Oncostatin M suppresses oestrogen receptor- α expression and is associated with poor outcome in human breast cancer

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Abstract

The most important clinical biomarker for breast cancer management is oestrogen receptor alpha (ER α). Tumours that express ER are candidates for endocrine therapy and are biologically less aggressive, while ER-negative tumours are largely treated with conventional chemotherapy and have a poor prognosis. Despite its significance, the mechanisms regulating ER expression are poorly understood. We hypothesised that the inflammatory cytokine oncostatin M (OSM) can downregulate ER expression in breast cancer. Recombinant OSM potently suppressed ER protein and mRNA expression *in vitro* in a dose- and time-dependent manner in two human ER+ breast cancer cell lines, MCF7 and T47D. This was dependent on the expression of OSM receptor beta (OSMR β) and could be blocked by inhibition of the MEKK1/2 mitogen-activated protein kinases. ER loss was also necessary for maximal OSM-induced signal transduction and migratory activity. *In vivo*, high expression of *OSM* and *OSMR* mRNA (determined by RT-PCR) was associated with reduced ER ($P < 0.01$) and progesterone receptor ($P < 0.05$) protein levels in a cohort of 70 invasive breast cancers. High *OSM* and *OSMR* mRNA expression was also associated with low expression of *ESR1* (ER, $P < 0.0001$) and ER-regulated genes in a previously published breast cancer gene expression dataset ($n = 321$ cases). In the latter cohort, high *OSMR* expression was associated with shorter recurrence-free and overall survival in univariate ($P < 0.0001$) and multivariate ($P = 0.022$) analyses. OSM signalling may be a novel factor causing suppression of ER and disease progression in breast cancer.

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Introduction

Oestrogen receptor alpha (ER α) is a central factor in breast cell biology and growth. As the primary transcription factor that mediates oestrogen signalling, ER is the linchpin of endocrine therapy and a feature that partly defines the various molecular breast cancer subtypes (Sotiriou & Pusztai 2009, Prat *et al.* 2010). While the mechanisms that regulate ER expression are clearly important for our understanding and clinical management of breast cancer, these remain poorly characterised.

Endocrine therapy is highly effective for the treatment of ER+ breast cancer (EBCTCG 2005). However, ~30% of primary tumours are ER– at

clinical presentation, constituting the principal mechanism of intrinsic resistance to endocrine therapy (Musgrove & Sutherland 2009). Among the tumours that are ER+ and respond initially to endocrine therapy, some develop acquired resistance through the suppression of ER expression, a mechanism that may account for up to 20% of resistant breast cancers (Musgrove & Sutherland 2009). Mechanisms to explain ER suppression include hypermethylation of the ER gene promoter (Stearns *et al.* 2007), destabilisation of ER mRNA (Reid *et al.* 2002), hypoxia (Cooper *et al.* 2004) and hyperactivation of mitogen-activated protein kinase (MAPK) signalling (Oh *et al.* 2001, Creighton *et al.* 2006, 2008, Bayliss *et al.* 2007,

Lopez-Tarruella & Schiff 2007). Prolonged growth of breast cells as mammospheres or upregulation of the transcription factors snail and slug may also cause ER suppression (Dhasarathy *et al.* 2007, Storci *et al.* 2010, Guttilla *et al.* 2012).

Leucocytes can influence malignant cells through various mechanisms, particularly cytokine release (Balkwill *et al.* 2005), and ER[−] breast tumours are generally enriched for intra-tumoral leucocytes compared with ER⁺ lesions (Teschendorff *et al.* 2007a). This raises the possibility that tumour-associated immune responses could influence ER expression. As many of the processes shown to influence ER are highly dynamic, the ER negativity of some tumours, whether observed at diagnosis or upon disease progression, might be reversible.

We and others have shown that oncostatin M (OSM), a cytokine of the interleukin 6 (IL6) family, promotes acquisition of aggressive features such as enhanced migration and invasiveness (Zhang *et al.* 2003, Holzer *et al.* 2004, Jorcyk *et al.* 2006, Underhill-Day & Heath 2006, West & Watson 2010). OSM is produced by leucocytes including T cells, monocytes and neutrophils (Tanaka & Miyajima 2003, Queen *et al.* 2005) and engages heterodimeric receptors involving gp130 and either the OSM receptor beta (OSMR β) or the leukaemia inhibitory factor receptor (LIFR). Signal transduction (Heinrich *et al.* 2003) is initiated by Janus kinases (JAKs) that engage the MAPK, signal transducer and activator of transcription 3 (STAT3) and phosphatidylinositol-3-kinase (PI3K (PIK3CA)) pathways, each of which has documented roles in breast tumour pathogenesis (Clevenger 2004, Dillon *et al.* 2007, Whyte *et al.* 2009). Based on the emerging recognition that immune activity can affect breast cancer biology (DeNardo & Coussens 2007), and a prior observation that OSM may influence ER in MCF7 cells (Grant *et al.* 2002), our primary aim in this study was to assess OSM signalling as a possible novel regulator of ER expression in breast cancer.

Materials and methods

Cell culture and cytokine stimulation

Human breast carcinoma cell lines MCF7, T47D and ZR75-1 (ATCC, Manassas, VA, USA) were cultured in DMEM with 5% FBS under standard conditions. Human OSM, IL6 and TNF- α (Peprotech, Rocky Hill, NJ, USA) were stored as 100 μ g/ml stocks in culture media and, unless otherwise specified, used at 100 ng/ml.

Chemical inhibitors and RNA interference

Inhibitors to MEKK1/2 (U0126 (Favata *et al.* 1998) and PD98059 (Dudley *et al.* 1995); Cell Signaling, Danvers, MA, USA), JAKs (JAK inhibitor I (Thompson *et al.* 2002)), Ras (FTI277 (Vogt *et al.* 1996)), c-jun N-terminal kinase (JNK) (JNK inhibitor VIII (Szczepankiewicz *et al.* 2006)), p38 MAPK (SB203580 (Cuenda *et al.* 1995)), PI3K (LY294002 (Vlahos *et al.* 1994)), EGFR (AG1478 (Oshero & Levitzki 1994)), mTOR (rapamycin) or NF- κ B (oridonin (Ikezoe *et al.* 2005) (Calbiochem, San Diego, CA, USA) were added to cultures 30 min before cytokine stimulation at doses of 10 μ M, with the exception of PD98059 (50 μ M), rapamycin (10 nM) and oridonin (10 μ g/ml). All inhibitors used in this study are established reagents for specific and effective disruption of their respective targets. Although the JAK inhibitor may suppress MAPK activity at the concentration used in this study, this was addressed by including it in all inhibition experiments as a positive control reagent for suppression of all aspects of OSM signalling. Gene knockdown was performed using ON-TARGET plus SMARTpool siRNA at a final concentration of 100 nM transfected with Dharmafect-4 (Dharmacon, Lafayette, CO, USA).

Western blots

Cells were prepared for immunoblotting as described previously (Al-Haddad *et al.* 1999). Protein concentrations were estimated using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Primary antibodies were ER α (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (1:3000; Stem Cell Technologies, Vancouver, BC, Canada), β -actin (1:3000; Abcam, Cambridge, MA, USA), phospho-STAT3 (Tyr705; 1:1000), phospho-AKT (Ser473; 1:500), phospho-ERK1/2 (Thr202/Tyr204; 1:500) and progesterone receptor (PR; 1:500; Cell Signaling). Secondary antibodies were HRP-conjugated bovine anti-rabbit and goat anti-mouse IgG (1:3000; Santa Cruz Biotechnology). STAT3 phosphorylation was used throughout this study as an indicator of OSM functionality (Kan *et al.* 2011). Band densitometry was performed using ImageJ.

Real-time quantitative PCR (RT-PCR)

Total RNA was extracted from cell lines and tissues using the Qiagen RNeasy mini kit (Qiagen) and quantified using an ND-1000 spectrophotometer. RNA was reverse transcribed using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD,

USA). RT-PCR was performed using Perfecta SYBR Green supermix (Quanta Biosciences) and an iCycler thermal cycler with a MyIQ real-time PCR detection system (Bio-Rad). Reactions were performed in triplicate for each sample. Data for all target genes were normalised to *RPL27* expression. Intron-spanning primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) and designed to have annealing temperatures of 60 °C. Primer sequences are as follows (listed 5′–3′): *RPL27* – forward, CAATCACCTAATGCCACAAG; reverse, TTCTTGCTGTCTTGATCTCTC; *ESR1* – forward, CGACTATATGTGTCCAGCCAC; reverse, CCTCTTCGGTCTTTTCGTATCC; *PGR* – forward, TCGCC-TTAGAAAGTGCTGTC; reverse, GCTTGGCTTT-CATTTGGAACG; *OSM* – forward, CTCGAAAGAG-TACCGCGTG; reverse, TCAGTTTAGGAACATC-CAGGC; and *OSMR* – forward, TCCCAATACC-ACAAGCACAG; reverse, GCAAGTTCCTGAGA-GTATCCTG.

ER functional assays

To assay responses to 17 β -estradiol (E_2 , 10 nM), cells were cultured in E_2 -free media (phenol red-free DMEM with 5% charcoal-dextran-stripped FBS) for 72 h before further treatment. To assess the effect of ER on cell migration, MCF7 cells were transfected with pcDNA3.1 or pcDNA3.1-ER α using Fugene 6 (Roche) at a Fugene:DNA ratio of 9 μ l:1 μ g. Two days later, cells were split into control or OSM treatment groups and on the following day seeded in triplicate onto 8 μ m pore polycarbonate filters in 24-well plates at a density of 50 000 cells/well using FBS as the chemoattractant. Cells were fixed 24 h later in 3.7% formaldehyde and stained with crystal violet. Cells on the upper membrane surface were removed with a cotton swab and migrated cells in four random fields counted under high power (200 $\times$) and averaged to produce one of the three replicate values.

Clinical cohorts

Two independent cohorts of human breast cancer were used. The first included 70 invasive breast carcinomas obtained from the Manitoba Breast Tumour Bank (MBTB), which operates with approval from the Research Ethics Board of the Faculty of Medicine, University of Manitoba. Cases were selected to represent several molecular subtypes (luminal-A, luminal-B, Her2 and basal-like and triple-negative non-basal (TNNB)). ER and PR status were previously determined by ligand-binding assay (LBA) and Her2, EGFR, CK5/6 and Ki67 status were determined by

immunohistochemistry in previous studies (Skliris *et al.* 2008, Blanchard *et al.* 2009). Luminal-A and -B tumours were defined as ER+ and/or PR+ (LBA scores of ≥ 10 and > 15 , respectively) and Her2– (IHC score $< 3+$); luminal-B tumours were additionally Ki67+ ($> 10\%$). Her2 tumours had an IHC clinical score of 3+. Basal-like tumours were triple negative for ER, PR and Her2 and were positive for CK5/6 and/or EGFR (IHC scores derived from the product of intensity and % positivity, as described previously (Wang *et al.* 2008)). TNNB tumours were triple negative but lacked CK5/6 and EGFR expression. Frozen tissue sections were used for RNA extraction and RT-PCR. Additional frozen tumour samples used to generate supplementary data were obtained from the BC Cancer Agency's Tumor Tissue Repository (REB certificate #H06-60001).

The second cohort was derived from a publically accessible microarray gene expression dataset (Prat *et al.* 2010), acquired from the University of North Carolina Microarray Database (UNC). Data from probes matching the same gene were collapsed by averaging (information regarding assay platforms and individual probes can be obtained from the Gene Expression Omnibus web site using accession number GSE18229). Only data from invasive carcinomas were assessed. Data for genes of interest were median normalised and converted to log₂ ratios before further analysis. For comparisons among *CD68*-low cases, we renormalised expression values to the *CD68*-low median before analysis.

Statistical analysis

All experiments were repeated at least thrice unless otherwise specified. Specific tests are specified in figure and table legends; all were two sided with significance established at < 0.05 . Univariate tests were performed using Prism 5.0 (GraphPad, La Jolla, CA, USA) and multivariate tests with Statistics 14 (SPSS, Chicago, IL, USA).

Results

OSM signalling suppresses ER expression

We began by studying the effect of OSM on three ER+ human breast cancer cell lines. Stimulation of both MCF7 and T47D cells with escalating doses of OSM revealed maximal suppression of ER at a dose of 100 ng/ml after 24 h of treatment. This corresponded to a reduction in ER protein by up to 95% in MCF7 cells and 85% in T47D cells (Fig. 1A). In contrast, OSM had no apparent effect on ER expression in a third ER+

Figure 1 Suppression of ER expression by OSM. (A) Western blot analysis of MCF7 and T47D cells treated for 24 h with 1–200 ng/ml of OSM. (B) RT-PCR assay for *ESR1* and *PGR* mRNA levels in MCF7 cells after 24 h of OSM treatment. Bars represent mean $\pm$ s.d. ***P < 0.001, Student's *t*-test. (C) Western blot comparison of the ER-suppressive effects of OSM vs IL6 when administered at 100 ng/ml to MCF7 cells. (D) Time-course western blot assay of MCF7 cells treated with 100 ng/ml OSM for 1–96 h.

cell line, ZR75-1 (data not shown). *ESR1* (ER) mRNA levels in MCF7 cells were also significantly reduced after 24 h of OSM stimulation, as were those of *PGR* (PR), a key target gene of ER transcriptional activity (Fig. 1B). Unlike OSM, stimulation with IL6, the prototypic cytokine of the OSM family, had comparatively little impact on ER expression (Fig. 1C). Time-course assays revealed that although OSM caused rapid (<1 h) activation of signalling effectors such as STAT3 and ERK1/2, ER protein levels did not noticeably diminish until after ~6 h of OSM treatment. With ongoing stimulation, ER levels remained stably suppressed for at least 96 h (Fig. 1D).

Suppression of ER by OSM depends on expression of OSMR

As OSM can engage both OSMR-gp130 and LIFR-gp130 receptor heterodimers, we assessed the degree of receptor specificity for the ER-suppressive activity of OSM by knocking down *OSMR* expression with siRNA. In MCF7 cells transfected with a control GFP-targeted siRNA, 24 h of OSM treatment caused an unexpected four- to five-fold increase in *OSMR* mRNA, but this was completely abrogated by transfection with *OSMR* siRNA. OSMR knockdown prevented

suppression of both *ESR1* and *PGR* by OSM (Fig. 2A). This was also evident at the protein level (Fig. 2C). As noted above, OSM had a robust impact on ER levels in MCF7 cells, a reduced effect on T47D cells and no observable impact on ZR75-1 cells. This is consistent with the level of *OSMR* expression in these cell lines; relative to MCF7 cells, T47D cells have comparable but lower levels of *OSMR* expression, while expression in ZR75-1 cells is nearly 100-fold lower (Fig. 2B). These data suggest that suppression of ER by OSM occurs principally via the OSMR-gp130 heterodimer.

ER suppression by OSM is reversible and dependent on MAPK signalling

To determine the persistence of ER suppression by OSM, we stimulated MCF7 and T47D cells for 48 h, withdrew OSM and cultured the cells for a further 24 h in cytokine-free media. Withdrawal of OSM caused full restoration of ER expression, indicating that the effect of OSM is transient in the absence of ongoing stimulation (Fig. 3A). To determine the signal transduction requirements for ER suppression, we individually attenuated the STAT3, PI3K and MAPK pathways before OSM treatment. As expected, JAK inhibition blocked downstream pathways and ER loss

Figure 2 OSM suppresses ER via the OSMR receptor chain. (A) Transfection of MCF7 cells with *OSMR* siRNA abrogates the suppressive effects of OSM on *ESR1* and *PGR* determined by RT-PCR. Bars represent mean $\pm$ s.d. $**P=0.001-0.01$, $***P<0.001$, Student's *t*-test. (B) Levels of *OSMR* and *ESR1* expression, with and without OSM treatment in three ER+ cell lines, MCF7, T47D and ZR75-1. Levels are expressed as means of triplicate RT-PCR experiments relative to those of untreated MCF7 cells, $\pm$ s.d. $**P=0.001-0.01$, $***P<0.001$, Student's *t*-test. (C) *OSMR* siRNA attenuates phosphorylation of STAT3 and loss of ER protein in MCF7 cells determined by western blot.

(Fig. 3B, left panel). Transfection of MCF7 cells with *STAT3*-specific siRNA failed to attenuate ER suppression (Fig. 3B, right panel), as did blockade of PI3K activity using LY294002 (Fig. 3B, left panel).

Treatment with the MEKK1/2 inhibitor U0126, however, partly restored ER expression (Fig. 3B, left panel), along with an alternative MEKK inhibitor, PD98059, and the farnesyltransferase inhibitor

Figure 3 ER suppression by OSM is reversible and depends on MAPK signalling. (A) Western blot of MCF7 cells treated for 48 h with OSM, followed by removal of cytokine and continued culture for up to 24 h. (B) Western blot analysis of MCF7 cells stimulated with 100 ng/ml OSM in the presence of specific inhibitors of JAK, MEKK and PI3K activity (left panel) or *STAT3* siRNA (right panel). (C) Treatment of MCF7 cells with the MEKK inhibitor U0126 blocks the morphological changes characteristic of OSM signalling. Original magnification 200 $\times$.

FTI277, a disruptor of Ras processing and downstream MAPK activity ([Supplementary Figure S1](#), see section on [supplementary data](#) given at the end of this article). Although OSMR physically associates and cooperates with EGFR, inhibition of EGFR with AG1478 did not affect ER nor did inhibition of the growth factor signalling mediators NF- κ B and mTOR using the NF- κ B DNA-binding inhibitors oridonin and rapamycin respectively ([Supplementary Figure S2](#), see section on [supplementary data](#) given at the end of this article). Similarly, although OSM can activate other MAPKs including JNK and p38 MAPK, inhibition of these pathways had no impact on ER ([Supplementary Figure S2](#)), suggesting that OSM-induced ER suppression may be specifically due to the ERK1/2 pathway. Intriguingly, blockade of ERK1/2 in MCF7 cells also prevented the morphological changes characteristic of OSM signalling, implying that ER suppression and the gain of motility-associated morphology may be mechanistically linked ([Fig. 3C](#)).

Suppression of ER is functionally important during OSM signalling

To determine whether suppression of ER by OSM was functionally relevant, we first assessed the ability of

OSM-stimulated cells to respond to the ER ligand, E_2 . In MCF7 and T47D cells that had been conditioned by growth in hormone-free conditions for 3 days and subsequently stimulated with E_2 , OSM-treated cells failed to respond to E_2 by upregulating expression of PR ([Fig. 4A](#) and [Supplementary Figure S3A](#), see section on [supplementary data](#) given at the end of this article). Cell proliferation in response to E_2 was similarly inhibited by OSM treatment, though in our experimental conditions this was statistically significant only for T47D cells ([Supplementary Figure S3B](#)). To assess the role of ER suppression in OSM-induced migration, we constitutively overexpressed ER in MCF7 cells and subjected them to transwell migration assays. Control-transfected cells displayed a sixfold increase in migration 48 h following OSM treatment. In contrast, migration was only modestly enhanced by OSM in ER-transfected cells ([Fig. 4B](#)), which displayed significantly reduced activation of STAT3 and ERK1/2, but not PI3K ([Fig. 4C](#)). Collectively, these data indicate that OSM can reduce ER expression below a functionally critical threshold and, furthermore, that suppression of ER may be required for full engagement of OSM-induced signal transduction and cell migration.

Figure 4 Functional relevance of ER suppression by OSM. (A) Western blot analysis of PR expression in MCF7 cells after 72 h of hormone withdrawal, followed by 48 h of 10 nM 17 β -estradiol treatment with or without 100 ng/ml OSM. (B) MCF7 cells transfected with empty vector or pcDNA3.1-ER for constitutive ER expression. After 48 h of transfection, cells were treated with 100 ng/ml OSM for 24 h and seeded onto 8 μ m pore filters in modified Boyden chamber assays. Transmigrated cells were counted 24 h later. Bars represent the averages ($\pm$ s.d.) of four individual filters, relative to the migration rate of unstimulated cells. ** $P=0.001$ –0.01, *** $P<0.001$, Student's t -test. (C, top) Western blot analysis of MCF7 cells treated as in panel B, with corresponding densitometric quantification of p-STAT3 and p-ERK1/2 levels (bottom; expressed as fold induction following OSM treatment in each transfection group). Bars represent mean ($\pm$ s.d.) of triplicate samples. Overall experiment was repeated once with similar results. * $P=0.01$ –0.05, ** $P=0.001$ –0.01, Student's t -test.

The OSM pathway correlates with suppression of ER *in vivo*

To investigate the association of OSM signalling with ER in human tumours, we examined *OSM* and *OSMR* expression by RT-PCR in a cohort of 70 invasive breast carcinomas (MBTB cohort; see Materials and methods section). Data were analysed using the median expression values as cutpoints to produce two patient groups: those high in both *OSM* and *OSMR* and those with low levels of one or both. This grouping was chosen on the assumption that both OSM and OSMR must be expressed for full activation of OSM signalling. Overall, 12% (4/32) of ER+ tumours were associated with high *OSM/OSMR* status compared with 45% (17/38) of ER– tumours (Fisher's exact test, $P=0.0041$), and ER and PR protein levels were reduced by seven- to eight-fold in the *OSM/OSMR*-high group ($P<0.05$, Fig. 5A). To determine whether this association was related to tumour differentiation, we compared the frequency of high *OSM/OSMR* between each of five major molecular subtypes of breast cancer (luminal-A, luminal-B, Her2, basal-like and TNNB subgroups). High *OSM/OSMR* status was rare in the luminal subtypes (<5% tumours) but was seen in 20–60% of tumours within the other subtypes. When compared with luminal-A tumours, *OSM/OSMR* expression was significantly more frequent within the Her2 and basal-like classes ($P=0.0135$ and 0.0029, respectively, Fisher's exact test, Fig. 5B). As high levels of growth factor signalling may independently contribute to suppression of ER (Lopez-Tarruella & Schiff 2007), a larger subset of Her2 subtype tumours was included within the cohort to assess the association between *OSM/OSMR* and ER independently of Her2 status. Within this small Her2+ subgroup, there was still a significant inverse correlation between *OSM/OSMR* and ER levels (Spearman $r=-0.626$, $P=0.0014$).

To validate these findings in a larger cohort, we examined data from previously published gene expression microarrays of over 300 invasive breast cancers ('Prat cohort' (Prat *et al.* 2010)). Due to the larger sample size of this dataset, we were able to consider three groups of tumours with respect to *OSM/OSMR* expression (using medians as cutpoints): those high in both *OSM* and *OSMR* (high/high, $n=77$), those high in one or the other (high/low, $n=161$) and those low in both (low/low, $n=83$). *ESR1* expression was substantially reduced in the high/low and high/high groups relative to the low/low group, with a clear trend towards lower *ESR1* as representation of the OSM pathway increased (Fig. 5C). This pattern

Figure 5 The OSM pathway is associated with defective ER signalling and aggressive phenotypes *in vivo*. (A) Mean ($\pm$ s.e.m.) ER and PR ligand-binding assay values from 70 breast cancer cases assayed by RT-PCR for expression of *OSM* and *OSMR*. * $P=0.01-0.05$, ** $P=0.001-0.01$, Mann-Whitney *U* test. (B) Frequency of high *OSM* and *OSMR* expression in the 70-case cohort, subdivided by inferred molecular subtypes (see Materials and methods section). Associated legend also applies to panel A. (C and D) Association of the OSM axis with molecular features in the Prat microarray cohort. (C) Association of *OSM/OSMR* expression status with *ESR1* and ER-regulated genes. Bars represent mean expression values $\pm$ s.e.m. * $P=0.01-0.05$, ** $P=0.001-0.01$, *** $P<0.001$, Mann-Whitney *U* test. Associated legend also applies to panel D. (D) Distribution of *OSM/OSMR* expression across molecular subtypes. In all cases, median expression values were used as the cutpoint to determine high vs low *OSM/OSMR* expression.

was also largely replicated with respect to four ER-regulated genes: *PGR* (PR), trefoil factor 1 or pS2 (*TFF1*), GATA-binding protein 3 (*GATA3*) and cyclin D1 (*CCND1*). This implies that the ER pathway as a whole, rather than simply ER alone, is disabled in tumours with robust OSM activity. Consistent with the MBTB cohort, high *OSM/OSMR* expression was, relative to the luminal-A subtype, strongly associated with the Her2, basal-like and claudin-low subtypes, each of which is notable for low hormone receptor expression (Fig. 5D; $P=0.0003$, 0.0007 and 0.0009 , respectively, χ^2 test). *OSM/OSMR*-high status was correlated with lower *ESR1* expression even when analysis was restricted to clinically ER- cases (data not shown). As with the MBTB cohort, clinically Her2+ tumours with high *OSM/OSMR* expression

had considerably reduced *ESR1* expression relative to low/low cases ($P=0.0184$, data not shown).

High OSMR expression is associated with poor prognosis

When the prognosis of the *OSM/OSMR* groups described above was assessed in the Prat cohort, we observed increased risk of recurrence in the high/low and high/high groups ($P=0.0323$; data not shown). However, when *OSM* and *OSMR* were assessed individually, only *OSMR* expression was associated with poor prognosis. To further explore this relationship, we first filtered the cohort by excluding cases with high (upper quartile) expression of the macrophage marker *CD68* (to increase the probability that assessed

OSMR expression was derived from malignant epithelium, as *OSMR* can be highly expressed in myeloid leucocytes (Dillon *et al.* 2004)). Cases high in *OSMR* (upper quartile) within this filtered cohort (total $n=241$) had a much greater risk of recurrence (HR = 5.06, 95% CI 2.49–10.29; $P<0.0001$; Fig. 6A) and overall mortality (HR = 5.64, 95% CI 2.63–12.11; $P<0.0001$). *OSMR*-high status was strongly associated with clinical ER – status ($P<0.0001$) but not clinical PR or Her2 status, nor lymph node metastasis, patient age, tumour grade or tumour size (Table 1). In multivariate Cox regression modelling of disease-free survival (DFS) involving the above parameters, *OSMR* was significantly associated with survival (HR = 2.75, 95% CI 1.15–6.55; $P=0.022$) along with lymph node and PR status (Table 1) and remained significant when molecular subtypes were included in the model ($P=0.025$). Indeed, *OSMR* was strongly prognostic even in the poor outcome basal-like ($P=0.0087$) and Her2 ($P=0.0001$) intrinsic subtypes (Supplementary Figure S4, see section on supplementary data given at the end of this article). *OSMR*-high status was also associated with loss of co-expression of *ESR1* and ER-regulated genes ($P<0.0001$; Fig. 6B). Among *OSMR*-high cases, those that retained high expression of both *ESR1* and *PGR* had a highly favourable prognosis relative to *ESR1/PGR*-suppressed cases ($n=41$, $P=0.0014$; Fig. 6C). This supports the concept that suppression of ER is required for OSM to fully activate an aggressive phenotype in breast cancer cells.

As the above *in vivo* observations could be attributable to enrichment of *OSM/OSMR* expression in the principally ER – basal-like, Her2 and claudin-low subtypes, we examined luminal subtype cases within the Prat cohort (>90% ER+) and observed relatively high levels of co-expression of *OSM* and *OSMR* to be associated with reductions in both *ESR1* ($P=0.0022$) and *GATA3* ($P=0.0009$) expression (Supplementary Figure S5, see section on supplementary data given at the end of this article), two key contributors to the luminal subtype definition (Sorlie *et al.* 2001). High *OSM/OSMR* status was also associated with poor prognosis ($P<0.0001$; Supplementary Figure S5), and these observations were not due to enrichment of *OSM/OSMR* expression in the luminal-B subtype.

OSM expression in breast tumours is associated with the innate immune compartment

Although OSM is considered as a product of leucocytes, direct evidence for this in breast cancer is lacking. To investigate this question, we examined

Figure 6 High *OSMR* expression is associated with poor prognosis. Cases in these analyses are those remaining after removal of *CD68*-high cases from the original cohort (see text). (A) Association of *OSMR* with 5-year disease-free survival. *OSMR*-high status is defined as the upper quartile of expression values. (B) Expression of ER-regulated genes (*ESR1*, *PGR*, *TFF1*, *CCND1* and *GATA3*) in the *OSMR*-high and -low subgroups. Cases are categorised according to the number of genes with expression values greater than the median. Significance determined by χ^2 test. (C) Clinical significance of hormone receptor loss in *OSMR*-high tumours. *OSMR*-high cases are categorised based on retention of both *ESR1* and *PGR* expression (> median) vs loss of one or both. Significance of survival curves was determined by the log-rank test.

Table 1 Prat cohort associations between *OSMR* and clinical parameters and their relationship with DFS in Cox regression modelling^a

Parameter	Association with <i>OSMR</i> ^b			Cox association with DFS (<i>n</i> =132) ^c		
	<i>OSMR</i> low	<i>OSMR</i> high	<i>P</i> value	Comparison	HR (95% CI)	<i>P</i> value
Patient age (year)						
<50	53	23	0.0762	<50 vs ≥50	0.48 (0.22–1.05)	0.067
≥50	90	20				
Tumour size						
T1	30	9	0.305	T3/4 vs T2 vs T1	1.67 (0.87–3.20)	0.122
T2	78	18				
T3–T4	35	15				
Tumour grade						
1–2	64	16	0.2848	3 vs 1–2	1.71 (0.72–4.08)	0.226
3	66	25				
Nodal status						
Negative	70	14	0.0805	Pos. vs neg.	2.85 (1.31–6.20)	0.008
Positive	74	29				
ER status						
Negative	39	26	<0.0001	Pos. vs neg.	1.00 (0.33–3.02)	1.00
Positive	101	16				
PR status						
Negative	68	22	0.1258	Pos. vs neg.	0.28 (0.10–0.84)	0.023
Positive	71	12				
Her2 status						
Negative	120	34	0.5197	Pos. vs neg.	1.84 (0.78–4.31)	0.161
Positive	27	10				
<i>OSMR</i> ^d	182	58	NA	High vs low	2.75 (1.15–6.55)	0.022

^aData reflect the cohort after removal of cases with *CD68* expression within the upper quartile.

^bComparisons calculated by two-sided Fisher’s exact or χ^2 tests as appropriate.

^cCalculated by Cox proportional hazards regression using the enter method.

^d*OSMR*-high cases are those with *OSMR* expression in the upper quartile.

OSM expression by RT-PCR in tumour tissues and cell lines. While *OSM* was readily detectable in breast tumour tissues, we did not detect expression in three breast cell lines (ER+ MCF7 and T47D cells and ER– MDA-MB-231 cells (Supplementary Figure S6A, see section on supplementary data given at the end of this article)). In the Prat cohort, we observed a significant association ($P<0.0001$) between *OSM* and expression of the pan-leucocyte marker *PTPRC* (CD45, Supplementary Figure S6B). Hierarchical clustering of cases with high expression of the immune cell markers *CD3Z* and/or *CD68* (upper quartile, $n=122$) using genes definitive for distinct leucocyte subtypes yielded expected myeloid/antigen presenting cell (APC), T cell, B cell and cytotoxic clusters. Of these, *OSM* grouped clearly with the APC subset (Supplementary Figure S6C). Analysis of individual genes showed strong associations between *OSM* and APC markers such as toll-like receptors, *CD14* and *CD163* but weak, non-existent or inverse relationships with lymphocyte markers (Supplementary Figure S6D). Thus, although this remains to be proven, innate leucocytes are a probable source of *OSM* in breast tumours.

Discussion

We have demonstrated that *OSM* drives the suppression of ER *in vitro* through a MAPK-dependent mechanism in breast cell lines and that this pathway is part of the pro-migratory phenotype stimulated by *OSM* in breast cancer. In human breast tumours, the *OSM* pathway is associated with reduced ER activity and poor prognosis. *OSM* signalling may therefore be a novel mechanism underlying ER suppression in breast cancer.

The phenotypic heterogeneity of breast cancer, of which ER expression is a key component, is currently explained by two main hypotheses. The first is a lineage-based model in which specific cells of origin (for example, luminal progenitor cells) are mutated and progress to malignancy along defined paths. The second invokes a stochastic model, whereby tumour cells are phenotypically plastic and adjust their behaviour in response to both mutagenic events and shifting environmental conditions. As an explanation for the evolution of ER– breast cancer, our data fit well with the latter hypothesis. Despite their luminal

phenotype, MCF7 and T47D cells rapidly down-regulate ER upon stimulation with OSM and adopt features typically associated with ER[−] disease, such as enhanced migration. Suppression of ER and oestrogen responsiveness may explain early observations that OSM reduces proliferation of breast tumour cells *in vitro* (Grant & Begley 1999). Upon withdrawal of OSM, hormone receptor suppression is reversible. These findings suggest that breast tumour cells *in vivo* may respond to external factors such as OSM by adopting a phenotype that, through activation of pathways such as MAPK, PI3K and STAT3, could afford them key advantages including improved survival, invasion and dissemination, independent of hormones. Upon cessation of stimulus, these cells could restore expression of ER. Such a model could explain the association between leucocyte infiltration and ER negativity, a phenomenon that is not currently understood (Teschendorff *et al.* 2007a). Furthermore, such a model would imply that some tumours characterised as low ER⁺ or ER[−] are in fact tumours in which dynamic cell-extrinsic factors serve to suppress ER expression. Indeed, we observed here that ER⁺ luminal-type breast tumours enriched for *OSM* and *OSMR* had lower ER expression and a prognosis similar to that of clinically ER[−] tumours. It should be noted that our data do not demonstrate that ER⁺ intrinsic subtypes can evolve directly into ER[−] subtypes due to OSM. Rather, because OSM signalling appears to be generally associated with depressed ER activity and poor prognosis in all subtypes, our data should only be interpreted as a demonstration that OSM signalling may be one among several possible mechanisms underlying ER suppression in breast cancer, regardless of intrinsic subtype.

The expression of *OSMR* varies among breast cancer cell lines and may explain much of the variation in their responsiveness to OSM. Compared with MCF7 cells, T47D cells had roughly half the level of *OSMR* mRNA (with a corresponding 30% reduction in the effect of OSM on ER expression), while ZR75 cells had nearly 100-fold less *OSMR* expression and no observable loss of ER in response to OSM (Fig. 2). Consistent with this notion is the observation that *OSMR* expression in breast tumours was more closely associated with clinical outcome than OSM. The reasons for variability in *OSMR* expression in breast tumours are not clear at this time but may be related to promoter methylation (Deng *et al.* 2009, Kim *et al.* 2009). Alternatively, the ability of OSM to induce *OSMR* expression (Fig. 2) suggests that varying *OSMR* levels *in vivo* may reflect local OSM concentrations in the tumour microenvironment. It has also been recently

shown that c-myc status serves as a determinant of the net cellular response to OSM in breast cell lines (Kan *et al.* 2011).

ER suppression following OSM stimulation was, in our experiments, dependent on Ras-mediated MAPK signalling, with no apparent requirement for STAT3 or PI3K activity. The involvement of MAPK signalling is consistent with the data from other studies. For example, MCF7 cells engineered to overexpress EGFR or constitutively active Her2, Raf or MEK exhibited oestrogen-independent growth, suppression of ER (Oh *et al.* 2001) and expression of a consistent set of MAPK-regulated genes that could accurately predict ER expression in human tumours (Creighton *et al.* 2006). Furthermore, ER suppression in this model was reversible and could be counteracted using MAPK inhibitors (Bayliss *et al.* 2007). Our work expands on these studies by identifying a physiologically relevant cytokine that potently activates MAPK-dependent ER suppression.

While various leucocyte subtypes are known to produce OSM, evidence that OSM is produced by tumour cells is inconclusive. A single study of its expression in breast cancer tissue was restricted to immunohistochemistry rather than mRNA expression (Garcia-Tunon *et al.* 2008). Our preliminary investigation of this issue indicated that OSM is absent in at least three commonly used breast cancer cell lines and that its expression *in vivo* correlates strongly with markers of innate leucocytes. Intriguingly, a recent study demonstrated that macrophage-conditioned media could suppress ER expression in MCF7 cells in a MAPK-dependent manner (Stossi *et al.* 2011). However, the specific factors mediating this observation were not identified. Therefore, OSM produced by tumour infiltrating leucocytes may constitute a novel cell-extrinsic mechanism of ER suppression. Further studies involving *in situ* hybridisation for *OSM* mRNA localisation in breast tissues and/or direct analysis of different cell types in fresh breast tumour specimens will be required to conclusively resolve this issue.

Relative to ER⁺ lesions, ER[−] breast tumours are enriched in leucocytes and cytokines (Chavey *et al.* 2007, Teschendorff *et al.* 2007a). Nevertheless, little is known regarding the direct effects of cytokines on ER expression. IL6 and tumour necrosis factor alpha (TNF- α) are reported to suppress ER expression (Bhat-Nakshatri *et al.* 2004, D'Anello *et al.* 2010), but other studies have presented conflicting results (Speirs *et al.* 2000, Rubio *et al.* 2006). In our hands, IL6 had little effect on ER expression (Fig. 1C). Clinically, IL6 has not emerged as a predictor of

response to endocrine therapy (Muss et al. 2007), nor has it shown great utility as a therapeutic target (Garber 2009). Preliminary data from our lab suggest that TNF- α does indeed suppress ER and that it can do so synergistically with OSM (NR West and PH Watson, unpublished observations). When we examined the expression of inflammatory cytokines and their receptors (OSM, IL6, LIF, IL1 and TNF- α) in the Prat dataset for associations with ER pathway expression and prognosis (as in Fig. 6), only *OSMR* and the TNF- α receptor (*TNFRSF1A*) associated with both parameters, though the *OSMR* relationships were considerably stronger (Supplementary Table S1, see section on supplementary data given at the end of this article). Thus, among inflammatory cytokine pathways, OSMR signalling may have a uniquely potent effect on breast tumour biology, for as-yet unknown reasons.

The correlation of OSMR with poor prognosis was not mirrored by its ligand, OSM, which may reflect a role for OSMR as the key limiting factor in this system. Alternatively, as a leucocyte product, the putative negative influence of OSM may be difficult to separate from the known beneficial impact of host immunity, particularly within ER – lesions (Teschendorff et al. 2007b, Rody et al. 2009). Notably, OSMR serves as a receptor for another cytokine, IL31, which is produced by activated Th2 cells (Dillon et al. 2004). Thus, IL31 could constitute an alternative OSMR ligand in tumours. The concept of innate leucocytes producing OSM and inducing aggressive changes in neighbouring tumour cells is consistent with the conventional mindset that these leucocytes are largely responsible for the various deleterious effects of anti-tumour immunity (Balkwill et al. 2005).

Future investigation of OSM as an ER modulator should involve *in vivo* pre-clinical models to further address the effect of OSM on tumorigenesis, ER expression and response to endocrine therapy. If such studies verify that OSM signalling promotes breast tumorigenesis, it is possible that this pathway could be targeted therapeutically. Translation to the clinic will require development of strategies for manipulation of either OSM production from macrophages or OSM reception and signalling through OSMR in tumour cells. Some existing chemotherapies are under investigation for their suppressive effects on myeloid cells (Naiditch et al. 2011) and new chemical and liposomal drug delivery systems that target macrophages are in development (Kelly et al. 2011, Needham et al. 2011). Development and clinical testing of monoclonal antibody-based therapies targeting IL6 signalling at both the ligand and the receptor level are already well

advanced (Jones et al. 2011), suggesting that OSM/OSMR signalling may be a feasible therapeutic target. Several features of OSM and OSMR make them attractive potential targets. First, by engaging multiple signalling cascades that potentially influence cell survival and migration, such as the STAT, MAPK and PI3K pathways (Heinrich et al. 2003), blockade of OSM signalling would impinge on each of these mechanisms. Secondly, because both gp130 and OSMR signal cooperatively with EGFR family receptors, blockade of OSMR could potentially attenuate EGFR/Her2 signalling (Grant et al. 2002). Thirdly, both antibody neutralisation of OSM and pharmacological inhibition of OSMR ligand binding would be feasible therapeutic mechanisms. Finally, because the effects of knocking out *Osm* and *Osmr* expression in mice appear mild (Fasnacht & Muller 2008), the side effects of targeting OSM signalling may be minimal. Further exploration of the OSM pathway as a potentially clinically relevant modulator of breast cancer is warranted.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-11-0326>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N R West and P H Watson designed the study and drafted the manuscript. N R West performed the experiments. N R West, L C Murphy and P H Watson contributed to manuscript revision and approved the final version.

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