

Cross-talk between HER2 and MED1 Regulates Tamoxifen Resistance of Human Breast Cancer Cells

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Abstract

Despite the fact that most breast cancer patients have estrogen receptor (ER) α -positive tumors, up to 50% of the patients are or soon develop resistance to endocrine therapy. It is recognized that HER2 activation is one of the major mechanisms contributing to endocrine resistance. In this study, we report that the ER coactivator MED1 is a novel cross-talk point for the HER2 and ER α pathways. Tissue microarray analysis of human breast cancers revealed that MED1 expression positively correlates most strongly with HER2 status of the tumors. MED1 was highly phosphorylated, in a HER2-dependent manner, at the site known to be critical for its activation. Importantly, RNAi-mediated attenuation of MED1 sensitized HER2-overexpressing cells to tamoxifen treatment. MED1 and its phosphorylated form, but not the corepressors N-CoR and SMRT, were recruited to the ER α target gene promoter by tamoxifen in HER2-overexpressing cells. Significantly, MED1 attenuation or mutation of MED1 phosphorylation sites was sufficient to restore the promoter recruitment of N-CoR and SMRT. Notably, we found that MED1 is required for the expression of not only traditional E2-ER α target genes but also the newly described EGF-ER α target genes. Our results additionally indicated that MED1 is recruited to the *HER2* gene and required for its expression. Taken together, these findings support a key role for MED1 in HER2-mediated tamoxifen resistance and suggest its potential usage as a therapeutic target to simultaneously block both ER α and HER2 pathways for the treatment of this type of endocrine resistant breast cancer. *Cancer Res*; 72(21); 5625–34. ©2012 AACR.

Introduction

Estrogen receptor (ER) α is the key mediator of estrogen functions in the breast and plays prominent roles in breast cancer (1–5). In fact, about 70% of all breast cancer patients have ER-positive tumors, whereas selective estrogen receptor modulators such as tamoxifen have been widely used in the treatment of these patients. Unfortunately, up to half of all ER-positive tumors either do not respond to this endocrine therapy or, after initial successful treatment, the tumors recur as endocrine-resistant breast cancer (6–10). It has been recognized that activation of the tyrosine kinase ErbB-2/HER2 is one of the major mechanisms contributing to the endocrine resistance (10–12). However, although blockage of HER2 with the monoclonal antibody trastuzumab (Herceptin) has been successfully used as a second-line treatment, again, resistance to this therapy is quite high. Hence, further development of

novel strategies to selectively block the activities of these pathways remains a major challenge for the treatment of human breast cancer.

HER2 is amplified and overexpressed in 20% to 30% of invasive breast cancers and has been implicated as a major player in both *de novo* and acquired tamoxifen resistance (7, 12–18). Several clinical studies have also indicated that HER2 overexpression is associated with a poor outcome in tamoxifen-treated patients. It was found that ectopic overexpression of HER2 in MCF-7 cells is sufficient to confer these cells with tamoxifen resistance (19). Further studies showed that mitogen-activated protein kinase (MAPK) activated by HER2 signaling can phosphorylate both ER α and its cofactors to enhance their activities (11, 16, 20–22). Significantly, this cross-talk between HER2 and ER α pathways has now been recognized as one of the key mechanisms that confers endocrine therapy resistance to human breast cancers.

Recent studies have established mediator subunit 1 (MED1) as a key ER α coactivator both *in vitro* and *in vivo* (23–30). It has been shown that ectopic MED1 expression is able to markedly enhance ER α functions, whereas knockdown of MED1 impairs both ER α -regulated transcription and estrogen-dependent growth of breast cancer cells. MED1 directly interacts with ER α through its 2 classical LxxLL motifs on its central region, whereas its C-terminus is likely to play important regulatory roles, as recent studies have shown that MED1 can be phosphorylated and activated by the MAP kinase pathway on threonines at the C-terminal 1,032 and 1,457 sites (31–34). Interestingly, further biochemical analyses indicated that

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MED1 exists only in a subpopulation of the Mediator complex with distinct subunit compositions (29). Importantly, most recent animal studies further revealed that MED1 is expressed only in selected cell types and plays rather cell-, tissue-, and gene-specific roles in mediating estrogen functions *in vivo* (30). Significantly, MED1 has been reported to be overexpressed in a high percentage (40%–60%) of human breast cancer cell lines and primary breast cancers (25, 35–37).

Consistent with previous reports that the *MED1* gene is located within the *HER2* amplicon on the chromosome 17q12 region and often coamplifies with *HER2* (25, 35), here we provided further evidence showing that MED1 protein expression levels correlate most strongly with the HER2 status of human breast cancer by tissue microarray (TMA) analyses. Significantly, our studies showed that MED1 is phosphorylated at the above-mentioned key MAP kinase sites by HER2 activation. Subsequent mechanistic studies supported a key role for MED1 and its cross-talk with HER2 in tamoxifen-induced coactivator/corepressor switch on the ER α target gene promoter. Moreover, we went on to further explore the underlying molecular mechanisms of MED1 in HER2-mediated tamoxifen resistance and found that MED1 is not only required for the expression of both E2-ER α and EGF-ER α target genes, but also for the optimal expression of HER2 itself.

Materials and Methods

Cell culture

The breast cancer cell lines MCF-7 and BT474 were purchased from American type culture collection and MCF-7/HER2 cells were described previously (38). Cell lines were authenticated on the basis of viability, recovery, growth, and morphology. The expression status of ER α , HER2, and MED1 was further confirmed by Western blot before they were used in the experiments. All cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) medium containing 10% FBS (Hyclone) at 37°C with 5% CO₂ in tissue culture incubators. The MAP Kinase inhibitor PD95089 and HER2 inhibitor AG825 were purchased from Calbiochem and EMD chemicals, respectively. 17 β -estradiol (E2) and 4-hydroxytamoxifen (TAM) were purchased from Sigma. For experiments involving E2 or TAM treatments, cells were routinely cultured in phenol red-free DMEM plus 10% charcoal-stripped FBS for at least 3 days before the treatments.

Plasmids and lentiviral vector preparation

The plasmid pERE-TK-Luc (ERE, estrogen response element), pRL-CMV, pcDNA3.1-MED1, pLKO.1-MED1 short hairpin RNA (shRNA), and pLKO.1-scramble shRNA were described previously (29). To generate lentiviral constructs expressing double-phosphomutant MED1 (T1032A and T1457A, termed DM MED1), PCR-mediated mutagenesis was conducted using pcDNA3.1-MED1 as a template. The PCR products were then cloned into lentiviral vector pCDH-CMV (System Biosciences) and the sequence confirmed by sequencing (Genewiz). High titer lentiviruses were generated by transient transfection of 293T cells vector only, pCDH-CMV-WT MED1 or pCDH-CMV-

DM MED1 with packaging constructs according to the manufacturer's instructions (System Biosciences).

Transient transfection and reporter gene assays

MCF-7 and MCF-7/HER2 cells were first plated in 24-well plates containing phenol red-free DMEM medium supplemented with 10% charcoal-stripped FBS. Control vector only (pcDNA3.1) or pcDNA3.1-MED1 was cotransfected with plasmids expressing ERE-TK-Luc reporter gene by using Lipofectamine 2000 (Invitrogen). pRL-CMV plasmids were also cotransfected to serve as a transfection efficiency control. Following the transfection, the cells were treated with TAM for 24 hours before harvest. A dual luciferase reporter assay system (Promega) was used to measure the luciferase activity.

Real-time reverse transcription PCR

Total RNA was isolated using RNeasy Mini Kits (Qiagen) and reverse transcribed using a SuperScript III first strand synthesis system (Invitrogen). Real-time PCR was then conducted using SYBR Green PCR Master Mix reagents (Roche) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the following primers: *cyclin D1*: 5'-CGCCCCACCCCTC-CAG-3' and 5'-CCGCCAGACCCTCAGACT-3'; *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*: 5'-CGGAGTCAACGGATTGGTCGTA-3' and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. The primers used to detect *TFF1*, *Myc*, *ACP6*, and *LIF* genes were as described (39, 40).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was conducted as described previously (29, 41). In brief, MCF-7 and MCF-7/HER2 cells were treated for 45 minutes with vehicle (ethanol), 100 nmol/L E2 or 1 μ mol/L TAM and immediately fixed by adding formaldehyde to the medium to a final concentration of 1%. After PBS washing, cells were harvested and the nuclear lysates were sonicated to generate an average DNA size of 0.5 to 1 kb. Immunoprecipitation experiments were then conducted with antibodies against MED1 (29), p-MED1 (34), N-CoR, and SMRT (Santa Cruz Biotechnology). Real-time PCR amplifications were conducted after reverse cross-linking and extraction of DNA from immunoprecipitated chromatin. The primers for *TFF1* promoter, ER α -binding site on *HER2* gene, *ACP6* promoter, and *LIF* enhancer2 all have been previously described (40, 42).

MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) assays were conducted as described previously with minor modifications (39). Two thousand cells per well were seeded in 96-well plates and treated with vehicle or tamoxifen at indicated concentrations in regular DMEM supplemented with 10% FBS for 7 days. MTT was added to the medium to a final concentration of 0.5 mg/mL and incubated for 30 minutes at room temperature. The medium was then removed and 0.2 mL DMSO was added. The absorbance of the converted dye was measured at 570 nm using a Synergy spectrophotometer (Biotek).

Immunohistochemistry staining

Immunohistochemistry (IHC) staining of human breast cancer TMA was carried out essentially as previously described (30). In brief, the slide was first deparaffinized and subjected to heat-induced antigen retrieval using citrate buffer. The tissue sections were then incubated with primary antibodies against MED1 overnight at 4°C, followed by extensive washes. The slide was subsequently treated with biotinylated anti-rabbit secondary antibody and then developed using avidin-conjugated horseradish peroxidase with diaminobenzidine as the substrate (VECTASTAIN Elite ABC kit, Vectorlab). Hematoxylin was used for counterstaining and the images were visualized and captured using axioplan imaging 2e microscope (Zeiss).

Statistical analysis of the data

All experiments were repeated at least 3 to 5 times and data expressed as average \pm SD. Statistical analyses of the data were conducted by pairwise Student's *t* test. Differences are considered statistically significant (*) if $P \leq 0.05$ and very significant (**) if $P \leq 0.01$. For TMA analyses, the staining intensity of MED1 in tumor cells was scored by a pathologist using a scale of 0 to 3 and analyzed by age-adjusted spearman correlation. The information on age, TNM, tumor grade, and HER2 status were provided by the manufacturer (BR962, 48 cases/96 cores, US Biomax).

Results

MED1 expression levels strongly correlate with HER2 status in human breast cancer

We have previously shown that MED1 is required for ER α -mediated transcription and breast cancer cell growth (29). MED1 has also been reported to be overexpressed in a high

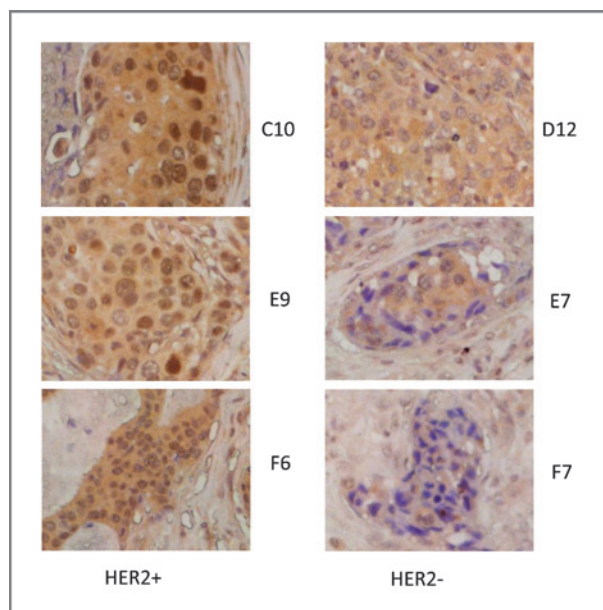


Figure 1. MED1 protein expression highly correlates with HER2 status of human breast cancer. Representative images of MED1 IHC staining of HER2-positive(+) and negative(-) human breast cancer samples are shown.

Table 1. Age-adjusted spearman analyses of MED1 staining with HER2 status, tumor grade and stage (TNM) using human breast cancer TMA

	Nuclear MED1		Cytosolic MED1	
	R	P	R	P
Grade	0.005	1	0.03	0.8
TNM	-0.15	0.3	0.06	0.7
HER2	0.35	0.007	0.02	0.9

percentage of human breast cancer cell lines (25, 35–37). To further explore the role of MED1 in breast cancer, we decided to assess the MED1 protein expression in human breast cancer by using TMA analysis. To achieve that, we carried out immunohistochemical staining of the human breast cancer TMA BR962 (US Biomax) using anti-MED1 antibodies (Fig. 1). The staining intensity of both nuclear and cytosolic MED1 in these tumor sections was scored using a scale of 0 to 3. By using age-adjusted spearman correlation, we found that nuclear, but not cytoplasmic, MED1 expression was highly correlated with HER2 status, but not with tumor grade or stage (TNM) of the human breast cancer tissue samples (Table 1).

HER2 overexpression enhances MED1 phosphorylation via activation of MAP kinase pathway

Previous studies have shown that MED1 can be phosphorylated and activated by the MAP kinase pathway (31–33). It is also known that the MAP kinase pathway is a key downstream pathway activated by HER2 amplification (15). Therefore, we decided to carry out experiments to determine whether HER2 activation could lead to MED1 phosphorylation. For this, we used an antibody generated specifically against phosphopeptides containing the phosphorylation site of MED1 (Threonine 1023; ref. 34). We first compared phosphor-MED1 levels using MCF-7 cells and BT474 cells that have HER2 amplification, and found that phosphorylated MED1 were present at significantly higher levels in BT474 cells (Fig. 2A). As BT474 cells may contain additional alterations that can also affect MED1 phosphorylation, we further used MCF-7/HER2 cells to determine whether HER2 overexpression alone is sufficient to enhance MED1 phosphorylation. Consistent with the results above, we found that ectopic overexpression of HER2 in MCF-7 cells was sufficient to induce MED1 phosphorylation at this MAPK site (Fig. 2B). Consistent with previous reports that MED1 phosphorylation could also increase its stability, we also found a slight increase of total MED1 protein levels in MCF-7/HER2 cells, whereas the mRNA levels of *MED1* was not affected (Fig. 2C). Importantly, AG825, a specific inhibitor for HER2, significantly inhibited MED1 phosphorylation in both BT474 and MCF-7/HER2 cells (Fig. 2D and E). Moreover, we found that treatment of MCF-7/HER2 cells with MAP kinase inhibitor PD98059 significantly decreased MED1 phosphorylation levels (Fig. 2F). Taken together, these data support a key role for

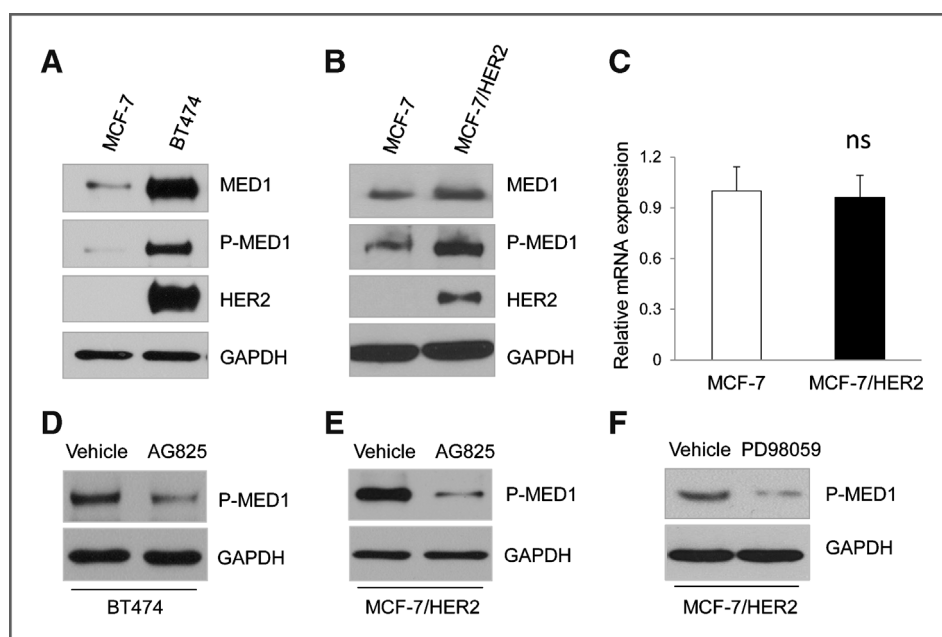


Figure 2. HER2 overexpression regulates MED1 phosphorylation through MAP kinase pathway. A and B, whole-cell extracts from MCF-7, BT474, and MCF-7/HER2 cells were prepared and subjected to Western blot analyses with anti-MED1, p-MED1, HER2, or GAPDH antibodies. C, total RNA was extracted from MCF-7 and MCF-7/HER2 cells, followed by real-time RT-PCR analyses of MED1 expression and normalization to GAPDH levels. ns, not significant. D and E, Western blot analyses of p-MED1 levels in BT474 (D) and MCF-7/HER2 (E) cells after control vehicle or 50 $\mu\text{mol/L}$ AG825 treatment. F, MCF-7/HER2 cells were treated with 25 $\mu\text{mol/L}$ PD98059 or vehicle for 24 hours, and subjected to Western blot analyses with anti-p-MED1 and anti-GAPDH (control) antibodies.

HER2 overexpression in mediating MED1 phosphorylation through the MAP kinase pathway.

Knockdown of MED1 sensitizes HER2 overexpression cells to tamoxifen treatment

HER2 overexpression has been recognized as a key mechanism in conferring endocrine resistance in human breast cancer. Our data above, indicating both coexpression and cross-talk between HER2 and MED1, led us to further test whether MED1 plays a role in the tamoxifen resistance of HER2-overexpressing cells. To achieve that, we infected the BT474 and MCF-7/HER2 cells with lentivirus expressing control scramble or MED1 shRNA and measured cell proliferation by MTT assay after control vehicle or tamoxifen treatments. As shown by Western blot analyses (Fig. 3A and B), MED1 shRNA treatment successfully knocked down MED1 protein expression in both cells. Importantly, we found that MED1 knockdown significantly sensitized these HER2-overexpressing cells to tamoxifen treatment, as compared with control scramble shRNA-treated cells (Fig. 3C and D Supplementary Fig S1A). As a control, we also conducted the same experiments using above-mentioned MCF-7 cells that do not express high levels of HER2. We found that these cells are already highly sensitive to tamoxifen treatment, as previously reported, and that MED1 knockdown does not significantly alter their sensitivity to tamoxifen (Supplementary Fig. S1B and S1C). These data support a key role for MED1 in mediating the tamoxifen resistance of HER2-overexpressing cells.

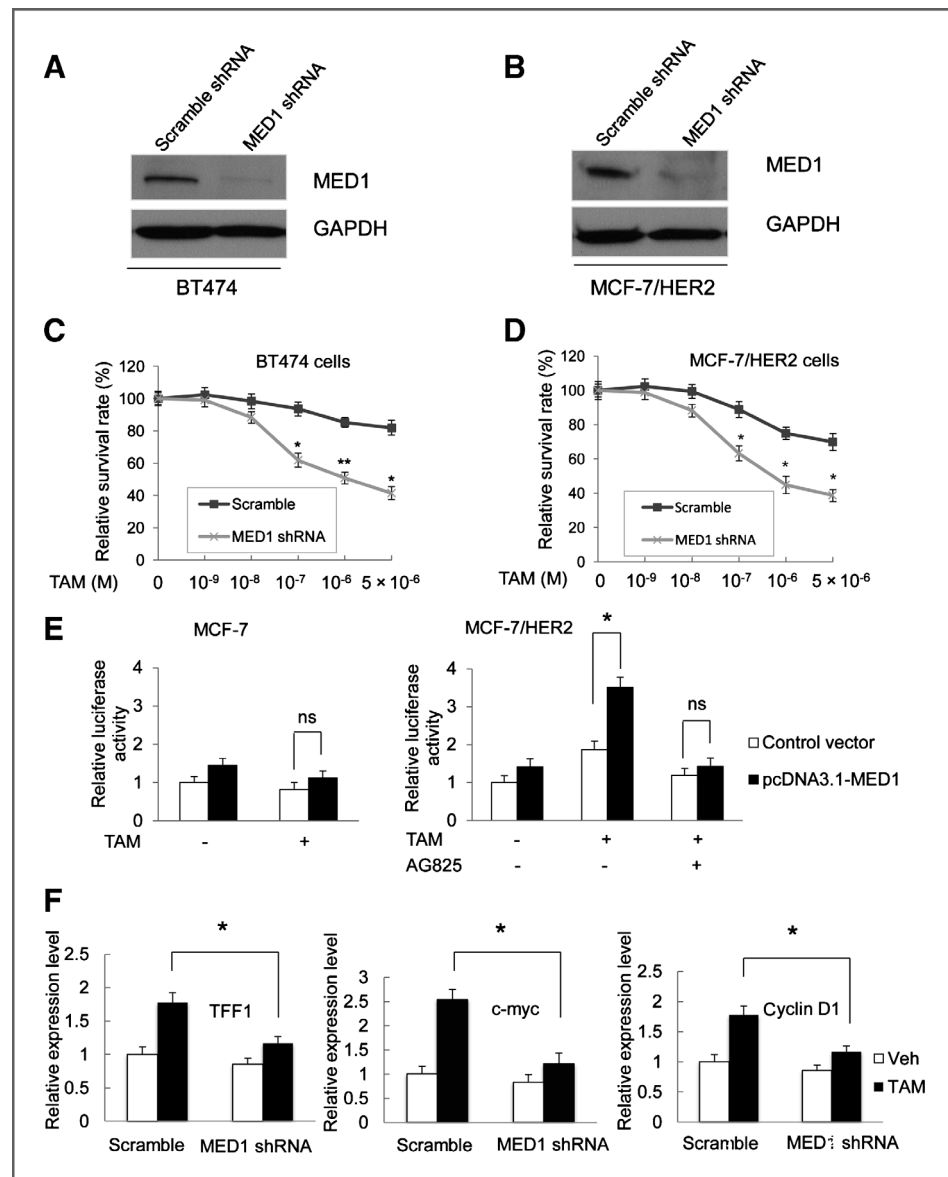
Previous studies have shown that tamoxifen plays an agonist role on ER α -mediated transcription in HER2-overexpressing cells (14, 16). Thus, we carried out to determine the effect of MED1 on ER α transcriptional activity and estrogen-responsive gene expression in response to tamoxifen. In agreement with previous studies, we found that tamoxifen could induce ER α transcriptional activity in MCF-7/HER2 cells but not in MCF-7

cells (Fig. 3E). Interestingly, we found that transient expression of MED1 could further enhance ERE-reporter expression by tamoxifen treatment in MCF-7/HER2 cells. Importantly, AG825 could totally abolish this tamoxifen induced ERE-reporter gene activation by MED1, indicating an HER2 dependency. In addition, we have also examined the requirement of MED1 for tamoxifen-induced expression of several well-known endogenous ER α target genes (*TFF1*, *cyclinD1*, and *c-Myc*) in these cells. As shown in Fig. 3F, our results indicate that MED1 is indeed required for the expression of tamoxifen-induced expression of these endogenous ER α target genes. Moreover, we also examined the potential effect of MED1 on the transcription of the ER α gene and found that MED1 knockdown does not affect the ER α mRNA level in MCF-7/HER2 cells (Supplementary Fig. S2).

MED1 inhibits the recruitment of N-CoR and SMRT to TFF1 promoter by tamoxifen in HER2-overexpressing cells

Transcriptional corepressors N-CoR and SMRT have been shown to play important roles in the antiproliferative action of tamoxifen in breast cancer cells through repression of ER α target genes in HER2-overexpressing cells (43–45). To gain a better understanding of the mechanism of how activation of MED1 phosphorylation by HER2 may lead to endocrine resistance, we conducted ChIP assays to examine the role of MED1 on the recruitments of N-CoR and SMRT on the promoter of above TFF1 gene, the most widely used and best-characterized estrogen-responsive gene. As expected, we found tamoxifen-induced promoter occupancy of N-CoR and SMRT but not MED1 or p-MED1 on the *TFF1* promoter in MCF-7 cells (Fig. 4A). However, we observed instead an increased occupancy of MED1 and p-MED1, but not N-CoR or SMRT on *TFF1* gene promoter in MCF-7/HER2 cell in the presence of tamoxifen. Importantly, treatment with HER2 inhibitor AG825 effectively

Figure 3. Knockdown of MED1 sensitizes HER2 overexpression cells to tamoxifen treatment. A and B, Western blot analyses of MED1 levels in BT474 and MCF-7/HER2 cells after control scramble or MED1 shRNA treatments. C and D, control or MED1 shRNA knockdown BT474 cells (C) or MCF-7/HER2 cells (D) were treated with vehicle (Veh) or indicated amount of TAM for 7 days. Cells were then harvested and assessed for cell proliferation by MTT assays. E, MCF-7 and MCF-7/HER2 cells were transfected with vector control pcDNA3.1 or pcDNA3.1-MED1, along with plasmids expressing ERE-TK-LUC reporter and PRL-TK (internal control), followed by vehicle (ethanol) or 1 μ M TAM treatment for 24 hours. The relative luciferase values are expressed as mean \pm SE. F, real-time RT-PCR was conducted to determine *TFF1*, *Myc*, and *cyclin D1* mRNA levels in control scramble or MED1 shRNA knockdown MCF-7/HER2 cells after normalization to that of GAPDH. (*, $P < 0.05$; **, $P < 0.01$).



restored the recruitment of N-CoR and SMRT to *TFF1* gene promoter by tamoxifen. Furthermore, we found that knockdown of MED1 by shRNA in these cells can also block this tamoxifen-induced occupancy of MED1 and p-MED1, and also restore tamoxifen-induced recruitment of N-CoR and SMRT (Fig. 4B). Moreover, we have carried out re-ChIP assay and confirmed that MED1 and ER α are acting on the same *TFF1* promoter in MCF-7/HER2 cells by tamoxifen (Supplementary Fig. S3A). Finally, re-ChIP assays again supported that gain of the interactions between N-CoR/SMRT and ER α occurred on the same *TFF1* promoter in a MED1-dependent manner (Supplementary Fig. S3B). These results are consistent with the hypothesis that MED1 phosphorylation by HER2 plays key roles in preventing tamoxifen-induced recruitment of N-CoR and SMRT to the promoter of ER α target genes in HER2-overexpressing cells. In addition, we found that knockdown of

MED1 also increases tamoxifen-induced recruitment of ER α corepressor HDAC1 but has no effect on the recruitment of either ER α itself or ER α coactivator CBP (Supplementary Fig. S4).

Wild type but not phosphor mutant MED1 prevents tamoxifen-induced N-CoR and SMRT recruitment

To further determine whether MED1 phosphorylation is required for the inhibition of the recruitment of these corepressors (N-CoR and SMRT) to the *TFF1* gene promoter in HER2-overexpressing cells, we further generated a double phosphomutant MED1 (DM MED1) with both threonines (T1032 and T1457) known to be activated by the MAP kinase pathway mutated to alanine (Fig. 5A). Lentivirus expressing WT and DM MED1 was then used to infect MCF-7/HER2 cells pretreated with MED1 shRNA for the rescue experiments. We found that

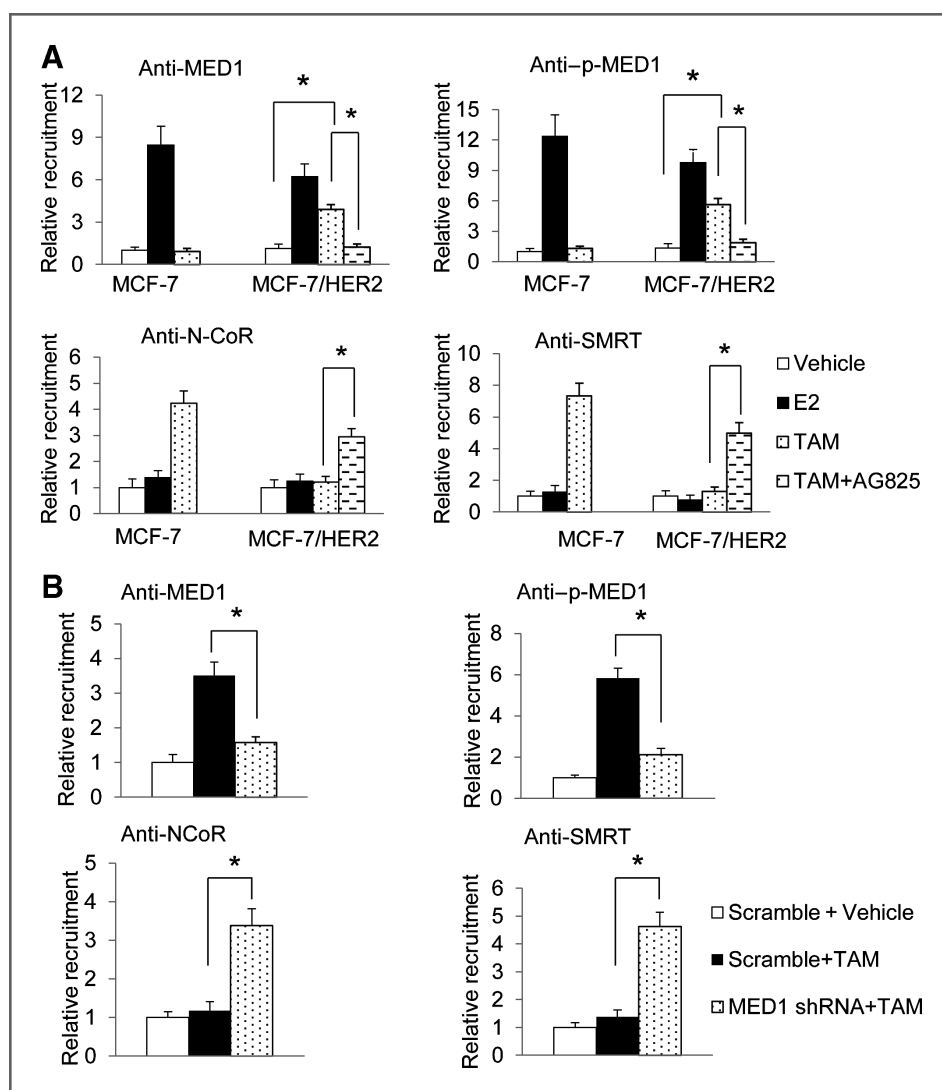


Figure 4. MED1 regulates the recruitment of N-CoR and SMRT to TFF1 promoter by tamoxifen. A, MCF-7 and MCF-7/HER2 cells were treated with 100 nmol/L E2, 1 μ mol/L TAM or 50 μ mol/L AG825 for 45 minutes. The soluble chromatin was then prepared and subjected to immunoprecipitation experiments using control IgG or antibodies against MED1, p-MED1, N-CoR, or SMRT. Immunoprecipitated DNA corresponding to the TFF1 promoter region was amplified and quantified by real-time PCR. B, chromatin immunoprecipitation experiments were carried out as in A by using control scramble or MED1 shRNA treated MCF-7/HER2 cells.

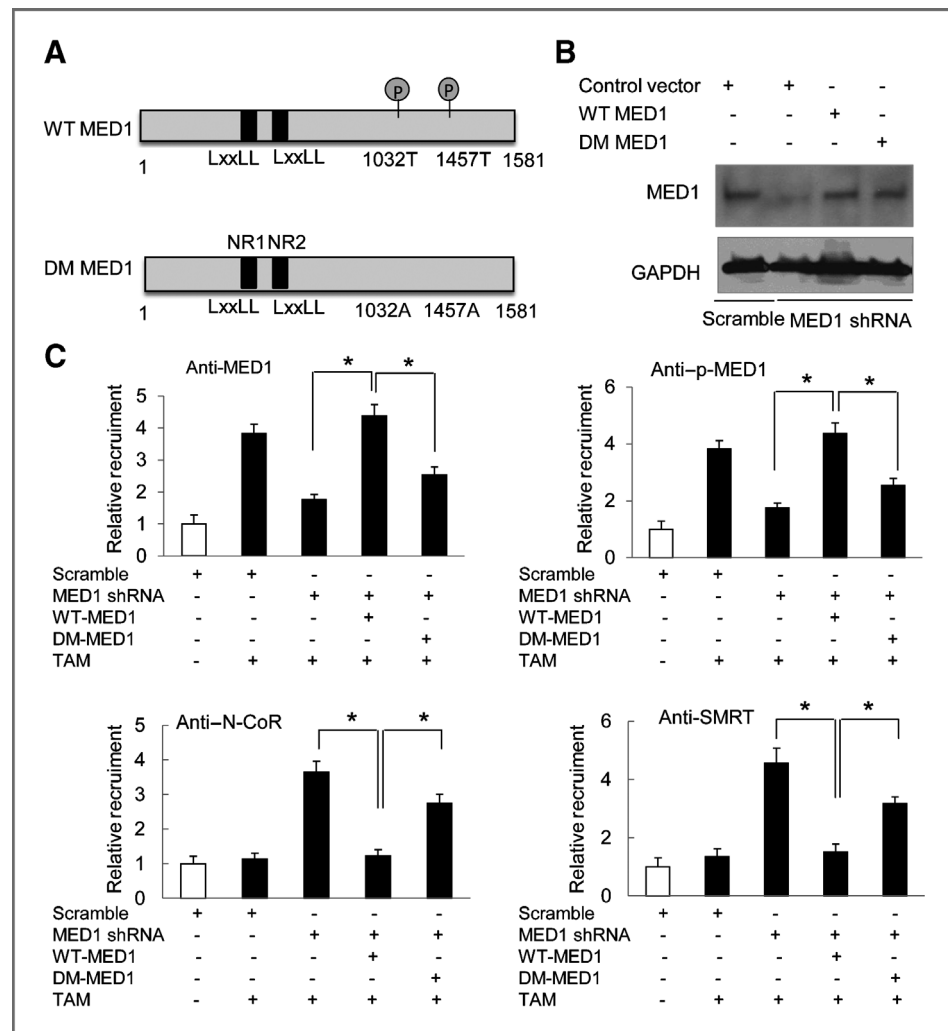
although both WT and DM MED1 express at a very similar level as shown by Western blot analyses (Fig. 5B), only WT MED1 but not DM MED1 can effectively restore the tamoxifen-induced recruitment of MED1 and p-MED1 to *TFF1* gene promoter (Fig. 5C). We also found that expression of WT MED1 completely abolishes tamoxifen-induced recruitments of N-CoR and SMRT to *TFF1* gene promoter. However, in contrast, expression of DM MED1 failed to prevent the promoter recruitments of either N-CoR or SMRT by tamoxifen. Taken together, these findings support the importance of MED1 phosphorylation sites on tamoxifen-induced recruitment of transcriptional corepressors N-CoR and SMRT in HER2-overexpressing cells.

MED1 regulates EGF-induced ER α target genes in MCF-7/HER2 cells

Most recently, a genome-wide ER α -cistrome analysis revealed that growth factors such as EGF can stimulate the binding of ER α to a distinct group of genes (e.g., *ACP6* and *LIF*; Fig. 6A; ref. 40). These genes were named EGF-ER α target genes as they are different from previously described E2-

induced ER α target genes (e.g., *TFF1* and *CyclinD*). Importantly, these EGF-ER α target genes are also overexpressed in HER2-positive breast cancers. To determine whether MED1 can also regulate the expression of this type of genes in HER2-overexpressing cells, we first examine the chromatin occupancy of MED1 on *ACP6* and *LIF* promoters. As shown in Fig. 6B, we found that MED1 is present on the promoter of *ACP6* and *LIF* genes. Importantly, the presence of MED1 on these promoters is significantly enhanced in HER2 overexpressing MCF-7/HER2 cells when compared with that of MCF-7 cells. Significantly, treatment with AG825 again totally abolished this increased recruitment of MED1 to the promoters of *ACP6* and *LIF* genes in MCF-7/HER2 cells. Next, we conducted real-time RT-PCR assays to determine whether MED1 is required for the expression of *ACP6* and *LIF* genes by knocking down MED1 in MCF-7/HER2 cells. As shown in Fig. 6C, we found MED1 shRNA but not control scramble shRNA treatment significantly inhibited the mRNA levels of both *ACP6* and *LIF* in MCF-7/HER2 cells. These, together with our above data (Fig. 3F), indicate that MED1 plays a key role in controlling the expression of both

Figure 5. MED1 phosphorylation is required for the recruitment of NCoR and SMRT to TFF1 promoter by tamoxifen. A, schematic diagram of wild-type (WT) MED1 and T1032A/T1457A double-mutated (DM) MED1. B, Western blot analyses of MED1 protein levels after infections with lentivirus expressing WT or DM MED1 into MCF-7/HER2 cells pretreated with control scramble or MED1 shRNA. C, cells described in B were further treated with 1 μ mol/L TAM or vehicle for 45 minutes and then subjected to ChIP assays using IgG (negative control) or antibodies for MED1, p-MED1, NCoR, or SMRT. (*, $P < 0.05$).



EGF-ER α target genes and traditional E2-ER α target genes in HER2 overexpression breast cancer cells.

MED1 is required for the optimal expression of *HER2* gene

Interestingly, it has been previously reported that ER α is able to directly regulate the *HER2* gene expression through its binding site within the *HER2* gene (ref. 42; also see diagram in Fig. 7A). As we have shown that MED1 functions as an ER α coactivator in both E2-ER α and EGF-ER α target genes, it raises an important question as to whether MED1 can also regulate the expression of *HER2*. To determine the role of MED1 in the regulation of *HER2* expression, we first conducted ChIP experiments to determine whether MED1 can bind to this ER α -binding region on the *HER2* gene. As shown in Fig. 7B, using IgG as a control, we found MED1 does bind to this ER α -binding region in both MCF-7 and BT474 cells. Furthermore, our data indicated that MED1 is present in a significantly higher level at this site in BT474 cells when compared with that of MCF-7 cells. Moreover, we found tamoxifen treatment can further increase the occupancy of MED1 at this ER α -binding site in

BT474 cells through ChIP and re-ChIP assays (Fig. 7C, Supplementary Fig. S5). Finally, to determine whether MED1 is required for *HER2* gene expression, we conducted further MED1 shRNA knockdown experiments using BT474 cells. The data show that MED1 shRNA, but not control scramble shRNA, effectively inhibits the mRNA and protein expression levels of *HER2* (Fig. 7D and E). Collectively, these data support a direct role for MED1 in regulating *HER2* gene expression.

Discussion

Through this study, we have established the cross-talk between *HER2* and MED1 and showed its roles in mediating tamoxifen resistance of human breast cancer cells. We found that: (i) MED1 protein expression highly correlates with *HER2* status in human breast cancer; (ii) *HER2* overexpression induces MED1 phosphorylation and knockdown of MED1 sensitizes *HER2*-overexpressing cells to tamoxifen; (iii) instead of known corepressors N-CoR and SMRT, MED1 and p-MED1 are recruited to ER α target gene promoters by tamoxifen in *HER2*-overexpressing cells; (iv) knockdown of MED1 or

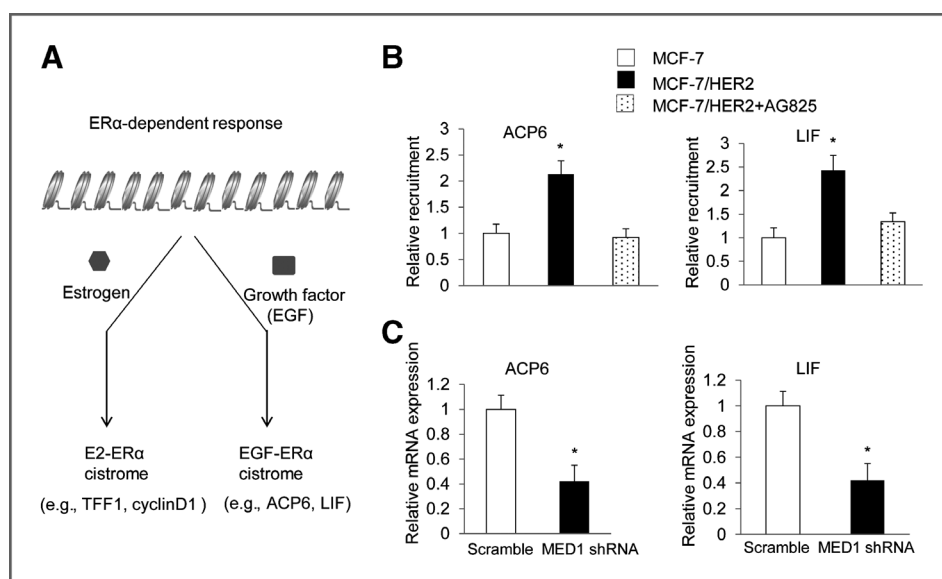


Figure 6. Knockdown of MED1 also inhibits the expression of EGF-ER α target genes. A, diagram for the regulation of ER α -dependent E2-ER α and EGF-ER α target genes by E2 and EGF. B, MCF-7 and MCF-7/HER2 cells were treated with 50 μ mol/L AG825 or vehicle for 1 hour and subject to chromatin IP experiments using control IgG or anti-MED1 antibodies. C, total RNA was extracted from MCF-7/HER2 cells treated with control scramble or MED1 shRNA, and the expression of EGF-ER α target genes ACP6 and LIF were measured by real-time RT-PCR and normalization to that of GAPDH. (*, $P < 0.05$).

mutation of MED1 phosphorylation sites is able to restore the tamoxifen-induced recruitment of N-CoR and SMRT; (v) MED1 is required for the expression of both E2-ER α and EGF-ER α target genes in HER2-overexpressing cells; and (vi) MED1 is also recruited to the *HER2* gene *cis*-regulatory element and

required for its optimal expression. Taken together, these findings support a key role for MED1 in HER2-mediated tamoxifen resistance in human breast cancer.

It has been previously reported that the *MED1* gene is localized on chromosome 17q12 and often coamplifies with *HER2* in human breast cancer cell lines and primary tumors examined (25, 35). Our studies here provided further evidence that MED1 protein expression levels strongly correlate with HER2 status by using human breast cancer TMAs. Importantly, we found that overexpression of HER2 alone is sufficient to induce MED1 phosphorylation at Thr (1032), a key site that is known to be critical for its functions, whereas blockage of HER2 or its downstream MAP kinase diminishes its phosphorylation levels in these cells. HER2 overexpression has been reported to be one of the major mechanisms for tamoxifen resistance of ER α -positive breast cancer cells (10, 11, 16, 20–22). Because our own and others' studies have established MED1 as a key transcriptional coactivator for ER α , we decided to further examine the role of MED1 in tamoxifen resistance of these cells. Indeed, we found that knockdown of MED1 significantly sensitizes the HER2-overexpressing cells to tamoxifen. Although our studies here focus on HER2, we should also mention that tamoxifen resistance has also been linked to a number of other kinases [e.g., epidermal growth factor receptor (EGFR), IGF1R, and c-Src; refs. 9, 46–48]. As they are also known to activate the MAP kinase pathway, it is conceivable that MED1 could also be phosphorylated and play a role in these kinase-mediated tamoxifen resistances.

In the presence of tamoxifen, ER α is known to preferentially bind and recruit transcriptional corepressors N-CoR and SMRT to target gene promoters. Importantly, the antiproliferative effect of tamoxifen critically depends on the recruitment of these corepressors, whereas reducing the levels of N-CoR and SMRT can instead convert tamoxifen into an agonist to stimulate endogenous ER target gene expression and cell growth (43–45). Interestingly, in this study, we found MED1 and p-MED1, but not N-CoR and SMRT, are recruited to the

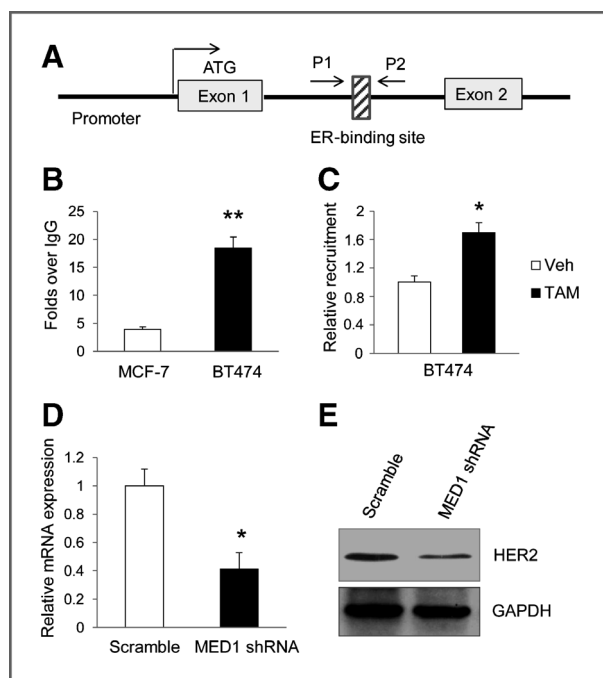


Figure 7. MED1 is present on the ER α -binding site of *HER2* gene and required for its expression. A, schematic representation of *HER2* gene sequence harboring the ER α -binding site. B, ChIP assays were conducted using MCF-7 and BT474 cells using control IgG or anti-MED1 antibodies. C, BT474 cells were treated with TAM or Veh for 1 hour and then subjected to ChIP assays as in B. D and E, real-time PCR and Western blot analyses for HER2 mRNA and protein expression levels, respectively, by using control scramble or MED1 shRNA-treated BT474 cells. (*, $P < 0.05$; **, $P < 0.01$).

ER α target gene promoter by tamoxifen in HER2-overexpressing cells. Importantly, blockage of HER2 signaling, knockdown of MED1, or mutations of both phosphorylation sites of MED1 all could effectively prevent the recruitment of MED1 and p-MED1, and restore N-CoR and SMRT to the promoter of ER α target gene. Taken together, these evidences strongly support a key role for HER2/MED1 cross-talk in tamoxifen resistance through affecting the preferential recruitment of MED1/p-MED1 versus N-CoR/SMRT by tamoxifen-bound ER α . However, one important question remaining is exactly how MED1 phosphorylation may lead to its recruitment to ER α target gene promoters by tamoxifen. One possibility is that it could be simply because of an increased MED1 protein level in these cells rendered by its phosphorylation-induced stabilization. Indeed, it has been previously proposed that the overall balance of coactivators and corepressors levels is an important determinant of the agonist or antagonist nature for tamoxifen (43–45). An alternative or maybe even complement possibility to this is that phosphorylation of MED1 may also lead to its conformation changes, which can in turn increase its accessibility or may even render higher affinity to tamoxifen-bound ER α . Nevertheless, future structural studies on ER α interactions with both phosphorylated and unphosphorylated MED1, in the presence of tamoxifen or estrogen, may be required to gain further deep insights into this important question.

Recent genome-wide ChIP-seq studies have found that activation of the growth factor signaling EGFR pathway by EGF could lead to the binding of ER α to EGF-ER α target genes, a distinct group of genes that are different from previously reported E2 induced E2-ER α target genes (40). Importantly, similar phenomena have most recently been further confirmed by using primary breast cancers from patients with different endocrine responses and clinical outcomes (49). It is known that amplified HER2 often forms complex with EGFR to activate its downstream signaling pathways. Importantly, these EGF-ER α target genes have been found to be over-expressed in HER2 overexpressing cells and are proposed to play important roles in mediating endocrine resistance of HER2 positive cells. Significantly, we found MED1 is not only recruited to E2-ER α target genes but also to EGF-ER α target genes, and are required for the expression of both these target genes. These results suggest that MED1 could potentially be used as an advantageous therapeutic target to block both these pathways for the treatment of tamoxifen resistant human breast cancer. Importantly, as MED1 functions at one of the

last steps right before transcription starts, targeting MED1 could effectively block these EGF-ER α and E2-ER α target genes, even if other HER2 and ER α downstream components, cofactors, or even ER α itself is misregulated or activated. Furthermore, as we have found that MED1 could also regulate the expression of HER2 itself, targeting MED1 could also simultaneously attenuate the activation of the HER2 pathway in these cells. Moreover, our most recently published data revealed a previously unexpected tissue- and gene-specific role for MED1 *in vivo*, and showed that disruption of MED1 resulted in an impaired estrogen response in breast, but not in uterus and bone (30). Most importantly, recent studies found that MED1 expression highly correlates with poor clinical outcome of breast cancer patients treated with endocrine therapy (49). Thus, targeting MED1 in a combined therapy with lower doses of tamoxifen may also result in selective inhibition of these pathways in the breast and overcome the severe adverse effects of currently used high dose tamoxifen regimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Cui, X. Zhang

Development of methodology: J. Cui

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Cui, J. Wang, S.-C. Wang, Q. Wang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Wu, X. Zhang

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Germer, J. Luo, S.-C. Wang

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