

Neutrophil Gelatinase–Associated Lipocalin: A Novel Suppressor of Invasion and Angiogenesis in Pancreatic Cancer

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Abstract

Neutrophil gelatinase–associated lipocalin (NGAL) is a 25-kDa secreted acute phase protein, which is also up-regulated in multiple cancers, including breast, lung, and pancreas. Recently, NGAL has been proposed as an early biomarker in pancreatic cancer (PaCa). However, its biological role in PaCa is unknown. In this study, we examined *in vitro* and *in vivo* the functional role of NGAL in PaCa. Well- to moderately differentiated PaCa cells (AsPC-1, BxPC-3, and Capan-2) expressed high levels of NGAL but moderately to poorly differentiated PaCa cells (PANC-1 and MIAPaCa-2) expressed undetectable NGAL levels. Immunohistochemistry of untreated tissue microarray showed specific NGAL staining in resected PaCa specimens ($P = 0.0167$). Stable NGAL overexpression (MIAPaCa-2 and PANC-1) significantly blocked PaCa cell adhesion and invasion *in vitro* and vice versa with stable PaCa clones (BxPC-3 and AsPC-1). Moreover, NGAL overexpression reduced focal adhesion kinase (FAK) tyrosine-397 phosphorylation in PaCa cells. Furthermore, NGAL overexpression potently decreased angiogenesis *in vitro* partly through reduced vascular endothelial growth factor (VEGF) production and vice versa. Stable NGAL overexpression or underexpression had no effect on PaCa cell survival, viability, and response to chemotherapeutic drugs. Finally, MIAPaCa-2 cells overexpressing NGAL reduced tumor volume ($P = 0.012$), local and distant metastasis ($P = 0.002$), and angiogenesis ($P = 0.05$) with no effect on K-67 proliferation index ($P > 0.1$) in an orthotopic nude mouse PaCa model. Collectively, our results suggest that NGAL reduces adhesion/invasion partly by suppressing FAK activation and inhibits angiogenesis partly by blocking VEGF production in PaCa cells. Thus, NGAL is a potential suppressor of invasion and angiogenesis in advanced PaCa. [Cancer Res 2008;68(15):6100–8]

Introduction

Neutrophil gelatinase–associated lipocalin (NGAL), also called lipocalin-2, belongs to the lipocalin protein family and was first purified from human neutrophils because of its association with

gelatinase (1). NGAL exists as a 25-kDa monomer, 46-kDa disulfide-linked homodimer, and 135-kDa disulfide-linked heterodimer with neutrophil gelatinase (2). NGAL is an immunomodulator because its expression is up-regulated in humans, typically in epithelial cells, under diverse inflammatory conditions, including appendicitis, inflammatory bowel disease, and diverticulitis (3). NGAL is also able to inhibit microorganism growth by interfering with siderophore-mediated iron acquisition (4). Moreover, NGAL can protect against acute ischemic renal injury and plays a role in cell survival (5, 6).

Elevated NGAL expression has also been observed in multiple human cancers including breast, colorectal, and ovarian cancers; however, the biological roles of elevated NGAL in cancer cells are not yet clear (3, 7–9). Recently, it was reported that NGAL suppressed cellular invasion and metastases in colon cancer and in Ras-transformed mouse mammary cells *in vitro* (10, 11). In contrast, NGAL is able to facilitate gastrointestinal mucosal regeneration by promoting cell migration (12). In breast cancer, NGAL expression is considered as a poor prognostic marker and is associated with invasive properties (13). However, in ovarian cancer, NGAL expression blocked epithelial to mesenchymal transition, one of the hallmarks of invasive neoplasia (8).

Multiple clinical studies have identified NGAL as a selective and an early biomarker in pancreatic cancer (PaCa) tissues using gene expression analyses (14–19). Recently, proteomic analysis of pancreatic exocrine secretions have also confirmed NGAL expression in PaCa (20). Given the fact that PaCa is the most deadly gastrointestinal cancer in the Western countries with poor survival rates (21), it is of utmost importance to understand the role of NGAL in this inevitably fatal disease. But, to date, nothing is known regarding NGAL functions in PaCa.

In the current study, we examined biological functions of NGAL in PaCa cells. We observed significant reduction in PaCa cells adhesion, invasion, and angiogenesis by NGAL overexpression and vice versa. Reduction of NGAL-induced cellular invasion is in part due to inhibition of focal adhesion kinase (FAK) phosphorylation and reduction of angiogenesis is in part due to inhibition of vascular endothelial growth factor (VEGF) secretion by these cells. However, we did not detect any effects of NGAL overexpression or underexpression on cell proliferation, survival, and sensitivity to chemotherapeutic agents. Finally, we showed that NGAL overexpression in an orthotopic model of PaCa significantly inhibited local/distant metastasis and reduced tumor microvessel density.

Materials and Methods

Cell lines and culture conditions. Human PaCa cell lines (AsPC-1, BxPC-3, Capan-2, PANC-1, and MIAPaCa-2) and human umbilical vascular

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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endothelial cells (HUVEC) were purchased from American Type Culture Collection and Lonza, respectively. Human papillomavirus E6/E7 immortalized human pancreatic ductal epithelial (HPDE) cell line was a kind gift from Dr. M. S. Tsao (Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada; ref. 22). AsPC-1, BxPC-3 and Capan-2 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 µg/mL streptomycin and 100 IU/mL of penicillin). PANC-1 and MIAPaCa-2 were cultured in DMEM supplemented with 10% FBS and antibiotics. HUVEC cells were cultured in endothelial growth medium-2 (Lonza). 293FT cells (from Invitrogen) were maintained in DMEM supplemented with 10% FBS and 500 µg/mL G418. HPDE cells were cultured in Keratinocyte-SFM medium supplemented with 50 µg/mL of bovine pituitary extract and 5 ng/mL of recombinant epidermal growth factor (Invitrogen). Cells were passaged at 80% confluency using 1 mmol/L EDTA-0.025% trypsin for 3 to 5 min.

Reagents and treatments. 5-fluorouracil (5-FU) was purchased from Calbiochem. Gemcitabine (Gemzar) was kindly supplied by Eli Lilly Co. Plasmids (pLenti-L6H with full-length NGAL and pLL3.7B-shNGAL) were gifts from Dr. Jang-Seong Kim (Mogan Biotechnology Research Institute, Yongin-city, Republic of Korea). Mouse monoclonal NGAL antibody was purchased from Antibody Shop. FAK, phospho-FAK, XIAP, and survivin antibodies were obtained from BD Biosciences. Antibodies to cMyc, cyclin D1, cIAP1, TRAF1, Bcl-xL, and Bcl-2 were purchased from Santa Cruz Biotechnology, Inc. Formalin-fixed, paraffin-embedded (FFPE) tissue microarray slides of human PaCa were obtained from the Department of Pathology at University of Texas M. D. Anderson Cancer Center (UTMDACC). 5-FU was dissolved in DMSO, and gemcitabine was dissolved in sterile PBS. The rest of the reagents used was of highest quality.

Cell viability assay. Cell viability was measured using the CellTiter Aqueous One Solution Cell Proliferation Assay kit (Promega). Briefly, treated or untreated cells were incubated with the reaction solution for 2 h at 37°C. The absorption at 490 nm was measured using a microplate reader. The results are presented as the percentage of controls.

Cell apoptosis assay. Cells were harvested, washed with cold PBS, resuspended in a solution containing 5 µL of recombinant Annexin V-FITC (Caltag) and 5 µg/mL of propidium iodide, and incubated for 15 min. Cells were analyzed with a Coulter F500 flow cytometer equipped with an argon laser (excitation wavelength of 488 nm and emission wavelengths used for propidium iodide and Annexin V were 620 and 525 nm, respectively). Cells stained with propidium iodide alone were considered necrotic, whereas cells stained with Annexin V were considered apoptotic.

Western blot. Proteins in the culture medium or cell lysates were separated by 10% or 12% SDS-PAGE. The separated proteins were electrophoretically transferred to Immobilon transfer membranes (Millipore) and incubated with a blocking solution, 5% dry milk in TBST [25 mmol/L Tris-HCl (pH 7.6), 200 mmol/L NaCl, and 0.15% Tween 20], for 1 h at room temperature. Target protein levels were measured by immunoblotting with the corresponding antibodies. Blots were washed thrice for 15 min each time at room temperature with TBST and then incubated for 1 h with secondary anti-mouse or anti-rabbit peroxidase-linked antibodies (1:10,000; Amersham) in a blocking solution. Blots were then washed (3 × 15 min). Bands were visualized by enhanced chemiluminescence (Amersham).

RNA isolation and reverse transcription-PCR. Total RNA from cells was isolated using the RNeasy mini kit (Qiagen). Reverse transcription-PCR (RT-PCR) was performed using a one-step kit (Invitrogen) for 35 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 45 s. NGAL primer was designed using Primer3 software according to the sequence of the NGAL gene, and β-actin primers were used as loading control. The amplified PCR products were resolved by 1.5% agarose gel electrophoresis containing ethidium bromide.

Generation of stable NGAL overexpression or NGAL-shRNA PaCa cell lines. To produce lentivirus for NGAL overexpression or NGAL-shRNA (shNGAL), pLenti-L6H plasmid or pLL3.7B-shNGAL plasmid, respectively, and control vector were cotransfected with ViroPower Packaging Mix into 293FT cells by using lipofectamine 2000 reagent (Invitrogen). Medium containing lentivirus with NGAL overexpression or underexpression was used to transduce PaCa cells 48 h after transfection. After transduction, cells

with stable expression of NGAL overexpression or shNGAL were selected by incubation in a medium containing blasticidin for at least 2 wk. NGAL expression of stable cell lines was further confirmed by Western blots.

Adhesion assay. The adhesion assay was performed by using an adhesion chamber. A coverslip coated with fibronectin (1 µg/mL) was placed in the bottom of the adhesion chamber, and another plain coverslip was placed on the top of that, separated by a rubber O-ring. A cell suspension (1×10^5 /mL in culture medium) was injected into the chamber. Cells that were seeded onto the fibronectin-coated coverslip were counted under an inverted microscope in 10 random low-power fields (×40) as the base number. After 10 min, the chamber was inverted and the cells bound to the fibronectin-coated surface were again counted in 10 random ×40 fields. The adhesion of cells was recorded as the percentage of the base number at seeding.

Invasion assay. The invasion capability of cells was determined by using a Matrigel-coated invasion chamber with 0.8-µm pore (BD Biosciences). A single-cell suspension (200 µL) containing 1×10^5 cells was added to the inner chamber after the addition of 600 µL of culture medium to the bottom chamber. After 16 h of incubation at 37°C in 5% CO₂, cells on the upper surface of the inner chamber were removed with cotton swabs. Invaded cells that adhered on the lower surface of the membrane were fixed, stained with Diff-Quik (Dade Behring), and counted.

HUVEC tube formation assay. HUVEC (1×10^4) in 100 µL of diluted conditioned medium (1:1 dilution) from NGAL overexpression or shNGAL clones were seeded onto 96-well plates that were precoated with Matrigel for 1 h. After 16 h, the total number of vascular tubes were counted under a microscope.

ELISA. Cells were cultured in a 100-mm dish (1.5×10^6 cells per dish) with 9 mL of culture medium containing 2% FBS for 24 h. The levels of VEGF in the culture medium were assayed by using a commercially available VEGF ELISA kit (R&D Systems).

Immunohistochemistry. FFPE slides were deparaffinized and immunohistochemistry (IHC) was performed as previously described (23). Monoclonal NGAL antibody (1:10) was used. Slides were developed with 3,3'-diaminobenzidine solution (Dako) for 2 to 5 min and counterstained with Harris's hematoxylin for 10 s. The presence of brown color indicated a positive staining of NGAL.

Ki-67 analysis. FFPE sections (5 µm) were stained with Ki-67 (rabbit monoclonal clone SP6; NeoMarkers) antibody as previously described (23). Results were expressed as percent of Ki-67⁺ cells ± SE per ×40 magnification. A total of 10 fields (×40) were examined and counted from each treatment groups ($n = 5$ per group). The values were compared using unpaired Student's *t* test.

Microvessel density. Ornithine carbamyl transferase (OCT)-fixed cryosections (5 µm) were stained with rat anti-mouse CD31 monoclonal antibody (BD Biosciences) as previously described (23). Areas of greatest vessel density were then examined under higher magnification (×100) and counted. Results were expressed as the mean number of vessels ± SE per high-power field (HPF or ×100). A total of 20 HPFs were examined and counted from each treatment groups ($n = 5$ per group). The values were compared using unpaired Student's *t* test.

Animals. Male athymic *nu/nu* mice (4-wk-old) were obtained from the breeding colony of the Department of Experimental Radiation Oncology at UTMDACC. Before initiating the experiment, we acclimatized all mice to a pulverized diet for 3 d. Our experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at UTMDACC.

Orthotopic PaCa model. MIAPaCa-2 cells stably transfected with NGAL and corresponding control vector were stably transduced with luciferase as previously described (24, 25). The animals were divided into 2 groups ($n = 5$ per group). The first group was injected with MIAPaCa-2 cells that are transfected only with vector and the second with MIAPaCa-2 cells that are transfected with NGAL. Mice were anesthetized with ketamine-xylazine solution, a small left abdominal flank incision was made, and (1×10^6) MIAPaCa-2 cells in 100 µL PBS were injected into the subcapsular region of the pancreas using a 27-gauge needle and a calibrated push button-controlled dispensing device (Hamilton Syringe Company). The abdominal wound was closed in one layer with wound clips (Braitree Scientific, Inc.).

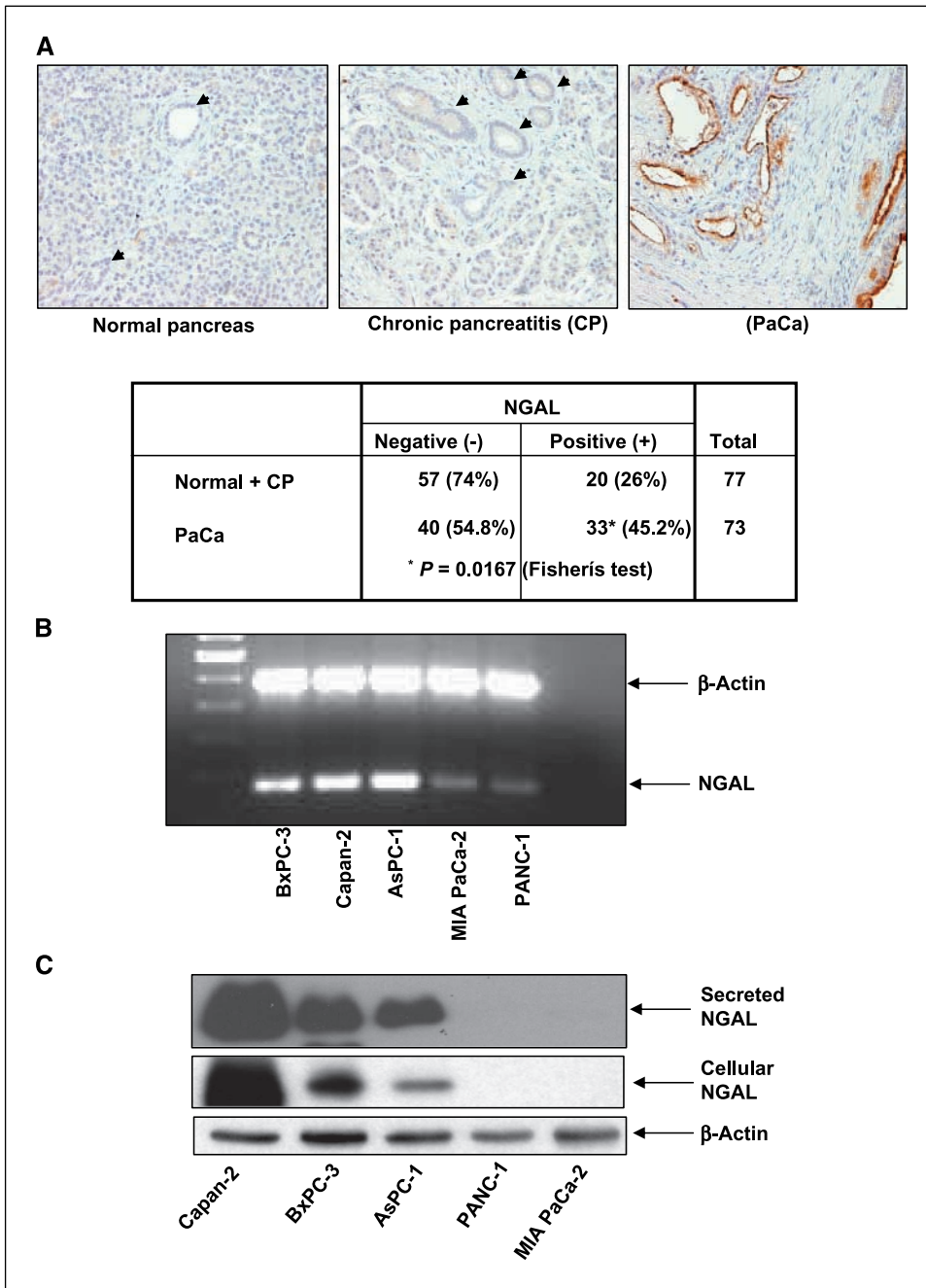


Figure 1. NGAL expression in PaCa tissue and PaCa cells. *A*, tissue microarrays from human PaCa (MDACC) were immunohistochemically stained by using a monoclonal NGAL antibody as described in Materials and Methods. The table shows a summary of NGAL IHC of the PaCa tissue microarrays. *B*, RT-PCR for NGAL expression by using NGAL-specific primers after total RNA extraction from PaCa cells as described in Materials and Methods. *C*, NGAL expression was determined by Western blots of protein lysates or culture medium from PaCa cells as described in Materials and Methods.

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Experimental protocol. One week after implantation, tumor volumes were monitored weekly by IVIS 200 (Xenogen) using a cryogenically cooled bioluminescence imaging system coupled to a data acquisition computer running Living Image software (Xenogen) as previously described (24, 25). Mice were imaged on day 7, 14, 21, and 28 after tumor cells implantation. The animals were sacrificed on 35th day after tumor cell implantation. Primary tumors in the pancreas were excised, and the final tumor volume was measured as $V = 2/3 \pi r^3$, where r is the mean of the three dimensions (length, width, and depth). All the measurements were compared among two groups using unpaired Student's t test. Half of the tumor tissue was FFPE for IHC (Ki-67) and routine H&E staining. The other half was divided into 2 sections: 1 portion snap frozen in liquid nitrogen and stored at -80°C and the other part fixed in OCT and then preserved for cryosections (CD31). H&E staining confirmed the presence of tumor(s) in each pancreas.

Results

NGAL expression in PaCa. To examine NGAL expression in human PaCa tissues, we performed immunohistochemical staining of FFPE tissue microarrays with anti-NGAL antibodies (Fig. 1A). We found that only 45% of PaCa tissues exhibited strong luminal and cytosolic staining of NGAL. Interestingly, 26% of adjacent chronic pancreatitis (CP) sections also showed NGAL staining. However, normal pancreatic ductal epithelial cells expressed no NGAL. Among PaCa staining, NGAL was detected predominantly in PanIN-1 and PanIN-2 lesions (data not shown).

The NGAL expression in PaCa cells was next evaluated by RT-PCR using total RNA extracted from 5 PaCa cell lines and NGAL-specific primers. As shown in Fig. 1B, all five cell lines

expressed NGAL. However, the expression of NGAL was dramatically higher in Capan-2, BxPC-3, and AsPC-1 cells than in MIA-PaCa-2 and PANC-1 cells. To confirm this result, cell lysates and conditioned medium from these cells were subjected to Western blot with anti-NGAL antibodies (Fig. 1C). Consistent with the RT-PCR results, Capan-2, BxPC-3, and AsPC-1 cells had high levels of both NGAL expression and secretion. However, the expression and secretion of NGAL at MIA-PaCa-2 and PANC-1 cells were undetectable, as well as in immortalized HPDE cells (data not shown).

Generation of NGAL-overexpressing and -underexpressing PaCa cells. MIA-PaCa-2 and PANC-1 cells with low basal NGAL expression were stably transduced with lentivirus overexpressing NGAL, designated as MIA-NGAL and PANC-NGAL, respectively. The expression of NGAL was detected by Western blotting. As shown in Fig. 2, MIA-NGAL and PANC-NGAL had very high levels of NGAL expression and secretion, whereas levels of NGAL expression and secretion were undetectable in control MIA-PaCa-2 and PANC-1 cells (designated as MIA-mock and PANC-mock). In addition, we also generated lentivirus-mediated stable expression of shNGAL in AsPC-1 and BxPC-3 cells with high basal NGAL expression, designated as AsPC-shNGAL and BxPC-shNGAL, respectively. The down-regulation of NGAL was also confirmed by Western blotting (Fig. 2).

Effects of NGAL on cell adhesion and invasion. Based on previous studies showing NGAL significantly inhibited metastasis in colon cancer (11) and blocked invasion of *H-Ras*-transformed 4T1 mammary cells (10), we investigated its possible role in PaCa cell adhesion and invasion. We first examined the adhering ability of PaCa cells that overexpress either NGAL or shNGAL by using fibronectin-coated adhesion chambers. We observed that the adhesion rates of MIA-mock and MIA-NGAL cells were $82.8\% \pm 2.7\%$ and $52.2\% \pm 3.7\%$, respectively, after 10 minutes of adherence to fibronectin (Fig. 3A, left). In contrast, the adhesion rates of BxPC-mock and BxPC-shNGAL cells were $54.1\% \pm 8.8\%$ and $99.2\% \pm 0.8\%$, respectively (Fig. 3A, right). Thus, NGAL blocked adhesion of PaCa cells to fibronectin-coated surface.

Next, we investigated whether NGAL blocked invasive capabilities of PaCa cells through Matrigel. We showed that NGAL overexpression suppressed the ability of MIA-PaCa-2 cells to invade through Matrigel by $55.7\% \pm 14\%$ (Fig. 3B, left). Conversely, NGAL down-regulation increased BxPC-3-shNGAL cellular invasion through Matrigel by $27\% \pm 1\%$ (Fig. 3B, right). Our results indicate that NGAL plays a significant role in adhesion and invasion of PaCa cells.

Effect of NGAL on FAK activation. FAK is an important regulator of cell adhesion and invasion (26). Having shown that NGAL overexpression inhibits PaCa cell adhesion and invasion, we sought to determine whether FAK activation was altered in these cells. Activation of FAK requires tyrosine phosphorylation at 397 (Y397; ref. 26). Therefore, we examined FAK activation by Western blotting using phospho-Y397-FAK-specific antibody. As shown in Fig. 3C, MIA-NGAL cells exhibited decreased level of FAK phosphorylation compared with MIA-mock cells. In contrast, BxPC-shNGAL cells had higher levels of FAK phosphorylation than did BxPC-mock cells. These results suggest that NGAL regulates cell invasion in part through the regulation of FAK phosphorylation.

Effects of NGAL on HUVEC tube formation *in vitro*. A previous report showed that NGAL blocked angiogenesis in *H-Ras*-transformed 4T1 cells (27), thus indicating a possible role of NGAL in PaCa angiogenesis. To determine the effects of NGAL on angiogenesis, conditioned medium from MIA-NGAL and PANC-NGAL cells

(containing high amounts of NGAL), and from BxPC-3-shNGAL (containing reduced NGAL) were used to assay for tube formation of HUVEC cells on Matrigel. Conditioned medium from MIA-NGAL (Fig. 4A) and PANC-NGAL (Supplementary Fig. S2B) significantly reduced the tube formation of HUVEC cells, by $69.5\% \pm 5\%$ and $68\% \pm 7.5\%$, respectively, compared with the conditioned medium from their control cells. In contrast, conditioned medium from BxPC-shNGAL increased the tube formation by 63.5% (Fig. 4B). Thus, our results confirm that NGAL inhibits HUVEC tube formation.

Effect of NGAL on VEGF production. As VEGF is a key molecular mediator of angiogenesis, we investigated whether NGAL could regulate VEGF production. To evaluate this possibility, we used an ELISA with conditioned medium from PaCa cells either overexpressing NGAL or shNGAL. VEGF secretions from MIA-NGAL and PANC-NGAL were 49% reduced ($P < 0.05$) compared with MIA-mock and PANC-mock (Supplementary Fig. S2C; Fig. 4C). Interestingly, addition of VEGF to the conditioned medium was not able to rescue HUVEC tube formation (Supplementary Fig. S2D). NGAL down-regulation in BxPC-3 cells (BxPC-shNGAL) significantly increased secretion of VEGF by 30.3% ($P < 0.05$) compared with controls (Fig. 4D). These data indicate that NGAL potentially blocks PaCa angiogenesis *in vitro* in part through reduction of VEGF secretion.

Effects of NGAL on proliferation and viability of PaCa cells. Previous studies showed that NGAL can stimulate cell proliferation (28) and tumor growth (29). We therefore investigated whether NGAL can affect PaCa cell proliferation and viability. No difference in cell proliferation was observed between MIA-NGAL and control, as determined by the CellTiter AQ assay (Fig. 5A). Similar results were obtained with PANC-NGAL cells (data not shown). Cell proliferation of BxPC-shNGAL also did not differ from that of BxPC-3-mock cells (Supplementary Figure S3A).

To further investigate whether manipulation of NGAL expression could affect cell viability, we measured apoptosis of MIA-NGAL, PANC-NGAL, AsPC-shNGAL, and BxPC-shNGAL and their corresponding control cells by flow cytometry after staining with Annexin V-FITC. Similar to the results for cell proliferation, up-regulation or down-regulation of NGAL had no effect on cellular apoptosis (Fig. 5B).

Western blots also confirmed no significant difference in the expression pattern of multiple proteins related to cell proliferation (c-Myc and cyclin D1), apoptosis, and survival (cIAP1, XIAP, survivin, TRAF-1, Bcl-xL, and Bcl-2) between PaCa cells with either

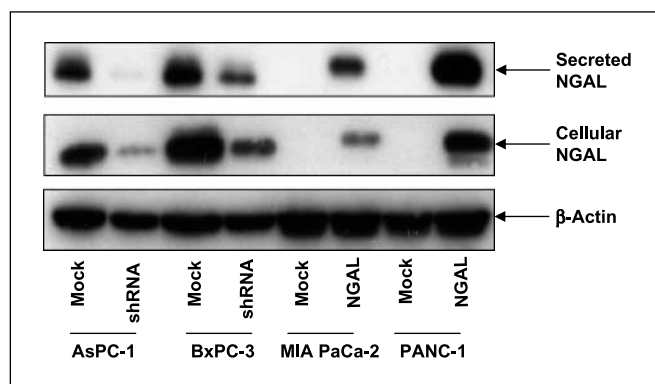


Figure 2. Validation of NGAL expression in stably transformed PaCa cells. Western blots show the expression and secretion of NGAL in stable PaCa cells either overexpressing NGAL or shNGAL as described in Materials and Methods.

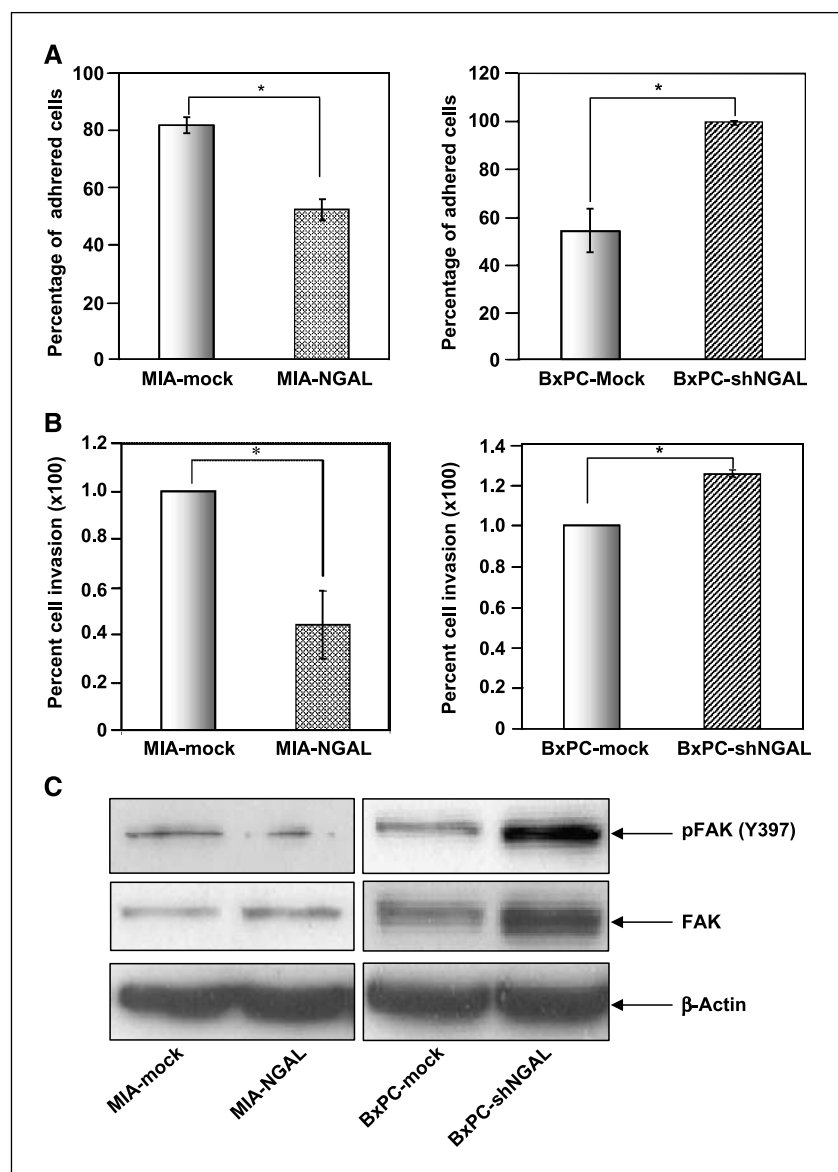


Figure 3. Effects of NGAL on PaCa cell adhesion and invasion. *A*, cell suspension (1×10^5 /mL) in culture medium either overexpressing NGAL (*left*) or shNGAL (*right*) was injected into an adhesion chamber coated with fibronectin. After 10 min, adhered cells were counted under a microscope, and the adhesion rate (percentage of adhered cells) was determined as described in Materials and Methods. *B*, cells (1×10^5) overexpressing NGAL (*left*) or shNGAL (*right*) were seeded onto a Matrigel-coated invasion chamber; 16 h later, invaded cells that adhered on the lower surface of the membrane were fixed, stained with Diff Quick set, and counted under a microscope as described in Materials and Methods. Representative fields are shown in Supplementary Figure S1A. *Columns*, mean of three independent experiments performed in triplicate; *bars*, SE. *, $P < 0.05$. *C*, Western blots showing phospho-FAK levels in the PaCa cells stably expressing either NGAL or shNGAL as described in Materials and Methods.

up-regulation or down-regulation of NGAL and their corresponding controls (Fig. 5C).

Effects of NGAL on sensitivity of PaCa cells to chemotherapy.

PaCa cells are inherently resistant to chemotherapy (30). Because NGAL is able to protect cells from the toxicity of certain chemicals (12) and apoptosis inducers (6), we sought to determine whether elevated NGAL expression in early PaCa plays a role in sensitivity to conventional chemotherapy. To test this idea, we treated cells with NGAL overexpression, shNGAL, or control vector with an increasing dose of gemcitabine for either 48 hours (BxPC-3) or 72 hours (MIA-PaCa-2). PaCa cell toxicity to gemcitabine was then determined by using the CellTiter AQ assay. We observed that sensitivity of PaCa cells with either up-regulated or down-regulated NGAL to gemcitabine did not differ from that of their controls (Supplementary Fig. S3B; Fig. 5D). Similar results were observed for 5-FU treatment (data not shown).

Effects of NGAL on PaCa growth and metastases *in vivo*.

Next, we examined whether NGAL overexpression can inhibit PaCa

growth, angiogenesis, and metastases *in vivo*. We used MIPaCa-2 cells either overexpressing NGAL (MIA-NGAL) or control vector (MIA-vector) stably transduced with luciferase for real-time bioluminescence imaging to monitor tumor volume and spread *in vivo* (Supplementary Fig. S4A and B). These cells were then orthotopically injected into the body of the pancreata of nude mice, and a week later, bioluminescence imaging was started once per week. Figure 6A (*left*) indicated the representative bioluminescence images of MIA-vector and MIA-NGAL on days 7, 14, 21, and 28 after orthotopic injection. Figure 6A (*right*) showed the significant difference ($P = 0.05$) in the bioluminescence area between the 2 groups ($n = 5$ per group). At the end of 35th day, the mice were euthanized and the final tumor volume was measured. As shown in Fig. 6B (*left*), MIA-NGAL tumors were significantly smaller than MIA-vector ($P = 0.012$). We calculated the metastasis score by tabulating the total number of visually recognizable of metastatic foci in liver, omentum/mesentery, and spleen per mouse after careful dissection. Figure 6B (*right*) showed the striking reduction

in the metastasis score of MIA-NGAL group compared with MIA-vector ($P = 0.002$). Next, we showed no difference in proliferation index among the two groups as determined by Ki-67 IHC (Supplementary Fig. S4C; Fig. 6C). However, there is a reduction in the microvessel density of MIA-NGAL tumors compared with control ($P = 0.05$) as determined by CD31⁺ staining of tumor explants (Supplementary Fig. S4D; Fig. S6D). Collectively, our data indicates that NGAL overexpression reduced tumor volume, angiogenesis, and metastatic spread of PaCa *in vivo*.

Discussion

NGAL, a member of the lipocalin family (1), is up-regulated in a number of pathologic conditions, including cancers (3). However, its importance in cancer progression remains unknown. In the current study, we investigated expression of NGAL in PaCa and possible roles of NGAL in PaCa progression. The main findings of the present study show that significant increase in NGAL expression occurs in the early stage of PaCa development and that overexpression of NGAL blocks cell adhesion, invasion, and angiogenesis of PaCa.

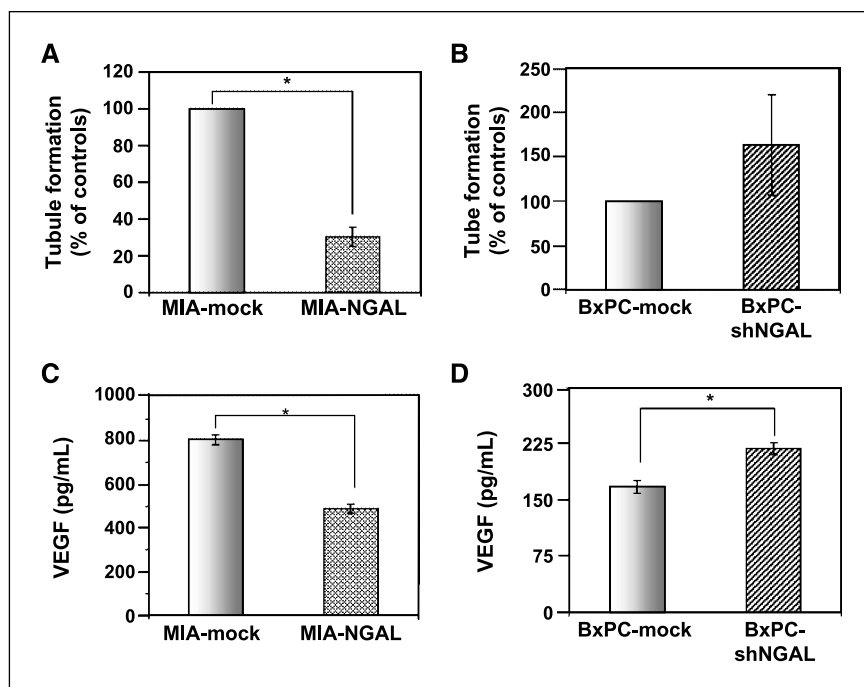
NGAL was described as a biomarker of cancer progression because of the association of increased NGAL expression with estrogen receptor-negative breast cancer cells (31). In addition, up-regulated NGAL expression was found only in the early stage of ovarian cancer (8). Our current study also suggests that NGAL could act as a biomarker for early stages of PaCa, as evidenced by the fact that well- to moderately differentiated PaCa cells (AsPC-1, BxPC-3, and Capan-2 cell lines) had very high levels of NGAL expression and secretion, whereas NGAL expression and secretion of poorly differentiated PaCa cell lines (MIAPaCa-2 and PANC-1) were undetectable. Furthermore, IHC showed that only 45% of surgically resectable PaCa tissues were NGAL positive. NGAL expression significantly increases in early PaCa but progressively gets reduced in late PaCa. We are currently investigating whether loss of NGAL expression in late stages is a potential biomarker of locally advanced/metastatic PaCa. Due to small sample size, we did not

observe any significant association between NGAL expression and stage or differentiation status of PaCa. We also observed NGAL expression in ducts from adjacent CP tissues. This is in agreement with earlier reports that NGAL is an inflammation-induced protein in multiple cell types (4). But it is not known whether NGAL expression in CP can predict further development of progressive advanced PanIN lesions that are pathognomonic of PaCa tissues (32).

Our current study showed that NGAL overexpression reduced PaCa cell adhesion and invasion *in vitro* and *in vivo*. In support of this idea, Lee and colleagues (11) reported that overexpression of NGAL blocked human colon cancer KM12SM cell invasion and liver metastasis. In contrast, Li and colleagues (33) reported that down-regulation of NGAL by antisense suppressed human esophageal carcinoma SHEEC cell invasion *in vivo*. These apparently conflicting observations could be due to distinct functions of NGAL in different cell types. One key difference is that the anti-invasive properties of NGAL noted are predominantly in oncogenic *K-Ras*-mediated transformed cells (colon and PaCa) unlike SHEEC, which was transformed by phorbol ester. This is further supported by a previous study showing that NGAL overexpression also suppressed Ras-transformed murine breast cancer 4T1 cell invasion and lung metastasis *in vivo* (10). Interestingly, in a study of gastrointestinal mucosal integrity and repair, administration of exogenous NGAL facilitated mucosal regeneration by promoting cell migration (12). This indicates that NGAL functions differently in the context of mucosal restitution.

The mechanisms by which NGAL regulates tumor metastasis are unclear. Study in human esophageal carcinoma SHEEC cells suggested that NGAL down-regulation suppressed cell invasion through reduction in matrix metalloproteinase (MMP)-9 activity (33). Overexpression of NGAL blocked Ras-transformed murine breast cancer 4T1 cell invasion and metastasis, possibly through the suppression of Ras-induced E-cadherin phosphorylation and subsequent inactivation (10). In PaCa, effects of NGAL on cell invasion are partly mediated through the regulation of FAK phosphorylation. This is supported by the fact that manipulation of

Figure 4. Effects of NGAL on PaCa angiogenesis and VEGF secretion. *A* and *B*, HUVEC cells in a conditioned medium from PaCa cells overexpressing NGAL (*A*) or shNGAL (*B*) and their controls were seeded onto the Matrigel-coated plate and incubated for 16 h. The tube formation was photographed and counted under a microscope as described in Materials and Methods. Representative fields are shown in Supplementary Fig. S2A. The results are expressed as the percentage of the control. *C* and *D*, the levels of VEGF in culture medium from PaCa cells overexpressing NGAL (*C*) or shNGAL (*D*) and their controls were detected by ELISA as described in Materials and Methods. Columns, mean of three independent experiments performed in duplicate; bars, SE. *, $P < 0.05$.



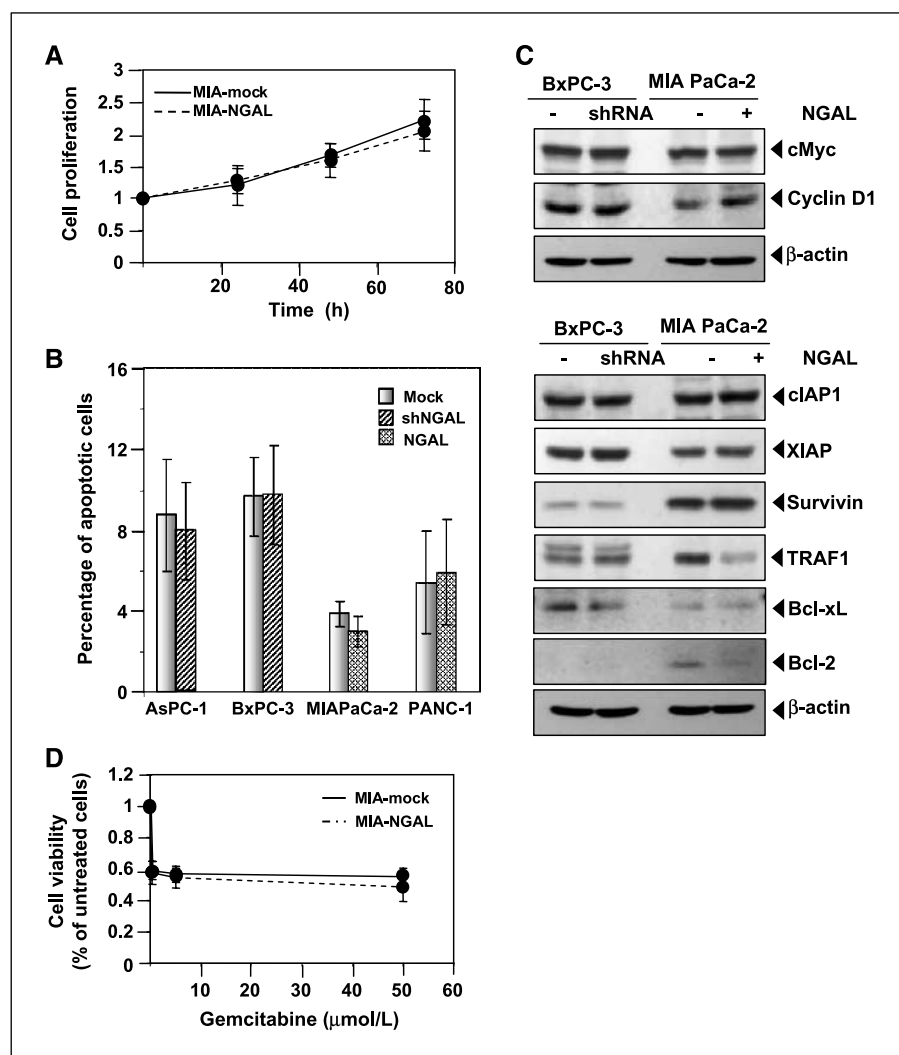


Figure 5. Effects of NGAL on proliferation, apoptosis, and the sensitivity of PaCa cells to chemotherapy. *A*, proliferation of PaCa cells overexpressing NGAL or shNGAL (Supplementary Fig. S3A) was determined by using the CellTiter Aqueous One Solution Cell Proliferation Assay kit as described in Materials and Methods. *B*, apoptosis in PaCa cells was determined by flow cytometry with Annexin V-FITC staining as described in Materials and Methods. *C*, Western blots show the expression of cell proliferation- and apoptosis-related proteins in stable PaCa cells that overexpress either NGAL or shNGAL. *D*, cells were treated with gemcitabine at the indicated doses for 72 h in MIA-NGAL and control cells, or 48 h in BxPC-shNGAL and control cells (Supplementary Fig. S3B). The viability of cells was measured by using the CellTiter Aqueous One Solution Cell Proliferation Assay kit as described in Materials and Methods. *Points*, mean of three independent experiments; *bars*, SE.

NGAL expression altered FAK phosphorylation at residue Y397, which is critical for FAK activation. Future studies will address the mechanism by which NGAL regulates FAK activation in PaCa. Furthermore, up-regulation or down-regulation of NGAL did not change the activity of MMP-9 in PaCa.⁷ It is well-known that FAK, a 125-kDa cytoplasmic tyrosine kinase, is an important molecule involved in tumor invasion and metastasis, including PaCa. For instance, down-regulation of FAK by RNA interfering inhibits the metastasis of human pancreatic adenocarcinoma cells (34). In addition, periostin promotes CFPanc1 PaCa cell invasion by an increase in the phosphorylation of FAK (35). Furthermore, FAK forms complexes with integrins and integrin-linked kinase. These complexes are also critical for mediating cellular adhesion, migration, and invasion. NGAL could potentially interact with FAK-integrin complexes either directly or indirectly and, thereby, affect migration and invasion of PaCa cells. Thus, FAK signaling pathways either directly or indirectly through interactions with integrin complexes may play a significant role in mediating effects of NGAL on PaCa invasion.

In addition to the involvement in metastasis, the results of our study also indicate that NGAL can inhibit angiogenesis in PaCa

cells, both *in vitro* and *in vivo*. This is in agreement with a previous report in Ras-transformed 4T1 cells (27). It is well-known that the angiogenic process depends on a net balance of positive and negative angiogenic factors in the tumor. One of the key molecules involved in angiogenesis is VEGF (36). PaCa cells overexpress a number of proangiogenic factors, including VEGF (37). An important mechanism by which NGAL overexpression may reduce PaCa angiogenesis is partly through the reduction of VEGF production or through blockade of VEGF action on HUVEC. NGAL could directly or indirectly interact with VEGF in the conditioned medium and prevents VEGF-VEGFR2-mediated signaling in HUVEC. In addition, perturbations of FAK-integrin-mediated signaling events could also lead to reduced HUVEC tube formation. Similarly, overexpression of NGAL dramatically suppressed Ras-induced VEGF production in 4T1 cells (27). Further studies will delineate whether the effects are at the transcriptional, translational, or posttranslational level of regulation. Of course, involvement of other angiogenic factors in NGAL-mediated inhibition of PaCa angiogenesis cannot be excluded.

One of the salient features in this article was validation of *in vitro* data on NGAL overexpression mediated reduction in invasion and angiogenesis in our orthotopic mouse model of PaCa. As expected, NGAL effects on tumor cell proliferation were not significant (Ki-67⁺ cells), but we observed a modest reduction in the final tumor

⁷ Z. Tong and S. Guha, unpublished observation.

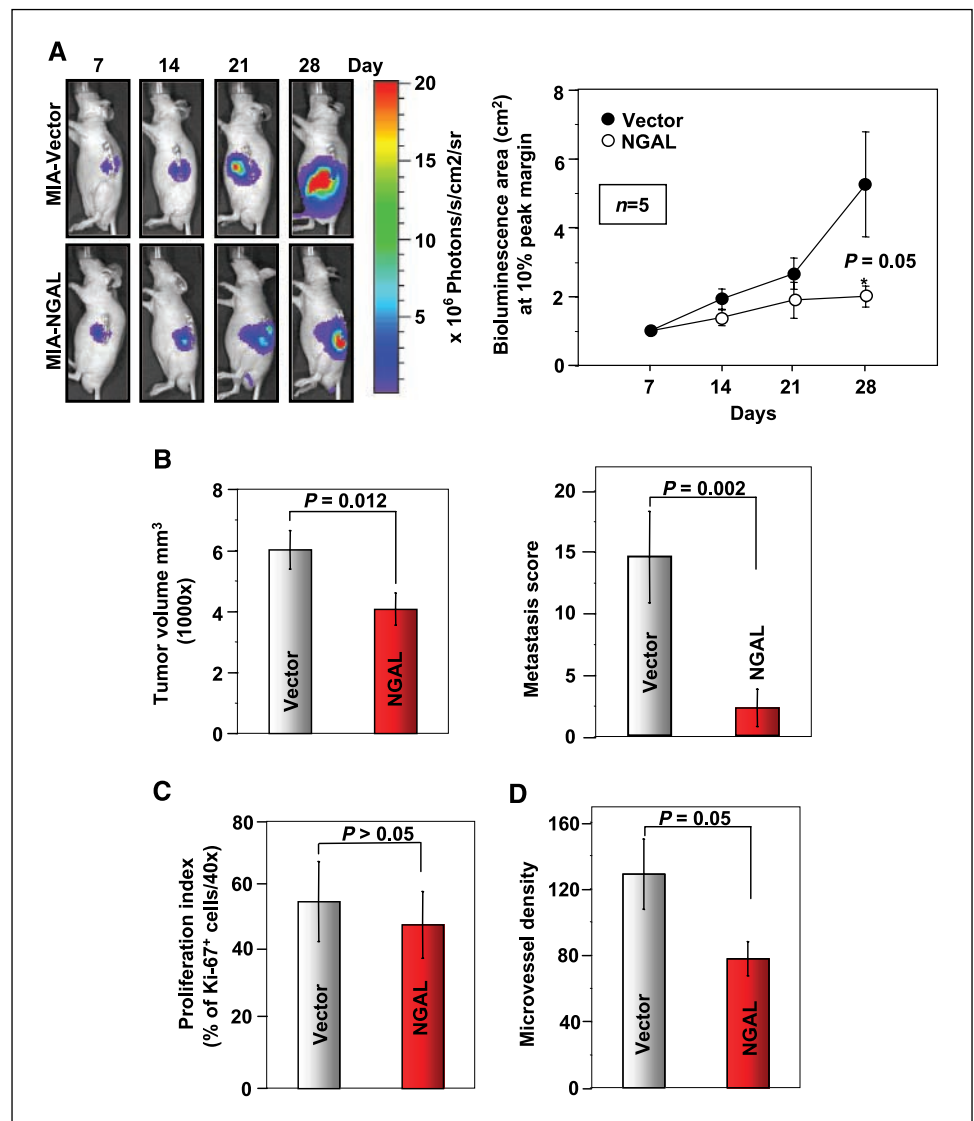
volume. This could be attributed in part to the reduction in angiogenesis as observed by reduction in tumor microvessel density (CD31⁺ cells). However, one cannot rule out the possibility that effects of NGAL on cell proliferation and/or apoptosis are dependent on cellular growth in a defined basement membrane or in an anchorage-independent manner. But the striking observation was the suppression of local and distant metastases by NGAL overexpression *in vivo*. Furthermore, using green and red fluorescent orthotopic models of PaCa metastasis *in vivo* (38–40), we will validate our findings with NGAL overexpression and underexpression clones. Taken together, our results suggest that NGAL could regulate multiple proteins including FAK-integrin signaling pathways, which play a significant role in the process of metastasis *in vivo*.

Accumulated evidence indicates that NGAL plays roles in the regulation of cell growth. For instance, up-regulated NGAL was highly correlated with the S-phase fraction in breast cancer (9), overexpression of NGAL stimulated breast tumor growth *in vivo* (29), and administration of exogenous NGAL to ischemic-injured kidney resulted in the enhanced tubule cell proliferation (28). In

PaCa, however, NGAL had no effects on cell proliferation, given that overexpression or down-regulation of NGAL did not change cell growth. Similar results were also reported in colon cancer (11). Thus, the effects of NGAL on cell proliferation may be cell type specific and context dependent.

NGAL is thought to be a survival factor due to induction of cellular apoptosis by NGAL antibody and increase in cellular resistance to PDK1 inhibitor by NGAL overexpression in lung cancer A549 cells (6). In MCF-7 breast cancer cells, up-regulation of NGAL also makes cells more resistant to the chemotherapeutic agent 4-HRP (41). However, data from the current study showed that NGAL has no effects on the viability of PaCa cells or their sensitivity to chemotherapeutic agents because the manipulation of NGAL expression, either up-regulation or down-regulation of NGAL expression, resulted in no changes in cell apoptosis or in gemcitabine-induced cell death compared with control cells. Similarly, apoptosis induced by several apoptotic inducers in thymocytes isolated from *Lcn2*^{-/-} mice had no significant different from that in wild-type thymocytes (42). Therefore, the effect of NGAL on cell sensitivity to chemotherapeutic agents may depend

Figure 6. Effects of NGAL on the growth of PaCa tumor and metastases *in vivo*. **A**, representative bioluminescence images of orthotopically implanted PaCa in live, anesthetized mice using Xenogen IVIS as described in Materials and Methods (*left*). *Right*, measurements of photons/s/cm²/steradian depicting bioluminescence area at 10% peak margin (mean ± SE) at indicated time points using Xenogen IVIS as described in Materials and Methods (*n* = 5). **B**, final tumor volumes (mean ± SE) measured on 35th day after orthotopic implantation of PaCa cells at autopsy using Vernier calipers and calculated using the formula $V = 2/3 \pi r^3$ (*n* = 5; *left*). *Right*, the metastasis score was calculated during autopsy and the composite score was shown in the figure (mean ± SE) as described in Materials and Methods (*n* = 5). **C**, quantification of Ki-67⁺ cells as described in Materials and Methods (*n* = 5). *Columns*, mean; *bars*, SE. Representative Ki-67⁺ IHC is shown in Supplementary Fig. S4C. **D**, quantification of CD31⁺ microvessel density as described in Materials and Methods (*n* = 5). *Columns*, mean of three independent experiments; *bars*, SE. Representative CD31⁺ IHC is shown in Supplementary Fig. S4C.



on the nature of the chemicals or cell type. That down-regulation of NGAL did not result in significant cellular apoptosis in the current study could also be due to the incomplete silencing of NGAL, which is enough to maintain cell viability.

In summary, well- to moderately differentiated PaCa cells and few resected PaCa tissues showed up-regulated NGAL expression. Overexpression of NGAL had no effects on PaCa cell viability or sensitivity to chemotherapy but did block PaCa cell adhesion, invasion, and angiogenesis. The involvement of NGAL in cell invasion and angiogenesis may be through the alteration of FAK phosphorylation and VEGF production, respectively. Therefore, loss of NGAL is a biomarker of PaCa progression, and manipulation of NGAL expression could control PaCa angiogenesis and metastasis.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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