RESEARCH

Effect of human epidermal growth factor on the tumor cell line A431: in vivo *analysis of tumor growth inhibition and gene expression*

✍ Isabel A Guillén[1], Hanlet Camacho[1], María E Fernández[1], Daniel O Palenzuela[1], Lincidio Pérez[2], María E Ochagavia[3], Julio A Ancizar[4], Ángela D Tuero[5], Laritza Gorovaya[4], Osmani Mendoza[4], Tamara Díaz[1], Julio R Fernández[1], Juan Roca[1], Karelia Cosme[4], Gerardo E Guillén-Nieto[6], Luis Herrera[7], Jorge Berlanga[8], Lidia I Novoa[1]

[1]Departamentos de Farmacogenómica, [2] Cáncer y [3] Bioinformática
Dirección de Investigaciones Biomédicas;
[4] Bioterio;
[5] Departamento de Estadísticas, Dirección de Ensayos Clínicos;
[6] Departamento de Vacunas, Dirección de Investigaciones Biomédicas;
[7] Dirección General;
[8] Departamento de Cicatrización y Citoprotección, Dirección de Investigaciones Biomédicas
Centro de Ingeniería Genética y Biotecnología, CIGB
Ave. 31 e/ 158 y 190, Playa, CP 10 600, La Habana, Cuba
E-mail: isabel.guillen@cigb.edu.cu

ABSTRACT

The historical tag of the epidermal growth factor (EGF) as carcinogenesis promoter has not been uniformly reproduced, since, in some experiments, malignant cells are bound to growth inhibition and apoptosis. The present study intends to obtain additional data on the interaction of EGF with cancer cells by analyzing its effect *in vitro* on the growth of cultured A431 cells, and *in vivo* on nude mice xenotransplanted with this cell line; identifying, in addition, the gene expression patterns for a selected group of genes related to EGF and cancer pathways in the solid tumor. EGF-treated animals showed significantly smaller tumor volumes than controls, suggesting cellular growth inhibition mediated by this factor. Similar results were obtained regarding the proliferation of cultured A431 when treated with 2.2, 33 and 165 nM of EGF. These results may imply a common mechanism for the EGF-mediated growth inhibition of A431 cells in both biological conditions (*in vivo* and *in vitro*). The action of EGF at nanomolar concentrations may trigger a transient recovery of the tumor suppressor ability of tumor cells through the reduction of the biological action of mutated TP53, cell cycle arrest due to a decrease in Cdk4 expression levels and the initiation of caspase through the activation of CASP9.

Keywords: epidermal growth factor, cancer, A431 cells, nude mice

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RESUMEN

Efecto del factor de crecimiento epidérmico humano en la línea celular tumoral A431: análisis de la inhibición del crecimiento tumoral y la expresión génica *in vivo*. El carácter de promotor de la carcinogénesis atribuido al factor de crecimiento epidérmico (FCE) no se ha reproducido uniformemente, pues las células malignas tratadas con esta molécula en algunos experimentos han mostrado inhibición del crecimiento y apoptosis. Se ofrecen datos adicionales de la interacción del FCE con células cancerosas, mediante el análisis de su efecto en el crecimiento de la línea celular A431 *in vitro* y en ratones atímicos xenotrasplantados con esta línea. Se estudiaron además los patrones de expresión de un grupo de genes relacionados con el FCE y el cáncer en los tumores sólidos de A431. Este estudio reveló que los animales tratados con FCE humano recombinante (FCEhr) desarrollaron volúmenes tumorales inferiores que los animales controles. Ello sugiere una inhibición del crecimiento celular mediada por el FCE. Resultados similares se obtuvieron *in vitro* al tratar las células A431 con FCEhr a 2.2, 33 y 165 nM. A estas concentraciones, el FCE recobra la propiedad supresora tumoral en las células cancerosas, posiblemente por la reducción de la acción biológica de la TP53 mutada, la inhibición del ciclo celular al disminuir la expresión del gen *cdk4*, y la iniciación de la vía de las caspasas mediante la activación de la expresión del gen CASP9. Tales hallazgos sugieren mecanismos comunes de inhibición del crecimiento de las células A431 *in vitro* e *in vivo*, mediados por el FCE.

Palabras clave: factor de crecimiento epidérmico, cáncer, células A431, ratones atímicos

*I*ntroduction

The epidermal growth factor (EGF) is a 53-amino acids polypeptide originally isolated from mouse salivary glands. EGF discovery was presided by its ability to stimulate epithelial growth and differentiation upon its injection to newborn mice. In extraembryonic life, the homeostatic preservation of epithelial cell populations by controlling cell growth, proliferation, and differentiation is one of its most remarkable physiological roles [1, 2].

It is likely that Stanley Cohen was the first to exogenously administer EGF in pharmacological concentrations to an animal model, thus evoking an expected

✍ Corresponding author

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clinical response while healing corneal burns in rabbits. This opened up the first pharmacological attempt to improve the healing process using the exogenous administration of a growth factor [3].

According to years of research in different biological systems, the exogenous administration of EGF at supra-physiological concentrations triggers two major pharmacological actions: cyto-protective and trophic-reparative [4]; which are critical for the survival and repair process of internal and external organs. However, the fact that some growth factors were identified in the conditioned medium of different cancer cell lines and the existing homology between some growth factors and/or their receptors and viral oncogenes engendered an early concern within the medical community. Accordingly, the events whereby EGF and other growth factors stimulate tissue repair are similar to those involved in tumorigenesis: cell proliferation, migration, survival, and *de novo* angiogenesis [5].

In contrast to other ligand family members, EGF itself is not an oncogene-derived product but its specific receptor is encoded by an oncogene (EGFR, ERBB1) endowed with tyrosine kinase activity. Several EGFR mutations correlate with tumor aggressiveness and patient's poor prognosis. Nowadays, EGFR constitutes a therapeutic target of central importance for most epithelial cancers [6, 7].

In contrast to the tumorigenic role of a deregulated EGFR, the co-carcinogenic effect of EGF appears controversial so far [7-9]. The EGF mediated-carcinogenic promotion has not proved reproducibility *in vivo* through different experimental settings and some data reveal an EGF-induced inhibition of several cancer cell lines of epithelial origin [10-13]. Parallely to the above basic findings, EGF emerged and progressed in the clinical arena. The yeast-made recombinant human molecule stands as a major therapeutic tool within the clinical possibilities of hard-to-heal lower extremity wounds in diabetics [14-16]. Within this realm, its intralesional administration has been demonstrated to trigger and sustain the healing process of poor-prognosis diabetic ulcers, which can reduce the rate of lower extremity amputations [14-16].

The present study is, therefore, aimed at providing further data on the impact of recombinant human EGF (rhEGF) in a broadly used human carcinoma line, examining the A431 cells proliferative response *in vitro* as the changes in tumor size *in vivo*. In addition, we studied the gene expression patterns of a selected group of genes related to EGF and cancer pathways in the solid xenografted tumors.

Materials and methods

Cell culture

The A431 human epithelial carcinoma cell line (donated by Dr. Belinda Sánchez from the Center of Molecular Immunology, Cuba) is a model cancer cell line over-expressing EGFR, often used in biomedical research to explore cancer pathways [10]. Cells were cultured in RPMI 1640 (Gibco-BRL®, Gaithersburg, MD, USA), supplemented with 10% fetal calf serum (PAA Laboratories, Germany), 1% L-glutamine, 50 µg/mL gentamycin (Sigma-Aldrich Corp, St Louis, MO, USA) at 37 °C in a 5% CO_2 atmosphere with 95% relative humidity. The cells were expanded up to 20×10^6 cells for flow cytometry experiments and for the inoculation of nude mice.

Flow cytometry in A431 cells

The expression of EGF receptor (EGFR) in cells was quantified by flow cytometry. The cells were removed from the culture flasks using a trypsin-EDTA solution and centrifuged at 1000 x *g* for 5 min; the pellet was washed then with phosphate-buffered saline (PBS) and suspended at a final concentration of 3×10^6 cells/mL in PBS containing 3% Bovine serum albumin and 10 µg/mL of the humanized anti-EGFR monoclonal antibody hR3 (Center of Molecular Immunology, Cuba) [17]. Samples without the antibody were included as controls. The mixture was incubated at 22 °C for 1 h with occasional gentle agitation, after which the cells were further washed three times with PBS by centrifugation. Then, they were incubated for 30 min in the dark with goat anti-human IgG antibodies labeled with fluorescein isothiocyanate (Sigma-Aldrich Corp, St Louis, MO, USA) diluted 1:60. Following three washes with PBS by centrifugation, the samples were resuspended in 1.5 mL of PBS and analyzed in a PAS III fluorescence-activated cell sorter (FACS) machine and using the program Flomax version 2.60 (Partec; Munster, Germany).

Effects of rhEGF on the growth of A431 cells *in vitro*

The rhEGF was obtained from a transformed *Saccharomyces cerevisiae* strain at the Center for Genetic Engineering and Biotechnology (CIGB), in Havana, Cuba [18]. This rhEGF preparation, composed of a mixture of the 52 and 51 aminoacid species, has been subjected to diverse preclinical studies [19] and is the active pharmaceutical ingredient of registered healing medications for topical use in acute lesions (Hebermin®) and for intralesional infiltration in chronic diabetic foot ulcers (Heberprot-P®). The A431 cells were seeded in 24-well plates at 25 000 cells/well using RPMI 1640 (Gibco-BRL®, Gaithersburg, MD, USA), supplemented with 10% Fetal Calf Serum (PAA), 1% L-glutamine and 50 µg/mL gentamycin (Sigma-Aldrich Corp, St Louis, MO, USA) and cultured at 37 °C in a 5% CO_2 atmosphere for 24 hours. After this time, the cells were washed with PBS and incubated with the same culture medium supplemented with different rhEGF concentrations (2.2, 33 and 165 nM) or EGF-free preparation (control composition: sucrose, dextran 40, disodium phosphate, sodium dihydrogen phosphate dehydrated, and water for injection). The cells were then cultured at 37 °C in a 5% CO_2 atmosphere for 72 h, after which they were removed from the wells using a trypsin-EDTA solution and counted using a hematocytometer.

Xenografts of human tumor cells into nude mice and evaluation of rhEGF administration effect

Twelve NIH nude mice (CIGB) were subcutaneously inoculated on the right dorsal region with 300 µL of RPMI 1640 medium containing 3×10^6 of A431 cells per animal. Once the tumor became visible and palpable (approximately 1-cm diameter) the mice were

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randomized (six animals for the rhEGF treatment group, the remaining six for control treatment). Both groups were injected near the tumor twice a week (approximately dose per kg and frequency to that used in rhEGF treatment of diabetic foot ulcers), using either 1 µg rhEGF or the equivalent volume of control solution per kg of bodyweight (in this case 20 ng of rhEGF (2.2 nM) or 100 µL of EGF-free preparation per 20 g of mice bodyweight), during 17 days. The results presented reflect the tumor growth and survival events from three experiments ex-temporarily and independently made.

The nude mice experiments were conducted under the observation of ethical procedures and the experimental protocol was reviewed and approved by the animal welfare committee of the CIGB

Tumor size (length and width) was measured every other day, and its diameter and ratio was calculated. The volume (V) of subcutaneous tumor was estimated using the following formula $V = (4/3)\ \pi r^3$ [20]. Once the administration scheme concluded, the animals were terminated and autopsied. Tumor samples were harvested for both: histopathology studies and to compare the gene expression profiles from the rhEGF-treated and control groups.

Histopathology

The specimens from the tumors (4 samples from treated animals and 3 samples from controls) were processed for histopathological study following routine techniques. Briefly, the samples were fixed in 10% buffered formalin, paraffin embedded, 5-µm sectioned and hematoxylin and eosin stained. Tumors were evaluated based on the following parameters: total cells in mitosis (TCM), tumor total cells (TTC), mitotic index (MI), total cells in apoptosis (TCA), apoptotic index (AI), mitosis-apoptosis ratio (MAR), total vessels neoformation (TVN) and neoformation vessels index (NVI). Ten randomly-selected fields with 100-cells each were used for quantifying all these parameters using 100x magnifications [21]. For the evaluation of these parameters the software Madip, version 4.0, and the following equations, as described Rodríguez *et al*. [22] were employed:

$$MI = \frac{TCM \times 100}{TTC} \qquad AI = \frac{TCA \times 100}{TTC}$$

$$MAR = \frac{MI}{AI} \qquad NVI = \frac{TVN \times 100}{TTC}$$

Isolation of RNA

Tumor biopsies were collected into RNAlater® (Ambion, AB applied Biosystem, USA) and stored at -20 °C for up to 2 weeks. The samples were then processed on a TissueLyser (Qiagen, Hilden, Germany); extracting total RNA with the TriReagent method (Ambion, AB applied Biosystem, USA). RNA purity and yield were determined using a NanoDrop Spectrophotometer (NanoDrop Technologies, USA), and RNA integrity number (RIN) was obtained on a Bioanalyzer analysis (Agilent, 2100, USA).

cDNA synthesis

Total RNA was treated with RQ1 RNase-Free DNase (Promega corporation, Fitchburg, Wisconsin, USA) in order to remove any DNA contaminants from the preparation. First strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using SuperScript™ II reverse transcriptase (Invitrogen technologies, Carlsbad, California, USA) and oligo-dT primers following fabricant´s instructions.

Selection of the gene set for Real-time-quantitative-PCR analysis

Gene expression of 49 genes selected from the EGF signaling cascade and cancer pathway (supplementary table for gene names, symbols and NCBI reference sequence transcript accession numbers) were analyzed, following the recommended signaling pathway in SABiosciences (Qiagen Company, Hilden, Germany). In order to characterize the selected set of genes on a genomic scale, software for generating molecular interaction networks and gene enrichment tools were used. The protein interaction network, composed of the direct interactions between the genes of the selected set, was generated with Cytoscape [23] using the BisoGenet plugin [24], using the whole set of databases available at the BisoGenet as primary source of protein interactions. This network was augmented by adding edges between genes coding for transcription factors and their target genes. This was done by compiling transcription regulation data from TRANSFAC® [25] (version 7.0, 2005) using its web interface [26], employing then this information to generate a Cytoscape .sif file. This file was imported into Cytoscape and merged with the protein interaction network created by using BisoGenet. Significant biological processes of Gene Ontology (GO) [27], were investigated with Cytoscape's plugin BiNGO [28], and significant pathways were identified with DAVID [29], KEGG [30] and BioCarta [31] databases as primary data sources on pathways. In both cases, a p-value of 0.05 was chosen as threshold for statistical significance.

Real-time-quantitative-PCR

A primer3 web application [32] was used to design Real-time-quantitative-PCR (qPCR) primers with a length of 22 bases and an average GC content of 50%. GAPDH, HMBS and YWHAZ were selected and tested as reference genes, due to the stability of their transcription levels across the sample set. We employed real-time PCR to analyze gene expression in the solid tumors of nude mice xenografted with A431 cells. A total of 4 animals from the rhEGF-treated group and three from the control were included, using two technical replicates per gene. The reactions were performed in a volume of 20 µL, including 10 µL of PCR Absolute™ QPCR SYBR Green Mix (Thermo Scientific), 6 µL of primers (70 nM) and 4 µL of cDNA diluted by a factor of 20. The reactions were performed in an optical detection rotor for 36 tubes at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. qPCR data analysis was performed with Capital Bio RT-Cycler series analysis software (version 2.001, CapitalBio Co, Ltd, Beijing, China). Relative quantification to untreated control and normalization with the reference genes were performed with REST 2009 version 2.0.13 (Qiagen GmbH) [33]. The

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LingRegPCR program (version 11.3, 2009; Amsterdam, the Netherlands) was used to estimate PCR efficiencies [34] and geNorm was used for the selection of the most stable reference genes from a set of tested genes in a given cDNA sample panel [35].

Statistical analysis

To study if significant differences existed in the tumor volume, A431 cell proliferation, and histophatological analysis between rhEGF-treated group and control, a normality analysis (Shapiro Wilk's test) and homogeneity of variance test (Levene's test) were carried out. Groups were compared using the Student's t-test (parametrical) or Mann-Whitney's U test (non-parametrical) applied to independent samples in the *in vivo* study, and Student's *t* test applied to independent samples in the *in vitro* study. In order to corroborate the results, due to small size of samples in each group, mean comparison by estimating confidence intervals or credibility to 95% and 90% by Bayesian Inference methods applied to independent samples were also done. The significance level chosen was 0.05. Statistical analyses were done using SPSS for Windows version 15.0, and EPIDAT for Windows version 3.1, Bayesian Module [36].

Results

Flow cytometry

In the FACS experiments, A431 human tumor cells were incubated with the hR3 monoclonal antibody against EGFR. Mab hR3 bound to 99.3% of the cells after subtracting the signal of the negative control, thus showing the high density of EGF receptors expressed by this cell line (Supplementary figure 1). This observation confirms previous reports that the A431 cell line expresses about 2.6×10^6 molecules of EGFR per cell [10].

Effect of different concentrations of rhEGF on the proliferation of A431 cells

In order to examine the effect of rhEGF on the inhibition of A431 proliferation, cells were exposed to different EGF concentrations, being 2.2 nM, the dose used in the treatment of diabetic foot ulcers. Cells were exposed to a range of doses of 2.2, 33 and 165 nM of rhEGF during 72 h; also including rhEGF-free preparation (control) and untreated cells controls. All the assayed rhEGF concentrations inhibited A431 cells proliferation. Graphical representation of cell proliferation and the results of statistical analysis are shown in figure 1. Growth of EGF-free preparation treated cells did not differ from untreated ones.

Tumor growth in mice

Changes in the subcutaneous solid tumor mass volumes were observed between rhEGF and control treated mice. On day 15, the control-treated group had an average tumor volume of 292 ± 27 mm^3, compared to 121 ± 10 mm^3 for the rhEGF-treated group. Tumor growth curves revealed the existence of large significant differences between the groups treated with control and rhEGF (Figure 2). Similar results of tumor growth between animals treated with rhEGF (1 µg/kg of animal body weight) and the control group were observed in two replication of this study using a larger number of animals and time-course treatment (Supplementary figure 2).

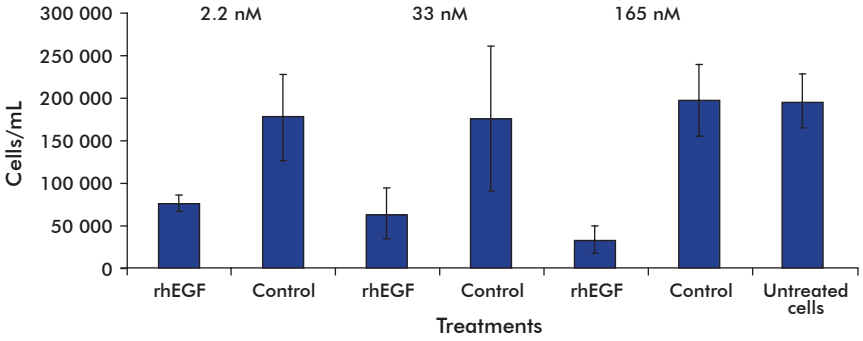

Figure 1. Effect of different concentrations of rhEGF on the proliferation of A431 cells. The data represent the number of cells (mean ± standard deviation) for each concentration of rhEGF compared pairedwise with the data obtained for the control. The cells counts were performed on day 3 (three replicas/treatment). In the one-tailed Student's t-test (parametrical) applied to independent samples, the p values for paired samples were: rhEGF 2.2 nM vs. control (p = 0.06); rhEGF 33 nM vs. control (p = 0.20) and rhEGF 165 nM vs. control (p = 0.013).

Epidermal growth factor prolongs survival time of tumor-bearing mice

It was observed that rhEGF prolonged the survival time of mice bearing A431 tumor xenografts vs. controls. Figure 3 and table 1 show the result of log-rank (Mantel-Cox) analysis in a study made using 3×10^6 cells xenotrasplanted/animal and treated with 20 ng of rhEGF or control. As shown in table 1, rhEGF-treated animals lived 5.87 weeks, but control animals lived 4.9 weeks, which was significantly different (p = 0.003). In this animal model, the 2.2 nM concentration of rhEGF prolonged the survival of animals one week with respect to the controls.

Histopathological study

The histopathological examination of the primary tumors, together with the calculation of the corre-

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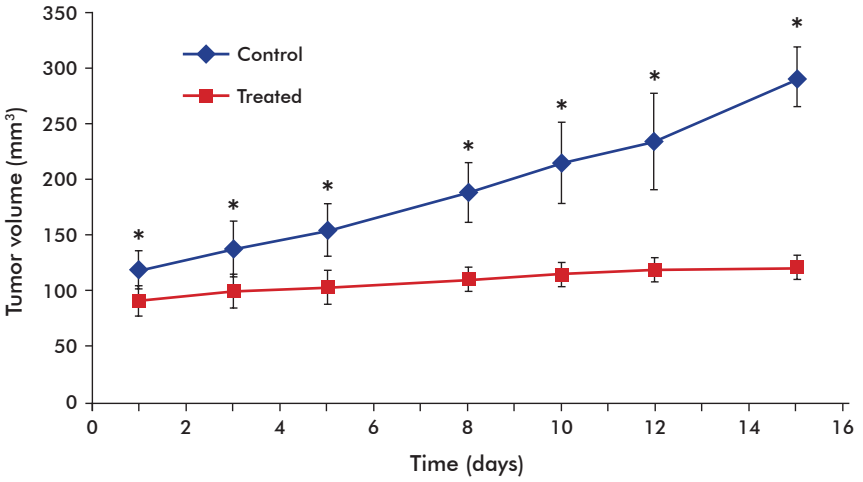

Figure 2. Tumor growth in the animals treated twice a week with 1 µg/kg of bodyweight of rhEGF or control over 15 days. Tumor volume (mean ± SD) at the end of the administration scheme was smaller in the animals treated with rhEGF compared to the controls (121 ± 10 vs. 292 ± 27, respectively). Statistical analysis was performed with the Bayesian Module of EPIDAT 3.1. Means were compared by estimating 95% confidence intervals by Bayesian inference methods. The significance level chosen was 0.05, with statistically significant differences in tumor volume indicated between the rhEGF-treated group and the control (*).

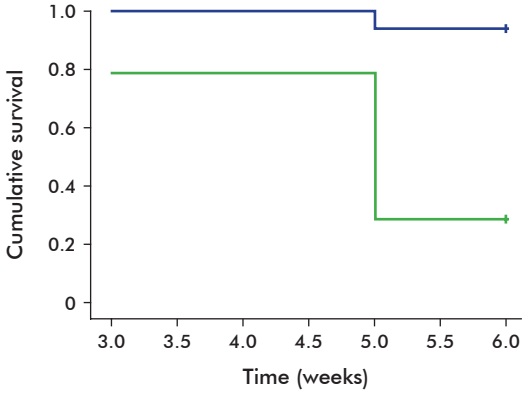

Figure 3. Survival curve of nude xenotransplanted mice with 3 x 10^6 cells/mL was evaluated using log-rank (Mantel-Cox) method using SPSS for Windows. Sixteen animals treated with rhEGF (blue line) and fourteen animals treated with placebo (green line) were followed by 6 weeks of treatment. Two animals treated with rhEGF died at week 5 and nine animals treated with placebo died during weeks 3 and 5 (p = 0.003).

sponding mitotic and mitotic/apoptotic indexes revealed the existence of significant differences in cell division between the groups. The mitotic index at 16 days showed that the proliferation status of cells sampled at these times had less number of cells in mitosis in relation to the total number of cells (Table 2). These indexes for the implanted tumors in the EGF-treated group were lower than in the control group.

Gene expression analysis in xenotrasplanted tumors (A431 cells) in nude mice

The isolated RNA appeared to meet the quality standard for further use in differential gene expression analyses by qPCR (data not shown). All the samples had RIN larger than 7. The qPCR technique was validated to demonstrate the absence of genomic DNA in the RNA preparations and the absence of inhibitors in the cDNA synthesized for the PCR reactions. A similar observation was found after the evaluation of references genes with the least variation in their expression levels between samples of rhEGF-treated group and the control. The use of qPCR addressed the identification of genes that could be involved in the observed cell proliferation inhibition *in vitro* and *in vivo* after the application of rhEGF. According to the results, 6% of the analyzed genes had significantly different expression levels between both treatment groups. Compared to the control animals, two of the three genes with significant differences had lower expression levels but the expression of the third was increased (Table 3). A molecular interaction network was designed using, as input, some of the most relevant genes that interact directly with Cdk4, TP53 and CASP9, genes that varied significantly their expression (Figure 4).

*D*iscussion

EGF is endowed with proliferative and cytoprotective abilities, promoting cell survival and tissue repair. There is a controversy about the role of EGF in cancer development because: its specific receptor is encoded by an oncogene, the EGFR is mutated in most epithelial cancers and the signals, produced by the interaction of EGF and its receptor to stimulate tissue repair such as proliferation, migration and angiogenesis, are the same signals that promote tumorigenesis [37].

Although an obvious concern still survives before the clinical use of EGF, a thorough examination of the international reported clinical interventions suggest that EGF treatments have been well tolerated (including systemic exposure) and safe within temporary biological windows enough for malignant processes' onset (4 to 15 years of follow up) [37].

Our study has shed further light on the safety profile of EGF, which at the effective clinically concentrations does not favor the development of tumors in A431 cells. We developed an A431 tumor-bearing nude mice model and it was validated through phenotypical and histological tests. A431 is a highly tumorigenic squamous carcinoma cell line that was chosen due to its large levels of EGFR expression, which provide a large dynamic range for testing the effect of different EGF concentrations on the activation of the EGFR and cancer signaling pathways [10].

In this study, we found that concentrations of rhEGF of 2.2, 33 and 165 nM produced cell proliferative inhibition of A431 during cultivation. In other words, rhEGF concentrations up to 16-fold higher than those reported by other authors produced an inhibitory effect on growth, similar to EGF at 0.1-10 nM [38-41]. For the *in vitro* study, the experimental point of 2.2 nM of rhEGF matches with the concentration used in the clinics during the therapy of chronic ulcers [14-16]. The results as a whole, suggest that rhEGF does not promote the proliferation of the A431 cell

Table 1. Survival results of nude xenotransplanted mice treated with rhEGF and control*

Groups	Estimate of survival time in weeks	Standard Error	95% confidence interval	
			Lower Bound	Upper Bound
Treated	5.9	0.08	5.71	6.03
Control	4.9	0.29	4.35	5.50
Overall	5.4	0.16	5.10	5.76

* Results were obtained by applying the log-rank (Mantel-Cox) test using SPSS for Windows to analyze the survival time of 16 animals treated with rhEGF and 14 used as controls. Events or deaths were 2 and 9 animals, respectively. Means for survival time denote how long the animals survived after the treatment with rhEGF or control. The estimation of means was limited to the largest survival time if it was censored.

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Table 2. Comparison of mitotic (MI) and apoptotic indexes (AI), mitosis-apoptosis ratio (MAR) and neoformation vessel index (NVI) means for each group

Treatment groups	MI	AI	MI/AI	NVI
Treated	1.21	0.43	3.17	25.62
rhEGF	0.68	0.3	1.75	23.70
95% CI†	(-4.29; -0.012)*	(-0.58; 0.79)	(-9.17; 33.83)	(-50.0; 21.70)

† 95% CI: 95% confidence intervals of the differences as determined by Bayesian statistics.
* Statistically significant differences between the treatment with rhEGF and the control.

Table 3. Human genes with significant differences in expression between the tumors of rhEGF treated animals and controls*

Gene symbol	P(H1)	FC
CASP9	0.01	1.194
TP53	0.015	0.675
Cdk4	0.027	0.787

*A complete list of FC and P(H1) values for the 49 analyzed genes on the animals treated with EGF is shown in supplementary table 1.
P(H1): Probability that the difference represented by the alternate hypothesis is due only to sampling error.
FC: Fold change. Values above and below the unit are up- and down-regulated, respectively.

line and, therefore, may not assist in tumorigenesis in these cells.

The high levels of EGFR in A431 cells appears to facilitate tumor growth *in vivo*, compared other tumor lines expressing lower levels of EGFR. These observations are consistent with the results of Barnes in 1982 [40] and Santon *et al*. in 1986 [41], which demonstrated that the concentration of EGFRs in A431 cells is directly proportional to their ability to grow as solid tumors in host animals. In line with this, the rhEGF-treated animal group showed significantly smaller tumor volumes than the control group, suggesting an inhibition of tumor growth mediated by rhEGF. We observed a similar behavior for A431 cells growing *in vitro* under the influence of nanomolar concentrations of rhEGF. These results might suggest that EGF promotes similar mechanisms in both biological scenarios (*in vivo* and *in vitro*), resulting in the growth inhibition of A431 cell line.

In addition to the tumor growth data, the histological analysis also suggests that rhEGF exerts an inhibitory, rather than stimulatory mitotic action on A431 tumor cells. The mitotic index, a critical parameter for cancer prevention and control, was lower in rhEGF-treated animals compared to the control, possibly due to cell cycle arrest arising from the repair of genetic material and the promotion of tumor cell death. These could be transient events that should be promoted by the rhEGF action on A431 tumors in athymic mice [42].

The rhEGF also favored the survival of animals inoculated with 3 million A431 cells, perhaps providing protective properties against tumor aggressiveness and invasiveness. As proposed by Amagase *et al*., EGF may play a role in preventing the metastasis of certain malignant neoplasms, thus prolonging the survival time of athymic nude mice bearing human xenografts [43].

Tumor phenotypic descriptions are accompanied by changes produced by EGF`s molecular mechanisms of action, which generate a cascade of intracellular signals that justify its biological effect and cellular response. The gene expression profile that characterized the treated group supports the above statement through genes that varied significantly their expression under the action of rhEGF (Figure 4).

Three genes (TP53, Cdk4 and CASP9) with relevance in cell cycle regulation and apoptosis showed significant differences in expression in the tumors of EGF-treated animals vs. control. These genes with subtle changes in their expression levels (fold-change lower than 2) could be indicating that they are more tightly regulated in its effect on the tumor biology formation [44].

The results showed that rhEGF at nanomolar concentrations inhibited the proliferation of A431 tumor cells both *in vitro* and *in vivo*. This cell line has a point mutation in the $TP53^{273.His}$ that contributes to malignant progression through genomic unstability due to the inhibition at the G1 arrest point [45]. $TP53^{273.His}$ is considered as a type I missense mutation, since it affects residues of the DNA-binding surface and disrupts the contact points between protein and DNA [46]. Some of the phenotypic effects of $TP53^{273.His}$ are: increase cell proliferation, increase growth density and induction of antiapoptotic activity [47]. The down-regulation of TP53 mRNA levels in tumors cells treated with rhEGF may be related with a reversible cell cycle arrest at the G1-S boundary. In this study, we observed a possible link between the effect of rhEGF and the down-regulation of two important cell cycle regulator genes, the $TP53^{273.His}$ and Cdk4, and their implication in the lengthening of the G1 to S phase transition, therefore inhibiting tumor growth. The results are not enough to further dissect and identify what genes or protein interactions lead to decrease gene expression levels for Cdk4, but the decrease in TP53 levels due to EGF may suggests a reduction of the oncogenic gain-of-function of the TP53 gene and a reprogramming of the activity of genes controlling the cell cycle, such as Cdk4.

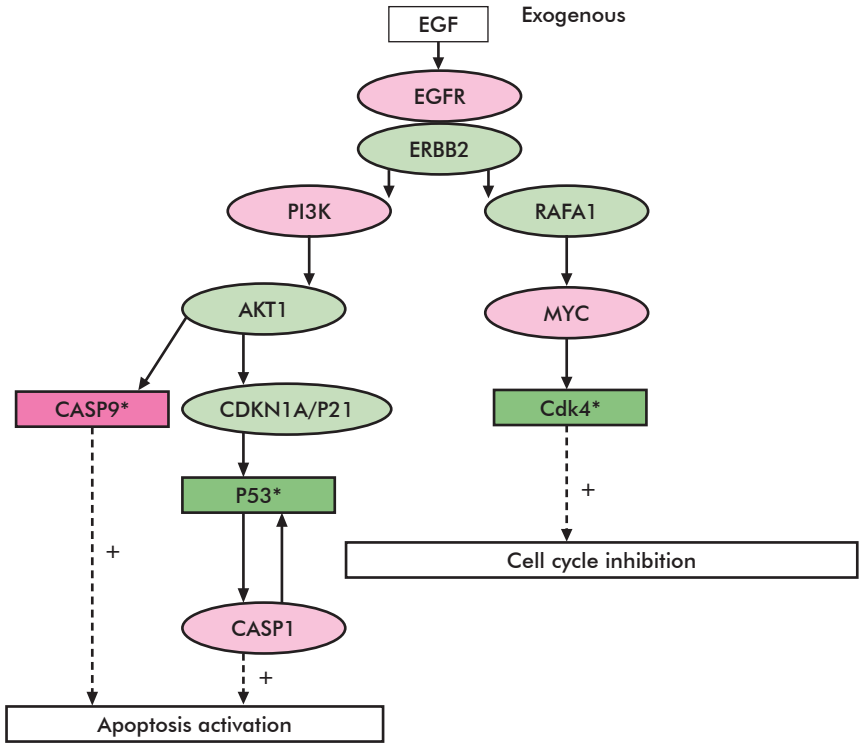

Figure 4. Schematic representation of some of the signaling pathway that may occur in A431 tumor cell line to the action of EGF in xenotrasplanted nude mice. Elipses in pink and green represent up- and down-regulated genes, respectively. Rectangles represent genes that significantly varied their expression levels (Cdk4, TP53 and CASP9). The dashed lines represent the biological function that could be mediated by the gene expression levels. The + symbol represent the positive action of up-regulated or down-regulated gene expression, on the promotion of cell cycle inhibition and stimulation of apoptosis events. Statistically significant differences in gene expression levels between the rhEGF-treated group and control are indicated (*). For more information about the gene expression levels, see supplementary table 1.

Another downstream component of the EGF regulatory cascade is Caspase-9, a gene for a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution phase of cell apoptosis and therefore, the restoration of some cell pathways through the action of EGF in tumor cells may drive them directly to cell death (apoptosis) [48].

Preliminarily, in the *in vivo* scenario, the action of rhEGF led to a transient recovery of tumor suppressor ability in tumor cells through the reduction of the biological action of mutated TP53, the arrest of cell cycle

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due to decreased levels of Cdk4 and the promotion of apoptosis through the activation of CASP9.

Our findings are not surprising. They rather support previous evidences documenting an EGF-mediated anti-tumorigenic effect at certain supraphysiological concentrations in A431 cells [38-40]. Other malignant cell lines as MX-1, UM-1 of mammary origin as the ES-4 derived from esophageal cancer are also inhibited by EGF in proportion to the concentrations injected, when transplanted to athymic recipients [42]. More recent studies have shown that EGF failed to enhance *in vitro* proliferation of three human gastric adenocarcinoma lines, as there were no effect on tumor growth when these cells were implanted in nude mice [49]. Curiously, for both the *in vitro* and *in vivo* approaches, the experimental protocols and the EGF doses have been largely used and known to facilitate proliferation of non-transformed cells. Lately, according to recent *in vitro* findings EGF induced more noticeable changes in A431 cells proliferation and death than cetuximab or gefitinib. Both, cells' arrest and death appeared far more prolonged and irreversible by EGF than that of the conventional anti-tumor drugs [50].

Conclusions

These results provide evidences in favor of the safe use of EGF in clinical practice, which at nanomolar concentrations prolongs the animal survival time and inhibited the proliferation of tumor in animal's xenotrasplanted with A431 cells. The findings of differential gene expression *in vivo* analysis reinforce these phenotypic observations. Important genes for the cell cycle regulation, as TP53, Cdk4 and CASP9 varied significantly their expression levels favoring cell cycle inhibition and apoptosis.

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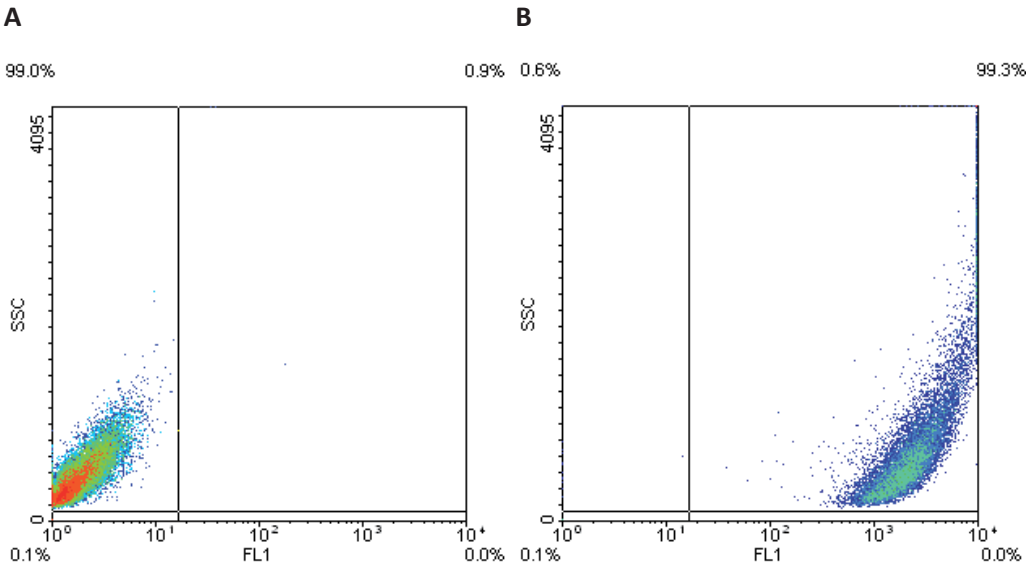
Supplementary Table. Complete list of p values and fold change (FC), for the 49 analyzed genes on the rhEGF-treated animals *vs.* controls

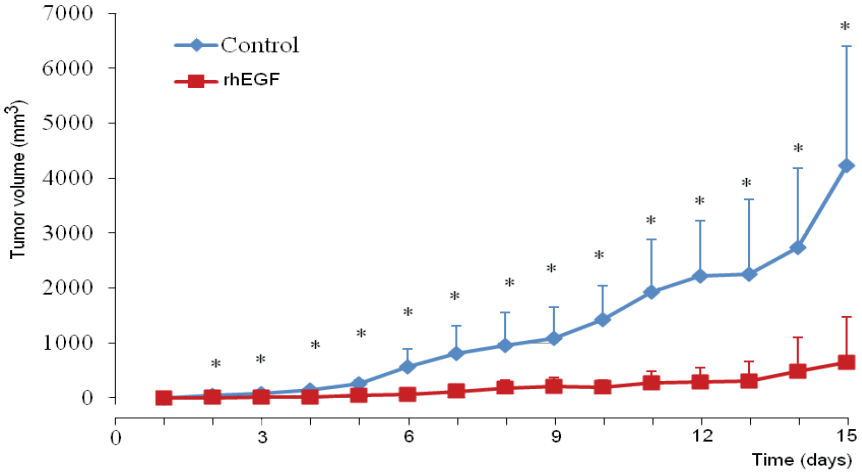
No.	Gene symbol	Gene name	NCBI Reference sequence transcript accession number	p values	FC
1	AKT1	v-akt murine thymoma viral oncogene homolog 1	NM_005163.2	0.557	0.907
2	ANGPT1	angiopoietin 1	NM_001146.3	0.481	0.784
3	BAD	BCL2-associated agonist of cell death	NM_004322.3	0.103	0.839
4	BAX	BCL2-associated X protein	NM_004324.3	0.122	1.442
5	BRCA1	breast cancer 1, early onset	NM_007294.3	0.386	1.34
6	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	NM_033292.2	0.089	1.347
7	CASP3	caspase 3, apoptosis-related cysteine peptidase	NM_004346.3	0.830	0.959
8	CASP8	caspase 8, apoptosis-related cysteine peptidase	NM_001080125.1	0.726	1.106
9	CASP9	caspase 9, apoptosis-related cysteine peptidase	NM_001229.3	0.010	1.194
10	CCNE1	cyclin E1	NM_001238.1	0.084	0.815
11	Cdk4	cyclin-dependent kinase 4	NM_000075.2	0.027	0.787
12	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389.4	0.411	0.856
13	CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits Cdk4)	NM_001262.2	0.117	1.175
14	CREB1	cAMP responsive element binding protein 1	NM_004379.3	0.427	1.182
15	EGFR	epidermal growth factor receptor	NM_005228.3	0.052	1.513
16	ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	NM_004448.2	0.513	0.910
17	FOS	FBJ murine osteosarcoma viral oncogene homolog	NM_005252.3	0.274	0.638
18	FOXO3	forkhead box O3	NM_001455.3	0.074	0.837
19	IGFBP3	insulin-like growth factor binding protein 3	NM_001013398.1	0.565	1.079
20	IRF1	interferon regulatory factor 1	NM_002198.2	0.878	0.978
21	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	NM_002203.3	0.426	1.233
22	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	NM_002211.3	0.948	1.009
23	MAP2K4	mitogen-activated protein kinase kinase 4	NM_003010.2	0.643	1.092
24	MCAM	melanoma cell adhesion molecule	NM_006500.2	0.165	1.181
25	MMP1	matrix metallopeptidase 1 (interstitial collagenase)	NM_002421.3	0.087	0.471
26	MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	NM_002423.3	0.344	1.163

27	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467.4	0.724	1.041
28	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_001165412.1	0.365	0.925
29	NGF	nerve growth factor (beta polypeptide)	NM_002506.2	0.815	1.044
30	NOTCH1	notch 1	NM_017617.3	0.462	1.188
31	PCNA	proliferating cell nuclear antigen	NM_002592.2	0.245	1.16
32	PDGFA	platelet-derived growth factor alpha polypeptide	NM_002607.5	0.182	0.807
33	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	NM_002608.2	0.496	1.112
34	PDGFRB	platelet-derived growth factor receptor, beta polypeptide	NM_002609.3	0.214	1.303
35	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	NM_181523.1	0.160	1.572
36	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	NM_002880.3	0.504	0.949
37	RASA1	RAS p21 protein activator (GTPase activating protein) 1	NM_002890.1	0.227	1.289
38	SERPINB5	serpin peptidase inhibitor, clade B (ovalbumin), member 5	NM_000321.2	0.706	0.920
39	SMAD2	SMAD family member 2	NM_005901.4	0.474	1.083
40	SMAD3	SMAD family member 3	NM_005902.3	0.122	0.641
41	STAT1	signal transducer and activator of transcription 1, 91kDa	NM_007315.3	0.250	0.769
42	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	NM_139276.2	0.260	0.841
43	SYK	spleen tyrosine kinase	NM_003177.4	0.223	0.840
44	TGFB1	transforming growth factor, beta 1	NM_000660.3	0.247	0.863
45	TGFB2	transforming growth factor, beta 2	NM_003254.2	0.251	1.259
46	TIMP1	TIMP metallopeptidase inhibitor 1	NM_003255.4	0.537	0.894
47	TP53	tumor protein p53	NM_000546.4	0.015	0.675
48	TWIST1	twist homolog 1 (Drosophila)	NM_000474.3	0.155	1.181
49	VEGFA	vascular endothelial growth factor A	NM_001025366.1	0.269	1.262



Supplementary Figure 1. Flow cytometry analysis of the human epidermoid carcinoma-derived cell line A431 using MAb hR3, specific for the EGF Receptor. The first dot plot (A) is the subclass and cellular auto-fluorescence control. The second dot plot (B) displays events that are positive for EGFR fluorescein isothiocyanate (blue dots). The experiment showed that the percentage of positive cells (or events) for EGFR expression was 99.3%.

A



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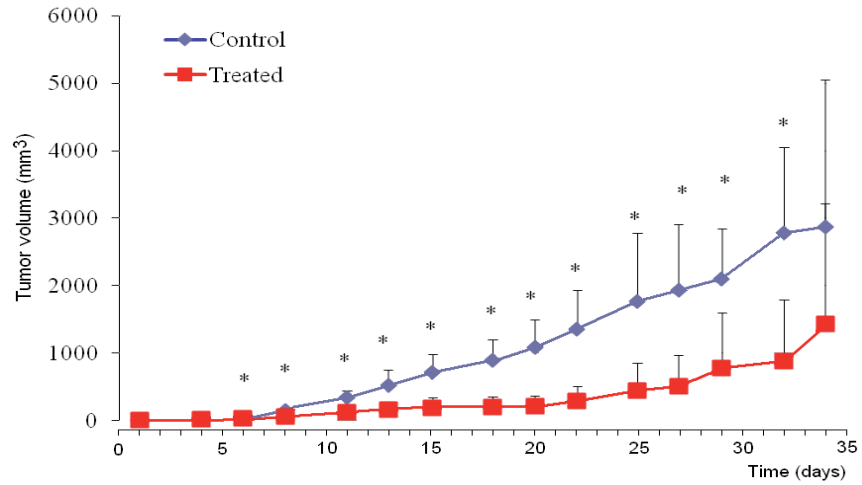

Supplementary Figure 2: Tumor growth in the xenotrasplanted animals with 2 × 10^6 (A) and 3 × 10^6 cells A431 (B). Animals with rhEGF or control were treated over a period of 34 days. Tumor volume (mean ± SD) at the end of the administration scheme was higher in the animals treated with control solution compared to those receiving rhEGF (4225 ± 2185 *vs*. 642 ± 828) for animals xenotrasplanted with 2 × 10^6 of A431 and (2863 ± 2194 *vs*. 1424 ± 1790), for animals xenotrasplanted with 3 × 10^6 of A431. Statistical analysis was performed with the Bayesian Module of EPIDAT 3.1 Mean comparison by estimating confidence intervals or credibility to 95% by Bayesian Inference methods. The significance level chosen was 0.05, with statistically significant differences in gene expression levels between the rhEGF-treated group and control indicated (*).