Chromatin, Gene, and RNA Regulation

MYC-Induced Epigenetic Activation of GATA4 in Lung Adenocarcinoma

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Abstract

Human lung cancer is a disease with high incidence and accounts for most cancer-related deaths in both men and women. Metastasis is a common event in non–small cell lung carcinoma (NSCLC), diminishing the survival chance of the patients with this type of tumor. It has been shown that MYC is involved in the development of metastasis from NSCLC, but the mechanism underlying this switch remained to be identified. Here, we focus on GATA4 as a MYC target in the development of metastasis with origin in lung adenocarcinoma, the most common type of NSCLC. Epigenetic alterations at the GATA4 promoter level were observed after MYC expression in lung adenocarcinoma in vivo and in vitro. Such alterations include site-specific demethylation that accompanies the displacement of the MYC-associated zinc finger protein (MAZ) from the GATA4 promoter, which leads to GATA4 expression. Histone modification analysis of the GATA4 promoter revealed a switch from repressive histone marks to active histone marks after MYC binding, which corresponds to active GATA4 expression. Our results thus identify a novel epigenetic mechanism by which MYC activates GATA4 leading to metastasis in lung adenocarcinoma, suggesting novel potential targets for the development of antimetastatic therapy. Mol Cancer Res; 11(2); 161–72. ©2012 AACR.

Introduction

Non–small cell lung carcinoma (NSCLC) is the most frequent type of lung cancer with high metastatic potential and a low cure rate. Cancer has been classified as a genetic disease ever since the discovery of retroviral oncogenes and the finding of frequent mutations in their cellular counterparts in human tumors. Growing evidence now suggests that epigenetic alterations are at least as common as mutational events in the development of cancer (1, 2). Tumor-specific promoter hypermethylation is well documented (1). Epigenetic silencing is also known to be a frequent event in NSCLC, that is, of p16, H-cadherin, death-associated protein (DAP) kinase 1 (DAPK1), 14–3–3 sigma, and the candidate tumor suppressor gene RASSF1A (3). However, comparatively little is known about the role of promoter hypomethylation in gene activation in cancer, especially in NSCLC.

We previously identified MYC as a metastasis inducer in a mouse model for NSCLC and in the human NSCLC cell line A549. On the basis of our observations in this mouse model, we suggested a lineage switch mechanism to be involved in progression to metastasis and identified GATA4 as a MYC-target in this context (4). Our earlier findings of RAF/MYC cooperation in the murine hematopoietic system revealed that MYC affects tumor cell reprogramming (5). Furthermore, amplifications and rearrangements of MYC genes were found in a fraction of human NSCLC (6).

The GATA family of transcription factors consists of 6 members, GATA1–6. These proteins have 2 highly conserved zinc-finger domains and recognize a consensus DNA-binding motif of (A/T)/GATA/(A/G; ref. 7). They are involved in the regulation of several biologic processes including organogenesis, differentiation, proliferation, and apoptosis (7). While the GATA1–3 proteins are important for the development of the nervous system and hematopoietic cell lineages, GATA4–6 are involved in organogenesis, especially of the heart, ovary, and extraembryonic tissues. In the murine lung, GATA6 drives the expression of the thyroid transcription factor TTF-1, which is an upstream effector necessary for surfactant protein C (Sp-C) expression (8). Furthermore, GATA6-Wnt signaling was shown to be important for epithelial stem cell development and airway

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regeneration (9). In contrast to GATA6, expression of GATA4 was detected neither in adult lung nor in lung adenomas induced by oncogenic C-RAF (4). GATA4 is, however, involved in the maintenance of the intestine of the adult mouse.

Here, we show that MYC induces epigenetic changes in the GATA4 promoter leading to its expression. Furthermore, MYC-dependent GATA4 upregulation was accompanied by site-specific demethylation and the acquisition of active histone modification marks in its promoter. Our results thus suggest a further autoregulation mechanism of GATA4 upon MYC expression.

Materials and Methods

Molecular cloning

The coding sequence of GATA4 was subcloned into the EcoRI site of the retroviral vector pEGZ/GFP/Neo by using the oligonucleotides 5'-CCGAATTCCGGATGTATCA-GAGCCTTGGCCATGG-3' and 5'-CCGAATTCGGGTTACCGAGTGATATTGTCCCC-3'. The resulting plasmid was confirmed by sequencing.

Cell culture and cell lines

The A549 and Phoenix amphotrophic cell lines were obtained from the American Type Culture Collection, where they are regularly verified by genotypic and phenotypic tests. All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO2 and harvested using 0.15% Trypsin-EDTA. For viral transfection, the MYC-ER virus encoding a fusion of the murine estrogen receptor (ER) and MYC-associated zinc finger protein (MAZ) shRNA and MYC-dependent GATA4 short hairpin RNA (shRNA), and MYC-associated zinc finger protein (MAZ) shRNA producing virus were independently produced in the Phoenix amphotropic packaging cell line. Ten micrograms of pBpuro c-MYC-ER (10), pLKO.1-puro-shGATA4 (MISSION shRNA Plasmid DNA from Sigma-Aldrich; Clone ID: NM_002052.2-1592s1c1) and pLKO.1-puro-shMAZ (MISSION shRNA Plasmid DNA from Sigma-Aldrich; Clone ID:NM_002383.1-761s1c1) were individually transfected into Phoenix amphotrophic cells using Lipofectamine (Invitrogen) according to manufacturer’s instructions. After 24 hours, the medium was changed. The viruses were produced overnight, and supernatant was then harvested and used for infection of A549 or A549 J5-1 (4) cells in the presence of 8 μg/mL of polybrene. Twenty-four hours after infection, infected cells were selected with 250 μg/mL of puromycin. For viral infection of A549 cells with pEZG-GATA4, the procedure described earlier was used. After infection, cells were selected with 500 μg/mL of zeocin. One week after selection, pools of 10 cells were seeded in a 96-well plate and for each pool GATA4 mRNA levels were measured. The pool number 11 showed the highest level of GATA4 mRNA and was used for further experiments. If mentioned, Trichostatin A (TSA) (Sigma-Aldrich) was added to the culture medium at a concentration of 50 ng/mL.

Soft agar assay

For the soft agar assay, the bottom agar was prepared using an autoclaved stock of 5% Sea Plaque Agarose, which was microwaved and mixed with DMEM to a final concentration of 0.5% agarose. Five milliliter of 0.5% agarose were poured in a 60-mm dish. For the top agar, the 5% stock was diluted to 0.6% agarose with DMEM and stored in a water bath at 40°C. Dilutions of the cells were prepared in 1 mL of medium (10,000 cells per 60-mm dish) and then mixed 1:1 with the 0.6% agarose. The mixture was poured on top of the solidified bottom agar. After solidification of the top agar, the dishes were incubated in a wet chamber at 37°C for 21 to 28 days, when the colonies number was counted.

Immunohistochemistry

Immunohistochemistry was conducted on paraffin sections. After deparaffinization and rehydration, sections were boiled in 10 mmol/L citrate buffer for 10 to 20 minutes for antigen retrieval. To quench the endogenous peroxidase activity, sections were incubated with methanol or PBS containing 1% to 3% H2O2. Nonspecific antibody binding was prevented by incubation with 5% of serum with 0.2% Triton X-100 in PBS for 1 hour at room temperature. After blocking, sections were incubated with the primary antibody [GATA4, sc-1237, Santa Cruz (1:200); TTF-1, M3575, Dako (1:200); Pro Sp-C, gift from Jeffrey A. Whitsett from the Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio (1:5,000)] overnight at 4°C. After washing, sections were incubated with the corresponding biotinylated secondary antibodies (Dako) at 1:200 to 1:600 for 1 hour at room temperature. For staining ABC reagent was applied (Vastastain Elite ABX Kit, Vector Laboratories) and color was developed with 3,3'-diaminobenzidine (DAB). For counterstaining hematoxylin was used. After dehydration, the stainings were mounted with entellan. For immunofluorescence staining, the following secondary antibodies (all obtained from Jackson ImmunoResearch Laboratories, at 1:200 dilutions) were used: donkey anti-goat Cy5 and donkey anti-rabbit Cy3.

Immunocytochemistry

Immunocytochemistry was conducted on cells grown on coverslips. After fixation using 4% PFA-PBS for 15 minutes at room temperature, cells were washed with PBS. To prevent unspecific antibody binding, cells were blocked with 4% serum, 0.2% Triton X-100 in PBS for 1 hour at room temperature. The primary antibody was diluted in 4% serum in PBS and applied overnight at 4°C [GATA4, sc-1237, Santa Cruz (1:250); GATA6, sc-9055, Santa Cruz (1:100)]. After washing with PBS, the cells were incubated with the secondary antibody for 2 hours at room temperature. The antibody was diluted in 4% serum in PBS (anti-goat-Alexa 647, 1:200; anti-rabbit-Cy3, 1:200). Before mounting with Mowiol, cells were counterstained using 4’,6-diamidino-2-phenylindole (DAPI).
Luciferase reporter assay

A 442-bp fragment upstream of the transcription start site of GATA4 was amplified from genomic DNA from HeLa cells using the following primers: 5′-AAAAAATCGAGGGA-CTAGCATCCAGCC-3′ and 5′-AAAAAAAGCTTGGCTGCAGCGCGGCGGAA-3′. The fragment was sub-cloned into XhoI and HindIII sites of the pGL3 Luciferase Reporter-Basic Vector (Promega) by enzymatic reaction. A549 and A549 J5-1 cells were transfected independently with the pGL3 vector containing the GATA4 promoter and with the pGL3 control vector containing luciferase under control of a SV40 promoter. Caco-2 cells were transfected with the same vectors and used as a positive control, as they normally express GATA4 (11). To measure luciferase activity, ONE-Glo Luciferase Assay System was used and conducted in 96-well plates according to the recommendations in the manual. The lumimeter was preheated to 37°C and the luminescence was measured with an integration time of 100 milliseconds.

Chromatin-immunoprecipitation

Cross-linked chromatin from A549 control cells or A549 J5-1 cells was prepared and immunoprecipitated as described previously (12). Chromatin immunoprecipitation (ChIP)-grade antibodies specific for H3K27me3 (07-449), H3K4me2 (07-030), and H3K4me3 (07-473) were purchased from Upstate. ChIP-grade antibodies specific for H3K9me2 (ab1220) and H3K9me3 (ab8898) were obtained from Abcam. Other antibodies used were against MAZ (Abcam ab85725), EZH2 (Cell Signalling AC22), DNMNT1 (active motive 39204), DNMNT3a (Abcam ab13888), DNMNT3b (active motive 39207), POLII (4H8, Abcam ab5408), GATA4 (Santa Cruz sc-1237), and GATA6 (Santa Cruz sc-9055). The chicken-v-MYC antibody was a gift from Klaus Bister from the Center for Molecular Biosciences, University of Innsbruck, Innsbruck, Austria. Immunoprecipitates were finally dissolved in 30 μL of Tris-EDTA (TE) buffer (10 mmol/L Tris–HCl pH 8, 1 mmol/L EDTA). One microliter was analyzed by real-time PCR using a primer pair specific for the GATA4 promoter region (CHIPGATA4_up 5′-CGGAGACCCCCCGACGTTG-3′; CHIPGATA4_lo 5′-CTCTCTACCTCCAGACACA-3′) in 10 μL PCR reactions, using the Absolute QPCR SYBR Green Mix (Thermo Scientific) and a Roche LightCycler 480. PCR conditions: 1 cycle: 95°C × 15 minutes; 42 cycles: 95°C × 15 seconds/60°C × 40 seconds, read; melting curve 65°C to 95°C, read every 1°C. Cycle threshold numbers for each amplification were measured with the LightCycler 480 software and enrichments were calculated as percentage of the input.

DNA-isolation from paraffin sections

After GATA4 staining, sections were air-dried and GATA4-positive and -negative tumor regions were scratched from the slide using a scalpel. Material was collected in 30 μL of lysis buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, 1% w/v Tween 20, 100 μg/mL proteinaseK) and incubated overnight at 37°C. For inactivation of proteinaseK samples were heated at 95°C for 20 minutes.

Bisulfite sequencing

For next generation, bisulfite-sequencing DNA from cultured cells was prepared using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer’s specifications. DNA from paraffin section was prepared as described earlier. 250 ng up to 1 μg of DNA was bisulfite-treated with the EpiTect Bisulfite Kit (Qiagen) according to manufacturer’s instructions. Deaminated DNA was amplified by PCR using the primer mentioned earlier but with the 454-adaptor sequence added and an identifier 4-base code for each tissue sample (here referred to as XXXX): 454-CGATA4-prom_up 5′-GCCCTCCCTCGGCCCATCA-GXXXXTATAAAATGGTTTGGTGATTATAG-3′/454-CGATA4-1_lo 5′-GCCCTGCCAGCCGGCTCA-GXXXXCCTACTACCTAAACATTTC-3′.

PCR conditions were as follows: 95°C for 3 minutes followed by 40 cycles at 95°C for 30 seconds, annealing temperature for 40 seconds and 72°C for 45 seconds, followed by a 3-minute incubation at 72°C. PCR products were subsequently purified using the QIAquick Gel Extraction Kit (Qiagen). For sequencing, equimolar amounts of all amplicons were combined in a single tube Roche 454 FLX. Standard sequencing was provided by the Genomics and Proteomics Core Facility of the DKFZ.

Real-time PCR

RNA was isolated from cultured cells using TriFast reagent (Peqlab). cDNA-synthesis was conducted using 1 μg of RNA per reaction with first strand cDNA synthesis kit (Fermentas). Quantitative PCR (qPCR) was conducted in a 20 μL reaction using Finzymes SYBER Green Master Mix (NEB) and Rox as a reference dye. The amplification efficiency was raised to the power of the threshold cycle (Ct) value to calculate the relative amount of the transcript. This gives the number of cycles necessary for the product to be detectable. The resulting value was normalized to the level of the housekeeping gene. Assays were conducted in triplicates in a StepOnePlus detection system (Applied Biosystems). The primer sequences used were for Chicken-v-MYC 5′-CGGCCCTTACCTGCGACGACC-3′ and 5′-GACCAAGCGACTGTGGTGG-3′, for hsGATA4 5′-CTCATGCGGCCAGTGAGGAG-3′ and 5′-CTGCGCTCCAGATGTTGTTG-3′, for hsGATA6 5′-TCCATGGGTTGCTTCAGCCA-3′ and 5′-CCCTGAGGTGTCTGCTTTGTG-3′, hsmucin2 5′-CGACTAACAATCTCGCGCTCGG-3′ and 5′-CGCAGGAGTACCTTGGGTTG-3′, for hsMYC 5′-GCCCTGGTGTCCTCATAGA-3′ and 5′-CAACATCATCTTCTCATCCTTCTGTCAGA-3′, and for β-actin 5′-TTCGTACCCAGGCTATGTGATGG-3′ and 5′-GCAATGCTGCTGGTACATGGTG-3′.

Ethics statement

All animal studies were approved by the Bavarian State authorities for animal experimentation.

Statistical analysis

Results are expressed as the mean ± SEM. One-way ANOVA was used to determine the differences among
several groups, followed by Student–Newman–Keuls test using GraphPad InStat software. Student t test was used for 2 group comparisons. For all tests, statistical significance was considered to be at \( P < 0.005 \).

Results

**GATA4 expression is accompanied by GATA4 promoter demethylation in c-MYC/KRas-mutant type II pneumocytes**

Previously, we observed a GATA6 to GATA4 switch in Sp-C-c-MYC and Sp-C-CRAF-BxB/Sp-C-c-MYC lung tumors and liver metastasis (4). In this work, we observed low or absent expression of pro Sp-C in GATA4-positive lung tumor regions from mice with the same genotype (Fig. 1A), which suggests that GATA4-positive tumor cells might become independent of any oncogenes under Sp-C promoter, including c-MYC, by an epigenetic mechanism. We have therefore surmised an epigenetic mechanism for the underlying mechanism of GATA4-induction in lung tumor.

We analyzed a 389-bp long CpG-rich region, which contains the promoter of the GATA4 gene (Supplementary Fig. S1A) by next generation bisulfite sequencing, using genomic DNA from GATA4-positive and -negative regions of a Sp-C-c-MYC/KRas–mutant lung tumor and a GATA4-positive liver metastasis (Supplementary Fig. S1B). We did not observe global methylation changes for the examined region but significantly reduced methylation levels for the CpG-sites 13 to 22 (with the exception of CpGs 15, 19, and 20—see Supplementary Table S1 for statistical tests) in the GATA4-positive tumor and metastasis material (Fig. 1B). Thus, this region showed clear hypomethylation in GATA4-expressing tissues.

**MYC is necessary to induce the expression of GATA4 and its target gene mucin2 in human lung adenocarcinoma cells**

If MYC expression is a prerequisite for induction of GATA4 expression in lung adenocarcinoma, the introduction of MYC into a GATA4-negative human lung adenocarcinoma cell line should lead to the expression of GATA4.

Figure 1. Ectopic expression of GATA4 in Sp-C-c-MYC/Ras–mutant lung adenocarcinoma is accompanied by site-specific GATA4 promoter demethylation. A, staining of mouse lung tumor sections for Sp-C and GATA4 shows mutually exclusive expression patterns between them. Lung tumor serial sections were stained for Sp-C and GATA4 (left and middle). GATA4-high/Sp-C-negative and GATA4-low/Sp-C-positive tumor regions were encircled with red and green dashed lines, respectively. Right, double immunofluorescence staining of a mouse lung tumor section for Sp-C and GATA4. Note that the majority of GATA4-positive tumor cells (red) are negative for Sp-C (green). DAPI (blue) shows nuclei. Scale bar, 50 \( \mu \)m. B, next generation bisulfite sequencing of the CpG-rich region near the GATA4 promoter (for exact primer locations see Supplementary Fig. S1A). DNA was isolated from lung tumor regions stained negatively and positively for GATA4 and from GATA4-positive liver metastasis, respectively. Sequencing results are shown as heatmaps in which each row represents one sequence read. Individual red boxes indicate methylated and green boxes indicate unmethylated CpG dinucleotides. Sequencing gaps are shown in white. CpGs showing the highest degree of change between DNA from GATA4-positive and –negative tumor tissue are boxed. The overall methylation of the region is 76% for both GATA4-positive and –negative lung tumors and 73% for the liver metastasis. A significant decrease in methylation of the CpGs 13-22 (boxed, 66% in GATA4-negative tissues) was observed in GATA4-positive tissue (44% in GATA4-positive tissues), KRas, KRas-mutant. For statistical tests see Supplementary Table S1. The DNA used for the next generation sequencing was isolated from GATA4-stained paraffin sections as illustrated in Supplementary Fig. S1B.
To test this possibility, we conducted qPCR and immunofluorescence staining on the chicken-v-MYC–expressing A549 J5-1 cells (4). As shown in Fig. 2A, the mRNA expression of GATA4 and its target gene mucin2 was significantly upregulated in MYC-expressing A549 cells.

and its target gene mucin2 (13). To test this possibility, we conducted qPCR and immunofluorescence staining on the chicken-v-MYC–expressing A549 J5-1 cells (4). As shown
Moreover, using an inducible cell line carrying a fusion of MYC with the ER, which is dependent on tamoxifen presence to be located to the nucleus, we could show GATA4 and mucin2 mRNA upregulation after 3 weeks of MYC induction, although MYC induction occurs as early as 1 week after 4-hydroxytamoxifen (OHT) addition (Fig. 2B). This suggests that epigenetic changes at the GATA4 promoter take effect at this time point.

In contrast to our in vivo data, GATA6 was also significantly upregulated in the A549 cells upon MYC-expression (Fig. 2A). This suggests that other factors might superimpose positive regulation. Furthermore, we conducted immunofluorescence staining for GATA4 and GATA6 and were able to detect both in the A549 J5-1 cells (Fig. 2C).

**GATA4 is necessary for MYC-induced anchorage-independent growth in lung adenocarcinoma cells**

The ability of cancer cells to form colonies without anchorage support is a key aspect of the tumor phenotype, particularly with respect to metastatic potential (14). A549 J5-1 cells showed an increased ability to form colonies in soft agar in comparison with the parental cell line (Fig. 3A and B). The inducible cell line A549 MYC-ER showed the same increased ability in the presence of tamoxifen (Fig. 3A and B). The knockdown of GATA4 in A549 in J5-1 (Fig. 3C) restored the behavior of wild-type A549 cells about anchorage-independent growth ability (Fig. 3D and E). These data suggest that GATA4 might be necessary for the transformation phenotype of MYC-expressing A549 cells. On the basis of these data, a newly prepared A549 cell line overexpressing GATA4 (Fig. 3F and G) was tested for the same feature, which showed that GATA4 alone is able to mimic MYC-induced ability to grow anchorage independently in A549 cells (Fig. 3H and I), strengthening the evidence that GATA4 is necessary for MYC-induced metastasis. While growing in adherent culture, the A549 GATA4-11 cells did not show a difference in proliferation when compared with A549 cells (data not shown).

**MYC is able to induce GATA4 promoter demethylation in human lung adenocarcinoma cells**

To test whether ectopic MYC expression is sufficient to induce demethylation of the GATA4 promoter in a human lung adenocarcinoma cell line, we conducted next generation bisulfite sequencing of the same region analyzed earlier in A549 and A549 J5-1 cells. Also, in A549 cells, the GATA4 promoter region was strongly methylated, which did not change significantly upon MYC expression (72% and 71%, respectively). Nevertheless, as shown in Fig. 4A and B, we observed a significant reduction in methylation at CpG sites 19 to 22 (with the exception of site 21—see Supplementary Table S2 for statistical tests) in these cell lines. These results thus confirm the data obtained in mouse lung tumors and strongly suggest that ectopic MYC expression is sufficient to induce local demethylation of the GATA4 promoter.

**Epigenetic signature of the GATA4 promoter**

To complement our analysis of epigenetic modifications in the GATA4 promoter, we mapped histone modifications by ChIP in the same region. Chromatin was prepared from A549 control and A549 J5-1 cells and precipitated with antibodies against histone H3 dimethylated at lysine 9 (H3K9me2), histone H3 trimethylated at lysine 9 (H3K9me3), histone H3 dimethylated at lysine 4 (H3K4me2), histone H3 trimethylated at lysine 4 (H3K4me3), and histone H3 trimethylated at lysine 27 (H3K27me3). Immuno precipitated DNA was analyzed by qPCR using a primer pair specific for the GATA4 promoter region. This PCR fragment of 372 bp spans a region starting with CpG 8 and extending about 200 bp into the GATA4 gene body. In agreement with the strong methylation of the promoter in A549 control cells, we found mainly repressive histone marks in the GATA4 promoter region (H3K9me3 and H3K27me3), most prominently H3K27me3, which would indicate repression by Polycomb group proteins (Fig. 5A). In contrast, in the MYC-expressing line J5-1, the levels of repressive marks were low, whereas both active H3K4 marks were enriched, indicating ongoing transcription at the GATA4 promoter. These results suggest a significant change in the epigenetic makeup of the GATA4 promoter upon MYC expression, which is characterized by local DNA hypomethylation and a switch from repressive to active histone marks.

**Protein occupancy in the GATA4 promoter region**

To identify proteins interacting with the GATA4 promoter in A549 control and A549 J5-1 cells, we analyzed a fragment of the GATA4 promoter region corresponding to the segment amplified by the bisulfite primers with the MatInspector software (15). This revealed the presence of a MYC-related E-box, the potential binding site for the MAZ, which is usually shared by the SP1 transcription factor (Supplementary Fig. S1C). In addition, we identified a Y-box and consensus sequences for the general transcription factor TFIID between CpGs 15 and 22. To validate these potential binding sites and to gain insight into the protein presence at the GATA4 promoter in control and MYC expressing cells, we conducted ChIP using antibodies against chicken-v-MYC (chk-MYC), GATA4, GATA6, MAZ, the 3 major DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b), the histone H3 lysine 27 methyltransferase Enhancer of Zeste 2 (EZH2) and the large subunit of the RNA-polymerase II (POL II) in chromatin prepared from A549 control cells and the J5-1 cell line. Immunoprecipitated DNA was analyzed by qPCR using the same primer pair for the analysis of histone modifications. In the repressed case (A549 control), we found strong binding of MAZ and EZH2 in the GATA4 promoter, which was paralleled by the presence of DNMT1 (Fig. 5B). In A549 J5-1, MAZ and EZH2 binding was greatly reduced, whereas chk-MYC (the protein expressed in this cell line) and GATA4 itself were found enriched on the GATA4 promoter. While DNMT3a and DNMT3b were detectable only at background levels, DNMT1 showed a significant interaction with the GATA4 promoter region (Fig. 5B). DNMT1 levels...
Figure 3. GATA4 is necessary for anchorage-independent growth induced by MYC. A, anchorage-independent growth of A549 MYC-ER cells in the presence of OHT. Colonies formed by A549 and A549 empty vector (EV) in the presence of OHT (controls), and A549 J5-1 and A549 MYC-ER in the presence of OHT, after 3 weeks in soft agar are shown. B, quantification of colonies formed by the different cell lines (conditions as indicated). A549 MYC-ER cells show, in the presence of OHT, a comparable ability to form colonies as A549 J-51 cells, indicating the induction of anchorage-independent growth by MYC. C, mRNA levels of human GATA4 in A549, A549 J5-1, and A549 J5-1 shGATA4 26. The shRNA-mediated knockdown of GATA4 in A549 J5-1 cells restored the low values of GATA4 mRNA levels of A549 wild-type cells. D, anchorage-independent growth of A549 J5-1 cells after knockdown of GATA4. Quantification of colonies formed by the 3 different cell lines indicated. The soft agar assay with the A549 J5-1 cells shows a highly significant reduction in the number of colonies after infection with the shRNA26-expressing virus. E, colonies formed by A549, A549 J5-1, and A549 J-51 shGATA4 26 cells after 3 weeks in soft agar are shown. F, A549 cells infected with the pEGZ/GATA4 vector show GATA4 expression. mRNA levels of GATA4 and EGFP in A549 cells infected with the plasmid pEGZ/GATA4 are shown. Pools of 10 cells were seeded in a 96-well plate after infection and selected with zeocin. The best candidate was pool number 11, showing the highest level of GATA4 mRNA levels and used for further experiments. G, immunocytochemistry for human GATA4 in A549 and A549 GATA4–11 cells. GATA4 and GFP expression was detected in the nucleus of A549 GATA4–11 cells. Red (Cy5) colored cells are GATA4-positive; green cells are GFP-positive; scale bars, 50 μm. H, growth in soft agar resulted in an increase of number and size of colonies in the case of A549 GATA 4–11 cells compared with the parental cell line A549. I, quantification of the colonies formed by A549 and A549 GATA4–11 showed a significant increase of the colonies formed in GATA4-expressing cells. All error bars represent the SD of the mean. Statistical differences between groups are indicated. ns, not significant.
Polycomb group—is usually kept repressed by MAZ-, DNMT1-, and GATA4 and its target mucin2 (Fig. 5E).

Figure 4. Site-specific demethylation of CpG dinucleotides in the promoter region of GATA4 upon MYC expression in A549 cells. Next generation bisulfite sequencing of the GATA4 promoter in A549 (A) and A549 J5-1 (B) cells (for exact primer locations see Supplementary Fig. S1A). Sequencing results are shown as heatmaps in which each row represents one sequence read. Individual red boxes indicate methylated and green boxes indicate unmethylated CpG dinucleotides. Sequencing gaps are shown in white. There is no significant change in the overall methylation status of the region (72% in A549 and 71% in A549 J5-1), but significant demethylation of the CpGs 19 to 22 (right box) was observed (49% in A549 vs. 37% in A549 J5-1). For statistical tests see Supplementary Table S2.

Discussion

A mutually exclusive expression of GATA4/GATA6 triggered by MYC was previously reported both in lung tumors and corresponding metastasis from animals expressing c-MYC in type II cells under the control of the Sp-C promoter, which suggests that this lineage switch occurring in the primary tumor is important for the development of c-MYC–induced metastasis (4).

In this work, we stained lung tumor tissues from animals with the same genotype and observed that GATA4-positive regions show low pro-Sp-C expression. This can represent loss of differentiation or reprogramming and suggests that these regions are presumably poor for any transgene expression that is under Sp-C promoter, such as c-MYC (Fig. 1A). These assumptions led us to the investigation of the epigenetic mechanism behind MYC-induced GATA4 expression suggested in this work.

MYC drives GATA4 expression in human lung adenocarcinoma cells

The upregulation of GATA4 and its functional target mucin2 was also detected in MYC-expressing A549 cells (Fig. 2A and B) and occurred 3 weeks after ectopic MYC expression (Fig. 2B), suggesting that a multistep mechanism might be involved in the changes induced by this oncogene. Accompanying GATA4 upregulation, MYC-expressing A549 cells showed a metastatic behavior (14), in particular increased ability to grow anchorage independently in soft agar, which was reverted after GATA4 knockdown (Fig. 3A,
D, E, H, and I). These data indicate the direct involvement of GATA4 in MYC-induced metastasis. In fact, GATA4 is a lineage selector gene, and its upregulation suggests dedifferentiation and loss of organ identity, which is in line with the theory that the metastatic process is a recapitulation of ontogeny (5). GATA4 promotes the expression of the antiapoptotic factor Bcl2 and cyclin D2 (17), increases proliferation and migration capacities in colorectal cancer (18), and its altered expression is correlated with a broad range of tumors emerging from gastrointestinal tract, lungs, ovaries, brain, and with poor prognosis in ovarian granulose cell tumors (19).

MYC induces GATA4 promoter demethylation
Growing evidence now suggests that epigenetic alterations at the promoter level are at least as common as mutational events in the development of cancer (1, 2). Tumor-specific promoter hypermethylation is well documented in NSCLC (1), but comparatively little is known about gene activation by promoter hypomethylation. In the present work, we show that GATA4 upregulation in lung adenocarcinoma is accompanied by site-specific demethylation of the CpG islands 13 to 22 of its own promoter upon MYC-expression in vitro, in primary tumors and in corresponding liver metastasis (Figs. 1B, 4A and B). Interestingly, this region

Figure 5. MYC and GATA4 bind to the GATA4 promoter upon expression of exogenous MYC in the A549 cells, whereas MAZ is displaced. A, Chip analysis of the GATA4 promoter region in control (A549) and MYC-expressing cells (A549 J5-1). Chip was repeated at least 3 times with chromatin from biologic replicates using antisera specific for H3K9me2, H3K9me3, H3K4me2, H3K4me3, and H3K27me3. Immunoprecipitated DNA was analyzed by real-time PCR and a primer specific for the GATA4 promoter region. Enrichments are shown as percentage of the total input. Error bars show the SD of the mean. B, Chip assay as in A monitoring occupancy of the GATA4 promoter by candidate proteins chicken-v-MYC (chik-MYC), GATA4, GATA6, MAZ, the 3 major DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b), EZH2, and the large subunit of the POL II. Binding of chicken-v-MYC and GATA4 on the GATA4 promoter was significantly higher in the MYC-expressing A549 J5-1 cells than in the parental cell line. Error bars show the SD of the mean. C, anchorage-independent growth of A549 cells after knockdown of MAZ. MAZ knockdown in A549 cells leads to GATA4 activation and increased anchorage-independent growth. Pictures from the colonies formed by A549 and A549 shMAZ 345 cells after 3 weeks in soft agar are shown. D, quantification of the colonies formed by A549 cells after infection with shRNA-producing virus 354. The soft agar assay plate with the A549 shMAZ 345 cells showed a highly significant increase in the number of colonies formed, when compared with the parental cell line A549. E, GATA4 and mucin2 mRNA levels were measured in A549 and A549 shMAZ 345 cells. Results showed a significant increase in both GATA4 and mucin2 mRNA levels in MAZ-depleted cells, compared with the parental cell line A549. Error bars show the SD of the mean. Statistical differences between groups as indicated.
contains an E-box, a potential binding site for MYC (20, 21), which was previously described as a key regulatory element of GATA4 expression in vitro (22) and in vivo (23). A Y-box sequence is located directly downstream. The Y-box–binding protein 1 regulates the expression of many important genes (24) and is involved in the development of metastasis in patients with gastric and breast cancer (25). This protein may bind to the hypomethylated region of the GATA4 promoter, and thus regulate its expression, or even act as a partner in metastasis development.

Although controversial, hypermethylation of GATA4 in lung cancer has been reported (18, 26). A study from Guo and colleagues on the methylation of GATA genes in lung cancer used several lung cancer cell lines revealing GATA4 silencing by hypermethylation in most of the tested cells, including A549 (26). Curiously, the only cells extracted from a metastatic tissue—H157—showed hypomethylation of their GATA4 promoter (26). Montavon and colleagues showed that the GATA4 promoter was kept unmethylated in all tissues collected from patients with a rapidly invasive ovarian cancer (high-grade serous ovarian cancer), whereas tumors from patients with less invasive ovarian cancers showed hypermethylation of the GATA4 promoter (27). This supports our observations that hypermethylation–dependent upregulation of GATA4 is involved in metastasis development. Because MYC preferentially binds to promoter regions and CpG-rich regions (21), our data highlight this oncogene as a key-player in epigenetic modifications occurring at GATA4 promoter level.

**MYC induces the enrichment of active histone marks at the GATA4 promoter**

DNA methylation acts in cooperation with histone tail modifications (28). In the present work, corresponding to the strong methylation of the GATA4 promoter of the A549 control, mainly repressive histone marks H3K9me3 (1), and most prominently trimethylation of lysine 27 of histone H3 (29) were found in control cells (Fig. 5A). The enrichment of H3K27me3 indicates repression by the Polycomb-repressive complex 2 (PRC2; ref. 30), which methylates H3K27 via its catalytic subunit—EZH2 (29, 30), which was indeed found to be enriched at the GATA4 promoter of A549 wild-type cells, in contrast to the low binding of EZH2 detected at the GATA4 promoter of MYC-expressing A549 cells. This supports our finding that GATA4 expression is repressed in A549 cells, and that this event is accompanied by an enrichment of the methylated target of PRC2, H3K27.

In contrast to the enrichment of repressive histone marks at the GATA4 promoter of wild-type A549 cells, in the MYC-expressing cell line J5-1, the levels of repressive marks are low, whereas both active H3K4 marks (1) are enriched, indicating ongoing transcription at the GATA4 promoter (Fig. 5A). The recruitment of POL II—the key rate-limiting transcription factor—further supports this observation.
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step in gene activation (31)—cooccurs with the enrichment of the active H3K4 marks (32) and was observed in MYC-expressing A549 cells. Altogether, these data show significant changes in the epigenetic landscape of the GATA4 promoter upon MYC expression leading to the transcriptional activation of GATA4.

MYC induces changes in protein occupancy at GATA4 promoter

Broad changes in protein occupancy at the GATA4 promoter of A549 cells upon MYC expression were also observed. Among other proteins tested, GATA4 markedly bound to its own promoter upon MYC expression. On the other hand, MAZ (16) and the already mentioned EZH2 protein were displaced from the GATA4 promoter in MYC-expressing A549 cells (Fig. 5B). Judging from the specific and very restricted hypomethylation observed in MYC-positive tumors or MYC-expressing A549, most likely also DNMT1 is displaced from or looses access to the region encompassing CpG13 to 22. Nevertheless, the resolution of the ChIP-method used (ca. 400 bp) is not suitable to resolve such restricted changes in DNMT1 levels. MAZ displacement might prevent maintenance methylation due to the absence of DNMT1-reCR, and therefore provoke very site-specific hypomethylation at the GATA4 promoter. Although the knockdown of MAZ in A549 cells did not lead to a very pronounced GATA4 upregulation, it led to a dramatic increase in mucin2 mRNA levels and in the ability of these cells to form colonies anchorage independently. These data suggest that moderate changes in GATA4 levels can result in strong upstream effects and highlight MAZ as a key-player in MYC-induced metastasis via GATA4 activation (Fig. 5C-E).

Indeed, a previous study from Bartoszewski and colleagues showed that the repression of the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) is maintained during endoplasmatic reticulum stress by recruitment of methyl-binding proteins and/or HDACs. The repressed state of CFTR depends on the binding of MAZ to a hypermethylated region of the promoter, showing methylation-specific repression by MAZ in this case as well (33).

Our findings suggest a scenario in which GATA4 is usually kept repressed by MAZ, DNMT1, and Polycomb group containing complexes. Upon MYC expression, chl- MYC interacts with its binding site (E-box) near the GATA4 promoter, which leads to MAZ and EZH2 displacement, and therefore MAZ-recruitment of DNMT1 to the MAZ-binding site and the E-box is blocked. Demethylation of these regions occurs as a consequence of replication proceeding in the absence of maintenance methylation, provided by DNMT1 (34), leading to subsequent GATA4 activation. Activated transcription of GATA4 might induce its autoregulation by interacting with its own promoter (Fig. 6). We propose a novel molecular mechanism for GATA4 activation in lung adenocarcinoma, which is dependent on epigenetic changes on its own promoter and leads to metastatic conversion. Moreover, we were able to identify GATA4 as a MYC target in malignant lung adenocarcinoma, and highlighted GATA4 as a potential target for antimitotic therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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