

## The NF- $\kappa$ B Inhibitor I $\kappa$ B $\alpha$ Negates Colon Cancer Cell Migration, Invasion, Proliferation and Tumor Growth

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### Abstract

*It is now well accepted that the NF- $\kappa$ B pathways are involved in inflammatory diseases, cancer development and progression in human solid tumors. The NF- $\kappa$ B signaling*

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*element I $\kappa$ B $\alpha$  was shown to inactivate NF- $\kappa$ B activity through sequestration of this transcription factor in the cytoplasm. In the present study, we investigated the impact of the I $\kappa$ B $\alpha$  on the invasive growth of human colon cancer cells HCT8/S11 stably transfected by this endogenous NF- $\kappa$ B inhibitor. We report that I $\kappa$ B $\alpha$  ectopic expression inhibited NF- $\kappa$ B promoter activity induced by the Y527Fsrc oncogene, and reduced HCT8/S11 cell migration in wound healing assays. Our data show that I $\kappa$ B $\alpha$  abrogated collagen type I invasion induced by the trefoil factors TFF1 and TFF3, but was ineffective on the invasive phenotype determined by leptin. Moreover, I $\kappa$ B $\alpha$  reduced HCT8/S11 cell proliferation in vitro and the growth of their corresponding tumor xenografts established in the athymic mice. Taken together our data demonstrated that the intrinsic NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  negates several transforming functions in human colon cancer cells. Our data provide the rationale for further preclinical and clinical studies based on therapeutic interventions targeting NF- $\kappa$ B pathway.*

**Keywords:** NF- $\kappa$ B promoter, src, TFF, leptin, wound healing, collagen type I, Ki-67.

### Introduction

Cancer is the third leading cause of death in the United Arab Emirates (UAE) and other major World regions<sup>1</sup>. Despite recent progress in the treatment of human solid tumors and leukemia, new strategies leading to the design of targeted anticancer drugs are expected to improve cancer patient survival. Nuclear NF- $\kappa$ B is a key transcription factor involved in normal development, inflammatory diseases, and cancer progression<sup>2</sup>. In many types of cancers including breast and colon cancer, the transcription factor NF- $\kappa$ B is constitutively activated<sup>3,4</sup>. The activity of NF- $\kappa$ B is regulated by the I $\kappa$ B proteins, which bind NF- $\kappa$ B and retain it in the cytoplasm. Thus, I $\kappa$ B $\alpha$ -inhibitor of NF- $\kappa$ B, induces the formation of the inactive heterotrimeric complex comprising p50/p65

and I $\kappa$ B $\alpha$ . Upon cellular stimulation, I $\kappa$ B is phosphorylated by the I $\kappa$ B kinase (IKK), ubiquitinated and degraded by the proteasome, allowing the nuclear translocation of the p50/p65 NF- $\kappa$ B complex to regulate transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules, and regulators involved in survival and apoptosis<sup>5, 6</sup>. Consistently, NF- $\kappa$ B activation is associated with transient and chronic inflammatory states linked to tumor promotion, progression and metastasis in several mouse models and clinical situations<sup>7</sup>.

Loss-of-function mutations in I $\kappa$ B- $\alpha$ , resulting in high constitutive nuclear activity of NF- $\kappa$ B, are characteristic of Reed–Sternberg cells in Hodgkin lymphoma<sup>8, 9</sup>. This implies a tumor suppression function for I $\kappa$ B. Moreover, defective I $\kappa$ B $\alpha$  were found in several solid tumors such as colon, breast, ovarian, pancreatic, bladder, prostate carcinomas and melanoma<sup>10</sup>. While ubiquitination-mediated proteolysis of I $\kappa$ B $\alpha$  by the 26S proteasome leads to NF- $\kappa$ B activation<sup>11</sup>, proteolysis of I $\kappa$ B $\alpha$  by caspase 3 inhibited NF- $\kappa$ B activation instead. This proteolysis generated an N-terminal truncated protein that can still bind to NF- $\kappa$ B and is resistant to TNF $\alpha$ -induced phosphorylation and degradation<sup>12</sup>. Interestingly, phosphorylation of I $\kappa$ B $\alpha$  by IKK or replacement of Ser32 and Ser36 with glutamates to mimic their phosphorylation prevented caspase-mediated proteolysis<sup>13</sup>. It appears then that phosphorylation of I $\kappa$ B $\alpha$  by IKK serves for proteasome-dependent proteolysis of I $\kappa$ B $\alpha$ , and avoids generation of a repressor-like fragment of I $\kappa$ B $\alpha$  by caspases.

In the present study, we investigated the impact of I $\kappa$ B $\alpha$  ectopic expression on several transforming functions explored in the HCT8/S11 human colonic cancer cell line. We examined the behavior of I $\kappa$ B $\alpha$ -transfected HCT8/S11 cells on cell proliferation, migration and invasion assays monitored *in vitro* with the proinvasive agents trefoil factors TFF1, TFF3, and leptin<sup>14, 15</sup>. In addition, we examined the influence of I $\kappa$ B $\alpha$  on the growth of HCT8/S11

tumor xenografts established in immunodeficient mice.

## Materials and Methods

### *Cell Culture, NF- $\kappa$ B Gene Reporter Assays, and Stable Transfections*

Human colorectal cancer cells HCT8/S11 were cultured in RPMI 1640 (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (Roche Molecular Biochemical's, Meylan, France). For transient transfections, HCT8/S11 colonic epithelial cells were seeded in six-well plates at a density of 75,000–200,000 cells/well. After overnight adhesion, cells were transfected with the Luciferase reporter constructs driven by NF- $\kappa$ B-dependent promoter, using the LipofectAMINE Reagent (Invitrogen). Where indicated, the transfection mixture was combined with plasmid vectors encoding src and I $\kappa$ B $\alpha$ . The src expression vector pSGT-srcY527F encoding the src oncogene and the corresponding control empty plasmid pSGT were provided by Dr. Serge Roche (CNRS UPR-1086 CRBM, Montpellier, France). Before assaying for luciferase activity, cells were washed with cold phosphate buffered saline (PBS) and scraped in the luciferase lysis buffer. Luciferase assays were performed using the Luciferase Assay System (Promega, Madison, USA). Values shown are mean  $\pm$  SEM of at least three independent experiments, each performed in triplicate.

For stable transfections, HCT8/S11 cells were plated for 24h in 60 mm culture dishes and transfected by lipofection (LipofectAMINE Reagent Plus, Invitrogen), according to the manufacturer's instructions. Cells were transfected with 4 $\mu$ g of the expression vector pECFP-I $\kappa$ B $\alpha$  carrying the neomycin resistance gene. The expression vector pECFP-I $\kappa$ B $\alpha$  was a gift from Dr. Johannes A. Schmid (Department of Vascular Biology and Thrombosis Research, University Vienna, Austria). The next day, cultures were split into two 100-mm-diameter dishes and selected for ten days in the culture medium supplemented with 400  $\mu$ g/ml of G418. Resistant colonies were ring-cloned as individual clones or pooled. Expression of the

transgene was determined by Western blotting using the  $\text{I}\kappa\text{B}\alpha$  mouse monoclonal antibody H4 raised against the GST- $\text{I}\kappa\text{B}\alpha$  human fusion protein (Santa Cruz, CA, USA).

#### *Wound Healing and Invasion Assays*

Cells were grown in six-well culture dishes until confluence and then incubated for 10 min in Moscona buffer (137 mM NaCl, 2.68 mM KCl, 11.9 mM  $\text{NaHCO}_3$ , 9.43 mM glucose, 0.176 mM  $\text{NaH}_2\text{PO}_4$ ), pH7.4. The buffer was sterilized by filtration through a 0.22 $\mu\text{m}$  filters and stored at -20°C until use. A scrape was made through the confluent monolayer with a plastic pipette tip of 1mm diameter, the Moscona buffer was removed, and the dishes were washed twice and incubated at 37°C in fresh RPMI containing 10% fetal calf serum. At the underside of each dish, a mark was made at three arbitrary places where the width of the wound was measured with an inverted microscope (objective x 4). Migration was expressed as the average  $\pm$  SEM of the difference between the measurements at time zero and the time points 4h, 6h, and 48h. The complete healing of the wound corresponds to 100%. The healing index corresponds to the % of the wound that is repaired per unit of time (h).

For invasion of collagen gels by HCT8/S11 and HCT8/S11-  $\text{I}\kappa\text{B}\alpha$  cells, six-well tissue culture dishes were filled with 1.35 ml of neutralized type I collagen (Upstate Biotechnology, Lake Placid, NY) and incubated overnight at 37°C to allow gelling. Parental and HCT8/S11-  $\text{I}\kappa\text{B}\alpha$  cells were harvested using Moscona buffer and trypsin/EDTA, and seeded on top of the collagen gels at the density of 300,000 cells per dish. Cultures were incubated for 24h at 37°C, in the presence or absence of the indicated agents. The number of invasive and superficial cells were counted in 12 fields of 0.157 mm<sup>2</sup> using an inverted microscope. The invasion index corresponds to the ratio of the number of cells invading the gel over the total number of cells counted in each field<sup>16</sup>. Recombinant human leptin was from R&D Systems Europe Ltd. (Oxon, UK). Purified recombi-

nant human TFF1 and TFF3 were provided by Dr. Lars Thim and Pr. Bruce Westley.

#### *Cell Proliferation and Tumor Growth Assays*

Parental and stable HCT8/S11- $\text{I}\kappa\text{B}\alpha$  cells were plated at the density of 25,000 cells into six-well tissue culture dishes supplemented with 10% FBS. At the time indicated, cancer cells were trypsinized, collected in 1 ml of medium, and counted by a Cell Coulter. Six-week-old athymic NMRI female nude mice (nu/nu, Elevage Janvier, France) were housed in filtered-air laminar flow cabinets and handled under aseptic conditions. Procedures involving animals and their care were conducted in conformity with Institutional guidelines that are in compliance with FMHS, national and international laws and policies. Parental and stable HCT8/S11- $\text{I}\kappa\text{B}\alpha$  cells were injected s.c. into the lateral flank of athymic nu/nu mice (3 X 10<sup>6</sup> cells in each xenograft, 7 to 8 animals in each group). Tumor volume (V) was calculated using the formula:  $V = 0.4 \times a \times b^2$ , with "a" being the length and "b" the width of the tumor. Tumor dimensions were measured with calipers every week for 9 weeks. Then, animals were sacrificed and the tumors excised, weighed and fixed for immuno-histochemical analysis. *For the immunohistochemical determination of the proliferating, five micrometer paraffin-embedded tissue sections were deparaffinized, microwaved during 5 min for antigen retrieval, and then incubated with the mouse mAb against Ki-67 antigen (DAKO, Copenhagen, Denmark, clone MIB-1, dilution 1:50) for 1h at room temperature. The samples were then washed and incubated with secondary antibody for 1h at room temperature, followed by incubation with the streptavidin-peroxidase complex. Ten high-power fields (0.159 mm<sup>2</sup>) per section from 4-5 tumor specimens of each group were examined microscopically. The average number of cells that stained positive for Ki-67 was evaluated in established control and  $\text{I}\kappa\text{B}\alpha$  -transfected HCT8/S11 xenografts.*

#### *Statistical Analysis*

Data are means  $\pm$  SEM for the number of experiments indicated. The statistical signifi-

cance between experimental values was assessed by the unpaired Student's t-test and  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Functional Expression of the $I\kappa B\alpha$ Transgene in HCT8/S11 Cells

First, we examined the impact of the NF- $\kappa$ B inhibitor  $I\kappa B\alpha$  on the activity of the NF- $\kappa$ B reporter gene in HCT8/S11 human colon cancer cells. Transient transfections were performed using the pSGT-srcY572F vector encoding the

oncogene *src*, together with the luciferase gene reporter construct driven by the NF- $\kappa$ B -dependent promoter. We have previously shown that this HCT8/S11 cell line exhibits a low level of *src* activity<sup>17</sup>. Consistently, introduction of the *src* oncogene induced a robust NF- $\kappa$ B activation (6.3 -fold) in HCT8/S11 cells, as compared with co-transfections performed with the control empty vector pSGT (Fig. 1A). As expected from previous studies showing the implication of *src* in NF- $\kappa$ B activation induced by the stress signals hypoxia and reactive oxy-

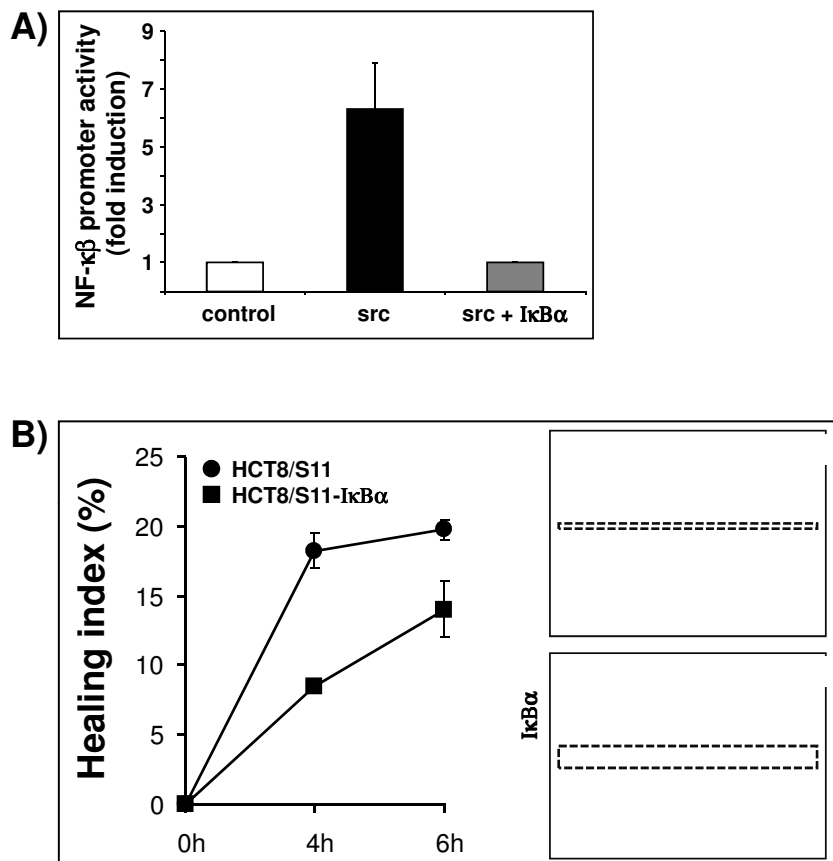


Fig. (1).  $I\kappa B\alpha$  down-regulates NF- $\kappa$ B promoter activity and wound healing in HCT8/S11 cells.

**A.** Reversion by  $I\kappa B\alpha$  of *src*-induced NF- $\kappa$ B promoter transactivation. Human cancer cells HCT8/S11 were transiently transfected by luciferase reporter constructs driven by the NF- $\kappa$ B-dependent promoter, alone or combined with expression vectors encoding *src* alone or combined with the intrinsic NF- $\kappa$ B inhibitor  $I\kappa B\alpha$ . Data are means  $\pm$  SEM of 3 independent experiments. **B.** Wounds were introduced in confluent monolayers of parental and stably transfected HCT8/S11-  $I\kappa B\alpha$  cells. Cells were then cultured at 37°C for 4, 6 and 48 h. The mean distance that cells traveled from the edge of the scraped area was measured in a blinded fashion, using an inverted microscope (4 x magnifications, insets). Data are means  $\pm$  SEM of 3 independent experiments.

gen species, as well as cellular adhesion to extracellular matrix components<sup>18</sup>, over-expression of  $\text{I}\kappa\text{B}\alpha$  abrogated the direct activation of NF- $\kappa\text{B}$  induced by the src oncogene in HCT8/S11 cells.

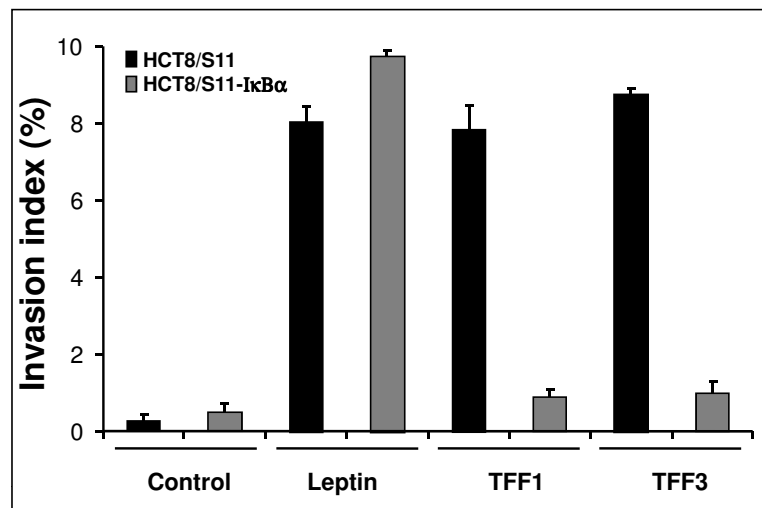
Thus, our data validate the functional insertion of  $\text{I}\kappa\text{B}\alpha$  in our HCT8/S11 model and encouraged us to establish a stably transfected HCT8/S11 cell line expressing high levels of the endogenous NF- $\kappa\text{B}$  inhibitor  $\text{I}\kappa\text{B}\alpha$ . The resulting cell line was designated HCT8/S11-  $\text{I}\kappa\text{B}\alpha$ . Expression of the tagged  $\text{I}\kappa\text{B}\alpha$  transgene was confirmed by western blot (not shown).

#### *Impact of $\text{I}\kappa\text{B}\alpha$ on Cancer Cell Migration, Invasion, Proliferation, and Tumor Xenograft Growth*

We next examined the effect of  $\text{I}\kappa\text{B}\alpha$  ectopic expression on cellular migration in HCT8/S11 cells. Using wound-healing experiments performed on sub-confluent cell cultures, we show that  $\text{I}\kappa\text{B}\alpha$  inhibited by 29-53% the migration of HCT8/S11-  $\text{I}\kappa\text{B}\alpha$  cells during the 4-6h short incubation time considered (*Fig. 1B, left*) suggesting that the inhibition of cellular migration is not due to inhibition of cell proliferation. Parental HCT8/S11 cells were able to achieve a complete wound healing within 48h, while

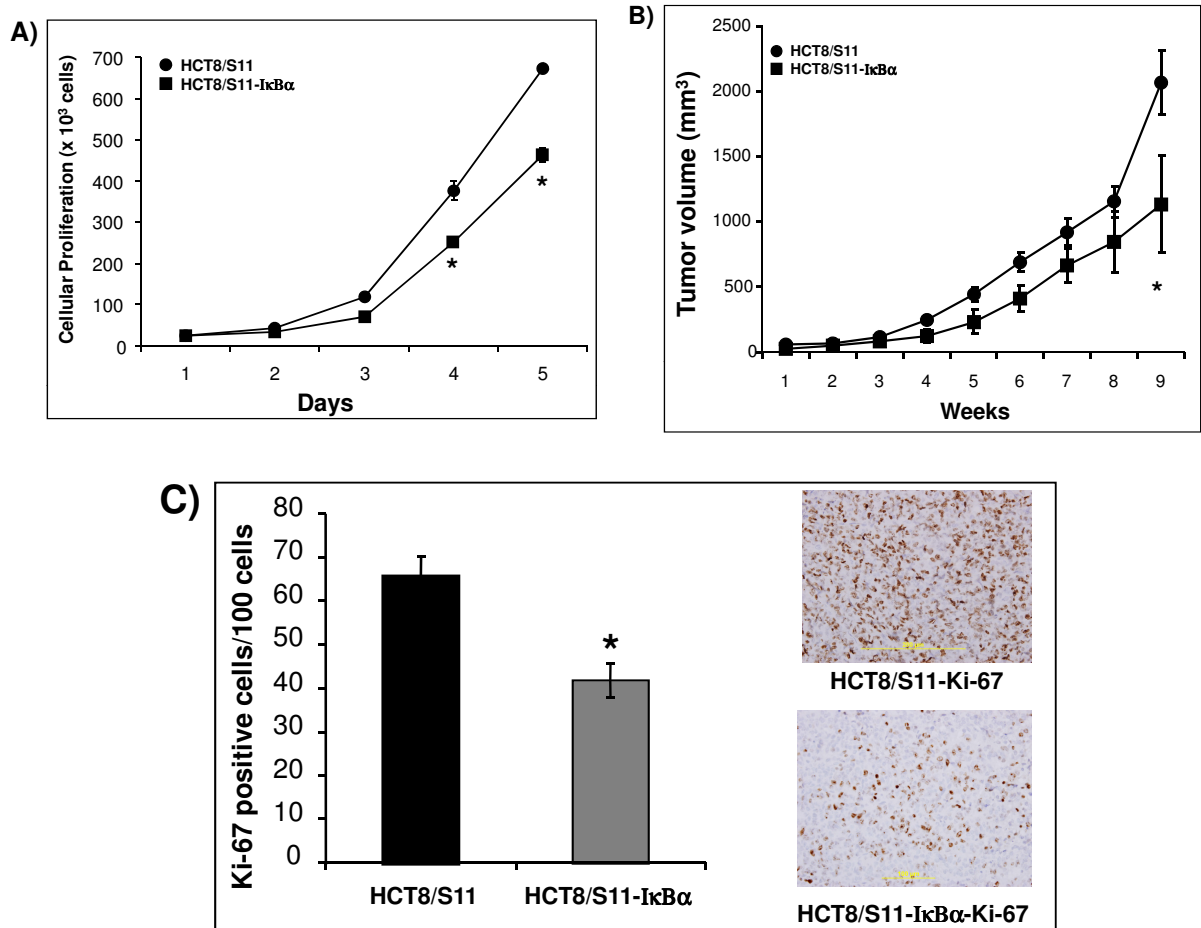
$\text{I}\kappa\text{B}\alpha$  cells were unable to fully colonize the wounds at this time period (*Fig. 1B, right*).

Although trefoil factors contribute to the immunity and mucosal protection in the normal gastrointestinal tract, it is now widely accepted that these secreted regulatory peptides are strongly induced during inflammatory diseases and cancer progression<sup>19</sup>. In agreement, TFFs play a pejorative role in several transforming functions associated with the neoplastic progression in cancer cells and tumors, including cellular scattering and invasion, survival and protection against anoikis-apoptosis, adenoma-adenocarcinoma transition, and angiogenesis<sup>20, 21</sup>. Indeed, we have previously shown that trefoil factors (TFF) and leptin induce the invasive phenotype in HCT8/S11 cells cultured on collagen type I gels<sup>14, 15</sup>. As shown in *Fig. 2*, the leptin invasive phenotype was not affected by  $\text{I}\kappa\text{B}\alpha$ , while this NF- $\kappa\text{B}$  inhibitor selectively impaired the invasive responses elicited by the trefoil factors TFF1 and TFF3. To test the ability of  $\text{I}\kappa\text{B}\alpha$  to interfere with cancer cell proliferation, parental HCT8/S11 cells and their  $\text{I}\kappa\text{B}\alpha$  transfected counterparts were compared for their growth rates. As shown in *Fig. 3A*, HCT8/S11-  $\text{I}\kappa\text{B}$  cells



*Fig. (2).  $\text{I}\kappa\text{B}\alpha$  ectopic expression selectively negates cellular invasion induced by pS2/TFF1 and intestinal trefoil factor /TFF3 in HCT8/S11 cells.*

Cellular invasion induced by leptin (100 ng/ml), TFF1 (100nM) and TFF3 (100nM) was measured in parental HCT8/S11 cells (control) and stably transfected HCT8/S11-  $\text{I}\kappa\text{B}\alpha$  cells. Data are means  $\pm$  SEM of 3 independent experiments.



**Fig. (3). Impact of IκBα ectopic expression on the proliferation of HCT8/S11 cancer cells in culture and on tumor xenografts in athymic mice.**

**A.** HCT8/S11 and HCT8/S11 IκBα stably transfected cells were seeded into six-well tissue culture dishes (25,000 cells /dish). Parental and IκBα -stably transfected cells were counted daily for 4 days. Data are means  $\pm$  SEM of 3 independent experiments. **B.** Tumor growth assay in immunodeficient mice injected S.C. with HCT8/S11 or HCT8/S11-IκBα cells (\* $P < 0.05$  versus HCT8/S11 xenografts). **C.** Immunohistochemical staining for Ki-67 expression in the corresponding HCT8/S11 and HCT8/S11-IκBα human colon cancer xenografts. Data are means  $\pm$  SEM of 3 independent experiments. Note differences in Ki-67 proliferation signals between HCT8/S11 and HCT8/S11-IκBα human colon cancer xenografts (\* $P < 0.05$  versus HCT8/S11 xenografts)

exhibit slower proliferation rates, as shown at days 4 and 5 in culture (24 and 31 % inhibition, respectively). This interesting data prompted us to investigate the impact of IκBα ectopic expression on the growth of HCT8/S11 human colon tumor xenografts in immunodeficient mice. As shown in Fig. (3B), IκBα ectopic expression led to significant inhibition (45%) of tumor growth at week 9 following heterotransplantation.

To test the role of IκBα on tumor cell proliferation in HCT8/S11 human colon xenografts,

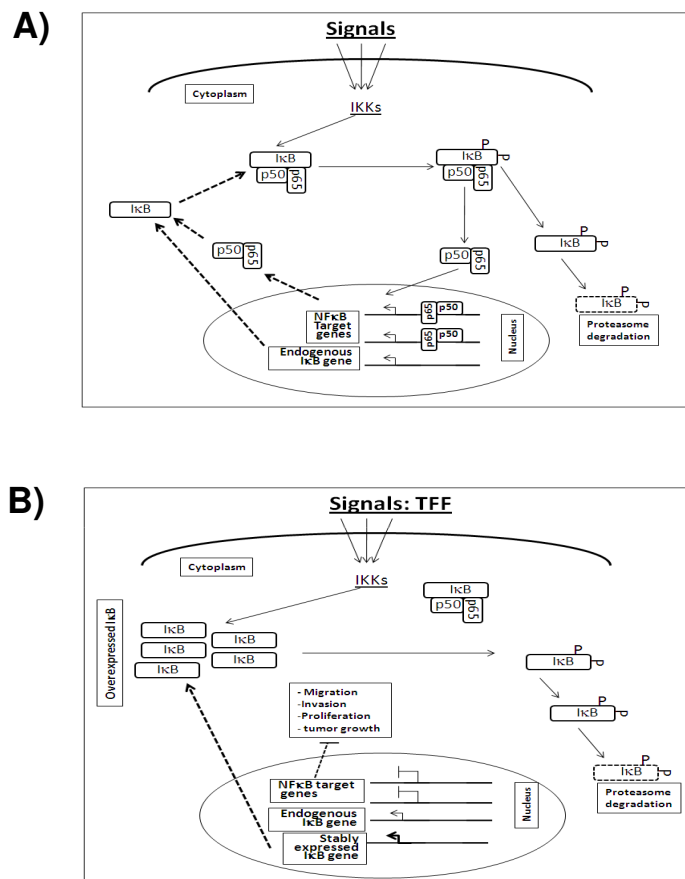
we performed a complementary study on the nuclear antigen Ki-67 by immunohistochemistry. This nuclear Ki-67 antigen, which is present in all phases of the cell cycle, except the Go phase, is considered as a classical marker in cells engaged in the cell proliferation cycle. There was a significant decrease in the mean number of Ki-67-positive cells in HCT8/S11-IκBα tumor xenografts (41.8  $\pm$  3.8 % vs. 65.8  $\pm$  4.6 %,  $P < 0.05$ ), as compared to the controls at 9 weeks (Fig. 3C). Thus, the inhibition of tumor xenografts growth induced by IκBα appears to

be due, at least in part, to a direct effect on tumor cell proliferation.

**Discussion**

Deregulated and constitutive activation of cell proliferation and survival signals constitute a critical mechanism underlying tumor development and cancer progression. Evidence suggests that aberrant activation of NF- $\kappa$ B and its downstream signaling pathways is responsible for the initiation of tumorigenesis including evasion from apoptosis, malignant transformation, sustained cell proliferation, metastasis, and angiogenesis<sup>22</sup>. A sustained activation of NF- $\kappa$ B contributes to the expression of proto-oncogenes c-myc and cyclin D1, which are responsible for both transformation initiation and tumorigenic proliferation<sup>22</sup>.

In the present study, we have shown that ectopic expression of the intrinsic NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  strongly inhibits NF- $\kappa$ B transactivation directly induced by src oncogene in HCT8/S11 human colon cancer cells. The src oncogene is known to be activated during the early and late stages of the neoplastic progression in human colon tumors<sup>23</sup>. Consistently, we demonstrated that blockade of the NF- $\kappa$ B pathways by I $\kappa$ B $\alpha$  led to the invalidation of several transforming functions in HCT8/S11 cells. We have shown that I $\kappa$ B $\alpha$  ectopic expression reduced the ability of HCT8/S11 cells to proliferate *in vitro* and *in vivo* as tumor xenografts in immunodeficient mice. Convergent data and reports also revealed that alterations in the adhesive properties of cancer cells are asso-



**Fig. (4). Diagram summarizing the NF- $\kappa$ B pathway in a cancer cell before (A) and following transfection with I $\kappa$ B $\alpha$  (B). Over-expression of I $\kappa$ B by stable transfection titrates IKKs out, leaving some I $\kappa$ B in the ternary complex unphosphorylated. This will prevent NF- $\kappa$ B nuclear translocation and thus maintaining a constitutive repression of the NF- $\kappa$ B responsive genes.**

ciated with invasive growth and tumor malignancy (data summarized in *Fig. 4*). Cellular adhesion to ECM components *via* their integrin receptors, intercellular adhesion, and activation of the actomyosin system in the cytoskeleton are known to play pivotal roles in the integration of the mechanisms leading to directed cellular movements during wound healing assays. Loss of E-cadherin and other intercellular adhesion molecules also correlate with cancer cell scattering, migration, and tumor cell invasion<sup>23</sup>. This loss of cell-cell adhesion appears to be a key event in acquisition of the invasive potential, because re-expression of E-cadherin suppresses the invasive growth of human tumors<sup>24,25</sup>. Consistently, it has been shown that NF- $\kappa$ B pathway can suppress E-cadherin expression *via* specific transcription repressors such as Snail, an NF- $\kappa$ B target gene<sup>26,27</sup>. Snail-induced E-cadherin loss and epithelial-mesenchymal transitions (EMT) are associated with the invalidation of the epithelial cell polarity, stimulation of cancer cell proliferation, and exacerbation of the invasive and metastatic potential in clinical human epithelial tumors<sup>28,29</sup>. Similarly, integrin ligation to ECM components was shown to activate the transcription factor NF- $\kappa$ B in multiple cell types<sup>18</sup>. Given the role of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  in inhibiting HCT8/S11 cell migration in the present study, one can postulate that this pathway has potential roles in the regulation of intercellular and cell-matrix adhesion mechanisms, as well as activation of the cytoskeleton during cancer cell migration and invasion. In coherence with this prediction, we have shown here that I $\kappa$ B $\alpha$  ectopic expression selectively negated HCT8/S11 cancer cell invasion induced by TFF1 and TFF3, but not by leptin, in collagen type I gels. Consistently, several studies indicate that both TFF expression and biological activities are controlled by NF- $\kappa$ B since several NF- $\kappa$ B binding sites are localized in the promoter regions of the three TFF genes<sup>19,29</sup>. In contrast, blockade of the NF- $\kappa$ B pathways is not associated with the reversion of the leptin invasive potential in our study, suggesting that alternative proinva-

sive pathways induced by this cytokine are still operational under NF- $\kappa$ B blockade. Indeed, several studies have identified the multiplicity of the leptin receptor signaling networks connected with cancer cell invasion and tumor angiogenesis. These include the PI3-kinase /AKT axis, MAPK ERK1/2 cascade, the Rho-GTPases, the JAK2 /STAT3 transcription signals, as well as several crosstalks engaged with other transmembrane receptors<sup>14</sup>. These canonical and alternative leptin pathways are inherent to cancer cell proliferation, survival, invasion and metastasis, as well as tumor angiogenesis and metastasis.

We propose that the NF- $\kappa$ B oncogenic pathway can be targeted to halt tumor invasive growth and progression to the metastatic stages. Several studies have addressed the design of putative NF- $\kappa$ B inhibitors as pharmacologic therapeutic agents in cancer patients. Anti-inflammatory drugs and natural compounds, such as curcumin and transveratrol, inhibit NF- $\kappa$ B by interfering with IKK activity<sup>30</sup>. Finally, proteasome inhibitors were also shown to prevent NF- $\kappa$ B activation by blocking the degradation of I $\kappa$ B. In summary, the results of our study provide new insight into the development of therapeutic strategies against colon cancer promotion and progression, using NF- $\kappa$ B interfering drugs in combinations with other anticancer agents targeting selected oncogenic pathways or genotoxins that disrupt the functional integrity of the DNA.

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