IKKβ/NFκBp65 activated by interleukin-13 targets the autophagy-related genes LC3B and beclin 1 in fibroblasts co-cultured with breast cancer cells

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Abstract. Interleukin-13 (IL-13), a Th2 cytokine, plays an important role in fibrosis, inflammation, tissue hyperresponsiveness and tumor development. Although studies have demonstrated that IL-13 exerts its roles through signal transducer and activator of transcription 6 (STAT6) signaling pathway, recent studies have revealed that I kappa B kinase (IKK)/nuclear factor kappa B (NFκB) pathway may also be involved in. The aim of this study was to investigate whether IL-13 delivers signals to IKKβ/NFκBp65 and whether autophagy genes are IL-13-induced the activation of NFκBp65 transcriptional targets in fibroblasts of breast tumor stroma. We examined the phosphorylation of IKKβ, the activation of NFκBp65 and NFκBp65-targeted autophagy genes in fibroblasts co-cultured with breast cancer cells under the condition of IL-13 stimulation. Results of this study showed that IL-13 induced IKKβ phosphorylation in the fibroblast line ESF co-cultured with breast cancer cell line BT474, and subsequently NFκBp65 was activated and aimed at beclin 1 and microtubule-associated protein 1 light chain 3 B (MAP1LC3B or LC3B) in these ESF cells. BMS345541, an inhibitor of IKK/NFκB pathway, significantly inhibited the IL-13-induced the activation of NFκB and also inhibited NFκB-targeted beclin 1 and LC3B expression. Our results suggest that IL-13 regulates beclin 1 and LC3B expression through IKKβ/NFκBp65 in fibroblasts co-cultured with breast cancer cells, and IL-13 plays role in activating IKKβ/NFκBp65.

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Introduction

Breast cancer is a complex disease with a long-term development and highly variable clinical prognosis, which are strongly associated with the stromal microenvironment. Fibroblasts are the predominant cells of tumor stroma (1), and play a major role in the initiation and progression of cancer (2,3). Cytokines are likely to have important effects on fibroblasts in tumor stroma; they may be involved in the activation of effector mechanisms that promote or limit certain functions of fibroblasts, which could potentially influence the tumor microenvironment.

Interleukin (IL)-13 is a T helper 2 (Th2) cytokine that participates in fibrosis, inflammation and the occurrence and development of cancer (4). Results of previous studies have confirmed the presence of IL-13 in breast cancer (5-7). The microenvironment of human breast tumor has been observed to display features of Th2 inflammation and fibrosis, whose pathologies can promote tumor development (8). IL-13-mediated hyperactive functions of breast stromal fibroblasts are associated with the malignant transformation of breast epithelial cells (7,9,10).

The increased expression levels of autophagy-related genes induced by IL-13 may be an abnormal event occurring in fibroblasts of tumor stroma, which may modify or alter the tumor stromal microenvironment and ultimately cause cells to become malignant. The association between autophagy and tumor development has been the subject of considerable attention from researchers (11,12). Autophagy involves nuclear factor (NF)κB, a transcription factor that is able to regulate the expression levels of autophagy-related genes (13,14). A study has shown that IL-13 induces inhibitor of κB kinase β (IKKβ) phosphorylation, and subsequently activates the p65 subunit of NFκB (NFκBp65) in bronchial smooth muscle cells *in vitro* (15). However, the molecular and cellular mechanisms contributing to the effect of IL-13 in breast tumors remain unclear.

The aim of the present study was to investigate whether the cytokine IL-13 signals to NFκBp65, and whether autophagy-related genes are induced by IL-13 via the activation of NFκBp65 as transcriptional targets in fibroblasts of breast tumor stroma.

Materials and methods

Cell culture and co-culture. The human fibroblast line CCC-ESF-1 (ESF) was obtained from the Cell Center of the Chinese Academy of Medical Sciences (Beijing, China). The human breast cancer cell line BT474 was acquired from the Cell Resource Center of the Chinese Academy of Sciences. ESF or BT474 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (GE Healthcare, Buckinghamshire, UK), 10 μg/ml streptomycin and 100 U/ml penicillin (both purchased form Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified 5% CO_2 atmosphere. ESF and BT474 cells were co-cultured using polycarbonate Transwell inserts (0.4 μm pore size; Corning Inc., Corning, NY, USA). ESF cells ($2x10^5$ cells/well) were plated at the bottom of 6 well companion culture plates and then allowed to adhere for at least 2 h without apical Transwell inserts. ESF cells plated in the wells were exposed to BT474 cell-conditioned media by placing the Transwell inserts plated with BT474 cells ($1.5x10^5$ cells/well) into these wells. This method allowed the ESF and BT474 cells to grow in the same medium without direct contact between them. Each experiment was repeated 3 times.

Treatment of cells. The quinoxaline derivative BMS345541 (Sigma-Aldrich, Beijing, China) is an inhibitor of IKKβ/NFκBp65. When ESF cells reached a confluence of 80-90%, they were cultured in serum-free DMEM for 4 h. Subsequently, cultured of co-cultured ESF cells were treated with BMS345541 (30 μmol/l) for 1 h, then treated with IL-13 (20 ng/ml; PeproTech, Rocky Hill, NJ, USA) for 30 min.

Western blot assay. ESF cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Nucleus or cytoplasm proteins were extracted using a protein extraction kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China). The protein content in each sample was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of protein were separated by electrophoresis on an 8% or 10% sodium dodecyl sulfate-polyacrylamide gel (Beyotime Institute of Biotechnology). The electrophoresed proteins were transferred to a nitrocellulose membrane (Beyotime Institute of Biotechnology); the 8% gel was run at 70-80 V and the 10% gel was run at 110-120 V. After overnight incubation at 4˚C in a blocking buffer (5% non-fat dry milk and 0.05% Tween-20 in Tris-buffered saline), the membranes were immunoblotted with antibodies against IKKβ (mouse monoclonal; 1:200; sc-271782), pIKKβ (rabbit polyclonal; 1:300; sc-21661), NFκBp65 (rabbit polyclonal; 1:500; sc-109) and GAPDH (rabbit polyclonal; 1:500; sc-365062) (all purchased from Santa Cruz Biotechnology, Dallas, TX, USA), beclin 1 (rabbit polyclonal; 1:500; AP1818b) and microtubule-associated protein 1 light chain 3β (MAP1LC3B or LC3B; rabbit polyclonal; 1:500; AP1802a) (both purchased from Abgent, Inc., San Diego, CA, USA) for 2 h at room temperature (RT). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at RT for 1 h. Finally, the membranes were developed using ECL plus (Beyotime Institute of Biotechnology). The electrophoresis results of the western blot were analyzed using ImageJ software (version 1.45; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription- quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the ESF cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific) and measured using an SMA400 spectrophotometer (Merinton Instrument, Ltd., Beijing, China) at wavelengths of 260 and 280 nm. The extracted RNA was reverse-transcribed into cDNA using a cDNA reverse transcription kit (Takara Bio, Inc., Kyoto, Japan). The first cDNA strand was synthesized at 25˚C for 5 min, 37˚C for 60 min and 70˚C for 5 min in a 20 μl reaction mixture containing 11 μl (0.6- 0.8 μg) of total RNA, 4 μl 5X RT buffer, 2 μl dNTP mixture (10 mM of each dNTP), 1 μl RNAse inhibitor (20 U/μl), 1 μl reverse transcriptase (200 U/μl) and 1 μl random primers (50 μM). Following the first- strand cDNA synthesis, the cDNA was stored at - 20˚C. qPCR analysis of the cDNA was performed in triplicate using IQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.) and SuperReal PreMix Plus (Tiangen Biotech Co., Ltd., Beijing, China) on a CFX connect Real-Time PCR detection system (Bio-Rad Laboratories, Inc.). Relative changes were calculated using the ΔΔCt method (16). The primer sequences used (Takara Biotechnology Co., Ltd., Dalian, China) were as follows: Beclin 1, forward: 5'AACCAA CGTCTTTAATGCAACCTTC3' and reverse: 5'AGCAGC ATTAATCTCATTCCATTCC3' (GenBank accession number NM_003766.3); LC3B, forward: 5'AACATGAGCGAGTTG GTCAAG3' and reverse: 5'GCTCGTAGATGTCCGCGAT3'; GAPDH forward: 5'AGAAGGCTGGGGCTCATTTG3' and reverse: 5'AGGGGCCATCCACAGTCTTC3' (GenBank accession number NM_022818.4). PCR products were analyzed using Bio-Rad CFX Manager software (version 1.6; Bio-Rad, Laboratories, Inc.).

Monodansylcadaverin (MDC) staining for the detection of autophagosomes. Glass slides were put into culture plates. When ESF cells cultured on the slides reached 80-90% confluence, the cells were pre-treated with BMS345541 (30 μmol/l), then treated with IL-13 (20 ng/ml). After washing, autophagosomes in the ESF cells were stained using MDC (Sigma-Aldrich) at RT for 20 min. The stained autophagosomes were observed using a laser confocal microscope (Zeiss LSM510-Meta confocal; Zeiss AG, Oberkochen, Gemany), and fluorescence intensity was analyzed using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA).

Statistical analysis. Experimental data were analyzed using SPSS Statistics software, version 17.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation. The differences between two groups were analyzed by the Student's *t*-test. A probability value (P) of <0.05 was considered to indicate a statistically significant difference.

Results

IL-13 induces IKKβ phosphorylation in co-cultured ESF cells. In order to determine whether IL-13 induces IKK

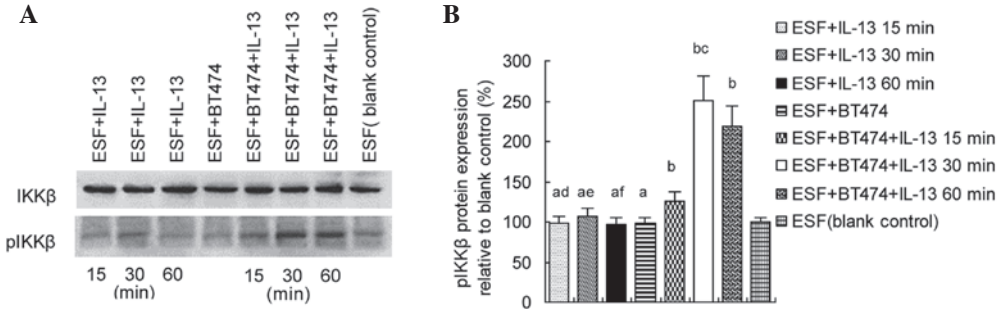

Figure 1. IL-13 induces IKKβ phosphorylation in ESF cells co-cultured with BT474 cells. (A) Western blotting results showing the expression of IKKβ and pIKKβ proteins in ESF cells. (B) Expression levels of pIKKβ as determined by densitometric analysis. [a]P>0.05 vs. the blank control group; [b]P<0.05 vs. with the blank control group; [c]P<0.05 vs. the ESF + BT474 + IL-13 15 min group or ESF + BT474 + IL-13 60 min group, respectively; [d]P<0.05 vs. the ESF+BT474+IL-13 15 min group; [e]P<0.05 vs. the ESF + BT474 + IL-13 30 min group; [f]P<0.05 vs. the ESF + BT474 + IL-13 60 min group. IL, interleukin; IKKβ, inhibitor of κB kinase β; pIKKβ, phosphorylated IKKβ.

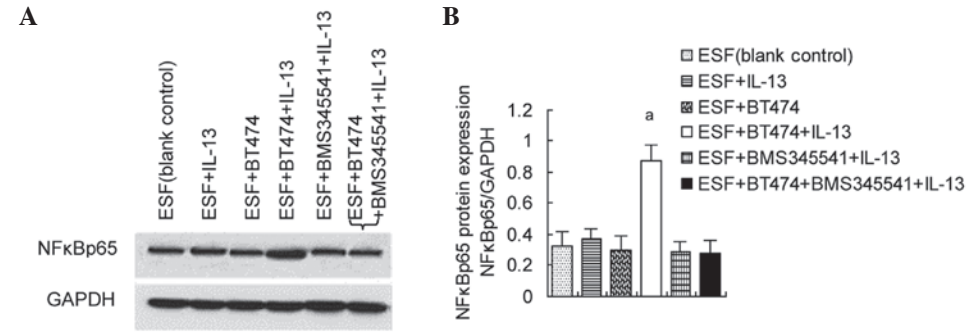

Figure 2. Upregulation of NFκBp65 in ESF cells co-cultured with BT474 cells by IL-13/pIKKβ. (A) Western blotting results showing the expression of NFκBp65 protein in ESF cells. (B) Expression levels of NFκBp65 as determined by densitometric analysis. [a]P<0.05 vs. each group, respectively. NFκBp65, p65 subunit of nuclear factor κB; IL, interleukin; pIKKβ, phosphorylated inhibitor of κB kinase β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (internal control).

phosphorylation in fibroblasts co-cultured with breast cancer cells, ESF cells were treated with IL-13 (20 ng/ml) for 15, 30 or 60 min. Western blot analyses were performed to detect pIKKβ protein in the ESF cells. The results showed that pIKKβ levels were significantly increased in the ESF + BT474 co-culture group treated by IL-13 compared with the blank ESF control (P<0.05), and were markedly higher in the ESF + BT474 + IL-13 30 min co-culture group than in the ESF + BT474 + IL-13 15 min or ESF + BT474 + IL-13 60 min co-culture groups (P<0.05; Fig. 1).

Upregulation of NFκBp65 levels in co-cultured ESF cells by IL-13/pIKKβ. The previous experiment showed that IL-13 can induce IKKβ phosphorylation in co-cultured ESF cells. In unstimulated cells, the activation of NFκB complexes is inhibited by inhibitor of NFκB (IκB) which binds to NFκB. pIKKβ can induce the phosphorylation and degradation of IκB, subsequently activating NFκB (17). Since stimulation with IL-13 can induce the phosphorylation of IKKβ, whether NFκBp65 in ESF cells is activated when stimulated by IL-13 was investigated. The results of western blot analysis showed that NFκBp65 levels were significantly increased in the ESF + BT474 co-culture group treated with IL-13 compared with those in untreated co-cultured cells. BMS345541, an inhibitor of IKKβ/NFκBp65, significantly inhibited the IL-13/pIKKβ-induced upregulation of NFκBp65 in co-cultured ESF cells (Fig. 2).

Upregulation of beclin 1 and LC3B in co-cultured ESF cells by IL-13/pIKKβ-induced NFκBp65 activation. Activated NFκB can translocate into the nucleus and activate target genes. In the present study, the expression levels of autophagy-related genes were examined to determine whether they are targeted by NFκBp65 when stimulated by IL-13. The results of western blot analysis showed that beclin 1 and LC3B expression levels were increased in co-cultured ESF cells following treatment with IL-13. BMS345541 significantly inhibited the expression of beclin 1 and LC3B targeted by IL-13/pIKKβ-induced NFκBp65 activation (Fig. 3A-D). The mRNA levels of beclin 1 and LC3B were also examined in co-cultured ESF cells using RT-qPCR. The results of RT-qPCR (Fig. 3E and F) were consistent with those of the western blot assay.

Autophagosomes are increased in co-cultured ESF cells by the IL-13-induced activation of NFκBp65/beclin 1 and LC3B. Finally, the formation of autophagosomes was examined. ESF cells were treated with BMS345541 and/or IL-13. Autophagosomes were stained using MDC, and observed by laser confocal microscopy. The results (Fig. 4) showed that the fluorescence intensity was significantly higher in the co- cultured ESF cells treated with IL-13 than in the blank control, untreated co- cultured ESF cells and IL-13-treated ESF cells, indicating that autophagosomes were increased in the ESF + BT474 + IL-13 group. Adding BMS345541 prior to IL-13 stimulation significantly inhibited the formation of

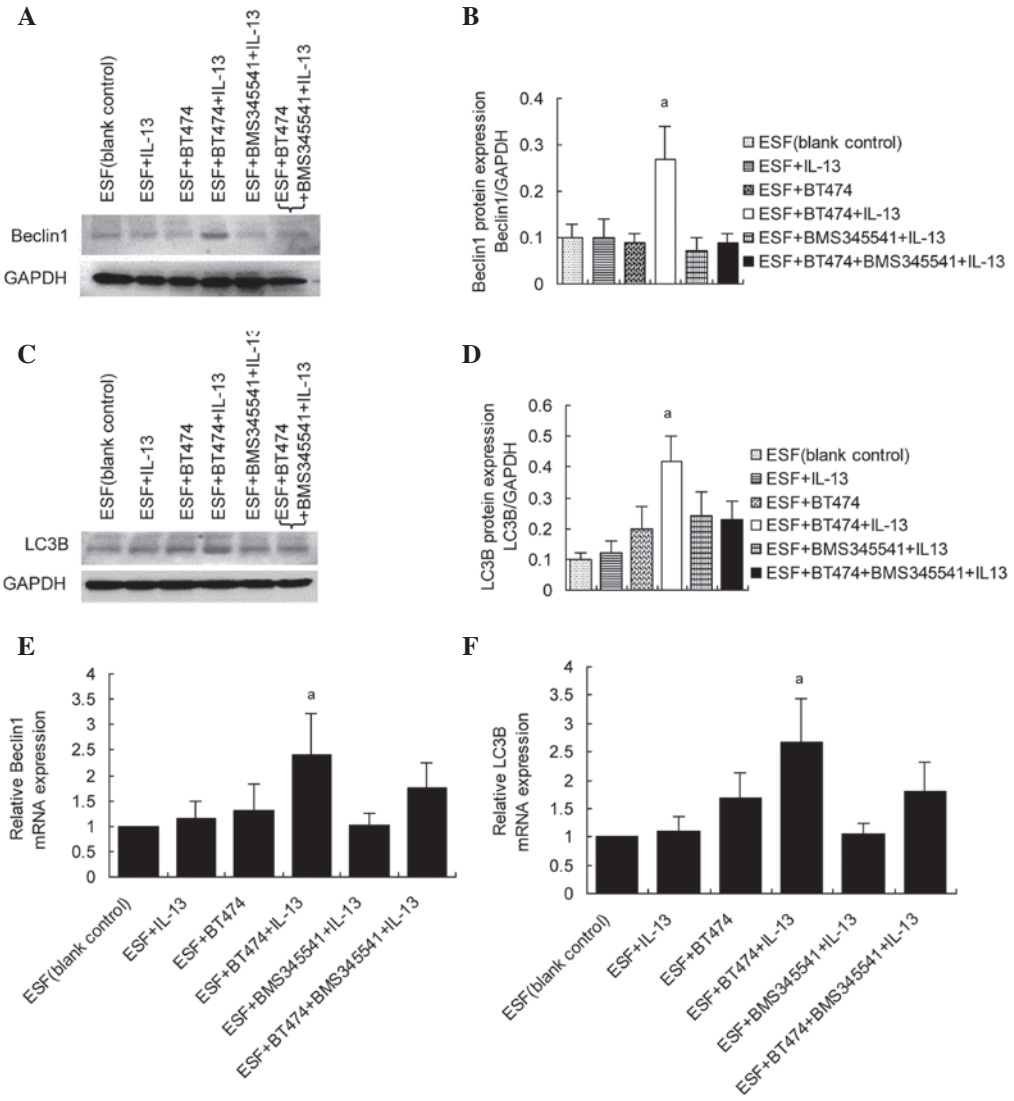

Figure 3. Upregulation of beclin 1 and LC3B in ESF cells co-cultured with BT474 cells by IL-13/pIKKβ-induced NFκBp65 activation. Western blots of (A) beclin 1 and (C) LC3B, and expression levels of (B) beclin 1 and (D) LC3B proteins as determined by densitometric analysis. mRNA levels of (E) beclin 1 and (F) LC3B were examined by RT-qPCR. [a]P<0.05 vs. each group, respectively. LC3B, microtubule-associated protein 1 light chain 3 β; IL, interleukin; pIKKβ, phosphorylated inhibitor of κB kinase β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (internal control).

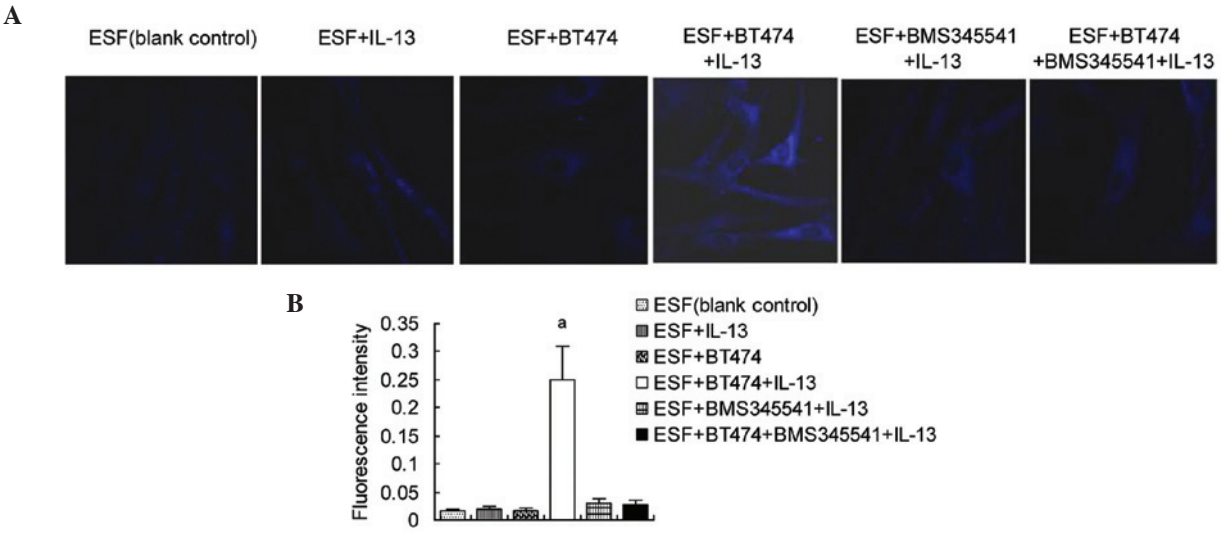

Figure 4. Activation of NFκBp65/beclin 1 and LC3B by IL-13/pIKKβ promotes formation of autophagosomes in ESF cells co-cultured with BT474 cells. (A) Autophagosomes (blue) in ESF cells stained with MDC and observed by laser confocal microscopy. Magnification, x1,000. (B) Fluorescence intensity of autophagosomes. [a]P<0.05 vs. each group, respectively. IL, interleukin; pIKKβ, phosphorylated inhibitor of κB kinase β; MDC, monodansylcadaverin.

autophagosomes in the co- culture, which was proportional to the expression levels of NFκBp65-targeted beclin 1 and LC3B.

Discussion

IL-13, a Th2 cytokine, is involved in allergic reactions, fibrosis, inflammation and tumor development (18). Studies of the IL-13 signaling pathway have focused on the signal transducer and activator of transcription 6 (STAT6) pathway (15,19). However, IL-13 is able to activate the IKK/NFκB signaling pathway, and autophagy can be activated by multiple conditions and signaling agents including cytokines (15,20). In the present study, the associations between IL-13, IKKβ/NFκBp65 and autophagy genes in fibroblasts co-cultured with breast cancer cells were investigated.

Fibroblasts are the predominant cells of the stromal microenvironment (1); they participate in the formation of stroma, and play an important role in stromal rebuilding or remodeling. Hyperactivity of fibroblasts can cause abnormalities of the components in stroma (21). Disorders of the immune system with abnormally high levels of IL-13 could lead to the abnormal activation of fibroblasts, which may be a significant cause of pathological changes of the stroma in the development of inflammation, autoimmune diseases and tumors. Breast diseases such as hyperplasia and tumors are closely associated with the IL-13-mediated abnormal active functions of fibroblasts in breast stroma. In the present study, a co-culture model of breast cancer cells with fibroblasts was established. This co-culture model allowed fibroblasts and breast cancer cells to grow in the same medium without direct contact between the two types of cells.

NFκB complexes are retained in a latent cytoplasmic form in unstimulated cells through binding to inhibitor of NFκB (IκB). Activation of IKK (the IκB kinase) complex induces the phosphorylation and subsequent degradation of IκB. Subsequently, free NFκB dimers translocate to the nucleus and activate target genes. NFκB, a pleiotropic transcription factor, can be activated by a diverse spectrum of modulating stimuli, linking a number of genetic targets (22). In the present study, ESF cells exhibited basic activation of IKKβ and NFκBp65, which may be a response to the essential growth or metabolism of cells, but the addition of IL-13 increased pIKKβ protein expression in fibroblasts co-cultured with breast cancer cells, indicating that the phosphorylation of IKKβ was induced by IL-13. Subsequently, the expression levels of NFκBp65 were upregulated, suggesting that NFκBp65 was activated by IL-13-induced pIKKβ. Evidence of mechanism was provided by the use of BMS345541, an inhibitor of the IKKβ/NFκBp65 signaling pathway, which inhibited the IKKβ/NFκBp65 response to IL-13 stimulation, suggesting that IL-13 plays the role of an upstream activator in the IKKβ/NFκBp65 signaling pathway.

NFκB is frequently activated by cytokines or stress and has either pro- or anti-autophagic functions, according to the cellular content (23). Direct molecular interactions exist between the canonical NFκBp65 activation pathway and the autophagic core machinery (24). The present study examined the consequences of NFκBp65 activation in fibroblasts co-cultured with breast cancer cells following stimulation with IL-13. The results showed that autophagy genes beclin 1 and LC3B were NFκBp65 transcriptional targets, which may be responsible for the increased quantity of autophagosomes in the fibroblasts co-cultured with breast cancer cells. The level of LC3B is proportional to the amount of autophagosomes (25). On the basis of these properties, western blots to quantify LC3B levels can be used to assess autophagosomal accumulation. Beclin 1, the mammalian ortholog of yeast Atg6, has a central role in autophagy. Beclin 1 can intervene at every major step in autophagic pathways, from autophagosome formation to autophagosome/endosome maturation (26). NFκBp65 can directly bind the beclin 1 promoter and upregulate its mRNA and protein levels (27).

Notably, the co-culture of fibroblasts with breast cancer cells fundamentally influenced the effects of IL-13 on the fibroblasts. The addition of IL-13 to the co-culture caused the occurrence of activation or regulation events, including the phosphorylation of IKKβ, the activation of NFκBp65, the upregulation of beclin 1 and LC3B, and the increase of autophagosomes in fibroblasts co-cultured with breast cancer cells, suggesting that modifications in tumor stroma can play a significant role in functional changes to, or oncogenic transformation of, fibroblasts.

In conclusion, this study highlights that IL-13 induced the activation of IKKβ/NFκBp65 and their regulatory events, including the upregulation of beclin 1 and LC3B expression, and the increase of autophagosomes in fibroblasts co-cultured with breast cancer cells. These results provide new evidence concerning the associations between IL-13, IKKβ/NFκBp65 and autophagy, indicating that IL-13 regulates the autophagy genes beclin 1 and LC3B through IKKβ/NFκBp65 in fibroblasts of breast tumor stroma, and that IL-13 may play role in the activation of IKKβ/NFκBp65.

Acknowledgements

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