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Conflict of interest: The authors have declared that no conflict of interest exists.
Abstract

The tumour microenvironment (TME) is reprogrammed by cancer cells and participates in all stages of tumour progression. The contribution of stromal cells to the reprogramming of the TME is not well-understood. Here we provide solid evidence of the role of the cytokine Oncostatin M (OSM) as central node for multicellular interactions between immune and non-immune stromal cells and the epithelial cancer cell compartment. Oncostatin M Receptor (OSMR) deletion in a multistage breast cancer model halted tumour progression. We ascribed causality to the stromal function of OSM axis by demonstrating reduced tumour burden of syngeneic tumours implanted in mice lacking OSMR. Single-cell and bioinformatic analysis of murine and human breast tumours revealed that OSM expression was restricted to myeloid cells, whereas OSMR was detected predominantly in fibroblasts and, to a lower extent, cancer cells. Myeloid-derived OSM reprogrammed fibroblasts to a more contractile and tumorigenic phenotype, elicited the secretion of VEGF and pro-inflammatory chemokines CXCL1 and CXCL16, leading to increased neutrophil and macrophage recruitment. Collectively, our data support that stromal OSM:OSMR axis reprograms the immune and non-immune microenvironment and plays a key role in breast cancer progression.
Introduction

The tumour microenvironment (TME), composed by different cell types (e.g. fibroblasts, adipocytes, endothelial and infiltrating immune cells), harbours complex cell interactions that are often manipulated and hijacked by tumour cells in every step of cancer progression (1). Tumour cells corrupt the local microenvironment and promote the recruitment of primarily immune-suppressor cells from circulation (1). In addition, growth factors released by cancer and stromal cells (including serum growth factors) have an important role in tumour proliferation and malignant progression (2). However, the contribution of stromal cells to the reprogramming of the tumour microenvironment is poorly understood. Cancer-associated fibroblasts (CAFs) are a key cell population in the tumour stroma. Apart from their very well-known functions in matrix deposition and extracellular matrix remodelling, CAFs have recently been shown to interact with the immune system by responding to and secreting chemokines and cytokines (3). They differ from non-cancer associated fibroblasts in multiple aspects and have distinctive properties including a particular cytokine and chemokine secretory profile (4). However, CAFs are very heterogenous and different subsets of functional fibroblasts have been proposed, some with predominant secretory functions and some with a prominent matrix remodelling phenotype (3).

Here, we discovered that the cytokine Oncostatin M (OSM) acts as a central regulator of the crosstalk between immune cells, CAFs and cancer cells; and that these immune-stromal-cancer cell interactions favour breast cancer progression and metastasis. OSM belongs to the IL6 family, which is considered one of the most important cytokine families in the process of tumorigenesis and metastasis (5). IL6 and OSM are acute-phase mediators of inflammation mainly produced by activated leukocytes. They can activate both epithelial and stromal cells to produce a wide array of additional inflammatory mediators (6). Nevertheless, the role of OSM in the TME remains unclear. OSM was first described as an anti-tumoral cytokine due to its...
anti-proliferative effect in melanoma and other cancer cells (7). However, in recent years, OSM has been associated with tumour progression as it induces EMT transition, cancer stem-like features, cell migration and metastasis in animal and cellular models (8, 9). High Oncostatin M receptor (OSMR) expression in clinical samples of glioblastoma, breast and cervical cancer correlates with decreased survival in those patients (10–12). However, the information regarding the role of OSM signalling in the TME is scarce, restricted to reports describing increased mRNA expression in this compartment (13, 14) and a role for OSM in macrophage M2 polarization (15).

In this study, we use samples from human primary breast tumours, transgenic and orthotopic mouse models of breast cancer, genetically modified mice lacking OSM signalling, single-cell analysis and in vitro cultures to demonstrate that OSM is a central node for multicellular interactions within the breast TME.
Results

Stromal OSM:OSMR axis promotes breast cancer progression

First, we set to study the contribution of OSM signalling in the genetic mouse model MMTV-
PyMT, widely used to study breast cancer progression in a fully competent tumour
microenvironment and immune system (16). We crossed OsMr deficient mice (KO) with
MMTV-PyMT as illustrated by the experiment scheme in Figure 1A. OsMr KO mice are viable
but show mild defects in acute inflammation, liver regeneration, thymic hypoplasia and net
metabolism of bone and fat (17), suggesting that OSMR deficiency is not completely
compensated. MMTV-PyMT:OsMr KO females showed a significant delay in tumour onset,
tumour growth and a reduced tumour burden at 14 weeks of age (Figure 1, B-D and
Supplemental Figure 1, A-D). Importantly, OSMR deletion also reduced the malignancy of the
tumours, assessed by histopathological analysis, as it reduced the percentage of mice with
malignant carcinomas and increased the proportion of mice with pre-malignant adenomas/
mammary intraepithelial neoplasia (MIN) or no tumours (Figure 1E and Supplemental Figure
1E, P value = 0.007 for Chi-square test comparing malignant lesions versus pre-malignant
lesions or no lesions). Interestingly, when compared to their controls, tumours in OSMR
deficient mice showed decreased levels of the extracellular matrix protein fibronectin,
predominantly produced by CAFs (4) (Figure 1, F and G) and increased levels of apoptosis, but
similar degree of proliferation (Supplemental Figure 1, F and G). Finally, OSMR deficiency
produced a remarkable reduction in the percentage of animals with lung metastasis (Figure 1,
H and I).

These results show that OSM signalling is causally associated with tumour aggressiveness but,
surprisingly, by using syngeneic cancer models, we found that this association requires, at least
in part, the presence of the OSM:OSMR axis in the tumour stroma. We injected TS1 cells,
derived from a MMTV-PyMT tumour (18), orthotopically into the mammary gland of syngeneic
*Osmr* deficient (KO) and wild-type (WT) control mice (Figure 2A). This model allows the assessment of the contribution of stromal OSM signalling to cancer progression as OSMR is only depleted in the tumour microenvironment while TS1 cancer cells express OSMR that can be activated by host-derived OSM (Supplemental Figure 2, A-C). Depletion of OSMR in the tumour microenvironment resulted in delayed tumour onset and tumour growth (Figure 2, B-E and Supplemental Figure 2, B and C) confirming that stromal OSMR signalling contributes to cancer progression. Conversely, OSMR depletion in cancer cells by CRISPR technique did not show any effect in tumour onset and tumour growth in WT animals (Supplemental Figure 2, D-G).

Analysis of published gene expression profiles of breast cancer demonstrated that both OSM and OSMR are increased in human breast cancer stroma, compared to cancer epithelial compartment and healthy stroma (Figure 2, F and G). A similar pattern of OSM:OSMR expression was observed in other cancer types including colorectal and ovarian cancers (Supplemental Figure 3A). We also observed that increased OSM mRNA levels associated with decreased disease-free survival in the Metabric (19) and Wang (20) breast cancer datasets (Supplemental Figure 3B). Analysis of TCGA data by Kaplan-Meier Plotter (21) showed that high OSM levels were significantly associated with worse overall survival in other cancer types (Supplemental Figure 3C).

**The OSM:OSMR signalling module exhibits a distinct microenvironment-restricted expression**

As we found an unexpected contribution of stromal OSM:OSMR axis to breast cancer progression, we performed single cell RNA-seq analysis of mammary tumours from the MMTV-PyMT model to decipher which cells were responsible to produce OSM and to express OSMR in the breast cancer context (Figure 3A). Our data indicate that the ligand *Osm* is almost exclusively expressed by the myeloid cell population, while the receptor *Osmr* is mainly...
expressed by the fibroblasts and some of the cancer epithelial clusters (Figure 3, A-C). The

*Osm:**Os_mr* signalling module exhibits a distinct microenvironment-restricted expression and it differs from the one observed for other cytokine-receptor pairs of the same family such as *Il6:*Il6r and *Lif:*Lifr (Figure 3, B and C), supporting that OSM exerts distinct and unique functions from other members of the family (22). *Il6st* (*GP130*) is the common subunit receptor for OSM, IL6, LIF and other cytokines of the family and is ubiquitously expressed (Figure 3, B and C), being the expression of the other receptor subunits more restricted and tightly regulated. RT-qPCR analysis of FACS-sorted breast TS1 orthotopic tumours (18) confirmed expression of *Osm* in the myeloid population and expression of *Os_mr* in fibroblasts and in cancer cells (Supplemental Figure 4, A-C). Similar results were obtained when analysing FACS sorted populations of MMTV-PyMT tumours (Supplemental Figure 4D). An identical pattern of *OSM:**OSMR* expression is maintained in the human setting, as demonstrated by RT-qPCR quantification in a large panel of human cell lines and analysis of RNA-seq data from the Human Protein Atlas (23) (Figure 4A and Supplemental Figure 5, A and B). *OSM* mRNA expression was restricted to undifferentiated and macrophage-like differentiated HL-60 cells (24) (Supplemental Figure 5A) and lymphoid and myeloid cell lines (Supplemental Figure 5B). Conversely, *OSMR* was only detected by RT-qPCR in breast cancer cells and fibroblasts, showing significantly higher expression in fibroblasts compared to epithelial cells (Figure 4A and Supplemental Figure 5A). Analysis of a battery of human cell lines (23) confirmed expression of *OSMR* only in epithelial, endothelial and fibroblast cell lines and not in immune cell lines (Supplemental Figure 5B). To prove the relevance of our previous findings in human cancer clinical data, we used the TIMER (25) and xCell (26) web resources to analyse the association between *OSM* and *OSMR* expression and TME composition in two different clinical breast cancer datasets (21, 27). TIMER analysis showed that *OSMR* mRNA expression significantly correlates with fibroblast enrichment in human breast cancer (Figure 4B), while *OSM* mRNA levels show the most significant associations with myeloid macrophage and
neutrophil populations (Figure 4C). This analysis also showed that OSM and OSMR mRNA expression inversely correlated with tumour cell purity. The OSMR and OSM associations with fibroblast and myeloid cell infiltration respectively, were validated by xCell in a different clinical dataset (Figure 4D). A similar pattern of OSM:OSMR expression was observed in FACS-sorted colorectal tumours (Supplemental Figure 5C). Altogether, our data reveal that OSM and OSMR are stroma-expressed molecules, and point to paracrine OSM:OSMR signalling in cancer, as ligand and receptor are expressed by different cell types in the tumour microenvironment.

**OSM activates CAFs promoting tumour progression**

As we previously observed that fibroblasts were the cell population with higher levels of OSMR within the tumour (Figures 3 and 4 and Supplemental Figures 4 and 5), we performed complementary in vitro and in vivo experiments to assess the effect of OSMR activation in mammary CAFs and normal fibroblasts derived from human breast tumours and reduction mammoplasty surgeries respectively (28). The ability to remodel the extracellular matrix is a hallmark of CAFs, that depends in the activation of actomyosin contractility (4). Importantly, OSM treatment enhanced the capacity of CAFs (CAF-173 and CAF-318) to contract collagen matrices and, interestingly, this effect was not observed in non-cancerous skin and breast fibroblasts (HS27 and RMF-31, respectively) (Figure 5, A and B). The effect of OSM in CAF contractility was blocked by pre-treatment with the highly potent inhibitor of Rho-associated kinase (ROCK) Y-27632 and could not be reproduced by LIF, a member of OSM cytokine family (Supplemental Figure 6A). In agreement, OSM activated molecular markers of actomyosin contractility (MLC2 and FAK) in CAF-173, but not in normal RMF-31 fibroblasts, and the effect was mediated by ROCK (Supplemental Figure 6, B-F). To further investigate the role of OSM in potentiating CAFs activation, we selected RMF-31 to be used as a model of normal breast fibroblasts and CAF-173 as a model of CAFs. In accordance with the contractility experiments, OSM promoted the growth of 3D CAF spheroids while it did not affect normal mammary
fibroblasts 3D spheroids (Figure 5, C and D). Similarly, OSM induced the expression of classical CAF markers such as *FAP, POSTN, VEGF* and *IL6* (4), only in CAF-173 CAFs, and not in normal RMF-31 fibroblasts (Figure 5E). Of interest, *OSMR* was similarly expressed in normal and CAFs (Figure 4A) and the pathway was functional in both cell types, as suggested by OSM induction of *OSMR* expression in both cell lines (Supplemental Figure 7A), a classical hallmark of OSMR activation (29). Gene set enrichment analysis (GSEA) of transcriptomic data of CAF-173 treated with OSM or vehicle, showed that OSM induced signatures related to fibroblast activation and JAK-STAT3 signalling, in agreement with increased STAT3 phosphorylation by OSM (Figure 5F, Supplemental Figure 7, B and C, and Supplemental Table 1). A transcriptional signature composed by the top differentially expressed genes by OSM in CAF-173 was enriched in the breast cancer stroma GSE9014 dataset compared to normal stroma (Supplemental Figure 7D). Importantly, the top 4 genes induced by OSM in CAF-173 (*SERPINB4, THBS1, RARRES1* and *TNC;* Supplemental Figure 7E) are associated with decreased overall survival in breast cancer patients (Figure 5G). In addition, *THBS1, RARRES1* and *TNC* levels correlate with *OSMR* expression in breast cancer clinical samples (Supplemental Figure 7F). These results indicate that OSM induces in CAFs the expression of pro-malignant genes, including fibroblast activation markers and genes associated with JAK-STAT3 signalling. Of interest, OSM induced changes in the transcriptome of CAF-173 that were different from the ones observed in OSM-activated MDA-MB-231 cancer cells (Supplemental Figure 8 and Supplemental Table 1), suggesting that OSM activates unique signalling pathways in CAFs.

Moreover, the changes induced by OSM in CAF-173 contributed to breast cancer malignancy as conditioned media from OSM-treated CAF-173 stimulated cancer cell migration in vitro (Supplemental Figure 9A). To test if the OSM-induced changes in CAFs contributed to breast cancer progression in vivo, we pre-treated CAF-173 CAFs with OSM or vehicle for 4 days in vitro and orthotopically co-injected them with MDA-MB-231 breast cancer cells into Athymic Nude-Foxn1nu mice as described in the experiment timeline in Figure 6A. Activation of
fibroblasts by OSM promoted tumour growth (Figure 6, B-D) and exhibited a trend to increase lung colonization (Figure 6E), assessed by qPCR analysis of human Alu DNA sequences in the lungs (30). The presence of metastasis in the lung of these mice was confirmed by vimentin staining of cancer cells (Supplemental Figure 9B). Conversely, OSMR downregulation by shRNA in CAF-173 delayed tumour onset and tumour growth at early stages when co-injected with MDA-MB-231 breast cancer cells ectopically expressing human OSM (Supplemental Figure 10, A-D). In addition, downregulation of OSMR in CAFs decreased IL6 expression in tumours, suggesting that OSM is inducing the expression of similar targets in vivo (Supplemental Figure 10E). Moreover, the tumours with OSMR silencing in CAFs showed reduced levels of GFP (Supplemental Figure 10E), suggesting reduced levels of CAFs in this experimental group, probably due to impaired CAF proliferation upon OSMR reduction, in line with the increased size of CAF spheres observed after OSMR activation (Figure 5, C-D). Together, our data prove that OSM:OSMR signalling activates CAFs and that this contributes to cancer progression.

**OSM signalling induces chemokine secretion and myeloid recruitment**

In an attempt to understand how OSMR activation in the stroma was inducing malignancy we deepened into our transcriptomic data of CAFs (CAF-173) treated with OSM. Microarray data indicated that pathways and signatures related to leukocyte chemotaxis and inflammatory response were significantly enriched by OSM (Figure 7, A and B). Interestingly, transcriptomic analysis of breast cancer cells (MDA-MB-231) activated by OSM showed enrichment of similar pathways (Supplemental Figure 11, A and B). These data suggested that, upon OSMR activation by OSM, both CAFs and cancer cells could be involved in shaping the tumour microenvironment by recruiting leukocytes to the tumour site. Analysis of a panel of 31 chemokines by antibody array showed that OSM induced expression of important chemoattractants (Figure 7C and Supplemental Figures 11C and 12). Some of these factors were exclusive of CAFs (mainly CXCL10 and CXCL12), others of cancer cells (mainly CXCL7 and
CCL20) and some factors, such as CCL2, were common for both cell types. Vascular endothelial growth factor (VEGF) can also modulate tumour immunity by inducing macrophage and myeloid-derived suppressor cells (MDSCs) recruitment (31) and we previously showed that it is an OSMR target (29). As seen in Figure 7D and Supplemental Figure 11D, VEGF levels were increased upon OSM treatment both in CAFs and tumour cells. As some of the OSM-induced chemokines are potent myeloid chemoattractants (e.g. VEGF, CCL2, CXCL12) (32, 33), we sought to determine whether OSMR activation influenced myeloid recruitment. Of interest, only conditioned media from OSM-treated CAF-173, and not from OSM-activated MDA-MB-231 cancer cells, promoted monocyte recruitment in vitro (Figure 8A and Supplemental Figure 11E). Accordingly, activation of CAFs by OSM resulted in increased number of tumour-associated F4/80 positive macrophages in vivo (Figure 8B). We also investigated if myeloid cell populations were altered in tumours after OSMR signalling abrogation and we quantified the number of F4/80 positive macrophages and Ly6G positive myeloid cells (1, 34, 35) in MMTV-PyMT:Osmr KO and control tumours. We observed that these two populations were reduced in MMTV-PyMT: Osmr KO tumours compared to Osmr WT tumours (Figure 8C). The decreased number of macrophages in MMTV-PyMT:Osmr KO tumours was confirmed by FACS analysis of CD45+ CD11b+ GR1med F4/80+ macrophages (Supplemental Figure 13, A and B). Of interest, there was no difference in the percentage of M2-like pro-tumoral (CD206+) and M1-like anti-tumoral (CD80+) macrophages in those tumours (Supplemental Figure 13C), suggesting that OSMR affects macrophage recruitment without altering their polarization. We did not observe a reduction of CD45+, CD11b+, GR1high neutrophils in Osmr KO tumours by FACS analysis (Supplemental Figure 13, A and B), suggesting that Ly6G staining in MMTV-PyMT:Osmr KO tumours may be marking neutrophils but also other myeloid-derived cells such as myeloid-derived suppressor cells (MDSCs) (36, 37). Marker analysis proved that most of the tumour-infiltrating neutrophils in MMTV-PyMT:Osmr WT and KO tumours exhibited a pro-tumoral and immunosuppressive phenotype assessed by CXCR4 and CCR5 positivity (38, 39) (Supplemental
Interestingly, tumour bearing MMTV-PyMT:Osmr KO mice compared to control mice showed reduced serum VEGF and CXCL16 levels and exhibited a trend towards a decrease in CXCL1 (Figure 8D), all factors being involved in myeloid cell recruitment (31, 40, 41). Our findings are clinically relevant as VEGF, CXCL1 and CXCL16 mRNA expression is associated with OSM and/or OSMR levels in breast cancer patients and with decreased overall survival (Figure 8, E and F). In summary, these results show that OSM:OSMR signalling in the stroma induces cytokine secretion and myeloid cell recruitment. As OSM is mainly expressed by myeloid cells (Figures 3 and 4 and Supplemental Figures 4 and 5), our data point to the existence of a feedback positive loop where OSM signalling induces the recruitment of more myeloid cells which will in turn secrete OSM within the tumour. Intriguingly, conditioned media from cancer cells pre-treated with OSM further increased OSM expression in macrophage-like differentiated HL-60 cells (Supplemental Figure 13E). We did not observe this effect with conditioned media from OSM-activated CAFs or with OSM itself. Therefore, we have discovered an unprecedented positive feed-forward loop between cancer cells, CAFs and myeloid cells in which 1) tumour infiltrating myeloid cells secrete OSM, 2) CAFs become activated promoting further myeloid cell recruitment and 3) OSM-induced secretome by cancer cells promote sustained OSM production by myeloid cells.

Analysis of OSM protein levels in 141 samples of early breast cancer samples confirmed the association between OSM expression and increased inflammation in a clinical setting (Figure 9, A and B). Inflammation was assessed by the pathologist as infiltration of inflammatory cells from all lymphoid and myeloid subtypes. Immunohistochemistry (IHC) staining confirmed that the tumour inflammatory infiltrate was composed, at least, by T cells (CD3*), macrophages (CD68*) and neutrophils (CD15*) (Supplemental Figure 14). We observed that OSM was mainly expressed by myeloid-like cells as determined by their larger size and more irregular shape (Figure 9A). Lymphoid cells, characterized by being smaller and round and by having a round nucleus with little cytoplasm, showed very low or negative OSM expression (Figure 9A).
Importantly, high OSM protein levels were associated with decreased overall survival in this dataset ($P=0.029$, Figure 9C).
Discussion

Cytokines are important players in inflammation, a process associated with tumour progression (42). Even the cancers not directly associated with persistent infections or chronic inflammation, such as breast cancer, exhibit tumour-elicited inflammation, which has important consequences in tumour promotion, progression and metastasis (5, 43) and facilitates the acquisition of cancer hallmarks (2). Understanding how inflammatory signals orchestrate pro-malignant effects in the different cell compartments within the tumour microenvironment is key to design new therapeutic strategies to target tumour-promoting inflammation. The oncogenic activity of inflammatory signals such as IL6 and OSM has been classically attributed to cell intrinsic mechanisms within the cancer cell. However, our results reveal a new key aspect of OSM:OSMR signalling that is instrumental for breast cancer progression beyond the epithelial compartment. Genetic and molecular analyses reveal that the tumour stroma responds to altered OSM production and signalling to influence breast cancer biology. Loss of OSMR in the non-tumoral tissue hampers tumour aggressiveness, thus demonstrating that tumour cell-extrinsic OSM signalling is a pivotal factor in breast cancer progression. Our study identifies the pro-inflammatory cytokine OSM as a crucial mediator of the crosstalk between different cell types within the tumour by activating an intriguing pro-tumoral “ménage-à-trois” between myeloid cells, CAFs and cancer cells.

Our single cell RNA-seq and FACS sorting analyses revealed that OSM and OSMR have a unique expression pattern in breast tumours, compared to other members of the IL6 cytokine family (Figures 3 and 4 and Supplemental Figures 4 and 5). While the ligand OSM was only expressed by the myeloid cell populations, we found that the receptor OSMR was mainly expressed by fibroblasts, cancer and endothelial cells. Whether there is one myeloid cell population mainly responsible for OSM production in breast cancer, or whether OSM is secreted by different immune cell types (including neutrophils, macrophages or even circulating monocytes)
remains to be determined. Of interest, a recent report identified OSM as one of the key signalling mediators of neutrophil-cancer cell interactions in pro-metastatic clusters of neutrophils and circulating tumour cells (CTCs) (44).

The cell population showing the most significant association with OSMR expression in human breast cancer samples is the CAF compartment, and our data point to an important role for this cell type in transducing OSM signalling within the TME. While the pro-tumoral effect of OSMR activation in cancer cells has been extensively described (10, 12, 45), little is known about the effects of OSM in the tumour stroma and our results shed light on the effects of OSM signalling in CAFs. It has been previously reported that OSM and its related cytokine LIF stimulate actomyosin contractility and matrix remodelling by oral SCC derived CAFs (46, 47). However, we did not observe any effect of LIF on collagen contraction assays in our experimental setting, maybe due to the particularities of the different protocols used. In addition to an effect in CAF contractility, we observed an increase in CAF proliferation and a pro-inflammatory phenotype in OSM activated CAFs. Moreover, the secretome of OSM-activated CAFs promoted cancer cell migration. To our knowledge, this is the first report describing that OSM induces the activation of a pro-inflammatory transcriptional programme in CAFs. In line with our results, a similar pro-inflammatory programme was described to be activated by OSM in intestinal stromal cells in inflammatory bowel disease (48). Importantly, our results reveal that OSMR activation in CAFs promotes the recruitment of OSM-producing myeloid cells to the tumour through OSM-induced secretion of chemokines, thereby inducing a feed-forward loop. It has been shown that blocking myeloid recruitment to the pre-metastatic niche with anti-Ly6G antibodies inhibits metastasis (49), and impairing recruitment of tumour-associated macrophages (TAMs) reduces tumour incidence and metastasis (50) in MMTV-PyMT mice. Thus, decreased numbers of Ly6G+ and F4/80+ myeloid cells may explain, at least in part, the strong antitumoral and anti-metastatic effect of OSMR depletion in the PyMT cancer model. Altogether, our data prove that OSMR activation in stromal CAFs could be
promoting tumour progression by different and complementary mechanisms, including increased matrix contractility and proliferation, activation of an inflammatory response, secretion of chemokines, and promotion of myeloid cell recruitment and cancer cell migration.

In summary, our results prove that OSM orchestrates an intriguing pro-tumoral crosstalk between myeloid cells, CAFs and cancer cells that has important consequences in tumour progression. Therapies aimed at modulating inflammatory responses in the tumour microenvironment have been of great interest in recent years (51). Of interest, targeting IL6 is problematic and anti-IL6 drugs have not yielded significant results against solid tumours in clinical trials (52, 53). Our results strongly support that therapeutic targeting of OSM signalling is a valid and worth exploring alternative to block tumour-promoting inflammation in cancer. OSM:OSMR signalling module exhibits a unique microenvironment-restricted expression pattern, distinct from the rest of the members of the family, supporting that OSM:OSMR targeting will potentially avoid the toxic effects of anti-IL6 drugs. OSM:OSMR interactions could be blocked by antibody based inhibition, a strategy that has had a major impact on cancer (54), which makes them a promising candidate for therapeutic targeting. Interestingly, anti-OSM humanized antibodies have proven to be safe and well tolerated (55) and are now in Phase 2 clinical trials for the treatment of inflammatory diseases, such as systemic sclerosis and Crohn's disease. Together, our findings further strengthen the case for the pre-clinical investigation of OSM:OSMR blocking antibodies as a targeted anti-cancer therapy.
Methods

Mouse studies. Generation of the congenic strain MMTV-PyMT:Osmr KO was accomplished by mating MMTV-PyMT mice (FVB/N-Tg(MMTV-PyVT)634Mul/J, The Jackson Laboratory), which spontaneously develop mammary tumours and lung metastases (56), with OSMR deficient mice: Osmr KO (B6.129S-Osmr<tm1Mtan>, Riken BRC) (57, 58). To transfer the transgenic Osmr KO line (with a C57BL/6J background) to the genetic background of the tumour-prone animals (FVB/NJ), the Osmr KO mice were previously backcrossed with FVB/NJ mice (Charles River) for 9 generations. Osmr WT (wild-type), HET (heterozygous) and KO (knockout) animals used for experiments were female littermates. Tumour onset was monitored by palpation and tumours were measured once a week using a calliper and volume was calculated as \((4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)\). Animals were culled at 14 weeks of age, once tumours in the control group reached the maximum allowed size. Tumour burden was calculated by adding the volume or the weight of all the tumours from the same animal. For whole-mount analysis of early lesions, abdominal mammary glands from 9 week-old MMTV-PyMT:Osmr KO and control female mice were spread out on a glass slide, fixed overnight in Carnoy’s solution, stained with Carmine Alum and cleared in ethanol and xylene. Pictures were taken with a Nikon D5000 at 60mm focal length. For the generation of syngeneic orthotopic tumours, 300,000 viable murine control or Osmr KO TS1 cells (derived from a MMTV-PyMT tumour in FVB/NJ mice (18)) in growth factor reduced (GFR) matrigel (1:1 ratio, Corning), were injected into the fourth right mammary fat pad of anesthetized (with 4% isoflurane) 6-8 week-old female FVB/NJ Osmr KO or control mice. For the orthotopic co-injections of human MDA-MB-231 breast cancer cells and CAF-173 CAFs, cells were injected into the fourth right mammary fat pad of anesthetized (with 4% isoflurane) 6 week-old female Athymic Nude-Foxn1nu immunocompromised mice (Envigo). In OSM activation experiments, CAF-173 were treated with 10 ng/mL OSM for 4 days, prior to co-injection with MDA-MB-231 (500,000 cells each cell line) in GFR matrigel (1:1 ratio). For OSMR knockdown experiments, 100,000 MDA-MB-231 hOSM cells and 500,000 CAF-173
shOSMR CAFs were co-injected in GFR matrigel (1:1 ratio). In all mouse experiments, animals were monitored 3 times a week and tumour growth measured using a calliper. Animals were culled once tumours reached the maximum allowed size. After animal culling, lungs were visually inspected for macroscopic metastases, and mammary glands and lungs were fixed in neutral buffered formalin solution (Sigma-Aldrich). Microscopic metastases were determined by H&E staining of formalin-fixed paraffin-embedded sections. Tumours were divided in portions for 1) preparation of tissue sections for H&E and IHC staining (fixed in formalin) and 2) protein and RNA extraction (snap frozen).

**Gene expression analyses of clinical datasets and bioinformatics analyses.** Disease-free survival (DFS) of patients based on OSM mRNA expression was calculated using data from the publicly available METABRIC (19) and Wang (20) datasets with the CANCERTOOL interface (59). Kaplan-Meier curves showing overall (OS) of patients from various cancer types according to the expression of different genes were obtained from Kaplan-Meier Plotter website (21). Expression values were stratified by median. RNA-seq data from 64 cell lines was retrieved from the Human Protein Atlas (23). RNA consensus normalized expression values were plotted for OSM and OSMR transcripts using GraphPad software. Associations between OSMR and OSM mRNA expression and infiltration of different cell types from the TME were analyzed by using xCell (26) on 1809 breast cancer samples from Kaplan-Meier Plotter website (21) and TIMER2.0 which incorporates 1100 breast cancer samples from TCGA (25). TIMER2.0 was also used to analyse gene expression correlations, after purity adjustment. All correlations were calculated with the Spearman’s rank correlation coefficient. Gene expression analyses of human tumour stroma and epithelia were retrieved from NCBI Gene Expression Omnibus (GEO): Finak (GSE9014, Breast) (60), Casey (GSE10797, Breast) (61), Yeung (GSE40595, Ovary) (62), Nishida (GSE35602, Colon) (63), and Calon (GSE39396, Colon) (64). For Affimetrix-based arrays, probe-to-gene mapping was performed using Jetset, while for the rest, highest variance
probes were selected. Unless otherwise stated, expression values for each gene were z-score normalized.

**Single-cell RNA sequencing (scRNA-seq).** Drop-seq dataset (65) raw data for MMTV-PyMT (WT) tumours were obtained from Valdes-Mora et al. (2021) (66). This subset was subsequently analyzed using Seurat (67) (v Seurat 3.2). Briefly, a total of 9,636 sequenced cells from 8 MMTV-PyMT tumours pass the QC filter, with <5% mitochondrial to nuclear gene content (65), and <8,000 molecules/cell as they potentially represented cell doublets. Downstream analysis was performed according to Butler et al. (2018) (67), using 30 principal components to build a Shared Nearest Neighbour (SNN) graph calculating k-nearest neighbour (Jaccard Index) for each cell, subsequent cluster calling and UMAP dimensional reduction projection (68).

**Cell culture.** Human breast cancer-associated (CAF-173, CAF-200, CAF-220 and CAF-318) and normal (RMF-31 and RMF-39) fibroblasts were derived from human breast tumours and reduction mammoplasty surgeries respectively, immortalized, tagged with GFP and cultured in collagen pre-coated flasks (28). The aforementioned human mammary fibroblasts, TS1 cells derived from primary tumours of the MMTV-PyMT mice model (18, 56), LM2 breast cancer cells (kindly donated by Dr Roger Gomis) and HS27 skin fibroblasts (kindly donated by Dr Ander Izeta) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin and streptomycin. HL-60 promyeloblast cell line, the human embryonic kidney cell line HEK293T and human breast cancer cell lines (MDA-MB-231, BT-549, HCC38, MDA-MB-157, SUM149PT, HCC1806, HCC70, MDA-MB-468, HCC1569, HCC1954, SK-BR-3, MDA-MB-453, CAMA-1, ZR-75-1, T47D, MCF-7, BT-474) were purchased from American Type Culture Collection (ATCC) and cultured following ATCC instructions. All cell lines were authenticated by short tandem repeat profiling (Genomics Core Facility at “Alberto Sols” Biomedical Research Institute) and routinely tested for mycoplasma contamination. HL-60
differentiation to macrophages and monocytes was achieved by adding 1nM of 12-O-Tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich) for 24 hours and 100 nM 1,25-(OH)2 vitamin D3 (Sigma-Aldrich) for 5 days, respectively. Recombinant human or murine OSM (R&D Systems) and human LIF (Millipore) was added to cells at 10 ng/mL unless otherwise specified.

**Generation of OSM overexpressing and OSMR knockdown cells.** MDA-MB-231 cells were stably transfected with 2 µg of pUNO1-hOSM expression construct (InvivoGen), using TurboFect™ followed by Blasticidin (Sigma-Aldrich) selection at 10 µg/mL. Control transfections were performed simultaneously using 2 µg of empty vector. For generation of murine TS1 Osmr knockout (KO) cells we used a CRISPR-Cas9D10A nickase strategy previously described (69). OSMR targeting guides (CTTAAAGTCTCGGGTTTCAC and GTGAAACCCGAGACTTTAAG) were cloned into an All-in-One backbone containing an EGFP-coupled Cas9 D10A nickase mutant, (AIO-GFP, Addgene). TS1 cells were transfected by nucleofection (Amaxa™ 4D-Nucleofector™, Lonza) with 2 µg of AIO-GFP plasmid containing OSMR targeting guides or the empty vector, and GFP+ cells were subjected to single cell FACS sorting 72h after. For OSMR knockdown in CAF-173 cells, pLKO-puro-shOSMR lentiviral vectors were purchased from Sigma-Aldrich (NM_003999.1-1342S21C1). Lentiviral infections were performed as previously described (70).

**Collagen gel contraction assays.** To assess the collagen remodelling capacity (71), fibroblasts were treated for 4 days with recombinant human OSM (R&D Systems) or LIF (Millipore) at 10 ng/mL or vehicle (PBS) before being embedded (250,000 cells per matrix) in collagen matrix (2mg/mL Rat tail collagen type I, Corning, in DMEM + 10% FBS) in the presence or absence of Y-27632 (10 µM, Tocris), and seeded in triplicate or quadruplicate in 24-wells plates. After polymerization, collagen gels were detached, and they were treated with OSM, LIF (both at 10ng/mL) or vehicle. Pictures were taken 48 hours later, and the area of collagen disks was analyzed using Fiji-Image J software.
**Cell migration assays.** MDA-MB-231 and CAF-173 cells were treated with a single dose of OSM (10 ng/mL) or vehicle (PBS) for 72h in serum-reduced media (2% FBS) for conditioned media (CM) generation. For breast cancer cell migration experiments, MDA-MB-231 cells were treated with the corresponding CM (diluted 1:2) for 72h and subjected to migration assays for 48h by seeding 25,000 cells at the top of 24-well transwell inserts (8 um pore, Corning). FBS was used as chemoattractant. Chambers were fixed in 10% formalin (20 min) and stained with crystal violet solution (20 min). For the quantification of migrated cells, crystal violet was solubilized with 600 ul of 1% SDS (30min) and absorbance was measured at 570nm. For monocyte migration experiments, 750,000 HL-60-derived monocytes were seeded at the top of transwell inserts (8 um pore, Corning) with 600 ul of the corresponding CM in the lower chamber. Cells were allowed to migrate for 3h and the number of migratory cells in the lower compartment was counted using a hemocytometer.

**3D fibroblast cell cultures.** Fibroblast spheres were formed seeding 8000 cells/well in 96-well ultra-low attachment Corning plates (Costar). Cells were treated with 30 ng/mL OSM or PBS for 3 (for transcriptomic microarray analysis) or 4 days (for RT-qPCR, Western blot analysis and quantification of spheres area). Pictures were taken using EVOS FL Cell Imaging System (ThermoFisher) and area of spheres was analyzed using Fiji-Image J software. Spheres were collected for RNA and protein analysis.

**Flow cytometry.** Freshly obtained tumours and mammary glands from 14-week-old MMTV-PyMT:Osmr KO, HET and WT mice were mechanically disrupted in 7 mL of digestion medium (Collagenase type 1, Merck) and incubated for 1 h at 37ºC. The single cell suspension was filtered through 70 μm cell strainer (Falcon) and treated with ACK lysis buffer (Invitrogen) for 3 minutes at room temperature (RT). Then, cells were stained with fluorochrome labelled antibodies described in Supplemental Table 2 and with DAPI (1/5000, ThermoFisher) in FACS buffer (eBioscience). Flow cytometry analysis was performed with a BD FACSsymphony flow
cytometer and data were analyzed on FlowJo (BD Biosciences). Gating strategy is shown in Supplemental Figure 13B. FACS sorting of TS1 GFP+ cells in CRISPR Cas9D10A nickase experiments was performed with a BD FACSJazz (2B74YG) cell sorter. For FACS sorting experiments of TS1-derived tumours, TS1 cells were injected orthotopically in FVB mice as described above, and 15 days after injection, freshly obtained TS1 tumours were dissociated into single cell suspension and stained with the antibodies described in Supplemental Table 2. Flow sorting was performed with a BD FACSaria II cell sorter. Gating strategy for experiments is shown in Supplemental Figure 4. A pool of 4 tumours from 4 animals was used for each sorting experiment. MMTV-PyMT tumours were sorted by Ferrari et al. (2019) (72) and RNA from FACS sorted tumours was kindly provided by Dr Fernando Calvo. Briefly, tumour populations were separated into fibroblasts (PDGFRA+), cancer (EPCAM+), immune (CD45+), endothelial cells (CD31+), and the remaining population (negative for all markers).

**Western blotting.** Cells and tumours were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail, and PhosSTOP, both from Roche). Total lysates were quantified by BCA (Pierce™ BCA Protein Assay Kit, Thermo Scientific), resolved by SDS/PAGE and transferred to nitrocellulose membranes. After blocking with 5% (wt/vol) nonfat dry milk in TBS-Tween, membranes were incubated with the corresponding antibodies (Supplemental Table 2) overnight at 4 °C. Secondary antibodies (Supplemental Table 2) were chosen according to the species of origin of the primary antibodies and detected by an enhanced chemiluminescence system (Bio-Rad). Densitometric analysis of the relative expression of the protein of interest versus the corresponding control was performed with Fiji-Image J software. Uncropped images used to display blots can be found in Supplemental material.

**DNA and RNA extraction, reverse transcription quantitative PCR (RT-qPCR) and transcriptomic analysis.** Lung genomic DNA was extracted from frozen lungs using the QIAmp
DNA mini kit (Qiagen) for qPCR analysis. RNA was obtained from snap frozen animal tissue or cell pellets and extracted using TRizol reagent (Invitrogen) or Recover all Total Nucleic Acid Isolation kit (Invitrogen), for RT-qPCR and microarray analysis, respectively. cDNA was obtained with the Maxima first strand cDNA synthesis kit (Thermo Scientific) with DNase treatment incorporated. qPCR was performed using Power SYBR Green PCR master mix (Applied Biosystems) and oligonucleotides sequences are described in Supplemental Table 3 (all purchased from Condalab). Expression levels of genes were determined using the ΔΔCt method (73) and normalized against 3 housekeeping genes optimized for each reaction (74). Human Alu sequences (30) were normalized against 18S housekeeping gene using primers capable of recognizing both human and mouse DNA. Microarray analysis was performed using Human Clariom S assay (ThermoFisher). RNA quality was evaluated using the 2100 Bioanalyzer (Agilent) and microarray chips were processed on the Affymetrix GeneChip Fluidics Station 450 and Scanner 3000 7G (Affymetrix) according to standard protocols (n=3 per experimental condition). Data were analyzed using the Transcriptome Analysis Console 4.0 (TAC). Genes with FDR<0.1 and fold change >|2| were considered significantly modulated. GO analysis was performed using Panther (75). GSEA was performed as previously described (76). FDR < 0.25 or 0.05 was regarded as statistically significant, depending on the type of permutations performed. We compiled the GSEA signatures used in Figures 5 and 7 and Supplemental Figures 7 and 11 from the Molecular Signatures Database (MsigDB) by the Broad Institute or they were manually curated from the literature. The gene list for each signature is publicly available at: http://software.broadinstitute.org/gsea/msigdb/search.jsp, Pein et al. (2020) (77) or in Supplemental Tables 4 and 5.

**Histopathology, immunohistochemistry (IHC) and immunofluorescence (IF) analyses.**

Histological analysis of murine tumours and lung metastasis was performed in H&E-stained sections. Immunohistochemical staining was performed in formalin-fixed paraffin-embedded sections using Novolink Polymer Detection Systems (Leica). Antigen retrieval was performed
using boiling 10 mM citrate buffer, pH 6.0, for 15 min. Endogenous peroxidase activity was inactivated by incubation with 3% hydrogen peroxide in methanol (15 min at RT). Tissue sections were incubated in a humidified chamber (overnight, 4 °C) using the antibodies described in Supplemental Table 2 diluted in Tris-buffered saline (TBS). For negative controls, primary antibodies were replaced by non-immune serum. After three rinses in TBS (5 min each), samples were incubated with the corresponding secondary antibody (Supplemental Table 2). After 30 min incubation, tissue sections were washed in TBS (5 min, 3 times) and immediately incubated for 30 min with streptavidin–peroxidase complex diluted 1/400 in TBS (Invitrogen). The chromogen was 3-30-diaminobenzidine (Vector Laboratories). Nuclei were counterstained with Harris haematoxylin for 1 min. Pictures were obtained using the Nikon Eclipse 80i microscope with a Nikon DS-5M camera incorporated. The number of positive cells and total cells per area was counted manually in 5-15 different areas of samples from 5-7 mice per experimental group, using Fiji-Image J software. For IF analysis, paraffin-embedded sections from murine lungs or cells fixed on a coverslip were permeabilized with 0.1% or 0.2% Triton X-100 and 0.01% SDS after performing antigen retrieval with citrate buffer for 15 min. The slides were then blocked with 3% BSA/PBS containing 3% normal goat serum at 0.1% Tween20 for 30 min - 3 h and stained overnight with the corresponding primary antibodies (Supplemental Table 2) followed by secondary antibody incubation (1 h at RT). F-actin was stained with Phalloidin CruzFluor™ 633 Conjugate (Santa Cruz). Nuclei were counterstained with DAPI (ThermoFisher). Finally, sections were mounted with Mowiol (ThermoFisher).

**Cytokine and chemokine analysis.** Cytokine and chemokine levels were analyzed in conditioned media from CAF-173 treated with OSM (30 ng/mL) or vehicle for 72 hours (n=4), and from MDA-MB-231-hOSM and corresponding control cells (n=6). A panel of 31 human chemokines was analyzed by Human Chemokine Array Kit (Proteome Profiler Array, R&D Systems), and VEGF levels were quantified by Human VEGF Quantikine ELISA Kit (R&D Systems) following the manufacturer’s instructions. Mouse VEGF, CXCL1 and CXCL16 levels on plasma
from 14-week-old MMTV-PyMT:Osr KO, HET and WT mice were analyzed by mouse Premixed Multi-Analyte Kit (Magnetic Luminex Assay, R&D Systems) following the manufacturer’s instructions. Detection was carried out with the MAGPIX® detector and data analysis was performed using the xPOTENT® software, both from R&D Systems.

**Tissue Microarrays.** Formalin-fixed and paraffin-embedded blocks of 141 tumour tissues from cases surgically resected at the University Hospital Basel between 1991 and 2013, and included in tissue microarrays (TMAs), were used for analysis of OSM protein expression in human samples. Complete histopathological information (Supplemental Table 6), date and cause of death, as well as date of local and/or distant relapse were available for all the patients. TMAs were generated by punching 1 mm spot of each sample. Tissue sections were subjected to a heat-induced antigen retrieval step prior to exposure to primary antibodies (Supplemental Table 2). Immunodetection was performed using the Roche Ventana BenchMark ULTRA IHC staining system, with DAB as the chromogen. Cases were reviewed by two independent pathologists and OSM staining was evaluated by the semiquantitative method of H-score (or “histo” score), used to assess the extent of immunoreactivity in tumour samples (78). Inflammation was semi quantitatively assessed by a pathologist as high or low tumour infiltration of immune cells according to their morphology.

**Statistics.** Statistical analyses were performed using GraphPad Prism or SPSS software. For Gaussians distributions, the student’s t-test (paired or unpaired) was used to compare differences between two groups. Welch’s correction was applied when variances were significantly different. One- or two-way ANOVA with post Tukey’s, Dunnett’s or Sidak’s multiple comparisons tests were used to determine differences between more than two independent groups. For non-Gaussian distributions, Mann–Whitney’s test was performed. Chi-square test was used to determine differences between expected frequencies. For Kaplan-Meier analysis the Log-rank (Mantel-Cox) test was used. $P$ values inferior to 0.05 were
considered statistically significant. Unless otherwise stated, results are expressed as mean values +/- standard errors (SEM).

**Data availability.** RNA-seq raw data were obtained from Valdes-Mora et al. (2021) (66) and is available in the GEO repository (GSE158677). The mRNA datasets generated during the current study are available in the GEO repository (GSE195787). Source data on uncropped Western blots are provided in Supplemental materials. The gene list for the fibroblast activation signature used in Figure 5 was derived from Sahai et al. (2020) (4) and is shown in Supplemental Table 4. The gene list for the CAF-173 OSM signature used in Supplemental Figure 7 includes the 233 genes differentially upregulated in CAF-173 upon OSM treatment and can be found in Supplemental Table 5. All other data files supporting the findings of this study are available from the corresponding author upon reasonable request.

**Study approval.** All patients whose samples were included in the TMAs have given written informed consent for their archival tissue to be used for scientific research in accordance with the Declaration of Helsinki, and the TMA construction was approved by the responsible local Ethical Committee EKBB (Ethikkommission beider Basel) number 361/12. All procedures involving animals were performed with the approval of the Biodonostia Animal Experimentation Committee and Gipuzkoa Regional Government, according to European official regulations.
Author Contributions

AMA, AA, PA, JR and IOQ performed all the cellular and molecular experiments. AMA, AA, JILV and MMC performed the animal experiments. JMF performed immunohistochemistry and analyzed mouse histopathology. JILV, SM and LP analyzed mouse immunostaining. FVM and DGO obtained and analyzed the scRNAseq data. AMA, LJ, NF, FC, LEM and AP performed FACS-Sorting and FACS analysis. AT and SEC collected and analyzed patient data, generated the TMAs, performed immunohistochemistry and interpreted and statistically analyzed the resulting data. RR and MMC interpreted and analyzed the TMA staining. AMA, AA, PA, NMM, FC, AC and MMC performed bioinformatic analysis. PFN and PB generated the fibroblast cell lines. PB, CV and AA performed and analyzed immunofluorescence staining. NC, IAL and AU contributed with experimental design. CMI and CHL contributed with experimental design and helped with supervision of the project. AMA, AA and MMC designed and supervised the study, analyzed the data and wrote the manuscript. Both AMA and AA contributed equally and have the right to list their name first in their CV and should be considered co-first authors. The order of co–first authors was determined by seniority in the laboratory. All authors gave final approval to the submitted and published versions of the manuscript.
Acknowledgements

We are grateful to the members of our laboratories for critical discussion of this work and to the Genomics and Histology Platforms and Animal Facility of the Biodonostia Health Research Institute and Onkologikoa Foundation for technical assistance and advice. We thank Dr Eva González-Suarez (CNIO, Madrid, Spain) and Dr William Muller (McGill University, Montreal, Canada) for providing the MMTV-PyMT mice, Dr Steve Jackson and Dr Valtteri Tulkki for providing CRISPR-Cas9D10A plasmids, and Dr Ander Izeta (IIS Biodonostia, San Sebastian, Spain) and Dr Roger Gomis (IRB, Barcelona, Spain) for providing the HS27 fibroblasts and LM2 cells respectively. This work was funded by Spanish Ministry of Science and Innovation - ISCIII (PI15/00623, PI18/00458, CP18/00076 and FI19/00193) and European Regional Development (FEDER) funds, Basque Department of Health (2017111011), Fundación SEOM (Beca SEOM-Font Vella), Fundación Gangoiti and Ikerbasque Basque Research Foundation. The group also received funds from the breast cancer patient’s charity Katxalin and from Roche Farma S.A. A.Araujo and A.Abaurrea are funded by Basque Government Doctoral Training Grants. JILV is funded by an AECC PhD Fellowship. FVM is supported by the Career Development Fellowship from the Cancer Institute New South Wales (2019/CDF002). AP’s research is funded by the European Research Council (ERC, ERC-2018-StG 804236-NEXTGEN-IO), and by the Spanish Ministry of Science and Innovation (PID2019-107956RA-I00 and RYC2018-024183-I). AC’s research is supported by the Basque Department of Industry, Tourism and Trade (Elkartek), the MICINN (PID2019-108787RB-I00 (FEDER/EU); Excellence Networks SAF2016-81975-REDT), European Training Networks Project (H2020-MSCA-ITN-308 2016 721532), the AECC (GCTRA18006CARR), Vencer el Cáncer Foundation, La Caixa Foundation (ID 100010434), under the agreement LCF/PR/HR17/ and the European Research Council (Consolidator Grant 819242). CIBERONC was co-funded with FEDER funds and funded by ISCIII. DGO is supported by the Cancer Council NSW project grant (RG18-03) and the National Breast Cancer Foundation Elaine Henry Fellowship (IIRS-21-096). FC is funded by Institute of Cancer Research, Spanish
Ministry of Science and Innovation (RYC-2016-20352 and RTI2018-096778-A-I00), Asociacion Española Contra el Cancer (LAB-AECC, LABAE19044CALV) and BBVA Leonardo Awards (IN[19]BBM_BAS_0076).
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Figure 1. Deletion of OSMR in the MMTV-PyMT model hampers tumour progression and reduces metastasis. (A) Experimental set-up of the in vivo experiment designed to assess the importance of OSMR signalling in disease progression of the MMTV-PyMT mouse model. F0,1 and 2: different filial generations. (B-D) Kaplan-Meier curves for tumour-free survival (B), tumour growth (C) and final tumour burden (D) in MMTV-PyMT:Osmr wild-type (WT), MMTV-PyMT:Osmr heterozygous (HET), and MMTV-PyMT:Osmr knockout (KO) mice. (E) Histopathological analysis of tumours at week 14. Graph represents percentage of mice bearing carcinomas, adenomas, hyperplasia and no lesions in mammary glands. * P value was determined comparing number of mice with malignant carcinoma vs. non-malignant phenotypes (adenoma, hyperplasia) and no lesions using Chi-square test. (F and G) Western blot (F) and densitometric analysis (G) of fibronectin (FN) protein levels in tumours at week 14 from animals of the different genotypes. (H) Percentage of animals with lung metastases at 14 weeks of age. * P value was determined comparing animals with metastasis (macro and micro) vs. non-metastasis using Chi-square test. (I) Representative pictures of lung metastases at week 14 in MMTV-PyMT:Osmr WT, HET and KO animals. Metastatic nodules are indicated with red arrows. Scale bar is 200 mm (upper and middle rows) and 50 mm (lower row). * P values were calculated using the Mantel-Cox test (B), two-way ANOVA with post Dunnett’s multiple comparison test (C) or one-way ANOVA test (D and G). ** P < 0.01; *** P < 0.001; **** P < 0.0001 KO vs WT and # P < 0.05 HET vs WT.
Figure 2. Stromal OSM:OSMR axis promotes breast cancer progression. (A) Experimental set-up of the in vivo experiment designed to assess the importance of OSMR signalling in the tumour microenvironment, in which TS1 cells were orthotopically injected into the mammary fat pad of Osmr wild-type (WT) and knockout (KO) mice. (B) Kaplan-Meier curves for tumour-free survival (B), tumour growth (C) and final tumour volume (D) and weight (E) after dissection of orthotopic tumours described in (A). Two independent experiments (exp 1 and 2) were performed, and the results were combined in B, D and E. (F and G) OSM and OSMR mRNA expression in paired cancer epithelial vs. cancer stroma (F, GSE10797) and normal stroma vs. cancer stroma breast cancer samples (G, GSE9014). Data were downloaded from GEO DataSets. P values were calculated using the Mantel-Cox test (B), two-way ANOVA with post Sidak’s multiple comparison test (C) or unpaired two-tailed t test (D-G). *** P < 0.001; **** P < 0.0001 for exp 1 and # P < 0.05; ## P < 0.01; #### P < 0.001 for exp 2.
Figure 3. The OSM:OSMR signalling module exhibits a distinct microenvironment-restricted expression. (A) UMAP plot showing cell clusters defined in each of the main cell lineages. Column legend depicts the main cell lineage of origin for each cluster, showing 7 clusters of epithelial origin, 6 immune and 4 stromal. LP: luminal progenitors, ECM: extracellular matrix, CAF: cancer-associated fibroblast, B: basal, ML: mature luminal. (B) Dot plot representing the expression level (red or blue jet) and the number of expressing cells (dot size) of the indicated genes in each cluster. (C) Feature UMAP plots showing the expression of the indicated genes in each of the main cell clusters.
**Figure 4. OSM and OSMR expression in human breast cancer microenvironment.** (A) mRNA expression levels of the indicated IL6 family members and associated receptors analyzed by RT-qPCR in a panel of breast cancer cell lines (n=18) and immortalized fibroblasts (n=6). In the OSMR graph, green and dark dots represent normal mammary fibroblasts and CAFs, respectively. P values were determined using the unpaired two-tailed t test. (B and C) Correlation of OSMR (B) and OSM (C) expression with tumour purity and infiltration level of indicated cell types in breast cancer samples. Data were downloaded from TIMER web platform, n=1100. Spearman correlation coefficients and P values are shown. (D) Truncated violin plots showing cell type enrichment of the indicated populations in breast tumours according to high (top quartile) or low (lowest quartile) OSM or OSMR expression. Data were obtained using xCell web resource on 1809 breast cancer samples from Kaplan-Meier Plotter website. P values were determined using Mann–Whitney’s test.
Figure 5. OSM activates cancer-associated fibroblasts (CAFs) in vitro promoting their contractility and proliferation. (A and B) Representative pictures of collagen contraction assays (A) and quantification of collagen disk areas (B) of fibroblasts pre-treated in monolayer with PBS or OSM. (C and D) Representative pictures (C) and area quantification (D) of 3D spheres proliferation assays of fibroblasts treated with PBS or OSM. Scale bar is 200 mm. In B and D, 2 independent experiments are plotted (Exp 1 and 2) and P values were calculated using the unpaired two-tailed t-test. (E) RT-qPCR analysis of mRNA levels of activation markers in normal (RMF-31) and cancer-associated (CAF-173) fibroblasts cultured in 3D with PBS or OSM. n=3 independent experiments. P values were determined using paired two-tailed t tests. (F) Gene set enrichment analysis (GSEA) showing enrichment of the indicated signatures in microarray data of CAF-173 treated with OSM. ES: enrichment score; NES: normalized enrichment score. (G) Kaplan-Meier curves showing overall survival (OS) for breast cancer patients according to the high or low expression in tumour samples of top 4 genes induced by OSM in CAF-173. Data were obtained using KM plotter website. P value was calculated using the Mantel-Cox test and high and low expression levels were stratified by median values.
Figure 6. OSM activates cancer-associated fibroblasts (CAFs) in vivo promoting tumour progression. (A) Experimental set up of the in vivo experiment designed to assess the contribution of OSMR activation in fibroblasts to cancer progression. CAF-173 were pre-treated with OSM or PBS for 4 days prior to injection and were co-injected with MDA-MB-231 (500,000 cells each cell line) in matrigel (1:1 ratio) in the mammary gland fat pad of nude mice. n=6 animals with MDA-MB-231+ CAF-173 PBS cells injected; and n=7 animals with MDA-MB-231+ CAF-173 OSM cells injected. (B-D) Tumour growth (B) and final tumour volume (C) and weight (D) after dissection of orthotopic tumours described in A. (E) Percentage of animals described in (A) with lung micrometastasis assessed using qPCR analysis of genomic human Alu sequences. Graph represents the percentage of animals with detectable qPCR signal and P value was calculated using the Chi-square test. P values were calculated using two-way ANOVA with post Sidak’s multiple comparison test (B) or the unpaired two-tailed t test in C-D. * P < 0.05; ** P < 0.01; **** P < 0.0001.
Figure 7. OSM:OSMR signalling in cancer-associated fibroblasts (CAFs) induces cytokine secretion. (A) Heatmap showing normalized mRNA expression of genes induced by OSM in CAF-173 and included in the indicated gene ontology (GO) pathway. (B) Gene set enrichment analysis (GSEA) showing enrichment of inflammatory hallmark signature in microarray expression data of CAF-173 spheres treated with OSM 30ng/mL for 4 days. ES: enrichment score; NES: normalized enrichment score. (C and D) Chemokine array analysis (C) and VEGF levels (D) in conditioned media from CAF-173 treated with PBS or OSM 30ng/mL for 72 hours. * P < 0.05; ** P < 0.01; *** P < 0.001. P values were determined using paired two-tailed t tests, n=4 independent experiments.
Figure 8. OSM:OSMR signalling induces myeloid recruitment. (A) Effect of conditioned media from CAF-173 treated with PBS (Control) or OSM 10 ng/mL for 72 hours on HL-60-derived monocytes migration, n=4 independent experiments. (B) Representative pictures and quantification of F4/80 immunohistochemistry staining in tumours derived from MDA-MB-231-CAF-173 co-injections described in Figure 6A. Quantification was performed by manual counting of positive cells per area in a total of 12-19 pictures per tumour and 4-7 tumours per group. Scale bar is 100 mm (large pictures) and 10 mm (inserts). (C) Representative pictures and quantification of F4/80 and Ly6G immunohistochemistry staining in tumours from MMTV-PyMT:Osmr wild-type (WT), heterozygous (HET), and KO mice at 14 weeks of age, described in Figure 1A. Quantification was performed by manual counting of positive cells per area in a total of 8 pictures per tumour and 5 tumours per group. Scale bar is 50 mm. (D) VEGF, CXCL1 and CXCL16 levels in plasma from MMTV-PyMT:Osmr WT, HET and KO mice at 14 weeks of age analyzed by Lumines assay. In A-D, P values between the different groups were determined using paired (A) or unpaired (B) two tailed t tests, one-way ANOVA (C) or one-way ANOVA with post Dunnett’s multiple comparison test (D). (E) Correlation of OSM and OSMR levels with VEGF, CXCL1 and CXCL16 expression in breast cancer samples. Data were downloaded from TIMER web platform, n=1100. Spearman correlation coefficients and P values are shown. (F) Kaplan-Meier curves showing overall survival (OS) for breast cancer samples according to the expression of VEGF, CXCL1 and CXCL16. Data were downloaded from KM plotter. P value was determined using the Mantel-Cox test and high and low expression levels were stratified by median value.
Figure 9. OSM expression associates with increased inflammation and decreased overall survival in human breast cancer samples. (A and B) Representative pictures (A) and quantification (B) of OSM immunohistochemistry staining in samples from breast cancer patients with high and low inflammation. Scale bar is 100 mm (upper row) and 50 mm (middle and lower rows). $P$ value was determined using Mann–Whitney’s test. (C) Kaplan-Meier curves showing overall survival (OS) for breast cancer patients analyzed in A and B, with high vs. low OSM expression. $P$ value was determined using the Mantel-Cox test and high and low expression levels were stratified by median value.