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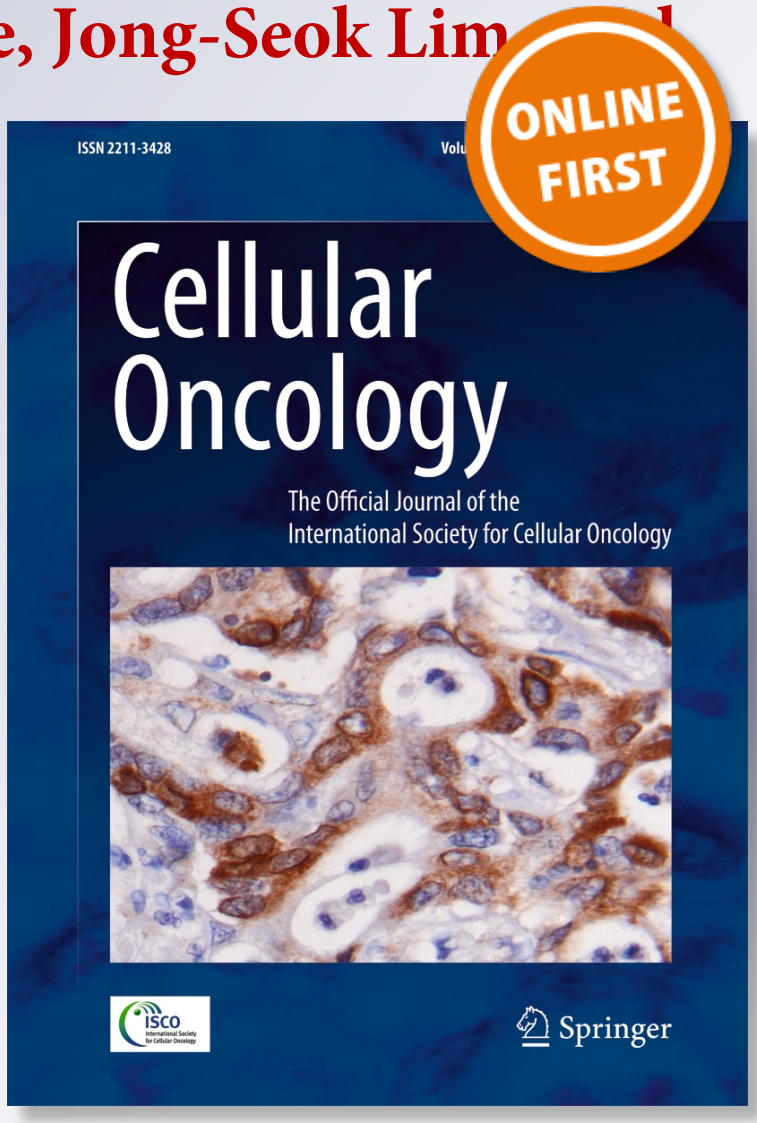

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ORIGINAL PAPER

Interleukin-32β stimulates migration of MDA-MB-231 and MCF-7cells via the VEGF-STAT3 signaling pathway

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Abstract
Background IL-32 is known to play an important role in inflammatory and autoimmune disease responses. In addition to its role in these responses, IL-32 and its different isoforms have in recent years been implicated in the development of various cancers. As of yet, the role of IL-32 in breast cancer has remained largely unknown.
Results By performing immunohistochemical assays on primary breast cancer samples, we found that the level of IL-32β expression was positively correlated with tumor size, number of lymph node metastases and tumor stage. In addition, we found that breast cancer-derived MDA-MB-231 cells exogenously expressing IL-32β exhibited increased migration and invasion capacities. These increased capacities were found to be associated with an increased expression of the epithelial mesenchymal transition (EMT) markers vimentin and Slug, the latter of which is responsible for the increase in vimentin transcription. To next investigate whether IL-32β enhances migration and invasion through a soluble factor, we determined the levels of several migration-stimulating ligands, and found that the production of VEGF was increased by IL-32β. In addition, we found that IL-32β-induced VEGF increased migration and invasion through STAT3 activation.
Conclusion The IL-32β-VEGF-STAT3 pathway represents an additional pathway that mediates the migration and invasion of breast cancer cells under the conditions of normoxia and hypoxia.

Keywords Interleukin-32 · VEGF · STAT3 · Breast cancer

J. S. Park and S. Y. Choi are equally contributed to this work.

J. S. Park · A. L. Jeong · S. Lee · S. Han · M.-S. Lee · J.-S. Lim · Y. Yang (✉)
Research Center for Women's Disease, Sookmyung Women's University, Seoul 140-742, South Korea
e-mail: yyang@sm.ac.kr

S. Y. Choi
Department of Surgery, Hallym University College of Medicine, Seoul, South Korea

J.-H. Lee
Department of Biochemistry, Kangwon National University, Chuncheon 200-701, South Korea

M. Lee · Y. Kwon
Department of Obstetrics and Gynecology, Hallym University, Seoul, South Korea

E. S. Nam
Department of Pathology, Hallym University College of Medicine, Seoul, South Korea

D. Y. Yoon
Department of Bioscience and Biotechnology, Konkuk University, Seoul, South Korea

1 Introduction

IL-32 is only expressed in primates and plays an important role in inflammatory and autoimmune disease responses [1–5]. It is conditionally produced by T lymphocytes, NK cells, epithelial cells, monocytes and keratinocytes [1, 6] and acts as a strong inducer of other proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8 [1, 7]. In recent years, IL-32 has been associated with various inflammatory diseases, including allergic rhinitis, myasthenia gravis, rheumatoid arthritis, liver inflammation and fibrosis [3, 8–10]. As a result of alternative mRNA splicing, six IL-32 splice variants have been identified (i.e., IL-32α, IL-32β, IL-32δ, IL-32γ, IL-32ε and IL-32ζ), [11] and the expression patterns of the corresponding isoforms were found to differ according to cell type. Initially, IL-32α and IL-32β were thought to be

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secretory proteins [1], but the existence of a non-secretory intracellular form was subsequently reported [12]. Currently, it is thought that IL-32 can exert its various functions via both intracellular and extracellular isoforms [13].

A growing body of evidence indicates that, in addition to its inflammatory function, IL-32 plays a role in tumor development. Studies on the role of IL-32 in tumor development have mainly focused on three isoforms, IL-32α, IL-32β and IL-32γ, because of their readily detectable expression levels. IL-32α and IL-32β were found to be highly expressed in gastric, lung, pancreatic, breast, brain and liver tumors [14–18], but their respective roles may vary in different tumors. IL-32α for example shows anti-tumor effects in human leukemia and colon cancer cells [19], whereas the proliferation of pancreatic cells is enhanced by IL-32α [14]. To understand these different effects, the IL-32-related signal transduction pathways should be elucidated in various different tumors.

High levels of IL-32 expression have previously been reported in breast tumors, but the role of IL-32 in these tumors has so far remained largely unexplored. Here, we found that IL-32β is the main isoform present in primary breast cancer cells. Based on this observation, we set out to assess the in vitro effects of IL-32β on the migration and invasion of breast cancer-derived cell lines. We found that exogenous IL-32β expression did not affect the proliferation of these cell lines but did increase their migration and invasion capacities. Because IL-32β was not found to be secreted by the breast cancer cell line MDA-MB-231, we hypothesized that intracellular IL-32β might stimulate growth factor secretion to exert its function. To test this, we assessed the putative IL-32β mediated secretion of EGF, IGF-1 and VEGF, growth factors known to enhance the migration and invasion of breast cancer cells. By doing so, we found that IL-32β specifically increases the secretion of VEGF, but not that of IGF-1 and EGF, and that this secreted VEGF enhances the migration and invasion of breast cancer cells through STAT3 activation.

2 Materials and methods

2.1 Cell cultures and reagents

Human MDA-MB-231, MCF-7 and HUVEC cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % heat-inactivated FBS (Life Technologies, Grand Island, NY, USA) at 37 °C in a humidified 5 % CO_2 incubator. Plasmids encoding IL-32β and anti-IL-32 antibody (K32-52) were obtained from Prof. Yoon at Konkuk University, Seoul, Korea [15]. Recombinant VEGF was purchased from Cell Signaling (Danvers, MA, USA), and a neutralizing polyclonal antibody against VEGF was purchased from Lab Vision (Fremont, CA, USA). The monoclonal antibodies against Slug, vimentin and STAT3 were purchased from Cell Signaling (Danvers, MA, USA).

2.2 Patient samples and tissue microarrays

We reviewed surgical pathology reports from breast cancer tissue samples collected between January 2001 and December 2005 at the Hallym University Kangdong Sacred Heart Hospital, as well as breast cancer samples from 138 patients with clinical follow-up, under Institutional Review Board approval. The interval from the date of surgery to the date of last contact (death or last follow-up) ranged from January 2001 to May 2012. Subsequently, tissue microarrays were generated using standard procedures and stained with anti-IL-32 antibody at a dilution of 1:100. An experienced pathologist, ES Nam, scored the immunohistochemical staining intensities according to the scoring system devised by Remmele and Stegner [20].

2.3 siRNAs and transfection

For the RNA interference assay, targeted small interfering RNA (siRNA) oligonucleotides were purchased from Samchully Pharmaceuticals (Seoul, Korea). The following sequences were used for the construction of the siRNAs: IL-32β siRNA #1, forward 5′-GCUCUCUGUCAG AGCUCUU-3′, reverse 5′-AAG-AGCUCUGACAG AGAGC-3′; IL-32β siRNA #2, forward 5′-GGCUUAUUAUGAGGAGCAGTT-3′, reverse 5′-CUGCUCCUCAUAAUAAGCCTT-3′; IL-32β siRNA #3; forward 5'-GGAGGACUUCAAAGAGTT-3', reverse 5'-CUCUUUGAAGUCGUCCUCCTT-3'; VEGF siRNA, forward 5'-CCUCCGAAAC CAUGAACUU-3, reverse 5'-AAGUUCAUGGUUUCGGAGG-3'; GFP siRNA, forward 5'-GUUCAGCGUGUCCGGCGAG-3', reverse 5'-CUCG-CCGGACACGCUGAAC-3'. A STAT3 siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The cells were transfected with 20 nM of siRNA using Lipofectamine™ LTX (Invitrogen, Carlsbad, CA, USA) and subjected to migration, invasion, immunoblot, and ELISA assays.

2.4 Isolation of sub-cellular fractions

Confluent monolayers of MDA-MB-231 cells were incubated with Buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 200 μl of 10 % IGEPAL CA-630, 1 mM DTT and a protease inhibitor cocktail) at room temperature for 10 min. The entire lysate was centrifuged at 15,000*g* for 3 min at 4 °C. The supernatant was saved as the cytosolic fraction. The remaining pellet was resuspended in Buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 10 % glycerol, 1 mM DTT and a protease inhibitor cocktail) and incubated with tapping for 1 h at 4 °C. The nuclear lysate was obtained by centrifuging at 15,000*g* for 5 min at 4 °C.

2.5 Immunoblot analyses

MDA-MB-231 and MCF-7 cell lysates were prepared after transfection with IL-32β plasmid or IL-32β siRNA, mixed with 5X sodium dodecyl sulfate (SDS) sample buffer, and sonicated for 15 sec. The sonicated samples were heated at 95 °C for 5 min and separated electrophoretically on a 10 % SDS-polyacrylamide gel. Subsequently, the proteins were transferred onto a 0.45-μm nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) for 2 h. The membrane was then incubated with anti-rabbit or anti-mouse IgG antibody conjugated to horseradish peroxidase (Assay Designs, Ann Arbor, MI, USA) at room temperature for 2 h. The proteins were visualized using an enhanced chemiluminescent substrate (Thermo Fisher Scientific, Logan, UT, USA) and analyzed using a LAS3000 luminescent image analyzer (Fuji Film, Tokyo, Japan). The protein bands were quantified using NIH ImageJ software.

2.6 Proliferation, invasion and migration assays

For the proliferation assay, MDA-MB-231 cells were transfected with the IL-32β-expression plasmid or an IL-32β siRNA, and then seeded into 96-well plates at a density of 1×10^4 cells per well. The proliferation of the cells was determined using an Ez-cytox cell viability assay kit (DaeiLab Service, Seoul, Korea) at 24, 48, and 72 h after plating of the cells. For the invasion assay, MDA-MB-231 cells were transfected with again the expression plasmids or siRNA and seeded on a matrigel-coated upper chamber. Cells that subsequently invaded the lower chamber of each plate were labeled with Calcein-AM (Santa Cruz, CA) after 24 h and measured with a Wallace 1420 Victor 3 plate reader (Victor 3 Perkin Elmer, Milano, Italy) at excitation of 485±10 nm and an emission of 520±10 nm. For the migration assay, a matrigel-uncoated chamber was used. The lower compartment of the transwell was filled with DMEM containing 10 % (v/v) FBS, and the migrated cells were labeled with Calcein-AM after 12 h and, subsequently, measured using a Wallace 1420 Victor 3 plate reader (Victor 3 Perkin Elmer, Milano, Italy).

2.7 HUVEC angiogenesis assay

MDA-MB-231 cells were transfected with IL-32β siRNA, and the culture medium was replaced with 2 ml of human umbilical vein endothelial cell (HUVEC) medium 15 h after transfection (Angiokit; TCS CellWorks, Buckingham, UK). After 24 h, the conditioned medium (CM) was harvested. The CM was mixed in a 1:1 ratio with fresh medium for the HUVECs and then added to the HUVECs cultured in fibroblasts (Angiokit; TCS CellWorks, Buckingham, UK). Tube formation was analyzed by measuring tubular length through light microscopy in a high-resolution field (Axiovert 405 M; Carl Zeiss, Feldbach, Switzerland). The extent of tube formation was estimated by assessing the branch point and overall length of the tube.

2.8 Statistical analyses

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA). For comparisons between more than two groups, data were analyzed using one-way analysis of variance (ANOVA) with Dunnett's for multiple comparisons. $*P<0.05$, $**P<0.01$ and $***P<0.001$ were considered benchmarks of significant differences.

3 Results

3.1 IL-32β is highly expressed in breast cancer cell lines and tissues

Although IL-32 has previously been found to be highly expressed in various cancers, the role of IL-32 in human breast cancer has so far remained unclear. To study the function of IL-32 in breast cancer, we first examined the expression levels of each IL-32 isoform in the non-tumorigenic breast cancer-derived cell lines MCF-10A and MCF-7 and the tumorigenic breast cancer-derived cell line MDA-MB-231. All cell lines were found to express different levels of IL-32β mRNA. MDA-MB-231 cells expressed IL-32γ mRNA together with IL-32β mRNA, whereas IL-32α and IL-32δ mRNAs were not detected in any of the cell lines tested (Fig. 1a). When the protein levels of IL-32β and IL-32γ were assessed using an anti-IL-32 antibody that allows the detection of all isoforms [15], IL-32β protein was readily detected in the MDA-MD-231 cells, whereas IL-32γ protein was not (Fig. 1a). Although the MCF-10A cells expressed detectable levels of IL-32β mRNA, IL-32β protein was not detected. These findings suggest that IL-32β protein expression may be regulated at the translational level. We next used RT-PCR to examine IL-32β transcript levels in primary breast cancer tissues and found an increase in IL-32β and IL-32γ expression compared to adjacent normal tissues (Fig. 1b). We next set out to examine IL-32β expression using a tissue microarray containing duplicate or triplicate samples from 138 breast cancer patients, in conjunction with immunohistochemistry. Each sample was scored blindly and classified as negative (score 0, 0–5 %), low (score 1, 6–25 %), intermediate (score 2, 26–50 %) or high (score 3, more than 51 %) intensity (Fig. 1c). The number of patients classified by the scoring system are listed in Table 1 and the TNM system was used as cancer staging system. A statistically significant correlation between IL-32β expression and tumor size was observed. Larger tumors showed stronger IL-32β expression intensities, whereas some patients with T1- and T2-stage tumors did not show any IL-32β expression (Fig. 1d). The IL-

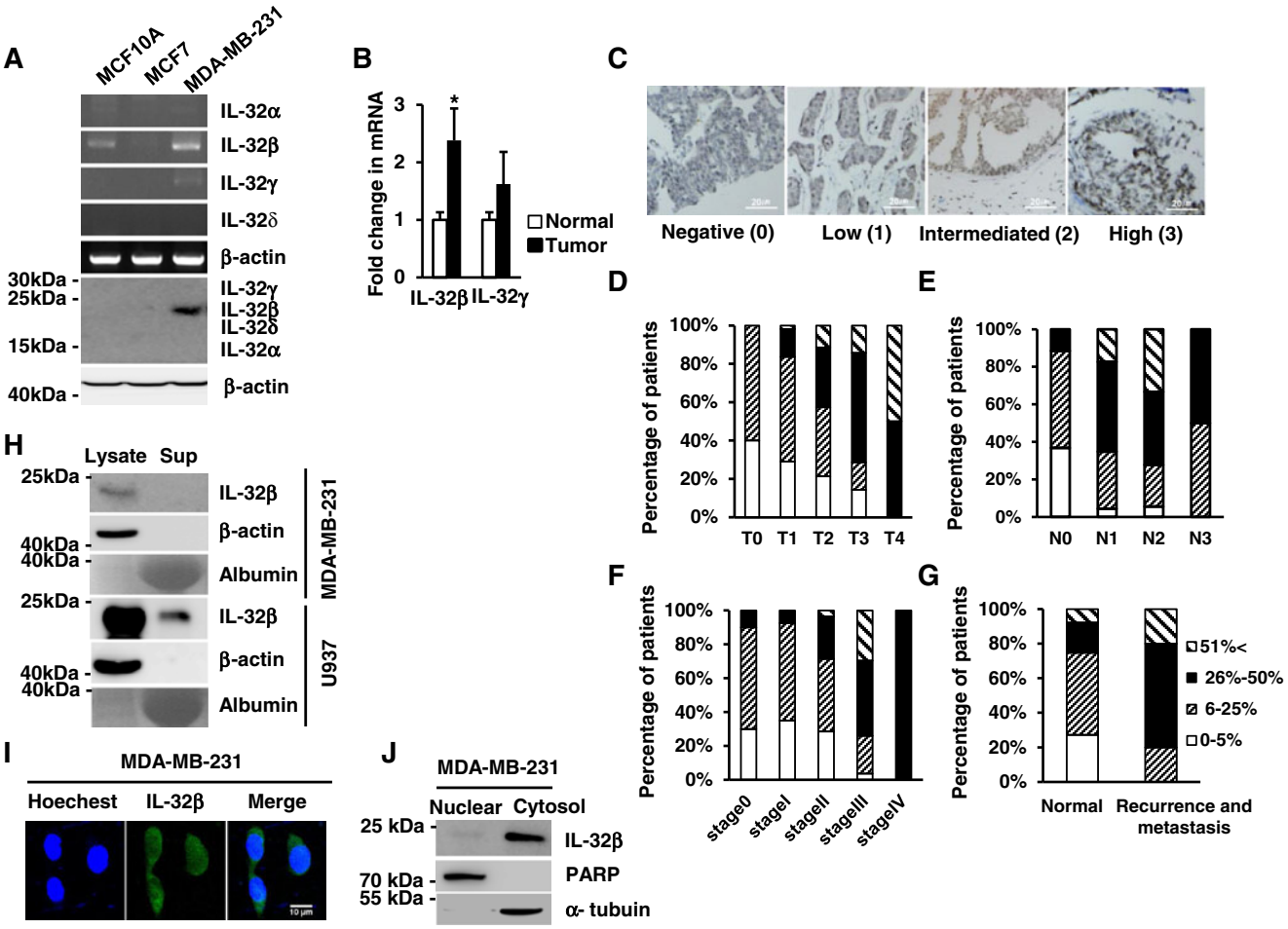

Fig. 1 Expression patterns and sub-cellular localization of IL-32β in breast cancer cell lines and tissues. **a** mRNA and protein levels of IL-32 isoforms detected by RT-PCR and immunoblotting. **b** Fold changes in IL-32 mRNA isoform expression detected by RT-PCR. **c** Representative photomicrographs of the grades of staining intensities used to score IL-32β expression in tissue microarrays. **d**–**g** The percentages of patient samples are indicated on the *y*-axis. The following indicators are plotted on the *x*-axis: (**d**) Tumor size; T0, no evidence of primary tumor; T1≤20 mm; 20<T2≤50 mm; T3≥ 50 mm; T4, direct extension to skin or chest wall, (**e**) Number of lymph node metastases, (**f**) Tumor stage, and (**g**) Follow-up data. **h** IL-32β expression levels in lysates and supernatants of MDA-MB-231 and U937 cells determined by immunoblotting. **i** Sub-cellular localization of IL-32β was determined by confocal microscopy. **j** IL-32β levels in cytosolic and nuclear fractions determined by immunoblotting

32β expression increased with increasing numbers of lymph node metastases, classified as N0 (0), N1 (1–3), N2 (4–9) or N3 (> 10) (Fig. 1e), and with increasing stage (stage 0, tumor in situ and N0; stage I, T0-T1 and N0- N1; stage II, T0-T3 and N0-N1; stage III, T0-T4 and N0-N3; stage IV, any T, any N, and M1; M, metastasis) (Fig. 1f). Finally, clinical follow-up data indicated that patients with recurrences and metastases exhibited increased IL-32β expression levels (Fig. 1g). Taken together, we found statistically significant correlations between IL-32β expression levels and tumor size, number of lymph node metastases and tumor stage.

Because IL-32 is known as a secretory protein [1], we set out to assess whether IL-32β is also secreted by MDA-MB-231 cells. By doing so, we found that IL-32β was not detectable in a concentrated culture supernatant, whereas a readily detectable level of IL-32β was found to be secreted by the control human histiocytic leukemia cell line U937 (Fig. 1h). Since we found that IL-32β is not secreted by the MDA-MB-231 cells, we next examined its sub-cellular localization. Using immunocytochemistry we found that IL-32β was primarily localized in the cytosol and weakly in the nucleus (Fig. 1i). We also examined the presence of IL-32β in the sub-cellular fractions by immunoblotting and, again, found that most of the IL-32β protein was localized in the cytosol (α-tubulin positive) and a barely detectable amount in the nucleus (PARP positive) (Fig. 1j).

3.2 IL-32β increases MDA-MB-231 cell migration and invasion

To determine the role of IL-32β in MDA-MB-231 cells, we first examined whether IL-32β affects proliferation, migration, and invasion using exogenous IL-32β over-expression and siRNA-mediated IL-32β knockdown. We found that IL-32β over-expression and knockdown did not affect the proliferative

Table 1 Number of patients classified by IL-32β intensities

Parameters (IL-32β intensity)	Negative (0–5 %)	Low (6–25 %)	Intermediate (25–50 %)	High (≥ 51 %)	*P*-value
Tumor size	34	59	33	11	0.001†
T0	4	6	0	0	
T1	16	30	8	1	
T2	13	22	19	7	
T3	1	1	4	1	
T4	0	0	2	2	
Lymph node metastasis	34	59	31	10	0.001†
N0	32	45	10	0	
N1	1	7	11	4	
N2	1	4	7	6	
N3	0	3	3	0	
Tumor Stage	34	59	33	10	0.001†
Stage 0	3	6	1	0	
Stage I	14	23	3	0	
Stage II	16	24	14	2	
Stage III	1	6	12	8	
Stage IV	0	0	3	0	
Follow -up data	35	58	31	11	0.071†
Normal	28	49	18	8	
Recurrence and metastasis	0	1	3	1	
Death	0	2	4	1	
Missed	7	6	6	1	

† *p*-value was calculated by chi-square test

capacity of the MDA-MB-231 cells during the indicated time intervals (Fig. 2a). Subsequently, we set out to examine the effect of IL-32β on the migration and invasion of MDA-MB-231 cells. After transfection of the cells with the IL-32β siRNA and expression plasmids, the IL-32β knockdown cells showed a decrease in migration and invasion, whereas the IL-32β over-expressing cells showed an increase in migration and invasion (Fig. 2b and c).

To identify the molecular players involved in the IL-32β-induced increases in migration and invasion, we examined the levels of the intermediate filament protein vimentin as an EMT marker [21]. Using immunoblotting, we found that vimentin expression was increased in the IL-32β over-expressing cells but decreased in the IL-32β knockdown cells (Fig. 2d). We next examined the expression of Slug, which is a well-known transcription factor involved in vimentin transcription, and found that it was increased in the IL-32β over-expressing cells and decreased in the IL-32β knockdown cells (Fig. 2d). Together, these results suggest that IL-32β-induced Slug stimulates vimentin expression and, ultimately, MDA-MB-231 breast cancer cell migration and invasion.

3.3 IL-32β increases VEGF production

Since MDA-MB-231 cells do not secrete IL-32β, we reasoned that cytosolic IL-32β may stimulate the secretion of a ligand that may be associated with the observed increases in migration and invasion. To assess this possibility, we examined the expression levels of several migration-stimulating ligands, including EGF, VEGF and IGF-1, using ELISA. By doing so, we found that the level of the VEGF protein (but not the EGF or IGF-1 proteins) was increased in IL-32β over-expressing cells, and decreased in IL-32β knockdown cells (Fig. 3a). To next determine whether the IL-32β-induced VEGF production is responsible for the enhanced migration, MDA-MB-231 cells were co-transfected with the IL-32β expression plasmid and a VEGF siRNA to block IL-32β-induced VEGF production and, subsequently, a migration assay was performed. We found that the increase in IL-32β-mediated migration, as well as the basal level of migration, was inhibited in the VEGF knockdown cells (Fig. 3b). To further assess whether the IL-32β-induced migration is mediated by VEGF, IL-32β over-expressing cells were treated with neutralizing anti-VEGF antibodies to block the activity of the secreted VEGF. We found that the IL-32β-enhanced migration was inhibited by treatment with these antibodies (Fig. 3c), strongly suggesting that the presence of IL-32β-induced VEGF increases MDA-MB-231 cell migration and invasion. Next, we examined whether conditioned media (CM) from IL-32β knockdown MDA-MB-231 cells impairs tube formation of HUVEC cells due to a lack of VEGF production. Through this assay, we found that the tube formation of

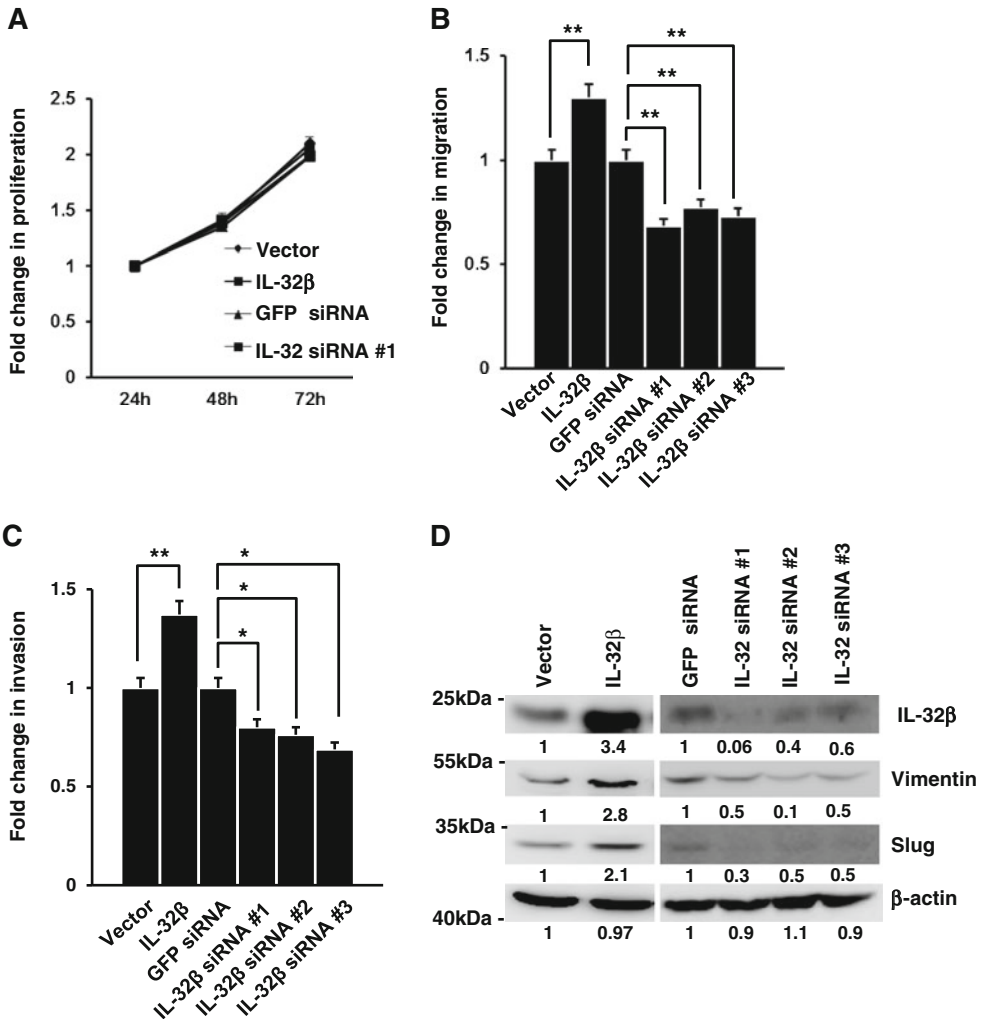

Fig. 2 Effect of IL-32β on migration and invasion in MDA-MB-231 cells. **a** MDA-MB-231 cells transfected with an IL-32β-expression plasmid and IL-32 siRNA #1. Cell proliferation was determined at the indicated time intervals. **b** MDA-MB-231 cells transfected with an IL-32β expression plasmid, a GFP siRNA and IL-32β siRNAs (#1, #2 and #3) seeded onto matrigel-uncoated wells to determine migration. Cell migration was measured using Calcein-AM after 12 h. The data shown represent one of three independent experiments carried out in triplicate. ** $p<0.01$. **c** MDA-MB-231 cells transfected with an IL-32β expression plasmid, a GFP siRNA and IL-32β siRNAs (#1, #2 and #3) seeded onto matrigel-coated wells to determine invasion. Cell invasion was measured using Calcein-AM after 24 h. The data shown represent one of three independent experiments carried out in triplicate. *$p<0.05$, ** $p<0.01$. **d** Protein levels of vimentin, Slug and IL-32β determined by immunoblotting 48 h after transfection. *Numbers* indicate the relative densities of each band

CM-treated HUVEC cells was dramatically reduced compared to that of control cells (Fig. 3d). These results substantiate our notion that the enhanced migration induced by IL-32β is due to the increased production of VEGF. In order to unravel how IL-32β increases VEGF production, IL-32β over-expressing MDA-MB-231 cells were treated with AKT, NF-κB, and Src inhibitors, since the corresponding signal transduction pathways are known to regulate VEGF expression in cancer cells [22, 23]. The resulting VEGF levels were measured by immunoblotting. We found that treatment with the Src inhibitor (but not the NF-κB and AKT inhibitors) decreased the IL-32β-mediated VEGF production (Fig. 3e). These results indicate that IL-32β increases VEGF production through activation of the Src signaling pathway.

3.4 Hypoxia-induced VEGF production is mediated by IL-32β

Hypoxic conditions are known to stimulate the production of VEGF [24] and, in turn, to increase the migration of MDA-MB-231 cells [25]. Thus, we set out to investigate whether IL-32β is related to hypoxia-induced VEGF production. To this end, MDA-MB-231 cells were exposed to hypoxic conditions for the indicated time intervals and, by doing so, increases in IL-32β and VEGF production were first observed 6 h after initiation of the hypoxic conditions (Fig. 4a). To assess whether the hypoxia-induced VEGF production is mediated by IL-32β, IL-32β knockdown cells were exposed to hypoxic conditions and VEGF levels were measured by ELISA. We found that the hypoxia-induced VEGF production was impaired in the IL-32β knockdown cells (Fig. 4b). These findings show that the hypoxia-induced VEGF production is mediated by intracellular IL-32β.

3.5 The IL-32β-VEGF-STAT3 pathway affects the migration of MDA-MB-231 cells

To determine the downstream signaling pathway of IL-32β-induced VEGF production, we measured STAT3 activation, since most VEGF functions are mediated by the stimulation of

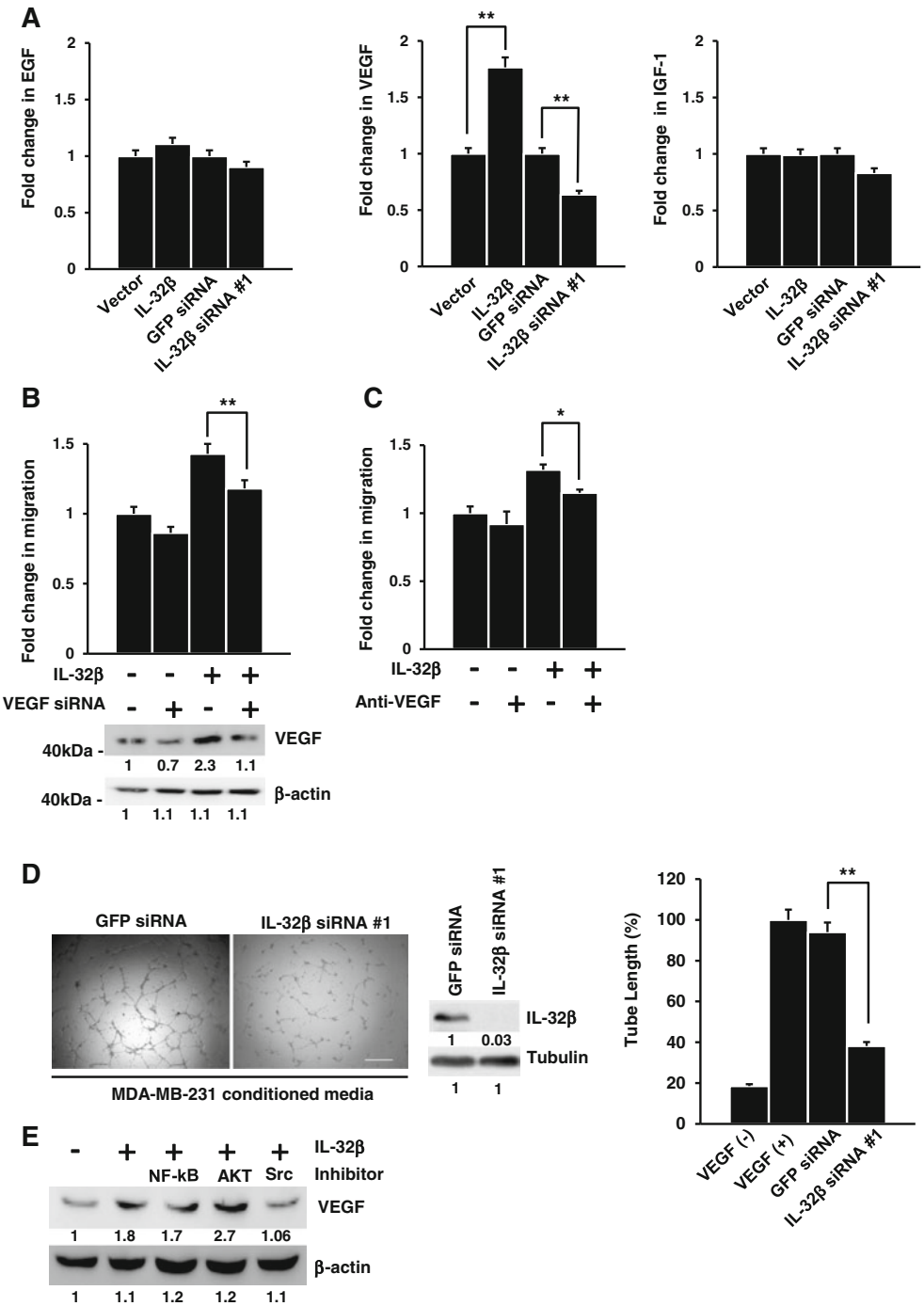

Fig. 3 IL-32β stimulates VEGF production and increases migration through the induction of VEGF production in MDA-MB-231 cells. **a** Levels of EGF, VEGF and IGF-1 in the culture supernatants measured by ELISA 48 h after transfection. **b** MDA-MB-231 cells co-transfected with 2.5 ug of IL-32β expression plasmid together with 20 nM of VEGF siRNA or alone, seeded onto matrigel-uncoated wells to determine migration 24 h after transfection. Cell migration was measured using Calcein-AM after an additional 24 h. The knockdown efficiency of VEGF siRNA was determined by immnoblotting. **c** Cells were transfected with 2.5 ug of IL-32β expression plasmid, and then 10 ug/ml of neutralizing anti-VEGF antibody was added to block VEGF function. Cell migration was measured using Calcein-AM 24 h after the treatment of anti-VEGF antibody.*$p<0.05$. **d** MDA-MB-231 cells were transfected with IL-32β siRNA, and CM was collected after 24 h. HUVEC cells were incubated for 10 h with VEGF and CM from IL-32β siRNA-treated MDA-MB-231 cells. Tube formation was measured at 40× magnification. Size bar=20 μm. **$p<0.01$. **e** IL-32β-transfected MDA-MB-231 cells were treated with 10 uM of AKT, NF-κB and Src inhibitors for 24 h, after which the protein levels of VEGF were determined by immunoblotting. *Numbers* indicate relative densities of each band

STAT3 [26, 27]. To this end, the cells were treated with recombinant VEGF and, subsequently, the levels of STAT3 phosphorylation were examined. We found that the level of STAT3 phosphorylation peaked at 15 min and returned to the basal level at 1 h after VEGF treatment (Fig. 5a). Since MDA-MB-231 cells express IL-32β, we reasoned that the level of phosphorylated STAT3 should be decreased by knocking down IL-32β. Indeed, we found that IL-32β knockdown cells exhibited a decreased STAT3 phosphorylation level (Fig. 5b). To verify that IL-32β-induced VEGF production is associated with the increased phosphorylation of STAT3, an IL-32β expression plasmid was transfected together with a VEGF siRNA and, subsequently, STAT3 phosphorylation was examined using immunoblotting. We found that the IL-32β-stimulated phosphorylation of STAT3 was blocked by VEGF siRNA treatment (Fig. 5c). To examine whether the IL-32β-VEGF-STAT3 signaling pathway is indeed associated with increased migration and invasion, MDA-MB-231 cells were simultaneously transfected with an IL-32β expression plasmid and a STAT3 siRNA. Clearly, these transfected cells

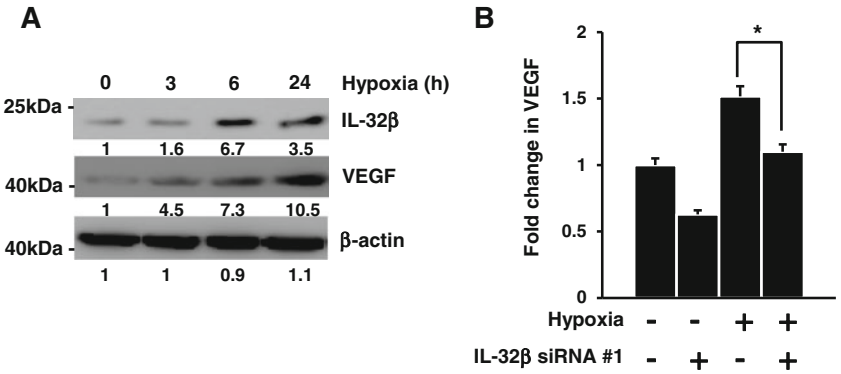

Fig. 4 IL-32β expression increases under hypoxic conditions. **a** MDA-MB-231 cells were placed in a hypoxic chamber during the indicated time intervals (0–24 h). The protein levels of IL-32β and VEGF were determined by immunoblotting. Numbers indicate relative densities of each band. **b** MDA-MB-231 cells were transfected with IL-32β siRNA #1 and placed in a hypoxic chamber for 24 h. Next, the levels of VEGF were measured by ELISA.*$p<0.05$

failed to exhibit IL-32β-mediated increases in migration (Fig. 5d) and invasion (Fig. 5e).

In order to compare the observed IL-32β activity with that of another breast cancer cell line, we simultaneously examined the effect of IL-32β on the migration of MCF-7 and MDA-MB-231 cells. When MCF-7 and MDA-MB-231 cells were transfected with IL-32β-expression plasmids and IL-32β siRNAs, respectively, the migration of MCF-7 cells was enhanced and the cells demonstrated morphologies typical of migrating cells, whereas IL-32β-depleted MDA-MB-231 cells became crowded (Fig. 6a and b). It is well known that a high level of E-cadherin expression inhibits tumor cell migration. We used this knowledge to determine whether IL-32β affects E-cadherin expression and found that the IL-32β-

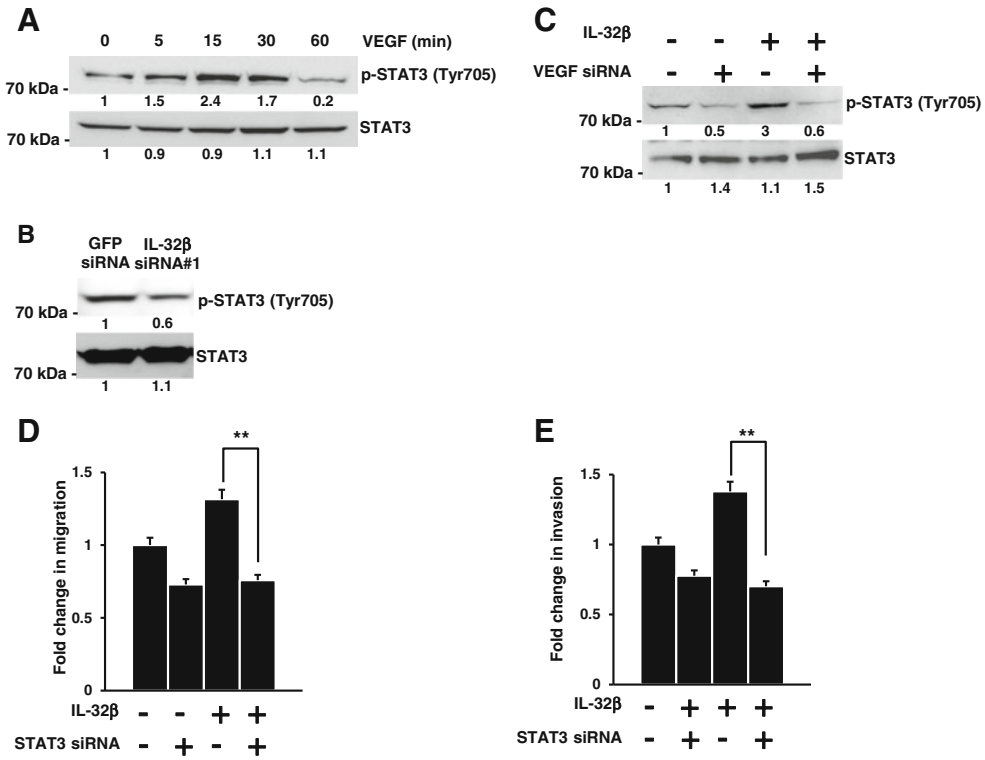

Fig. 5 IL-32β-induced VEGF activates STAT3 in MDA-MB-231 cells. **a** MDA-MB-231 cells were treated with recombinant VEGF, and the levels of phosphorylated STAT3 were determined. **b** MDA-MB-231 cells were transfected with GFP and IL-32β siRNA, and the levels of phosphorylated STAT3 were determined. **c** MDA-MB-231 cells were transfected with an IL-32β expression vector alone or together with a VEGF siRNA, and the levels of phosphorylated STAT3 and STAT3 were determined. Numbers indicate relative densities of each band. **d** MDA-MB-231 cells were transfected with an IL-32β expression vector alone or together with a STAT3 siRNA and then seeded onto matrigel-uncoated wells for a migration assay. **$p<0.01$. **e** MDA-MB-231 cells were transfected with an IL-32β expression vector alone or together with a STAT3 siRNA and cell invasion was measured using Calcein-AM 24 h after the treatment. **$p<0.01$

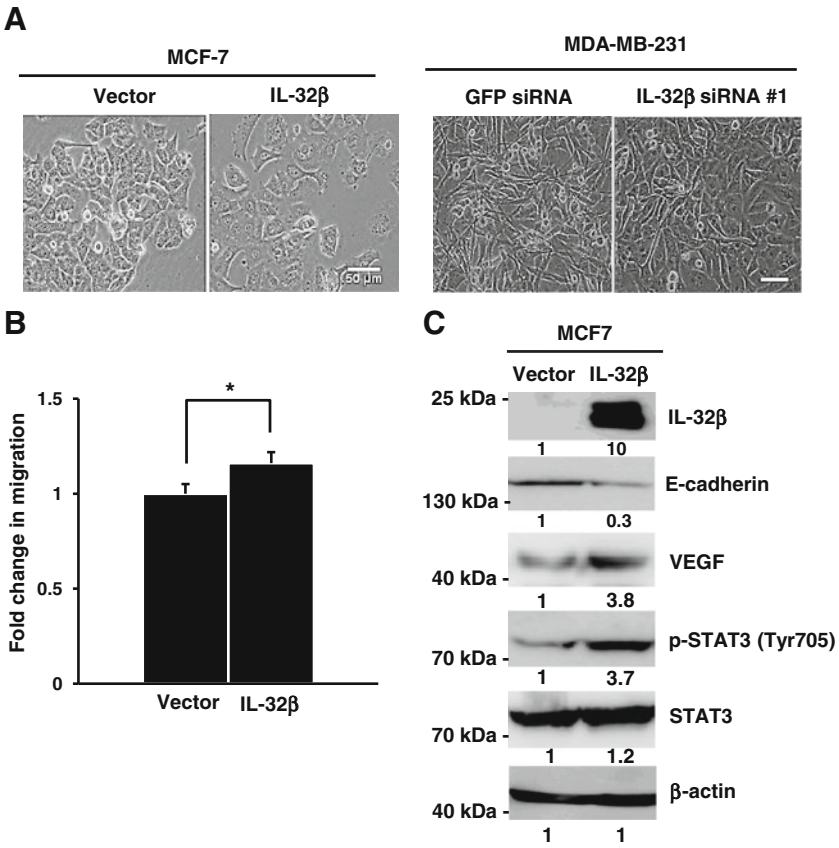

Fig. 6 IL-32β-induced VEGF activates STAT3 in MCF-7 cells. **a** MDA-MB-231 and MCF-7 cells were transfected with an IL-32β expression vector, and their morphology was assessed using light microscopy. **b** MCF-7 cells were transfected with an IL-32β expression vector and seeded onto matrigel-uncoated upper wells for a migration assay. Cell migration was measured using Calcein-AM 24 h after the treatment.*$p < 0.05$. **c** MCF-7 cells were transfected with an IL-32β expression vector, and the protein levels of E-cadherin, VEGF, STAT3 and phospho-STAT3 were determined. *Numbers* indicate relative densities of each band

transfected MCF-7 cells exhibited strongly decreased levels of E-cadherin and increased levels of VEGF (Fig. 6c). Since we found that VEGF-induced STAT3 activation is associated with an increased migration of MDA-MB-231 cells, the level of STAT3 phosphorylation was examined in the MCF-7 cells. The IL-32β-transfected MCF-7 cells showed a clear increase in STAT3 phosphorylation (Fig. 6c), indicating that IL-32β expression is similarly involved in the migration of MCF-7 cells.

4 Discussion

Previously, a high IL-32 expression in primary breast cancer tissues was observed in 10 of 14 cases examined using RT-PCR analysis [17], and a web-based database reports that out of 489 cases of invasive breast carcinoma listed, 24 cases showed IL-32 amplification (The cBio Cancer Genomics Portal, http://www.cbioportal.org). Using tissue microarray analysis, we here showed that IL-32β expression was significantly increased in advanced breast cancer stages compared to earlier stages. Recently, it was reported that tumor depth and lymph node metastases develop more frequently in IL-32-positive gastric cancer patients [28]. By combining these results, it appears that IL-32β is increasingly expressed during the progression of breast and gastric cancers. Initially, IL-32 was found to be secreted when human peripheral blood mononuclear cells were stimulated with Con A [1], but later it was found that certain cell types do not secrete IL-32 [6, 11, 29, 30]. Here, we show that even hypoxia-induced IL-32β was not secreted. Thus, we consider it warranted to ascertain whether the serum concentrations of IL-32β are elevated in cancer patients, as well as which cell type is responsible for such elevated levels. In addition, the putative role of IL-32β in tumor-microenvironment interactions remains unclear, as IL-32β may be secreted by immune cells, such as U937 cells, but not by certain tumor cells.

Others have found that knocking down IL-32 expression in HS5 stroma cells largely reduces the secretion of VEGF [31] and that, in contrast, knocking down IL-32 expression in normal human bronchial epithelial cells significantly increases the secretion of VEGF and that, in addition, the culture supernatants of these latter cells enhance in vitro angiogenesis [12]. Which isoform of IL-32 is expressed in normal human bronchial epithelial cells remains, however, to be determined in order to be able to explain the differential regulation of the effects of IL-32 on VEGF production. In this study, we show that IL-32β increases VEGF production in both MDA-MB-231 and MCF-7 cells, similar to HS5 stromal cells. It has also been reported that the expression levels of IL-6, IL-8 and

VEGF in both tumor-infiltrating leukocytes and tumor cells are positively correlated with IL-32β expression within the same cell population [16]. These data support our finding that IL-32β stimulates VEGF production. On the other hand, since IL-32β-activated Src increases VEGF production (Fig. 3e), it is possible that the activated Src-induced VEGF binds to the VEGF receptor 2 (VEGFR2) and, by doing so, contributes to the proliferation, migration and invasion of breast cancer cells. Upon the binding to its receptor, VEGF is able to activate Src kinase and p38 MAPK, after which the activated Src can phosphorylate STAT3, leading to dimerization of phosphorylated STAT3 [32, 33]. Dimerized STAT3 in the nucleus increases VEGF production [34]. Thus, VEGF and STAT3 may form an autocrine amplifying loop through Src activation by IL-32β.

Many aggressive breast cancer cell lines express vimentin [35]. We found that the MDA-MB-231 cell line expresses relatively high levels of vimentin when compared to the MCF-7 cell line. Nevertheless, we found that exogenous over-expression of IL-32β in MDA-MB-231 cells further increased the level of vimentin, which is consistent with a report in the literature showing that the amount of vimentin is positively correlated with the migratory capacity of breast cancer cells [36]. The E-cadherin repressor Slug positively regulates vimentin expression [21]. Since we found that exogenous over-expression of IL-32β increased both Slug and vimentin expression, it is likely that IL-32β increases the migration of MDA-MB-231 cells through the Slug-vimentin signaling pathway. In addition to Slug, Twist has also been reported to act as an important EMT-associated transcription factor [37]. However, since MDA-MD-231 cells do not express Twist, Slug is likely to be the main transcription factor associated with the IL-32β-VEGF-Slug-vimentin signaling pathway underlying the migration of MDA-MB-231 cells. In MCF-7 cells, exogenous IL-32β expression resulted in decreased levels of E-cadherin. Thus, IL-32β appears to be associated with pathways underlying both EMT and migration in breast cancer cells.

The progression from normal cells to cancer cells is, among others, influenced by environmental and extracellular factors. The extracellular factors involved in this process include inflammatory cytokines, among which IL-6 is found to be up-regulated in epithelial cancers, such as breast and prostate cancers [38, 39]. IL-6 activates NF-κB, which plays a causative role in malignant transformation and progression [40, 41]. Furthermore, IL-32 is known to stimulate NF-κB for the production of proinflammatory cytokines and to be induced through the activation of NF-κB by IL-1β or LPS [30]. Thus, next to IL-6, IL-32β represents an important link between inflammation and cancer.

In summary, we have shown for the first time that a high level of IL-32β expression in breast cancer cells imposes increased migration and invasion capacities on these cells, and we uncovered the mechanism underlying this IL-32β-mediated increase in migration and invasion. In addition, we found that IL-32β-induced VEGF stimulates the activation of STAT3, which in turn increases migration and invasion.

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Conflict of interest All authors declare no conflicts of interest.

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