Title
The Role of SOCS3 and CXCL1 in Metastasis and Radiation Response of Solid Tumors

Author
Jiang, Sisi

Publication Date
2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The Role of SOCS3 and CXCL1 in Metastasis and Radiation Response of Solid Tumors

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biomedical Physics

by

Sisi Jiang

2014

ABSTRACT OF THE DISSERTATION

The Role of SOCS3 and CXCL1

in Metastasis and Radiation Response

of Solid Tumors

by

Sisi Jiang

Doctor of Philosophy in Biomedical Physics

University of California, Los Angeles, 2014

Professor Nicholas Cacalano, Chair

Suppressor of Cytokine Signaling (SOCS) proteins are inhibitors of cytokine signaling that function via the Janus kinase (JAK)/ signal transducers and activators of transcription (STAT) pathway. Our lab has previously demonstrated that SOCS3-deficient mouse embryonic fibroblasts (MEFs) display enhanced sensitivity to DNA damaging agents, but the molecular mechanism of this effect is not well understood. In this study, we have confirmed SOCS3 as a radioprotector in fibroblasts and human solid tumors and identified a novel mechanism through which SOCS3 exerts its radioprotective effect. SOCS3 regulates the DNA damage response by promoting p21 induction and controlling STAT3-mediated proliferation. We have found that the transcriptional repressor BCL6 is targeted by SOCS3/STAT3 and this novel axis is responsible

for specific control of the G1-S transition, as SOCS3-deficient cells display normal S-phase checkpoint. Further, by overexpressing SOCS3 in human pancreatic cancer cell lines that signal through insulin-like growth factor (IGF) receptor and the RON receptor tyrosine kinase, we have determined that the radioprotective effect of SOCS3 supercedes its ability to block specific kinase pathways that increase radiation resistance in tumor cells.

In addition to these novel effects of SOCS3 on the DNA damage response, we have also identified radiation-independent functions for SOCS3 in aggressive, metastatic solid tumors. Initial cDNA microarray studies in SOCS3 knockout MEFs revealed that the pro-inflammatory C-X-C family chemokines CXCL1 and CXCL2 are underrepresented in SOCS3-deficient fibroblasts, due to transcriptional repression by uncontrolled STAT3 signaling. On the basis of these observations, we have identified three molecular subtypes of human pancreatic ductal adenocarcinoma (PDA) based on SOCS3 expression and CXCL1/2 dependence. We have stratified human PDA cell lines into three groups in which SOCS3 is either highly expressed, partially repressed, or severely repressed/totally silenced in response to IL-6 or oncostatin M (OSM). Repression of the SOCS3 gene in PDA is not CpG island methylation-dependent, suggesting a possible novel mechanism through which SOCS3 expression is silenced in PDA. Our laboratory has shown that ectopic expression of SOCS3 can promote CXCL1 gene activation and tumor aggressiveness in the molecular subtype with partial SOCS3 repression but not the others. Thus, the three molecular subtypes of PDA that we have identified display distinct biological behaviors, responses to SOCS3 overexpression and CXCL1-dependence. Using an orthotopic xenograft model of human PDA in immunodeficient mice, we demonstrated the *in vivo* relevance of the STAT3/SOCS3/CXCL1 axis in tumor aggressiveness and metastatic behavior. Our results suggest a novel mechanism for controlling pro-inflammatory signaling in

human solid tumors and that SOCS3 can regulate both metastasis and radiation sensitivity through distinct pathways. Our ability to stratify human PDA on the basis of SOCS3/STAT3/CXCL1/2 dependence has implications for the development of individualized therapy for cancer patients. By targeting STAT3, SOCS3, as well as CXCL1, we can regulate metastatic behavior in solid tumors, and increase radiation sensitivity of tumors in the clinic.

Keywords: JAK/STAT signaling, cytokine, chemokine, SOCS3, CXCL1, DNA damage response, metastasis, radiation therapy, pancreatic ductal adenocarcinoma.

The dissertation of Sisi Jiang is approved.

William McBride

Keisuke Iwamoto

Anahid Jewett

Nicholas Cacalano, Committee Chair

University of California, Los Angeles

2014

Table of Contents

List of Figures

List of Table

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my PhD advisor, Dr. Nicholas Cacalano for his excellent guidance, immense knowledge, patience and caring during my graduate studies at UCLA. I would never be able to finish my dissertation without his help and support.

I would also like to gratefully and sincerely thank my PhD committee members Dr. William McBride, Dr. Kei Iwamoto and Dr. Anahid Jewett for their helpful career advice and insightful suggestions in general. I will forever be thankful to Dr. William McBride for financially supporting me during these past five years.

I would also like to take this time to thank Biomedical Physics Interdepartmental Graduate Program and Department of Molecular and Cellular Oncology at UCLA for helping me to develop my background in Molecular and Cellular Oncology, Molecular and Medical Imaging and Therapeutic Medical Physics as well as all of the funding they were able to provide to me in order to make this dissertation possible.

I would like to specially thank Dr. Jewett and her graduate student Helen, as well as Dr. Gatti and his postdoc researcher Dr. Shareef for helping me with my experiments.

I am also very fortunate to have support from Dr. Jamieson and her lab at UCSD for my research.

Lastly, I would like to give my thanks to my parents Haixiang Jiang and Yixia Yang, because they were always supporting me and encouraging me with their best wishes.

VITA/BIOGRAPHICAL SKETCH

Tsinghua University Beijing, China	B.S.	Biological Sciences and Biotechnologies	2004-2008

I. Introduction

1. JAK/STAT Signaling Pathway and SOCS Proteins

1.1 JAK/STAT signaling pathway

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway is the principal signaling mechanism that regulates a variety of cytokines and growth factors. It is well known that cytokine signaling mediated by the JAK/STAT pathway plays essential roles in differentiation, maturation, proliferation and apoptosis of a various types of cells. A variety of ligands and their receptors stimulate the JAK/STAT pathway. Intracellular activation occurs when ligand binding induces the multimerization of receptor subunits. For signal propagation through either homodimers or heteromultimers, the cytoplasmic domains of two receptor subunits must be associated with JAK tyrosine kinases. JAK activation occurs upon ligand-mediated receptor multimerization because two JAKs are brought into close proximity, allowing trans-phosphorylation. The activated JAKs subsequently phosphorylate additional targets, including both the receptors and the major substrates, STATs, which are latent transcription factors that reside in the cytoplasm until activated. Phosphorylated STATs enter the nucleus and once in the nucleus, dimerized STATs bind specific regulatory sequences to activate or repress transcription of target genes. Thus the JAK/STAT cascade provides a direct mechanism to translate an extracellular signal into a transcriptional response (Reviewed by Jason S, 2004). The JAK/STAT pathway is often mutated or aberrantly activated in multiple human cancers, and correlates with increased proliferation and tumor aggressiveness (J. N. Ihle, 1995; V. Boundy, L. Li and N, Kaur, 2002; E. B. Haura, 2005; R. J. Leeman, 2006).

1.2 SOCS proteins

There are three major classes of negative regulator of JAK/STAT pathway: SOCS (suppressors of cytokine signaling), PIAS (protein inhibitors of activated stats) and PTPs (protein tyrosine phosphatases) (reviewed by Greenhalgh and Hilton, 2001). The SOCS protein family includes eight members: the Cytokine-inducible SH-2 containing protein (CIS) and SOCS1-7 (Hilton DJ, 1998). Each SOCS protein contains three distinct domains: an N-terminal domain with low conservation, a conserved central Src homology (SH)-2 domain and a more highly conserved SOCS box at the C-terminus (Alexander, 2002). In addition, a small kinase inhibitory region (KIR) located N-terminal to the SH2 domain has been identified for SOCS1 and SOCS3, which is responsible for inhibition of JAK. There are 4 possible steps that SOCS proteins inhibit cytokine signaling: (1) block STATs recruitment to the cytokine receptor by masking STAT binding sites of the receptor, (2) target proteins for proteasomal degradation via ubiquitination (3) bind to JAKs and inhibiting their kinase, or (4) target JAKs for degradation via the proteasome (Yoshimura A, 2012). The SOCS complete a classical negative feedback loop in the JAK/STAT signaling: activated STATs stimulate transcription of the SOCS genes and the resulting SOCS proteins bind phosphorylated JAKs and their receptors to turn off the pathway (Jason S, 2004; T. A. Endo, T. Naka and R. Starr, 1997).

SOCS1 and SOCS3 inhibit not only STATs but also other signaling pathways such as Ras/ERK and PI3K, which affect cell proliferation, survival, and differentiation (Lu, 2006; Madonna, 2008). Interestingly, SOCS3 is tyrosine phosphorylated upon cytokine or growth factor stimulation, and phosphorylated Y221 of SOCS3 interacts with p120-RasGAP, resulting in a sustained activation of ERK. Although SOCS proteins inhibit growth factor responses, tyrosine

phosphorylation of SOCS3 can ensure cell survival and proliferation through the Ras pathway (Cacalano, 2001).

In addition to their classic function as negative regulators of JAK/STAT pathway, recent studies using gene-disrupted mice have revealed that SOCS proteins play unexpected and important roles in many immunological processes, atherosclerosis, metabolism, and cancer (Reviewed by Akihiko Yoshimura, 2012).

2. SOCS3 in DNA Damage Response

2.1 DNA damage response and signaling pathways

The DNA damage response evolved primarily as a way for cells to deal with chromosome instability, errors in DNA replication and reactive oxygen species (ROS) that result in DNA breakage (Reviewed by Stephen P. Jackson and Jiri Bartek, 2009). This response gives the cell two choices: undergo cell cycle arrest, repair the damage, and re-enter the cell cycle, or undergo programmed cell death, if the extent of the damage is too great. The importance of this pathway cannot be overstated: spontaneous mutations in the DNA damage response pathway lead to a number of human DNA breakage syndromes, immunodeficiencies, susceptibility to cancer, and sensitivity to radiation. Among the most well studied mutations are those occurring in ATM (Ataxia Telangiectasia), FANCD2 (Fanconi's anemia), BRCA1/2 (breast cancer), p53, p21, and NBS1 (Nijmegen Break Syndrome) (Moldovan and D'Andrea, 2009; David W. Meek, 2009).

Ionizing radiation (IR) activates the same response, and is a key determinant of tumor cell responses to cancer therapy. Not surprisingly, signal transduction pathways, genetic mutations and epigenetic events in tumors that modulate the DNA damage response greatly affect the outcome of radiotherapy. A Major obstacle to effective radiation therapy is the accumulation of

mutations that can directly impair the DNA damage response or effectively antagonize it. A number of receptor tyrosine kinase families as well as cytoplasmic kinases are often constitutively active in tumors and can inhibit the effectiveness of radiotherapy. A number of activated signaling pathways such as JAK/STAT, ERK MAPK, mTOR and PI3K/Akt are linked to radioresistance in tumors by promoting DNA repair or increasing the expression of anti-apoptotic molecules that interfere with IR-induced cell death (Matusoka S, 2007; Kastan M.B., 2008). Several of these pathways are targets for pharmacologic inhibitors that can synergize with radiotherapy.

The STAT3 transcription factor is an oncogenic signaling protein/transcription factor that can drive cellular proliferation and promote cell survival in cancer. One component of STAT3-mediated oncogenesis is its ability to disrupt the expression of cell cycle inhibitory proteins. The p21 (Cip-1/Waf1) promoter has three STAT binding sites which are capable of recruiting STAT3. Recently, it was demonstrated that STAT3 represses p21 expression by directly binding the p21 promoter, but failing to recruit the histone acetylase CBP300 and RNA polymerase, suggesting that STAT3 can act as direct transcriptional inhibitor of p21 (B. Benjamin, 2002). In another study, STAT3 has also been shown to directly repress p53 gene expression (G. Niu, 2005). STAT3 may also negatively regulate p21 by indirect mechanisms as well. The STAT3 target genes c-Myc and BCL6 have been shown to repress p21 gene expression and activate cell cycle progression (S. Mukherjee, 2005; T. Shirogane, 1999; A.L. Gartel, 2003; H.A. Coller, 2000; Gisela F. Claassen, 2000; Phan RT, 2005). Therefore, STAT3 is capable of acting as a radioprotector or radiosensitizer, depending on cellular context. In particular, the degree to which STAT3 signaling is uncontrolled may determine which pathway the cell takes following

radiation exposure. In this study, we show that SOCS3 plays a key role in the regulation of STAT3-dependent radiation outcomes.

2.2 SOCS3 as a radioprotector

Our laboratory has previously identified a novel and unexpected function for SOCS3 in the DNA damage response. We have found that SOCS3 has a radioprotective effect, as SOCS3-deficient fibroblasts display enhanced sensitivity to DNA damaging agents in a clonogenic survival assay. We demonstrated that SOCS3-deficient cells failed to undergo G1 cell cycle arrest in response to radiation. Further, we demonstrated that this defect is likely due to uncontrolled STAT3 signaling in irradiated cells, which results in repression of p21 gene expression (J. C. Sitko, 2008).

In this study, we further investigate the molecular mechanisms by which STAT3 represses p21 expression in mouse embryonic fibroblasts (MEFs) as well as human cancer cell lines following DNA damage. We demonstrate that SOCS3 regulates DNA damage response by inhibiting STAT3 activation, resulting in repressed expression of STAT3 target genes BCL6 and c-Myc, and increased induction of p21. We have also found that the SOCS3/STAT3/BCL6 axis is responsible for specific control of the G1-S transition, as SOCS3-deficient cells display normal S-phase checkpoint and SOCS3 expression does not affect CDC25A degradation or SMC1 phosphorylation following ionizing radiation. Our findings indicate that the balance between proliferation and cell cycle arrest following DNA damage requires strict regulation of the STAT3 transcription factor. Thus, SOCS3 may be a key factor in checkpoint control following DNA damage. In addition to ionizing radiation, we report that SOCS3 also protects MEFs from UV irradiation by *in vitro* clonogenic survival assay. We extend this finding to human cancer cell lines and confirm the radioprotective effect of SOCS3 in non-small cell lung cancer (NSCLC)

cell lines. Besides, overexpression of SOCS3 in NSCLC lines enhanced resistance to DNA damaging agents such as Cisplatin. We further analyzed radiation responses in human pancreatic cancer cells where SOCS3 has been shown to target specific radioprotective kinase pathways. We found that SOCS3 remained radioprotective despite its ability to block IGF-1R and RON receptor tyrosine kinase signaling and biological responses of tumor cells. Since tumor cells have altered responses to ionizing radiation compared to non-transformed cells, targeting the STAT3-SOCS3 pathway may modulate radiation sensitivity and be of therapeutic benefit.

3. SOCS3 in Tumors

3.1 SOCS3 as tumor suppressor

There have been a growing number of studies on the role of SOCS protein family in the oncogenesis of solid and haematological tumors. So far, several laboratories, including ours, have identified defects in SOCS1 and SOCS3 expression in human tumors, indicating an important role of SOCS proteins in cancer pathogenesis (H. Yoshikawa, 2001; R. Rottapel, 2002; O. Galm and H. Nagi, 2003; H. Zhou, 2007). Aberrant hypermethylation of promoter regions in CpG islands has been shown as the principal mechanism which may result in SOCS gene silencing and subsequent loss of negative feedback control on the JAK/STAT signaling pathway in tumor cells (Laird P.W., 2003).

Expression of SOCS3 is normally induced by cytokines through JAK/STAT signaling in normal cells, which in turn inhibits JAK activity and subsequent STAT3 activation. In addition to its physiologic role in inhibiting cytokine signaling, SOCS3 may also function as an important tumor suppressor gene by blocking the transforming activity of oncogenic forms of STAT3. Several studies supported the role of SOCS3 as a tumor suppressor gene in melanoma (T. Tokita,

2007), non-small cell lung cancer (B. He, 2004), head and neck squamous cell carcinoma (C. Rossa, 2012), and liver cancer (H. Ogata, 2006) and associated the silencing of SOCS3 expression by epigenetic mechanisms with the occurrence and progression of these tumors (T. Tokita, 2007; B. He, 2004; C. Rossa, 2012; H. Ogata, 2006).

Lung cancer has been the leading cause of cancer-related deaths in the United States. According to American Cancer Society (ACS), lung cancer accounts for more deaths than any other cancer in both men and women. An estimated 159,480 deaths, accounting for about 27% of all cancer deaths, were expected to occur in 2013. Lung cancer is classified as small cell (15%) or non-small cell (84%) for the purposes of treatment. Annual screening with chest x-ray has not been shown to reduce lung cancer mortality. Because the disease has usually spread by the time it is discovered (American Cancer Society, 2013). This disease is often diagnosed at an advanced stage, which greatly impairs treatment efforts using surgery, radiation, and chemotherapy either alone or in various combinations.

The involvement of SOCS3 in human non-small cell lung cancer (NSCLC) tumorigenesis has been well studied. During the development of NSCLC, the negative regulation of STAT3 activation is suppressed because of the silencing of SOCS3 by promoter hypermethylation. This may result in cells becoming more sensitive to aberrant growth stimulating signals, functioning through the JAK/STAT pathway, which lead to cell growth and survival. It has been reported that restoration of SOCS3 in lung cancer cells where SOCS3 was methylation-silenced resulted in the down-regulation of active STAT3, induction of apoptosis, and growth suppression (B. He, 2003). The high prevalence of SOCS3 promoter hypermethylation supports targeted therapies using the JAK/STAT pathway for treatment of NSCLC. Reactivation of SOCS3 can functionally restore the endogenous negative regulatory machinery of the cytokine-signaling cascade, thus

restoring the apoptotic machinery in these cancer cells. It has been suggested that SOCS3 targeted therapy may be used to sensitize NSCLC cells to traditional chemotherapies (B. He, 2004).

3.2 SOCS3 in pancreatic cancer

Despite the fact that many studies support a role for SOCS3 as a tumor suppressor gene in various types of cancer, the exact role of SOCS3 is complex. For example, overexpression of SOCS3 associated with decreased survival in a cohort of patients with *de novo* follicular lymphoma (Krishnadasan R, 2006). While hypermethylation mediated reduction in SOCS3 expression has been observed in malignant human melanoma (Tokita T, 2007), constitutive SOCS3 expression has also been shown to confer a proliferative advantage to a human melanoma cell line (Komyod W, 2007).

Pancreatic cancer is one of the deadliest cancer types with the worst prognosis of any adult malignancy (Lowy, 2003; Shields, 2011). Most pancreatic cancer patients will die within the first year of diagnosis, and just 6% will survive five years. The pancreas contains two types of glands: the exocrine and endocrine glands. The exocrine glands produce enzymes that help digest food; the endocrine glands produce important hormones such as insulin, which regulates blood sugar levels. Exocrine and endocrine cells form completely different types of tumors with distinct risk factors, symptoms, diagnostic tests, treatment, and survival rates. Exocrine tumors are by far the most common type of pancreatic cancer, representing about 95% of cases (American Cancer Society, 2013). The causes of pancreatic cancer are not well understood. Early stage pancreatic cancer usually has no symptoms. When symptoms do occur, the tumor has usually spread to surrounding tissues or distant organs. The high mortality rate from pancreatic cancer is a result of the high incidence of metastatic disease at the time of diagnosis. The frustrating lack of

significant clinical advancements in the treatment of metastatic pancreatic cancer remains one of medical oncology's biggest disappointments (Nieto, 2008).

It has been reported that in non-small cell lung cancer and pancreatic cancer, malignant cells can rapidly acquire activities that confer both infiltration and colonization competence, as implied by the short time between primary tumor diagnosis and metastatic relapse in these two diseases (Don X. Nguyen, 2009). So far, metastasis and resistance to radiotherapy are two important problems that determine patient outcomes and are still very difficult to overcome clinically. It is for this reason that much effort has been dedicated toward the identification of novel signaling pathways as well as biomarkers of metastatic disease to more fully understand the molecular basis of human PDA pathogenesis and to develop biological targeting agents for use in the clinic. Recently, the function of concomitant inflammation in pancreatic ductal adenocarcinoma (PDA) has garnered increasing interest, because pro-inflammatory markers in the serum, such as interleukin (IL)-1β and IL-6, are associated with outcomes in PDA (Ebrahimi et al., 2004; Sawai et al., 2003). Additionally, chronic pancreatitis is a risk factor for PDA, supporting the involvement of concomitant inflammation in pancreatic oncogenesis (Lowenfels et al., 1993). STAT3 activation in PDA has been documented in human tissues and pancreatic cancer cell lines and reported to support the malignant phenotype of human pancreatic cancer (Scholz, A., 2003). A recent study has demonstrated that higher STAT3 phosphorylation level correlates with the markers of aggressiveness of pancreatic cancer cell lines (K. Patel, 2014). It has also been reported that STAT3/SOCS3 activation by IL-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia (PanIN) and development of pancreatic cancer. Aberrant activation of STAT3 through homozygous deletion of SOCS3 in the pancreas accelerates PanIN progression and PDA development (M. Lesina, 2011).

RON, also referred to as macrophage stimulating 1-receptor (MST1R), is a receptor tyrosine kinase (RTK) of the hepatocyte growth factor (HGF)/Met receptor family primarily expressed on epithelial cells and macrophages (Reviewed by Wagh, 2008). The ligand for RON is hepatocyte growth factor-like (HGFL) protein and is also known as macrophage stimulating protein (MSP). Binding of HGFL to RON activates RON and leads to the induction of a variety of intracellular signaling cascades that leads to cellular growth, motility and invasion. RON has been identified as an overexpressed protein and a potential novel therapeutic target in pancreatic cancer (Jocelyn Logan-Collins, 2010). Studies of the expression and function of RON in pancreatic cancer have identified that RON receptor is highly expressed in several human pancreatic cell lines, including AsPC1, BxPC3, CFPAC-1, FG, and L3.6 pl (Camp, 2007). In one report, 93% of the human pancreatic cancer tissues showed overexpression of RON relative to normal ductal epithelium (Camp, 2007). Several strategies are currently being undertaken to inhibit RON as a potential therapeutic target; current strategies include the use of RON blocking proteins, small interfering RNA (siRNA), monoclonal antibodies, and small molecule inhibitors. In total, these data suggest that RON is a critical factor in tumorigenesis and that inhibition of this protein, alone or in combination with current therapies, may prove beneficial in the treatment of cancer patients (Wagh, 2008).

Several studies have identified insulin-like growth factor receptor (IGF1-R) as a RON partner (Potratz,J.C, 2010; D.V. Jaquish, 2011) and showed that IGF-1-induced STAT3 activation is at least partially dependent on RON signaling (D.V. Jaquish, 2011). Our laboratory has recently demonstrated that SOCS3 inhibited both IGF-1R and RON signaling in human PDA cell lines, indicating the involvement of SOCS3 in PDA pathogenesis. Despite some circumstantial data suggesting that SOCS3 may act as a tumor suppressor in some specific animal models of PDA,

nothing is currently known about its role in human pancreatic cancer and no studies have been published that describe SOCS3 expression patterns or defects in human PDA. In order to further explore the role of SOCS3 in pancreatic cancer cell growth, proliferation, differentiation, and invasion, our lab has conducted both *in vitro* and *in vivo* research on a panel of human PDA cell lines.

We examine the epigenetic regulation of SOCS3 expression in a panel of PDA cell lines as well as the effect of overexpressing SOCS3 in PDA cell lines on pancreatic tumor growth, metastasis and response to ionizing radiation. We identify PDA cell lines into three distinct molecular subtypes based on expression of SOCS3 in response to IL-6/OSM stimulation. In the PDA cell line FG, SOCS3 is highly expressed upon IL-6/OSM stimulation and overexpression of SOCS3 in FG does not affect tumor growth. On contrast, re-introduction of SOCS3 into PDA cell lines 2.8 and PL12 in which SOCS3 expression is severely low or totally repressed inhibits tumor growth and metastasis, indicating a tumor suppressor role of SOCS3 in these cell lines. In another PDA cell line AsPC1, SOCS3 expression is partially repressed. Our *in vitro* and *in vivo* data demonstrate that overexpression of SOCS3 in AsPC1 results in increasing cell migration, malignant tumor growth, aggressive metastasis as well as enhancing resistance to ionizing radiation and genotoxic agents. Interestingly, we do not identify SOCS3 promoter hypermethylation in PDA cell lines by methylation-specific PCR (MS-PCR), suggesting that SOCS3 repression in human PDA is via mechanism(s) other than promoter CpG island methylation. Although our data indicated the involvement of SOCS3 in PDA pathogenesis, the molecular mechanism(s) by which SOCS3 regulate pancreatic tumor progression and invasion still remains to be clarified.

4. CXCL1 in Tumors

4.1 CXCL1 in tumor metastasis

By performing gene expression microarray assay using WT and SOCS3 KO mouse embryonic fibroblasts (MEFs), we identified a few genes that were under-represented in SOCS3 KO MEFs, one of which caught our interest is the pro-inflammatory C-X-C chemokine, CXCL1.

It is now well understood that chemokines play a number of functional roles in tumor biology, including cellular transformation, tumor growth, homing, and metastasis (Richmond A, 2004; Payne AS, 2002). The C-X-C chemokine CXCL1, previously designated as melanoma growth stimulatory activity/growth related protein (MGSA/GRO), has been shown to play an important role in tumorigenesis and angiogenesis and its overexpression has been associated with tumor progression. CXCL1, and its receptor CXCR2, are expressed by melanoma cells as well as tumors from breast, bladder and prostate, and it has been shown that autocrine CXCL1-CXCR2 signaling can drive tumor cell migration and increase metastatic potential (H. Kawanishi, 2008; Swarnali Acharyya, 2012).

In normal immunologic physiology, CXCL1 is expressed by macrophages, neutrophils and epithelial cells and functions as a neutrophil chemoattractant to sites of infection (Moser B, 1990; Schumacher C, 1992). Therefore, it is not surprising that CXCL1 secretion by solid tumors often results in neutrophil infiltration of the tumor microenvironment. Indeed, it has been shown that CXCL1 specifically induces the migration of neutrophils, drives them to leave the bloodstream and enter into the surrounding tissue (Schumacher C, 1992). It is becoming increasingly clear that tumor-associated neutrophils (TAN) play a major role in cancer biology. There is suggestive evidence for a role for TAN in enhanced disease progression in specific human tumors (Kuang D.M., 2011; Wislez M., 2003). And the infiltration of neutrophils in the tumor microenvironment

often correlates with poor prognosis (de Visser et al., 2006). It has also been reported that TANs can produce soluble factors, including pro-inflammatory mediators such as S100A8 and S100A9, as well as metalloproteases MMP7 and MMP9, which contribute to promote destruction of the extracellular matrix (ECM), tumor metastasis, and angiogenesis. Several studies showed that MMP-13 was highly expressed in invasive bladder tumor tissue (H. Kawanishi, 2008; Bostrom PJ, 2000), and a glioma cell line overexpressing CXCL1 showed an increase in motility and invasiveness and that CXCL1-transfected cells showed increased expression of MMP-2 (Zhou, 2005). Interestingly, both CXCL1 and TANs have been described in pancreatic tumors and cell lines, yet their function in the pathogenesis of PDA is poorly understood.

4.2 Regulation of CXCL1 expression

CXCL1 has also been characterized as one of the many chemokines involved in radiation response (Van der Meeren A, 2003; Dhawan P, 2002). It is indicated that radiation-induced enhancement of CXCL1 may contribute to tumor growth and survival during subsequent radiation response (Prakash C, 2005). Increased expression of CXCL1 has been attributed to constitutive activation of NF-κB through mitogen-activated protein kinase (MAPK) signaling (Dhawan P, 2002). Although chemotherapy and radiation are used to conquer cancer, they also produce reactive oxygen species (ROS) in the tumor microenvironment, which in turn activates NF-κB and thus leads to the production of factors like C-X-C chemokines involved in tumor progression (Reuter et al., 2010). Recently, CXCL1 and CXCL2 were revealed to be at the center of a chemotherapy-triggered process leading to chemoresistance and metastasis in breast cancer. *In vitro* experiments revealed that chemotherapy induced TNF-α production from endothelial

and other stromal cells, which in turn caused CXCL1/2 up-regulation in cancer cells via NF-κB activation and resulted in metastasis and chemoresistance (Swarnali Acharyya, 2012).

Another key regulator of CXCL1 is STAT3. It has been reported that STAT3 signaling via the IL-6 common receptor subunit gp130, negatively regulates production of a number of inflammatory genes, including chemokines such as CXCL1 (T Wang, 2004; A. Matsukawa, 2005; C. A. Fielding, 2008). Of greatest interest to our laboratory is the fact that SOCS3 negatively regulates STAT3 and therefore may modulate CXCL1 expression in cancer though the STAT3 pathway. We have shown that the SOCS3 gene is silenced in a subset of pancreatic cancer cell lines, which results in repression of CXCL1 production, presumably due to increased STAT3 activation. Thus, we hypothesize that SOCS3-regulated CXCL1 expression may control cell migration and metastasis in human pancreatic cancer.

We have found that restoration of SOCS3 confers to up-regulated expression of CXCL1 and MMP7 in non-small cell lung cancer (NSCLC) cell line H1703 and PDA cell line AsPC1. While overexpressing SOCS3 in FG, PL12, and 2.8 does not affect CXCL1 secretion. Further, using an orthotopic xenograft model of human PDA in immunodeficient mice, we demonstrate that immunodeficient mice injected with AsPC1 SOCS3 have a shorter survival time and develop more aggressive disease compared to mice injected with control cells. Overall, our results suggest a novel mechanism for controlling pro-inflammatory signaling in human solid tumors and that SOCS3 can regulate both metastasis and radiation sensitivity through distinct pathways. SOCS3 and CXCL1 may constitute interesting therapeutic targets for pancreatic cancer. By targeting STAT3, SOCS3, as well as CXCL1, we can inhibit metastatic behavior in solid tumors, and increase radiation sensitivity in radiation therapy.

II. *In vitro* studies on the role of SOCS3 in DNA damage response in mouse embryonic fibroblasts (MEFs) and human cancer cell lines

1. Materials and Methods

1.1 Materials

Cell lines and cell culture. Mouse embryonic fibroblasts (MEFs), human non-small cell lung cancer (NSCLC) cell lines A549, H1703 and H460M, human pancreatic ductal adenocarcinoma (PDA) cell line AsPC1 were used in this study. MEFs (WT, SOCS1 KO and SOCS3 KO) were cultured in Dulbecco's modified Eagle's medium or DMEM (Cat. #10-013-CV, CORNING cellgro, Mediatech. Inc) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich) and 100 µg/ml of Antibiotic Antimycotic Solution (Cat. #30-004-CI, CORNING cellgro, Mediatech. Inc) at 37°C in 6% CO_2. Human cancer cell lines were cultured in RPMI 1640 (Cat. #10-040-CV, CORNING cellgro, Mediatech. Inc) supplemented with 10% FBS and 100 µg/ml of Antibiotic Antimycotic Solution at 37°C in 6% CO_2. 0.25% Trypsin-EDTA (Cat. #25-053-CI, CORNING cellgro, Mediatech. Inc) was used to lift the cells.

1.2 Methods

Genetic reconstitution of human cancer cell lines. We have previously generated C-terminal FLAG epitope tagged cDNAs encoding WT SOCS3 and a Y705F dominant negative (DN) mutant of STAT3 in the pMX-IRES-GFP retroviral expression vector which generates a bicistronic message containing SOCS3 (or DNSTAT3) followed by a picornavirus internal ribosome entry site (IRES) followed by the gene for green fluorescent protein (GFP) (Zhou Hong, 2007; John Sitko, 2008). In this study, we continued to use the pMXs-IRES-GFP (also known as

pMXs-IG) retroviral vector (Cell Biolabs, Inc.) to deliver exogenous genes into genome of human cancer cell lines. The pMXs-SOCS3-IRES-GFP plasmid was made by Dr. Cacalano and used for stable transfection of PA317 retrovirus packaging cells. Supernatant from the stable cell line PA317 expressing SOCS3 was used to infect MEFs and human cancer cell lines for at least 24 hours with 8 μg/ml of polybrene (Millipore). Retrovirus infection was repeated for at least two times. Once GFP positive cells were detected under the fluorescence microscope, cells were sorted by flow cytometry (UCLA JCCC) and SOCS3 positive cells were collected.

Clonogenic survival assay. Human NSCLC cell lines A549, H1703 and H460M were irradiated with X-ray at 0, 1, 2, 4, 6 and 8 Gy and then plated in 10 cm dishes. The parental and SOCS3+ NSCLC and PDA cell lines were irradiated with X-ray at 0, 1, 2, 4, 6 Gy and then plated in 10 cm dishes. WT and SOCS3 KO MEFs were irradiated using Ultraviolet B or medium wave light at 0, 10, 20, 40, 60, 80 and 100 mJ/cm^2 (wavelength: 315 nm - 280 nm, energy per photon: 3.94 - 4.43 eV). The cells were then plated in 10 cm dishes and incubated for about two weeks to let the colonies grow. The NSCLC cell lines were treated with Cisplatin at 0, 0.84, 1.67 and 3.33 μg/ml for 6 hours and then plated in 10 cm dishes in complete RPMI. AsPC1 and AsPC1 SOCS3 were plated in 10 cm dishes with complete RPMI 1640 containing Gemcitabine at 0, 0.5, 1, 5, 10, 20 nM. The cells were left to grow for about two weeks and surviving colonies were stained with 0.05% w/v crystal violet/methanol for 30 minutes and counted to generate survival fraction data at each dose of radiation, Cisplatin or Gemcitabine.

2. Results and Discussion

2.1 Results

SOCS3 silencing sensitizes human non-small cell lung cancer cells to ionizing radiation

In a previous study, our laboratory determined that SOCS3 has a radioprotective effect, as SOCS3+ cells display greater viability in a clonogenic survival assay after exposure to ionizing radiation than SOCS3-deficient cells. We irradiated MEFs from WT and SOCS3 KO and found that cells from the knockout were significantly more sensitive to radiation (data not shown). Further, we extended these findings to a human non-small cell lung cancer (NSCLC) model. As shown in Figure 1, in agreement with our studies in SOCS3 KO MEFs, we found that two human NSCLC cell lines that silence SOCS3 expression (H1703 and H460M) are significantly more radiosensitive than A549 in which SOCS3 expression is normal, suggesting that SOCS3 is a radioprotector in human solid tumors.

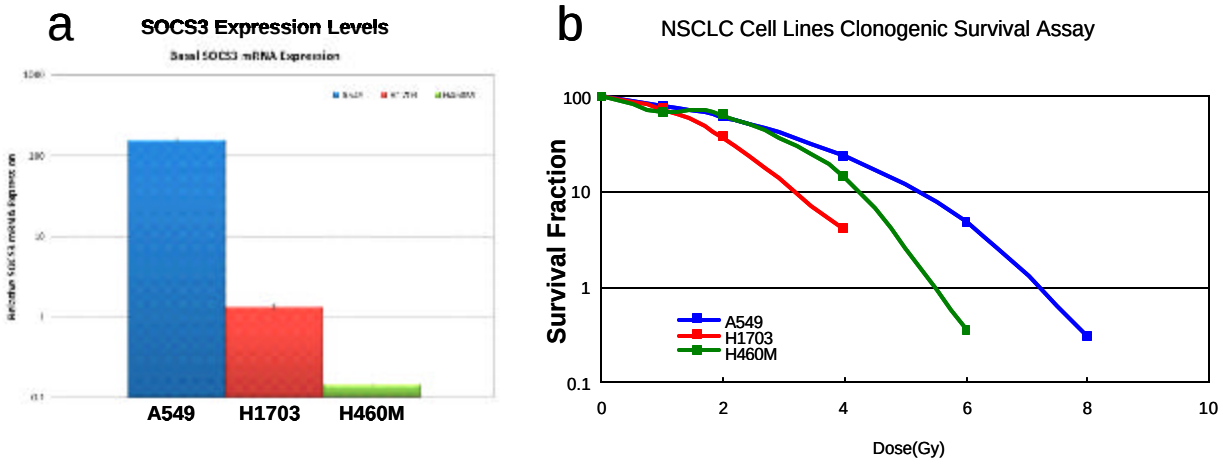


Figure 1. SOCS3-deficient non-small cell lung cancer cells are IR-sensitive. (a) Analysis of SOCS3 mRNA expression levels in NSCLC cell lines. (b) Clonogenic survival assay showed that human NSCLC cell lines H1703 and H460M, which silence SOCS3 expression, are more radiosensitive than A549, which expresses normal levels of SOCS3.

Overexpression of SOCS3 in NSCLC and PDA cell lines enhances resistance to IR

We have demonstrated that SOCS3 has a radioprotective effect in MEFs and NSCLC cell lines. Next, we further confirmed this effect by overexpressing SOCS3 in NSCLC cell lines H460M and H1703 in which SOCS3 expression is silenced. As shown in Figure 2, re-introduction of

SOCS3 into H460M and H1703 enhanced cell resistance to IR. We have also identified this effect of SOCS3 in human pancreatic cancer cell lines (see below).

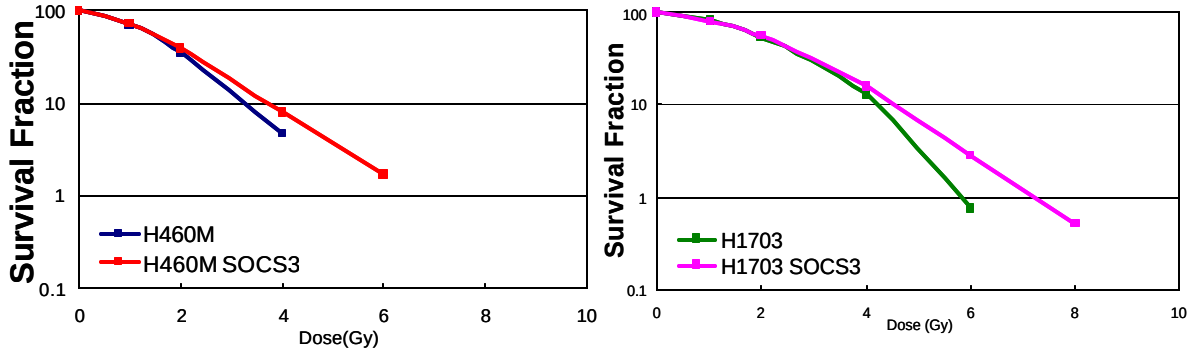


Figure 2. Clonogenic survival assay showed that restoring SOCS3 in NSCLC cell lines increased resistance to IR.

SOCS3-deficient MEFs are more sensitive to UV irradiation

Our lab has previously demonstrated that SOCS3 acted as a radioprotector for MEFs following ionizing radiation. We further characterized resistance of WT and SOCS3 KO MEFs to UV irradiation. As shown in Figure 3, WT MEFs are more resistant to UV irradiation compared with SOCS3 KO MEFs. Our data indicated that SOCS3 can protect MEFs from both IR and UV.

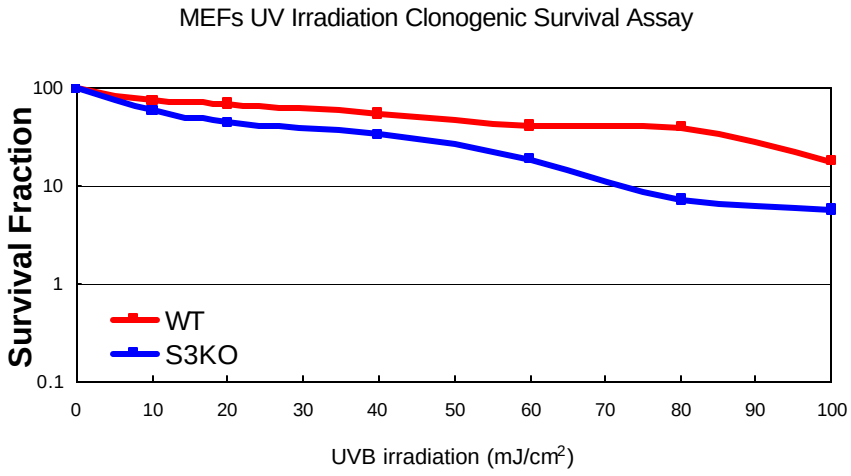


Figure 3. Clonogenic survival assay showed that SOCS3 protected MEFs from UV irradiation. SOCS3-deficient fibroblasts are more sensitive to UV irradiation compared to WT MEFs.

Overexpression of SOCS3 in NSCLC and PDA cell lines enhances resistance to DNA damaging agents

We then further investigate the effect of overexpressing SOCS3 in NSCLC and PDA cell lines in response to DNA damaging agents.

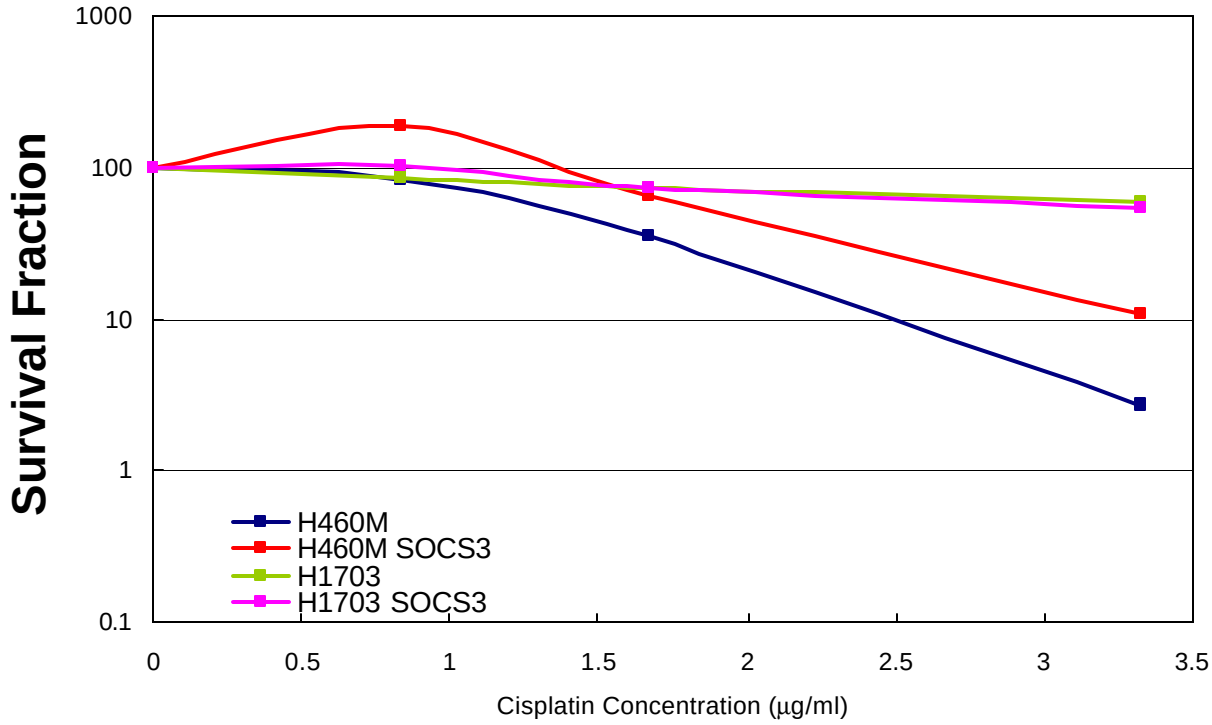


Figure 4. Clonogenic survival assay showed that overexpression of SOCS3 in NSCLC cell lines enhanced resistance to Cisplatin.

As shown in Figure 4, we treated the parental and SOCS3+ NSCLC cell lines with Cisplatin and characterized the cell viability by performing *in vitro* clonogenic survival assay. We found that restoration of SOCS3 in NSCLC cell lines increased their resistance to Cisplatin.

We also examined the effect of overexpressing SOCS3 in the pancreatic cancer cell line AsPC1 in response to ionizing radiation and DNA damaging agents by *in vitro* clonogenic survival assay. As shown in Figure 5, overexpression of SOCS3 in AsPC1 protected cells from ionizing radiation and enhanced cell resistance to Gemcitabine treatment.

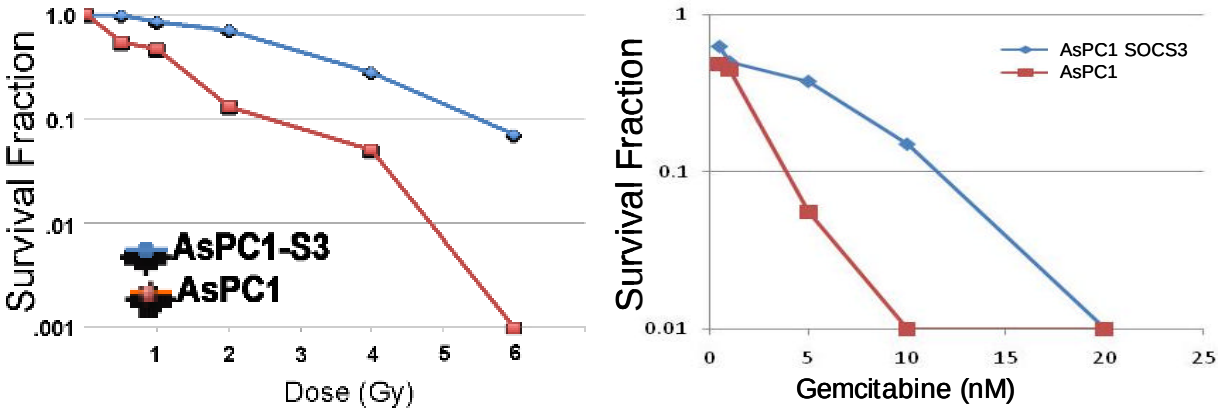


Figure 5. Clonogenic survival assay showed that overexpression of SOCS3 in AsPC1 resulted in enhanced resistance to IR and Gemcitabine.
Left Panel. Overexpression of SOCS3 in AsPC1 increased cell resistance to IR.
Right Panel. Overexpression of SOCS3 in AsPC1 increased cell resistance to Gemcitabine.

2.2 Discussion

Our lab has previously investigated the function of SOCS3 in the regulation of DNA damage response by using mouse embryonic fibroblasts (MEFs). In this study, we identify that SOCS3 can protect MEFs from both ionizing radiation and UV irradiation. We also further confirm the radioprotective effect of SOCS3 in human non-small cell lung cancer and pancreatic ductal adenocarcinoma. We demonstrate that human NSCLC cell line A549 in which SOCS3 expression is normal, are more radioresistant than H460M and H1703 in which SOCS3 is silenced. Restoration of SOCS3 in NSCLC cell lines H460M, H1703 and PDA cell line AsPC1 increased resistance to radiation of the cells. We also investigate the effect of SOCS3 restoration in response to other DNA damaging agents by clonogenic survival assay. Restoration of SOCS3 in H460M and H1703 resulted in enhanced cell resistance to the chemotherapy drug Cisplatin. In addition, we also revealed that overexpressing SOCS3 in AsPC1 increased cell resistance to Gemcitabine, which has been commonly used as chemotherapy in various cancers including pancreatic cancer, non-small cell lung cancer, bladder cancer and breast cancer.

Our data further confirmed the radioprotective effect of SOCS3 in both MEFs and human solid tumor cell lines. Besides, we also demonstrated that ectopic expression of SOCS3 in NSCLC and PDA cell lines drives resistance to genotoxic agents such as Cisplatin and Gemcitabine. Our lab has previously demonstrated that SOCS3-deficient cells failed to undergo G1 cell cycle arrest in response to radiation, and this defect is likely due to uncontrolled STAT3 signaling in irradiated cells, which results in repression of p21 gene expression (John Sitko, 2008). In the context of cancer, it seems counterintuitive that SOCS3, an inhibitor of STAT3 activation, would be overexpressed in some radioresistant cancers, in particular those that display constitutive STAT3 activation. Our data suggest a possible mechanism for this phenomenon, and argue that targeting SOCS3 in tumors may improve responses to radiotherapy. We are going to further identify the mechanism(s) underlying the regulation of the DNA damage response by SOCS3 and to assess the potential for targeting SOCS3 as a novel anti-cancer therapy.

3. Conclusion

Our data reveal that overexpression of SOCS3 in human NSCLC and PDA cells enhanced resistance to ionizing radiation and genotoxic agents. Since tumor cells have altered responses to ionizing radiation compared to non-transformed cells, targeting the STAT3-SOCS3 pathway may modulate radiation sensitivity and be of therapeutic benefit.

III. *In vitro* studies on the molecular mechanism(s) by which SOCS3 promotes p21 induction and the role of SOCS3 in intra-S-phase checkpoint following ionizing radiation in MEFs and NSCLC cell lines

1. Materials and Methods

1.1 Materials

Cell lines and cell culture. MEFs (WT, SOCS1 KO, SOCS3 KO and SOCS3 KO DN STAT3), human NSCLC cell lines H460M, H460M SOCS3, H1703 and H1703 SOCS3 were used in this study. All the cell lines were maintained as described above.

1.2 Methods

Western blot. MEFs and human cancer cell lines were irradiated with X-ray and harvested at 0, 1, 2, 4, 6, 8 hours following irradiation by direct addition of ice cold PBS and scraping. After centrifugation, the cell pellets were resuspended with Brij lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl pH7.5, 2 mM EDTA, 0.875% Brij 97 (Sigma), 0.125% Igepal CA-630 (Sigma), 10 µM Aprotinin (Cat. No. 190779, ICN Biomedicals Inc), 10 µM Leupeptin Hemisulfate (Cat. No. 151553, MP Biomedicals), 10 µM Pepstain, 1 mM Phenylmethylsulfonyl fluoride (PMSF) (Sigma), 1 mM Sodium Vanadate and 1 mM NaF. Cells were lysed for 15 minutes on ice before centrifuging at 10,000 rpm for 10 minutes at 4 °C to remove nuclei.

Nuclear lysates were prepared according to a published protocol (Nancy C. Andrews, 1991). Briefly, approximately 10^6 of MEFs or human cancer cells were scraped into 1.5ml of cold phosphate-buffered saline (PBS). The cell suspension was then transferred to a microfuge tube. Cells were pelleted for 10 seconds and resuspended in 400 µl cold Buffer A (10 mM HEPES-

KOH pH7.9 at 4 ^{o}C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol or DTT, 0.2 mM PMSF) by flicking the tube. The cells were allowed to swell on ice for 10 minutes, and then vortexed for 10 seconds. Samples were centrifuged for 10 seconds, and the supernatant fraction was discarded. The pellet was resuspended in 50 µl of cold Buffer C (20mM HEPES-KOH pH7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 minutes for high-salt extraction. Cellular debris was removed by centrifugation for 2 minutes at 4^{o}C and the supernatant fraction (containing DNA binding proteins) was stored at -70 ^{o}C. Whole cell lysates and nuclear lysates were prepared by the addition of sample buffer and boiling for 5 minutes. Protein sample buffer was prepared by adding 50 µl βME (Bio-Rad, Cat. # 161-0710) to 950 µl Laemmli Sample Buffer (Bio-Rad, Cat. #161-0737). Cell lysates were diluted 1: 1 with the sample buffer.

Proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Bradford, MA). The membranes were probed with appropriate antibodies diluted 1:1000 in PBS plus 0.1% Tween-20 and 3% BSA (Sigma). Primary antibodies were bound at 4°C overnight with gentle shaking, washed 3 times with PBS-Tween 20, 10 minutes each, and probed with a 1:5000 dilution of either horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, Buckinghamshire, UK) as appropriate. After washing, the proteins were detected by the chemiluminescent substrate WesternDura (Pierce, Rockford, IL) using FUJI LAS3000 Digital Imaging System (Dr. Nishimura's lab of UCLA Dental School). The antibodies used in this study were: anti-BCL6, anti-c-Myc, anti-CDC25A, anti-Actin, anti-phospho-SMC1 and anti-SMC1 (Cell Signaling or Santa Cruz).

Analysis of radioresistant DNA synthesis (RDS). We analyzed RDS in MEFs following ionizing radiation according to a published protocol (C.H. Lai, 2004). Briefly, approximately

5×10^6 of WT, SOCS3 KO and SOCS3 KO DN STAT3 MEFs were incubated in complete DMEM with 0.4 μCi/ml [^{14}C] thymidine (NEC-568 methyl-^{14}C thymidine, 0.1 mCi/ml, Perkins Elmer, Boston, MA) overnight at 37^{o}C prior to irradiation. Cells were then harvested and irradiated with 0, 2, 5, 10, 25 and 50 Gy, to establish a dose-response curve. Following irradiation, cells were incubated at 37^{o}C for one hour and then pulsed with 4 μCi/ml [^{3}H] thymidine (NET-027X methyl-^{3}H thymidine, 1.0 mCi/ml, Perkins Elmer, Boston, MA) at final concentration per well in a 96 well round bottom plate (Costar type 3799 round bottom, Corning, NY) for an additional 60 minutes. Cells were harvested using Comi-12 cell harvester (Melocular Devices, Corp., Sunnyvale, CA), air dry at least three hours before counting. 2 ml of scintillation fluid (ScintiSafe 30% LSC-cocktail, Fisher Scientific, Fair Lawn, NJ) was prepared for each sample in the 7 ml glass scintillation vial (Fisher Scientific, Fair Lawn, NJ) with the filter mat (1.5 μm without binding material, Molecular Devices, Corp., Sunnyvale, CA) face up towards the vial cap. The samples were counted in a Packard 2900TR Liquid Scintillation Analyzer (Perkin-Elmer, Boston, MA; Department of Human Genetics, UCLA). The ratio of incorporated ^{3}H to ^{14}C was used for quantification and to normalize the samples for variation in DNA recovery. The non-irradiated ^{3}H/^{14}C ratio was set as 100% to calculate the percentages of every irradiated ratio as portion of the non-irradiated. Each treatment was performed in triplicate for statistical analysis.

2. Results and Discussion

2.1 Results

SOCS3 promotes p21 induction by repressing BCL6 and c-Myc expression

Our lab has previously investigated the function of SOCS3 in the regulation of DNA damage response by using MEFs. We identified that SOCS3 has a radioprotective effect by promoting DNA damage repair, supporting p21 expression and G1 cell cycle arrest (John Sitko, 2008). We will further investigate the molecular mechanism(s) by which SOCS3 promotes p21 induction.

There are several potential pathways that could result in p21 repression in cells lacking SOCS3, one of which would involve a STAT3 target gene that acts to block radiation-induced p21 expression and G1 cell cycle arrest. The proto-oncogene BCL6 is an intriguing candidate for the p21 effect because its main function is to repress p21 induction during the DNA damage that occurs in germinal center B cells that are actively rearranging B cell antigen receptor genes (Phan, R. T., 2005). BCL6 is of particular interest in our system because STAT3 is known to bind the BCL6 silencer region and activate its transcription (Masafumi Arima, 2008). Our finding that STAT3 phosphorylation is uncontrolled in SOCS3 KO MEFs suggests that BCL6 may be aberrantly triggered in cells lacking the SOCS3 inhibitory pathway.

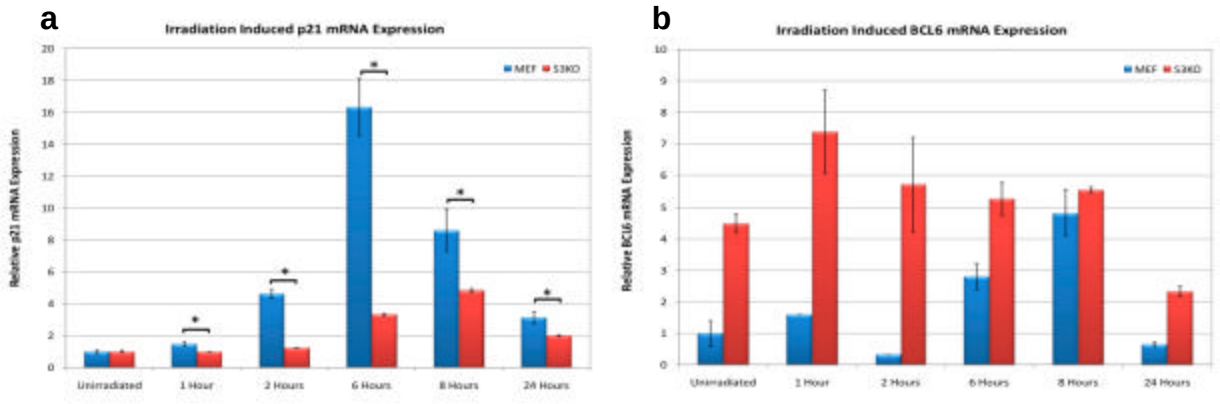


Figure 6. Analysis of p21 and BCL6 mRNA expression following IR in WT and SOCS3 KO MEFs. (a) Analysis of radiation-induced p21 mRNA expression in MEFs. (b) Analysis of BCL6 mRNA expression in MEFs following IR.

Initially, we analyzed BCL6 and p21 mRNA expression following ionizing radiation in MEFs. As shown in Figure 6 (a), we identified that radiation-induced p21 expression is impaired in

SOCS3 KO MEFs, consistent with our previous studies that SOCS3-deficient fibroblasts failed to undergo G1 cell cycle arrest due to repression of p21 induction following IR. We then analyzed BCL6 expression in MEFs following irradiation. As shown in Figure 6 (b), we found that BCL6 mRNA is overexpressed in SOCS3 KO MEFs, supporting the idea that STAT3 target genes may function as p21 inhibitor in DNA damage response following irradiation.

We also examined expression of BCL6 protein in MEFs following ionizing radiation. As shown in Figure 7, BCL6 expression in SOCS3 KO cells is higher basally and following irradiation compared to WT MEFs, consistent with a model that aberrant activation of BCL6 may play a role in suppression of radiation-induced p21 expression in SOCS3-deficient cells. Not surprisingly, we found that in SOCS3 KO MEFs that express a dominant negative STAT3, BCL6 expression is completely silenced, confirming that in our system, STAT3 is the major factor that controls BCL6 expression in response to IR.

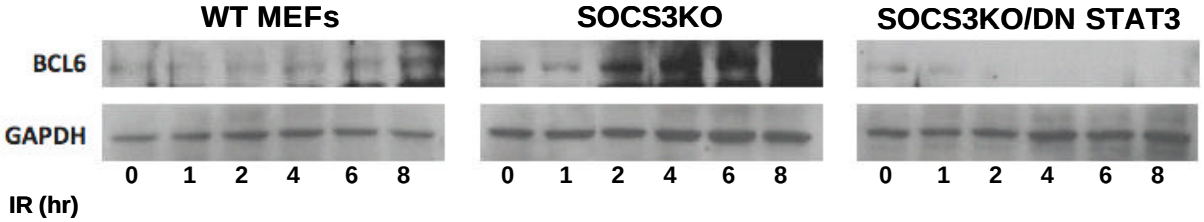


Figure 7. Immunoblot analysis of whole cell lysates from MEFs following IR showed that BCL6 was overexpressed in SOCS3-deficient MEFs.

Importantly, we have extended these observations to human tumor cell lines. In Figure 8, we analyzed SOCS3 and BCL6 mRNA expression in two non-small cell lung cancer (NSCLC) cell lines that display SOCS3 gene methylation and silencing (H1703 and H460M, columns 2 and 3), as well as the cell line A549 (column 1), which does not silence SOCS3 expression. Consistent with our data in MEFs, we found an inverse relationship between SOCS3 and BCL6 expression levels in the NSCLC cells (BCL6 levels were highest in cells that repressed SOCS3 expression),

suggesting that human tumors that silence SOCS3 may also harbor a defect in radiation-induced p21 expression and display enhanced sensitivity to IR.

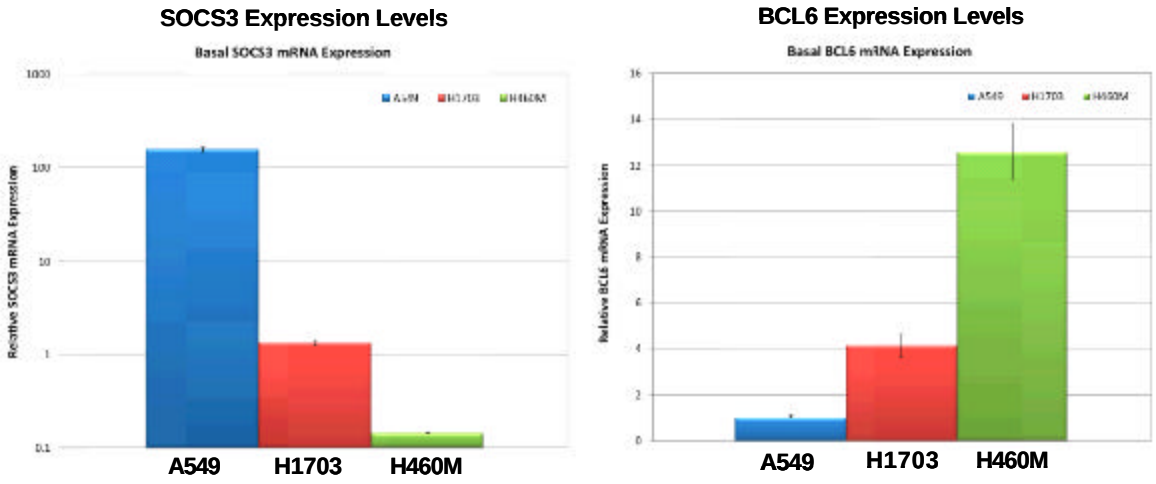


Figure 8. Analysis of SOCS3 and BCL6 mRNA expression in human NSCLC cell lines.

In addition to BCL6, the c-Myc proto-oncogene, another STAT3 target gene, has been shown to repress p21 gene expression and activate cell cycle progression. We then investigated expression of c-Myc in MEFs and NSCLC cell lines and observed that c-Myc protein level is elevated in SOCS3 KO MEFs (Figure 9, left panel). In human NSCLC cell lines, we observed higher c-Myc mRNA level in H1703 and H460M which silence SOCS3 expression, while A549 in which SOCS3 expression is normal, displayed the lowest level of c-Myc mRNA (Figure 9, right panel).

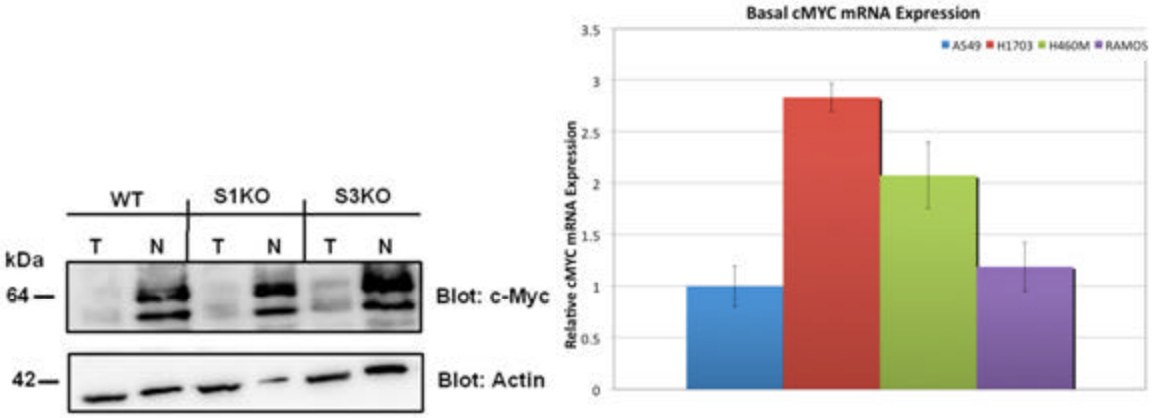


Figure 9. Analysis of c-Myc protein and mRNA expression in MEFs and human NSCLC cell lines.

Left Panel. Immunoblot analysis of nuclear lysates from MEFs showed that c-Myc protein expression was up-regulated in SOCS3-deficient MEFs.
Right Panel. Analysis of mRNA expression in NSCLC cell lines showed an inverse relationship between SOCS3 and c-Myc expression levels.

SOCS3-deficient MEFs undergo normal intra-S-phase checkpoint following IR

Our lab has previously demonstrated that SOCS3-deficient cells failed to undergo G1 cell cycle arrest in response to radiation. Next, we determine whether SOCS3 regulates radiation-induced intra-S-phase checkpoint by performing radioresistant DNA synthesis (RDS) analysis using WT and SOCS3 KO MEFs as well as SOCS3 KO MEFs expressing a Y705 dominant negative mutant of STAT3 (S3KO DNST3).

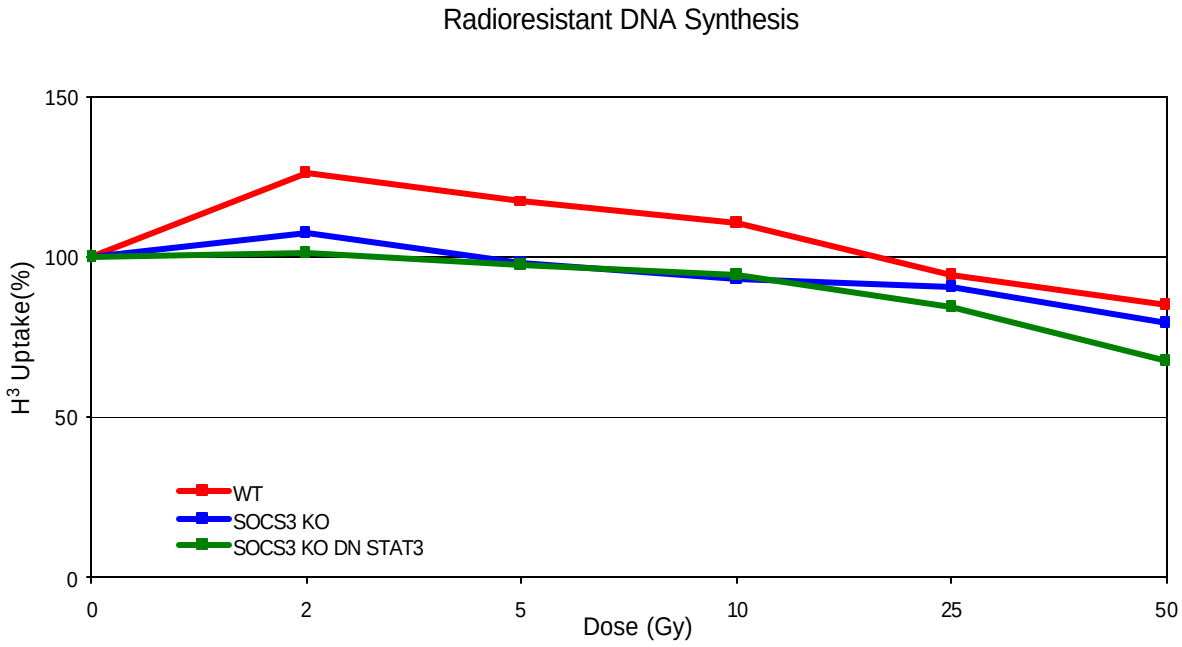


Figure 10. Radioresistant DNA synthesis analysis of WT, SOCS3 KO and SOCS3 KO DN STAT3 MEFs following ionizing radiation.

As shown in Figure 10, we observed reduced [^{3}H] thymidine incorporation in SOCS3 KO MEFs relative to WT MEFs, indicating that SOCS3 KO MEFs undergo S-phase checkpoint following

IR. We also identified reduced RDS in SOCS3 KO MEFs expressing DN STAT3 compared to WT MEFs. Our data suggest that there is no difference between the three cell lines in their RDS responses, indicating that the S-phase checkpoint in SOCS3-deficient cells is normal.

To complement the RDS assay, we also analyzed S-phase checkpoint by measuring CDC25A phosphorylation and degradation biochemically. Normal S-phase checkpoint is characterized by loss of CDC25A protein levels due to proteasome-mediated degradation. In agreement to our finding of RDS, we observed degradation of CDC25A protein in SOCS3 KO MEFs following ionizing radiation (Figure 11).

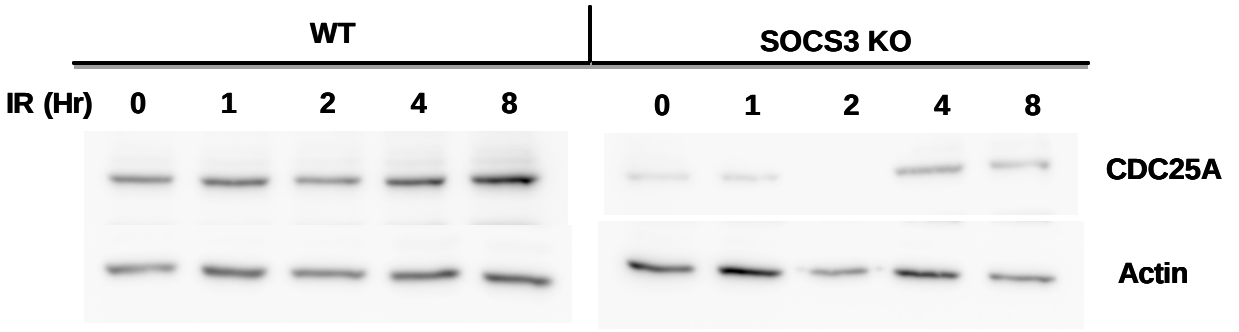


Figure 11. Immunoblot analysis of whole cell lysates from MEFs showed degradation of CDC25A following ionizing radiation.

We also examined phosphorylation of SMC1 in MEFs and NSCLC cell lines following IR. Structural maintenance of chromosomes (SMC) proteins (SMC1, SMC3) are evolutionarily conserved chromosomal proteins that are components of the cohesin complex, necessary for sister chromatid cohesion. These proteins may also function in DNA damage repair. It has been reported that SMC1 is a component of the DNA damage response network that functions as an effector in the ATM/NBS1-dependent S-phase checkpoint pathway.

As shown in Figure 12, we found that SMC1 phosphorylation is not affected by SOCS3 in MEFs and NSCLC cell lines following ionizing radiation, which is also in agreement with our RDS data that SOCS3 KO MEFs undergo normal S-phase checkpoint.

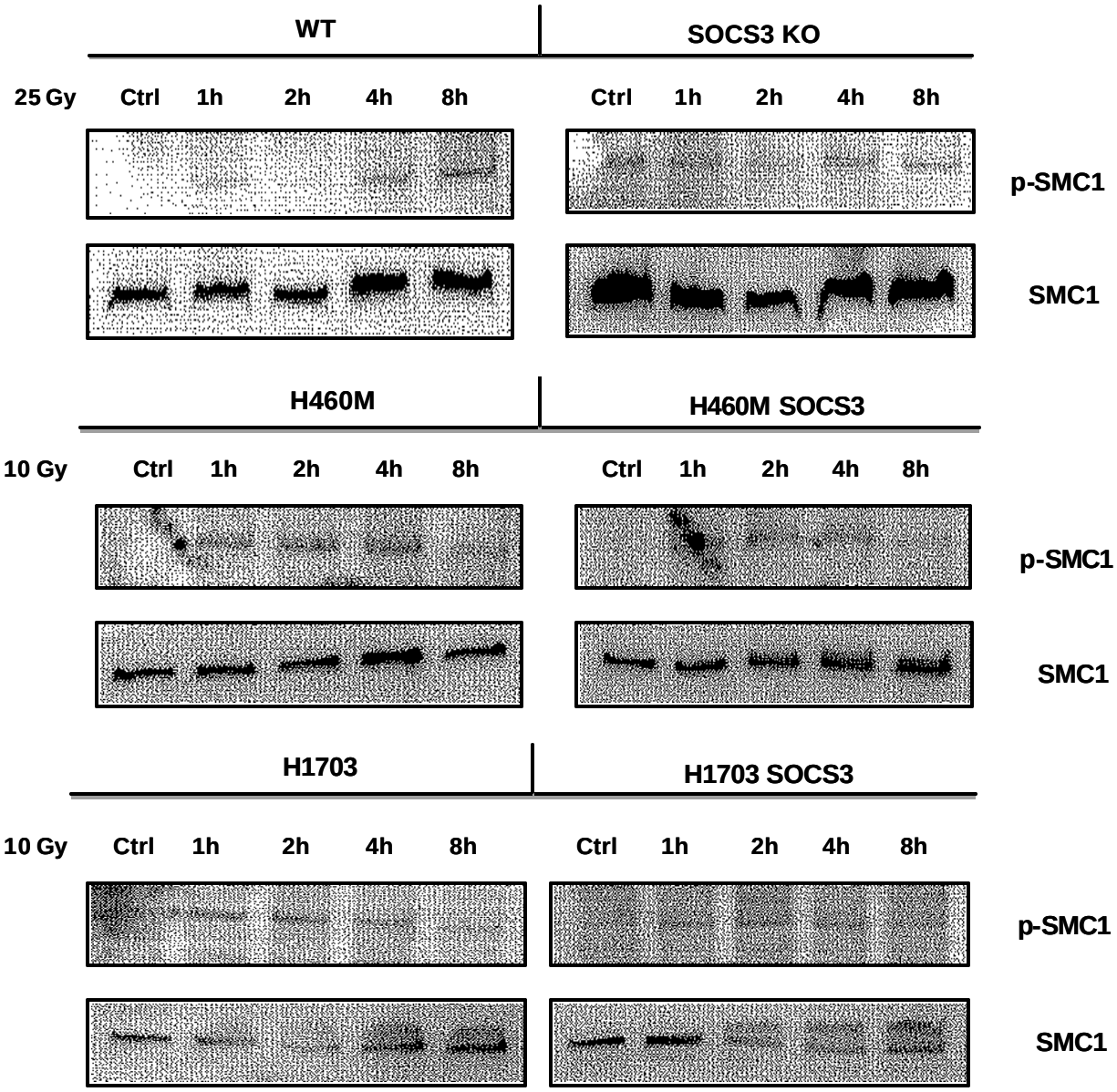


Figure 12. Immunoblot analysis of whole cell lysates from MEFs and NSCLC cell lines showed that SMC1 phosphorylation is not affected by SOCS3 expression following IR.

2.2 Discussion

Our laboratory has identified a novel and unexpected function for SOCS3 in the DNA damage response, that SOCS3 has a radioprotective effect in MEFs and human solid tumors. We have previously demonstrated that SOCS3-deficient cells failed to undergo G1 cell cycle arrest in

response to radiation, likely due to uncontrolled STAT3 signaling in irradiated cells, which results in repression of p21 gene expression. The STAT3 transcription factor is an oncogenic signaling protein/transcription factor that can drive cellular proliferation and promote cell survival in cancer. One component of STAT3-mediated oncogenesis is its ability to disrupt the expression of cell cycle inhibitory proteins. The p21 (Cip-1/Waf1) promoter has three STAT binding sites which are capable of recruiting STAT3. Recently, it was demonstrated that STAT3 represses p21 expression by directly binding the p21 promoter, but failing to recruit the histone acetylase CBP300 and RNA polymerase, suggesting that STAT3 can act as direct transcriptional inhibitor of p21. STAT3 may also negatively regulate p21 by indirect mechanisms as well. One STAT3-dependent gene that can repress p21 expression is the BCL6 proto-oncogene. BCL6 is generally associated with germinal center B cells and functions to suppress p21-mediated cell cycle arrest in response to the DNA damage that occurs during immunoglobulin gene rearrangement. However, there are reports of aberrant BCL6 gene activation in solid tumors, such as breast cancer, and may play a role in disease pathogenesis (Logarajah S, 2003; Pinto AE, 2009). BCL6 is of particular interest in our system because STAT3 is known to bind the BCL6 silencer region and activate its transcription. Our finding that STAT3 phosphorylation is uncontrolled in SOCS3KO MEFS suggests that BCL6 may be aberrantly triggered in cells lacking the SOCS3 inhibitory pathway. Indeed, our cDNA microarray analysis of MEFs has identified BCL6 as an overrepresented gene in SOCS3 KO MEFs. We analyzed BCL6 expression in WT and SOCS3 KO MEFs and found that both mRNA and protein expression of BCL6 are up-regulated in SOCS3 KO MEFs following IR. In addition, we also investigated expression of the proto-oncogene c-Myc, another target gene of STAT3 which function as a transcriptional repressor of p21. We identified that the protein expression level of c-Myc is

elevated in SOCS3 KO MEFs. Importantly, we revealed an inverse relationship between SOCS3 and BCL6/c-Myc mRNA expression levels in human NSCLC cell lines, indicating that human tumors that silence SOCS3 may also have a defect in radiation-induced p21 expression and display enhanced sensitivity to IR. Overall, our data indicated that one of the molecular mechanisms by which SOCS3 promotes p21 induction in DNA damage response following IR is through inhibition of BCL6 and c-Myc expression.

We also determined that SOCS3-deficient MEFs undergo normal S-phase checkpoint by RDS assay. Immunoblot analysis showed degradation of CDC25A and phosphorylation of SMC1 in MEFs and NSCLC cells following ionizing radiation, indicating that S-phase checkpoint is not affected by SOCS3.

3. Conclusion

Our data suggest a novel mechanism through which SOCS3 exerts its radioprotective effect. SOCS3 regulates the DNA damage response by promoting radiation-induced p21 expression and controlling STAT3-mediated proliferation. We have identified that the transcriptional repressor BCL6 is targeted by SOCS3/STAT3 and this novel axis is responsible for specific control of the G1-S transition, as SOCS3-deficient cells display normal S-phase checkpoint.

IV. *In vitro* studies on SOCS3 regulating IGF-1R and RON signaling in MEFs and human PDA cell lines

1. Materials and Methods

1.1 Materials

Cell lines and cell culture. WT and SOCS3 KO MEFs, human PDA cell lines AsPC1, AsPC1 RON shRNA, AsPC1 SOCS3, FG, FG RON shRNA, FG SOCS3, PL12 and PL12 SOCS3 were used in this study. All the cell lines were maintained as described above.

1.2 Methods

Western blot. MEFs and human cancer cell lines were seeded in 10 cm dishes (Cat. #430167, Corning) or 6-well plates (Cat. #353046, Falcon, Corning Incorporated) and starved in media with 0.2% FBS overnight. On the next morning, cells were stimulated with 100 ng/ml of IGF-1 for 15 minutes, 30 minutes, 1 hour, 2 hours and 4 hours. Whole cell lysates were prepared as described above. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes, blotted and detected by the chemiluminescent substrate WesternDura. The antibodies used in this study were: anti-phospho-ERK, anti-ERK, anti-phospho-Akt, anti-Akt.

Real-time cell analysis (RTCA). In order to determine the role of SOCS3 in IGF-1 and MSP induced cell proliferation in PDA cell lines, we performed RTCA of parental and SOCS3+ PDA cells using the impendance-based label-free real-time xCELLigence Dual Plate (RTCA DP) system (Acea and Cambridge Biosciences). This system is a highly flexible, low throughput instrument for the monitoring of cell migration and invasion in particular, as well as other cellular processes, such as cell proliferation, cytotoxicity, adhesion, viability, functional

signaling and modulation of barrier function in real time without the incorporation of labels or reporters. This instrument analyzes cell functions in real time using non-destructive, reagent free impedance measurements. Real time monitoring detects short term events which endpoint assays can easily miss. The instrument uses impedance measurements to monitor cell growth or death and morphology changes using 5000 to 60000 cells per well, depending on the cell line.

Clonogenic survival assay. Cells were harvested using 0.25% Trypsin-EDTA and irradiated with X-ray at 0, 0.5, 1, 2, 4, 6 and/or 8 Gy, then plated in 10 cm dishes and incubated at 37°C in 6% CO_2. The cells were left to grow for about two weeks and surviving colonies were stained with 0.05% w/v crystal violet/methanol for 30 minutes and counted to generate survival fraction data at each dose of radiation.

2. Results and Discussion

2.1 Results

SOCS3 inhibits phosphorylation of ERK and Akt upon IGF-1 stimulation in MEFs and PDA cell lines

The effects of SOCS3 on the DNA damage response are a novel function for this molecule and represent a general regulatory axis that involves fundamental components of both the signal transduction machinery (STAT3) and the radiation response (p21). However, SOCS3 has been shown to behave as a tumor suppressor in some solid tumors and we wished to determine whether SOCS3 could induce radiation sensitivity if it is targeting specific radioprotective kinase pathways in tumor cells. In other words, in the context of tumors displaying SOCS3-sensitive kinase pathway activation, which function of SOCS3 would be dominant: kinase-specific inhibition and radiosensitivity or radioprotection?

To answer this question, we chose to express SOCS3 in human pancreatic cancer cell lines that display activation of SOCS3-sensitive receptor kinases, the insulin-like growth factor receptor IGF-1R and the RON receptor tyrokine kinase. Using this system, we have extended our understanding of SOCS3 in the DNA damage response and have uncovered important radiation-independent functions of SOCS3 in aggressive, metastatic solid tumors.

IGF-1 is one of the most potent natural activators of the Akt signaling pathway. Besides, IGF-1 receptor activation is also coupled to the stimulation of a family of MAP kinases, including extracellular-signal-regulated kinase ERK-1 and -2, Jun kinase (JNK)-1 and -2 and p38 MAP kinase (Reviewed by Luigi Laviola, 2007).

The RON receptor tyrosine kinase has been found to overexpress in the majority of pancreatic cancers. In pancreatic cells, RON is an important K-Ras effector and RON ligand can enhance migration/invasion and apoptotic resistance (Jocelyn Logan-Collins, 2010). The ligand for RON is hepatocyte growth factor-like (HGFL) protein and is also known as macrophage stimulating protein (MSP). Binding of MSP to RON activates RON and leads to the induction of a variety of intracellular signaling cascades including PI3K/Akt and ERK MAPK pathways (Thomas R, 2007; Camp ER, 2007).

Recently, IGF-1R has been identified as a novel RON interactant in pancreatic cancer cell lines, and confirmed to play an essential role in pancreatic cancer cell migration induced by insulin-like growth factor 1 (IGF-1). In particular, IGF-1 induces rapid phosphorylation of RON, but RON signaling did not activate IGF-1R indicating unidirectional signaling between these RTKs (Dawn V. Jaquish, 2011). It has been reported that in pancreatic cancer cells, STAT3 is activated in response to IGF-1, in a RON-dependent manner, while in contrast, IGF-1R activates Akt independent of RON (D.V. Jaquish, 2011). RON signaling has also been identified to regulate

the expression of STAT3 in pancreatic tumor xenografts (Jocelyn Logan-Collins, 2011) and other tumor types (Iwama A, 1996).

Immunoprecipitation studies have revealed that SOCS3 associated with IGF-1R upon IGF-1 stimulation, indicating its inhibitory role on IGF-1 pathway upon association with the receptor (Dey BR, 2000). Of great interest to our laboratory is the role of SOCS3 in regulating IGF-1R and RON signaling in human pancreatic ductal adenocarcinoma (PDA) cell lines (illustrated in Figure 13 a).

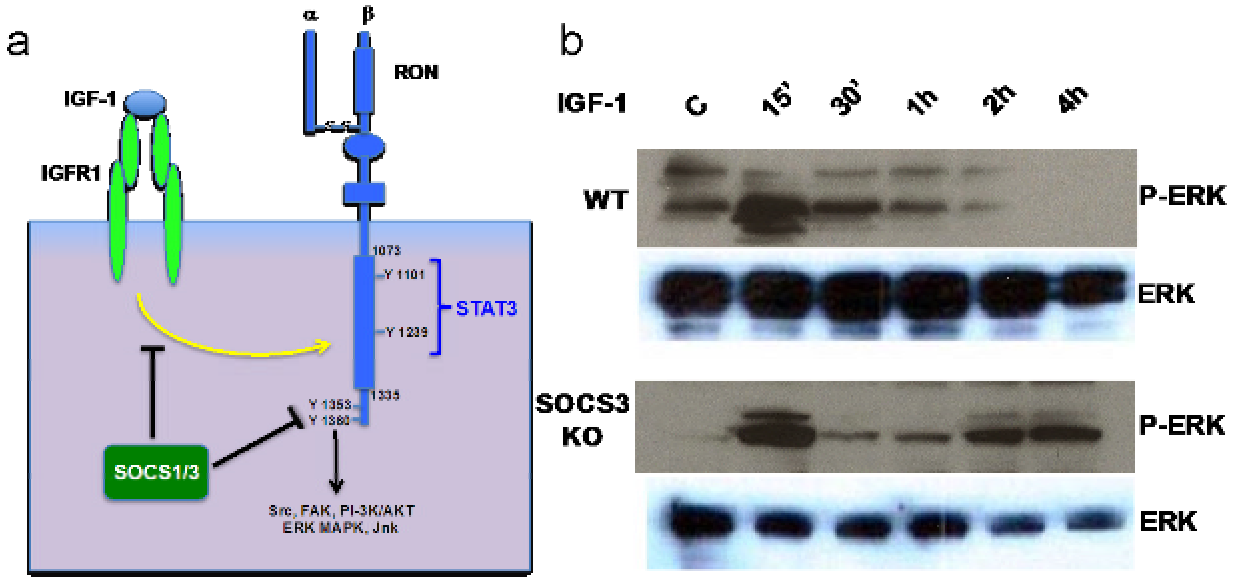


Figure 13. SOCS3 inhibited downstream signaling pathways mediated by IGF-1R or IGF-1R through RON. (a) SOCS3 associated with IGF-1R upon IGF-1 stimulation, indicating its inhibitory role on IGF-1 pathway. The IGF-1R/RON-signaling axis in pancreatic cancer cells involves activation of STAT3, which is inhibited by SOCS3. (b) IGF-1 signaling is enhanced in SOCS3-deficient fibroblasts. MEFs from WT and SOCS3 KO mice were stimulated with 100 ng/ml IGF-1 for the indicated time points and protein extracts were analyzed by western blotting for phosphorylated ERK MAP kinase. SOCS3-deficient fibroblasts display extended kinetics of ERK phosphorylation in response to IGF-1 relative to WT MEFs.

We first investigated the phosphorylation of ERK in response to IGF-1 stimulation in WT and SOCS3 KO MEFs. As shown in Figure 13 (b), we found that SOCS3-deficient fibroblasts displayed extended kinetics of ERK phosphorylation in response to IGF-1 relative to WT MEFs, indicating an inhibitory role of SOCS3 on IGF-1 signaling.

Next, we extended to examine the activation of downstream signaling pathways upon IGF-1 stimulation in human PDA cell lines. As shown in Figure 14, we found that overexpression of SOCS3 in PDA cell line ASPC1 resulted in suppressed phosphorylation of Akt in response to IGF-1, while the parental cell line AsPC1 displayed enhanced level and extended kinetics of Akt and ERK phosphorylation.

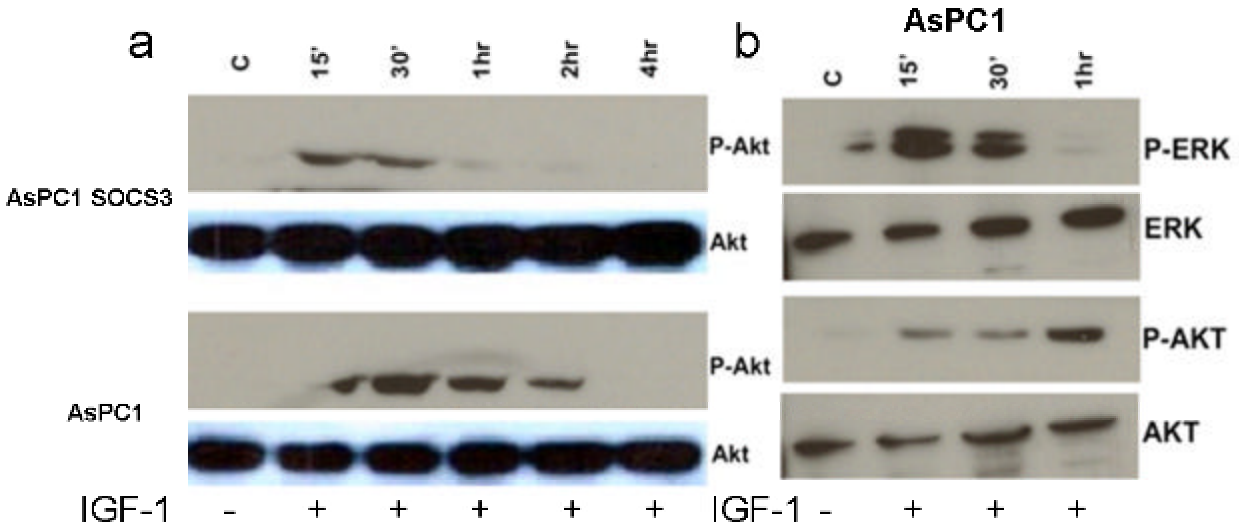


Figure 14. Overexpression of SOCS3 in PDA cell line AsPC1 inhibited IGF-1 signaling. (a) Immunoblot analysis of whole cell lysates from PDA cell lines showed that phosphorylation of Akt upon IGF-1 stimulation was suppressed in AsPC1 overexpressing SOCS3 compared to the parental cell line. (b) AsPC1 displayed extended kinetics of ERK and Akt phosphorylation in response to IGF-1 stimulation.

Overexpression of SOCS3 in human PDA cell lines inhibits IGF-1 and MSP induced cell proliferation

It has been reported that following IGF-1 stimulation, human coronary artery smooth muscle cells (hCASMCs) overexpressing SOCS3 were susceptible to a higher degree of apoptosis and became resistant to IGF-1 induced proliferation (Kriti Rakesh, 2006). We further identified the effect of overexpressing SOCS3 in IGF-1 and/or MSP induced cell proliferation in PDA cell lines by performing real-time cell analysis (RTCA). RTCA was performed by impedance

measuring, which is expressed as the cell index (CI), a parameter of cell viability (Acea and Cambridge Biosciences). The cell growth curves were automatically recorded on the xCELLigence System in real time (Figure 15).

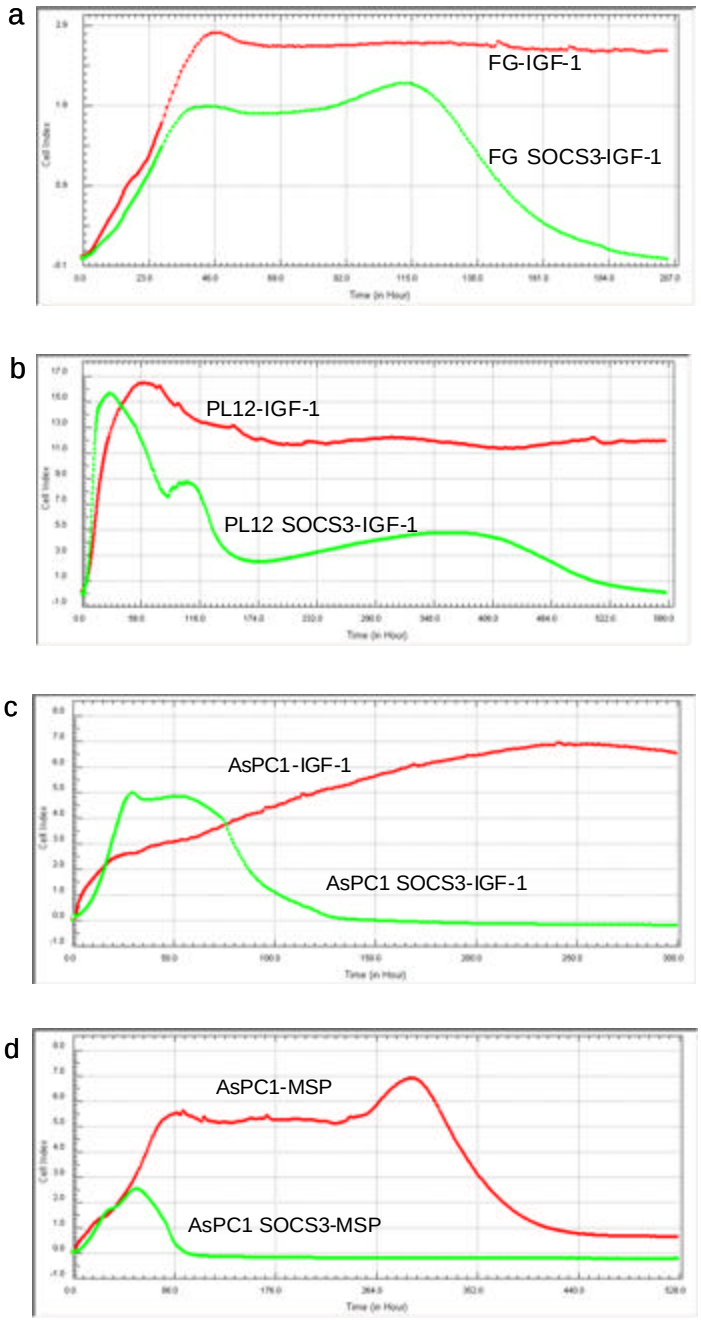


Figure 15. Overexpression of SOCS3 in PDA cell lines inhibited IGF-1 and MSP induced cell growth. (a), (b) and (c) Overexpression of SOCS3 in FG, PL12 and AsPC1 inhibited IGF-1 induced cell growth. (d) Overexpression of SOCS3 in AsPC1 inhibited MSP induced cell growth.

As shown in Figure 15 (a), (b) and (c), we observed inhibited cell growth in response to IGF-1 with overexpression of SOCS3 in PDA cell lines FG, PL12 and AsPC1. Importantly, we identified that overexpression of SOCS3 in AsPC1 also inhibited MSP induced cell proliferation as shown in Figure 15 (d), indicating that SOCS3 can negatively regulate both IGF-1R and RON signaling in PDA cell lines. Both IGF1-R and RON-activated pathways have been shown to be radioprotective. Thus, we wanted to determine the effects of receptor inhibition on the radiation response by genetic ablation via shRNA knockdown or through biological targeting of the receptors via SOCS3 overexpression.

Silencing of RON by shRNA-mediated suppression sensitizes PDA cell lines to IR, while overexpression of SOCS3 which blocks RON signaling protects PDA cell lines from IR

It has been reported that small hairpin ribosomal nucleic acid (shRNA)-mediated silencing of RON in pancreatic cancer xenografts inhibited their growth, primarily by increasing susceptibility to apoptosis and by sensitizing them to Gemcitabine treatment (Jocelyn Logan-Collins, 2010). We further examined the effect of silencing RON by shRNA-mediated suppression on radiosensitivity in PDA cell lines.

We performed clonogenic survival assays using FG, FG RON shRNA and AsPC1, AsPC1 RON shRNA. As shown in the upper panels of Figure 16, we found that silencing of RON by shRNA-mediated suppression sensitized the PDA cell lines to ionizing radiation, suggesting that RON signaling pathway is radioprotective in PDA cells.

Meanwhile, we have also identified the radioprotective effect of SOCS3 in PDA cell lines as shown in the lower panels of Figure 16, indicating that although SOCS3 can target a specific

radioprotective pathway, its ability to be a radioprotector through the STAT3/BCL6 signaling overrides the negative effects on RON signaling in PDA.

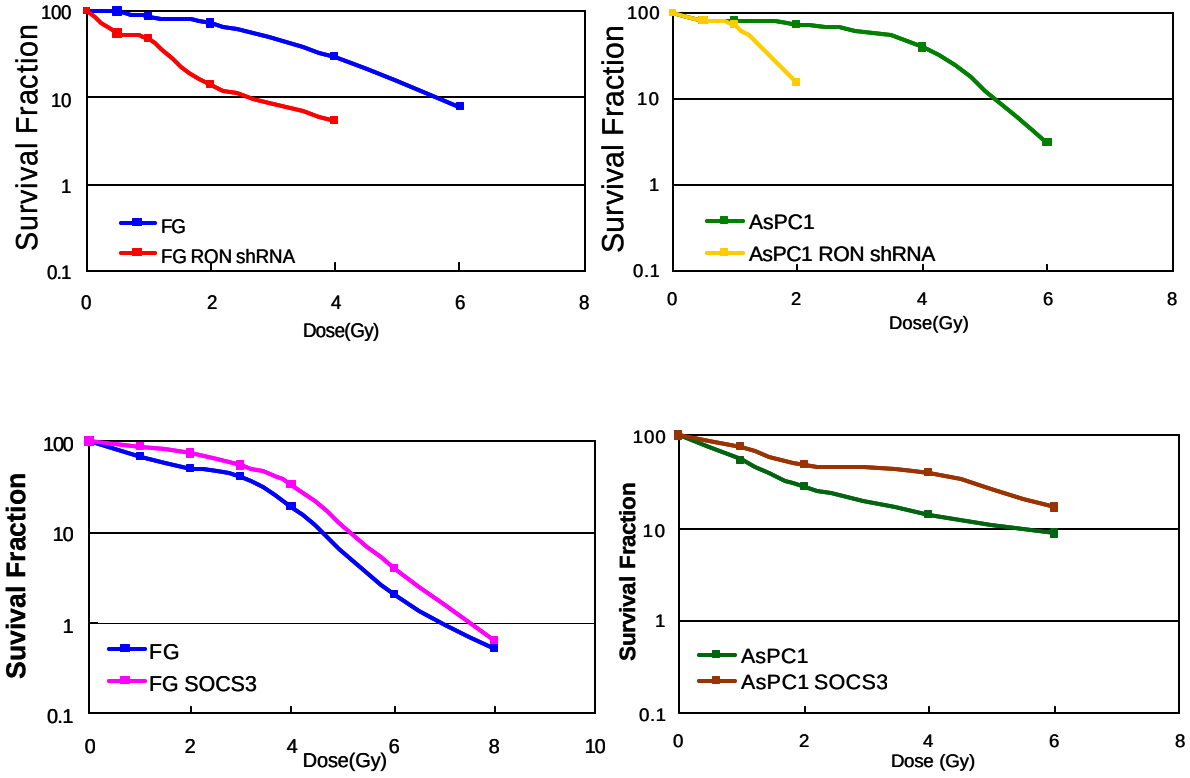


Figure 16. *In vitro* clonogenic survival assay showed that silencing of RON by shRNA-mediated suppression radiosensitized PDA cell lines while SOCS3 protected PDA cell lines from IR.
Upper Panels. Silencing of RON by shRNA-mediated suppression in PDA cell lines increased radiosensitivity.
Lower Panels. Overexpression of SOCS3 protected PDA cell lines from IR.

2.2 Discussion

In Section III, we have identified a novel mechanism by which SOCS3 regulates the DNA damage response. Initially thought to be a tumor suppressor and possibly a radiosensitizer, we have shown that SOCS3 is actually a radioprotector due to its control of the STAT3-BCL6 axis in the DNA damage response. This is a generalized effect of SOCS3 on STAT3 activation.

However, one question is whether SOCS3 can act as a tumor suppressor and radiosensitizer if it is targeting a specific kinase pathway that is known to be active in a tumor and can radioprotect. In order to explore this possibility, we next choose to express SOCS3 in human pancreatic cancer cell lines that display activation of SOCS3-sensitive receptor kinases, IGF-1R and RON.

While RON overexpression appears to be a hallmark of many human cancers, the mechanisms by which RON induces tumorigenesis and metastasis are still unclear. Recent studies have identified several RON protein interactants in pancreatic cancer cells and demonstrated receptor cross talk (Follenzi,A, 2000; Peace,B.E, 2003). The insulin-like growth factor receptor (IGF-1R) has been identified and confirmed to interact with RON in pancreatic cancer cell lines. And it has been demonstrated that the IGF-1R/RON-signaling axis in pancreatic cancer cells involves activation of STAT3 (D.V. Jaquish, 2011). Both the RON and IGF-1R receptors represent attractive potential targets for pancreatic cancer therapy as they are commonly overexpressed and mediate oncogenic phenotypes in pancreatic cancer cells.

Both IGF-1 and MSP act as potent activators of the PI3K/Akt and ERK MAPK signaling pathways in pancreatic cancer. It has been previously reported that STAT3 is activated in response to IGF-1 (Kriti Rakesh, 2006), and immunoprecipitation studies have revealed that SOCS3 associated with IGF-1R upon IGF-1 stimulation, indicating its inhibitory role on IGF-1 pathway upon association with the receptor (Dey BR, 2000).

We have identified the inhibitory function of SOCS3 on IGF-1 signaling in MEFs and PDA cell lines. We found that SOCS3-deficient MEFs displayed increased level and extended kinetics of ERK phosphorylation in response to IGF-1 stimulation relative to WT MEFs. Overexpression of SOCS3 in PDA cell line AsPC1 resulted in suppressed phosphorylation of Akt compared with the parental cell line. Additionally, we revealed that overexpression of SOCS3 inhibited IGF-1

induced cell proliferation in PDA cell lines FG, PL12 and AsPC1. Importantly, we further confirmed the inhibitory effect of SOCS3 on PDA cell proliferation in response to the RON ligand MSP. Our finding that overexpression of SOCS3 inhibited IGF-1 and MSP induced cell proliferation suggested SOCS3 as a negative regulator of both IGF-1R and RON signaling in PDA cell lines. While this data indicated SOCS3 may function as a tumor suppressor in human PDA, we identified a discrepancy in radiation responses of PDA cell lines. We have previously identified that PDA cell lines overexpressing SOCS3 which display reduced responses to MSP are more radioresistant than the parental cell lines, indicating a radioprotective role of SOCS3 in PDA cell lines, while silencing of RON by shRNA-mediated suppression increased radiosensitivity in PDA cell lines. Our data suggest that SOCS3 can target a specific radioprotective kinase pathway but its ability to be a radioprotector through the STAT3/BCL6 pathway is more important. shRNA of RON is a radiosensitizer but SOCS3, which blocks RON responses, is still a radioprotector in PDA cells. In the next sections, we describe a novel, radiation-independent role for SOCS3 in the regulation of chemokine expression and tumor aggressiveness in pancreatic cancer.

3. Conclusion

Our data reveal that SOCS3 negatively regulate IGF-1R and RON signaling in MEFs and PDA cell lines. Overexpression of SOCS3 in PDA cell lines inhibits IGF-1 and MSP induced cell proliferation but increases cell resistance to ionizing radiation, while PDA cell lines that silence RON by shRNA-mediated suppression are more radiosensitive relative to the parental cell lines. Our finding indicates that the radioprotective effect of SOCS3 supercedes its ability to block specific radioprotective kinase pathways in pancreatic cancer cells.

V. Analysis of SOCS3 expression and STAT3 activation in MEFs and human cancer cell lines

1. Materials and Methods

1.1 Materials

Cell lines and cell culture. WT and SOCS3 KO MEFs, human PDA cell lines AsPC1, AsPC1 SOCS3, BxPC3, PL12, 2.8, 2.13, 4.14, FG as well as NSCLC cell lines H1703, H1703 SOCS3, H460M and H460M SOCS3 were used in this study. All the cell lines were maintained as described above.

1.2 Methods

Reverse Transcriptase-PCR (RT-PCR). Human cancer cell lines were starved in 0.2% serum media overnight and stimulated with 20 ng/ml of IL-6 or one of the IL-6 family of cytokines, the oncostatin M (OSM) for 1 hour. Total RNA was isolated by using TRIzol Reagent (Cat. #15596-018, Invitrogen) or RNeasy Mini Kit (Cat. #74104, Qiagen) or PureLink RNA Mini Kit (Cat. #12183020, life technologies). Quantification of the total RNA was performed by using Nanodrop ND-1000 Spectrophotometer (UCLA Gonda Genomics Core). The first-strand cDNA was made by SuperScript Reverse Transcriptase III (Cat. #18080-400, Invitrogen) and used as templates for PCR to detect target genes using the Taq DNA polymerase (Cat. #M0273L, New England Biolabs) and Deoxynucleotide Solution Mix (Cat. #N0447S, New England Biolabs). Reactions products were analyzed on a 2% agarose gel (Cat. #A9539-500G, SIGMA) with 0.5 mg/l of Ethidium Bromide Solution (Cat. No. 15585-011, Invitrogen). The electrophoresis gel was analyzed using the FUJI LAS3000 Digital Imaging System (Dr. Nishimura's lab of UCLA

Dental School). Primers were ordered from Invitrogen and reconstituted in sterile water to provide a 0.2 mM working concentration. Primers were designed with the Invitrogen OligoPerfect Designer to amplify a 150-200 base pair internal sequence for each target. The primer sequences used in this study are as follows: SOCS3 fwd 5'-CTC AAG ACC TTC AGC TCC AA-3'; SOCS3 rev 5'-TTC TCA TAG GAG TCC AGG TG-3'; human β-Actin fwd 5'-GGA CTT CGA GCA AGA GAT GG-3'; β-Actin rev 5'-AGC ACT GTG TTGGCGTACAG-3'.

Methylation-specific PCR (MSP). Genomic DNA was obtained from the PDA cell lines. MSP was applied to investigate the methylation status of the promoter regions of the SOCS3 gene. After an initial bisulfite treatment to modify the DNA, PCR was performed to distinguish methylated from unmethylated DNA as described (Herman JG, 1996). Briefly, 2 µg of genomic DNA were denatured with 0.3 M NaOH. In all, 10mM hydroquinone and 3M sodium bisulfite were added and incubated at 50°C for 16 hours. Afterwards, modified DNA was purified using the Wizard DNA purification resin (Qiagen, Hilden, Germany) followed by desulfonating in 0.3 M NaOH and subsequent ethanol precipitation and resuspension in 30-50 µl water. MSP was performed using specific primers and conditions previously described (Herman JG, 1996). Briefly, a 20 µl reaction volume containing 150 ng bisulfite modified DNA, 1×PCR buffer, 1.5 mM $MgCl_2$, 0.16 µM dNTPs, 0.25 µM specific primer mix and 1 unit Taq enzyme was used. PCR was carried out by an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 40 seconds, annealing at 63°C (methylation) or 62°C (unmethylation) for 40 seconds, and elongation at 72°C for 40 seconds. Cycling was completed by a final elongation step at 72°C for 10 minutes. The PCR products were applied for electrophoresis on a 2% agarose gel, stained with Ethidium Bromide and visualized under UV

illumination. Placental DNA treated with methyltransferase was used as a positive control for methylation.

The methylation-specific and unmethylation-specific primers were designed using the Methyl Primer Express software v1.0 (ABI) according to a previously published protocol and adopted to the specific conditions of PDA (B He, 2003; A Weber, 2005; S Zhang, 2008). Sequences of methylation-specific primers of SOCS3 were 5'-TAT ATA TTC GCG AGC GCG GTT T-3' (forward) and 5'-CGC TGC GCC CAG ATG TT-3' (reverse); sequences of unmethylation-specific primers were 5'-TGT GGT GGT TGT TTA TAT ATT TGT GAG TGT GGT T-3' (forward) and 5'-CAA CCA ACA ATA ACC CAC ACT ACA CCC A-3' (reverse). Sequences of methylation-specific primers in the exon 2 of SOCS3 were 5'-TTC GAG GTG TTC GAG TAG TC-3' (forward) and 5'- AAC GAT CTT CCG ACA AAA AT-3' (reverse); sequences of unmethylation-specific primers were 5'-TTT TTT GAG GTG TTT GAG TAG TT-3' (forward) and 5'-AAC AAT CTT CCA ACA AAA ATA CT-3' (reverse), and the lengths of PCR products were both 159 bp (S Zhang, 2008). Sequences of methylation-specific primers of SOCS3 were 5'-GTA GTG CGT AAG TTG TAG GAG AGC-3' (forward) and 5'-GTA AAA AAA TAA CGC TAA TCC GAA-3' (reverse); sequences of unmethylation-specific primers were 5'-TAG TGT GTA AGT TGT AGG AGA GTG G-3' (forward) and 5'-GTA AAA AAA TAA CGC TAA TCC GAA-3' (reverse). The lengths of PCR products were 139 bp and 134 bp respectively (A Weber, 2005).

Western blot. MEFs and human cancer cell lines were seeded in 10 cm dishes (Cat. #430167, Corning) or 6-well plates (Cat. #353046, Falcon, Corning Incorporated) and starved in media with 0.2% FBS overnight. On the next morning, cells were stimulated with 20 ng/ml of IL-6 or HGF or OSM for 30 minutes, 1 hour, 2 hours and 4 hours. Whole cell lysates were prepared as

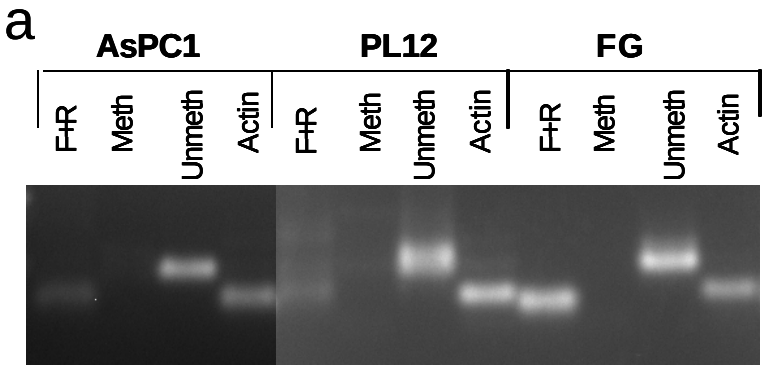
described above. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes, blotted with antibodies and detected as described above. The antibodies used in this study were: p-STAT3 (Y705) (3E2) Mouse mAb (Cat. #9138S, Cell Signaling Technology), STAT3 (124H6) Mouse mAb (Cat. #9139S, Cell Signaling Technology), p-STAT1 (Y701) (58D6) Rabbit mAb (Cat. #9167S, Cell Signaling Technology), STAT1 Rabbit Ab (Cat. #9172S, Cell Signaling Technology).

2. Results and Discussion

2.1 Results

Pancreatic ductal adenocarcinoma (PDA) cell lines express various levels of SOCS3 in response to IL-6 or OSM

Lacking of SOCS3 expression in H1703 has been reported to be correlated with hypermethylation in CpG islands of the functional SOCS3 promoter by using methylation-specific PCR (MSP) (He B, 2007). Since SOCS3 expression defects in cancer are almost exclusively due to promoter methylation, we initially screened PDA cell lines for SOCS3 promoter methylation by MSP.



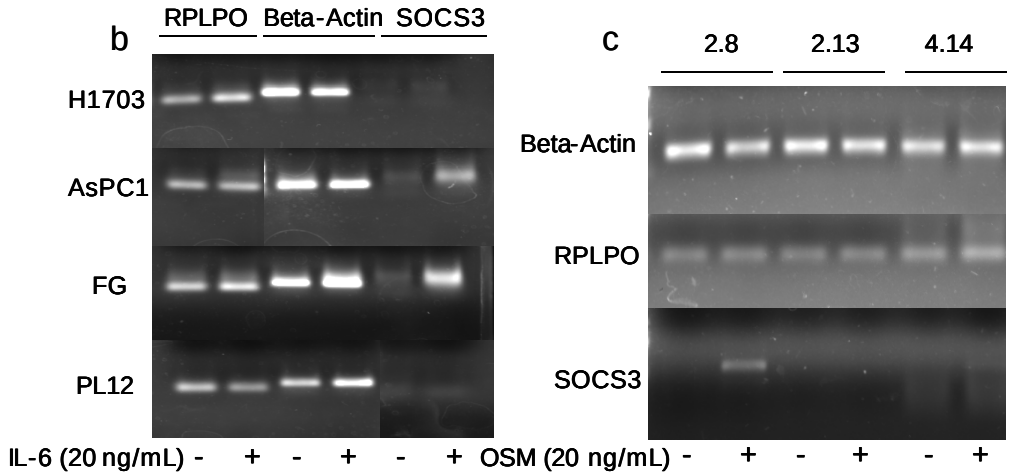


Figure 17. Analysis of SOCS3 promoter methylation and cytokine-induced expression of SOCS3 in PDA and NSCLC cell lines.
(a) Methylation-specific PCR (MSP) of genomic DNA from PDA cell lines AsPC1, PL12 and FG. Bands in lanes labeled "Unmeth" are unmethylated DNA product amplified with unmethylation-specific primers. Bands in lanes labeled "Meth" are methylated DNA product amplified with methylation-specific primers. (b) and (c) PDA cell lines AsPC1, FG, PL12, 2.8, 2.13, 4.14 and one NSCLC cell line H1703 were evaluated for SOCS3 expression upon 20 ng/mL of IL-6 or OSM stimulation at 1 hour.

Surprisingly, we did not identify significant SOCS3 promoter methylation in AsPC1, PL12 or FG by MSP, as shown in Figure 17 (a).

Next we screened the PDA cell lines for SOCS3 gene induction following stimulation with members of the IL-6 family of cytokines, which are known to activate SOCS3 expression in normal tissue. As shown in Figure 17 (b) and (c), IL-6 or OSM stimulation failed to induce SOCS3 expression in human NSCLC cell line H1703 in which SOCS3 expression is known to be silenced by promoter hypermethylation. For PDA cell lines, we found that SOCS3 expression in response to IL-6 or OSM is silenced in PL12, 2.13 as well as 4.14. While in FG, AsPC1 and 2.8, endogenous SOCS3 expression can be induced by IL-6 or OSM to different degrees.

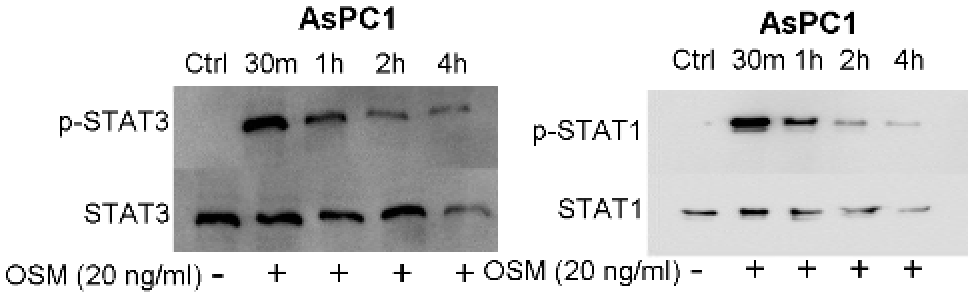
Our findings identified three distinct molecular subtypes of PDA cell lines based on expression of SOCS3 in response to IL-6/OSM stimulation. In the PDA cell line FG, endogenous expression of SOCS3 is high after stimulation with IL-6. While in AsPC1, we identified intermediate level

of SOCS3 induction in response to IL-6, suggesting partially repression. On contrast, in 2.8, PL12, 2.13, and 4.14, SOCS3 expression is low or totally repressed upon IL-6 or OSM stimulation. In particular, we report that SOCS3 is down-regulated through mechanisms other than promoter methylation in PDA cell lines.

Phosphorylation of STAT3 and STAT1 upon OSM stimulation is various in PDA cell lines

In previous studies, it was demonstrated that genetic silencing of SOCS3 results in abnormally high levels of STAT3 and STAT1 tyrosine phosphorylation (H. Yasukawa, 2003; R. Lang, 2003; B.A.Croker, 2003; Y. Lu, 2006). Mutations in the JAK/STAT signaling pathway have often been reported to be associated with cancer. For example, constitutive activation of STAT3 has been known to be correlated with cell proliferation in breast carcinoma (Zhang F, 2003) and non-small cell lung cancer (NSCLC) (B. He, 2003).

We have identified three molecular subtypes of human PDA cell lines represented by the cell lines FG (strong SOCS3 induction), AsPC1 (partially repressed), and PL12/2.8 (SOCS3 low or totally repressed). As shown in Figure 18, we have shown that for at least a subset of the cell lines with low induction or total repression of SOCS3, cytokine-induced STAT3 activation is prolonged (in the cell line PL12 with total SOCS3 repression), while STAT3 phosphorylation decays over 4 hours in the other two subtypes.



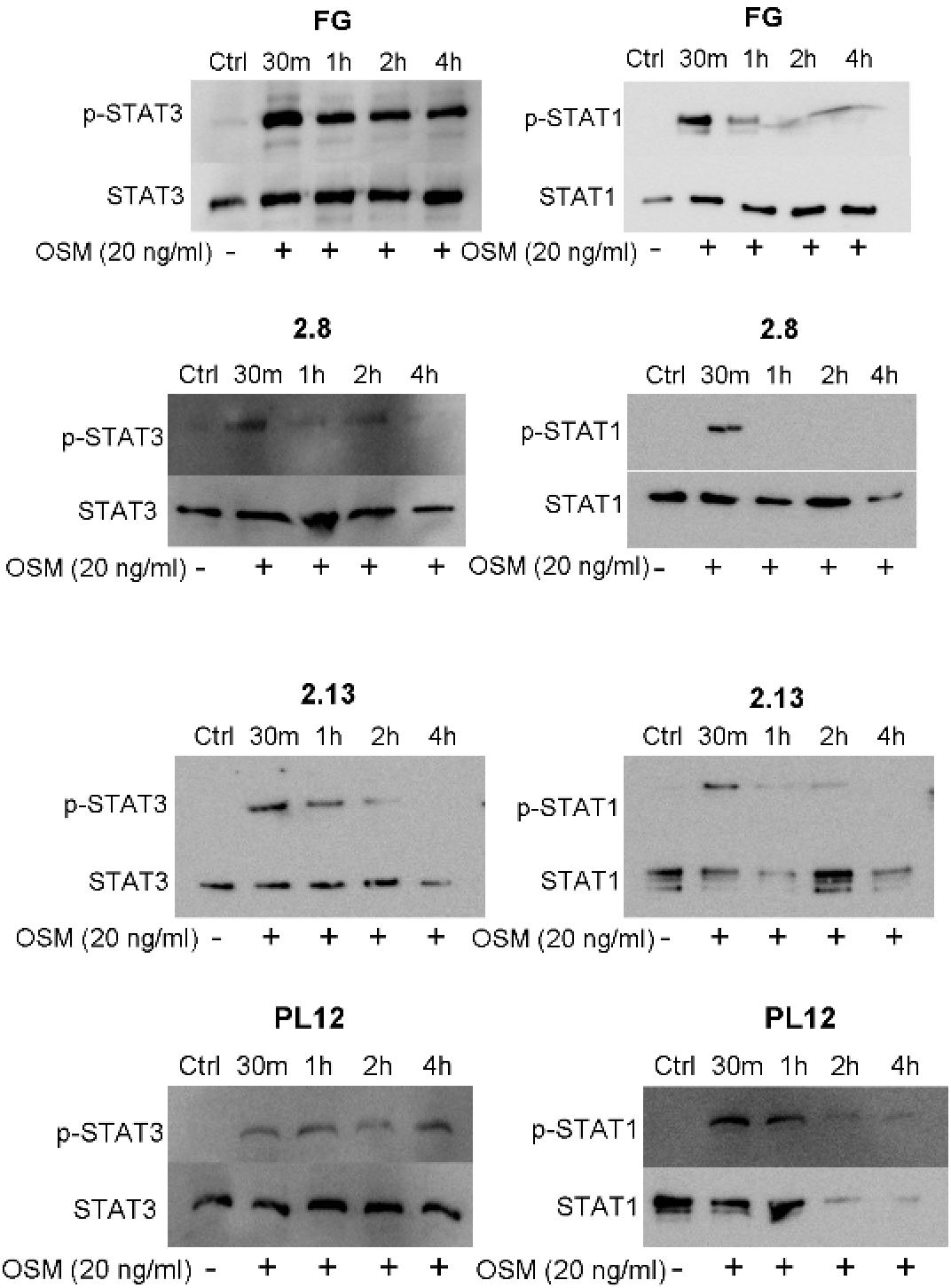


Figure 18. Immunoblot analysis of whole cell lysates from PDA cell lines showed various level of STAT3 and STAT1 phosphorylation upon OSM stimulation. PDA cells were plated in 6-well plates and starved in 0.2% serum media for overnight. Cells were stimulated with OSM at 20 ng/ml and lysed at different time points after stimulation. Whole cell lysates were analyzed using an anti-phospho-STAT3 Mouse mAb and anti-phospho-STAT1 Rabbit mAb.

We also investigated STAT1 phosphorylation upon OSM stimulation in PDA cell lines. As shown in Figure 18, we found that STAT1 was also rapidly phosphorylated in response to OSM stimulation, and its activation was down-regulated in all of the PDA cell lines. However, there are some differences among the cell lines, most notably that AsPC1 displayed a slightly more robust and prolonged phospho-STAT1 signal than the other cell lines.

Restoration of SOCS3 in NSCLC and PDA cells in which SOCS3 is repressed results in down-regulation of activated STAT3 and STAT1 upon OSM stimulation

In order to further address the role of SOCS3 in regulating activation of STAT3, we have also evaluated the phosphorylation of STAT3 in WT and SOCS3 KO MEFs. As shown in Figure 19, STAT3 was constitutively activated in unstimulated SOCS3 KO MEFs. Upon stimulation with IL-6 or HGF, we observed an up-regulation of STAT3 phosphorylation in SOCS3 KO MEFs.

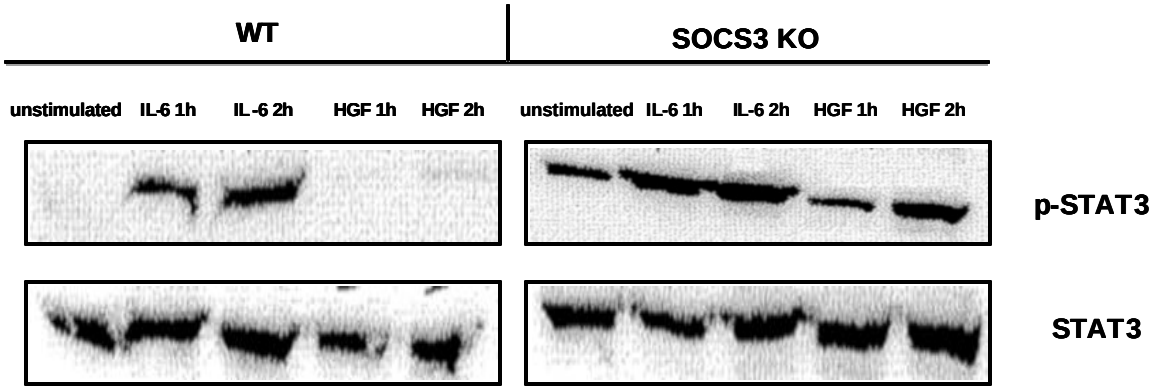


Figure 19. Immunoblot analysis of whole cell lysates from MEFs identified constitutively activated STAT3 in SOCS3-deficient fibroblasts. STAT3 phosphorylation was found to be constitutively activated and up-regulated in response to IL-6 and HGF in SOCS3 KO MEFs, while in WT MEFs, phosphorylation of STAT3 was not observed until stimulation with IL-6.

We further extended our research to human cancer cell lines. We found that restoration of SOCS3 in NSCLC cell lines H460M and H1703 in which SOCS3 expression is methylation-silenced suppressed phosphorylation of STAT3, as shown in Figure 20. We then continued to

inspect phosphorylation of STAT3 in human PDA cells. We chose AsPC1 for gain of function experiments by SOCS3 overexpression. As shown in Figure 20, restoration of SOCS3 in H1703 and AsPC1 resulted in down-regulation of STAT3 phosphorylation in response to OSM stimulation at 30 minutes. Our findings suggested that silencing of SOCS3 is one of the important mechanisms of constitutive activation of the JAK/STAT pathway in human solid tumors.

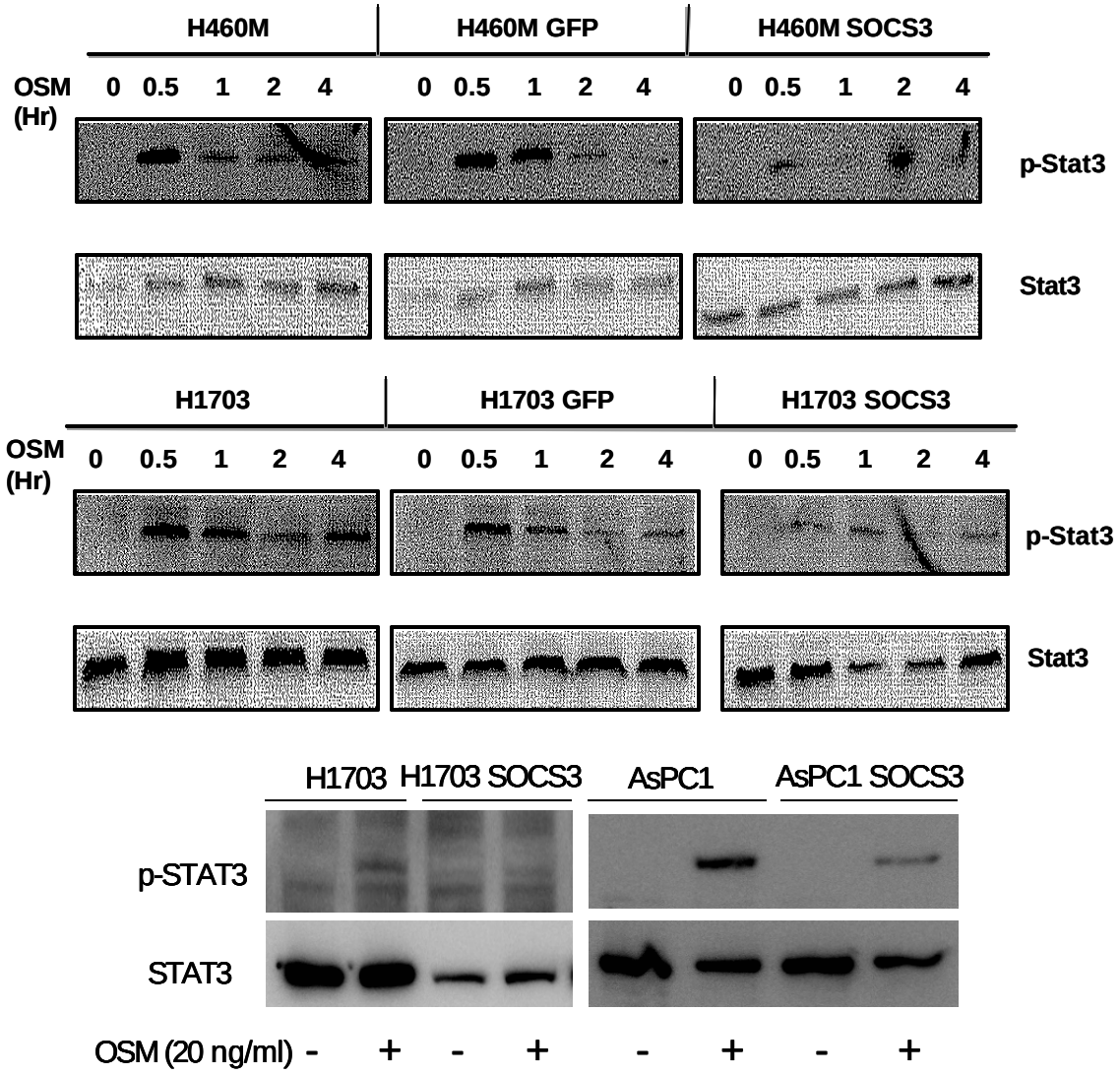


Figure 20. Immunoblot analysis of whole cell lysates from NSCLC and PDA cell lines showed that overexpression of SOCS3 inhibited STAT3 phosphorylation in response to OSM. Approximately 1×10^{7} cells were starved overnight, stimulated with 20 ng/ml of OSM, and protein extracts were analyzed by western blot with an anti-phospho-STAT3 antibody.

We also examined STAT1 phosphorylation upon OSM stimulation in human NSCLC cell lines. As shown in Figure 21, STAT1 was rapidly but transiently activated in response to OSM stimulation in human NSCLC cell lines, while overexpression of SOCS3 in NSCLC cell lines resulted in an even stronger suppression of STAT1 phosphorylation.

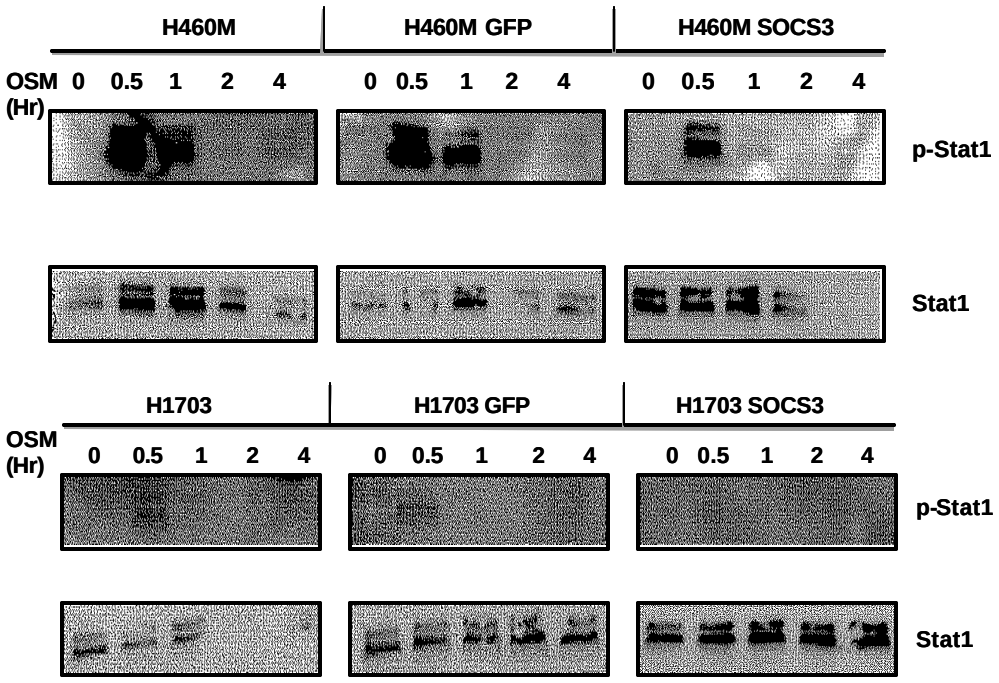


Figure 21. Immunoblot analysis of whole cell lysates from NSCLC cell lines upon OSM stimulation showed that STAT1 phosphorylation was suppressed by overexpression of SOCS3.

Phosphorylation of STAT3 is up-regulated in SOCS3 KO MEFs following IR

We next characterized the cells for signaling abnormalities in response to ionizing radiation. As shown in Figure 22, SOCS3 KO MEFs displayed constitutive STAT3 phosphorylation as well as enhanced and extended kinetics of STAT3 activation after exposure to ionizing radiation compared to WT MEFs.

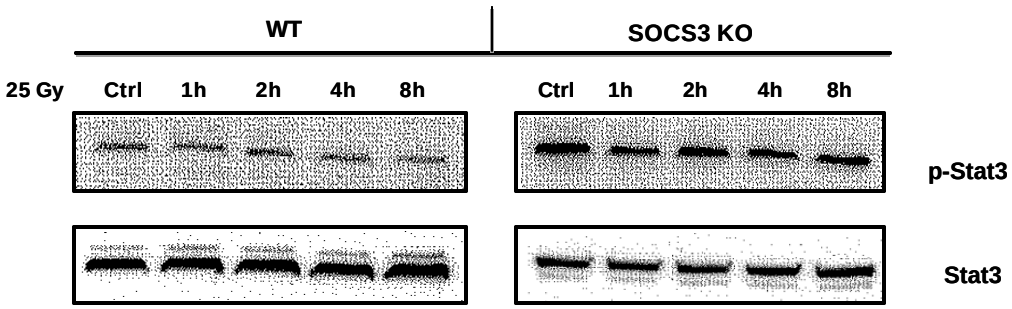


Figure 22. Immunoblot analysis of whole cell lysates from MEFs showed that phosphorylation of STAT3 was up-regulated in SOCS3-deficient fibroblasts following IR.

2.2 Discussion

In recent years, the Suppressors of Cytokine Signaling (SOCS) proteins have been widely studied for their role in the development of different cancers. SOCS3 has been demonstrated as a tumor suppressor gene in non-small cell lung cancer (He B, 2004). It has been reported that silencing of SOCS3 by promoter methylation is one of the important mechanisms of constitutive activation of the JAK/STAT pathway in cancer pathogenesis. Restoration of SOCS3 in lung cancer cells where SOCS3 was methylation-silenced resulted in the down-regulation of active STAT3, induction of apoptosis, and growth suppression (He B, 2003).

Initially, we screened a panel of pancreatic ductal adenocarcinoma (PDA) cell lines for defects in SOCS3 expression. Surprisingly, we found that none of the cell lines tested displayed any significant SOCS3 promoter CpG island methylation, despite the finding by our lab and others that the SOCS1 gene is often methylated and silenced in human PDA (data not shown). However, despite lack of SOCS3 promoter methylation, we indeed discovered a wide range of cytokine-inducible SOCS3 expression levels which allowed us to roughly group the cell lines into three categories, based on this molecular criteria: 1. High levels of cytokine-inducible SOCS3 expression, represented by the FG cell line; 2. Intermediate SOCS3 levels (partial SOCS3 repression), represented by AsPC1, and 3. Low or completely repressed SOCS3

induction, represented by the cell lines 2.8 and PL12. We also identified two other cell lines, 2.13 and 4.14 in which SOCS3 appears to be completely repressed following cytokine stimulation and therefore also fall into category 3. Perhaps the most surprising finding was that although promoter hypermethylation has been identified as the principal mechanism to silence SOCS3 expression and associated with the tumor suppressor effect of SOCS3 in solid tumors including lung cancer and head and neck squamous carcinoma, SOCS3 repression in PDA appeared to be independent of epigenetic regulation. There are very few cases of SOCS gene mutation or deletion in solid tumors, thus we may have identified a novel phenomenon in pancreatic cancer that leads to SOCS3 repression.

microRNAs (miRNAs) are small non-coding RNAs that bind to complementary sequences on target mRNAs, often silencing gene expression. miRNAs have emerged to play important roles in tumorigenesis and drug resistance.

microRNAs that target SOCS3	microRNAs overexpressed in pancreatic cancer
MiR-203	MiR-10a
miR-19a	MiR-10b
MiR-483-5a	MiR-21
miR-221	MiR-17-5p
miR-19	**MiR-203**
miR-30	MiR-222
miR-802	MiR-196a
Mirc1 (miR-17-92)	MiR-190
Mirc3 (miR-106b-25)	MiR-186

Table 1. Overexpression of miR-203 indicates a potential mechanism for loss of SOCS3 expression in human pancreatic cancer.

Importantly, several recent studies have reported that miRNAs may regulate JAK/STAT signaling via control of SOCS expression (Collins As, 2013; Ying C, 2014), indicating that possible mechanisms for SOCS3 repression may include overexpression of miRNAs that target

SOCS3. As listed in Table 1, miR-203 which targets SOCS3 has been reported to be overexpressed in a variety of human solid tumors including breast cancer (Peng Ru, 2011) and pancreatic cancer, and has been associated with poorer survival (Thomas G., 2010).

Next, we explored whether differential SOCS3 expression in PDA cell lines had consequences on cytokine-inducible STAT3 activation. The representative cell lines studied most in this work are FG, AsPC1 and PL12, and these cell lines indeed displayed unique STAT3 phosphorylation kinetics in response to stimulation with gp130-using cytokines. We found that FG and AsPC1 (subtypes 1 and 2), in which SOCS3 expression can be induced by OSM stimulation, inducible STAT3 phosphorylation decayed significantly over the 4 hour time course of the experiment. In contrast, the PDA cell line PL12 in which SOCS3 is silenced displayed extended kinetics of STAT3 phosphorylation. This finding is of great interest as it suggests that PDA tumors can be stratified on the basis of SOCS3 expression, which might reflect their degree of STAT3 dependence or "addiction".

There is variability within the groups, however, as we have also found that one of the cell lines displaying SOCS3 repression, 2.13 showed more "normal" phospho-STAT3 decay kinetics following cytokine stimulation, unlike the PL12 cell line. This may reflect involvement of other inhibitory mechanism(s) regulating the JAK/STAT pathway in this cell line.

Genetic silencing of SOCS3 results in abnormally high levels of both STAT3 and STAT1 tyrosine phosphorylation. Thus, we also examined phosphorylation of STAT1 in response to OSM in PDA cell lines. Although STAT1 is mainly activated by interferons (IFNs), we also identified activation of STAT1 upon OSM stimulation in the PDA cell lines. Our data indicate that, with some variability, STAT1 phosphorylation follows an induction and decay phase in all of the cell lines, suggesting that STAT1 signaling is not as dramatically affected by variability in

SOCS3 expression as STAT3. It is of note, however, that the AsPC1 cell line appears to display slightly more robust and extended STAT1 phosphorylation compared to the other cell lines, which may affect the regulation of chemokine expression and tumor aggressiveness (see below). We have also identified constitutively activated STAT3 even in unstimulated SOCS3-deficient fibroblasts. Overexpression of SOCS3 in NSCLC and PDA cell lines inhibited STAT3 and STAT1 phosphorylation upon OSM stimulation, confirming that silencing of SOCS3 is one of the important mechanisms of constitutive activation of the JAK/STAT pathway in human solid tumors. Importantly, we have shown that STAT3 phosphorylation is up-regulated in SOCS3 KO MEFs following IR compared to WT MEFs, which is in agreement with our previous studies that SOCS3 regulates DNA damage response by controlling STAT3 activation after exposure to IR. Overall, we found that there are a range of defects in SOCS3 expression among human PDA cell lines, with concomitant effects on STAT3 signaling. These results suggest that SOCS3 dysregulation may play a role in pancreatic cancer pathogenesis and that PDA tumors may display distinct biological behaviors based on how strongly they repress SOCS3 expression. We next attempted to answer this question using an orthotopic xenograft model of human PDA.

3. Conclusion

We have identified three distinct molecular subtypes of human PDA, in which cytokine-inducible SOCS3 is either highly expressed, or partially repressed, or severely repressed/totally silenced in response to IL-6 or OSM stimulation. Repression of SOCS3 gene in PDA is not CpG island methylation-dependent, suggesting a possible novel mechanism through which SOCS3 expression is silenced in PDA. We have shown that silencing of SOCS3 is one of the important mechanisms of constitutive activation of the JAK/STAT pathway in human solid tumors. The three molecular subtypes of PDA display distinct STAT3 signaling patterns in response to

cytokine stimulation and may indicate differential STAT3 dependence or "addiction" among these different tumor classes.

VI. cDNA microarray and gene expression analysis of MEFs and human cancer cell lines

1. Materials and Methods

1.1 Materials

Cell lines and cell culture. MEFs (WT and SOCS3 KO), human PDA cell lines AsPC1, AsPC1 SOCS3, BxPC3, BxPC3 SOCS3, FG, FG SOCS3 as well as NSCLC cell lines H460M, H460M SOCS3, H1703 and H1703 SOCS3 were used in this study. All the cell lines were maintained as described above.

1.2 Methods

Gene expression microarray. Total RNA was isolated from WT and SOCS3 KO MEFs by TRIzol Reagents (Invitrogen), quantificated by Nanodrop ND-1000 Spectrophotometer (UCLA Gonda Genomics Core) and used for gene expression microarray analysis. The MouseRef-8 v2.0 Expression BeadChip (Illumina) was prepared by UCLA DNA Microarray Center and the project data was analyzed using GenomeStudio software (Illumina).

Reverse Transcriptase-PCR (RT-PCR). Total RNA of MEFs and human cancer cell lines was isolated by using TRIzol Reagent. First strand cDNA was made as described above and used as template for PCR to detect expression of target genes. The sequences of primers used in this study were human USP18: 5'-GGG CAG TAT GAG CTT TTT GC-3' (forward); 5'-TAT GCA

GTT TCC TGC CAG TG-3' (reverse); human PARP14: 5'-ACC AGA TGC AAA TGG GAG AA-3' (forward); 5'-GGT TCT TTG AAG GAG GCA CA-3' (reverse); human CXCL8: 5'-GTG CAG TTT TGC CAA GGA GT-3' (forward); 5'-CTC TGC ACC CAG TTT TCC TT-3' (reverse); human NRP1: 5'-GAA AAA TGC GAA TGG CTG AT-3' (forward); 5'-AAT GGC CCT GAA GAC ACA AC-3' (reverse); human NRP2: 5'-ACA CCA AGT GAG AGG CCA AC-3' (forward); 5'-CGA TGT TCC CAC AGT GTT TG-3' (reverse); human TWIST1: 5'-GTC CGC AGT CTT ACG AGG AG-3' (forward); 5'-TGG AGG ACC TGG TAG AGG AA-3' (reverse); human SLUG: 5'-CTT TTT CTT GCC CTC ACT GC-3' (forward); 5'-ACA GCA GCC AGA TTC CTC AT-3' (reverse); human SNAI1 (snail): 5'-GCC TTC AAC TGC AAA TAC TGC-3' (forward); 5'-TGA CAT CTG AGT GGG TCT GG-3' (reverse); human Zeb1: 5'-GAG GAG GAG GAG GAG GAA GA-3' (forward); 5'-GCT TGA CTT TCA GCC CTG TC-3' (reverse); human NANOG: 5'-ACC TTC CAA TGT GGA GCA AC-3' (forward); 5'-GAA TTT GGC TGG AAC TGC AT-3' (reverse); human CD47: 5'-TAT TGC GGC GTG TAT ACC AA-3' (forward); 5'-GAT TGG AAG CCA CAA ATT TCA-3' (reverse); human CALR: 5'-AGG TCA AGT CTG GCA CCA TC-3' (forward); 5'-CCT CCT CTT TGC GTT TCT TG-3' (reverse); murine IGF2: 5'-GCT TGC CAA AGA GCT CAA AG-3' (forward); 5'-CTG ATG GTT GCT GGA CAT CT-3' (reverse); murine CXADR: 5'-AAC CAA GTC CCC AGT GAA GA-3' (forward); 5'-ATA GAC CCG TCC TTG CTC TG-3' (reverse); murine PKC alpha: 5'-TGG CAA AGG AGC AGA AAA CT-3' (forward); 5'-ACT GGG GGT TGA CAT ACG AG-3' (reverse); murine GDF15: 5'-CCT GCA GCC TGA CAA GGT-3' (forward); 5'-GGC CAC CAG GTC ATC ATA AG-3' (reverse); murine RIPK4: 5'-CAC TGC ACC TGG CTG CTC-3' (forward); 5'-CTC TGG CCT CCT TGG AAC TT-3' (reverse); murine PSCA: 5'-TTT GGG CAA GAA GAA CAT CA-3' (forward); 5'-ACA GCA ACA GGC TGC AGA G-3' (reverse);

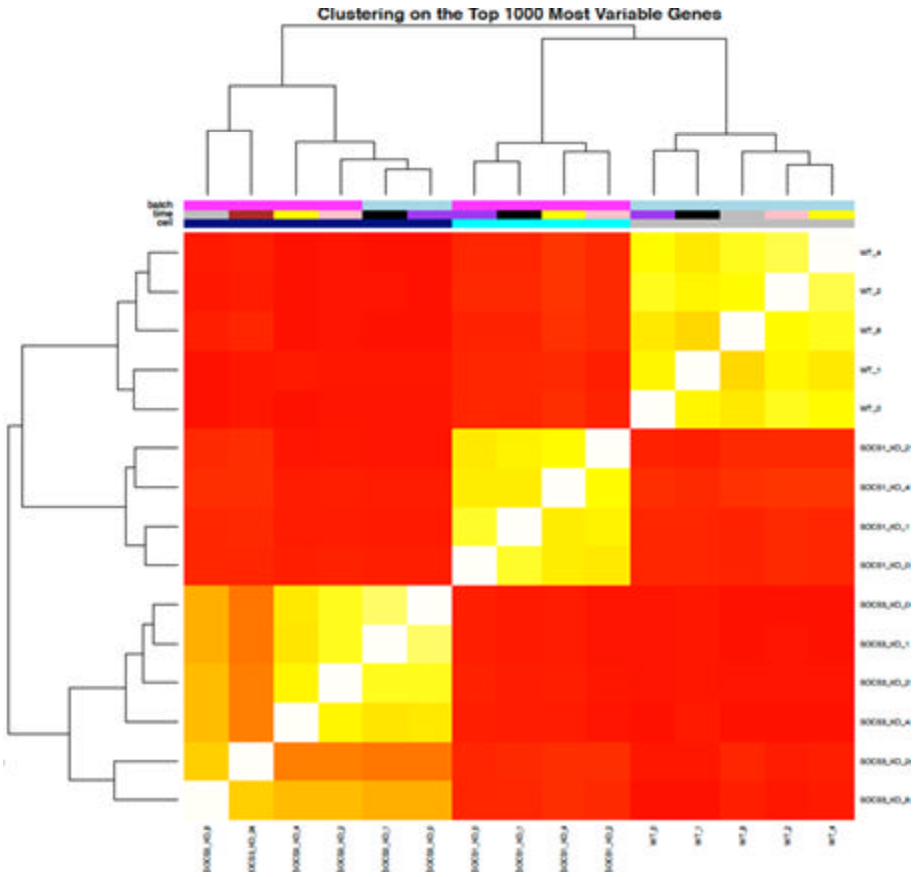
murine Heme Oxygenase: 5'-TGC TAG CCT GGT GCA AGA TA-3' (forward); 5'-ATG GCA TAA ATT CCC ACT GC-3' (reverse); murine GST: 5'-ATT TTG ACC TTG CTG CCT GT-3' (forward); 5'-AAG GGC TTC CTC AGT TTC TTG-3' (reverse).

2. Results and Discussion

2.1 Results

cDNA microarray analysis of WT and SOCS3 KO MEFs

In order to further investigate the potential role of SOCS3 and SOCS3 molecular subtypes in human PDA pathogenesis, we performed a gene expression microarray analysis using WT and SOCS3 KO MEFs to identify possible SOCS3 target genes that might play a role in PDA aggressiveness and/or metastasis *in vivo.*



Genes Underrepresented in SOCS3KO MEFs

Gene	Function	Fold Repression
Gata2	Transcription Factor	5.0
Hoxb2/4/5/6/7	Transcription Factors	4.8
Nid2 (osteonidogen)	Basement Membrane Protein	4.2
USP18	Ubiqutinating Enzyme	4.2
PARP14	DNA Repair	4.1
MSX1	Transcription Factor	4.0
CXCL1	**Chemokine**	**4.0**
CXCL2	**Chemokine**	**4.0**
STK39	Kinase	3.9
RIPK4	Kinase	3.9
Protein Kinase C alpha	Kinase	3.7
Txnip	Thioredoxin interacting protein	3.7
ISGF3	Transcription factor	3.5
G1p2	ISG15 Ubuquitin-like modifier	3.5

Genes Overrepresented in SOCS3KO

Gene	Function	Fold Induction
Receptor Accessory Protein (Reep3)	Increases expression of GPCR	12.2
Prostate Stem Cell Antigen	Upregulated in Cancer	7.5
Translation Initiation Factor 2	Increases Protein Translation	6.5
IGF2	Signaling Receptor	5.0
Wnt7a	Proliferation/Cell Survival	4.2
Gdf15	Growth Factor	4.0
Shh3	Cell Cycle Control	4.0
Bcl6	p21 inhibitor	3.2
Eif2s3y	Translation Initiation Factor	4.0
Clcn7	Chloride Ion Channel	3.6
Zfp52	Zinc Finger Protein	
GATA2	Transcription Factor	
Ddr1	Discoidin Domain Receptor Tyrosine Kinase	

Figure 23. cDNA microarray analysis of genes differentially regulated in WT and SOCS3 KO MEFs.

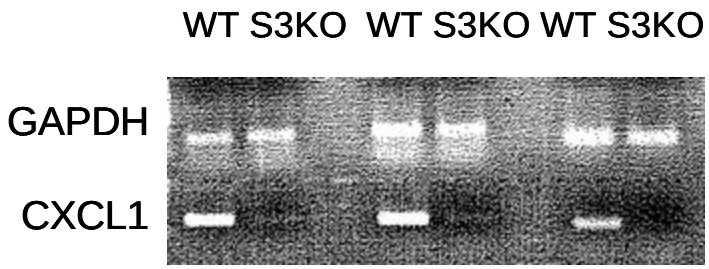
By performing cDNA microarray analysis, we were able to identify a panel of genes that are abnormally expressed in SOCS3 KO MEFs as shown in Figure 23. Genes that are overrepresented in SOCS3 KO MEFs include Reep3, PSCA, TIF2, IGF2, Wnt7a, Gdf15, Shh3,

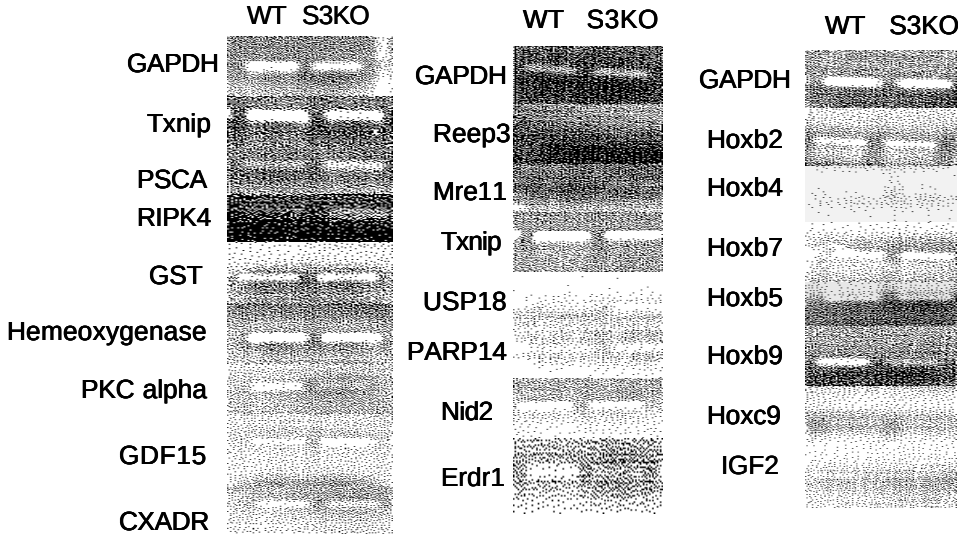
BCL6, Eif2s3y and Clcn7, which are involved in cell proliferation, cell survival and cell cycle control.

Genes that are underrepresented in SOCS3 KO MEFs include Gata2, Hoxb2/4/5/6/7, Nid2, USP18, PARP14, MSX1, CXCL1/2, STK39, RIPK4, PKC alpha, Txnip, ISGF3, and G1p2, which function as transcription factors, kinases or chemokines.

Gene expression analysis of MEFs, NSCLC and PDA cell lines by RT-PCR

We then extended to investigate the expression of these candidate genes identified in cDNA microarray analysis by performing RT-PCR.





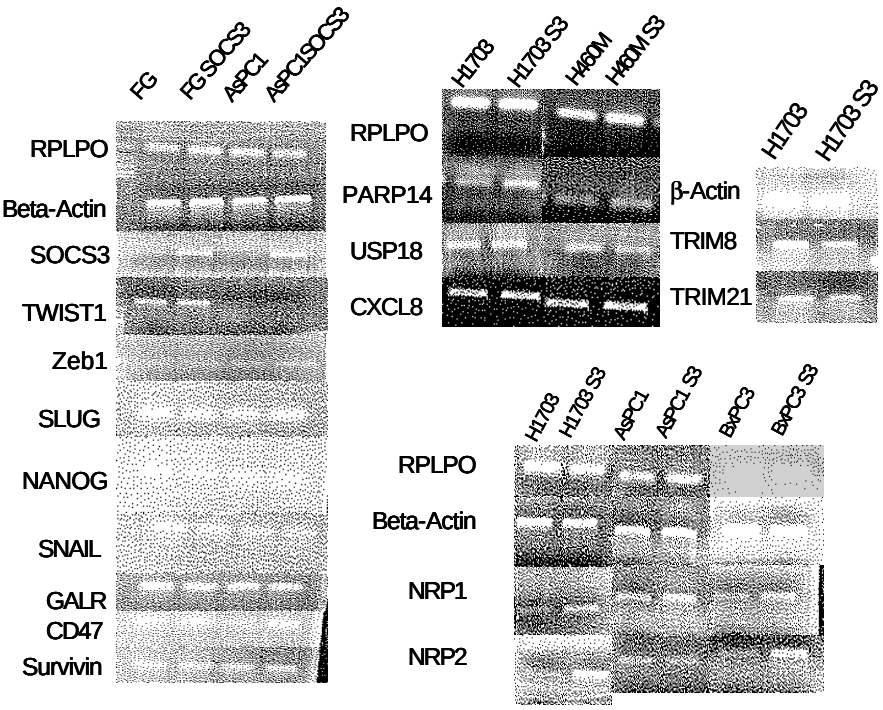


Figure 24. MEFs, NSCLC and PDA cell lines were screened for mRNA expression of a panel of genes identified in cDNA microarray analysis.

As shown in Figure 24, We found that CXCL1, Txnip, PKC alpha, CXADR, Erdr1 and Hoxb9 are underrepresented in SOCS3 KO MEFs, while PSCA, RIPK4, GDF15 and Mre11 are overrepresented in SOCS3 KO MEFs. We did not identify significant Reep3 mRNA expression in WT or SOCS3 KO MEFs.

For human cancer cell lines, we found that PARP14 is underrepresented in H1703 which silenced SOCS3. NRP1 and NRP2 are underrepresented in H1703, AsPC1 and BxPC3. We also identified that TRIM8 and TRIM21 are overrepresented in H1703. Moreover, we found that TWIST1 is silenced in AsPC1 and AsPC1 SOCS3, but not in FG and FG SOCS3.

2.2 Discussion

In cDNA microarray analysis of MEFs, we identified that BCL6, which functions as a p21 inhibitor, is overrepresented in SOCS3 KO MEFs. This finding is in agreement with our previous data of western blot and mRNA analysis confirming an inverse relationship between SOCS3 and BCL6 expression levels.

We also identified underrepresented CXCL1 and CXCL2 in SOCS3 KO MEFs by cDNA microarray analysis and/or RT-PCR. It has been reported that STAT3 negatively regulates CXCL1 production via the IL-6 common receptor subunit gp130. CXCL1 has been shown to play an important role in tumorigenesis and angiogenesis and its overexpression has been associated with tumor progression. Of greatest interest to our laboratory is the fact that SOCS3 negatively regulates STAT3 and therefore may modulate CXCL1 expression in cancer though the STAT3 pathway. Next, we will further explore the function of SOCS3 and CXCL1 in PDA pathogenesis.

Some of the genes identified in cDNA microarray analysis and RT-PCR represent interesting research targets in the future. For example, the poly ADP-ribose polymerase (PARP) superfamily of proteins is characterized by the presence of the PARP catalytic domain and consists of 17 members. These proteins are implicated in a number of cellular processes including, DNA damage repair, transcription regulation, telomere cohesion, and energy metabolism (Schreiber, V., 2006; Kim, M. Y., 2005). Recently, PARP14 has been reported to function as a transcriptional switch for STAT6-dependent gene activation (Purvi Mehrotra, 2011).

Neuropilins NRP1 and NRP2 are membrane-bound coreceptors to a tyrosine kinase receptor for both vascular endothelial growth factor (VEGF) and semaphoring family members. NRP1 plays

versatile roles in angiogenesis, axon guidance, cell survival, migration and invasion. NRP2 may play a role in cardiovascular development, axon guidance and tumorigenesis.

We also found that TWIST1 was expressed in FG and FG SOCS3, but silenced in AsPC1 and AsPC1 SOCS3. It has been reported that TWIST plays an essential role in cancer metastasis. Overexpression of TWIST or methylation of its promoter is common in metastatic carcinomas. Hence targeting TWIST has a great promise as a cancer therapeutic. TWIST is activated by a variety of signal transduction pathways, including Akt, STAT3, MAPK, Ras and Wnt signaling. Activated TWIST up-regulates N-cadherin and down-regulates E-cadherin, which are the hallmarks of EMT. Moreover, TWIST plays an important role in some physiological processes involved in metastasis, like angiogenesis, invadopodia, extravasation and chromosomal instability. TWIST also protects cancer cells from apoptotic cell death. In addition, TWIST is responsible for the maintenance of cancer stem cells and the development of chemotherapy resistance. TWIST1 is extensively studied for its role in head and neck cancers. TWIST1 has been shown to be involved in evading apoptosis, making the tumor cells resistant against chemotherapeutic drugs like Cisplatin. Moreover, TWIST1 has been shown to be expressed under conditions of hypoxia, corresponding to the observation that hypoxic cells respond less to chemotherapeutic drugs. Another process in which TWIST1 is involved is tumor metastasis. The underlying mechanism is not completely understood, but it has been implicated in the up-regulation of matrix metalloproteinases and inhibition of TIMP. Recently, targeting TWIST has gained interest as a target for cancer therapeutics. The inactivation of TWIST by small interfering RNA or chemotherapeutic approach has been demonstrated *in vitro*. Moreover, several inhibitors which are antagonistic to the upstream or downstream molecules of TWIST

signaling pathways have also been identified. Therefore the role of TWIST1 in human pancreatic cancer remains an interesting research target in the future.

3. Conclusion

By performing cDNA microarray analysis and RT-PCR, we have identified a panel of potential candidate genes that are differentially regulated between tumor cell lines such as TWIST1, as well as genes that are differentially regulated in WT and SOCS3 KO MEFs and tumors including CXCL1 and CXCL2, which makes them prime candidates for SOCS3-dependent effects in PDA pathogenesis.

VII. Analysis of the expression and function of CXCL1 in MEFs and human cancer cell lines

1. Materials and Methods

1.1 Materials

Cell lines and cell culture. MEFs (WT and SOCS3 KO), human NSCLC cell lines H460M, H460M SOCS3, H1703 and H1703 SOCS3 as well as PDA cell lines AsPC1, AsPC1 SOCS3, PL12, PL12 SOCS3, FG, FG SOCS3, BxPC3, BxPC3 SOCS3, 2.8 and 2.8 SOCS3 were used in this study. All the cell lines were maintained as described above.

1.2 Methods

CXCL1 ELISA. The Quantikine ELISA kit for Human CXCL1/GROα (R&D Systems) was used to examine expression of CXCL1 in the cancer cell lines. Because there is no species cross-reactivity of this kit, human GROα levels in culture media containing 10% bovine or fetal bovine serum can be assayed without interference. About 50,000 cells in 100 µl of media were seeded in a 96-well plate. On the next day, 200 µl of cell culture supernatants were added to a microplate coated with a mouse monoclonal antibody against human CXCL1/GROα (R&D Systems). The samples were incubated for 1.5 hours at room temperature and then washed three times with wash buffer. 200 µl of GROα conjugate was added to the plate which was then incubated for 1 hour at 4°C and washed again. The plate was then developed by adding 200 µl of Substrate Solution to each well, incubated for 15 minutes, added 50 µl of Stop Solution and then read by a microplate reader at 450 nm, 540 nm and 570 nm (Dr. McBride's lab of UCLA DMCO).

Reverse-Transcriptase PCR (RT-PCR). RT-PCR was carried out as described above. In this study, we designed three sets of primers to detect mRNA expression of each target gene. Three sets of CXCL1 primers are: CXCL1 1 fwd 5'-AGG GAA TTC ACC CCA AGA AC-3'; CXCL1 1 rev 5'-TAA CTA TGG GGG ATG CAG GA-3'; CXCL1 3 fwd 5'-CAC CCC AAG AAC ATC CAA AG-3'; CXCL1 3 rev 5'-TAA CTA TGG GGG ATG CAG GA-3'; CXCL1 5 fwd 5'-AGG GAA TTC ACC CCA AGA AC-3'; CXCL1 5 rev 5'-ACT ATG GGG GAT GCA GGA TT-3'; Three sets of human MMP7 primers are: MMP7 1 fwd 5'-TGC TCA CTT CGA TGA GGA TG-3'; MMP7 1 rev 5'-TGG GGA TCT CCA TTT CCA TA-3'; MMP7 2 fwd 5'-GAC AGG TCT CGG AGG AGA TG-3'; MMP7 2 rev 5'-TGG GGA TCT CCA TTT CCA TA-3'; MMP7 5 fwd

5’-GTC TCG GAG GAG ATG CTC AC-3’; MMP7 5 rev 5’-TAC CCA AAG AAT GGC CAA GT-3’; Three sets of CXCR2 primers are: CXCR2 2 fwd 5’-ATT CTG GGC ATC CTT CAC AG-3’; CXCR2 2 rev 5’-AAA GGA AGG CCT GCT GTC TT-3’; CXCR2 4 fwd 5’-GAG ATT CTG GGC ATC CTT CA-3’; CXCR2 4 rev 5’-AAA GGA AGG CCT GCT GTC TT-3’; CXCR2 5 fwd 5’-CAG AAG TTT CGC CAT GGACT-3’; CXCR2 5 rev 5’-AGT GGA AGT GTG CCC TGA AG-3’.

Real-time cell analysis (RTCA). We performed RTCA using AsPC1 and AsPC1 SOCS3 to determine the role of SOCS3 and presumably CXCL1 in cell migration *in vitro*. This system is a highly flexible, low throughput instrument for the monitoring of cell migration and invasion in particular by analyzing cell functions in real time using non-destructive, reagent free impedance measurements. Briefly, each well of the top chamber of a microplate was coated with 50 µl of 1:50 diluted (with media containing 2% FBS) low concentration Matrigel and incubated for 15 minutes in incubator or 1 hour at room temperature. 160 µl of 2% FBS media was added to the bottom chamber wells carefully to avoid bubbles. The top and bottom chambers were then assembled and incubated at 37°C for 1 hour. A total of 100,000 cells was added to each well of the top chamber and then incubated for 30 minutes at room temperature. The xCELLigence DP softwell was used to measure the migration of cells in real time.

Genetic reconstitution of human NSCLC and PDA cell lines. In order to further explore the role of CXCL1 in tumor progression and metastasis, we continued to use the pMXs-IRES-GFP (also known as pMXs-IG) retroviral vector (Cell Biolabs, Inc.) to deliver CXCL1 into genome of human cancer cell lines. The pcDNA 3.1(-) CXCL1 was kindly granted by Dr. Nakamura. The CXCL1 fragment was cut by BamHI (Cat. #R3136S, New England Biolabs) first, blunted with T4 DNA polymerase (Cat. #M0203S, New England Biolabs), then cut with XhoI (Cat. #R0146S,

New England Biolabs). The gel purified fragment containing CXCL1 cDNA was then cloned into the pME18S vector which was cut with PstI (Cat. #3140S, New England Biolabs) first, blunted and then cut with XhoI. T4 DNA ligase (New England Biolabs) was used for ligation and the ligation product was transformed into competent E.coli cell line DH5α (Cat. #18265-017, Invitrogen). The positive single clone was obtained by Ampicilin (100 mg/ml) selection. The pME-CXCL1 construct was then cut with BamHI and NotI (Cat. #R3189S, New England Biolabs) and cloned into pMXs-IG. Since pMXs-IG is a low copy number plasmid, the competent E.coli cell line TG1 (Zymo Research) with high transformation efficiency was used for the transformation after ligation. The positive clone of pMXs-CXCL1-IRES-GFP was used to transfect the package cell line Ampho cells using Effectene Transfection Reagent (Qiagen) which then produces high-titer, replication-incompetent viruses. The supernatant was collected, centrifuged, and passed through an 80 micron syringe filter (Corning), and applied to the cancer cell lines with treatment of 80 μg/ml of polybrene (Cat. #TR-1003-G, Millipore). Cells were infected twice at least and then sorted by GFP expression (UCLA JCCC).

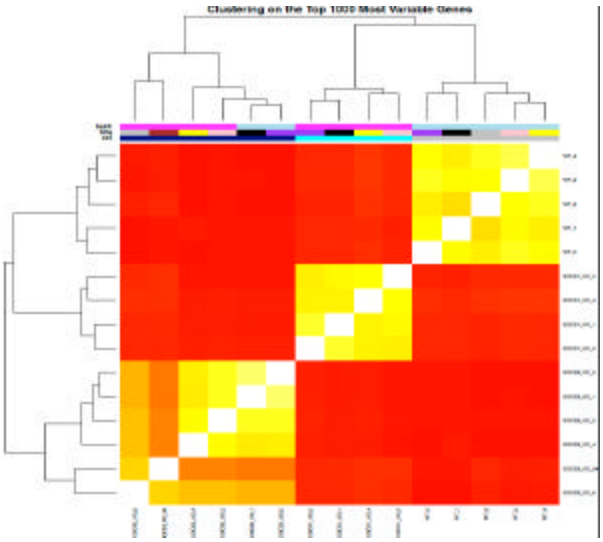
Clonogenic survival assay. We investigated the role of CXCL1 in radiation responses of tumors by performing *in vitro* clonogenic survival assay. The NSCLC cell lines H1703 and H1703 SOCS3 were starved overnight in media with 0.2% FBS, then harvested with Trypsin and counted. H1703 SOCS3 were treated with anti-CXCL1 antibody (R&D Systems) at 100 ng/ml, while H1703 were treated with recombinant human CXCL1 (R&D Systems) at 20 ng/ml. The cells were then incubated for 1 hour and irradiated with X-ray at 0, 1, 2, 4 and 6 Gy. Two hours after irradiation, cells were plated in 10 cm dishes in 10% FBS medium. The cells were left to grow for about two weeks and stained with 0.05% w/v crystal violet/methanol for 30 minutes and then counted to generate survival fraction data at each dose of radiation.

2. Results and Discussion

2.1 Results

Expression of CXCL1 is down-regulated in SOCS3-deficient fibroblasts and NSCLC cell lines

Our findings in Section V suggest that there is heterogeneity of SOCS3 expression in human PDA that may play a role in pancreatic cancer pathogenesis and might also indicate differential response to therapy among different molecular subtypes of tumors. In order to explore this possibility, we examined cDNA microarray data from WT and SOCS3-deficient MEFs to identify potential SOCS3/STAT3 target genes that may regulate PDA pathogenesis.



Genes Underrepresented in SOCS3KO MEFs

Gene	Function	Fold Repression
Gata2	Transcription Factor	5.0
Hoxb2/4/5/6/7	Transcription Factors	4.8
Nid2 (osteonidogen)	Basement Membrane Protein	4.2
USP18	Ubiqutinating Enzyme	4.2
PARP14	DNA Repair	4.1
MSX1	Transcription Factor	4.0
CXCL1	**Chemokine**	**4.0**
CXCL2	**Chemokine**	**4.0**
STK39	Kinase	3.9
RIPK4	Kinase	3.9
Protein Kinase C alpha	Kinase	3.7
Txnip	Thioredoxin interacting protein	3.7
ISGF3	Transcription factor	3.5
G1p2	ISG15 Ubuquitin-like modifier	3.5

Figure 25. cDNA microarray analysis of MEFs showed that the C-X-C chemokines CXCL1 and CXCL2 are underrepresented in SOCS3-deficient fibroblasts.

cDNA microarray analysis of WT and SOCS3 KO MEFs showed that a couple of genes are underrepresented in the SOCS3 KO MEFs, one of which caught our interest is the C-X-C chemokine CXCL1, as shown in Figure 25. CXCL1 expression is known to be repressed by STAT3 (T Wang, 2004; A. Matsukawa, 2005; C. A. Fielding, 2008). CXCL1 is of particular

interest in our system because SOCS3 negatively regulates STAT3 and therefore may modulate CXCL1 expression in human solid tumors. Next, we further analyzed the expression of CXCL1 mRNA and protein in MEFs and human cancer cell lines.

As shown in the left panel of Figure 26, CXCL1 mRNA expression is down-regulated in SOCS3 KO MEFs which is in agreement with the result of cDNA microarray analysis. We then further extended our research into human cancer cell lines. As shown in the right panel of Figure 26, CXCL1 expression is silenced in H1703, while restoration of SOCS3 into H1703 increased the expression of CXCL1. We also found that CXCR2, the receptor of CXCL1, was expressed in both parental and SOCS3+ cell lines. Importantly, our RT-PCR data revealed that the expression of matrix metalloproteinase-7 (MMP7) was also up-regulated in H1703 with restoration of SOCS3. Proteins of the matrix metalloproteinase (MMP) family are found to be involved in the breakdown of extracellular matrix in metastasis. It has been reported that restoration of SOCS3 into tumor cells which epigenetically silence SOCS3 has effect on tumor cell migration and invasion (C. Rossa, 2012). Our findings indicated that a possible mechanism of SOCS3 regulating tumor cell migration involves up-regulation of CXCL1 and/or MMP7 expression by SOCS3.

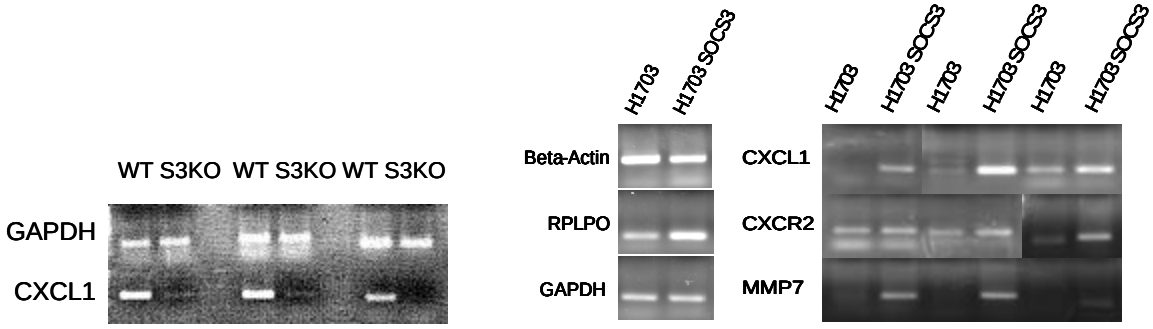
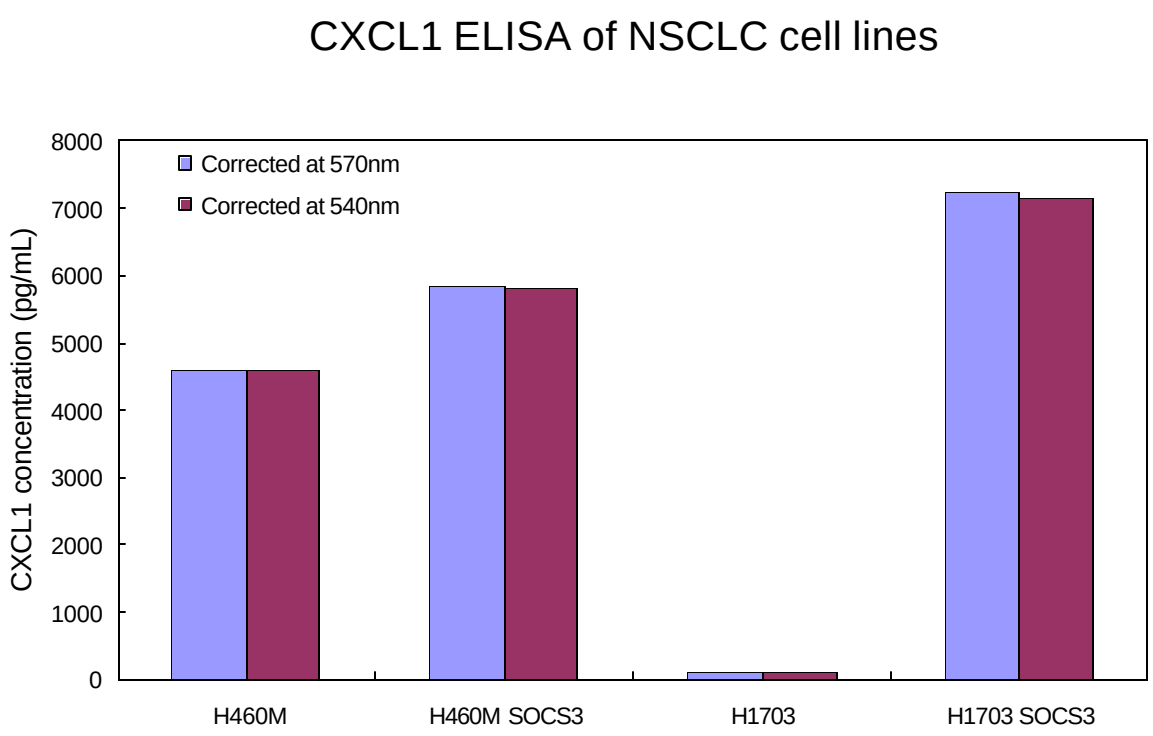


Figure 26. CXCL1 mRNA expression is down-regulated in SOCS3-deficient fibroblasts and NSCLC cell lines.
Left Panel. CXCL1 mRNA expression is dramatically down-regulated in SOCS3 KO MEFs.

Right Panel. NSCLC cell line H1703 which epigenetically silences SOCS3, displays lower level or total repression of CXCL1 and MMP7 mRNA expression. Re-introduction of SOCS3 into H1703 up-regulates expression of CXCL1 and MMP7.

Overexpression of SOCS3 in PDA cell lines differentially regulates CXCL1 expression according to SOCS3 molecular subtype

We then examined the expression of CXCL1 protein in NSCLC cell lines H460M and H1703 as well as PDA cell lines AsPC1, PL12, 2.8, BxPC3 and FG, both parental and SOCS3+ by ELISA. As shown in the upper panel of Figure 27, in agreement with our RT-PCR studies, secretion of CXCL1 was increased in the two NSCLC cell lines with restoration of SOCS3. In particular, there was almost no CXCL1 secretion in H1703, while re-introduction of SOCS3 dramatically increased CXCL1 expression, consistent with a model that SOCS3 can modulate CXCL1 expression by inhibiting STAT3 signaling.



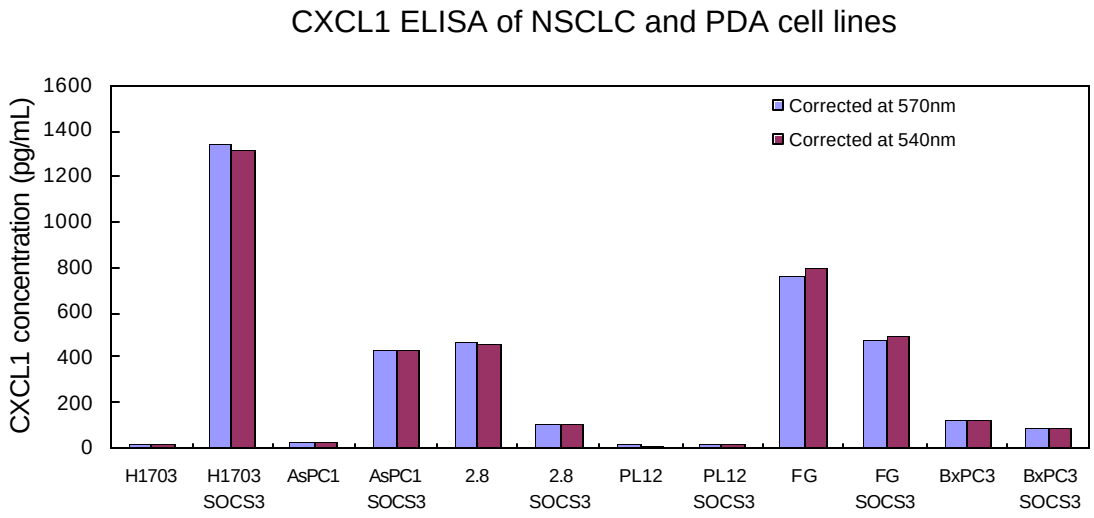


Figure 27. Expression of CXCL1 in NSCLC and PDA cell lines is evaluated by ELISA. 200 μl of cell culture supernatants from NSCLC and PDA cell lines, both parental and SOCS3+, were added to a microplate coated with a mouse monoclonal antibody against human CXCL1/GROα (R&D Systems). The samples were incubated for 1.5 hours at room temperature and then washed. 200 μl of GROα conjugate was added to the plate which was then incubated for 1 hour at 4°C and washed again. The plate was then developed by adding 200 μl of substrate solution, incubated for 15 min, added 50 μl of stop solution and then read by a microplate reader at 450nm, 540nm and 570nm.

We have identified three distinct molecular subtypes of human PDA based on SOCS3 expression in response to IL-6/OSM stimulation. Our CXCL1 ELISA data revealed that the three groups of PDA cell lines displayed unique responses in CXCL1 secretion with overexpression of SOCS3. As shown in lower panel of Figure 27, in FG (subtype 1, SOCS3 expression is high), 2.8 and PL12 (subtype 3, SOCS3 expression is low or repressed), we observed either no effect or reduced CXCL1 secretion in response to overexpression of SOCS3. These data suggest that the SOCS3/STAT3/CXCL1 axis is not critical in these subgroups of tumors and that other STAT3 targets are more important in these cells for disease pathogenesis.

On the other hand, the molecular subtype represented by AsPC1 (partial SOCS3 repression) demonstrated a completely different behavior that re-introduction of SOCS3 resulted in

increased CXCL1 expression, indicating that the SOCS3/STAT3 signaling machinery may play a major function in the regulation of CXCL1 expression in this cell line.

Overexpression of SOCS3 slightly increases cell migration of AsPC1

CXCL1 has been shown to be up-regulated in several cancers such as melanoma cells and is associated with enhanced growth, ability to form tumors in nude and SCID mice, and enhanced metastatic capacity in melanoma tumors (Balentien, 1991; Singh, 1994 and 1995; Schadendorf, 1993).

We next determined whether SOCS3/CXCL1 can regulate migration and invasion through a Matrigel matrix using a real-time cell analysis (RTCA) platform. We found that migration and invasion in AsPC1 cells are slightly enhanced by overexpression of SOCS3. However, we could not block the enhanced migration with anti-CXCL1 antibodies or a small molecule CXCR2 inhibitor, suggesting that SOCS3 may not directly affect the ability of cells to migrate to distal sites (data not shown).

Blocking CXCL1 with anti-CXCL1 antibody sensitizes H1703 SOCS3 to radiation, while treating H1703 with recombinant human CXCL1 increases radiation resistance

CXCL1 has also been reported to be involved in radiation response of tumor cells and may contribute to tumor growth and survival (Prakash C, 2005). In order to identify the role of CXCL1 in radiation response, we treated H1703 and H1703 SOCS3 with 20 ng/ml of recombinant human CXCL1 and anti-CXCL1 antibody respectively. As shown in Figure 28, we found that CXCL1 acted as a radioprotector in H1703 and blocking CXCL1 with anti-CXCL1 antibody sensitized H1703 SOCS3 to ionizing radiation.

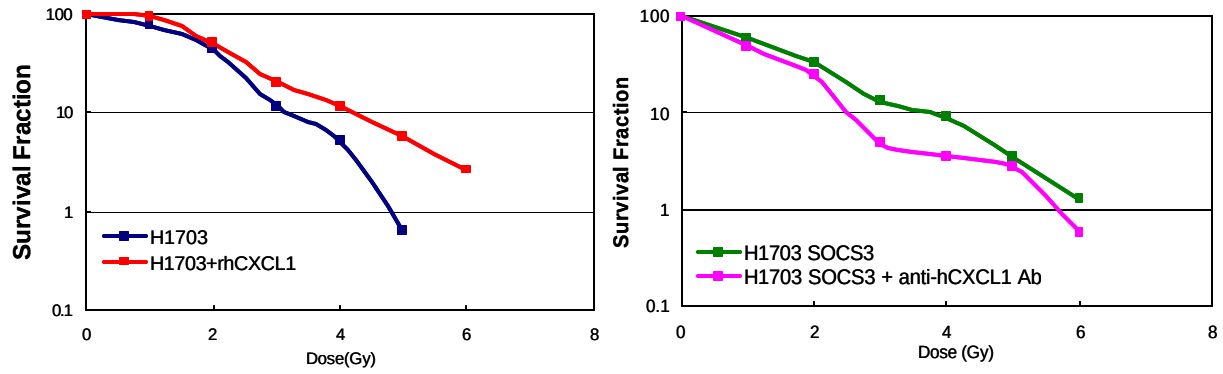


Figure 28. Clonogenic survival assay showed that anti-CXCL1 antibody sensitized H1703 SOCS3 to IR, while recombinant CXCL1 protected H1703 from IR.

2.2 Discussion

Our previous gene expression microarray analysis of MEFs showed that the C-X-C chemokine CXCL1 is underrepresented in the SOCS3 KO MEFs. We then further investigated CXCL1 expression in human cancer cell lines. Indeed, we found that re-introduction of SOCS3 into NSCLC cell lines H460M and H1703 increased the expression of CXCL1 mRNA and/or protein. We also found that CXCR2, the receptor of CXCL1, was expressed in both parental and SOCS3+ cell lines. Importantly, we identified an up-regulation of MMP7 expression with re-introduction of SOCS3 in H1703. These data suggested a possible mechanism of SOCS3 affecting PDA invasion and metastasis is through regulation of CXCL1 and MMP7 expression.

By performing CXCL1 ELISA, we have identified that the three molecular subtypes of human PDA with differential SOCS3 expression responded uniquely to SOCS3 re-introduction in CXCL1 expression. In subtypes 1 and 3 (SOCS3 high and SOCS3 low/repressed), we observed either no effect or reduced CXCL1 expression in response to SOCS3, indicating that the SOCS3/STAT3/CXCL1 axis is not critical in these subgroups of tumors and that other STAT3 targets are more important in these cells for disease pathogenesis. In contrast, the molecular

subtype represented by AsPC1 (partial SOCS3 repression) demonstrated a completely different behavior that re-introduction of SOCS3 resulted in increased CXCL1 expression. Our findings suggest that stratification of PDA tumors on the basis of SOCS3 or CXCL1 expression may predict response to biological targeting of STAT3 and/or C-X-C chemokines. One possible interpretation of this data is that if CXCL1 is important for PDA pathogenesis, SOCS3 might behave as a tumor suppressor in subgroups 1 or 3 but may have a tumor promoting role in cells from subgroup 2.

CXCL1 has also been reported to be involved in radiation response. Previous studies showed that CXCL1 is up-regulated following irradiation in HNSCC cell line UM-SCC6 and identified to contribute to tumor growth and survival during radiation (P. Chinnaiyan, 2005). We next further explored the role of CXCL1 in radiation response by performing clonogenic survival assay. We identified that CXCL1 may function as a radioprotector in human tumor cells and blocking CXCL1 with anti-CXCL1 antibody may sensitize tumor cells to ionizing radiation.

3. Conclusion

We have demonstrated that the three molecular subtypes of PDA with differential SOCS3 expression display distinct responses in CXCL1 secretion by ELISA. Overexpression of SOCS3 up-regulates CXCL1 expression in the molecular subtype with partial SOCS3 repression but not the others. Our data suggest that PDA cells can be stratified based on SOCS3 expression and CXCL1-dependence which might indicate distinct biological behaviors among these different molecular subtypes of cells in PDA pathogenesis.

VIII. *In vivo* studies on the role of SOCS3 and CXCL1 in PDA pathogenesis using an orthotopic xenograft model

1. Materials and Methods

1.1 Materials

Cell lines and cell culture. Human PDA cell lines AsPC1, AsPC1 SOCS3, PL12, PL12 SOCS3, FG, FG SOCS3, 2.8 and 2.8 SOCS3 were used in this study. All the cell lines were maintained as described above.

Mouse models. To establish orthotopic tumors, cells were trypsinized and suspended at a concentration of 1×10^8 cells/ml in phosphate-buffered saline (PBS). An equal volume of Matrigel (Cat. #356234, Becton Dickinson) was added to the cells and kept on ice until injection. Severe combined immunodeficient (SCID) mice (NOD/SCID; IL-2γc-/-, lacking T, B and natural killer cells) were anesthetized with ketamine (100 mg/kg) and xylzine (10 mg/kg), and then the pancreas exposed through an abdominal incision (laparotomy). Tumor cells were transferred by direct injection of the single cell suspension into the pancreas. After tumor transplantation, all mice were monitored at least twice weekly for disease progression by abdominal palpation and for signs of morbidity such as ruffled fur, and hunched posture. Moribund mice were euthanized by CO_2 inhalation. For survival studies, mice were followed until death or euthanized when signs of morbidity are evident.

1.2 Methods

Analysis of tumor progression. A separate cohort of mice harboring tumors will undergo repeat laparotomy to directly measure tumor size at two, four, and six weeks after implantation. Tumor

volume will be calculated based on the formula for ellipsoid volume, $V = 4/3 \times \pi \times (a/2)(b/2)(c/2)$ where a, b and c represent the maximal length, width and height, in millimeters, respectively. Mice will be sacrificed and analyzed histologically and for liver and lung metastasis, as well as for infiltration of peri-pancreatic lymph nodes.

Histologic analysis. Pancreatic tumors, livers, and lungs will be harvested from mice at three and six weeks after orthotopic tumor implantation. Tissues will be embedded in paraffin and representative sections stained with H&E. For immunohistochemistry, sections will be antigen-retrieved in rodent decloaker antigen retrieval solution (Biocare Medical) inside of a pressure cooker, and sections will be blocked and incubated with polyclonal rabbit anti-cytokeratin-19 (CK19), a marker of pancreatic ductal adenocarcinoma cells (AbCam) at 1/200 dilution, followed by detection using a Rabbit on Rodent Polymer detection kit (Biocare Medical). We will also analyze sections for neutrophil infiltration with antibody against CD11b, which is expressed on surface of neutrophils, as well as MMP2, MMP7 and MMP9. Sections will be mounted with Faramount aqueous mounting medium (Dako). Microscopic images will be taken with a V2.4 Nuance Multispectral Imaging VIS Flex Camera system (CRI) using an Olympus BX51 microscope (Olympus America Inc.).

2. Results and Discussion

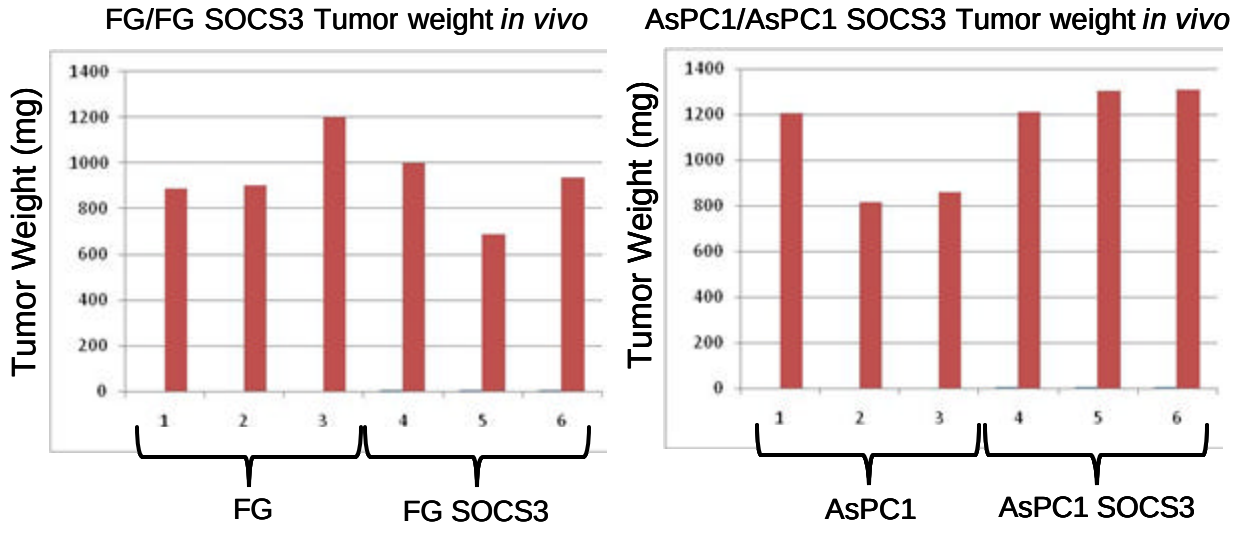
2.1 Results

Re-introduction of SOCS3 into PDA cell lines has various effects on tumor growth and metastasis in immunodeficient mice

We evaluated the growth and migration characteristics of human PDA cell lines, both with and without SOCS3 re-introduction, *in vivo* using an orthotopic xenograft model of human PDA in

immunodeficient mice. We have identified three distinct molecular subtypes of human PDA cell lines based on SOCS3 expression in response to IL-6/OSM stimulation. The representative cell lines are FG, in which SOCS3 expression is high after stimulation; AsPC1, in which SOCS3 expression is partially repressed in response to IL-6; 2.8 and PL12, in which SOCS3 is low or totally repressed. Interestingly, our *in vivo* data revealed that the three groups of PDA cell lines respond uniquely to SOCS3 re-introduction in tumor xenografts growth and metastasis.

As shown in Figure 29, overexpression of SOCS3 in the PDA cell line FG had no effect on tumor xenografts growth. Immunodeficient mice that were injected with FG or FG SOCS3 both developed huge tumors after six weeks. Meanwhile, overexpression of SOCS3 in AsPC1 contributed to tumor xenografts growth *in vivo*. Immunodeficient mice that were injected with AsPC1 SOCS3 developed larger tumors compared to those injected with the parental cells. On the other hand, re-introduction of SOCS3 into 2.8 and PL12 resulted in inhibition of tumor growth *in vivo*, as we observed reduced tumor weight of xenografts from immunodeficient mice injected with 2.8 SOCS3 or PL12 SOCS3 relative to mice injected with the parental cell lines.



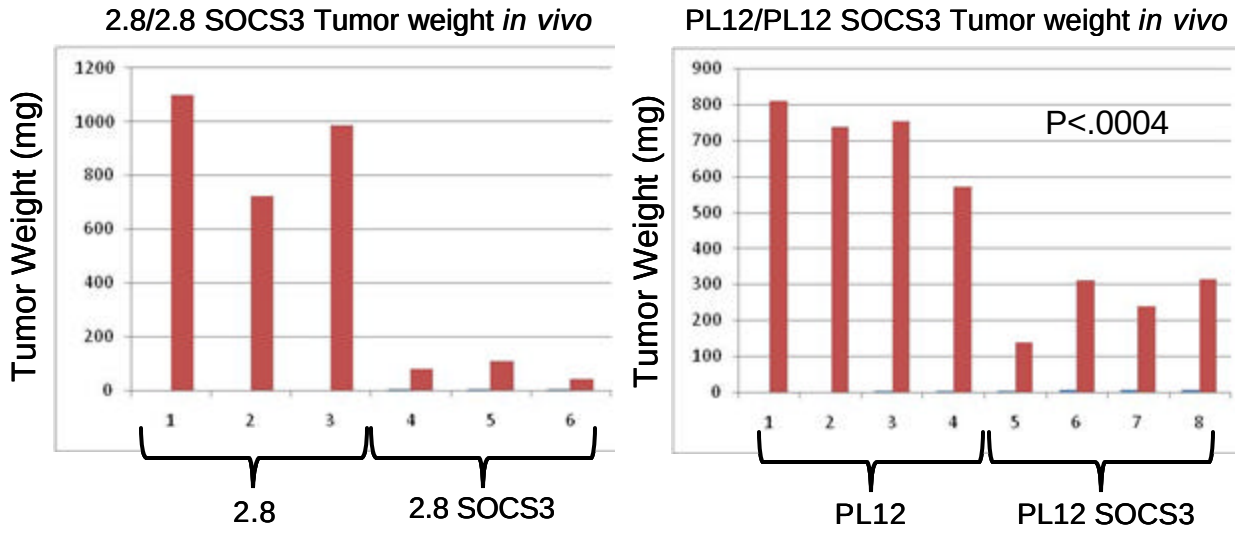
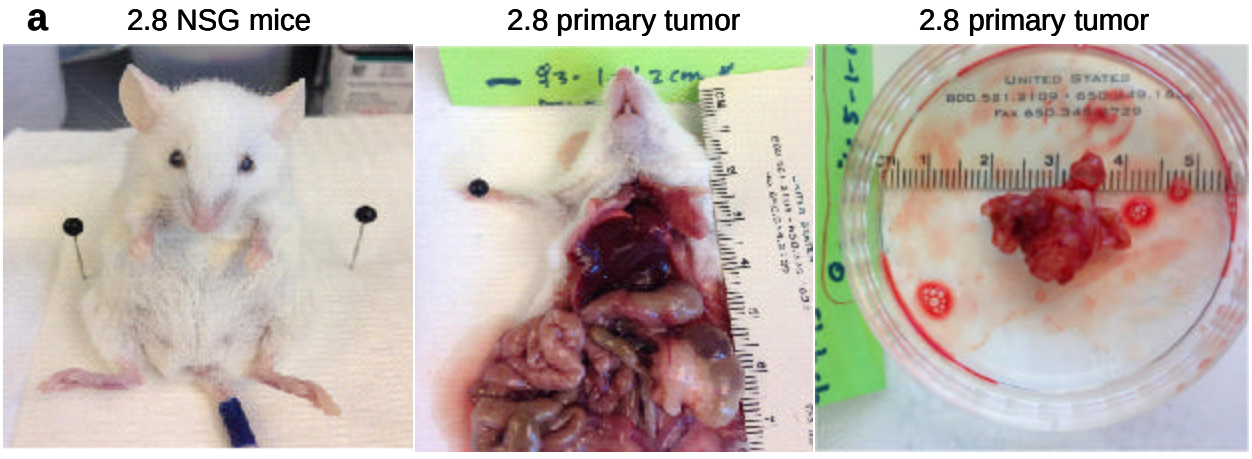


Figure 29. Human PDA cell lines respond uniquely to SOCS3 re-introduction in tumor weight of orthotopic xenografts from immunodeficient mice.

Histological analysis revealed that primary tumor xenografts from immunodeficient mice injected with 2.8 measured over 2 cm (Figure 30 a) while re-introduction of SOCS3 into 2.8 resulted in significant loss of tumor weight (Figure 29) and volume shrinkage (data not shown) in immunodeficient mice.



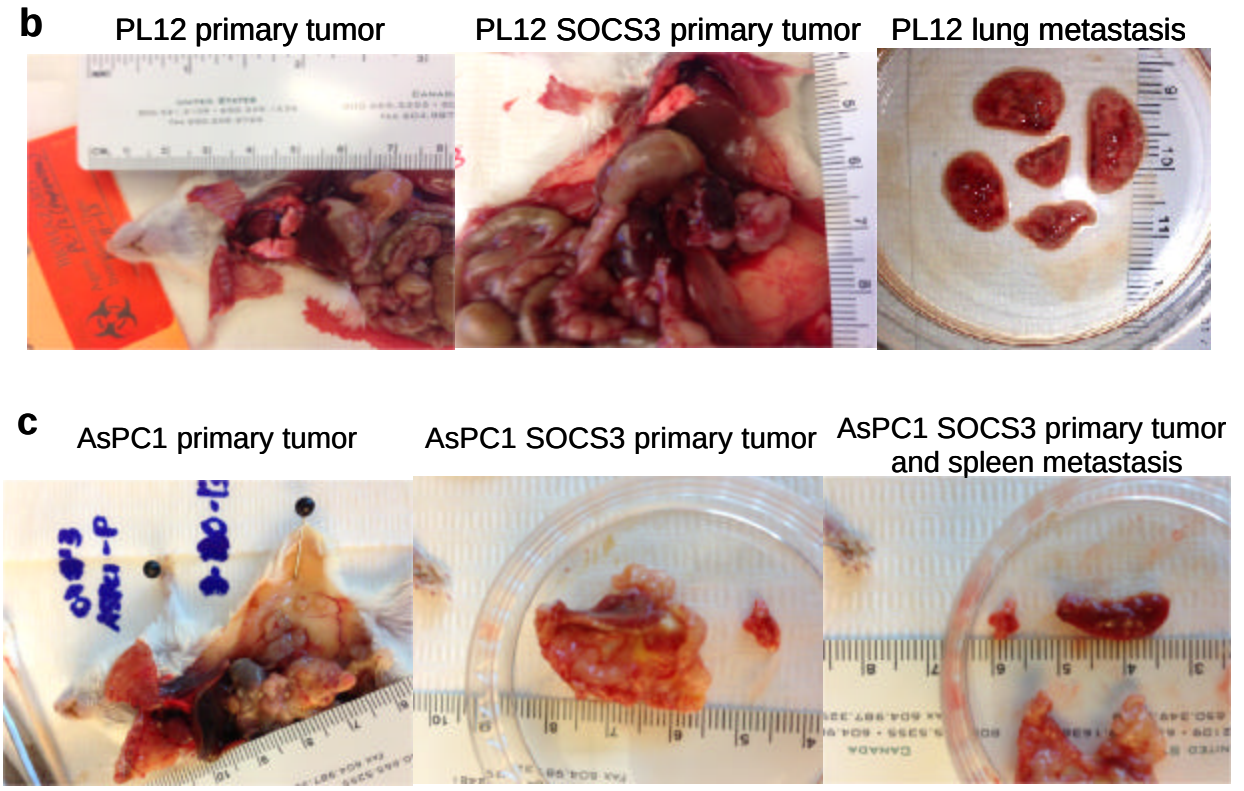


Figure 30. Histological analysis of tumor xenografts and metastasis from immunodeficient mice injected with human PDA cell lines. (a) Representative images and quantification of tumor xenografts developed in NSG mice injected with 2.8. (b) Representative images and quantification of primary tumor and lung metastasis developed in NSG mice injected with PL12 and PL12 SOCS3. (c) Representative images and quantification of primary tumor and spleen metastasis in NSG mice injected with AsPC1 and AsPC1 SOCS3.

As shown in Figure 30 (b), we observed metastasis to lung and liver in immunodeficient mice injected with PL12, but did not identify detectable metastasis in mice injected with PL12 SOCS3, suggesting re-introduction of SOCS3 into PL12 also inhibited tumor metastasis *in vivo.* Overall, this data supported SOCS3 as a tumor suppressor gene in PDA cell lines PL12 and 2.8.

On the other hand, we found that immunodeficient mice injected with AsPC1 SOCS3 developed larger primary tumor relative to those injected with AsPC1. We also observed metastasis to lung, liver, spleen and kidneys in immunodeficient mice injected with both AsPC1 and AsPC1 SOCS3 as shown in Figure 30 (c). Next, we further investigated responses of AsPC1 to SOCS3 overexpression in tumor development and metastasis *in vivo.*

Immunodeficient mice injected with AsPC1 SOCS3 have a shorter life time, develop malignant tumors and display aggressive tumor invasion

We further examined the growth/migration characteristics of the PDA cell line AsPC1 and AsPC1 SOCS3 *in vivo* using an orthotopic xenograft model of human PDA in immunodeficient mice. AsPC1 is of special interest to us because we revealed an unexpected role of SOCS3 in this cell line that re-introduction of SOCS3 into AsPC1 promoted tumor growth *in vivo*. We have demonstrated that overexpression of SOCS3 in AsPC1 up-regulated CXCL1 secretion. We then further extended to evaluate the involvement of SOCS3/STAT3/CXCL1 signaling axis in PDA tumor metastasis *in vivo.*

As shown in Figure 31 (a), we found that immunodeficient mice injected with FG and FG SOCS3 did not show a significant difference in survival time. On contrast, NSG mice injected with AsPC1 SOCS3 had a shorter survival time and developed more malignant tumors compared to mice injected with AsPC1 (Figure 31 b-e).

We also identified more aggressive liver metastasis in mice injected with AsPC1 SOCS3 compared to those injected with AsPC1 (Figure 31 f and g).

Importantly, hematoxylin and eosin (H&E) stained primary tumors from NSG mice injected with AsPC1 SOCS3 showed infiltrating neutrophils (Figure 31 h and i). Tumor-associated neutrophils (TAN) have been shown to play a major role in enhanced disease progression in human tumors.

We also identified CD11b-positive cells in tumor xenografts developed in immunodeficient mice injected with AsPC1 SOCS3 (Figure 31 j-l). CD11b belongs to the integrin family and is expressed on neutrophils, monocytes, natural killer cells, and a subset of lymphocytes. CD11b expressed on neutrophils and monocytes has been extensively investigated and reported to play an important role in the migration of these subsets of leukocytes.

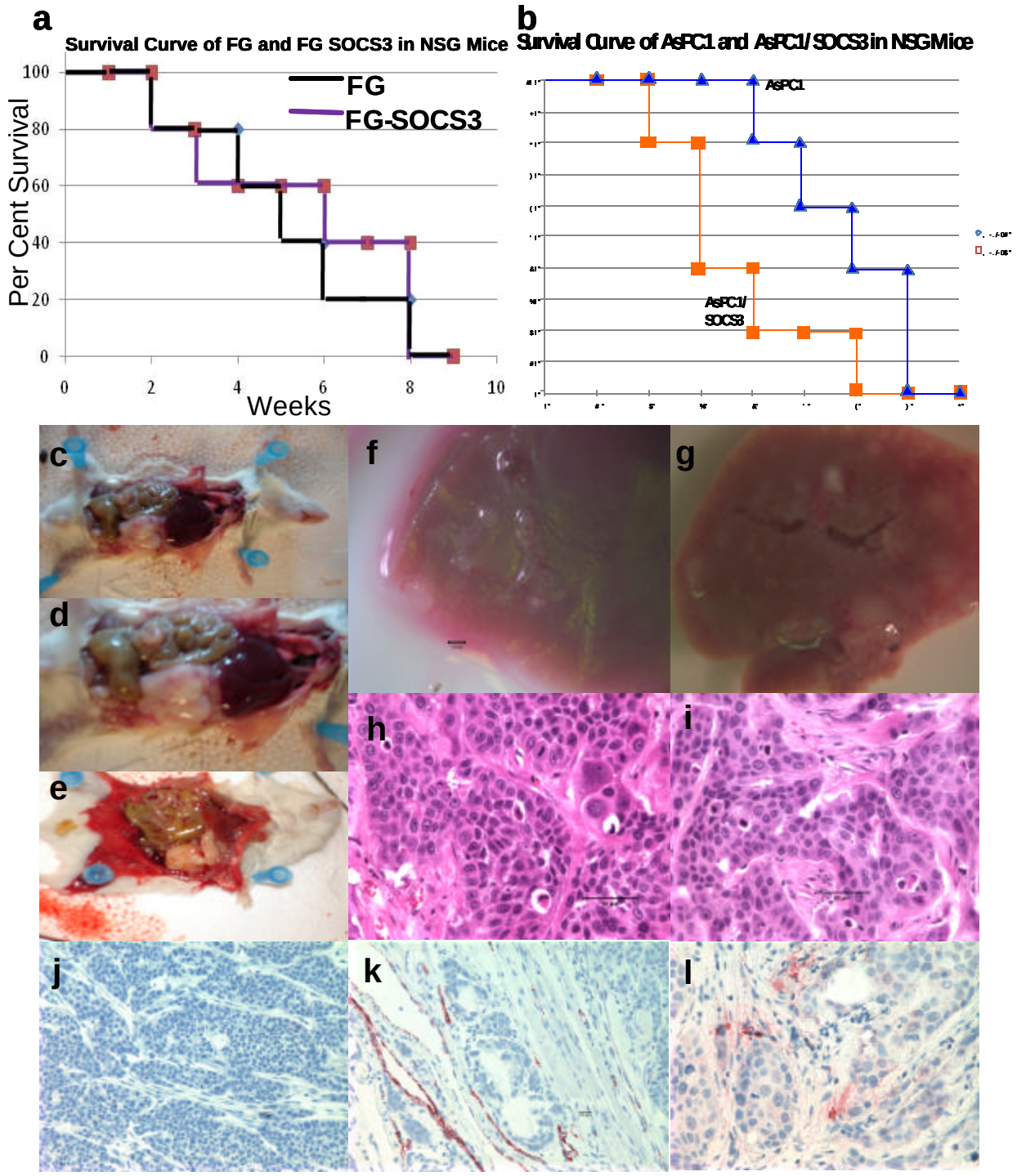


Figure 31. Survival assay, histological and immunohistochemistry analysis of pancreatic tumor xenografts and metastasis developed in immunodeficient mice. Pancreatic tumors were harvested from mice after orthotopic tumor implantation of human PDA cell lines AsPC1 and AsPC1 SOCS3. (a) Survival study of NSG mice that are injected with FG and FG SOCS3. (b) Survival study of NSG mice that are injected with AsPC1 and AsPC1 SOCS3. (c), (d) and (e) Histological analysis of pancreatic tumor xenografts from NSG mice that are injected with AsPC1 and AsPC1 SOCS3. (f) Representative images of liver metastasis in NSG mice injected with AsPC1. (g) Representative

images of liver metastasis in NSG mice injected with AsPC1 SOCS3. (h) and (i) Representative images of H&E-stained primary tumor sections from NSG mice injected with AsPC1 SOCS3 showing infiltrating neutrophils. (j) Representative images of primary tumor sections from NSG mice injected with AsPC1 stained with CD11b antibody. (k) and (l) Representative images of primary tumor sections from NSG mice injected with AsPC1 SOCS3 showing CD11b-positive cells.

Our finding that CXCL1 is up-regulated in AsPC1 SOCS3 indicated a possible mechanism through which SOCS3 can regulate pro-inflammatory recruitment of neutrophils to the tumor microenvironment. It has also been reported that autocrine CXCL1-CXCR2 signaling can drive tumor cell migration and increase metastatic potential. Overall these data suggested that overexpression of SOCS3 in AsPC1 may contribute to PDA pathogenesis by promoting tumor growth and metastasis through up-regulation of CXCL1 secretion.

2.2 Discussion

Despite recent advances in our understanding of pancreatic ductal adenocarcinoma (PDA), it remains a poorly understood, devastating disease that is largely resistant to all standard treatment modalities. Much effort has been made to identify novel tumor suppressor genes, biomarkers of metastatic behavior, and targets for molecular therapeutics that can improve prognosis and quality of life for PDA patients.

Pro-inflammatory signaling induced by chemokines and cytokines have been identified to be involved in pancreatic oncogenesis. The pro-inflammatory C-X-C family chemokine CXCL1 has been shown to be up-regulated in several cancers such as melanoma cells and is associated with enhanced growth, ability to form tumors in nude and SCID mice, and enhanced metastatic capacity in melanoma tumors (Balentien, 1991; Singh, 1994 and 1995; Schadendorf, 1993). Antibodies to CXCL1 or its receptor, CXCR2, can block these processes (Norgauer, 1996;

Schadendorf, 1993). Secreted CXCL1 has also been reported as a potential mediator and marker of the tumor invasion of bladder cancer (H. Kawanishi, 2008). In addition to the autocrine CXCL1-CXCR2 signaling which has been shown to drive tumor cell migration and increase metastatic potential (H. Kawanishi, 2008; Swarnali Acharyya, 2012), CXCL1 has also been reported to induce the infiltration of neutrophils to the tumor microenvironment which produce soluble factors including pro-inflammatory mediators as well as metalloproteases, and confer to increased motility and invasiveness of tumor cells (H. Kawanishi, 2008; Bostrom PJ, 2000; Zhou, 2005). Both CXCL1 and neutrophils have been described in pancreatic tumors and cell lines. Despite the finding that CXCL1 and CXCL2 are expressed in a significant fraction of human PDA tissues and are known to promote metastasis in bladder and breast cancer, very little is know of its regulation and function in the progression of PDA.

Our *in vivo* data revealed that human PDA cell lines response variously to SOCS3 re-introduction in tumor xenografts growth and metastasis in NSG mice. Overexpression of SOCS3 in FG did not affect tumor growth *in vivo*. While restoration of SOCS3 in PDA cell lines 2.8 and PL12 inhibited tumor growth and metastasis *in vivo*. We identified lung and liver metastasis in NSG mice injected with PL12, but not in those injected with PL12 SOCS3. Overall, this data supported the role of SOCS3 as a tumor suppressor gene in PL12 and 2.8.

On the other hand, overexpression of SOCS3 in AsPC1 resulted in up-regulation of CXCL1 expression. In an animal survival assay, we identified that NSG mice that were injected with AsPC1 SOCS3 displayed shorter lifetime, developed more malignant tumors and showed aggressive invasion and tumor metastasis compared to those injected with AsPC1. Histological analysis of tumor sections showed CD11b expression in tumor xenografts of mice injected with AsPC1 SOCS3, indicating recruitment of neutrophils into the tumor microenvironment. It may

seem controversial that overexpression of SOCS3, a STAT3 inhibitor, would contribute to tumor progression and invasion in NSG mice. Our data suggested a possible mechanism of SOCS3 affecting PDA progression and metastasis is through regulation of CXCL1 and/or MMP expression. The elevated level of CXCL1 secretion in AsPC1 SOCS3 may contribute to PDA pathogenesis by autocrine activation of the CXCR2 receptor expressed on tumor cells and pro-inflammatory recruitment of neutrophils to the tumor microenvironment.

Overall, our work has offered a novel clue in terms of how to stratify tumors and patients. Some recent studies point to an important general role for STAT3 in PDA pathogenesis and suggest therefore that SOCS3 is a tumor suppressor. However, our data suggest that the situation is more complex and depends on cellular context. We have been able to stratify human PDA cell lines into at least three molecular subgroups based on SOCS3 expression/repression and therefore we suggest that each group has a distinct level of addiction to STAT3 signaling and CXCL1 dependence. Subgroup 1, in which SOCS3 expression is not deregulated, does not respond to SOCS3 overexpression and therefore is likely to be the least dependent on STAT3 signaling for disease progression. Subgroup 3, which severely or completely represses SOCS3 expression, appears to be highly STAT3-addicted. Thus, SOCS3 expression in these cells results in greatly impaired growth and metastasis *in vivo*. Lastly, subgroup 2, represented by AsPC1, appears to partially control STAT3 signaling in order to exploit a selective advantage imparted by CXCL1 expression. In these cells, inhibition of STAT3 signaling via SOCS3 increases tumor aggressiveness and metastatic behavior due to elevated CXCL1 levels. These findings clearly have important implications for the design of therapies. For example, if one wants to use a STAT3 inhibitor, it might only work in cells like PL12 and 2.8 but might make the disease worse with cells like AsPC1, and may have no effect on FG- like cells.

3. Conclusion

Our laboratory has identified three distinct molecular subtypes of PDA based on SOCS3 expression and CXCL1/2 dependence in response to IL-6 or OSM. We have shown that ectopic expression of SOCS3 can promote CXCL1 gene activation and tumor aggressiveness in the molecular subtype with partial SOCS3 repression but not the others. Thus, the three molecular subtypes of PDA that we have identified display distinct biological behaviors, effects of SOCS3 overexpression and CXCL1 dependence in tumor progression and metastasis. Using an orthotopic xenograft model of human PDA in immunodeficient mice, we have demonstrated the *in vivo* relevance of the STAT3/SOCS3/CXCL1 axis in tumor aggressiveness and metastatic behavior. Our results suggest a novel mechanism for controlling pro-inflammatory signaling in human solid tumors and that SOCS3 can regulate both metastasis and radiation sensitivity through distinct pathways. Our ability to stratify human PDA on the basis of SOCS3/STAT3/CXCL1/2 dependence has implications for the development of individualized therapy for cancer patients. Overall, our data suggest that SOCS3 and CXCL1 may constitute potential therapeutic targets for pancreatic cancer.

References

Akihiko Yoshimura, Mayu Suzuki, Ryota Sakaguchi, Toshikatsu Hanada and Hideo Yasukawa. SOCS, inflammation, and autoimmunity. Fronties in Immunology. 12 March 2012. doi: 10.3389/fimmu.2012.00020.

Anette Weber, Ulrich R Hengge, Walter Bardenheuer, Iris Tischoff, Florian Sommerer, Annett Markwarth, Andreas Dietz, Christian Wittekind and Andrea Tannapfel. SOCS-3 is frequently

methylated in head and neck squamous cell carcinoma and its precursor lesions and causes growth inhibition. Oncogene (2005) 24, 6699–6708.

American Cancer Society. Cancer Facts & Figures 2013. Atlanta: American Cancer Society; 2013.

Alexander, W. S. (2002). Suppressors of cytokine signalling (SOCS) in the immune system. Nat. Rev. Immunol. 2, 410-416.

A. Matsukawa, S. Kudo, T. Maeda, K. Numata, H. Watanabe, K. Takeda, S. Akira, T. Ito. 2005. Stat3 in resident macrophages as a repressor protein of inflammatory response. J. Immunol. 175: 3354-3359.

A.L. Gartel, K. Shchors, Exp. Cell Res. 283 (1) (Feb 1 2003) 17–21.

B.A. Croker, D.L. Krebs, J.G. Zhang, S.Wormald, T.A. Willson, E.G. Stanley, L. Robb, C.J. Greenhalgh, I. Forster, B.E. Clausen, N.A.Nicola, D.Metcalf, D.J. Hilton, A.W.Roberts,W.S. Alexander, Nat Immunol. 4 (2003) 540.

Balentien, E., Mufson, B. E., Shattuck, R. L., Derynck, R., Richmond, A. (1991) Effects of MGSA/GRO alpha on melanocyte transformation. Oncogene 6, 1115–1124.

Benjamin Barré, Sylvie Avril and Olivier Coqueret. Opposite Regulation of Myc and p21waf1 Transcription by STAT3 Proteins. Published, JBC Papers in Press, November 15, 2002, DOI 10.1074/jbc.M210422200.

Bostrom PJ, Ravanti L, Reunanen N, et al. Expression of collagenase-3 (matrix metalloproteinase-13) in transitional-cell carcinoma of the urinary bladder. Int J Cancer 2000; 88:4179 (23).

C. A. Fielding, R. M. Mcloughlin, L. Mcleod, C. S. Colmont, M. Najdovska, D. Grail, M. Ernst, S. A. Jones, N. Topley, B. J. Jenkins. IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. J. Immunol. 2008. 181: 2189-2195.

Cacalano, N. A., Sanden, D., and Johnston, J.A. (2001). Tyrosine-phosphorylated SOCS-3 inhibits STAT activation but binds to p120 RasGAP and activates Ras. Nat. Cell Biol. 3, 460–465.

Camp, E. R., Yang, A., Gray, M. J., Fan, F., Hamilton, S. R., Evans, D. B., et al. (2007). Tyrosine kinase receptor RON in human pancreatic cancer: Expression, function, and validation as a target. Cancer 109, 1030–1039.

Chih-Hung Lai, Helen H. Chun, Shareef A. Nahas, Midori Mitui, Kristin M. Gamo, Liutao Du, and Richard A. Gatti. Correction of ATM gene function by aminoglycoside-induced read-through of premature termination codons. Proc Natl Acad Sci U S A. Nov 2, 2004; 101(44): 15676–15681.

Collins, AS; McCoy, CE; Lloyd, AT; O'Farrelly, C; Stevenson, NJ. miR-19a: An Effective Regulator of SOCS3 and Enhancer of JAK-STAT Signalling. PLoS One, 2013 vol. 8(7).

David W. Meek. Tumour suppression by p53: a role for the DNA damage response? Nature Revies. Volume 9. October 2009 (715).

Dawn V. Jaquish, Peter T. Yu, Andrew M. Lowy et al. IGF1-R signals through the RON receptor to mediate pancreatic cancer cell migration. Carcinogenesis. Aug 2011; 32(8): 1151-1156.

Dhawan P, Richmond A. A novel NF-nB-inducing kinase-MAPK signaling pathway up-regulates NF-nB activity in melanoma cells. J Biol Chem 2002; 277:7920–8. Epub 2001 Dec 28.

Don X. Nguyen, Paula D. Bos, Joan Massague. Metastasis: from dissemination to organ-specific colonization. Nature 2009. (9) 274.

de Visser, K.E., Eichten, A., and Coussens, L.M. (2006). Paradoxical roles of the immune system during cancer development. Nat. Rev. Cancer 6, 24–37.

Dey BR, Furlanetto RW, Nissley P. Suppressor of cytokine signaling (SOCS)-3 protein interacts with the insulin-like growth factor-I receptor. Biochem Biophys Res Commun. 2000 Nov 11;278(1):38-43.

E.B. Haura, J. Turkson, R. Jove. Nat Clin. Pract Oncol. 2 (2005) 315.

Ebrahimi, B., Tucker, S.L., Li, D., Abbruzzese, J.L., and Kurzrock, R. (2004). Cytokines in pancreatic carcinoma: correlation with phenotypic characteristics and prognosis. Cancer 101, 2727–2736.

Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, Matsumoto A, Tanimura S, Ohtsubo M, Misawa H, Miyazaki T, Leonor N, Taniguchi T, Fujita T, Kanakura Y, Komiya S and Yoshimura A. (1997). Nature 387, 921-924.

Follenzi,A. et al. (2000) Cross-talk between the proto-oncogenes Met and Ron. Oncogene, 19, 3041–3049.

Fridlender, Z. G. et al. Polarization of tumor-associated neutrophil phenotype by TGF-ß: "N1" versus "N2" TAN. Cancer Cell 16, 183–194 (2009)

Feld, R., Rubinstein, L. V. & Weisenberger, T. H. Sites of recurrence in resected stage I non-small-cell lung cancer: a guide for future studies. J. Clin. Oncol. 2, 1352–1358 (1984).

Gisela F. Claassen and Stephen R. Hann. A role for transcriptional repression of $p21^{CIP1}$ by c-Myc in overcoming transforming growth factor β-induced cell-cycle arrest. PNAS. August 15 2000. Vol. 97 No. 17 (9498-9503).

Greenhalgh, C. J. and Hilton, D. J. (2001). Negative regulation of cytokine signaling. Journal of Leukocyte Biology 70, 348-356.

Guilian Niu, Kenneth L. Wright, Yihong Ma, Gabriela M. Wright, Mei Huang, Rosalyn Irby, Jon Briggs, James Karras, W. Douglas Cress, Drew Pardoll, Richard Jove, Jiangdong Chen and Hua Yu. Role of Stat3 in Regulating p53 Expression and Function. Mol. Cell. Biol. September 2005 vol. 25 no. 17 7432-7440.

He B, You L, Uematsu K, Zang K, Xu Z, Lee AY, Costello JF, McCormick F, Jablons DM. SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc Natl Acad Sci U S A. 2003 Nov 25; 100(24):14133-8.

Herman JG, Graff JR, Myö hä nen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996, 93:9821-6.

Hilton DJ, Richardson RT, Alexander WS, Viney EM, Willson TA, Sprigg NS, Starr R, Nicholson SE, Metcalf D and Nicola NA. Twenty proteins containing a C-terminal SOCS box form five structural classes. Proc Natl Acad Sci USA 1998; 95: 114-119.

Hiroaki Kawanishi, Yoshiyuki Matsui, Masaaki Ito, et al. Secreted CXCL1 Is a Potential Mediator and Marker of the Tumor Invasion of Bladder Cancer. Clin Cancer Res 2008;14:2579-2587.

H.A. Coller, C. Grandori, P. Tamayo, T. Colbert, E.S. Lander, R.N. Eisenman, T.R. Golub, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 3260.

H. Nagai, T. Naka, Y. Terada, T. Komazaki, A. Yabe, E. Jin, O. Kawanami, T. Kishimoto, N. Konishi, M. Nakamura, et al. J. Hum. Genet. 48 (2003) 65.

H. Yoshikawa, K. Matsubara, G. S. Qian, P. Jackson, J. D. Groopman, J. E. Manning, C. C. Harris, J.G. Herman. Nature Genetics. 28 (2001) 29.

H. Yasukawa, M. Ohishi, H. Mori,M.Murakami, T. Chinen, D. Aki, T. Hanada, K. Takeda, S.Akira, M.Hoshijima, T.Hirano, K.R.Chien, A. Yoshimura, Nat.Immunol. 4 (2003) 551.

Huber, A.R., Kunkel, S.L., Todd III, R.F., Weiss, S.J., 1991. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science 254, 99–102.

Horton, L.W., Yu, Y., Zaja-Milatovic, S., Strieter, R.M., Richmond, A., 2007. Opposing roles of murine duffy antigen receptor for chemokine and murine CXC chemokine receptor-2 receptors in murine melanoma tumor growth. Cancer Res. 67, 9791–9799.

Iwama A, Yamaguchi N, Suda T. STK/RON receptor tyrosine kinase mediates both apoptotic and growth signals via the multifunctional docking site conserved among the HGF receptor family. EMBO J 1996;15:5866–75.

Jason S. Rawlings, Kristin M. Rosler and Douglas A. Harrison. The JAK/STAT signaling pathway. Journal of Cell Science 117, 1281-1283. Published by The Company of Biologists 2004. doi:10.1242/jcs.00963

J. N. Ihle. Nature 377 (1995) 591.

Jocelyn Logan-Collins, Ryan M. Thomas, Peter Yu, Dawn Jaquish, Evangeline Mose, Randall French, William Stuart, Rebecca McClaine, Bruce Aronow, Robert M. Hoffman, Susan E. Waltz, and Andrew M. Lowy. Silencing of RON Receptor Signaling Promotes Apoptosis and Gemcitabine Sensitivity in Pancreatic Cancers. Cancer Res; 70(3) February 1, 2010.

Kastan, M. B. DNA damage responses: mechanisms and roles in human disease. 2007 G.H.A. Clowes Memorial Award Lecture. Mol. Cancer Res. 6, 517–524 (2008).

Kim, M. Y., Zhang, T., and Kraus, W. L. (2005) Genes Dev. 19, 1951–1967.

Krishnadasan R, Bifulco C, Kim J, Rodov S, Zieske AW and Vanasse GJ. Overexpression of SOCS3 is associated with decreased survival in a cohort of patients with de novo follicular lymphoma. Br J Haematol 2006; 135: 72-75.

Komyod W, Bohm M, Metze D, Heinrich PC and Behrmann I. Constitutive suppressor of cytokine signaling 3 expression confers a growth advantage to a human melanoma cell line. Mol Cancer Res 2007; 5: 271-281.

Kripa Patel, Anita Kollory, Asami Takashima, Sibaji Sarkar, Douglas V. Faller, Sajal K. Ghosh. MicroRNA let-7 downregulates STAT3 phosphorylation in pancreatic cancer cells by increasing SOCS3 expression. Cancer Letters 347 (2014) 54–64.

Kriti Rakesh, Cheng Gang, Jianhui Yang, Devendra K Agrawal. Abstract 1670: Over-expression of SOCS3 Negatively Regulates STAT3 Activation and Attenuates IGF-1 Mediated Human Coronary Artery Smooth Muscle Cell Proliferation. (Circulation. 2006; 114:II_325.) © 2006 American Heart Association, Inc.

Kuang, D. M. et al. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. J. Hepatol. 54, 948–955 (2011).

L. Li, P. E. Shaw. J. Biol. Chem 277 (2002) 17397.

Laird, P. W. (2003) Nat. Rev. Cancer 3, 253–266.

Logarajah S, Hunter P, Kraman M, Steele D, Lakhani S, Bobrow L, Venkitaraman A, Wagner S. BCL-6 is expressed in breast cancer and prevents mammary epithelial differentiation. Oncogene. 2003 Aug 28;22(36):5572-8.

Lowy AM. (2003) Transgenic models of pancreatic cancer.Int J Gastrointest Cancer. 33:71-78.

Lowenfels, A.B., Maisonneuve, P., Cavallini, G., Ammann, R.W., Lankisch, P.G., Andersen, J.R., Dimagno, E.P., Andren-Sandberg, A., and Domellof, L. (1993). Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. N. Engl. J. Med. 328, 1433–1437.

Lu,Y., Fukuyama,S., Yoshida,R., Kobayashi,T., Saeki,K., Shiraishi, H., Yoshimura,A., and Takaesu,G. (2006). Loss of SOCS3 gene expression converts STAT3 function from anti-apoptotic to pro-apoptotic. J. Biol. Chem. 281, 36683–36690.

Luigi Laviola, Annalisa Natalicchio and Francesco Giorgino. The IGF-I Signaling Pathway. Current Pharmaceutical Design, 2007, 13, 000-000.

Madonna,S., Scarponi,C., DePita,O., and Albanesi,C. (2008). Suppressor of cytokine signaling 1 inhibits IFN-gamma inflammatory signaling in human keratinocytes by sustaining ERK1/2 activation. FASEB J. 22, 3287–3297.

Marina Lesina, Magdalena U. Kurkowski, Katharina Ludes, Stefan Rose-John, Matthias Treiber, Akihiko Yoshimura, Wolfgang Reindl, Bence Sipos, Shizuo Akira, Roland M. Schmid, and Hana Algu. Stat3/Socs3 Activation by IL-6 Transsignaling Promotes Progression of Pancreatic Intraepithelial Neoplasia and Development of Pancreatic Cancer. Cancer Cell. DOI 10.1016/j.ccr.2011.03.009.

Mark M. Song and Ke Shuai. The Suppressor of Cytokine Signaling (SOCS) 1 and SOCS3 but Not SOCS2 Proteins Inhibit Interferon-mediated Antiviral and Antiproliferative Activities. 10.1074/jbc.273.52.35056 December 25, 1998. The Journal of Biological Chemistry, 273, 35056-35062.

Masafumi Arima, Takeshi Fukuda, and Takeshi Tokuhisa. Role of the Transcriptional Repressor BCL6 in Allergic Response and Inflammation. WAO Journal 2008;115Y122.

Matsuoka, S. et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316, 1160–1166 (2007).

Murphy, P.M., Tiffany, H.L., 1991. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. Science 253, 1280–1283.

Moldovan GL, D'Andrea AD. 2009. How the fanconi anemia pathway guards the genome. Annu Rev Genet 43: 223–249.

Moser B, Clark-Lewis I, Zwahlen R, Baggiolini M (May 1990). Neutrophil-activating properties of the melanoma growth-stimulatory activity. J. Exp. Med. 171 (5): 1797–802.

Nancy C.Andrews and Douglas V.Faller. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Research, Vol. 19, No. 9. 2499. 1991 Oxford University Press.

Norgauer, J., Metzner, B., Schraufstatter, I. (1996) Expression and growth-promoting function of the IL-8 receptor beta in human melanoma cells. J. Immunol. 156, 1132–1137.

Nieto, J., Grossbard, M. L. & Kozuch, P. Metastatic pancreatic cancer 2008: is the glass less empty? Oncologist 13, 562–576 (2008).

N. Kaur, A. L. Wohlhueter, S. W. Halvorsen. Cell Signaling 14 (2002) 419.

Oh H, Siano B, Diamond S. (2008) Neutrophil isolation protocol. J. Vis. Exp 10:3791

O. Galm, H. Yoshikawa, M. Esteller, R. Osieka, J. G. Herman. Blood 101 (2003) 2784.

Ogata H, Kobayashi T, Chinen T, Takaki H, Sanada T, et al. (2006) Deletion of the SOCS3 gene in liver parenchymal cells promotes hepatitis-induced hepatocarcinogenesis. Gastroenterology 131: 179–193.

Payne AS, Cornelius LA: The role of chemokines in melanoma tumor growth and metastasis, J Invest Dermatol 2002, 118(6):915-922.

Peace,B.E. et al. (2003) Cross-talk between the receptor tyrosine kinases Ron and epidermal growth factor receptor. Exp. Cell Res., 289, 317–325.

Peng Ru, Robert Steele, Eddy C. Hsueh, and Ratna B. Ray. Anti-miR-203 Upregulates SOCS3 Expression in Breast Cancer Cells and Enhances Cisplatin Chemosensitivity. Genes Cancer. Jul 2011; 2(7): 720–727.

Phan RT, Saito M, Basso K, Niu H, Dalla-Favera R. BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. Nat Immunol. 2005 Oct; 6(10):1054-60. Epub 2005 Sep 4.

Pinto AE, André S, Silva G, Vieira S, Santos AC, Dias S, Soares J. BCL-6 oncoprotein in breast cancer: loss of expression in disease progression. Pathobiology. 2009;76(5):235-42. doi: 10.1159/000228899. Epub 2009 Oct 2.

Potratz,J.C. et al. (2010) Synthetic lethality screens reveal RPS6 and MST1R as modifiers of insulin-like growth factor-1 receptor inhibitor activity in childhood sarcomas. Cancer Res., 70, 8770–8781.

Prakash Chinnaiyan, Shyhmin Huang, Geetha Vallabhaneni, Eric Armstrong, Sooryanarayana Varambally, Scott A. Tomlins, Arul M. Hinnaiyan, and Paul M. Harari. Mechanisms of Enhanced Radiation Response following Epidermal Growth Factor Receptor Signaling Inhibition by Erlotinib (Tarceva). Cancer Res 2005; 65: (8). 3328.

Purnima K.Wagh, Belinda E. Peace, and Susan E.Waltz. Met-Related Receptor Tyrosine Kinase Ron in Tumor Growth and Metastasis. Advances in CANCER RESEARCH. DOI: 10.1016/S0065-230X(08)00001-8.

Purvi Mehrotra, Jonathan P. Riley, Ravi Patel, Fang Li, Le'erin Voss, and Shreevrat Goenka. PARP-14 Functions as a Transcriptional Switch for Stat6-dependent Gene Activation. THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 3, pp. 1767–1776, January 21, 2011.

Raman, D., Sobolik-Delmaire, T., Richmond, A., 2011. Chemokines in health and disease. Exp. Cell Res. 317, 575–589.

R. Rottapel, S. Ilangumaran, C. Neale, J. La Rose, J. M. Ho, M H. Nguyen, D. Barber, P. Dubreuil, P. de Sepulveda. Oncogene 21 (2002) 4351.

R.J. Leeman, V.W. Lui, J.R. Grandis. Expert Opin. Biol. Ther. 6 (2006) 231.

Reuter, S., Gupta, S.C., Chaturvedi, M.M., Aggarwal, B.B., 2010. Oxidative stress, inflammation, and cancer: how are they linked? Free Radic. Biol. Med. 49, 1603–1616.

Richmond A, Fa GH, Dhawan P, Yang J: How do chemokine/chemokine receptor activators affect tumorigenesis? Novartis Found Symp 2004, 256:74-89; discussion 89-91, 106-11, 266-9.

R. Starr, T.A. Willson, E.M. Viney, L.J. Murray, J.R. Rayner, B.J. Jenkins, T.J. Gonda, W.S. Alexander, D. Metcalf, N.A. Nicola, D.J. Hilton. Nature 387 (1997) 917.

R. Lang, A.L. Pauleau, E. Parganas, Y. Takahashi, J. Mages, J.N. 1hle, R. Rutschman, P.J. Murray, Nat Immunol. 4 (2003) 546.

Rossa C Jr, Sommer G, Spolidorio LC, Rosenzweig SA, Watson DK, et al. (2012) Loss of Expression and Function of SOCS3 Is an Early Event in HNSCC: Altered Subcellular Localization as a Possible Mechanism Involved in Proliferation, Migration and Invasion. PLoS ONE 7(9): e45197. doi:10.1371/journal.pone.0045197.

S. Mukherjee, S.E. Conrad, J. Biol. Chem. 280 (2005) 17617.

Sasi W, Jiang WG, Sharma A, Mokbel K. Higher expression levels of SOCS 1,3,4,7 are associated with earlier tumour stage and better clinical outcome in human breast cancer. BMC Cancer 10: 178 (2010).

Sawai, H., Funahashi, H., Matsuo, Y., Yamamoto, M., Okada, Y., Hayakawa, T., and Manabe, T. (2003). Expression and prognostic roles of integrins and interleukin-1 receptor type I in patients with ductal adenocarcinoma of the pancreas. Dig. Dis. Sci. 48, 1241–1250.

Schiller JH, Harrington D, Belani CP, et al. Eastern Cooperative Oncology Group. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med. 2002; 346:92–98.

Scholz, A., Heinze, S., Detjen, K.M., Peters, M., Welzel, M., Hauff, P., Schirner, M., Wiedenmann, B., and Rosewicz, S. (2003). Activated signal transducer and activator of transcription 3 (STAT3) supports the malignant phenotype of human pancreatic cancer. Gastroenterology 125, 891–905.

Schreiber, V., Dantzer, F., Ame, J. C., and de Murcia, G. (2006) Nat. Rev. Mol. Cell Biol. 7, 517–528.

Schumacher C, Clark-Lewis I, Baggiolini M, Moser B (November 1992). High- and low-affinity binding of GRO alpha and neutrophil-activating peptide 2 to interleukin 8 receptors on human neutrophils. Proc. Natl. Acad. Sci. U.S.A. 89 (21): 10542–6.

Shields DJ, Murphy EA, Desgrosellier JS, Mielgo A, et al. (2011) Oncogenic Ras/Srccooperativity in pancreatic neoplasia.Oncogene. 30:2123-2134.

Sitko JC, Yeh B, Kim M, Zhou H, Takaesu G, Yoshimura A, McBride WH, Jewett A, Jamieson CA, Cacalano NA. Cell Signal. 2008 20 (12):2221-30.

Singh, R. K., Gutman, M., Radinsky, R., Bucana, C. D., Fidler, I. J. (1994) Expression of interleukin 8 correlates with the metastatic poten- tial of human melanoma cells in nude mice. Cancer Res. 54, 3242–3247.

Singh, R. K., Gutman, M., Reich, R., Bar-Eli, M. (1995) Ultraviolet B irradiation promotes tumorigenic and metastatic properties in primary cutaneous melanoma via induction of interleukin 8. Cancer Res. 55, 3669 –3674.

Schadendorf, D., Moller, A., Algermissen, B., Worm, M., Sticherling, M., Czarnetzki, B. M. (1993) IL-8 produced by human malignant melanoma cells *in vitro* is an essential autocrine growth factor. J. Immunol. 151, 2667–2675.

Siyang Zhang, Dawei Guo, Lili Jiang, Qingfu Zhang, Xueshan Qiu and Enhua Wang. SOCS3 inhibiting migration of A549 cells correlates with PYK2 signaling *in vitro*. BMC Cancer 2008, 8:150.

Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA and Hilton DJ. (1997). Nature 387, 917-921.

Stephen P. Jackson and Jiri Bartek. The DNA-damage response in human biology and disease. Vol 461. 22 Octorber 2009. doi:10.1038. nature08467.

T. Shirogane, T. Fukada, J.M.Muller, D.T. Shima, M. Hibi, T. Hirano, Immunity 11 (1999) 709.

Thomas R, Toney K, Fenoglio-Preiser C, Revelo-Penafiel MP, et al. The RON receptor tyrosine kinase mediates oncogenic phenotypes in pancreatic cancer cells and is increasingly expressed during pancreatic cancer progression. Cancer Res 2007;67:6075–82.

Thomas Greither, Lukasz F. Grochola, Andrej Udelnow, Christine Lautenschla¨ger, Peter Wu¨rl, Helge Taubert. Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. Int. J. Cancer: 126, 73–80 (2010).

Tokita T, Maesawa C, Kimura T, Kotani K, Takahashi K, et al. (2007). Methylation status of the SOCS3 gene in human malignant melanomas. Int J Oncol 30: 689–694.

Tuli, R, A. Surmak Jr., E. C. Ford, E. Tryggestad, J. Wong, T. L. DeWeese, J. M. Herman; Johns Hopkins University School of Medicine, Baltimore, MD Biolluminescene image-guided irradiation and tumor monitoring in a preclinical cancer mouse model. (2011) J Clin Oncol 29: 2011 (suppl 4; abstr 192).

T. Wang, G. Niu, M. Kortylewski. L. Burdelya, K. Shain, S. Zhang, R. Bhattacharya, D. Gabrilovich, R. Heller, D. Coppola, et al. 2004. Regulation of the innate and adaptive immune responses by Stat3- signaling in tumor cells. Nat. Med. 10: 48-54.

T.A. Endo, M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, T. Miyazaki, N. Leonor, T. Taniguchi, T. Fujita, Y. Kanakura, S. Komiya, A. Yoshimura. Nature 387 (1997) 921.

T. Naka, M. Narazaki, M. Hirata, T. Matsumoto, S. Minamoto, A. Aono, N. Nishimoto, T. Kajita, T. Taga, K. Yoshizaki, S. Akira, T. Kishimoto. Nature 387 (1997) 924.

V. Boudny, J. Kovarik. Neoplasma 49 (2002) 349.

Van der Meeren A, Vandamme M, Squiban C, Gaugler MH, Mouthon MA. Inflammatory reaction and changes in expression of coagulation proteins on lung endothe- lial cells after total-body irradiation in mice. Radiat Res 2003;160:637–46.

Weber A, Hengge UR, Bardenheuer W, Tischoff I, Sommerer F, et al. SOCS-3 is frequently methylated in head and neck squamous cell carcinoma and its precursor lesions and causes growth inhibition. Oncogene 24: 6699–6708 (2005).

Wislez, M. et al. Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death. Cancer Res. 63, 1405–1412 (2003).

Y. Lu, S. Fukuyama, R. Yoshida, T. Kobayashi, K. Saeki, H. Shiraishi, A. Yoshimura, G. Takaesu, J. Biol. Chem. 281 (2006) 36683.

Ying, C; Sui-xin, L; Kang-ling, X; Wen-liang, Z; Lei, D; Yuan, L; Fan, Z; Chen, Z. MicroRNA-492 reverses high glucose-induced insulin resistance in HUVEC cells through targeting resistin. Mol Cell Biochem, 2014 vol. 391 pp. 117-25.

Yoshimura A, Suzuki M, Sakaguchi R, Hanada T, Yasukawa H. SOCS, Inflammation, and Autoimmunity. Front Immunol 2012; 3:20; PMID:22566904.

Zhang F, Li C, Halfter H, et al. Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. Oncogene 2003; 22:894-905.

Zhou H, Miki R, Eeva M, Fike FM, Seligson D, Yang L, Yoshimura A, Teitell MA, Jamieson CA, Cacalano NA. Clin Cancer Res. 13 (2007) 2344.

Zhou Y, Zhang J, Liu Q, et al.The chemokine GRO-a (CXCL1) confers increased tumorigenicity to glioma cells. Carcinogenesis 2005; 26:2058(68).