Lipocalin 2 diminishes invasiveness and metastasis of ras transformed cells

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Running title: Lipocalin 2 as a metastasis inhibitor

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Lipocalin 2, an iron-siderophore binding protein, converts embryonic kidney mesenchyme to epithelia. We found that lipocalin 2 could also convert 4T1-ras transformed mesenchymal tumor cells to an epithelial phenotype, increase E-cadherin expression and suppress cell invasiveness in vitro and tumor growth and lung metastases in vivo. The ras-MAPK pathway mediated the epithelial to mesenchymal transition (EMT) in part by increasing E-cadherin phosphorylation and degradation. Lipocalin 2 antagonized these effects at a point upstream of raf activation. Lipocalin 2 action was enhanced by iron-siderophore. These data characterize lipocalin 2 as an epithelial inducer in ras malignancy and a suppressor of metastasis.

Downregulation of epithelial proteins and the induction of mesenchymal proteins (EMT) (1,2-5,6) enhances the metastatic potential of epithelial tumors (7-9) while reactivation of epithelial genes reverses the malignant phenotype (MET) (10). We hypothesized that an endogenous epithelial inducer (21), lipocalin 2 (also called siderocalin, Ngal, 24p3, uterocalin, neu related lipocalin) could stimulate the epithelial phenotype in Ras transformed cells and reverse their metastatic potential.

Lipocalin 2 is a member of a superfamily of carrier proteins (11) that is expressed in granulocytic precursors (12) as well as in numerous epithelia cell types (13,14). Crystallography showed that the protein is a carrier of iron bound to a siderophore, which is a small organic molecule produced by bacteria (20). Both recombinant and mammalian expressed lipocalin 2 (16) induce the de novo expression of E-cadherin, the formation of polarized epithelia, and the development of tubules in embryonic mesenchyme in an iron dependent fashion (17). Though lipocalin 2 is highly expressed upon polyoma, SV 40 or neu transformation and after malignant transformation of the breast, lung, colon and pancreatic epithelia (12,13), its functional role in this context is unknown. Here we suggest that the protein regulates the epithelial characteristics of malignant cells as it does for embryonic mesenchyme. This activity might result from iron transport or signaling through unknown receptors (18).

To test these hypotheses, we added purified lipocalin 2 or lipocalin 2 vectors to ras transformed 4T1 mouse mammary tumor cells. These cells are known to metastasize to bone, liver and lung tissue in a pattern similar to that found in human breast cancer (19). However, introduction of lipocalin 2 reversed ras induced EMT, reduced tumor growth and dramatically suppressed metastasis. In lipocalin 2 treated cells, E-cadherin was rescued from proteasomal degradation by inhibition of ras-MAPK signaling. This protection was iron dependent.

Experimental Procedures

Plasmids, virus constructs, lipocalin 2 proteins, antibodies and signaling inhibitors - The human lipocalin 2 cDNA (Genbank accession #
BC033089) with a C-terminus HA tag was PCR amplified and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The constitutively active form H-ras A12-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 purchased from the Guthrie cDNA Resource Center (Sayre, PA). Constitutively active from of MEK (MEK-DD) and Lac-Z adenoviral vectors were gifts from Dr. E. O’Leary (Harvard Institute of Medicine, Boston, MA). MEK-DD cDNA was a gift from Dr. H. Iba (Tokyo University, Tokyo, Japan).

Recombinant mouse lipocalin 2 (accession # NM 008491) was expressed as GST-fusion protein in BL21 strain of E. coli (Stratagene, La Jolla, CA), which does not synthesize siderophore (20,21). Ferric sulfate (Sigma-Aldrich, St. Louis, MO) was added in the culture medium at 50 µM. The protein was isolated using Glutathione Sepharose 4B beads (Amersham Bioscience, Piscataway, NJ), eluted with thrombin (Sigma-Aldrich, St. Louis, MO), and further purified with gel filtration (Superdex 75, Amersham Biosciences, Piscataway, NJ). Iron-loaded (Lipo:Sid:Fe) and iron-unloaded lipocalin 2 (Lipo:Sid) were prepared by mixing the recombinant protein with iron-loaded and iron-unloaded forms of a bacterial siderophore enterochelin (EMC Microcollections, Tübingen, Germany) in PBS at room temperature for 60 min. Unbound siderophore was removed with Microcon YM-10 (Millipore, Bedford, MA). The recombinant protein diluted in culture medium was sterilized before addition to the cells using 0.22 µm filters (Millipore, Cork, Ireland).

The following reagents were purchased from 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 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 FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA); anti-GAPDH antibody (Chemicon International Inc, Temecula, CA); anti-Hakai antibody (Zymed Laboratories, San Francisco, CA); proteasome inhibitor MG132 (Boston Biochemistry, Cambridge, MA); deferoxamine mesylate salt (Sigma-Aldrich Co., St. Louis, MO).

**Stable cell lines** - 293T and 4T1 cells (ATCC, Manassas, VA) were cultured in DMEM, 10% FCS and seeded (10⁶/100-mm dish) 12 h prior to transfection with Fugen6 reagent (32.5 µl, Roche Pharmaceuticals, Nutley, NJ) and retroviral construct (10 µg, CA-H-ras-pBabe or empty-pBabe). Ten ml of condition medium were collected at 48 hrs and diluted 1:1 with DMEM 10% FCS and added to the 4T1 cells (10⁶/100-mm dish) for 48 hrs, followed with selective medium containing hygromycin (Invitrogen). 8-10 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. R cells (clone 1) were transfected with lipocalin 2-pcDNA3.1, and selected with neomycin and screened for lipocalin 2-(HA tagged) using anti-HA antibody (Santa Cruz). RL (double transfectant) clone (clone 6) which showed the highest level of lipocalin 2 expression was used for further studies. Supplemental Figure A shows that the H-ras A12 DNA is present in RL cells and that the transcript is expressed in these cells i.e. RL cells are indeed a double transfectant and have not merely lost expression of the mutant ras gene.

**Immunodetection** - Cells were stained as described previously (22) and images acquired with a DeltaVision system (Applied Precision, Issaquah, WA) equipped with an Axiovert 100 microscope (Carl Zeiss Microlmaging Inc., Shelton, CT) and a Photometrics 300 series scientific-grade cooled CCD camera, reading 12-bit images, and using the 63 /1.4 NA plan-NeoFluar objective. For immunoprecipitation and immunoblotting, tissues were weighed, diced, soaked in ice cold RIPA buffer with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml Aprotinin, 1 mM Na₃VO₄, 1 mM NaF, homogenized on ice, centrifuged at 10,000g for 10 min at 4°C and the supernatant fluid collected as
total cell lysate. Cultured cells were washed, scraped and solubilized in a lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1% aprotinin and 1 mM PMSF. After 20 minutes on ice, the 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, Carlsbad, CA), followed by electroblotting onto a polyvinylidenedifluoride membrane (PVDF). Protein bands were detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL) (23).

**Luciferase assay** - After transient transfection of the plasmids, cells were incubated for 20 h in 10% FCS and luciferase activity in the cell lysates was determined using a luminometer normalized by sea-pansy luciferase activity under the control of the thymidine kinase promoter. The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI) (24).

**In vitro invasion assay** - Polycarbonate membranes (6.5 mm diameter, 8 μm pore size) of Transwells (Coster, NY) were coated with Matrigel® (BD Biosciences, Franklin Lakes, NJ) and cells were seeded (10^6 cells/100 μl) with DMEM including 0.1% serum. 16 hours later, cells were fixed, stained with Giemsa solution, and the upper surface of each membrane was scraped with a cotton swab. Cells that had reached the lower surface of the membrane (migrated cells) were counted in 20 random fields using a light microscope (x400).

**Semi-quantitative reverse transcriptase - polymerase chain reaction (RT-PCR)** - Total RNA was isolated from 4T1 cells in vitro using the SV Total RNA Isolation system (Promega, Madison, WI). Tissue RNA was collected with TRIzol® (GibcoBRL, Gaithersburg, MD). RT-PCR was performed on the Perkin Elmer GeneAmp PCR system 2400 using Omniscript (Qiagen, Valencia, CA) for reverse transcription reaction, and Taq DNA polymerase (Qiagen) and primers for mouse E-cadherin (5’-TGCCCAAGAAAATGAAAGG-3’ and 5’-AATGGCAGGAATTTGCAATC-3’), GAPDH (5’-ACAGTCTTTCTGAGTTGC-3’ and 5’-CCCATTCCCGTCCTCCAG-3’) and HA-tagged lipocalin 2 (5’-GGAGTACTTCAAGATCA-3’ and 5’-GAAAGCATAGTCTGGACGCATAG-3’) for DNA amplification. The PCR conditions were established for DNA amplification in the linear range. RT-PCR products were analyzed on 1% agarose gels.

**In vivo assay for primary tumor growth and pulmonary metastases** - 10^7 4T1- (EV, R and RL) cells were injected subcutaneously in Balb/c mice (25). Though this model is not the standard orthotopic model used, we have used it extensively in our laboratory to study metastases in lung. Primary tumor volume (V) = a · b · b / 2, where a represents the minimum and b the maximum tumor diameter. After 3 weeks, lung weights and the number of metastatic nodules on the lung surface were evaluated.

**Statistical Analysis** - All values are expressed as mean ± S.E. A one tailed Student’s t test was used to identify significant differences in multiple comparisons. A level of P<0.05 was considered statistically significant.

**Results**

**Lipocalin 2 reverses the ras transformed phenotype.** - We chose a syngeneic spontaneously metastasizing murine breast cancer model (4T1 cell line) and accelerated its metastatic potential by introduction of constitutively active mouse H-ras mutant A12 using retrovirus. While 4T1 cells infected with an empty vector (EV) grew in a cobblestone-shaped pattern (Fig. 1A, top left), 4T1-ras (R) cells were spindle-shaped and did not form clusters at low confluency (Fig. 1A, top middle). To assess the effects of lipocalin 2 expression on ras transformation, we generated stable clones of 4T1-ras cells expressing lipocalin 2 (RL) by transfection of a lipocalin expression plasmid (lipocalin 2-pcDNA3.1). Compared to R cells, the RL cells (Fig. 1A, top right) reverted to an epithelial morphology and grew appositionally (similar to EV cells), re-expressed E-cadherin and suppressed the expression of mesenchymal vimentin. (Figs. 1A, lower panel, and 1B). In contrast, E-cadherin mRNA remained unchanged (Fig. 1C), suggesting that the effect of ras and
lipocalin were post-transcriptional. Expression of E-cadherin in RL cells was dependent on the dose of lipocalin 2-pcDNA3.1 expression vector (transiently introduced in a population of R cells), on a conditioned medium containing lipocalin 2 (Figs. 1D, E), as well as on recombinant lipocalin 2 protein. Indeed stable lipocalin 2 expression (RL) almost completely reversed (by approximately 76%) ras induced invasiveness in vitro (Fig. 2A).

To determine whether lipocalin 2 could alter growth of tumors in vivo we injected EV, R or RL cells subcutaneously in the backs of Balb/c mice, and assessed primary and metastatic tumor size at 1, 2 and 3 weeks post-inoculation. Primary tumors of R cells were significantly larger than lipocalin 2 cells (RL) or control cells (EV) (Fig. 3A). Indeed, lipocalin 2 reversed the invasion of adjacent muscle seen in tumors derived from R cells (Fig. 3B). Just like control EV cells, RL tumors were solid, compact and condensed (they could be “shelled out”). Indeed, RL tumors had more E-cadherin and less vimentin than R cells, making them similar to control tumors (EV cells; Fig. 3C). Most dramatically, the number of metastatic pulmonary nodules was reduced by 80% in RL cells compared to R cells (Figs. 3E, F, G) and lung weights were less. All of these effects were likely post-transcriptional; though mRNA for E-cadherin seemed downregulated in the R versus EV tumors (Fig. 3D) loading differences (note the GAPDH “controls”) make this effect less pronounced and more consistent with the in vitro data (Fig. 1C). Taken together, we find that lipocalin 2 enhanced the epithelial phenotype and inhibited metastasis of ras transformed cells.

MAPK signaling: activation by ras and suppression by lipocalin 2 - Ras has multiple downstream effectors (26). It activates raf, which in turn activates MEK, leading to the phosphorylation of MAPK. To clarify the ras pathway of EMT, we assessed the effect of a MEK inhibitor (U0126) and a PI3K inhibitor (LY294002) on R cells. As shown in Fig. 4, the MEK inhibitor reversed ras-induced EMT, but the effect of the PI3K inhibitor was partial, at best. Because U0126 can inhibit MEK5 in addition to the MEK1/2 (being referred to here as MEK), we infected R cells with an adenovirus carrying a dominant negative form of MEK1 and found the same results as those obtained with U0126 (data not shown). These data indicate that ras-MEK signaling is essential for EMT.

To determine whether lipocalin 2 reverted ras-induced EMT by interfering with MEK signaling, we added purified lipocalin 2 protein (iron-loaded with siderophore, Lipo:Sid:Fe) to R cells and found that ras induced phosphorylation of raf, MEK and ERK1/2, was largely abrogated, but that total ras expression was unchanged (Fig. 5A). Signaling events downstream of ERK activation were then monitored with a multi-copy serum-response element (SRE) - luciferase construct introduced into EV, R and RL (Fig. 5B). RL and EV cells gave comparable levels of luciferase activity, but this was only about half to two-thirds of the transcription found in R cells. Just like R cells treated with exogenous protein (Fig. 5A), R cells infection with recombinant adenovirus carrying lipocalin 2, but not GFP, reduced SRE-luciferase activity, MEK and ERK1/2 phosphorylation, without altering ras expression (data not shown). These data indicate that ras-MEK is modulated by lipocalin 2.

To localize the effect of lipocalin on ras-MAPK signaling, we utilized an adenovirus and expression plasmid encoding a constitutively active MEK (MEK-DD) (27). MEK-DD adenoviral infection of EV cells led to increased SRE-luciferase activity (increased MAPK activity; data not shown). Importantly, constitutively active MEK resulted in a concentration dependent EMT, as ascertained by cell shape and colony morphology (Fig. 5C) and by expression of E-cadherin protein (Fig. 5D) in RL cells, indicating that MEK-DD was dominant over the effect of lipocalin 2. Consistent with this idea, MEK-DD also increased SRE-luciferase activity in EV cells but lipocalin 2 protein (Lipo:Sid:Fe) was unable to inhibit this effect (Fig. 5E, lanes 1,4, and 5). On the other hand, lipocalin 2 protein downregulated SRE-luciferase activity resulting from transfection of a constitutively active form of H-ras V12 (CA-H-ras) (Fig. 5E, lanes 1,2, and 3), as would be expected from the data with stable clones in Fig. 5B. Also, lipocalin 2 cDNA transfection induced E-cadherin expression in EV cells, but this effect...
Lipocalin 2 inhibits ras induced E-cadherin phosphorylation and degradation - To determine how lipocalin might affect ras mediated EMT, we focused on the expression of E-cadherin and its relationship to MAPK signaling. Lipocalin 2 is likely to modulate E-cadherin expression on a post-transcriptional level because it did not affect E-cadherin mRNA levels (Figs. 1C and 3D) nor did it enhance E-cadherin promoter transcriptional activity (data not shown). Indeed, we found that E-cadherin is powerfully regulated by proteosomal mediated degradation, because proteasome inhibitor MG132 (0.5 nM) for 2 days increased E-cadherin protein in R cells (Fig. 6B, lanes 3 and 4) and in EV cells (Fig. 6B, lanes 1 and 2). In contrast, MG132 only slightly increased E-cadherin in RL cells (Fig. 6B, lanes 5 and 6), suggesting that E-cadherin degradation was already inhibited, and implicating lipocalin 2 in the process. There was also no significant difference in GAPDH protein expression, showing specificity and lack of toxicity of MG132. Further, it is likely that regulation of E-cadherin by proteosomal degradation is relevant to ras mediated EMT, because MG132 reverted R cells to an epithelial phenotype (Fig. 6A).

E-cadherin degradation is mediated by phosphorylation at the binding site for p120 and then recognition by Hakai (28), which targets the protein for ubiquitination and proteosomal degradation. However Hakai expression was unchanged by ras transformation or by lipocalin 2 expression (Fig. 6C). However, we found that E-cadherin phosphorylation was higher in R cells than in either EV or RL cells or R cells treated with the MEK inhibitor U0126 (Fig. 6D, top panel), in a pattern inversely correlated with E-cadherin protein levels (Fig. 6D, second panel), but unaccounted for by changes in E-cadherin mRNA levels (Fig. 6D, third panel). Hence, E-cadherin phosphorylation is a target of ras signaling in 4T1 cells, that MEK activation - critical for EMT - is also responsible (directly or indirectly) for E-cadherin phosphorylation, and that lipocalin 2 impinges on the ras-MAPK pathway, suppressing E-cadherin phosphorylation, and presumably decreasing its turnover.

Role of iron - Because the inductive activity of lipocalin 2 is markedly enhanced by loading the protein with iron (17), we tested the effect of iron on E-cadherin expression and MAPK signaling. Deferoxamine mesylate (2 - 5 µM; DFO), an iron chelating agent that can deplete iron from the intracellular pool (29), changed the morphology of RL cells to a mesenchymal phenotype and suppressed E-cadherin expression (Fig. 7A) indicating that iron was necessary for E-cadherin expression. Indeed the effect of lipocalin 2 preparations on R cell epithelial morphology (see Supplemental Fig. B) and E-cadherin expression correlated with iron carriage (Lipo:Sid:Fe>Lipo:Sid>Lipo; Fig. 7B and Supplemental Fig. C) and was dose dependent. (It should be noted that because the affinity of the siderophore for iron is so high Kd=10^{-49} (30), it is likely that the unloaded siderophore partially loaded with iron from the culture media). The same rank order was found the phosphorylation state of ERK1/2 (Fig. 7C) in cells treated with the lipocalins. In contrast to these results, simply adding iron (ferric ammonium sulfate; 50 µM) to R cells did not change their phenotype. Hence the data demonstrate that lipocalin 2 inhibits ras mediated transformation, by upregulating E-cadherin through an inhibition of MAPK signaling in an iron dependent manner, but iron alone is insufficient to reverse EMT.

Discussion

In this report we demonstrate that lipocalin 2 can alter the invasive and metastatic behavior of ras transformed breast cancer cells - in vitro and in vivo - by reversing the EMT inducing activity of ras, through restoration of E-cadherin expression, via effects on the ras-MAPK signaling pathway. The data are consistent with overexpression models of E-cadherin which prevents invasiveness...
of human carcinoma cell lines (4,10,31,32). However, to the best of our knowledge, there has never been a soluble factor which can upregulate E-cadherin and reverse metastatic phenotype in vitro and in vivo.

**Lipocalins, cancer and lipocalin 2’s effects on EMT in tumor cells** - Increased expression of lipocalin 2 accompanies numerous transformations (induction by polyoma, SV40, phorbol ester and the neu oncogene), and human carcinomas (colorectal, hepatic, pancreas, breast), but the action of the protein has been obscure (reviewed in (33) with the exception of β2μ-globulin in inducing renal cancer (34). One report using antisense RNA in an esophageal cancer cell line implanted in an animal suggests that lipocalins are tumor promoters in vivo (35), and lipocalin 2 may promote slightly the proliferation of estrogen receptor negative mammary cells in vitro (36). However using a large variety of assays we find a protective role for lipocalin 2 during ras mediated transformation and metastasis in vitro and in vivo. Indeed the lipocalin 2 produced smaller, more coherent tumors of higher density (similar weight but different cell types), with less regional invasion and dramatically fewer metastases in vivo as assessed by lung weight, by the number of nodules on the lung surface, as well as by histology. Consistent with this anti-metastatic action of lipocalin 2, two reports have noted the loss of lipocalin 2 expression at metastatic (colon) carcinoma sites (13,14), in contrast to abundant expression in the primary location. Moreover, our data are consistent with lipocalin 2’s actions on embryonic mesenchyme.

**Lipocalin 2 signaling** - Lipocalins may stimulate cell growth and development by binding to cell surface receptors (37) and activating downstream targets (18) and/or by delivering to cells small molecules such as retinoids (38). For lipocalin 2, no receptor has been identified nor have intracellular signaling events been defined. We demonstrate that lipocalin 2 regulates ras signaling.

Numerous pathways have been defined downstream of ras activation (26,39). In human tumors, ras activation typically occurs as a result of ras mutations, leaving it in a constitutively active state. The two signaling pathways studied as ras effectors include the ras-MAPK and the PI3K/Akt pathways, but we found that ras mediated EMT could be reversed by a MEK inhibitor, suggesting that the classical ras-MAPK pathway was critical for the maintenance of EMT in 4T1-ras cells. Lipocalin 2 protein reduced the phosphorylation level of raf, MEK and ERK1/2 and the downstream activation of a reporter consisting of concatemers of the serum response element but could not reduce SRE driven luciferase activity in the presence of a constitutively active form of MEK, suggesting that the point of lipocalin action on the ras-MAP kinase pathway was downstream of ras and upstream of MEK. Taken together with the raf phosphorylation data and the lack of change in ras expression levels, we suggest that lipocalin 2’s point of action lies between ras and raf activation. Studies using constitutively active raf would be needed to confirm this finding and the use of a raf mutant constitutively targeted to the cell membrane would help to further refine the point of action of lipocalin 2. It is unlikely that lipocalin exerts its action on the level or activity of the recently defined proteins IMP, KSR and RKIP as these are felt to act downstream of raf (40,41).

Given the effect of lipocalin 2 on ras-MAPK signaling, we examined the phosphorylation state of E-cadherin and discovered that the ras pathway phosphorylated E-cadherin. Phosphorylation was commensurate with a decrease in absolute levels of E-cadherin and conversely both lipocalin 2 as well as the MEK inhibitor markedly downregulated E-cadherin phosphorylation, while increasing the level of protein expression. Hence MEK promotes E-cadherin phosphorylation and conversely lipocalin 2 inhibits this pathway. Phosphorylation of E-cadherin appeared to be a critical signal for degradation, because Hakai, a ubiquitin ligase recognizes phosphorylated E-cadherin and targets it for proteasomal disposal. Consistent with this pathway, the proteasome inhibitor MG132 upregulated E-cadherin in EV cells as well as in R cells, but had minor effects on RL cells, (which might have been the result of pre-inhibition of E-cadherin degradation by lipocalin 2) and reverted the mesenchymal phenotype, suggesting that the proteasome is essential for ras induced transformation. Similar findings in HGF
and Src-induced MDCK transformation have been reported by Nigam et al (42) and this pathway is consistent with recent studies that showed that activation of the MAPK pathway promotes degradation of the β-subunit of the epithelial Na⁺ channel (ENaC) by the proteasome pathway (43).

The iron requirement for lipocalin 2’s MET promoting activity - The effect of lipocalin 2 on E-cadherin expression was enhanced by the siderophore- and even more so by the iron-siderophore-lipocalin 2 complex. Similar data were obtained in embryonic rat mesenchyme (17,21). In both of these cases, the activity of the complex might be ascribed to the siderophore, to the iron, or to the combination of any of these components with the carrier protein. First, it is most likely that the iron siderophore form is the effector, rather than the unloaded siderophore. This is because in both ras transformed cells and embryonic mesenchyme the iron loaded form had greater activity than the iron unloaded form. Second, it is very likely that some of the iron free siderophore-lipocalin 2 complex became partially loaded with iron in the cultures, because of its great avidity for iron (30). These data indicate that iron enhances the actions of lipocalin 2. In fact, in preliminary experiments, when we substituted iron with gallium, a metal that binds enterochelin siderophores (30), but does not undergo redox reactions that characterize iron, the induction of E-cadherin in mesenchyme was greatly diminished.

One possible explanation for the data then, is that iron delivery is itself sufficient to modulate E-cadherin levels, particularly because the addition of DFO inhibited E-cadherin expression in RL cells. In agreement with this notion, DFO was found to induce phosphorylation of ERK1/2 (44). However, supplying iron to R cells, in excess of the culture media, did not upregulate E-cadherin. Further, there is a report that iron overload decreases E-cadherin mRNA (45). Hence it appears that different parts of the E-cadherin pathway have different sensitivities to iron loading: the ERK1/2 mediated pathway of E-cadherin degradation is iron suppressible, but de novo synthesis of E-cadherin is iron-insensitive. Hence, lipocalin 2 may modulate E-cadherin degradation by iron delivery, but it may be necessary to invoke a second lipocalin 2 mediated signal that initiates changes in E-cadherin levels. Indeed, recent work by Devireddy et al. showed that lipocalin 2 suppressed ATF5 expression in lymphocytes (18), suggesting iron independent signaling by the protein. However, there are very few proteins that are known to be truly bifunctional, and the role of the lipocalin as a carrier protein has not been previously addressed, nor was ATF5 modulated by lipocalin 2 in embryonic kidney (J. Barasch, unpublished). Comparing ligand to carrier protein based signaling will require mutagenesis of the lipocalin 2 calyx to abolish binding of ligands, or possibly the use of the gallium-siderophore to block iron mediated signaling.

Future studies

Many interesting questions remain. Is there a cell-surface receptor for lipocalin? Further studies on lipocalin mediated signaling events would be aided by the identification of such a molecule. Will lipocalin 2 reverse other actions of ras besides EMT? For example, ras can induce an angiogenic phenotype through upregulation of VEGF and this effect appears to be reversed by lipocalin 2 (manuscript in preparation). Moreover, ras induced EMT in MDCK cells is also reversed by lipocalin 2, so the studies described here are not unique to the 4T1 breast cancer line. Interestingly, lipocalin 2 appears to revert EMT changes induced by other agents, such as TGF-β (data not shown). The mechanism by which lipocalin exerts these effects is under investigation as are the gene targets for its action. Finally, it is known that the lipocalin promoter is inducible both by glucocorticoids and by estrogen (36,46). Thus screens for small molecules that might induce endogenous lipocalin 2 could be undertaken. Such drugs could be of therapeutic benefit and may be easier to use than lipocalin 2 protein.

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Fig. 1 Ras-induced EMT in 4T1 cells and effects of lipocalin 2
Clones EV, R and RL were analyzed. (A) Upper panel shows phase contrast and lower panel fluorescent images for E-cadherin by confocal microscopy. (B) Western blot analysis of clones. EV, R and RL cells blotted with antibodies to E-cadherin, vimentin and GAPDH. (C) Results of RT-PCR analysis for E-cadherin and GAPDH. (D) R cells were transiently transfected with lipocalin 2 pcDNA3.1 and E-cadherin protein level was analyzed. R cells were seeded in a 6-well plate and transfected with lipocalin 2 by Fugen6 at 40% confluency. After 48 h, cells were trypsinized, respread on 6-well plate and transfected again in the same conditions. After 72 h, cells were harvested and analyzed by western blotting. Transfected amounts of lipocalin 2 pcDNA3.1 were 0, 1, 2 µg/well (lanes 1-3 respectively) and lane 4 (EV) represents 4T1-EV cells as a control. Total amount of transfected cDNA was equalized with the empty vector pcDNA3.1. (E) R cells were cultured with conditioned medium (CM) containing lipocalin 2 produced from 293T cells transfected with lipocalin 2-pcDNA3.1. EV cells were seeded in a 6-well plate with 1ml/well containing 10% FCS with DMEM and 2 ml of CM was added at 10% confluency. After 72 h, cells were harvested and analyzed by western blotting. CM is a mixture of media from 293T cells transfected with lipocalin 2 and from 293 cells transfected with empty vector (pcDNA3.1). Amount of media from lipocalin 2-transfected 293T cells was 0, 1, 2 ml for lanes 1-3 respectively with total amount of media equalized by addition of media from empty-vector transfected 293T cells. Lane 4 (EV) represents EV cells as a control. GAPDH serves as a loading control.

Fig. 2 Invasion migration assay using each stable clone of 4T1
Polycarbonate membranes of Transwells were coated with Matrigel® and cells were seeded. Sixteen hours later, cells were fixed, stained with Giemsa solution, and counted for each of the stable clones EV, R and RL.

Fig. 3 Lipocalin 2’s effects on 4T1 primary tumor growth and metastasis and on lung metastasis
4T1 clones (EV, R and RL) were suspended in PBS and injected subcutaneously in the backs of Balb/c mice. (A) primary tumor size was calculated based upon measurements at 1, 2 and 3 weeks. (B) Hematloxalin and eosin (H & E) staining of tumor sections. White arrow in the middle shows muscle tissue into which tumor has invaded. (C) Western blot of lysate from primary tumor for the antigen indicated. (D) RT-PCR for each primary tumor. Top lane shows the expression of lipocalin 2 mRNA in the RL stable cell clone using primers directed against the HA tag in the lipocalin 2 cDNA. (E&F) Lung weight (E) and the number of metastatic nodules on the lung surface (F) were evaluated. (G) H & E
staining of lung sections.

**Fig. 4** Effects of PI3K and MEK inhibitors on ras-induced EMT
(A) Fluorescent images for E-cadherin staining in R cells by confocal microscopy. R cells (left panel) were incubated with the PI3K inhibitor (LY294002, 10µM) (middle panel) and MEK inhibitor (U0126, 10µM) (right panel). (B) Western blotting of E-cadherin and GAPDH for each condition.

**Fig. 5** Lipocalin 2’s effects on ras-MAPK signaling
(A) Effects of lipocalin 2 on phosphorylation state of ras-MAPK signaling molecules. EV and R cells were starved in DMEM without serum for 48 h. During this time, half of the R cells were incubated with 50 µg/ml of lipocalin 2 protein with iron-loaded siderophore (R + Lipo:Sid:Fe), after which all cells were incubated with 10% FCS containing DMEM for 20 min and then harvested for western blotting with phosphospecific antibodies. (B) Using 4T1 clones (EV, R and RL), SRE-luciferase assay was performed after the 48 h incubation in serum free DMEM. Ratio of renilla luciferase to sea-pansy luciferase is shown on the ordinate. (C&D) RL cells in a 6-well plate were infected with an adenovirus carrying the MEK dominant active form (MEK-DD) and a Lac-Z adenovirus at the indicated multiplicities (MOI) in 2% serum including DMEM medium for 48 h. Cells were then trypsinized, respread on 6-well plate at 5-10% confluency, and incubated with 10% serum including DMEM medium. Phase contrast images showing RL with 0, 200 and 400 MOI of MEK-DD adenovirus (right, middle and right panel respectively). All images (C) were taken at 24 h later after the final plating. Cell lysates (D) were collected for western blotting 48 h after the final plating. (E) Using 4T1-EV cells with or without Lipo:Sid:Fe, SRE-luciferase assay was performed. Plasmids coding for the constitutively active form of H-ras V12 (CA-H-ras) and/or a constitutively active form of MEK pcDNA3.1 (MEK-DD) were transfected as indicated 2 h before the protein loading. 24 h later, cells were incubated in serum free DMEM in the presence of Lipo:Sid:Fe for another 24 h.

**Fig. 6** Proteasome inhibitor effects on ras-induced EMT and effects of ras, lipocalin 2, and an MEK inhibitor on E-cadherin phosphorylation.
(A) Morphology of R cells treated with proteasome inhibitor MG132 (0.5 nM) for 48 h. (B) Stable clones (EV, R and RL) were analyzed by western blotting with or without proteasome inhibitor MG132 (48 h). (C) Hakai protein expression levels in 4T1 clones were analyzed by western blotting. (D) E-cadherin phosphorylation, protein level and mRNA levels in EV, R, and RL cells and R cells treated with the MEK inhibitor (U0126).

**Fig. 7** Iron requirement for lipocalin 2’s functions
(A) RL cells were incubated with DFO for 48 h. Phase contrast pictures and western blotting for E-cadherin and GAPDH are shown. The DFO concentrations were 0, 2, 5 µM (left, middle and right panels or lanes, respectively). (B) E-cadherin expression in R cells incubated with Lipo:Sid:Fe (lanes 7, 8), Lipo:Sid (lanes 5,6), Lipo (lanes 3,4) or PBS (lanes 1,2). The protein concentrations were 15 µg/ml (lanes 4, 6, 8) or 50 µg/ml (lanes 3, 5, 7). (C) Effects of lipocalin 2 formulations on ERK phosphorylation. R cells at 50% confluency on 6-well plate were incubated in 0% serum including DMEM for 48 h with PBS or lipocalin 2. Cells were stimulated with 10% serum containing DMEM for 20 min and cell lysates were collected for western blotting with phospho-ERK and total ERK antibodies.

**Fig. 8** Summary of lipocalin 2’s effects on ras induced signaling
This schematic shows (1) that lipocalin 2 antagonizes ras signaling at a point upstream of raf activation in the ras-MAPK pathway, and (2) that activation of the ras-MAPK pathway leads to phosphorylation of E-cadherin due to the action of MEK or a downstream kinase.
**Fig. 1; Hanai et al. 2005**

**A**

![Images of cell cultures and immunofluorescence](Fig.1_A.png)

**B**

<table>
<thead>
<tr>
<th>EV</th>
<th>R</th>
<th>RL</th>
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<tr>
<td>E-cad</td>
<td>vimentin</td>
<td>GAPDH</td>
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**D**

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<td>Lipocalin 2 cDNA (μg/well)</td>
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<tr>
<td>E-cad</td>
<td>GAPDH</td>
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**C**

<table>
<thead>
<tr>
<th>EV</th>
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<tbody>
<tr>
<td>E-cad mRNA</td>
<td>GAPDH mRNA</td>
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**E**

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<tr>
<td>Lipocalin 2 CM (ml/well)</td>
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</tr>
<tr>
<td>E-cad</td>
<td>GAPDH</td>
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Fig. 2; Hanai et al. 2005

![Graph showing invasion assay results with EV, R, and RL groups compared.](http://www.jbc.org/)

- EV: n=7
- R: n=7
- RL: n=7

P < 0.01 for comparisons between R and the other groups.
Fig. 3; Hanai et al. 2005

A

B

EV

R

RL

C

EV

R

RL

E-cad
vimentin
GAPDH

D

EV

R

RL

Exogeneous lipocalin 2
mRNA
E-cadherin mRNA
GAPDH mRNA

E (g)

Lung weight

P<0.05

P<0.05

EV

n=5

R

n=4

RL

n=5

F

number of lung metastasis

P<0.01

P<0.01

EV

n=5

R

n=4

RL

n=5

G

EV

R

RL
Fig. 4; Hanai et al. 2005

![Image of Western blots and immunofluorescence images showing R, R + LY294002, and R + U0126 conditions with E-cad and GAPDH expression.]

- R
- R + LY294002
- R + U0126

Western blot analysis with bands for E-cad and GAPDH.
Fig. 5; Hanai et al. 2005

A

<table>
<thead>
<tr>
<th>EV</th>
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<th>R + Lipo:Sid:Fe</th>
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<tbody>
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<td>p-MEK</td>
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<td>GAPDH</td>
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B

Luciferase activities (ratio)

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<tr>
<th>EV</th>
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<th>RL</th>
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<tbody>
<tr>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
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</table>

n=4

C

RL

RL+ MEK-DD 200MOI

RL+ MEK-DD 400MOI

D

MEK-DD (MOI)
Lac-Z (MOI)
E-cad
GAPDH

E

* = Lipo:Sid:Fe

Luciferase activities (ratio)

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<thead>
<tr>
<th>CA-H-ras</th>
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<td>P&lt;0.01</td>
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n=4
Fig. 6; Hanai et al. 2005

A

R

R + MG132 (0.5 nM)

B

<table>
<thead>
<tr>
<th>EV</th>
<th>R</th>
<th>RL</th>
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<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
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</table>

MG132 (0.5 nM)

E-cad

GAPDH

C

EV  R  RL

Hakai

GAPDH

D

EV  R  RL  R+U0126

IP:E-cad

Blot:P-Tyr

Blot:E-cad

E-cad mRNA

GAPDH mRNA
Fig. 7; Hanai et al. 2005

A

RL

RL + DFO 2 μM

RL + DFO 5 μM

E-cad

GAPDH

B

1 2 3 4 5 6 7 8

E-cad

GAPDH

C

PBS Lipo:Sid:Fe Lipo:Sid Lipo

p-ERK1/2

ERK1/2
Fig. 8; Hanai et al. 2005

Lipocalin 2

ras → raf

MEK

ERK

phospho-E-cad

degradation
Supplemental data A; Hanai et al. 2005

(1) Semi-quantitative RT-PCR

(2) PCR of genomic DNA

Downloaded from http://www.jbc.org/ by guest on September 1, 2017
Supplemental data B; Hanai et al. 2005

<table>
<thead>
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<th>Lipocalin 2 cDNA (μg/2ml)</th>
<th>MEK-DD (MOI)</th>
<th>Lac-Z (MOI)</th>
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<th>GAPDH</th>
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Supplemental data C; Hanai et al. 2005

R + Lipo:Sid:Fe

R + Lipo:Sid

R + Lipo

R + PBS
Supplemental Figure Legends

(A) Confirmation of exogeneous ras expression in R and RL cells
(1) Using mRNA from 4T1 cells (EV, R and RL), semi-quantitative RT-PCR was performed to detect both endogeneous H-ras and the exogeneously introduced H-ras A12 allele. The primer set used was 5’-CAGAATACAGCTTGGTGTTG-3’ and 5’-CATGCAGCCAGGACCCTCTCATC-3’. The arrow denotes the composite of the H-ras and H-ras A12 mRNA signal at 550 bp, since the primer set cannot distinguish these two. The increased signal in the R and RL cells compared to that in EV cells is likely from the H-ras A12 transfected gene, suggesting that the RL cells have not lost expression of H-ras A12.
(2) PCR of genomic DNA for H-ras A12 was performed in R and RL cells using the same primer set. The endogeneous H-ras genomic DNA contains several exons and introns between the location of these primers so that only the exogeneously integrated H-ras A12 DNA is amplified with the resultant band of 550 bp. The H-ras A12 DNA was detected only in the R and RL cells.

(B) R cells converted to an epithelial phenotype by lipocalin 2 transfection are reverted to a mesenchymal state by MEK-DD.
R cells at 40% confluency on 6-well plates were transfected with lipocalin 2-pcDNA3.1 at the indicated dose (µg/2ml) using Fugen6 and incubated for 48 h. Cells were trypsinized and replated in 6-well plates and were transfected again under the same conditions. Cells were trypsinized and infected with MEK-DD adenovirus or a Lac-Z adenovirus in the same conditions as in Fig. 5D. Cell lysates were collected for western blotting at 48 h after the final plating.

(C) Phase contrast images showing R cells treated with various lipocalin 2 formulations in the same conditions as in Fig. 7C.
Lipo:Sid:Fe, Lipo:Sid and Lipo proteins were used at a concentration of 50 µg/ml.
Lipocalin 2 diminishes invasiveness and metastasis of ras transformed cells
Jun-ichi Hanai, Tadanori Mammoto, Pankaj Seth, Kiyoshi Mori, S. Ananth Karumanchi, Jonathan Barasch and Vikas P. Sukhatme

J. Biol. Chem. published online February 3, 2005

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