Lipocalin 2 Antagonizes the Proangiogenic Action of Ras in Transformed Cells

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Abstract

Lipocalin 2 is an iron-binding secreted protein that converts embryonic kidney mesenchyme to epithelia. Previously, we reported that lipocalin 2 could revert 4T1-ras-transformed mesenchymal tumor cells to a more epithelial phenotype, increase E-cadherin expression, and suppress cell invasiveness *in vitro* and *in vivo*, indicating that lipocalin 2 is a metastasis suppressor. Here, we show that lipocalin 2 can suppress the ras-induced expression of vascular endothelial growth factor in 4T1 cells via down-regulation of ras mitogen-activated protein kinase and ras phosphatidylinositol-3-kinase signaling. In addition, the expression of thrombospondin-1 (an antiangiogenic molecule) was increased in tumors formed by 4T1-ras cells into which lipocalin 2 was stably introduced. Tumor angiogenesis, assessed via an intradermal tumor angiogenesis assay, was also suppressed by lipocalin 2. We also show that caveolin-1 is a critical mediator of this activity. These data provide new insights into the action of lipocalin 2 and raise the possibility that the administration of lipocalin 2 may be useful for inhibiting tumor angiogenesis, in addition to suppressing tumor metastasis, in cancers which show ras activation. (Mol Cancer Res 2006;4(11):821 – 9)

Introduction

Down-regulation of epithelial junctional proteins and the induction of mesenchymal proteins, part of a process known as epithelial to mesenchymal transition (EMT; refs. 1-6), enhances the metastatic potential of epithelial tumors (7-9), whereas reversal of the process (MET) via reactivation of epithelial genes can reverse the malignant phenotype (10). We have previously shown that an endogenous epithelial inducer (11), lipocalin 2 (also referred to as siderocalin, Ngal, 24p3, uterocalin, or neu-related lipocalin), induces an epithelial phenotype in ras-transformed cells and reverses their metastatic potential. Here, we hypothesized that this endogenous epithelial inducer could also function as an antiangiogenic factor.

Lipocalin 2 is a member of a superfamily of carrier proteins (12) that is expressed in granulocytic precursors (13) as well as in numerous epithelial cell types (14, 15). Very recently, its murine receptor was identified (16). Both overexpressed and endogenous lipocalin 2 (17), induced the *de novo* expression of E-cadherin, the formation of polarized epithelia, and the development of tubules in the embryonic mesenchyme in an iron-dependent fashion (18). It was therefore logical to ask whether lipocalin 2 could promote MET in transformed cells. We chose a breast cancer model; 4T1 mouse mammary tumor cells in a syngeneic host in which metastasis to bone, liver, and lung are known to occur, in a pattern similar to that found in human breast cancer (19). Somewhat surprisingly, the addition of purified lipocalin 2 to cultured ras-transformed 4T1 mouse mammary tumor cells (20) led to the reversal of ras-induced EMT, reduced tumor growth, and dramatically suppressed metastasis. In lipocalin 2 – treated cells, E-cadherin was rescued from proteasomal degradation by the inhibition of ras mitogen-activated protein kinase (MAPK) signaling. In view of these effects on *in vivo* tumor growth and metastasis, we asked whether lipocalin 2 might also regulate the angiogenic activity of tumor cells. To test this hypothesis, we focused primarily on vascular endothelial growth factor (VEGF) expression, which is known to be induced by ras activation. The introduction of lipocalin 2 down-regulated VEGF at both mRNA and protein levels via the inhibition of ras-MAPK and phosphatidylinositol-3-kinase (PI3K) signaling. Moreover, caveolin-1 was found to be critical in mediating both the MET and antiangiogenic functions of lipocalin 2.

Results

We have recently shown that lipocalin 2 reverses ras-induced transformation and metastatic potential in a syngeneic, spontaneously metastasizing, murine breast cancer model (4T1 cells; ref. 20). Because ras transformation is known to promote angiogenesis (21), we explored whether lipocalin 2 would also reverse this action of ras. Ras is known to up-regulate the production of VEGF (22, 23), a potent proangiogenic protein, important for endothelial cell survival, proliferation and migration, and to show the antiangiogenic protein thrombospondin-1 (TSP-1). We show here that lipocalin 2 antagonizes these proangiogenic activities of ras in the 4T1 cell line system, *in vitro* and *in vivo*.

Lipocalin 2 Reduces Ras-Induced VEGF Production in 4T1 Cells In vitro

To determine the effects of lipocalin 2 on angiogenesis, we used three stable clones of 4T1 cells: infected with empty retroviral control (EV cells), retrovirally infected with constitutively active mouse H-ras mutant A12 (R cells), and R cells transfected with a lipocalin 2 expression plasmid (RL cells) as previously described (20). Lipocalin 2 expression in RL cells and secretion in conditioned medium was detected by Western blotting (Fig. 1A). As shown in Fig. 1E, the expression of mouse lipocalin 2 receptor was detected in all EV, R, and RL cell types. These reverse transcription-PCR (RT-PCR) products were proven to be amplified from mRNAs because they were not detected in the reaction mixtures without reverse transcription. We also confirmed specific amplification from the target mRNAs by sequencing the PCR products (data not shown).

We evaluated VEGF production from these stable cell lines by an ELISA assay. VEGF secretion from 4T1 cells (EV) was dramatically up-regulated ($\sim$10-fold) by ras-transformation (R), but was suppressed ($\sim$7.5-fold) nearly to baseline in the lipocalin 2 (RL) transfectants (Fig. 1B). We also examined VEGF production using the conditioned medium of 293T cells transfected with lipocalin 2 cDNA and empty vector control. Lipocalin 2 secretion from transfected 293T cells under different incubation conditions were determined by Western blotting (Fig. 1C), and we selected 2 day–incubated conditioned medium for the VEGF ELISA assay. VEGF secretion from R cells was down-regulated by the lipocalin 2–conditioned medium (Fig. 1D).

Lipocalin 2 Down-Regulates the Expression of Angiogenic Factors in 4T1 Tumor Cells In vivo

To determine whether lipocalin 2 could affect the expression of antiangiogenic factors *in vivo*, we injected EV, R, or RL cells subcutaneously in the backs of BALB/c mice and dissected the primary tumors at 3 weeks postinoculation. As shown previously (20), E-cadherin and vimentin protein expression varied reciprocally in the three tumor types. We then assessed the expression of VEGF and TSP-1 by Western blotting in each tumor tissue. In the primary tumors of R cells, a significantly larger amount of VEGF protein was observed as compared with tumors derived from EV cells, an increase that was completely abrogated in RL cells (Fig. 2). Moreover, the antiangiogenic protein TSP-1 was down-regulated by ras in the primary tumors
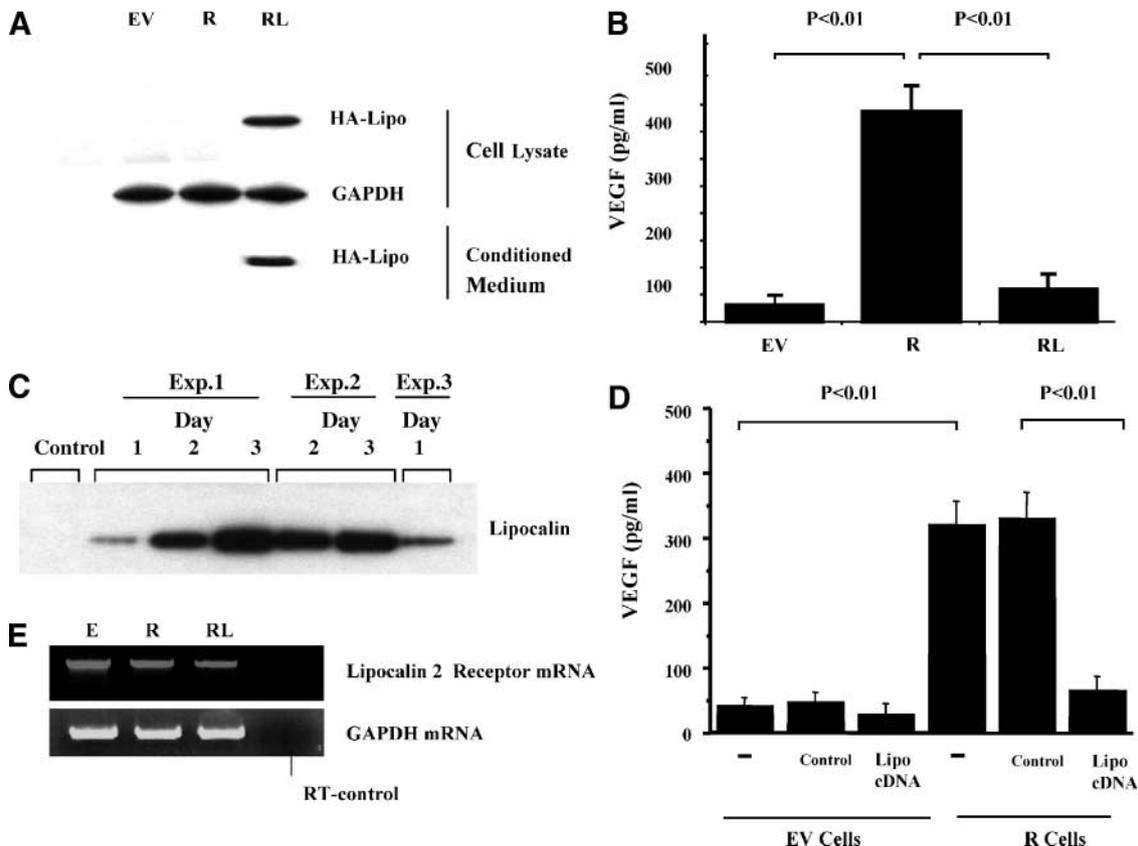

FIGURE 1. VEGF secretion from 4T1 cell clones and lipocalin 2 receptor mRNA expression in 4T1 cell clones. **A.** Lipocalin 2 expression and secretion from 4T1 cell clones was determined by Western blotting. **B.** Conditioned media of 4T1 clones (*EV, R,* and *RL*) cultured in a six-well plate were collected after 2 days of incubation. VEGF levels were determined by ELISA. VEGF secretion was stimulated $\sim$10-fold by ras transformation (*R cells*) and down-regulated ($\sim$7.5-fold) by lipocalin 2 (*RL cells*). **C.** Lipocalin 2 secretion in the conditioned medium of 293T cells transfected with lipocalin 2 cDNA and mock control by Fugene 6 (Roche) is shown at different incubation times. Experiments 1, 2, and 3 are from independent transfection and days 1, 2, and 3 reflects the times after each transfection. **D.** VEGF ELISA assay results using the 2-day incubation-conditioned medium of transfected 293T cells. **E.** RT-PCR for each 4T1 clone. Lipocalin 2 receptor mRNA levels in EV, R, and RL cells.
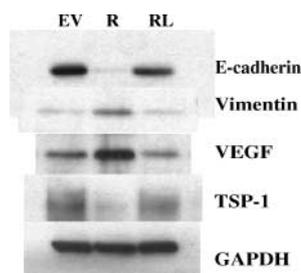
FIGURE 2. VEGF and TSP-1 expressions in each 4T1 primary tumor *in vivo*. 4T1 clones (*EV, R,* and *RL*) were suspended in PBS and injected subcutaneously in the backs of BALB/c mice. Three weeks later, each primary tumor tissue was dissected out, weighed, diced, and soaked in ice-cold radioimmunoprecipitation assay buffer with 1 mmol/L of phenyl-methylsulfonyl fluoride, 1 μg/mL of aprotinin, 1 mmol/L of Na$_3$VO$_4$, 1 mmol/L of NaF, homogenized on ice, centrifuged at 10,000 × *g* for 10 minutes at 4°C and the supernatant fluid collected as total cell lysate. Western blot of lysate from each primary tumor for the antigen is shown. GAPDH served as a loading control. The E-cadherin, vimentin, and GAPDH data is from our previous article (20).

of R cells, also in accordance with previous data (24), and returned in RL cells to the levels noted in EV cells. These data indicate the *in vivo* antiangiogenic activity of lipocalin 2 by its effects on the expression of two angiogenic molecules.

Lipocalin 2 Inhibits Intradermal Tumor Angiogenesis

To determine whether lipocalin 2 could alter angiogenesis *in vivo*, we used an intradermal tumor angiogenesis assay. Each clone of 4T1 cells was inoculated intradermally in the flanks of BALB/c mice. After 48 hours, tumors were located with a dissecting microscope, and the capillaries oriented toward the tumors and surrounding them were observed (Fig. 3). These capillaries were most strikingly noted for the R tumors (Fig. 3). This *in vivo* data was consistent with our *in vitro* VEGF and *in vivo* TSP-1 data.

Both MAPK and PI3K Signaling are Involved in Ras-Induced VEGF Production in 4T1 Cells

Next, we elected to explore the mechanism by which lipocalin 2 alters VEGF expression. First, we wished to ascertain which signaling pathways known to stimulate VEGF expression in other cell types and known to be ras effectors (25, 26) were applicable to our 4T1 systems. We used both MEK and PI3K inhibitors to address this question. In R cells, each inhibitor alone reduced VEGF production in a dose-dependent manner, with a maximum inhibition of ∼50% (Fig. 4). However, combined blockade of these pathways showed >90% inhibition (Fig. 4).

Lipocalin 2 Down-Regulates AKT Phosphorylation in 4T1 Cells

We have previously shown that lipocalin 2 down-regulates MEK and ERK phosphorylations (20). We therefore explored whether lipocalin 2 affects PI3K signaling. Lipocalin 2 down-regulates ras-induced phosphorylation of AKT (Fig. 5A). However, lipocalin 2 does not down-regulate the insulin-like growth factor I–induced phosphorylation of AKT (Fig. 5B), suggesting that the effect of lipocalin 2 on AKT phosphorylation shows specificity to ras signaling.

Lipocalin 2 Reduces the Expression of VEGF mRNA in 4T1 Cells In vitro

Having noted lipocalin 2's effects on VEGF protein expression and secretion, we asked whether these effects were secondary to changes in VEGF mRNA levels. We tested the effects on VEGF mRNA using three stable cell clones of 4T1 cells. Ras-transformation augmented VEGF mRNA level and lipocalin 2 reduced this up-regulation (Fig. 6A), paralleling our VEGF protein data (Figs. 1 and 2).

Both MAPK and PI3K Signaling Pathways Affect the Expression of VEGF mRNA in 4T1 Cells

We also observed the synergistic inhibition of VEGF mRNA expression by the PI3K inhibitor and the MEK inhibitor (Fig. 6A). These are consistent with the ELISA data (Fig. 4).

Lipocalin 2's Inhibitory Effect on VEGF mRNA Expression is Reversed by the Activation of MAPK and PI3K Signaling

Using signaling inhibitors, we showed that VEGF mRNA expression, as well as VEGF secretion, is regulated by both PI3K and MAPK signaling and that lipocalin's effects (Fig. 6A, *lane 3*) seem to mimic the effects seen with combined blockade of MEK and PI3K (Fig. 6A, *lane 6*). To show whether lipocalin 2 functions upstream or downstream of MEK and PI3K activation by ras and ras-induced VEGF expression, we used constitutively active forms of MEK (MEK-DD) and AKT (CA-AKT) and assessed VEGF mRNA (Fig. 5C). CA-AKT and

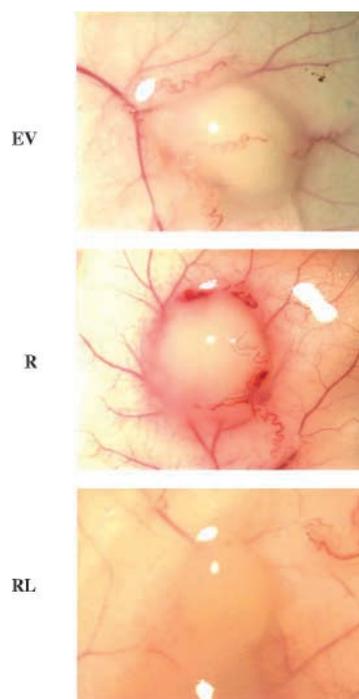**FIGURE 3.** Intradermal tumor angiogenesis. 4T1 clones (*EV, R*, and *RL*) were inoculated intradermally in the flanks of mice. After 48 hours, the skin around the inoculation sites was removed and the capillaries oriented toward the tumors were assessed. Representative picture for each condition (from four experiments) is shown. Lipocalin 2 down-regulated tumor angiogenesis stimulated by ras *in vivo*.
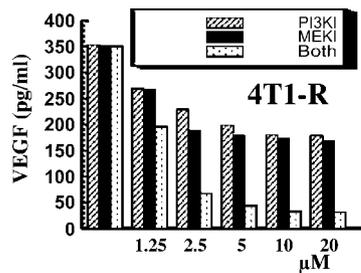
FIGURE 4. VEGF induction in ras-transformed cells is regulated by MEK and PI3K. Conditioned media from R cells (cultured in a six-well plate) treated with the MEK inhibitor and the PI3K inhibitor (2 days of incubation) were analyzed by ELISA for VEGF concentration. When both inhibitors were used together, the total dose was equally split between the two inhibitors. For example, in the 10 μmol/L data for "both", 5 μmol/L of MEK and 5 μmol/L of PI3K inhibitors were used.

MEK-DD reversed the inhibitory effect of lipocalin 2 on VEGF mRNA, with CA-AKT being the most potent. These effects were also confirmed at the level of VEGF secretion by ELISA assay (Fig. 7A), although it seemed to be in contrast with the VEGF mRNA data, suggesting that costimulation of the two pathways together gave maximal VEGF secretion.

Role of Iron

It has been reported that the EMT-inducing activity of lipocalin 2 is markedly enhanced in the presence of iron (18, 20). To determine the effect of iron on lipocalin 2–induced inhibition of angiogenesis, we used deferoxamine mesylate (2-5 μmol/L), an iron chelating agent. Deferoxamine mesylate, which depletes iron from the intracellular pool (27), partially reversed the inhibitory effect of lipocalin 2 on VEGF secretion (Fig. 7B). On the other hand, there was no significant effect of deferoxamine mesylate on VEGF secretion in either EV or R cells. The inhibition of VEGF secretion in RL cells shows that iron is necessary for the full antiangiogenic action of lipocalin 2.

Lipocalin 2 Up-Regulates the Expression of Caveolin-1 in 4T1 Cells

Because caveolin-1 expression is known to affect a number of signaling pathways, and others have described caveolin-1 loss following ras transformation (28), we sought to tie in lipocalin 2 signaling with ras and caveolin-1 expression. Using the stable clones of 4T1 cells, we found that in the process of ras transformation, caveolin-1 expression is diminished, consistent with previous findings (28, 29), and that lipocalin rescued this loss of caveolin-1 (Fig. 8A). In the RL cells, the epithelial phenotype was lost in a dose-dependent manner by the inhibition of caveolin-1 expression using adenoviral infection of a caveolin-1 antisense construct (Fig. 8B), suggesting that caveolin-1 is necessary for the EMT-reversing function of lipocalin 2. Reduction in caveolin-1 expression also led to a decrease in E-cadherin expression, as noted previously (28). Interestingly, VEGF expression increased dramatically as caveolin-1 expression decreased, and moreover, there was a concomitant activation of pMEK and pAKT. These data implicate caveolin-1 function in both the EMT inhibitory and antiangiogenic activities of lipocalin 2.

Caveolin-1 Overexpression Is Not Sufficient to Drive MET

Next, we assessed whether caveolin-1 is sufficient to induce MET. We increased the expression of caveolin-1 in R cells by adenoviral infection of a caveolin-1 construct. We found that caveolin-1 did not result in a morphologic change in the R cells (data not shown), nor was E-cadherin expression increased (Fig. 8C), suggesting that caveolin-1 is not sufficient to cause MET.

Discussion

This article extends our previous findings that lipocalin 2 down-regulates tumor metastasis (20). Similar observations in a colon cancer model metastatic to liver have recently been reported (30). The novel findings shown here are (*a*) that lipocalin 2 inhibits ras-induced tumor angiogenesis through the down-regulation of VEGF expression and the up-regulation of TSP-1 expression in 4T1 cells, (*b*) that there is a synergistic role of MAPK and PI3K signaling in the regulation of VEGF expression, both of which are down-regulated by lipocalin 2, and (*c*) that caveolin-1 is involved in lipocalin's antimetastatic and antiangiogenic functions.

Lipocalin and Angiogenesis

The induction of an angiogenic phenotype by oncogene activation or through the loss of tumor suppressor gene function has been well-described (24, 31, 32). For example, ras and src are known to induce angiogenic proteins and to repress endogenous inhibitors of angiogenesis. Ras causes potent induction of VEGF, partly mediated through the PI3K pathway (21, 33). Similarly, the loss of a tumor suppressor could lead to the up-regulation of proangiogenic pathways. For example, loss of the von Hippel-Lindau tumor suppressor has been known to up-regulate VEGF through the stabilization of HIF-1α (34). In most cases, however, the detailed mechanisms by which the gain of a dominantly active oncogene or the loss of a tumor suppressor leads to a proangiogenic state have not been well-defined. We have shown in this report that ras up-regulates the production of VEGF in cultured 4T1 cells. In our double-stable transfectants of 4T1 with lipocalin 2 and ras, this induction was largely abrogated (Fig. 1). This effect was also noted *in vivo* (Fig. 2). Moreover, we found that lipocalin 2 inhibits tumor

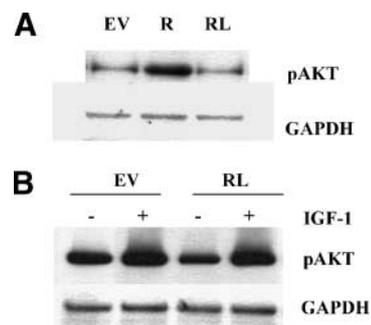**FIGURE 5.** Lipocalin 2 down-regulates ras-induced AKT phosphorylation, but not insulin-like growth factor I–induced (*IGF-I*) AKT phosphorylation. Western blot for phospho-AKT (*pAKT*). **A.** Lysate from each 4T1 clone (*EV, R,* and *RL*) was analyzed. **B.** Lysate from EV or RL cells treated with or without insulin-like growth factor I (15 ng/mL, 10 minutes) was analyzed. GAPDH served as a loading control.
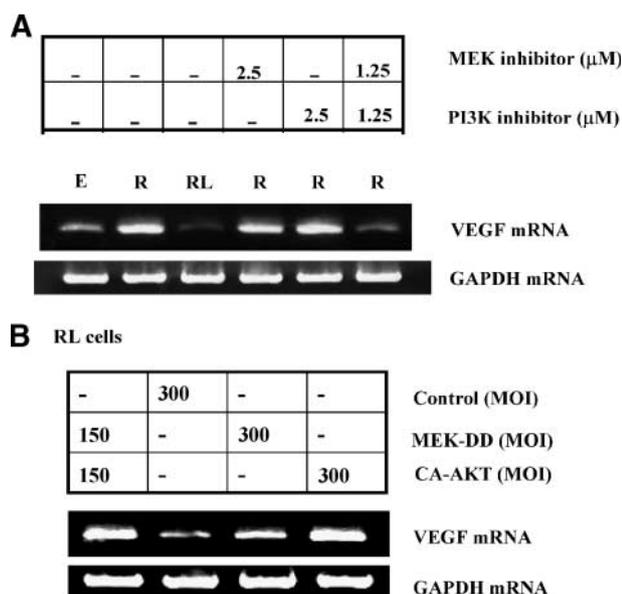
A

MEK inhibitor (μM)	PI3K inhibitor (μM)
–	–
–	–
–	–
2.5	–
–	2.5
1.25	1.25

E   R   RL   R   R   R

VEGF mRNA

GAPDH mRNA

B RL cells

Control (MOI)	MEK-DD (MOI)	CA-AKT (MOI)
–	150	150
300	–	–
–	300	–
–	–	300

VEGF mRNA

GAPDH mRNA

FIGURE 6. VEGF mRNA expression in 4T1 cell clones and regulation by MEK and PI3K. RT-PCR for each 4T1 clone. **A.** R cells incubated with 10% serum including DMEM in a six-well plate were treated with the MEK inhibitor and PI3K inhibitor at the indicated doses for 24 hours. Total RNA was isolated and RT-PCR was done for VEGF and lipocalin 2 receptor mRNA. GAPDH served as a loading control. **B.** RL cells in a six-well plate were infected with an adenovirus carrying the MEK dominant active form (*MEK-DD*), constitutively active AKT (*CA-AKT*), and a Lac-Z adenovirus at the indicated multiplicities (*MOI*) in 2% serum including DMEM for 48 hours. Cells were then trypsinized, replated on six-well plates at 5% to 10% confluency, and incubated with 10% serum including DMEM for 24 hours. Total RNA was isolated and RT-PCR was done for VEGF mRNA. GAPDH served as a loading control.

angiogenesis *in vivo* (Fig. 3). Thus, lipocalin 2 reversed the proangiogenic ras-induced state in 4T1 cells *in vitro* and *in vivo*. This is the first report that a MET inducer can inhibit angiogenesis, and is consistent with previous studies showing that some EMT inducers promote angiogenesis (35-37). Although oncogenesis is often accompanied in tumor cells by both EMT and a proangiogenic phenotype, e.g., ras and HIF-1α can mediate both effects, there was no a priori reason to expect that lipocalin 2 would reverse the effects of ras on angiogenesis. Another known effect of ras is to repress TSP-1, an endogenous inhibitor of angiogenesis (23, 24, 38, 39). We were able to show *in vivo* that lipocalin 2 reversed this action of ras as well (Figs. 2 and 3). In summary, lipocalin 2 can antagonize multiple effector pathways by which ras induces angiogenesis. These data are consistent with the lack of effect of ras or lipocalin 2 on the *in vitro* proliferation of 4T1 cells (data not shown).

Regulation of VEGF Expression by MAPK and PI3K in Tumor Cells

VEGF regulation is complex: at the transcriptional level, several response elements such as HIF-1, SP-1, AP2, Egr-1, and STAT sites have been identified (25). It has been reported that ras-MAPK affects VEGF transcription through the SP1 site. Moreover, ras-PI3K signaling is known to affect HIF-1 regulation (26, 40), which in turn, regulates VEGF transcription. Furthermore, the PI3K pathway has been implicated in regulating the activity of RNA binding (AU sequence binding)

proteins such as HuR, thereby affecting VEGF mRNA stability (41). In 4T1 cells, ras-induced VEGF secretion was partly inhibited by either a MEK inhibitor or a PI3K inhibitor, but was markedly inhibited by both when given together, i.e., we could see the synergistic effect of both pathways in the expression of VEGF in tumor cells. Such synergism from the use of PI3K and MAPK inhibitors has been reported when angiogenic factors were induced in myeloma, melanoma, and gastric cancer cells (42-44).

Iron Requirement for the Antiangiogenic Activity of Lipocalin 2

We have previously shown (20) that iron is critical to mediate the full effect of lipocalin 2 on the inhibition of invasiveness and metastasis. Figure 7 shows that the same holds for lipocalin 2's ability to inhibit angiogenesis, at least as assessed by VEGF secretion.

The Involvement of Caveolin-1 in the Action of Lipocalin 2

Caveolin-1 may influence multiple signaling pathways that relate to cell growth, differentiation, and angiogenesis. Caveolin-1 gene disruption promotes tumorigenesis and enhances metastasis (45), and Lu et al. reported that EGF
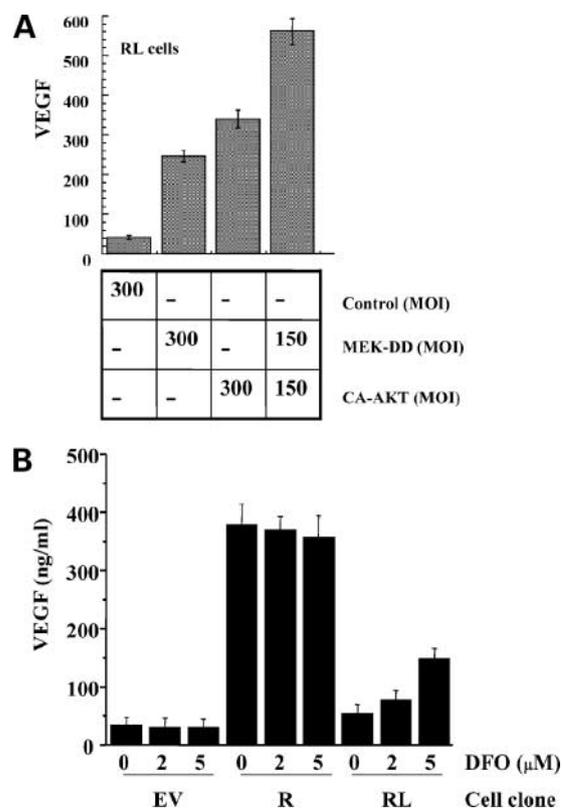
A

RL cells

VEGF

Control (MOI)	MEK-DD (MOI)	CA-AKT (MOI)
300	–	–
–	300	–
–	–	300
–	150	150

B

VEGF (ng/ml)

0	2	5	0	2	5	0	2	5	DFO (μM)
EV			R			RL			Cell clone

FIGURE 7. Down-regulated VEGF secretion by lipocalin 2 in RL cells can be rescued with constitutively active MEK and AKT. **A.** Conditioned media from RL cells infected with constitutively active MEK and AKT adenoviral vectors were analyzed by ELISA for VEGF concentration. **B.** 4T1 cells (*EV, R*, and *RL*) were incubated with deferoxamine mesylate (DFO) for 48 hours. VEGF secretion detected by ELISA is shown. The deferoxamine mesylate concentrations were 0, 2, and 5 μmol/L as indicated.
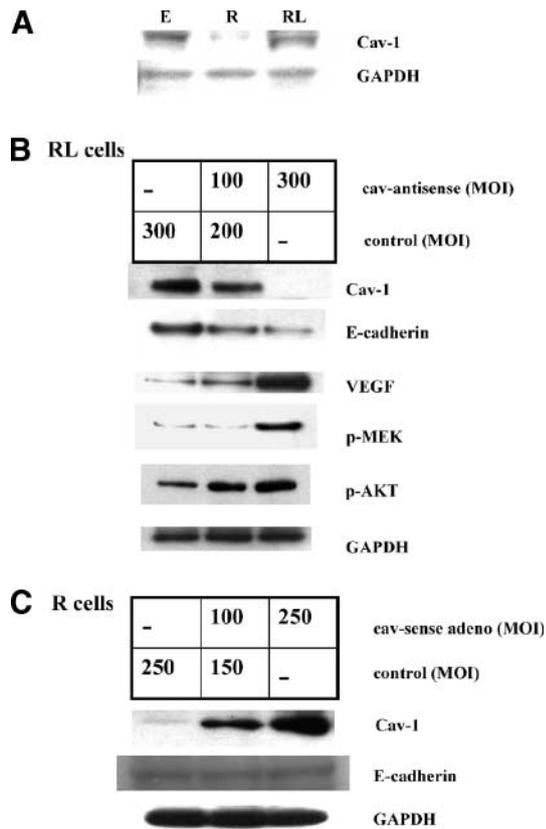
FIGURE 8. Involvement of caveolin-1 in the MET-inducing and antiangiogenic function of lipocalin 2. Western blot of 4T1 cell lysate. **A.** Caveolin-1 expression in clones EV, R, and RL. **B.** RL cells in a six-well plate were infected with an adenovirus carrying the caveolin-1 antisense or a Lac-Z adenovirus at the indicated multiplicities (*MOI*) in 2% serum including DMEM for 48 hours. Cells were then trypsinized, replated on six-well plates at 5% to 10% confluency, and incubated with 10% serum including DMEM for 24 hours. Lysate was collected for Western blot analysis. **C.** R cells were infected with an adenovirus carrying caveolin-1 sense and a Lac-Z in the same condition as in (**B**). Lysate was collected for Western blot analysis.

down-regulates caveolin-1 causing loss of E-cadherin and the invasion of tumor cells (28). Moreover, caveolin-1 over-expression in oncogenically transformed cells suggests that it can act as a tumor suppressor protein (46). On the other hand, up-regulated caveolin-1 is a prognostic variable for poor survival in patients with metastatic cancer (47-49). Hence, the function of caveolin-1 seems to be dependent on the developmental stages of the tumor. In the early stages of tumor development, it might act as a tumor suppressor molecule, and in the late and advanced stages of tumor development, it seems to promote invasiveness. In view of these observations, we explored caveolin-1's role in both EMT and angiogenesis in our 4T1 cell model. We also asked whether lipocalin 2's effects on inducing MET and as an antiangiogenic factor were mediated through increased expression of caveolin-1.

We found that lipocalin 2 up-regulates caveolin-1 in ras-transformed 4T1 cells (Fig. 8A) and noted that caveolin-1 down-regulation causes up-regulation of ras-MAPK signaling (Fig. 8B), which is consistent with previous reports (50, 51). Our data on PI3K signaling is, however, inconsistent with that

of Cohen et al. (51), although this may be the result of the different cell types used. Down-regulation of caveolin-1 also decreased E-cadherin expression which was up-regulated by lipocalin 2 in ras-transformed 4T1 cells, i.e., down-regulation of caveolin-1 interfered with lipocalin 2's function in reversing EMT. Moreover caveolin-1 down-regulation caused the up-regulation of VEGF, which was suppressed by lipocalin 2. Our data suggests that caveolin-1 is necessary for MET induction and the antiangiogenic functions of lipocalin 2 in 4T1 tumor cells.

However, caveolin-1 induction alone was not sufficient to cause MET because caveolin-1 up-regulation by adenoviral infection was not enough to reverse ras-induced EMT (Fig. 8C). This data suggests that lipocalin 2 reverses ras-induced EMT via negatively affecting the ras-MAPK signaling not only through up-regulation of caveolin-1 but also through other poorly defined caveolin-1-independent pathways (Fig. 9).

In conclusion, in addition to the antimetastatic function of lipocalin 2, the present study shows that lipocalin 2 represses ras-induced expression of VEGF in 4T1 cells through the down-regulation of ras-MAPK and ras-PI3K signaling. Our study also highlights the role of caveolin-1 in both the antiangiogenic and antimetastatic functions of lipocalin 2.

Materials and Methods
Mice
BALB/c nude mice were purchased from Charles River Laboratories (Boston, MA). The mice were caged under specific pathogen–free conditions with access to sterilized food and water.

Plasmids, Viral Constructs, Antibodies, and Signaling Inhibitors
The human lipocalin 2 cDNA (GenBank accession no. BC033089) with a COOH terminus HA tag was PCR-amplified and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The constitutively active form of H-ras A12 in the pBabe retroviral vector and empty-pBabe were gifts from Dr. M. Ewen (Dana-Farber Cancer Institute, Boston, MA). Another constitutively active form of ras plasmid (H-ras V12-pcDNA3.1) was
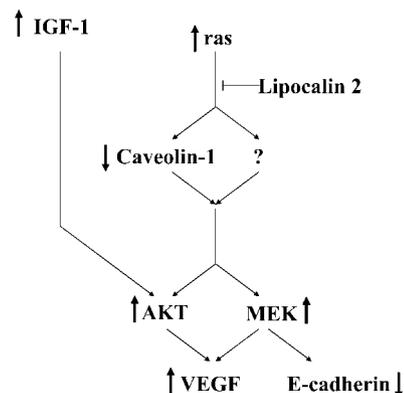

FIGURE 9. The signaling pathway from ras activation to VEGF induction and E-cadherin down-regulation. "?", unknown caveolin-1-independent pathway (see text).

purchased from the Guthrie cDNA Resource Center (Sayre, PA). The constitutively active from of MEK (MEK-DD) and Lac-Z adenoviral vectors were gifts from Dr. Eileen O'Leary (Harvard Institute of Medicine, Boston, MA). Caveolin-1 and antisense caveolin-1 adenoviral vectors were gifts from Dr. Timothy C. Thompson (Baylor College of Medicine, Houston, TX). AKT adenoviral vectors were gifts from Dr. Kenneth Walsh (Boston University, Boston, MA; ref. 52).

The following reagents were purchased from their respective companies: anti-ras antibody (Oncogene Research Products, San Diego, CA); anti-raf, anti-phospho-raf, anti-MEK1/2, anti-phospho-MEK1/2, anti-ERK1/2 and anti-phospho-ERK1/2 antibodies, anti-AKT and anti-phospho-AKT antibodies, and MEK (U0126) and PI3K inhibitors (LY294002; Cell Signaling Technologies, Beverly, MA); anti-E-cadherin and PY20 anti-P-Tyr monoclonal antibodies (BD Transduction Laboratories, Deerfield, IL); anti-vimentin monoclonal antibody and anti-caveolin-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Chemicon International, Inc., Temecula, CA); and insulin-like growth factor I (Upstate, Temecula, CA). Anti-TSP-1 antibody was a gift from Dr. Jack Lawler (Beth Israel Deaconess Medical Center, Boston, MA).

Stable Cell Lines

293T and 4T1 cells (10^6 cells; American Type Culture Collection, Manassas, VA) were cultured in 100 mm dishes with culture medium (DMEM containing 10% FCS) 12 hours before transfection. Retroviral constructs (10 μg, CA-H-ras-pBabe or empty-pBabe) were introduced into the cells by using the Fugene 6 transfecting reagent (32.5 μL; Roche Pharmaceuticals, Nutley, NJ). Forty six hours later, 10 mL of conditioned medium was filtered under sterile conditions and added to the 4T1 cells (10^6/100 mm dish) after dilution with (1:1) culture medium (DMEM containing 10% FCS). After 48 hours, virus-containing medium was replaced with medium containing hygromycin for selection of stable clones: 8 to 10 single clones [4T1-ras (R) or 4T1-EV (EV)] were selected. A single clone (clone 1) from the R group was used for further studies. Similarly, a single clone (clone 1) from the EV group was selected. Stable R cells (clone 1) were transiently transfected with lipocalin 2-pcDNA3.1, and cells were selected one more time with neomycin to obtain stable RL cells. RL (double transfectant) clones were tested for lipocalin 2 (HA tagged) expression using anti-HA antibody. The RL clone (clone 6) with the highest level of lipocalin 2 expression was used for further studies (20).

Measurement of VEGF Levels by ELISA

Conditioned media of 4T1 cells were collected after 2 days of incubation. Murine VEGF levels were determined in duplicate using a commercially available sandwich ELISA kit (R&D Systems, Minneapolis, MN), with an affinity-purified polyclonal antibody specific for mouse VEGF which was precoated onto a microplate. Results were compared with a standard curve of mouse VEGF with a lower detection limit of 7 pg/mL. A model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA) was used to measure light intensity correlating with VEGF binding.

Immunodetection

For immunoprecipitation and immunoblotting, tissues were weighed, diced, soaked in ice-cold radioimmunoprecipitation assay buffer with 1 mmol/L of phenylmethylsulfonyl fluoride, 1 μg/mL of aprotinin, 1 mmol/L of Na_3VO_4, 1 mmol/L of NaF, homogenized on ice, centrifuged at $10,000 \times g$ for 10 minutes at 4°C, and the supernatant fluid collected as total cell lysate. The cultured cells were washed, scraped, and solubilized in a lysis buffer containing 20 mmol/L of Tris-HCl (pH 7.5), 150 mmol/L of NaCl, 0.5% Triton X-100, 1% aprotinin, and 1 mmol/L of phenylmethylsulfonyl fluoride. After 20 minutes on ice, cells were pelleted by centrifugation and the supernatants were used as a cell lysate. Cell lysates or immunoprecipitated cell lysates were separated by PAGE (NuPAGE gels; Invitrogen), followed by electroblotting onto a polyvinylidene-difluoride membrane. Protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Co., Rockford, IL; ref. 53).

Semiquantitative RT-PCR

Total RNA was isolated from 4T1 cells *in vitro* using TRIzol (Life Technologies, Gaithersburg, MD). The integrity of RNA was validated on agarose gel and RNA purity was confirmed by spectrophotometry. RT-PCR was done using the Perkin-Elmer GeneAmp PCR system 2400 using Omniscript (Qiagen, Valencia, CA). First-strand cDNA synthesis was done using the Superscript First Strand Synthesis System (Invitrogen). Two micrograms of each RNA sample was reverse transcribed using oligo-dT priming and the control reaction was prepared for every sample in which the reverse transcriptase was omitted. PCR was done using Taq DNA polymerase (Qiagen) using primers for mouse VEGF (5′-GTA CCT CCA CCA TGC CAA GT-3′ and 5′-GCG AGT CTG TGT TTT TGC AG-3′), GAPDH (5′-ACAGTCTTCTGAGTGGCA-3′ and 5′-CCCATCAC-CATCTTCCAG-3′), and mouse lipocalin 2 receptor (5′-GGC TCT TCG GTT TCT CCT and GAA TGG CAT GGG CAA TAA AG) for DNA amplification (sequence information was obtained from Dr. Michael R. Green's laboratory). PCR amplification was achieved by an initial 94°C incubation for 5 minutes followed by 25 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with 72°C for 7 minutes as an extension time. These PCR conditions were established for DNA amplification in the linear range. PCR products were analyzed on 1.5% agarose gels.

Intradermal Tumor Angiogenesis Assay

Tumor angiogenesis was assessed as previously described (54-56). 4T1 clones (EV, R, and RL) were cultured and harvested when they were in exponential growth. Cells were washed thoroughly and resuspended with PBS. Cells (2×10^6) were injected intradermally into the flanks of mice. After 2 days of inoculation, the mice were euthanized using carbon dioxide and the skin around the tumor was removed using a dissecting microscope.

In vivo Assay for Primary Tumor

4T1 cells (10^7; EV, R and RL) were injected subcutaneously in BALB/c mice (57). After 3 weeks, primary tumor was dissected and evaluated (20).

Statistical Analysis

All values are expressed as mean ± SE. A one-tailed Student's *t* test was used to identify significant differences in multiple comparisons. $P < 0.05$ was considered statistically significant.

Acknowledgments

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