Estrogen receptorβ2 regulates interlukin-12 receptorβ2 expression via p38 mitogen-activated protein kinase signaling and inhibits non-small-cell lung cancer proliferation and invasion

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Received January 17, 2014; Accepted July 25, 2014

DOI: 10.3892/mmr.2015.3366

Abstract. Lung cancer is one of the most common types of cancer and is the leading cause of cancer-related mortality worldwide. Estrogens are known to be involved in the development and progression of non-small-cell lung cancer (NSCLC). These effects are initially mediated through binding of estrogen to estrogen receptors (ERs), in particular ERβ2. Our preliminary studies demonstrated that ERβ2 and interleukin-12 receptorβ2 (IL-12Rβ2) expression are correlated in NSCLC. The present study investigated the expression of these proteins in NSCLC cells and how changes in their expression affected cell proliferation and invasion. In addition, it aimed to explore whether p38 mitogen-activated protein kinase (p38MAPK) is involved in the regulation of IL-12Rβ2 expression by ERβ2. An immunocytochemical array was used to observe the distribution of ERβ2 and IL-12Rβ2. Co-immuoprecipitation was employed to observe the interaction between p38MAPK and IL-12Rβ2, by varying the expression of ERβ2 and p38MAPK. Western-blot analysis and reverse transcription-polymerase chain reaction assays were used to investigate the mechanism underlying ERβ2 regulation of IL-12Rβ2 expression. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, scratch wound healing and Transwell assays were used to investigate the impact of ERβ2 on proliferative, invasive and migratory abilities of NSCLC cells. ERβ2 was predominantly found in the cytoplasm and nucleus, whilst IL-12Rβ2 was largely confined to the cytoplasm, although a degree of expression was observed in the nucleus. Compared with normal bronchial epithelial cells, IL-12Rβ2 and ERβ2 were overexpressed in the NSCLC cell groups. Coimmuoprecipitation demonstrated an interaction between p38MAPK and IL-12Rβ2. ERβ2 appeared to upregulate IL-12Rβ2 expression and inhibition of p38MAPK attenuated this effect. ERβ2 and IL-12Rβ2 expression inhibited the proliferation, metastasis and invasion of NSCLC cell lines, but knockout of IL-12Rβ2, even in the presence of ERβ2, led to an increase in NSCLC cell proliferation and invasiveness. In conclusion, to the best of our knowledge this study is the first to demonstrate that IL-12Rβ2 may be important in the mechanisms underlying ERβ2 inhibition of NSCLC development, and that this interaction may be mediated via p38MAPK.

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Key words: estrogen receptor β2, interleukin-12 receptorβ2, non-small-cell lung cancer, proliferation and invasion, A549, LTEP-a2 and H358, p38 mitogen-activated protein kinase

Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide (1). Lung adenocarcinoma is the most common form of NSCLC (1,2). Owing to its complex tumorigenesis, lung adenocarcinoma is difficult to treat and its course in individual patients is hard to predict. In order to identify therapeutic targets and prognostic biomarkers, research on lung adenocarcinoma has focused on a number of key molecules, in particular, certain growth factor receptors, such as epidermal growth factor receptor and insulin-like growth factor 1 receptor (3,4). Recent studies have reported that binding of estrogen to estrogen receptors (ERs) in NSCLC may affect progression of this disease, thus offering a potential target for treatment (5,6).

Estrogens regulate a number of biological processes, including cell differentiation and cell proliferation, by binding to two receptors, ERα and ERβ. It has been postulated that the latter may be the key estrogen receptor in NSCLC progression (7,8). Recent studies have shown that ERβ has a number of subtypes (5,9). In a recent study, we found that overexpression of ERβ2 was observed in NSCLC cell lines, and may indicate the earlier stage of tumor development in prostate cancer progression (10). However, the mechanism by which ERβ2 influences NSCLC progression remains unclear. A number of the most recent studies suggest that p38MAPK signaling may be an important mediator of the effect of ERβ2 on NSCLC (11,12). Further research is required in order to investigate the mechanism by which ER activates nuclear transcription of certain genes.

The biological functions of human interleukin 12 (IL-12) are known to be mediated by the IL-12 receptor (IL-12R), which is composed of β1 and β2 subunits, that possess high affinity and responsiveness to IL-12. The β2 subunit is hypothesized to be the primary molecule involved in IL-12 signal transduction, and may function as a tumor suppressor protein (13,14). A number of studies have investigated the importance of IL-12Rβ2 in lung adenocarcinoma (15,16). IL-12Rβ2-deficient mice were shown to develop lung adenocarcinoma with a poor prognosis. However, the mechanism through which IL-12Rβ2 influences NSCLC progression is unclear. Other studies (17-22) have shown that IL-12 activates downstream molecules via binding to IL-12R. These molecules include p38MAPK, indicating that there may be an interaction between p38MAPK and IL-12R. A recent study provided further evidence that IL-12Rβ2 and ERβ2 are co-expressed in NSCLC (22).

Despite evidence demonstrating a correlation between IL-12Rβ2 and ERβ2 expression in NSCLC, the mechanisms by which these molecules affect progression of this disease remains obscure. This study aimed to explore the association between IL-12Rβ2 and ERβ2 *in vitro,* and to investigate whether p38MAPK affects the expression of IL-12Rβ2

Materials and methods

Cell lines and reagents. The human NSCLC cell lines (A549, LTEP-a2 and H358) and human normal bronchial epithelial cells (HBE, and NC004) were obtained from the Shanghai Institute of Cell Biology (Shanghai, China). They were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere of 5% CO_2. Cells were cultured in a phenol-red free medium supplemented with 5% charcoal stripped FBS (Sangon Biotech Co., Ltd, Shanghai, China) for at least 18 h prior to transfection. The ERβ2-specific mouse anti-human monoclonal antibody (57/3; Serotec, Kidlington, UK) and p38MAPK-specific rabbit anti-human monoclonal antibody (4511; Cell Signaling Technology Inc., Danvers, MA USA) were conserved in our laboratory. The p38MAPK inhibitor (SB203580) was purchased from Calbiochem (San Diego, CA, USA).

Plasmid construction and transfection. ERβ2-, p38MAPK- and IL-12Rβ2- (9,14) expressing plasmids (p3xFlag-ERβ2, p3XFlag-p38MAPK and pXJ40-Myc-IL-12Rβ2) were produced through the ligation of polymerase chain reaction (PCR)-generated inserts into p3xFlag-CMV-7.1-2 or pXJ40-Myc-SOX4, as appropriate. The small hairpin (sh) IL-12Rβ2 plasmids were purchased from the national RNAi core Facility located at the Institute of Molecular Biology/Genomic Research Center (Academia Sinica, Taipei, China).

The purified p3xFlag- ERβ2/ p38MAPK and p XJ40-Myc-IL-12Rβ2 plasmids were transfected into 70% confluent A549, LTEP-a2 and H358 cells, using Lipofectamine® 2000 (Invitrogen Life Technologies) reagents in a total volume of 1 ml of Opti-MEM (Invitrogen Life Technologies), as described in a previous study (23). A549, LTEP-a2 and H358 cells were co-transfected with 1 mg p3xFlag-p38MAPK, pXJ40-Myc-IL-12Rβ2, p3xFlag-empty or pXJ40-Myc-empty plasmids.

Cell proliferation and Transwell invasion assays. After transfection (12 h), cells were seeded in triplicate at a density of $5x10^3$ cells per 96-well plates. The following day, cells were treated with 10 mM estrogen (E2) dissolved in ethanol. At 24, 48 and 72 h after E2 treatment, 10 μl of a modified 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide solution (MTT; Dojindo, Kumamoto, Japan) was added to the culture and reaction mixtures were incubated at 37˚C for 2 h. In order to detect migration, cells were placed in transwell chambers (Forma™; Thermo Fisher Scientific, Waltham, MA, USA) at $2x10^4$ cells/well. The lower transwell chamber contained 10% FBS as a chemoattractant. For the invasion assay, the bottom of the culture inserts (8-mm pores) were coated with 30 μl of the mixture containing serum-free RPMI-1640 and Matrigel™ (1:8; BD Biosciences, Bedford, MA, USA). This was allowed to solidify at 37˚C overnight. After 24 h, cells that had migrated or invaded through the membrane were fixed with 95% alcohol and stained with crystal violet. The number of migrated or invaded cells was quantified by counting five independent symmetrical visual fields under an Olympus BX51 microscope at 200x magnification (Olympus Corp., Tokyo, Japan).

Scratch wound-healing assay. Cells were seeded onto six-well tissue culture dishes ($4x10^6$ cells/well) and grown to 95% confluence. Each confluent monolayer was wounded linearly using a 200 μl pipette tip and washed three times with phosphate-buffered saline (PBS). Thereafter, cell morphology and movement was observed and photographed at 200x magnification [Olympus E-P5 (14-42mm II R) Olympus Corp.] at 0, 12 and 24 h.

Immuocytochemical detection. NSCLC cells not treated with E2 were initially fixed in 1.5% agarose and incubated for 15 min at room temperature (RT). Sections (3 μm) from the paraffin block were placed on to adhesive-coated slides. In a heated antigen retrieval process (24), the slides were placed in an EDTA buffer (pH 8.0) and heated for 2 min in a steamer. The slides were incubated overnight at 4˚C with monoclonal mouse anti-human anti-ERβ2 (Serotec) and polyclonal goat anti-human anti-IL-12 Rβ2 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies (25) in bovine serum albumin prior to incubating with the secondary antibodies rabbit anti-mouse immunoglobulin G (IgG) k-chain specific antibody (SAB3701212; 1:100, Sigma-Aldrich) or rabbit anti-goat IgG F(ab')2 (SAB3700244; 1:100; Sigma-Aldrich) at RT for 20 min. Color was developed in 3,3'-diaminobenzidine (DAB) solution for 10 min followed by counterstaining with Harris hematoxylin. Cells were dehydrated, coverslipped and reviewed under an Olympus BX51 light microscope (400x; Olympus Corp.), and the mean percentage of ERβ2 and IL-12 Rβ2 positive cells was counted in 10 high power fields in each group.

Western blot analysis. Cells were homogenized in a radioimmunoprecipitation assay (RIPA) buffer containing 10% protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA), and protein concentrations were then quantified using a BioRad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal quantities of proteins were separated by

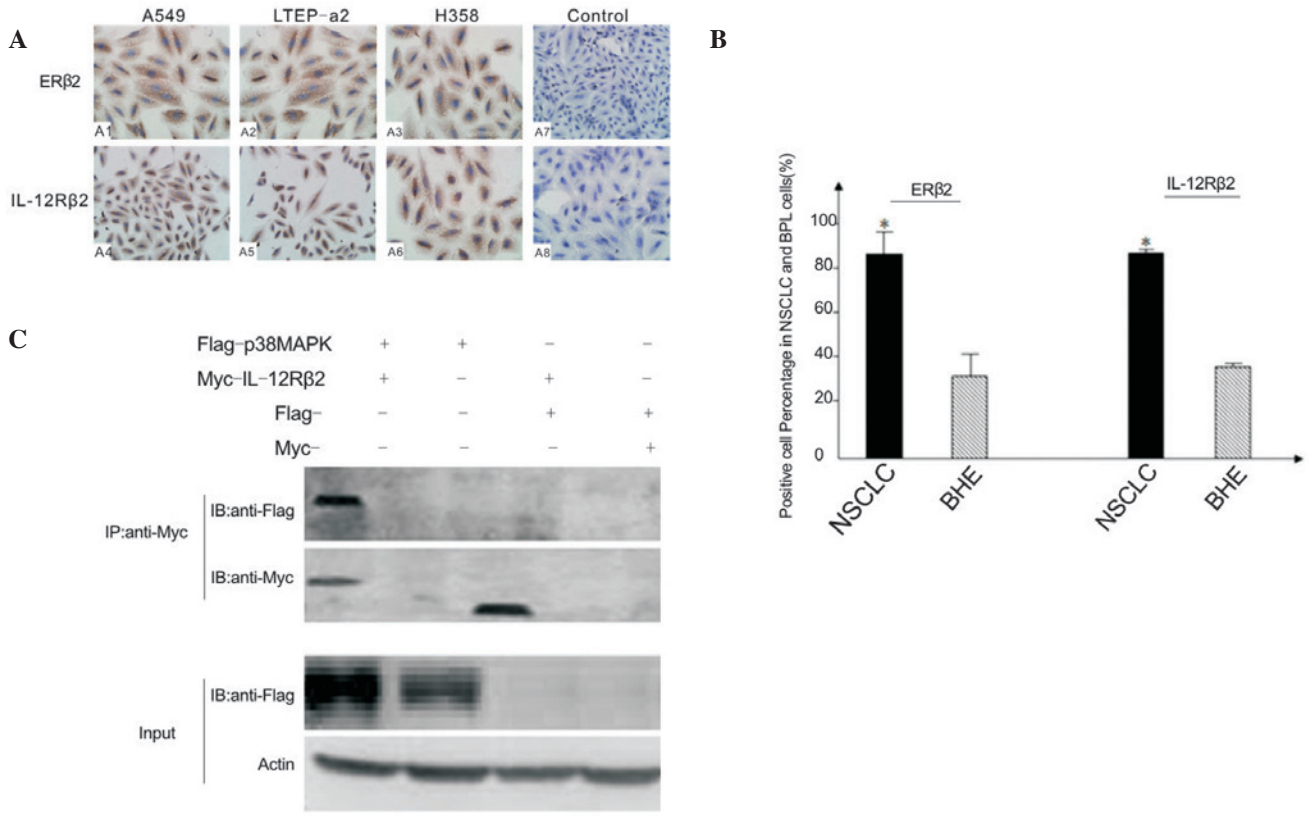

Figure 1. (A) Expression of ERβ2 and IL-12Rβ2 in human NSCLC cell lines A549, LTEP-a2 and H358, and human normal bronchial epithelial cells (control). Transfected cells were incubated with antibodies against ERβ2 and IL-12Rβ2 overnight. (B) Quantification of immunocytochemical detection of ERβ2 and IL-12Rβ2. The mean percentage of ERβ2 and IL-12Rβ2 positive cells in the NSCLC and BHE groups were counted in 10 high power fields in each group. *P<0.05 compared with BHE. (C) Coimmunoprecipitation assay. Input rows indicate the positive control (IgG as negative control, not shown). Via construction of Flag-tagged p38MAPK and Myc-tagged IL-12Rβ2, coimmunoprecipitation confirmed that there was an interaction between p38MAPK and IL-12Rβ2 specifically. ERβ2, estrogen receptor-β2; IL-12Rβ2, interleukin-12 receptor-β2; NSCLC, non-small-cell lung cancer, BHE, bronchial (human) epithelium; p38MAPK, p38 mitogen-activating protein kinase; IL-12Rβ2, interleukin-12 receptorβ2.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Primary goat anti-human polyclonal anti-IL-12Rβ2 antibodies (E-20; Santa Cruz Biotechnology Inc.) were then applied to the membranes according to the manufacturer's instructions. The membranes were washed and treated with the appropriate horseradish peroxidase-conjugated secondary antibodies (rabbit anti-goat IgG F(ab')2; 1:100; Sigma-Aldrich). A similar process was conducted for β-actin using the mouse monoclonal anti-actin antibody (A3854; Sigma-Aldrich). The results were visualized with chemiluminescence (West Dura; CPS160; Sigma-Aldrich).

Semiquantitative reverse transcription (RT-qPCR) analysis. RNA was prepared with TRIzol (Life Technologies, Rockville, MD, USA). For semiquantitative RT-qPCR, complementary (c)DNA was reverse-transcribed from total RNA with oligo(dT)16 primers and murine leukemia virus reverse transcriptase (PerkinElmer, Wellesley, MA, USA). The quantities of cDNA were adjusted by quantifying the level of actin DNA. Then, the same quantities of cDNA normalized to the actin were amplified for IL-12Rβ2, using the following primers: Forward: 5'-ATCCATGCGCCTGCTAAC-3' and reverse: 5'-GAGTGTTTGAGAGGCCTTTTCTG-3'.

Co-immunoprecipitation. After transfection (12 h), each group of cells was treated with mock (ethanol) for 24 h. Protein (500 μg) from the cell lysates was incubated with 2 μg anti-Myc antibody or normal rabbit IgG (Santa Cruz Biotechnology Inc.) for 16 h at 4˚C. To each sample, 20 μl of protein A/G-agarose beads was added (Santa Cruz Biotechnology), incubated for 1 h and washed three times with RIPA buffer. Then, the complex was resolved on 10% SDS-PAGE, transferred to the membrane and blotted with anti-Flag (1 μg/ml, Sigma-Aldrich) or anti-Myc antibody. Membranes were incubated with enhanced chemiluminescence reagent (Super Signal West Pico; Pierce, Rockford, IL, USA) and exposed to autoradiographic film (Kodak, Rochester, NY, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. The quantitative data were analyzed using Student's t-test or one-way analysis of variance. All statistical tests were two-sided. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of ERβ2 and IL-12Rβ2 is increased in NSCLC. To investigate the association between ERβ2 and IL-12Rβ2,

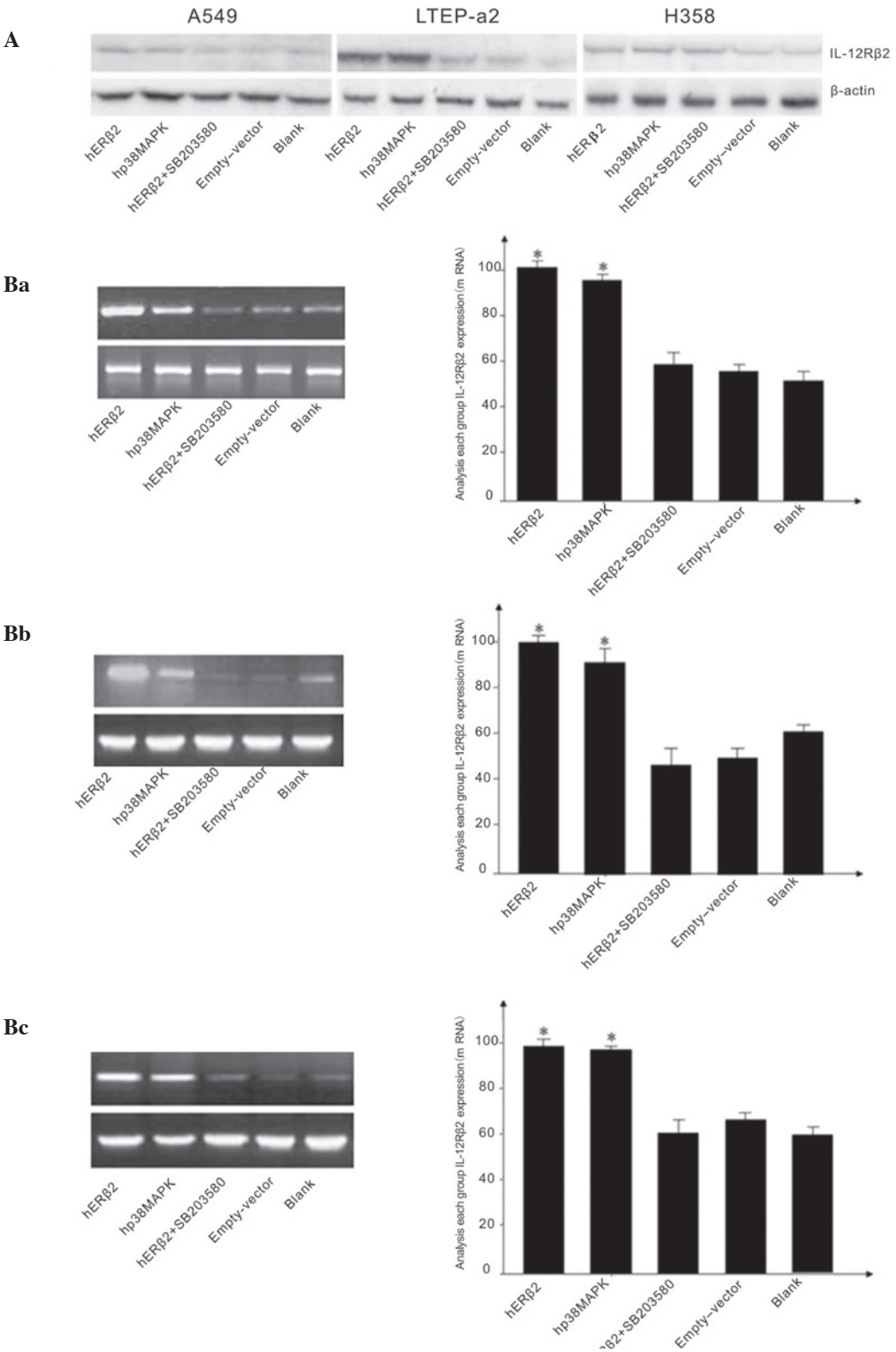

Figure 2. (A) Western blotting showing expression of IL-12Rβ2 in human NSCLC cell lines A549, LTEP-a2 and H358, with β-actin as a control. Expression shown for cells containing p3x-ERβ2, p3x-p38MAPK, psx-ERβ2+SB203580, blank and empty vectors. (B) Levels of IL-12Rβ2 expression in the NSCLC cell lines with the vectors indicated. (Ba) cell line A549, (Bb) cell line LTEP-a2, (Bc) cell line H358. Levels of IL-12Rβ2 cDNA were quantified using semi-quantitative reverse transcription-polymerase chain reaction. *P<0.05 vs. empty-vector control group. IL-12Rβ2, interleukin-12 receptorβ2; p38MAPK, p38 mitogen-activating protein kinase; ERβ2, estrogen receptor β2; SB203580, p38MAPK inhibitor; NSCLC, non-small-cell lung cancer.

their expression and distribution was analyzed with immuno-cytochemical technology. ERβ2 was predominantly found in the cytoplasm and the nucleus in all three NSCLC cell lines as shown in Fig. 1A. IL-12β2 expression was largely confined to the nucleus. Compared with the normal bronchial cell line, the percentage of cells positive for ERβ2 and IL-12 Rβ2 was increased by 82.23% and 82.0%, respectively (Fig. 1B), demonstrating that ERβ2 and IL-12Rβ2 protein are

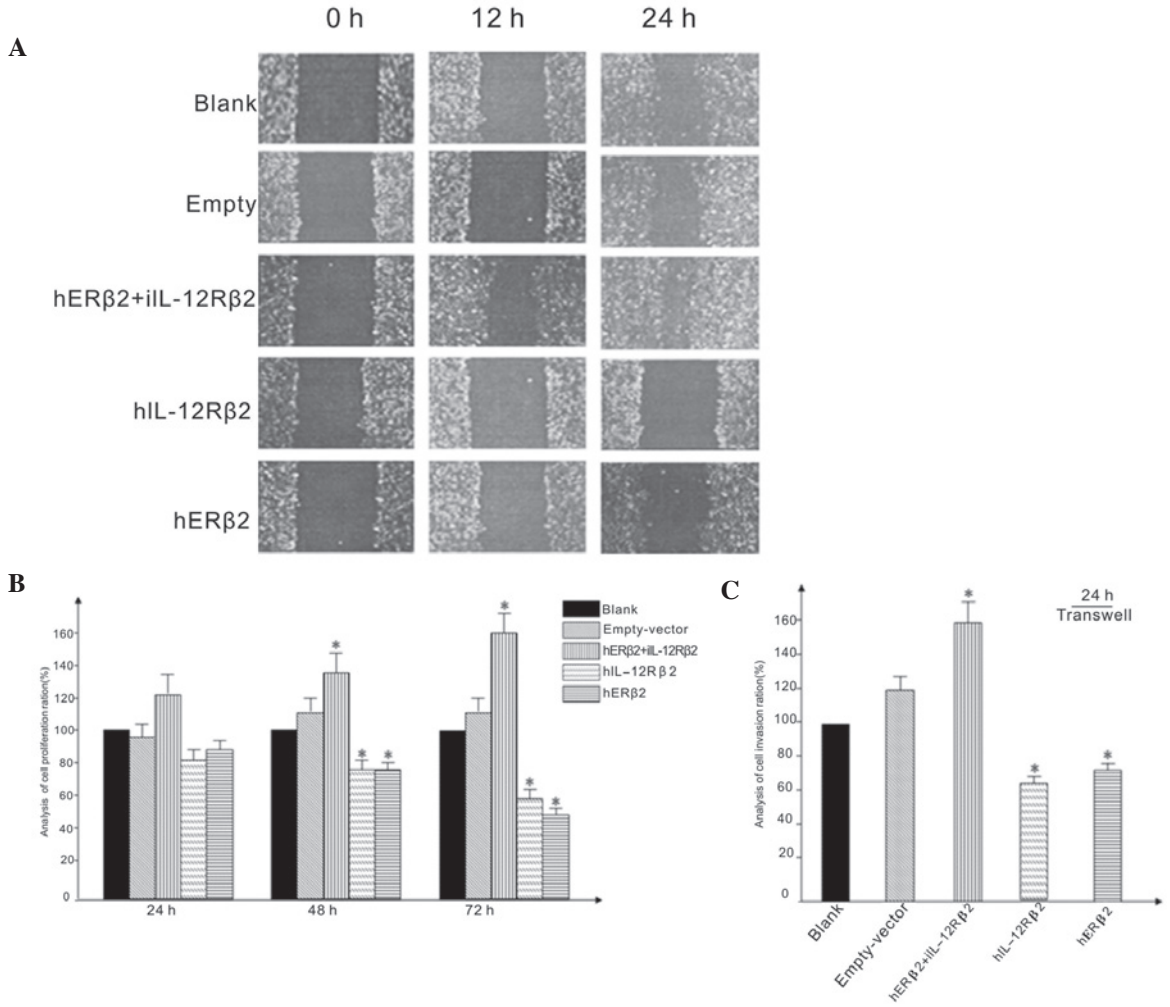

Figure 3. (A) Scratch wound-healing assay. Effect of altering levels of expression of ERβ2 and IL-12Rβ2 in NSCLC cell lines. Cell layers were wounded with a pipette tip, and morphology and movement was noted and photographed at 0, 12 and 24 h. (B) Levels of cell proliferation at 24, 48 and 72 h in NSCLC cell lines transfected with hERβ2+IL-12Rβ2, hIL-12Rβ2, hERβ2, blank and empty vectors. Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide assay. (C) Extent of cell migration in cell transfected with the plasmids noted. Transwell invasion assay was used to assess cell invasiveness. Numbers of migrated cells were counted in five fields in each group. *P<0.05 vs. empty-vector control group. ERβ2, estrogen receptorβ2; IL-12Rβ2, interleukin-12 receptorβ2; NSCLC, non-small-cell lung cancer.

overexpressed in NSCLC cell lines, which is in agreement with previous studies (5,8,16,22).

ERβ2 regulates IL-12Rβ2 expression via p38MAPK signaling

To investigate the roles and mechanism of ERβ2 further, the correlation between ERβ2 and IL-12Rβ2 was investigated. In the co-immunoprecipitation assay, certain proteins were constructed and groups were set, i.e., Flag-p38MAPK and Myc-IL-12Rβ2. Through the analyses, an interaction between p38MAPK and IL-12Rβ2, rather than a mixture, was observed, indicated due to the fact that the interaction molecular weight was slightly greater than the sum of their weights (Fig. 1C).

Western blotting and RT-qPCR analysis were performed to assess IL-12Rβ2 expression in the three NSCLC cell lines, each containing p3x-ERβ2, p3x-38MAPK, p3x-ERβ2+ SB203580, blank or empty vectors. Expression of the IL-12Rβ2 protein was increased in the p3x-ERβ2 and p3x-p38MAPK groups compared with the other groups (Fig. 2A and B). Fig. 2B shows that there was a significant increase in IL-12Rβ2 gene expression in the p3x-ERβ2 and p3x-38MAPK-containing cells compared with p3x-ERβ2+ SB203580-, blank- or empty vector-containing cells. This suggests that p38MAPK is required to enable the upregulation of IL-12Rβ2 by ERβ2.

ERβ2 inhibits NSCLC progression via IL-12Rβ2. There has been controversy over the role of ERβ2 in certain cancers, for example a number of studies have reported that E2 may promote lung adenocarcinoma development as well as insulin-like growth factor type-1 (3,4,7,8). This study demonstrated a potentially protective effect of ERβ2 in NSCLC, but showed that this effect did not persist, and indeed, was reversed, in the absence of IL-12Rβ2. An MTT assay was used to detect cell proliferation in each group. It was found that compared with the blank group, cell proliferation in the p3x-ERβ2 and pXJ40-IL-12Rβ2 groups was reduced. This effect was only apparent after 48 h, and persisted at 72 h. However, there was only minimal inhibition of proliferation in the p3x-ERβ2 and pXJ40-IL-12Rβ2 groups compared with the blank and empty vector groups at 24 h (Fig. 3B). Notably, an opposite effect was observed in the p3x-ERβ2+sh-IL-12Rβ2 group, where

proliferation was observed to increase significantly compared with the blank and empty vector groups.

The transwell assay was used to measure invasiveness of the cell lines. Invasiveness was observed to be significantly reduced in the p3x-ERβ2 and pXJ40-IL-12Rβ2 groups compared with the p3x-ERβ2+sh-IL-12Rβ2, blank and empty vector groups (Fig. 3C). By contrast, the p3x-ERβ2+sh-IL-12Rβ2 12Rβ2 group showed greater invasiveness compared with the blank and empty vector groups. This suggests that ERβ2/IL-12Rβ2 signaling may be important in regulating the progression of NSCLC.

Discussion

Estrogen is a hormone secreted predominantly by the ovaries to promote the development of the female reproductive system and the proliferation of the endometrium as part of the menstrual cycle (26,27). The biological effect of estrogen is achieved through binding to ERs, which are comprised of two subtypes, ERα and ERβ. Through them, E2 activates downstream molecules, such as MAPK (28). In certain studies (9,10,11), ERβ has been shown to be a key protein involved in multiple functions, including as a ligand (E2, or specific estrogen receptor β agonists), activation of ERβ (ERβ1, 2 or 5), regulation of nuclear proteins (AF-1/2, SRC-1, NF-κB, CyclinE and c-Myc) and expression via MAPK signaling (6,9,10). The ERβ isoform has been extensively investigated in certain types of cancer, including lung and breast cancer. Studies have demonstrated that there are three ERβ isoforms that are overexpressed in NSCLC, and ERβ2 appears to be particularly important (22,10). The correlation between p38MAPK and IL-12R was investigated in other studies (21,22,29-31), which demonstrated that IL-12 induces the activation of certain downstream molecules and that its functions are mediated through IL-12R, which is known to be activated by ERK and p38MAPK. ERβ2 and IL-12Rβ2 appear to be co-expressed in NSCLC tissue (22).

Immunocytochemical technology identified that ERβ2 and IL-12Rβ2 are overexpressed in NSCLC cell lines compared with a normal bronchial epithelia tissue cell group. This is in agreement with previous studies (22). In order to investigate the ERβ2 mechanism and the correlation between them, certain interfering protein expression methods were used. The present study showed that high expression of ERβ2 or IL-12Rβ2 protein led to a significant reduction in NSCLC cell proliferation and invasiveness. Furthermore, when IL-12Rβ2 protein expression was eliminated and ERβ2 expression remained high this effect was reversed, such that proliferation and invasiveness were significantly increased compared with the blank and empty vector groups. These results suggest that ERβ2 may alter NSCLC progression via its effect on IL-12Rβ2. Through the observation and analysis of the results, further assays were performed. The interaction between IL-12Rβ2 and p38MAPK was confirmed. In addition, IL-12Rβ2 expression appeared to be correlated with p38MAPK expression, such that the effect of ERβ2 on the upregulation of IL-12Rβ2 was inhibited by administration of a p38MAPK inhibitor.

The present study demonstrated that ERβ2 may regulate downstream molecules via p38MAPK signaling, one of which may be IL-12Rβ2. Further studies are required to elucidate the details of the interaction between p38MAPK and IL-Rβ2 and identify other molecules involved in this pathway in the context of NSCLC. The results suggest that ERβ2 acts via p38MAPK/ IL-12Rβ2 signaling, which may indicate that the co-expression of IL-12Rβ2 and ERβ2 may be associated with a more favorable prognosis.

In conclusion, the current study provides support for further research into the role of ERβ2 in NSCLC, including the correlation between ERβ2 and IL-12Rβ2, and further investigation into the importance of p38MAPK in this interaction.

Acknowledgements

The authors would like to thank Mr. He-Xiao Tang (Department of Thoracic Surgery, Tongji Hospital, affiliated to Tongji Medical College of Hua Zhong University of Science and Technology) who provided partial data, and Dr Chun-Hua Su (Department of Thoracic Surgery, The First Hospital of Sun-Yat sen University) who conducted the statistical analysis.

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