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Variation in IL6ST cytokine family function and the potential of IL6 trans-signalling in ERα positive breast cancer cells

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ABSTRACT

High expression of the transmembrane receptor IL6ST (gp130) has been identified as a predictive biomarker of endocrine treatment response in ERα-positive breast cancers. To investigate its function further in this disease, this study evaluated the expression, function and signalling of IL6ST in ERα-positive breast cancer cell lines and investigated crosstalk between ERα and IL6ST. IL6ST was differentially expressed in ERα-positive breast cancer cell lines (low in MCF-7, high in ZR751 and T47D), while multiple soluble isoforms of IL6ST were identified. IL6ST is the common signal transducing receptor component for the IL6ST family of cytokines and the effects of seven IL6ST cytokines on these cell lines were studied. These cytokines caused differential growth and migration effects in these cell lines e.g. MCF-7 cells were growth-stimulated, while ZR751 cells were inhibited by IL6 and OSM.. Activation of the STAT and ERK pathways is associated with these responses. Evidence to support trans-signalling involved in cell growth and migration was obtained in both MCF-7 and ZR751 models. Interaction between cytokines and estrogen on ERα-positive cell lines growth were analysed. High expression of IL6ST (in ZR751) may lead to growth inhibition by interacting cytokines while lower expression (in MCF-7) appears associated with proliferation. High IL6ST expression is consistent with a more beneficial clinical outcome if cytokine action contributes to anti-estrogen action.

1. Introduction

Interleukin-6 signal transducer (IL6ST) (gp130) is a subunit of several receptor complexes for the interleukin-6 (IL6) family of cytokines. This cytokine family includes interleukin-6 (IL-6), oncostatin M (OSM), interleukin-11 (IL-11), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1) and interleukin-27 (IL-27) [1–3]. These cytokines bind first to their specific receptor (α subunit) to form a complex which then recruits IL6ST to generate either homo- or hetero-dimers; in turn this leads to activation of downstream signalling pathways which include Janus kinase / signal transducer and activator of transcription (JAK/STAT), the PI3K/Akt, and the Ras-ERK1/2 MAPK cascade pathways [1]. Interleukin-6 and IL-11 bind to specific non-signalling receptors (IL-6R and IL-11R respectively) which then interact with IL6ST to initiate signalling, while OSM, LIF and IL27 bind to their receptors (OSM-R, LIF-R and WXS-1 respectively) which are capable of signalling as well as via IL6ST [1,2] (Supplementary Fig. 1). Ciliary neurotrophic factor and CT-1 bind to their specific receptors (CNTFR and CT-1R respectively) but also interact with LIF-R in addition to IL6ST (Supplementary Fig. 1) [1,2].

We are not aware of previous studies that have explored in detail the functionality of IL6ST in estrogen receptor positive (ER+) breast cancer cell lines. Of the IL6ST interacting cytokines, most studies in breast cancer to date have focussed on IL-6 [4–8] and OSM [9–14] while less attention has been paid to other IL6 family members. Interleukin-6 is a key cytokine in breast cancer and its signalling processes play critical roles in tumour promotion. IL-6 signalling can operate through either classic (via membrane-bound IL-6R) or trans-signalling (via soluble IL-6R) mechanisms which are activated via IL6ST [2,3]. *trans*-Signalling has been shown to contribute significantly to the biology of IL-6 because it dramatically expands the spectrum of target cells for IL-6 which do not express membrane-bound IL-6R and targeting the IL-6 trans-signalling pathway could inhibit signalling [2]. Expression of soluble IL6-R (sIL-6R) has been demonstrated in human breast cancer cell lines and patient

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cancers providing the potential for IL-6 trans-signalling to mediate the effects of IL-6 in breast cancer cells [15,16]. However, there is limited knowledge about sIL-6R and soluble IL6ST (sIL6ST) in breast cancer cell lines with respect to their expression and roles [17]. At least four different forms of human sIL6ST (sgp130) with different molecular weights have been reported [18 –21]. These soluble forms can be generated either by proteolytic cleavage "shedding" or by alternative splicing but the cellular origin is poorly understood. Most significantly, they can act as potent inhibitors of trans-signalling [3,22]. The soluble forms of IL6ST which occur naturally have been detected at relatively high concentration up to 390 ng/ml in human serum [23].

The majority of human breast tumours are initially dependent upon estrogen to support their growth with the highest incidence of breast cancer occurring in postmenopausal women. Estrogen synthesis in peripheral tissues, including normal and malignant breast tissues, can be regulated by cytokines such as IL-6. Expression levels of aromatase, estradiol 17β-hydroxysteroid dehydrogenase and estrone sulfatase activities are all increased by IL-6 [24,25]. Furthermore, a positive correlation has been found between IL-6 and ERα expression in breast cancers [26]. IL6ST expression is predictive of outcome to endocrine therapy in breast cancer [27]. It is a component molecule of two separate assays predicting response to endocrine therapy in breast cancer, namely the Endopredict test [28] and the Edinburgh EndoAdjuvant2 Clinical test [29]. It is unclear as to why IL6ST expression helps predict response and further studies are required.

In the present study, we investigated the expression of both full length and soluble forms of IL6ST and its associated cytokine receptors in ER+ breast cancer cell lines. We investigated the possible impact of IL6ST family cytokines in regulating the growth and migration of breast cancer cell lines. Furthermore, we studied the possible role of IL-6 trans-signalling in these cell lines in order to advance understanding of this pathway in breast cancer. We assessed the possible effects of IL6ST cytokines on influencing the response to estrogen and studied further interactions between the IL6ST and ERα pathways. Finally, we knocked down the expression of IL6ST in a breast cancer cell line to assess impact on growth response, cell signalling and transcription. These studies provide further insight into the interaction between IL6ST interacting cytokines and the estrogen signalling pathway in breast cancer and offer an indication as to why high IL6ST expression is a predictor of good endocrine response.

2. Materials and methods

2.1. Cell lines and culture condition

The ERα-positive MCF-7, ZR-75-1 and T47D breast cancer cell lines were cultured in DMEM (phenol red positive, Gibco) supplemented with 10% heat-inactivated FBS serum and 1% penicillin / streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. The MCF-7/LCC1 and MCF-7/LCC9 cell lines were grown in DMEM (phenol red free, Gibco) supplemented with 5% double charcoal-stripped serum, glutamine (0.3 mg/mL), and 1% penicillin/streptomycin in a humidified atmosphere 5% CO_2 at 37 °C and were a kind gift from Professor Bob Clarke, Georgetown University [30,31].

2.2. Cell viability analysis by sulforhodamine B (SRB) assay

Cells were harvested by trypsinisation, then seeded at densities of 1–2 × 10^5 cells/ml into 96-well cell culture plates and incubated for 48 h in the cell culture conditions described above. They were then treated with 100 ng/ml of each cytokine and incubated for 5 days. Cells were then fixed using 25% cold trichloroacetic acid (Sigma) and incubated for 1 h at 4 °C. Plates were washed, air-dried, and stained with sulforhodamine B (Sigma) dye (0.4% solution in 1% acetic acid). After being washed with 1% acetic acid, Tris buffer (10 mM, pH 10.5) was added to each well 1 h prior to the optical density (OD) being read using an iMRAK microplate Reader (BIO-RAD) reader at 540 nm. Cytokines were obtained from PeproTech and all other reagents from Sigma-Aldrich unless specified. Recombinant Human GP130 Fc Chimera was from R&D System (671-GP-100) while sIL6-R was from Preprotech. BAZ was obtained from Tocris and 4-OHT from Sigma.

2.3. Scratch wound cell migration assay

The migration assay was performed using the IncuCyte® Live Cell Analysis Imaging System. Prior to initiating a 96-well migration assay, cells were grown to 90% confluence. Then, homogenous wound scratches were created by the WoundMaker tool which is a 96-pin mechanical device. Cells were then treated with cytokines as indicated in 1% FBS and incubated in the Incucyte ZOOM Live-cell analysis system (Essen BioScience). The wound density images were taken every 3 h and the results analysed using IncuCyte S3 software.

2.4. Protein expression analysis by western blotting

Protein lysates (40 μg) were electrophoretically resolved on 7.5% SDS-PAGE and transferred overnight onto nitrocellulose membranes (Millipore). After transfer, membranes were blocked with LiCor Odyssey Blocking Buffer for 1 h before probing with the appropriate primary antibody overnight at 4 °C. Primary antibodies used for western blotting were as follows: IL6ST (CD130 /GP130) (Thermo Fisher), phospho-STAT3 (Tyr705), STAT3, P44/42 MAPK (ERK1/2), phospho-P44/42 MAPK (ERK1/2) (Cell Signalling) and used at 1:1000 dilution. Antibodies targeting ERα (ProteinTech) and PR (Epitomics) were used at 1:250 dilution, IL6R (Themo Fisher) at 1:500 dilution and OSMR (Santa Cruz) and LIFR (Santa Cruz) at 1:100 dilution. Alpha-Tubulin (Abcam Ab 7291) and β-Actin (Abcam 8227) antibodies were used as loading controls (at 1:10000 dilution). The membranes were incubated with fluorescently labelled secondary antibodies diluted with Odyssey Blocking Buffer and then scanned on a Li-Cor Odyssey scanner, and the fluorescence value (integrated intensity, I.I.) corresponded with the detected protein expression levels.

2.5. Analysis of IL6ST variants by RT-PCR

Primers to measure expression levels of soluble IL6ST isoforms were designed and these sequences were as follows:

Full-length, AACACATCTGGCCTAATGTTCC and TACCACTGC TGTGTCCTTCAG; RAPS, TCCACCCGATCTTCATTCACTG and AAGGAGGCAATGTTATCTTCATAGGT; E10, TCCATCCCATACTCAAG GCTAC and TCACAGATACAAACCTTGAAAGTCAC; Dimont, GCAGCATACACAGATGAAGGTG and TAAGCTGTAAGGTCCTCGTTGG; Sharkey, ACTACCCCAAAGTTTGAATT and TCACCTCACACTCCTCCAAGG; All gp130, GAACAGCATCCAGTGTCACC and CATTTTCTTCCCCTCGT TCAC.

RT-PCR was processed using a 7900HT Fast Real-Time PCR System. A Power SYBR Green RNA-to-CT™ 1-Step Kit was used to perform reverse transcription and amplification of RNA samples extracted as described above. Total reaction volume of 10 μl contained: 5 μl 2× SYBR Green master mix, 1 μl primer mix (1 μM); 0.08 μl RT Mix; 1.92 RNase-free water and 1 μl of RNA (5 ng). Real time cycler conditions were RT: 48 °C for 30 min; PCR: initial activation 95 °C for 10 min followed by 40 cycles of denaturation 95 °C for 15 s finally annealing and extension 60 °C for 1 min. A standard curve of 5 points for each primer set was

used to quantify RNA samples. Each sample was run in triplicate. Comparison of gene expression between samples were performed after normalization of all values to the geometric mean of three 'housekeeping genes" CCSER2, SYMPK and ANKRD17, which have been demonstrated to be less variable in breast cancer cell lines and tissues [32]. Primers used were: CCSER2, GACAGGAGCATTACCACCTCAG and CTTCTGAGCCTGGAAAAAGGGC; SYMPK, CTTCACCAAGGTTGTGCTGGAG and GCGCTTGAAGATCAGGTCTCGA; ANKRD17, CAAATGGTGGACACCTCGATGTG and CTAAGTAGCGCACCACCTTCAC.

2.6. IL6ST siRNA knockdown

Knockdown of IL6ST was performed using SMARTpool: ON-TARGETplus IL6ST siRNA (Dharmacon, L-005166-00-0005) with a mixture of four sequences targeting four different isoforms of IL6ST Oligonucleotides and transfection reagents were purchased from Dharmacon and IL6ST siRNA transfection was carried out following the manufacturer's instructions.

2.7. Reverse phase protein array analysis

Cells within wells, in biological triplicate, were washed with PBS and lysed as described [33]. All lysates were normalised to 2 mg/ml and a dilution series comprising four serial twofold dilutions of each sample was performed. The diluted samples were printed onto nitrocellulose-coated slides (Grace Bio-Labs) in technical triplicate under conditions of constant 70% humidity using the Aushon 2470 array platform (Aushon Biosystems). Slides were hydrated in blocking buffer (Thermo Fisher Scientific) and then incubated with validated primary antibodies (all diluted 1:250 in blocking buffer) (Supplementary Table 1). Bound antibodies were detected by incubation with anti-rabbit DyLight 800-conjugated secondary antibody (New England BioLabs). An InnoScan 710-IR scanner (Innopsys) was used to read the slides, and images were acquired at the highest gain without saturation of the fluorescence signal. The relative fluorescence intensity of each sample spot was quantified using Mapix software (Innopsys). The linear fit of the dilution series of each sample was determined for each primary antibody, and median relative fluorescence intensities from the 4 dilution points were calculated. Signal intensities were normalised by global sample median normalization.

2.8. Modulation of IL6ST expression using CRISPR/CAS9

Lentiviral transduction which contains eSpCas9, guide RNA (sgRNA) and selection marker (Puromycin) in an All- In-One vector is manufactured by GenScript. Three sgRNAs were selected using genome-wide databases containing pre-validated guide RNA (gRNA) sequences designed by the Broad Institute of Harvard and MIT [50] and which target different exons were chosen to silence IL6ST expression. The sequences of these gRNAs for IL6ST were gRNA 1: TTTGAGTTGCATTGTGAACG (Exon 5), gRNA 2: AAGGAGCAATATACTATCAT (Exon 4) and gRNA3: ATTCGCTGTATGAAGGAAGA (Exon 8).

2.9. Lentiviral particle generation

The plasmid DNA (lentiviral vector) was first amplified and isolated using a QIAGEN Plasmid Maxi Kit (Qiagen, 12,163), as per manufacturer's instructions. The production of viral particles was carried out by transfection of the 293 T cell line. Cells were seeded in a 10 cm dish at 140–180 $\times$ 10^4 cells, grown in DMEM media containing 10% FBS and antibiotics and maintained at 37 °C in 5% CO_2 throughout the virus production period. Next day or when cells reached 90–95% confluence, the transfection mixture for each plate was prepared in 500ul of Optimum media. The DNA mixture was mixed thoroughly and incubated for 10 min, which was then added to the cells in dropwise manner and the dish was incubated overnight. Following the incubation period, the medium containing the transfection mixture was replaced with 10 ml fresh medium and dish was incubated for 48 h. The first batch of virus was then harvested from each dish and stored at 4 °C. Then 10 ml of fresh media was added to harvest the second batch after overnight incubation. Finally, the two virus batches were mixed and filtered through a 0.45 μm low protein binding Durapore filter (Millipore) to remove cell debris. Final viruses were then aliquoted and stored in −80 °C.

2.10. Viral transduction

ER+ breast cancer cell lines were seeded in 6 wells plates and grown in DMEM media containing 10% FBS and antibiotics and maintained at 37 °C in 5% CO_2. Once they reached 50% confluence, media was replaced with 2 ml virus plus polybrene (1:1000). The plates were then wrapped with parafilm and spun at room temperature at 1000 rpm for 10 min. The plates were then incubated overnight. Next day, the growth medium was changed to fresh media and incubated at 37 °C overnight to allow the cells to recover. Next day, an appropriate selection marker (antibiotic) was added. Puromycin was used at 2 μg/ml and blasticidin at 10 μg/ml for a period of 4–6 days, until all uninfected control cells had died.

2.11. Gene expression analysis

RNAseq gene expression analysis was performed to investigate the transcriptome expression of ZR751- control (CAS9) and ZR751- IL6ST-KO (CAS9-CRISPR) cell lines. The experiment was performed under 5% charcoal stripped FCS and 4 groups were treated as follows: untreated control, IL-6 (100 ng/ml), E_2 (1nM) + IL-6, and IL-6 + E_2 + 4-OHT (0.1 μM). Three replicates for each condition were prepared and collected after 8 h. In order to analyse the RNAseq data, the Rank Products function within TMeV software package with *p*-value being set at 0.01. The two cell lines were compared at each time point separately. The genes in each cluster were analysed for common pathways and biological processes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) online resource.

2.12. Statistical analysis

All statistical analyses were performed using GraphPad Prism V.8. The comparisons between groups were performed using ordinary one-way ANOVA. Data are expressed as the mean ± SD. All differences and associations were considered statistically significant if *p*-value was below 0.05.

3. Results

3.1. ER+ breast cancer cell lines express varying levels of IL6ST

The variation of IL6ST gene expression level along with expression of ESR1 (ERα) and the estrogen-responsive progesterone receptor (PR) was first evaluated in an integrated dataset from three separate datasets of panels of breast cancer cell lines (ArrayExpress (E-TABM-194) and NCBI GEO (GSE10890, GSE12777). This analysis revealed that IL6ST mRNA is differentially expressed across breast cancer cell lines but is not clearly associated with ESR1 (ERα) or PR expression (Fig. 1A). Three ERα-positive cell lines (MCF-7, T47D, ZR751) were chosen for further study. These cell lines possess varying expression levels of IL6ST with T47D and ZR751 having relatively high levels and MCF-7 cells having relatively low levels of IL6ST mRNA (Fig. 1B). Western blot analysis was consistent with this bioinformatic analysis indicating that the full-length protein form of IL6ST (130KDa) is more highly expressed in T47D, and ZR751 cells than in MCF-7 cells with other bands at lower molecular weight size (100 kDa) (Fig. 1C). To confirm that these bands were IL6ST, siRNA targeting IL6ST was used to knockdown IL6ST. The three cell lines were transfected with SMARTpool: ON-TARGETplus IL6ST siRNA

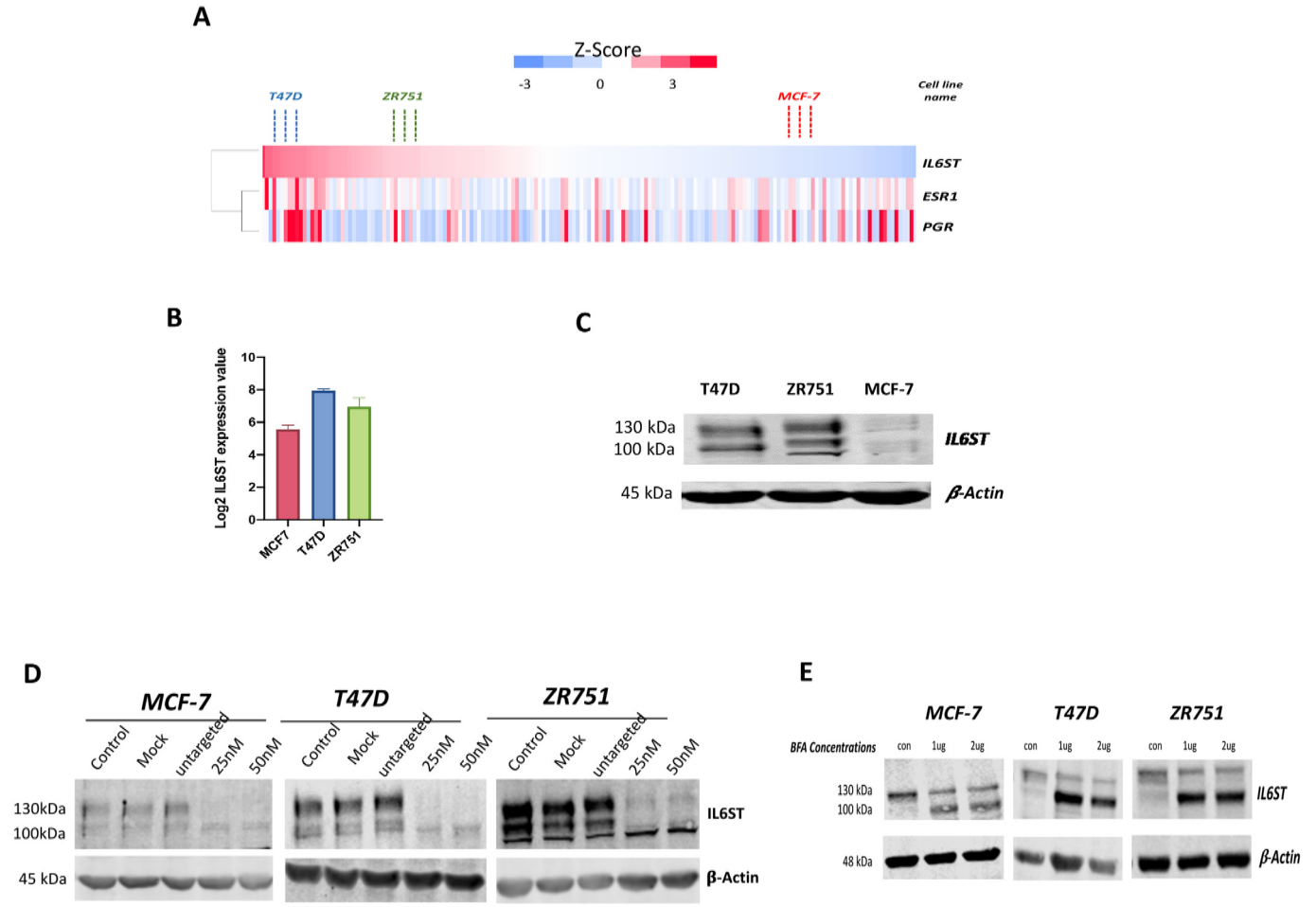

Fig. 1. Expression of IL6ST and its isoforms across breast cancer cell lines.
A. Heatmap showing the expression of IL6ST for three breast cancer cell lines alongside expression of ESR1 and PGR genes. Heatmap colour are log2 mean-centred values, with red indicating high value and blue indicating low value. The small bar indicates the value of heatmap colour intensity.
B. Bar chart shows the Log2 IL6ST mRNA expression value in the cell lines using values exported from the panel of three studies of breast cancer cells shown in the previous figure. Error bars are S.D.
C. Western blot of IL6ST expression in the cell lines.
D. Western blot of the cell lines treated with IL6ST SMARTpool siRNA. All cells were treated with IL6ST siRNA at final concentrations 25uM and 50uM alongside three controls. The control represents cell without any transfection, mock control represents cells with transfection agent only and no siRNA while untargeted control represents cells transfected with 25uM untargeted siRNA. Whole lysates were collected after 24 h. The Thermo polyclonal antibody was used to detect IL6ST and β-Actin was used as the loading control. The molecular weight of each target is indicated.
E. Western blot analysis of whole cell lysates of the breast cancer cell lines cultured without and with different concentration of BFA as indicated for 6 h. The lysates were subjected to immunoblotting using IL6ST antibody. β-Actin was used as loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(25uM and 50uM) with a mixture of four sequences targeting four different isoforms of IL6ST. After 24 h transfection, the results of this experiment are consistent with both bands corresponding to IL6ST since both markedly decreased in the presence of siRNA at 25uM and 50uM concentrations compared with the control transfection (Fig. 1D). ZR751 cells contain an extra band on western analysis, but since it is not removed in the presence of IL6ST-targeted siRNA, it is unlikely to be IL6ST. However if it is a related molecule with similarities in sequence, we cannot rule out the possibility that this may contribute to the difference in cellular response between the cell lines as described below. The mature 130 kDa IL6ST is reported to be a highly glycosylated protein as it has 11 potential N-linked glycosylation sites in the extracellular domains of the predicted 101 kDa IL6ST [2]. Therefore, it was feasible that the 100 kDa band seen in the previous results could be a de-glycosylated form of IL6ST. To test this, the three breast cancer cell lines were treated with two different concentrations of Brefeldin A (BFA), an inhibitor of N-glycosylation [34]. This drug produced a shift in expression of IL6ST with reduction in expression of the higher (130 kDa) band and an increase in expression of the lower (100 kDa) band (Fig. 1E). This is consistent with the upper band representing the glycosylated form of IL6ST and the lower band, the un-glycosylated or minimally core glycosylated IL6ST.

3.2. *IL6ST cytokine family demonstrates variable biological responses in ER+ breast cancer cell lines*

The actions of seven exogenously added cytokines (IL-6, OSM, LIF, IL-11, CT-1, CNTF, IL-27) were then investigated to assess the variability of response on the growth of MCF-7, T47D, and ZR751 cell lines. Diverse proliferative responses were observed after five days treatment compared with the control (Fig. 2A). All IL6ST cytokine family members tested, except IL-27, produced an increase in the growth of MCF-7 cells. For T47D cells, no significant effect on proliferation was observed after treatment with IL6, CT-1, LIF or IL-27. Interestingly, OSM caused a

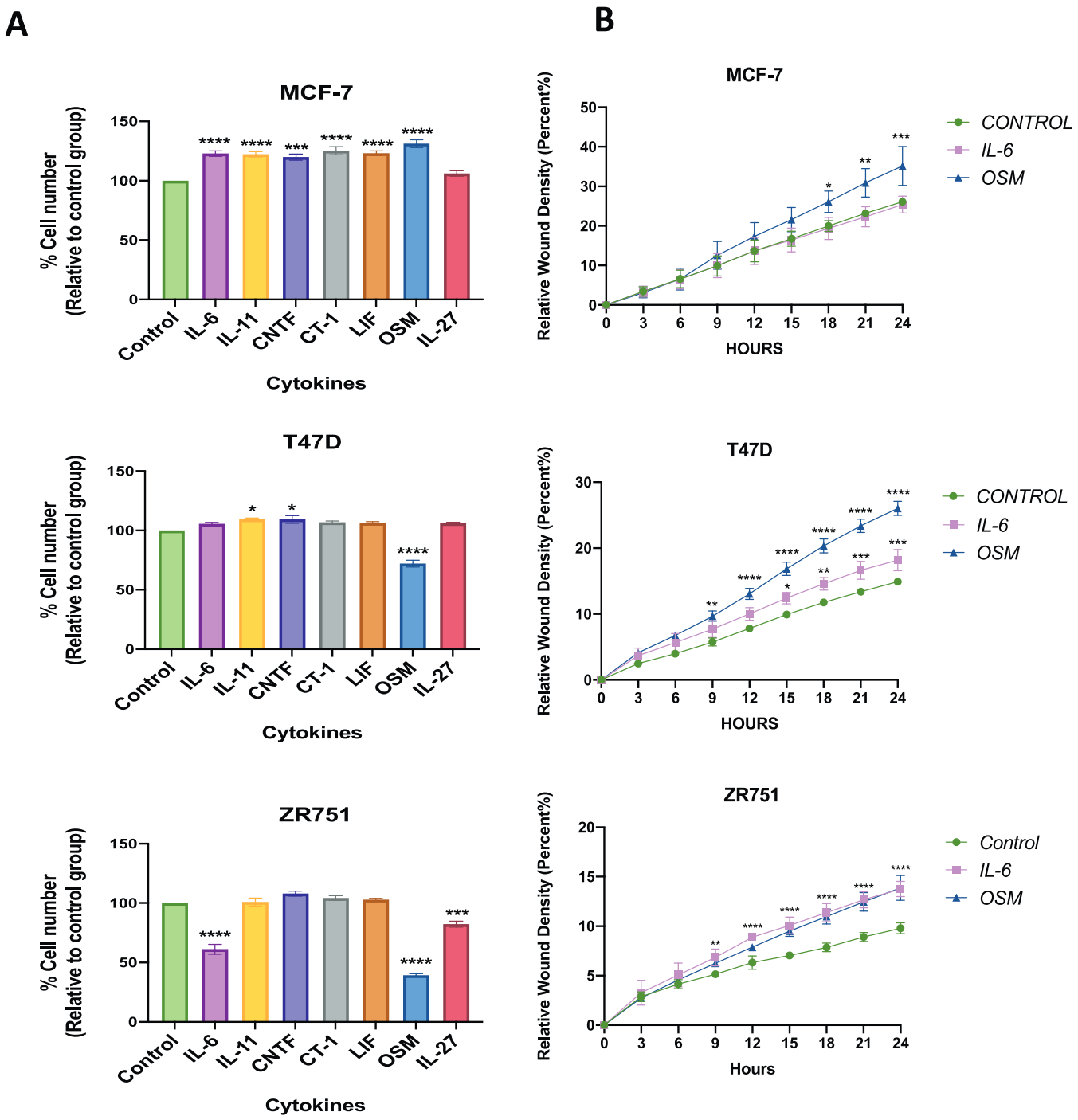

Fig. 2. Differential functional responses to IL6ST cytokines across a panel of breast cancer cell lines.
A.The effect of IL6ST cytokines on the growth of breast cancer cell lines. IL-6, IL-11, CNTF, CT-1, LIF, OSM, and IL-27 (100 ng/ml) were added to cell lines for 5 days and then assessed by SRB assays. This graph shows pooled results with each data point representing the mean from biological quadruplicate experiments for each treatment condition (6 technical replicates per concentration), normalised to vehicle controls, and error bars representing standard error (SE). Data are mean ± SE, where $*p < 0.05$, $**p = 0.001$, $***p = 0.0001$, $****p < 0.0001$.
B. The effect of IL-6 and OSM on the migration of cell lines. Cells were seeded in 10% FBS near to confluence onto 96 wells plate. After 24 h homogenous wound scratches were created by WoundMaker tool (which is a 96-pin mechanical device). Cells were then treated with 100 ng/ml of each cytokine as indicated in the figure legend in 1% FBS and incubated in IncuCyte S3 live-cell analysis system for 24 h. The wound density images were taken every 3 h and the results were analysed using IncuCyte S3 software. This graph shows pooled results with each data point representing the mean from triplicate biological experiments for each treatment condition (6 technical replicates per concentration) and error bars representing standard deviation (SD). Data are mean ± SD, where $*p < 0.05$, $**p = 0.001$, $***p = 0.0001$, $****p < 0.0001$.

significant inhibition, while both IL-11 and CNTF caused a minor stimulation in T47D cells growth. In contrast, growth inhibition was seen in ZR751 cells in the presence of IL-6, OSM and IL-27, whereas the other cytokines had no effect on growth (Fig. 2A).

Since the IL-6 cytokine family had differential effects on the growth of ER+ breast cancer cell lines, it was of interest to assess whether there would be similar diversity on migration. Interleukin-6 and OSM were evaluated against the 3 cell lines (Fig. 2B). Interleukin-6 stimulated migration of both T47D and ZR751 cell lines but had no significant effect on the migration of MCF-7 cells. Oncostatin M stimulated migration of all three cell lines. The relative effect of OSM on T47D cells was greater than on the other two cell lines.

As shown above, the IL6ST cytokines produced functional diversity and exerted different biological activities on ERα + breast cancer cell lines. Expression of IL-6R, OSMR and LIFR were all demonstrated in the three cell lines using Western analysis (Supplementary Fig. 1). It was feasible that downstream signalling transduction pathways might be differentially activated by the cytokines in the cell lines consistent with the different functional responses. In previous cell line studies, IL-6 activation of STAT3 has been associated with growth inhibition [35,36] while activation of MAPK has been shown to inhibit this signalling [37]. Therefore, IL-6 was used to assess STAT and MAPK activation in the three cell lines at 5, 30 and 60 min. Interleukin-6 increased both phospho-STAT3 (Tyr705) as well as phospho-p42/p44 (ERK1/ERK2) (Thr202/Tyr204) expression in all cell lines for this early time period (Fig. 3). Further studies will be needed to assess whether there are differences in the balance of signalling via these two pathways.

3.3. The potential of trans-signalling pathway in ER+ breast cancer cell lines

To investigate the possibility of trans-signalling in these cell lines (Supplementary Fig. 2), the combination of recombinant IL-6 (100 ng/ml) and sIL-6R (100 ng/ml) were studied using SRB proliferation assays performed over five days. Interleukin-6 growth stimulation in MCF-7 cells was increased by combination with sIL-6R, while the growth inhibition in ZR751 produced by IL-6 was further enhanced by sIL-6R consistent with trans-signalling. No significant effects on growth were observed in T47D cells (Fig. 4A).

To evaluate the possible role of soluble IL6ST as a potential inhibitor of trans-signalling, the sgp130FC fusion protein was used [38]. ZR751 cells were either treated with (i) IL-6,(ii) a combination of IL-6 and sIL-6R or (iii) different concentrations of sgp130Fc (fusion protein) (1 or 2μg) in the presence of both IL-6 and sIL-6R. The results revealed that sgp130Fc blocked the enhancement effect of the combination of IL-6 and sIL-6R (Fig. 4B).

The modulation of proliferation that was seen above led us to investigate the trans-signalling effect on the migration of the three cell lines. Cells were treated with either IL6 alone or IL6 in the presence of sIL6R -which model classic and trans-signalling conditions respectively and were compared with untreated cells. A two-way ANOVA revealed that there was a significant increase in migration rate of T47D cells with IL-6 + sIL-6R while ZR751 cells showed no enhanced response compared to IL-6 alone. No significant effects on migration were seen for MCF-7 cells (Fig. 4C).

The presence of known soluble IL6ST isoforms (Supplementary Fig. 3) was investigated by qRT-PCR as these might influence the trans-signalling pathway (by inhibition). Relative quantification of known IL6ST alternatively spliced isoforms was examined under basal conditions (10% FBS) by performing qRT-PCR using specific primer sets for each transcript (see Materials and Methods). These primers were designed against sequences indicated in Supplementary Fig. 3. Both soluble forms and the full-length form of IL6ST are differentially expressed across the three cell lines. The full-length transcript was the most abundant form detected in all cell lines, however, the E-10 and RAPS soluble forms were also highly expressed. The Diamant and Sharkey isoforms were the least abundant forms across the cell lines (Supplementary Fig. 3).

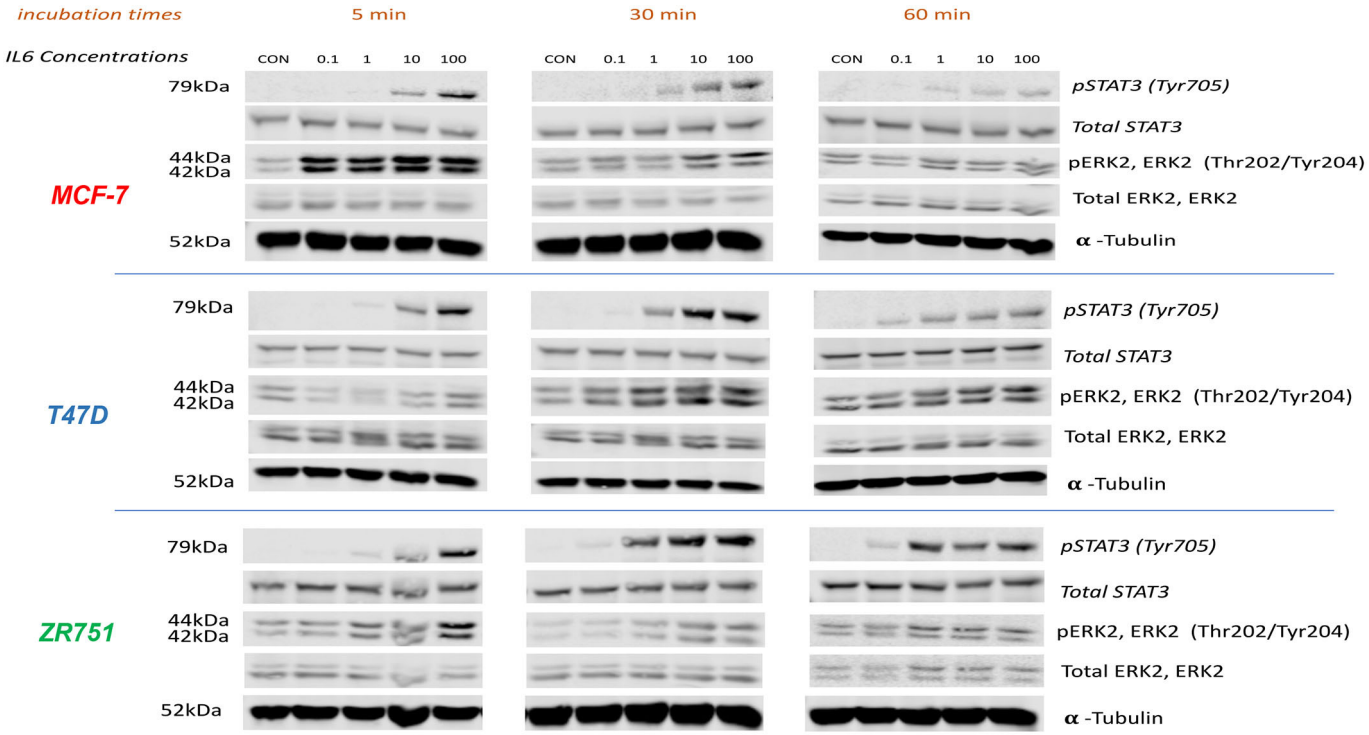

Fig. 3. Interleukin-6 activates both STAT3 and ERK pathways in the breast cancer cell lines.
Lysates of three cell lines cells treated with different concentrations (0.1-100 ng/ml) of IL-6 and collected after specific time points as indicated. Western blotting was performed to detect STAT3 Tyr705 phosphorylation, Total STAT3, p44–42-ERK1/2 phosphorylation and Total ERK. The molecular weight of each target shown is indicated and α-Tubulin used as the loading control.

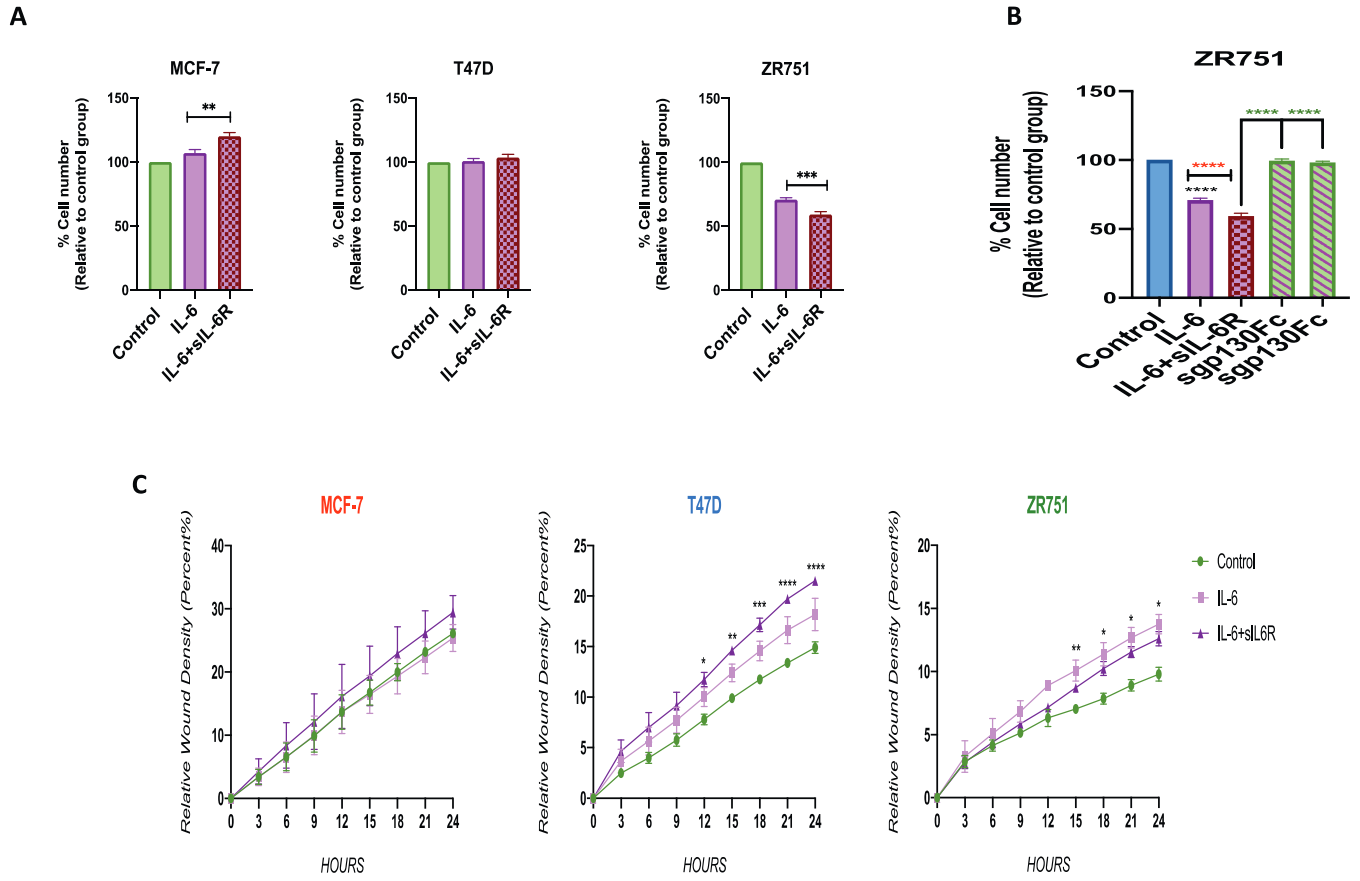

Fig. 4. Interleukin 6 mediated classic and trans-signalling in breast cancer cell lines.
A. Combination of sIL6R and IL-6 enhance proliferation in MCF-7 and inhibition in ZR751 cells. Cells were cultured with 10% FBS then treated with 100 ng/ml IL-6, as indicated, without or with 100 ng/ml of sIL-6R and incubated for 5 days. Each data point represents the mean from biological quadruplicate experiments for each treatment condition (6 technical replicates per concentration), normalised to vehicle controls, and error bars represent standard error (SE). IL-6 effect was examined by comparing the results with vehicle treated samples, while the combination of IL-6 and sIL-6R was examined by comparing the results with IL-6 treated samples. Data are mean (±SE), where $^{**}p = 0.001$, $^{***}p = 0.0001$.
B.The inhibitory effect of sg130Fc in the ZR751 cell line. Cells were cultured with 10% FBS then treated as indicated for 5 days. This graph shows pooled results with each data point representing the mean from biological quadruplicate experiments for each treatment condition (6 technical replicates per concentration), normalised to vehicle controls, and error bars represent standard error (SE). IL-6 effect was examined by comparing the results with vehicle treated samples, while the combination of IL-6 and sIL-6R was examined by comparing the results with IL-6 samples. The effect of sgp130Fc (1st bar = 1μg; 2nd bar = 2μg) in combination with IL-6 and sIL-6R was analysed by comparing the results with the combination of IL-6 and sIL-6R only. Values are mean (±SE), where $^{*}p < 0.05$, $^{**}p = 0.001$, $^{***}p = 0.0001$, $^{****}p < 0.0001$.
C.The effect of trans-signalling pathway on the migration of the three cell lines. Cells were seeded in 10% FBS near to confluence onto 96 wells plate. After 24 h homogenous wound scratches were created as described in Materials and Methods. Cells were then treated with 100 ng/ml IL-6 as indicated in the figure in 1% FBS and incubated in IncuCyte S3 live-cell analysis system for 24 h. The wound density images were taken every 3 h and the results were analysed using IncuCyte S3 software. This graph shows pooled results with each data point representing the mean from triplicate biological experiments for each treatment condition (6 technical replicates per concentration) and error bars represent standard deviation (SD). Data are mean (±SE), where $^{*}p < 0.05$, $^{**}p = 0.001$, $^{***}p = 0.0001$, $^{****}p < 0.0001$.

3.4. Impact of estrogen on cytokine response

In order to assess the possible interaction of IL6ST cytokines and estrogen, both the panel of 3 ER+ breast cancer cell line models and two endocrine-resistant MCF-7 cell line models (MCF-7/LCC1 and MCF-7/LCC9) emulating the clinical development of acquired endocrine were used. Unlike the estrogen-dependent MCF-7 breast cancer cell line, the MCF-7/LCC1 (LCC1) cell line is estrogen-independent but still sensitive to selective estrogen receptor modulators (SERM) drugs [30], while the MCF-7/LCC9 (LCC9) cell line is fully estrogen, and SERM resistant [31]. This was confirmed by using two types of SERM which were bazedoxifene (BAZ) and 4-hydroxytamoxifen (4-OHT) (Supplementary Fig. 4).

Experiments were undertaken in the absence and presence of estrogen (1 nM 17β-estradiol) to assess if the presence of E_2 influenced the cell line response to cytokines. In the absence of estrogen, despite expressing similar levels of full-length IL6ST (Supplementary Fig. 4), both LCC1 and LCC9 cell lines responded differently to IL6ST cytokines when compared to their parent MCF-7 cell line (Fig. 5A). MCF-7 and LCC1 cell growth increased significantly as a result of IL-6 and LIF stimulation, while both cell lines showed no additional effect of IL-6 under condition that are associated with trans-signalling (i.e. in the presence of sIL-6R). In contrast, LCC9 showed no response to these conditions. Surprisingly, LCC1 and LCC9 were growth inhibited by OSM in contrast to the parental MCF-7 cell line which as expected showed an increase in growth. The same experiment was also performed with T47D and ZR751 cells. As expected, a significant decrease in growth with IL-6 and OSM in ZR751 cells was observed, while the IL-6 trans-signalling pathway caused a slight enhancement in inhibition. However, no effect on T47D cell growth were seen under these conditions.

In the presence of E_2, the stimulation effect of IL-6 and OSM in MCF-7 cells was eliminated (Fig. 5B). However, LCC1 cells showed significant inhibition after exposure to either IL-6 or OSM. In LCC9 cells, IL-6 had

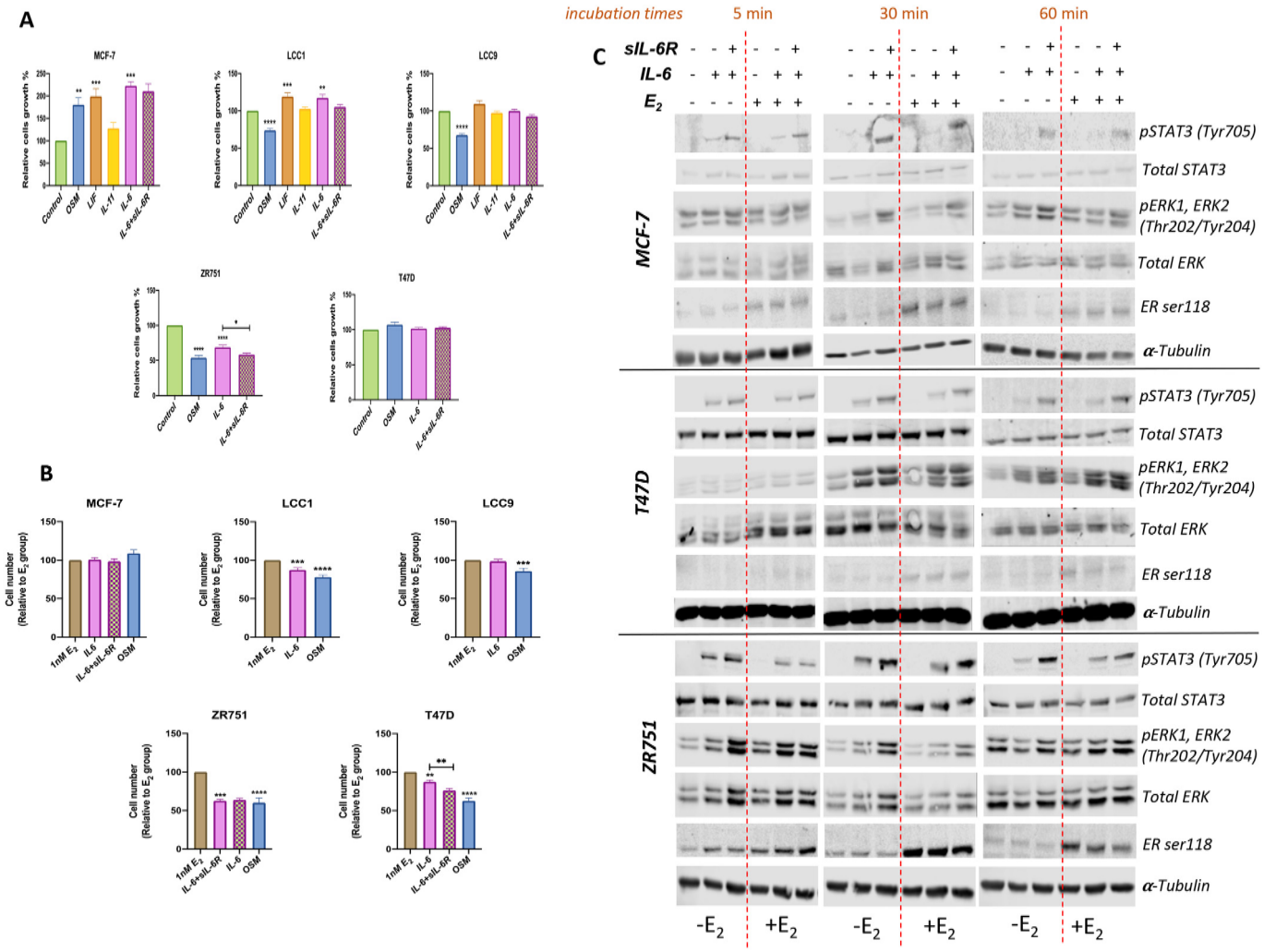

Fig. 5. Altered proliferation and cell signalling responses to IL6ST cytokines in endocrine-sensitive and resistant breast cancer cell lines.

A.The effect of IL6ST cytokines in the absence of estrogen. Cells were grown in 5% charcoal stripped FCS and treated with 100 ng/ml cytokine, as indicated, in the absence of E_2 and incubated for 5 days. Cells were also treated with 100 ng/ml IL-6 with and without 100 ng/ml of sIL-6R as indicated. This graph presents pooled results with each data point representing the average from quadruplicate biological experiments for each treatment condition (6 technical replicates per concentration), normalised to controls (untreated), and error bars represent standard error (SE). Data are mean (±SE), where $^{*}p < 0.05$, $^{**}p = 0.001$, $^{***}p = 0.0001$, $^{****}p < 0.0001$.

B.The effect of IL6ST cytokines in the presence of 10 nM E2. Cells were grown in 5% charcoal stripped FCS and treated with 100 ng/ml cytokines, as indicated, in the absence of E2 and incubated for 5 days. Cells were also treated with 100 ng/ml IL-6 with and without 100 ng/ml of sIL-6R as indicated. This graph presents pooled results with each data point representing the average from quadruplicate biological experiments for each treatment condition (6 technical replicates per concentration), normalised to controls (untreated), and error bars represent standard error (SE). Data are mean (±SE), where $^{*}p < 0.05$, $^{**}p = 0.001$, $^{***}p = 0.0001$, $^{****}p < 0.0001$.

C.The effect of estrogen on classic and trans-signalling pathways in breast cancer cell lines. Cells were seeded in 5% Charcoal Stripped serum overnight. Lysates of the three cell lines treated with (100 ng/ml) of IL6 alone and a combination of IL6 and IL6R 100 ng/ml each in the presence and absence of E_2 conditions collected after 5, 30 and 60 min. The western blot shown is representative of STAT3 Tyr705 phosphorylation, total STAT3, phospho-p42/p44 (ERK1/ERK2) MAPK (Thr202/Tyr204) and total ERK. The molecular weight of each target shown is indicated and α-Tubulin was used as a loading control. Representative blot shown of $n = 3$.

no significant effect, while OSM caused reduction in growth. In the presence of E_2, IL6 produced a decrease in cell proliferation as a result of IL-6 classic signalling pathway or OSM treatment in ZR-75-1 cells, however the trans-signalling enhancement in ZR751 cells was not seen. Surprisingly, T47D cells showed a slight inhibition under classic signalling conditions, and OSM, while trans-signalling conditions enhanced this inhibition.

Western blot analysis of the signalling at 5, 30 and 60 min indicated similar effects on pSTAT3 (Tyr705) as well as phospho-p42/p44 (ERK1/ERK2) (Thr202/Tyr204) under classic and trans-signalling conditions in the presence and absence of E_2 (Fig. 5C) in MCF-7, ZR751 and T47D cells. The effect of E_2 on signalling was demonstrated by increased expression of phospho-ERα (Ser118).

3.5. IL-6 modulates different downstream signalling pathways in MCF-7 and LCC9 cell lines

As a consequence of the differential IL-6 growth responses in MCF-7 and LCC9 cell lines, further investigation was undertaken to understand the effect of IL-6 on signalling pathways by using Reverse Phase Protein Arrays (RPPAs) to assess phosphoprotein and total protein expression levels of downstream signalling pathways. Both MCF-7 and LCC9 cell lines were grown in 5% charcoal stripped FCS in the absence of E_2 and lysates were collected after 30 min and 24 h.

The STAT pathways were activated by IL-6 stimulation in the MCF-7 cell line, but not in the LCC9 line (Fig. 6A). In MCF-7 cells, a significant increase in the expression of phospho-STAT1 (Tyr701), phospho-STAT3

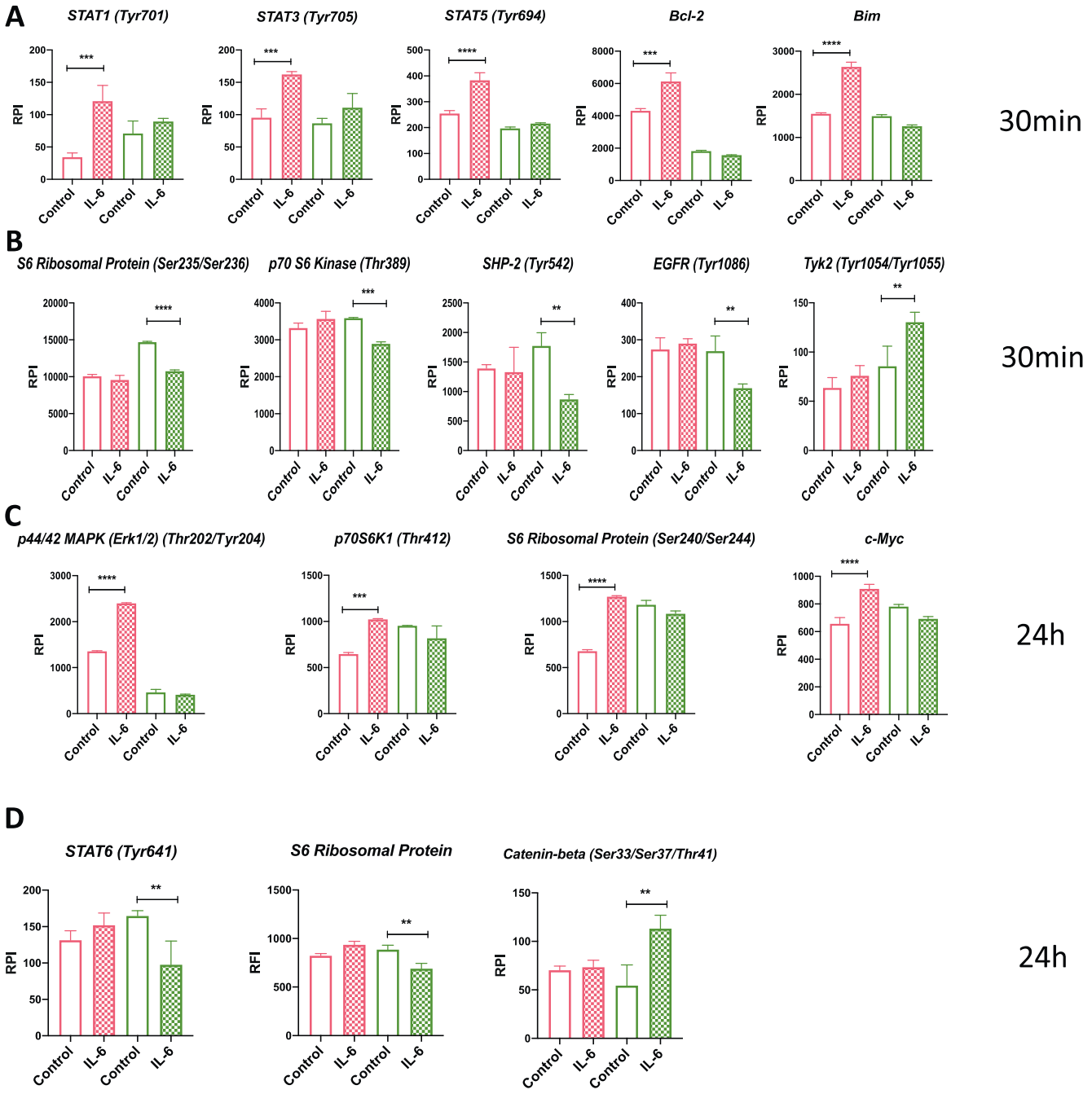

Fig. 6. Differences in IL-6 modulated signalling between endocrine-sensitive and resistant breast cancer cells.
Effects of IL-6 (100 ng/ml) on selected signalling components in the MCF-7 (red) and LCC9 (green) cell line as measured by RPPA. Differential effects in the MCF-7 but not LCC9 cell line are shown at 30 min (A) and 24 h (C). Differential effects in the LCC9 cell line but not MCF-7 are shown at 30 min (B) and 24 h (D). This graph presents pooled results with each data point representing the average from triplicate biological samples for each treatment condition. The relative fluorescence intensity (RFI) was obtained by comparing Data are mean (±SE), where $*p < 0.05$, $**p = 0.001$, $***p = 0.0001$, $****p < 0.0001$ ns = not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Tyr705), and phospho-STAT5 (Tyr694) was observed after 30 min. Similarly, increases were observed in the expression of selected apoptosis markers BCL-2 and Bim (Fig. 6A). However, two proteins of the downstream mTOR signalling pathway S6 Ribosomal protein and S6 Ribosomal protein (Ser235, Ser236) and p70 S6 Kinase (Thr389) signals were significantly reduced after IL-6 stimulation in LCC9 cells after 30 min. Also, expression of both SHP-2 (Tyr542) and EGFR (Tyr1086) were decreased, while Tyk2 (Tyr1054/Tyr1055) expression was increased after 30 min of IL-6 exposure (Fig. 6B).

After 24 h of IL-6 exposure, MCF-7 cells showed an increase of expression in the following proteins - p44/42 MAPK (ERK1/2) (Thr202/Thr185, Tyr204/Tyr187), p70SK1 (Thr412), S6 Ribosomal protein (Ser235, Ser236) and c-Myc but not in LCC9 cells (Fig. 6C). In contrast, LCC9 demonstrated a significant reduction in expression of STAT6 (Tyr641) and S6 Ribosomal protein, whereas the expression of Catenin-beta (Ser33/Ser37/Thr41) was dramatically increased (Fig. 6D).

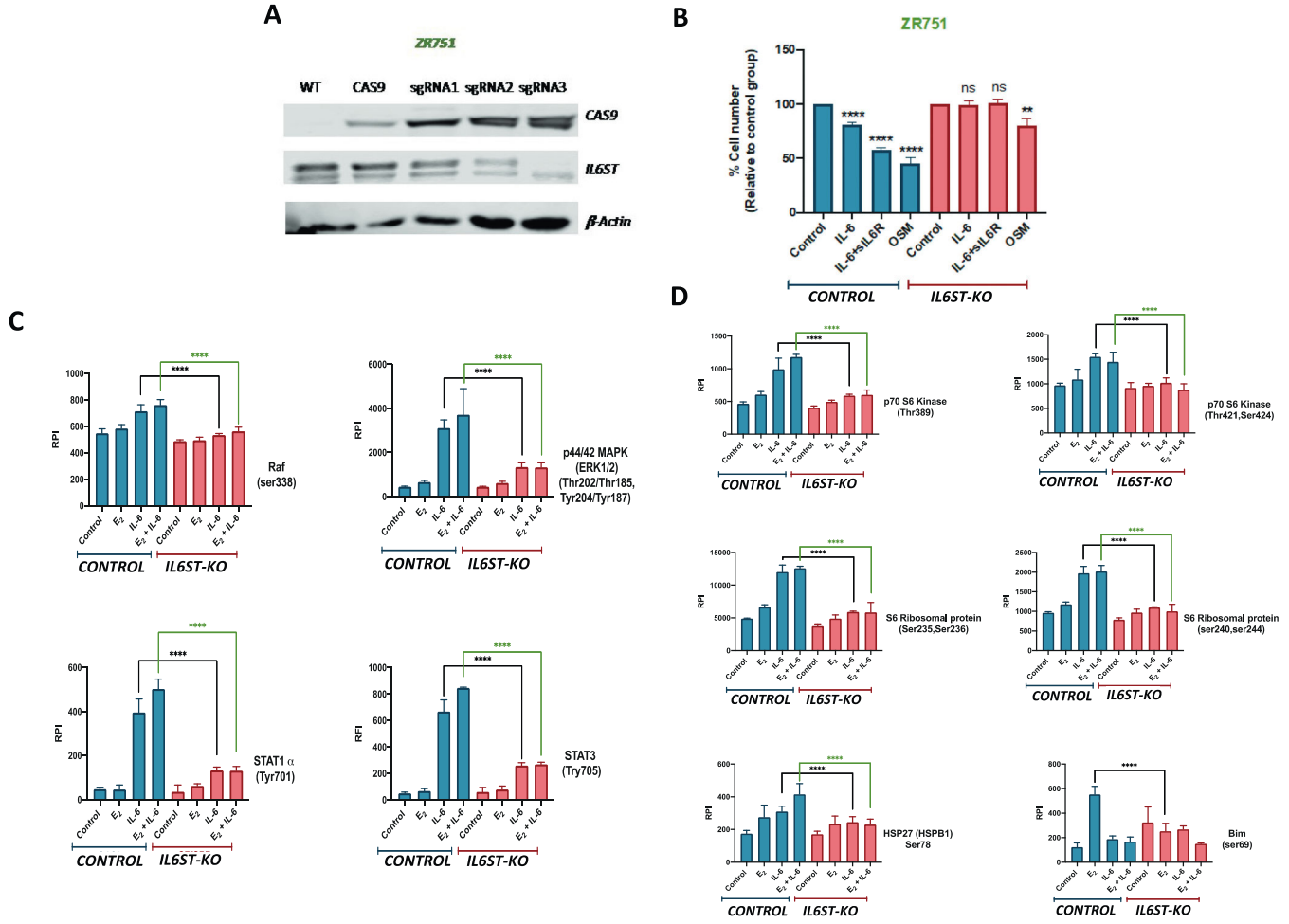

Fig. 7. The effect of silencing IL6ST on function and signalling in ZR751 cells.
A.Validation of IL6ST CRISPR-CAS9 gene editing. Western blot analysis of whole cell lysates of ZR751 cells transduced with All- In-One vector. The lysates were subjected to immunoblotting using anti-IL6ST antibody and CAS9 antibody. β-Actin was used as loading control. WT indicates wild type.
B.Effect of silencing IL6ST on cell growth: Cells were grown in 10% FBS and treated with 100 ng/ml concentrations of IL-6, sIL-6R and OSM and incubated for 5 days. This graph presents pooled results with each data point representing the mean from biological quadruplicate experiments for each treatment condition (6 technical replicates per concentration), normalised to controls (untreated), and error bars representing standard error (SE). The proliferation was examined by comparing the untreated (control) with cytokine treated cells as indicated. The Data are mea n(±SE), where *p < 0.05, **p = 0.001, ***p = 0.0001, ****$p < 0.0001$.
C.The effect of silencing IL6ST on both Raf/MAPK and STAT pathways. RPPA was undertaken as described in Materials and Methods. This graph presents pooled results with each data point representing the mean from technical triplicate samples for each treatment condition. The relative phosphorylation intensity was examined by comparing IL-6 and E_2 + IL-6 groups of CAS9 cells with the same group in CAS9-CRISPR cells as indicated. The Data are mean (±SE), where *p < 0.05, **p = 0.001, ***p = 0.0001, ****p < 0.0001.
D.The effect of silencing IL6ST on mTORC1 pathway and other targets. RPPA was undertaken as described in Materials and Methods. This graph presents pooled results with each data point representing the average from technical triplicate samples for each treatment condition. The relative phosphorylation intensity was examined as indicated. The Data are mean (±SE), where *p < 0.05, **p = 0.001, ***p = 0.0001, ****p < 0.0001

3.6. *Effect of IL-6 and OSM on the expression of ER and PR*

The effect of IL-6 and OSM on the protein expression of ERα and PR in the three ER+ cell lines was next investigated to probe the connection between IL6ST and ER signalling pathways. Interleukin-6 suppressed expression of ER and PR protein in ZR751 cells after 3 days, whereas an increase in PR expression was seen in MCF-7 cells. No effect was seen on PR expression in T47D cells, although ER expression appeared decreased (Supplementary Fig. 4). Oncostatin M decreased ER expression in both T47D and ZR751 cells after 24 h, while a modest change in ER expression was seen in the MCF-7 cell line. In contrast, OSM increased PR expression in both MCF-7 and ZR751 cells (Supplementary Fig. 4).

3.7. *The effect of silencing IL6ST on function, signalling and gene expression in ZR751 cells*

To further assess the nature of the growth inhibition produced by IL6 in ZR751 cells, a CRISPR knockdown model of IL6ST was generated. After lentiviral transduction of All- In-One vector into ZR751 cells and puromycin selection, pools of sgRNA3 surviving cells demonstrated near

complete knockout of IL6ST and this clone was used in subsequent experiments (Fig. 7A). Pool sgRNA2 ZR751 surviving cells presented about 60% IL6ST knock down, while pool sgRNA1 showed IL6ST expression similar to the wild type and CAS9 cells. To ensure that each pool of sgRNAs or crRNAs contain CAS9, the western blot membrane was blotted with CAS9 antibody. All CRISPR cell pools alongside the CAS9 cells (negative control) showed a strong expression of CAS9 (Fig. 7A).

Growth assays were carried out to assess the effect of IL-6 (classic and trans-signalling pathways) and OSM on the growth of CRISPR-IL6ST-KO (CAS9) cells compared to control (CAS9) cells. Reduction of IL6ST in the IL6ST-KO cell line resulted in elimination of the action of both IL-6 signalling pathways and reversal of the effect of OSM (Fig. 7B).

To assess the effects of IL6ST knockdown on cell signalling, RPPA was used for ZR751 control and IL6ST-KO cell lines cultured in 5% charcoal stripped FCS under control, E_2 (1nM), IL-6 (100 ng/ml) and E_2 + IL-6 conditions (Fig. 7C). The lysates were collected after 30 min. As shown in Fig. 7C, the phosphorylation level of p44/42 MAPK (ERK1/2) (Thr202/Thr185, Tyr204/Tyr187), Raf (Ser338), STAT3 (Tyr705) and STAT1 (Tyr701) as expected were significantly increased as a result of IL-6 alone and IL-6 + E2 stimulation in control cells. The phosphorylation levels of these targets were dramatically reduced in IL6ST-KO cells. Furthermore, members of the mTORC1 pathway were affected as a result of knocking out IL6ST. These targets included p70 S6 kinase (Thr389), p70 S6 kinase (Thr421, Ser424), S6 Ribosomal protein (Ser235, Ser236), and S6 Ribosomal protein (Ser240, Ser244) (Fig. 7D). The phosphorylation of these targets was dramatically increased as a result of both IL-6 and IL-6 + E_2 exposure in control cells, while their phosphorylation was dramatically reduced in IL6ST-KO cells.

RNAseq gene expression analysis was performed to investigate the transcriptome expression of ZR751-control and ZR751-IL6ST-KO cell lines. The experiment compared untreated control, IL-6 (100 ng/ml), E_2 (1nM) + IL-6, and IL-6 + E_2 + 4-OHT (0.1 μM). Samples were collected after 8 h and analysed by RNAseq, the Rank Products function within TMeV software package with *p*-value being set at 0.01. Differentially expressed genes (DEGs) are shown in Table 1 and in Supplementary Fig. 5 after specific treatments.

Comparison of gene expression between untreated control ZR751-control and ZR751-IL6ST-KO cell lines revealed 1913 genes more lowly expressed and 1870 genes more highly expressed in the control than in the IL6ST-KO line. Pathway and biological process analysis (DAVID) identified that genes more highly expressed in the control cell line included genes associated with the JAK/STAT pathway, apoptotic pathways and cell adhesion pathways, while genes more highly expressed in the IL6ST-KO cell line included genes associated with cell cycle, cell proliferation and G2/M transition of mitotic cell cycle.

Treatment with IL-6 stimulated the expression of genes associated with the EGFR signalling pathway and JNK pathway in the ZR751-control cell line (Table 1). However, this cytokine stimulation in ZR751 IL6ST-KO cells was associated with genes related to DNA replication and mitotic cell cycle. In both cell lines IL-6 stimulation resulted in DEGs associated with the MAPK pathway (Table 1).

Stimulation with E_2 + IL-6 in the control cell line enriched genes that were related to the p53 signalling pathway, PI3K-Akt signalling, cell cycle and G1/S transition of mitotic cell cycle. In the IL6ST-KO cells this stimulation affected genes of the EGFR signalling pathway, cell motility and regulation of cell adhesion. In both cell lines, this stimulation modulated genes associated with response to estradiol (Table 1).

Treatment with IL-6 + E_2 + 4OH-TAM identified DEGs associated with genes targeting ER in control cells. In IL6ST-KO cells this treatment modulated genes associated with cell proliferation and with positive regulation of cell-cell adhesion (Table 1).

4. Discussion

This study used cell line models to further explore the role of IL6ST function and signalling within ERα breast cancer cells. Cell line model

Table 1
Differentially expressed genes identified in the ZR751-CAS9 Control and ZR751-IL6ST-KO (CRISPR) cell lines after treatment.

Treatment	*Cell line*	*Enrichment analysis results*	*Genes*	*p-value*
IL-6	CAS9 CONTROL	Positive regulation of EGFR signalling pathway	*TNIP2 FLT4 COPS5 FZD8 MLKL ITCH ADORA2B ROR2 MAP2K1 AGER MAP4K4 MAP3K4 LTBR DAB2IP IRAK4 TRAF4 MDFIC MAP3K5 STK3 SH3RF1 HDAC3 GADD45G IRAK2*	10^{-1}
		Positive regulation of JNK cascade	*STK3 LTBR MAP3K5 GADD45G FLT4 TRAF4 DAB2IP*	10^{-2}
		Stress-activated MAPK cascade	*MMP9 ARAP1 SHKBP1 PLAUR GPR37 PDGFB OSM*	10^{-5}
		Positive regulation of MAPK cascade	*IGFBP4 DUSP19 TNFRSF25 FGFR3 FLT4 RELT SORBS3 CDK10 FLT3*	10^{-2}
	IL6ST-KO	DNA replication	*BRCA1 DDX11 POLA2 BOD1L1 RFC2 RAD51 POLB MCM6 ORC1 CDC7 MCM5 POLA1 MCM4 RNASEH2A CCNE1 POLD2 ZPR1 MCM9 BCL6 MSH6 ID3 MCM8 CHTF18 INO80 POLN RFC3 SDE2 GINS4 CHEK1 EME1 CHAF1B DBF4B E2F7 POLL MCM7 CHAF1A ATAD5 POLE GINS3 WDHD1 BRCA1 POLA2 NCAPH2 SPDL1 RAD51 TRIP13 GTSE1 WDR62 MCM6 RBL1 ORC1 TFAP4 CDC7 MCM5 PDGFB VRK1 MYBL2 BMP7 CDC25B POLA1 MCM4 CCNE1 CDK6 EZH2 NCAPG ZPR1 BCL6 ID2 MSH6 NCAPH MCM8 XRCC3 SBDS PIN1 INO80 STK33 CLIP1 TOP2A NES MEIS2 PSRC1 NUDT15 HECW2 ASCL1*	10^{-8}
		Mitotic cell cycle	*EFCAB11 NUF2 CCNB3 CEP78 CHEK1 HAUS1 SPC25 TRIM36 XPC EME1 FBXW5 SON DBF4B CCNF POC1A SUGT1 E2F7 ZFYVE19 MCM7 MIS12 CNTROB TRIAP1 PKIA RAD9A SPHK1 ATAD5 ANAPC2 POLE PIDD1 SEPT1 KNTC1 CDK10 MYBL1 PLK5 DYNC1H1 SFI1 PIM3 WDHD1 PRC1 PPP1R10 HAUS7 HAUS3 CDK11B*	10^{-6}
IL-6 + E_2	CAS9 CONTROL	PI3K-Akt signalling pathway	*DDIT4 NR4A1 FGFR2 CCNE1 CASP9 EPHA2 FGF13 CCNE2 GHR GNB5 BRCA1 CDK6 IL7 ITGA10 FGFR3 RBL2 CCND1 SGK2 NRAS STK11 ITGA1 CDK2*	10^{-1}
		Response to estradiol	*GHR TXNIP CASP9 CTGF CCND1 AREG KCNJ11 DNMT3A MMP15 EZH2 DUSP1*	10^{-2}
		Cell cycle	*PKMYT1 CCNE1 CHEK1 CDC14A SFN CCNE2*	10^{-3}

(*continued on next page*)

Table 1 (*continued*)

Treatment	*Cell line*	*Enrichment analysis results*	*Genes*	*p-value*
			CDK6 MCM5 E2F2 CCND1 RBL2 ORC6 E2F1 MCM7 TGFB3 CDK2 PKMYT1 CCNE1 TYMS	
		G1/S transition of mitotic cell cycle	*DHFR CDT1 PLK3 CCNE2 POLA1 CDK6 MCM5 CCND1 ORC6 PLK2 MCM7 CDK2 POLE2*	10^{-4}
		p53 signalling pathway	*CCNE1 CHEK1 CASP9 CCNG2 SFN TP73 CCNE2 SESN1 CDK6 RCHY1 CCND1 SESN3 CDK2*	10^{-4}
		Negative regulation of cell migration	*INSR ADRA2A CREB3 THBS1 MYLK FGR ACVR1 ATP8A1NTRK3*	10^{-1}
		EGFR signalling pathway	*CSK NRAS PIK3C2A PAG1 GRB7 GAREM1*	10^{-1}
		Response to estradiol	*DNMT3B BAD SOCS2 PTCH1 CASP9 MBD3 CASP3 ASS1 ESRRA*	10^{-2}
		Positive regulation of cell adhesion	*PTPRJ C1QBP TFE3 RHOD CXCL12 TMEM102 ADAM8*	10^{-3}
	IL6ST-KO	Cell motility	*SEMA3G PIK3C2A SEMA3B WAS CDH1 LIMA1 SLC12A2 CLASP1 PIH1D3 BAMBI HIF1A MAPK3 CFAP69 FSCN3 CXCL12 SPOCK2 BMPR1A PFN1 C1QBP PEX7 PDE4D DPYSL3 ARHGEF2 MIIP F3 SNAI1 BBS2 OVOL2 BCL11B MGAT3 FGF13 ITGB4 UNK C5AR2 TNS3 PIK3C2G ASCL1 SORD TNFRSF11A GRB7 PTPRF ARHGAP18 SHROOM2 PTPRJ ADGRL3 SPATA4 ADAM8 NUS1 ELMO1 ABR IL24 ADORA1 CITED2 BMPER TP53INP1 DNAAF2 RET DAPK3 SEMA6B LDLRAD4 P2RY1 PLEKHG5 RHOD NET1 FAM89B FUT7 TMEM102 FAM110C MIXL1 KRT16 CCK DNAH17 NANOS1 HRH1 TMSB4X CKLF PRAG1*	10^{-3}
IL6 + E_2 + 4OH-TAM	CAS9 CONTROL	Protein targeting to ER	*TRAM2 RPL6 RPL34 RPS12 SSR3 SPCS2 RPL21 RPS15A RPS6 RPS3A RPL7A ZFAND2B RPS7 RPL4 RPL10A RPS18*	10^{-5}
	IL6ST-KO	Positive regulation of cell-cell adhesion	*LEF1 KIF26B SOX2*	10^{-2}

systems were first selected which contained either high (ZR751 and T47D) or low (MCF-7) levels of IL6ST expression. In these cell lines, IL6ST is heavily glycosylated as has been observed in other cancer cell systems e.g. ovarian cancer cells [39].

Differential growth responses to IL6ST interacting cytokines were observed in the cell lines with the high IL6ST expressing ZR751 cells demonstrating growth inhibition to IL6, OSM and IL-27, while low-IL6ST expressing MCF-7 cells were growth-stimulated by IL-6, OSM, LIF, IL-11, CT-1, CNTF but not IL-27. Growth of T47D cells was unresponsive to these cytokines. Variable results have been reported for the responsiveness of these cell lines to this cytokine family. MCF-7 cells have previously been reported to be growth stimulated by IL-6 (e.g. [4]) while other reports indicate growth inhibitory effects of IL-6 in breast cancer cell lines [40] as observed for ZR751 cells or no response [41] as seen here for T47D cells. In contrast, more consistent effects were observed on migration with IL-6 stimulating migration in both ZR751 and T47D cells, while OSM stimulated migration in all three cell lines.

We next investigated cell signalling pathways downstream of IL6ST. Interleukin-6 clearly stimulated both the STAT and ERK pathways in all three cell lines. In melanoma cells, IL-6 growth inhibition has been shown to be STAT3-dependent [36]. An ERK-mediated mechanism for inhibiting IL-6-induced JAK-STAT signalling has also been demonstrated [37]. If the STAT3 pathway programmes for growth inhibition while the ERK pathway programmes for proliferation [35 – 37], the balance of signalling may determine outcome. Interestingly, T47D cells had good activation of both pathways consistent with a neutral effect on growth, but perhaps relevant to cell migration. High IL6ST expression in ER+ breast cancers is an indicator of improved response to endocrine therapy in patients [27–29] and if cytokines exert a growth inhibitory response in this context this may contribute to a more favourable outcome.

The potential for IL-6 trans-signalling was next investigated in the cell lines. Addition of sIL6-R enhanced both the growth stimulatory effect of IL-6 in MCF-7 and the growth inhibitory effect in ZR751 cells respectively consistent with trans-signalling. Both STAT3 and ERK activation were increased by the combination of IL-6 and sIL6-R compared to IL-6 alone. As it is reported that sIL6-R is expressed by breast cancer cells [15,16], this mode of signalling therefore is likely to be operating alongside classic IL6R-mediated signalling in breast cancers [16]. It has been hypothesized that the body can be protected from overstimulation by IL-6 trans-signalling by use of the buffer of sIL-6R and soluble IL6ST [42]. Expression of soluble IL6ST isoforms [18–21] was detected by RT-PCR in the cell lines indicating the presence of soluble IL6ST and to the best of our knowledge these isoforms have not been reported previously in breast cancer cells. To test whether a soluble form of IL6ST might influence growth, sgp130Fc was added to ZR751 cells in the presence of IL-6 and sIL6-R and was able to reverse the trans-signalling growth inhibition. Cell migration was also increased under trans-signalling conditions in T47D cells and while trans-signalling activation has been shown to increase cell motility in ovarian and prostate cells [43,44], to our knowledge, this is the first report showing the role of trans-signalling in ER+ breast cancer cell lines migration. Together, these results support a potential role for IL-6 trans-signalling in the regulation of breast cancer cell growth and migration.

Since IL6ST expression is an important determinant of anti-estrogen response within clinically applied gene signatures [27–29], the potential associations between IL6ST signalling and endocrine response was next investigated. In the absence of estrogen, the growth stimulatory effects of the cytokines were lost in the resistant cell lines when the estrogen / anti-estrogen sensitive MCF-7 cell line was compared with its resistant LCC9 counterpart. Use of RPPA to study downstream signalling supported these results and indicated loss of IL6-induced STAT and ERK pathway signalling despite the presence of IL6ST in LCC9 cells. Under estrogen-stimulated conditions, the stimulatory effect of IL-6 was minimal in MCF-7 cells. IL6ST interacting cytokines such as IL-6 and OSM modulate expression of both ER and PR and these in turn may influence estrogen response and this is in agreement with a previous report [45].

Finally, IL6ST expression was knocked down using CRISPR in ZR751 cells to assess the functional and cell signalling changes associated with loss of IL6ST. As expected, growth inhibition by IL-6 and OSM was lost or markedly attenuated in the knockdown cell line, while IL-6 activation of the STAT and ERK pathways was markedly reduced. Gene expression analysis indicated changes in key cell signalling pathways. Interleukin-6 activation of key pathway signalling such as the EGF receptor and JNK pathways were differentially modulated in the control cells while genes

associated with DNA replication and mitotic control were more greatly affected in the knockdown CRISPR cells. Addition of estrogen to IL-6 resulted in changed gene expression in PI3K, p53 pathways and cell cycle control for the control cells while effects on genes linked to cell migration, adhesion and cell motility were observed for the knockdown cell line.

In conclusion, these studies have shown that the IL6ST cytokine interacting response can produce either a stimulating or inhibitory response which may be dependent on expression level of IL6ST.There is potential for trans-signalling in addition to classical signalling in these breast cancer cells and moreover, soluble IL6ST isoforms are produced by these cancer cells. Knockout of IL6ST markedly reduces growth response and intracellular signalling and modifies the expression of key cell signalling, cell cycle and proliferation genes. A higher level of IL6ST expression is associated with cytokine inhibition of growth which may contribute to an enhanced anti-estrogen growth inhibition in a clinical setting. This would be consistent with high IL6ST expression acting as a biomarker and predictor of favourable endocrine response.

Data availability statement

The data presented in this study are available on request from the corresponding author.

Author contributions

D.M., S.P.L and A.H·S conceived and designed the experiments, D.M. and K.M. conducted the experiments, D.M., K.M., N.M., A.T., A.H·S and S.P.L analysed the results. All authors contributed to the writing and review of the manuscript.

Additional information

The authors declare no competing interests.

Our colleague Dr. Andrew H Sims sadly passed away during the preparation of this manuscript; he had been pivotal in the design and analysis of this study and had contributed to the drafting and preparation of this manuscript.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2022.110563.

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