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Anti-tumoral activity of the human-specific duplicated form of α7-nicotinic receptor subunit in tobacco-induced lung cancer progression

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ABSTRACT

Objectives: Tobacco smoking is strongly correlated with the onset and progression of non-small cell lung cancer (NSCLC). By activating α7 nicotinic acetylcholine receptors (α7-nAChRs) in these tumors nicotine and its tobacco-derived nitrosamine, NNK, contribute to these oncogenic processes. Here, we investigated whether the human-specific duplicated form of the α7-nAChR subunit (dupα7) behaves as an endogenous negative regulator of α7-nAChR-mediated tumorigenic activity induced by tobacco in NSCLC cells, similarly to its influence on other α7-nAChR-controlled functions in non-tumor cells.

Methods: Two human NSCLC cell lines, lung adenocarcinoma (A549) and squamous cell carcinoma of the lung (SK-MES-1), both wild-type or with stable overexpression of dupα7 ($A549^{dup\alpha7}$ or SK-MES-$1^{dup\alpha7}$), were used to investigate *in vitro* anti-tumor activity of dupα7 on nicotine- or NNK-induced tumor progression. For this purpose, migration, proliferation or epithelial-mesenchymal transition (EMT) were examined. The anti-tumor effect of dupα7 on nicotine-promoted tumor growth, proliferation or angiogenesis was also assessed *in vivo* in an athymic mouse model implanted with $A549^{dup\alpha7}$ or A549 xenografts.

Results: Overexpression of dupα7 in both cell lines almost completely suppresses the *in vitro* tumor-promoting effects induced by nicotine (1 μM) or NNK (100 nM) in wild-type cells. Furthermore, in mice receiving nicotine, $A549^{dup\alpha7}$ xenografts show: (i) a significant reduction of tumor growth, and (ii) decreased expression of cell markers for proliferation (Ki67) or angiogenesis (VEGF) compared to A549 xenografts.

Conclusion: Our study demonstrates, for the first time, the *in vitro* and *in vivo* anti-tumor capacity of dupα7 to block the α7-nAChR-mediated tumorigenic effects of tobacco in NSCLC, suggesting that up-regulation of dupα7 expression in these tumors could offer a potential new therapeutic target in smoking-related cancers.

1. Introduction

Lung cancer is the leading global cause of cancer deaths, with non-small cell lung cancer (NSCLC) accounting for 75–85% of all lung cancer cases [1]. Lung adenocarcinoma and squamous cell carcinoma of the lung are the two major histological types of NSCLC. Cigarette smoking is an important risk factor for many types of cancers, including NSCLC, which is understandable because tobacco smoke contains more than 70 known carcinogens that will eventually initiate carcinogenesis [2,3]. In parallel with the mutagenic and cytotoxic effects of these

Abbreviations: α7-nAChR, α7 nicotinic acetylcholine receptor subtype; dupα7, human-specific duplicated form of the α7-nAChR subunit; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HRP, horseradish peroxidase; IHC, immunohistochemistry; MEK, mitogen-activated protein kinase; NNK, nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyrydyl)-1-butanone; NSCLC, non-small cell lung cancer; $p90^{RSK}$, MAPK-activated protein kinase-1; PVDF, polyvinylidene difluoride; qPCR, real-time quantitative PCR; Raf-1, RAF proto-oncogene serine/threonine-protein kinase; Rb, retinoblastoma tumor suppressor protein; SCLC, small cell lung cancer; VEGF, vascular endothelial grown factor

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carcinogens, nicotine, the addictive component of tobacco, and its carcinogenic derivative nitrosamine 4-(methylnitrosamino)-1-(3-pyrydyl)-1-butanone (NNK) also contribute to the initiation and progression of lung cancer by activating nicotinic acetylcholine receptors (nAChRs) expressed in these tumor cells [4–7].

nAChRs are complex structures having five subunits. Several alpha (α3-α7, α9) and beta (β2 and β4) nAChR subunits have been identified in human NSCLC cell lines and in primary lung tumors from NSCLC patients [see Ref. [8] and references therein [9],]. Therefore, the above tumors can express several nAChR subtypes composed of identical subunits (homomeric α7- and α9-nAChRs) or of different α and β subunits (heteromeric α3β4α5-, α3β2α5- and α4β2-nAChRs). Despite this diversity of nAChR subtypes, the α7 subtype is recognized as being the main trigger for the nicotine-mediated proliferative, pro-angiogenic and pro-metastatic effects in human NSCLC [10–13]. These nicotine effects result from α7-nAChR-mediated downstream activation of several oncogenic signaling pathways that, in the case of NSCLC cells, involve the MEK/ERK, Akt and Rb-Raf-1/phospho-ERK/phospho-p90RSK pathways [see Ref [6,14]. and references therein]. Accordingly, the α7-nAChR subtype could be considered as a target for lung cancer prevention and/or therapy.

The α7 subunits that make up the α7-nAChR in humans are encoded by the *CHRNA7* gene located on the long arm of chromosome 15 (15q13-q14). This gene is partially duplicated in the same chromosome and, after its fusion with the *FAM7A* gene, generates a new chimeric gene (*CHRFAM7A*), which is unique to the human genome since it is not found in other higher primates [15,16]. This chimeric gene encodes for a truncated α7 nAChR subunit form, dupα7, which shares all the structural elements of the ancestral protein except for a substantial part of the N-terminal region containing the signal peptide and the agonist-binding domain [15]. Furthermore, dupα7 and α7 subunits are naturally expressed in the same human cell types and tissues, including neurons, epithelial and immune cells [see Ref [17]. and references therein] and primary NSCLC tumors [9].

The functional role of the above human-specific duplicated gene was long unidentified until we demonstrated that its product negatively regulated α7-nAChR activity in *Xenopus* oocytes [18], a finding corroborated shortly afterwards by others [19]. It is noteworthy that the blocking effect of dupα7 on α7-nAChR function in amphibian cells has also been found in mammalian cells, as we have recently reported in murine macrophages, where dupα7 overexpression attenuated the known anti-inflammatory effect controlled by the α7-nAChRs naturally expressed in these cells [20]. Given the prominent role of α7-nAChRs in tobacco-induced lung cancer progression and also the fact that dupα7 is natively expressed in primary tumors from NSCLC patients [9], it is feasible that this latter subunit may act as an endogenous suppressor of the growth and progression of the above tumors by interfering with their α7-nAChR responsiveness. To date, there have been no data in the literature on this issue.

We addressed this lack of information by performing *in vitro* and *in vivo* tumorigenic assays after stable overexpression of an epitope-tagged dupα7 construct (dupα7.pcDNA3.1/Myc-His) in human NSCLC cell lines (A549 and SK-MES-1) and in A549 xenografts in nude mice. The construct, prepared in our laboratory, is crucial for the selection of cells with stable dupα7-Myc transfection since, otherwise, the high homology in the peptide sequences between the dupα7 and α7 subunits makes commercially-available antibodies cross-react with both subunits, preventing the immunocytochemical identification and selection of clones with stable dupα7 overexpression.

Here, we demonstrate the anti-tumor activity of the duplicated form of the α7 subunit by blocking, both *in vitro* and *in vivo*, the tobacco-promoted oncogenic effect of the ancestral protein that makes up the α7-nAChR.

2. Material and methods

2.1. Cell cultures and reagents

Human NSCLC cell lines (A549 and SK-MES-1) were purchased from American Type Culture Collection (ATCC) and maintained respectively in RPMI 1640 (Gibco, Thermo Fischer Scientific, Waltham, MA, USA) or MEM (Sigma-Aldrich; St. Louis, USA) media supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified incubator at 37 °C. Nicotine and NNK were purchased from Sigma-Aldrich and Toronto Research Chemicals (ON, Canada), respectively. The following primary antibodies were used: mouse anti-Myc (Roche, Mannheim, Germany); rabbit anti-β-catenin (Cell Signaling Technology, Danvers, MA, USA); mouse anti-vimentin, mouse anti-fibronectin, goat anti-β-actin, rabbit anti-VEGF and rabbit anti-α7 (Santa Cruz Biotechnologies, CA, USA); and mouse anti-Ki67 (Dako, Santa Clara, CA, USA).

2.2. Cell transfection and selection of stable dupα7-Myc transfectants

The dupα7.pcDNA3.1/Myc-His construct used for transfection was prepared in our laboratory as described elsewhere [20]; it contains the full-length human dupα7 cDNA sequence in frame with the Myc-His tag. The A549 or SK-MES-1 cells were transfected either with the above plasmid or with the corresponding empty-vector (pcDNA3.1/Myc-His) using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer´s instructions. Forty-eight hours after transfection, the cells were trypsinized and plated in culture medium supplemented with the aminoglycoside antibiotic, geneticin (600 μg/ml; Sigma-Aldrich), for the selection of stably-transfected cells (A549$^{dup\alpha7}$ or SK-MES-1$^{dup\alpha7}$). Positive clones were double-confirmed by: 1) quantitative real-time PCR (qPCR) as detailed below; or 2) immunocytochemistry combined with confocal microscopy using the anti-Myc (1:200) primary antibody followed by the Alexa Fluor 488 goat anti-mouse IgG [1:400; Molecular Probes (Invitrogen, USA)] secondary antibody as described elsewhere [20].

2.3. qPCR assay of nAChR subunit gene expression

Techniques for RNA extraction and *α7* or *dupα7* gene expression analysis from cells or tumor tissues by qPCR from reverse-transcribed RNA using the SYBR green-based assays (Bio-Rad, Hercules, CA) and the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA), have been described elsewhere [9,20,21]. The following set of primers were used for PCR amplification of the corresponding transcript: α7, forward 5´-GCTGCAAATGTCTTGGACAGAT and reverse 5´-AACAGTCTTCA − CCCCTGGATAT; dupα7, forward 5´- CAATTGCTAA TCCAGCATTTGTGG and reverse 5´ − CCCAGAAGAATTCACCAACACG. The next pair of primers [forward 5′-TGATCAAGGGAAAGATGACCA and reverse 5′-AACCCTCTTGCAATCGAAAA] was employed to amplify the human D esterase gene (*ESD*), which was selected as an endogenous control for the PCR reaction since, among the housekeeping genes, that gene seems to have the most stable expression in NSCLC [22,23]. Cycling conditions for PCR were: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Analysis of the melting curves demonstrated that each pair of primers amplified a single product. Relative changes in α7 or dupα7 mRNA expression in A549$^{dup\alpha7}$ or SK-MES-1$^{dup\alpha7}$ cells were assessed by the $2^{-\Delta\Delta Ct}$ method using the corresponding mRNA expression in non-transfected cells (A549 or SK-MES-1) as calibrator (set to a value of 1).

2.4. Wound-healing assay in vitro

This assay serves to detect cell migration. Control or transfected NSCLC cells were seeded in 6-well plates (3.5×10^5 or 8×10^5 cells per well, respectively) and grown to 90% confluence (≈ 24 h). Subsequently, the cell monolayer was scratched vertically with a sterile

pipette tip and the wells washed with PBS to remove the detached cells. The cells were then incubated or not with nicotine, NNK or 10% FBS (positive control) to induce cell migration. Images of the wound were captured at 0 and 24 h after scratching using a DXM1200 F digital camera attached to a personal computer, with a Nikon TE2000-S microscope.

2.5. Transwell assay in vitro

The cell migration assay was also performed using the two-well Transwell Boyden Chamber (Corning-Costar, Cambridge, MA, USA) with a polycarbonate membrane of 8 μm pores per the manufacturer's instructions. A total of 5×10^4 cells were placed in the upper chambers in serum-free medium containing or not nicotine or NNK; the lower chambers were loaded with complete medium (10% FBS). After 24 h, the non-invasive cells retained in the upper chambers were removed with a cotton swab, while the cells that had migrated across the basement membrane and attached to its lower face were fixed and their nuclei stained with DAPI before cell counting with the ImageJ software of the confocal microscope *Leica TCS SP5*.

2.6. Cell proliferation assay in vitro

Cell proliferation was evaluated using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Life Technologies/Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, control or transfected A549 or SK-MES-1 cells were plated on 12-mm glass coverslips (1.1×10^4 or 1.7×10^4 cells/coverslip, respectively) and maintained in a serum-starved medium for 24 h. Then cells were incubated with 5-ethylnyl-2´-deoxyuridene (EdU) for 24 h while undergoing treatment with nicotine, NNK or 10% FBS (positive control) to induce proliferation. Subsequently, cells were fixed, permeabilized and incubated with the Click-iT reaction cocktail to detect the EdU signal before staining nuclei with DAPI. The coverslips were visualized in a *Leica TCS SP5* confocal microscope that automatically counted the number of EdU-positive cells in the microscope field with the ImageJ software.

2.7. Western blot analysis

After being subjected to the indicated treatments, cells were lysed and protein concentration determined in the cell lysates as described elsewhere [20,24]. Proteins were resolved by 10% SDS/PAGE gel electrophoresis, transferred to a PVDF membrane (Millipore) and analyzed by immunoblot after overnight incubation of the blots at 4 °C with the following primary antibodies [anti-α7 (1:500 dilution) or anti-β-catenin, anti-vimentin or anti-fibronectin (at 1:1000 dilution)]. The anti-β-actin antibody (1:1000) was incubated for 1 h at room temperature. Proteins were detected by blot incubation (1 h) with the appropriate HRP-conjugated secondary antibody (1:5000, Santa Cruz Biotechnologies, CA, USA). The resulting bands were detected using the ECL Select Western Blotting Detection Reagent (Amersham, GE Healthcare, UK).

2.8. Mice model and in vivo tumor xenograft assays

This study was approved by the Animal Care and Use Committee of the Universidad Autonoma de Madrid (UAM) in accordance with the EU Directive 2010/63/EU for animal experiments. Animals were housed under pathogen-free conditions in the Animal Resource Facilities of our institution (Instituto de Investigaciones Biomédicas Alberto Sols-CSIC-UAM). Five- to six-week-old female NU-Foxn1nu athymic mice were purchased from Charles River Laboratories (Wilmington, MA, USA). A suspension of wild-type A549 cells or of $A549^{dup\alpha7}$ cells (2×10^6 cells/mouse) was subcutaneously injected into the left flank of each mouse. Ten days later, each group of mice inoculated with one or the other cell variant was randomly distributed into two subgroups (5–6 mice/subgroup) according to whether they were going to receive nicotine or not in drinking water containing saccharin (2%). Mice were monitored once every three days to evaluate the growth rate of tumor volume, which was determined in each measurement using the following formula: volume (mm^3) = (D^2 x d) x 0.52, where (D) and (d) are the longest and shortest tumor diameters, respectively. After 27 days of nicotine treatment, all mice were sacrificed and their tumors excised, photographed, measured, fixed in 4% PFA and embedded in paraffin for subsequent immunohistochemistry (IHC) analysis.

2.9. IHC analysis

IHC assays to examine the expression of proliferation or angiogenesis markers in the above tumor xenografts were performed on serial Sections (3.5 μm thick) using a Dako commercial kit (EnVision™ FLEX System kit) following the manufacturer's instructions. Briefly, deparaffinized tissue slices were subjected to antigen retrieval and endogenous peroxidase blockade. Slices were then incubated with the anti-Ki-67 (Ready-to-Use) or anti-VEGF (dilution 1:50) primary antibodies followed by incubation with a secondary polyvalent antibody from Dako (goat anti-rabbit and anti-mouse Ig-HRP; 1:200). The target antigens marked by the respective primary antibodies showed the brown color generated by incubating slices with the EnVision™ FLEX DAB + Substrate Chromogen System (Dako). Sections were hematoxylin and eosin counterstained using the EnVision FLEX Hematoxylin reagent and mounted with the Dako Mounting Medium. Section images were taken using a Nikon TE2000-S microscope with ImageJ software.

2.10. Data and statistical analysis

Data normalization was used for evaluation of the nicotine or NNK effects on cell migration measured by the transwell migration test (Fig. 3B) or on epithelial-mesenchymal transition measured by Western blot (Fig. 5B), where the untreated (blank or control) cell value was set to 100% or 1, respectively. The Mann-Whitney U test was used for comparisons between two groups, and one-way ANOVA followed by the Newman-Keuls *post-hoc* test (parametric data) for multiple comparisons (Fig. 6A and 6C). Data were represented as mean ± SEM of at least five independent experiments, except for Western blots in Fig. 1 (n = 2) and Fig. 5 (n = 3). A p value ≤ 0.05 was considered statistically significant. Statistical analyses were performed using Prism software, version 5 (GraphPad).

3. Results

3.1. Stable overexpression of dupα7-Myc in NSCLC cells does not alter the expression level of endogenous α7 mRNA or protein

Cells from lung adenocarcinoma (A549) or squamous cell carcinoma of the lung (SK-MES-1) were used for these and the subsequent *in vitro* experiments since, in addition to representing the two major histological types of NSCLC, both cell types express α7-nAChRs whose activation by nicotine or NNK promotes tumor growth and development [11,12,25,26]. The first experiments in this study were designed to rule out the possibility that dupα7 overexpression could inhibit the endogenous expression of α7 since, if this were the case, the interpretation of our results would change radically. Thus, we analyzed the expression of dupα7 and α7, at the mRNA or protein level, by qPCR and immunocytochemistry combined with confocal microscopy or Western blot, in both cell types stably-transfected with dupα7-Myc ($A549^{dup\alpha7}$ or $SK\text{-}MES\text{-}1^{dup\alpha7}$) or in the wild-type cells (A549 or SK-MES-1) that were going to be used throughout our study. The results show that, in comparison to wild-type cells, the overexpression of dupα7-Myc in stably-transfected cells (Figs. 1A and B) did not modify the endogenous expression of α7 mRNA or protein in either of the two NSCLC cell lines tested (Figs. 1A and C).

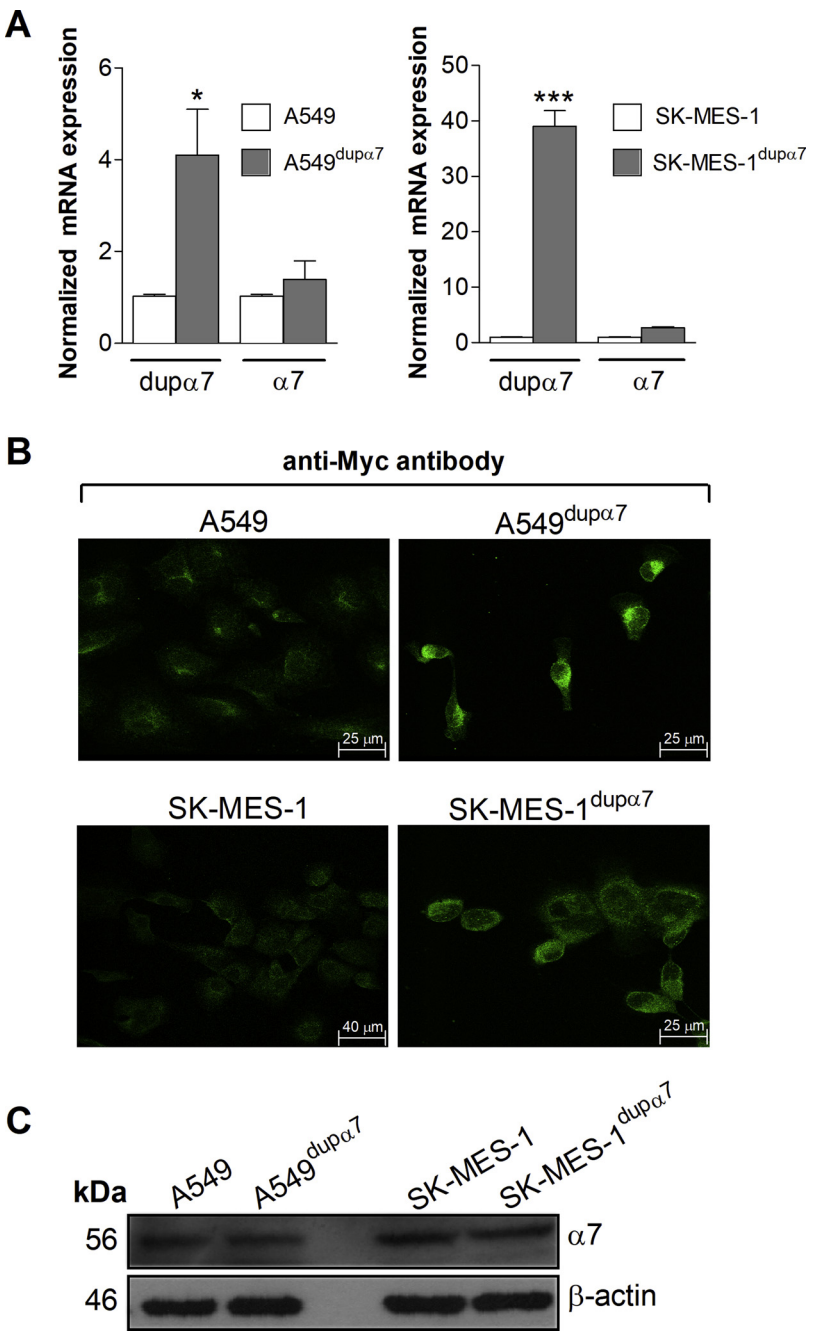

Fig. 1. The endogenous expression of α7 in human NSCLC cell lines remains unchanged after stable overexpression of dupα7. The figure reflects the expression of native α7, at the mRNA or protein level, in parallel with the corresponding expression of dupα7 mRNA (native plus foreign) or dupα7-Myc protein (foreign) in the four cellular variants that were going to be used throughout present study. These variants correspond to cells stably-transfected with dupα7-Myc ($A549^{dup\alpha7}$ or SK-MES-$1^{dup\alpha7}$) or to non-transfected wild-type cells (A549 or SK-MES-1). (A) Normalized expression of dupα7 or α7 messengers determined by qPCR in the above cell variants. Data show mean ± SEM of three independent cell cultures. *$p < 0.05$ and ***$p < 0.001$ compared to non-transfected cells. (B) Confocal images showing the successful overexpression of the dupα7-Myc subunit in $A549^{dup\alpha7}$ or SK-MES-$1^{dup\alpha7}$ cells compared to expression in wild-type cells; the subunit was detected by cell incubation with the anti-Myc primary antibody followed by the appropriate Alexa Fluor 488 secondary antibody. (C) Immunoblot evaluating the α7-nAChR subunit expression in the above four cellular variants using β-actin as a loading control; the blot is representative of two independent experiments. Specific bands corresponding to α7 protein expression were detected with the anti-α7 primary antibody and the appropriate secondary (HRP)-conjugated antibody.

3.2. Stable overexpression of dupα7 in NSCLC cells prevents in vitro cell migration induced by nicotine or NNK

To get an insight into the functional effect of dupα7 on the oncogenic activity induced by nicotine or NNK in NSCLC cells, we analyzed the effects of both tobacco smoke constituents on migration in non-transfected (wild-type) cells or in cells with stable dupα7 overexpression ($A549^{dup\alpha7}$ or SK-MES-$1^{dup\alpha7}$). The wound-healing assay performed on wild-type cells showed significantly increased migration after 24 h of stimulation with nicotine (1 μM), NNK (100 nM) or 10% FBS (Fig. 2) compared to the corresponding unstimulated cells (Blank). The overexpression of dupα7 in the transfected cells significantly reduced the pro-migratory effect of nicotine or NNK, but not that due to FBS. This last observation ruled out the possibility that dupα7 overexpression alters the migratory capacity of these cells. Moreover, the pro-migratory response to any of the three stimuli tested in the cells overexpressing the empty-vector ($A549^{pcDNA3.1}$ or SK-MES-$1^{pcDNA3.1}$) was indistinguishable from that found in the corresponding wild-type cell variant (Fig. 2B). The transwell migratory assay performed with the same cell variants and treatments described above yielded similar results: the increase in cell migration promoted by nicotine or NNK (24 h) in wild-type cells was abolished in $A549^{dup\alpha7}$ or SK-MES-$1^{dup\alpha7}$ cells (Fig. 3).

3.3. Nicotine and NNK lose their ability to stimulate in vitro cell proliferation in NSCLC cells overexpressing dupα7

Changes in cell proliferation elicited by nicotine or NNK due to dupα7 overexpression were assessed by an EdU assay performed on the above NSCLC cell lines. Fig. 4A shows typical confocal images of non-proliferating cells (blue nuclear staining with DAPI) or EdU positive proliferating cells (magenta color staining) obtained in each of the four

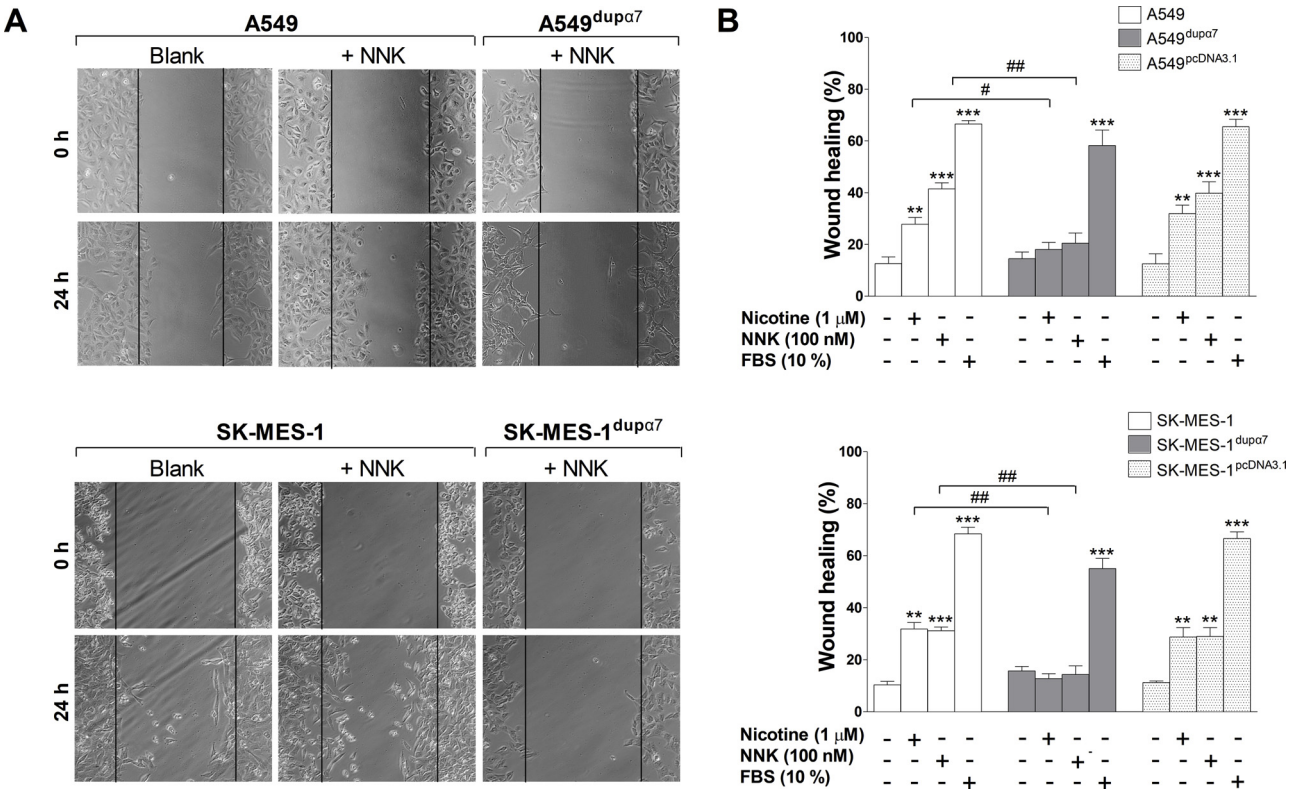

Fig. 2. Overexpression of dupα7 in NSCLC cell lines inhibits *in vitro* cell migration induced by nicotine and NNK. Migration rate in A549 cells (top) or SK-MES-1 cells (bottom) was determined by the wound-healing assay. (A) Representative microscope images (10X) of the wound captured at 0 and 24 h after scratching in wild-type cells (A549 and SK-MES-1) or in dupα7 overexpressing cells (A549^{dupα7} and SK-MES-1^{dupα7}) after 24 h stimulation with NNK (100 nM). Blank: cells not stimulated. (B) Pooled results of the migration rate induced by nicotine, NNK or FBS in wild-type cells or in cells that overexpress dupα7 or the empty vector (A549$^{pcDNA3.1}$ and SK-MES-1$^{pcDNA3.1}$). The migration rate, expressed as percentage of wound healing (scratch closure), was calculated according to the next equation: % of wound healing = [(a-b/a)x100], where (a) is the distance between both edges of the wound at 0 h and (b) at 24 h. Data show mean ± SEM for the cell migration induced by the above three stimuli tested in five independent cell cultures assayed in duplicate. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the corresponding non-stimulated cell variant. $^{\#}$p < 0.05 and $^{\#\#}$p < 0.01 after comparing the indicated groups.

cell variants used here (A549, SK-MES-1, A549^{dupα7} or SK-MES-1^{dupα7}) in the different conditions tested. Fig. 4B represents means ± SEM of the number of proliferating cells, expressed as a percentage of the total number of cells found in the microscope field, in response to each stimulus. A significant increase in proliferation was found in non-transfected cells of both cell types after 24 h of incubation with nicotine (1 μM), NNK (100 nM) or 10% FBS. Once again, the proliferative effect induced by nicotine or NNK was completely lost in A549^{dupα7} or SK-MES-1^{dupα7} cells, whereas that of FBS (10%) persisted in cells overexpressing dupα7.

3.4. Overexpression of dupα7 in NSCLC cells prevents the epithelial-mesenchymal transition (EMT) elicited by nicotine or NNK in vitro

EMT is the trans-differentiation of stationary epithelial cells into motile mesenchymal cells; it facilitates the motility of individual cells and the acquisition of an invasive phenotype. Changes in expression of epithelial (β-catenin) or mesenchymal (vimentin and fibronectin) markers in response to nicotine or NNK caused by overexpression of dupα7 were evaluated by Western blot in A549^{dupα7} or SK-MES-1^{dupα7} cells and in the corresponding wild-type cells, using β-actin as the loading control protein. Fig. 5A shows representative blots of the expression of the above markers induced by 48 h of incubation of each cell variant with nicotine (1 μM) or NNK (100 nM). Fig. 5B shows pooled immunoblot results, expressed as the marker/β-actin ratio for each cell variant, taking the ratio in non-stimulated (control) cells as 1; bars show the mean values ± SEM obtained in different cell cultures. Results reveal reduced expression of β-catenin accompanied by increased expression of vimentin and fibronectin in A549 or SK-MES-1 upon nicotine or NNK incubation compared with their respective non-stimulated cell variants (Control). In contrast, in the case of A549^{dupα7} or SK-MES-1^{dupα7} cells, changes in the expression of the above markers induced by nicotine or NNK were either abolished (β-catenin) or the opposite (decreased expression of vimentin or fibronectin) of those observed in the corresponding wild-type cell variant subjected to the same treatment.

3.5. Overexpression of dupα7 suppressed the nicotine-mediated tumorigenic effect in vivo

Next, we proceeded to evaluate whether the *in vitro* findings were reproduced *in vivo* in a nude mouse xenograft model. In this model, wild-type A549 cells or A549^{dupα7} cells were inoculated into the left flanks of the mice, which would then receive or not receive nicotine (1 μM) in their drinking water. The volumes of the xenograft tumors generated in the four subgroups were measured over the 37 days of the study. Tumor volumes (mean ± SEM) in the two wild-type A549 xenograft subgroups (receiving nicotine or not) reflected a progressive and significantly higher growth rate than that observed in the two subgroups of A549^{dupα7} xenografts subjected to the same conditions (Fig. 6A). In fact, volumes in the two latter subgroups remained quite small and stable throughout the study. Furthermore, the final tumor volumes (mean ± SEM) determined in the four subgroups after the completion of the study revealed that nicotine produced significant differences between the two wild-type subgroups but not between the two subgroups with dupα7 overexpression (Fig. 6C). Fig. 6B shows the

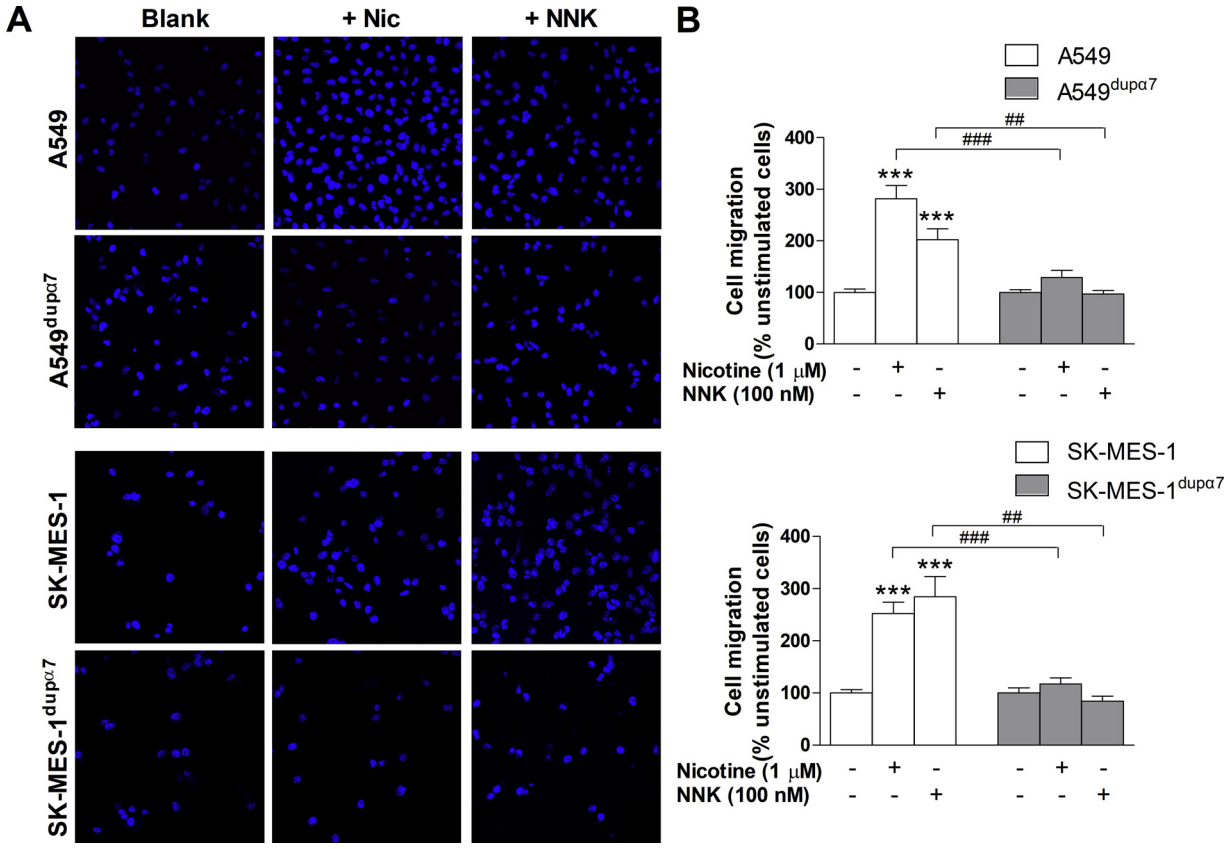

Fig. 3. Overexpression of dupα7 in NSCLC cell lines suppresses the *in vitro* pro-migratory effects of nicotine or NNK. Cell migration in A549 cells (top) or SK-MES-1 cells (bottom) was determined by the transwell migration assay. (A) Representative confocal images (40X) of the migration rate in wild-type cells (A549 or SK-MES-1) or in cells overexpressing dupα7 (A549^{dupα7} or SK-MES-1^{dupα7}) after 24 h stimulation with nicotine (1 μM) or NNK (100 nM). Blank: unstimulated cells. (B) Bar charts representing the pooled results of cell migration, expressed as a percentage of the spontaneous migration by the same unstimulated cell variant (considered 100%), in response to nicotine or NNK. Values show mean ± SEM from five independent cell cultures; 2-3 microscopic fields were randomly selected for migration analysis in each cell culture. ***$p < 0.001$ compared to its corresponding unstimulated cell variant. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ after comparing the indicated groups.

photos corresponding to four representative mice tested for each subgroup as well as their corresponding tumors excised immediately after their sacrifice. The IHC analysis reveals a clear increase in the expression of the cellular markers for proliferation (Ki67) and angiogenesis (VEGF) in the A549 xenograft tumors of mice receiving nicotine compared to those that did not (Fig. 6D). The effect of nicotine on the expression of the two markers was suppressed in the A549^{dupα7} xenografts. Collectively, our latest findings reveal that dupα7 overexpression in lung adenocarcinoma cells suppresses tumor growth and progression *in vivo*, just as it does *in vitro*.

4. Discussion

The present study shows the first experimental evidence that dupα7, a human-specific duplicated form of the α7-nAChR subunit, interferes with the tobacco-activated tumorigenic activity mediated by α7-nAChRs in human NSCLC cell lines (A549 or SK-MES-1). Specifically, we show that dupα7: (1) significantly inhibits *in vitro* cell migration, proliferation and EMT induced by nicotine or NNK in both cells lines; and (2) strongly restrains nicotine-induced tumor growth and increased expression of tumor markers (Ki67 or VEGF) in A549 xenografts transplanted into nude mice.

Tobacco smoking is the main risk factor for the development of many types of tumors, including lung cancer and its two major categories: NSCLC and small cell lung cancer (SCLC). Smoking-related tumor cells express several nAChR subtypes with different pharmacological sensitivities and, depending on tumor type, specific pathophysiological functions. Thus, homomeric α9-nAChRs play an important role in the progression of human breast cancer [27], whereas α7-nAChR is the main subtype responsible for the nicotine-mediated oncogenic effects in human NSCLC [10–13]. Stimulating α7-nAChRs in smoking-related tumor cells leads to downstream activation of multiple signaling cascades promoting cancer cell survival, proliferation, migration, angiogenesis and metastasis in a tumor-specific manner [4–7].

We reported previously that the dupα7 subunit interferes with various α7-nAChR-controlled functions in cell types as different as *Xenopus* oocytes or murine macrophages [18,20]. Here, we identify a new function for dupα7 in human NSCLC cells: it negatively regulates α7-nAChR-mediated oncogenic activity induced by nicotine or NNK, two tobacco smoke constituents. Furthermore, the inhibitory effect of dupα7 on α7-nAChR function is not due to a reduction of α7 expression in the cells since both α7 mRNA or protein levels in wild-type cells do not change in cells with stable dupα7 overexpression (Fig. 1).

As has been previously reported in lung, breast or gastric tumor cells [5,13,28,29], our data show that the above constituents of tobacco smoke increase *in vitro* migration of non-transfected A549 or SK-MES-1 cells, an effect that was abolished in these cells upon dupα7 overexpression (Figs. 2 and 3). Furthermore, dupα7 expression is only effective against stimuli (nicotine or NNK) that activate cell migration through α7-nAChRs but not against other types of stimuli unrelated to these receptors, such as FBS. It is noteworthy that the dupα7 effect on α7-nAChR-mediated migration is not reproduced in cells overexpressing the empty-vector (A549$^{pcDNA3.1}$ and SK-MES-1$^{pcDNA3.1}$) (Fig. 2), indicating that dupα7, and not the vector, interferes with α7-

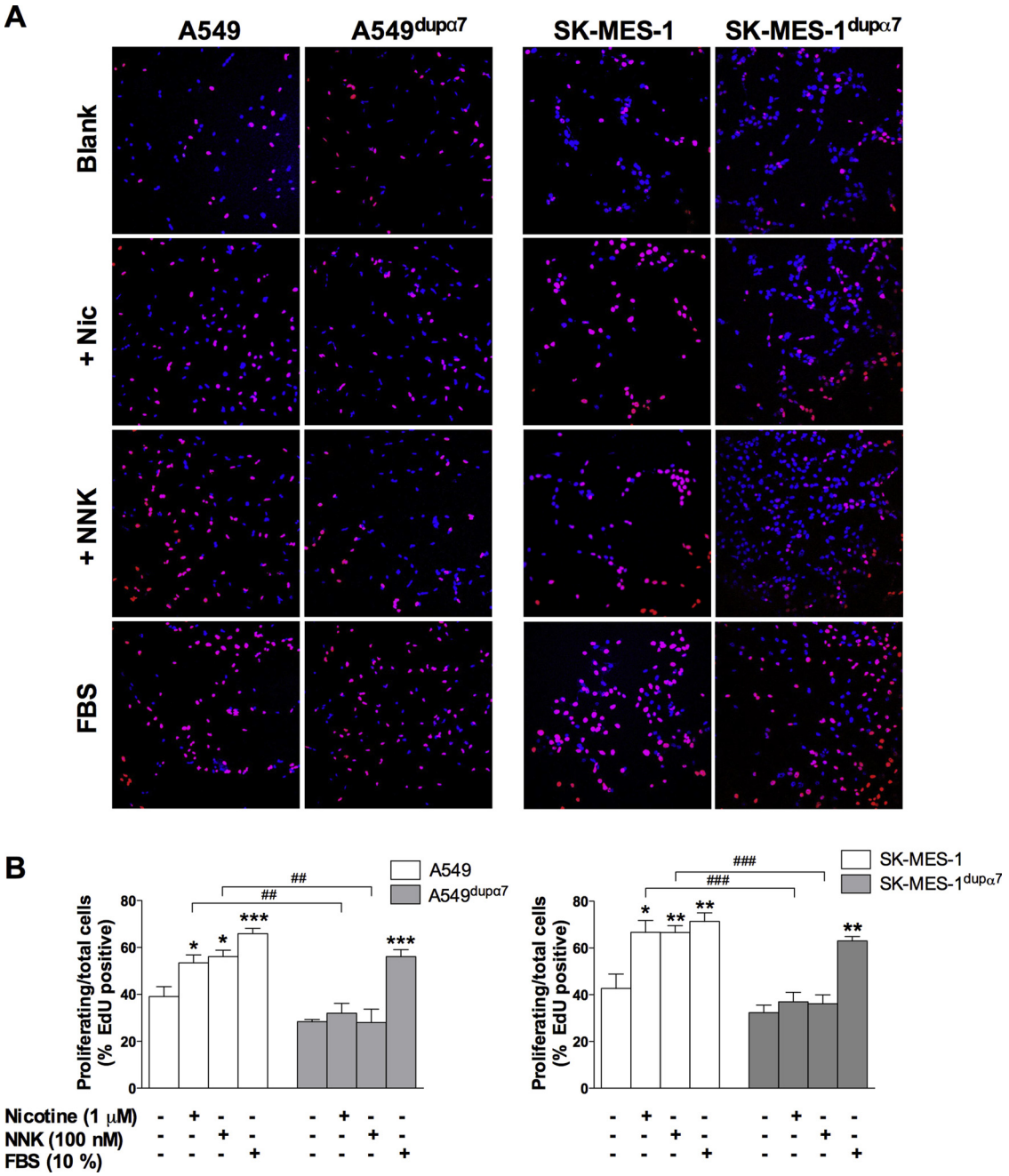

Fig. 4. The nicotine- or NNK-induced *in vitro* proliferative effects in NSCLC cells were abolished in cells overexpressing dupα7. The EdU assay combined with cell nucleus staining with DAPI makes it possible to count the number of proliferating (magenta stained) and non-proliferating (blue stained) cells in the microscopic field. (A) Representative confocal images (20X) of the proliferative effects of nicotine (1 μM), NNK (100 nM) or FBS (10%) in wild-type cells (A549 or SK-MES-1) or in cells overexpressing dupα7 (A549$^{dup\alpha7}$ or SK-MES-1$^{dup\alpha7}$). Blank: non-stimulated cells of each cell variant. (B) Bar charts showing pooled results of proliferating cells with respect to the total number of cells in the microscope field, expressed as the percentage of Edu positive cells, for each cell variant and indicated treatment. Values represent mean ± SEM of five independent cell cultures; 3–4 microscopic fields were randomly selected for proliferation analysis in each cell culture. *$p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$ compared to the corresponding non-stimulated cell variant; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.01$ after compared the indicated bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

nAChR function.

Our study also shows that nicotine and NNK induce *in vitro* proliferation of both wild-type NSCLC cell lines (Fig. 4), an effect that has been previously reported in the same cells and in other cancer cell lines from stomach, pancreas and breast [28,30,31]. The nicotine or NNK effects promoting cell proliferation were abolished in A549$^{dup\alpha7}$ and SK-MES-1$^{dup\alpha7}$ cells in our study (Fig. 4). Once again, dupα7 expression did not affect the proliferative effect of FBS. The last part of our *in vitro* study reveals that nicotine and NNK reduce the expression of β-catenin (an epithelial marker) and increase expression of vimentin and fibronectin (two mesenchymal markers) in non-transfected A549 and SK-MES-1 cells; the effects induced by the two stimuli were abolished in cells overexpressing dupα7 (Fig. 5). Changes in the expression of epithelial/mesenchymal markers induced by nicotine or NNK in wild-type cells like those found in our study are a hallmark of EMT, a process that is associated with invasiveness, metastasis and poor prognosis. Thus, our results agree with previous observations in patients with lung cancer showing that tobacco use favors EMT [32]. Interestingly, our

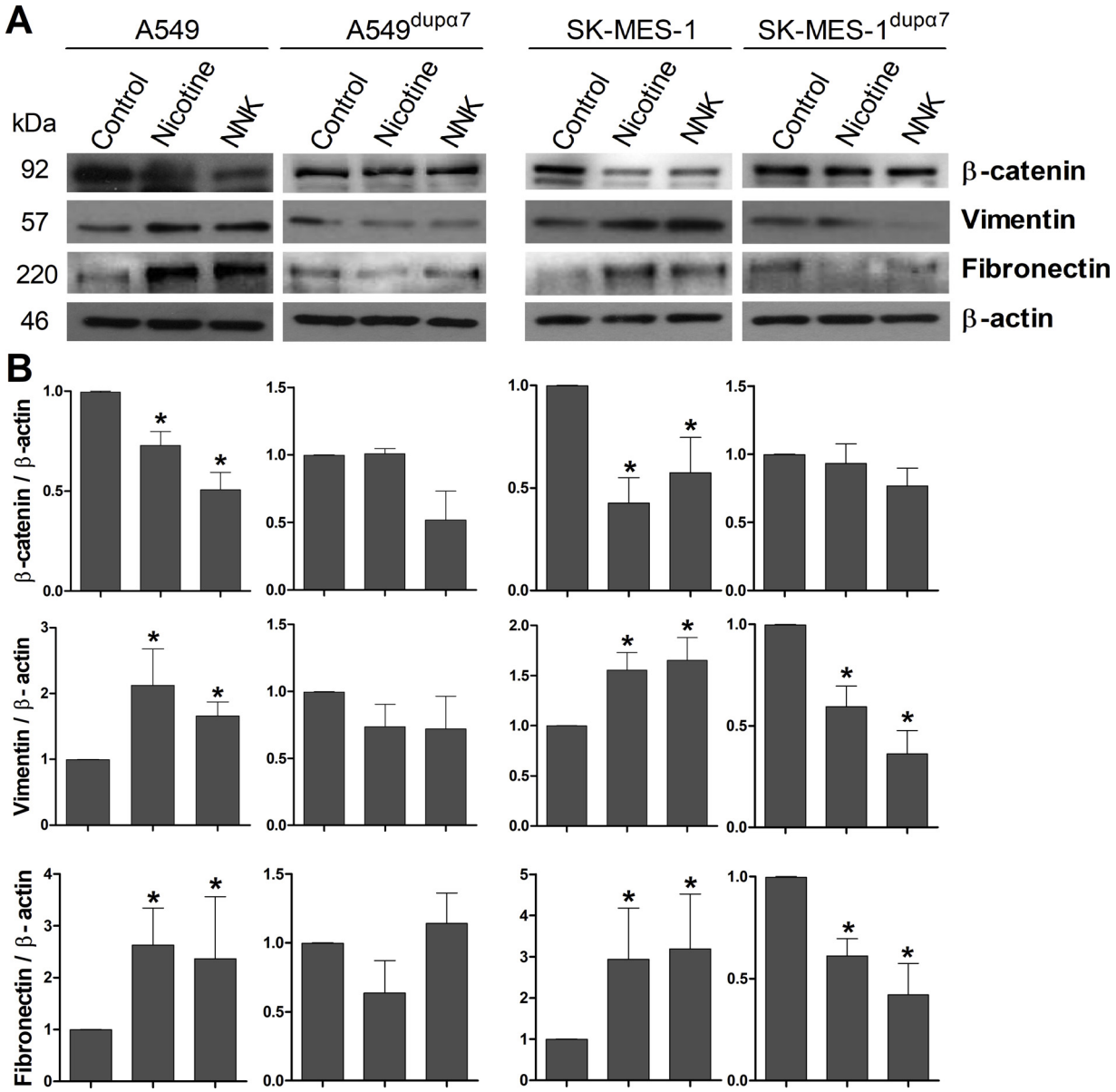

Fig. 5. Overexpression of dupα7 in NSCLC cells abolishes the *in vitro* EMT induced by nicotine or NNK. (A) Representative immunoblots of the expression changes in epithelial (β-catenin) and mesenchymal (vimentin and fibronectin) markers in response to nicotine or NNK caused by overexpression of dupα7 in the A549 or SK-MES-1 cells. Cell cultures were incubated with nicotine (1μM) or NNK (100 nM) for 48 h. Control: unstimulated cells from each variant. β-actin was included as a loading control. (B) Pooled immunoblot results showing the marker/β-actin expression ratio for each cell variant and experimental condition, taking the ratio in control cells as 1. The bar graphs show the mean values ± SEM obtained in three different cell cultures for each cell variant. *$p < 0.05$ vs. untreated (control) cells of the same cell variant.

finding concerning nitrosamine as an EMT inductor represents the first demonstration of such an effect in NSCLC cells, although it has been previously reported in gastric cancer cells [29].

Previous studies have shown that blockade of α7-nAChR with specific antagonists or siRNA prevents the stimulatory effects of nicotine and NNK on proliferation, migration, EMT or angiogenesis in several cancer cell lines [13,29,31,33]. Nicotine and NNK are α7-nAChR agonists, and the affinity of nitrosamine for the receptor is 1,300 times greater than that of nicotine [34]. Since we did not find significant differences in the *in vitro* tumorigenic effects between nicotine and NNK, we can conclude that both agents produce all the above oncogenic effects mainly through α7-nAChRs expressed in A549 or SK-MES-1 cells.

The present *in vitro* findings are corroborated in our *in vivo* study, where we found that nicotine promotes tumor growth of A549 xenografts transplanted into nude mice (Figs. 6A-6C). This nicotine effect on tumor growth agrees with previous data obtained in xenografts corresponding to different tumor cell lines, including lung, breast, bladder or hepatic carcinoma cell lines [35–38]. Moreover, the histological analysis of A549 xenografts in our *in vivo* study reveals the increased expression of the angiogenesis (VEGF) and proliferative (Ki67) markers in response to nicotine (Fig. 6D), a finding already reported in xenografts derived from adenocarcinoma or SCLC cell lines [39–41]. Here, we report, for the first time, that dupα7 overexpression in A549 xenografts completely suppresses tumor growth and the increased expression of tumor progression markers *in vivo*. Although we still do not know the mechanism by which dupα7 interferes with the α7-nAChR function in NSCLC cells, we have already reported that duplicated subunits assembled with ancestral subunits to form heteromeric dupα7/α7-nAChRs in the neuroendocrine GH4C1 cell line [20].

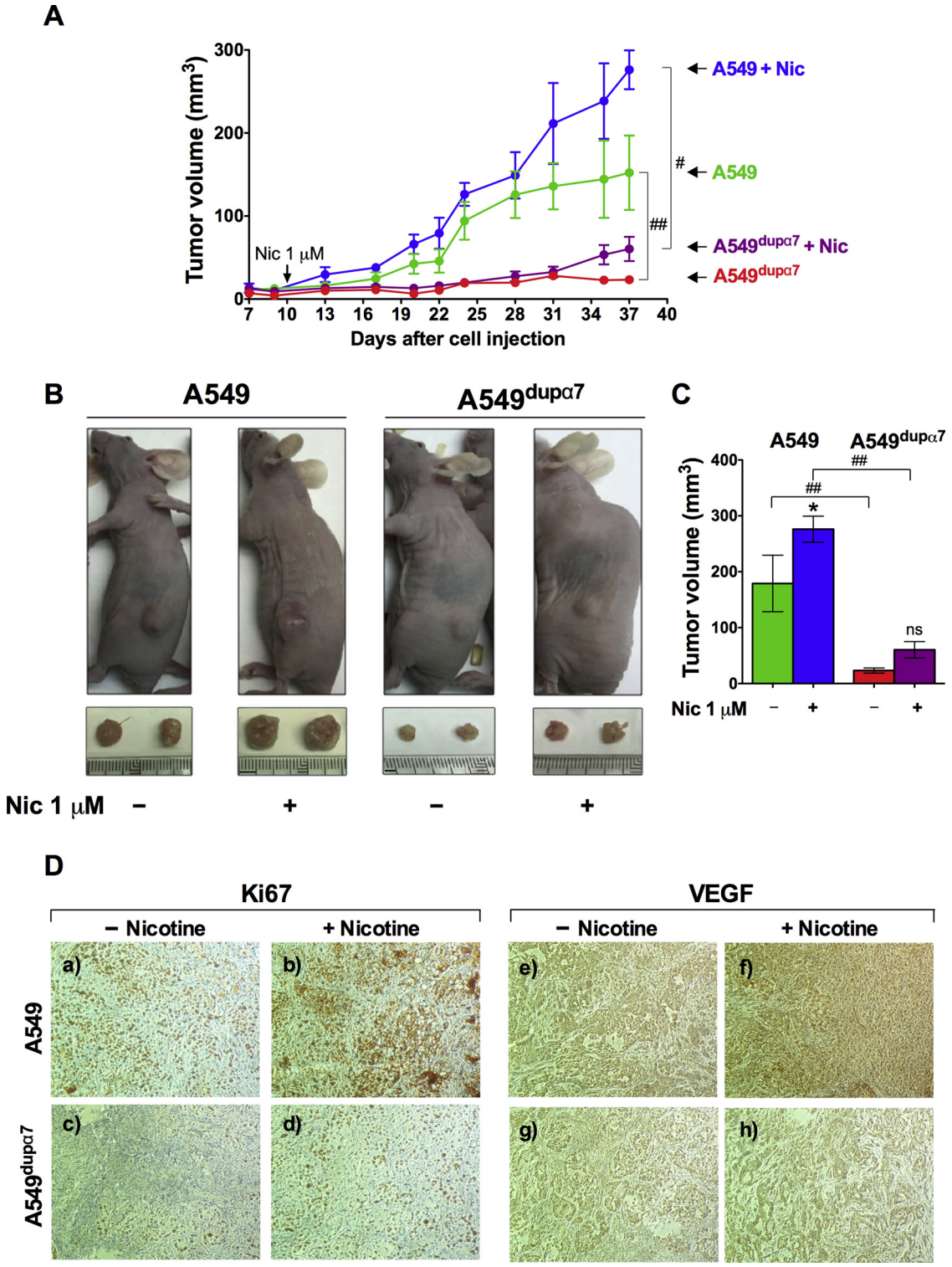

Fig. 6. Overexpression of dupα7 in a nude mouse A549 xenograft model suppressed the nicotine-mediated tumorigenic effect *in vivo*. Wild-type cells (A549) or dupα7 overexpressing cells ($A549^{dup\alpha7}$) were innoculated into the left flanks of the mice, which would then receive nicotine or not in the drinking water. There were four mouse subgroups (5–6 mice/subgroup) in the study. (A) Tumor volume growth (mean ± SEM) in the four subgroups over the 37 days of the study; tumor volume was expressed in mm^3. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ after multiple comparative analysis of the indicated mice subgroups. (B) Photos of four representative mice from each subgroup as well as their corresponding tumors excised immediately after the sacrifice of the animal. (C) Final tumor volumes (mean ± SEM) determined in the four subgroups after the completion of the study. $^{*}p < 0.05$, significantly different from the wild-type A549 xenograft subgroup that did not receive nicotine; ns, no significant differences between the two $A549^{dup\alpha7}$ xenograft subgroups (receiving nicotine or not). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, significant differences between the indicated subgroups. (D) Microscopic images of expression levels for proliferation (Ki67) and angiogenesis (VEGF) markers, staining (brown) by IHC, in a representative xenograft tumor from each subgroup.

As expected from the structural characteristics of dupα7, these heteromeric receptors exhibit worse migration from endoplasmic reticulum to the cell membrane, as well as less sensitivity to agonists than homomeric α7-nAChRs and thus, reduced functionality [18,20].

It should be noted that SCLC cells express high levels of α7-nAChRs, which have been identified as central regulators of nicotine- or NNK-induced proliferation and migration by these cells through stimulation of the release of autocrine growth factors serotonin, mammalian bombesin and, possibly, other neuropeptides [see Ref. [42] and references therein]. Given that the strength of association with smoking is stronger for SCLC than for lung adenocarcinoma [43], one of the two major histological types of NSCLC, it is feasible that the tumor suppressor effect of dupα7 on α7-nAChR activity that we have just revealed in A549 cells may also be applicable to SCLC tumors. Additional experiments with SCLC cell lines will be necessary to contrast the above proposal.

In conclusion, our study shows, both *in vitro* and *in vivo*, that overexpression of dupα7 in human NSCLC cell lines inhibits the oncogenic function of the tobacco-activated ancestral subunit that makes up α7-nAChR. Our finding is noteworthy, since to date few functions have been attributed to human-specific duplicate genes, even though these genes have been recognized as a primary source of evolutionary innovation [44]. Eichler's group was one of the first to report a compendium of human-specific gene duplications identified [45]. Their study, which compiles 88 complete gene duplications that have occurred since the divergence of humans and chimpanzees, includes the *CHRFAM7A* gene. However, unlike other genes in the above study that provide genetic redundancy with respect to the parent gene, our data indicate that the *CHRFAM7A* gene acquires a function that differs from that of the ancestral gene (*CHRNA7*) and that negatively regulates its oncogenic activity. Interestingly, the new function of the *CHRFAM7A* gene, revealed here, is quite like the function recently attributed to another human-specific gene (*SRGAP2C*), the result of a partial duplication of the ancestral gene (*SRGAP2*) expressed in brain [46]. According to their study, the protein resulting from the duplicated gene would form a heterodimer with that of the ancestral gene, thereby sequestrating it and, thus, inhibiting its function. Inhibiting *SRGAP2* function would have resulted in a greater density of dendritic spines and the consequent evolutionary advantage for the development of the neocortex in humans. Whether dupα7 naturally expressed in human primary tumors from NSCLC patients has a pathophysiological role does require further studies; in fact, we have recently reported that dupα7 gene expression is down-regulated whereas that of α7 is up-regulated in the above tumors compared to their paired healthy lung specimens [9]. Thus, there is a possibility that the deficient expression of the endogenous duplicated subunit along with the overexpression of the ancestral subunit in the tumor would facilitate the oncogenic process promoted by tobacco smoking in NSCLC patients, suggesting that up-regulation of dupα7 expression in these tumors could offer a potential new therapeutic target in these patients.

Conflicts of interest statement

The authors declare that they have no conflicts of interests.

Acknowledgements

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