No Effect of NGAL/lipocalin-2 on Aggressiveness of Cancer in the MMTV-PyMT/FVB/N Mouse Model for Breast Cancer

Elisabeth P. Cramer¹, Andreas Glenthøj¹, Mattias Häger¹, Anna Juncker-Jensen², Lars H. Engelholm², Eric Santoni-Rugiu³, Leif R. Lund⁴, Ole D. Laerum².⁵, Jack B. Cowland¹*¹, Niels Borregaard¹*¹

¹ The Granulocyte Research Laboratory, Department of Hematology, Copenhagen University Hospital, Copenhagen, Denmark, ² Finsen Laboratory, National University Hospital, Rigshospitalet, University of Copenhagen, Denmark, ³ Department of Pathology, National University Hospital, Rigshospitalet, University of Copenhagen, Denmark, ⁴ Department of Cellular and Molecular Medicine, University of Copenhagen, Denmark, ⁵ The Gade Institute, Section of Pathology, University of Bergen, Norway

Abstract

NGAL/lipocalin-2 is a siderophore-binding protein that is highly expressed in several cancers. It is suggested to confer a proliferative advantage to cancer cells. Its expression has been correlated with aggressiveness of breast cancer as assessed in mouse breast cancer models. This was recently confirmed in two mouse models of spontaneous breast cancer in wild-type and lipocalin-2-deficient mice. We used a similar strategy using a different mouse strain. Lipocalin-2-deficient mice and mouse mammary tumor virus-polyoma middle T antigen (MMTV-PyMT) mice were crossed into the same FVB/N background. All mice developed tumors by week 8. The mice were sacrificed on week 13 and tissue was processed for biochemical and histological analysis. The total tumor volume and number of metastases were quantitated in 26 lipocalin-2-deficient mice and 34 wild-type controls. Lipocalin-2 expression in tumors of MMTV-PyMT-positive and wild-type mice was assessed by quantitative real-time PCR and by immunohistochemistry. The expression of the lipocalin-2 receptors 24p3R and megalin and of Mmp-9, transferrin receptor, and Bdh2 (a producer of a mammalian siderophore) were quantitated by real-time PCR. No significant difference was observed between wild-type and lipocalin-2-deficient mice. Lipocalin-2 was highly expressed in tumors from wild-type mice, but the expression did not correlate with tumor size. No effect of lipocalin-2 was observed with respect to time to tumor appearance, total tumor volume, or to the number of metastases. Histology and gelatinolytic activity of the mammary tumors did not differ between wild-type and lipocalin-2-deficient mice. We conclude that NGAL/lipocalin-2 does not invariably affect the aggressiveness of breast cancers as assessed in mouse models, thus questioning the role of lipocalin-2 in cancer development.

Introduction

NGAL, neutrophil gelatnine associated lipocalin, was so named by its discoverers as a lipocalin present in human neutrophil specific granules, in part covalently associated with gelatinase B/ matrix metalloproteinase-9 (MMP-9) [1,2]. Highly homologous proteins are found in other species such as exFAB in fowls [3] and neu-related lipocalin induced in rat mammary tumors by the neu oncogene [4]. The mouse orthologue was identified as an oncogene and termed 24p3 [5] but has also been termed major urinary protein [6] and siderocalin [7]. These different names are united in the term lipocalin-2 encoded by the Lcn2/Lnu2 gene in man and mouse, respectively.

Soon after the discovery of lipocalin-2 as a constituent of neutrophil specific granules, lipocalin-2 was found to be highly up-regulated in epithelial cells at sites of inflammation [8–10] and to be highly expressed in some cancers [4,11–13]. Lipocalin-2 has also received significant attention as an early and sensitive marker of damage to kidney tubule epithelial cells [14–17]. A function of lipocalin-2 in the innate immune defense against bacteria was demonstrated shortly after the discovery of lipocalin-2 as a siderophore-binding protein [7,18,19], as mice deficient in lipocalin-2 are more susceptible to infections by E. coli [20–22], K. pneumonia [23], and M. tuberculosis [24,25] than their wild-type litter controls.

Lipocalin-2 has been inferred as an important regulator of apoptosis in myeloid cells [26]. This is claimed to depend on the ability of cells to take up lipocalin-2 via the 24p3-receptor [27]. Such uptake would result in apoptosis if lipocalin-2 is iron-deplete and promote growth if lipocalin-2 is iron-replete. The bacterial enzyme Ent A synthesizes the siderophore constituent 2,3-dihydroxy benzoic acid (DHBA). A mammalian homologue named BDH2 was recently described which generates 2,5-DHBA, the long sought for endogenous siderophore responsible for lipocalin-2 mediated...
iron trafficking in mammalian cells [28]. Catechol, a metabolite of tyrosine and other organic compounds, was identified as an alternative endogenous siderophore candidate [29]. The role of lipocalin-2 in controlling myeloid cell apoptosis is, however, questioned by the lack of obvious alterations in myelopoiesis of lipocalin-2-deficient mice [21] and in in vitro studies of the effect of lipocalin-2 on isolated human myeloid cells [30].

The involvement of lipocalin-2 in tumor genesis has particularly been studied in breast cancer, where lipocalin-2 (NGAL) expression is associated with poor prognosis in human primary breast cancer [31] and an increased urinary level of lipocalin-2 correlates with aggressiveness of the cancer [32]. The proposed mechanisms by which lipocalin-2 promotes growth and metastasis of breast cancer cells are multiple. Association of lipocalin-2 with MMP-9 was shown to induce allosteric activation of the enzymatic mechanisms by which lipocalin-2 promotes growth and metastasis [32]. Since the genetic background influences the development of tumor formation with MMP-9 seems to be part of the mechanism. Our study used the same MMTV-PyMT mouse model as Berger et al. [38], but the results differ substantially, as we do not observe any significant effect of lipocalin-2 on any parameter associated with tumor growth and metastasis despite a brisk up-regulation of lipocalin-2 in the breast cancer cells. In addition, our study addresses whether the lipocalin-2 receptors and Bdh2 are expressed in the tumors.

Materials and Methods

Ethics statement

All animal experiments were conducted at The Department of Experimental Medicine, University of Copenhagen and National University Hospital, Rigshospitalet, Copenhagen, Denmark, in accordance with both institutional and national guidelines (Danish Animal Experiments Inspectorate, permission number 2007/561–1353). The review board at the Faculty of Health Science, University of Copenhagen, approved this study (P0599). An observer unaware of the genotypes of the mice performed all experimental evaluations. The mice were inspected daily and palpated once a week. No experiments were performed on live mice. Humane endpoint was set at tumor size influencing the general well being or behavior of the mice.

Mice breeding

Congenic heterozygous male FVB/N-MMTV-PyMT mice were mated with heterozygous female Lcn2 knock-out mice (Lcn2+/−) [20] that had been back-crossed to the FVB/N strain for 8 generations (N8). Their male FVB/N-PyMT, Lcn2+/− offspring (F1) were mated with FVB/N-Lcn2+/− (N9) females to generate the PyMT, Lcn2+/+ (n = 34); PyMT, Lcn2−/− (n = 26); Lcn2+/+ (n = 5), and Lcn2−/− (n = 7) mice used throughout the study (Figure S1).

Quantification of primary tumors

Mice were examined weekly for mammary tumor onset by palpation for nodules in all 10 mammary glands. Tumor volume was assessed by measuring the length (L) and width (W) of individual tumors with a caliper and calculated by the formula V(tumor) = π L W^2/6 [40]. The individual tumor volumes were summed to give the total tumor volume in each mouse.

Tissue processing

Mice were anaesthetized by intraperitoneal administration of 1:1 mixture of Hypnorm (Janssen-Cilag Ltd) and Midazolam (Roche) and tissue fixed by intracardiac perfusion with 10 ml cold PBS followed by 10 ml of freshly prepared PBS with 4% parafomaldehyde (PFA). A blood sample was obtained by heart puncture prior to perfusion-fixation and EDTA-plasma was isolated by centrifugation (2000 g, 30 minutes, 4°C) and stored at −20°C. The tumor located in the fourth breast gland on the left hand side was excised prior to PFA perfusion and stored at −80°C for later RNA purification or extraction for western blotting and zymography. Following PFA perfusion, the corresponding tumor on the right hand side was placed in PFA for paraffin embedding and histological examination. The lungs were removed and placed in PFA for further fixation followed by processing and immunohistochemical staining as described in [41].

Quantification of metastases

The volume of metastases in lungs was determined by a computer-assisted stereological method on hematoxylin eosin-stained sections of the lungs as described previously in [42].

Immunohistochemical staining

Immunohistochemical detection of lipocalin-2 and MMP-9 was performed as described in [22]. Antibodies used were goat anti-mouse lipocalin-2 (1:100, AF1857, R&D Systems) and rabbit anti-MMP-9 (1:2000, ab38898, Abcam). Immunohistochemical staining of formalin-fixed paraffin-embedded primary tumors for E-cadherin, vimentin, and alpha smooth muscle actin (α-SMA) was performed using the following antibodies: Rabbit anti-mouse E-cadherin (1:500, ab92547, Abcam), rabbit anti-mouse Vimentin (1:500, ab92547, Abcam), and rabbit anti-mouse α-SMA (1:500, ab5694, Abcam). The specimens for this procedure were selected among PyMT, Lcn2+/+ and PyMT, Lcn2−/− mice with large and small total volumes of primary tumors, large, intermediate, and small total volumes of metastases, and among mice with large, intermediate, and small numbers of metastases respectively. In total 16 specimens of primary tumors underwent immunohistochemical staining for E-cadherin, vimentin, and α-SMA. Sections of primary tumors were deparaffinized, hydrated, and antigen retrieval was performed by 10 minutes incubation with proteinase K at 37°C for E-cadherin-staining and 10 minutes of heating at 98°C in citrate buffer, pH 6 for vimentin and α-SMA staining. Endogenous peroxidase activity was blocked with 1% H2O2. Sections were incubated overnight with primary antibodies diluted in Dako Antibody Diluent (S3022, Dako). Sections were subsequently incubated for 45 minutes with secondary antibody, EnVision™-System HRP-Labelled Polymer Anti-rabbit (K4003, Dako), developed with Vector NovaRED (SK-4800, VWR International), counterstained with Mayer’s Hematoxylin, and finally dehydrated and mounted. The slides were examined under
a BX51 microscope (60x/1.40 PlanApo oil objective) with Olympus DP70 photo system and analySIS software 5.0 (Olympus) or Leica DM 2500 microscope with Leica DFC 425 camera and software.

**Histology**

The tumors were diagnosed on coded specimens using the classification of PyMT tumors by Lin et al. [43], and employed by Almholt et al. [44]. In short, the four tumor stages are: Hyperplasia, Adenoma, Early Carcinoma, and Late Carcinoma.
The patterns of stromal formation were also recorded. Evaluation of sections stained for E-cadherin, vimentin, and α-SMA were evaluated on coded specimens.

Isolation of murine granulocytes for Western blot
Two FVB/N wild-type mice were euthanized by cervical dislocation and the spleens were removed and homogenized in PBS with 4% fetal calf serum (FCS) with a mortar and a pestle. The homogenate was filtered through a 70 μm nylon mesh (352550, BD Biosciences) and kept on ice. Following centrifugation (300 g for 4 minutes, 4°C), the supernatant was removed and the pellet resuspended in Pharm Lyse (BD Biosciences) for lysis of erythrocytes. Lysis was terminated by addition of PBS with 4% FCS and the cells were pelleted by centrifugation (300 g for 4 minutes, 4°C) and subsequently resuspended in PBS with 4% FCS. Granulocytic cells were isolated by immunomagnetic sorting using the magnetic-activated cell sorting (MACS) system and Gr-1-Biotin antibody (130-092-332, Miltenyi or 51-01212J, BD Pharmingen) according to the manufacturer’s instructions (Miltenyi). Separation was performed on a MACS LS column (Miltenyi).

Figure 2. Representative immunohistochemical stainings of mammary tumors. A, staining for E-cadherin in a PyMT, Lcn2−/− mouse. B, staining for E-cadherin in a PyMT, Lcn2+/− mouse. C, staining for α-SMA in a PyMT, Lcn2+/+ mouse. D, staining for α-SMA in a PyMT, Lcn2−/− mouse. E, staining for vimentin in a PyMT, Lcn2−/− mouse. F, staining for vimentin in a PyMT, Lcn2+/− mouse. Original magnification x400. Abbreviations: PyMT: MMTV-PyMT. α-SMA: Alpha smooth muscle actin.

doi:10.1371/journal.pone.0039646.g002
Preparation of human samples for Western blot

Granulocytes were isolated from peripheral blood by use of Lymphoprep (Axis-Shield PoC AS) and treated with 5 mM diisopropyl fluorophosphate (Calbiochem). Granulocytes were resuspended in disruption buffer (100 mM KCl, 1 mM Na2ATP, 3.5 mM MgCl2, 10 mM PIPES, pH 7.2) with 0.5 mM phenylmethylsulfonylfluoride (PMSF) added. Cells were disrupted by nitrogen cavitation at 600 psi for 5 minutes and collected in 1.5 mM EGTA [45,46]. The cavitate was centrifuged at 400 g for 15 minutes to remove nuclei and unbroken cells. The supernatant (S1), which contains the granules, was analyzed further by Western blot as described below.

Tumor extraction

Four large tumors from PyMT, Lcn2+/+ mice and four tumors of corresponding sizes from PyMT, Lcn2−/− mice were selected for extraction. The tumors were kept on ice and homogenized with a blender in 5 μl of lysis buffer (150 mM NaCl, 50 mM Trizma base, 0.5% deoxycholic acid, 0.1% SDS, 1% NP-40) per mg weight of tissue. After 15 minutes of incubation on ice the

Table 1. Histological diagnoses of H+E-stainings of the breast tumors.

| Selection criteria                  | Histology, wild type | Histology, knock out |
|-------------------------------------|----------------------|----------------------|
| Largest total volume of primary tumors | LC                   | LC                   |
| Smallest total volume of primary tumors | LC                   | LC                   |
| Largest number of metastases        | LC                   | LC                   |
| Smallest number of metastases       | LC                   | EC                   |
| Largest total volume of metastases  | LC                   | LC                   |
| Smallest total volume of metastases | LC                   | LC                   |

Tumors diagnosed were selected by the above listed criteria.

LC = Late Carcinoma, EC = Early Carcinoma.

doi:10.1371/journal.pone.0039646.t001

Figure 3. Growth of primary tumors in PyMT, Lcn2+/+ and PyMT, Lcn2−/− mice. A, Lcn2-deficiency has no effect on tumor onset. Data shown are percentage of mice without palpable mammary tumors on the indicated weeks visualized by a Kaplan-Meier plot. Curves did not differ statistically significantly, log-rank test, p = 0.57 (n = 33 for PyMT, Lcn2+/+ and n = 25 for PyMT, Lcn2−/−). B, average of total volume of mammary tumors versus age. No statistical significant difference between PyMT, Lcn2+/+ and PyMT, Lcn2−/− mice at week 13, t-test after logarithmic transformation, p = 0.21 (n = 29 for PyMT, Lcn2+/+ and n = 19 for PyMT, Lcn2−/−). C, numbers of tumor positive glands per mouse as determined by palpation at week 10 where the first mouse out of all the mice presents with all mammary glands tumor positive. No significant difference in tumor positive mammary glands, Mann-Whitney U-test, P = 0.85 (n = 33 for PyMT, Lcn2+/+ and n = 25 for PyMT, Lcn2−/−). Horizontal bars indicate medians. Abbreviations: PyMT: MMTV-PyMT.

doi:10.1371/journal.pone.0039646.g003
tumor suspensions were centrifuged at 13,500 rpm for 10 minutes at 4°C and the supernatants were collected and stored at −20°C.

**Western blot**

The samples were diluted in sample buffer with or without reducing agents and boiled for 5 minutes. Electrophoresis was performed according to standard procedures on a 4–12% Nu Page Bis-Tris gradient gel (Invitrogen) as described in [22]. The antibodies used were goat anti-mouse lipocalin-2 (1:1000, AF1857, R&D Systems), rabbit anti-mouse MMP-9 (1:5000, ab38898, Abcam), mouse anti-human lipocalin-2 (1:1000, [47]) or rabbit anti-human MMP-9 (1:1000, [46]), followed by HRP-conjugated secondary antibodies: Rabbit anti-goat (1:1000, P0449, Dako), goat anti-rabbit (1:1000, P0448, Dako), or rabbit antismouse (1:1000, P0260, Dako). The membranes were developed by chemiluminescence using SuperSignal West Pico Chemiluminescence Substrate (Pierce) according to the manufacturer's instruction and analyzed on a Bio-Rad Chemidoc (Bio-Rad).

**Zymography**

Samples in 4% glycerol, 1% SDS, 0.125% bromophenol blue, 125 mM Tris-HCl, pH 6.8 were loaded on precast 10%, 1 mm gelatin zymogram gels (Novex Invitrogen EC61752 BOX) and run at 25 mA, washed in 2.5% Triton X-100, 20 mM Tris-HCl, pH 7.8, 5 mM CaCl$_2$, 1 μM ZnCl$_2$, stained for 30 min in 0.5% Coomassie, 10% acetic acid 30% ethanol, and destained in 10% acetic acid, 30% ethanol. For inhibition of metalloproteinase activity, 5 mM EDTA and 2 mM 1,10-phenantroline were added during the overnight incubation [48].

**RNA purification and quantification**

Tumors were homogenized and suspended in TRIzol Reagent (Invitrogen) for RNA isolation according to the manufacturer’s instruction. mRNA expression was determined by real-time PCR using the TaqMan method as described in [9]. The FAM-labeled probes used were Lcn2: Mm01324470_m1, Mmp9: Mm0044941_m1, Megalin (Lrp2): Mm01328171_m1, 24p3R (Scl22a1): Mm00480680_m1, and Bdh2: Mm00459075_m1. For internal normalization we used HEX-labeled Gapdh probe: 4326317E (all from Applied Biosystems).

**Results**

We chose the **MMTV-PyMT**-model on a FVB/N background for the study of spontaneous breast cancer development as tumor development is very uniform and predictable with time, thus offering minimal variation in tumor development and progression [49,50]. The breeding strategy is shown in Figure S1. The progression of mammary tumors was quantified as described in Materials and Methods and the mice were sacrificed at week 13.

Figure 1 (A, C, E) shows the expression of lipocalin-2 in tumors of the MMTV-PyMT and **MMTV-PyMT Lcn2** knock-out mice (from now on referred to as PyMT-mice). A uniform strong immunohistochemical staining for lipocalin-2 was observed in the tumor cells of wild-type mice whereas no labeling was observed in the knock-out mice. MMP-9 was observed in the few neutrophils present and no difference was observed between wild-type and **Lcn2** knock-out mice (Figure 1 B, D, F). Histological examination showed that all tumors except one were Late Carcinomas. The only Early Carcinoma was from the group with lowest numbers of metastases, indicating low malignant potential. No obvious differences in the stroma were observed between the two groups. In late Carcinomas, fibrous strands of collagen tissue were seen between tumor nodules, and tumors were otherwise surrounded by loose connective tissue or fatty tissue. Focal squamous differentiation was seen in one Late Carcinoma, a feature which is not considered to alter the malignant potential. In the other Late Carcinomas, smaller and larger cystic necroses or partly cystic glands were seen. Hence, no difference in indices of malignancy was observed between wild-type and knock-out mice as determined by histological examination (Figure 1 G, H and table 1).
Moreover, we performed immunohistochemical staining for the luminal marker E-cadherin, the myoepithelial marker α-smooth muscle actin (α-SMA), and the mesenchymal marker vimentin. E-cadherin was negative in all tumors (Figure 2 A, B) but clearly positive in adjacent normal mammary glands and epidermis (Figure S2 A). α-SMA was also negative in all tumors, but some positivity was seen in tumor capsule and vessel wall (Figure 2 C, D). Normal mammary glands with myoepithelial cells and vessel walls stained positive for α-SMA as expected (Figure S2 B). Vimentin stained positive in tumor capsule and in small connective tissue strands between tumor nodules, but the core of the tumor nodules was negative (Figure 2 E, F). This is

**Figure 5. Quantitative real-time PCR analysis of mRNA levels in mammary tumors/glands.** Expression levels of each marker are shown relative to the value found in tumors of PyMT, Lcn2+/- mice, which is given the value 1. The cycle threshold (Ct) value of the PyMT, Lcn2+/- mouse with the highest expression of each marker is: Lcn2 (Ct = 20), Mmp-9 (Ct = 34), 24p3R (Ct = 28), Tfrc (Ct = 27), and Bdh2 (Ct = 35). Data for the PyMT, Lcn2+/- and PyMT, Lcn2-/- mice are the mean expression in tumors of nine mice each (each measured in triplicate). Data for the Lcn2+/- and Lcn2-/- mice represent the mean expression in mammary glands of two mice each (each measured in triplicate). The vertical error bars represent the standard deviations. Abbreviations: PyMT: MMTV-PyMT.

doi:10.1371/journal.pone.0039646.g005
conformable with the observations performed on H+E-stained sections where very little stroma is seen in tumors. Overall we saw no differences in staining for E-cadherin, vimentin and α-SMA between tumors from PyMT, Lcn2+/+ mice, p = 0.68 (n = 27). PyMT, Lcn2+/+ mice are indicated by dots and Lcn2+/+ mice are indicated by a square. Solid line indicates best fitted line and dashed lines indicate 95% confidence bands. Abbreviations: PyMT: MMTV-PyMT.

Figure 6. Plasma-lipocalin-2 (p-LCN2) analyzed by ELISA. There is no correlation determined by linear regression between the total volume of mammary tumors at week 13 and p-LCN2 in PyMT, Lcn2+/+ mice, p = 0.68 (n = 27). PyMT, Lcn2+/+ mice are indicated by dots and Lcn2+/+ mice are indicated by a square. Solid line indicates best fitted line and dashed lines indicate 95% confidence bands. Abbreviations: PyMT: MMTV-PyMT.

doi:10.1371/journal.pone.0039646.g006
Both in plasma and in tumor extracts, but with no difference in the patterns between wild type and Lcn2 knock-out mice. No activity with a MW higher than the MW of monomeric human MMP-9 was observed in tumors. All gelatinolytic activity was inhibited by EDTA and phenantrolin (data not shown).

Discussion

Our results show that lipocalin-2 is dispensable for tumor development judged by any of the parameters used for determination of the clinical aggressiveness of cancers, such as differentiation grade, time to cancer development, number of mammary glands involved, size of primary tumors, and number and size of metastases in this mouse model of breast cancer induced by the mouse mammary tumor virus-polyoma middle T antigen. This is in contrast to two recent reports using much the same approach as here to study the role of lipocalin-2 in breast cancer development. The first report, where tumor development was driven by the EshB2 oncogene [39], showed both faster onset of tumor genesis, higher primary tumor numbers, larger volume of primary tumors, as well as a higher number of metastases and larger volume of metastases in lipocalin-2 expressing mice. The second report, where the tumors were driven by the polyma-middle T antigen as in our study, showed no effect of lipocalin-2 expression on number of metastases, but did show an effect on number of primary tumors, the volume of primary tumors, and on the time of onset of tumor genesis [38]. The mice used for examining the effect of EshB2-induced mammary tumors was obtained by breeding Lcn2+/+/ (C57BL/6) mice with MMTV-ErbB2 (FVB/N) mice. The mixed and non-homogeneous genetic background of the offspring from the cross between the two mouse strains (C57BL/6 and FVB/N) in combination with the smaller sample sizes analyzed in the EshB2-study might explain some of the differences between the finding and the data presented here.

The other study examining PyMT-induced mammary tumors was performed in both C57BL/6 and 129/Ola mice (both backcrossed for at least 10 generations), while we used mice on a FVB/N background backcrossed for 8 generations. It is possible that this may have had an effect on the outcome. The FVB/N strain has been demonstrated to be more susceptible than C57BL/6 with respect to tumor genesis in the PyMT model [49,53,54]. The FVB/N background has therefore been used by us for several studies of mammary tumor growth and metastases because it offers a very uniform and predictable mammary tumor development by the tumor virus-polyoma middle T antigen [55], thus optimizing the ability to test the effect of agents that interfere with tumor development, in particular such that inhibit tumor growth and metastases [44,56]. Finally, one could envision that the specific knock-out mouse model used might influence the results. Berger et al. used a knock-out mouse constructed by themselves [21].

Figure 7. Western blot of human granulocytes from peripheral blood, mouse granulocytes from the spleen, and tumor extracts. Lipocalin-2 (LCN2) is unable to form a heterodimer with MMP-9 in mouse. There is no difference in the post translational modification of LCN2 in tumors compared to myeloid cells in mouse. A, samples analyzed under non-reducing conditions. In humans the LCN2:Lcn2 and the MMP-9:MMP-9 homodimers and the LCN2:MMP-9 heterodimer are identified. In addition the MMP-9 monomer is seen. In contrast no heterodimer between LCN2 and MMP-9 is seen in the mouse, only the LCN2 monomer and both the monomeric and dimeric forms of MMP-9 are seen. B, samples analyzed under reducing conditions. The LCN2:MMP-9 heterodimer as well as the LCN2:Lcn2 and MMP9:MMP9 homodimers are no longer identifiable after breakage of disulfide bindings. Merely the LCN2 monomer and the MMP-9 monomer are identified in both humans and mouse. C, left panel, samples analyzed under non-reducing conditions. Right panel, reducing conditions. Only the LCN2 monomer and no heterodimer is identified under both conditions. D, left panel, samples analyzed under non-reducing conditions. The MMP-9 monomer is seen, and in some of the tumors even the homodimer is identified. Right panel, reducing conditions. The MMP-9 monomer is seen along with high molecular weight (HMW) bands of unknown origin. The HMW bands are seen in both PyMT, Lcn2+/− and PyMT, Lcn2−/− mice and therefore cannot be the lipocalin-2:MMP-9 heterodimer. All blots are shown in full-length size. Samples are loaded in the same order in C and D as in Figure 8. Abbreviations: WT and KO tumors: Tumors from wild type and lipocalin-2 knock-out mice. Gr-1+ Gr-1 positive cells purified from the spleen of two FVB/N mice.

doi:10.1371/journal.pone.0039646.g007

Figure 8. Gelatin zymography of MMP-9 activity. No difference in gelatinolytic activity is observed between PyMT, Lcn2+/− and PyMT, Lcn2−/− mice, either in tumors or in plasma. A, zymography of tumors from PyMT, Lcn2+/+ (WT) and PyMT, Lcn2−/− (KO) mice with purified human MMP-9 (hMMP-9) as standard [2]. B, zymography of plasma from WT and KO mice along with human plasma and a molecular weight standard. Tumor and plasma samples from mice are loaded in the same order as in Figure 7 C and D.

doi:10.1371/journal.pone.0039646.g008

Figure 7. Western blot of human granulocytes from peripheral blood, mouse granulocytes from the spleen, and tumor extracts. Lipocalin-2 (LCN2) is unable to form a heterodimer with MMP-9 in mouse. There is no difference in the post translational modification of LCN2 in tumors compared to myeloid cells in mouse. A, samples analyzed under non-reducing conditions. In humans the LCN2:Lcn2 and the MMP-9:MMP-9 homodimers and the LCN2:MMP-9 heterodimer are identified. In addition the MMP-9 monomer is seen. In contrast no heterodimer between LCN2 and MMP-9 is seen in the mouse, only the LCN2 monomer and both the monomeric and dimeric forms of MMP-9 are seen. B, samples analyzed under reducing conditions. The LCN2:MMP-9 heterodimer as well as the LCN2:Lcn2 and MMP9:MMP9 homodimers are no longer identifiable after breakage of disulfide bindings. Merely the LCN2 monomer and the MMP-9 monomer are identified in both humans and mouse. C, left panel, samples analyzed under non-reducing conditions. Right panel, reducing conditions. Only the LCN2 monomer and no heterodimer is identified under both conditions. D, left panel, samples analyzed under non-reducing conditions. The MMP-9 monomer is seen, and in some of the tumors even the homodimer is identified. Right panel, reducing conditions. The MMP-9 monomer is seen along with high molecular weight (HMW) bands of unknown origin. The HMW bands are seen in both PyMT, Lcn2+/− and PyMT, Lcn2−/− mice and therefore cannot be the lipocalin-2:MMP-9 heterodimer. All blots are shown in full-length size. Samples are loaded in the same order in C and D as in Figure 8. Abbreviations: WT and KO tumors: Tumors from PyMT, Lcn2+/+ or PyMT, Lcn2−/− mice respectively. Gr-1+: Gr-1 positive cells purified from the spleen of two FVB/N mice.

doi:10.1371/journal.pone.0039646.g007

Figure 8. Gelatin zymography of MMP-9 activity. No difference in gelatinolytic activity is observed between PyMT, Lcn2+/− and PyMT, Lcn2−/− mice, either in tumors or in plasma. A, zymography of tumors from PyMT, Lcn2+/+ (WT) and PyMT, Lcn2−/− (KO) mice with purified human MMP-9 (hMMP-9) as standard [2]. B, zymography of plasma from WT and KO mice along with human plasma and a molecular weight standard. Tumor and plasma samples from mice are loaded in the same order as in Figure 7 C and D.

doi:10.1371/journal.pone.0039646.g008

Lipocalin-2 in Mouse Breast Cancer

Both in plasma and in tumor extracts, but with no difference in the patterns between wild type and Lcn2 knock-out mice. No activity with a MW higher than the MW of monomeric human MMP-9 was observed in tumors. All gelatinolytic activity was inhibited by EDTA and phenantrolin (data not shown).

Discussion

Our results show that lipocalin-2 is dispensable for tumor development judged by any of the parameters used for determination of the clinical aggressiveness of cancers, such as differentiation grade, time to cancer development, number of mammary glands involved, size of primary tumors, and number and size of metastases in this mouse model of breast cancer induced by the mouse mammary tumor virus-polyoma middle T antigen.

This is in contrast to two recent reports using much the same approach as here to study the role of lipocalin-2 in breast cancer development. The first report, where tumor development was driven by the EshB2 oncogene [39], showed both faster onset of tumor genesis, higher primary tumor numbers, larger volume of primary tumors, as well as a higher number of metastases and larger volume of metastases in lipocalin-2 expressing mice. The second report, where the tumors were driven by the polyma-middle T antigen as in our study, showed no effect of lipocalin-2 expression on number of metastases, but did show an effect on number of primary tumors, the volume of primary tumors, and on the time of onset of tumor genesis [38]. The mice used for examining the effect of EshB2-induced mammary tumors was obtained by breeding Lcn2−/− (C57BL/6) mice with MMTV-ErbB2 (FVB/N) mice. The mixed and non-homogeneous genetic background of the offspring from the cross between the two mouse strains (C57BL/6 and FVB/N) in combination with the smaller sample sizes analyzed in the EshB2-study might explain some of the differences between the finding and the data presented here.

The other study examining PyMT-induced mammary tumors was performed in both C57BL/6 and 129/Ola mice (both backcrossed for at least 10 generations), while we used mice on a FVB/N background backcrossed for 8 generations. It is possible that this may have had an effect on the outcome. The FVB/N strain has been demonstrated to be more susceptible than C57BL/6 with respect to tumor genesis in the PyMT model [49,53,54]. The FVB/N background has therefore been used by us for several studies of mammary tumor growth and metastases because it offers a very uniform and predictable mammary tumor development by the tumor virus-polyoma middle T antigen [55], thus optimizing the ability to test the effect of agents that interfere with tumor development, in particular such that inhibit tumor growth and metastases [44,56]. Finally, one could envision that the specific knock-out mouse model used might influence the results. Berger et al. used a knock-out mouse constructed by themselves [21].
conclude that the role of lipocalin-2 in breast cancer progression is questionable.

Supporting Information

Figure S1 Breeding strategy used for generating the mice used in the experiments. Congenic heterozygous male FVB/N-MMTV-PyMT mice were mated with female FVB/N-Lcn2+/− mice back-crossed to the FVB/N strain for 8 generations (N8). Their male FVB/N-PyMT, Lcn2+/− offspring (F1) were mated with FVB/N-Lcn2+/− (N9) females to generate the FVB/N-PyMT, Lcn2+/−; FVB/N-PyMT, Lcn2−/−; FVB/N Lcn2+/+, and FVB/N, Lcn2−/− mice used throughout the study. Abbreviations: PyMT: MMTV-PyMT.

(TIF)

Figure S2 Positive control stainings for E-cadherin and α-SMA. A. E-cadherin staining of the dermis and epidermis of a PyMT, Lcn2+/+ mouse as positive control to Figure 2 C, D. Original magnification x400. B. α-SMA staining of a primary tumor and adjacent vessels of a PyMT, Lcn2+/+ mouse as positive control to Figure 2 C, D. Original magnification x200. Abbreviations: PyMT: MMTV-PyMT.

(TIF)

Figure S3 Tumor growth of the fastest and the slowest growing mammary gland pairs in PyMT, Lcn2+/+ and PyMT, Lcn2−/− mice. A, average of tumor volume in gland pair number one versus mouse age. B, average of tumor volume in gland pair number two versus mouse age. No statistical significant difference between PyMT, Lcn2+/+ and PyMT, Lcn2−/− mice at week 13, t-test after logarithmic transformation, p = 0.60 for gland pair number one and p = 0.51 for gland pair number two (n = 29 for PyMT, Lcn2+/+ and n = 20 for PyMT, Lcn2−/−).

(TIF)

Figure S4 Quantitative real-time PCR analysis of mRNA levels in mammary tumors/glands. A. Lcn2, B. Mmp9, C. 24p3R, D. Tirc. E. Bdh2. The vertical error bars represent standard deviations. The mean expressions are shown in three PyMT, Lcn2+/+ mice with small tumors (1), three with intermediate size tumors (2) and three with large tumors (3), and the mean expressions are shown in three PyMT, Lcn2−/− mice with small tumors (4), three with intermediate size tumors (5) and three with large tumors (6) located to the fourth mammary gland on the left hand side. The PyMT negative mice were selected randomly and number (7) and (8) represent two mice each. Expression levels of each marker are shown relative to the value found in small tumors of PyMT, Lcn2+/+ mice, which is given the value 1. Tumor volumes: 1) 0.07 cm3, 0.15 cm3, 0.17 cm3 2) 0.47 cm3, 0.55 cm3, 0.68 cm3 3) 0.82 cm3, 0.98 cm3, 1.50 cm3 4) 0.02 cm3, 0.08 cm3, 0.09 cm3 5) 0.50 cm3, 0.54 cm3, 0.63 cm3 6) 0.73 cm3, 0.98 cm3, 1.33 cm3 Abbreviations: PyMT: MMTV-PyMT.

(TIF)

Acknowledgments

The technical assistance of Charlotte Horn, Tessa Hornøyd, Margit Bæksted, Agnieszka Ingvorsen, and Mette M. Andersen is greatly appreciated. We thank Maria Torp Larsen and Stine Novrup Clemmensen for their great assistance in immunomagnetic sorting and Western blotting.

Author Contributions

Conceived and designed the experiments: JBC NB LRL. Performed the experiments: EC MH ES. Analyzed the data: EC AG OD. Contributed reagents/materials/analysis tools: LHE AJ. Wrote the paper: NB JBC EC.
References

1. Kjeldsen L, Bainton DF, Sengelov H, Borregaard N (1994) Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. Blood 83: 799–807.

2. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N (1995) Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem 270: 10425–10432.

3. Canceldda FD, Malpeli M, Genni C, Di M, V, Bet P, et al. (1996) The developmental regulatory avian b32 lipocalin is an extracellular fatty acid binding protein. J Biol Chem 271: 20161–20169.

4. Stoeza SP, Gould MN (1995) Overexpression of neu-related lipocalin (NRL) in neu-initiated but not ras or chemically initiated rat mammary carcinomas. Oncogene 11: 2233–2244.

5. Flower DR, North AC, Atwood TK (1991) Mouse oncoprotein 24p3 is a member of the lipocalin protein family. Biochem Biophys Res Commun 180: 69–74.

6. Beynon RJ, Hurst JL (2003) Multiple roles of major urinary proteins in the house mouse, Mus domesticus. Biochem Soc Trans 31: 142–146.

7. Holmes MA, Paulene W, Jide X, Ratledge C, Strong RK (2005) Siderocalin mediates an innate immune response to bacterial infection by sequestrating iron. J Biol Chem 280: 182: 4947–4956.

8. Cowland JB, Sorensen OE, Sehested M, Borregaard N (2003) Neutrophil gelatinase-associated lipocalin is up-regulated in human epithelial cells by IL-1 beta, but not by TNF-alpha. J Immunol 171: 6630–6639.

9. Karlsen JR, Borregaard N, Cowland JB (2010) Induction of neutrophil gelatinase-associated lipocalin by co-stimulation with interleukin-17 and tumor necrosis factor-alpha is controlled by IkappaB-zeta but not by C/EBP-beta nor C/EBP-delta. J Biol Chem 285: 14088–14100.

10. Mallbris L, O’Brien KP, Hulthen A, Sandstedt B, Cowland JB, et al. (2002) Neutrophil gelatinase-associated lipocalin is a marker for dysregulated keratinocyte differentiation in human skin. Exp Dermatol 11: 584–591.

11. Nielsen BS, Borregaard N, Bandgaard JB, Timrihel S, Sehested M, et al. (1996) Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases. Gut 38: 414–420.

12. Friedl A, Stoesz SP, Buckley P, Gould MN (1999) Neutrophil gelatinase-associated lipocalin in normal and neoplastic human tissues. Cell type-specific pattern of expression. Histochem J 31: 433–441.

13. Stoeza SP, Friess A, Haag JD, Landstrom MJ, Clark GM, et al. (1998) Heterogeneous expression of the lipocalin NGAL in primary breast cancers. Int J Cancer 79: 565–572.

14. Mishra J, Ma Q, Qian J, Cui J, Yang, et al. (2004) Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol 15: 3073–3082.

15. Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, et al. (1996) Identification of inbred mouse strains harboring genetic modifiers of mammary tumor phenotype. J Natl Cancer Inst 88: 1305–1310.

16. Liu JS, Zheng M, Mietzner TA, et al. (2009) Lipocalin 2 functions as a mammalian siderophore synthase by an enzyme with a bacterial homolog involved in iron acquisition. Cell 141: 1006–1017.

17. Bao G, Clifton M, Hoette TM, Mori K, Deng SX, et al. (2010) Iron traffics in circulation bound to a siderocalin (NGAL)-catalyzed complex. Nat Chem Biol 6: 602–609.

18. Klaussen P, Nieminen CU, Cowland JB, Krabbke K, Borregaard N (2005) On myeloid and macrophage gelatinase-associated lipocalin is not involved in apoptosis or acute response. Eur J Haematol 75: 332–340.

19. Bauer M, Eckhoff JC, Gould MN, Mundhenke C, Maas, et al. (2008) Neutrophil gelatinase-associated lipocalin (NGAL) is a predictor of poor prognosis in human primary breast cancer. Breast Cancer Res Treat 108: 389–397.

20. Yang J, Biezenberg DR, Rodig SJ, Doiron R, Clifton MC, et al. (2009) Lipocalin 2 promotes breast cancer progression. Proc Natl Acad Sci U S A 106: 3913–3918.

21. Kuhnen SJ, Zolotars Z, Nadel D, Adzick NS, Matz PG, et al. (2001) The human neutrophil lipocalin supports the allostERIC activation of matrix metalloproteinases. J Eur J Biochem 288: 1918–1928.

22. Sevriich L, Schurig U, Sasse K, Gadja M, Werner F, et al. (2010) Synergistic antitumor effects of combined cathepsin B and cathepsin Z deficiencies on breast cancer progression and metastasis in mice. Oncogene 29: 34–46.

23. Vaalioja O, Korovin M, Gadja M, Brodoseil F, Bjoik L, et al. (2008) Reduced tumour cell proliferation and delayed development of high-grade mammary carcinomas in catsdeficient mice. Oncogene 27: 4181–4190.

24. Berger T, Cheung CC, Eas A, Mak TW (2009) Erlotinib down-regulation of lung Department and release of human neutrophil gelatinase, confirming the existence of the Lenv2 gene in mice suppresses primary mammary tumor formation but does not decrease lung metastasis. Proc Natl Acad Sci U S A 107: 2995–3000.

25. Leng X, Hanon K, Han H, Wei L, et al. (2009) Inhibition of lipocalin 2 impairs breast tumorigenesis and metastasis. Cancer Res 69: 3579–3584.

26. Euhus MS, Hudd C, LaRegina MC, Johnson FE (1986) Tumor measurement in nude mice. J Surg Oncol 31: 229–234.

27. Juncker-Jensen A, Romer J, Pennington J, Lund LR, Almhol K (2009) Spontaneous metastasis in matrix metalloproteinases 3-deficient mice. Mol Carcinog 48: 618–625.

28. Nielsen BS, Lund LR, Christensen B, Jørgensen M, Usher PA, et al. (2001) A precise and efficient stereological method for determining murine lung metastasis volumes. Am J Pathol 158: 1397–1401.

29. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, et al. (2003) Progession to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. J Am Pathol 163: 2115–2126.

30. Almhol K, Juncker-Jensen A, Laurum OD, Dans K, Almhol K (2009) Metastasis is strongly reduced by the matrix metalloproteinase inhibitor Galardin in the MMTV-PymT transgenic breast cancer model. Mol Cancer Ther 7: 2738–2747.

31. Borregaard N, Heiple JM, Simons ER, Clark RA (1983) Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translation during activation. J Cell Biol 97: 52–61.

32. Biermann J, Jorgensen O, Aske A, Norgaard B (1992) Subcellular localization and release of human neutrophil gelatinase, confirming the existence of separate gelatinase-containing granules. Biochem J 279 (P 2): 603–610.

33. Juncker L, Koch C, Amerlits K, Borregaard N (1996) Characterization of two ELISAs for NGAL, a newly described lipocalin in human neutrophils. J Immunol Methods 198: 155–164.

34. Muzzoni A, Mannello R, Fay FR, Tori GA, Papa S, et al. (2007) Zymography analysis and characterization of MMP-2 and -9 forms in human sound dentin. J Dent Res 86: 446–449.

35. Lichten T, Le VT, Williams M, Muller W, Klein-Szanto A, et al. (1996) Identification of ashed mouse strain harbors genetic modifiers of mammary tumor age of onset and metastatic progression. Int J Cancer 77: 604–614.

36. Guy CT, Bradford RD, Muller WJ (1992) Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metatstic disease. Mol Cell Biol 12: 454–462.

37. Hvidberg V, Jacobsen C, Strong RK, Cowland JB, Moestrup SK, et al. (2005) The endocytic receptor megalin binds the iron transporting neutrophil-gelatinase-associated lipocalin with high affinity and mediates its cellular uptake. FEMS Lett 576: 773–777.

38. Liu Q, Nielsen-Hamilton M (1995) Identification of a new acute phase protein. J Biol Chem 270: 22565–22570.

39. Davies CA, Magliorne J, Manner CK, Young D, Cardwell RD, et al. (2007) Evidence of MMP backgrounds on breast tumor phenotype in inducible nitric oxide synthase deficient mice. Transgenic Res 16: 193–201.

40. Martin MJ, Carter KJ, Jean-Philippe SR, Chang M, Mohashery S, et al. (2008) Effect of ablation or inhibition of stromal matrix metalloproteinase-9 on lung
metastasis in a breast cancer model is dependent on genetic background. Cancer Res 68: 6251–6259.

55. Almholt K, Green KA, Juncker-Jensen A, Nielsen BS, Lund LR, et al. (2007) Extracellular proteolysis in transgenic mouse models of breast cancer. J Mammary Gland Biol Neoplasia 12: 83–97.

56. Almholt K, Lund LR, Rygaard J, Nielsen BS, Dano K, et al. (2005) Reduced metastasis of transgenic mammary cancer in urokinase-deficient mice. Int J Cancer 113: 525–532.

57. Fernandez CA, Yan L, Louis G, Yang J, Kutok JL, et al. (2003) The matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients. Clin Cancer Res 11: 5390–5395.