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Paracrine SLPI Secretion Upregulates MMP-9 Transcription and Secretion in Ovarian Cancer Cells

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Abstract

Objectives—Secretory leukocyte protease inhibitor (SLPI) is amplified in serous ovarian cancer. We have dissected its function, showing it is a survival factor for ovarian cancer and promotes tumorigenesis and paclitaxel-resistance. We hypothesized that the protease inhibitory function was responsible for modulating SLPI's invasive capacity.

Methods—Stable HEYA8 ovarian cancer transfectants expressing vector, wild type SLPI, and protease inhibitor null (F-)SLPI were examined in vitro and in xenografts. Invasion, enzyme activity, and MMP production and function assays were applied. SLPI and MMP immunoexpression were graded on tissue microarray and clinical samples. Statistical comparisons used unpaired T test and ANOVA, where appropriate.

Results—SLPI and F-SLPI cells caused greater parenchymal and peritoneal dissemination over control cells in xenografts and invasion assays ($p < 0.001$). MMP-9 protease activity was increased in SLPI and F-SLPI cells over control. SLPI, but not F-SLPI, inhibited plasmin activity, necessary for MMP-9 activation and release, and inhibited activation of MMP-9. However, paradoxically, both induced quantitative MMP-9 transcription ($p < 0.05$) and protein ($p < 0.008$), yielding an increased net MMP-9 activity in the face of plasmin inhibition. SLPI and MMP-9 expression were strongly correlated in serous ovarian cancers ($r^2 = 0.986$) and a set of ovarian cancers ($p < 0.02$). SLPI expression was greater in serous than endometrioid ovarian cancers ($p = 0.04$).

Conclusions—SLPI stimulates ovarian cancer invasion, modulated in part by its serine protease inhibitory activity attenuating MMP-9 release. However, SLPI induction of MMP-9, independent of protease inhibition activity, is greater yielding a net pro-invasive behavior. These findings further support SLPI as a molecular target for ovarian cancer.

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Keywords

ovarian cancer; SLPI; MMP-9; metalloproteinase

INTRODUCTION

Expanding knowledge of ovarian cancer biology has led to improved understanding of the molecular mechanisms of disease and advancement of targeted therapeutics [1, 2]. Tumor, stromal, and vascular cells in the ovarian cancer microenvironment initiate and respond to autocrine and paracrine stimulation with promotion of cell survival and invasive behavior [3]. We have shown that secretory leukocyte protease inhibitor (SLPI), known to be amplified in ovarian cancer [4–8], is a survival factor for ovarian cancer and its increased expression is associated with growth, metastasis, and chemoresistance advantages in vitro and in vivo [9–11]. Increased metastatic activity was found in cells expressing an engineered dominant SLPI mutation that neutralized its protease inhibitory site and activity. We hypothesized that SLPI reduced activity or release of a protease necessary for invasion, and that loss of the protease inhibitory activity of SLPI resulted in augmented invasive capacity and we focused on the most prominent protease in ovarian cancer, matrix metalloproteinase-9 (MMP9).

MMPs have been described as drivers of invasion and angiogenesis in vitro, in vivo, and in patients [12, 13]. MMPs 2 and 9 are activated and released from the cell by the serine protease plasmin. They are secreted in pre-pro forms and then cut into lower molecular weight active forms [14, 15]. Both these MMPs are found on the cell surface in a complex with tissue inhibitor of metalloproteinases (TIMP) 1 and/or 2, and MT1-MMP [16].

SLPI, a small molecular weight secreted whey acidic protein (WAP), is a selective serine protease inhibitor [17]. We and others have shown recombinant and secreted SLPI inhibits elastase activity [10, 11, 18]. Trypsin, chymotrypsin, and cathepsin G have also been described as SLPI substrates [18]. Leu72 is the site required for elastase inhibition [17]. We hypothesized that serine protease inhibition by SLPI would regulate release of free MMPs facilitating ovarian cancer invasive potential. We report that SLPI regulated both production and release of MMP-9, with little effect on MMP-2. MMP release was reduced in cells overexpressing wild type SLPI relative to those expressing the mutant SLPI, consistent with SLPI inhibition of plasmin, reducing MMP-9 membrane release. The correlation between increased SLPI and increased MMP-9 was confirmed in the Gynecologic Oncology Group (GOG) ovarian cancer tissue microarray.

MATERIAL AND METHODS

Cells, transfection, and SLPI silencing

A panel of ovarian cancer cell lines was initially screened for baseline expression of SLPI protein by immunoblot analysis [9, 10]. HEY-A8, a low SLPI expresser, and high expressers SKOV3 ovarian cancer cells (generous gift from Dr. G. Mills, MD Anderson Cancer Center, Houston TX) were selected. HEY-A8 were subjected to stable forced expression of a 3'HA-tagged SLPI, or a Leu72Phe mutation (F-SLPI) and were maintained with G418 as described [10, 11]. SLPI was silenced with SMARTpool SLPI siRNA (Dharmacon, Lafayette, CO) with optimized concentrations and transfection conditions, incubated for 72 hours prior to analyses. A confirmed SMARTpool non-targeting siRNA control was used [9]. Recombinant human SLPI (R&D Systems, Minneapolis, MN) was added to cells as described.

In vivo metastasis

Human ovarian cancer xenograft experiments using the validated SLPI and protease-inhibitor null SLPI mutant series HEY-A8 cells were done under National Cancer Institute Animal Care and Use Committee approval as described [10]. Review of pathology and analysis of the presence of serosal caking and macroscopic serosal liver metastases was done.

Matrigel invasion assay

HEY-A8 SLPI cell series was subjected to Biocoat Matrigel invasion assay according to manufacturer's instructions (Becton Dickinson Biosciences, Bedford, MA). Serum was used as the chemoattractant. Methanol-fixed, invaded cells were stained with crystal violet and number of invaded cells determined. Results presented as background-subtracted mean and SEM of 3 replicate experiments.

Assessment of gelatinase activity

Serum-starved cells were incubated in basal medium with 0.5% ITS (RPMI/ITS) for 24 hours after which conditioned medium (CM) was collected, clarified of cellular debris, aliquoted, and frozen at -80°C until use [19]. CM was separated on 10% gelatin SDS-PAGE gels (Invitrogen, Carlsbad, CA) under non-reducing conditions and zymograms resolved as reported. Zymograms were scanned and activity of MMP-2 and -9 measured by densitometry. MMP-2 and -9 CM concentrations were determined by commercial immunoassay according to manufacturer's protocol (Calbiochem, San Diego, CA). Experiments were repeated at least 3 times.

Quantitative PCR (Q-PCR)

Quantitative PCR was performed to assess MMP gene expression. Total RNA isolated from SKOV3 and HEY-A8 control and transfectants was reversed transcribed and analyzed at the NCI Center for Cancer Research Q-PCR Core Laboratory using established TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA) and calculated using the comparative Ct method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an internal control for normalization. Experiments were at least duplicated and values reflect the calculated average.

Plasmin activity/activation assay

Plasminogen (50 – 500 $\mu\text{g/ml}$) was added to serum-starved ITS-incubated cells for a 4 hour period after which CM was collected for measure of plasmin activity; plasminogen-free CM served as the control. Plasmin activity was measured using a modified Chromozym PL assay (Roche Applied, Mannheim, Germany) with CM (10 μl) added to the plasmin substrate. Substrate cleavage and 4-nitroaniline formation was followed over time at 405nm at 37°C . A standard plasmin curve was generated with purified human plasmin (Roche Applied, Mannheim, Germany). Experiments were repeated at least 3 times.

Clinical samples and tissue microarray analysis

Samples obtained from primary surgery for advanced stage epithelial ovarian cancer from patients treated on a clinical trial of cisplatin, cyclophosphamide, and paclitaxel [20, 21] were stained for MMP-9 and SLPI; specimens and treatment were consented on an National Cancer Institute IRB-approved protocol. The available subset of 18 cases were scored as described [20, 22].

NIH Office of Human Subjects Research approval was obtained prior to use of GOG Tissue Bank tissue microarrays of anonymized epithelial ovarian cancers. These arrays contained

replicate tissue samples from 179 ovarian cancers of endometrioid or serous histology spanning stages I - IV and grades 1–3 (<http://www.biopathologycenter.org/TMA.aspx>). The formalin-fixed paraffin embedded tissue arrays were deparaffinized in xylene followed by graded ethanol solutions, and hydration. Target Retrieval Solution citrate pH6.0 (Dako North America, Carpinteria, CA) and a 20 minute microwave treatment was used for antigen retrieval. Endogenous peroxide was blocked with 3% hydrogen peroxide in PBS for 15 minutes. Slides were incubated with primary antibodies (monoclonal mouse MMP-2 and MMP-9, 1:50, Calbiochem, San Diego, CA) and goat anti-human SLPI (R&D Systems, Minneapolis, MN) at room temperature for 1 hour, washed, exposed to HRP-conjugated secondary and color developed with DAB solution. Gill's hematoxylin was used for counterstain. Similar conditions were used to evaluate SLPI and MMP-2 and -9 expression in samples from HEY-A8 ovarian cancer xenograft specimens from replicate experiments previously reported [10]. Slides were read blinded to the stage and grade (TMAs), or outcome (mouse experiments). Stained slides were imaged with an Aperio CS imaging system utilizing a 20X objective (Aperio, Inc, Vista CA). Images of the TMA were organized in a Spectrum database (Aperio Inc.) and analyzed with a positive pixel count algorithm. The average intensity of the pixels defined as positive staining by the algorithm was utilized as the final output for all stains.

Statistical analyses

Comparative analyses were done with unpaired Student's t test or one-way ANOVA as appropriate to the examination (Excel, Microsoft, Seattle, WA). Mouse experiment results were analyzed by χ -squared analysis. The power trend line analysis was done using Prism (GraphPad Software, La Jolla, CA) to examine the relationship between SLPI and MMP-9 staining of the protocol samples. Image analysis output data were combined with clinicopathologic data provided by GOG within Excel for analysis.

RESULTS

Increasing solid tumor and parenchymal metastases in SLPI and F-SLPI HEY-A8 transfectant xenografts

We previously reported ovarian cancer cells overexpressing SLPI and SLPI null-inhibitor mutants have greater cell growth and survival compared to mock-transfected cells and produced invasive disease with orthotopic xenografts [10]. We showed increased Ki-67 index with overexpression of SLPI and the protease-inhibitor null mutants. Patients with microscopic and small volume residual disease (e.g. millimeter-sized nodules) have been shown to have better overall outcomes [23]. We thus analyzed the spectrum of small peritoneal nodules v. tumor masses in the xenografts finding SLPI overexpression was associated with more and larger tumor nodules and liver metastases (Fig. 1A; $p \leq 0.002$ and ≤ 0.04). Peritoneal surface studding was not SLPI-sensitive. These quantitative differences led us to hypothesize that SLPI expression promotes invasive behavior in ovarian cancer cells.

SLPI expression reduces Matrigel invasion

We sought to determine if SLPI's invasive behavior in vivo could be modeled in vitro. SLPI expressors had statistically significantly less invasion than mock transfectants (ANOVA, $p = 0.0001$; Fig. 1B). Expression of protease-null SLPI reversed the inhibitory effects of SLPI approximately 40% ($p < 0.001$). We theorized reduced invasion in SLPI-expressing cells was due to SLPI neutralization of serine protease activity necessary to release and activate matrix metalloproteinases.

Wild type SLPI inhibits plasmin activity

We began dissection of SLPI effects on invasion by determining its effect on the serine protease plasmin. We demonstrated an approximately 35% reduction of plasmin activity using CM from SLPI-expressing ovarian cancer cells; protease-null SLPI partially abrogated this inhibition, proportionately the same amount as they reversed the inhibition of invasion [10, 11]. Cells cultured in vitro do not inherently produce plasminogen, thus addition of plasminogen during cell conditioning was required. We examined the effect of the added plasminogen at concentrations up to 500ug/ml. Safe and reproducible cell and SLPI function was observed at concentrations up to 200ug/ml for a 4hr period (data not shown). Since WT SLPI CM produced only a 35% reduction of plasmin activity, recombinant human (rh)SLPI also was examined. Figure 2 demonstrates a dose-dependent loss of plasmin cleavage function in the presence of rhSLPI, albeit incomplete in the presence of up to 1ug/ml rhSLPI, suggesting that multiple factors may be involved in regulation of plasmin activity. The decreased invasive activity of the SLPI transfectants can be explained by proportionate SLPI inhibition of plasmin activity; however, elucidation of other potential mechanisms resulting in the invasive in vivo lesions required further investigation.

SLPI increases MMP-9 secretion yet moderates its activation

MMP9 activity is necessary and sufficient to promote invasion in vitro and in vivo [24]. We investigated production and secretion of MMPs 2 and 9, respectively. SLPI overexpression increased MMP-9 quantity and activity demonstrated by zymogram of CM (Fig. 3A). A progressive increase in MMP-9 activity and quantity was observed with wild-type SLPI overexpression and the SLPI mutant cells. Secreted MMP-9 was quantified by ELISA and confirmed the differential SLPI secretion ($p=0.007$, SLPI v. control; $n=4$) and greater concentration from F-SLPI cells (v. control; $p=0.002$, $n=4$; Fig. 3B). Negligible amounts of MMP-2 were observed in CM from all cells (data not shown). Increasing concentrations of rhSLPI, a non-glycosylated bacterial product, did not induce MMP-9 secretion in wild type HEY-A8 cells, suggesting that native and/or intracellular SLPI is required.

MMP-9 activity requires cleavage to its active form. Plasmin cleaves MMP-9 at the cell surface resulting in its activation and release [15]. We proposed modulation of plasmin activity was responsible at least in part for the invasive activity of the protease-null mutant SLPI xenografts. Secretion and activation of MMP-9 from cells exposed to plasminogen was examined. Increase cleaved MMP-9 was observed in a plasminogen dose-dependent manner (Fig. 4). A statistically greater fold change in MMP-9 activation occurred in control and mutant F-SLPI compared to SLPI at 250 μ g/ml plasminogen concentration ($p\leq 0.04$); F-SLPI was statistically different than SLPI overexpressors at the lowest plasminogen concentration tested, 50 μ g/ml ($p=0.01$).

SLPI regulates MMP-9 transcription

We investigated MMP-9 transcription to solve the paradox of increased MMP-9 in the absence of active plasmin cleavage in the wild type SLPI overexpressors. A 225% (± 82) increase in MMP-9 transcription was observed in SLPI transfectants compared with control cells ($p<0.05$; Fig. 5). The role of SLPI was confirmed in the SKOV3 line, a high SLPI producer, by silencing SLPI. Reducing SLPI caused marked and significant reduction in MMP-9 transcription in siSLPI SKOV3 cells compared to control (Fig. 5B).

SLPI and MMP-9 expression are correlated in aggressive papillary serous ovarian carcinoma tissue samples

The xenograft and in vitro findings suggest that SLPI is active in the invasive and aggressive behavior of serous epithelial ovarian cancer. A pilot examination of 18 well annotated

primary surgical specimens from a series of protocol clinical cases showed MMP-9 expression was statistically associated with SLPI quantity ($p=0.02$; Pearson correlation) [20, 22]. We further investigated this relationship using the Gynecologic Oncology Group tissue microarray of anonymized serous and endometrioid epithelial ovarian cancers, containing all stages and grades. SLPI expression was strongly correlated with MMP-9 expression in scoreable TMA spots ($r^2=0.986$; Fig. 6A, B), and despite the small quantities of MMP-2 induced in culture, a relationship between SLPI and MMP-2 was observed in the serous cancer cases ($r^2=0.79$; $n=45$). SLPI expression was statistically significantly greater in serous cancers than endometrioid ($p<0.04$; Fig. 6C). No statistically significant difference in MMP-9 expression was observed between serous and endometrioid cases (Fig. 6D).

DISCUSSION

Invasion of the basement membrane by tumor cells is a hallmark signature of cancer. We reported previously and herein that overexpression of SLPI is associated with increased malignancy and invasiveness, and this occurs independently of the protease inhibition function [10]. We thus hypothesized that the increased invasiveness was due to an imbalance of activity in the local microenvironment as a response to the increased serine protease inhibitor, SLPI. Paradoxically, overexpression of SLPI resulted in more parenchymal invasion and peritoneal nodularity *in vivo*, independent of the protease inhibitor function. The protease inhibitor null SLPI (F-SLPI) cells were more invasive through Matrigel *in vitro* compared with the SLPI overexpressors, but still less invasive than control transfectants. This led us to investigate mechanisms of serine protease-independent invasion. Secreted and recombinant SLPI inhibited plasmin activity but was attenuated by the neutralizing mutation, Leu72Phe (F-SLPI). The contradiction was explained in part by the discovery that both SLPI and F-SLPI induced MMP-9 gene and protein expression, without proportional induction of MMP-9 function. The protease inhibition activity of wild type SLPI reduced plasmin activation and subsequent MMP-9 cleavage and activation. A correlation between SLPI and MMP-9 and MMP-2 expression in ovarian cancer tissues was observed, linking the laboratory findings to clinical behavior. These findings further credential SLPI as a molecular target in epithelial ovarian cancer.

Our results were counterintuitive in several ways. We found the SLPI effects *in vitro* to be contrary to its effects *in vivo* in ovarian and lung cancer xenografts [10, 25]. SLPI is a known inhibitor of multiple serine proteases, and is upregulated during times of stress [18]. Target proteases include neutrophil elastase, cathepsin G, and plasmin, all of which are, or are related to, extracellular matrix proteases. It would be expected that the *in vitro* reduction in invasion would translate *in vivo* to reduced metastatic potential, but that did not occur. Our observations are complemented by other reports. Zhang et al demonstrated SLPI inhibition of MMP-1, -9 and -7 production through the inflammatory PGE_2 –cAMP pathway in monocytes [26–29]. Growth and growth-like factors, oncogenes, hormones, and cytokines transcriptionally regulate MMP-9 in cancer cells [13, 16, 28, 30]. Many of these are induced through the same pro-inflammatory and stress axes that induce SLPI production. This concept was also demonstrated in a wound-healing model where SLPI-null mice had increased elastase activity and increased matrix proteolysis, through loss of regulatory serine protease inhibitory activity [3, 31]. Plasmin and MMPs have been shown to be activate and important in vascular remodeling, suggesting another target for SLPI [32]. Together, these data argue that the *in vivo* effects of SLPI may be important in its identification as a druggable target.

Degradation of the basement membrane occurs by secreted and locally activated proteases; they may be secreted and/or activated by cancer cells and/or activated stromal and inflammatory cells within the tumor [12]. Matrix metalloproteinases are secreted as pre-

zymogens, and require cleavage for activation to the pro and active forms [33, 34]. The serine protease plasmin is a primary protease that cleaves, activates, and releases MMP-9 from the cell membrane [27, 32, 34–36]; it is also a primary target of SLPI [18]. We confirm that both secreted and recombinant SLPI inhibit plasmin activity *in vitro* and that activity is markedly attenuated by the neutralizing mutation Leu72Phe (F-SLPI). However, only the physiologic SLPI moiety produced by and secreted from the wild type and transfected cells stimulated MMP-9, presenting another paradox. This may be explained in that the post-translational modifications of SLPI, such as its recognized glycosylation, may be important for some of its physiologic roles.

As an explanation for the paradoxical increase in invasive behavior *in vivo*, we posited then proved that SLPI had a regulatory affect on MMP quantity through transcriptional upregulation. SLPI has been documented in the nucleus of ovarian cancer cells [9], although SLPI lacks a nuclear localization signal [37]. Possible direct nuclear activities have been reported in inflammatory cells during infection. We have shown that SLPI is both cytoplasmic and near the surface intracellularly, then rapidly secreted in large quantities [9, 11]. How SLPI moves into the nucleus and what its function there may be remains unknown. SLPI may either directly or indirectly induce transcription of MMP-9. This coupled with its extracellular interaction with plasmin regulates MMP-9 activation and release. Thus, the net pro-invasive effect of SLPI on MMP-9 results from both increased gene transcription and protein production and secretion, tempered in part by SLPI inhibition of plasmin activity required to cleave the pro peptide from MMP-9.

The survival effects of SLPI and protease-null SLPI in ovarian cancer are contrary to the SLPI alarm anti-protease activity associated with regulation of inflammation and infection. SLPI has an important role in wound healing, where it tempers physiologic proteolysis [31]. SLPI has been shown to reduce infectivity of viruses, including herpes simplex virus [38], and it is secreted into mucosal and other fluids in addition to its presence in the local microenvironment of cancer and inflammation [18, 39]. The many roles of SLPI, both as an inducible pro-inflammatory cytokine, and as a pro-survival and pro-invasion factor, suggest that modulation of its activity may be therapeutically important in inflammation and infection as well as in ovarian cancer.

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References

1. Annunziata CM, Azad N, Dhamoon AS, Whiteley G, Kohn EC. Ovarian cancer in the proteomics era. *Int J Gynecol Cancer*. 2008; 18:1–6. [PubMed: 18336391]
2. Kohn EC, Azad N, Annunziata C, Dhamoon AS, Whiteley G. Proteomics as a tool for biomarker discovery. *Dis Markers*. 2007; 23:411–7. [PubMed: 18057524]
3. Angelov N, Moutsopoulos N, Jeong MJ, Nares S, Ashcroft G, Wahl SM. Aberrant mucosal wound repair in the absence of secretory leukocyte protease inhibitor. *Thromb Haemost*. 2004; 92:288–97. [PubMed: 15269824]
4. Bingle L, Singleton V, Bingle CD. The putative ovarian tumour marker gene HE4 (WFDC2), is expressed in normal tissues and undergoes complex alternative splicing to yield multiple protein isoforms. *Oncogene*. 2002; 21:2768–73. [PubMed: 11965550]
5. Kobel M, Kalloger SE, Boyd N, McKinney S, Mehl E, Palmer C, Leung S, Bowen NJ, Ionescu DN, Rajput A, Prentice LM, Miller D, Santos J, Swenerton K, Gilks CB, Huntsman D. Ovarian

- carcinoma subtypes are different diseases: implications for biomarker studies. *PLoS Med.* 2008; 5:e232. [PubMed: 19053170]
6. Tanner MM, Grenman S, Koul A, Johannsson O, Meltzer P, Pejovic T, Borg A, Isola JJ. Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. *Clin Cancer Res.* 2000; 6:1833–9. [PubMed: 10815905]
 7. Tapper J, Butzow R, Wahlstrom T, Seppala M, Knuutila S. Evidence for divergence of DNA copy number changes in serous, mucinous and endometrioid ovarian carcinomas. *Br J Cancer.* 1997; 75:1782–7. [PubMed: 9192982]
 8. Tsukishiro S, Suzumori N, Nishikawa H, Arakawa A, Suzumori K. Use of serum secretory leukocyte protease inhibitor levels in patients to improve specificity of ovarian cancer diagnosis. *Gynecol Oncol.* 2005; 96:516–9. [PubMed: 15661245]
 9. Simpkins FA, Devoogdt NM, Rasool N, Tchabo NE, Alejandro EU, Kamrava MM, Kohn EC. The alarm anti-protease, secretory leukocyte protease inhibitor, is a proliferation and survival factor for ovarian cancer cells. *Carcinogenesis.* 2008; 29:466–72. [PubMed: 17916899]
 10. Devoogdt N, Rasool N, Hoskins E, Simpkins F, Tchabo N, Kohn EC. Overexpression of protease inhibitor-dead secretory leukocyte protease inhibitor causes more aggressive ovarian cancer in vitro and in vivo. *Cancer Sci.* 2009; 100:434–40. [PubMed: 19154415]
 11. Rasool N, LaRochelle W, Zhong H, Ara G, Cohen J, Kohn EC. Secretory leukocyte protease inhibitor antagonizes paclitaxel in ovarian cancer cells. *Clin Cancer Res.* 2010; 16:600–9. [PubMed: 20068074]
 12. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature.* 2001; 411:375–9. [PubMed: 11357145]
 13. Curran S, Murray GI. Matrix metalloproteinases in tumour invasion and metastasis. *J Pathol.* 1999; 189:300–8. [PubMed: 10547590]
 14. Corcoran ML, Hewitt RE, Kleiner DE Jr, Stetler-Stevenson WG. MMP-2: expression, activation and inhibition. *Enzyme Protein.* 1996; 49:7–19. [PubMed: 8796994]
 15. Devy L, Noel A, Baramova E, Bajou K, Trentesaux C, Jardillier JC, Foidart JM, Jeannesson P. Production and activation of matrix metalloproteinase-9 (MMP-9) by HL-60 promyelocytic leukemia cells. *Biochem Biophys Res Commun.* 1997; 238:842–6. [PubMed: 9325178]
 16. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem.* 2003; 253:269–85. [PubMed: 14619979]
 17. Eisenberg SP, Hale KK, Heimdal P, Thompson RC. Location of the protease-inhibitory region of secretory leukocyte protease inhibitor. *J Biol Chem.* 1990; 265:7976–81. [PubMed: 2110563]
 18. Williams SE, Brown TI, Roghanian A, Sallenne JM. SLPI and elafin: one glove, many fingers. *Clin Sci (Lond).* 2006; 110:21–35. [PubMed: 16336202]
 19. Kohn EC, Jacobs W, Kim YS, Alessandro R, Stetler-Stevenson WG, Liotta LA. Calcium influx modulates expression of matrix metalloproteinase-2 (72-kDa type IV collagenase, gelatinase A). *Journal of Biological Chemistry.* 1994; 269:21505–11. [PubMed: 8063786]
 20. Annunziata CM, Stavnes HT, Kleinberg L, Berner A, Hernandez LF, Birrer MJ, Steinberg SM, Davidson B, Kohn EC. Nuclear factor kappaB transcription factors are coexpressed and convey a poor outcome in ovarian cancer. *Cancer.* 2010; 116:3276–84. [PubMed: 20564628]
 21. Sarosy GA, Hussain MM, Seiden MV, Fuller AF, Nikrui N, Goodman A, Minasian L, Reed E, Steinberg SM, Kohn EC. Ten-year follow-up of a phase 2 study of dose-intense paclitaxel with cisplatin and cyclophosphamide as initial therapy for poor-prognosis, advanced-stage epithelial ovarian cancer. *Cancer.* 2010; 116:1476–84. [PubMed: 20091841]
 22. Annunziata CM, Kleinberg L, Davidson B, Berner A, Gius D, Tchabo N, Steinberg SM, Kohn EC. BAG-4/SODD and associated antiapoptotic proteins are linked to aggressiveness of epithelial ovarian cancer. *Clin Cancer Res.* 2007; 13:6585–92. [PubMed: 18006758]
 23. Winter WE 3rd, Maxwell GL, Tian C, Carlson JW, Ozols RF, Rose PG, Markman M, Armstrong DK, Muggia F, McGuire WP. Prognostic factors for stage III epithelial ovarian cancer: a Gynecologic Oncology Group Study. *J Clin Oncol.* 2007; 25:3621–7. [PubMed: 17704411]
 24. Moser TL, Young TN, Rodriguez GC, Pizzo SV, Bast RC Jr, Stack MS. Secretion of extracellular matrix-degrading proteinases is increased in epithelial ovarian carcinoma. *Int J Cancer.* 1994; 56:552–9. [PubMed: 8112891]

25. Devoogdt N, Hassanzadeh Ghassabeh G, Zhang J, Brys L, De Baetselier P, Revets H. Secretory leukocyte protease inhibitor promotes the tumorigenic and metastatic potential of cancer cells. *Proc Natl Acad Sci U S A*. 2003; 100:5778–82. [PubMed: 12732717]
26. Belotti D, Calcagno C, Garofalo A, Caronia D, Riccardi E, Giavazzi R, Taraboletti G. Vascular endothelial growth factor stimulates organ-specific host matrix metalloproteinase-9 expression and ovarian cancer invasion. *Mol Cancer Res*. 2008; 6:525–34. [PubMed: 18403633]
27. Farina AR, Coppa A, Tiberio A, Tacconelli A, Turco A, Colletta G, Gulino A, Mackay AR. Transforming growth factor-beta1 enhances the invasiveness of human MDA-MB-231 breast cancer cells by up-regulating urokinase activity. *Int J Cancer*. 1998; 75:721–30. [PubMed: 9495240]
28. Uttamsingh S, Bao X, Nguyen KT, Bhanot M, Gong J, Chan JL, Liu F, Chu TT, Wang LH. Synergistic effect between EGF and TGF-beta1 in inducing oncogenic properties of intestinal epithelial cells. *Oncogene*. 2008; 27:2626–34. [PubMed: 17982486]
29. Zhang Y, DeWitt DL, McNeely TB, Wahl SM, Wahl LM. Secretory leukocyte protease inhibitor suppresses the production of monocyte prostaglandin H synthase-2, prostaglandin E2, and matrix metalloproteinases. *J Clin Invest*. 1997; 99:894–900. [PubMed: 9062347]
30. St-Pierre Y, Couillard J, Van Themsche C. Regulation of MMP-9 gene expression for the development of novel molecular targets against cancer and inflammatory diseases. *Expert Opin Ther Targets*. 2004; 8:473–89. [PubMed: 15469396]
31. Ashcroft GS, Lei K, Jin W, Longenecker G, Kulkarni AB, Greenwell-Wild T, Hale-Donze H, McGrady G, Song XY, Wahl SM. Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. *Nat Med*. 2000; 6:1147–53. [PubMed: 11017147]
32. Lijnen HR. Plasmin and matrix metalloproteinases in vascular remodeling. *Thromb Haemost*. 2001; 86:324–33. [PubMed: 11487021]
33. Noel A, Gilles C, Bajou K, Devy L, Kebers F, Lewalle JM, Maquoi E, Munaut C, Remacle A, Foidart JM. Emerging roles for proteinases in cancer. *Invasion Metastasis*. 1997; 17:221–39. [PubMed: 9876217]
34. Chen JM, Aimes RT, Ward GR, Youngleib GL, Quigley JP. Isolation and characterization of a 70-kDa metalloprotease (gelatinase) that is elevated in Rous sarcoma virus-transformed chicken embryo fibroblasts. *J Biol Chem*. 1991; 266:5113–21. [PubMed: 1848240]
35. Knauper V, Will H, Lopez-Otin C, Smith B, Atkinson SJ, Stanton H, Hembry RM, Murphy G. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *J Biol Chem*. 1996; 271:17124–31. [PubMed: 8663255]
36. Dolo V, D'Ascenzo S, Violini S, Pompucci L, Festuccia C, Ginestra A, Vittorelli ML, Canevari S, Pavan A. Matrix-degrading proteinases are shed in membrane vesicles by ovarian cancer cells in vivo and in vitro. *Clin Exp Metastasis*. 1999; 17:131–40. [PubMed: 10411105]
37. Taggart CC, Cryan SA, Weldon S, Gibbons A, Greene CM, Kelly E, Low TB, O'Neill SJ, McElvaney NG. Secretory leukoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding. *J Exp Med*. 2005; 202:1659–68. [PubMed: 16352738]
38. Fakioglu E, Wilson SS, Mesquita PM, Hazrati E, Cheshenko N, Blaho JA, Herold BC. Herpes simplex virus downregulates secretory leukocyte protease inhibitor: a novel immune evasion mechanism. *J Virol*. 2008; 82:9337–44. [PubMed: 18667508]
39. Weldon S, Taggart CC. Innate host defense functions of secretory leukoprotease inhibitor. *Exp Lung Res*. 2007; 33:485–91. [PubMed: 18075823]

RESEARCH HIGHLIGHTS

- SLPI is overexpressed and functionally important in EOC.
- SLPI causes increased invasive potential of EOC in vivo and in vitro through MMP9.
- Autocrine and paracrine SLPI induces MMP9 transcription and production.

Figure 1. Differential invasiveness with wild type and F-SLPI overexpression

A. Increased invasiveness in vivo with SLPI and F-SLPI: solid tumor nodules and liver metastases ($p \leq 0.002$, and 0.04 , χ^2 , respectively). B. Invasion assays of stable bulk transfectants of empty vector, SLPI, or protease inhibitor null F-SLPI. Invasion was reduced ($p < 0.0001$, ANOVA) in wild type SLPI expressers and less so in F-SLPI. Mean $\pm$ SEM.

Figure 2. SLPI reduces plasmin serine protease inhibition

Chromozym assay was used to assess plasminogen cleavage. A) rhSLPI; B) CM from control, SLPI, and F-SLPI cells. These data are representative of triplicate experiments.

Figure 3. SLPI induces MMP-9 production

HEY-A8 cells incubated in serum-free medium and CM spun free of cells and subjected to gelatin zymography (A) or ELISA quantitation, Mean±SEM (B). A marked increase in MMP-9 production was seen in F-SLPI over SLPI or control.

Figure 4. Reduced activation of MMP-9 in SLPI transfectants exposed to plasminogen
 A. Greater quantities of plasminogen were required in wild type SLPI transfectants in order to see the activation band. Arrows: 92kDa pro form and 88kDa active form. B. MMP-9 activation is inhibited by wild type SLPI. Statistically significantly greater fold change in the activated MMP-9 88kDa form is demonstrated in aggregate data from 4 experiments.

Figure 5. Both wild type and mutant SLPI induce MMP-9 gene expression

Real-time PCR was used to quantitate MMP-9 transcription. A. Significant induction by expression of either SLPI or F-SLPI in HEY-A8 cells. B. Silencing of SLPI in high-SLPI expressing SKOV3 cells is associated with significant inhibition of SLPI message. Inset: SLPI protein expression with scramble or SLPI-targeted siRNA on cellular (lysate) and secreted (CM) SLPI.

Figure 6. SLPI expression correlates with MMP-9 expression in serous epithelial ovarian cancer tissue samples

Protein expression was evaluated by immunohistochemistry in serial recuts of the GOG serous ovarian cancer TMA. Tissue staining was evaluated as described in Methods. A) Photomicrographs of immunohistochemistry: a) SLPI; b) MMP-2; c) MMP-9; d) Negative Control. All images 125X magnification. B) SLPI expression is directly correlated with MMP-9 expression (pink) and MMP-2 expression (blue) in serous ovarian cancer. C, D Expression of SLPI (C) and MMP-9 (D) is greater in serous ovarian cancer than endometrioid ovarian cancer.