HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2012 October 19.

Published in final edited form as:

Oncogene. 2012 April 19; 31(16): 2017–2027. doi:10.1038/onc.2011.382.

GATA3 Inhibits Lysyl Oxidase Mediated Metastases of Human Basal Triple-Negative Breast Cancer Cells

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Abstract

Discovery of mechanisms that impede the aggressive and metastatic phenotype of human basal triple-negative type breast cancers (BTNBC) could provide novel targets for therapy for this form of breast cancer that has a relatively poor prognosis. Previous studies have demonstrated that the expression of GATA3, the master transcriptional regulator of mammary luminal differentiation, can reduce the tumorigenicity and metastatic propensity of the human BTNBC MDA-MB-231 cell line (MB231), although the mechanism for reduced metastases was not elucidated. We demonstrate through gene expression profiling that GATA3 expression in 231 cells resulted in the dramatic reduction in the expression of Lysyl oxidase (LOX), a metastasis-promoting matrix remodeling protein, in part, through methylation of the LOX promoter. Suppression of LOX expression by GATA3 was further confirmed in the BTNBC Hs578T cell line. Conversely, reduction of GATA3 expression by siRNA in luminal BT474 cells increased LOX expression. Reconstitution of LOX expression in 231-GATA3 cells restored metastatic propensity. A strong inverse association between high LOX and low GATA3 expression was confirmed in a panel of 51 human breast cancer cell lines. Similarly, human breast cancer microarray data demonstrated that high LOX/low GATA3 expression is associated with the BTNBC subtype of breast cancer and poor patient prognosis. Expression of GATA3 reprograms BTNBC to a less aggressive phenotype and inhibits a major mechanism of metastasis through inhibition of LOX. Induction of GATA3 in BTNBC cells or novel approaches that inhibit LOX expression or activity could be important strategies for treating BTNBC.

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Conflict of Interest. The authors report no conflict of interest.

Keywords

GATA3; basal triple-negative breast cancer; lysyl oxidase; differentiation; gene expression profiling

Introduction

Although primary tumors in cancer patients are often successfully treated, the emergence of metastases generally heralds a poor prognosis and is responsible for over 90% of cancer patient deaths (Gupta *et al.*, 2006). High-throughput gene expression profiling and molecular subtype clustering have been highly effective for predicting the propensity of a breast tumor to metastasize with poor patient outcome. Based on hierarchical clustering analyses, breast tumors have been classified into distinct subtypes (Basal-like A and B, ErbB2+, Normal Breast-like, Luminal A, B and C) (Sorlie *et al.*, 2003; Hennessy *et al.*, 2009). Patients with basal-type tumors lacking estrogen receptor (ER), progesterone receptor (PR), and ErbB2 - referred to as basal triple-negative breast cancer (BTNBC) - have a worse prognosis compared to patients with more differentiated, less metastatic tumors expressing markers of the luminal lineage, including the transcription factors GATA3 and ER (Sorlie *et al.*, 2003; Neve *et al.*, 2006; Perou *et al.*, 2000). These observations suggest that the constellation of genes responsible for the specification of the luminal or basal subtype of breast cancer may also promote or inhibit metastatic potential. Although gene signatures have been invaluable for defining categories of breast cancer metastatic propensity and patient outcome (Wang *et al.*, 2005; van de Vijver *et al.*, 2002; van't Veer *et al.*, 2002), elucidating the molecular mechanisms governing metastatic propensity remains a critical challenge.

Human breast cancer cell lines recapitulate many important molecular features of breast cancer and have been classified into three of the major tumor subtypes – luminal, basal A and basal B - based on microarray analyses (Neve *et al.*, 2006). Breast cancer cell lines clustering within the luminal subtype, such as BT474, exhibit limited invasive properties compared to cell lines of the basal subtype including the MDA-MB-231 (MB231) cell line, which clusters within the Basal B subtype (Neve *et al.*, 2006). Since the MB231 cell line displays many critical biological and molecular features of BTNBC, it has been extensively used as an important model to study this form of breast cancer.

While distinct subtypes of breast cancer have been delineated, few studies have explored whether a potential plasticity exists for tumor cells of one subtype to trans-differentiate into another subtype and what factors would lead to such a phenotypic shift. Previous studies have demonstrated that overexpression of the mammary luminal transcription factor GATA3 in BTNBC cells could reduce tumorgenicity and metastases. However, no mechanism has been reported that accounts for how GATA3 expression reduces the metastatic propensity of BTNBC cells *in vivo*. In this study, we have determined that the suppression of Lysyl oxidase (LOX) expression by GATA3 is a major mechanism for the reduction of metastases.

Expression of GATA3 is intimately associated with the luminal subtype of breast cancer and its expression is highly correlated with ER expression and many genes associated with the

luminal subtype (Sorlie *et al.*, 2003; Perou *et al.*, 2000; Usary *et al.*, 2004). GATA3 is generally absent or minimally expressed in basal subtypes of breast cancer including MB231 cells. Recently, GATA3 was shown to be essential for normal mammary gland development and luminal cell differentiation (Kouros-Mehr *et al.*, 2006a; Asselin-Labat *et al.*, 2007). Conditional knock-out of GATA3 in mammary epithelial cells resulted in abnormal mammary duct formation (Kouros-Mehr *et al.*, 2006a; Asselin-Labat *et al.*, 2007). Retroviral expression of GATA3 in mammary progenitor cells induced expression of luminal differentiation markers (Asselin-Labat *et al.*, 2007). Thus, GATA3 appears to be a key factor in determining the biological characteristics of mammary luminal epithelial cells and breast cancers with a luminal phenotype.

In this study, we demonstrate through gene expression profiling that GATA3 induces numerous transcriptional alterations affecting differentiation, metastasis, interactions with the extracellular matrix and paracrine signaling. Further, we determined that GATA3 reduces the expression of many metastasis-related genes including macrophage-colony-stimulating factor (CSF-1), which is a potent chemoattractant for macrophages promoting metastatic progression. Importantly, we demonstrate that the repression of LOX expression by GATA3 is a key mechanism for the GATA3-mediated inhibition of metastases. LOX expression in breast cancer has been shown to be associated with reduced overall survival and distant metastasis-free survival in ER negative patients (Erler *et al.*, 2006b). The lack of GATA3 expression resulting in elevated LOX expression in human BTNBC may account for the highly metastatic nature of this form of breast cancer and suggests that LOX is an important target for therapy.

Results

GATA3 reduces MB231 cell proliferation in 3D culture, primary tumor outgrowth and metastases and alters cell morphology and cytoskeletal organization

GATA3 protein was ectopically expressed in MB231 cells via transduction with lentivirus expressing GATA3 (231-GATA3) (Supplementary Figure 1a). In order to confirm that our MB231 cells containing an empty lentiviral vector (231-Empty) and our 231-GATA3 cells exhibited similar growth and metastatic characteristics as were previously reported, we determined their growth characteristics both *in vitro* and *in vivo*. We observed no differences in apoptosis by ELISA assay for cytoplasmic histone-associated-DNA-fragments between 231-Empty and 231-GATA3 cells (Supplementary Figure 1b). Pulse-chase BrdU labeling revealed that GATA3 over-expression in MB231 did not affect proliferation in 2D cultures (Supplementary Figure 1c) as previously reported (Yan *et al.*, 2010). However, we demonstrate for the first time that in 3D culture using Cultrex® basement membrane extract (BME), 231-GATA3 cells were significantly less proliferative compared to 231-Empty control cells ($p<0.001$; Figure 1a). Thus, differences in the rates of cell proliferation between 231-Emtpy and 231-GATA3 may not necessarily only be due to intrinsic cellular changes but appear to also result from GATA3 altering cell interactions with the extracellular matrix (ECM). In 2D culture, 231-Empty cells maintained a spindle, elongated morphology, whereas 231-GATA3 cells were larger and cuboidal (Supplementary Figure 1d). In 3D culture using BME, 231-Empty cells appeared invasive by protruding into the BME matrix

to form interconnected networks of cells, whereas the 231-GATA3 cells appeared less invasive without extended protrusions and formed more tightly organized, rounded clusters (Figure 1b).

Similarly, in xenograft studies, primary tumor outgrowth of 231-GATA3 cells was significantly delayed compared to 231-Empty cells when orthotopically transplanted into mammary fat pads (Supplementary Figure 2a) with a concomitant ∼40% increase in survival of mice (Supplementary Figure 2b). Histologically, 231-Empty tumors were characterized primarily by spindyloid cells whereas tumors arising from 231-GATA3 cells appeared primarily epithelioid (Supplementary figure 2c).

We further confirmed that during early lesion development, tumors arising from 231-GATA3 cells expressed a more differentiated phenotype than tumors from 231-Empty cells. 231-GATA3 tumors were immunoreactive for GATA3, E-cadherin and cytokeratin 8 by immunohistochemistry compared to 231-Empty tumors which were negative for these markers (Supplementary Figure 2d). Interestingly, there appears to be strong selective pressure against the expression of GATA3 as the tumors grow. Thus, over time, tumors arising from the 231-GATA3 cells lose GATA3 expression and the associated changes. Advanced tumors showed similar immunostaining for both Ki67 and TUNEL in mice receiving either 231-Empty or 231-GATA3 injections (data not shown) Lungs from mice receiving orthotopic implantations of the cells were collected and visualized by immunofluorescence but we did not observe GFP positive lung lesions at the time when mice were sacrificed due to significant primary tumor burden.

Although we did not observe a statistically significant difference in the number of 231-GATA3 cells compared to 231-Empty cells invading through Matrigel *in vitro* using the Boyden chamber assay (Supplementary Figure 3a), there was a dramatic increase in the clearing of tail vein injected 231-GATA3 cells in the lungs compared to 231-Empty cells within the first 24 hrs following tail vein injection (Supplementary Figure 3b). At 24 hours, there was an approximately 75% reduction in the number of 231-GATA3 cells in the lungs compared to the number of cells in the lungs 2 hours post-injection, whereas at the same time points there was an approximately 20% increase in the number of 231-Empty cells in the lungs (Supplementary Figure 3b). This suggests that GATA3 greatly reduces the ability of MB231 cells to initially survive in the lung metastatic site. Furthermore, mice tail vein injected with 231-GATA3 cells had a statistically significant 9-fold reduction in total metastatic burden in the lung compared to mice injected with the 231-Empty cells 2-months after injection ($p<0.05$; Figure 1c). The observed reduced metastatic burden in the lungs of mice receiving 231-GATA3 cells was the result of a reduced number and smaller size of lesions as observed by immunofluorescence (Supplementary Figure 3c) and by quantitation of H&E staining (Supplementary Figure 4a) by a pathologist. We previously demonstrated that this method of using immunofluorescence to detect GFP labeled cells in whole lungs by single cell whole organ microscopy (SCOM) is extremely sensitive and quantitative (Barkan *et al.*, 2008).

We additionally quantitated the percentage of lung area occupied by metastatic lesions based upon Ki-67 staining using Apiro Image Analysis Software. This similarly revealed that

GATA3 expression significantly reduced metastatic burden compared to 231-Empty cells. We further characterized the lung lesions from mice 2-months after they received either 231-Empty or 231-GATA3 cells for proliferation and apoptosis by Ki67 and TUNEL staining and observed no statistical differences between these two cohorts (data not shown).

GATA3 profoundly alters the transcriptome of MB231 cells with a concomitant reduction in expression of metastasis-associated genes

Gene expression profiling analyses revealed that the expression of 1273 probe sets were altered between 231-GATA3 and 231-Empty cells (776 up- and 497 down-regulated in 231-GATA3 cells with fold change ≥ 1.5 and $p < 0.001$, false discovery rate (FDR) of 3% (Supplementary Dataset 1) and that several biological processes were altered (Supplementary Figure 5).

Microarray analysis further revealed that LOX, a gene functionally involved in cell adhesion, extracellular matrix remodeling, migration and metastasis (Erler *et al.*, 2006b; Erler *et al.*, 2009), was the gene most down-regulated by GATA3. We investigated whether the dramatic reduction in metastatic propensity of 231-GATA3 cells was the result of GATA3 dependent inhibition of LOX expression. Quantitative-real time-PCR (Q-RT-PCR) confirmed that LOX expression was reduced by 70% in 231-GATA3 cells compared to 231-Empty cells ($p<0.01$; Figure 2a). We further confirmed at the protein level that GATA3 expression resulted in a reduction of LOX expression. 231-Empty and 231-GATA3 cell pellets were analyzed for GATA3 and LOX expression by IHC. Whereas 231-Empty cells were negative for GATA3 expression, LOX expression was clearly demonstrable (Figure 2b). However, most 231-GATA3 cells exhibited strong nuclear staining for GATA3, but LOX expression was not detectable (Figure 2b). Similarly, early 231-GATA3 primary tumors exhibited less LOX expression by IHC compared to 231-Empty tumors (Supplementary Figure 2d). Similarly analyses were performed on metastatic lesions in the lung. Lung lesions arising from 231-Empty lacked nuclear GATA3 staining by IHC whereas 231-GATA3 lung lesions showed positive GATA3 staining (Supplementary Figure 6a). Furthermore, lung lesions from 231-Emtpy cells expressed LOX protein by IHC, whereas 231-GATA3 metastatic lesions stained poorly for LOX (Supplementary Figure 7a).

When GATA3 was expressed in another BTNBC cell line, Hs578T, LOX expression was reduced by 30% ($p<0.05$; Figure 2a), further demonstrating that GATA3 could suppress LOX expression. Furthermore, 231-GATA3 cells had significantly reduced LOX catalytic activity compared to 231-Empty cells, consistent with the reduction in LOX expression ($p<0.01$; Figure 2c).

To additionally confirm that GATA3 regulates LOX expression in breast cancer cells, we knocked-down GATA3 expression using siRNAs and measured LOX expression. 75% knock-down of GATA3 in the luminal, GATA3-positive breast cancer cell line BT474 increased LOX expression over 4- fold (confirmed using two different siRNAs) (Figure 2d). These findings suggest that GATA3 can regulate LOX expression in both basal and luminal breast cancer subtypes.

GATA3 inhibits LOX expression through DNA methylation

Methylation of the LOX promoter in 231-GATA3 cells was significantly increased compared to control cells (Figure 2e). Although treatment with the methylation inhibitor 5-AZA diminished promoter methylation of LOX in 231-GATA3 cells to levels similar to that of 231-Empty, LOX expression measured by Q-RT-PCR in 231-GATA3 cells treated with 5-AZA was not completely restored to levels observed in 231-Empty cells treated with 5-AZA (Figure 2e) suggesting that GATA3 also regulates LOX expression through methylation-independent pathways. Although there was a trend for reduced LOX expression in 231-Empty cells with 5-AZA treatment compared to vehicle, these differences were not statistically significant and may have arisen from some toxicity effects of the drug during the 4 day treatment period. We observed no changes in GATA3 expression with 5-AZA treatment in 231-Empty (data not shown).

GATA3 reduces macrophage recruitment to metastatic lesions and CSF-1 expression

Since myeloid cells recruitment has been shown to be an important component of metastatic progression especially in the promotion of metastases by LOX, we investigated whether GATA3 expression was also associated with changes in cytokine expression related to myeloid recruitment. Our microarray analysis identified an almost 2 -fold reduction of CSF-1 expression (a key chemokine that recruits macrophages) in 231-GATA3 cells compared to control cells (see below). This was confirmed by ELISA showing a 40% reduction in secreted CSF-1 by 231-GATA3 compared to 231-Empty cells ($p<0.001$; Figure 3a). Reduced secretion of granulocyte-macrophage-CSF (GM-CSF) in 231-GATA3 cells ($p<0.01$) was also observed, although total levels were lower compared to those of CSF-1. There was no change in secreted macrophage migration inhibitory factor (MIF; Figure 3a).

Since we observed a reduction in secreted CSF-1 in 231-GATA3 cells compared to 231-Empty and macrophages have been shown to be an important component of the metastatic process (Condeelis *et al.*, 2006), we quantitated macrophage recruitment in the lungs of mice injected with 231-Empty or 231-GATA3 cells by flow cytometry. Lungs from mice tail-vein injected with 231-GATA3 cells were infiltrated with about 50% fewer mature tumor associated macrophages (F4/80+/Gr-1-) compared to the lungs of mice receiving 231-Empty cells (53% F4/80+/Gr1- cells for 231-Empty vs. 29% for 231-GATA3, $p<0.05$; Figure 3b). There was no change in the percent of CD11b+/Gr-1+ immune cells recruited (Figure 3b).

GATA3 increases the pattern of luminal cell-type gene expression

Using a previously identified gene signature that categorizes the human breast cancer cells into Luminal, Basal A or Basal B subtypes (Neve *et al.*, 2006), we combined our microarray data with data from the 51 breast cancer cell line dataset of Neve *et al.* to perform hierarchical clustering of all of the cell lines using 249 unique signature genes available from both platforms (see Supplementary Materials and Methods). 231-Empty cells, as expected, clustered within the highly invasive basal B subtype, whereas the 231-GATA3 cells clustered within the luminal subtype (Supplementary Figure 8a). GATA3 reduced the expression of 76 named genes associated with the basal phenotype and increased the expression of 46 named genes associated with the luminal phenotype (Supplementary

Dataset 2). Among the genes up-regulated by GATA3 expression were members of the claudin family, claudin 3 and claudin 4, whose low expression is characteristic of the claudin-low subtype of breast cancer (Hennessy *et al.*, 2009). Q-RT-PCR confirmed that GATA3 altered the expression of several signature genes that distinguish the Luminal, Basal A and Basal B phenotypes towards the luminal phenotype (ANK3, CLDN3, CLDN4, KRT19, EPCAM, TSPAN13, ERBB3, FSCN1 and HMGA2) (Supplementary Figure 8b). Western blot confirmed increased expression of cytokeratin-18 and re-expression of E-cadherin in 231-GATA3 (Supplementary Figure 8c).

LOX and GATA3 are inversely expressed in breast cancer cells

To address whether LOX and GATA3 expression in breast cancers may be inversely associated, we performed a retrospective analysis of the previously published microarray data for 51 breast cancer cell lines (Neve *et al.*, 2006). GATA3 expression is inversely associated with LOX expression ($p<0.001$; Figure 4a and b) with the luminal subtype cell lines expressing high GATA3 and low LOX, whereas LOX expression was high in the more invasive basal subtypes (basal B > basal A) lacking GATA3 expression (Figure 4c).

Re-expression of LOX in 231-GATA3 cells reverses metastatic propensity

231-GATA3 cells were transduced with lentiviral vectors expressing control RFP (231-GATA3-Empty) or both LOX and RFP (231-GATA3-LOX) and examined for their metastatic potential *in vivo*. Overexpression of LOX in 231-GATA3 cells was confirmed by Q-RT-PCR (Figure 5a). LOX protein levels were increased in 231-GATA3-LOX cells compared to 231-GATA3-Empty cells as determined by IHC (Figure 5b). Similarly, LOX activity was increased in 231-GATA3-LOX cells compared to 231-GATA3-Empty cells (Figure 5c). However, 231-GATA3-LOX cells maintained their cuboidal morphology and continued to express E-cadherin (Supplementary Figure 9a and b).

We observed no differences in the rates of proliferation in 2D or 3D or in invasive potential by Boyden chamber invasion assay between 231-GATA3-Empty and 231-GATA3-LOX *in vitro* (data not shown). Most importantly, SCOM analysis revealed that mice tail vein injected with 231-GATA3-LOX cells exhibited a statistically significant marked increase in total lung metastatic burden of more than 5-fold compared to 231-GATA3-Empty cells ($p<0.05$; Figure 5d) that was similar to that of 231-Empty cells (Figure 1c). This was further validated by image quantitation of Ki-67 expression and H&E staining of metastatic lung lesions using Apirio Image Analysis software (Supplementary Figure 4b) which demonstrated an approximately 8-fold increase in metastatic burden due to increased size and number of lesions in 231-GATA3-LOX cells compared to 231-GATA3-Empty cells. Importantly, this demonstrates that the reduction in metastatic potential of tumor cells by the suppression of LOX by GATA3 can be restored by the reexpression of LOX.

There was a selection against GATA3 as the metastatic lesions progressed consistent with our model that GATA3 reduces metastatic potential. GATA3 expression was still detected in some of the lung metastatic lesions from both 231-GATA3-Empty and 231-GATA3-LOX cells (Supplementary Figure 6b). Metastatic lung lesions from 231-GATA3-Empty cells

exhibited minimal LOX expression whereas metastatic 231-GATA3-LOX lesions showed strong LOX expression by IHC (Supplementary Figure 7b).

We determined that the great majority of genes whose expression was initially altered by GATA3 were not affected by reexpression of LOX in MB231. In fact, only nine named genes dysregulated by GATA3 were expressed in the opposite direction by reexpression of LOX (adrenomedullin, fibronectin, MMP1, MMP12, anterior gradient homolog 2, IL7R, , neural precursor cell expressed - developmentally down-regulated 4-like, RNA binding protein with multiple splicing, and chordin-like 1). Thus, the effect of LOX appears to be more specific for promoting a more metastatic phenotype than globally affecting the transcriptome.

As in the previous tail-vein injection experiment, we observed no lung metastasis by immunofluorescence in the mice receiving orthotopic implantations of 231-GATA3-Empty vs. 231-GATA3-LOX cells.

Patients expressing a high LOX/GATA3 ratio have poor prognosis

Retrospective statistical analyses of the NKI patient microarray database (n=295) (van de Vijver *et al.*, 2002) revealed higher LOX expression in the basal-subtype of breast cancer compared to the luminal A ($p<0.001$) and luminal B types ($p<0.01$), whereas GATA3 was lower in the basal-subtype compared to the luminal A ($p<0.001$) and the luminal B ($p<0.001$) (Figure 6a). Importantly, an inverse correlation between LOX and GATA3 expression was also demonstrated across the breast cancer subtypes ($r= -0.3$; $p<0.001$; Figure 6b), consistent with our results for the 51 breast cancer cell lines. Although we observed an inverse association between GATA3 and LOX expression in patients, there were some tumors expressing relatively high or low levels of both GATA3 and LOX. Therefore additional factors may be involved in regulating the expression of LOX in breast cancer patients. These retrospective data along with our breast cancer cell line data support a model whereby breast cancers that express low GATA3 (clustering with the basal subtype), and express elevated LOX have an increased metastatic potential. GATA3 expression (and possibly ER expression) in luminal tumors appears to override the survival effects of high LOX expression. A large portion of basal ER negative tumors that express very low levels of GATA3 express high levels of LOX. Kaplan-Meier analysis using the above database revealed that patients that display a low GATA3/high LOX expression pattern have significantly reduced survival compared to patients with a low GATA3/low LOX expression pattern ($p <0.01$; Figure 6c). Thus, LOX may serve as a predictor of survival in patients with low GATA3 expression. Even in cases where tumors expressed high levels of LOX, the concominant expression of GATA3 was shown to improve survival (Figure 6c), thus GATA3 expression may have a dominant protective role to prolong survival that overcomes high LOX expression through other mechanisms.

Discussion

This study has identified a key mechanism for GATA3-induced inhibition of metastatic propensity of BTNBC, an aggressive form of breast cancer with poor prognosis. We have demonstrated that the expression of GATA3 induces global changes to the transcriptome

associated with a significant reduction in metastatic propensity and extended survival of mice in xenograft studies. While GATA3 has previously been shown to reduce metastases of the MB231 cells (Yan *et al.*, 2010; Dydensborg *et al.*, 2009), this study identified a major mechanism for the GATA3-induced inhibition of metastases through the down-regulation of LOX. GATA3 has been shown to be a key developmental transcription factor in the hematological system and during mammary luminal epithelial cell development (Kouros-Mehr *et al.*, 2006a; Zhou *et al.*, 2003; Kouros-Mehr *et al.*, 2006b). The expression of GATA3 is a defining property of luminal type breast cancers, whereas it is minimally expressed in basal type breast cancers.

We observed that many genes that have been previously shown to be involved in metastatic progression were coordinately down-regulated by GATA3, including Fascin homologue 1 (FSCN1), Chemokine receptor 4 (CXCR4), Mannosidase, alpha, class 1A, member 1 (MAN1A1), Tenascin C (TNC) and CSF-1. These genes were previously identified to be part of a lung metastasis signature in MB231 cells (Minn *et al.*, 2005) suggesting that the expression of GATA3 in BTNBC cells inhibits the expression of genes that promote invasion and dissemination. Although GATA3 was previously shown to reduce the metastatic potential of MB231 or the MB231 variant LM2-4175 cell line that is highly metastatic to the lung in mice (Yan *et al.*, 2010; Dydensborg *et al.*, 2009), the responsible factor(s) responsible for the marked reduction of metastases *in vivo* was not identified. Neither study found and confirmed an *in vivo* mechanism through which GATA3 over-expression inhibits metastases as presented in our study. We demonstrate for the first time that repression of LOX by GATA3 is a major mechanism resulting in the inhibition of metastases and that re-establishment of LOX expression in the 231-GATA3 cells restored the metastatic phenotype.

Several mechanisms may be involved through which LOX affects metastases. Intracellular active LOX facilitates migration and invasiveness in breast cancer cells through a hydrogen peroxide mediated mechanism that results in the phosphorylation and activation of Src/FAK pathways (Payne *et al.*, 2005). Activated LOX secreted into the extracellular environment plays an important role in potentiating metastatic tumor cell growth through the cross-linking of several collagen types and elastins in the extracellular matrix (Erler *et al.*, 2009; Kagan *et al.*, 2003; Payne *et al.*, 2007; Levental *et al.*, 2009). Most importantly, inhibition of LOX enzymatic activity in orthotopically implanted MB231 cells eliminates lung metastases (Erler *et al.*, 2006b; Bondareva *et al.*, 2009). More recently, LOX was found to also activate FAK and promote invasiveness in an integrin β1 dependent mechanism involving collagen cross-linking and tissue stiffening (Levental *et al.*, 2009).

We also observed that while over-expression of LOX significantly increased lung metastasis by tail-vein injection of 231-GATA3 cells, there was a paradoxical reduction in primary tumor outgrowth. This is consistent with another study where over-expression of LOX in the gastric cancer cell line, MKN28, reduced primary tumor growth in a xenograft model (Kaneda *et al.*, 2004). Treatment of MB231 xenografts with BAPN, shLOX or an inhibitory LOX antibody reduced metastasis to the lung but did not affect primary tumor growth (Erler *et al.*, 2006a), whereas inhibition of LOX catalytic activity in uveal melanoma significantly reduced cellular invasion (Abourbih *et al.*, 2010). The mechanisms responsible for these

differences in response to LOX expression between the primary and metastatic sites remain unknown, but may be attributed to the dual role of LOX as a tumor suppressor and as a tumor promoter. The function of LOX is likely dependent on the cellular context (Payne *et al.*, 2007), the biological activity of its propeptide (Palamakumbura *et al.*, 2009; Grimsby *et al.*, 2010) and perhaps the metastatic site. Although we have only studied the effect of GATA3 and LOX in the lung, LOX might also affect metastasis at other organs.

LOX is inactivated by methylation in human gastric cancer and methylation status was associated with loss of LOX mRNA expression in gastric cancers (Kaneda *et al.*, 2004). However, mechanisms responsible for LOX methylation are still unknown. Here, we provide evidence implicating changes in the DNA methylation status of the LOX promoter partially responsible for the reduced expression of LOX upon over-expression of GATA3. Preliminary analyses of the genome-wide methylation patterns by microarray indicates that regions in the 5′ regulatory region and first exon indicate a significant increase in methylation in the 231-GATA3 cells compared to empty cells. These results will require further validation and functional analyses to more precisely define the role of methylation in regulating LOX expression.

Although our studies demonstrated that GATA3 alone is sufficient to reduce LOX expression through changes in methylation which may be direct or indirect, future studies are required to gain further insights into the underlying mechanism that results in methylation of the LOX promoter and subsequent suppression of LOX expression. It is also likely that in addition to its effect on methylation, GATA3 alters the expression of other genes that positively or negatively regulate the transcription of LOX or its post-translational stability. Chip-on-Chip studies did not identify GATA3 binding sites in the LOX promoter suggesting that GATA3 does not directly bind to and inhibit the LOX promoter (Paul Meltzer, personal communication). While LOX showed increased methylation resulting in reduced expression, E-cadherin showed reduced methylation at the DNA promoter upon GATA3 over-expression (data not shown). Therefore, the GATA3 dependent changes in the epigenome appear to be gene specific.

Our results suggest that expression of GATA3 in the mammary gland may promote global changes in gene expression resulting in the expression of genes involved in luminal differentiation, and in the repression of genes associated with the basal subtype through epigenetic modifications such as alterations in methylation patterns. We demonstrated increased LOX expression associated with the more invasive basal B subtype in breast cancer cell lines and with the basal subtype in breast cancer patients who have a poorer overall survival compared to patients with the luminal A subtype (van de Vijver *et al.*, 2002). Although GATA3 can regulate LOX expression, GATA3 may not be the only factor that regulates LOX expression. In addition to LOX, Basal B cells likely have additional factors that could contribute to metastasis. Most importantly, our retrospective analysis revealed that LOX expression is critical at predicting survival in patients with reduced GATA3 expression.

Expression of GATA3 in MB231 cells also resulted in important changes in how the cells interacted with the ECM. Many genes altered by GATA3 expression are extracellular or

plasma membrane proteins which may be responsible for the observed reduced proliferation of 231-GATA3 cells in 3D cultures and their more organized compacted spherical structure in 3D cultures compared to 231-Empty cells.

Additionally, the expression of GATA3 led to changes in the transcription of genes that induce important paracrine effects in the stroma. Recruitment of macrophages at the metastatic site has been shown to be a critical component for metastatic growth (Condeelis *et al.*, 2006). CSF-1 secretion was significantly reduced in 231-GATA3 cells compared to 231-Empty cells which may be responsible for our observed reduction in macrophage infiltration into the lungs of 231-GATA3 tail vein injected mice compared to 231-Empty. In addition, we observed dramatically increased clearing of tumor cells in the lung within the first 24 hrs of tail vein injection of 231-GATA3 cells compared to control cells suggesting that GATA3 may reduce the ability of cells to survive during early stages of tumor infiltration at the metastatic site. It is also possible that the expression of GATA3 may inhibit additional paracrine factors required for recruitment of macrophages. Taken together, our data suggest that GATA3 alone is sufficient to alter molecular events that can regulate metastasis.

It is, therefore, conceivable that tissue- or subtype-specific transcription factors responsible for promoting global changes in the tumor transcriptome, may be critical targets that account for the heterogeneous nature of tumors, predict patient outcome and most importantly, may become valuable novel therapeutic targets. The data presented here provide strong evidence indicating that the induced expression of GATA3 or the inhibition of LOX activity may be worthy therapeutic approaches for the reduction of metastasis in breast cancer.

Materials and Methods

Cell lines, transfection and lentivirus infection

MB231, BT474 and Hs578T cells were obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA). Cells were negative for mycoplasma. See Supplementary Materials and Methods for experimental details.

Methylation-specific PCR—Cells were treated with vehicle or 5-aza-2′-deoxycytidine (5-AZA) (Sigma, St. Louis, MO, USA) for 4 days prior to DNA isolation. Details for methylation-specific PCR are provided in Supplementary Materials and Methods.

Mice, necropsy and ex-vivo imaging

All animal work was carried out in accordance with the guidelines of the Animal Care and Use of Laboratory Animals (NIH publication No. 86-23, 1985) under an approved animal protocol. Xenograft studies were performed using 6-8 week old female SCID or NOD/SCID mice (NCI Frederick or Jackson Laboratories). Details for animal work are provided in Supplementary Materials and Methods.

LOX activity

LOX activity was measured as the fluorometric β-aminopropionitrile (BAPN) inhibitable LOX activity assay using Amplex red (Palamakumbura *et al.*, 2002), See Supplementary Materials and Methods for a detailed description.

Immunoblotting and Antibodies

Cells were lysed in ice-cold RIPA buffer for Western blot analyses as described previously (Hoenerhoff *et al.*, 2009). GATA3 (Santa Cruz, Santa Cruz, CA, USA), and β-actin (Sigma) antibodies were used.

3D culture and Proliferation assay

Cells were cultured in growth factor-reduced 3D Cultrex® Basement Membrane Extract (BME; Trevigen, Helgerman, CT, USA) as previously described with minor modifications (Barkan *et al.*, 2008; Barkan *et al.*, 2010). Cells were cultured in complete media and media was replenished every 2 days. Proliferation was measured as previously described in (Barkan *et al.*, 2008) at 2, 5, 8 and 12 days after seeding by CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) (Promega Madison, WI, USA).

Immunofluorescence and confocal

Cells grown in 3D culture were imaged by confocal microscopy as previously described (Barkan *et al.*, 2008). Briefly, cells were cultured in 8-well chamber glass slides pre-coated with Cultrex®. For f-actin staining, cells were incubated over night with Alexa-Fluor 488 Phalloidin (Molecular Probes, Eugene, Oregon, USA) and mounted with VECTASHIELD® Mounting Medium with 4′-6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). The slides were imaged using a Leica confocal microscope (Leica Microsystems AG).

Flow analysis

Cell cycle profiles were assayed by 5-bromo-2-deoxyuridine (BrdU) pulse labeling and flow cytometric analysis were performed as previously described (Chu *et al.*, 2005). For myeloid analysis, mice were tail-vein injected with 1 million cells and lungs were harvested after 2 months. See Supplementary Materials and Methods for experimental details.

Microarray data processing

Total RNA was isolated by Trizol® (Invitrogen, Carlsbad, CA, USA) from 231-Empty and 231-GATA3 for microarray analysis. See Supplementary Material and Methods for detailed descriptions. Data deposited in GEO [Reviewer access only: URL http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE24249].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Lalage Wakefield and Li Yang for helpful scientific discussions. We thank Lara El-Touny, Dalit Barkan, Zi-Yao Liu, TingHu Qiu, Anthony Vieira, Christina Bennett, Christine Tomlinson, Steven Austin, Christian Mustroph and Wei-Chu Lai for technical assistance; Paul Meltzer for sharing unpublished data; the LRBGE Fluorescence Imaging Core; and Chand Khanna for the use of fluorescent microscopy equipment; Julie Foley and Norris Flagler for technical assistance with image analysis. This research was supported in part by the Intramural Research Program, Center for Cancer Research, NCI, NIH. IMC acknowledges support from the Department of Defense Breast Cancer Research Program (W81XWH-10-2-0030).

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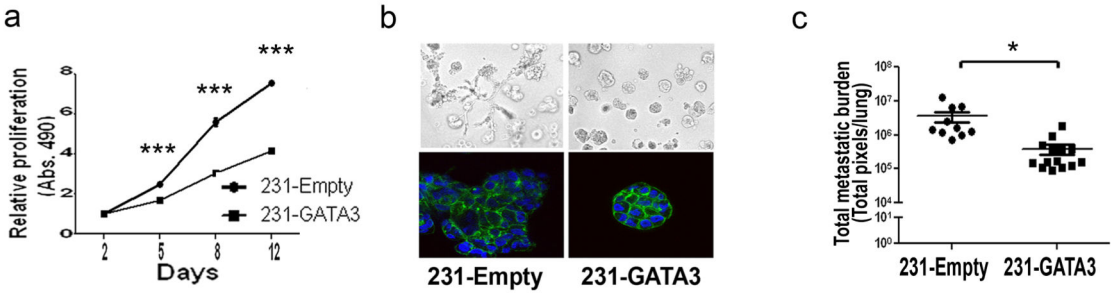

Figure 1.

GATA3 over-expression reduces proliferation in 3D culture and experimental metastasis in mice. (**a**) 231-Empty and 231-GATA3 cells were seeded on 3D Cultrex® for 12 days. 231-GATA3 cells show reduced proliferation as measured by MTS (mean +/- SEM). (**b**) Top panels, bright field images; lower panel, confocal microscopy of cells on 3D Cultrex® fixed and stained with DAPI (blue) for nuclear localization and phalloidin (green) for f-actin. (**c**) Lung lesions of mice injected by tail vein with 231-Emtpy and 231-GATA3 cells. Lungs were imaged by fluorescent microscopy with total metastatic burden calculated per lung.

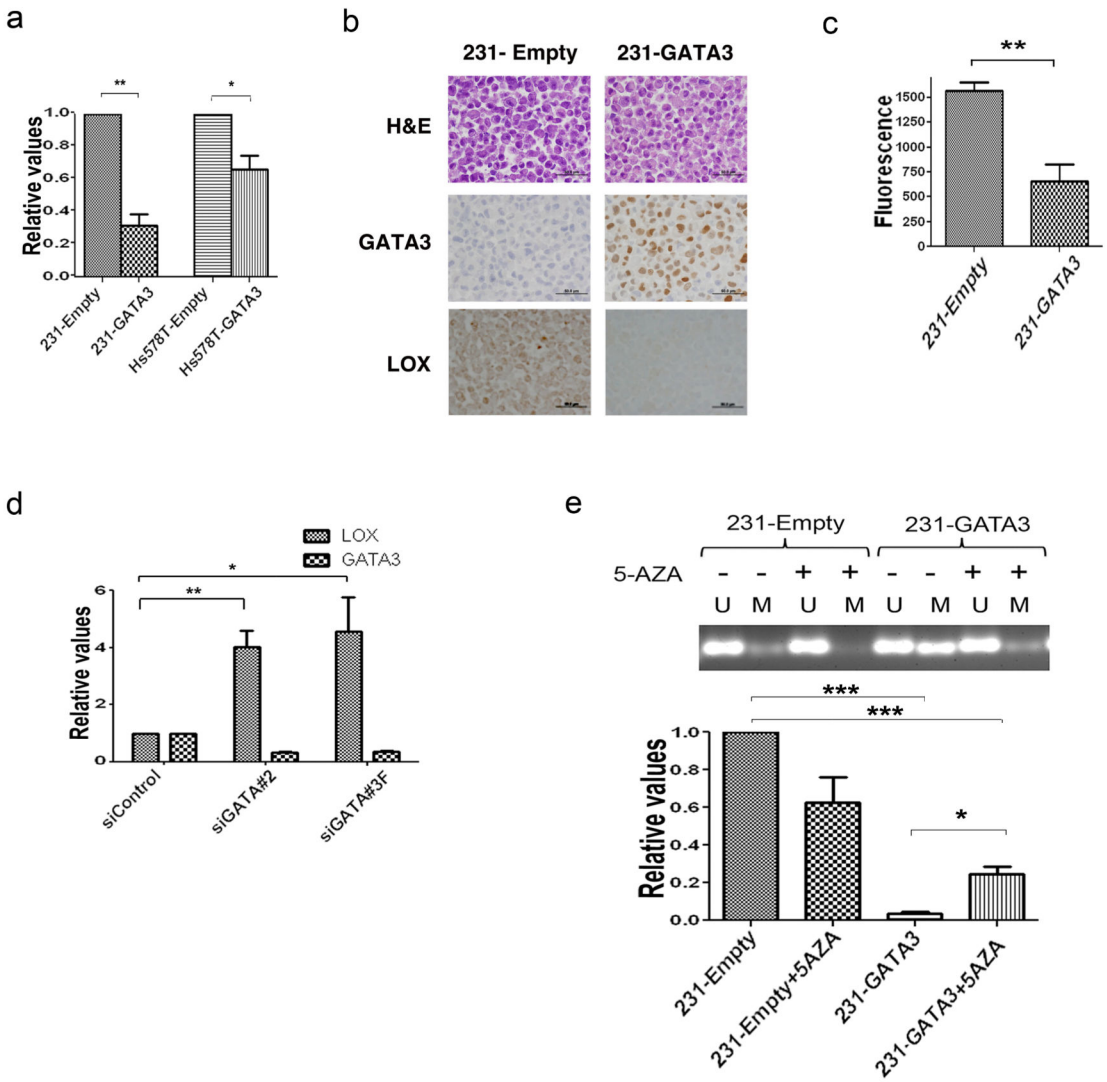

Figure 2.

GATA3 regulates LOX expression in breast cancer cells in part through LOX promoter methylation. (**a**) Relative LOX expression by Q-RT-PCR. Samples were normalized to cyclophilin B. Over-expression of GATA3 in MB231 and Hs578T cells reduces LOX mRNA expression. (**b**) Immunohistochemical staining of cell pellets confirmed positive staining for GATA3 in only 231-GATA3 cells and positive staining for LOX only in 231-Empty cells. (**c**) Relative LOX activity in the media of 231-Empty and 231-GATA3 cells measured as the increase in fluorescence over BAPN containing controls. Relative activity measured at 2400 seconds (40 min). (**d**) Relative LOX and GATA3 mRNA expression measured by Q-RT-PCR. BT474 cells were transfected with GATA3 siRNA for 72 hrs prior to RNA isolation. (**e**) Cells were treated with 5-AZA for 4 days prior to DNA or mRNA isolation. Top panel, PCR of the LOX promoter using LOX unmethylated (U) or methylated (M) specific primers. Lower panel, relative LOX expression by Q-RT-PCR. Treatment of 231-GATA3 cells with 5-AZA increased LOX mRNA expression.

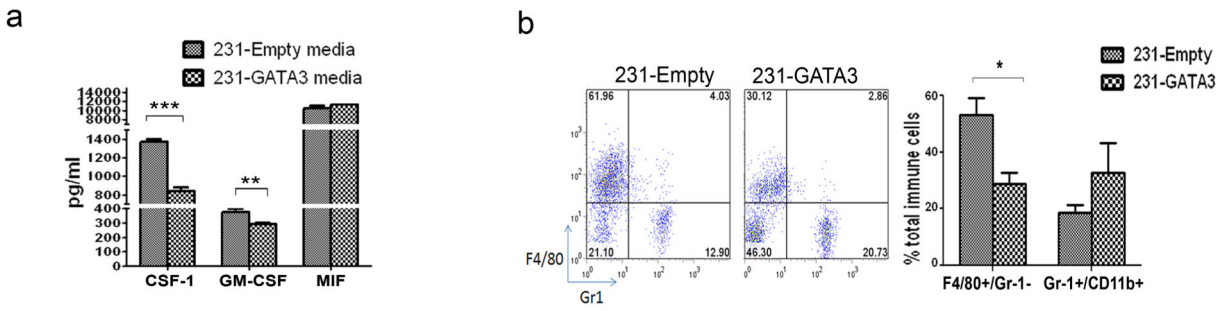

Figure 3.

GATA3 reduces macrophage recruitment to the lung (**a**) ELISA of media collected from 231-Empty and 231-GATA3 cells. 231-GATA3 cells showed reduced secretion of CSF-1 and GM-CSF. (**b**) Flow cytometric analyses of immune cells collected from lungs of tail-vein injected mice (n=4). Cells were labeled with anti CD45, F4/80, Gr1 or CD11b antibodies. Lungs collected from mice injected with 231-GATA3 cells showed reduced F4/80+/Gr1- recruitment.

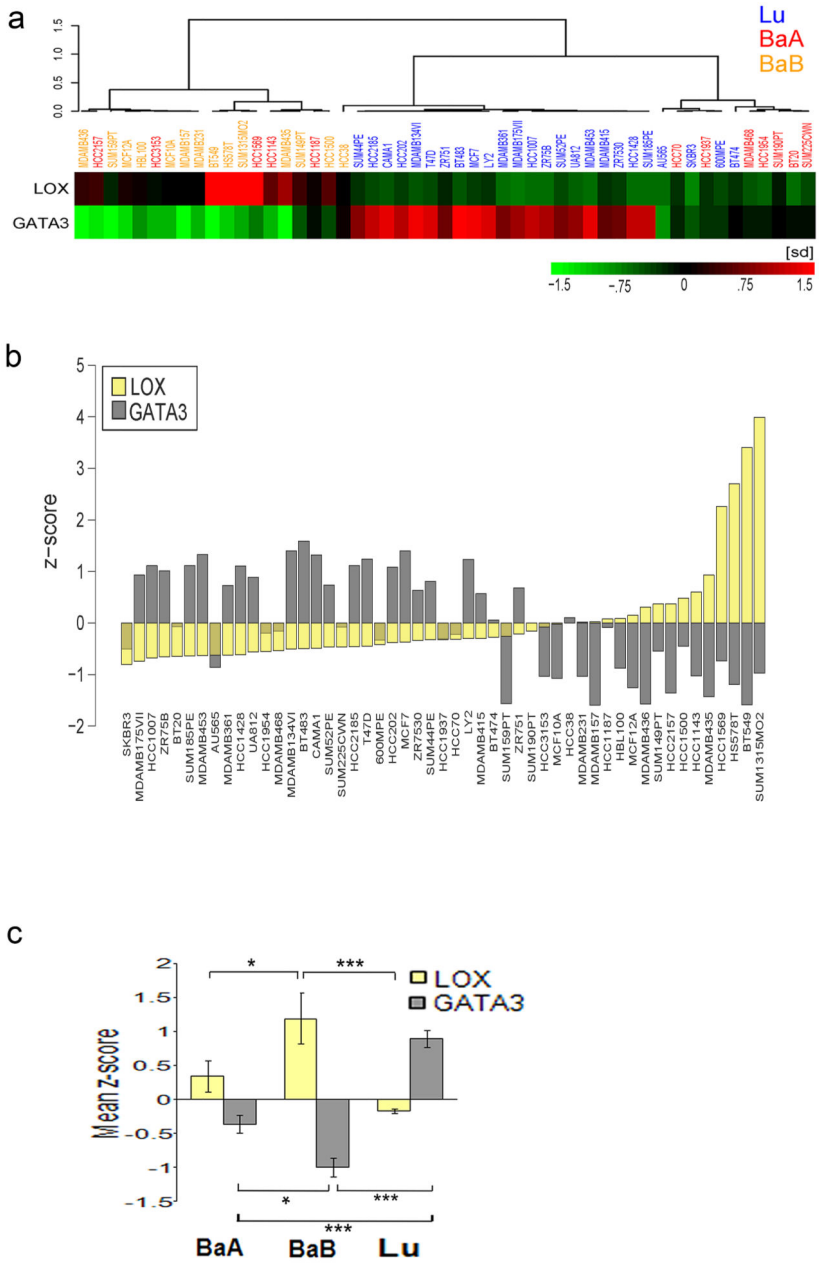

Figure 4.

Analysis of the Neve *et al.* 51 breast cancer cell line microarray database for LOX and GATA expression (Neve *et al.*, 2006). (**a**) Heatmap of LOX and GATA3 expression in breast cancer cell lines. The displayed expression of each gene was standardized with Z-score. The hierarchical clustering used 1-uncentered correlation distance metric and average linkage. (**b**) Relative GATA3 and LOX expression in breast cancer cell lines arranged in order of increasing LOX expression (Pearson's correlation coefficient r=-0.53, P<0.001). (**c**) Relative expression of GATA3 as represented by Z-score (see Supplementary Materials and Methods). GATA3 is enriched in luminal breast cancer cells whereas LOX is enriched in Basal B cells.

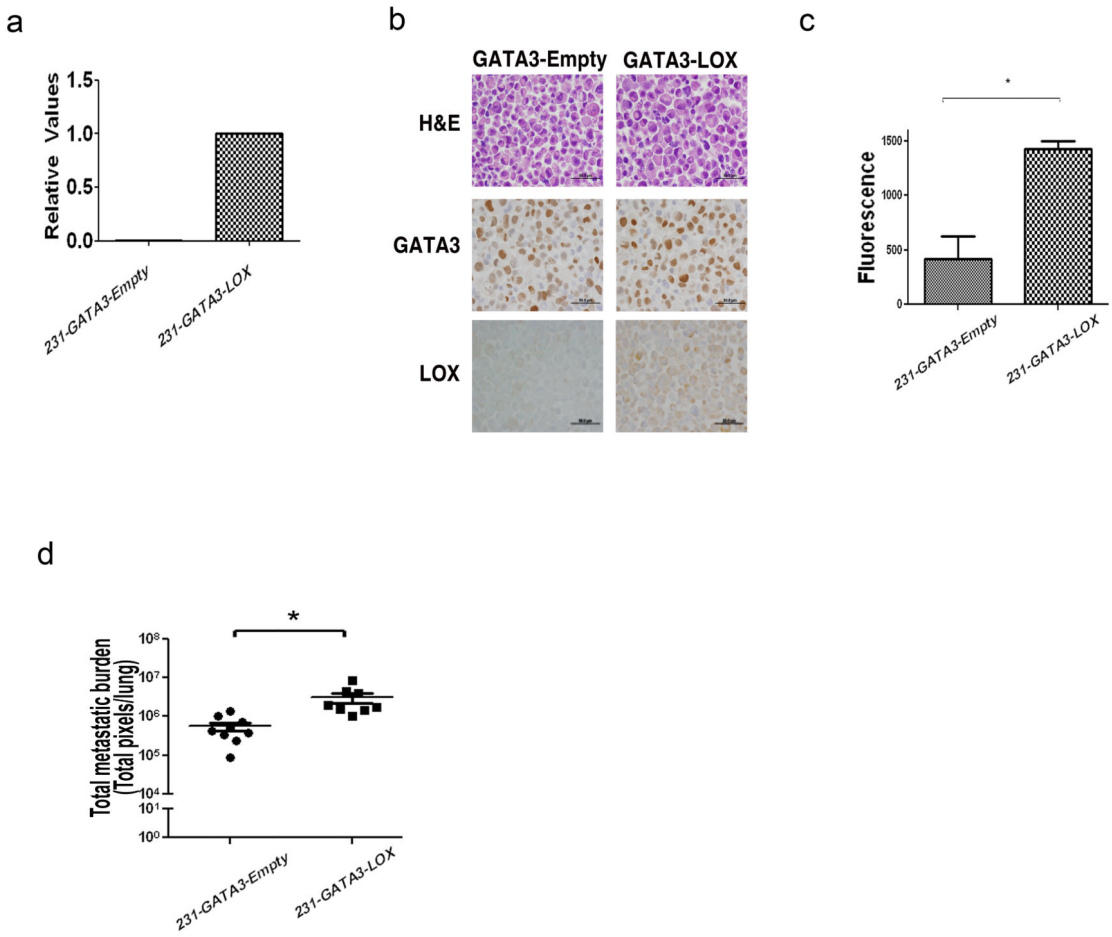

Figure 5.

Re-expression of LOX in 231-GATA3 cells increased metastatic potential of 231-GATA3 cells. (**a**) Lentiviral transduction of 231-GATA3 cells with LOX increases LOX expression in 231-GATA3 cells. Relative LOX expression by Q-RT-PCR. (**b**) Immunohistochemical staining of cell pellets confirmed positive staining for GATA3 in 231-GATA3-Empty and 231-GATA3-LOX cells and positive staining for LOX in only 231-GATA3-LOX cells. (**c**) Relative LOX enzymatic activity measured at 2400 seconds (40 min). (**d**) Mice tail-vein injected with 231-GATA3-Empty and 231-GATA3-LOX cells with lungs collected after 2 months. Lungs imaged by fluorescent microscopy with total metastatic burden calculated per lung.

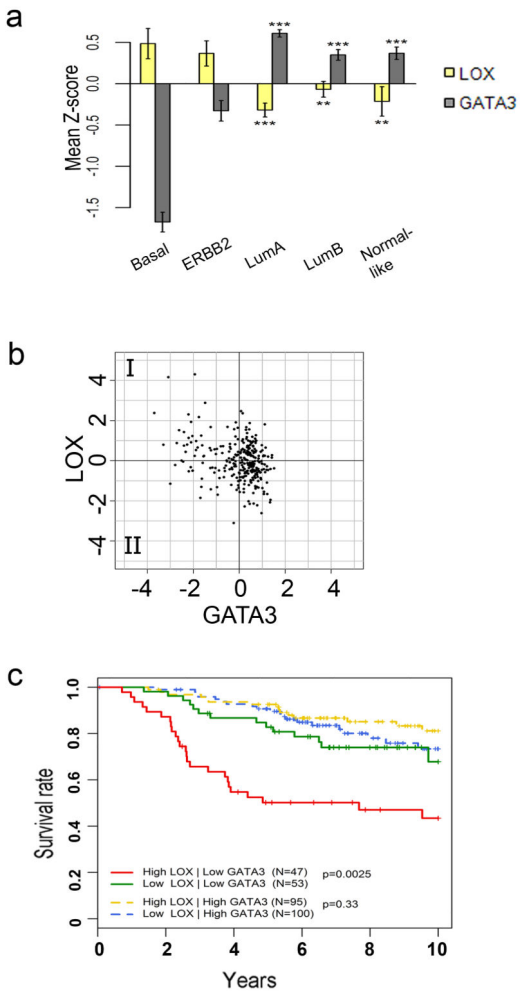

Figure 6.

Retrospective microarray analysis of breast cancer patient microarray data from Van de Vijver *et al*, (van de Vijver *et al.*, 2002). (**a**) GATA3 is associated with the Luminal A and luminal B subtype whereas LOX is enriched in the Basal subtype. (**b**) Correlation between LOX (y-axis) and GATA3 (x-axis) among breast cancer patients (n=295). GATA3 and LOX are inversely correlated (Pearson's correlation coefficient r=-0.30, P<0.001). (**c**) Kaplan-Meier survival curves showing that patients with high LOX and reduced GATA3 expression (quadrant I in (b) above) had significantly reduced overall survival (HR=2.65, p<0.01) compared to patients with low LOX and Low GATA3 (quadrant II in **(b)** above). High and low are defined as above or below median expression as depicted in (b). The log-rank test p-values are indicated. The interaction between LOX and GATA3 was statistically significant (P<0.05).