (1, 2). This is not particularly surprising given that NF-κB controls expression of a number of proinflammatory factors (cytokines, chemokines ,and growth factors), secreted by cancer cells in the tumor microenvironment, that substantially contribute to tumor development (3, 4). 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 innovative perspectives in the treatment of tumors.

We have shown that NF-κB is strongly activated in human anaplastic thyroid carcinomas (ATCs) (5). To study the role of NF-κB in thyroid cancer, we 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 from an increased susceptibility to drug-induced apoptosis (5).

The neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, is a member of a large family of lipocalins, a group of small extracellular proteins with great functional diversity (6). 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 a matrix metalloproteinase-9 (MMP-9) (7). NGAL is thought to be an acute phase protein (8) whose expression is up-regulated in human epithelial cells under different inflammatory conditions, such as inflammatory bowel disease (9). Elevated NGAL expression has also been shown in different human tumors including breast (10), lung (11), colon (9), ovary (12), and pancreas (13) carcinomas. However, the precise role of NGAL has not been well defined. Several studies have suggested that NGAL is a potent bacteriostatic agent that has siderophore-mediated sequestering of iron (14, 15), and that it is capable of protecting MMP-9 from autodegradation, thereby favoring the metastatic potential of cancer cells (16). As a secreted binding protein, it has been reported that the murine ortholog, 24p3, plays a crucial role in IL-3 deprivation-induced apoptosis by regulating intracellular iron delivery, very likely after interaction with a 24p3 receptor (17). Indeed, the human NGAL protects A459 and MCF7 cells from apoptosis induced by PDK1 inhibitors (18). Last, NGAL induces cell proliferation by promoting the iron-dependent metabolism of nucleotides for DNA synthesis (19).

The cellular system FRO/FRO IκBαM represents an excellent model for the identification of NF-κB-regulated factors that, when secreted in the extracellular milieu, could play a role in thyroid cancer. Thus, we used a different proteomic approach to analyze 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. We show that knocking down NGAL expression blocks the ability of FRO cells to form colonies in soft agar and tumors in nude

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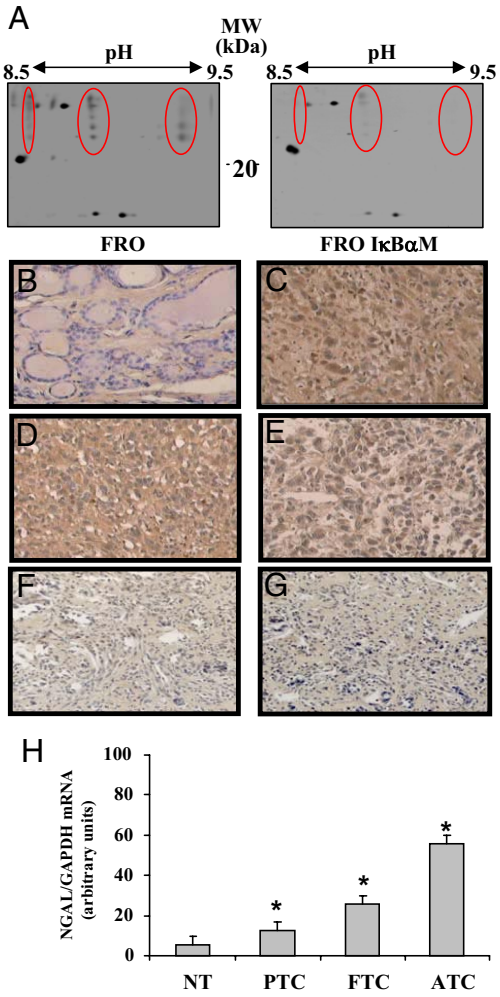

Fig. 1. Analysis of NGAL expression. Two-dimensional gel electrophoresis showing expression of NGAL in the conditioned medium of FRO IκBαM cells, as compared with parental counterpart FRO (*A*). Localization of NGAL *in situ* was determined by immunohistochemistry in sections from normal thyroid tissue (*B*) and three different ATCs (*C–E*). Lack of specific tissue staining was seen when either secondary Abs (*F*) or blocked Abs (*G*) were used. Magnification: ×400. (*H*) 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 ($n = 3$). *, $P < 0.0005$.

mice and, therefore, increases their susceptibility to apoptosis. In addition, we show that the prosurvival activity of NGAL is mediated by its ability to bind and transport iron inside the cells.

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. Among the proteins whose expression decreased in the extracellular medium of FRO IκBαM cells, as compared with FRO, at least three groups were examined because of their strong reduction (Fig. 1 *A*, circled spots). The MALDI-TOF peptide mass fingerprint analysis identified all spots as NGAL, whose modifications after translation account for the multiple species that show variable molecular weight and pI values in the 2-DE gel.

Northern and Western blot assays confirmed the decreased expression of NGAL in FRO IκBαM cells, as compared with parental FRO cells [supporting information (SI) Fig. S1 *A–C*]. To confirm that NF-κB was controlling NGAL expression, we knocked down p65 expression in FRO cells by using siRNA and detected a decreased NGAL expression by Western blot analysis(Fig. S1*D*). In addition, WT and $p65^{-/-}$ mouse embryonic fibroblasts (MEF) were treated with IL1β or IL17, and the NGAL expression was detected by Northern and Western blot assays (Fig. S1 *E* and F). No induction of NGAL was detected in absence of p65. Reexpression of p65 in $p65^{-/-}$ MEF restored NGAL expression (Fig. S1*G*).

It was demonstrated that NF-κB controls NGAL expression via the inducible cofactor IκBζ (20, 21). We then investigated, by using chromatin immunoprecipitation, whether, in FRO cells, IκBζ was also bound to the NGAL promoter. As shown in Fig. S2, IκBζ was bound to the NGAL promoter together with the p50 and the p52 subunits of NF-κB.

Immunohistochemical 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 specimen and specimen from different thyroid carcinomas by immunostaining. As shown in Table 1 and Fig. 1 *B–G*, although NGAL expression was not detected in normal thyroid, NGAL immunostaining was positive for papillary, follicular and ATC. The levels of NGAL expression increased with the malignant phenotype of tumors, reaching the highest intensity in the anaplastic carcinomas (Table 1 and Fig. 1 *B–G*), in parallel with NF-κB basal activity in the same types of thyroid tumors (5). Real time PCR analysis confirmed the increased expression on the NGAL mRNA in primary human thyroid cancer (Fig. 1*H*).

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. Fig. 2*A* shows the levels of NGAL protein in two of the clones analyzed (indicated as siRNA NGAL 2/2 and 2/6) in FRO cells, either transfected with the empty vector (FRO Neo cells) or transfected with a control siRNA

Table 1. Immunohistochemical analysis of NGAL expression in normal and pathological human thyroid tissues

Histological type of thyroid samples	Number of total cases analyzed	Number of positive cases/ Number of total cases analyzed	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

Human specimens were 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.

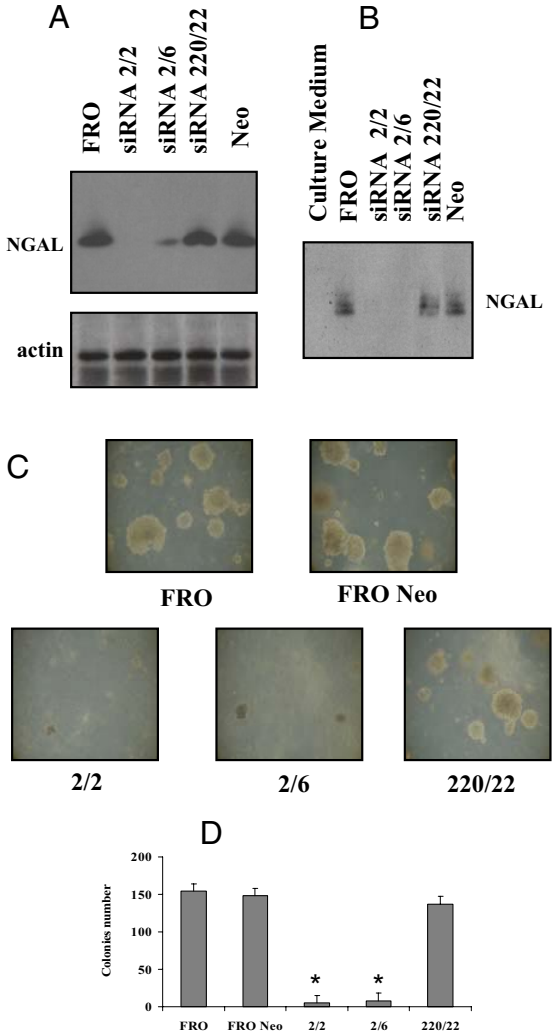

Fig. 2. Inhibition of NGAL expression by siRNA and *in vitro* oncogenic activity of the siRNA clones. The expression of NGAL in FRO cells, stably transfected with either siRNA plasmids (2/2, 2/6), control siRNA plasmid (220/22), or empty vector (Neo), was determined by Western blot analysis on total cell lysates (*A*) and on conditioned media (*B*). (*C*) Colony formation assay. Colonies >50 cells were scored after 2 weeks incubation at 37°C (*D*). Magnification: ×200. *, $P <$ 0.0001 (2/2 and 2/6 vs. FRO, FRO-Neo, and 220/22).

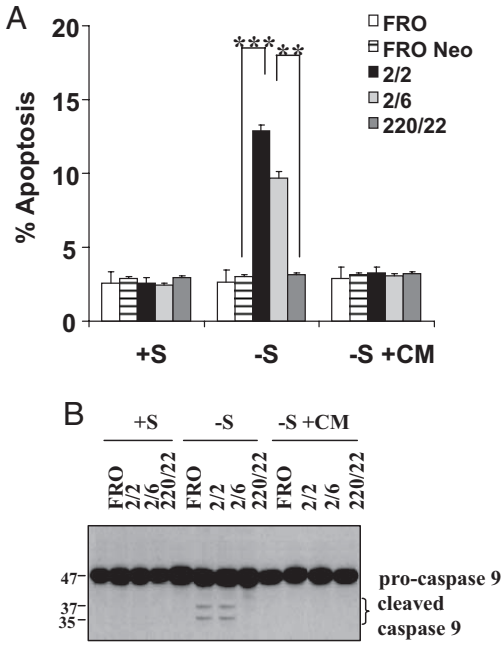

Fig. 3. NGAL protects FRO cells from apoptosis induced by serum-withdrawal. 2.5×10^5 cells per well were seeded in 6-well culture plates and grown either in the presence of serum (+*S*), in the absence of serum (−*S*), or in serum-free conditioned medium from FRO cells (−*S* + *CM*). Cell death was assessed by propidium iodide staining (*A*) and by Western blot analysis (*B*). Results were mean ± SD of at least three separate experiments. **, $P < 0.0005$; ***, $P < 0.0001$.

(indicated as siRNA NGAL 220/22). Fig. 2*B* shows the levels of secreted NGAL. These clones were tested for their ability to grow in semisolid medium and to form tumors in nude mice. In *in vitro* experiments, whereas 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. 2 *C* and *D*). In *in vivo* assays, the injection of parental FRO or 220/22 cells into nude mice induced tumor formation in 12 of 12 mice, whereas the injection of 2/2 or 2/6 cells induced tumor formation in 1 of 6 and 4 of 6 mice, respectively (Table 2). The tumors developed from 2/2 and 2/6 cells were 4-fold and 2-fold smaller than those formed after injection of parental cells, respectively (Table 2). Notably, 2 of 4 tumors from 2/6 cells showed NGAL staining after immunohistochemical analysis, indicating that the expression of the siRNA plasmid was lost. We also blocked NF-κB in FRO cells by transient overexpression of IκBαM. In the absence of functional NF-κB, FRO cells did not form colonies in soft agar. Reexpression of NGAL partially rescued the ability of FRO cells to form colonies in soft agar (Fig. S3 *A–C*).

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% apoptosis, as assessed by propidium iodide staining (Fig. 3*A*). 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. 3*A*). This effect was reverted by the addition of the conditioned medium of either

Table 2. *In vivo* tumor growth induced by FRO Neo cells and FRO siRNA NGAL clones

Cell type	Tumor incidence	Tumor volume average, cm^3	Tumor weight average, g
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

2×10^7 cells were injected s. c. on a flank of each 6-week-old nude mouse. Tumor weight, diameter, and volume values were measured and determined as described in the *SI Text*. Two of four tumors developed by mice injected with FRO siRNA 2/6 cells showed partial NGAL staining after immunohistochemical analysis. ***, $P < 0.0001$ (FRO 2/2 vs. FRO); **, $P < 0.001$ (FRO 2/6 vs. FRO).

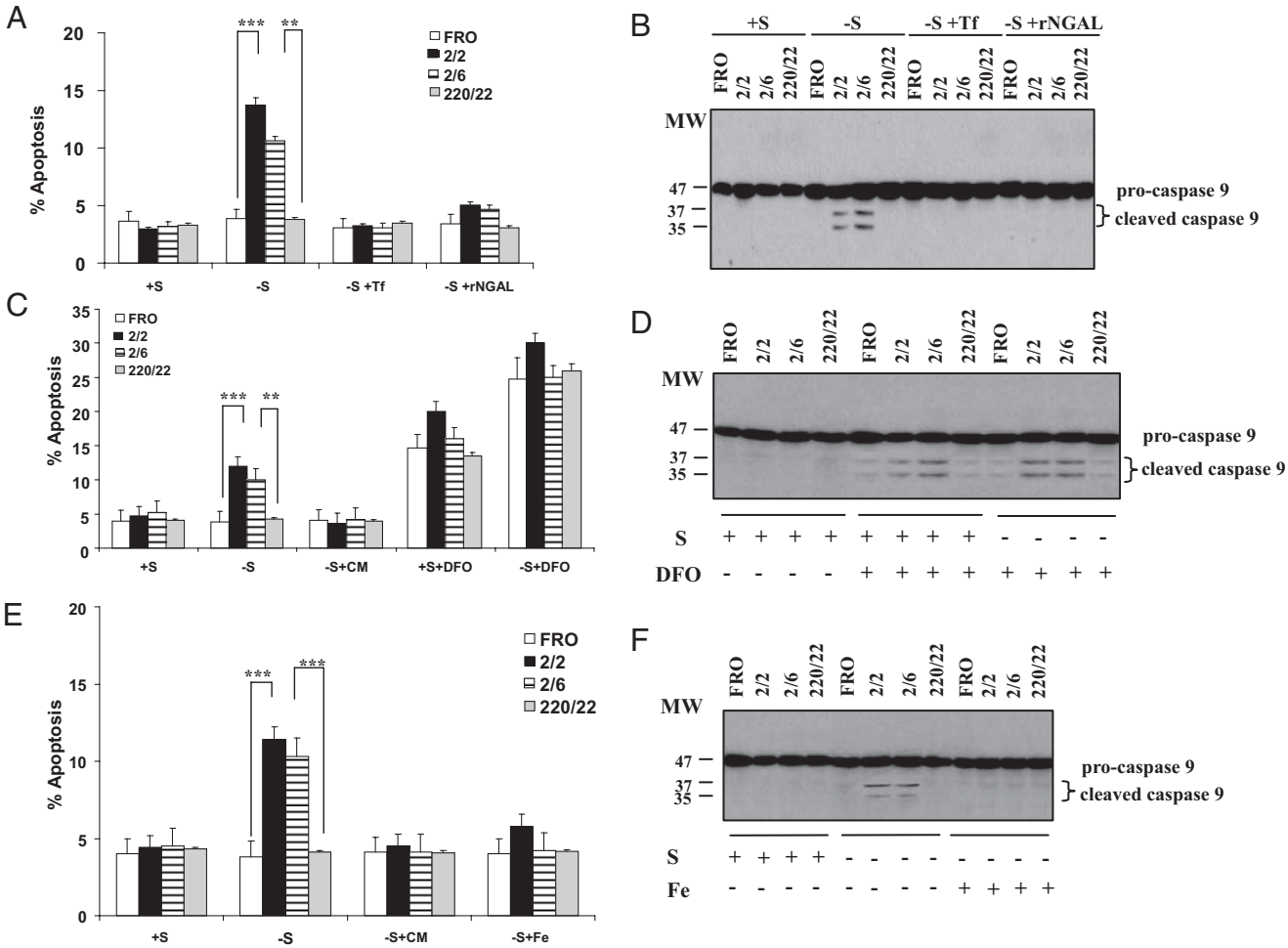

Fig. 4. Iron mimics the role of NGAL-mediated survival of FRO cells. 2.5×10^5 cells per well were seeded in 6-well culture plates and grown in different experimental conditions, as indicated. Cell death was either assessed by propidium iodide staining (*A*, *C*, and *E*) or by Western blot analysis (*B*, *D*, and *F*). Results were mean ± SD of at least three separate experiments. S, serum; CM, conditioned medium; Tf, transferrin; rNGAL, recombinant NGAL; DFO, deferoxamine; Fe, iron. **, $P < 0.0005$; ***, $P < 0.0001$.

FRO cells, FRO Neo cells, 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. S3*D*). To confirm the activation of the apoptotic machinery, we analyzed by using Western blot analysis, the caspase-9 activity in parental FRO cells and NGAL siRNA clones grown in the same experimental conditions used in the propidium iodide assay (Fig. 3*B*). Serum deprivation induced cleavage of caspase-9 in 2/2 and 2/6 clones but not in parental FRO cells (Fig. 3*B*). Also, in this case, incubation of NGAL siRNA clones with the conditioned medium of FRO cells, FRO Neo cells, or 220/22 cells restored their survival (Fig. 3 *A* and *B*). These data strongly suggest that NGAL is a survival factor for thyroid carcinoma cells.

A dissection of the apoptosis pathways is illustrated in Fig. S4. In the NGAL siRNA clones grown in absence of serum, the initiating caspases-2 and -9 and the execution caspases-3 and 7 are activated, whereas the activity of other caspases (such as the initiating-, receptor-associated caspase-8) are not. This effect is reverted by the addition of either recombinant NGAL or iron (Fig. S4*A*). A very similar pattern of caspases activation is detected in FRO or 220/22 cells incubated with deferoxamine (DFO). Iron deprivation also resulted in the decrease of mitochondrial membrane potential (Fig. S4*B*) and in the release of cytochrome C (Fig. S4*C*). 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.

NGAL-Mediated Intake of Extracellular Iron Accounts for the Antiapoptotic Activity of NGAL in FRO Cells. One of the properties of NGAL is an ability to deliver iron from the extracellular milieu into the cells. Because it has been described that iron could play an important role in cancer and apoptosis (22), we tested whether the increased susceptibility of siRNA NGAL clones to cell death was because of the decreased NGAL-mediated delivery of iron. We first analyzed the ability of iron-loaded transferrin and NGAL to rescue siRNA NGAL clones from serum deprivation-induced apoptosis. As reported in Fig. 4, both propidium iodide staining (*A*) and Western blot anti-caspase-9 (*B*) showed that addition of transferrin or recombinant NGAL to the serum-free medium restored the survival of siRNA clones.

To further confirm that NGAL acts as a 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. 4 *C* and *D*). However, $FeCl_3$ produced the opposite effect; it restored the resistance of siRNA NGAL clones to serum deprivation-induced apoptosis (Fig. 4 *E* and *F*).

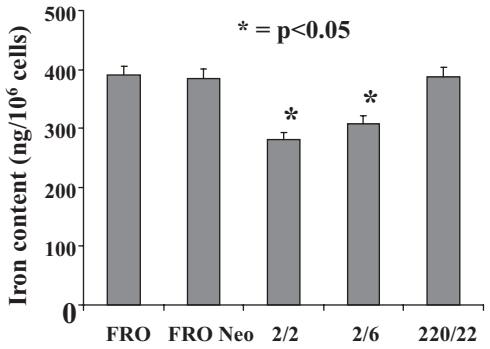

Fig. 5. Intracellular iron content of the different FRO cell lines. Colorimetric analysis of intracellular iron concentration is shown. Results were mean ± SD of at least three separate experiments, performed in triplicate. Significantly different from controls. *, $P < 0.05$.

Because 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. 5). The colorimetric assay showed that 2/2 and 2/6 clones lacked at least 20–30% of iron content ($P < 0.05$) compared with FRO, FRO Neo, and 220/22 cells, thus suggesting that the absence of NGAL determined a decrease of iron uptake in the clones.

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 FRO cell line and other poorly differentiated thyroid cancer cell lines (Fig. 1*A* and data not shown), is highly expressed in primary human ATC (Fig. 1 *B–E* and Table 1), and acts as a survival factor for thyroid cancer cells (Figs. 3 and 4). The prosurvival activity of NGAL is mediated by its ability to bind iron and to transport it inside the cells (Figs. 4 and 5). These findings show that NGAL is a critical effector of NF-κB-mediated oncogenic activity, defines a prosurvival function of NGAL, and highlights iron as a central controller of cell survival.

Various types of cancers express high levels of NGAL, including colon, pancreas, breast, bladder, and liver (9–13). NGAL represents the human homolog of the rat neu-related lipocalin, a gene that was shown to be overexpressed in HER-2/neu oncogene-induced rat mammary tumors (23). 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 the apoptosis that was induced by a PDK1 inhibitor whereas the decreased expression of NGAL by siRNA had opposite effects (18). Our results confirm that NGAL is a surviving factor for cancer cells, and we 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.* (17). 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 (22). Cancer cells have a higher requirement for iron than normal cells because 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 (24). The importance of keeping the levels of iron uptake constant 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, hypotransferrinemic mice (25, 26) and humans (27) have defects in central nervous system development and hematopoiesis when most epithelial organs are normal. Likewise, the mice lacking the transferrin receptor 1 initiate organogenesis but succumb to the effects of anemia (28). Given that iron is necessary for all of the cells, there must be other pathways for iron acquisition in epithelial cells. One such pathway is mediated by NGAL. In our experimental model, we observed that cells knocked down for NGAL expression showed a decrease of ≈20–30% iron content, and that this decrease still allowed cell survival. However, whether either 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 knockdown cells to survive in the absence of serum. Similarly, NGAL knockout mice are vital and do not show gross developmental defects, although they succumb to bacterial infection (29).

Many studies have demonstrated that iron chelators, such as DFO, have effective anticancer activity (30). Iron is essential for the catalytic activity of ribonucleotide reductase (an enzyme mediating the conversion of all four ribonucleotides to their deoxyribonucleotide counterparts), which is the rate-limiting step of DNA synthesis (31). 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 (32). HIF-1 is primarily regulated by specific PHDs that initiate its degradation via the von Hippel-Lindau tumor suppressor protein. 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 up-regulated by the addition of DFO. Moreover, the activity of a HIF1-responsive promoter parallels the level of HIF-1 protein (A.I., F.P., and A. Leonardi, unpublished observation). Because NF-κB is a central component in the hypoxic response that positively regulates HIF-1α expression (33), it is tempting to speculate that the involvement of NGAL as an iron transporter, and its opposing effect on HIF-1α expression, could be a part of an autoregulatory loop that has inhibitory function between NF-κB and HIF-1 to control tumor progression.

Modulation of cell survival may not be the only way NGAL influences the behavior 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 (16). This is supported by the evidence that the level of NGAL expression correlates with the clinical outcome of the patients and the depth of the tumor invasion (17). We have evidence that a complex between NGAL and MMP-9 also exists in transformed thyroid cells, although neither the exact role that such a complex plays in thyroid cancer, nor whether or not the activity of MMP-9 is affected by the presence of NGAL, is known (A.I. and A. Leonardi, unpublished observations).

In other experimental systems, NGAL has been shown to induce expression of E-cadherin to promote formation of polarized epithelia and diminish invasiveness of ras-transformed

cell lines (34). In our experimental system, NGAL seems to have a different role. In fact, ectopic expression of NGAL in a normal thyroid cell line, or its knockdown in transformed cells, does not alter the expression of E-cadherin or vimentin (data not shown).

Our results, in addition to identifying NGAL as a potential target for therapeutic intervention, also strengthen 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 iron of a component critical for various cellular processes and induces apoptosis.

Materials and Methods

Cell cultures and biological reagents, processing of conditioned media, two-dimensional gel elecrophoresis, mass spectrometry analysis, immunohistochemical analysis, Northern and Western blot experiments, *in vitro* and *in vivo* tumorigenicity assays, measurements of apoptosis, quantification of iron content, and statistical analysis were performed as described in the *SI Text*.

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