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IKKα Promotes the Progression and Metastasis of Non-Small Cell Lung Cancer Independently of its Subcellular Localization

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ABSTRACT

Lung cancer is the leading worldwide cause of cancer mortality, however, neither curative treatments nor substantial prolonged survival has been achieved, highlighting the need for investigating new proteins responsible for its development and progression. IKKα is an essential protein for cell survival and differentiation, which expression is enhanced in human non-small cell lung cancer (NSCLC) and correlates with poor patient survival, appearing as a relevant molecule in lung cancer progression. However, there are not conclusive results about its role in this type of cancer. We have recently found that IKKα performs different functions and activates different signaling pathways depending on its nuclear or cytoplasmic localization in tumor epidermal cells. In this work, we have studied the involvement of IKKα in lung cancer progression through the generation of lung cancer cell lines expressing exogenous IKKα either in the nucleus or in the cytoplasm. We demonstrate that IKKα signaling promotes increased cell malignancy of NSCLC cells as well as lung tumor progression and metastasis in either subcellular localization, through activation of common protumoral proteins, such as Erk, p38 and mTor. But, additionally, we found that depending on its subcellular localization, IKKα has non-overlapping roles in the activation of other different pathways known for their key implication in lung cancer progression: while cytoplasmic IKKα increases EGFR and NF-κB activities in lung tumor cells, nuclear IKKα causes lung tumor progression through c-Myc, Smad2/3 and Snail activation. These results suggest that IKKα may be a promising target for intervention in human NSCLC.

1. Introduction

Lung cancer is the leading cause of cancer mortality in the world. Non-small cell lung cancer (NSCLC) is the most frequent type of lung cancer (representing 85% of all cases) and entails a poor survival rate, with <15% of patients surviving more than five years [1]. NSCLC comprises several types of cancer, being the two main types lung adenocarcinomas (ADC; 65%) and squamous cell carcinomas (SCC; 5%). It is noticeable that despite administration of standard chemotherapeutic agents, survival of lung cancer patients has not substantially improved in the last 30 years [2]. This is due in part to the fact that most patients are diagnosed in advanced stages, where the option of surgical treatment (the most effective therapeutic strategy), is not possible, and to the large number of patients who develop primary and secondary resistance to current therapies. Additionally, lung cancer is a very aggressive tumor, often producing distant metastases, mainly in bones, brain and liver and, more locally, in other lobes of the lungs themselves [3]. This makes the identification of new targets for lung cancer therapy an imperative issue.

Among the molecules that have been found to play an important role in the development and progression of lung cancer are the epidermal growth factor (EGF) and its receptor (EGFR). It is estimated than 43–89% of lung tumors overexpress EGFR [4], more frequently in

Abbreviations: NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SCC, squamous cell carcinoma; NMSC, non melanoma skin cancer.

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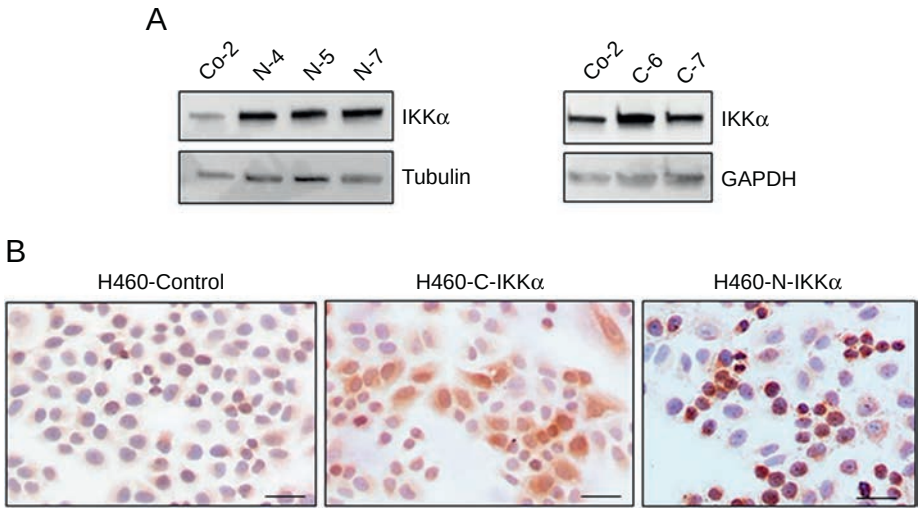

Fig. 1. Generation of the C-and N-H460-IKKα clones. (A) Western blots showing increased levels of IKKα in the C (cytoplasmic) –and-N (nuclear)-H460– IKKα cells. The expression in different selected clones is shown. Tubulin and GAPDH were used as loading controls. (B) Immunohistochemical analysis of the expression of the IKKα protein in the H460 cells of the three genotypes. Note the elevated cytoplasmic expression of IKKα in the C-H460-IKKα cells transfected with the β-actin-C-IKKα construct. Nuclear expression of IKKα is observed in N-H460-IKKα cells, transfected with the β-actin-N-IKKα construct. In the H460-Control cells, transfected with the empty vector, low expression of IKKα is detected. Scale bar: 40μm.

squamous cell carcinomas (70%) than in ADC (50%) [5]. Also, activating mutations in the tyrosine kinase (TK) domain of the EGFR gene have been detected in 15–20% of NSCLC patients and in even up to 40–60% of ADC patients [6]. The activation of EGFR has pleiotropic effects, highlighting its contribution to the immune escape of tumors, the increase of proliferation, the suppression of autophagy and the enhancement of cell migration of tumoral cells, which contribute to the increase of invasive capacity of lung tumors. In those patients

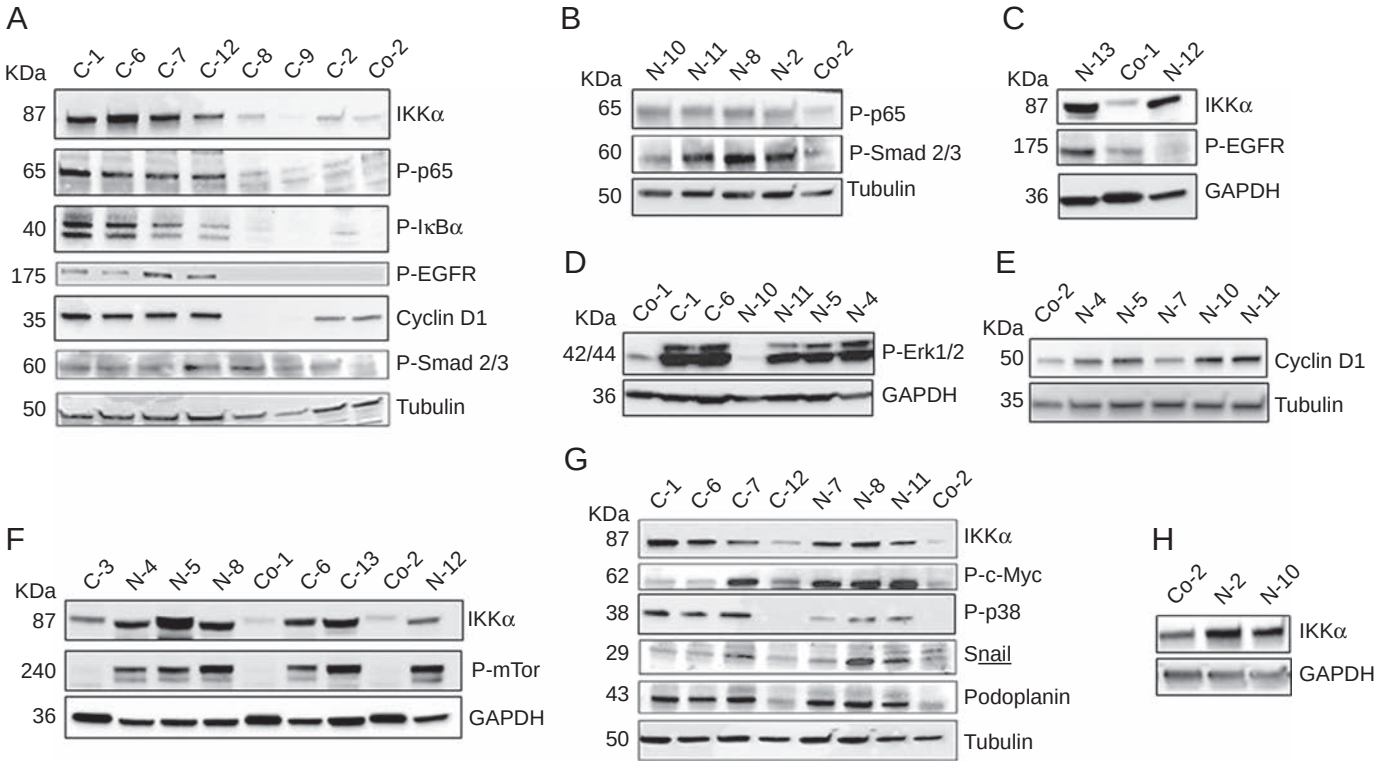

Fig. 2. Molecular characterization of the different isolated clones that overexpress IKKα in the cytoplasm or in the nucleus of the H460 cells. (A) WB of total protein extracts showing the hybridization of different C-H460-IKKα pools with the indicated antibodies. Note that those clones with increased levels of IKKα (C-1, C-6, C-7 and C-12) exhibit overactivation of the classical NF-κB pathway (elevated levels of P-p65 and P-IκBα); as well as activation of EGFR (measured as P-EGFR). They also present increased levels of Cyclin D1 expression with respect to the values observed in the Control-H460 cells and those of the clones that do not express the transgene (C-8, C-9 and C-2). (B) Levels of P-p65 in the N-H460-IKKα cells are similar to those of Control cells, indicating that no enhanced activation of the NF-κB is observed when IKKα is expressed in the nucleus of the H460 cells. Enhanced P-Smad2/3 is also detected in the N-H460-IKKα mice, while it is not enhanced in the C-H460-IKKα cells (A). (C) WB showing overactivation of EGFR in the C-H460-IKKα cells, but not in the N-H460-IKKα ones. (D) Increased activation of Erk1/2 is observed in both types of cells overexpressing IKKα. (E) Levels of Cyclin D1 are increased in the N-H460-IKKα clones regarding the values in the Control-H460 cells. (F) Overactivation of mTor (increased levels of P-mTor) is detected in both C-and-N-H460-IKKα cells. (G) N-H460-IKKα cells present increased levels of P-c-Myc and Snail. Increased levels of P-p38 and Podoplanin are observed in the C-and-N-H460-IKKα cells. (H) Levels of IKKα expression in the N-H460-IKKα pools N-2 and N-10 are shown. Tubulin and GAPDH are used as loading control. Different C– H460-IKKα, N-H460-IKKα and Control-H460 pools are shown.

where EGFR is activated, inhibitors of TK activity (TK inhibitors) have been used; however, in spite of a good and prolonged initial response of the patients, in practically all cases acquisition of resistance to the inhibitors is observed. This is likely due, on the one hand to the activation of the mTOR protein (which, being involved in the regulation of transcription, proliferation and cell death, yields a higher tumor progression and lower survival); and on the other hand to the rapid hyperactivation of NF-κB after treatment with TK inhibitors, which limits the success of therapy against EGFR [7]. In fact, the activation of NF-κB appears as a relevant mechanism in the progression of lung cancer, and several groups have described the inhibition of lung tumor growth when the activation of NF-κB is prevented [8,9].

Another common event that occurs in human lung cancers is amplification and activation of c-Myc, that is seen in >30% of lung ADC patients [10], causing an increase in proliferation, cell survival, genetic instability, angiogenesis and metastasis. In addition, c-Myc activation is associated with poor prognosis and aggressive and invasive phenotype.

The induction of the expression of other proteins, such as Snail and Podoplanin, which promote the epithelial-mesenchymal transition (EMT), thus favoring an invasive phenotype and metastasis, has also been described in lung cancer [11,12].

A protein that has recently been found to play an important role in NSCLC is IKKα, a member of the NF-κB signaling cascade: it is part of the IκB kinase complex (IKK), which is composed of two kinases, IKKα and IKKβ, and a regulatory subunit called IKKγ or NEMO. In mammals NF-κB consists of five ubiquitously expressed members (p50, p52, p65/RelA, RelB and c-Rel) that form homo or heterodimers [13]. In its inactive state NF-κB is found in the cytosol bound to inhibitory proteins of the IκB family and can be activated by numerous stimuli, such as inflammatory cytokines, growth factors, carcinogens and tumor promoters. In the classical NF-κB pathway, the activated IKK complex phosphorylates IκBα, which is ubiquitinated and degraded via the proteasome, leading to the activation of NF-κB and the subsequent translocation into the nucleus, where activates its target genes [14].

In addition to its cytoplasmic localization, IKKα is also found in the nucleus of cells, where it plays an essential role in processes of differentiation, apoptosis and cell cycle, and promotes tumor progression and metastasis [15–19].

While the role of IKKα as a tumor promoter is firmly demonstrated in some types of cancer, such as breast and prostate cancers [20,21], in other cases (lung and nonmelanoma skin cancer (NMSC), contradictory results have been obtained. To solve this controversy about the role of IKKα in its development and progression, we have recently generated new models of transgenic mice, which express IKKα either in the nucleus or in the cytoplasm of keratinocytes. This approach has allowed us to discover that IKKα acts as tumor promoter in NMSC in either localization, although by different mechanisms i.e., whereas cytoplasmic IKKα mainly activates the classical NF-κB and EGFR signaling pathways in tumor epidermal cells, IKKα of nuclear localization induces overexpression of c-Myc [19].

In the case of lung cancer, some studies point to IKKα as a tumor suppressor in lung SCC and ADC [22,23]. However, it has been recently reported that IKKα (total IKKα, i.e., both in nuclear and cytoplasm localizations) promoted lung cancer growth [24]. This report also describes that the upregulation of IKKα expression is associated with decreased patient survival and that its overexpression may predict poor clinical outcome in lung ADC patients [24]; in this, case, the authors propose that nuclear localization of IKKα is necessary for lung ADC growth; however, they do not determine the mechanisms through which nuclear IKKα acts, nor study whether cytoplasmic IKKα plays a role in lung ADC as well. Therefore a new approach is needed to clarify the function of IKKα in lung cancer and to discern the role of nuclear and cytoplasmic IKKα in lung cancer progression. Here, we have generated NSCLC cells that express exogenous IKKα in the nucleus or in the cytoplasm. This approach has allowed us to determine that IKKα acts in either location as a tumor promoter of NSCLC, largely favoring tumor

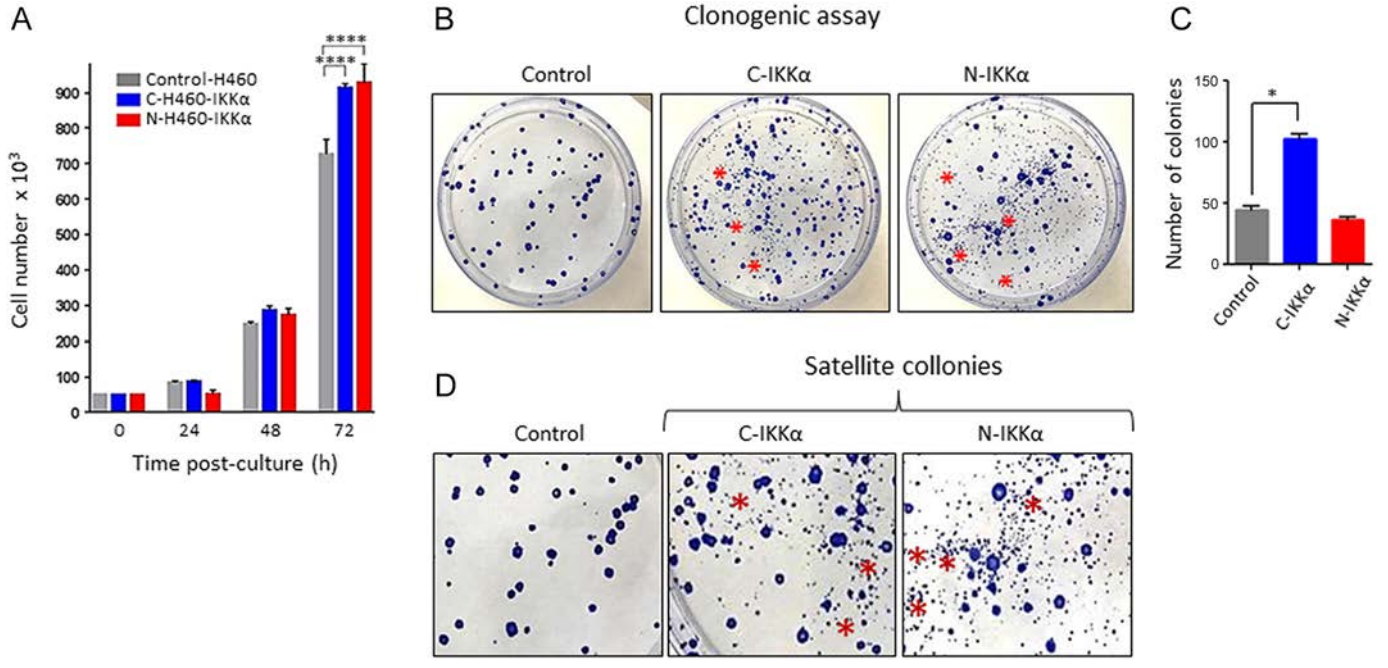

Fig. 3. Analysis of cell proliferation and clonogenic capacities of the H460 cells of the three genotypes. (A) 50.000 cells were seeded (point 0) and counted at the indicated times per triplicate. Note the increased proliferation rate of both C-and-N-IKKα pools 72 h post-seeding respect to the Control-H460 cells (**** $P < .0001$). Statistical significance was determined using the Bonferroni's multiple comparison test. (B) Representative images of a colony forming assay. Plates seeded in triplicate with 600 cells of each genotype are shown. (C) Increased number of colonies formed by the C-H460-IKKα cells respect to Control-H460 and N-H460-IKKα cells (*$p < .05$; Student's t-test). (D) Higher magnification of colonies in (B) showing the appearance of small and numerous satellite colonies (red asterisk) both in C-and-N-H460-IKKα cells while they were not detected in the Control-H460 cells. Cells were stained with Coomasie blue 14 days after seeding. Experiments were preformed three times.

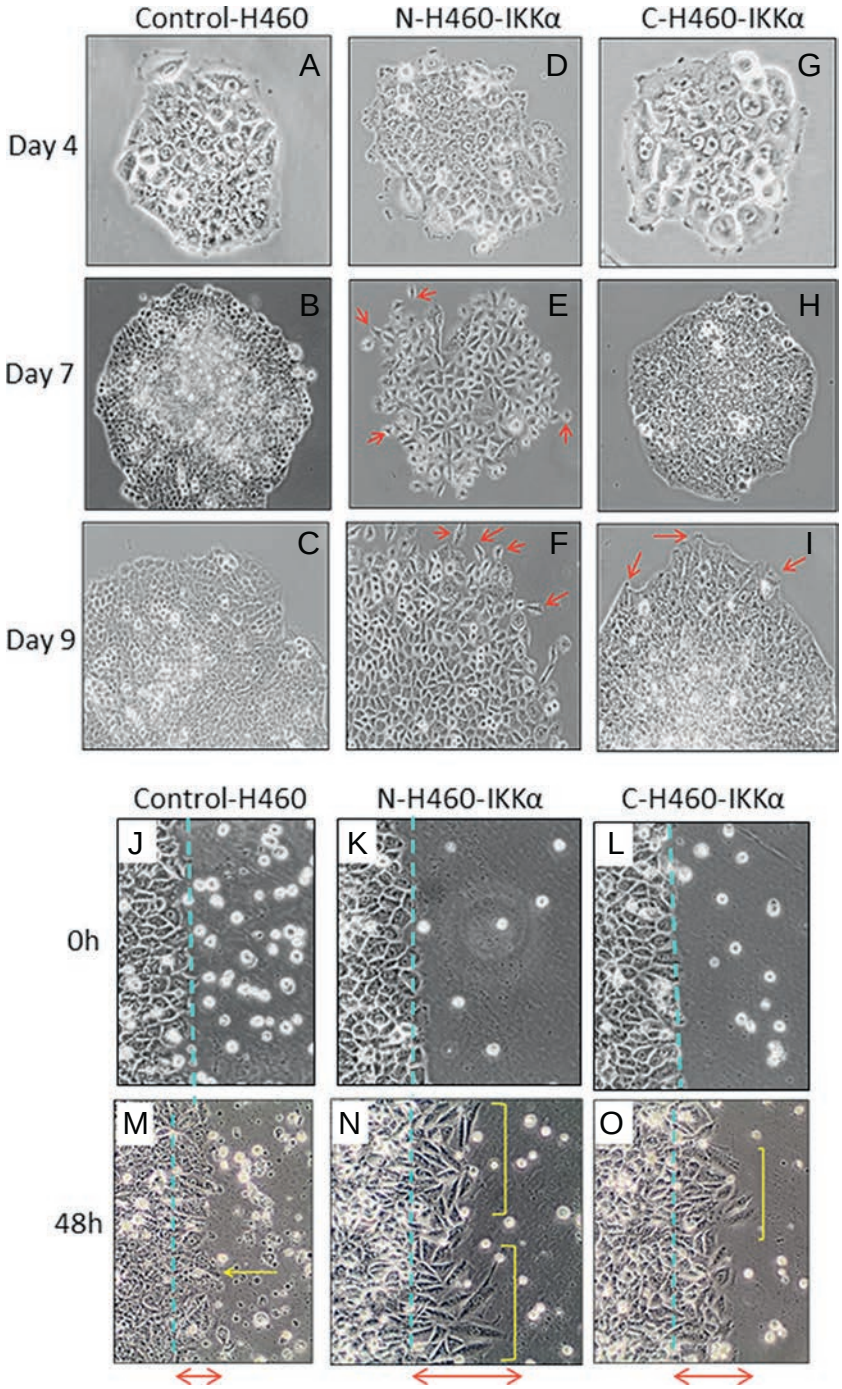

Fig. 4. Increased capacity of migration of C-and-N-H460-IKKα cells. (A-I) Representative images showing the appearance of the colonies formed by cells of the three genotypes at different time points after seeding (4, 7 and 9 days). Plates were seeded in triplicate with 300 or 600 cells of each genotype (images correspond to 600 cells). Observe the compact colonies with well-defined borders formed by the Control-H460 cells (A-C). Colonies formed by the N-H460-IKKα cells appear loose and show abundant migratory cells moving away from the core of the colony (red arrows); this behavior was observed from day 4 onwards (D-F). Colonies formed by the C-H460-IKKα cells showed an intermediate phenotype between that of Control and N-IKKα cells: some migratory cells in the periphery of the colonies are detected. The number of migratory cells increase at large time points, i.e., at 7 and 9 days (G-I). (J-O) Wound healing assay. Cell pictures at the time of the scratch (0h) are presented (J-L); also, migration 48 h after the 'scratch' wound is shown: note the increased migration of both the C-and-N-IKKα cells (N, O). Discontinued blue lines indicate the position in which the migration initiates in each cell type. Yellow brackets show the migration front in the C-and-N-H460-IKKα cells; in Control-H460 only isolated migratory cells (yellow arrows) were detected (M); double headed red arrows show the migration distance.

progression and metastasis. Interestingly, although IKKα induces both in the nucleus and in the cytoplasm some common proteins already known for its relevance in NSCLC promotion (such as mTor and Podoplanin); it also activates, depending on its subcellular localization, different key pathways for lung cancer progression, i.e., cytoplasmic IKKα activates EGFR and NF-κB pathways and nuclear IKKα induces c-Myc, P-Smad2/3 and Snail among others.

2. Experimental Procedure

2.1. DNA Constructs

The C-and N-IKKα constructs have been previously described [19]. Briefly, both of them contain the sequence of the human IKKα gene cDNA but while in the N-IKKα construct an extra NLS (nuclear

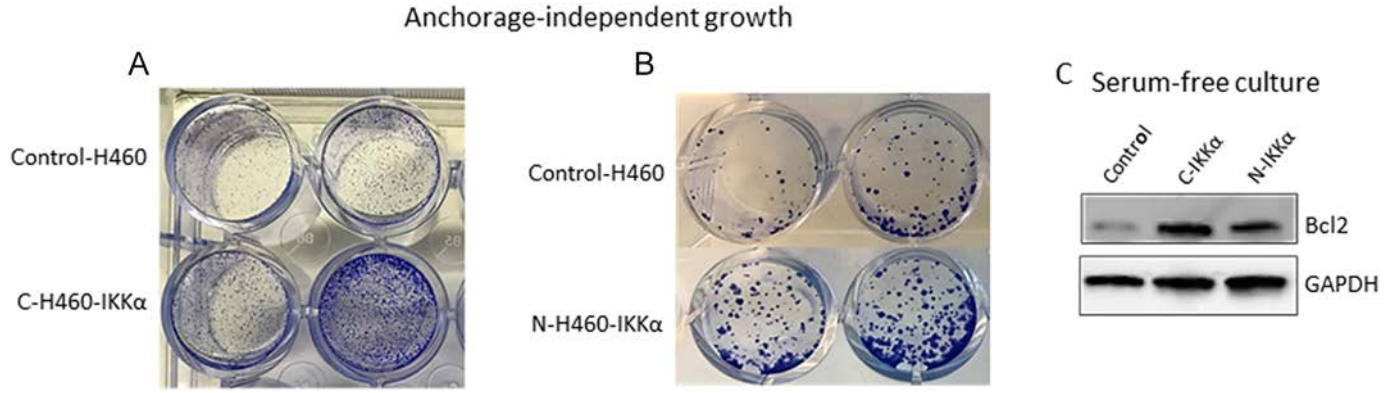

Fig. 5. Increased cell survival of C-and-N-H460-IKKα cells. (A, B) Anchorage-independent growth assay showing the increased capability of the H460 cells overexpressing IKKα either in the cytoplasm or in the nucleus to survive after 24 h in suspension. Cells were fixed and stained with Coomassie blue. (C) Western blot of total protein extracts from H460 cells of the three genotypes grown in absence of serum for 6 days. An increase in the expression of the anti-apoptotic protein Bcl-2 is observed in both the C-and-N-H460-IKKα cells. GAPDH was used as loading control. Experiments were performed three times per duplicate. The duplicate wells at 5A and 5B show the result of two different experiments performed on distinct days.

localization signal) was added in 5′, in the C-IKKα construct the internal NLS site was removed. The original NLS signal of IKKα is located approximately in the middle of the protein. The NLS that we added in the N-IKKα construct was in the N-terminal part, immediately after the ATG. Both constructs were subcloned in the pRC vector containing the βActin promoter [25]. The empty pRC-βActin vector was used as a Control (Control-H460 cells). All constructs confer resistance to G418.

2.2. Cells, Culture Conditions and Transfection Assays

H460 cells come from the ATCC and have been provided by Dr. Luis Paz-Ares, Hospital 12 de Octubre, Madrid, Spain. They were culture in RPMI-10% FCS, permanently transfected using Lipofectamine 3000 (*Life Technologies*) and selected using G418 (0.8 mg/ml, BioNova).

2.3. Cell Proliferation Assay

5×10^4 cells/p60 were seeded in complete medium (RPMI-10% FCS). At 24, 48 and 72 h cells were trypsinized and counted. Three experiments per triplicate were performed.

2.4. Colony Forming Assays

A total of 3 and 6×10^2 cells were seeded per duplicate in RPMI-10% FCS in p100 plates. Medium was replaced every 4 days. Growing colonies were photographed at different time points. Cells were fixed, stained with Coomasie blue and the number of colonies counted. Experiments were performed three times.

2.5. Wound Healing Assays

Cells growing in monolayer cultures were incubated 2 h at 37 °C in RPMI plus 5 mg/ml of mitomycin C; then washed with PBS. After 1 h, a 'scratch' wound was created in vitro by scraping the cell monolayer with a sterile pipette tip. Healing was measured at 48 and 72 h post-scratch.

2.6. Cell Suspension Growth

24-well plates were covered with 0.9% agarose. After 1 h, 5×10^4 cells were added in 1.5 ml RPMI without FCS. After 24 h, multiwells were seeded with 600 ml aliquots of the cellular suspension and fed with RPMI-10% fetal bovine serum for 24 h. Cells were fixed, stained with Coomasie blue and the number of colonies counted. Experiments were performed three times.

2.7. Growth in Serum-free Medium

Cells were seeded into p60-plates in complete medium. After 24 h, the medium was replaced by serum-free RPMI. Cells were collected 6 days later.

2.8. Xenograft Model of Lung Carcinogenesis and Metastasis

Tumors were induced in immunodeficient mice (Rag2/IL2RG Double Knockout (R2G2), generously donated by Envigo, Spain; and in Hsd-Athymic Nude (Harlan, Barcelona, Spain). 2×10^6 H460 cells of each of the three genotypes (Control, C-IKKα and N-IKKα) were inoculated subcutaneously on both flanks of mice. Four R2G2 and six nude mice were injected with cells from each of the genotypes. Tumors were measured with an external caliper and their volume was calculated as $(4\pi/3) \times (\text{width}/2)2 \times (\text{length}/2)$. For metastasis experiments lungs were collected 29 or 36 days post-injection (two weeks after the removal of the tumors). Experimental procedures were performed according to European and Spanish laws and approved by our institution's Ethics Committee.

2.9. Ethics Statement

All animal experimental procedures were performed according to European and Spanish laws and regulations and approved by our institution's ethics committee (PROEX 182/15).

2.10. Western Blot Analysis

Total protein extracts (30 μg) were subjected to SDS/PAGE. The separated proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL; BioRad, France) and probed with antibodies against IKKα (NB100–56704 Novus Biologicals); c-Myc (Biolegend, CA, USA); GAPDH, P-Erk1/2, Podoplanin, P-Smad2/3, Snail (Santa Cruz Biotechnology, Inc. Europe); α-Tubulin (Sigma- Aldrich, MO, USA); P-mTor, P-IkBa, P-EGFR (Tyr1176), P-p38 (Cell Signaling Technology, USA); P-c-Myc, P-p65 (Abcam) and Cyclin D1 (Neomarkers). In all cases samples were subjected to luminography with the Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Illinois, USA).

2.11. Immunohistochemical Staining

Cells were fixed in methanol/acetone (1/1). Murine tumors were fixed in 10% buffered formalin and embedded in paraffin. Cells and

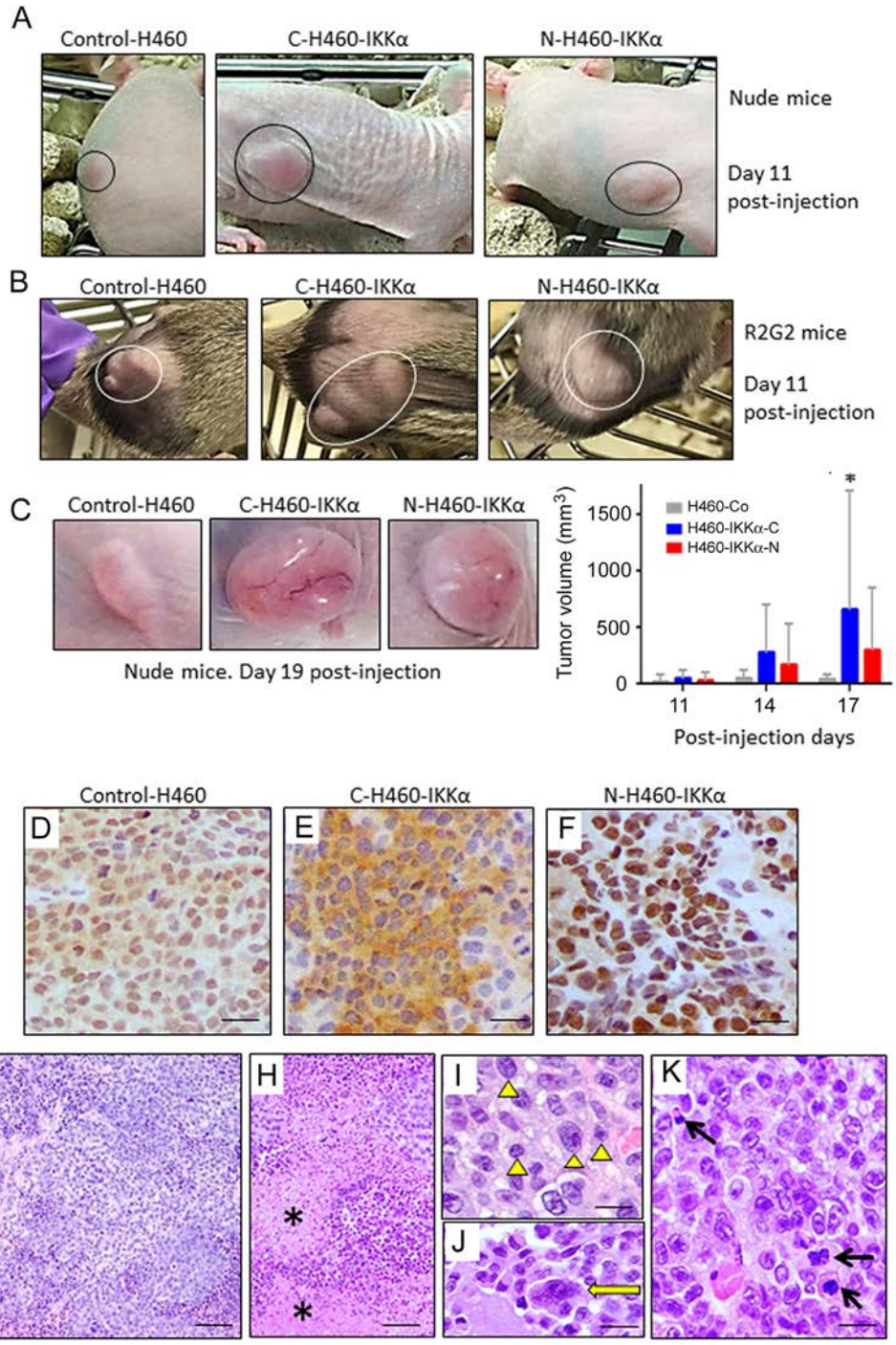

Fig. 6. Overexpression of IKKα both in the nucleus and cytoplasm of NSCLC cells enhances the growth of lung tumors in vivo. (A, B) Representative images showing tumors developed on the flanks of nude (A) and R2G2 (B) mice 11 days post-injection of the indicated type of H460 cells. Note the large size of tumors originated by inoculation of both C-and-N-H460-IKKα cells in both strains of immunodeficient mice. Each cell type was injected into six nude mice and four R2G2 mice. (C) Larger size of tumors that overexpress IKKα. Left: detail of tumors photographed on day 19-post-injection. Observe the larger size and the presence of blood vessels visible to naked eye in both the C-and-N-H460-IKKα tumors. Right: a representative graph of tumor growth curve of xenograft assay in nude mice is shown. Tumor growth was followed for 19 days and measured with an external caliper. Statistical significance was determined using the Bonferroni's multiple comparison test (* $P < .05$). (D-F) Immunochemistry with an anti-IKKα-specific antibody shows enhanced staining in the cytoplasm of the C-H460-IKKα tumors (E) and in the nucleus of the N-H460-IKKα ones (F). Lower expression of IKKα is detected in tumors derived from the Control-H460 cells (D). (G) Representative image showing the appearance of the ADCs derived from the H460 cells of the three genotypes (a C-H460-IKKα tumor is shown). (H-K) Representative example of areas of necrosis (asterisk in H); anisocaryosis (yellow triangles in I); multinucleated giant cell (yellow arrow in J), and aberrant mitosis (black arrows in K) in C-H460-IKKα tumors. Scale bars: 50μm (D-F); 250 μm (G-H); 40 μm (I-K).

tumor sections were stained with antibodies against IKKα, PECAM-1 (Santa Cruz and Novus Biologicals); K8 (Troma I; DSHB); TTF1 (Epitomic) and BrdU (Roche, Mannheim, Germany). Tumor and lung sections were stained with H&E and histopathological evaluation was performed by two experimented observers: MJFA, specialized in human pathology and RAGF, a veterinarian expert in animal pathology.

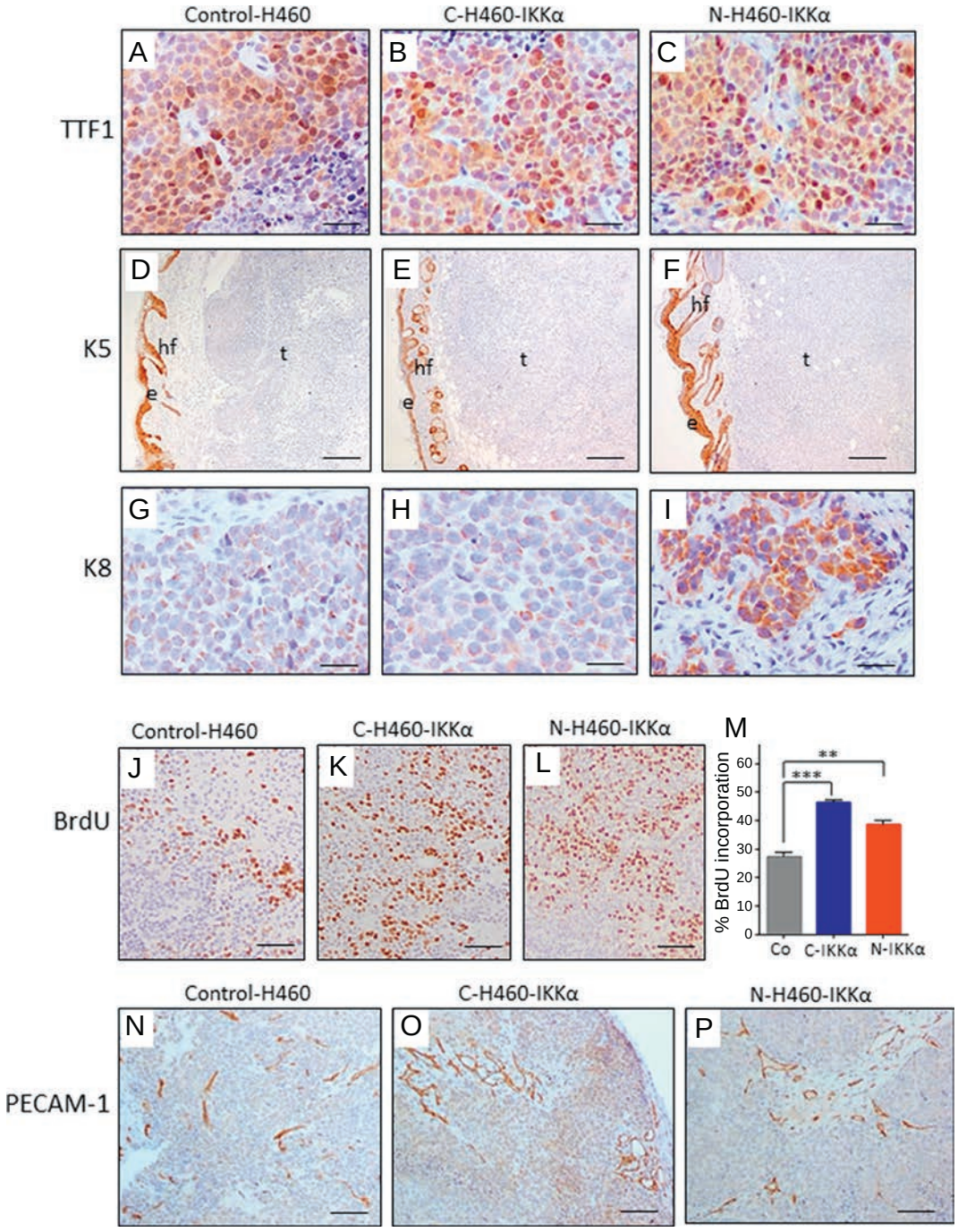

Fig. 7. Increased levels of IKKα in NSCLC cells lead to the development of lung tumors of augmented malignant features. (A-C) Representative images showing nuclear expression of TTF1, a marker of lung ADC, in tumors derived from H460 cells of the three genotypes. (D-F) Tumors (t) derived from H460 cells of the three genotypes do not express K5. Observe K5 positive staining in the epidermis (e) and hair follicles (hf) surrounding the tumor masses. (G-I) Increased expression of keratin K8 in the N-H460-IKKα tumors (I). (J-L) Immunohistochemistry showing the increased BrdU incorporation in the ADC derived from both the C-and-N-H460-IKKα cells; (M) graphic representation of the percentage of proliferating cells (BrdU positive) per field at magnification x10 in each type of cells. Statistical analysis was performed using the Student's *t*-test (***$p < .001$; **$p < .01$). (N-P) Representative image of PECAM-1 immunostaining showing the blood vessel pattern in the lung tumors. Observe the lacunar appearance of vessels in the C-and-N-H460-IKKα tumors (O, P), in contrast with the narrower vessels of Control-H460 tumors (N). Scale bars: 120 μm (A-C); 350 μm (D-F); 160 μm (G-I); 250 μm (J-L); 300 μm (O-P).

3. Results

3.1. Analysis of IKKα Overexpression in the Cytoplasm or in the Nucleus of H460 Cells Transfected with the C-IKKα and N-IKKα Constructs Respectively

We have used the H460 cell line of NSCLC, widely used in studies of lung cancer, to analyze the role of nuclear (N) and cytoplasmic (C) IKKα in the growth and progression of lung ADC. To this end, H460 cells were permanently transfected with constructs containing human N-IKKα or C-IKKα under the control of the β-Actin promoter. After transfection, to minimize any potential effect of clonal selection we selected different pools formed by 4–50 distinct G418-resistant colonies expressing the transgenic IKKα in the cytoplasm (C-H460-IKKα cells) or in the nucleus (N-H460-IKKα cells). Western blotting analysis showed that all N-H460-IKKα pools, and 8 out of 12C-H460-IKKα pools overexpressed IKKα (Fig. 1A and Fig. 2A). Some C-H460-IKKα pools which did not express the construct were used as controls (Fig. 2A).

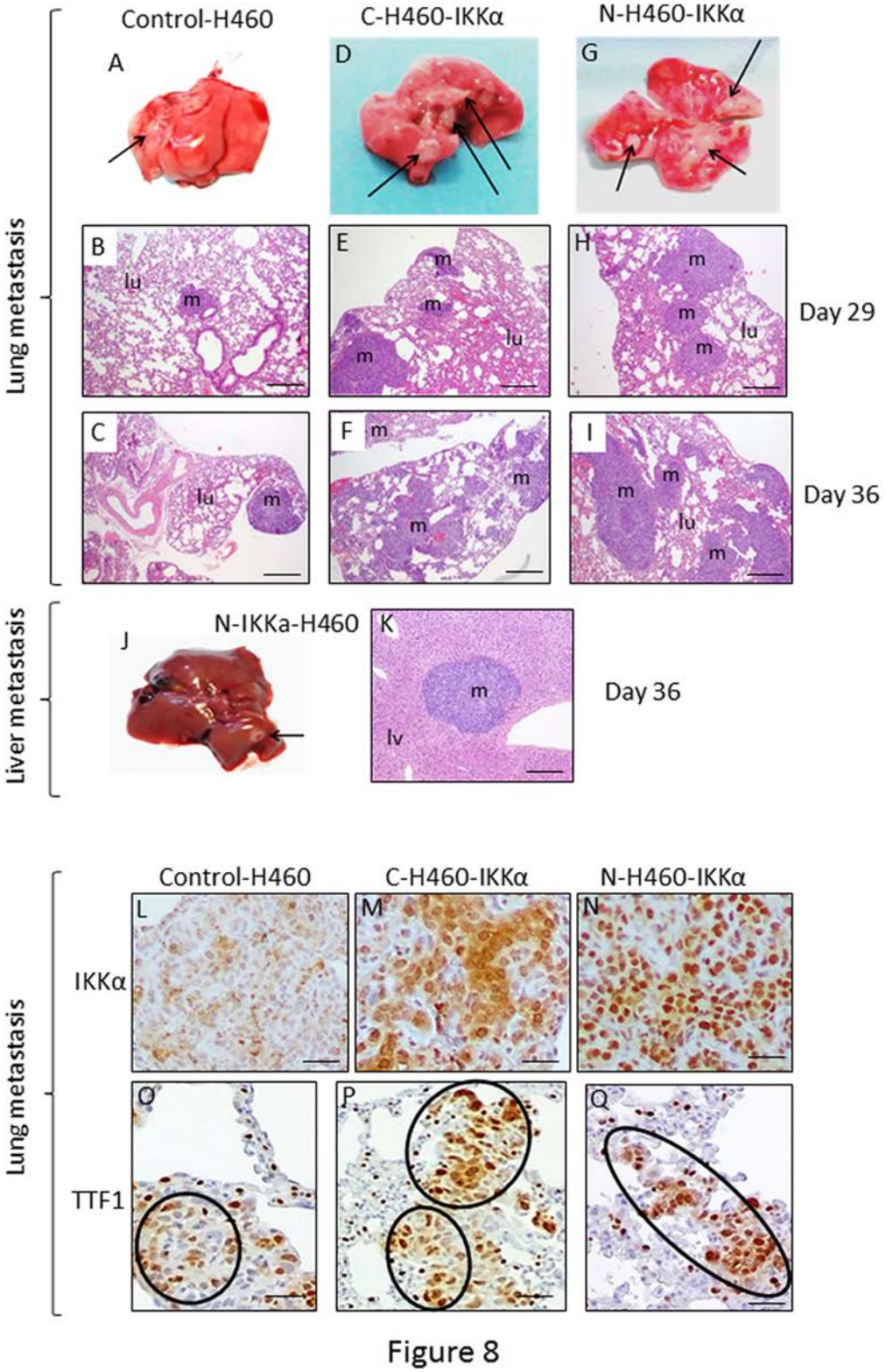

Figure 8

Fig. 8. Increased metastatic capacity of the C-and-N-H460-IKKα adenocarcinomas. (A-K) Macroscopic and histological analysis of the lungs (A-I) and liver (J, K) of mice injected subcutaneously with H460 cells of the three genotypes. Observe the less frequent development of lung metastasis (black arrow) in immunodeficient mice injected with the Control-H460 cells (A) compared to the abundant foci of lung metastasis (whitish formations, black arrows in D, G) in both C-and-N-IKKα lungs. (B, C) Representative images showing the histology of lungs of mice injected with the Control-H460 cells; observe the scarce number and low size of the tumor metastasis (m). (E, F, H, I) Abundant and large metastasis (m) were detected in the lungs of mice inoculated with C-H460-IKKα (E, F) or N-H460-IKKα (H, I) cells. One metastasis was found in the liver of a mice injected with N-H460-IKKα cells (J, K). (L-N) Immunostaining showing increased expression of IKKα in the cytoplasms of the metastasis of mice injected with C-H460-IKKα cells (M) and in the nuclei of metastasis of mice inoculated with N-H460-IKKα cells (N). Expression of IKKα was also detected in the metastasis of mice receiving Control-H460 cells (L). (O-Q) Immunostaining showing the nuclear expression of TTF1, in the metastasis of the lungs of mice injected with the H460 cells of the three genotypes. lu: lung tissue; m: metastasis; lv: liver tissue. Scale bars: 350 μm (B, C, E, F, H, I, K); 120 μm (L-N); 100 μm (O-Q).

Immunohistochemical staining confirmed overexpression of IKKα in the N-and-C-H460-IKKα pools that showed increased levels of IKKα (Fig. 1B); additionally, it also showed that the C-H460-IKKα and N-H460-IKKα cells expressed correctly the transgene, i.e., it was detected in the cytoplasm of the C-H460-IKKα cells and in the nucleus of the N-H460-IKKα ones, while the Control-H460 cells (containing the empty vector) showed lower levels of IKKα expression (Fig. 1B).

3.2. Overexpression of IKKα, both in the Nucleus and in the Cytoplasm of NSCLC Cells, Leads to the Activation of Protumoral Proteins

We analyzed by WB the levels of expression and activation of proteins recognized as relevant for the development of and/or progression of NSCLC to determine whether they resulted affected by the altered expression of IKKα.

We found overactivation of the classical NF-κB pathway (measured as an increase in the levels of P-p65 and P-IκBα) in the C– H460-IKKα pools C-1, C-6, C-7 and C-12 that showed increased expression of the IKKα protein (Fig. 2A); however, no activation of NF-κB was detected in those pools in which the transgene was not expressed (C-8, C-9 and C-2 pool) and therefore showed levels of IKKα similar to those of Control-H460 cells; consequently, these results indicate that the activation of NF-κB is due to the presence of transgenic IKKα protein. As expected, no enhanced activation of the NF-κB pathway was observed in the N-H460-IKKα pools (Fig. 2B).

Increased activation of EGFR was also detected in cells overexpressing IKKα in the cytoplasm (Fig. 2A), while no differences were found in N-H460-IKKα cells (Fig. 2C). Activation of the Erk1/2 (Fig. 2D) and p38 (Fig. 2G) signaling pathways and overexpression of Cyclin D1 (Fig. 2A, E) were detected both in the C-and-N-H460-IKKα cells. Hyperactivation of the mTOR pathway (measured as increased levels of P-mTOR) was noticed as well in both H460-IKKα-overexpressing cells (Fig. 2F). The expression of Podoplanin and Snail, two relevant proteins promoting cell tumor migration and metastasis were analyzed and we observed that the expression of Podoplanin was augmented in both the C-and-N-H460-IKKα cells (Fig. 2G), whereas Snail was mainly increased in the cells overexpressing nuclear IKKα (Fig. 2G). Hyperactivation of Smad2/3 (Fig. 2A,B) and c-Myc (Fig. 2G) was specifically found in the N-H460-IKKα cells.

3.3. Both Nuclear and Cytoplasmic IKKα Provides Greater Proliferative, Migratory and Survival Capacities to Lung Cancer Cells

The molecular alterations found in C-H460-IKKα and N-H460-IKKα cells suggest an increase in their malignancy; therefore, we performed different tests to analyze whether the expression of transgenic IKKα induces changes in the behavior of C-and-N-H460– IKKα cells in culture. Since, as shown in Fig. 2, all pools of both C-and-N-H460-IKKα that express the transgene showed similar molecular alterations, we chose one of each genotype for further analysis (pools C-H460-IKKα-1 (C-1); N-H460-IKKα-8 (N-8), and Control-H460–2 (Control-2)). First we investigated their proliferative capacity. Growth curves at different time points showed that after 72 h in culture the number of cells was significantly higher in the cells overexpressing nuclear or cytoplasmic IKKα, suggesting that they had greater proliferative capacity (Fig. 3A). We also examined the colony-forming efficiency and found that the C-H460-IKKα cells formed a greater number of colonies than Control and N-H460-IKKα cells (Fig. 3C). Although no differences were found in the size of the colonies formed by the three types of H460 cells, we noted the presence of a large number of very small colonies in both the C-and-N-H460-IKKα cells corresponded to “satellite colonies” that had escaped from the primary colony (Fig. 3B, D), suggesting that overexpression of IKKα provides cells with an increased ability to migrate, mainly when IKKα is expressed in the nucleus. The analysis of the morphology of the colonies formed in these experiments showed that those from Control-H460 cells were compact an exhibited a well-defined shape (Fig. 4A-C); by contrast, colonies formed by N-H460-IKKα cells were loose and showed an imprecise contour, with abundant isolated cells migrating from the colony (Fig. 4D-F). This behavior was observed at early time post-seeding (4 days, Fig. 4D) and was more pronounced at later time points (7–9 days of culture, Fig. 4E, F). C-H460-IKKα colonies showed an intermediate appearance between Control and N-H460-IKKα cells, showing in long-term cultures (9 days) the presence of cells that migrated from the colony (Fig. 4I). Therefore, migration of the isolated IKKα-overexpressing cells from the colonies is in agreement with the observed formation of “satellite colonies” in the C-and-N-H460-IKKα cells.

To further analyze the effect of nuclear and cytoplasmic IKKα expression on H460 cell motility we performed in vitro wound healing assays. Cells growing in monolayer cultures were subjected to a ‘scratch’ wound; as a result it was found that both N-and-C-H460-IKKα cells formed large and abundant foci of migration, constituting practically a continuous migratory cell front at 48 h after scratching (Fig. 4N, O); by contrast, in the ‘scratch’ wound of the Control-H460 cells only the presence of few isolated migratory cells were observed (Fig. 4M). Additionally, the N-and-C-H460-IKKα cells achieved greater migration distance.

The increased cell proliferation and migration exhibited by the N-and-C-H460-IKKα cells are characteristics suggestive of enhanced malignant progression; another marker indicative of increased aggressiveness of tumor cells is their anchorage-independent growth [26]. Thus, we examined in suspension cultures the anchorage-independent growth capacity of the H460 cells of the three genotypes. We found that IKKα-overexpressing cells exhibited increased capability to grow independently of anchoring after 24 h in suspension, i.e., C-and-N-H460-IKKα cells formed larger numbers of colonies, indicating that a greater number of cells had survived in suspension and, subsequently, were able to form colonies upon adhering to the substrate (Fig. 5A, B). We also verified that in a stress situation, such as culture in the absence of serum, cells of both genotypes, C-H460-IKKα and N-H460-IKKα, had an increased capacity to survive, likely as a result of their increased expression of the anti-apoptotic protein Bcl-2 (Fig. 5C). The induction of Bcl-2 expression in the H460-overexpressing IKKα cells is a marker of enhanced malignancy, as it has been found that Bcl-2 is markedly increased in lung cancer patient biopsies; additionally, its overexpression has been related with resistance against EGFR-TK inhibitors [27].

Therefore, the increased ability to proliferate, migrate and survive found in the C-and-N-H460-IKKα cells suggest that overexpression of IKKα in either of its subcellular localization confers greater malignancy to the H460 cells.

3.4. IKKα Signaling both in Nucleus and Cytoplasm of NSCLC Cells Favors Lung Tumor Growth in Xenograft Models of Carcinogenesis

To determine whether the expression of the exogenous IKKα in the nucleus or in the cytoplasm of lung tumor cells also enhances tumorigenicity in vivo, we used a xenograft model. We subcutaneously injected H460 cells of the three genotypes into immunodeficient mice (pools C-H460-IKKα-1 (C-1); N-H460-IKKα-8 (N-8), and Control-H460-2 (Control-2) were inoculated). Two independent assays were performed in R2G2 and nude mice that yielded similar results: the latency period of the tumors was of 9 days in both the C-and-N-H460-IKKα cells and 11 days in the Control-H460 cells. At 11 days after injection, tumors were clearly visible in the three groups of mice injected with each type of H460 cells, being larger those from H460 cells overexpressing IKKα (Fig. 6A, B); these differences were also maintained at more advanced time points (Fig. 6C). Similar growth tumor rates were observed in both strains of immunodeficient mice used. Tumors were collected on day 15–20-post-injection. We verified by immunohistochemical staining the correct expression of the transgene in the tumors, i.e., we detected the cytoplasmic expression of IKKα in the C-H460-IKKα tumors (Fig. 6E), and the nuclear expression of IKKα in the N-H460-IKKα

tumors (Fig. 6F). In the Control-H460 tumors a low expression of IKKα was detected (Fig. 6D).

The histological analysis revealed that tumors of the three genotypes of H460 cells resembled lung ADCs, showing the presence of nests or solid masses separated by scarce vascularized stroma (Fig. 6G). Among them, the C-H460-IKKα tumors showed histological features of greater aggressiveness, characterized by large areas of necrosis (Fig. 6H), high frequency of anisokariosis (variation in the size of the nuclei, considered a marker of cellular atypia, Fig. 6I), frequent anisocytosis, presence of multinucleated giant cells (Fig. 6J) and aberrant mitosis (Fig. 6K).

We confirmed the diagnosis of pulmonary ADCs by immunohistochemistry with an anti-TTF1 (Thyroid Transcription Factor 1) antibody in the tumors of the three genotypes (Fig. 7A-C), while staining with the keratin K5, characteristic of lung SCCs, was negative for all three tumor types (Fig. 7D-F). The expression of K8, which we and others have found to be positively regulated by IKKα ([28], and Alameda et al., in preparation) was higher in the N-H460-IKKα tumors (Fig. 7G-I), being K8 upregulation considered a marker of increased malignancy in different types of cancer; indeed we have reported that K8 induces the formation of NMSC [29]. The analysis of the proliferation rate, measured as the percentage of BrdU-positive cells in the tumors collected on day 20 after injection, revealed that the C-and-N-H460-IKKα tumors were significantly more proliferative than Control tumors (Fig. 7J-M), which was in agreement with the higher size of the IKKα-overexpressing tumors shown in Fig. 6. No apoptosis was detected in any case (not shown).

As observed in Fig. 6C, tumors arisen from both C-and-N-IKKα cells had a reddish appearance and showed the presence of blood vessels visible to naked eye. Therefore, we analyzed the pattern of blood vessels staining in the tumors using a PECAM-1 antibody and found that vessels from the IKKα-overexpressing xenotransplants were lacunar and presented a greater caliber than those in Control-H460 tumors (Fig. 7N-P). These pattern of blood vessels in the IKKα-overexpressing tumors is suggestive of higher malignancy, since the existence of highly vascularized tumors has been significantly correlated in NSCLC patients with worse survival and increased capacity to metastasize [30].

Therefore, our results indicate that in agreement with the increased malignancy of the C-and-N-H460-IKKα cells in culture, NSCLC tumors derived from them also presented features of higher aggressiveness.

3.5. IKKα Increases the Metastatic Capacity of Lung ADC Cells when Localized either in the Nucleus or in the Cytoplasm

To confirm the augmented malignancy of the C-and-N-H460-IKKα cells in vivo, we performed metastasis experiments: after resection of the subcutaneous tumors (at day 15 or 20 post-inoculation of the cells), animals were kept alive for two more weeks with the aim of analyzing the appearance of possible metastases. At 29 and 36 days post-injection animals were sacrificed. We observed macroscopically the presence of whitish foci in the lungs that were histologically confirmed to be metastases (Fig. 8A, D, G). In mice receiving the Control-H460 cells metastases were scarce and small (Fig. 8A–C). In contrast, large whitish metastatic areas were noted in the lungs of the mice injected with the C-or-N-H460-IKKα cells (Fig. 8D, G; E-F; H-I). In the liver a metastasis was found in one N-H460-IKKα mouse (Fig. 8J, K). Immunohistochemical staining confirmed the expression of cytoplasmic IKKα in the metastasis of the mice receiving the C-H460-IKKα cells (Fig. 8M) and the nuclear expression of IKKα in the metastasis of tumors inoculated with the N-H460-IKKα cells (Fig. 8N). Positive staining with the TTF1-specific antibody were detected in the metastasis (Fig. 8 O–Q), similar to the TTF-1 staining observed in the H460 subcutaneous tumors of the three types of cells.

Thus our results confirm the increased metastatic capacity of NSCLC cells that overexpress IKKα either in the cytoplasm or in the nucleus.

4. Discussion

The analysis of the H460 cells expressing exogenous IKKα in the nucleus or in the cytoplasm has been a valuable tool to solve the controversy that had arisen regarding the role that IKKα plays in lung ADC development since some studies suggest that IKKα is necessary for lung ADC development and progression [24] while others propose its role as a tumor suppressor of this type of cancer [22,23]. Our results show that IKKα plays a protumoral role in lung ADC. Although recently the relevance of the nuclear localization of IKKα for lung ADC growth has been proposed [24,31], these studies however do not include the analysis of the mechanisms underlying the role of nuclear IKKα in the progression of lung ADC; neither is explored whether IKKα of cytoplasmic localization also plays a role in this pathology. Here we show that regardless of its subcellular localization, IKKα promotes lung tumor growth and metastasis.

We have found that the mechanisms through which IKKα-overexpression leads to increased malignancy of NSCLC include the upregulation of common pathways relevant for lung cancer progression and metastasis, such as the overexpression of Cyclin D1 and Podoplanin and the activation of Erk1/2, p38 and mTor signaling. Additionally, the specific localization of IKKα in the nucleus or in the cytoplasm of NSCLC cells activates different pathways, i.e. while the expression of C-IKKα results in the overactivation of the EGFR and NF-κB pathways, nuclear IKKα enhances c-Myc and Smad2/3 activation, and the levels of expression of Snail. All these signaling pathways are known to be relevant for the progression of NSCLC. Thus, it is known that EGFR and NF-κB pathways are activated in numerous lung cancer patients [4]. Indeed, in NSCLC, overexpression or mutations in the EGFR gene have been observed in 43–89% of cases [6]. The sustained activation of EGFR and its downstream targets in lung tumor cells results in cell proliferation and anti-apoptosis and is thought to yield more aggressive tumor phenotypes [32]; accordingly, some studies have shown that EGFR activation in NSCLC is associated with reduced survival [33], frequent lymph node metastasis and poor chemosensitivity [34]. Although anti-EGFR treatments are available in the clinic, however, they frequently cause the hyperactivation of NF-κB which in turn triggers resistance to EGFR inhibitors [7] and in addition contributes to tumor progression, since as lung tumorigenesis progresses, the activation of the canonical NF-κB is maintained serving as a survival signal that contributes substantially to the resistance to cell death [35]. The important role of NF-κB in lung cancer is also due to its critical function in the initiation of NSCLC, since it has been shown that its activation causes spontaneous lung cancer in vivo even in the absence of oncogene manipulation or carcinogen exposure [9,36]. Therefore, the activation of both pathways in the C-H460-IKKα cells could explain their increased malignancy and the larger size and metastatic capacity of the ADC derived from them. The increased activity of c-Myc, Smad2/3 and Snail found in the N-H460-IKKα cells has also been recognized for playing a prominent role in NSCLC progression. c-Myc is amplified in >30% of ADC [10], causing increased proliferation, cell survival, angiogenesis and metastasis. Therefore, the hyperactivation of c-Myc could be responsible, at least in part, for the increase in malignancy of the N-H460-IKKα cells, providing them with the proliferative, survival and migratory advantages observed. But in addition, overexpression of nuclear IKKα produces the activation of Smad2/3 which is implicated in anchorage-independent growth and in the survival of circulating lung tumor cells, favoring the EMT; accordingly, it has been reported that the activation of Smad2/3 is involved in lung tumor growth and in lung and liver metastatic nodule formation in mice [37]. We have also detected overexpression of Snail in the N-H460-IKKα cells, which is also increased in NSCLC patients and has been associated with tumor progress and recurrence, metastasis and poor prognosis [38,39], predicting a shorter survival in NSCLC patients [40].

All the other common pathways that are activated in both H460-IKKα-overexpressing cells, i.e. Erk1/2, p38 and mTor; as well as the

induction of the expression of Cyclin D1 and Podoplanin, have been also recognized as having a prominent role in the progression of lung cancer, i.e., increased Erk1/2 phosphorylation has been reported in a subset of NSCLC cell lines [41]; also, activated p38 was consistently increased in lung tumor compared with normal tissue in human samples, suggesting a role for this pathway in malignant cell growth [42]. The mTOR axis is dysregulated and activated in 74% of human NSCLC [43], particularly in ADC, and plays a critical role in mediating proliferative and survival signals in lung cancer cells favoring NSCLC progression [44] and resistance to chemotherapy and EGFR inhibitors [45]. Podoplanin is also known to enhance lung cancer cell growth in vivo [46]; it is involved in tumor progression through the induction of platelet aggregation, facilitating hematogenous dissemination; indeed, Podoplanin has been shown to be expressed in circulating tumor cells and to promote pulmonary metastasis [47]. There is also evidence implicating the deregulation of cyclin D1 in the pathogenesis of NSCLC, being cyclin D1 frequently overexpressed in tumors and in pre-invasive bronchial lesions [48]; thus, it is considered to be involved in tumorigenesis of NSCLC from early stage and could be a predictive molecular marker for poor prognosis in resectable NSCLC patients [49].

Therefore, accordingly with the activation of the above mentioned pathways in C-and-N-H460-IKKα cells, both types of cells exhibit an increased capacity to form colonies, as well as higher proliferation, migration and survival abilities. Consequently, xenograft experiments showed that tumors derived from both types of IKKα-overexpressing cells are more proliferative, have histological and biochemical markers of greater malignancy and show increased capacity to metastasize.

It is remarkable that these results are similar to those obtained when the C-IKKα or N-IKKα constructs were expressed in tumor epidermal cells: we observed that in the cytoplasm, IKKα induced the activation of NF-κB and EGFR and the overexpression of Cyclin D1; being the consequences of these changes an increase in the malignancy of the skin tumors derived from them. Also, we found the activation of c-Myc in tumor epidermal cells expressing the N-IKKα construct that consequently yielded, in xenografts experiments, skin SCC of increased aggressiveness [19]; additionally, we have also detected the induction of Snail and Podoplanin in skin equivalents of human keratinocytes overexpressing IKKα [50] .

The finding that the likely signaling pathways through which IKKα induces tumor progression in both skin and lung cancer are similar is very interesting, since it suggests the possibility that the mechanisms by which IKKα induces tumor progression in these neoplasms could be generalized to other tumor types (breast, prostate, pancreas, etc.). It will be interesting to analyze the possible activation of these pathways in additional tumors.

Our results reveal that alterations in IKKα signaling in either the nucleus or the cytoplasm of the H460 cells provoke lung cancer progression and metastasis. This is very relevant as NSCLC is the leading cause of cancer death worldwide. Moreover, although surgical resection is the treatment of choice for early-stage NSCLC, frequent tumor recurrence and metastasis occur, being the main obstacles for long-term survival [51]. Also, resistance to chemotherapy and molecular target therapies are common in the treatment of lung cancer, being the cell survival and increased cell migration the major underlying mechanisms to these events [52]. Thus, the identification of molecular markers related to metastasis may predict the prognosis and survival in patients with NSCLC. We provide evidence that in each localization both in the nucleus and the cytoplasm IKKα promotes cell survival and cell migration of lung ADC cells, indicating that the overexpression of IKKα in lung cancer cells strongly increases their malignancy and promotes tumor metastasis, suggesting that IKKα may be a potential new target for intervention in lung cancer in humans. Our results are reinforced by the fact that recently the upregulation of IKKα expression in lung ADC in humans has been described [24], as well as its association with overall decreased survival of lung cancer patients [31]. It has also been suggested that levels of IKKα expression may be a useful tool to predict poor clinical outcome in lung ADC patients [31].

Therefore, our findings help in understanding the progression of human lung ADC and are very promising from a clinical point of view since they have identified the IKKα protein as a relevant molecule in the promotion and metastasis of NSCLS cancer. We propose that IKKα could be a good target to address more specific treatments for this type of neoplasia that causes the highest number of cancer deaths in the world.

5. Conclusions

In conclusion, our data indicate:

1. IKKα located either in the nucleus or in the cytoplasm of non-small cell lung cancer cells promotes enhanced tumor progression and metastasis.
2. IKKα overexpression induces in the nucleus and the cytoplasm of NSCLC cells the activation of common signaling pathways relevant for the development and promotion of lung cancer. Additionally, IKKα induces other specific protumoral molecules, depending on its subcellular localization.
3. IKKα activates the classical NF-κB and the EGFR pathways in the cytoplasm of the H460 cells.
4. Nuclear localization of IKKα in H460 cells promotes the activation of c-Myc, Smad2/3 and Snail.
5. IKKα provides in its two subcellular localizations, increased proliferative, clonogenic, survival and migration capacity to NSCLC cells, possibly as a consequence of the activation of the aforementioned pathways.
6. IKKα signaling increases, in its two localizations, nuclear and cytoplasm, the malignancy of tumors developed in preclinical xenograft models in immunodeficient mice, as well as the metastasis of the NSCLC cells.

Conflict of Interest

None.

Acknowledgments

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