

ORIGINAL ARTICLE

Lipocalin 2 performs contrasting, location-dependent roles in APCmin tumor initiation and progression

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Evidence that lipocalin 2 (LCN2) is oncogenic has grown in recent years and comes from both animal models and expression analysis from a variety of human cancers. In the intestine, LCN2 is overexpressed in colitis patients and its overexpression is a negative prognostic indicator in colorectal cancer. Functionally, LCN2 has a number of different activities that may contribute to its oncogenic potential, including increasing matrix metalloproteinase activity, control of iron availability and stimulating inflammation. In this report, we examined APCmin intestinal tumorigenesis in an LCN2-deficient background. We found that the loss of LCN2 increased tumor multiplicity specifically in the duodenum, suggesting a potential tumor-suppressive activity. Concurrently, however, LCN2 increased the average small intestinal tumor size particularly in the distal small intestine. We found that this increase was correlated to tumor iron(II) content, suggesting that an iron-scavenging role is important for LCN2 oncogenic activity in the intestine.

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INTRODUCTION

Lipocalin 2 (LCN2; a.k.a. 24p3, NGAL, uterocalin and MSFI) is a potent oncogene in mammary tumorigenesis in mice.^{1,2} Aberrant expression of LCN2 has also been noted in human cancers³ including breast,^{4–7} gastric,^{8–10} esophageal,¹¹ renal,¹² brain¹³ and colorectal^{14,15} cancers. Additionally, *Lcn2* is upregulated in response to various other stresses in different organs including involution in the breast,¹⁶ reperfusion in the kidney¹⁷ and colitis.¹⁸

Functionally, LCN2 has a number of different activities that may contribute to its oncogenic potential (reviewed in Rodvold *et al.*¹⁹). First, LCN2 binds to matrix metalloproteinase (MMP)9 and sustains its activity by reducing the rates of autolysis.^{9,20,21} For several forms of cancer, the presence of this complex is the key attribute of aberrant LCN2 expression.^{6,9,11} Second, LCN2 controls iron availability. For this activity, it was first found to bind to bacterial iron siderophores and thus inhibit bacterial iron uptake and growth.^{22–25} More recently, candidate mammalian iron siderophore(s) as well as LCN2 receptor(s) suggest that LCN2 may also mediate a host iron-scavenging strategy.^{26–30} Finally, LCN2 is proposed to have proinflammatory properties that are potentially independent of its other activities.^{31,32}

MMP activity, bacterial exposure and inflammation are all factors that have a role in intestinal tumorigenesis. The mouse intestinal tumor model APCmin (reviewed in Moser *et al.*³³) develops intestinal tumors at multiples of 10–120 per mouse and is sensitive to altered host innate immunity,³⁴ the presence of gut microbes^{35,36} and MMP deficiency, including loss of MMP9.^{37,38}

Here, we examined whether LCN2 has a more general role in tumorigenesis by examining APCmin intestinal tumorigenesis in an LCN2-deficient background. We found that LCN2 affected neither multiplicity nor total tumor sizes in either the small intestines or colon. Surprisingly, LCN2 deficiency increased tumor

multiplicity specifically in the duodenum, suggesting a potential tumor-suppressive activity. Concurrently, LCN2 promoted average small intestinal tumor growth in the distal small intestine. We found that this increase was not correlated to immune activities but rather to tumor iron(II) content. In contrast with mammary tumor models, however, LCN2 deficiency gave only a modest effect, suggesting it is not a robust oncogene in intestinal tumorigenesis.

RESULTS

LCN2 expression is increased in APCmin tumors

The evidence that LCN2 is oncogenic in several cancers prompted us to examine potential changes in *Lcn2* expression in small intestinal adenomas from *Apc*^{min/+} mice. We harvested RNA from six individually isolated adenomas from different sections of small intestines in different mice as well as normal small intestinal epithelia from wild-type mice of the same colony. After reverse transcription, we quantified *Lcn2* mRNA expression by quantitative real-time PCR. We found that adenomas had an average fivefold increase of *Lcn2* mRNA over normal epithelia (Figure 1; $P = 0.015$), suggesting that LCN2 might promote small intestinal tumorigenesis.

LCN2-deficient mice have normal intestinal development

Before investigating any potential effects of LCN2 deficiency on intestinal tumorigenesis in the APCmin model, we wanted to establish that intestinal development in the LCN2-deficient animal was normal. We therefore analyzed the small intestines of mice that were wild-type (+/+) and nullizygous (–/–) for *Lcn2* for a panel of cell lineage markers of intestinal epithelia development (Supplementary Figure S1). We found normal morphology of intestinal tissue and expected quantities of goblet cells,

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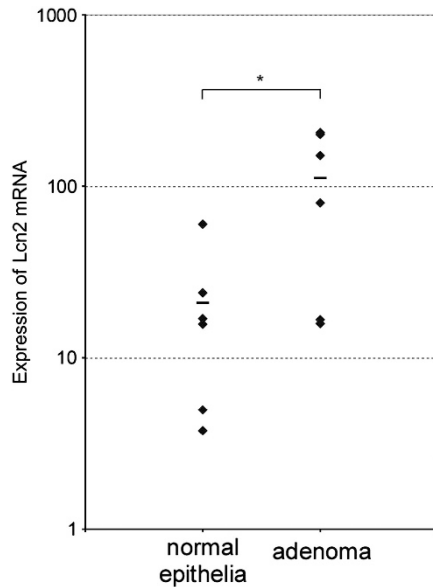


Figure 1. Enhanced *Lcn2* expression in *Apc*^{min/+} adenomas. *Lcn2* mRNA expression was analyzed in normal small intestinal epithelia and in small intestinal adenomas from *Apc*^{min/+} mice by quantitative RT-PCR. Values normalized to *Rps2* gene. **P* < 0.05 in Student's *t*-test analysis.

enterochromaffin cells, paneth cells and absorptive enterocytes. Hence, we conclude that intestinal development in the LCN2-deficient mouse is likely normal.

LCN2 is not a strong oncogene in intestinal tumorigenesis

To investigate how LCN2 may impact intestinal tumors in mice, we bred the *Lcn2*-null allele into the *Apc*^{min/+} background. A cohort of nine sex-matched littermate pairs of *Apc*^{min/+}::*Lcn2*^{+/+} and *Apc*^{min/+}::*Lcn2*^{-/-} mice were generated and aged. Mice were killed at times determined by experimental endpoints and/or advanced age. Genotype-blinded analysis on each pair was then performed. Intestinal tumor loads, defined as the sum of maximum tumor lengths, were examined with small intestinal tumors and colon tumors considered separately based on recognized differences.^{39,40} No consistent differences were evident in tumor multiplicity (Figure 2a) or total tumor load (Figure 2b) in the small intestines or for tumor incidence, multiplicity or load in the colon (Supplementary Table S1). Thus, unlike mouse mammary tumorigenesis where LCN2 deficiency severely attenuates disease progression, intestinal tumors are relatively refractory to LCN2 status.

LCN2 does not affect invasiveness of APCmin tumor tissue

On the basis of earlier suggestion that LCN2 promoted tumorigenesis in mammary tissue by increasing invasiveness and epithelia–mesenchymal transition (EMT),^{2,41} we examined the morphology of intestinal neoplasias. Consistent with previously published results,^{42,43} *Apc*^{min/+} adenomas demonstrated a variegated β -catenin staining pattern whereby more intense and nuclear-enriched staining localizes to regions of abnormal crypt morphology, multilayered growth and nuclear atypia (Supplementary Figure S2A). Importantly, however, we found no evidence of epithelial invasion of the basement membrane in either LCN2-competent or -deficient tumors. Furthermore, when we examined EMT markers *Twist* and *Slug*, we found no aberrant LCN2-dependent mRNA expression of these markers in adenomas (Supplementary Figure S2B). Thus, in contrast to reported LCN2-dependent EMT causing increased metastasis of mammary

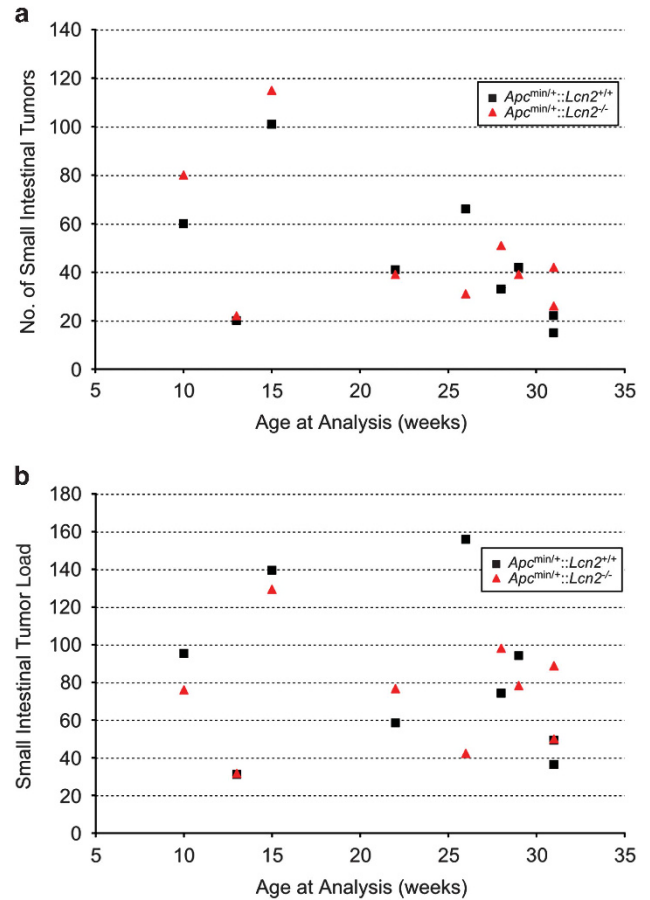


Figure 2. LCN2 deficiency does not affect multiplicity or total load of *Apc*^{min/+} small intestinal tumorigenesis. Sex-matched littermate pairs of LCN2-competent (black squares) or LCN2-deficient (red triangles) were harvested at various ages and scored for (a) multiplicity of small intestinal tumors and (b) total small intestinal tumor load. Each data point reflects an individual mouse of a pair harvested at indicated age. No statistically significant differences were determined by paired Student's *t*-test.

tumors, we found no evidence for LCN2-dependent EMT or invasion in the non-metastatic APCmin model.

LCN2 inhibits duodenal tumor incidence in *Apc*^{min/+} mice

In order to more fully examine the impact of *Lcn2* deletion on the APCmin phenotype, we examined the small intestines in equal-lengthed quintile segments⁴⁴ numbered from proximal to distal as shown in Figure 3a. In this analysis, we found that LCN2-deficient mice had an increased multiplicity of tumors in the S1 segment (*P* = 0.012) representing the duodenum (Figure 3b). Furthermore, consistent with increased multiplicity of tumors, the total tumor load in the S1 segment was increased (*P* = 0.019). No other differences in tumor multiplicity or total tumor load across the other quintile segments were evident in this analysis. Thus, in contrast to mammary tumorigenesis where incidence was decreased in the absence of LCN2, in the duodenum LCN2 seems to function as a weak tumor-suppressor protein.

LCN2 promotes small intestinal adenoma progression

In order to better understand the impact on LCN2 on APCmin adenoma growth, we normalized total tumor load across the small intestines by the number of tumors present, thus providing an average tumor length. When we compared average tumor lengths of LCN2-competent and -deficient mice, we found that LCN2-

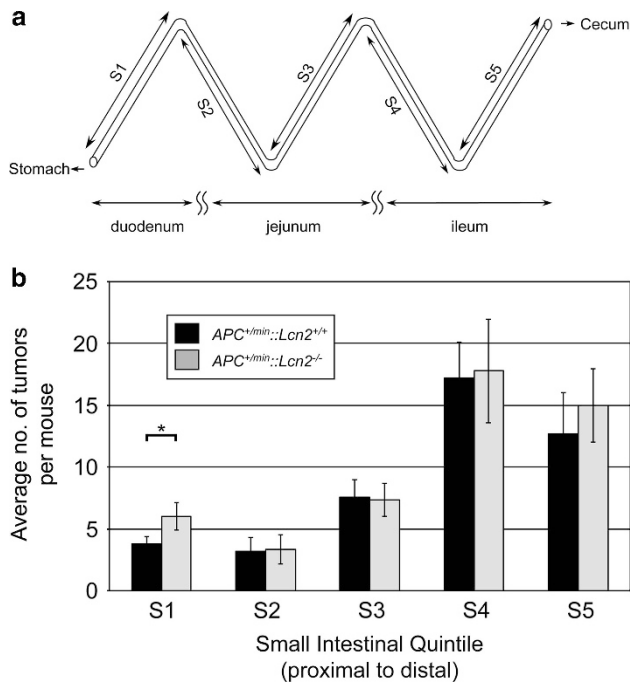


Figure 3. LCN2 decreases duodenal tumorigenesis. (a) Diagram of five equal-length segments of the small intestine used in examination. 'S1' to 'S5' are numbered from proximal to distal. (b) Average tumor counts of LCN2-competent and LCN2-deficient in the different small intestinal segments. * $P < 0.05$ in paired Student's *t*-test analysis.

deficient adenomas were consistently smaller ($P = 0.007$). To demonstrate this effect, we generated histograms displaying the numbers of adenomas of increasing sizes for each of the paired samples (Figure 4a and Supplementary Figure S3). Evident here is that, although the total number of tumors will vary, the sizes of tumors in *Lcn2^{-/-}* mice shifts toward a smaller size. Additionally when average small intestinal tumor sizes are plotted against age at harvest (Figure 4b), there is an apparent trend for greater divergence in average adenoma size with increased animal age. Thus, consistent with *Lcn2* expression providing an oncogenic function in APCmin adenomas, LCN2 deficiency inhibits tumor progression in the small intestine.

To clarify where in the small intestine that LCN2 may function in tumor progression we examined changes in average tumor sizes as a function of location (Figure 5a). With the exception of the relatively infrequent tumors in quintile S2, we found that average size of tumors from LCN2-deficient mice in the other quintiles was smaller than from LCN2-competent mice. Indeed, loss of LCN2 in quintile S5 had a statistically significant effect in isolation ($P = 0.034$). This indicates that, although the requirement for LCN2 in tumor growth is broadly seen across the small intestine, it is most important in the distal small intestine.

LCN2-dependent tumor promotion is likely not due to inflammation

The reported roles of inflammation in tumor initiation and progression on the APCmin phenotype^{34,45,46} as well as of LCN2 in promoting inflammation prompted us to examine whether LCN2 status was altering the inflammation profile in these mice. To do so, we examined plasma cytokine levels as well as splenic leukocytes to look for potential differences. The cytokine profiles (Supplementary Table S2) demonstrated a statistically significant

difference in interleukin (IL)13 expression ($P = 0.018$) associated with LCN2 deficiency, but no other significant changes in systemic cytokines. Additionally, splenocyte analysis could not identify any statistically significant differences in any of the cell lineage populations measured (Supplementary Table S3).

In order to exclude that restricted local inflammation was responsible for LCN2-dependent tumor promotion, we examined mRNA levels for inflammation-related genes in three pairs of size- and location-matched, S5-derived adenomas. Although some mRNA cytokine quantities were reduced in the LCN2-deficient adenomas, namely *Tnfa*, *Il10*, *Il6* and *Cox2*, we found no statistically significant differences in qRT-PCR signal for any mRNA examined (Supplementary Figure S4A). In addition, we tested possible deficiencies in immune infiltration of the size-matched adenomas by testing mRNA of macrophage and T-cell markers, *Itgam* and *Cd3e*, respectively (Supplementary Figure S4B). We found no clear difference in these markers of immune infiltration, suggesting that inflammatory processes are not likely altered due to LCN2 deficiency in these tumors. Taken together, we conclude that inflammation is not an important factor in LCN2-dependent promotion of small intestinal tumor size in the *Apc^{min/+}* background.

LCN2-deficient tumors have reduced iron content

The identification of a mammalian iron siderophore²⁸ suggests that LCN2 may function as a mammalian iron-scavenging strategy. In order to examine whether LCN2 may promote small intestinal tumor size by increasing iron availability to the tumor, we examined iron content in the tumor. As stored iron, predominantly existing as iron(III) in ferritin, may be more susceptible to physiological fluctuations,^{47,48} we chose to focus on iron(II) where LCN2 also has a demonstrated effect.⁴⁹ From paired mice, we excised size- and location-matched adenomas and quantified iron(II) content by atomic-absorption spectrometry. We found that adenomas from *Lcn2^{-/-}* mice had a statistically significant reduction in iron(II) content compared with those from *Lcn2^{+/+}* mice ($P = 0.011$, $n = 10$). To better examine the nature of this defect, we segregated the adenomas based on their location of excision (Figure 5b).

The data suggest that, in *Apc^{min/+};;Lcn2^{+/+}* mice, iron(II) content is higher as you move distally down the small intestine. Adenomas from *Apc^{min/+};;Lcn2^{-/-}* mice had a consistently lower iron(II) content and a reduced difference in iron content between proximal and distal regions. Interestingly, the largest difference was evident in S5 adenomas, where LCN2 had the greatest effect on average tumor size (Figure 5a). This suggests that LCN2-dependent iron uptake into adenomas may be providing a tumor growth advantage particularly in the region of the distal small intestine.

DISCUSSION

By breeding the *Lcn2* deficiency into the APCmin intestinal tumor model, we sought to examine whether LCN2 is oncogenic in mouse tumors in organs apart from mammary glands. We found that LCN2 fulfills a complex role in small intestinal tumorigenesis depending on the location examined. In the duodenum, LCN2 inhibits adenoma multiplicity, whereas in the ileum, LCN2 stimulates tumor growth. Hence, LCN2 concurrently performs both tumor-suppressive and oncogenic activities in separate small intestinal compartments. Surprisingly, the effect of LCN2 on intestinal tumorigenesis does not seem to correlate to altered inflammatory signals as suggested by very few evident changes in plasma cytokine levels or splenic cell lineages. In contrast, its effect on tumor growth did correlate well with total iron(II) in the adenomas, suggesting that an iron-scavenging role is important for LCN2 oncogenic activity in the intestine.

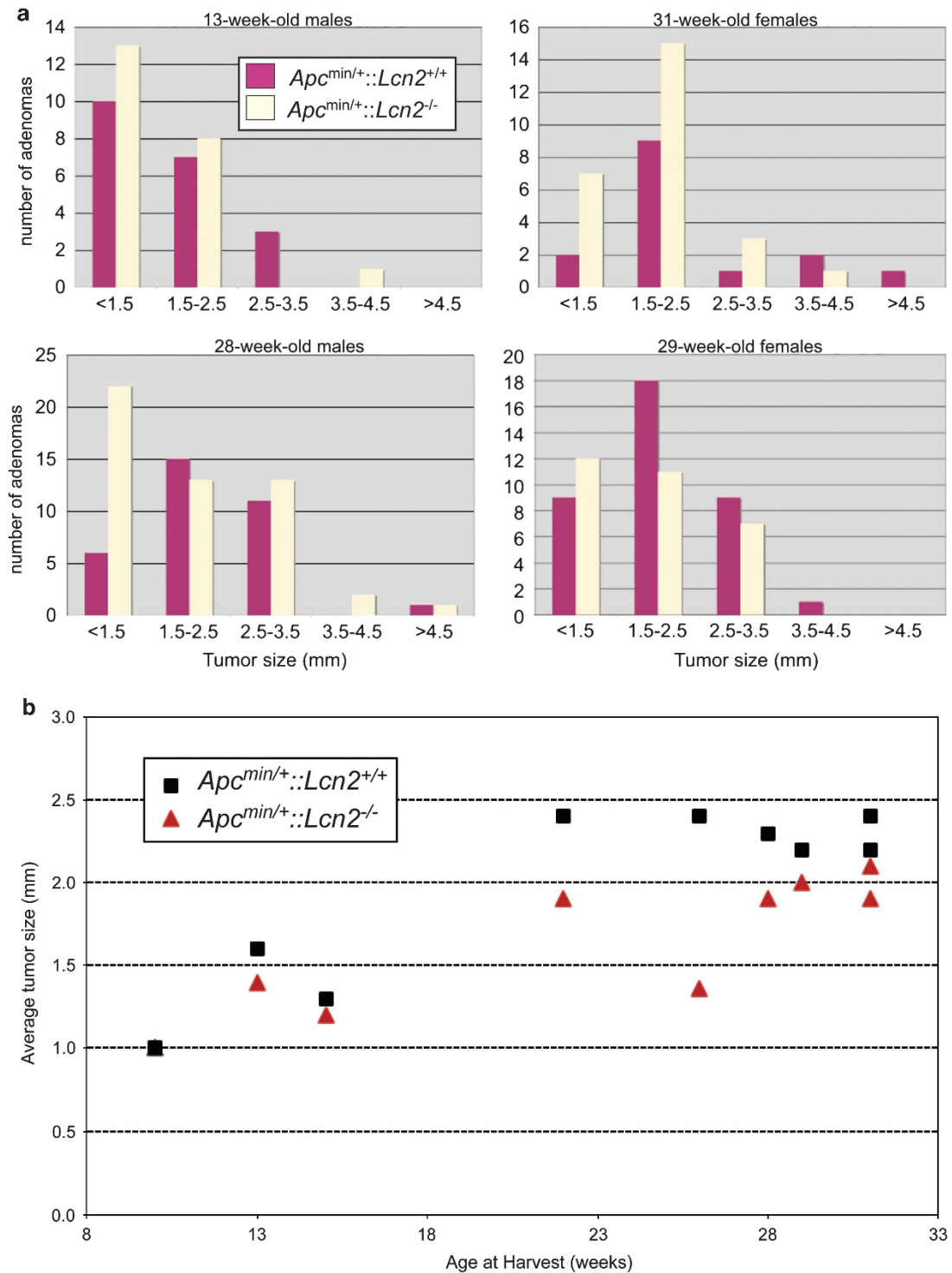


Figure 4. LCN2 promotes average small intestinal tumor size. **(a)** Representative histograms of small intestinal tumors of indicated sizes from different sex-matched littermate pairs of LCN2-competent and -deficient *Apc^{min/+}* mice. Additional histograms in Supplementary Figure S3. **(b)** Average small intestinal adenoma size per animal graphed as a function of age for LCN2-deficient and LCN2-competent *Apc^{min/+}* mice. Cumulative difference in average small intestinal tumor size determined to be statistically significant by paired Student's *t*-test ($P = 0.007$).

The broad array of human cancers that demonstrate aberrant LCN2 expression suggests that LCN2 can widely promote tumorigenesis. From a number of these studies, it is an enhanced expression LCN2 complex with MMP9 that is the aberrant hallmark. Only a few of these studies indicate that aberrant expression of LCN2 may originate at the tumor itself. When we examined *Lcn2*

mRNA expression in normal versus adenoma tissue, we found that *Lcn2* was indeed overexpressed in the neoplasia.

It is as yet unclear how LCN2 may function as a tumor suppressor in the duodenum. We hypothesize that this phenomenon may relate to the reported antibacterial activity of LCN2. Among the reported activities for LCN2, only a defense against bacterial

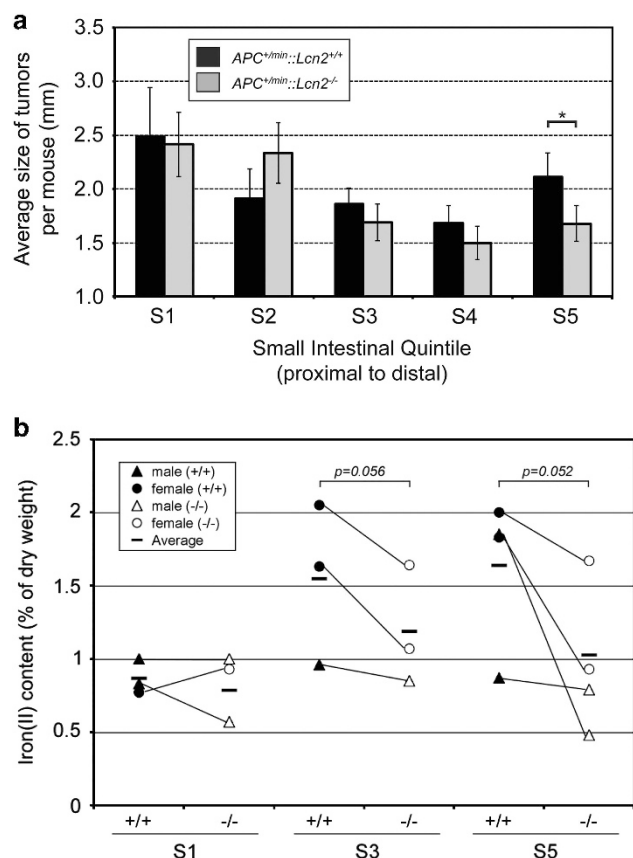


Figure 5. LCN2 promotion of small intestinal growth correlates with iron(II) uptake. **(a)** Average tumor size is graphed by small intestinal location; **(b)** Iron(II) content from size-matched adenomas from paired mice are graphed by location of excision. * $P < 0.05$ by paired Student's *t*-test.

colonization would be predicted to be tumor suppressive. Inflammation induction by LCN2 is marked by increases in certain cytokines and enhanced MMP9 activity. Our analysis of cytokine levels in *Apc*^{min/+};*Lcn2*^{-/-} mice only showed a statistically significant difference in IL13. Expression analysis of the adenomas, however, suggests that there was no difference in immune infiltration by T cells or macrophages. We therefore suggest that inflammation is not critical to the reduction of tumor growth we see in the distal small intestines of these mice. As the small intestine represents a special anti-inflammatory milieu, LCN2 inflammatory signals might be dampened in contrast to other tissues.

MMP9 has been shown to promote tumorigenesis in the APCmin model. In contrast to LCN2, however, MMP9 affected multiplicity but not size of small intestinal adenomas.³⁷ The discrepancy between these phenotypes suggests that LCN2 is also unlikely to be functioning through enhanced MMP9 activity to promote tumorigenesis in the small intestine. Intriguingly, LCN2 as a prognostic indicator in human CRC does not correlate with MMP9 expression, indicating that there is likely another function involved.¹⁴

Our data tentatively suggest that LCN2-aided iron uptake might be a critical tumor-promoting activity in APCmin adenomas. Although LCN2 has been reported to increase intracellular iron in cultured cells,^{27,29,30,49} this is the first evidence that LCN2 can impact tissue iron homeostasis *in vivo*.

In our model, loss of LCN2 results in a modest reduction in average tumor size in the small intestine, particularly in the distal small intestine. The finding that *Lcn2*^{-/-} mice have adenomas with less iron(II) content than *Lcn2*^{+/+}, particularly in the distal

small intestine, induces us to propose that LCN2 performs an iron-scavenging role that promotes adenoma growth. Given the more anaerobic environment of the distal small intestine relative to the proximal, this higher iron(II) content may provide an important energetic advantage in epithelial cell electron transport activities. In general, however, we found a far less dramatic effect of LCN2 on tumor growth than seen in mammary tumor models.

MATERIALS AND METHODS

Mice

Apc^{min} (Jackson Laboratory, Bar Harbor, ME, USA) and *Lcn2*-null⁵⁰ alleles in C57BL6 pure-bred background were mated to generate pairs of sex-matched littermate *Apc*^{min/+};*Lcn2*^{+/+} and *Apc*^{min/+};*Lcn2*^{-/-} pairs. Pairs were maintained in individually ventilated cage with 4–6% protein-irradiated diet and *ad libitum* water. Animals were mated and monitored under protocols SHS/447 and SHS/489 authorized by the Singhealth Institutional Animal Care and Use Committee. Genotyping primer sequences are provided in Supplementary Table S4.

Quantitative RT-PCR

RNA was extracted from normal epithelia of *Apc*^{+/+} mice and from adenomas of *Apc*^{min/+} mice, with or without *Lcn2* deficiency, using a standard protocol and the RNeasy kit (Qiagen, Hilden, Germany), quantified and reverse-transcribed using a Superscript II first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA). cDNA samples were then used as templates for quantitative real-time PCR using a ABI 7900HT detection system and SYBR Green (Applied Biosystems, Foster City, CA, USA). Data were normalized to the 40S ribosomal protein S2 (*Rps2*) gene. Primer sequences are provided in Supplemental Table S4.

Histology

Intestinal samples or adenomas were fixed in 10% buffered formalin overnight and processed for paraffin embedding. Adenomas were dyed with Tissue Marking dyes (Cancer Diagnostics Inc., Morrisville, NC, USA) before embedding. Serial 5 μm sections, where available, were stained with hematoxylin and eosin, Alcian blue, anti-Chromogranin A (Immunostar, Hudson, WI, USA; cat. #20085), anti-Lysozyme (Dako, Glostrup, Denmark; cat. #A0099), anti-L-FABP (kindly provided by Dr J Gordon, Washington University) or anti-β-Catenin (Becton Dickinson, Franklin Lake, NJ, USA; cat. # 610154) using standard protocols.

Tumor analysis

Mice were killed with carbon dioxide asphyxiation and opened for removal of blood, spleen and intestine. Whole intestine was cut longitudinally, cleaned with phosphate-buffered saline (PBS), and fixed in PBS-buffered 10% formalin solution. Tumor counting was performed using dissection microscope. Only clearly defined adenomas exceeding 0.5 mm in size were counted as tumors.

Plasma analysis

Blood was collected from mice immediately post-mortem in heparin-coated tubes and centrifuged to remove cells. Plasma was removed to a new tube and frozen at -80 °C until analysis. Cytokines were quantified using the Bioplex-pro mouse cytokine 23-plex array (Bio-Rad Laboratories, Hercules, CA, USA) using standard protocols.

Flow cytometry

Splenic leukocytes were isolated by blending whole spleens to single-cell suspension, filtration in 4 °C PBS, followed by erythrocyte lysis using ammonium chloride. Leukocytes were then stained with specified lineage-specific antibodies (Becton Dickinson) and examined on FACScaliber cytometer (Becton Dickinson).

Iron(II) testing

Size- and location-matched adenomas from formalin-fixed intestines of sex-matched littermates were excised and washed with distilled water twice and maintained in distilled water at 4 °C overnight. Iron(II) content in the adenomas was then assayed blinded of genotype by SGSTL Pte. Ltd., Singapore.

Statistical analysis

Except the cases noted immediately below, all statistical analyses were performed using paired Student's *t*-test of sex-matched littermate pairs. Where *Lcn2*^{+/+} and *Lcn2*^{-/-} tumors were compared, samples were further selected from sex-matched littermates by pairing approximate size and intestinal location. *Lcn2* expression comparing intestinal expression in normal versus tumor tissue was analyzed using homoscedastic Student's *t*-test. Potential alterations in colon tumorigenesis were determined to be not statistically significant by the nonparametric Mann-Whitney *U*-test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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