A Putative Role for Psoriasin in Breast Tumor Progression

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Abstract

Psoriasin (S100A7) was identified as a gene highly expressed in psoriatic keratinocytes and highly and more frequently expressed in ductal carcinoma in situ (DCIS) than in invasive breast carcinomas (IBC), suggesting a potential role in tumor progression. Psoriasin expression is associated with poor prognostic factors in both DCIS and IBC. Several putative functions have been proposed for psoriasin in various disease types, but none of these can fully explain its involvement in breast tumor progression. Here, we show that down-regulation of endogenous psoriasis expression via stable short hairpin RNAs in a human IBC cell line (MDA-MB-468) increases cell migration and invasion without influencing cell proliferation and survival in vitro but inhibits tumor growth in vivo. These seemingly paradoxical results are potentially explained by the dramatic up-regulation and down-regulation of matrix metalloproteinase-13 and vascular endothelial growth factor (VEGF), respectively, observed in cells with decreased psoriasin levels compared with controls. Correlating with this, high psoriasis expression in human IBC is associated with increased angiogenesis and worse clinical outcome, and psoriasin mRNA levels are coordinately regulated with VEGF and other genes related to hypoxia and mitochondrial reactive oxygen species (ROS). Based on these results, we propose that psoriasin may play a role in breast tumor progression by promoting angiogenesis and enhancing the selection for cells that overcome its anti-invasive function. This hypothesis may explain why psoriasis expression is highest in high-grade and/or estrogen receptor-negative tumors, as these are associated with increased hypoxia and ROS, a setting in which the angiogenic effects of psoriasis are most important. (Cancer Res 2005; 65(24): 11326-34)

Introduction

Psoriasin (S100A7) is a small calcium binding protein of the S100 protein family that was originally identified as one of the most abundant proteins in psoriatic keratinocytes (1). Subsequent studies showed that psoriasin expression is up-regulated in abnormally differentiating keratinocytes, squamous carcinomas of different organs, and in a subset of breast tumors (2–6). We and others also identified psoriasin as one of the few genes that is highly and more frequently expressed in ductal carcinoma in situ (DCIS) than in invasive breast carcinomas, suggesting a potential role in tumor progression (3, 6–9). Other members of the S100 gene family have also been shown to be associated with breast tumor progression; most notably, S100A4 has been implicated in promoting cell invasion and metastasis, and correlating with this, its expression is associated with worse outcome in breast cancer (10–15).

In human invasive breast carcinomas, the expression of psoriasin has been shown to correlate with unfavorable histopathologic features, including estrogen receptor (ER) negativity and poor clinical outcome (4, 7, 9). Similarly, in in situ carcinomas, psoriasis expression is more frequent in high nuclear grade, ER-negative, comedo DCIS with higher risk of local recurrence (16).

Psoriasis protein was found to be secreted but also present in the cytoplasm and the nuclei of cells expressing it (4, 7, 17, 18). Correlating with its secreted nature, there is evidence that psoriasin can function as a chemotactic factor for CD4+ lymphocytes in the skin, and more recently, it has been implicated in the antibacterial defense mechanism of the skin (18, 19). In MCF-10A human immortalized mammary epithelial cells, the expression of psoriasin is undetectable in exponentially growing cells but is dramatically up-regulated by growth factor deprivation, loss of attachment to extracellular matrix, and prolonged confluence (7). Because all these conditions influence cell proliferation and survival, psoriasis may play a role in the regulation of these pathways. Supporting this hypothesis, psoriasis was found to interact with JAB1, a component of the COP9 signalosome that is involved in multiple signal transduction pathways, including the regulation of E3 ubiquitin ligases and the JUN/AP1 transcription factors (20). Overexpression of psoriasin in MDA-MB-231 breast cancer cells was shown to influence the intracellular distribution and activity of JAB1 and enhance tumorigenesis and metastasis (20). In a yeast two-hybrid screen, psoriasis was also found to interact with RanBP, a RAN-GTP binding protein localized to the centrosomes, but the physiologic relevance of the association of psoriasis with RanBP is undefined (21).

We sought to address the function of psoriasin in human breast cancer by down-regulating its expression in the MDA-MB-468 cell line using stable short hairpin RNA (shRNA). We and others have previously determined that MDA-MB-468 cells have very high endogenous levels of psoriasis protein and mRNA even when grown in standard culture conditions (7, 8, 20). Compared with control MDA-MB-468 cells, stable pools of clones with a near complete lack of psoriasis expression showed no difference in cell proliferation and survival but had enhanced soft agar growth, migration, and invasion in vitro and decreased tumorigenicity in nude mice. Correlating with these phenotypic changes, cells expressing psoriasis shRNA had...
increased and decreased expression of matrix metalloproteinase-13 (MMP-13) and vascular endothelial growth factor (VEGF), respectively, both in vitro and in vivo. Moreover, expression of psoriasin in primary breast tumors was associated with increased angiogenesis and poor clinical outcome.

Materials and Methods

Tissue microarrays and immunohistochemical analyses. Tissue microarrays (TMA) were purchased from Imgenex (San Diego, CA) or constructed at the University of Michigan Cancer Center as described previously (22). Immunohistochemical analyses of psoriasin, MBI1 (Ki67), and CD31 expression were done essentially as described (9) using monoclonal anti-psoriasin (7), anti-MBI1 (DakoCytomation, Carpinteria, CA), and anti-CD31 (clone JC70A, DakoCytomation) antibodies. On the TMA with clinical follow-up data, psoriasin expression was scored using a previously validated scale as negative, weak, moderate, or strong (22). The TMA from Imgenex (50 breast tumors) with CD31 and psoriasin staining was scanned using an ACIS II (version 2) imaging system (Chromavision Medical Systems, Inc., San Juan Capistrano, CA). Quantitation of staining intensity and calculation of the positively stained area/tumor were done essentially as described (23). Briefly, the intensity of the staining (brown area) was determined using proprietary software and compared with the counterstain used as background. Based on this calculation, we set up positive-negative thresholds, quantitated the stained/ unstained areas, and calculated the percent staining using this formula: (stained area / (stained + unstained area), for each spot on the TMA.

The TMA from Imgenex (50 breast tumors) with CD31 and psoriasin staining was determined using proprietary software and analyzed using real-time PCR and the LightCycler instrument (Roche Applied Science, Indianapolis, IN), together with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Indianapolis, IN), to ensure high efficiency of transfection. Stable clones expressing the short hairpin RNA constructs were selected using the compound was added at a concentration of 100 μM/L in both the top and bottom wells of the assay at the time the cells were added. All of the above-described experiments were repeated at least thrice.

Mouse xenograft experiments. Exponentially growing MDA-MB-468 pLKO-puro and Pso-RNAi-1-3 cells were harvested, and 2.5 × 106 cells per well and assayed 36 hours later, whereas for migration assays, we used 2.5 × 105 cells per well and determined cell numbers 12 hours later. To test the effect of the MMP-13 inhibitor CL-82198 (Calbiochem, San Diego, CA), the compound was added at a concentration of 100 μM/L in both the top and bottom wells of the assay at the time the cells were added. All of the above-described experiments were repeated at least thrice.

The xenograft experiments were repeated twice with essentially the same results.

RNA extraction, cDNA synthesis, and quantitative real-time PCR. Total RNA was prepared from cultured cells and tumor xenografts using the RNeasy Mini kit (Qiagen, Valencia, CA) and a guanidium/cesium chloride protocol (8), respectively, whereas cDNA was synthesized using SuperScriptII RNase H-Reverse Transcriptase (Invitrogen) as recommended by the manufacturer. The relative expression of VEGF and MMP-13 in MDA-MB-468 pLKO-puro and Pso-RNAi-2 cells and the xenografts derived from them were analyzed using real-time PCR and the LightCycler instrument (Roche Applied Science, Indianapolis, IN), together with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science), according to the manufacturer's instructions. Primers for VEGF and MMP-13 were designed by OligoPerfect Designer (Invitrogen) and verified by them were analyzed using real-time PCR and the LightCycler instrument (Roche Applied Science, Indianapolis, IN), together with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science), according to the manufacturer's instructions. Primers for VEGF and MMP-13 were designed by OligoPerfect Designer (Invitrogen) and verified by in silico PCR (http://www.urogene.org). Primers for S100A7 and β-actin were as previously described (4, 26). Two microliters of cDNA were added to 18 μL of PCR Master mixture [including 3 mmol/L of MgCl2, 0.5 mmol/L of each of S100A7, β-actin, and MMP-13: and 0.6 mmol/L of VEGF (primers) and 2 μL of LightCycler Fast Start Master SYBR Green], the protocol consisted of a 10-minute denaturation step at 95°C and 40 to 45 cycles of amplification at 95°C for 10 seconds; at 56°C and 58°C (S100A7 and β-actin) for 5 seconds; and 72°C for 8 seconds (β-actin and MMP-13), 9 seconds (VEGF), and 10 seconds (S100A7), respectively. After amplification, melting curves were obtained to verify the specificity of the amplification reaction, and the products were also run on a 2% agarose gel. Quantitative analysis was done with the LightCycler software version 3.5 (Roche Applied Science), which estimates the crossing point for each sample. The crossing point values determined for MMP-13, VEGF, and S100A7 were normalized to β-actin (endogenous control). The relative expression of all three genes in the Pso-RNAi-2 cells was determined in relation to the expression in the control pLKO-puro cells. Data is expressed as means ± SD.

Statistical analyses. To study the association between psoriasin expression and cellular phenotype in vitro, experimental groups were compared with control with t test. For the mouse xenograft experiments, because the weights of the two tumors in a single mouse were correlated, the sum of the two tumor weights was used as the end point. Two groups of animals were compared using the exact Wilcoxon rank sum test, stratified by experiment when the data from the two experiments were combined.
More than two groups were compared using the approximate Kruskal-Wallis test. To explore the relationship between psoriasin expression and patients’ characteristics, contingency tables were generated, and Fisher’s exact test was done for each covariate on available data without imputing for missing data. To compare clinical outcomes of survival and time to tumor recurrence between patients with psoriasin expressed and unexpressed, the log-rank test was done. Nominal *P* values are reported, without adjustment for multiple comparisons. K-nearest neighbor (KNN) analysis of breast cancer gene expression data to identify genes coexpressed with psoriasin was done as previously described (27, 28).

**Results and Discussion**

**Generation of cells expressing psoriasin short hairpin RNA.** Previous studies analyzing the function of psoriasin in breast cancer used a cell line exogenously overexpressing psoriasin (20). Because exogenous overexpression can lead to nonphysiologic expression levels and the results could be influenced by the choice of cell type, eliminating the expression of a gene in the cell that expresses it endogenously is more likely to yield physiologically relevant results. Thus, to dissect the functional relevance of psoriasin expression in breast cancer, we derived stable clones from the MDA-MB-468 human breast cancer cell line that expressed control or psoriasin shRNA. We designed three different shRNAs corresponding to different areas of the psoriasin mRNA. Pso-RNAi-1 targets the 5' untranslated region, whereas Pso-RNAi-2 and Pso-RNAi-3 target the proximal and distal portion of the coding region, respectively. In addition, we also designed shRNAs corresponding to CXCL12 and CXCL14 as controls, because neither of these genes are expressed in MDA-MB-468 cells at levels detectable by Northern blot analysis (29). All of these shRNAs were subcloned into the plKO-puro construct and used for the establishment of stable clones. The effectiveness of the shRNAs was confirmed by immunoblot analysis of cell extracts prepared from pools of cells following selection. Pso-RNAi-2 and to a lesser degree Pso-RNAi-1 dramatically down-regulated psoriasin protein levels, whereas Pso-RNAi-3 and the control CXCL12 and CXCL14 shRNAs had no significant effect on psoriasin expression (Fig. 1A). Because we derived pools of stable clones to avoid clonal selection, we also did immunocytochemical analysis of the cells to determine intercellular heterogeneity within the pools. We found that the expression of psoriasin was uniformly down-regulated in the Pso-RNAi-1 and Pso-RNAi-2 cells, whereas the plKO-puro and Pso-RNAi3 and control shRNA cells all expressed high levels of psoriasin (Fig. 1B; data not shown).

**Effect of psoriasin on cell proliferation, survival, migration, and invasion in vitro.** Next, we analyzed the association between psoriasin expression and cellular behavior in vitro using the above-described stable pools. We found no significant difference between the proliferation of control plKO-puro and Pso-RNAi-1-3 cells (Fig. 1C). Similarly, there was no significant difference in the sensitivity of control and psoriasis shRNA-expressing cells to various apoptosis-inducing stimuli, including chemotherapeutic agents, serum and glucose deprivation, and oxidative stress induced by menadione and hydrogen peroxide (data not shown).

However, we observed statistically significantly increased colony numbers in soft agar assays using the Pso-RNAi-1 (P = 0.01) and Pso-RNAi-2 (P = 0.0001) cells compared with controls, suggesting that down-regulation of psoriasin in MDA-MB-468 cells enhances anchorage-independent growth (Fig. 1D). The influence of psoriasin expression on cell migration and invasion was assessed using a modified Boyden chamber assay (25). There was also a consistent and significant increase in the motility and invasion of cells with decreased psoriasin levels, suggesting that psoriasin may have a negative effect on cell migration and invasion (Fig. 1E and F). Interestingly, overexpression of psoriasin in MDA-MB-231 cells was reported to increase cell proliferation, motility, and invasion, although these effects were very modest in vitro (1.2- to 1.5-fold difference compared with controls; ref. 20). Similarly, overexpression of S100A4 in mouse mammary epithelial cells derived from neu-transgenic mice leads to increased motility and invasion, but this was not correlated with increased proteolytic degradation of the extracellular matrix (30). Although we do not know the reason for the apparent discrepancy between our and previously reported results, the use of different cell types and exogenous overexpression instead of down-regulation of endogenous psoriasis expression could explain the observed differences in the in vitro behavior of the cells.

During breast tumor development, one of the most critical transitions is the progression from in situ to invasive carcinoma. Immunohistochemical analyses of adjacent DCIS and invasive breast tumors show decreased psoriasin expression in the invasive areas, suggesting that down-regulation of psoriasin may play a role in the in situ to invasive carcinoma progression (4, 6, 31) potentially by relieving psoriasis-mediated inhibition of migration and invasion of the cancer cells. Our results showing increased migration, invasion, and soft agar growth following down-regulation of psoriasin in MDA-MB-468 cells support this hypothesis. However, because the MDA-MB-468 cells were derived from a metastatic breast carcinoma, our results may not be directly interpretable for DCIS tumors. Thus, understanding the function of psoriasin in the in situ to invasive carcinoma progression requires further studies.

**Effect of psoriasin on in vivo tumorigenicity in nude mice.** To determine if psoriasin expression also correlates with invasion and cell growth in vivo, control plKO-puro and Pso-RNAi-1, Pso-RNAi-2, and Pso-RNAi-3 cells were injected s.c. into the flank of female nude mice, and tumor growth and metastasis were assessed. Control plKO-puro and Pso-RNAi-3 [ineffective RNA interference (RNAi)] cells generated tumors at nearly all injection sites (17 of 20 and 9 of 10, respectively) after 10 weeks of growth, and these tumors were first noted at 2 to 3 weeks after injection. In mice injected with Pso-RNAi-1 and Pso-RNAi-2 cells, several injection sites did not result in tumors or developed tumors that were barely measurable (only 6 of 20 injections resulted in measurable size tumors). These tumors also appeared somewhat later after injection (3–4 weeks) and grew much slower than the controls (Fig. 2A). Analysis of tumor weight at 10 weeks after injection revealed that down-regulation of psoriasin in the Pso-RNAi-1 and Pso-RNAi-2 cells dramatically reduced tumorigenicity (Fig. 2B). The mean tumor weight combined from two independent experiments in the control plKO-puro group was 0.19 g, whereas in Pso-RNAi-1 and Pso-RNAi-1 groups was 0.05 and 0.07 g, respectively. Wilcoxon rank sum test determined that both of these experimental groups were statistically significantly different from controls (P = 0.05 and P = 0.05 for Pso-RNAi-1 and Pso-RNAi-2, respectively). Tumors derived from cells with the ineffective psoriasin (Pso-RNAi-3) or irrelevant control (CXCL12) shRNA did not differ significantly from the plKO-puro controls (Fig. 2A and B; data not shown).

Microscopic examination of the tumors resulting from the different experimental groups did not reveal differences in histologic appearance, although the larger tumors in the control and
Pso-RNAi-3 groups tended to have more extensive necrotic areas (data not shown). Immunohistochemical analysis of psoriasin expression in the tumors confirmed the stable down-regulation of psoriasin in the Pso-RNAi-1 and Pso-RNAi-2 cells and uniformly high psoriasin levels in pLKO-puro and Pso-RNAi-3 cells (Fig. 2C). Staining for MIB1 (Ki67) expression, a marker of proliferating cells, determined that all tumors were very highly proliferative irrespective of the expression of psoriasin (Fig. 2D). This result correlates with the results of our in vitro cell proliferation assays that also did not reveal differences among the different groups (Fig. 1C).

Figure 1. Generation and characterization of MDA-MB-468 cells expressing psoriasin shRNAs. A, immunoblot analysis of psoriasin expression in control MDA-MB-468 pLKO-puro, three different psoriasin shRNA-expressing (Pso-RNAi-1, Pso-RNAi-2, and Pso-RNAi-3), and two control shRNA (CXCL12 and CXCL14) cells. RNAi-3 did not effectively knock down psoriasin protein levels, whereas RNAi-1 and RNAi-2 almost completely diminished psoriasin expression. Extracts were probed for β-tubulin expression to indicate equal loading. B, immunocytochemical analysis of psoriasin expression in control MDA-MB-468 pLKO-puro and three different psoriasin shRNA-expressing cells. This examination confirmed the Western blot results and also indicated that the stable pools of cells are homogeneously positive (pLKO-puro and Pso-RNAi-3) or negative (Pso-RNAi-1 and Pso-RNAi-2) for psoriasin expression. C, results of a representative in vitro proliferation assay showing no statistically significant differences among the growth rates of control pLKO-puro and Pso-RNAi-1, Pso-RNAi-2, and Pso-RNAi-3 cells. Points, mean cell counts in three independent wells; bars, SD. Experiment was done more than three times with identical results. D, down-regulation of psoriasin expression leads to increased soft agar growth in MDA-MB-468 cells. Quantitative summary of the result of a representative soft agar assay. Columns, mean colony counts; bars, SD. P = 0.01, Pso-RNAi-1 and P = 0.0001, Pso-RNAi-2 cells compared with controls (statistically significant differences). One representative experiment done in triplicate. Experiment was repeated thrice with identical results. Down-regulation of psoriasin expression increases cell migration (E) and invasion (F). Columns, means of a representative experiment done in triplicate; bars, SD. Ps of statistically significant differences compared with pLKO-puro cells. Experiment was repeated thrice with the same results.
We also assessed if the expression of psoriasin influences metastatic behavior; however, we did not see grossly evident lung and other major organ metastases in any of the experimental groups. The MDA-MB-468 cell line is not highly metastatic, and the tumors were only allowed to grow to a relatively small size and short time (10 weeks). However, analysis of lung micrometastases using reverse transcription-PCR (RT-PCR) and primers specific for human DNA (hHPRT gene) revealed micrometastasis in all mice in the control but not in the Pso-RNAi-1 and Pso-RNAi-2 groups (data not shown). This result is in agreement with prior studies showing increased number of abdominal lymph node metastases in mice injected with psoriasin overexpressing MDA-MB-231 cells compared with controls (20).

Effect of psoriasis on the expression of vascular endothelial growth factor and matrix metalloproteinase-13. To begin to dissect the molecular bases of the observed in vitro and in vivo phenotypic differences between control and psoriasin shRNA-expressing cells, we analyzed the expression of genes involved in angiogenesis and invasion. Previous data suggested an association between psoriasin expression and VEGF and MMP-13 mRNA levels in human breast cancer cells (20). Specifically, exogenous overexpression of psoriasin in MDA-MB-231 cells led to increased activator protein-1 (AP-1) transcriptional activity and subsequent increase in the RNA levels of two of its target genes VEGF and MMP-13 (20). To determine if such association also exists in our experimental system, we analyzed the expression of VEGF and MMP-13 by real-time RT-PCR in exponentially growing control and Pso-RNAi-2 cells cultured in vitro. We also analyzed the expression of psoriasin itself as a control for the assay and ACTB as a reference gene. We confirmed the downregulation of psoriasin mRNA in the Pso-RNAi-2 cells compared with control pLKO-puro cells validating the reliability of the assay.

Figure 2. Effect of psoriasis expression on tumor growth in nude mice. A, representative mice at 10 weeks after injection from each experimental group. Compared with the control pLKO-puro and Pso-RNAi-3 groups, several mice in the Pso-RNAi-1 and Pso-RNAi-2 groups lacked visible tumors. B, columns, mean tumor weight of each experimental group of mice combined from two independent experiments (20 injections total); bars, SD. In each experiment, five mice per group were used, and each mouse was injected at two sites. Down-regulation of psoriasis expression in the Pso-RNAi-1 and Pso-RNAi-2 cells significantly diminished tumor size. P values of statistically significant differences compared with tumors generated from pLKO-puro cells. C, immunohistochemical analysis of psoriasis expression in the xenografts confirmed the stability and effectiveness of the shRNAs. D, immunohistochemical analysis of MIB-1 (Ki67) expression in the xenografts did not reveal significant differences in the proliferation rates of the cells, consistent with the results of the in vitro growth assays.
and also the stability of the clones and the efficiency of shRNA (Fig. 3A). Correlating with previous data, we found statistically significant ($P = 0.02$) down-regulation of VEGF in the Pso-RNAi-2 cells compared with controls; however, the expression of MMP-13 was statistically significantly ($P = 0.02$) increased (Fig. 3A). Although the up-regulation of MMP-13 is the opposite of that expected based on prior results, it is consistent with our observation that the invasiveness of the Pso-RNAi-2 cells is increased compared with controls (Fig. 1F). To better understand the contribution that the induction of MMP-13 expression may make to the migration and invasion of the MDA-MB-468 cells, we examined the effect of a specific inhibitor of MMP-13 in the in vitro migration/invasion assay (32). We found that the MMP-13 inhibitor had no appreciable effect on the migration of the

Figure 3. Effect of psoriasin levels on the expression of VEGF and MMP-13. A, real-time PCR analysis of the expression of psoriasin, VEGF, and MMP-13 in control pLKO-puro (black column) and Pso-RNAi-2 (white column) cells. Expression of MMP-13 was statistically significantly ($P = 0.02$) increased, whereas that of VEGF was decreased ($P = 0.02$) in Pso-RNAi-2 cells compared with controls. Expression of psoriasin was also analyzed and was significantly decreased in the Pso-RNAi-2 cells ($P = 0.04$). Each sample was analyzed in duplicate in three independent PCR reactions. Values obtained in control pLKO-puro cells were designated as 1, and values corresponding to Pso-RNAi-2 cells were normalized to this from the same run. Columns, means; bars, SD. Ps were determined using paired Student's $t$ test following normalization to $\beta$-actin levels. B, effect of a specific MMP-13 inhibitor (CL-82198) on the migration and invasion of control (pLKO-puro) and psoriasin RNAi (Pso-RNAi-2) cells. Inhibitor had no effect on the migration of the cells nor did it influence invasion of control cells. However, a significant reduction in invasion was detected in the psoriasin RNAi cells. Columns, mean cell counts in three independent wells; bars, SD. Experiment was done more than three times with identical results. Ps of statistically significant differences compared with pLKO-puro and untreated Pso-RNAi-2 cells. C, real-time PCR analysis of the expression of VEGF and MMP-13 in two representative independent tumors derived from control pLKO-puro (T1 and T2) and Pso-RNAi-2 (T3 and T5) cells. PCR reactions and calculations were done as described above, except that pLKO-puro-T1 was used as reference. VEGF levels were statistically significantly lower in both Pso-RNAi-2 tumors (T3: $P = 0.005$ and T5: $P = 0.03$) compared with control, whereas MMP-13 levels were statistically significantly increased (T3: $P = 0.03$ and T5: $P = 0.001$). The two control pLKO-puro tumors (T1 and T2) were not statistically significantly different from each other for the expression of these two genes (VEGF: $P = 0.2$ and MMP-13: $P = 0.3$). D, immunohistochemical analysis of CD31 endothelial cell marker expression to assess the degree of angiogenesis in a representative set of three ductal carcinomas. The same tumors were also analyzed for the expression of psoriasin. There was a positive correlation between CD31 and psoriasin expression. Top, two tumors show high psoriasin expression and high blood vessel density; bottom, psoriasin-negative tumor is largely CD31 negative. E, relationship between psoriasin expression and clinical outcome. Kaplan-Meier survival curve depicting association between psoriasin expression and death due to breast cancer in 108 patients. Patients with psoriasin-positive tumors (red line) had shorter disease-specific survival than patients whose tumors were negative for psoriasin (green line), but this was of borderline statistical significance ($P = 0.05$).
pLKO-puro or Pso-RNAi-2 cells (Fig. 3B). However, inhibition of MMP-13 led to a significant ($P = 0.003$) reduction in the invasive capacity of the Pso-RNAi-2 cells while having little effect on that of the pLKO-puro cells (Fig. 3B). This provides further support for the hypothesis that up-regulation of MMP-13 plays a role in the increased invasive ability of the Pso-RNAi-2 cells. The differences observed between the present results and those of a prior study (20) may be attributable to differences in the techniques used; the published report overexpressed psoriasin in a cell line that does not normally make detectable levels of the protein. In contrast, here, shRNA was used to knockdown psoriasin expression in a cell line with high endogenous protein high levels. In general, a loss-of-function approach is felt to provide a more physiologic read out of a protein's function than that derived from overexpression.

To determine if the differences between control and Pso-RNAi-2 cells in VEGF and MMP-13 levels are maintained during in vivo tumor growth, we repeated the real-time PCR analysis using two representative tumor samples from both control and Pso-RNAi-2 groups. Analysis of the tumor samples gave identical results to what we found in the cells cultured in vitro. Tumors generated from the Pso-RNAi-2 cells showed statistically significantly decreased VEGF and increased MMP-13 mRNA levels (Fig. 3C).

Thus, the decreased VEGF levels could be partially responsible for the decreased tumor take and growth observed following the injection of the Pso-RNAi-2 cells. Interestingly, psoriasin itself seems regulated by reactive oxygen species (ROS) because its expression is increased in cells treated with a low concentration of hydrogen peroxide in MCF10A mammary epithelial cells (33). Moreover, the induction of its expression by detachment from the extracellular matrix can be completely eliminated by antioxidant treatment or bcl-2 overexpression or by inhibiting nuclear factor-kB activation (33). Because hypoxic conditions lead to increased ROS levels, psoriasin may play a role in the up-regulation of VEGF and induction of angiogenesis under these conditions. Alternatively, psoriasin and VEGF may be regulated by the same transcriptional mechanisms. High-grade comedo DCIS tumors, which frequently overexpress psoriasin, have high rates of hypoxia, apoptosis, and necrosis, potentially causing the up-regulation of psoriasin in these tumors (34). Overexpression of psoriasin subsequently may increase the levels of VEGF and enhance angiogenesis. Correlating with this hypothesis, high-grade comedo DCIS is associated with increased angiogenesis (35). To further explore the physiologic function of psoriasin in human breast cancers, we analyzed its expression in microarray data from 89 invasive breast carcinomas (27), and using a KNN algorithm (28), we identified genes whose expression most highly correlated with the expression of psoriasin (Table 1). A surprisingly high fraction (26%) of these 50 highest ranked genes is localized to chromosome 1q, a chromosomal arm where psoriasin itself and an S100 gene cluster are located, potentially suggesting coregulation of the genes or underlying genetic change. Gain of 1q is one of the most common and earliest genetic events in breast cancer (36). Confirming previous data showing coregulation of psoriasin with S100A9 and other S100 proteins (33), S100A9 and S100A8 were most closely correlated with psoriasin expression (Table 1). Interestingly, VEGF itself was identified by this analysis, further strengthening the association between psoriasis and VEGF expression. In addition, a significant fraction (26%) of the 50 genes represented mitochondrial proteins involved in energy metabolism. This suggests that these genes are coordinately regulated with psoriasin in invasive breast cancers and provides further evidence linking psoriasin to ROS and angiogenesis.

**Psoriasin expression in human primary breast carcinomas.** The decreased VEGF levels in MDA-MB-468 cells with decreased psoriasin levels and the high expression of psoriasin in high-grade, comedo DCIS associated with increased VEGF levels and blood vessel density (35) suggested that psoriasin may play a role in angiogenesis. To determine if there is an association between psoriasis expression and angiogenesis in human breast tumors, we did immunohistochemical analysis of psoriasin and CD31 (an endothelial cell-specific marker) expression on a tissue microarray composed of 49 invasive breast carcinomas and 10 normal breast tissue and fibroadenomas. Based on this analysis, we found a statistically significant ($P = 0.02$) positive correlation between psoriasin expression and blood vessel density (determined by quantitating the intensity and fraction of CD31-positive cells/tissue spot). Representative psoriasin and CD31 staining results are depicted in Fig. 3D.

To analyze the relationship between psoriasis expression and clinical outcome in breast cancer, we did immunohistochemical analysis of a tissue microarray composed of 138 cases of invasive breast tumors with clinical follow-up data. The median follow-up was 138 months; a detailed description of the patient cohort has been previously reported (37). We examined the association between psoriasin expression and various tumor characteristics, including the expression of estrogen and progesterone receptors (ER and PR) and HER2, size of tumor, total number of positive lymph nodes, clinical stage, tumor grade, presence of angioinvasion, and age of patients. We found that the expression of psoriasin was statistically significantly more frequently observed in ER-negative ($P = 0.00006$), PR-negative ($P = 0.000002$), HER2-positive ($P = 0.02$), and high-grade (0.001) tumors. In addition, patients with psoriasin-positive tumors were more likely to be under 50 years of age ($P = 0.01$). There was no statistically significant association between the expression of psoriasin and other tumor and patient characteristics (data not shown).

Analysis of time to recurrence suggested that patients with tumors that lacked psoriasis expression survived longer than patients with psoriasis positive tumors, but this did not reach statistical significance ($P = 0.06$; Fig. 3E). Similarly, investigation of the association between psoriasis expression and overall survival revealed a positive association between psoriasis expression and decreased survival, but this was of borderline statistical significance ($P = 0.05$). This result is consistent with previous studies showing association between poor clinical outcome and psoriasis expression in invasive breast carcinomas (38).

In summary, here, we report that down-regulation of psoriasis in invasive breast cancer cells increases anchorage-independent growth, cell motility, and invasion in vitro, while decreasing tumorigenicity in vivo. These effects are potentially due to decreased VEGF and increased MMP-13 levels observed following down-regulation of psoriasis expression. Consistent with these results, we observed that psoriasis expression is associated with increased blood vessel density in human breast cancer. Based on these findings, we propose that modulation of psoriasis expression and/or function is an important step in the progression of breast cancer from the in situ to invasive stage.

The ability of psoriasis to negatively influence cell migration, invasion, and anchorage-independent growth must be overcome during tumor progression, although the mechanism may be highly dependent on the particular molecular subtype of breast cancer. For many cancers in transition, this is accomplished via down-regulation of psoriasis expression. This scenario is consistent with
the observation that in many breast carcinomas the expression of psoriasin is markedly higher in DCIS compared with adjacent invasive tumors. However, in a subset of invasive cancers (e.g., high-grade and/or ER-negative tumors), hypoxia and ROS are dominant factors, and the positive effects of psoriasin expression, particularly proangiogenic effects, may outweigh the negative effects or the cells may become resistant to them, and the tumor therefore retains high psoriasin expression. This hypothesis is supported by data showing that psoriasin expression is induced by ROS (33) and our observation of coordinated expression of

<table>
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<th>Rank</th>
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</tr>
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<tbody>
<tr>
<td>1</td>
<td>AA586894 Psoriasin (S100A7)</td>
<td>1q21</td>
</tr>
<tr>
<td>2</td>
<td>W72424 Calgranulin B (S100A9)</td>
<td>1q21</td>
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<tr>
<td>4</td>
<td>X14487 Acidic (type I) cytookeratin 10</td>
<td>19q13</td>
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<td>5</td>
<td>U91932 Adaptor-related protein complex 3, sigma 1 subunit (AP3S1)</td>
<td>5p22</td>
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<td>6</td>
<td>AA142942 Chromosome 14 open reading frame 147 (C1orf147)</td>
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<td>7</td>
<td>AF002668 Putative fatty acid desaturase MLD</td>
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<td>AF027208 Prominin 1 (PROM1)</td>
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The list of 50 genes ranked highest by KNN analysis in breast cancer gene expression data with psoriasin

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NOTE: Genes in boldface encode proteins implicated in ROS/mitochondrial function. VEGF is in boldface and capitalized. KNN rank order, Genbank ID no., gene description, and chromosomal localization are listed for each gene.

the observation that in many breast carcinomas the expression of psoriasin is markedly higher in DCIS compared with adjacent invasive tumors. However, in a subset of invasive cancers (e.g., high-grade and/or ER-negative tumors), hypoxia and ROS are dominant factors, and the positive effects of psoriasin expression, particularly proangiogenic effects, may outweigh the negative effects or the cells may become resistant to them, and the tumor therefore retains high psoriasin expression. This hypothesis is supported by data showing that psoriasin expression is induced by ROS (33) and our observation of coordinated expression of
psoriasin and ROS-associated genes in breast cancers. Certainly, our data and prior reports indicate that the role of psoriasin in \textit{in situ} and invasive cancer is complex and likely differs in distinct breast cancer subtypes. A full understanding of the role of psoriasin in breast cancer will thus require further studies.

**Acknowledgments**

Received 5/2/2005, revised 9/1/2005; accepted 10/7/2005.

**Grant support:** National Cancer Institute grant ROI CA107469 (C.G. Kleer) and Specialized Program in Research Excellence in Breast Cancer at Dana-Farber/Harvard Cancer Center grant CA89393 and Department of Defense Breast Cancer Center of Excellence grant DAMD17-02-1-0692 (K. Poljak).

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We thank Dr. Andrea Richardson (Department of Pathology, Brigham and Women's Hospital, Boston, MA) for generously providing the breast cancer gene expression data used for the KNN analysis, Dr. William Hahn (Dana-Farber Cancer Institute) for providing us with the pLKO-puro construct, Dr. William Sellers (Dana-Farber Cancer Institute) for providing us the RNAi selection software, Jaana Lahti-Domenici and Minna Allinen for their help with the generation of the psoriasin and control shRNA constructs, Robert Kim and Mark Rubin for their help with the Chromavision imaging system, and members of the Polyak laboratory for critical reading of the article and constructive criticism throughout the execution of this project.

**References**

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