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A Negative Feedback Regulatory Loop Associates the Tyrosine Kinase Receptor ERBB2 and the Transcription Factor GATA4 in Breast Cancer Cells

Guoqiang Hua,[1,2,3] Bing Zhu,[1,2,3,4] Fréderic Rosa,[1,2,3] Nicolas Deblon,[1,2,3,5] José Adélaïde,[1,2,3] Brigitte Kahn-Perlès,[1,2,3] Daniel Birnbaum,[1,2,3] and Jean Imbert[1,2,3]

[1]Université de la Méditerranée; [2]Institut Paoli-Calmettes; [3]Institut National de la Santé et de la Recherche Médicale, U891, Centre de Recherche en Cancérologie de Marseille, Marseille, France; [4]Department of Pathology, University of Texas Medical Branch, Galveston, Texas; and [5]CMU/Dpt PHYM, Geneva, Switzerland

Abstract

Overexpression of the *ERBB2* gene, linked to genomic and transcriptional amplifications, is a poor prognosis indicator in 25% to 30% of breast cancers. In contrast to some well-documented genomic amplifications, molecular mechanisms leading to *ERBB2* transcriptional overexpression remain poorly characterized. Gene expression analyses of breast cancer have characterized distinct transcriptional signatures allowing a molecular classification of breast carcinoma. Coexpression of the *ERBB2* and *GATA4* genes was originally observed in tumors. Both genes are essential for cardiovascular development and *GATA4* has been proposed to control the transcription of critical genes for the differentiation and the function of myocardium. We determined that *ERBB2*-targeted small interfering RNA repressed both *ERBB2* and *GATA4* genes, whereas *GATA4*-targeted small interfering RNA repressed *GATA4* and activated *ERBB2* transcription. Transfected GATA4-expressing construct repressed *ERBB2* promoter. Phylogenetic foot printing revealed multiple putative GATA4 binding sites conserved in mammals within the *ERBB2* promoter region. Chromatin immunoprecipitation showed that *GATA4* binds specifically to several *ERBB2* gene noncoding regions. Electrophoretic mobility shift assay revealed GATA4 binding to a well-conserved consensus motif. Site-directed mutagenesis confirmed the role of this new regulatory element for the activity of the *ERBB2* gene enhancer. In agreement with a repressor role of GATA4 on *ERBB2* gene expression balanced by *ERBB2* activation of the *GATA4* gene, a negative correlation between the relative levels of *ERBB2* and *GATA4* mRNA was observed in breast cancer cell lines and breast tumor samples. We propose that the negative feedback loop linking *ERBB2* and *GATA4* plays a role in the transcriptional dysregulation of *ERBB2* gene expression in breast cancer. (Mol Cancer Res 2009;7(3):402–14)

Introduction

The *ERBB2* gene encodes a tyrosine kinase receptor belonging to the epidermal growth factor receptor family (1). Amplification and overexpression of this receptor is a poor prognosis indicator observed in 25% to 30% of breast cancers but also in other types of cancer with variable proportions (2). Its detection might also predict resistance to chemotherapy. In breast cancer with *ERBB2* amplification, current treatments include humanized monoclonal antibody trastuzumab (Herceptin) directed against *ERBB2*, which blocks the activity of the receptor by poorly defined mechanisms. However, resistance to trastuzumab is frequent and this drug can trigger some cardiotoxicity (3-5).

Overexpression of *ERBB2* was originally attributed to genomic amplification, but it appeared rapidly that increased transcription was observed in all analyzed tumor cells (6). These observations indicate that overexpression at the transcriptional level can precede gene amplification and contribute to the severity of the disease (7). More recently, several reports showed that various transcriptional and post-transcriptional mechanisms contribute to increased levels of *ERBB2* transcript and protein in cancer cells (8-10). In this context, a precise and complete definition of the regulatory elements and their cognate transcription factors that control *ERBB2* gene transcription is a major challenge.

ERBB2 gene transcription is under the control of at least two promoters separated by 12 kb (11). Although the distal promoter remains poorly defined, several regulatory elements have been characterized within the proximal promoter and its 5′-flanking sequence up to the 6 kb upstream of the major transcription start site (TSS; refs. 8, 12-16; Fig. 1A). A set of studies suggest that several associated transactivators, or transrepressors such as FOXP3 and PEA3 (10, 17), are involved in the increased

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Note: Current address for G. Hua, F. Rosa, B. Kahn-Perlès, and J. Imbert: Institut National de la Santé et de la Recherche Médicale, U928, TAGC, Marseille Cedex 09, F-13288, France.

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Requests for reprints: Jean Imbert, Institut National de la Santé et de la Recherche Médicale, N928, TAGC 163, avenue de Luminy, case 928, Marseille Cedex 09, 13288 France. Phone: 33-491-828-703; Fax: 33-491-828-701. E-mail: jean.imbert@inserm.fr

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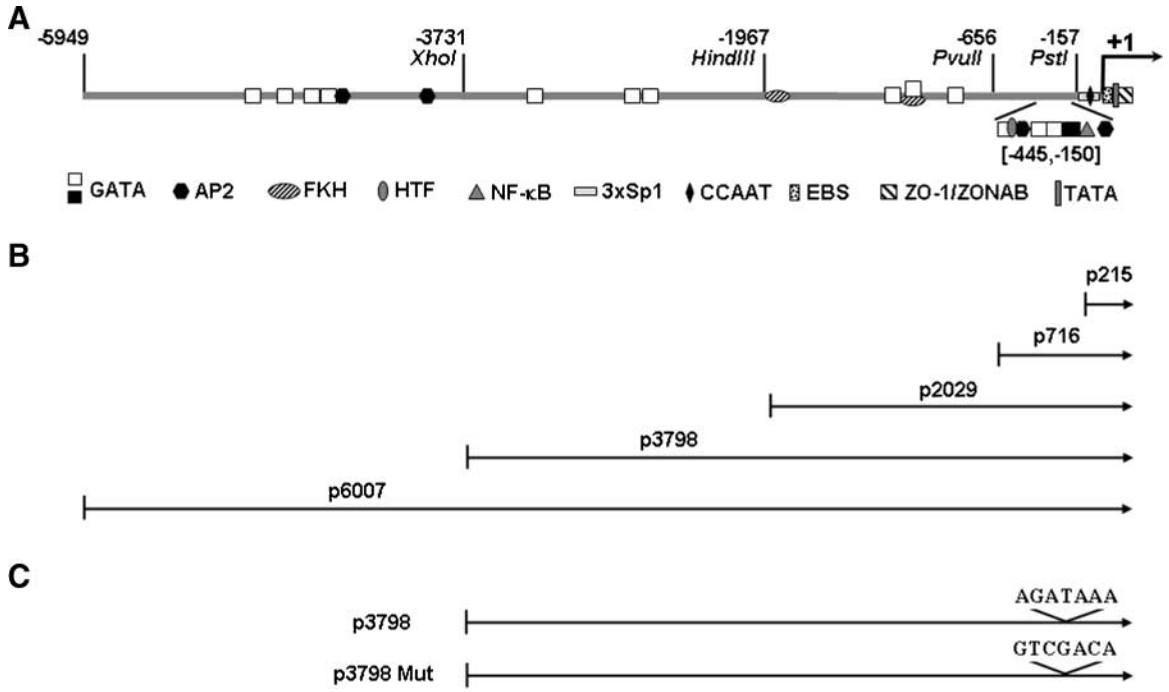

FIGURE 1. Schematic representation of the different promoter fragments of the human *ERBB2* gene. **A.** Map of the region 5′ upstream region [-5949, +104] of the human *ERBB2* gene. Numbering above the map line refers to the major TSS according to RefSeq NM_000448. Known and putative regulatory elements located on the map line (5′-3′ listing): 13 putative (*open squares,* [-4955, -4943], [-4769, -4757], [-4565, -4560], [-4551, -4539], [-3320, -3315], [-2727, -2715], [-2694, -2682], [-1221, -1216], [-1102, -1097], [-843, -831], [-466, -460], [-445, -440], and [-386, -381]) and 1 functional GATA (*closed square,* [-286, -281]); 4 AP-2 ([-4447, -4438], [-3946, -3938], [-439, -430], and [-158, -150]; refs. 7, 13, 14); 2 FOXP3/FKH ([-1924, -1908] and [-1114, -1098]; ref. 10), 1 HTF binding site ([-460, -419]; ref. 18); 1 nuclear factor-κB ([-243, -234]); 3 Sp1/GC-box ([-82, -65], [-46, -28], and [-6, +8]; ref. 55); 1 CCAAT box ([-15, -11]; ref. 56); 1 EBS ([+27, +32]; refs. 17, 57); 1 TATA box ([+34, +38]; ref. 55); and 1 ZO-1/ZONAB ([+60, +83]; ref. 58). Symbols corresponding to the regulatory elements are below the map line. **B.** Representation of the *ERBB2* promoter regions inserted in the luciferase reporter vector used to generate the data illustrated in Fig. 3 (13). Numbering relative to RefSeq entry NM_004448 differs from the original report, which refers to an alternative TSS (57): p215 [-157, +104], p716 [-656, +104], p2209 [1967, +104], p3798 [-3731, +104], p6007 [-5949, +104]. **C.** Representation of the p3798 luciferase vector containing a wild-type or mutated GATA binding site [-286, -281] used to generate data illustrated in Fig. 6.

transcription of the *ERBB2* gene in breast cancer cells (8, 12-14, 18-21). Among the sequence-specific transcription factors bound specifically to the proximal *ERBB2* promoter, only AP-2 and ETS factor family members are required for a maximal promoter activity in transient transfection assays and are associated with *ERBB2* gene overexpression in breast cancers (7, 12, 22, 23). It was also proposed that the regulatory regions of the *ERBB2* gene involved in its overexpression in epithelial breast cancer cells might be different from those contributing to its overexpression in colon and ovarian cancers (8, 9).

Gene expression analyses of breast cancers with various pathophysiologic and/or prognostic features have characterized several sets of coregulated genes that define transcriptional signatures, allowing a precise molecular classification of breast cancers in subtypes (24). In one study, the gene encoding the transcription factor GATA4 was identified as one of the 29 overexpressed genes differentially expressed in tumors associated with *ERBB2* overexpression (24, 25). Interestingly, *ERBB2* and *GATA4* are essential for cardiovascular development (26-28) and it has been proposed that *GATA4* controls the transcription of critical genes for both differentiation and function of the myocardium. The transcriptional activity of *GATA4* is activated via the mitogen-activated protein kinase pathway in cardiomyocytes (29) and the ERBB2 receptor triggers several transduction pathways including the mitogen-activated protein kinase pathway (2). However, no direct functional link has been established thus far between *ERBB2* and *GATA4*. Consequently, we have hypothesized the existence of a direct functional interaction between these two major regulators of mammalian development and cell biology. Using a combination of functional and interaction studies, we show here that the *ERBB2* gene is a direct target of GATA4. We propose that the *GATA4* gene is activated by the ERBB2 receptor, whereas the *ERBB2* gene is repressed by the transcription factor GATA4 through a negative feedback regulatory loop.

Results

GATA4 Can Repress ERBB2, Whereas ERBB2 Can Activate GATA4

To investigate the functional relations between *ERBB2* and *GATA4*, RNA interference (RNAi) assays were carried out in the breast cancer cell line BT-474, which overexpresses both *ERBB2* and *GATA4*. A 10- and 2-fold decrease of *ERBB2* and *GATA4* RNA levels were observed, respectively, when human *ERBB2* Stealth select RNAi (Invitrogen) were transiently transfected in BT-474 cells (Fig. 2A). Immunoblotting of lysates from small interfering RNA (siRNA)-transfected cells

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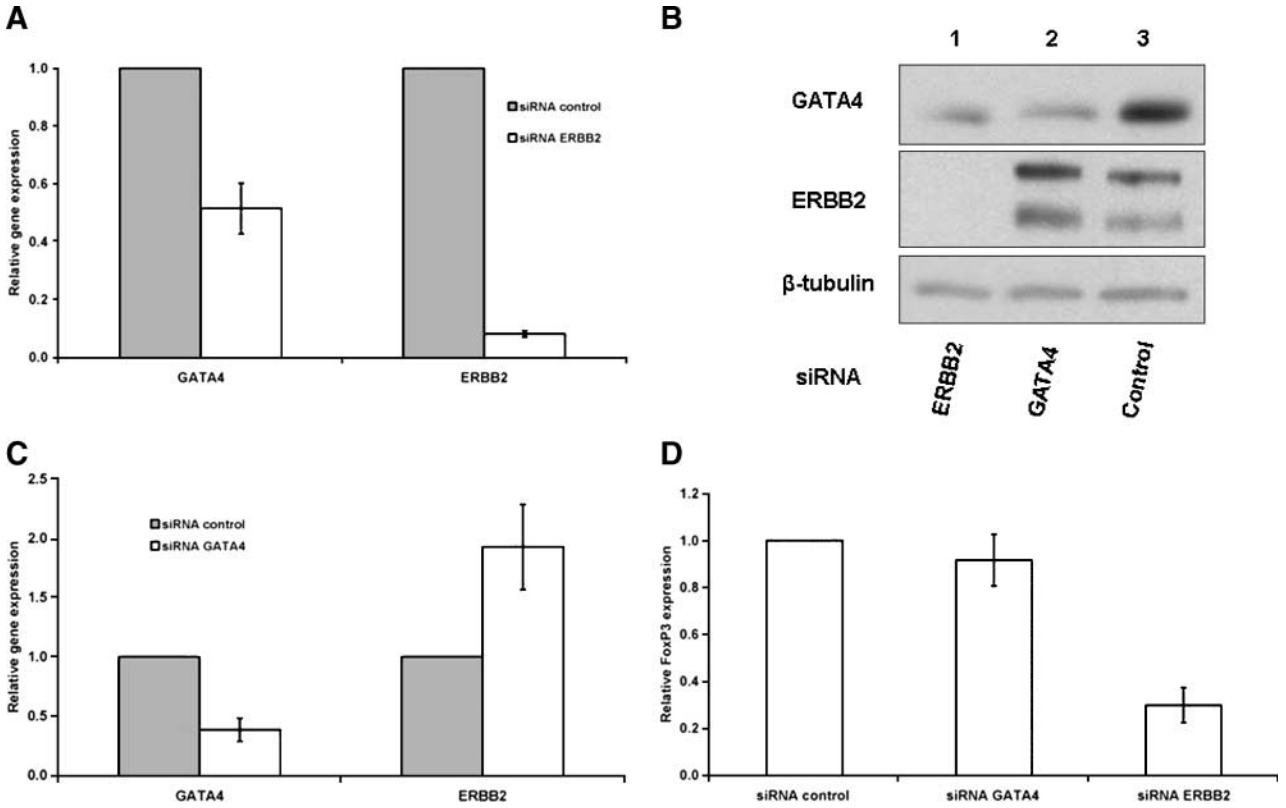

FIGURE 2. ERBB2 activates *GATA4* and GATA4 represses *ERBB2* in BT-474 cells. **A.** Effect of transfection of *ERBB2* siRNA on *GATA4* and *ERBB2* expression. Relative *GATA4* and *ERBB2* RNA levels on treatment with either human *ERBB2* Stealth RNAi (HSS103333 and HSS103334) or matched negative controls (Med and Lo GC, respectively) determined qRT-PCR (*empty* and *gray columns*, respectively). *Y axis,* RGE normalized against TBP levels as described in Materials and Methods. Mean ± SE of 6 independent assays. **B.** ERBB2 and GATA4 protein levels determined by immunoblotting of cell lysates treated by *ERBB2* (*lane 1*), *GATA4* (*lane 2*), or control (*lane 3*) siRNA using GATA4 and ERBB2 antibodies. The amount of β-tubulin was used as loading control. **C.** Effect of transfection of *GATA4* siRNA on *GATA4* and *ERBB2* expression. Relative *GATA4* and *ERBB2* RNA levels on treatment with either human *ERBB2* Stealth RNAi (HSS104005 and HSS104007) or matched negative controls (Lo and Med GC, respectively) determined by qRT-PCR (*empty* and *gray columns*, respectively). *Y axis,* RGE normalized against TBP level as described in Materials and Methods. Mean ± SE of 12 independent assays. **D.** Effect of transfection of *GATA4* and *ERBB2* siRNA on *FOXP3* gene expression. RNA samples tested were the same as used in **A** and **C**.

confirmed the abolition of the ERBB2-specific signal in the presence of *ERBB2* siRNA and the dramatic reduction of *GATA4* compared with matched control (Fig. 2B, *lanes 1* and *3*). In contrast, *GATA4* Stealth select RNAi induced a 2.5-fold increase of *ERBB2* RNA level while producing a 2-fold inhibition of *GATA4* RNA (Fig. 2C). Accordingly, immunoblotting of lysates from *GATA4* siRNA-transfected cells revealed a significant decrease of GATA4 protein level, whereas siRNA treatment significantly increased ERBB2 protein level (Fig. 2B, *lane 2*). Neither set of Stealth select RNAi affected the expression of the gene coding for β-tubulin and TBP (Fig. 2B; data not shown). Because FOXP3 has been recently identified as a major negative regulator of *ERBB2* gene expression in mammary tumors (10), we wondered whether this transcription factor of the forkhead family might be involved in *GATA4*-dependent *ERBB2* gene repression. As shown in Fig. 2D, *GATA4* siRNA had no effect on *FOXP3* gene expression, whereas *ERBB2* siRNA induced a 3-fold reduction of *FOXP3* RNA. These results excluded apparently the hypothesis of an indirect inhibition of the *ERBB2* gene by GATA4 via the repression of the *FOXP3* gene transcription and added FOXP3 in the list of transcription factors activated by ERBB2 signaling (30). Altogether, these results suggested the presence of a regulatory loop linking the *ERBB2* and *GATA4* genes in BT-474 cells where GATA4 can directly repress *ERBB2* and ERBB2 can activate *GATA4*.

GATA4 and ERBB2 mRNA Expression Levels Are Negatively Correlated

Results obtained with *ERBB2* and *GATA4* siRNA were unexpected when one considers our original observation based on transcriptome analysis using human cDNA microarrays. We have indeed reported previously a transcriptional signature including correlated *ERBB2* and *GATA4* overexpression in a set of breast cancer cell lines and tumor samples (25). Therefore, real-time quantitative reverse transcription-PCR (qRT-PCR) analyses were done to determine the relative levels of *ERBB2* and *GATA4* mRNA in 17 breast cancer cell lines and 17 breast carcinoma samples. The normal breast epithelial cell line HME-1 was used as a reference to measure the relative gene expression (RGE) of both genes. *ERBB2* RGE was negatively correlated to *GATA4* RGE with a Spearman rank correlation coefficient equal to -0.515 in breast cancer cell lines (Fig. 3A)

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and -0.517 in tumor samples (Fig. 3B). These significant negative correlations ($P = 0.035$ and 0.034, respectively) were in agreement with the increased level of *ERBB2* when *GATA4* was repressed by specific siRNA. They are also in agreement with the hypothesis of a negative feedback loop of regulation linking *ERBB2* and *GATA4*. The discrepancy with our original cDNA microarray observations (25) is most probably due to the difference in sensitivity between the two methods employed. It is well documented indeed that qRT-PCR is more sensitive in detecting a relative change than microarrays, which underestimate sometimes dramatically the actual expression change (31, 32). This is particularly true for the low expressed genes such as *GATA4* in our experiments. Consequently, it was not a posteriori unexpected to observe a coexpression of *ERBB2* and *GATA4* because *ERBB2* activates *GATA4*, although it was not possible to determine precisely their relative changes using DNA microarrays.

GATA4 Represses the Transcriptional Activity of the Human ERBB2 Gene Promoter and Its 5′-Flanking Sequences

To evaluate whether GATA4 can directly act on *ERBB2* gene transcription, we used luciferase gene reporter vectors containing *ERBB2* enhancer/promoter fragments of increasing size (Fig. 1B; ref. 33) and a mammalian expression vector for human GATA4 (34). The series of *ERBB2* enhancer/promoter fragments were first transiently transfected alone in two breast cancer cell lines with *ERBB2* gene amplification, BT-474 (RGE *ERBB2* = 25 and *GATA4* = 304) and HCC-1954 (RGE *ERBB2* = 22 and *GATA4* = 0.01), and one without, HCC-1806 (RGE *ERBB2* = 0.05 and *GATA4* = 126). As shown in Fig. 4A, the p3798 plasmid sustained the strongest luciferase activity normalized to that sustained by p215 containing a human *ERBB2* minimal promoter fragment, which has been shown to direct similar activity in a set of related breast cancer cell lines

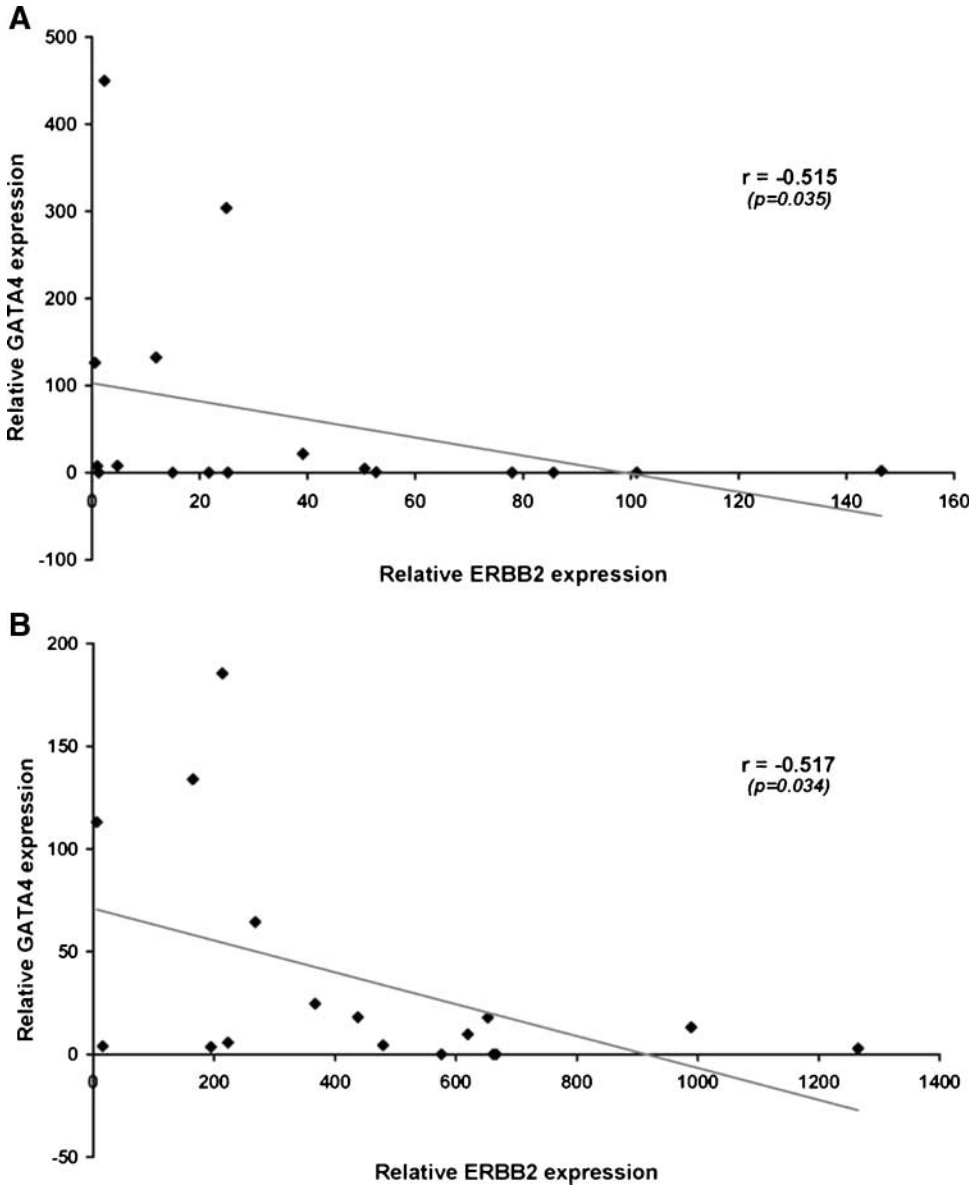

FIGURE 3. Inverse correlation of *GATA4* and *ERBB2* RNA levels in 17 breast cancer cell lines (**A**) and in 17 breast carcinoma samples (**B**) revealed by qRT-PCR. *X* and *y axes,* RGE levels of *ERBB2* and *GATA4* RNA, respectively, in breast cancer cell lines or tumor samples normalized to the levels of *ERBB2* and *GATA4* RNA measured in the normal breast epithelial cells HME-1 as described in Materials and Methods. Closed lozenges, normalized gene expression values determined for each cell line as described in Materials and Methods. Gray line, linear regression of Pearson. The coefficient of correlation *r* was determined and its statistical significance was tested using the nonparametric Spearman rank correlation test.

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(13). However, our results differ from original reports (13, 33) where p3798 displayed a very weak transcriptional activity when compared with p716 or p6007 in the same breast cancer cell line. Each luciferase vector was sequenced to eliminate any mix-up (data not shown) and observations were highly reproducible in at least three independent experiments. A difference linked to the normalization used for our assays was also excluded (dual luciferase assay versus protein concentration). It shall also be noted that some significant variations using the same set of vectors were presented in at least two reports (compare Fig. 2 in 33 and Fig. 1 in 13), but there is no clear explanation at this stage for these discrepancies.

When the set of *ERBB2* enhancer/promoter fragments were cotransfected together with a GATA4 expression vector in BT-474 cells, the corresponding activities were dramatically reduced when compared with the activities observed in the absence of artificially overexpressed GATA4. This repressive effect was observed on all constructs, except p215, as well as on the unrelated pNF-κB-Luc control plasmid (Fig. 4B and C). The same repressive effect was also observed when the cotransfection was done in HCC-1954 cells, which do not express *GATA4* (Fig. 4D). No effect was observed when the cotransfection assays were done in the *ERBB2*-negative HCC-1806 cells, which overexpress *GATA4* (RGE = 126). Most probably, the strong endogenous GATA4-negative signal in this cell line precluded any effect of further overexpression. Interestingly, an even higher *GATA4* overexpression was detected in BT-474 cells (RGE = 304), although *ERBB2* was significantly active, indicating the presence of stronger positive signals that stimulated *ERBB2* gene transcription in this breast cancer cell line.

Altogether, our results suggested that GATA4 can act directly on several locations within the *ERBB2* promoter 5′-flanking sequence from nucleotides -5949 to -156 relative to the major TSS characterized in the RefSeq record NM_004448.

Human ERBB2 Gene Contains Several Conserved Putative GATA Binding Sites

To determine whether the 5′-flanking sequence of the *ERBB2* gene contains putative binding sites for members of

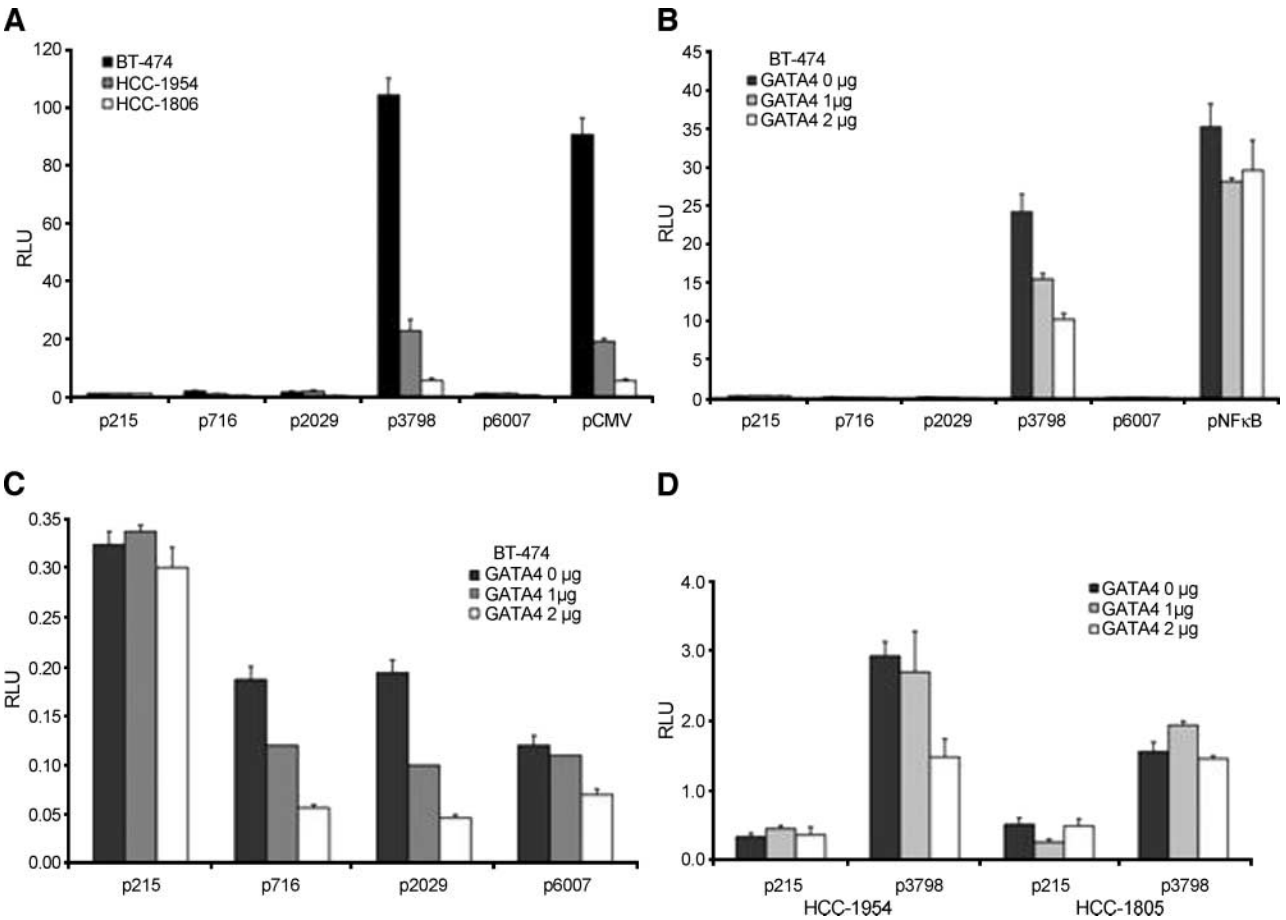

FIGURE 4. Overexpression of transfected GATA4 represses *ERBB2* proximal promoter in breast cancer cell lines. Relative luciferase activities observed in BT-474, HCC-1954, and HCC-1806 cells transfected by a series of luciferase reporter vectors containing *ERBB2* promoter fragments of increasing sizes with or without increasing amount of a GATA4 expression vector. The construct pCMV-Luc containing the high activity enhancer derived from the human cytomegalovirus was used as positive control and the construct pNF-κB-Luc as a GATA4-independent control. Mean ± SE fold induction above the level induced by the p215 vector of at least three independent experiments. **A.** Transcriptional activity of a series of *ERBB2* promoter constructs in three breast cancer cell lines. **B** and **C.** Same series of results in BT-474 cells but restricted in **C** to the data obtained with p215, p716, p2029, and p6007 to emphasize the effects of GATA4 overexpression. **D.** Overexpression of GATA4 with p215 and p3798 in HCC-1954 cells.

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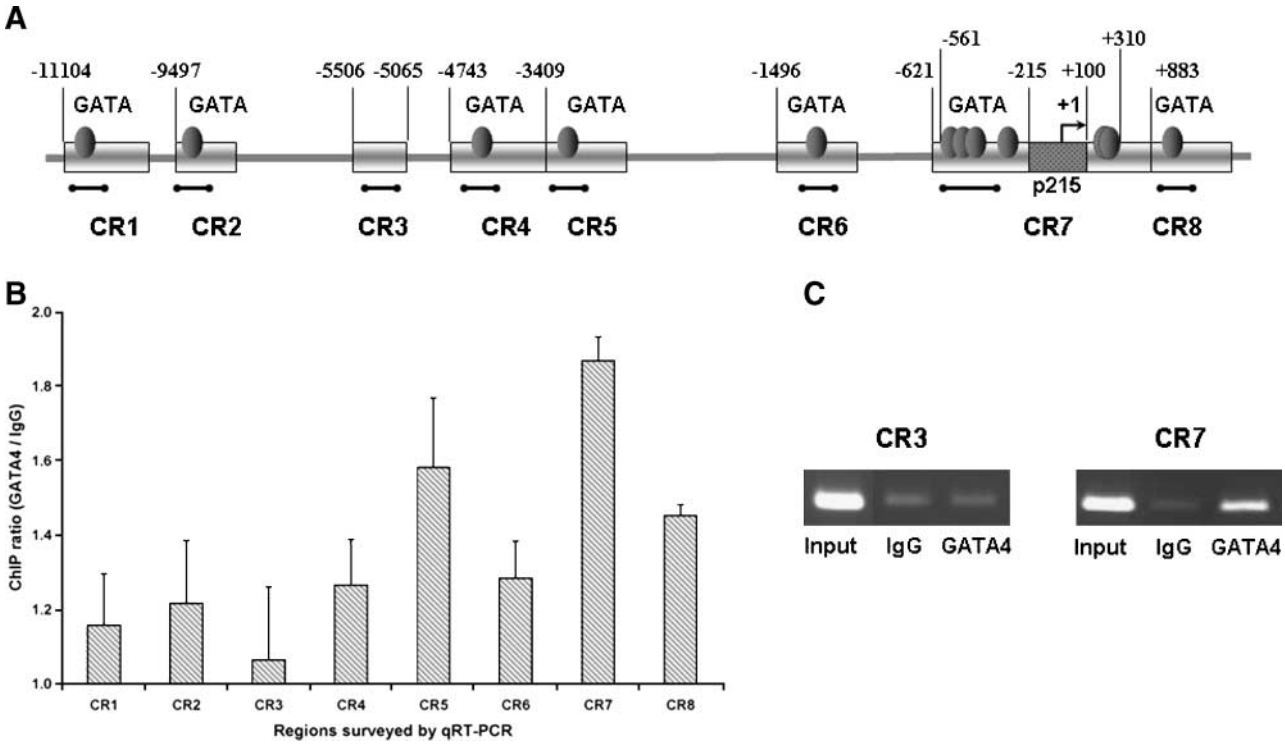

FIGURE 5. GATA4 is recruited *in vivo* at several locations of *ERBB2* 5′-flanking sequence in BT-474 breast cancer cells. **A.** Representation of the 5′-end flanking sequence of the human *ERBB2* gene showing the eight conserved regions between *Homo sapiens* and *Mus musculus*. Putative GATA binding sites found within seven of eight conserved regions. Pointed lines below the schematic map correspond to the fragments amplified by the chromatin immunoprecipitation-specific primers listed in Table 1. **B.** Quantitative chromatin immunoprecipitation assays done using a GATA4 monoclonal antibody as described in Materials and Methods. Mean ± SE of 3 to 6 independent experiments. **C.** Representative semiquantitative chromatin immunoprecipitation assays done with the *GATA4*-less CR3 region and the *GATA4*-containing CR7 region using either a GATA4 monoclonal antibody or a matched IgG_{2a} control as indicated below the lanes.

the GATA transcription factor family, a comparison between human and mouse gene sequences was done combined with a search for GATA binding sites. Eight conserved regions were characterized by analysis of the corresponding sequences of the human and murine *ERBB2* genes using the online software PipMaker[6] (Fig. 5A). Several potential binding sites for GATA factors were identified in seven of eight conserved regions by a search combining several methods (consensus WGATAR, Nucleotide Position Weight Matrix, etc.). Interestingly, the nucleotide [-156, +100] region, corresponding to the minimal promoter region included in the p215 construct, did not contain any identifiable GATA consensus site in agreement with the absence of effect of overexpressed GATA4 in transient transfection assay. Similarly, the nucleotide [-5446, -5065] CR3 region did not contain any GATA consensus site, whereas six GATA putative binding sites were found within CR7 located at nucleotide [-561, +310].

GATA4 Is Bound In vivo *to Several Locations in the ERBB2 Gene Enhancer/Promoter Region*

In vivo recruitment of *GATA4* was tested by chromatin immunoprecipitation assay in BT-474 cells. After cross-linking and sonication, the chromatin extract was immunoprecipitated by a commercial, GATA4-specific mouse monoclonal antibody and the corresponding DNA fragments were purified as described previously (35). Portions of the seven characterized conserved regions containing putative GATA binding sites plus GATA-less CR3 were amplified by PCR using specific primer pairs (Table 1) and quantified using SYBR Green chemistry and a Light Cycler 2 (Roche Diagnostics). As illustrated in Fig. 5B and C, several conserved regions were bound *in vivo* by GATA4 including the proximal regulatory promoter region included in CR7 and, to a less extent, CR4, CR5, and CR8. As expected, no significant recruitment of GATA4 was observed within CR3 devoid of any GATA consensus site but also in CR1, CR2 and CR6, notwithstanding the presence of bona fide GATA consensus sites. This confirms that not all consensus binding sites are accessible *in vivo* for the recruitment of a given sequence-specific transcription factor. Altogether, these results identified *in vivo* binding of GATA4 in several locations within the *ERBB2* gene enhancer/promoter region, in accordance with a direct role of this transcription factor in the regulation of human *ERBB2* gene transcription.

GATA4 Represses the ERBB2 Gene Enhancer/Promoter through a Positive Regulatory Element

Although chromatin immunoprecipitation assays revealed *in vivo* recruitment of GATA4 at several locations within the

[6] pipmaker.bx.psu.edu

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Table 1. qRT-PCR Oligonucleotide Primers Specific for the Eight Conserved Regions in *ERBB2* Gene and an Irrelevant Genomic Control Region Analyzed by Chromatin Immunoprecipitation

ERBB2 gene	Orientation	Sequence	PCR Product Size (bp)
CR1	Forward	5′-ATCCTCTCCCTGCTCACCTC-3′	164
	Reverse	5′-GGCTACTTCTTACTCATTCCAACC-3′	
CR2	Forward	5′-TGGTGGAAGTGGGAGTAGAGA-3′	223
	Reverse	5′-TGAAGCCAAATACAAGTTAGGAAG-3′	
CR3	Forward	5′-GTTCCTCGTCTCCTCTTCCTTG-3′	324
	Reverse	5′-TAGCTTTGCTTTGCCACCTG-3′	
CR4	Forward	5′-CTGCCCCTTTGCTGTCCT-3′	167
	Reverse	5′-AGGCTTGAGGTGTCCCTTTG-3′	
CR5	Forward	5′-ATCTCAAGGCTCAAGGTTCCTC-3′	323
	Reverse	5′-TCCAGGAGTCACTGGTTCATC-3′	
CR6	Forward	5′-AGCACATGGAAGCAAGTTTAG-3′	163
	Reverse	5′-CCCAGCCAAGAATGCAG-3′	
CR7	Forward	5′-TCCTTTCGATGTGACTGTCTCC-3′	339
	Reverse	5′-CTAAATGCAGAGGCTGGTGACT-3′	
CR8	Forward	5′-GCTTAGGGACTGTGCTCTGTG-3′	220
	Reverse	5′-TGGACAGATGGGTCAGGATAC-3′	
Control	Forward	5′-ATGGTTGCCACTGGGGATCT-3′	174
	Reverse	5′-TGCCAAAGCCTAGGGGAAGA-3′	

ERBB2 gene enhancer promoter, they cannot define which putative GATA binding sites are functional. For example, four GATA potential binding sites were identified within the 338-bp fragment nucleotide [-525, -187] amplified by PCR (Fig. 6A). Among these four putative binding sites, the WGATAR motif nucleotide [-286, -281] was strictly conserved between human and mouse. In addition, this GATA consensus was almost identical to a functional GATA4-specific binding site identified in the human *IL-5* gene promoter (Fig. 5B; ref. 36). Therefore, we designed a 21-bp oligonucleotide probe covering this WGATAR motif to perform electrophoretic mobility shift assays (EMSA) using nuclear extracts from BT-474 cells (Fig. 6B). Similar migration profiles were observed with both *IL-5* and *ERBB2 GATA* wild-type probes including five major retarded protein-DNA complexes (Fig. 6C, *lane 2*; data not shown). Competition using an excess of unlabeled wild-type double-stranded oligonucleotides abolished the three C1, C2, and C3 complexes (Fig. 6C, *lanes 3* and *5*), which were not affected by the corresponding mutant competitors (Fig. 6C, *lanes 4* and *6*). This showed that the three C1, C2, and C3 complexes are specific to the WGATAR motif present in the *ERBB2 GATA* probe.

The reaction mixtures were incubated with antibodies directed against five members of the GATA family (GATA1-4 and GATA6) to determine which of these factors may contribute to the formation of the specific protein-DNA complexes. As shown in Fig. 6D (*lane 7*), only the GATA4-specific antibodies retarded migration of C2 complex observed with the wild-type *ERBB2 GATA* probe. Accordingly, only GATA4 antibodies slowed down the migration of the corresponding complex detected using the wild-type *IL-5 GATA* probe (Fig. 6D, *lane 10*; data not shown).

To determine whether the bona fide GATA4 binding site at nucleotide [-286, -281] is transcriptionally activated, this site was abrogated by site-directed mutagenesis in the luciferase construct p3798 (Fig. 1C). Wild-type and mutated constructs were then transiently transfected in BT-474. As illustrated in Fig. 6E, the transcriptional activity was dramatically reduced in the p3798 Mut construct. The lack of further repression in the presence of coexpressed GATA4 evidenced that an intact WGATAR motif is required for GATA4-mediated repression of the *ERBB2* enhancer/promoter region. No significant differences were observed when using a low-activity p716 wild-type and p716 Mut plasmids (data not shown).

Altogether, these results strongly indicate that GATA4 mediates *ERBB2* down-regulation through a bona fide WGATAR motif embedded in a positive regulatory element required for elevated expression of the *ERBB2* gene.

Discussion

The molecular mechanisms leading to *ERBB2* overexpression in human cancers remain poorly characterized, except for the well-documented genomic amplification. However, *ERBB2* gene amplification alone is apparently not sufficient to explain all cases of RNA and protein overexpression (6) because a significant proportion of human cancers with increased expression of *ERBB2* do not exhibit gene amplification (37-39). There are converging lines of evidence that an increase of *ERBB2* mRNA levels per gene copy depends on *ERBB2* gene transcription. Several regulatory elements located near the major TSS of the *ERBB2* gene promoter have been characterized previously as well as some more distal elements up to 6 kb in the 5′-flanking sequence and in the *ERBB2* gene first intron (8, 12-14, 18-21). Altogether, data suggested their involvement in the increased transcription of the *ERBB2* gene observed in breast cancer cells. Two of the cognate transcription factors associated with the previously characterized regulatory elements have been identified as negative regulators of *ERBB2* expression in breast cancer. The ETS DNA-binding protein PEA3 targets specifically a DNA motif on the *ERBB2* gene promoter and down-regulates its promoter activity, but there is no clear evidence that genetic lesions of PEA3 can cause *ERBB2* overexpression (17). The transcription factor FOXP3 recently characterized as a X-linked breast cancer tumor suppressor in mice and humans represses transcription of the *ERBB2* gene via interaction with forkhead DNA-binding motifs in the *ERBB2* promoter (10). Although the functional relationships between these negative and positive transacting factors

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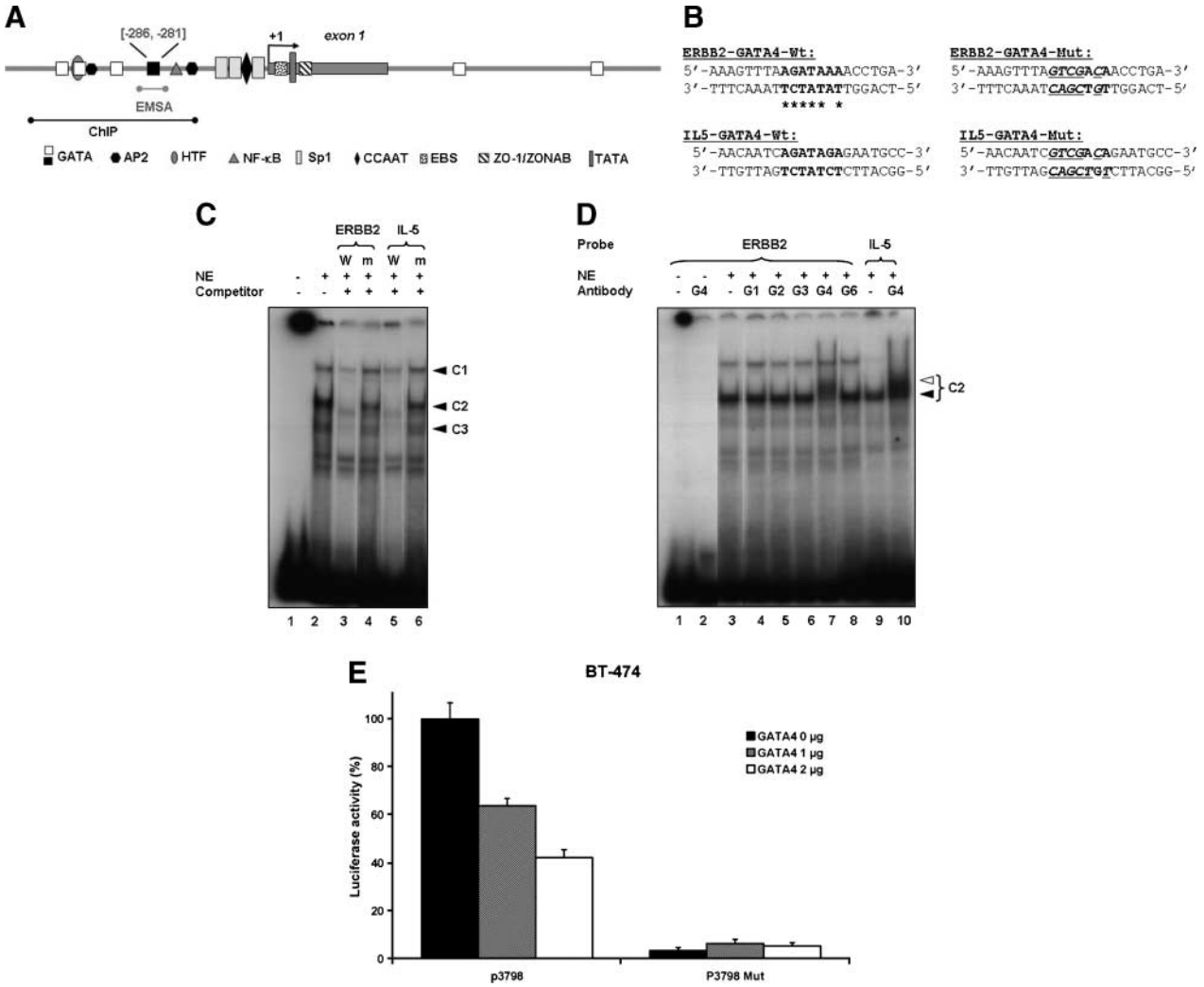

FIGURE 6. GATA4 represses *ERBB2* promoter activity though a bona fide GATA4 binding site acting as a positive regulatory DNA motif. **A.** Map of the region CR7 located at nucleotide [-561, +310] showing the previously reported regulatory elements (see Fig. 1A for details) as well as the 6 putative GATA binding sites (*G*) identified *in silico*. Closed square, conserved WGATAR motif located at nucleotide [-286, -281]. Major TSS (*+1*) and the first exon (*gray box*). The fragment located at nucleotide [-525, -187] amplified by PCR for the chromatin immunoprecipitation assays and the oligonucleotide probe nucleotide [-294, -274] used for the EMSA are shown below the map line as well as the symbols corresponding to the regulatory elements located on the map line. **B.** Sequences of the double-stranded oligonucleotide probes used for EMSA corresponding to the conserved putative GATA4 motif of the *ERBB2* gene (*ERBB2-GATA4*-Wt) and the functional GATA4 binding site of the human *IL-5* promoter described previously (ref. 36; *IL5-GATA4*-Wt) and their matched inactive substitution mutants (*ERBB2-GATA4*-Mut and *IL5-GATA4*-Mut, respectively). **C.** EMSA using BT-474 nuclear extracts and the radiolabeled *ERBB2-GATA4*-Wt probe in the absence (*lane 2*) or presence of a 50-fold molar excess of wild-type (*lanes 3* and *5*) and mutated (*lanes 4* and *6*) unlabeled double-stranded competitors as indicated above the lanes. Lane 1, migration of the radiolabeled probe in the absence of nuclear cell extract. Right, migration of the three specific protein-DNA complexes C1, C2, and C3 (*closed arrowheads*). **D.** Characterization of the *ERBB2*-GATA4 and *IL-5*-GATA4 motif binding proteins. BT-474 nuclear extracts were incubated 15 min at 4°C in the absence (*lanes 3* and *9*) or presence of GATA-specific antibodies (*lanes 4-8* and *10*) before addition of the radiolabeled *ERBB2-GATA4* and *IL5-GATA4*-Wt probes (*lanes 1-8* and *9-10*, respectively) as indicated above the lanes. Lanes 1 and 2, migration of the radiolabeled probe *ERBB2-GATA4*-Wt without nuclear cell extract in the absence or presence of GATA4 monoclonal antibody. Right, migration of the GATA4-containing complex C2 and its shifted position in the presence of GATA4 antibody (*closed* and *open arrowheads*, respectively). **E.** Luciferase reporter gene activity driven by either wild-type or a mutated p3798 construct (Fig. 1C) transiently transfected in BT-474 cells in absence or presence of overexpressed GATA4. Percentage ± SE luciferase activity relative to the activity of the p3798T without overexpressed GATA4 defined as 100.

remain largely unexplored, these results illustrate altogether the complexity of *ERBB2* gene transcriptional regulation that requires further investigation. In this context, we provide here evidence that the transcription factor GATA4 can also act as a transcriptional repressor of the *ERBB2* gene in breast cancer cells through its direct binding to *ERBB2* regulatory sequences.

Interestingly, the three best characterized transrepressors of the *ERBB2* gene (FOXP3, PEA3, and GATA4) appear to act through three positive regulatory elements embedding their cognate binding sites (see Fig. 3E and G in ref. 10, Fig. 3A in ref. 17, and Fig. 6D and E). This suggests the intervention of unidentified transactivators acting on these bifunctional regulatory elements. Accordingly, our EMSA has evidenced the presence of three specific protein-DNA complexes, whereas only the C2 complex was displaced by GATA4-specific antibodies, suggesting the presence of other sequence-specific transcription

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factors. The elucidation of the underlying molecular mechanisms requires further investigation to identify the transactivators associated to these bifunctional regulatory elements and their role in the dysregulations of *ERBB2* in cancer cells.

Our siRNA assays excluded an indirect effect of GATA4 via a putative action on the expression of the *FOXP3* gene because *GATA4* siRNA did not affect significantly *FOXP3* RNA levels. We also wondered whether cofactors of the FOG family that modulate the activity of the GATA transcription factors in mammals (40) might be involved. Although a role of the GATA4 partner FOG2/ZFPM2 (41) appears excluded in BT-474 cell line, which does not overexpress the corresponding gene (data not shown), an involvement of FOG1/ZFPM1 in GATA4-dependent repression of *ERBB2* is a worth investigating possibility (42).

A repressor role for GATA4 is in agreement with previous observations on this transcription factor, which can act as both a transcriptional activator and a repressor depending on various factors such as the target gene, the cell type, the interactions with other sequence-specific transcription factors, or even the localization of its binding site relative to the core promoter. For example, GATA4 activates the gene coding for P450c17 by direct interaction with SP1 (34), the *Grp78/BiP* gene in cooperation with ATF6 in embryonic heart (43), as well as the *IL-5* gene in T lymphocytes (36). In contrast, GATA4 acts as a repressor of the α2(I) collagen (*COL1A2*) via its binding to both proximal promoter and intronic elements (44, 45). Furthermore, GATA4 binds two sites of the *FGF3* promoter acting as positive and negative regulatory elements (46).

Inhibition of *GATA4* and *FOXP3* by *ERBB2*-targeted siRNA enlarges the list of transcription factors involved in ERBB2 signaling, which already include multiple factors such as FOS/JUN, EGR1, MYC, ELK, and SP1 (30). Altogether, these results and our own observations show that the transcriptional control of *ERBB2* is much more complex than expected and require further detailed investigation before designing any credible or risk-limited ''transcriptional'' therapy directed against either *ERBB2* regulatory elements and/or their cognate sequence-specific transcription factors.

GATA4 transcriptional activity is activated by the mitogen-activated protein kinase pathway in cardiomyocytes (29) and the ERBB family triggers several signaling pathways including this pathway (2). Although the activation of GATA4 by the mitogen-activated protein kinase pathway subsequent to *ERBB2* overexpression in breast cancer cells requires further exploration, it was reported that *GATA4* gene expression is induced by ERBB4 (47). Because GATA4 negatively regulates *ERBB2* expression, an *ERBB2/ERBB4/GATA4* regulatory loop might explain why co-overexpression of *ERBB4* with *ERBB2* in mammary carcinoma is associated with a more favorable clinical outcome than overexpression of *ERBB2* alone (48). Similarly, the regulatory networks that associate the multiple transcription factors activated by the ERBB2 receptor and the *ERBB2* gene itself need extensive investigation to determine which are direct targets and what are the putative regulatory loops linking these genes.

Trastuzumab, a humanized anti-ERBB2 antibody, is a major therapeutic agent for patients with overexpressed ERBB2 receptor on cancer cells. This treatment blocks the activity of the receptor by multiple and partially defined mechanisms, but resistance to trastuzumab is frequent (3). Some secondary cardiac effects have also been observed with this agent and more particularly when associated with anthracyclines (4, 5, 49). Studies in animals and cell culture have provided some insight into the mechanisms of trastuzumab-induced decrease of cardiac contractile function (for review, see ref. 49). Gene targeting studies and conditional deletion of *ERBB2* in mice have shown that *ERBB2* is essential for maintenance of normal cardiac structure and function. Collectively, published data suggest that one role of *ERBB2/4* signaling is to dynamically regulate sarcomere structure. In contrast, overexpression of the transcription factor GATA4, known to be important in the regulation of cardiac sarcomeric protein expression, protects the heart against anthracycline toxicity in a mouse model (50). It remains to establish whether the *ERBB2/GATA4* regulatory loop, solely characterized here in a breast cancer cell model, has any role in mature heart before trying to elucidate these apparently opposite outcomes.

Considering the major role played by *ERBB2* and *GATA4* in the development of cardiovascular system (26, 51), our results provide some new clues to further investigate the molecular basis of trastuzumab cardiotoxicity. The complex transcriptional regulation of the *ERBB2* gene and the transcriptional regulatory loop that associates this epidermal growth factor receptor and the cardiac-specific transcription factor GATA4 may indeed interfere with trastuzumab therapy. A precise and complete definition of these regulatory mechanisms is required for better understanding and fighting resistance to treatments targeting *ERBB2* as well as limiting their cardiotoxicity.

Materials and Methods

Cell Lines and Breast Carcinoma Samples

The nononcogenic human primary mammary epithelium cells HME-1 (Clontech) and the breast cancer cell lines BT-474, BT-483, HCC-202, HCC-1569, HCC-1806, HCC-1954, MDA-MB-175, MDA-MB-361, MDA-MB-453, SK-BR-3, UACC-812, ZR-75-30, and Br-Ca-Mz-01 (American Type Culture Collection[7]) and SUM-185, SUM-190, SUM-206, and SUM-225 (University of Michigan[8]; ref. 52) were grown according to the recommendations of the supplier. Seventeen breast carcinoma samples were obtained from women treated at Institut Paoli-Calmettes. *ERBB2* status of each sample was previously measured by comparative genomic hybridization array, transcriptome analysis, or immunohistochemistry (Table 2). Twelve breast carcinoma samples were *ERBB2* positive, whereas 5 samples were *ERBB2* negative according to these criteria.

Plasmids and Transient Transfection Experiments

The *ERBB2* reporter vectors containing promoter fragments of increasing sizes (13, 33) and GATA4 expression vector (34) were kindly provided by Dr. Rosita Winkler (Molecular Oncology Laboratory, University of Liege) and Dr. Walter L.

[7] http://www.atcc.org
[8] http://www.cancer.med.umich.edu/breast_cell/production

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Table 2. *ERBB2* and *GATA4* Gene Copy Numbers in Breast Cancer Carcinoma Biopsies and Cell Lines

Tumor Samples	Comparative Genomic Hybridization Gene Copies		Cell lines	Comparative Genomic Hybridization Gene Copies	
	ERBB2	GATA4		ERBB2	GATA4
9934	7	2	BT-474	26	2
7462	7	1	BT-483	2	1
12710	9	1	MDA-MB-453	5	1
13591	8	2	HCC-1806	2	2
8035	2	1	ZR-75-30	30	1
2933	4	1	MDA-MB-175	2	1
7780	4	1	UACC-812	24	1
13008	17	1	HCC-1569	50	1
9948	21	1	SUM-225	104	1
12781	15	1	SUM-185	1	1
8584	7	2	SK-BR-3	17	1
10982	2	2	SUM-206	2	1
9983	6	1	MDA-MB-361	6	1
9725	4	2	SUM-190	72	1
6604S	2	1	HCC-202	14	1
9840	2	2	HCC-1954	71	1
9745	2	1	Br-Ca-Mz-01	2	2
			HME-1	2	2

Miller (Department of Pediatrics and The Metabolic Research Unit, University of California at San Francisco), respectively. The pCMV-Luc and pNF-κB-Luc (Clontech) plasmids were used as positive controls. Cells were transfected using FuGENE 6 reagent (Roche Diagnostics). Cells (1×10^5-3×10^5) were seeded in six-well plates (Falcon 3046; BD Biosciences) and treated with a FuGENE/DNA ratio of 3 μL/2 μg for 48 h in complete medium. Sample (1 μg) of each reporter construct was cotransfected with 0.4 μg *Renilla* plasmid (pTK-RL; Promega) and 0.6 μg pUC19. Cells were harvested and lysed and luciferase activities were measured according to the manufacturer's instructions (dual luciferase reporter gene assay kit; Promega). *ERBB2* reporter vector activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity and then expressed for each construct as the ratio to the minimal *ERBB2* promoter vector (p215). GATA4 expression vector (0-0.2 μg) was cotransfected with 1 μg sample of each reporter construct, 0.4 μg pTK-RL, 0.3 μg pUC19, and 0.3 to 0.1 μg pNF-κB-Luc. Transfections were done in triplicate and repeated at least in three independent experiments.

RNAi Assay

Three 25-mer duplex siRNA to target *ERBB2* and *GATA4*, respectively, were obtained from a commercial source (Stealth select RNAi; Invitrogen). All siRNA duplexes (10 nmol/L) and Stealth RNAi Negative Control duplexes were transfected to 3×10^5 cells in six-well plates by Lipofectamine RNAiMAX (Invitrogen) for 48 h at 37°C in a CO_2 incubator according to the manufacturer's instructions. Gene knockdown was confirmed by qRT-PCR and immunoblotting. Inhibition effects were observed from all siRNA duplexes and the siRNA that induced the highest inhibition were used in three independent experiments.

qRT-PCR

Total RNA from cell lines was extracted using RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. Total RNA from tumor samples was extracted as described previously (53). RNA was quantified using a Nanodrop 1000 spectrophotometer device. RT-PCR was done using SuperScript II reverse transcriptase (Invitrogen) and random hexamer primers. DNA was quantified using LC FastStart DNA Master SYBR Green I and read with a Light Cycler 2 instrument (Roche Diagnostics) according to the manufacturer's instructions. The primers used for qRT-PCR analysis are listed in Table 3. The precise amount of total cDNA added to each reaction mix and its quality are both generally difficult to

Table 3. qRT-PCR Oligonucleotide Primers Used to Determine the Relative RNA Expression Level of *TBP, GATA4, ERBB2*, and *FOXP3* Genes

mRNA	Orientation	Sequence	Product Size (bp)
TBP	Forward	5′-CTTGTGCTCACCCACCAAC-3′	228
	Reverse	5′-GGAGGCAAGGGTACATGAGA-3′	
GATA4	Forward	5′-TCCAAACCAGAAAACGGAAG-3′	224
	Reverse	5′-CATCGCACTGACTGAGAACG-3′	
ERBB2	Forward	5′-GGGAAACCTGGAACTCACCT-3′	216
	Reverse	5′-CAGGGGTGGTATTGTTCAGC-3′	
FOXP3	Forward	5′-TACTTCAAGTTCCACAACATGCGACC-3′	202
	Reverse	5′-CGCACAAAGCACTTGTGCAGACTCAG-3′	

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assess. Therefore, the relative expression level of the gene of interest was computed with respect to the internal standard TBP to normalize for variations in RNA quality and the amount of input cDNA. Threshold cycles (Ct) were determined for quantification of the input target number.

For comparison between different cell lines and tumor samples, the target gene expression level (*ERBB2* or *GATA4*) in each cell line and tumor samples was normalized using a calibrator RNA sample extracted from the HME-1 cell line. For RNAi assay, the RGE was calculated as a percentage of the measured mRNA level in cells transfected with target siRNA compared with cells transfected with matched control siRNA. Data were normalized against the *TBP* signal and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of a given gene (54).

Immunoblotting

For immunoblotting, 10^5 BT-474 cells were lysed in 10 μL lysis buffer [25 mmol/L Tris (pH 7.9), 1% (w/v) SDS, 1 mmol/L DTT] and fractionated on a 7.5% SDS-polyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane. Specific polypeptides were revealed with the rabbit polyclonal anti-ERBB2 (Santa Cruz Biotechnology), mouse monoclonal anti-GATA4 (Santa Cruz Biotechnology) at a dilution of 1:200 in TBS-Tween 20 containing 5% nonfat milk, and mouse monoclonal anti-β-tubulin (Abcam) at a dilution of 1:1,000 in TBS-Tween 20 containing 5% bovine serum albumin and then reacted with secondary antibodies at a dilution of $1{:}10^4$ in the same buffer.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation assay was done using the Chromatin Immunoprecipitation Assay kit (Upstate). In brief, 2×10^6 BT-474 cells were treated by 1% formaldehyde for 15 min at 37°C, and the cross-linking reaction was stopped by the addition of 125 mmol/L glycine incubating 10 min at room temperature. Cells were rinsed twice with ice-cold 1× PBS containing protease inhibitors (Complete Protease Inhibitor Cocktail; Roche Diagnostics), scraped, and collected. After centrifugation, the cell pellet was lysed 10 min on ice in SDS lysis buffer supplemented with protease inhibitor cocktail. The chromatin was sheared by sonication to an average DNA length of 200 to 1,000 bp using a Sonics ultrasonic processor (750 W). The sonicated chromatin supernatant was diluted with Dilution Buffer containing protease inhibitor cocktail and subjected to a 30 min preclearing by incubation with salmon sperm DNA/protein A agarose-50% slurry beads at 4°C under rotation. The beads were pelleted, and the precleared chromatin supernatant was immunoprecipitated with 3 μg of either a matched nonimmune IgG control (mouse IgG_{2a}; BD Pharmingen) or a specific antibody (mouse monoclonal anti-GATA4; Santa Cruz Biotechnology) at 4°C overnight under rotation. Immunocomplexes were collected by 1 h incubation with salmon sperm DNA/protein A agarose-50% slurry at 4°C under rotation. Complexes were washed once, sequentially, in a low-salt buffer, high-salt buffer, and low LiCl buffer and twice in TE buffer. The antibody-protein-DNA complexes were eluted twice in 250 μL elution buffer (0.1 mol/L $NaHCO_3$, 1% SDS) by medium power vortexing and rotation for 15 min at room temperature. The combined eluates as well as the input sample (1% of the amount used in the immunoprecipitation procedure) were cross-link reversed by heating at least 4 h at 65°C in the presence of 200 mmol/L NaCl. After proteinase K digestion, the DNA fragments were extracted using phenol/chloroform and ethanol precipitated. qRT-PCR using LC FastStart DNA Master SYBR Green I and Light Cycler 2 instrument (Roche Diagnostics) and semiquantitative PCR were done with selected primer pairs (Table 1). For each experimental sample, the amount of target and endogenous reference was determined from a standard curve. The standard curve was constructed with 4-fold serial dilutions of input from the BT-474. The results from specific antibodies were reported to that observed in the corresponding IgG samples. A region between the *GAPDH* and *CNAP1* genes was used as the negative control to standardize the results from all the conserved regions (ChIP-It protocol; Active Motif).

EMSA

Complementary oligonucleotides carrying a GATA motif related to the GATA4-specific binding site of the *IL-5* gene promoter (36) and corresponding to nucleotide [-294, -274] of the wild-type *ERBB2* promoter (5′-AAAGTTTAAGA-TAAAACCTGA-3′ and 3′-TTTCAAATTCTATATTGGACT-5′) was labeled with [γ-^{32}P]ATP and T4 kinase. Labeled oligonucleotides were annealed and purified by Chroma Spin Columns (STE buffer; Clontech). Nuclear extracts from BT-474 cells (16.8 μg proteins) were incubated for 5 min at 20°C with 0.25 μg poly[(dI-dC)/(dI-dC)], 5 mmol/L HEPES, 1 mmol/L KCl, 49 mmol/L NaCl, 1 mmol/L EDTA, 5 mmol/L DTT, 4% glycerol, 1% Ficoll, 50 pg/mL pUC19, and 50-fold molar excess of unlabeled wild-type or mutated GATA binding site double-stranded competitors (5′-AAAGTTTAGTCGA-CAACCTGA-3′ and 3′-TTTCAAATCAGCTGTTGGACT-5′). Radiolabeled probe (0.5 ng; 5×10^5 cpm) was then added to the reaction mixture for 10 min at 20°C. The complexes were resolved on a 5% polyacrylamide gel in TBE buffer [0.025 mol/L Tris base, 0.022 mol/L boric acid, and 0.5 mmol/L EDTA (pH 8.3)] at 200 V. The gel was dried and analyzed by autoradiography. Supershift assay was done by incubating antibodies against GATA1 to GATA4 and GATA6, before the addition of radiolabeled probe, with the reaction mixture for 15 min at 4°C.

Site-Directed Mutagenesis

Site-directed mutagenesis was carried out using QuikChange II Site-Directed Mutagenesis Kit (Stratagene). PCR was done in *ERBB2* reporter vectors using following primers: forward 5′-gatgcaagctccccaggaaagtttagtcgacaacctgagacttaaaagggtgt-3′ and reverse 5′-acacccttttaagtctcaggttgtcgactaaactttcctggg-gagcttgcatc-3′. PCR products were digested by *Dpn*I restriction enzyme and transformed in XL1-Blue supercompetent cells. Mutated *ERBB2* luciferase reporter vectors were confirmed by sequencing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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