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14. ABSTRACT
Underlying mechanisms that account for the increased risk of aggressive, metastatic disease associated with basal type breast cancers compared to the more differentiated, luminal tumor subtype have not been well established. Our work demonstrates that the transcription factor GATA3, essential for luminal differentiation during mammary gland development, is sufficient to promote global changes in basal triple-negative breast cancer (BTNBC) cells resulting in both 1) reduced metastasis via LOX downregulation and 2) acquisition of luminal features, thus offering a direct link between these two processes. GATA3 promoted global alterations of the transcriptome of BTNBC cells resulting in molecular and cellular changes associated with a more differentiated, luminal tumor subtype and a concomitant reduction in primary tumor growth, lung metastasis, and macrophage recruitment at the metastatic site. Importantly, we demonstrate that the inhibition of metastases by GATA3 results from the suppression of lysyl oxidase (LOX) expression, a metastasis promoting matrix remodeling protein, via epigenetic changes. MDA-MB-231 breast cancer cells overexpressing GATA3 showed increased methylation at the LOX promoter compared to control cells. Expression of LOX and GATA3 in breast cancer cells were inversely correlated. Most importantly, elevated LOX and reduced GATA3 expression levels predicted poor survival in breast cancer patients. Thus, altering transcription factor expression that promotes differentiation may be an important approach to mitigate aggressive tumor characteristics and to identify therapeutic targets such as LOX for the prevention or treatment of metastatic disease.

15. SUBJECT TERMS
GATA3, Lysyl oxidase, triple-negative breast cancer, metastasis

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INTRODUCTION

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. Given that patients presenting with a luminal breast cancer phenotype tend to have a better prognosis than those with a basal subtype, we explored whether BTNBC MB231 cells 1) could be reprogrammed to express luminal features through the over-expression of the luminal transcription factor GATA3; 2) if reprogramming would result in a less aggressive and less metastatic phenotype; and 3) if so, what molecular mechanisms would account for these changes? 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. 2006; 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. Here, we demonstrate that the expression of GATA3 in human BTNBC cells results in a profound reprogramming of the BTNBC cells into a more luminal phenotype with a concomitant dramatic reduction in their tumorigenic and metastatic propensity. Gene expression profiling identified hundreds of GATA3-induced transcriptional alterations affecting differentiation, metastasis, interactions with the extracellular matrix and paracrine signaling. Further, we determine 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 lysyl oxidase (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 and Giaccia 2006). 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.

BODY

Below I have described a detailed description of the experiments and results obtained as I was addressing the tasks proposed for the first two years of the grant. For those 2 years, I proposed to address task 1 and part of task 2, 3 and 4. I have also appended a manuscript that is currently published in Oncogene and I have referenced experiments from that paper in the following report.

GATA3 reprograms MB231 cells to a more luminal subtype (task 1a)

GATA3 negative cells were transduced with lentivirus expressing GATA3-IRES-eGFP (231-GATA3) and cells were sorted for GFP-expressing cells. We first confirmed that GATA3 protein was expressed in 231-GATA3 cells by Western blot (Figure 1A). We observed no change in cell death as measured by Elisa (Figure 1B). We then performed gene expression profiling analyses to determine the effect of GATA3 expression on the transcriptome of MB231 cells. The expression of 1273 probe sets were found to be altered between 231-GATA3 and control 231-Empty cells (776 up- and 497 down-regulated in 231-GATA3 cells with fold change $\geq$ 1.5 and $p < 0.001$, false discovery rate (FDR) reaches 3%. A previous study of 51 human breast cancer cell lines identified a 305 gene signature that distinguishes the cells into Luminal, Basal A or Basal B subtypes (Neve *et al.,* 2006). Analysis using the Ingenuity software, revealed several network of genes altered upon GATA3 overexpression (Figure 2). We applied a gene centering method with z-score conversion of our microarray data combined with similarly converted data from Neve *et al.* dataset to perform hierarchical clustering of all of the cell lines using 249 unique signature genes available from both platforms. 231-Empty cells, as expected, clustered within the highly invasive basal B subtype, whereas the 231-GATA3 cells clustered within the luminal subtype (Figure 3A). GATA3 reduced the expression of 82 genes associated with the basal phenotype and increased the expression of 48 genes associated with the luminal phenotype. Interestingly, the expression pattern of 231-Empty cells showed some differences compared to the MB231 cells used in the Neve *et al.* dataset. This may be due to clonal differences between the MB231 cells used by Neve *et al*. and our lab, or as a result of the transduction of the MDA-MB-231 cells with the GFP expressing lentivirus.

Consistent with the transition of 231-GATA3 cells to express genes associated with a more luminal phenotype. We also observed increased protein expression by Western blot of cytokeratin-18 and re-expression of E-cadherin in 231-GATA3 (Figure 3B). Altogether, these findings suggest that the transcription factor, GATA3 alone is sufficient to promote critical global changes resulting in the reprogramming and differentiation of the Basal B MB231 cells towards a more luminal, less aggressive phenotype.

GATA3 promotes expression of epithelial markers and loss of mesenchymal markers in MB-231 cells (Task1a)

Our analyses of microarray data comparing 231-Empty cells with 231-GATA3 cells demonstrated a significant reduction in gene expression associated with EMT and an increase in

expression of genes associated with mesenchymal-to-epithelial transition (MET). We observed an increase in expression of Tetraspanin 13, Occludin, Zona-occludin 1, Claudin 3 and Claudin 4 by Q-RT-PCR analysis (Figure 4A), which are genes associated with an epithelial or less aggressive phenotype. In contrast, the expression of genes associated with a mesenchymal phenotype were reduced including Cadherin-11, Snail1, Snail2, Twist, Zeb1, Vimentin, Versican, Fascin homolog 1, CXCR4 and Fibronectin 1 (Figure 4B). These changes in gene expression are indicative of MET.

Loss of E-cadherin expression is considered a hallmark of EMT (Yang and Weinberg 2008). MB-231 cells lack E-cadherin expression and exhibit a more mesenchymal phenotype. Immunofluorescence for E-cadherin and GATA3 protein revealed expression of E-cadherin in the cytoplasmic membrane only in 231-GATA3 cells, but no detectable expression in control 231-Empty cells (Figure 5A). Furthermore, western blot analysis also revealed reduction of β-catenin in 231-GATA3 cells compared to 231-Empty cells (Figure 5B). Altogether, these changes are indicative of transition towards a more epithelial phenotype.

Ectopic expression of GATA3 in MB231 alters cell morphology and cytoskeletal organization in 2D and 3D cultures and reduces proliferation of cells in 3D but not 2D culture. (Task 1b)

For task1b, we investigated whether the ectopic expression of GATA3 could alter the biological phenotype of MB231 cells (Neve *et al.,* 2006). GATA3 induced significant morphological changes in MB231 cells both in 2D and 3D cultures compared to control cells. 231-Empty cells maintained a spindle, elongated morphology, whereas 231-GATA3 cells were larger and cuboidal in shape when grown in 2D culture (Figure 6A). These findings are consistent with those of Yan and colleagues (Yan *et al.,* 2010) who demonstrated that GATA3 can induce morphological changes in 231 cells in 2D culture. In addition to changes in morphology in 2D, we observed changes in the structural organization of 231-GATA3 cells when cultured in 3D using Cultrex® basement membrane extract (BME). Elongated 231-Empty cells appeared invasive by protruding into the BME matrix with a tendency to form interconnected networks of cells, whereas the 231-GATA3 cells remained rounded, tightly organized clusters and failed to form extended protrusions through the BME (Appendix 1, Figure 1B). These morphological changes promoted by GATA3 in 3D cell cultures are characteristic of a less invasive, more organized cell phenotype (Kenny *et al.,* 2007).

GATA3 was shown to directly modulate expression of genes regulating the cell cycle (Pei *et al.,* 2009; Molenaar *et al.,* 2010) and GATA3 over-expression reduced proliferation in 293T cells (Usary *et al.,* 2004). Thus, we investigated whether GATA3 could alter proliferation of MB231 cells. Pulse-chase BrdU labeling revealed that GATA3 over-expression in MB231 did not affect proliferation in 2D cultures with 37% and 38% of cells in S-phase for 231-Empty and 231-GATA3 cells, respectively (Figure 6B). These findings are consistent with Yan *et al.* for 2D cultures (Yan *et al.,* 2010). However, in 3D Cultrex®, 231-GATA3 cells were significantly less proliferative compared to 231-Empty control cells ($p<0.001$; Appendix 1, Figure 1A). Thus, differences between 231-Emtpy and 231-GATA3 rates of cell proliferation may not necessarily be due to intrinsic cellular changes but may be the result of GATA3 altering the interaction of the cells with the extracellular matrix (ECM).

GATA3 reduces primary tumor outgrowth and metastatic potential of xenografts (task 1c)

For task 1c, we proposed to investigate whether GATA3 influences tumor growth and metastasis in xenografts. Primary tumor outgrowth of 231-GATA3 cells was significantly delayed compared to 231-Empty cells when orthotopically transplanted (Figure 7A). Tumor growth of 231-GATA3 cells to a volume of 1 cm^3 was delayed by 20 days compared to 231-Empty cells. Concomitant with these findings, we observed an improvement in cumulative survival of mice implanted with 231-GATA3 cells compared to those implanted with 231-Empty cells. Median survival was extended approximately 40% (from 53 to 73 days) for mice receiving 231-GATA3 cells compared to mice receiving the control cells (Figure 7B). Histologically, sections of 231-Empty tumors were characterized primarily by bundles and streams of spindyloid cells with scant to moderate eosinophilic fibrillar cytoplasm and large oval to elongate hyperchromatic nuclei with coarsely clumped marginated chromatin (Figure 7C). This spindyloid population was admixed with smaller numbers of round to ovoid neoplastic cells with scant eosinophilic amorphous cytoplasm and round to reniform eccentric hyperchromatic nuclei. Sections of tumors arising from 231-GATA3 cells displayed a less prominent spindyloid phenotype, with a predominance of cells exhibiting a round, or epithelioid appearance (Figure 7C). Often a spindyloid population was present centrally within GATA3 tumors, but it was not a predominant feature as compared to tumors arising from 231-Empty cells.

In addition to orthotopic tumor growth, MB231 cells form metastatic lesions in the lung of tail-vein injected NOD/SCID mice. Thus, we examined the effect of GATA3 in altering the metastatic potential of MB231 cells *in vivo*. 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 (Figure 8A), 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 (Figure 8B). 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 (Figure 8B). 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 ($p<0.05$; Appendix 1, 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 (Figure 8C).

GATA3 reduces LOX expression in MB231 and Hs578T cells and knock down of GATA3 in BT474 increases LOX expression (task 1a)

Our microarray analyses revealed reduction of LOX expression in 231-GATA3 compared to 231-Empty cells. Since LOX was shown previously to be involved in breast cancer metastasis to the lung and in tissue remodeling (Erler *et al.,* 2006; Erler *et al.,* 2009), we investigated whether the dramatic reduction in metastatic propensity of 231-GATA3 cells was the result of

GATA3 dependent inhibition of LOX expression. Reduced LOX expression in 231-GATA3 cells was confirmed by Q-RT-PCR. LOX expression was reduced by 70% in 231-GATA3 cells compared to 231-Empty cells ($p<0.01$; Appendix 1, Figure 2A). When GATA3 was expressed in another BTNBC cell line, Hs578T, LOX expression was reduced by 30% ($p<0.05$; Appendix 1, Figure 2A). Furthermore, 231-GATA3 cells had significantly reduced LOX catalytic activity compared to 231-Empty cells, consistent with the reduction in LOX expression ($p<0.01$; Appendix 1, Figure 2C).

To further confirm that GATA3 regulates LOX expression in breast cancer cells, we knocked-down GATA3 expression in the luminal, GATA3-positive breast cancer cell line BT474 and measured LOX expression. We initially used a lentivirus construct expressing shRNA for GATA3. We used 5 different RNAi sequences and sorted cells after transduction. The siRNA sequence that resulted in best knock down of GATA3 also reduced slightly the proliferation of cells. However, as we passaged the cells, we observed loss of knock down and GATA3 was reexpressed in our knock down cells. There was a strong selection against the knock down of GATA3. Thus, we decided to do transient transfections with siRNA for GATA3. Knock-down of GATA3 was successfully achieved with two different siRNA sequences resulting in a 76% and 75% reduction in GATA3 expression for siGATA#2 and siGATA#3F, respectively. LOX expression was increased 4-fold and 4.5-fold with siGATA#2 and siGATA#3F, respectively (Appendix 1, Figure 2D). These findings suggest that GATA3 can regulate LOX expression in both basal and luminal breast cancer subtypes.

GATA3 reduces macrophage recruitment to metastatic lesions and CSF-1 expression (Task 1c)

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 ($p<0.001$; Appendix 1, 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; Appendix 1, 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 and Pollard 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, $p<0.05$; Appendix 1, Figure 3B). There was no change in the percent of CD11b+/Gr-1+ immune cells recruited (Appendix 1, Figure 3B).

Re-expression of LOX in 231-GATA3 cells reverses metastatic propensity (Task 1c)

To investigate whether the loss of LOX is responsible for the reduced metastatic potential observed in tail vein injected mice with 231-GATA3 compared to 231-Empty cells, we restored LOX expression in 231-GATA3 cells and assayed for metastatic potential. 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 (Appendix 1, Figure 5A). LOX activity was increased in 231-GATA3-LOX cells compared to 231-GATA3-Empty cells as measured by a LOX activity assay (Appendix 1, Figure 5C).

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 ($p<0.05$; Appendix 1, Figure 5D) that was similar to that of 231-Empty cells. This was further validated by image quantitation of Ki-67 expression and H&E staining of metastatic lung lesions using Apirio Image Analysis software (Figure 9) 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 re-expression 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 (Figure 10). Metastatic lung lesions from 231-GATA3-Empty cells exhibited minimal LOX expression whereas metastatic 231-GATA3-LOX lesions showed strong LOX expression by IHC (Figure 11).

GATA3 inhibits LOX expression through DNA methylation (Task 2)

For Task 2, we wanted to investigate mechanisms of inhibition of LOX mediated by GATA3. Originally, we investigated whether GATA3 could directly bind to the LOX promoter resulting in direct inhibition of LOX expression. However, our Chromatin immunoprecipitation studies did not reveal any GATA3 binding sites at putative consensus GATA3 binding regions within the 4kb region of the LOX promoter (data not shown). Therefore, we decided to explore the possibility that GATA3 may indirectly regulate LOX expression through epigenetic modifications of the LOX promoter. To investigate this, we did a Methylation Specific PCR (MSP) at the LOX promoter. Methylation of the LOX promoter in 231-GATA3 cells was significantly increased compared to control cells (Appendix 1, Figure 2E, top panel). 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 (Appendix 1, Figure 2E, lower panel) 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.

LOX and GATA3 are inversely expressed in breast cancer cells (Task 3)

Our results suggest that GATA3 plays an important role in modulating LOX expression suggesting that LOX and GATA3 expression in breast cancer cells may be inversely associated. To address this possibility, we performed a retrospective analysis of the previously published microarray data for 51 breast cancer cell lines (Neve et al. 2006). Our analyses confirmed that GATA3 expression was inversely associated with LOX expression ($p<0.001$; Appendix 1, Figure 4A&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 (appendix 1, Figure 4C).

Patients expressing a high LOX/GATA3 ratio have poor prognosis (Task 3)

To further investigate whether the inverse association between LOX and GATA3 observed in breast cancer cell lines is also observed in breast cancer patients, we performed a retrospective study of previously accessible microarray datasets of breast cancer patients. 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 ($p<0.001$) (Appendix 1, Figure 6A). Importantly, an inverse correlation between LOX and GATA3 expression was also demonstrated across the breast cancer subtypes ($r= -0.3$; $p<0.001$; Appendix 1, 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. 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 ($p <0.01$; Appendix 1, 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 (Appendix 1, Figure 6C), thus GATA3 expression may have a dominant protective role to prolong survival that overcomes high LOX expression through other mechanisms.

MB231 cells are sensititve to the Src inhibitor AZD0530 (Task 4)

Since LOX has been shown to activate the Src pathway (Payne et al. 2005) and we observed increased LOX expression in basal breast cancer cells, we wanted to investigate the effects of the Src inhibitor, AZD0530, in the basal MB231 cell line. MB231 cells were treated with 5 uM AZD0530 and migration was observed in a scratch assay. MB231 cells migrated towards the

scratched area within 1 day in the vehicle control cells, in contrast treatment with AZD0530 resulted in inhibition of migration (Figure 12A). Proliferation was measured by CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) assay and we observed that AZD0530 could efficiently kill 50% of the cells with 5 uM (Figure 12B). Future studies utilizing different combinations including Vorinostat and the LOX inbhitor and different basal breast cancer cell lines will determine the optimal combination and concentration that effectively will kill basal breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

Overexpression of the transcription factor, GATA3 in MDA-MB-231 cells (231-GATA3) results in a more luminal phenotype and a concomitant reduction in the expression of metastasis-associated genes compared to control 231-Empty cells.

231-GATA3 cells showed altered morphology and cytoskeletal organization in 2D culture and in 3D cultrex

231-GATA3 and 231-Empty cells have similar proliferation rates in 2D but 231-GATA3 shows reduced proliferation in 3D cultrex compared to 231-Empty

Orthotopic implantations of 231-GATA3 cells showed reduced primary tumor growth in xenografts compared to 231-Empty cells

231-GATA3 cells showed similar invasive potential by Boyden chamber compared to 231-Emtpy but we observed reduced lung metastatic potential in xenografts compared to 231-Empty cells

231-GATA3 reduces LOX expression in MDA-MB-231 and in another triple negative breast cancer cell line, Hs578T

Transient knock down of GATA3 in the luminal GATA3 positive BT474 cell lines, increases GATA3 expression.

GATA3 alters global gene expression profile of MB231 resulting in a reduction in the expression of genes associated with metastasis and EMT and reexpression of E-cadherin

GATA3 inhibits LOX expression through increased methylation levels at the LOX DNA promoter in MB231 cells

GATA3 reduces the secretion of macrophage colony stimulating factor and of Granulocyte-macrophage-colony stimulating factor in MB231 cells and ages to the metastatic lesion and reduces CSF-1 in cells

LOX and GATA3 expression are inversely expressed in human breast cancer cells

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

Patients expressing elevated ratios of LOX/GATA3 have worst survival compared to patients that express low levels of LOX or high levels of GATA3.

AZD treatment of MB231 cells reduces migration compared to vehicle treated cells

REPORTABLE OUTCOMES

Oral Presentations

1. **Isabel Chu,** Aleksandra Michalowski and Jeffrey Green. GATA3 inhibits lysyl oxidase-mediated metastases of human basal TN BrCa cells. 2nd Joint Meeting on Hereditary Breast and Ovarian Cancers: lessening the burden NYU Cancer Institute, September 14-17, 2011, New York, New York.

2. **Isabel Chu**, Aleksandra M. Michalowski, and Jeffrey E. Green. GATA3 promotes Luminal reprogramming in MDA-MB-231 breast cancer cells and reduces metastasis through Suppression of Lysyl Oxidase. 2011. July 15th, 2011. Miller School of Medicine. Braman Breast Cancer Center. Miami, Florida.

3. **Isabel Chu**, Aleksandra M. Michalowski, Mark Hoenerhoff, Kornelia M. Szauter, Dror Luger, Misako Sato, Akira Oshima, Katalin Csiszar and Jeffrey E. Green. GATA3 Induces Luminal Reprogramming of Human Basal Triple Negative Breast Cancer Cells and Inhibits Metastases through Suppression of Lysyl Oxidase. 2011. Center for Cancer Research Fellows Young Investigators Colloquium, Williamsburg, Virginia.

4. **Isabel Chu**, Aleksandra Michalowski and Jeffrey Green. Trans-differentiation of breast cancer by GATA3 reduces primary growth and metastasis. 2010. Center for Applied Medical Research, University of Navarra, Pamplona, Spain.

Abstracts

1. **Isabel M. Chu**, Aleksandra Michalowski, Kornelia M. Szauter, Katalin Csiszar, and Jeffrey E. Green. Gata3 induces luminal reprogramming of basal triple negative breast cancer cels and inhibits metastases through suppression of lysyl oxidase. Era of Hope Department of Defense Breast Cancer Research Program. August 2-5, 2011, Orlando, Florida.

2. **Isabel M. Chu,** Aleksandra Michalowski and Jeffrey Green. Trans-differentiation of breast cancer by GATA3 reduces primary growth and metastasis. Frontiers in Tumor progression. Oct 24-28, 2010. Madrid, Spain

Published manuscript:

IM Chu, AM Michalowski, M Hoenerhoff, KM Szauter, D Luger, M Sato, K Flanders, A Oshima, K Csiszar and JE Green. 2012. GATA3 inhibits Lysyl oxidase- mediated metastases of human basal triple-negative breast cancer cells, Oncogene. **31,** 2017–2027.

Microarray database

We deposited in GEO the microarray database for 231-Empty and 231-GATA3 http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE24249].

Employment Opportunity:

I was offered a Scientist II position at Cell Signaling Technology, Inc in the target validation/Cancer Discovery team to investigate potential targets for cancer and coordinate the development of therapeutic antibodies for these targets. The position will start on May 7 2012.

CONCLUSION

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. 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. 2006; Zhou and Ouyang 2003; Kouros-Mehr and Werb 2006). 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 and EMT were coordinately down-regulated by GATA3. We demonstrate 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.

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.

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 and Li 2003; Payne et al. 2007; Levental et al. 2009). 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.

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APPENDIX 1

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ORIGINAL ARTICLE

GATA3 inhibits lysyl oxidase-mediated metastases of human basal triple-negative breast cancer cells

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Discovery of mechanisms that impede the aggressive and metastatic phenotype of human basal triple-negative-type breast cancers (BTNBCs) could provide novel targets for therapy for this form of breast cancer that has a relatively poor prognosis. Previous studies have demonstrated that 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 small interfering RNA in luminal BT474 cells increased LOX expression. Reconstitution of LOX expression in 231-GATA3 cells restored metastatic propensity. A strong inverse association between LOX and 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 BTNBCs 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 BTNBCs.
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Keywords: GATA3; basal triple-negative breast cancer; lysyl oxidase; differentiation; gene expression profiling

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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 and Massague, 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; and luminal A, B and C) (Sorlie *et al*., 2003; Hennessy *et al*., 2009). Patients with basal-type tumors lacking estrogen receptor (ER), progesterone receptor and ErbB2—referred to as basal triple-negative breast cancer (BTNBC)—have a worse prognosis compared with patients with more differentiated, less metastatic tumors expressing markers of the luminal lineage, including the transcription factors GATA3 and ER (Perou *et al*., 2000; Sorlie *et al*., 2003; Neve *et al*., 2006). 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 (van de Vijver *et al*., 2002; van't Veer *et al*., 2002; Wang *et al*., 2005), 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, show limited invasive properties compared with 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). As the MB231 cell line shows 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 tumorigenicity 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 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 (Perou *et al*., 2000; Sorlie *et al*., 2003; 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 knockout 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 or in late carcinomas induced the expression of luminal differentiation markers (Asselin-Labat *et al*., 2007; Kouros-Mehr *et al*., 2008). 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 (ECM) 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 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*., 2006). 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 through 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 showed 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 for cytoplasmic histone-associated DNA fragments between 231-Empty and 231-GATA3 cells (Supplementary Figure 1b). Pulse–chase 5-bromo-2-deoxyuridine labeling revealed that GATA3 overexpression in MB231 did not affect proliferation in two-dimensional cultures (Supplementary Figure 1c) as reported previously (Yan *et al*., 2010). However, we demonstrate for the first time that in three-dimensional (3D) culture using Cultrex Basement Membrane Extract, 231-GATA3 cells were significantly less proliferative compared with 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 be caused only by intrinsic cellular changes, but appear to also result from GATA3 altering cell interactions with the ECM. In two-dimensional culture, 231-Empty cells maintained a spindle, elongated morphology, whereas 231-GATA3 cells were larger and cuboidal (Supplementary Figure 1d). In 3D culture using Basement Membrane Extract, 231-Empty cells appeared invasive by protruding into the Basement Membrane Extract 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).

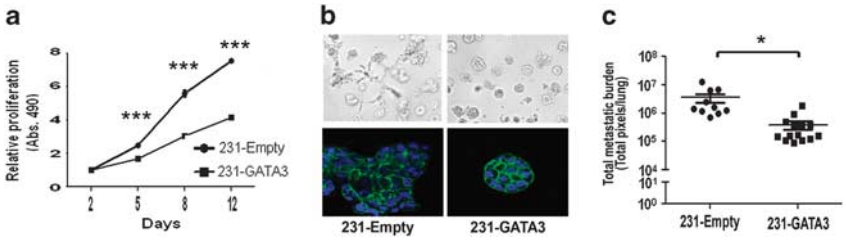

Figure 1 GATA3 overexpression reduces proliferation in 3D culture and experimental metastasis in mice. (**a**) 231-Empty and 231-GATA3 cells were seeded on 3D Cultrex for 12 days. 231-GATA3 cells show reduced proliferation as measured by MTS (mean ± s.e.m., $***P<0.001$). (**b**) Top panels, bright-field 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 fluorescence microscopy, with total metastatic burden calculated per lung ($*P<0.05$).

Similarly, in xenograft studies, primary tumor outgrowth of 231-GATA3 cells was significantly delayed compared with 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 (IHC) as compared with 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 231-GATA3 cells lose GATA3 expression and the associated changes. Advanced tumors showed similar immunostaining for both Ki-67 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 green fluorescent protein-positive lung lesions at the time when mice were killed because of significant primary tumor burden.

Although we did not observe a statistically significant difference in the number of 231-GATA3 cells as compared with 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 with 231-Empty cells within the first 24 h following tail vein injection (Supplementary Figure 3b). At 24 h, there was an approximately 75% reduction in the number of 231-GATA3 cells in the lungs compared with the number of cells in the lungs 2 h after 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 ninefold reduction in total metastatic burden in the lung compared with 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 hematoxylin and eosin staining (Supplementary Figure 4a) by a pathologist. We previously demonstrated that this method of using immunofluorescence to detect green fluorescent protein-labeled cells in whole lungs by single-cell, whole-organ microscopy is extremely sensitive and quantitative (Barkan *et al*., 2008).

We additionally quantitated the percentage of lung area occupied by metastatic lesions based on Ki-67 staining by using the Apiro Image Analysis Software. This similarly revealed that GATA3 expression significantly reduced metastatic burden as compared with 231-Empty cells. We further characterized lung lesions from mice 2 months after they received either 231-Empty or 231-GATA3 cells, for proliferation and apoptosis by Ki-67 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 the expression of metastasis-associated genes

Gene expression profiling analyses revealed that the expression of 1273 probesets was altered between 231-GATA3 and 231-Empty cells (776 up- and 497 down-regulated in 231-GATA3 cells, with fold change $\geqslant 1.5$ and $P<0.001$, and a false discovery rate 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, ECM remodeling, migration and metastasis (Erler *et al*., 2006; Erler *et al*., 2009), was the gene most down-regulated by GATA3. We investigated whether the dramatic reduction in the 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 with 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 showed strong nuclear staining for GATA3, but LOX expression was not detectable (Figure 2b). Similarly, early 231-GATA3 primary tumors showed less LOX expression by IHC compared with 231-Empty tumors (Supplementary Figure 2d). Similar 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 with 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

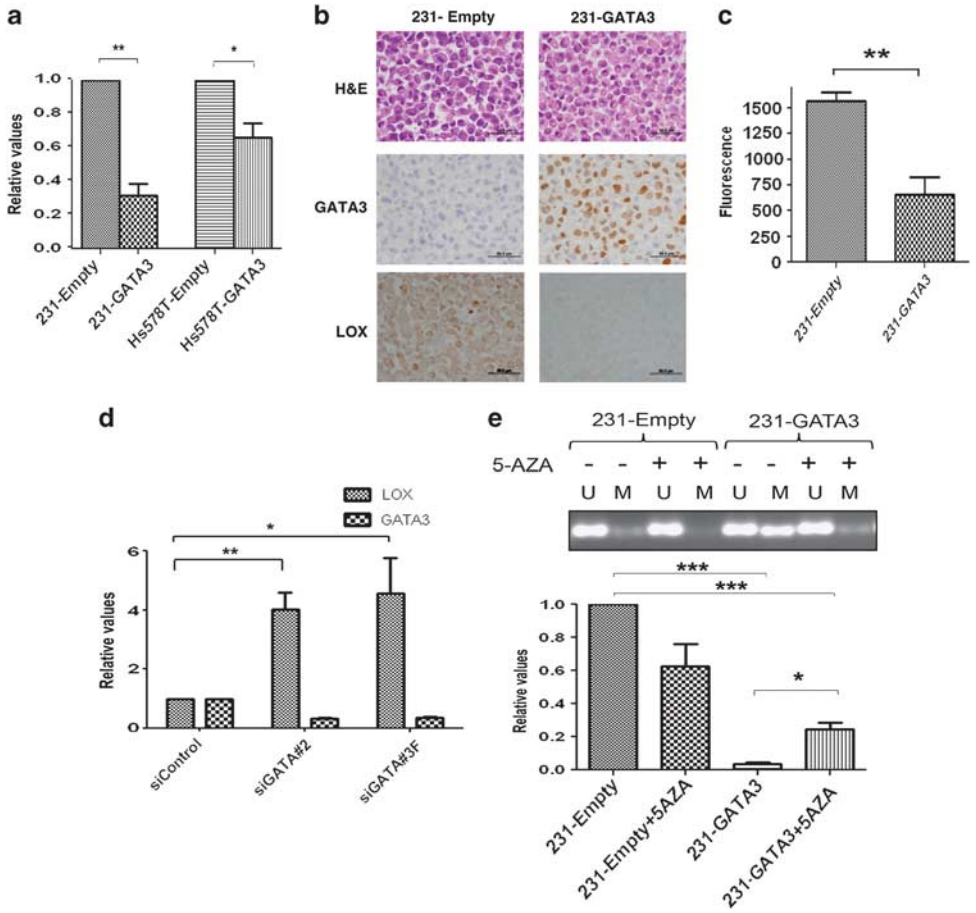

Figure 2 GATA3 regulates LOX expression in breast cancer cells in part through LOX promoter methylation. (**a**) Relative LOX expression by Q-RT–PCR. Samples were normalized to cyclophilin-B. Overexpression of GATA3 in MB231 and Hs578T cells reduces LOX mRNA expression (**$P<0.01$, *$P<0.05$). (**b**) 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 medium of 231-Empty and 231-GATA3 cells measured as the increase in fluorescence over β-aminopropionitrile-containing controls. Relative activity measured at 2400 s (40 min, **$P<0.01$). (**d**) Relative LOX and GATA3 mRNA expression measured by Q-RT–PCR. BT474 cells were transfected with GATA3 small interfering RNA for 72 h prior to RNA isolation (*$P<0.05$, **$P<0.01$). (**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 (***$P<0.001$, *$P<0.05$).

GATA3 expression using small interfering RNAs and measured LOX expression. Seventy-five percent knockdown of GATA3 in the luminal, GATA3-positive breast cancer cell line BT474 increased LOX expression over four-fold (confirmed using two different small interfering RNAs) (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 as compared with control cells (Figure 2e). Although treatment with the methylation inhibitor 5-aza-2′-deoxycytidine (5-AZA) diminished the promoter methylation of LOX in 231-GATA3 cells to levels similar to that in 231-Empty cells, 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 using 5-AZA treatment as compared with 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 using 5-AZA treatment in 231-Empty cells (data not shown).

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

As myeloid cell 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 twofold reduction of CSF-1 expression (a key chemokine that recruits macrophages) in 231-GATA3 cells as compared with control cells (see below). This was confirmed by ELISA showing a 40% reduction in secreted CSF-1 by 231-GATA3 as compared with 231-Empty cells ($P<0.001$; Figure 3a). Reduced secretion of granulocyte–macrophage CSF in 231-GATA3 cells ($P<0.01$) was also observed, although total levels were lower compared with those of CSF-1. There was no change in the secreted macrophage migration-inhibitory factor (Figure 3a).

As we observed a reduction in secreted CSF-1 in 231-GATA3 cells as compared with 231-Empty cells, and macrophages have been shown to be an important component of the metastatic process (Condeelis and Pollard, 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−) as compared with the lungs of mice receiving 231-Empty cells (53% F4/80+/Gr-1− cells for 231-Empty versus 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 data set of Neve *et al*. to perform hierarchical clustering of all of the cell lines by 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 upregulated 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 toward 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 cells (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 ($P<0.001$; Figures 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 red fluorescent protein (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 as compared with 231-GATA3-Empty cells as determined by IHC (Figure 5b). Similarly, LOX activity was increased in 231-GATA3-LOX cells as compared with 231-GATA3-Empty cells (Figure 5c). However, 231-GATA3-LOX cells maintained their cuboidal morphology and continued to express E-cadherin (Supplementary Figures 9a and b).

We observed no differences in the rates of proliferation in two-dimensional or 3D culture, or in invasive potential by Boyden chamber invasion assay, between 231-GATA3-Empty and 231-GATA3-LOX *in vitro* (data not shown). Most importantly, single-cell, whole-organ microscopy analysis revealed that mice tail vein-injected with 231-GATA3-LOX cells showed a statistically significant marked increase in total lung

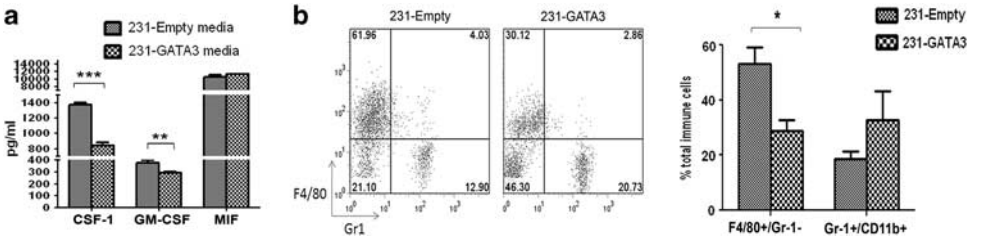

Figure 3 GATA3 reduces macrophage recruitment to the lung (**a**) ELISA of medium collected from 231-Empty and 231-GATA3 cells. 231-GATA3 cells showed reduced secretion of CSF-1 and granulocyte–macrophage-CSF (***$P<0.001$, **$P<0.01$). (**b**) Flow-cytometric analyses of immune cells collected from the lungs of tail vein-injected mice ($n=4$). Cells were labeled with anti-CD45, F4/80, Gr-1 or CD11b antibodies. Lungs collected from mice injected with 231-GATA3 cells showed reduced F4/80+/Gr-1− recruitment (*$P<0.05$).

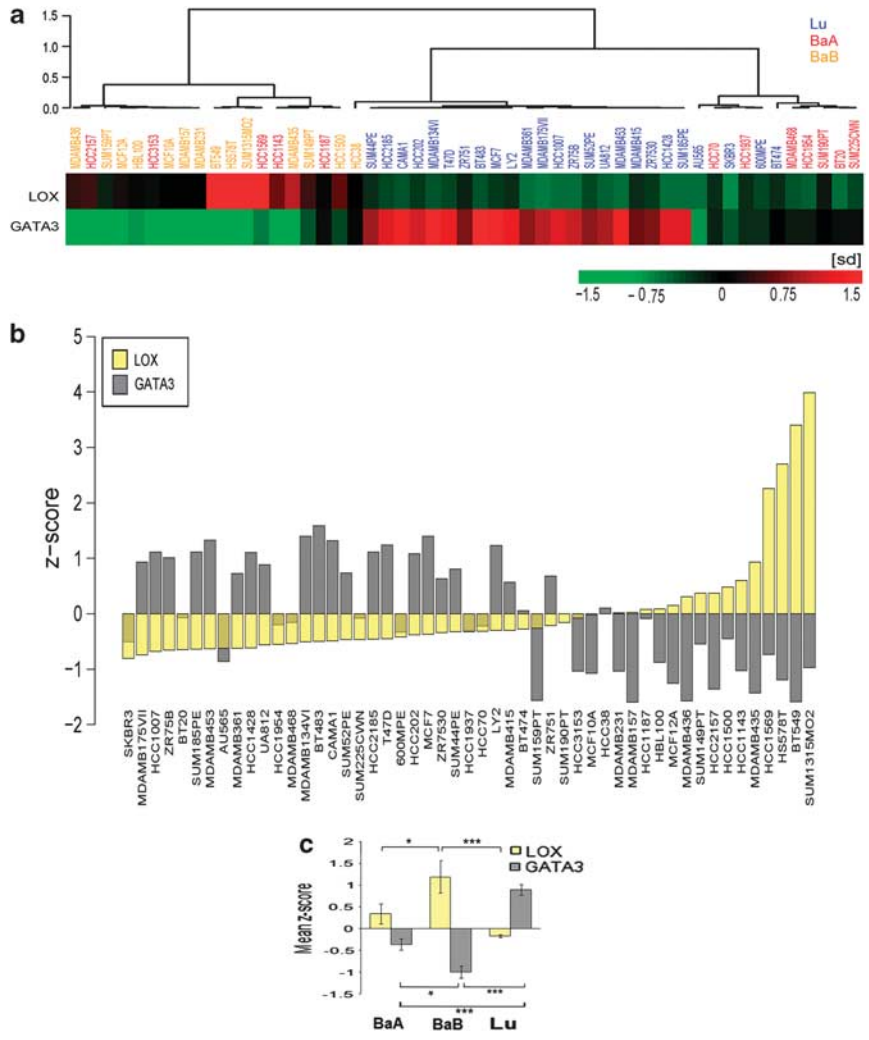

Figure 4 Analysis of the Neve *et al*. 51 breast cancer cell line microarray database for LOX and GATA expression (Neve *et al*., 2006). (**a**) Heat-map of LOX and GATA3 expression in breast cancer cell lines. The displayed expression of each gene was standardized using *Z*-score. The hierarchical clustering 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 (*$P<0.05$, ***$P<0.001$).

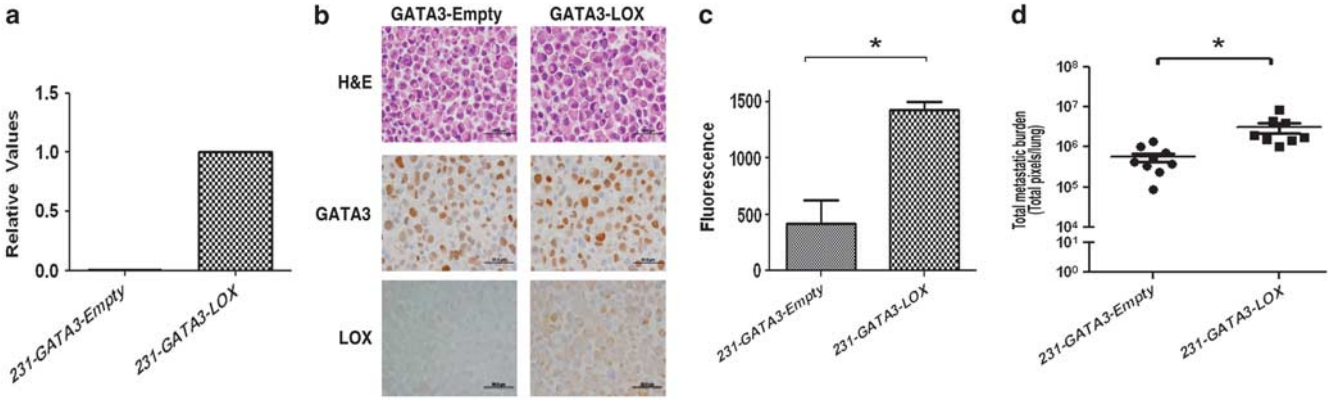

Figure 5 Re-expression of LOX in 231-GATA3 cells increased the metastatic potential of 231-GATA3 cells. (**a**) Lentiviral transduction of 231-GATA3 cells with LOX increases LOX expression in 231-GATA3 cells. Relative LOX expression by Q-RT–PCR. (**b**) Immunohistochemical staining of cell pellets confirmed 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 s (40 min, *$P<0.05$). (**d**) Mice tail-vein-injected with 231-GATA3-Empty and 231-GATA3-LOX cells, with lungs collected after 2 months. Lungs imaged by fluorescence microscopy, with total metastatic burden calculated per lung (*$P<0.05$).

metastatic burden of more than five-fold compared with 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 hematoxylin and eosin staining of metastatic lung lesions using the Apirio Image Analysis software (Supplementary Figure 4b), which demonstrated an approximately eightfold increase in metastatic burden owing to increased size and number of lesions in 231-GATA3-LOX cells as compared with 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 re-expression 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 showed 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 re-expression of LOX in MB231. In fact, only nine named genes dysregulated by GATA3 were expressed in the opposite direction by re-expression of LOX (adrenomedullin, fibronectin, MMP1, MMP12, anterior gradient homolog-2, IL7R, neural precursor cell expressed–developmentally downregulated 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 mice receiving orthotopic implantations of 231-GATA3-Empty versus 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 as compared with the luminal-A ($P<0.001$) and luminal-B types ($P<0.01$), whereas GATA3 was lower in the basal subtype compared with 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

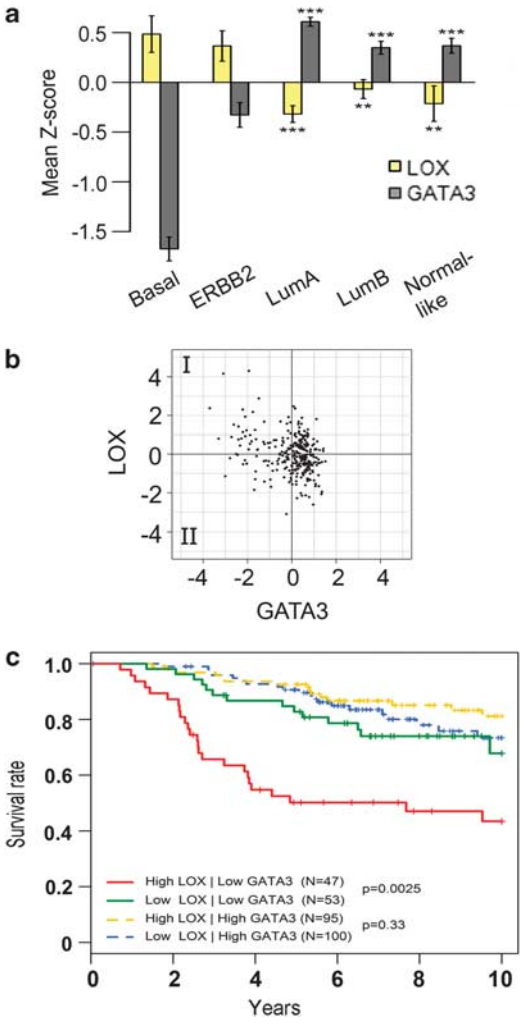

Figure 6 Retrospective microarray analysis of breast cancer patient microarray data from van de Vijver *et al*. (2002). (**a**) GATA3 is associated with the luminal-A and luminal-B subtype, whereas LOX is enriched in the basal subtype (***$P<0.001$, **$P<0.01$). (**b**) Correlation between LOX (y-axis) 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 panel **b** above) had significantly reduced overall survival (hazard ratio = 2.65, $P<0.01$) compared with patients with low LOX and Low GATA3 (quadrant II in panel **b** above). High and low are defined as above or below median expression as depicted in panel **b**. Log-rank test P-values are indicated. The interaction between LOX and GATA3 was statistically significant ($P<0.05$).

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 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 show a low GATA3/high LOX expression pattern have significantly reduced survival compared with

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 concomitant 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 the GATA3-induced inhibition of the metastatic propensity of BTNBC, an aggressive form of breast cancer with poor prognosis. We have demonstrated that 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 the metastases of MB231 cells (Dydensborg *et al*., 2009; Yan *et al*., 2010), this study identified a major mechanism for the GATA3-induced inhibition of metastases through downregulation of LOX. GATA3 has been shown to be a key developmental transcription factor in the hematological system and during mammary luminal epithelial cell development (Zhou and Ouyang, 2003; Kouros-Mehr *et al*., 2006a; Kouros-Mehr and Werb, 2006b). 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 shown previously to be involved in metastatic progression were coordinately downregulated by GATA3, including Fascin homolog-1 (FSCN1), chemokine receptor-4 (CXCR4), mannosidase, alpha, class-1A, member-1 (MAN1A1), tenascin-C 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 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 (Dydensborg *et al*., 2009; Yan *et al*., 2010), the 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 overexpression 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/focal adhesion kinase (FAK) pathways (Payne *et al*., 2005). Activated LOX secreted into the extracellular environment has an important role in potentiating metastatic tumor cell growth through cross-linking of several collagen types and elastins in the ECM (Kagan and Li, 2003; Payne *et al*., 2007; Erler *et al*., 2009; Levental *et al*., 2009). Most importantly, inhibition of LOX enzymatic activity in orthotopically implanted MB231 cells eliminates lung metastases (Erler *et al*., 2006; 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, whereas overexpression 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 overexpression 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 β-aminopropionitrile, shLOX or an inhibitory LOX antibody reduced metastasis to the lung but did not affect primary tumor growth (Erler *et al*., 2006), 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 to be partially responsible for the reduced expression of LOX upon overexpression 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 as compared with 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 the 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). Whereas LOX showed increased methylation resulting in reduced expression, E-cadherin showed reduced methylation at the DNA promoter upon GATA3 overexpression (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 as compared with 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 as compared with 231-Empty cells.

Additionally, 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 and Pollard, 2006). CSF-1 secretion was significantly reduced in 231-GATA3 cells as 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 as compared with 231-Empty. In addition, we observed dramatically increased clearing of tumor cells in the lung within the first 24 h of tail vein injection of 231-GATA3 cells as compared with 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 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 induced expression of GATA3 or inhibition of LOX activity may be worthy therapeutic approaches for the reduction of metastasis in breast cancer.

Materials and methods

Cell lines, transfection and lentiviral 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 (Sigma, St Louis, MO, USA) for 4 days prior to DNA isolation. Details of methylation-specific PCR are provided in the Supplementary Materials and methods.

Mice, necropsy and ex vivo *imaging*
All animal work was performed 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 by using 6- to 8-week-old female SCID or NOD/SCID mice (NCI, Frederick, MD, USA or Jackson Laboratories, Bar Harbor, ME, USA). Details of animal work are provided in the Supplementary Materials and methods.

LOX activity
LOX activity was measured as the fluorometric β-aminopropionitrile-inhibitable LOX activity assay by using Amplex red (Palamakumbura and Trackman, 2002). See Supplementary Materials and methods for a detailed description.

Immunoblotting and antibodies
Cells were lysed in ice-cold radioimmunoprecipitation assay buffer for western blot analyses as described previously (Hoenerhoff *et al*., 2009). GATA3 (Santa Cruz Biotechnology, 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 (Trevigen, Gaithersburg, MD, USA) as described previously, with minor modifications (Barkan *et al*., 2008, 2010). Cells were cultured in complete medium and medium was replenished every 2 days. Proliferation was measured as described previously by Barkan *et al*. (2008) at 2, 5, 8 and 12 days after seeding by CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA).

Immunofluorescence and confocal microscopy
Cells grown in 3D culture were imaged by confocal microscopy as described previously (Barkan *et al*., 2008). Briefly, cells were cultured in eight-well chamber glass slides pre-coated with Cultrex. For f-actin staining, cells were incubated overnight with Alexa-Fluor-488 phalloidin (Molecular Probes, Eugene, OR, 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, Buffalo Grove, IL, USA).

Flow-cytometric analysis
Cell-cycle profiles were assayed by 5-bromo-2-deoxyuridine pulse labeling and flow-cytometric analysis were performed as described previously (Chu *et al*., 2005). For myeloid analysis, mice were tail-vein-injected with one 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 cells for microarray analysis. See Supplementary Material and methods for detailed descriptions. Data have been deposited in GEO (reviewer access only: URL http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc = GSE24249).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

SUPPORTING DATA

FIGURES

A **B**

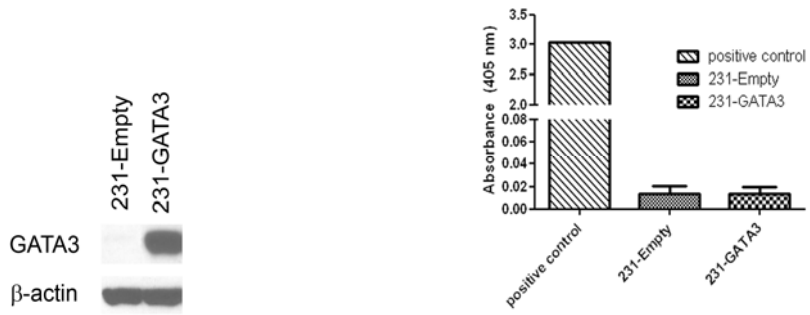


Figure 1. GATA3 overexpression in MB-231 cells. (A) MB231 cells were transduced with a lentivirus system to express GATA3 (231-GATA3) or control vector (231-Empty). Western blot demonstrates expression of GATA3 in 231-GATA3 cells but not 231-Empty cells. β-actin was used as a loading control. **(B)** 231-Empty and 231-GATA3 cells show equal basal levels of cell death in 2D culture conditions as measured by ELISA assay for cytoplasmic histone-associated-DNA-fragments.

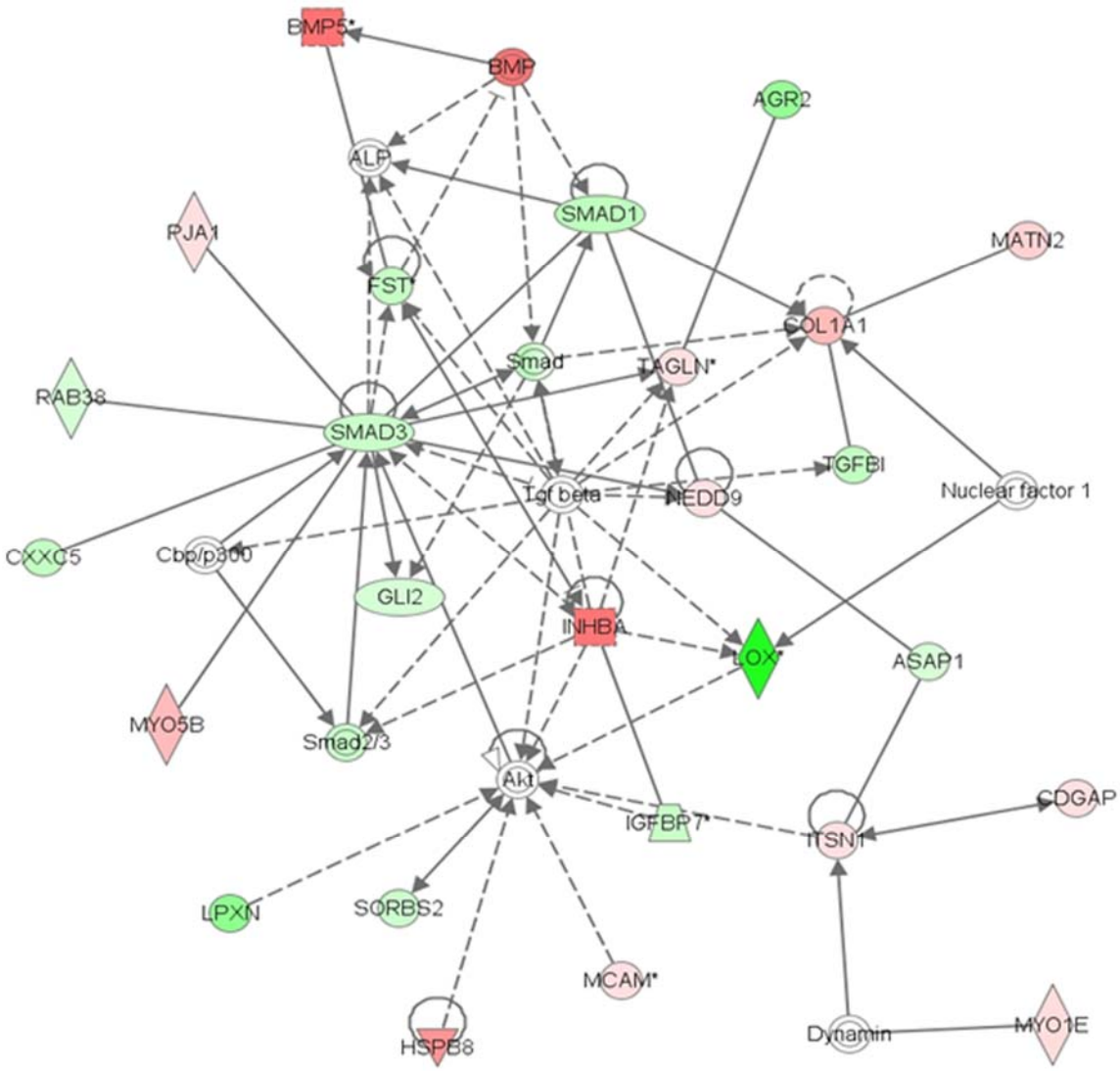
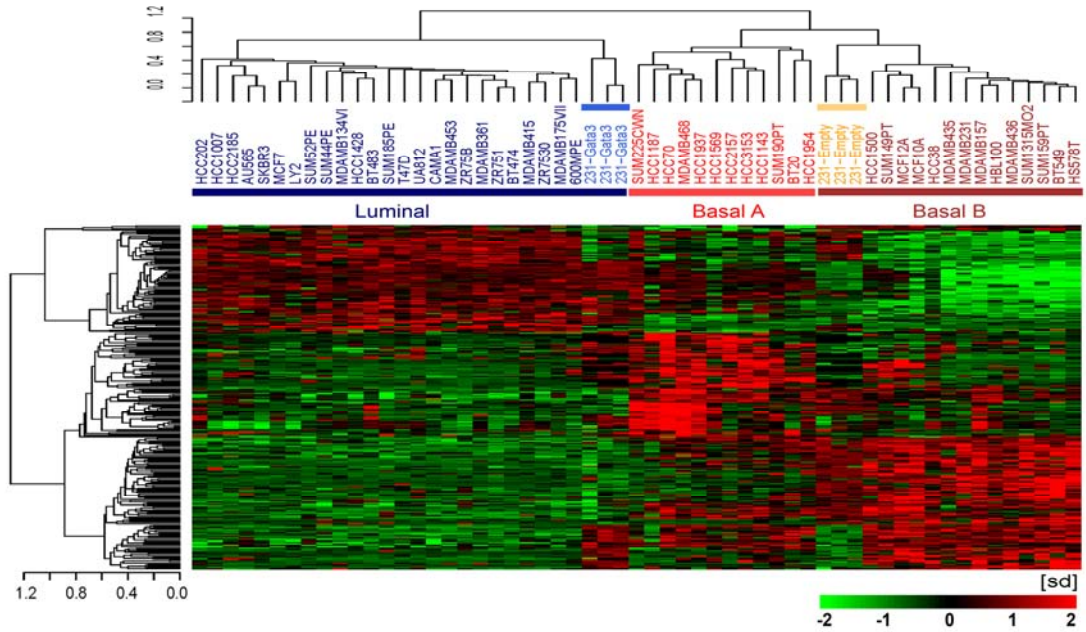


Figure 2. Ingenuity pathway analysis of 231-Empty and 231-GATA3. 231-Emtpy and 231-GATA3 cells were analysed by microarray and genes that were differentially expressed were input in Ingenuity. Genes in Green are downregulated in 231-GATA3 cells and genes in red are upregulated. 231-GATA3 reduces genes in LOX and TGF-beta pathway.

A



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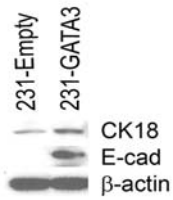
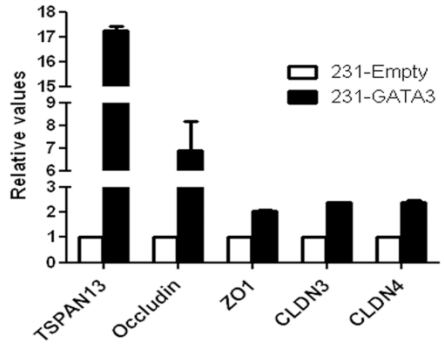


Figure 3. Microarray analysis of 231-Empty vs. 231-GATA3 cells. (A) Hierarchical clustering analysis of 51 breast cancer cell lines (Neve *et al.*, 2006) and the 231-Empty and 231-GATA3 cells. Two hundred forty nine unique genes from the subtype predictor signature (Neve *et al.*, 2006) are included in the analysis (multiple microarray probes per gene were reduced to the probe with the highest median intensity across samples). The heatmap gene expression values are relative differences within the respective datasets (gene-wise Z-score transformation in the 51 cell line collection and the 231-Empty and 231-GATA3 cells). The two-way hierarchical clustering of the combined data sets employs a distance metric of one minus Pearson's correlation coefficient and average linkage algorithm. **(B)** Western blot showing CK18 and E-cadherin in 231-Emtpy vs. 231-GATA3. b-actin was used as a loading control. There is re-expression of E-cadherin and increased CK18 in 231-GATA3 vs. 231-Empty cells.

A



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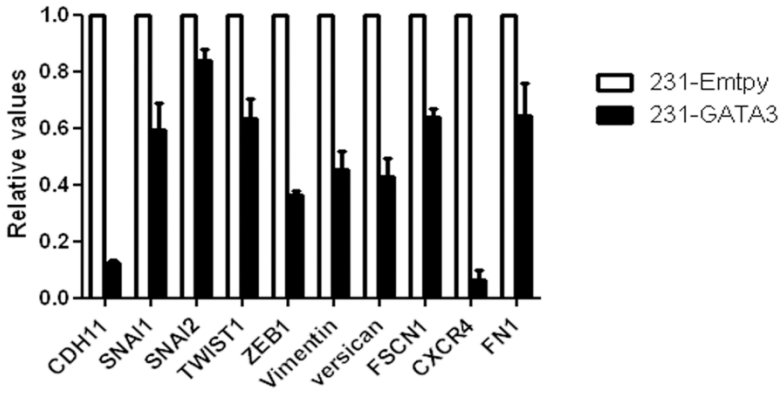


Figure 4. GATA3 over-expression in MB231 cells promotes EMT. (**A**) Q-RT-PCR demonstrates increased expression of epithelial markers in 231-GATA3 cells compared to 231-Empty cells. Samples were normalized to cyclophilin B. (**B**) Q-RT-PCR showing that the relative expression of mesenchymal and metastatic markers is reduced in 231-GATA3 cells compared to 231-Empty cells. Samples were normalized to cyclophilin B

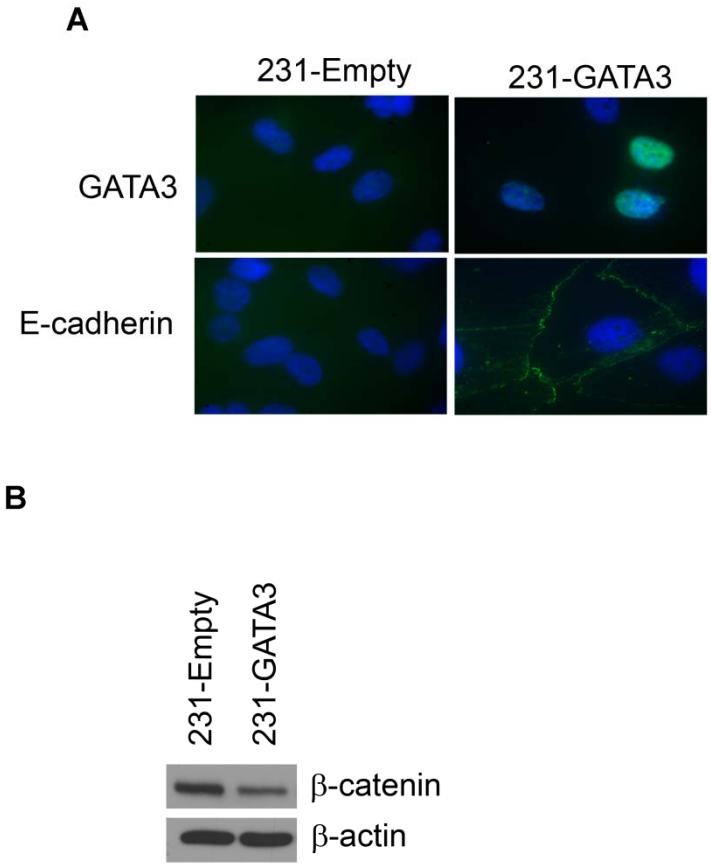
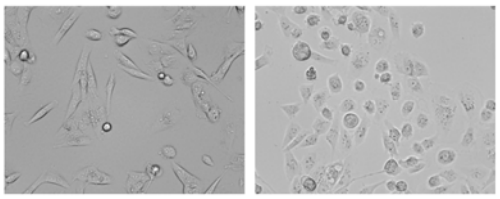


Figure 5. **231-GATA3 cells express E-cadherin and have reduced β-catenin.** (**A**) Immunofluorescence of 231-Empty and 231-GATA3 cells for GATA3 and E-cadherin. GATA3 over-expression in MB-231 results in re-expression of E-cadherin associated with the cell membrane. (**B)** Western blot showing reduced expression of β-catenin in 231-GATA3 cells compared to 231-Empty cells. β-actin was used as loading control.

A



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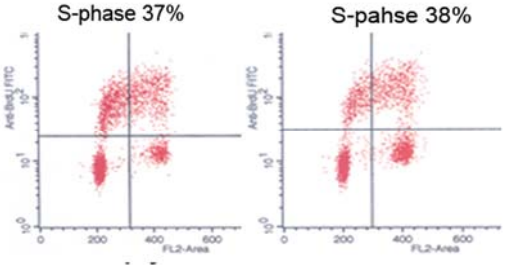
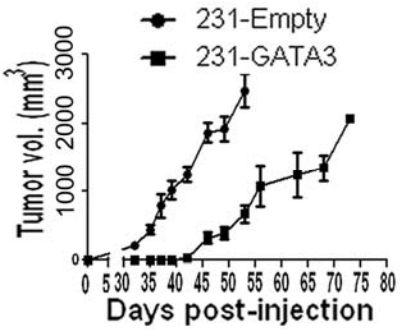


Figure 6. GATA3 alters morphology of MB231 cells (A) Bright field images of 231-Empty and 231-GATA3 cells grown with complete media in 2D culture. (**B**) Flow cytometric analysis showing cell cycle profiles of 231-Empty vs. 231-GATA3 cells. There is no difference in %S-phase in both cell lines.

A



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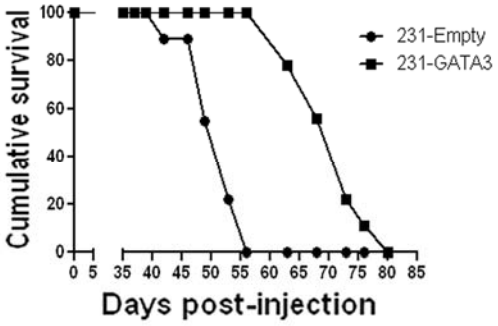

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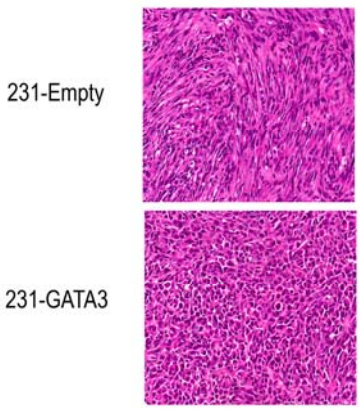

Figure 7. **Orthotopic implantation of 231-Empty and 231-GATA3 cells.** (**A**) 231-GATA3 exhibit reduced primary tumor growth compared to 231-Empty cells (tumor size measured every 2-4 days (vol.=l x w^2 x 0.4). (**B**) Increased survival of mice receiving 231-GATA3 vs. 231-Empty cells. Mice were euthanized when the tumor reached 2 cm in diameter. (**C**) H&E staining. Primary 231-GATA3 mammary tumor xenografts display a more prominent epithelioid phenotype compared to a predominant spindyloid appearance of 231-Empty mammary xenografts.

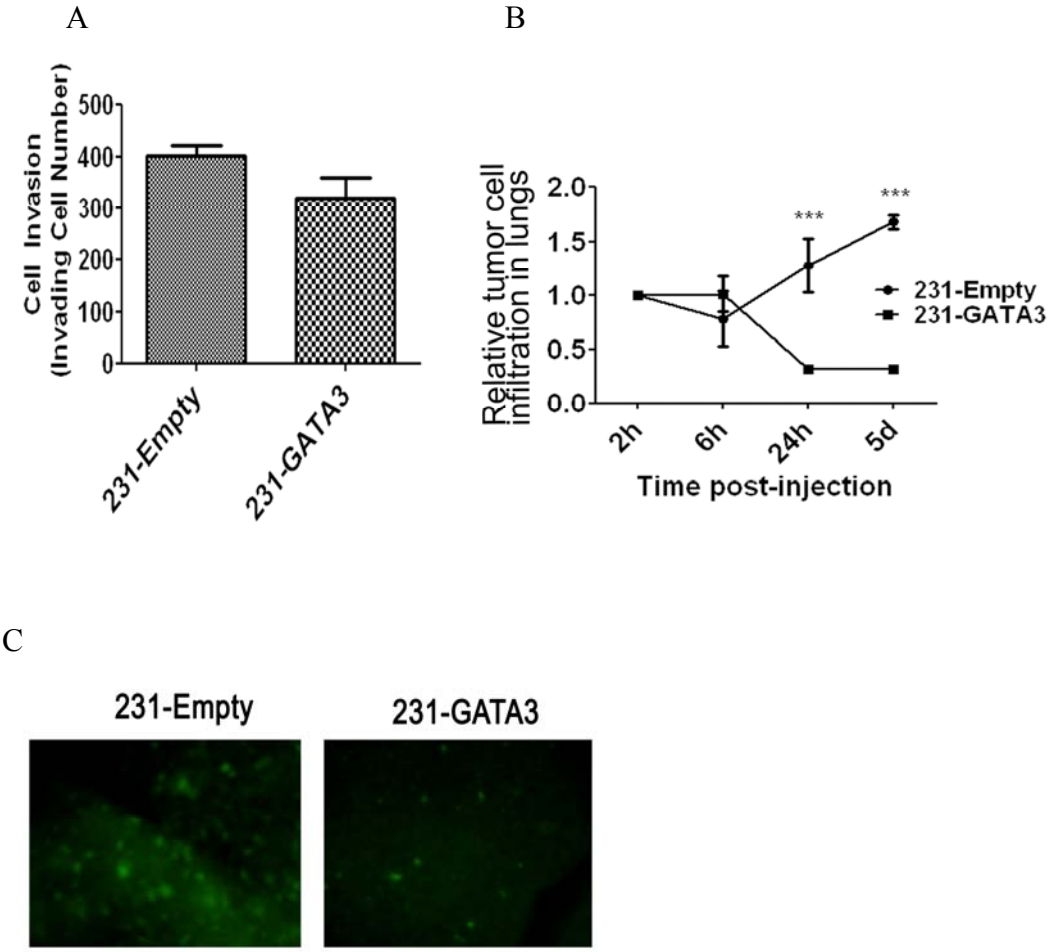

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Figure 8. Reduced lung metastases in mice receiving 231-GATA3 cells by tail vein injection compared to 231-Empty cells. (A) Boyden Chamber assay of 231-Empty and 231-GATA3 cells showed a non-statistically significant trend for reduced number of cells invading through a Matrigel coated chamber. (B) Relative tumor cell infiltration in lungs as measured by immunofluorescence of lungs from tail vein injected mice with 231-Empty or 231-GATA3 cells expressing GFP. Lungs were collected at the indicated times. (C) Immunofluorescence picture of the lung of 231-Empty and 231-GATA3 tail vein injected mice

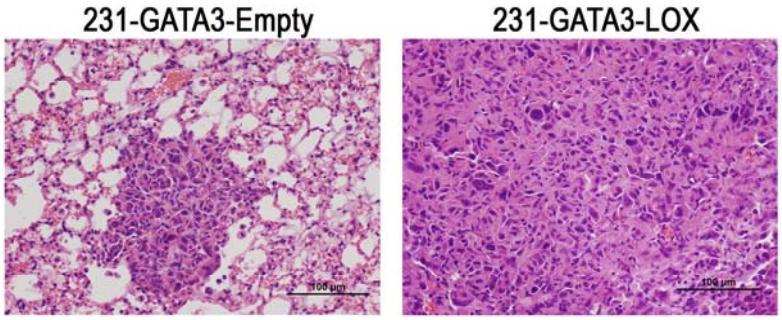

Figure 9. H&E staining of metastatic lung lesions. Staining of 231-GATA3-Empty and 232-GATA3-LOX lung lesions from tail vein injected mice.

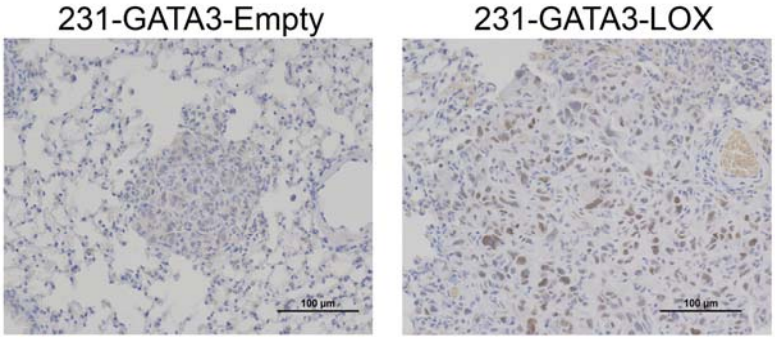

Figure 10. GATA3 staining of metastatic lung lesions. Immunohistochemical staining for GATA3 of 231-GATA3-Empty and 232-GATA3-LOX lung lesions from tail vein injected mice.

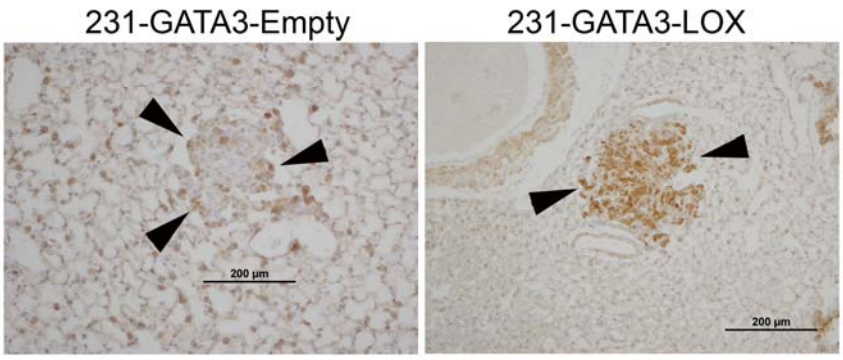

Figure 11. **LOX staining of metastatic lung lesions**. Immunohistochemical staining for LOX of 231-GATA3-Empty and 232-GATA3-LOX lung lesions from tail vein injected mice.

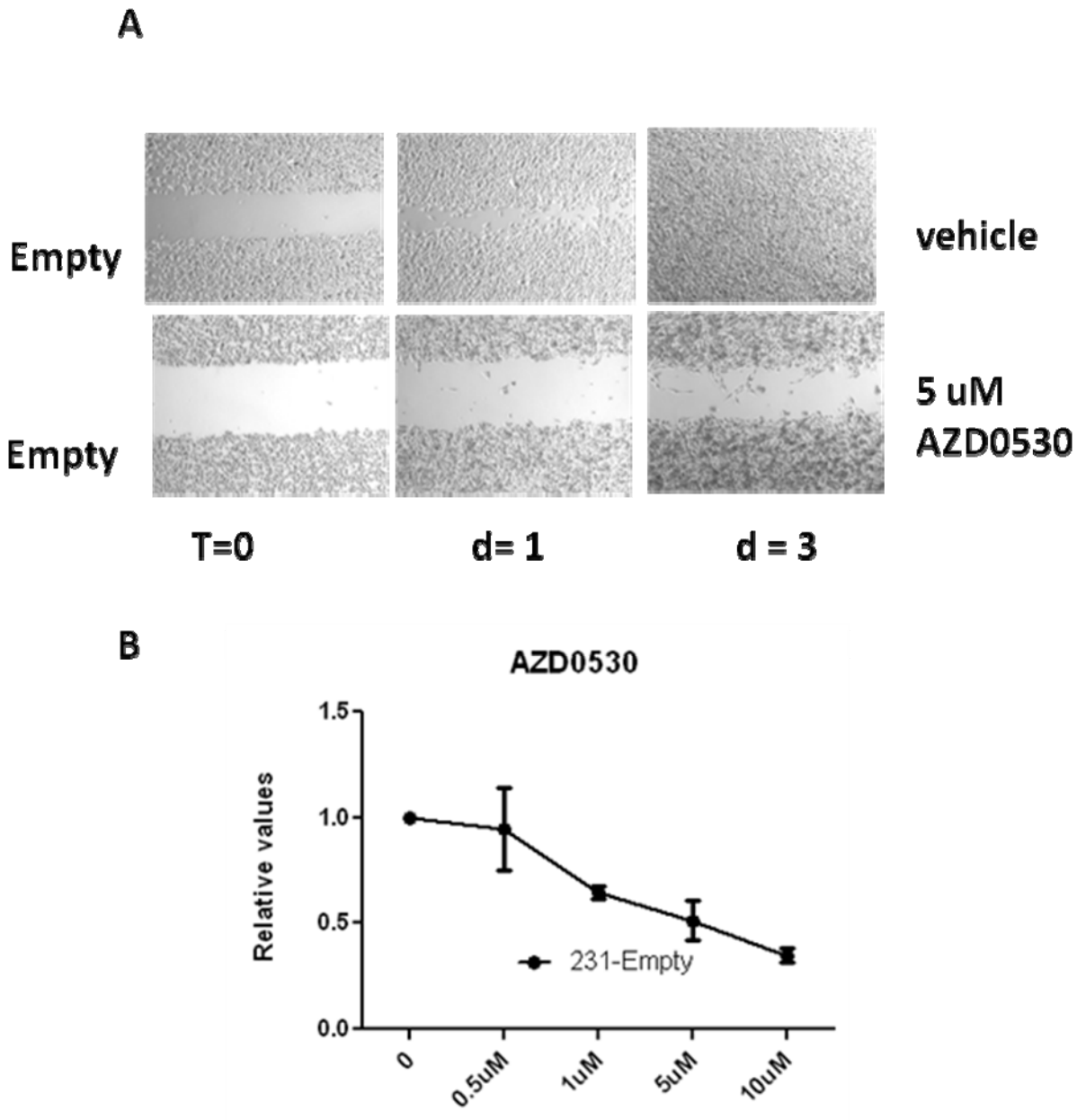

Figure 12. The Src inhibitor, AZD0530, inhibits migration and proliferation in MB231 cells. (**A)** MB231 cells were treated with 5 μM of AZD0530. MB231 vehicle treated cells migrated towards the clear scratched area within 24 hours. However, MB231 cells treated with AZD0530 failed to migrate. (**B)** MTS assay of MB231 cells treated with increasing concentrations of AZD0530. AZD0530 inhibits proliferation of MB231 cells