Upregulation of Neutrophil Gelatinase–Associated Lipocalin by ErbB2 through Nuclear Factor-κB Activation

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Abstract

ErbB2 (HER2, neu) is a receptor tyrosine kinase overexpressed in about 25% of invasive breast carcinomas. Neutrophil gelatinase–associated lipocalin (NGAL) is a secreted glycoprotein expressed in a variety of cancers, including breast carcinomas. NGAL can inhibit erythroid cell production, leading to anemia. Anemia usually occurs in cancer patients and negatively affects quality of life. However, current treatment for cancer-related anemia has potential complications. ErbB2, NGAL, and anemia have all been associated with increased metastasis and poor prognosis in breast cancer patients, although the relationship between ErbB2 and NGAL expression is not clear. Here, using breast cancer cell lines *in vitro* and transgenic mice carrying the activated c-neu oncogene driven by a mouse mammary tumor virus (MMTV-neu) *in vivo*, we show that ErbB2 overexpression leads to NGAL upregulation, which is dependent on nuclear factor-κB (NF-κB) activity. MMTV-neu transgenic mice developed anemia after tumor onset, and anemia progression could be partially arrested by a NF-κB inhibitor and ErbB2-targeted therapy. Taken together, upregulation of NGAL by ErbB2 through NF-κB activation is involved in cancer-related anemia, and the ErbB2, NF-κB, and NGAL pathways may serve as potential therapeutic targets for cancer-related anemia. [Cancer Res 2009;69(24):9163–8]

Introduction

ErbB2 overexpression has been correlated with poor prognosis and increased incidence of metastasis in breast cancer patients (1). Anemia commonly occurs in breast cancer patients and is also associated with poor prognosis, in addition to having profound negative effect on patients' quality of life (2). Current treatment for cancer-related anemia is blood transfusion or using erythropoiesis-stimulating agents; each has a potential risk of severe complications (3). Blood transfusions can result in infectious disease transmission and transfusion-related reactions (3), and recent phase III trials revealed that erythropoiesis-stimulating agents increased the risk of developing venous thromboembolism and decreased survival rates in anemic cancer patients (4). Therefore, it is necessary to develop new treatment modalities for cancer patients with anemia.

Neutrophil gelatinase–associated lipocalin (NGAL; also known as *LCN2* or lipocalin 2) is a 25-kDa monomer capable of homodimerization and heterodimerization with neutrophil gelatinase (5). NGAL, identified as a component of neutrophil granules, inhibits bacterial growth by depleting their intracellular iron stores (6). NGAL is also a promising biomarker of kidney injury that predicts acute renal impairment (7). NGAL overexpression is found in a variety of cancers, including breast carcinomas (8), and is a predictor of poor prognosis and increased risk of metastasis in breast cancer patients (8). Additionally, NGAL was shown to induce apoptosis of primary bone marrow cells, including erythroid progenitor cells, and inhibit erythroid cell production leading to anemia (9–12). ErbB2, NGAL, and anemia have all been associated with poor prognosis in breast cancer patients. However, the association between ErbB2 and NGAL is debatable (8, 13). We postulated that ErbB2 overexpression may upregulate NGAL and play a role in cancer-related anemia. Indeed, we found that ErbB2 overexpression upregulates NGAL in a nuclear factor-κB (NF-κB)–dependent manner. Additionally, MMTV-neu transgenic mice developed anemia after mammary tumor onset around a mean time of 24 weeks. Furthermore, NGAL expression and anemia progression could be partially inhibited by blocking the NF-κB pathway or by ErbB2-targeted therapy.

Materials and Methods

Cell lines and antibodies. 231BR.vec, 231BR.erbB2, MCF7.vec, and MCF7.erbB2 cells were described previously (14, 15). Antibodies used are NGAL (HYB 211-01, Antibodyshop), NGAL (M-145, Santa Cruz), Neu (C-18, Santa Cruz), β-actin (Sigma), phosphor-IκBα (Cell Signaling), and IκBα (Cell Signaling). Immunoblotting was done as previously described (16).

Plasmids. The pGL3-NGAL-267 plasmid was obtained by PCR amplification of genomic DNA using forward primer 5′-CCACATACAGGGCAATCAGA-3′ and reverse primer 5′-GATTTCAGGGCCGAGGAAG-3′. This PCR fragment was ligated into pCR 2.1-TOPO II TA cloning vector (Invitrogen), then cloned into pGL3-basic (Promega) using the *Hin*dIII and *Xho*I restriction enzymes. The pGL3-NGAL-267(−267 to +69) plasmid contains 336 bp from the region upstream of the translational start site on the *LCN2* gene. All plasmids were sequenced across the multiple cloning sites of pGL3-basic to verify sequence integrity. Truncated deletion promoter plasmids, pGL3-NGAL-154(−154 to +69) and pGL3-NGAL-138(−138 to +69), were generated by PCR using pGL3-NGAL-267 as the template with the primers 5′-CTCTGTCTTGCCCAATCCTG-3′ and 5′-CCTGACCAGGTGCAGAAATC-3′, respecitively.

Conditioned media. Conditioned media were collected after the indicated breast cancer cells were cultured for 48 h in serum-free DMEM/F-12 to 80% to 90% confluency and concentrated using centrifugal filter units (Millipore).

Drug treatments. Indicated cells were treated with 2 μg/mL trastuzumab (Genentech, Inc.) or human normal IgG (Jackson ImmunoResearch Laboratories, Inc.) for 6 h and with NF-κB inhibitor, Bay 11-7082 (Sigma), for 4 h. Conditioned media were collected 48 h later and assayed as indicated.

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Luciferase assays. Luciferase assays were done as previously described (17).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay was done as previously described (17). Antibodies used were IgG, histone H1, and NF-κB subunits p50 and p65 (all from Santa Cruz). Coprecipitated DNA (2 μL) was analyzed by PCR (30–35 cycles) using specific primers, 5′-AAGGCACAGAGGGAGTCGTTGTCC-3′ and 5′-AAGAGGAGGTGGCGAGTGAGAGGC-3′, to the NF-κB binding region of the NGAL promoter.

Immunohistochemistry. Immunohistochemistry was done as previously described (16).

Animal experiments. MMTV-neu transgenic mice were obtained from Dr. William Muller (McGill University, Montreal, Quebec, Canada). MMTV-polyoma middle T antigen transgenic mice (PyMT) and FVB wild-type mice were obtained from The Jackson Laboratory. Tail-vein blood (200 μL) from mice was collected in microcontainers containing EDTA at the indicated time. Whole blood was processed for complete blood cell (CBC) counts by the Veterinary Laboratory Medicine Core Facility, Department of Veterinary Medicine and Surgery at The University of Texas M.D. Anderson Cancer Center. MMTV-neu mice were palpated for mammary tumors twice a week. Bay 11-7082 (5 mg/kg) was dissolved in 0.5% methyl cellulose and injected i.p. three times a week for 2 wk (200 μL/mouse). The same amount of DMSO dissolved in 0.5% methyl cellulose was used as the control for Bay 11-7082. The monoclonal antibody (mAb) 7.16.4 (2 mg/kg) was injected i.p. twice a week for 2 wk, and mouse IgG was used as a control. Protein extraction from mammary tumors was done as previously described (16).

Statistical analysis. Statistical differences were assessed with paired *t* test, unpaired *t* test, or one-way ANOVA as indicated. $P < 0.05$ was considered statistically significant.

Results

ErbB2 overexpression leads to increased NGAL expression *in vitro* and *in vivo*. To determine if overexpression of ErbB2 induces NGAL expression, we compared NGAL protein levels in conditioned media from MDA-MB-231BR and MCF7 human breast cancer cells stably overexpressing ErbB2 (231BR.erbB2 and MCF7.erbB2) or vector controls (231BR.vec and MCF7.vec). We found that ErbB2 high-expressing 231BR.erbB2 and MCF7.erbB2 cells secreted more NGAL than ErbB2 low-expressing vector control cells after serum starvation (Fig. 1*A*). To further investigate whether ErbB2 overexpression induces NGAL, we compared the NGAL expression in mammary tumors from MMTV-neu transgenic mice with that from MMTV-PyMT transgenic mice. Mammary tumors from MMTV-neu mice had higher NGAL expression than those from MMTV-PyMT mice (Fig. 1*B*), indicating ErbB2 overexpression induced NGAL expression.

To determine whether ErbB2 was required for NGAL upregulation, we treated 231BR.vec and 231BR.erbB2 cells with trastuzumab, an ErbB2 antibody, to inhibit ErbB2 signaling. Indeed, trastuzumab treatment reduced NGAL production along with ErbB2 downregulation in 231BR.erbB2 cells compared with the IgG control (Fig. 1*C*). Next, we tested if downregulation of ErbB2 in MMTV-neu mice could lead to decreased NGAL expression. When tumors became palpable, MMTV-neu mice were injected either with mAb 7.16.4 targeting the rat neu oncogene or with mouse IgG. Similar to trastuzumab treatment of 231BR.erbB2 cells, mAb 7.16.4 treatment of tumor-bearing MMTV-neu mice led to ErbB2 downregulation and reduced NGAL expression compared with IgG-treated controls (Fig. 1*D*). Together, these data indicate that ErbB2 overexpression induces NGAL upregulation.

ErbB2 upregulates NGAL expression in a NF-κB–dependent manner. To investigate the mechanism of ErbB2-mediated NGAL upregulation, we examined NGAL mRNA levels in 231BR.vec and 231BR.erbB2 cells by reverse transcription-PCR (RT-PCR) after serum starvation. The 231BR.erbB2 cells had increased NGAL mRNA levels compared with 231BR.vec cells (Fig. 2*A*). To determine whether increased NGAL mRNA was due to transcriptional upregulation, we cloned the proximal region of NGAL promoter, known to be involved in NGAL transcriptional regulation, into a luciferase reporter (pGL3-NGAL-267; Fig. 2*B*). Relative luciferase activity was

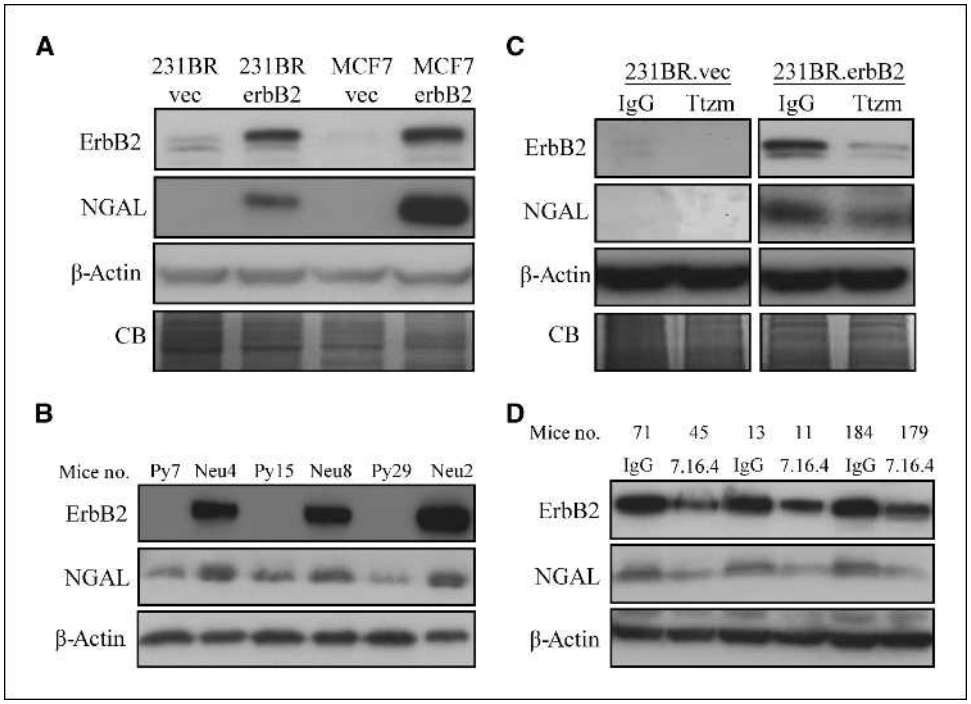

Figure 1. NGAL protein expression correlates with ErbB2 status *in vivo* and *in vitro*. *A*, Western blot analysis revealed that breast cancer cell lines stably overexpressing ErbB2 secreted more NGAL in conditioned media than did vector control cell lines. *B*, mammary tumors from MMTV-neu mice had higher NGAL expression than did tumors from MMTV-PyMT mice. *C*, following trastuzumab (*Ttzm*) treatment, Western blot analysis in 231BR.vec and 231BR.erbB2 cells confirmed that NGAL production was dependent on high ErbB2 expression levels. *D*, after ErbB2 is downregulated by mAb 7.16.4, NGAL protein expression in mammary tumors decreased in MMTV-neu transgenic mice. *CB*, Coomassie blue staining.

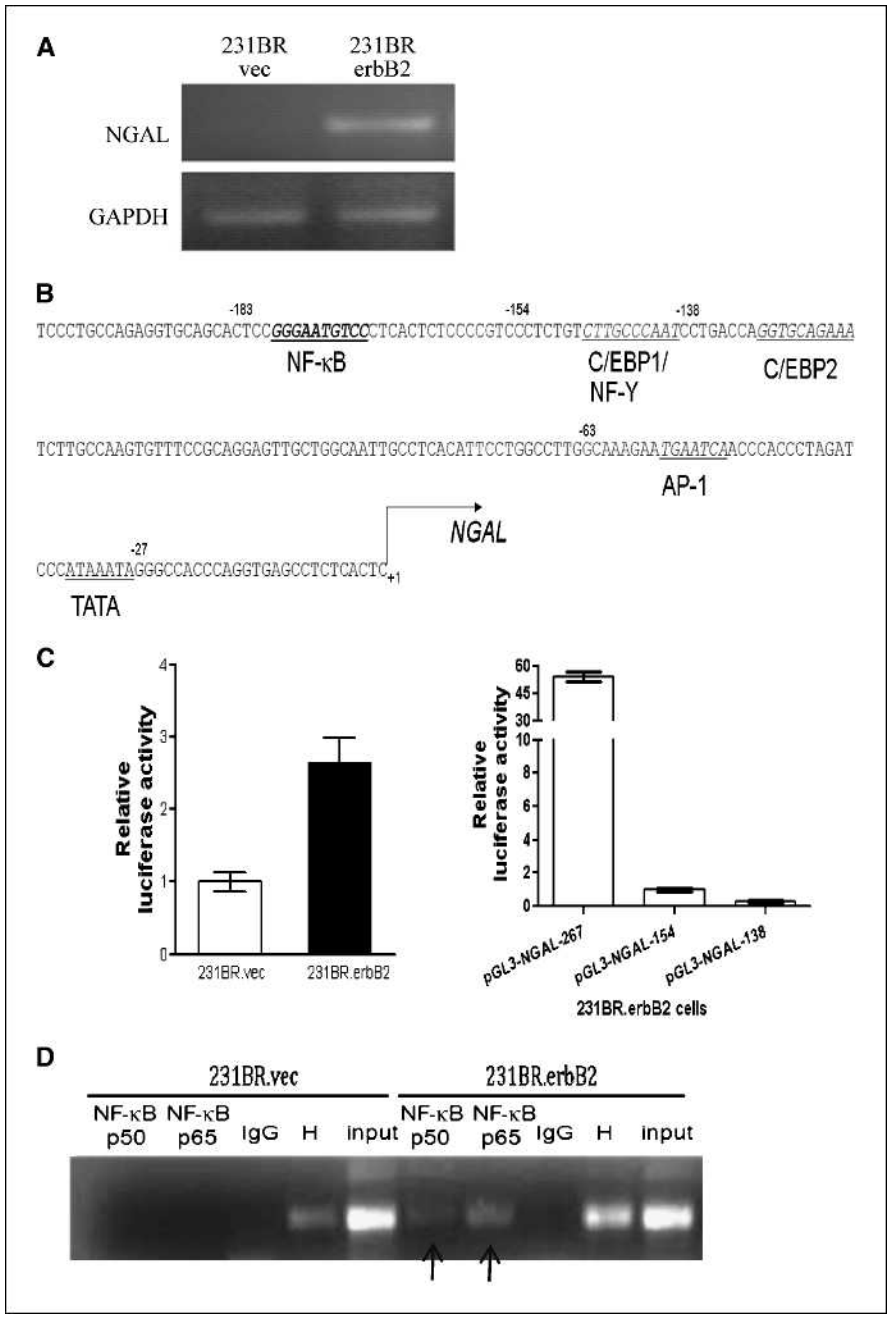

Figure 2. ErbB2 overexpression leads to increased NGAL production through increased NF-κB activation, resulting in transcriptional upregulation. *A,* RT-PCR showed NGAL mRNA levels increased in 231BR.erbB2 cells compared with 231BR.vec cells. *B,* DNA sequencing shows truncation of the NGAL promoter at the 5′ end; putative regulatory consensus binding sites of the NGAL gene are also indicated. *C, left,* 231BR.erbB2 cells transfected with pGL3-NGAL-267 had higher luciferase activity than 231BR.vec cells transfected with pGL3-NGAL-267. *Right,* luciferase activity is shown relative to the −267 to −154 NGAL promoter regions. *Columns,* mean; *bars,* SD. *D,* chromatin immunoprecipitation assay revealed that NF-κB subunits p65 and p50 were recruited to the NGAL promoter in 231BR.erbB2 cells. Histone H1 and IgG immunoprecipitations served as positive and negative controls, respectively.

higher in 231BR.erbB2 cells transfected with pGL3-NGAL-267 than in 231BR.vec cells, indicating NGAL upregulation at the transcriptional level (Fig. 2*C, left*). To further identify the critical promoter region(s) for NGAL upregulation, we generated two NGAL promoter 5′ deletion constructs, pGL3-NGAL-154 and pGL3-NGAL-138. 231BR.erbB2 cells failed to induce NGAL promoter–driven luciferase activity when NGAL promoter is deleted to −154 (Fig. 2*C, right*), indicating that the sequence between −267 and −154 is necessary for ErbB2-mediated NGAL transcriptional upregulation. Among the sequences between −267 and −154, the sequences between −180 and −171 has a homology to the NF-κB binding-site consensus sequence, 5′-GGG(A/G)NNT(C/T)CC-3′ (18). Indeed, chromatin immunoprecipitation assay showed that NF-κB subunits p50 and p65 bind to the NGAL promoter in 231BR.erbB2 cells after serum starvation (Fig. 2*D*).

To further determine the contribution of NF-κB in ErbB2-mediated NGAL upregulation, we treated 231BR.erbB2 cells with Bay 11-7082, which inhibits IκBα degradation and blocks NF-κB translocation from the cytoplasm to the nucleus. Bay 11-7082 treatment decreased NGAL levels in the conditioned media from 231BR.erbB2 cells by inhibiting NF-κB binding to the NGAL promoter (Fig. 3*A* and *B*). Furthermore, treatment of tumor-bearing MMTV-neu mice with Bay 11-7082 reduced phospho-IκBα, NF-κB nuclear localization, and NGAL expression in mammary tumors relative to control-treated mice (Fig. 3*C* and *D*). These data indicate that NF-κB signaling is important for ErbB2-mediated NGAL upregulation.

Cancer-related anemia can be partially rescued by blocking the NF-κB pathway and by ErbB2-targeted therapy. To test if anemia occurs in tumor-bearing MMTV-neu mice, we collected blood from the tail veins and analyzed the CBC counts before mammary tumor development at 16 wk of age and again when mammary tumors were palpable at 24 wk. As controls, we also counted CBC from the wild-type mice at 24 wk of age. After tumor onset, MMTV-neu mice had significantly ($P < 0.01$) lower hemoglobin levels than did wild-type mice and MMTV-neu mice before their tumors appeared (Fig. 4*A*). To investigate whether blocking the NF-κB pathway can impede anemia progression in tumor-bearing MMTV-neu mice and MMTV-PyMT mice, we treated mice i.p. with Bay 11-7082 or placebo. CBC counts before and after treatment showed that anemia progression could be partially reversed by Bay 11-7082 treatment in MMTV-neu mice, but not in MMTV-PyMT mice (Fig. 4*B*). Additionally, the ErbB2-targeted therapy with mAb 7.16.4 also partially arrested the anemia progression in tumor-bearing MMTV-neu mice (Fig. 4*C*).

Discussion

In this study, we found that ErbB2 induces NGAL expression through NF-κB activation, which contributes to anemia in mice with ErbB2/neu-overexpressing tumor; whereas a NF-κB inhibitor and ErbB2-targeted therapy can decrease NGAL expression in ErbB2/neu-overexpressing mammary tumor and partially reverse anemia progression. Previous studies reported that NGAL can induce erythroid progenitor cell apoptosis, inhibit RBC production, and lead to anemia (10–12). NF-κB is also responsible for impaired erythropoietin synthesis, which contributes to the development of cancer-related anemia (19). Our findings are consistent with previous reports; thus, we propose that both NGAL and NF-κB may play critical roles in cancer-related anemia, especially in ErbB2/neu-overexpressing tumor-related anemia. Anemia negatively influences cancer patients' overall quality of life and worsens their prognosis. Unfortunately, current treatment for cancer-related anemia has limitations. Because NF-κB inhibitor is currently under clinical trials (20) and ErbB2-targeted therapy (trastuzumab) is routinely used for patients with ErbB2-overexpressing breast cancers (1), our data provide a rational base for using these targeted therapies as potential new treatments for cancer-related anemia, which could have an impact on breast cancer patient care. Meanwhile, further studies are necessary to elucidate the underlying mechanism of NGAL and NF-κB in cancer-related anemia and identify better therapeutic targets, as the NF-κB inhibitor or ErbB2-targeted therapy had partial efficacy in treating cancer-related anemia in MMTV-neu mice. In conclusion, our data provided evidence that NF-κB plays a pivotal role in ErbB2-mediated NGAL upregulation and cancer-related anemia. This pathway may serve as a promising therapeutic target itself and provide insight for further identification of other targets.

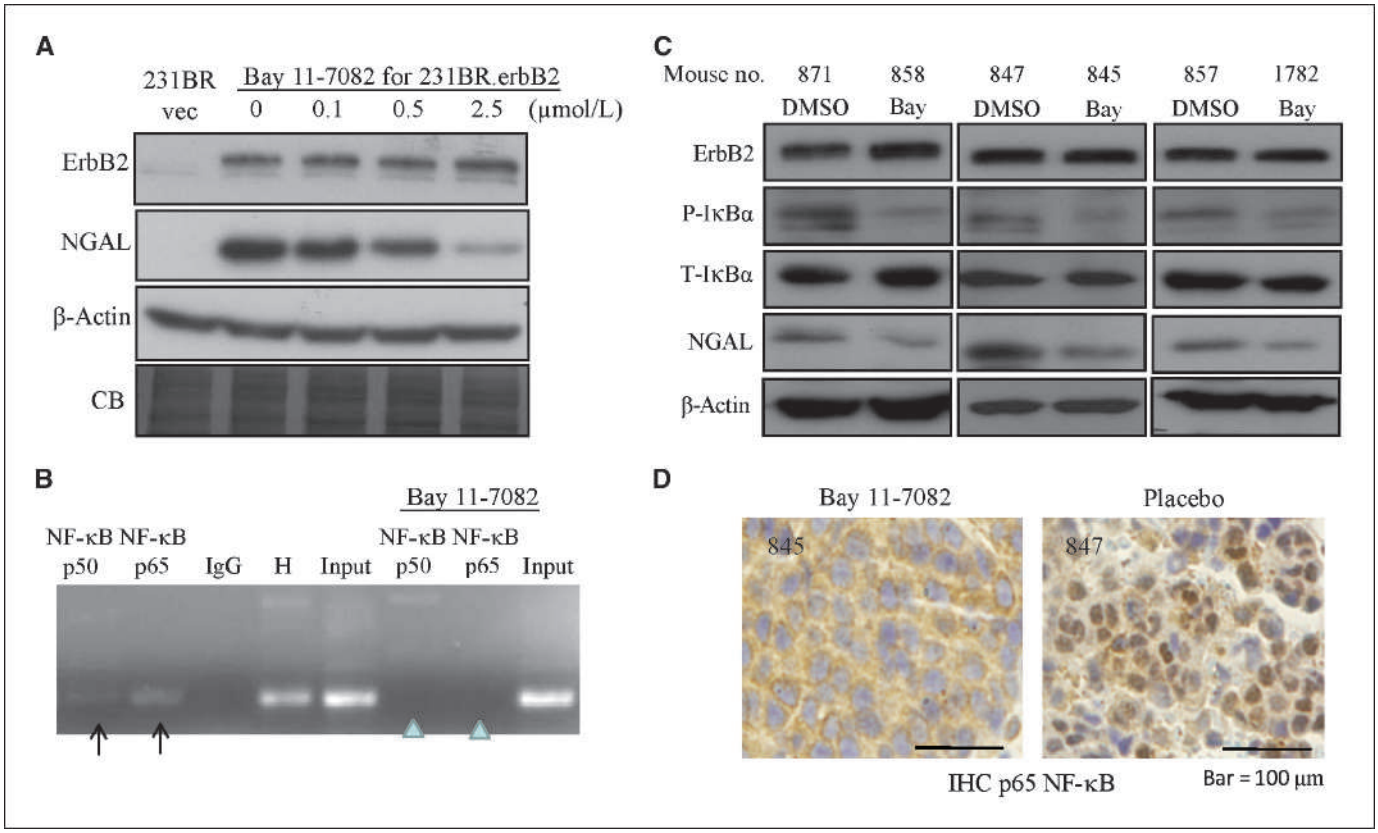

Figure 3. NGAL protein expression decreased after blocking NF-κB activity using Bay 11-7082 *in vitro* and *in vivo*. *A,* Bay 11-7082 treatment decreased NGAL levels in the conditioned media from 231BR.erbB2 cells. *B,* chromatin immunoprecipitation assay revealed that Bay 11-7082 inhibited NF-κB binding to the NGAL promoter in 231BR.erbB2 cells. *C,* treatment of tumor-bearing MMTV-neu mice with Bay 11-7082 reduced phospho-IκBα and NGAL expression in mammary tumors relative to control-treated mice. *D,* NF-κB subunit p65 nuclear staining decreased in tumor-bearing MMTV-neu mice treated with Bay 11-7082. *IHC,* immunohistochemistry.

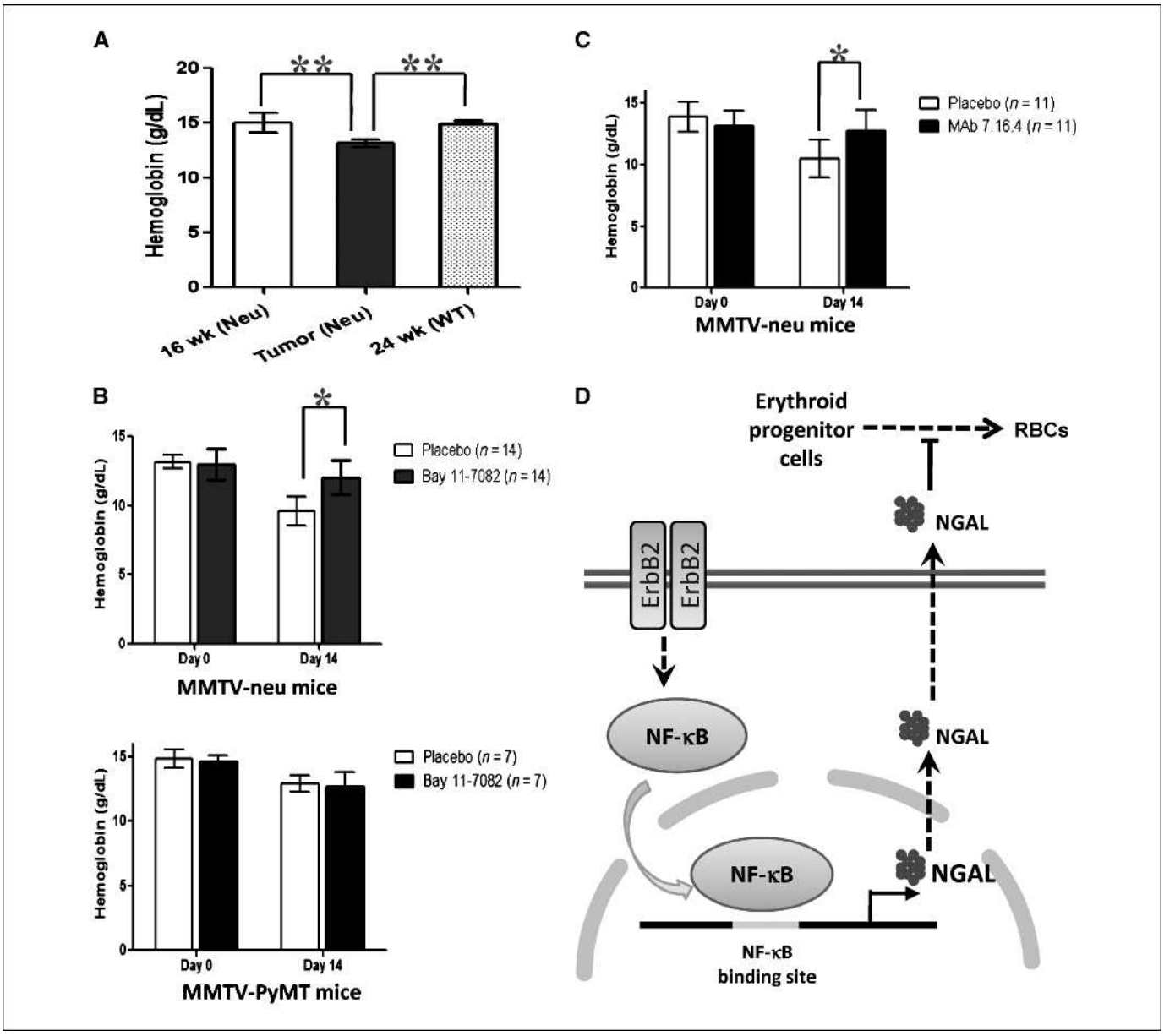

Figure 4. *A,* compared with MMTV-neu mice ($n = 38$) at 16 wk (before tumor onset) and wild-type (*WT*) mice ($n = 7$) at 24 wk, hemoglobin levels of tumor-bearing MMTV-neu mice ($n = 38$) showed significant anemia after tumor onset. *B,* anemia progression after tumor onset was partially arrested by Bay 11-7082 in MMTV-neu mice (*top*), but not in MMTV-PyMT mice (*bottom*). *C,* mAb 7.16.4 also partially arrested the anemia progression in tumor-bearing MMTV-neu mice. *D,* scheme for ErbB2-mediated NGAL upregulation through NF-κB signaling. *Columns,* mean; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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