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c-MYC Functions as a Molecular Switch to Alter the Response of Human Mammary Epithelial Cells to Oncostatin M

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Abstract

Cytokines play an important role in creating an inflammatory microenvironment, which is now considered a hallmark of cancer. While tumor cells can exploit cytokine signaling to promote growth, invasion and metastasis, the response of normal and premalignant epithelial cells to cytokines present in a developing tumor microenvironment remains unclear. Oncostatin M (OSM), an IL-6 family cytokine responsible for STAT3 activation, has been implicated in cancer development, progression, invasion and metastasis. Paradoxically, OSM can also suppress the growth of normal cells and certain tumor-derived cell lines. Using isogenic human mammary epithelial cells (HMEC) at different stages of neoplastic transformation, we found that OSM signaling suppressed c-MYC expression and engaged a p16- and p53-independent growth arrest that required STAT3 activity. Inhibition of STAT3 activation by expressing a dominant-negative STAT3 protein or a STAT3-shRNA prevented the OSM-mediated arrest. In addition, expression of c-MYC from a constitutive promoter also abrogated the STAT3-mediated arrest, and strikingly, cooperated with OSM to promote anchorage-independent growth (AIG), a property associated with malignant transformation. Cooperative transformation by c-MYC and OSM required PI3K and AKT signaling, demonstrating the importance of multiple signaling pathways downstream of the OSM receptor in defining the cellular response to cytokines. These findings identify c-MYC as an important molecular switch that alters the cellular response to OSM-mediated signaling from tumor suppressive to tumor promoting.

Introduction

Clinical and epidemiological data has associated an inflammatory microenvironment with cancer development (1). Most tumors show evidence of infiltrating immune and inflammatory cells, and chronic inflammatory disorders are known to increase the overall risk of cancer development. Importantly, inflammation is often observed during early stages in the transformation process, however, there remains debate over whether the inflammatory cells and the cytokines they produce in the developing tumor microenvironment act to

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inhibit or facilitate tumor development (2). The IL-6 family of cytokines, which includes IL-6, Oncostatin M (OSM) IL-11, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), and cardiotrophin-like cytokine (CLC) are secreted by immune cells, stromal cells, and epithelial cells, and regulate diverse processes (3). Although each of the IL-6 family cytokines signals through a distinct receptor complex, they share the gp130 receptor subunit (4). Activation of gp130 kinase activity results in activation of the STAT3 transcription factor, and the Mitogen-Activated Protein Kinase (MAPK) and Phosphatidylinositol 3-Kinase (PI3K) signaling cascades (4). The gp130 family of cytokines are highly pleiotropic in normal development, yet an overlapping role for these cytokines in cancer continues to emerge, with studies now implicating autocrine and paracrine IL-6, LIF and OSM-mediated gp130 activation as important mediators of tumor progression and metastasis (4–7).

OSM was originally identified based upon its ability to inhibit the proliferation of melanoma cells (8), an observation that has since been confirmed in breast cancer cells, lung cancer cells, glioma, and neuroblastoma (3). In humans, OSM and LIF bind to the LIF β -gp130 receptor, while OSM binds uniquely to the OSMR β -gp130 receptor. (3, 9). The growth inhibitory properties of OSM are engaged by the STAT3-mediated suppression of the c-MYC gene, and the resulting hypothesis from early studies of OSM was that OSM signaling was tumor suppressive and may be exploited as a potential cancer therapy (10, 11). However, OSM stimulates the proliferation of normal dermal fibroblasts, Kaposi's sarcoma cells, and plasmacytoma cells, and can promote breast cancer cell migration and invasiveness (3, 12). In addition, subsequent studies have confirmed the paradoxical effects of OSM in normal and preneoplastic lung epithelial cells, with OSM suppressing the proliferation of normal lung epithelial cells, while increasing the proliferation of preneoplastic lung cells (13). To date, an explanation for the contradictory responses to OSM remains elusive.

We describe here, the effects of OSM on human mammary epithelial cells (HMEC) at various stages of neoplastic transformation. Since STAT3 is an oncogene, we hypothesized that sustained STAT3 activation (by persistent gp130 activation) would promote a p53- and p16-independent oncogene-induced senescence (OIS) similar to RAS, MOS or STAT5 (14–16). However, we found that the OSM-mediated growth arrest occurs independently of the p16 and p53 tumor suppressors, and required efficient down-regulation of c-MYC gene expression by STAT3. Inhibition of STAT3 using a dominant-negative protein or an shRNA targeting STAT3 expression or expression of c-MYC from a constitutive promoter prevented efficient OSM-mediated growth suppression. Importantly, not only did HMEC constitutively expressing c-MYC fail to undergo an arrest in response to OSM, they actually gained the capacity for anchorage-independent growth (AIG), a hallmark of transformed cells. The transformed phenotype conferred by OSM treatment was dependent on PI3K-AKT signaling, since inhibition of either PI3K or AKT suppressed OSM-mediated AIG. Our results provide an understanding of the paradoxical nature of OSM signaling, and suggest that c-MYC is an important molecular switch that alters the cellular response to OSM from tumor suppressive to tumor promoting. Understanding the tumor suppressive barriers that are engaged during the OSM-mediated growth arrest may provide a foundation for future therapies aimed at reengaging these hidden limits to proliferation as a cancer therapy.

Methods

Cell lines and culture conditions

Finite lifespan, post-selection HMEC 48R, batch S (17) and 184 were provided by Dr. Martha Stampfer (Lawrence Berkeley National Laboratory). The non-immortalized 48R cells were expanded and used between passage 10 and passage 16 for the studies performed here and the non-immortalized 184 cells were used between passage 9 and passage 15. Cells were grown in a humidified atmosphere containing 5% CO₂ in Medium 171 with mammary epithelial growth supplement (MEGS; Invitrogen). Senescence Beta-Galactosidase Staining Kit #9860 was purchased from Cell Signaling and the standard protocol was performed.

Plasmids and retroviral infection

The following plasmids were obtained from Addgene: pBpuro-RasV12 (Addgene plasmid 1768); pBabe-Puro-MEKDD (Addgene plasmid 15268); pWZL-Blast-Myc (Addgene plasmid 1074). LPCX-STAT3 (wild-type), LPCX-STAT3C and LPCX-STAT3-Y705F were obtained from Dr. George Stark (The Cleveland Clinic Foundation, Cleveland, Ohio) (18). LHCX-MEK1-K97M was a generous gift from Dr. Damu Tang (McMaster University, Hamilton, Ontario, Canada) (19). shSTAT3 and shControl was kindly provided by Dr. Giorgio Inghirami (New York University School of Medicine, New York, New York) (20). pWZL-puro-MF-p110a (21) and other shRNA constructs and protocols for virus production and infection of cells have been described elsewhere (22).

Growth Assays

For growth assays in Figures 1A, 2A, 3A, 4C, and S4, cells were initially plated at 400,000/10-cm plate and treated with OSM, TGF- β , or IL-6 and the soluble IL-6 receptor (PeproTech, Rocky Hill, NJ) at the indicated concentrations for 4 days. Multiple plates of OSM-treated cells were used to obtain enough cells for the Western blots that correspond with the growth assays. After 4 days of OSM treatment, the cells were then trypsinized and plated at 50,000 cells/well of a 6-well plate in triplicate with OSM or TGF- β , and given fresh medium containing cytokines every 48 hours for an additional 6 days. Cell number was quantified with a Coulter Counter. For growth assays presented in Figures 5A and S3, 25,000 cells were plated per well in a 6-well plate and grown in the presence or absence of OSM or IL-6 and the soluble IL-6 receptor at the indicated concentrations for the indicated times. MK-2206, an Akt inhibitor, was purchased from ChemieTek (Indianapolis, IN). LY-294002, a PI3K inhibitor, was purchased from LC Labs (Woburn, MA). All experiments were performed in triplicate and the mean $\pm$ standard deviation is shown. Each figure is representative of at least three independent experiments.

Anchorage-independent growth assays

For AIG assays, HMEC (2×10^5) were suspended in 0.6% type VII agarose in complete MCDB170 medium (Sigma) and plated onto 60mm plates coated with 1.2% type VII agarose in complete MCDB170 medium. The agarose was made by mixing 2X agarose with 2X Mammary Epithelial Basal Medium MCDB 170 (from powder: US biological) supplemented with 0.1 mM phosphoethanolamine, 0.1 mM ethanolamine, and mammary

epithelial growth supplement (MEGS: Invitrogen). The medium was changed every 3 days in the presence of the indicated cytokines or chemical inhibitors and the plates were analyzed after 3 weeks. To quantify colonies, each plate was scanned using an automated multi-panel scanning microscope, and the digital images analyzed using MetaMorph image quantification software. All experiments were performed in triplicate and the mean $\pm$ standard deviation is shown. Each figure is representative of at least three independent experiments.

Western analysis

Whole cell extracts were prepared by incubating cell pellets in lysis buffer as described (21). Equal protein amounts were separated by SDS-PAGE (8–12.5% acrylamide) and transferred to PVDF membranes (Millipore), and Immunoblotted with the indicated antibodies. Antibodies to p53 (DO-1), STAT3 (C-20), p21 (C19) were from Santa Cruz Biotechnology; the phospho-STAT3 (Tyr705) was obtained from Cell Signaling; the antibody to Actin (C4) was from Neomarkers; and antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and HDM2 (2A10) were from Calbiochem. Primary antibodies were detected with goat anti-mouse or goat anti-rabbit conjugated to horseradish peroxidase.

Results

OSM treatment induces a p16- and p53-independent growth arrest in human mammary epithelial cells

OSM has been reported to induce growth arrest or proliferation in a variety of normal and tumor-derived cancer cells, although the reason for the divergent responses is not understood (3). We reasoned that differences in key tumor suppression pathways may account for the conflicting results obtained with OSM in previous studies. For example, the growth arrest engaged following dysregulated oncogene expression, including constitutive STAT5, RAS, and MOS (17–20), can be inhibited by disabling p16 or molecular components of the DNA damage response (DDR), including ATM, CHK2 or p53. Since STAT3 is an oncogene, we hypothesized that sustained STAT3 activation would also promote a p53-dependent oncogene-induced growth arrest similar to STAT5, RAS, or MOS. To test this hypothesis, an shRNA targeting p53 was expressed in post-selection HMEC (referred to here as 48RS), which lack p16 protein expression due to promoter methylation (23), and the response to persistent STAT3 activation was examined following long-term OSM treatment.

The absence of p53 expression and function was confirmed in cells expressing the p53-shRNA using Western analysis and functional growth assays [Supplemental Fig. 1 and (24)]. After confirming the suppression of p53, the control 48RS and shp53-48RS were treated with OSM and growth was assessed after 10 days. Treatment of both 48RS and shp53-48RS cells resulted in a similar level of STAT3 phosphorylation (P-STAT3; Fig. 1A), an enlarged, vacuolated cellular morphology (Fig. 1B), and an 80% decrease in cell number relative to untreated cells, similar to TGF- β treatment (Fig. 1C). In addition, the OSM treated cells had considerable senescence-associated (SA) β -galactosidase activity resulting in the characteristic blue staining upon incubation of the OSM-treated cells with bromo-chloro-indolyl-galactopyranoside [X-gal; (Fig. 1B)]. The presence of SA β -galactosidase

activity is consistent with our conclusion that the decrease in cell number following OSM treatment was due to a decrease in proliferation. Moreover, we confirmed that the decrease in cell number observed following OSM treatment was not due to increased cell detachment, as has been reported for invasive breast cancer cells treated with OSM (12, 25), by measuring the number of detached cells in control and OSM-treated cultures 48 hours after treatment (Supplemental Fig. 2).

To further define the kinetics of the OSM-mediated arrest, a time-course experiment was performed in which cells were treated with OSM for 2 to 9 days. A decrease in proliferation was observed with as little as 48 hours of OSM treatment, and was increasingly evident throughout the time-course (Supplemental Fig. 3). We conclude that unlike the arrest observed following constitutive STAT5 activation (15), which is dependent on ATM and p53 signaling, persistent STAT3 activation induced by OSM engages a p53-independent growth arrest. Since most tumors have inactivated p53 signaling, understanding this unique p53-independent arrest engaged by OSM may provide opportunities for novel therapies aimed at re-engaging this proliferative barrier as a cancer therapy.

OSM suppresses HMEC growth more efficiently than IL-6

OSM is a member of the Interleukin-6 (IL-6) family of cytokines, which signal through a common gp130 receptor subunit (4). IL-6 itself has been extensively implicated in cancer and is also a known activator of STAT3 (6). To compare OSM and IL-6, and determine whether IL-6 also suppresses the growth of HMEC, the 48RS-shp53 cells were treated with IL-6 (with or without soluble IL-6 receptor) at 50 or 100 ng/mL, and compared to OSM treatment at 2, 10, or 100 ng/mL after 10 days. Again, OSM treatment induced a significant growth inhibition (80% inhibition at 10 ng/mL and 95% inhibition at 100 ng/mL), while IL-6 treatment induced only a moderate growth inhibition (10% inhibition at 50 ng/mL and 50% inhibition at 200 ng/mL; Fig. 2A). In agreement with the results obtained using standard 2-dimensional culture, acini formation of 48RS-shp53 cells in 3-dimensional laminin-rich basement membrane (Matrigel) was strongly inhibited by OSM (70% inhibition at 25 ng/mL) and weakly inhibited (25% inhibition at 25 ng/mL) by IL-6 (Supplemental Fig. 4). In addition, IL-6 treatment resulted in a less robust phosphorylation of STAT3-Y705 relative to OSM, with maximal STAT3 phosphorylation observed at the lower 50 ng/mL dose (Fig. 2B). A time-course comparison of OSM (10 ng/mL) and IL-6 (50 ng/mL) treatment demonstrated that STAT3 phosphorylation was strongly induced 15 minutes after OSM treatment, and to a lesser extent after IL-6 treatment (Fig. 2C). However, after one hour of OSM or IL-6 treatment there was a significant decrease in phosphorylated STAT3, consistent with the negative feedback regulation of STAT3 by SOCS3 and PIAS3 [Fig. 2C; (26, 27)]. In both the long-term and short-term analyses, IL-6 was clearly less efficient at inducing and maintaining STAT3 phosphorylation, even when used at a significantly higher dose, likely explaining the decreased ability of IL-6 to suppress growth. Furthermore, while the suppression of STAT3 phosphorylation is robust and occurs rapidly (within 1 hour), sustained STAT3 phosphorylation is evident in the OSM-treated samples between 1 and 10 days (Fig. 2C and 2D).

OSM-mediated growth suppression is STAT3-dependent

Activation of the OSM/gp130 receptor results in the phosphorylation of STAT3, which permits its dimerization and movement to the nucleus, where it transcriptionally regulates a myriad of genes (6, 28). In addition, OSM/gp130 receptor activation also results in the activation of MAPK and PI3K signaling cascades (4). To ensure that STAT3 is responsible for the OSM-mediated arrest, shp53-48RS cells expressing dominant negative proteins targeting each gp130-activated pathway (DN-STAT3, DN-MEK, and DN-PI3K) were created. Upon treatment with OSM, there was a clear abrogation of the OSM-mediated growth arrest in the shp53/DN-STAT3-48RS cells (5% decrease in cell number following OSM treatment) compared to the control shp53-48RS (61% decrease), shp53/DN-MEK-48RS (57% decrease) and shp53/DN-PI3K-48RS (80% decrease; Fig. 3A). Western analysis confirmed that cells expressing DN-STAT3 were no longer able to phosphorylate STAT3 after OSM treatment, while cells expressing DN-MEK or DN-PI3K had phosphorylated STAT3 levels comparable to control HMEC in response to OSM (Fig. 3B). A similar loss of OSM-mediated growth suppression was observed using shp53-HMEC expressing a shRNA targeting STAT3, clearly implicating STAT3 as an important mediator of the OSM growth arrest (Supplementary Fig. 5).

c-MYC functions as a molecular switch to alter the response of HMEC to OSM

c-MYC is a transcription factor, and one of the most potent and frequently dysregulated oncogenes in human cancers (29, 30). OSM-mediated transcriptional suppression of the c-MYC gene has been reported in breast cancer cells that are sensitive to OSM-mediated growth arrest. Moreover, suppression of c-MYC expression in response to OSM is STAT3-dependent, since dominant negative STAT3 prevented c-MYC suppression (10, 11). We confirmed that c-MYC expression was suppressed in shp53-48RS cells treated with OSM using quantitative real-time PCR (Fig. 4A), and created isogenic shp53-48RS expressing c-MYC from a constitutive promoter (shp53/MYC-48RS, Fig. 4B). Indeed, the OSM-mediated arrest was largely prevented in the 48RS-shp53/MYC cells (20% inhibited) compared to the control 48RS-shp53 cells (80% inhibited), implicating STAT3-mediated c-MYC repression as an important mechanism of the arrest (Fig. 4C). We confirmed that constitutive c-MYC expression did not prevent the phosphorylation of STAT3-Y705 by OSM (Supplementary Fig. 6). Taken together, our results suggest that downstream STAT3 effects, rather than upstream gp130 receptor or JAK kinase activity, were inhibited by constitutive c-MYC expression.

We have recently shown that c-MYC expression contributes to a transformed phenotype when combined with an aberrant growth signal, such as mutant RAS-G12V (24). We hypothesized that once the OSM/STAT3-mediated growth arrest was dismantled, additional OSMR/gp130-mediated signaling (which includes PI3K-ATK or MAPK) might cooperate to transform the HMEC. To test this hypothesis, the shp53-48RS derivatives were plated into soft agar with or without OSM and anchorage-independent growth (AIG), a property associated with malignant transformation, was assayed. IL-6 treatment was also tested for comparison. In the absence of c-MYC expression, there was no observable AIG, whereas c-MYC expression conferred a slight increase in AIG (Fig. 4D). Treatment of shp53/MYC-48RS cells with 10 ng/mL or 25 ng/mL OSM induced significant AIG, with a slight

inhibition of AIG at the higher dose of OSM (Fig. 4D). In contrast to the weak effect observed with IL-6 in the growth suppression assays, IL-6 efficiently induced AIG following treatment with either 10 or 25 ng/mL. Combined addition of OSM and IL-6 did little to either enhance or inhibit AIG (Fig. 4D).

To confirm that our findings are not specific to one HMEC culture, we repeated our experiments in a second post-selection strain derived from an independent reduction mammaplasty, referred to here as patient 184. An shRNA targeting p53 was delivered to the 184 HMEC and Western analysis confirmed the knockdown of p53 protein levels in the shp53-184 and the abrogation of p53-dependent transactivation of target genes HDM2 and p21 in response to Nutlin-3, a p53 stabilizing compound (Supplementary Fig. 7). Similar to the 48RS derivatives, both control 184 and 184-shp53 cells were growth inhibited by OSM treatment, and largely insensitive to IL-6 treatment (Fig. 5A and 5B). The growth of shp53-184 cells expressing c-MYC from a constitutive promoter (shp53/MYC-184) was unaffected by either OSM or IL-6. Upon plating the shp53-184 and shp53/MYC-184 cells into agar to assess AIG, we again observed that both OSM and IL-6 cooperated with c-MYC to promote a transformed phenotype. As expected, control shp53-184 lacking constitutive c-MYC expression failed to form colonies when treated with OSM or IL-6. Our collective results using HMEC from two independent patients suggest that c-MYC is an important molecular switch that alters the cellular response to OSM from tumor suppressive to tumor promoting, providing an understanding of the paradoxical nature of OSM signaling.

OSM-mediated transformation of c-MYC expressing HMEC is PI3K-AKT-dependent

Following ablation of the p16 and p53 tumor suppressors, and constitutive c-MYC expression, 48RS HMEC can be readily transformed by numerous aberrant growth signals, including mutant RAS-G12V (24, 31). We hypothesized that, once c-MYC was constitutively expressed and unable to be suppressed by OSM/STAT3, the additional, well-documented growth promoting properties of STAT3 would serve to drive HMEC transformation. To test this, shp53/MYC-48RS cells expressing wild-type or constitutively active STAT3 (STAT3-C), or RAS-G12V (used as a positive control (24)) were created and plated into soft agar. Surprisingly, expression of either STAT3 protein failed to promote AIG in the shp53/MYC-48RS cells (Fig. 6A). In addition, expression of dominant-negative STAT3 did not suppress OSM-mediated AIG (Supplementary Fig. 8). Therefore, we next examined whether OSM/gp130-mediated signaling to additional downstream effectors, which include PI3K or MAPK, contributed to the AIG of shp53/MYC-48RS cells following OSM treatment. Shp53/MYC-48RS cells expressing constitutively active PI3K (CA-PI3K), MEK (CA-MEK), or RAS-G12V were created and plated into soft agar in the absence or presence of OSM. Expression of CA-PI3K or CA-MEK in the absence of c-MYC resulted in weak AIG, which was not influenced by OSM (Fig. 6B and 6C). In contrast, expression of CA-PI3K with c-MYC promoted AIG as efficiently as RAS-G12V even in the absence of OSM. Moreover, addition of OSM to shp53/PI3K/MYC-48RS or shp53/RAS/MYC-48RS cells was unable to facilitate greater AIG compared to the absence of OSM (Fig. 6B and 6C). This observation argues that OSM functions to enhance AIG by activating PI3K signaling, which cannot be elevated beyond the activity imparted by CA-PI3K or RAS-G12V. In contrast to CA-PI3K, co-expression of CA-MEK with c-MYC was unable to

promote AIG alone, but again, resulted in an increase in AIG following OSM treatment similar to the control cells (Fig. 6B and 6C). To further define the requirement for PI3K/AKT signaling in the OSM-mediated AIG, shp53/MYC-48RS cells were plated into soft agar and treated with OSM, together with a PI3K inhibitor (LY-294002) or an AKT inhibitor (MK-2206). Treatment with LY-294002 or MK-2206 resulted in a dose-dependent suppression of OSM-mediated AIG (Fig. 6D). Taken together, we propose that the combination of constitutive c-MYC expression and OSM-mediated PI3K-AKT signaling, but not constitutively active STAT3, cooperate to drive a transformed phenotype.

The presence of OSM has been reported in the tumor interstitial fluid (TIF) that perfuses the breast carcinoma microenvironment (32). Furthermore, in a recent microarray experiment to identify stromal gene expression signatures, Finak et. al performed laser capture microdissection to isolate tumor stroma and matched normal stroma from human breast cancers (33). Analysis of their expression data identified OSM mRNA as significantly upregulated in the tumor-associated stroma compared to normal stroma (Fig. 7A). Furthermore, within the tumor stroma, higher OSM mRNA levels correlated a significant increase in the risk of tumor recurrence (Fig. 7B; (33)). Our cell culture data suggests that the presence of OSM within a developing tumor microenvironment, as described by Finak et. al would suppress growth at the initial stages of the transformation process, but following dysregulated c-MYC expression within the epithelial cells, stromal-derived OSM would promote tumor growth.

Discussion

Human cell transformation models have delineated numerous pathways involved in cancer initiation and progression; however, they have largely ignored the contribution of signaling initiated by microenvironmental factors. The breast tumor microenvironment is complex, and involves many cell types including stromal fibroblasts, myoepithelial cells, endothelial cells and a number of immune cells [dendritic cells, macrophages, granulocytes, natural killer cells, and mast cells; (2)] in addition to the tumor cells themselves. These microenvironment components secrete soluble signaling mediators that act in a paracrine or autocrine fashion and include growth factors, cytokines and chemokines that can alter an incipient cancer cells proliferation, apoptosis, differentiation, migration, and invasion programs (34). Our studies have investigated the effects of OSM, an IL-6 family cytokine, on normal and premalignant HMEC growth and transformation. Our results have important implications in our understanding of breast cancer initiation, progression, and metastasis, and may ultimately influence how breast cancer is managed therapeutically.

The presence of OSM in the breast and prostate tumor microenvironment has been documented, with increasing OSM protein concentrations observed during cancer progression (7, 32). OSM in the microenvironment may originate from tumor-associated macrophages, neutrophils, tumor cells, or additional cancer stromal components (2, 33, 35), and may contribute to tumor progression in a number of ways. First, OSM mRNA is significantly upregulated in microdissected breast tumor-associated stroma compared to normal stroma, and those tumors harboring the highest level of stromal OSM mRNA have the greatest risk of tumor recurrence [Fig. 7; (33)]. Second, OSM-positive macrophages are

predominantly localized at the advancing, infiltrative margins of carcinomas, which may implicate OSM in tumor invasion (36). Third, in addition to OSM in the tumor microenvironment, the OSM receptor (OSMR) is also frequently overexpressed in cervical and ovarian carcinomas, and is associated with adverse clinical outcome (37, 38). Finally, the widely used chemotherapy drug cisplatin induces peritoneal and bone marrow-derived macrophages to induce significant OSM secretion (39, 40). Together, these studies and others suggest that OSM signaling is oncogenic and capable of promoting hyperplasia, and that OSM induction by cancer therapies (such as cisplatin) may have unexpected and adverse effects on tumor growth (3, 12, 25, 39, 40).

Paradoxically however, OSM was originally identified based on its ability to inhibit the proliferation of melanoma cells (8), an observation since confirmed in HMEC and other cancer cell lines (3, 41). The reasons OSM causes some cells to cease proliferating while others acquire a more transformed phenotype upon OSM exposure is not well understood, but has significant clinical implications (3). For example, the responsiveness of short-term cultures of a patient's melanoma cells to Oncostatin M and/or IL-6 can predict the patient's overall survival following immunotherapy (42). Our findings are consistent with the conclusions of this study, and provide a mechanism to explain why tumors that retain at least a partial response to OSM/IL-6 are less aggressive when compared to tumors that have efficiently inhibited the growth suppressive function of OSM/STAT3.

We propose that early breast hyperplasia engages microenvironmental responses that lead to persistent OSM/STAT3-signaling and a tumor suppressive arrest. In support of this hypothesis, recent studies have identified unknown non-proliferative or proliferative breast lesions in 53% of women who underwent elective mammoplasty reduction surgery. It is likely that these early-stage lesions are constrained by tumor suppressive pathways that may involve OSM/STAT3 signaling to suppress c-MYC. An involvement of OSM/STAT3 in restraining the growth of early breast lesions would have significant implications for therapies currently being used to suppress STAT3 and its upstream activator JAK2. Such inhibitors may actually prevent the STAT3-mediated tumor suppression that is maintaining these breast lesions in a non- or low-proliferative state and promote cancer progression. When taken together, the data support a model whereby the suppression of c-MYC is a critical molecular determinant of whether persistent OSM/STAT3 signaling suppresses or enhances HMEC growth.

In a recent study, we defined a p16- and p53-independent senescence response that was engaged in HMEC upon oncogenic RAS expression. Similar to our findings with OSM, RAS-mediated senescence could be prevented by constitutive c-MYC expression (24). We suggest that the growth suppression engaged in HMEC by persistent OSM/STAT3 or RAS signaling is an inherent tumor suppressive response that is frequently dismantled during the transformation process by c-MYC gene amplification or protein overexpression [Fig. 7C; (29)]. Importantly, dysregulation of c-MYC would prevent the tumor suppressive decrease in c-MYC mRNA by the OSM/STAT3-signaling axis while allowing additional gp130-activated signals to drive the transformation process (Fig. 7C). We originally hypothesized that STAT3 activation would cooperate with c-MYC to promote a transformed phenotype, given the numerous STAT3 target genes with oncogenic properties (18). However, it was

clear that neither wild-type or constitutively active STAT3 were sufficient to take the place of OSM in promoting a transformed phenotype in HMEC. Furthermore, expression of dominant-negative STAT3 failed to suppress OSM-mediated AIG, arguing that additional signaling from the OSMR/gp130 receptor complex is required for transformation. Both PI3K and MAPK activation were obvious candidates, but only constitutively active PI3K cooperated with c-MYC to drive HMEC transformation, leading us to conclude that PI3K-AKT signaling is the alternative transforming signal generated from OSM receptor activation. Consistent with this conclusion, inhibition of either PI3K or AKT suppressed the ability of OSM to promote AIG. One might predict that PI3K-AKT inhibitors would be effective at suppressing the growth of tumors harboring hyperactive gp130-signaling. Interestingly, while IL-6 was a less efficient activator of STAT3 and an inefficient suppressor of HMEC proliferation, it cooperated significantly with constitutive c-MYC expression to promote AIG. The reason for the discrepancy between the growth suppressive and growth promoting phenotypes is unclear, but not unprecedented given the pleiotropic effects of the IL-6 family.

The paradoxical nature of OSM signaling is reminiscent of TGF- β signaling, which also acts as a tumor suppressor or tumor promoter, depending upon the context of receptor activation. In the case of TGF- β signaling, the differential response of normal, hyperplastic, and transformed epithelial cells is also often explained by the diverse signals generated by TGF- β receptor activation, which include SMAD-dependent and -independent pathways [involving TAK1, NFkB, JNK, MAPK, PI3K/AKT and mTOR; (43)]. Our observations provide an explanation for the disparate results commonly observed following the treatment of various cell lines with OSM. We propose that both the level of c-MYC expressed in a cell and its ability to be effectively suppressed will dictate whether cells arrest or continue proliferating. Once a cell dismantles the tumor suppressive response engaged by persistent STAT3 activation and continues proliferating in the presence of OSM, the additional signaling emanating from PI3K, MYC, and STAT3 will allow for premalignant expansion and ultimately full transformation (Fig. 7C). While our studies show that STAT3 is not sufficient to cooperate with c-MYC in HMEC transformation, it is clear that many tumor-derived cells have high levels of phosphorylated STAT3 and require sustained STAT3 activity for survival. STAT3 may be responsible for inhibiting apoptosis, or promoting invasion and metastasis, due to BCL-xL and matrix metalloproteinase induction, respectively. In the experiments described here, we did not measure these STAT3 effectors and therefore cannot define their involvement in our model.

The studies presented here led us toward the identification of a physiologically appropriate set of three genetic events (p16 and p53 suppression and constitutive c-MYC expression) that cooperate with OSM, a cytokine present in the breast tumor microenvironment, to consistently drive HMEC transformation. Continued refinement of human cell transformation models are providing important clues into how the growth suppressive barriers in normal cells can be overcome. Expanding these studies to include microenvironmental signals, as described here, is an important next step. Understanding how OSM and other microenvironmental factors impinge on the tumor suppressor/oncogene interactions within the cellular circuitry will provide a foundation for future therapies aimed at reengaging growth suppressive signaling as a cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. OSM induces a p16- and p53-independent growth arrest

Post-selection HMEC (48R batch S; 48RS) were infected with amphotropic retroviruses encoding a shRNA against p53 (shp53-48RS). A, Control 48RS and shp53-48RS cells were treated with OSM (10 ng/mL) or TGF-β (10 ng/mL) for 10 days. Western analysis was performed for STAT3, phospho- STAT3 (Y705) and ACTIN (as a loading control). B, shp53-48RS cells were treated with OSM (25 ng/mL) or left untreated for 96 hours and stained for the presence of senescence-associated β-galactosidase activity (which results in blue coloration). C, Control 48RS and shp53-48RS cells were treated with OSM (10 ng/mL) or TGF-β (10 ng/mL) for 10 days and cell number was quantified using a Coulter counter.

Fig. 2. OSM induces a more efficient growth arrest than IL-6

A, shp53-48RS cells were treated with increasing doses of OSM (2, 10, and 100 ng/mL) or IL-6 (50 and 200 ng/mL) for 10 days and cell number was quantitated. B, shp53-48RS cells treated with OSM or IL-6 for 10 days at the indicated concentrations. Cell lysates were analyzed by the Western method for STAT3 (Y705) phosphorylation. C and D, shp53-48RS cells treated with OSM at 10 ng/mL or IL-6 at 50 ng/mL for the indicated times and analyzed by the Western method for STAT3 (Y705) phosphorylation.

A

B

Fig. 3. OSM induces a STAT3-dependent growth arrest

shp53-48RS cells were infected with amphotropic retroviruses encoding DN-STAT3, DN-PI3K, DN-MEK, or control vector. A, Control, DN-STAT3, DN-PI3K, and DN-MEK cells were treated with OSM (10 ng/mL) for 10 days and cell number was quantified. B, Cells expressing DN-STAT3, DN-PI3K, and DN-MEK were treated with 10 ng/mL OSM for 10 days and analyzed by Western analysis for STAT3 and phospho-STAT3 (Y705).

Fig. 4. Constitutive c-MYC expression alters the cellular response to OSM-mediated signaling from tumor suppressive to tumor promoting in shp53-48RS cells

A, shp53-48RS were treated with OSM (10 ng/mL) for 10 days. Total RNA was subjected to real-time PCR analysis for the c-MYC gene. B, shp53-48RS were infected with amphotropic retroviruses encoding c-MYC or control vector and analyzed by Western analysis for c-MYC expression. C, shp53-48RS and shp53/MYC-48RS cells were treated with OSM (10 ng/mL) for 10 days and cell number was quantified. D, shp53-48RS and shp53/MYC-48RS were plated into soft agar and treated with OSM or IL6 at the indicated concentrations for 3 weeks.

Fig. 5. Constitutive c-MYC expression alters the cellular response to OSM-mediated signaling from tumor suppressive to tumor promoting in shp53-184 cells

A and B, Control 184, shp53-184, and shp53/MYC-184 cells were treated with increasing doses of OSM (10 or 25 ng/mL) or IL-6 (10 or 25 ng/mL) for 5 days and cell number was quantified. C, shp53-184 and shp53/MYC-184 were plated into soft agar and treated with OSM or IL6 at the indicated concentrations for 3 weeks.

Fig. 6. PI3K-AKT signaling is required for cooperative transformation by c-MYC and OSM
A, shp53/MYC-48RS were infected with amphotropic retroviruses encoding RAS-G12V (as a control), wild-type STAT3 (WT) or constitutively active STAT3 (C) as indicated. The shp53/MYC-48RS derivatives, along with shp53-48RS cells (Control), were plated into soft agar for 3 weeks before being quantified. B and C, shp53-48RS and shp53/MYC-48RS were infected with amphotropic retroviruses encoding constitutively active (CA)-PI3K, CA-MEK, or RAS. Cells were plated into soft agar and treated with OSM (10 ng/mL) for 3 weeks before being quantified and photographed. D, shp53-48RS and shp53/MYC-48RS were plated into soft agar and treated with OSM (10 ng/mL) together with LY-294002 or MK-2206 at the indicated concentrations for 3 weeks before being quantified and photographed.

Fig. 7. Breast tumor stroma expresses elevated OSM mRNA

A, Oncomine data showing OSM mRNA levels in normal breast stroma (N) or breast cancer stroma (C). B, Comparison of the level of OSM mRNA in breast tumor stroma from patients with and without tumor recurrence after 5 years. C, Model implicating the regulation of c-MYC expression as a central determinant of the cellular response to OSM.